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

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TB patients express high IL-4 levels and exhibit a compartment-specific skewed Th2/Th1 response. *In vitro*, IL-4 subverts mycobacterial containment in *M. tuberculosis*-infected human macrophages indicating its potential utility as an immunotherapeutic target. bit.ly/2HclAOH

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

Blood and/or bronchoalveolar lavage fluid (BAL) were obtained from participants with pulmonary tuberculosis (TB) (n=23) and presumed latent TB infection (LTBI) (n=22). Messenger RNA expression levels of interferon (IFN)-γ, IL-4 and its splice variant IL-4δ2 were determined by real-time PCR. The effect of human recombinant (hr)IL-4 on mycobacterial survival/containment ($CFU{\cdot}mL^{-1}$) 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 ($CD4^+IFN\text{-}\gamma^+$ $p<0.001$; $CD4^+TNF\alpha^+$ $p=0.01$). Blocking IL-4 significantly neutralised mycobacterial containment ($p=0.03$), $CD4^+IFN\gamma^+$ levels ($p=0.03$) and Treg expression ($p=0.03$).

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

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Introduction

Tuberculosis (TB) has killed more than 1 billion people over the past 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 only 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* is a robust T-helper type 1 (Th1) response. Although interferon (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 (BCG) and MVA85A, prioritised 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. tuberculosis*-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. tuberculosis* 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 interleukin (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 (IL-4R) 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 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 (hr)IL-4 on mycobacterial survival in *M. tuberculosis*-infected human macrophages.

Methods

Participant recruitment

Newly diagnosed (<2 weeks of anti-TB therapy), drug-sensitive pulmonary TB patients were recruited from four primary care clinics in Cape Town. TB diagnosis was microbiologically confirmed by mycobacteria growth indicator tube (MGIT) liquid culture. Presumed latently TB-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 IFN-γ 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 bronchoalveolar lavage sample processing

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

RNA extraction, reverse transcription and quantitative real-time PCR

RNA was extracted from whole blood and BAL cells using the PAXgene blood RNA kit (PreAnalytiX, Hombrechtikon, Switzerland) and RNeasy Plus kit (Qiagen), respectively. Following RNA quality assessment and reverse transcription, transcribed cDNA was amplified using quantitative (q)PCR using primers and probes specific to IFN-γ, IL-4 and IL-4δ2 (supplementary table E1) from published literature [6]. Values

were normalised to a validated reference gene, human acidic ribosomal protein (HuPO) [6]. Full methodological details are provided in the supplementary material.

Expression, purification and bioactivity assessment of hrIL-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 supplementary material.

Mycobacterial containment assay

A mycobacterial (*M. tuberculosis*) 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. tuberculosis* 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 h at a multiplicity of infection (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; 12 $\mu g \cdot mL^{-1}$) with or without hrIL-4 to generate pre-primed effector T-cells (Teff cells). After 6 days, H37*Rv*-infected MDMs and Teff cells were co-cultured for 48 h. Appropriate controls performed in duplicate included a reference control containing H37*Rv*-infected MDMs only and a positive *M. tuberculosis* containment control containing H37*Rv*-infected MDMs co-cultured with PPD pre-primed Teff cells. The effect of IL-4 was assessed by adding various concentrations (5, 20, 100 $ng \cdot mL^{-1}$) of hrIL-4 (day 1) together with PPD to PBMCs for 6 days to generate PPD+IL-4 Teff cells prior to co-culture with infected MDMs. After co-culturing for 48 h, intracellular H37*Rv* was released by lysis of infected MDMs and plated on Middlebrook 7H10 agar. Colonies were counted and expressed as $CFU \cdot mL^{-1}$. In order to normalise the data to account for interpatient variability, the percentage *M. tuberculosis* containment was also reported, defined as the change in *M. tuberculosis* survival compared to the reference control (H37*Rv*-infected MDMs only; see supplementary table E3 for experimental details):

$$100 - \left[\frac{\text{Experimental Condition } (CFU \cdot mL^{-1})}{\text{Reference control } (CFU \cdot mL^{-1})} \times 100\right] = \%M.tuberculosis \text{ containment}$$

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

Adherent (MDMs) and non-adherent (Teff cells) cellular fractions in the mycobacterial containment assay were analysed by flow cytometry to determine the mechanisms contributing to the IL-4 modulation of *M. tuberculosis* containment. Adherent cells were lifted by treatment with cold 0.5% EDTA and gentle scraping. Cells were stained using fluorescent-labelled 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, tumour necrosis factor (TNF)-α and IL-10 (BD Biosciences, Franklin Lakes, NJ, USA; BioLegend, San Diego, CA, USA; and eBiosciences, Waltham, MA, USA). Cells were acquired on an LSRII flow cytometer and analysed using FACSDiva software (BD Biosciences).

Neutralisation of hrIL-4

The effect of neutralising IL-4 was assessed by adding anti-IL-4 antibody (20 $\mu g \cdot mL^{-1}$; Abcam, Cambridge, UK) to hrIL-4-containing interventions (day 1) in the mycobacterial containment assay. The effects on *M. tuberculosis* containment and associated cellular mechanisms were determined by colony counting ($CFU \cdot mL^{-1}$) and flow cytometry, respectively (supplementary tables E3 and E4). We also performed similar preliminary experiments to determine the effect of blocking IL-4R using anti-IL-4 receptor antibodies (10 $\mu g \cdot mL^{-1}$; 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, San Diego, CA, USA).

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 (supplementary material) in whole blood and BAL cells of TB patients (n=23 and n=8, respectively) and presumed LTBI controls (n=22 and n=7, respectively). IFN-γ expression levels (median, interquartile range (IQR) per 10^6 copies of HuPO) in BAL was approximately six-fold higher compared to whole blood in both TB (6783 (2452–24 918) per 10^6 copies *versus* 1721 (692–3161) per 10^6 copies, respectively; p=0.005) and LTBI (6281 (2570–10 438) per 10^6 copies *versus* 1871 (1131–3521) per 10^6 copies, respectively; p=0.02; figure 1a). IL-4 mRNA levels were higher in TB patients *versus* LTBI controls in whole blood (126 (45–232) per 10^6 copies *versus* 42 (16–98) per 10^6 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). In addition, the IL-4/IFN-γ expression ratio, thought to represent the Th2/Th1 balance, was higher in whole blood of TB patients compared to LTBI controls (0.046 (0.021–0.155) per 10^6 copies *versus* 0.019 (0.010–0.036) per 10^6 copies; p=0.01), but not in BAL cells. The IL-4/IFN-γ ratio was much lower in BAL compared to whole blood 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; supplementary figure E4) 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 (supplementary 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 (supplementary figure E6). IL-13

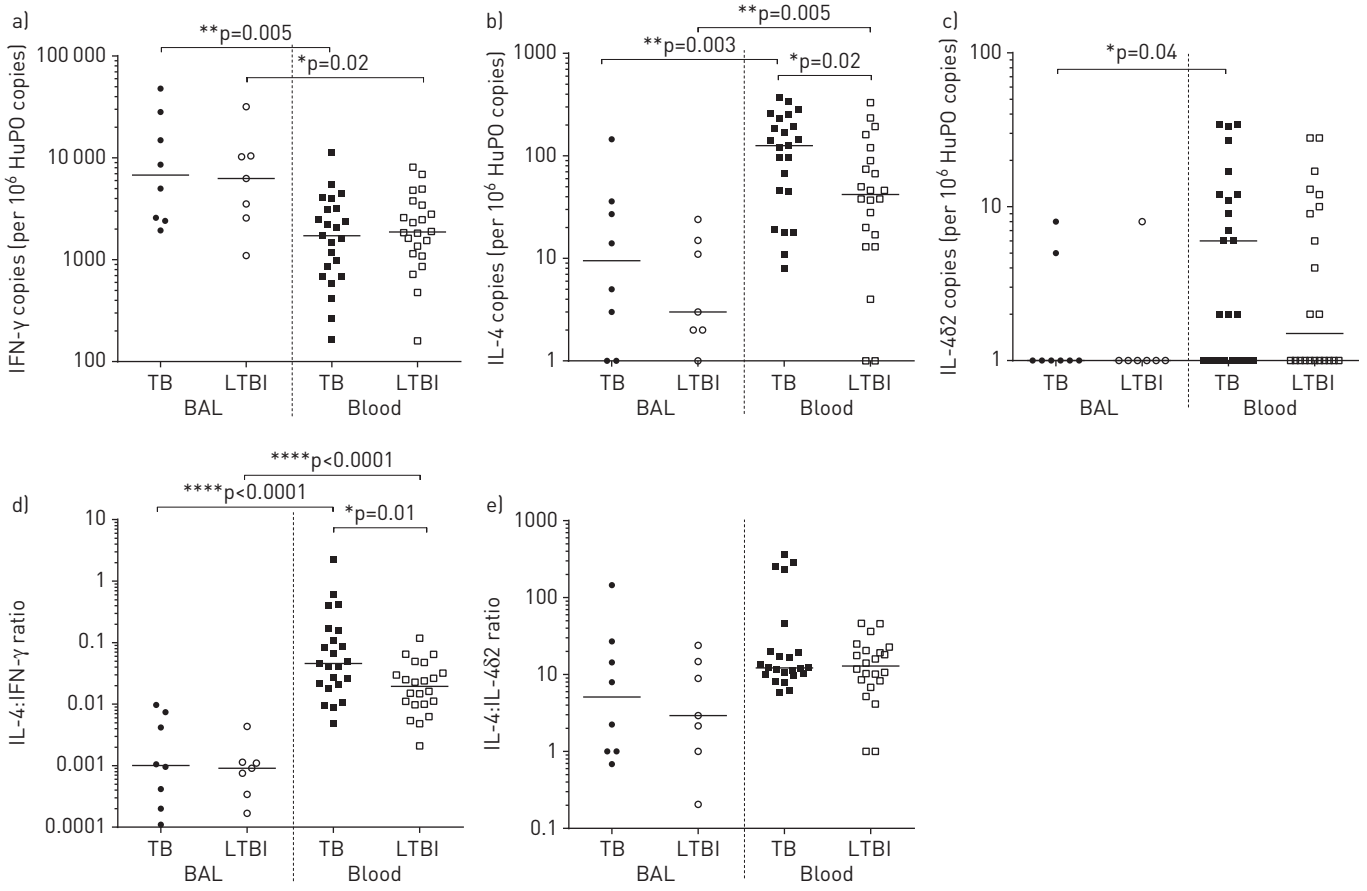

FIGURE 1 mRNA expression levels of a) interferon (IFN)-γ, b) interleukin (IL)-4 and c) IL-4δ2, and cytokine expression ratios of d) IL-4/IFN-γ and e) IL-4/IL-4δ2 in cells of bronchoalveolar lavage (BAL) and peripheral whole blood from patients with pulmonary tuberculosis (TB; BAL n=8; blood n=23) and presumed latently TB-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 human acidic ribosomal protein (HuPO) (validated reference gene). Statistical analyses between groups were performed using the Mann–Whitney test and p<0.05 was deemed significant. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

protein levels were detectable, but also low, and there were no differences in expression levels between TB patients and LTBI controls (supplementary figure E7).

IL-4-primed effector cells can subvert mycobacterial containment

In TB patients (n=8), the addition of PPD Teff cells to infected MDMs reduced the median *M. tuberculosis* from 30.7×10^3 CFU·mL^{-1} to 15.7×10^3 CFU·mL^{-1} compared to infected MDMs only (p=0.008; figure 2a), equivalent to a 48% increase in *M. tuberculosis* containment (p=0.008; figure 2b).

In the PPD+IL-4 Teff interventions, there was a significant increase in median CFU·mL^{-1} at 5–100 ng·mL^{-1} hrIL-4 (29.1×10^3 to 43.8×10^3, p=0.008; figure 2a) compared to the PPD Teff control in TB patients (n=8). This equated to a decrease in percentage *M. tuberculosis* containment at each of the hrIL-4 concentrations (1%, −73% and −33%, respectively *versus* 48%, p=0.008; figure 2b). The percentage *M. tuberculosis* containment was significantly lower at 100 ng·mL^{-1} compared to 5 ng·mL^{-1} 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^{-1} increased in the PPD+IL-4 Teff interventions at 5–100 ng·mL^{-1} hrIL-4 (45.6×10^3 to 108.3×10^3) compared to the PPD Teff control (28.0×10^3; supplementary figure E10A). This resulted in decreased percentage *M. tuberculosis* containment at each hrIL-4 concentration compared to the control (64%, 18% and −6%, respectively *versus* 72%; supplementary figure E10B). However, the differences between the PPD+IL-4 Teff interventions and PPD Teff 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 (supplementary table E5).

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

In order to determine the effect of hrIL-4 on cellular biomarker expression, cells (MDMs and Teff cells) were harvested from the mycobacterial containment assay and analysed using flow cytometry.

Regulatory T-cells

The gating strategy for identification of regulatory T cells (Tregs) ($CD3^+CD4^+CD25^+FoxP3^+$) is shown in figure 3a. The median percentage Treg expression (IQR) was significantly increased in the PPD+IL-4 Teff

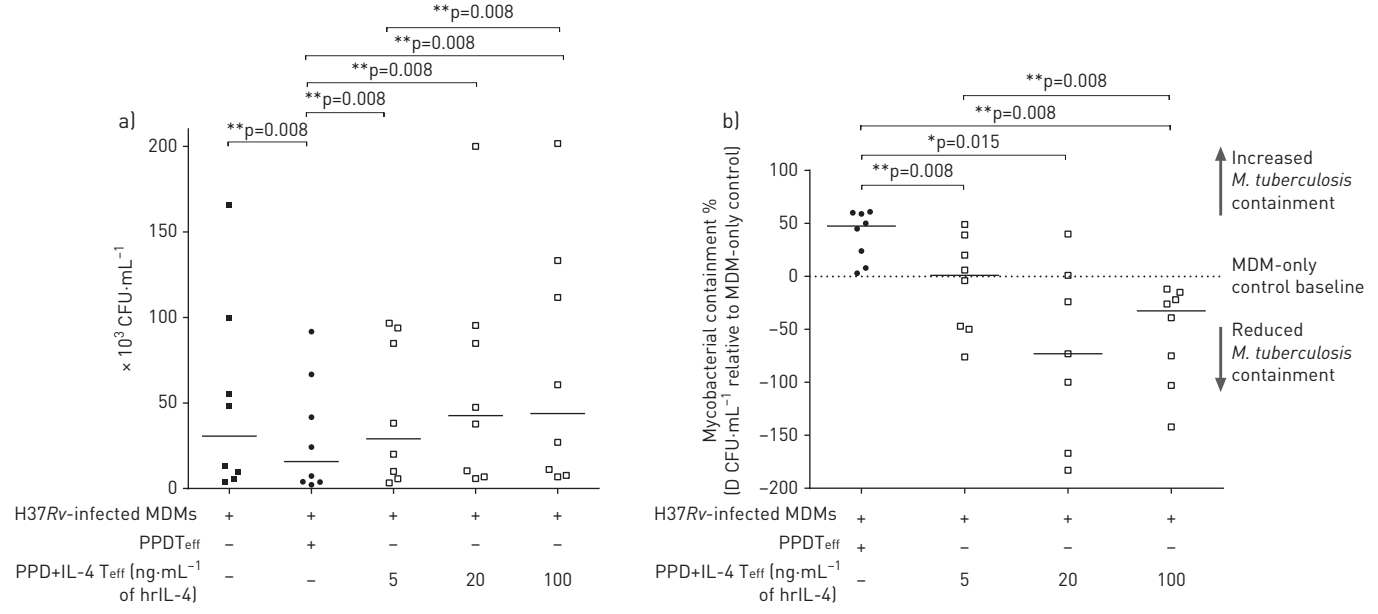

FIGURE 2 The effect of human recombinant interleukin (hrIL)-4 on mycobacterial containment in infected monocyte-derived macrophages (MDMs) from pulmonary tuberculosis patients (TB; n=8). A mycobacterial containment assay was performed where H37*Rv*-infected MDMs were cultured by themselves or co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation alone (PPD Teff), or PPD and hrIL-4 (PPD+IL-4 Teff) at concentrations of 5, 20 and 100 ng·mL^{-1} hrIL-4. Results are expressed as a) median CFU·mL^{-1} in TB patients and b) the percentage mycobacterial (*M. tuberculosis*) containment, defined as the change in *M. tuberculosis* survival compared to the "H37*Rv*-infected MDMs only" control, were also determined. Increased *M. tuberculosis* containment indicates a reduction in *M. tuberculosis* survival, whereas decreased *M. tuberculosis* containment indicates an increase in *M. tuberculosis* survival. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and p<0.05 was deemed significant.

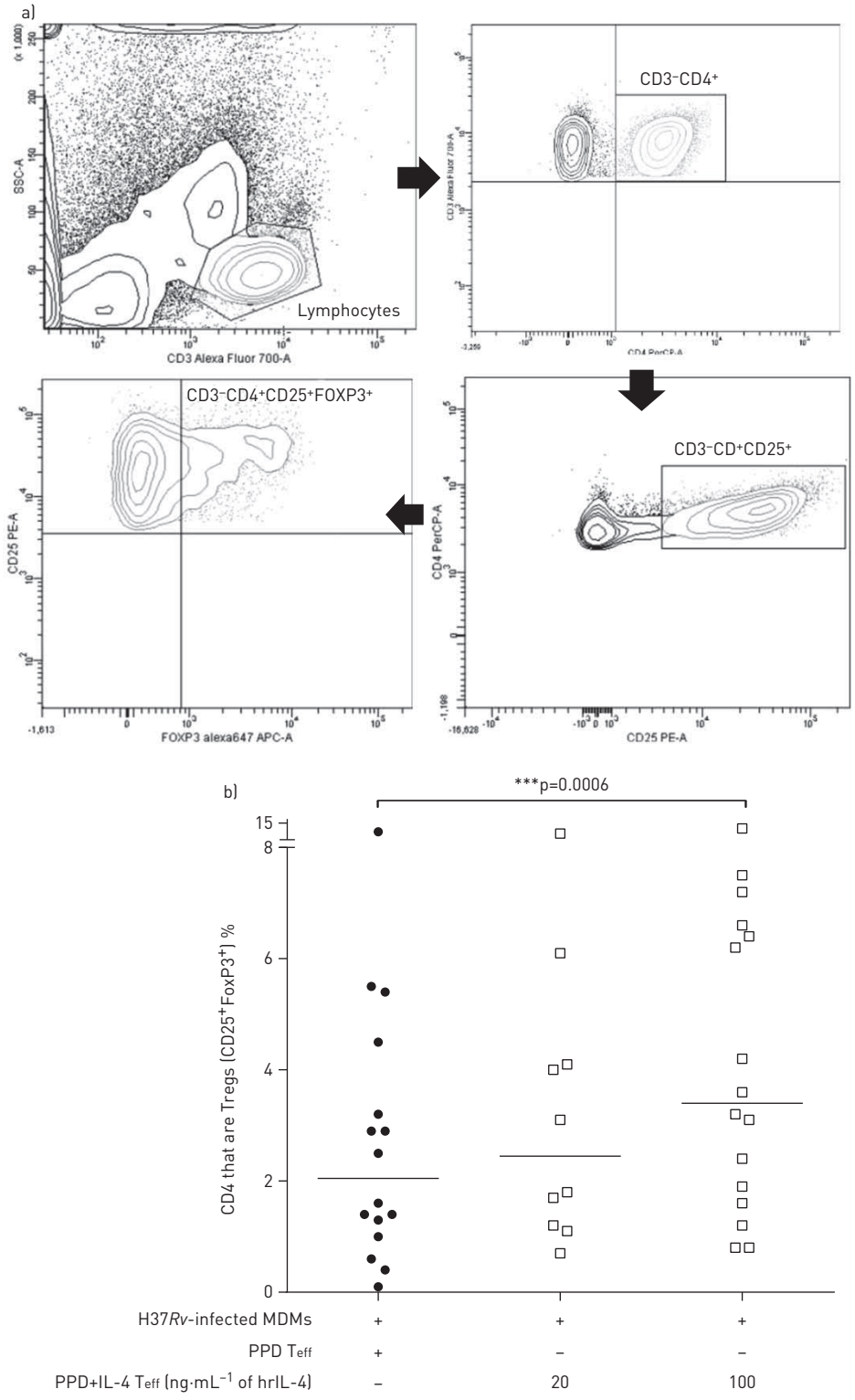

FIGURE 3 The effect of human recombinant interleukin (hrIL)-4 on regulatory T-cell (Treg) expression in a mycobacterial containment co-culture assay as measured by flow cytometry. a) The flow cytometry gating strategy for identification of Tregs ($CD3^+CD4^+CD25^+FoxP3^+$) within the lymphocyte population; and b) the frequency (%) of Tregs in the co-cultured lymphocyte population of the mycobacterial containment assay in pulmonary tuberculosis patients. H37*Rv*-infected monocyte-derived macrophages (MDMs) were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (PPD Teff), or PPD and hrIL-4 (PPD+IL-4 Teff) at concentrations of 20 $ng \cdot mL^{-1}$ (n=10) and 100 $ng \cdot mL^{-1}$ (n=16) hrIL-4. Tregs were expressed as a percentage of $CD4^+$ lymphocytes. Statistical analysis was performed using the Wilcoxon matched-pairs signed rank test and $p<0.05$ was deemed significant.

intervention (100 ng·mL^{-1} hrIL-4; n=16) compared to the PPD Teff control (4.2 (1.2–10.5)% *versus* 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 TNF-α) 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 100 ng·mL^{-1} hrIL-4 (n=16; 1.2 (0.7–3.8)%), compared to the PPD Teff 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 TNF-α expression was less pronounced than $CD4^+$IFN-γ expression. Lower median $CD4^+$TNF-α^+ expression was observed in the PPD+IL-4 Teff intervention at 100 ng·mL^{-1} hrIL-4 compared to the PPD Teff control (1.4 (0.5–3.1)% *versus* 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 Teff control compared to the infected MDMs-only well (12.3 (4.6–21.2)% *versus* 23.8 (5.8–45.5)%, respectively, p=0.02). However, DC-SIGN expression increased in the PPD+IL-4 Teff intervention at 100 ng·mL^{-1} hrIL-4 (16.8 (5.8–21.5)%, p=0.02; figure 4d).

Neutralisation of IL-4

The effect of neutralising rIL-4 was determined in TB patients (n=6). Addition of anti-IL-4 antibody (20 µg·mL^{-1}) to the PPD+IL-4 Teff intervention at 20 ng·mL^{-1} reduced the CFU·mL^{-1} to levels similar to the PPD Teff control (76.5×10^3 to 32.4×10^3 CFU·mL^{-1}, p=0.03; figure 5a). Consequently, the percentage *M. tuberculosis* 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; 20 µg·mL^{-1}) antibody was tested in the PPD+IL-4 Teff intervention at 100 ng·mL^{-1} hrIL-4 only (limitations in sample amount prevented testing of 20 ng·mL^{-1} hrIL-4). 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 Teff control. No significant effect on TNF-α 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 (supplementary figure E12).

Discussion

The key findings of this study are as follows. 1) In blood, but not BAL, TB patients exhibited higher IL-4 mRNA expression and a higher IL-4/IFN-γ ratio compared to LTBI controls; 2) overall, IL-4δ2 expression levels were very low; 3) responses were compartmentalised (higher IFN-γ, lower IL-4 and a lower IL-4/IFN-γ ratio in the lungs *versus* blood) in both TB patients and LTBI controls; 4) hrIL-4 can subvert *M. tuberculosis* containment in human macrophages, and these effects were concentration-dependent; 5) IL-4-driven mycobacterial containment was associated with an increased Treg frequency, reduced $CD4^+$Th1 cytokine expression and increased macrophage DC-SIGN expression; and 6) these effects were reversed upon neutralisation 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. tuberculosis*-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]). In addition, host-directed therapies exhibit tremendous potential, either to shorten treatment regimens or expand the limited treatment options available for those with highly drug-resistant TB [2, 35].

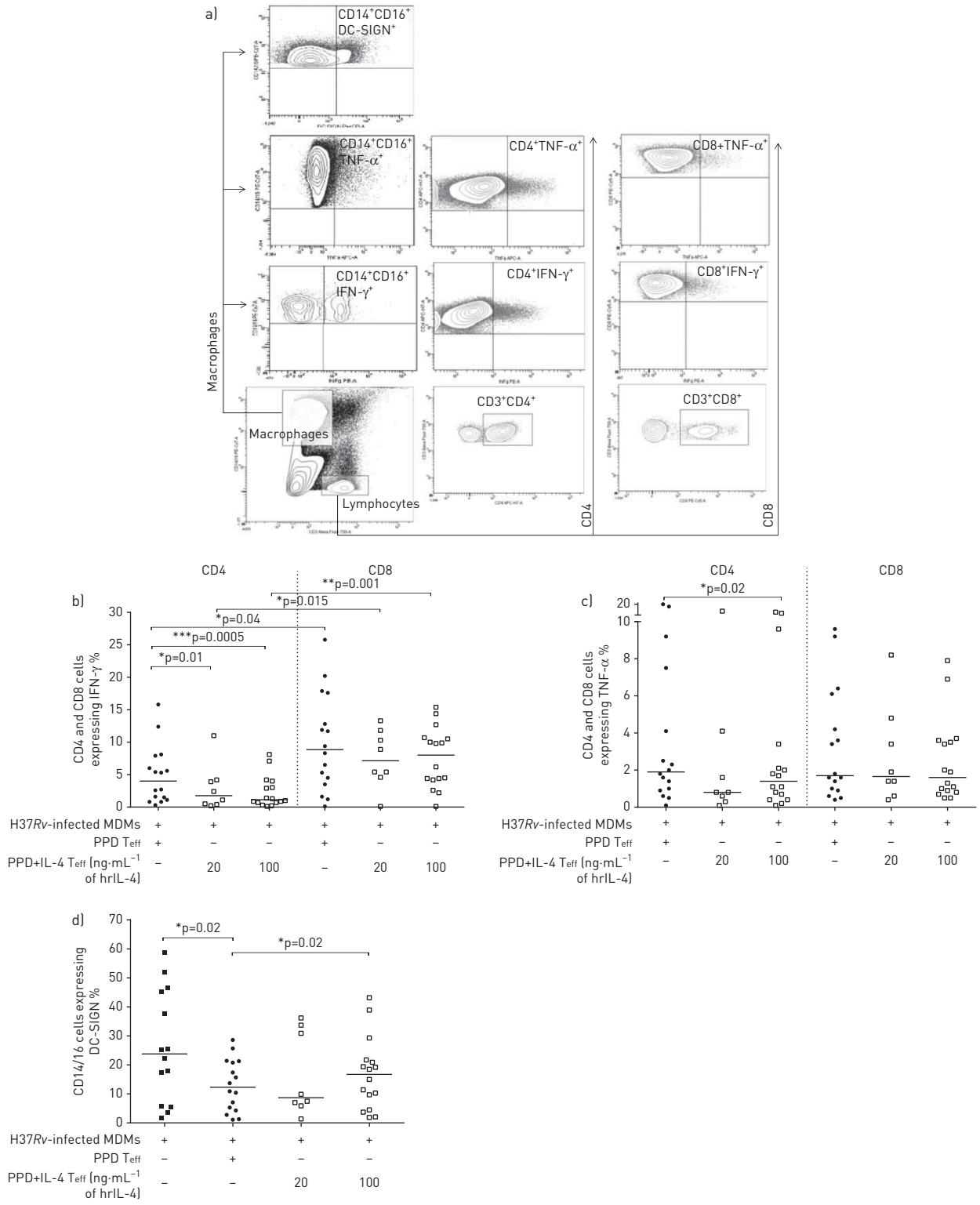

FIGURE 4 The effect of human recombinant interleukin (hrIL)-4 on Th1 cytokine expression in lymphocytes and dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) expression in macrophages in a mycobacterial containment assay as measured by flow cytometry. a) The flow cytometry gating strategy for identifying interferon (IFN)-γ and tumour necrosis factor (TNF)-α within the lymphocyte ($CD4^+$ and $CD8^+$) population and DC-SIGN expression within the macrophage ($CD14^+CD16^+$) population. The percentages of $CD4^+$ and $CD8^+$ lymphocytes expressing b) IFN-γ, c) TNF-α and $CD14^+CD16^+$ macrophages expressing d) DC-SIGN in cells harvested from a mycobacterial containment assay from pulmonary tuberculosis patients. H37*Rv*-infected monocyte-derived macrophages (MDMs) were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (PPD Teff), or PPD and hrIL-4 (PPD+IL-4 Teff) at concentrations of 20 $ng{\cdot}mL^{-1}$ (n=10) and 100 $ng{\cdot}mL^{-1}$ (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.

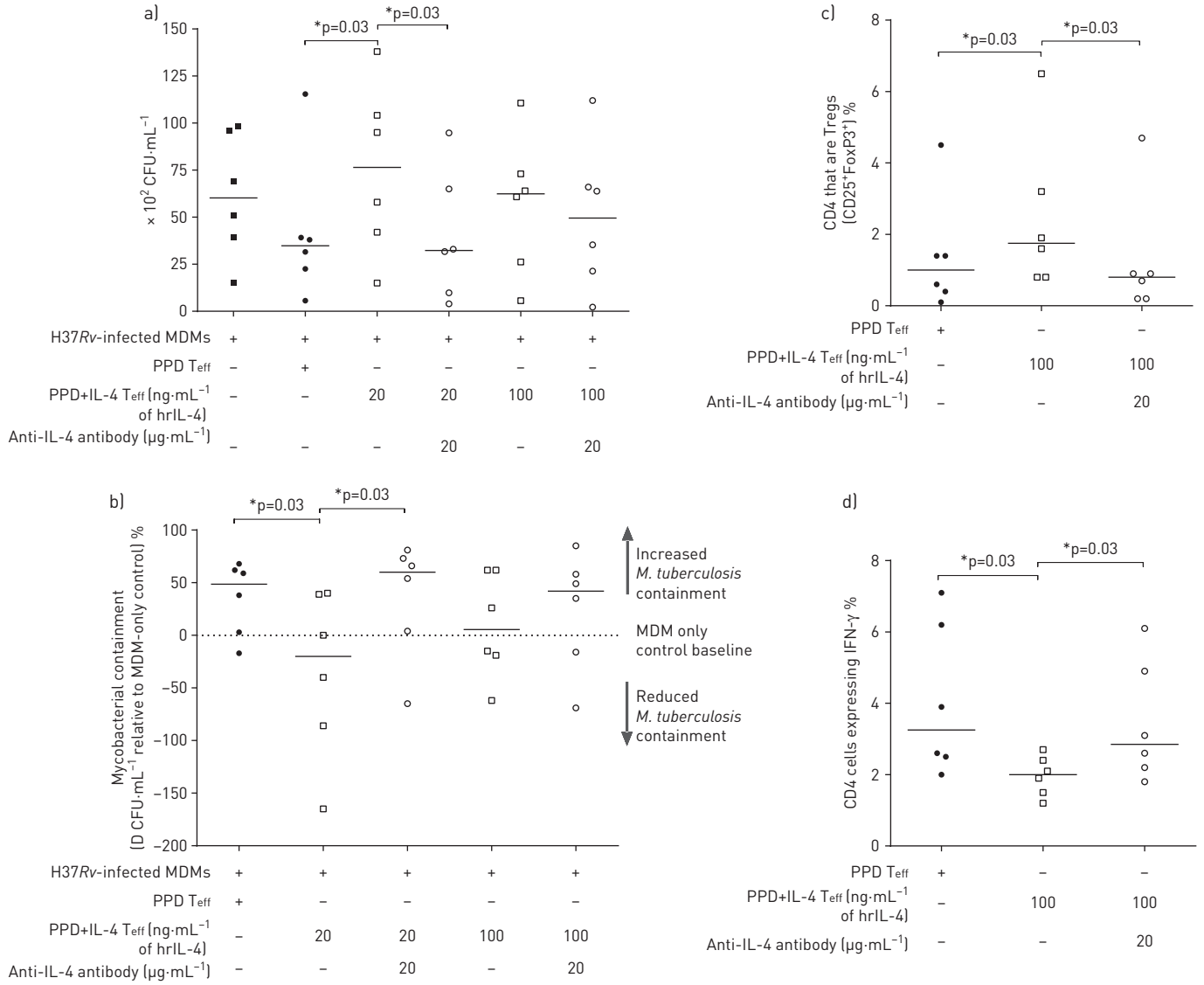

FIGURE 5 The effect of neutralising human recombinant interleukin (IL)-4 on mycobacterial containment and on cytokine and regulatory T-cell (Treg) expression in a mycobacterial containment assay. H37*Rv*-infected monocyte-derived macrophages (MDMs) were co-cultured with peripheral blood mononuclear cells pre-primed with purified protein derivation (PPD) alone (PPD Teff) or PPD and hrIL-4 (PPD+IL-4 Teff) at 20 and 100 ng·mL^{-1} hrIL-4. To the IL-4 interventions, anti-IL-4 antibody (20 µg·mL^{-1}) was added at the same time as hrIL-4 (day 1). In tuberculosis patients (n=6), mycobacterial containment was measured in terms of a) CFU·mL^{-1} and b) percentage mycobacterial (*M. tuberculosis*) containment, defined as the change in *M. tuberculosis* survival compared to the "H37*Rv*-infected MDMs only" control. Increased percentage *M. tuberculosis* containment indicates a reduction *M. tuberculosis* survival, whereas decreased percentage *M. tuberculosis* containment indicates an increase in *M. tuberculosis* survival. The percentage of CD4$^+$ lymphocytes that were c) Treg (CD25$^+$FoxP3$^+$) and expressing d) interferon (IFN)-γ were also determined within the system (cells co-cultured within the *M. tuberculosis* 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.

Interestingly, dupilumab, 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 IL-4δ2 expression observed in this 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 (supplementary figures E6 and E7). 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 signalling pathways, they can have distinct expression profiles and functions in Th2-driven conditions [42], but whether this is 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 IL-4δ2 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 IL-4δ2 are likely to hinder the development of an antibody-based diagnostic assay. These include sub-ELISA expression levels ([44, 45] and supplementary 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 IL-4δ2 protein has only been described in asthma [49], but its mechanism of action and precise function remain 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. tuberculosis* survival in infected MDMs by PPD pre-primed effectors (PPD Teff 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. tuberculosis* containment by 50–120%. The addition of IL-4 to unprimed T-cells before commitment to a specific T-cell lineage creates a Th2-polarising 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 polarisation.

A similar reduction in mycobacterial containment was observed in LTBI participants, but this failed to reach statistical significance ($p=0.06$), probably because there were fewer sample numbers in this group. Furthermore, no differences were observed when TB and LTBI groups were compared directly (supplementary 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]. 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. tuberculosis* replication [59]). Although producing very little IL-10 (supplementary 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 increased in the PPD+IL-4 Teff intervention. DC-SIGN facilitates entry of *M. tuberculosis* into phagocytic cells and is a hallmark of alternatively activated M2 macrophages. A number of *M. tuberculosis* components that act *via* DC-SIGN can drive host immunoregulatory mechanisms [61]. In our model, M2 polarisation probably occurs 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. tuberculosis* containment.

Preliminary experiments indicate that blocking of the IL-4 receptor resulted in a similar effect to neutralisation of IL-4 on mycobacterial containment, Treg frequency and IFN-γ expression, although no significant differences were observed, probably due to the limited sample numbers. Previous evidence indicates that IL-4Rα-mediated signalling is associated with tissue pathology in TB-infected mice [18] and the development of necrotising 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. First, the hrIL-4 concentrations used in the *M. tuberculosis* 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 1) recombinant proteins tend to be less stable and active compared to their naturally produced counterparts [63]; 2) 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; 3) proteins expressed in insect cell systems may undergo inappropriate N-glycosylation patterns, which can affect protein function and stability [64]; and 4) quantification methods, including the Bradford assay, fail to distinguish between functional and nonfunctional recombinant protein. Moreover, the rIL-4 concentrations used in our *M. tuberculosis* containment assays were derived from those used in the ^{3}H-thymidine T-cell proliferation assay (supplementary figure E9C) which produced a significant effect, and other *in vitro* stimulation experiments have used similar IL-4 concentrations [66]. Second, 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. tuberculosis* containment assay [25]. Third, we only performed preliminary functional experiments to determine the effect of IL-4R on *M. tuberculosis* 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. tuberculosis* containment. Fourth, 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 (supplementary figure E4) also showed a compartment-specific effect similar to the complete dataset outlined in figure 1 (characterised by elevated IFN-γ levels in the lungs, whereas IL-4 levels were increased in the peripheral blood).

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

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