

**1 Th22 cells are a major contributor to the mycobacterial CD4+ T cell**  
**2 response and are depleted during HIV infection**

3

4

5 Rubina Bunjun<sup>1,2</sup>, Fidilia M.A. Omondi<sup>1,2</sup>, Mohau S. Makatsa<sup>1,2</sup>, Tracey L. Müller<sup>1,2</sup>, Caryn  
6 S.L. Prentice<sup>1,2</sup>, Robert J. Wilkinson<sup>1,3,4,5,6</sup>, Catherine Riou<sup>1,2,3</sup>, Wendy A. Burgers<sup>1,2,3</sup>

7

8

9 <sup>1</sup>Institute of Infectious Disease and Molecular Medicine, <sup>2</sup>Department of Pathology, and  
10 <sup>3</sup>Wellcome Centre for Infectious Diseases Research in Africa, University of Cape Town,  
11 Observatory, 7925, South Africa; <sup>4</sup>Department of Medicine, University of Cape Town,  
12 Observatory, 7925, South Africa; <sup>5</sup>Department of Medicine, Imperial College London, W12  
13 0NN, United Kingdom; <sup>6</sup>The Francis Crick Institute, London NW1 1AT, United Kingdom.

14

15 **FOOTNOTES:**

16 **Funding:** This project is part of the EDCTP2 programme supported by the European Union  
 17 (EU)'s Horizon 2020 programme (Training and Mobility Action TMA2016SF-1535 –  
 18 CaTCH-22, to WAB). Additionally, WAB was funded by the SAMRC, NRF SA (92755)  
 19 and NHLS Trust (2016-2DEV04). RB was a Carnegie Corporation Fellow and received PhD  
 20 funding from the University of Cape Town and the Canada Africa Prevention Trials (CAPT)  
 21 Network. CR is funded by the EDCTP2 programme supported by the EU's Horizon 2020  
 22 programme (Training and Mobility Action TMA2017SF-1951– TB-SPEC, to CR). RJW is  
 23 supported by the Wellcome Trust (203135 and 104803), NIH (U01 AI115940), the Francis  
 24 Crick Institute (Cancer Research UK, MRC UK and Wellcome FC0010218), NRF SA  
 25 (96841) and SAMRC (SHIP). The funders had no role in study design, data collection and  
 26 analysis, decision to publish, or preparation of the manuscript. The views expressed are those  
 27 of the authors, and the funders are not responsible for any use that may be made of the  
 28 information contained herein.

30 **Correspondence:** Wendy Burgers, Institute of Infectious Disease and Molecular Medicine,  
 31 Faculty of Health Sciences, University of Cape Town, Observatory 7925, South Africa  
 32 ([wendy.burgers@uct.ac.za](mailto:wendy.burgers@uct.ac.za)).

34 **Potential conflicts of interest:** The authors of this manuscript do not have commercial or  
 35 other associations that pose a conflict of interest.

37 **Manuscript information:**

38 Running title: HIV depletes mycobacteria-specific Th22 cells

39 Abstract: 250/250 words; Figures: 6, Tables: 1; Supplementary Figures: 3

# 41 ABSTRACT

42 HIV-1 infection substantially increases the risk of developing tuberculosis (TB). Some  
 43 mechanisms, such as defects in the Th1 response to *Mycobacterium tuberculosis* (*M.tb*) in  
 44 HIV-infected individuals have been widely reported. However, Th1-independent mechanisms  
 45 also contribute to protection against TB. To identify a broader spectrum of defects in TB  
 46 immunity during HIV infection, we examined IL-17 and IL-22 production in response to  
 47 mycobacterial antigens in individuals with latent TB infection (LTBI) and HIV co-infection.  
 48 Upon stimulating with mycobacterial antigens, we observed a distinct CD4+ T helper lineage  
 49 producing IL-22 in the absence of IL-17 and IFN- $\gamma$ . Th22 cells were present at high  
 50 frequencies in response to mycobacterial antigens in blood and contributed up to 50% to the  
 51 CD4+ T cell response to mycobacteria, comparable in magnitude to the IFN- $\gamma$  Th1 response  
 52 (median 0.91% and 0.55%, respectively). Phenotypic characterization of Th22 cells revealed  
 53 that their memory differentiation was similar to *M.tb*-specific Th1 cells (*i.e.* predominantly  
 54 early-differentiated CD45RO+CD27+ phenotype). Moreover, CCR6 and CXCR3 expression  
 55 profiles of Th22 cells were similar to Th17 cells, while their CCR4 and CCR10 expression  
 56 patterns displayed an intermediate phenotype between Th1 and Th17 cells. Strikingly,  
 57 mycobacterial IL-22 responses were three-fold lower in HIV-infected individuals compared  
 58 to uninfected individuals, and the magnitude of responses correlated inversely with HIV viral  
 59 load. These data provide important insights into mycobacteria-specific T helper subsets and  
 60 suggest a potential role for IL-22 in protection against TB during HIV infection. Further  
 61 studies are needed to fully elucidate the role of IL-22 in protective TB immunity.

## 62 INTRODUCTION

63

64 Tuberculosis (TB) is the leading cause of death from an infectious agent, claiming 1.6  
 65 million lives in 2017, with 10 million new TB cases that year (1). This considerable burden  
 66 of disease, along with a host of challenges in diagnosing, treating and managing TB,  
 67 emphasize its significance as a global health threat. Although TB is curable and successful  
 68 treatment outcomes are typically >80%, cure is achieved less frequently with drug  
 69 resistant TB (56%), and outcomes during HIV co-infection are worse (2). Importantly,  
 70 cure does not lead to protection from re-infection or disease reactivation. HIV-infected  
 71 persons are particularly vulnerable to developing TB, with an estimated increase in risk of  
 72 20-30 fold (3). The widespread introduction of ART has coincided with only a modest  
 73 decline in TB in regions most affected by HIV (4), as TB risk still remains elevated in  
 74 HIV-infected persons compared to HIV-uninfected persons, despite immune  
 75 reconstitution (5).

76 The development of an effective TB vaccine is hampered by a lack of  
 77 understanding of correlates of immune protection (6), particularly the functional and  
 78 phenotypic characteristics of effector T cells that mediate control of *Mycobacterium*  
 79 *tuberculosis* (*M.tb*), and how this immune response might be balanced by  
 80 immunoregulatory T cell populations to limit inflammation and avoid pathology. The  
 81 recent demonstration of the first candidate TB vaccine capable of protecting adults from  
 82 pulmonary TB with an efficacy of 54% (7), provides the field with an opportunity to  
 83 define correlates of vaccine protection, and has the potential to uncover unique insights  
 84 into immunological control of TB.

85 TB and HIV co-infection presents us with a further prospect to improve our  
 86 understanding of the mechanisms of immune control of *M.tb*, by identifying how HIV

renders the immune response to *M.tb* defective, leading to increased risk of TB disease. CD4<sup>+</sup> T cells and specifically the Th1/IFN- $\gamma$  response to *M.tb* are critical for protective immunity to TB (8). Most studies of HIV-TB co-infection focus on Th1 immunity, and have demonstrated depletion or dysfunction of *M.tb*-specific Th1 responses in both blood (9–12) and the airways (13–15) during HIV infection.

However, there is evidence of a role for IFN- $\gamma$ -independent mechanisms in immune control of TB (16) that may also contribute to, or synergize with, Th1 responses to TB. Recently, we characterized the profile of Th subsets specific for *M.tb* using lineage-defining transcription factors, revealing the broad spectrum of Th subsets involved in mycobacterial immunity, demonstrating that the inflammatory environment associated with HIV infection skewed these profiles (17). Th17 cells form part of this spectrum of *M.tb*-specific Th responses, and are believed to play an important role in immune protection from TB (18). Suppression of Th17-related genes was recently shown to be associated with progression to TB disease in *M.tb*-infected adolescents (19). In line with this, *M.tb*-specific IL-17-producing CD4<sup>+</sup> T cells were significantly depleted in HIV-infected individuals from a TB-endemic area, compared to HIV-uninfected individuals (20).

Whilst IL-17 responses in *M.tb* immunity have been relatively well-studied (21–26), IL-22 responses have been overlooked in part due to their classification as a Th17 cytokine from studies in mice (27). In humans, however, IL-22 is produced by a distinct subset of CD4<sup>+</sup> T cells (28–30), termed “Th22 cells”. IL-22 is a member of the IL-10 family of cytokines, and functions mainly to protect tissues from inflammation and infection, through stimulating proliferation and repair, and the production of antimicrobial peptides (31). Until recently, IL-22 was thought to be dispensable for control of *M.tb*, since deficiency or neutralization of IL-22 in mice had no effect control of *M.tb* using lab strains H37Rv and Erdman (32–35). However, the recent observation that IL-22 deficient mice infected with a

clinical strain of *M.tb* (HN878) had an impaired ability to control *M.tb*, leading to increased bacterial burden and greater dissemination of infection (36), has triggered renewed interest in IL-22 and its role in TB control.

Given the paucity of data on *M.tb*-specific IL-22 CD4<sup>+</sup> responses, and the knowledge that HIV infection results in the preferential targeting and depletion of Th22 cells (37), we sought to characterize HIV-induced defects in adaptive immunity to *M.tb*, with a focus on Th22 cells. Our findings highlight the large contribution IL-22 makes to the human CD4<sup>+</sup> T cell response to TB (equivalent in magnitude to the IFN- $\gamma$  response), with *M.tb*-specific Th22 cells being entirely distinct from Th1 and Th17 cells. Moreover, we show for the first time that *M.tb*-specific Th22 cells are depleted during HIV co-infection to a similar extent as Th1 responses. These findings emphasize the potential importance of this understudied CD4<sup>+</sup> Th subset in TB immunity, and suggest that the loss of *M.tb*-specific Th22 cells may contribute to the increased risk of TB during HIV infection.

## MATERIALS AND METHODS

### Study Participants

Volunteers were recruited from Cape Town, South Africa, and fell within the following groups: ART naive HIV-seropositive persons with CD4 counts >400 cells/mm<sup>3</sup> (n=25; median age 31; 96% female) and HIV-seronegative persons (n=25; median age 23; 60% female). HIV RNA levels were determined using an Abbott m2000 RealTime HIV-1 assay and blood CD4 counts by the Flow-CARE™ PLG CD4 test. All volunteers were TB sensitized based on a positive IFN- $\gamma$  release assay (IGRA; Quantiferon, Cellestis), and active TB was excluded, based on symptoms and radiological evidence.

Healthy donors were recruited from the University of Cape Town, South Africa. Participants were >18 years of age, weighed >55 kg, did not have any chronic disease, did not use immunosuppressive medication and were not pregnant or lactating. These studies were approved by the Research Ethics Committee of the University of Cape Town (158/2010, 279/2012). All participants provided written, informed consent.

### **Whole blood stimulation assays**

Venous blood was collected and processed within 4 hours. Whole blood stimulation was performed as previously described (38) with the following antigens: Bacillus Calmette-Guerin (BCG; MOI of 4; SSI), Purified Protein Derivative (PPD) of *M. tuberculosis* (20µg/ml; Statens Serum Institute), ESAT-6 and CFP-10 peptide pools (4µg/ml), *M. tuberculosis* whole cell lysate (10µg/ml; BEI Resources) or PMA and Ionomycin (0.01µg/ml and 1µg/ml, respectively, Sigma), in the presence of anti-CD28 and anti-CD49d (1µg /ml each). Unstimulated cells were incubated with co-stimulatory antibodies only. Brefeldin A (BFA, 10µg/ml; Sigma) was added 7 hours after the onset of stimulation, and five hours after BFA addition, cells were either stained immediately, or red blood cells were lysed, the cell pellet stained with a violet viability dye, ViViD (Molecular Probes), fixed with FACS Lyse (BD Biosciences) and cryopreserved in 10% DMSO in FCS.

### **Antibody Staining and Flow Cytometry**

Cryopreserved or freshly stimulated whole blood was stained as previously described (15). For intracellular markers, cells were permeabilized with Perm/Wash buffer (BD Biosciences) and then stained intracellularly. Cells were stained with the following antibodies: CD3 APC-H7 (SK7; BD Biosciences), CD4 PE-Cy5.5 (S3.5; Invitrogen), CD4 ECD (T4; Beckman Coulter), CD8 QDot705 (3B5; Invitrogen), CD45RO ECD (UCLH1; Beckman Coulter),

CD27 PE-Cy5 (1A4CD27; Beckman Coulter), CXCR3 PE-Cy7 (1C6/CXCR3; BD Biosciences), CCR6 BV605 (11A9; BD Biosciences), CCR4 BV510 (L291H4; Biolegend), CCR10 PE (1B5; BD Biosciences), KLRG1 PE-vio770 (REA261; Miltenyi Biotec), CD26 FITC (M-A261; BD Biosciences), IFN- $\gamma$  Alexa700 (B27; BD Biosciences), IL-17 Alexa488 (N49-653; BD Biosciences), IL-17 FITC (BL-168; Biolegend), IL-22 PE (22URTI; e-Bioscience) or IL-22 eFluor450 (22URTI; e-Bioscience). Cells were acquired on a BD Fortessa using FACSDiva software and data analysed using FlowJo (TreeStar) and Pestle and Spice (39). A positive cytokine response was defined as twice background and a net response >0.025%, and all data are reported after background subtraction. A minimum of 30 cytokine-positive events was required for memory or chemokine receptor phenotyping.

## Statistical Analysis

Statistical analyses were performed using Prism 7 (GraphPad). Non-parametric tests (Mann-Whitney U test, Wilcoxon matched pairs test and Spearman Rank test) were used for all comparisons. Kruskal-Wallis with Dunn's post-test was used for multiple comparisons. A p value of <0.05 was considered significant.

## RESULTS

### IL-22 responses are a major component of the CD4+ mycobacterial response

We examined CD4+ T cell cytokine profiles in response to a range of mycobacterial antigens in 25 healthy, HIV-uninfected persons sensitized by *M. tuberculosis* (*M.tb* IGRA+; **Table 1**). **Figure 1A** shows representative flow cytometry plots of IFN- $\gamma$ , IL-22 and IL-17 CD4+ responses to *M. bovis* BCG, *M.tb* PPD and a pool of ESAT-6 and CFP-10 peptides from



*M.tb.* As expected, CD4<sup>+</sup> T cell IFN- $\gamma$  responses to BCG were detected in all donors (median 0.55%, IQR: 0.28-1.46%; **Figure 1B**). Remarkably, IL-22 accounted for the greatest proportion of the CD4<sup>+</sup> response to BCG (median 0.91%, IQR: 0.52-1.24%). In fact, the frequency of IL-22<sup>+</sup> cells was greater than IFN- $\gamma$  in 75% of participants. IL-17 CD4<sup>+</sup> responses to BCG were significantly lower (median 0.11%, IQR: 0.06-1.66%) than both IFN- $\gamma$  ( $p=0.007$ ) and IL-22 ( $p=0.0008$ ). Stimulation with *M.tb* PPD led to the detection of a similar IFN- $\gamma$  response as BCG (median 0.74%), with comparatively lower frequencies of PPD-specific IL-22<sup>+</sup> CD4<sup>+</sup> T cells (median 0.21%;  $p=0.02$ ) and IL-17<sup>+</sup> cells (median 0%;  $p<0.0001$ ) (**Figure 1B**). The ESAT-6/CFP-10 response was dominated by IFN- $\gamma$  (median 0.07%), with low to undetectable IL-17 and IL-22 responses (medians of 0%). Taken together, these data demonstrate that different mycobacterial antigen preparations result in detection of different CD4<sup>+</sup> T cell cytokine profiles. Of note, IL-22 made a substantial contribution to the anti-mycobacterial CD4<sup>+</sup> response, with responses equivalent to or greater than the IFN- $\gamma$  response to BCG.

## **Most CD4<sup>+</sup> T cells producing IL-22 do not make IFN- $\gamma$ and IL-17**

We next focused on the high magnitude IL-22 response detected to BCG, to further characterize IL-22 CD4<sup>+</sup> responses and their relationship with IFN- $\gamma$  and IL-17. There was a highly significant positive correlation between IFN- $\gamma$  and IL-22 responses to BCG ( $p<0.0001$ ,  $r=0.830$ ; **Figure 2A**). The frequency of IL-17<sup>+</sup> CD4<sup>+</sup> T cells also correlated with both IFN- $\gamma$  and IL-22 production ( $p=0.039$ ,  $r=0.424$  and  $p=0.005$ ,  $r=0.559$ , respectively; data not shown). Given these associations between IFN- $\gamma$ , IL-22 and IL-17, we examined the co-expression patterns of the cytokines following BCG stimulation (**Figure 2B**). The majority of BCG-responding CD4<sup>+</sup> T cells produced only IL-22 (median 47%; IQR: 36.6-59.6), whilst CD4<sup>+</sup> cells secreting IFN- $\gamma$ -alone made up a median of 37% (IQR: 27.1-47.4). There was minimal

co-expression of IL-22 with both IL-17 (median 0.5%) and with IFN- $\gamma$  (median 6.4%). When examining all CD4<sup>+</sup> T cells producing IL-22, a median of 78% produced IL-22 alone (IQR: 71.1-89.2%), while 14% and 1.5% co-expressed IFN- $\gamma$  or IL-17, respectively (data not shown). We also investigated IL-22 production in combination with other cytokines and found low or negligible co-expression with TNF- $\alpha$ , IL-2 and IL-21 (medians 0.3%, 0.5% and 3%, respectively, data not shown). Our data reveal that the large proportion of BCG-specific IL-22 was produced predominantly by CD4<sup>+</sup> T cells secreting IL-22 in the absence of either IL-17 or IFN- $\gamma$ , consistent with being a distinct 'Th22' lineage (28–30).

### Phenotypic characteristics of mycobacteria-specific IL-22-producing CD4<sup>+</sup> T cells

In order to characterize the Th22 subset in more detail, we determined the memory differentiation profile of mycobacteria-specific Th22 cells (*i.e.* those producing IL-22 alone) compared to cells producing only IFN- $\gamma$  or IL-17. **Figure 3A** shows representative flow cytometry plots of CD45RO and CD27 expression on total CD4<sup>+</sup> cells with overlays of BCG-specific cytokine-producing CD4<sup>+</sup> T cells (IFN- $\gamma$ , IL-22 or IL-17 alone). The memory profile of BCG-specific CD4<sup>+</sup> T cells was comparable, regardless of their cytokine secretion profile, with approximately 79% having an early differentiated phenotype (ED: CD45RO<sup>+</sup>CD27<sup>+</sup>, comprising central and transitional memory cells). Of the remaining cells, a median of ~17% were late differentiated (LD: CD45RO<sup>+</sup>CD27<sup>-</sup>, comprising effector memory and intermediate cells), with few terminally differentiated (TD: CD45RO<sup>-</sup>CD27<sup>-</sup>; ~0.3%) or naïve-like (CD45RO<sup>-</sup>CD27<sup>+</sup>; ~2 %) cells (**Figure 3B**). Thus, CD4<sup>+</sup> T cells producing IFN- $\gamma$ , IL-22 or IL-17 shared a similar memory differentiation phenotype.

To further characterize the phenotype of the different cytokine-producing subsets, we examined chemokine receptor expression profiles on CD4<sup>+</sup> cells producing IFN- $\gamma$ , IL-22 or IL-17. For these studies, we stimulated whole blood with *M.tb* whole cell lysate. To ensure

that we were measuring similar cytokine responses, we compared IFN- $\gamma$ , IL-22 and IL-17 induced by each antigen and found highly comparable frequencies of CD4<sup>+</sup> T cell responses in the same donors (**Supplemental Figure S1**). **Figure 4A** shows representative flow cytometry plots of *M.tb*-specific CD4<sup>+</sup> T cell production of IFN- $\gamma$ , IL-22 and IL-17 overlaid onto chemokine receptor expression profiles (CXCR3, CCR6, CCR4 and CCR10). Whilst a majority of IFN- $\gamma$ -producing cells expressed CXCR3 (median 61.5%), a sizable fraction also expressed CCR6 (median 54.5%), with a low proportion expressing CCR4 (median 4.1%) and negligible CCR10 (median 0.5%; **Figure 4B**). In contrast, CD4<sup>+</sup> cells producing IL-22 were almost all CCR6 positive (median 94.7%), and compared to cells producing IFN- $\gamma$ , significantly fewer expressed CXCR3 (median 27.7%), and significantly more expressed CCR4 and CCR10 (median 23.3% and 2.7%, respectively). Th17 cells (IL-17<sup>+</sup>) shared comparable expression profiles for CCR6 and CXCR3 (medians 96.1% and 17.2%, respectively), but a higher proportion expressed CCR4 (median 50.8%) and CCR10 (median 5.8%) compared to Th22 cells. Of note, cells co-producing IFN- $\gamma$  and IL-22 had a similarly high expression of CCR6 as Th22 and Th17 cells, but were otherwise intermediate between IFN- $\gamma$ <sup>+</sup> and Th22 for the remaining chemokine receptors (**Supplemental Figure S2A**). These findings demonstrate distinct patterns of chemokine receptor expression on different cytokine-producing subsets. These data are consistent with previous descriptions (28, 30), but also highlight the substantial overlap in chemokine receptor expression between T helper subsets producing distinct cytokines.

We also investigated the homing potential of *M.tb*-specific CD4<sup>+</sup> Th subsets using KLRG1 and CD26. Killer cell lectin-like receptor G1 (KLRG1)-expressing cells appear to be retained within lung blood vasculature, while KLRG1<sup>-</sup> cells migrate to the lung parenchyma (40). Dipeptidyl peptidase IV (CD26) is involved in enzymatic chemokine modification that enhances T cell migration (41, 42). Expression of these markers was significantly different

between Th subsets (**Supplementary Figure S2B**). Th22 cells were characterized by a near absence of KLRG1 expression compared to Th1 and Th17 cells. In contrast, 50% of Th22 cells expressed CD26, compared to a median of 34% of Th1 cells and 11% of Th17 cells (**Supplemental Figure S2B**). These data suggest that *M.tb*-specific Th22 are endowed with a distinct homing potential compare to Th1 and Th17 cells.

### **The effect of HIV infection on the Th22 response to mycobacteria**

Th1 responses to *M.tb* are impaired or reduced during HIV infection (3). However, little is known about the effect of HIV co-infection on the Th22 response to *M.tb*. Hence, we examined IFN- $\gamma$ , IL-22 and IL-17 responses to BCG and PPD in 25 HIV-infected individuals with a median CD4 count of 619 cells/mm<sup>3</sup> (IQR: 532.5-782) and a median plasma viral load of 6.38 x10<sup>3</sup> copies/ml (IQR: 3.55-16.45 x10<sup>3</sup>; **Table 1**). Consistent with previous reports, the frequency of *M.tb*-specific CD4<sup>+</sup> T cells producing IFN- $\gamma$  was significantly lower in HIV-infected participants compared to uninfected participants in response to BCG (p=0.0004, medians 0.12% and 0.55%, respectively; **Figure 5A**). Notably, the IL-22 response to BCG was also lower in HIV-infected individuals, to a similar degree as the IFN- $\gamma$  response (p=0.0005; medians 0.28% and 0.91%, respectively). Additionally, IL-17 responses were also significantly lower in HIV-infected individuals in response to BCG compared to the HIV-uninfected group (p<0.0001, medians 0% and 0.11%, respectively). After adjusting for CD4 count, these differences became even more evident (**Figure 5B**), despite the relatively well-preserved CD4<sup>+</sup> T cell numbers in our HIV-infected cohort. HIV-infected participants had 8-fold (p<0.0001) and 3-fold (p=0.0003) fewer CD4<sup>+</sup> T cells producing IFN- $\gamma$  or IL-22, respectively, compared to uninfected participants. There were also fewer cells producing IL-17 in HIV-infected individuals (median 0; p<0.0001). Similar results were obtained for IFN- $\gamma$  and IL-22 in response to PPD (**Supplemental Figure S3A and B**). Overall, HIV-infected

participants had lower *M.tb*-specific IFN- $\gamma$ , IL-22 and IL-17 responses. Whilst the decrease in *M.tb*-specific IFN- $\gamma$  and IL-17 responses during HIV infection has been reported, we report here a striking loss of *M.tb*-specific CD4<sup>+</sup> T cells producing IL-22.

To further investigate the impact of HIV on BCG-specific Th22 responses, we measured the amount of IL-22 produced per cell, using median fluorescent intensity (MFI). The MFI of IL-22 was significantly lower in HIV-infected individuals compared to uninfected individuals ( $p < 0.0001$ ; medians 4169 and 6215, respectively; **Figure 5C**), whereas no differences in the MFI of IFN- $\gamma$  and IL-17 was observed. This suggests that HIV may have a unique effect on Th22 cells in response to BCG. However, we found no differences in the MFI of any cytokines produced in response to PPD (**Supplemental Figure S3C**).

To investigate whether the lower cytokine responses to mycobacterial antigens in HIV-infected individuals related to clinical parameters, the association between IFN- $\gamma$ , IL-22 and IL-17 responses and CD4 count or plasma viral load was examined. We observed a significant positive correlation between both the IFN- $\gamma$  and IL-22 response to BCG and CD4 count ( $p = 0.04$ ,  $r = 0.43$ ; and  $p = 0.004$ ,  $r = 0.57$ , respectively; **Figure 6A**). Likewise, in response to PPD, IFN- $\gamma$  ( $p = 0.03$ ,  $r = 0.45$ ) and IL-22 ( $p = 0.004$ ,  $r = 0.57$ ) correlated directly with CD4 count (data not shown). This suggests that the decrease in these responses could be a consequence of overall CD4<sup>+</sup> T cell depletion, despite the relatively narrow CD4 count range and modest CD4 decreases in our study (84% of participants had CD4 counts  $> 500$  cells/mm<sup>3</sup>). No association between the frequency of IL-17 and CD4 count was observed for either BCG (**Figure 6A**, bottom panel) or PPD (data not shown). Finally, there were no significant associations between plasma viral load and IFN- $\gamma$  or Th17 responses to BCG; **Figure 6B**) or any cytokine in response to PPD (data not shown). However, the frequency of

Th22 cells responding to BCG was significantly inversely correlated with plasma viral load (p=0.006, r=-0.54, **Figure 6B**, middle panel).

Overall, we demonstrate the detrimental effect of HIV infection on CD4<sup>+</sup> T helper subsets in response to mycobacteria. In particular, the Th22 subset exhibited both a decrease in the magnitude of the response to mycobacteria, and a defect in IL-22 production on a per cell basis. Furthermore, unlike the other cytokine-producing subsets examined (Th1 and Th17), the frequency of Th22 cells correlated inversely with HIV viral load, suggesting a direct relationship between HIV infection and the loss of Th22 cells specific for mycobacteria.

## DISCUSSION

Th1/IFN- $\gamma$  responses are needed for an effective response to TB (8), however a range of immune mechanisms beyond Th1 immunity may also contribute to protection from TB (6). Since HIV-infected individuals are considerably more susceptible to TB disease (3), key components required for effective immune control of *M.tb* are likely to be defective in these individuals, and we sought to identify these. In addition to IFN- $\gamma$ /Th1 immunity, this study examined IL-17 and IL-22 responses to mycobacteria in *M.tb*-sensitized, HIV-infected and uninfected individuals. Consistent with previous studies, we identified distinct populations of CD4<sup>+</sup> T cells expressing IFN- $\gamma$ , IL-17 or IL-22 in response to mycobacterial antigens (43, 44). The IL-22 response was unexpectedly abundant, contributing up to 50% of the mycobacterial response measured using these three cytokines, and the source was a distinct subset of CD4<sup>+</sup> T cells producing IL-22 alone. Importantly, IL-22 response was impaired in HIV-infected individuals in both magnitude and function, suggesting that depletion of this subset may contribute to TB risk.

IL-22 has classically been characterized as a Th17-related cytokine, since in mice it is co-secreted with IL-17 and has overlapping functions with IL-17 (27). However, IL-22 is a member of the IL-10 family (45), and in humans IL-22 is not co-expressed with IL-17 (28–30). Consequently, ‘Th22’ cells were proposed as a novel CD4<sup>+</sup> T helper cell lineage in humans, with shared but distinct features and functions compared to Th17 cells. To date, the role of IL-17 in *M.tb* immunity has been well-studied (21–24, 26). Here, we found that IL-17 responses made only a modest contribution to the total mycobacterial response in *M.tb*-exposed individuals, consistent with previous reports (20, 43). In contrast, we detected ample mycobacteria-specific IL-22 production from CD4<sup>+</sup> T cells in the absence of IL-17 (and IFN- $\gamma$ ), consistent with a distinct Th22 subset and in agreement with earlier observations in LTBI and TB disease (43, 46). Phenotypic profiling demonstrated that whilst their memory differentiation phenotype was similar to that of Th1 and Th17 cells, the bulk of Th22 cells expressed CCR6, with expression frequencies of CXCR3, CCR4 and CCR10 intermediate between Th1 and Th17 cells, somewhat consistent with published reports (28, 30). *M.tb*-specific Th22 cells were also characterized by higher CD26 and absent KLRG1 expression compared to both Th1 and Th17 cells. Altogether, these characteristics emphasize the shared and unique features of mycobacteria-specific Th22 cells relative to Th1 and Th17 cells, which may relate to distinct homing capabilities.

The previously unappreciated, sizeable contribution Th22 cells make to the mycobacterial response prompts the question of whether Th22 responses play a role in protective immunity against *M.tb*. Previous studies demonstrated that deficiency or neutralization of IL-22 in mice did not affect control of laboratory strains of *M.tb* (H37Rv and Erdman) (32–35). However, renewed interest in IL-22 has been garnered since the observation that IL-22 deficient mice infected with a hypervirulent clinical strain of *M.tb* (HN878) have an impaired ability to control the infection, resulting in both increased



bacterial burden and greater dissemination of infection (36). Additional evidence from a range of models suggest that IL-22 may indeed participate in TB immunity. IL-22 has been found at sites of TB disease; soluble IL-22 and IL-22 transcripts were elevated in the airways, lung tissue, granuloma, and in pleural and pericardial effusions during TB disease (43, 46–50). Along with IFN- $\gamma$ , IL-22 was one of the strongest genes upregulated in bovine TB (51), and gene expression signatures revealed that IFN- $\gamma$  and IL-22 were the dominant correlates of protection from bovine TB in blood in BCG-vaccinated cattle (52). Human genetic studies demonstrated the association between increased susceptibility to TB and a single nucleotide polymorphism in the IL-22 promoter that decreased IL-22 expression (50).

If IL-22 is involved in TB immunity, how might it mediate a protective function? IL-22 functions as a key regulator of tissue-specific antimicrobial immunity (31). The receptor for IL-22 is a heterodimer consisting of the IL-10R2 and the IL-22R, and expression is primarily restricted to non-hematopoietic cells, particularly epithelial cells in the skin, digestive tract and respiratory tract (31). IL-22 has been shown to be essential for mediating protective immunity to a range of extracellular and intracellular bacteria, such as *Klebsiella* and *Chlamydia* in the lung and *Citrobacter* in the intestine (53–56). Neutralization of IL-22 led to bacterial dissemination, exacerbated pathology, and lower Th1 and Th17 responses in the lung (55). The protective role at barrier sites appears to be mediated by three distinct functions, namely; maintenance of barrier integrity by promotion of epithelial homeostasis, stimulating epithelial proliferation and preventing apoptosis, as well as enhancing mucin production and tight junction formation; inducing antimicrobial peptides such as  $\beta$ -defensins; and regulating chemokine secretion from epithelial cells to co-ordinate recruitment of immune cells, such as neutrophils, to inflamed tissue (27, 29, 55, 57). Indeed, Treerat and colleagues demonstrated that the TB-protective role of IL-22 resulted from the secretion of S100 and Reg3 $\gamma$  from epithelial cells, and induction of CCL2 that mediated macrophage



recruitment to the infected lung (36). It is worth noting that several studies have independently documented IL-22R expression on *M.tb*-infected monocyte-derived macrophages (MDMs), as well as macrophages in TB granulomas in humans and non-human primates (36, 58, 59). Consistent with these findings, IL-22 from CD4<sup>+</sup> T cells and innate cells, as well as recombinant IL-22, reduced mycobacterial replication in MDMs by improving phagolysosome fusion (36, 58–60). These data suggest that a direct effector function for IL-22 in limiting mycobacterial growth cannot be ruled out.

HIV-infected individuals remain one of the most vulnerable populations at risk of TB (3). The early depletion of *M.tb*-specific Th1 responses, considered fundamental to TB immunity, has been reported during HIV infection (9, 10). Here, we investigated the relative effect of HIV on Th22 and Th17 responses to mycobacteria compared to Th1 responses. An important and novel finding from our study was that the mycobacteria-specific Th22 response was depleted during HIV infection, to a similar extent as Th1/IFN- $\gamma$  responses. Several studies have described a global and preferential loss of Th22 and Th17 cells during HIV/SIV infection, leading to mucosal gut damage and systemic immune activation, driving HIV disease progression (37, 61–64). The CCR6<sup>+</sup>CD4<sup>+</sup> T cell subset (within which all Th22 and Th17 cells reside) is more permissive to HIV infection and replication, and is enriched for HIV DNA (65–67). Elevated expression of HIV co-receptors CCR5 and CXCR4 has been reported on CCR6<sup>+</sup>CD4<sup>+</sup> T cells, which could facilitate HIV entry (68). In addition, post-entry mechanisms appear to create a more permissive cellular environment for HIV replication in CCR6-expressing cells, demonstrated by specific transcriptional signatures favoring HIV replication (69–71). We report here that higher HIV plasma viral load correlates with lower frequencies of Th22 cells specific for mycobacteria, consistent with a mechanism of direct, preferential infection of Th22 cells by HIV. Overall, multiple mechanisms may contribute to the loss of Th22, Th17 and Th1

subsets specific for *M.tb* (72, 73), and their combined depletion may contribute to TB risk during HIV infection.

Our new findings add to a growing body of evidence in support of a role for IL-22 in protective immunity to TB. However, a number of questions remain unanswered. Does IL-22 contribute to protective immunity to TB, or only during infection with specific clinical strains, or during HIV infection, when multiple immunological defects manifest? Does IL-22 assume a direct effector or indirect regulatory role in immunity to TB, or both? Does the inflammatory context dictate whether IL-22 might be beneficial to the host or pathological (74)? Ultimately, will it be necessary to induce Th22 responses for a TB vaccine to be effective? Notwithstanding these gaps in our knowledge, our study highlights the substantial contribution that Th22 cells make to mycobacterial immunity, and the importance of further elucidating the role of IL-22 in the control of *M.tb* infection and disease.

## ACKNOWLEDGEMENTS

We thank the study participants for providing samples and for their time and commitment to the study, and to the clinical staff at the Ubuntu HIV-TB clinic. We thank Dr Shaun Barnabas and Rene Goliath for phlebotomy. We thank Mrs Kathryn Norman for administrative assistance. We are grateful to BEI Resources, NIAID, NIH, for the following reagent: *Mycobacterium tuberculosis*, Strain H37Rv, Whole Cell Lysate, NR-14822.

## AUTHOR CONTRIBUTIONS

434   Conceived and designed the experiments: WAB, CR and RJW. Performed the experiments:  
435   RB, FMAO, MSM, TLM and CSLP. Analyzed the data: RB, FMAO, SMM and WAB. Wrote  
436   the paper: RB and WAB. All authors approved the final manuscript.

## REFERENCES

1. World Health Organization. 2018. *Global tuberculosis report 2018*.
2. El-Sadr, W. M., D. C. Perlman, E. Denning, J. P. Matts, and D. L. Cohn. 2001. A Review of Efficacy Studies of 6-Month Short-Course Therapy for Tuberculosis among Patients Infected with Human Immunodeficiency Virus: Differences in Study Outcomes. *Clin. Infect. Dis.* 32: 623–632.
3. Esmail, H., C. Riou, E. du Bruyn, R. P.-J. Lai, Y. X. R. Harley, G. Meintjes, K. A. Wilkinson, and R. J. Wilkinson. 2018. The Immune Response to *Mycobacterium tuberculosis* in HIV-1-Coinfected Persons. *Annu. Rev. Immunol.* 36: 1–36.
4. GBD. 2018. The global burden of tuberculosis: results from the Global Burden of Disease Study 2015. *Lancet Infect. Dis.* 18: 261–284.
5. Lawn, S. D., L. Myer, D. Edwards, L.-G. G. Bekker, and R. Wood. 2009. Short-term and long-term risk of tuberculosis associated with CD4 cell recovery during antiretroviral therapy in South Africa. *AIDS* 23: 1717–1725.
6. Sakai, S., K. D. Mayer-Barber, and D. L. Barber. 2014. Defining features of protective CD4 T cell responses to *Mycobacterium tuberculosis*. *Curr. Opin. Immunol.* 29: 137–142.
7. Van Der Meeren, O., M. Hatherill, V. Nduba, R. J. Wilkinson, M. Muyoyeta, E. Van Brakel, H. M. Ayles, G. Henostroza, F. Thienemann, T. J. Scriba, A. Diacon, G. L. Blatner, M.-A. Demoitié, M. Tameris, M. Malahleha, J. C. Innes, E. Hellström, N. Martinson, T. Singh, E. J. Akite, A. Khatoon Azam, A. Bollaerts, A. M. Ginsberg, T. G. Evans, P. Gillard, and D. R. Tait. 2018. Phase 2b Controlled Trial of M72/AS01 E Vaccine to Prevent Tuberculosis. *N. Engl. J. Med.* 379: 1621–1634.
8. O’Garra, A., P. S. Redford, F. W. McNab, C. I. Bloom, R. J. Wilkinson, and M. P. R. Berry. 2013. The immune response in tuberculosis. *Annu. Rev. Immunol.* 31: 475–527.
9. Geldmacher, C., A. Schuetz, N. Ngwenyama, J. P. Casazza, E. Sanga, E. Saathoff, C.

Boehme, S. Geis, L. Maboko, M. Singh, F. Minja, A. Meyerhans, R. A. Koup, and M. Hoelscher. 2008. Early depletion of *Mycobacterium tuberculosis*-specific T helper 1 cell responses after HIV-1 infection. *J. Infect. Dis.* 198: 1590–8.

10. Geldmacher, C., N. Ngwenyama, A. Schuetz, C. Petrovas, K. Reither, E. J. Heeregrave, J. P. Casazza, D. R. Ambrozak, M. Louder, W. Ampofo, G. Pollakis, B. Hill, E. Sanga, E. Saathoff, L. Maboko, M. Roederer, W. A. Paxton, M. Hoelscher, and R. A. Koup. 2010. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. *J. Exp. Med.* 207: 2869–81.

11. Day, C. L., D. A. Abrahams, L. D. Harris, M. van Rooyen, L. Stone, M. de Kock, and W. A. Hanekom. 2017. HIV-1 Infection Is Associated with Depletion and Functional Impairment of *Mycobacterium tuberculosis*-Specific CD4 T Cells in Individuals with Latent Tuberculosis Infection. *J. Immunol.* 199: 2069–2080.

12. Strickland, N., T. L. Müller, N. Berkowitz, R. Goliath, M. N. Carrington, R. J. Wilkinson, W. A. Burgers, and C. Riou. 2017. Characterization of *Mycobacterium tuberculosis*-Specific Cells Using MHC Class II Tetramers Reveals Phenotypic Differences Related to HIV Infection and Tuberculosis Disease. *J. Immunol.* 199: 2440–2450.

13. Kalsdorf, B., T. Scriba, K. Wood, C. L. Day, K. Dheda, R. Dawson, W. A. Hanekom, C. Lange, and R. J. Wilkinson. 2009. HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria. *Am. J. Respir. Crit. Care Med.* 180: 1262–1270.

14. Jambo, K. C., E. Sepako, D. G. Fullerton, D. Mzinza, S. Glennie, A. K. Wright, R. S. Heyderman, and S. B. Gordon. 2011. Bronchoalveolar CD4+ T cell responses to respiratory antigens are impaired in HIV-infected adults. *Thorax* 66: 375–82.

15. Bunjun, R., C. Riou, A. P. Soares, N. Thawer, T. L. Müller, A. Kiravu, Z. Ginbot, T. Oni, R. Goliath, B. Kalsdorf, F. von Groote-Bidlingmaier, W. Hanekom, G. Walzl, R. J. Wilkinson, and W. A. Burgers. 2017. Effect of HIV on the Frequency and Number of

487 *Mycobacterium tuberculosis*–Specific CD4<sup>+</sup> T Cells in Blood and Airways During Latent *M.*  
488 *tuberculosis* Infection. *J. Infect. Dis.* 216: 1550–1560.

489 16. Gallegos, A. M., J. W. J. van Heijst, M. Samstein, X. Su, E. G. Pamer, and M. S.  
490 Glickman. 2011. A gamma interferon independent mechanism of CD4 T cell mediated  
491 control of *M. tuberculosis* infection in vivo. *PLoS Pathog.* 7: e1002052.

492 17. Riou, C., N. Strickland, A. P. Soares, B. Corleis, D. S. Kwon, E. J. Wherry, R. J.  
493 Wilkinson, and W. A. Burgers. 2016. HIV Skews the Lineage-Defining Transcriptional  
494 Profile of *Mycobacterium tuberculosis*–Specific CD4<sup>+</sup> T Cells. *J. Immunol.* 196: 3006–  
495 3018.

496 18. Shen, H., and Z. W. Chen. 2018. The crucial roles of Th17-related cytokines/signal  
497 pathways in *M. tuberculosis* infection. *Cell. Mol. Immunol.* 15: 216–225.

498 19. Scriba, T. J., A. Penn-Nicholson, S. Shankar, T. Hraha, E. G. Thompson, D. Sterling, E.  
499 Nemes, F. Darboe, S. Suliman, L. M. Amon, H. Mahomed, M. Erasmus, W. Whatney, J. L.  
500 Johnson, W. H. Boom, M. Hatherill, J. Valvo, M. A. De Groote, U. A. Ochsner, A. Aderem,  
501 W. A. Hanekom, D. E. Zak, and other members of the ACS cohort study team. 2017.  
502 Sequential inflammatory processes define human progression from *M. tuberculosis* infection  
503 to tuberculosis disease. *PLoS Pathog.* 13: e1006687.

504 20. Murray, L. W., I. Satti, J. Meyerowitz, M. Jones, C. B. Willberg, J. E. Ussher, D.  
505 Goedhals, J. Hurst, R. E. Phillips, H. McShane, C. van Vuuren, J. Frater, C. van Vuuren, and  
506 J. Frater. 2018. Human Immunodeficiency Virus Infection Impairs Th1 and Th17  
507 *Mycobacterium tuberculosis*–Specific T-Cell Responses. *J. Infect. Dis.* 217: 1782–1792.

508 21. Gopal, R., L. Monin, S. Slight, U. Uche, E. Blanchard, B. A. Fallert Junecko, R. Ramos-  
509 Payan, C. L. Stallings, T. A. Reinhart, J. K. Kolls, D. Kaushal, U. Nagarajan, J. Rangel-  
510 Moreno, and S. A. Khader. 2014. Unexpected role for IL-17 in protective immunity against  
511 hypervirulent *Mycobacterium tuberculosis* HN878 infection. *PLoS Pathog.* 10: e1004099.

- 512 22. Gopal, R., Y. Lin, N. Obermajer, S. Slight, N. Nuthalapati, M. Ahmed, P. Kalinski, and S.  
513 A. Khader. 2012. IL-23-dependent IL-17 drives Th1-cell responses following *Mycobacterium*  
514 *bovis* BCG vaccination. *Eur. J. Immunol.* 42: 364–373.
- 515 23. Gopal, R., J. Rangel-Moreno, S. Slight, Y. Lin, H. F. Nawar, B. A. Fallert Junecko, T. A.  
516 Reinhart, J. Kolls, T. D. Randall, T. D. Connell, and S. A. Khader. 2013. Interleukin-17-  
517 dependent CXCL13 mediates mucosal vaccine-induced immunity against tuberculosis.  
518 *Mucosal Immunol.* 6: 972–984.
- 519 24. Khader, S. A., G. K. Bell, J. E. Pearl, J. J. Fountain, J. Rangel-Moreno, G. E. Cilley, F.  
520 Shen, S. M. Eaton, S. L. Gaffen, S. L. Swain, R. M. Locksley, L. Haynes, T. D. Randall, and  
521 A. M. Cooper. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T  
522 cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat.*  
523 *Immunol.* 8: 369–377.
- 524 25. Okamoto Yoshida, Y., M. Umemura, A. Yahagi, R. L. O'Brien, K. Ikuta, K. Kishihara,  
525 H. Hara, S. Nakae, Y. Iwakura, and G. Matsuzaki. 2010. Essential role of IL-17A in the  
526 formation of a mycobacterial infection-induced granuloma in the lung. *J. Immunol.* 184:  
527 4414–22.
- 528 26. Domingo-Gonzalez, R., S. Das, K. L. Griffiths, M. Ahmed, M. Bambouskova, R. Gopal,  
529 S. Gondi, M. Muñoz-Torrico, M. A. Salazar-Lezama, A. Cruz-Lagunas, L. Jiménez-Álvarez,  
530 G. Ramirez-Martinez, R. Espinosa-Soto, T. Sultana, J. Lyons-Weiler, T. A. Reinhart, J.  
531 Arcos, M. de la Luz Garcia-Hernandez, M. A. Mastrangelo, N. Al-Hammadi, R. Townsend,  
532 J.-M. Balada-Llasat, J. B. Torrelles, G. Kaplan, W. Horne, J. K. Kolls, M. N. Artyomov, J.  
533 Rangel-Moreno, J. Zúñiga, and S. A. Khader. 2017. Interleukin-17 limits hypoxia-inducible  
534 factor 1 $\alpha$  and development of hypoxic granulomas during tuberculosis. *JCI insight* 2: 1–20.
- 535 27. Liang, S. C., X.-Y. Tan, D. P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M.  
536 Collins, and L. A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells

537 and cooperatively enhance expression of antimicrobial peptides. *J. Exp. Med.* 203: 2271–9.

538 28. Duhon, T., R. Geiger, D. Jarrossay, A. Lanzavecchia, and F. Sallusto. 2009. Production of  
539 interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat.*  
540 *Immunol.* 10: 857–63.

541 29. Eyerich, S., K. Eyerich, D. Pennino, T. Carbone, F. Nasorri, S. Pallotta, F. Cianfarani, T.  
542 Odorisio, C. Traidl-Hoffmann, H. Behrendt, S. R. Durham, C. B. Schmidt-Weber, and A.  
543 Cavani. 2009. Th22 cells represent a distinct human T cell subset involved in epidermal  
544 immunity and remodeling. *J. Clin. Invest.* 119: 3573–85.

545 30. Trifari, S., C. D. Kaplan, E. H. Tran, N. K. Crellin, and H. Spits. 2009. Identification of a  
546 human helper T cell population that has abundant production of interleukin 22 and is distinct  
547 from T(H)-17, T(H)1 and T(H)2 cells. *Nat. Immunol.* 10: 864–71.

548 31. Sonnenberg, G. F., L. A. Fouser, and D. Artis. 2011. Border patrol: regulation of  
549 immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat. Immunol.*  
550 12: 383–90.

551 32. Wilson, M. S., C. G. Feng, D. L. Barber, F. Yarovinsky, A. W. Cheever, A. Sher, M.  
552 Grigg, M. Collins, L. Fouser, and T. A. Wynn. 2010. Redundant and pathogenic roles for IL-  
553 22 in mycobacterial, protozoan, and helminth infections. *J. Immunol.* 184: 4378–90.

554 33. Khader, S. A., L. Guglani, J. Rangel-moreno, R. Gopal, a Beth, F. Junecko, J. J.  
555 Fountain, C. Martino, J. E. Pearl, M. M. Tighe, Y. Lin, S. Slight, J. K. Kolls, T. A. Reinhart,  
556 T. D. Randall, A. M. Cooper, and B. A. F. Junecko. 2011. IL-23 is required for long-term  
557 control of *Mycobacterium tuberculosis* and B cell follicle formation in the infected lung. *J.*  
558 *Immunol.* 187: 5402–7.

559 34. Behrends, J., J. Renauld, S. Ehlers, and C. Hölscher. 2013. IL-22 is mainly produced by  
560 IFN $\gamma$ -secreting cells but is dispensable for host protection against *Mycobacterium*  
561 *tuberculosis* infection. *PLoS One* 8: e57379.



35. Segueni, N., E. Tritto, M. L. Bourigault, S. Rose, F. Erard, M. Le Bert, M. Jacobs, F. Di Padova, D. P. Stiehl, P. Moulin, D. Brees, S. D. Chibout, B. Ryffel, M. Kammüller, and V. F. Quesniaux. 2016. Controlled *Mycobacterium tuberculosis* infection in mice under treatment with anti-IL-17A or IL-17F antibodies, in contrast to TNF $\alpha$  neutralization. *Sci. Rep.* 6: 1–17.
36. Treerat, P., O. Prince, A. Cruz-Lagunas, M. Muñoz-Torrico, M. A. Salazar-Lezama, M. Selman, B. Fallert-Junecko, T. A. Reinhardt, J. F. Alcorn, D. Kaushal, J. Zuñiga, J. Rangel-Moreno, J. K. Kolls, and S. A. Khader. 2017. Novel role for IL-22 in protection during chronic *Mycobacterium tuberculosis* HN878 infection. *Mucosal Immunol.* 10: 1069–1081.
37. Kim, C. J., A. Nazli, O. L. Rojas, D. Chege, Z. Alidina, S. Huibner, S. Mujib, E. Benko, C. Kovacs, L. Y. Y. Shin, A. Grin, G. Kandel, M. Loutfy, M. Ostrowski, J. L. Gommerman, C. Kaushic, and R. Kaul. 2012. A role for mucosal IL-22 production and Th22 cells in HIV-associated mucosal immunopathogenesis. *Mucosal Immunol.* 5: 670–80.
38. Hanekom, W. A., J. Hughes, M. Mavinkurve, M. Mendillo, M. Watkins, H. Gamielien, S. J. Gelderbloem, M. Sidibana, N. Mansoor, V. Davids, R. A. Murray, A. Hawkrige, P. A. J. Haslett, S. Ress, G. D. Hussey, and G. Kaplan. 2004. Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency in field studies. *J. Immunol. Methods* 291: 185–95.
39. Roederer, M., J. L. Nozzi, and M. C. Nason. 2011. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. *Cytometry. A* 79: 167–74.
40. Sakai, S., K. D. Kauffman, J. M. Schenkel, C. C. McBerry, K. D. Mayer-Barber, D. Masopust, and D. L. Barber. 2014. Cutting Edge: Control of *Mycobacterium tuberculosis* Infection by a Subset of Lung Parenchyma-Homing CD4 T Cells. *J. Immunol.* 192: 2965–9.
41. Iwata, S., N. Yamaguchi, Y. Munakata, H. Ikushima, J. F. Lee, O. Hosono, S. F. Schlossman, and C. Morimoto. 1999. CD26/dipeptidyl peptidase IV differentially regulates the chemotaxis of T cells and monocytes toward RANTES: Possible mechanism for the

switch from innate to acquired immune response. *Int. Immunol.* 11: 417–426.

42. Ikushima, H., Y. Munakata, S. Iwata, K. Ohnuma, S. Kobayashi, N. H. Dang, and C. Morimoto. 2002. Soluble CD26/dipeptidyl peptidase IV enhances transendothelial migration via its interaction with mannose 6-phosphate/insulin-like growth factor II receptor. *Cell. Immunol.* 215: 106–110.

43. Scriba, T. J., B. Kalsdorf, D.-A. Abrahams, F. Isaacs, J. Hofmeister, G. Black, H. Y. Hassan, R. J. Wilkinson, G. Walzl, S. J. Gelderbloem, H. Mahomed, G. D. Hussey, and W. A. Hanekom. 2008. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J. Immunol.* 180: 1962–70.

44. Ye, Z. J., Q. Zhou, M. L. Yuan, R. H. Du, W. B. Yang, X. Z. Xiong, B. Huang, and H. Z. Shi. 2012. Differentiation and recruitment of IL-22-producing helper T cells stimulated by pleural mesothelial cells in tuberculous pleurisy. *Am. J. Respir. Crit. Care Med.* 185: 660–669.

45. Dumoutier, L., J. Louahed, and J. C. Renauld. 2000. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. *J. Immunol.* 164: 1814–1819.

46. Qiu, Y., Y. Huang, J. Chen, D. Qiao, G. Zeng, and J. Cai. 2013. Depletion of IL-22 during culture enhanced antigen-driven IFN- $\gamma$  production by CD4(+)T cells from patients with active TB. *Immunol. Lett.* 150: 48–53.

47. Matthews, K., K. A. Wilkinson, B. Kalsdorf, T. Roberts, A. Diacon, G. Walzl, J. Wolske, M. Ntsekhe, F. Syed, J. Russell, B. M. Mayosi, R. Dawson, K. Dheda, R. J. Wilkinson, W. A. Hanekom, and T. J. Scriba. 2011. Predominance of interleukin-22 over interleukin-17 at the site of disease in human tuberculosis. *Tuberculosis* 91: 587–93.

48. Semple, P. L., A. B. Binder, M. Davids, A. Maredza, R. N. van Zyl-Smit, and K. Dheda. 2013. Regulatory T cells attenuate mycobacterial stasis in alveolar and blood-derived

macrophages from patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 187: 1249–58.

49. Yao, S., D. Huang, C. Y. Chen, L. Halliday, G. Zeng, R. C. Wang, and Z. W. Chen. 2010. Differentiation, distribution and gammadelta T cell-driven regulation of IL-22-producing T cells in tuberculosis. *PLoS Pathog.* 6: e1000789.

50. Zhang, G., X. Chen, L. Chan, M. Zhang, B. Zhu, L. Wang, X. Zhu, J. Zhang, B. Zhou, and J. Wang. 2011. An SNP selection strategy identified IL-22 associating with susceptibility to tuberculosis in Chinese. *Sci. Rep.* 1: 1–20.

51. Aranday-Cortes, E., P. J. Hogarth, D. A. Kaveh, A. O. Whelan, B. Villarreal-Ramos, A. Lalvani, and H. M. Vordermeier. 2012. Transcriptional profiling of disease-induced host responses in bovine tuberculosis and the identification of potential diagnostic biomarkers. *PLoS One* 7: e30626.

52. Bhujra, S., E. Aranday-Cortes, B. Villarreal-Ramos, Z. Xing, M. Singh, and H. M. Vordermeier. 2012. Global Gene Transcriptome Analysis in Vaccinated Cattle Revealed a Dominant Role of IL-22 for Protection against Bovine Tuberculosis. *PLoS Pathog.* 8: 8–15.

53. Basu, R., D. B. O’Quinn, D. J. Silberger, T. R. Schoeb, L. Fouser, W. Ouyang, R. D. Hatton, and C. T. Weaver. 2012. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. *Immunity* 37: 1061–75.

54. Zheng, Y., P. A. Valdez, D. M. Danilenko, Y. Hu, S. M. Sa, Q. Gong, A. R. Abbas, Z. Modrusan, N. Ghilardi, F. J. de Sauvage, and W. Ouyang. 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat. Med.* 14: 282–289.

55. Aujla, S. J., Y. R. Chan, M. Zheng, M. Fei, D. J. Askew, D. A. Pociask, T. A. Reinhart, F. McAllister, J. Edeal, K. Gaus, S. Husain, J. L. Kreindler, P. J. Dubin, J. M. Pilewski, M. M. Myerburg, C. A. Mason, Y. Iwakura, and J. K. Kolls. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat. Med.* 14: 275–81.

56. Peng, Y., and X. Gao. 2014. Interleukin-22 Promotes T Helper 1 (Th1)/Th17 Immunity in

637 Chlamydial Lung Infection. *Mol. Med.* 20: 109–119.

638 57. Aujla, S. J., and J. K. Kolls. 2009. IL-22: a critical mediator in mucosal host defense. *J.*

639 *Mol. Med.* 87: 451–4.

640 58. Dhiman, R., M. Indramohan, P. F. Barnes, R. C. Nayak, P. Paidipally, L. V. M. Rao, and

641 R. Vankayalapati. 2009. IL-22 produced by human NK cells inhibits growth of

642 *Mycobacterium tuberculosis* by enhancing phagolysosomal fusion. *J. Immunol.* 183: 6639–

643 45.

644 59. Zeng, G., C. Y. Chen, D. Huang, S. Yao, R. C. Wang, and Z. W. Chen. 2011. Membrane-

645 bound IL-22 after de novo production in tuberculosis and anti-*Mycobacterium tuberculosis*

646 effector function of IL-22+ CD4+ T cells. *J. Immunol.* 187: 190–9.

647 60. Dhiman, R., S. Venkatasubramanian, P. Paidipally, P. F. Barnes, A. Tvinnereim, and R.

648 Vankayalapati. 2014. Interleukin 22 inhibits intracellular growth of *Mycobacterium*

649 *tuberculosis* by enhancing calgranulin A expression. *J. Infect. Dis.* 209: 578–87.

650 61. Brenchley, J. M., M. Paiardini, K. S. Knox, A. I. Asher, B. Cervasi, T. E. Asher, P.

651 Scheinberg, D. A. Price, C. A. Hage, L. M. Kholi, A. Khoruts, I. Frank, J. Else, T. Schacker,

652 G. Silvestri, and D. C. Douek. 2008. Differential Th17 CD4 T-cell depletion in pathogenic

653 and nonpathogenic lentiviral infections. *Blood* 112: 2826–35.

654 62. Klatt, N. R., and J. M. Brenchley. 2010. Th17 cell dynamics in HIV infection. *Curr.*

655 *Opin. HIV AIDS* 5: 135–40.

656 63. Page, E. E., L. Greathead, R. Metcalf, S.-A. Clark, M. Hart, D. Fuchs, P. Pantelidis, F.

657 Gotch, A. Pozniak, M. Nelson, A. Boasso, B. Gazzard, and P. Kelleher. 2014. Loss of Th22

658 Cells Is Associated With Increased Immune Activation and IDO-1 Activity in HIV-1

659 Infection. *J. Acquir. Immune Defic. Syndr.* 67: 227–35.

660 64. Ryan, E. S., L. Micci, R. Fromentin, S. Paganini, C. S. McGary, K. Easley, N. Chomont,

661 and M. Paiardini. 2016. Loss of Function of Intestinal IL-17 and IL-22 Producing Cells

662 Contributes to Inflammation and Viral Persistence in SIV-Infected Rhesus Macaques. *PLoS*  
663 *Pathog.* 12: 1–22.

664 65. Gosselin, A., P. Monteiro, N. Chomont, F. Diaz-Griffero, E. A. Said, S. Fonseca, V.  
665 Wacleche, M. El-Far, M.-R. Boulassel, J.-P. Routy, R.-P. Sekaly, and P. Ancuta. 2010.  
666 Peripheral Blood CCR4+CCR6+ and CXCR3+CCR6+ CD4+ T Cells Are Highly Permissive  
667 to HIV-1 Infection. *J. Immunol.* 184: 1604–1616.

668 66. Gosselin, A., T. R. Wiche Salinas, D. Planas, V. S. Wacleche, Y. Zhang, R. Fromentin,  
669 N. Chomont, É. A. Cohen, B. Shacklett, V. Mehraj, M. P. Ghali, J.-P. Routy, and P. Ancuta.  
670 2017. HIV persists in CCR6+CD4+ T-cells from colon and blood during antiretroviral  
671 therapy. *AIDS* 31: 35–38.

672 67. Monteiro, P., A. Gosselin, V. S. Wacleche, M. El-Far, E. A. Said, H. Kared, N.  
673 Grandvaux, M.-R. Boulassel, J.-P. Routy, and P. Ancuta. 2011. Memory CCR6+CD4+ T  
674 Cells Are Preferential Targets for Productive HIV Type 1 Infection Regardless of Their  
675 Expression of Integrin  $\beta 7$ . *J. Immunol.* 186: 4618–4630.

676 68. Alvarez, Y., M. Tuen, G. Shen, F. Nawaz, J. Arthos, M. J. Wolff, M. A. Poles, and C. E.  
677 Hioe. 2013. Preferential HIV Infection of CCR6+ Th17 Cells Is Associated with Higher  
678 Levels of Virus Receptor Expression and Lack of CCR5 Ligands. *J. Virol.* 87: 10843–10854.

679 69. Bernier, A., A. Cleret-Buhot, Y. Zhang, J. P. Goulet, P. Monteiro, A. Gosselin, S.  
680 DaFonseca, V. S. Wacleche, M. A. Jenabian, J. P. Routy, C. Tremblay, and P. Ancuta. 2013.  
681 Transcriptional profiling reveals molecular signatures associated with HIV permissiveness in  
682 Th1Th17 cells and identifies peroxisome proliferator-activated receptor gamma as an  
683 intrinsic negative regulator of viral replication. *Retrovirology* 10: 160.

684 70. Cleret-Buhot, A., Y. Zhang, D. Planas, J. P. Goulet, P. Monteiro, A. Gosselin, V. S.  
685 Wacleche, C. L. Tremblay, M. A. Jenabian, J. P. Routy, M. El-Far, N. Chomont, E. K.  
686 Haddad, R. P. Sekaly, and P. Ancuta. 2015. Identification of novel HIV-1 dependency factors

in primary CCR4 + CCR6 + Th17 cells via a genome-wide transcriptional approach.

*Retrovirology* 12: 1–23.

71. Planas, D., Y. Zhang, P. Monteiro, J. Goulet, A. Gosselin, N. Grandvaux, T. J. Hope, A.

Fassati, J. Routy, and P. Ancuta. 2017. HIV-1 selectively targets gut-homing CCR6+CD4+ T

cells via mTOR-dependent mechanisms. *JCI insight* 2: 1–21.

72. Favre, D., J. Mold, P. W. Hunt, B. Kanwar, P. Loke, L. Seu, J. D. Barbour, M. M. Lowe,

A. Jayawardene, F. Aweeka, Y. Huang, D. C. Douek, J. M. Brenchley, J. N. Martin, F. M.

Hecht, S. G. Deeks, and J. M. McCune. 2010. Tryptophan catabolism by indoleamine 2,3-

dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Sci. Transl.*

*Med.* 2: 32ra36.

73. Klatt, N. R., J. D. Estes, X. Sun, A. M. Ortiz, J. S. Barber, L. D. Harris, B. Cervasi, L. K.

Yokomizo, L. Pan, C. L. Vinton, B. Tabb, L. A. Canary, Q. Dang, V. M. Hirsch, G. Alter, Y.

Belkaid, J. D. Lifson, G. Silvestri, J. D. Milner, M. Paiardini, E. K. Haddad, and J. M.

Brenchley. 2012. Loss of mucosal CD103+ DCs and IL-17+ and IL-22+ lymphocytes is

associated with mucosal damage in SIV infection. *Mucosal Immunol.* 5: 646–657.

74. Sonnenberg, G. F., M. G. Nair, T. J. Kirn, C. Zaph, L. A. Fouser, and D. Artis. 2010.

Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-

17A. *J. Exp. Med.* 207: 1293–1305.

706 **Table**

707 Table 1: Characteristics of study participants

HIV-uninfected (n=25)		HIV-infected (n=25)		
PID	CD4 count (cells/mm <sup>3</sup> )	PID	CD4 count (cells/mm <sup>3</sup> )	Viral Load (RNA copies/ml)
1032	ND	1086	1449	4250
1035	1459	1151	988	1848
1052	1412	1075	965	5922
1031	1169	1150	894	311
1023	1120	1006	802	4141
1070	1028	1039	790	4521
1024	939	1080	774	14100
1025	915	1152	749	<40
1057	871	1154	714	2954
1058	866	1018	681	4614
1054	832	1143	656	618
1094	827	1073	632	12274
1028	814	1084	619	9192
1033	813	1134	599	6383
1011	801	1079	591	32485
1095	760	1153	571	9697
1038	743	1137	560	18797
1072	741	1141	552	9826
1047	680	1076	543	908
1061	674	1045	522	59125
1015	659	1129	510	4559
1049	655	1074	478	10093
1001	631	1142	441	544849
1066	621	1126	433	32994
1010	580	1020	406	31145
<b>Median</b>	813		619	6383
<b>IQR</b>	675.5-933		532.5-782	3548-16449

708

709

710

## FIGURE LEGENDS

### Figure 1: CD4+ T cell cytokine responses to mycobacterial antigens in latent TB

**infection. (A)** Representative flow cytometry plots of the production of IFN- $\gamma$ , IL-22 and IL-17 from CD4+ T cells after stimulation with *M. bovis* BCG, *M.tb* PPD and ESAT-6/CFP-10 peptides, in one study participant. UNS corresponds to the unstimulated control. The frequency of cytokine-producing cells is shown as a percentage of the total CD4+ T cell population, after gating on live, CD3+ lymphocytes. **(B)** Individual IFN- $\gamma$  (blue), IL-22 (red) or IL-17 (green) responses to BCG, PPD or ESAT-6/CFP-10 (n=25). The frequency of cytokine-producing cells is shown as a percentage of the total CD4+ T cell population, after gating on live, CD3+ lymphocytes. Each dot represents one individual. Data are shown as box and whisker (interquartile range) plots and horizontal bars represent the median. Statistical comparisons were performed using a Kruskal-Wallis and Dunn's multiple comparison test. \*p $\leq$ 0.05, \*\*p $\leq$ 0.01, \*\*\*p $\leq$ 0.001, \*\*\*\*p $\leq$ 0.0001

### Figure 2: The relationship between IL-22, and other cytokines produced in response to

**BCG. (A)** The relationship between the frequency of CD4+ T cells producing IFN- $\gamma$  and IL-22 in response to BCG (n=24). Each dot represents an individual. Statistical analyses were performed using a non-parametric Spearman rank correlation. **(B)** Populations of CD4+ T cells producing different combinations of IFN- $\gamma$ , IL-22 and IL-17 in response to BCG. The pie charts indicate the proportion of cytokine combinations that makes up the BCG response. Each slice of the pie represents a specific subset of cells, defined by a combination of cytokines shown by the color at the bottom of the graphs. Data are shown as box and whisker (interquartile range) plots and horizontal bars represent the median.



**Figure 3: Memory profiles of CD4+ T cells producing IFN- $\gamma$ , IL-22 or IL-17 in**

**response to BCG. (A)** Representative flow cytometry plots of total CD4+ memory subset distribution in one individual based on CD45RO and CD27 staining. Naïve: CD45RO-CD27+, early differentiated (ED: CD45RO+CD27+), late differentiated (LD: CD45RO+CD27-) and terminally differentiated (TD: CD45RO-CD27-). The overlays indicate the antigen specific CD4+ T cells producing IFN- $\gamma$  (blue), IL-22 (red) or IL-17 (green). The frequencies of each subset are indicated. **(B)** The memory distribution of cells producing IFN- $\gamma$  (blue), IL-22 (red) or IL-17 (green) in response to BCG (n=20, 25 and 7, respectively). Only individuals with a positive cytokine response and more than 30 cytokine events were included in the phenotyping. Each dot represents one individual. Data are shown as box and whisker (interquartile range) plots and horizontal bars represent the median. Statistical comparisons were performed using a Kruskal-Wallis and Dunn's multiple comparison test.

**Figure 4: Chemokine receptor expression of CD4+ T cells producing IFN- $\gamma$ , IL-22 or**

**IL-17 in response to *M.tb* whole cell lysate. (A)** Representative flow cytometry plots of the expression of CCR6, CCR4, CXCR3 and CCR10 on total CD4+ T cells in one individual. The overlays indicate the antigen specific CD4+ T cells producing IFN- $\gamma$  (blue), IL-22 (red) or IL-17 (green). The frequencies of each subset are indicated. **(B)** The chemokine receptor distribution of cells producing IFN- $\gamma$  (blue), IL-22 (red) or IL-17 (green) in response to *M.tb* lysate (n=19, 19 and 11, respectively). Only individuals with a positive cytokine response and more than 30 cytokine events were included in the phenotyping. Each dot represents one individual. Data are shown as box and whisker (interquartile range) plots and horizontal bars represent the median. Statistical comparisons were performed using a Kruskal-Wallis and Dunn's multiple comparison test. \*p $\leq$ 0.05, \*\*p $\leq$ 0.01, \*\*\*p $\leq$ 0.001, \*\*\*\*p $\leq$ 0.0001

761

762 **Figure 5: CD4+ T cell responses to BCG in HIV-infected and uninfected individuals. (A)**

763 The individual IFN- $\gamma$ , IL-22 or IL-17 responses in HIV uninfected or infected individuals in  
764 response to BCG (n=24 in each group). **(B)** The cytokine frequency adjusted for CD4 count  
765 in HIV-infected and HIV-uninfected individuals in response to BCG. **(C)** The median  
766 fluorescent intensity (MFI) of IFN- $\gamma$  in response to BCG (n=24 and n=15 for HIV-uninfected  
767 and infected, respectively) The MFI of IL-22 in response to BCG (n=23 and n=20 for HIV-  
768 uninfected and infected, respectively) The MFI of IL-17 in response to BCG (n=22 and n=8  
769 for HIV-uninfected and infected, respectively). For each cytokine, MFI was only graphed for  
770 individuals with positive cytokine responses. HIV-uninfected participants are shown with  
771 open circles and HIV-uninfected individuals with closed circles. Each dot represents one  
772 individual. Data are shown as box and whisker (interquartile range) plots and horizontal bars  
773 represent the median. Statistical comparisons were performed using a non-parametric Mann  
774 Whitney test. \*p $\leq$ 0.05, \*\*p $\leq$ 0.01, \*\*\*p $\leq$ 0.001, \*\*\*\*p $\leq$ 0.0001

775

776 **Figure 6: The relationship between clinical parameters and IFN- $\gamma$ , IL-22 or IL-17 CD4+**

777 **T cell responses to BCG in HIV-infected individuals.** The association between IFN- $\gamma$   
778 (blue), IL-22 (red) or IL-17 (green) responses to BCG and **(A)** CD4 count or **(B)** viral load.  
779 Each dot represents an individual (n=24). The dotted line indicates linear regression for  
780 statistically significant correlations, highlighted in bold. Statistical analyses were performed  
781 using a non-parametric Spearman rank correlation.

782

783 **Supplemental Figure S1: Cytokine responses to BCG and *M.tb* whole cell lysate.**

784 Comparison of the frequencies of CD4+ T cells producing IFN- $\gamma$ , IL-17 and IL-22 in  
785 response to BCG (circles) and *M.tb* whole cell lysate (triangles) in healthy donors (n=8). The

frequency of cytokine-producing cells is shown as a percentage of the total CD4<sup>+</sup> T cell population, after gating on live, CD3<sup>+</sup> lymphocytes. Statistical comparisons were performed using a non-parametric matched pairs Wilcoxon test.

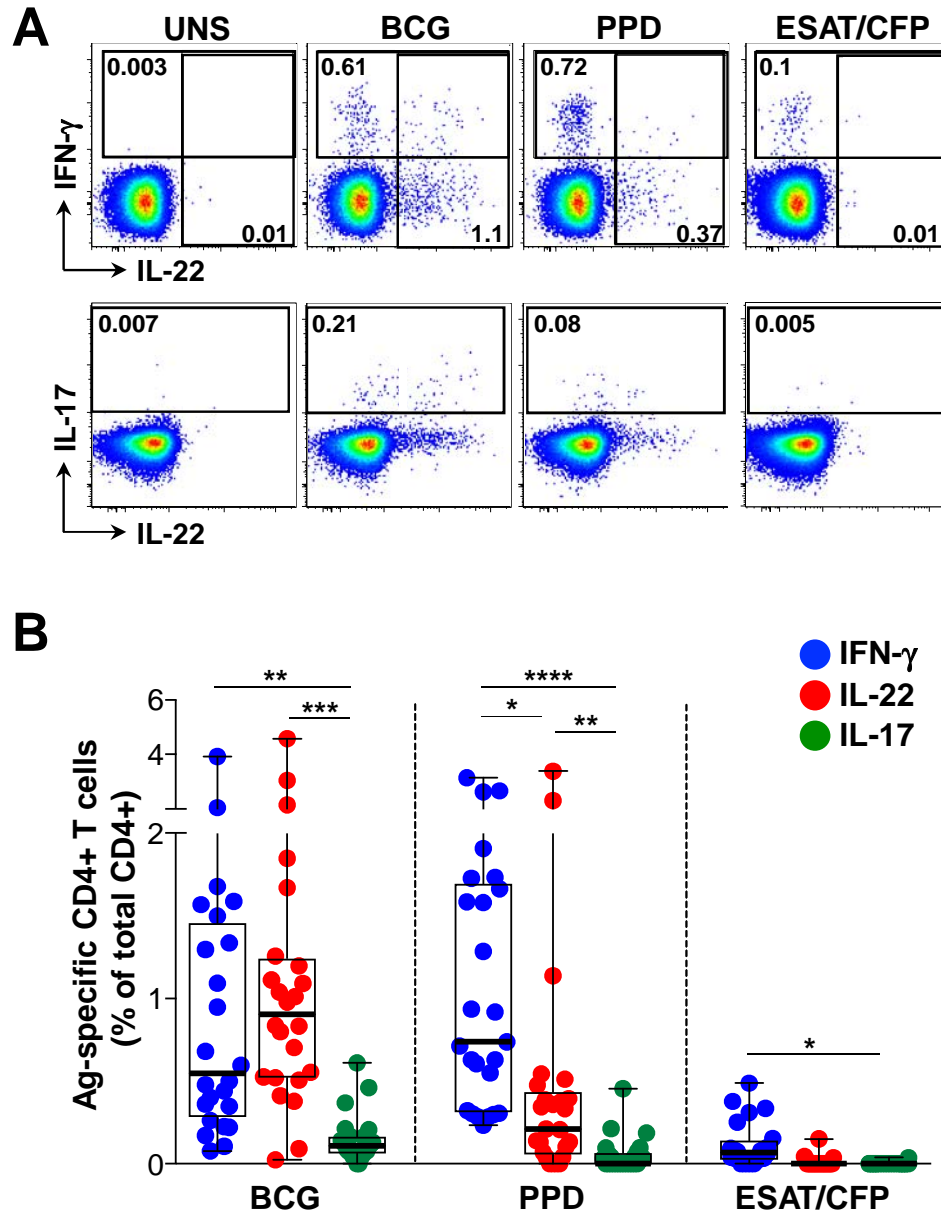
**Supplemental Figure S2: Phenotypic profiles of Th subsets.** (A) The chemokine receptor distribution of cells co-producing IFN- $\gamma$  and IL-22 (n=14) in response to *M.tb* lysate. Horizontal bars represent the median. (B) KLRG1 and CD26 expression on *M.tb*-specific Th1, Th22 and Th17 cells. Representative overlay plots showing KLRG1 and CD26 expression on total CD4<sup>+</sup> T cells (grey), IFN- $\gamma$ <sup>+</sup> (blue), IL-22<sup>+</sup> (red) and IL-17<sup>+</sup> (green) cells in response to *M.tb* lysate (top panel). Expression of KLRG1 and CD26 shown as box and whisker (interquartile range) plots (bottom panel) and horizontal bars represent the median (n=19). Statistical comparisons were performed using a Kruskal-Wallis and Dunn's multiple comparison test. Only individuals with a positive cytokine response and more than 30 cytokine events were included in the phenotyping. Each dot represents one individual.

**Supplemental Figure S3: CD4<sup>+</sup> T cell responses to PPD in HIV-infected and uninfected individuals.** (A) The individual IFN- $\gamma$ , IL-22 or IL-17 responses in HIV uninfected (n=25) or infected individuals (n=24) in response to PPD. (B) The cytokine frequency adjusted for CD4 count in HIV-infected and HIV-uninfected individuals in response to PPD. (C) The median fluorescent intensity (MFI) of IFN- $\gamma$  in response to PPD (n=25 and n=24 for HIV-uninfected and infected, respectively). The MFI of IL-22 in response to PPD (n=21 and n=18 for HIV-uninfected and infected, respectively). The MFI of IL-17 in response to PPD (n=12 and n=12 for HIV-uninfected and infected, respectively). For each cytokine, MFI was only plotted for individuals with positive cytokine response. HIV-uninfected participants are shown with open circles and HIV-uninfected individuals with closed circles. Each dot represents one

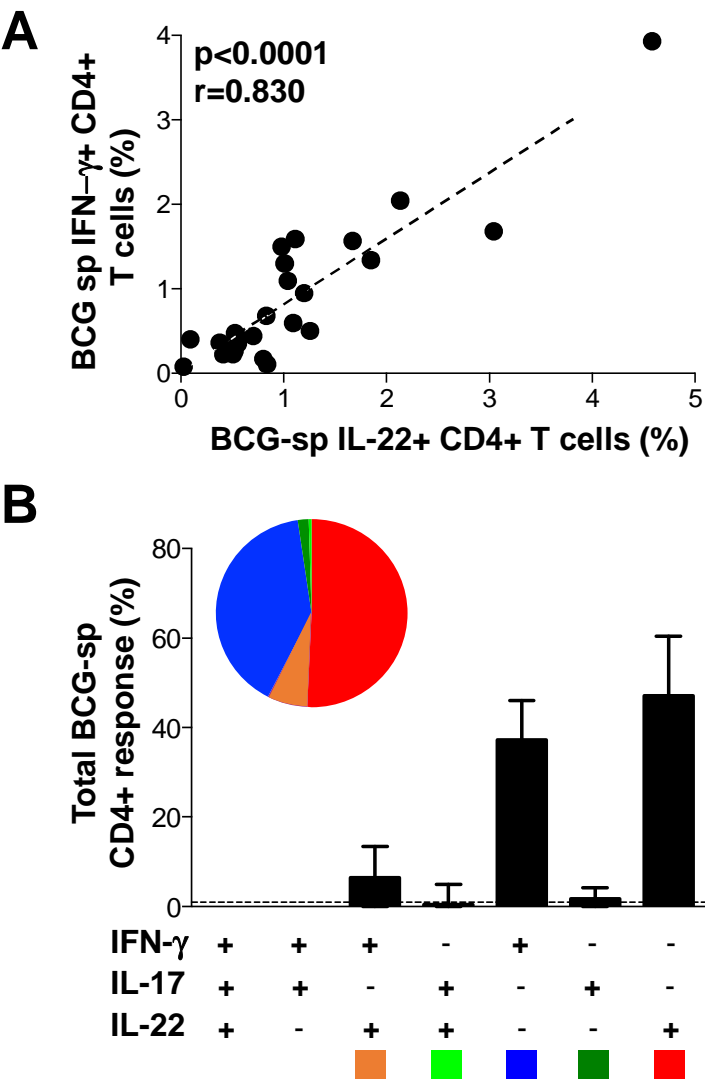
811 individual. Data are shown as box and whisker (interquartile range) plots and horizontal bars  
812 represent the median. Statistical comparisons were performed using a non-parametric Mann  
813 Whitney test.

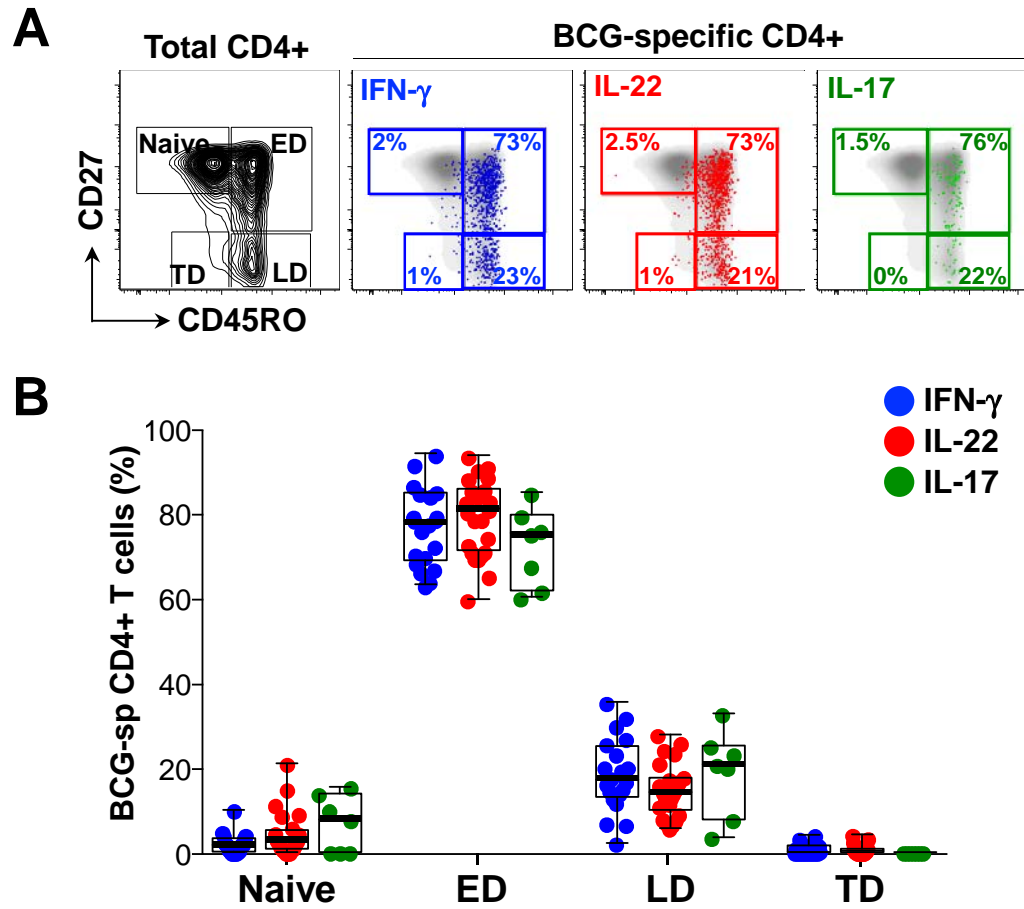
# FIGURE 1

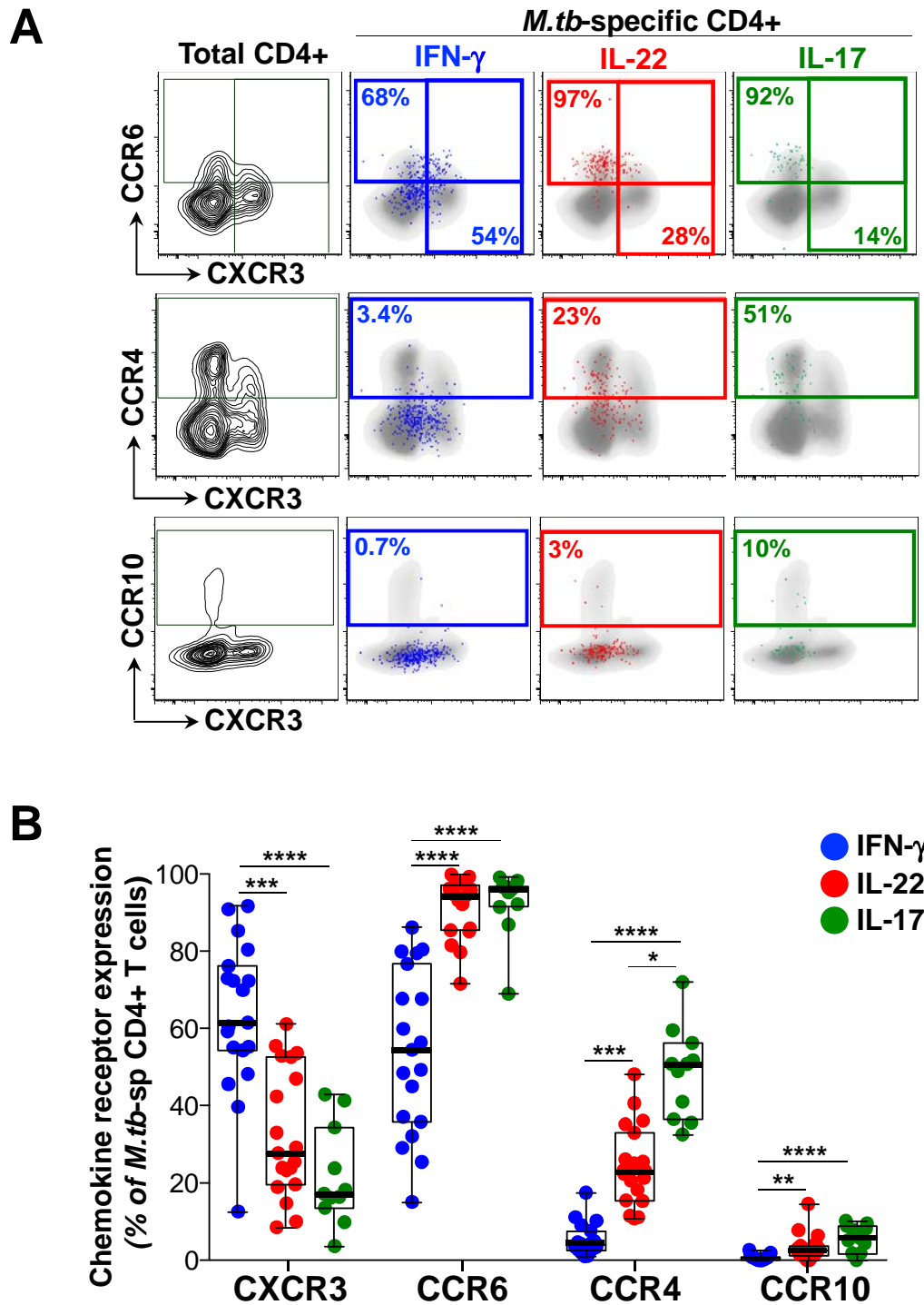
Bunjun *et al.*



**FIGURE 2**  
**Bunjun et al.**

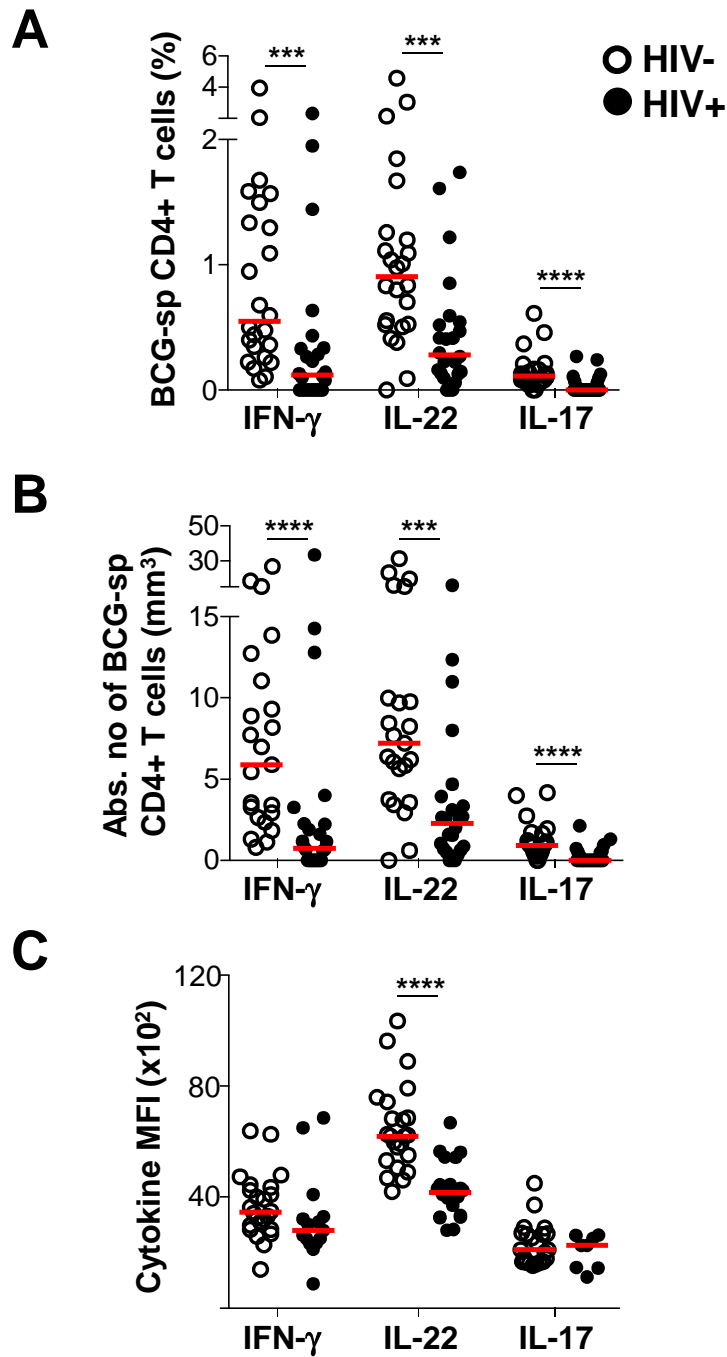








# FIGURE 5 Bunjun et al.



# FIGURE 6 Bunjun et al.

