Activation-Induced Marker Expression Identifies *Mycobacterium tuberculosis*–Specific CD4 T Cells in a Cytokine-Independent Manner in HIV-Infected Individuals with Latent Tuberculosis

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Abstract

HIV infection is a significant risk factor for reactivation of latent *Mycobacterium tuberculosis* infection (LTBI) and progression to active tuberculosis disease, yet the mechanisms whereby HIV impairs T cell immunity to *M. tuberculosis* have not been fully defined. Evaluation of *M. tuberculosis*–specific CD4 T cells is commonly based on IFN-γ production, yet increasing evidence indicates the immune response to *M. tuberculosis* is heterogeneous and encompasses IFN-γ–independent responses. We hypothesized that upregulation of surface activation-induced markers (AIM) would facilitate detection of human *M. tuberculosis*–specific CD4 T cells in a cytokine-independent manner in HIV-infected and HIV-uninfected individuals with LTBI. PBMCs from HIV-infected and HIV-uninfected adults in Kenya were stimulated with CFP-10 and ESAT-6 peptides and evaluated by flow cytometry for upregulation of the activation markers CD25, OX40, CD69, and CD40L. Although *M. tuberculosis*–specific IFN-γ and IL-2 production was dampened in HIV-infected individuals, *M. tuberculosis*–specific CD25$^+$OX40$^+$ and CD69$^+$CD40L$^+$ CD4 T cells were detectable in the AIM assay in both HIV-uninfected and HIV-infected individuals with LTBI. Importantly, the frequency of *M. tuberculosis*–specific AIM$^+$ CD4 T cells was not directly impacted by HIV viral load or CD4 count, thus demonstrating the feasibility of AIM assays for analysis of *M. tuberculosis*–specific CD4 T cells across a spectrum of HIV infection states. These

data indicate that AIM assays enable identification of *M. tuberculosis*–specific CD4 T cells in a cytokine-independent manner in HIV-uninfected and HIV-infected individuals with LTBI in a high-tuberculosis burden setting, thus facilitating studies to define novel T cell correlates of protection to *M. tuberculosis* and elucidate mechanisms of HIV-associated dysregulation of antimycobacterial immunity.

INTRODUCTION

Globally, one-fourth of the world is burdened by latent *Mycobacterium tuberculosis* infection (LTBI) and therefore at risk for developing active tuberculosis (TB) disease (1). Ten million new cases of TB disease and 1.5 million deaths were reported in 2018 (2). HIV infection greatly increases the risk of reactivation of LTBI and progression to TB disease (2, 3), although the mechanisms whereby HIV impairs immune control of *M. tuberculosis* infection have not been fully elucidated.

HIV infection results in CD4 T cell depletion, and increasing evidence indicates that *M. tuberculosis*–specific CD4 T cells are either preferentially depleted (4–7) and/or have impaired functional capacity in HIV-infected individuals. Decreased frequencies of *M. tuberculosis*–specific CD4 T cells producing Th1, Th2, and Th17 cytokines have been described in HIV-infected individuals compared with those not infected with HIV (4, 7–10). *M. tuberculosis*–specific CD4 T cells in HIV-infected individuals also have a heightened level of activation (4, 11) and profoundly impaired proliferative capacity (4, 12).

Currently available tools for detecting LTBI include tuberculin skin test and the IFN-γ release assays (IGRA) (13), which detect immune sensitization to *M. tuberculosis* Ags. IGRA positivity relies on detection of IFN-γ-producing T cells to CPF-10 and ESAT-6 peptides, yet increasing evidence highlights the importance of IFN-γ-independent immune responses contributing to the host immune response following *M. tuberculosis* exposure and infection (14). Novel approaches to identify Ag-specific T cells in a cytokine-independent manner have been developed, based on surface expression of activation-induced markers (AIM). AIM assays for coexpression of CD25/OX40 and CD69/CD40L have been described for detection for human CD4 T cells specific for several viral and bacterial Ags (15–22). In AIM assays, surface expression of activation markers on live cells is evaluated by flow cytometry, without the need for fixation and permeabilization of cells for intracellular cytokine staining, thus facilitating sorting of live Ag-specific CD4 T cells for downstream applications. $CD25^{+}OX40^{+}$ CD4 T cells to CFP-10/ESAT-6 peptides have been reported in a small study of $IGRA^{+}$ adults in the United States, which indicated positive CD25/OX40 AIM assay responses in each individual with LTBI tested (15). However, AIM assays have not been evaluated for detection of *M. tuberculosis*–specific CD4 T cell responses in $IGRA^{+}$ and $IGRA^{-}$ individuals in high-TB burden settings. Moreover, the effect of HIV coinfection on detection of *M. tuberculosis*–specific CD4 T cell responses has not been evaluated, nor has the relationship between HIV viral load and CD4 count on AIM assay detection of *M. tuberculosis*–specific CD4 T cells been determined.

To address these issues, we conducted AIM assays and evaluated cytokine production profiles in $IGRA^{-}$ and $IGRA^{+}$ Kenyan adults, with and without HIV infection. We found

evidence of dampened *M. tuberculosis*–specific IFN-γ and IL-2 production in HIV-infected individuals, although AIM[+] *M. tuberculosis*–specific CD4 T cells are detectable in both HIV-infected and HIV-uninfected Kenyan adults. The frequency of *M. tuberculosis*–specific AIM[+] CD4 T cells did not correlate directly with either HIV viral load or absolute CD4 count, suggesting that *M. tuberculosis*–specific AIM assay performance is not directly impacted by these parameters of HIV disease progression. The data presented in this study provide support for the use of AIM assays as a sensitive and specific tool to isolate *M. tuberculosis*–specific CD4 T cells in people living with HIV and facilitate downstream studies defining mechanisms of HIV-associated impairment of CD4 T cell immunity to TB.

MATERIALS AND METHODS

Study participants and sample collection

Blood samples were collected from individuals 18 y of age at the Kenya Medical Research Institute (KEMRI) Clinical Research Center in Kisumu, Kenya. Study participants included HIV-uninfected and HIV-infected adults with a normal chest x-ray, no symptoms of TB disease, and no history of diagnosis or treatment for active TB. *M. tuberculosis* infection status was evaluated by IGRA using a QuantiFERON-TB Gold test (QFT; QIAGEN). Individuals with a positive IGRA result (TB Ag-Nil 0.35 IU/ml) were defined as having LTBI. Individuals with a TB Ag-Nil response <0.35 IU/ml were defined as IGRA[−]. HIV testing was done using the Diagnostic Kit for HIV (1 + 2) Ab V2 (Shanghai Kehua Bio-Engineering). All HIV-infected participants were antiretroviral therapy–naive at the time of enrollment and had absolute CD4 T cell counts >200 cells/μl. Sputum samples were collected from all HIV-infected participants to exclude the possibility of subclinical TB; all sputum samples were negative for *M. tuberculosis* by smear microscopy, Xpert MTB/RIF, and liquid culture. All subjects provided written informed consent for participation in the study, which was approved by the KEMRI Scientific and Ethics Review Unit and the Emory University Institutional Review Board.

PBMC isolation and Ag stimulation

Blood was collected in sodium heparin Vacutainer CPT Mononuclear Cell Preparation Tubes (BD Biosciences). PBMC were isolated via density gradient centrifugation, cryopreserved, and stored in LN_2. PBMC were thawed as previously described (23), suspended in R10 media (RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine), and rested at 37°C for 4 h. Pure functional-grade anti-CD40 Ab (0.5 μg/ml; Miltenyi Biotec) was then added, and cells were stimulated with the following Ags: pooled, overlapping 15-mer peptides corresponding to the sequences of CFP-10 and ESAT-6 (1 μg/ml) and human CMV (HCMV) pp65 (1 μg/ml). *M. tuberculosis* H37Rv whole cell lysate was obtained from BEI Resources (National Institute of Allergy and Infectious Diseases, National Institutes of Health). HCMV pp65 peptide pool was obtained from the National Institutes of Health AIDS Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) (24–26). PBMC were stimulated with staphylococcal enterotoxin B (SEB; 1 μg/ml; Toxin Technology) as a positive control. PBMC incubated in R10 media with no Ag were used as a negative control. PMBC were incubated at 37°C for 16 h, after which supernatants were

harvested for cytokine quantification by Luminex (described below), and cells were analyzed by flow cytometry (described below).

Abs and AIM expression by flow cytometry

Stimulated cells were washed with PBS and stained with Zombie NIR Fixable Viability Dye (BioLegend). Cells were surface stained with CD3–Brilliant Violet 711 (UCHT1), CD4–Brilliant Violet 570 (RPA-T4), CD8-PerCP-Cy5.5 (SK1), CD25-PE-Cy7 (MA251), OX40 (CD134)-PE (ACT35), CD69-PE/Dazzle 594 (FN50), and CD40L (CD154) –Brilliant Violet 421 (24–31). All Abs were obtained from either BioLegend or BD Biosciences. Cells were washed and suspended in PBS with 2% paraformaldehyde.

Cytokine quantification

The following cytokines were measured in PBMC supernatants using a ProcartaPlex Immunoassay (Invitrogen) according to the manufacturer's instructions: IFN-γ, IL-2, IL-4, IL-10, IL-17AF, IL-21, IL-22, and TNF-α. Immunoassay plates were read using a Luminex MAGPIX system with xPONENT software (version 4.2) and analyzed using MILLIPLEX Analyst 5.1 software.

Flow cytometry and data analysis

Cells were acquired on a BD LSRII flow cytometer with BD FACSDiva software (v8.0) and analyzed using FlowJo software (v9.9.6). Compensation was calculated using single-stained anti-mouse Ig,κ beads (BD Biosciences). Doublet cell populations were excluded by plotting forward scatter area versus forward scatter height. Viable lymphocytes were defined as Zombie NIR-low cells. Combinations of cells expressing AIM markers were determined using Boolean gating in FlowJo.

Data analysis and statistics

Functionality scores of CD4 T cells expressing AIM markers were determined via the R package Combinatorial Polyfunctionality Analysis of Single Cells (COMPASS) (27). COMPASS incorporates the cell counts for each possible AIM subset in the Ag-stimulated and unstimulated conditions and uses a Markov chain Monte Carlo algorithm to generate a functionality score, which is defined as the proportion of Ag-specific AIM subsets expressed for a given subject among all possible AIM subsets (27). GraphPad Prism (version 8.1.2) and R programming software (28) were used to perform statistical analyses. Correlograms were created using the R package corrplot. Ag-specific AIM marker expression and cytokine levels are shown after subtraction of background levels in the negative control condition. The nonparametric Mann–Whitney U test was used to compare differences between groups. Correlations were evaluated using Spearman rank-order correlation, and p values <0.05 were considered to be statistically significant.

RESULTS

Study participants

Blood samples were collected from 75 adults enrolled in Kisumu, Kenya (Table I). There were no significant differences in CD4 T cell counts or HIV plasma viral loads between HIV-infected individuals in the IGRA⁻ and IGRA⁺ groups. The TB Ag IFN-γ response as measured in the QFT assay was lower in HIV-infected IGRA⁺ individuals compared with HIV-uninfected IGRA⁺ individuals (Fig. 1A, Table I), despite equivalent IFN-γ production between the two IGRA⁺ groups following stimulation with the mitogen PHA (Supplemental Fig. 1A).

AIM assay identifies M. tuberculosis–specific CD4 T cells in HIV-infected and HIV-uninfected individuals

Lower IFN-γ responses by QFT in HIV-infected individuals suggested possible deficiency in cytokine production by *M. tuberculosis*–specific CD4 T cells in HIV-infected individuals. To evaluate the frequency of *M. tuberculosis*–specific CD4 T cells in HIV-infected individuals in a cytokine-independent manner, we next evaluated whether upregulation of activation markers can be used to identify *M. tuberculosis*–specific CD4 T cells in both HIV-uninfected and HIV-infected individuals. PBMCs were stimulated for 16 h with *M. tuberculosis* Ags (CFP-10/ESAT-6 peptide pool and *M. tuberculosis* whole cell lysate), HCMV pp65 peptide pool, and SEB, followed by analysis of Ag-induced coexpression of CD25/OX40 and CD69/CD40L (Fig. 1B). In all four participant groups, stimulation with SEB induced robust expression of each of the activation markers (Supplemental Fig. 1B), thus suggesting that CD4 T cell AIM expression is not inherently impaired in the setting of either LTBI and/or HIV infection.

Consistent with IGRA responses, the frequencies of CD25⁺OX40⁺ and CD69⁺CD40L⁺ CD4 T cells to CFP-10/ESAT-6 were higher in IGRA⁺ individuals compared with IGRA⁻ individuals (Fig. 1C). Moreover, there were significant positive correlations between the magnitude of the IGRA response and the percentage of CFP-10/ESAT-6–induced CD25⁺OX40⁺ and CD69⁺CD40L⁺ CD4 T cells in both HIV-infected and HIV-uninfected individuals (Fig. 1D, Supplemental Fig. 2A). By contrast with IGRA responses, the frequencies of CFP-10/ESAT-6–specific AIM⁺ CD4 T cells were not significantly different between HIV-infected and HIV-uninfected IGRA⁺ individuals, thus suggesting that the AIM assay facilitates detection of *M. tuberculosis*–specific CD4 T cells that may otherwise be missed by evaluation of IFN-γ production alone. The frequencies of AIM⁺ CD4 T cells were similar across the four participant groups following stimulation with HCMV pp65 peptide pool (Supplemental Fig. 3A), thus indicating that the observed differences in CFP-10/ESAT-6–induced AIM expression in IGRA⁻ and IGRA⁺ individuals are reflective of *M. tuberculosis* infection status.

To evaluate the AIM assay to *M. tuberculosis* Ags beyond the IGRA Ags CFP-10 and ESAT-6, we measured AIM expression following stimulation with *M. tuberculosis* lysate. Consistent with the greater number of potential CD4 T cell epitopes in the lysate, frequencies of AIM⁺ CD4 T cells to *M. tuberculosis* lysate were higher than to CFP-10/

ESAT-6 peptide pool (Fig. 1B, 1E). However, the frequencies of *M. tuberculosis* lysate-induced AIM+ CD4 T cells were less differentiated among the four groups compared with CFP-10/ESAT-6 (Fig. 1C, 1E), likely reflecting detection of mycobacteria-specific CD4 T cell responses primed by bacille Calmette-Guérin (BCG) vaccination, which is routine in Kenya (29), and/or community level TB and nontuberculous mycobacteria exposure. There was a significant positive correlation between IGRA responses and the frequency of *M. tuberculosis* lysate-induced AIM+ CD4 T cells among all participants evaluated (Fig. 1F), although the correlation coefficients were lower than observed with CFP-10/ESAT-6. When HIV-infected and HIV-uninfected individuals were evaluated separately, correlations between IGRA and *M. tuberculosis* lysate-induced AIM+ CD4 T cells remained significant for HIV-uninfected individuals and not HIV-infected individuals (Supplemental Fig. 2B).

Frequencies of CFP-10/ESAT-6–specific AIM+ CD4 T cells do not correlate directly with HIV viral load or CD4 count

We next evaluated the relationship between HIV viral load and absolute CD4 count with the frequency of Ag-specific AIM+ CD4 T cells (Fig. 2). As anticipated, there was a strong inverse correlation between viral load and CD4 count. No significant correlations were found with either viral load or CD4 count and the frequency of CFP-10/ESAT-6–specific AIM+ CD4 T cells, although there was a positive correlation between absolute CD4 count and the frequency of *M. tuberculosis* lysate-induced CD69+CD40L+ CD4 T cells. By contrast with *M. tuberculosis*–specific responses, there was a significant inverse correlation between HIV viral load and the frequencies of AIM+ HCMV-specific CD4 T cells. Taken together, these data demonstrate that detection of AIM+ *M. tuberculosis*–specific CD4 T cells is feasible in HIV-infected individuals across a range of viral loads and CD4 counts, unlike HCMV-specific AIM+ CD4 T cells, which decline in frequency with increasing HIV viral load.

HIV-infected and HIV-uninfected individuals with LTBI have similar functionality scores in the AIM assay

To more comprehensively evaluate the impact of HIV coinfection on detection of *M. tuberculosis*–specific CD4 T cell responses in the AIM assay, we used COMPASS, a Bayesian hierarchical mixture model (27), to incorporate all combinations of Ag-induced CD25, OX40, CD69, and CD40L expression and generate an overall functionality score for each participant that summarizes CD4 T cell AIM expression profiles (Fig. 3). Consistent with the higher number of potential CD4 T cell epitopes in *M. tuberculosis* lysate than peptide pools, higher CD4 T cell AIM functionality scores were found in *M. tuberculosis* lysate-stimulated cells, compared with CFP-10/ESAT-6 and HCMV pp65 (Fig. 3, Supplemental Fig. 3B). There were no differences overall in CD4 T cell AIM functionality scores following Ag stimulation of PBMCs from HIV-uninfected versus HIV-infected IGRA+ individuals. Last, we conducted a principal component analysis of AIM expression profiles and found that HIV-uninfected and HIV-infected IGRA+ individuals could not be clearly differentiated by CD4 T cell AIM expression, regardless of Ag stimulation (Fig. 3, Supplemental Fig. 3B). Taken together, these data indicate that AIM assays for CD25/OX40 and CD69/CD40L coexpression detect *M. tuberculosis*–specific CD4 T cells in a cytokine-

independent manner and that coexpression of these activation markers is not significantly compromised in our HIV-infected cohorts.

HIV infection modifies M. tuberculosis–specific cytokine production profiles in individuals with LTBI

We next used the PBMC supernatants from the AIM assay to further probe the effect of HIV infection on *M. tuberculosis*-specific cytokine production profiles and evaluate the relationship between cytokine profiles and AIM+ CD4 T cells. Using a Luminex assay, we measured production of Th1 (IL-2, IFN-γ, and TNF-α), Th2 (IL-4), Th17 (IL-17AF, IL-21, and IL-22), and regulatory (IL-10) cytokines in AIM assay supernatants. IL-4 and IL-21 were detectable in each participant group following stimulation with SEB, although there were no differences in SEB-stimulated IL-4 or IL-21 production capacity in the four participant groups (data not shown). IL-4 and IL-21 were not detectable above background in any participant group following stimulation with *M. tuberculosis* Ags (data not shown).

IFN-γ was detected at the highest concentrations following stimulation with CFP-10/ESAT-6 in IGRA+ individuals compared with the other cytokines measured, although there was a trend for lower IFN-γ production in HIV-infected individuals (Fig. 4A). CFP-10/ESAT-6–specific IL-2 production was significantly reduced in HIV-infected IGRA+ individuals compared with HIV-uninfected individuals (Fig. 4A). There was no evidence of diminished IL-2 production capacity to HCMV pp65 or SEB in HIV-infected individuals (Fig. 4C, 4D), suggesting lower IL-2 production capacity is specific for *M. tuberculosis.*

When stimulated with *M. tuberculosis* lysate, PBMCs from HIV-infected IGRA+ individuals exhibited lower levels of IFN-γ, IL-17AF, and IL-22 compared with HIV-uninfected individuals (Fig. 4B). However, IFN-γ, IL-17AF, and IL-22 production following stimulation with SEB and HCMV pp65 was similar between HIV-uninfected and HIV-infected individuals (Fig. 4C, 4D), thus indicating that production of these cytokines is not inherently impaired in HIV-infected IGRA+ individuals, but may be diminished specifically in their immune response to *M. tuberculosis.* By contrast, TNF-a production to *M. tuberculosis* lysate was significantly higher in HIV-infected individuals than HIV-uninfected individuals (Fig. 4B), thus indicative of an enhanced proinflammatory response to *M. tuberculosis* in HIV-infected individuals.

HIV infection alters the relationship between M. tuberculosis–specific CD4 T cell AIM expression and cytokine production in individuals with LTBI

To further evaluate how HIV infection may influence the relationship between the frequency of *M. tuberculosis*–specific AIM+ CD4 T cells and cytokine production profiles in individuals with LTBI, we evaluated the correlations between CFP-10/ESAT-6–specific CD4 T cell AIM expression and cytokine levels (Fig. 5, Supplemental Fig. 4). We focused this analysis on CFP-10/ESAT-6–specific CD4 T cells because CFP-10/ESAT-6 peptides have been shown to stimulate predominately CD4 T cells in individuals with LTBI (30, 31), whereas *M. tuberculosis* lysate will stimulate cytokine production from a broader range of lymphocytes within PBMCs. As anticipated, there was a significant positive correlation

between the frequency of CD25$^+$OX40$^+$ CD4 T cells and CD69$^+$CD40L$^+$ T cells in HIV-infected and HIV-uninfected individuals (Fig. 5).

In both HIV-uninfected and HIV-infected IGRA$^+$ individuals, the frequency of CFP-10/ESAT-6–specific AIM$^+$ CD4 T cells correlated positively with Th1 cytokine production (IFN-γ, TNF-α, and IL-2). In HIV-uninfected individuals, CFP-10/ESAT-6–specific AIM$^+$ CD4 T cells also correlated positively with IL-22 production (Fig. 5A, Supplemental Fig. 4). However, CFP-10/ESAT-6–specific AIM$^+$ CD4 T cells from HIV-infected individuals displayed no correlation with IL-22 and instead correlated strongly with IL-10 production (Fig. 5B, Supplemental Fig. 4). No relationship between CFP-10/ESAT-6–specific AIM$^+$ CD4 T cells and IL-17AF production was found in either group. Taken together, these data suggest that *M. tuberculosis*–specific AIM$^+$ CD4 T cells correlate with Th1 cytokine production and also reveal differential associations between CFP-10/ESAT-6–specific AIM$^+$ CD4 T cells and IL-22 and IL-10 production according to HIV infection status.

DISCUSSION

Although *M. tuberculosis* infection is commonly detected by IGRAs, there is mounting evidence that T cell responses to *M. tuberculosis* are highly heterogenous and include important roles for IFN-γ–independent T cells (14, 32, 33). We used a cytokine-independent AIM assay to identify *M. tuberculosis*–specific CD4 T cell responses in both HIV-uninfected and HIV-infected individuals with LTBI in a high-TB burden setting. We demonstrated that although quantitative IFN-γ production by IGRA is significantly diminished in HIV-infected compared with HIV-uninfected individuals, the frequency of *M. tuberculosis*–specific AIM$^+$ CD4 T cells is not significantly different between these two groups. Importantly, the frequency of *M. tuberculosis*–specific AIM$^+$ CD4 T cells is not directly associated with either HIV viral load or absolute CD4 count, thus demonstrating feasibility for use of the AIM assay in isolating *M. tuberculosis*–specific CD4 T cells from people living with HIV across a spectrum of disease states.

Conventional methods for the detection of Ag-specific CD4 T cells, such as intracellular cytokine staining, ELISA, and ELISPOT, rely on cytokine production for the detection of Ag-specific cells. More recently, live-cell, cytokine-independent methods have been developed to detect Ag-specific CD4 T cells via surface coexpression of activation markers, including CD25, OX40, CD69, and CD40L (15,17, 34, 35). AIM assays for CFP-10/ESAT-6–induced CD25/OX40 expression have been reported in a small number of HIV-uninfected, non–BCG-vaccinated IGRA$^+$ adults in the United States (15); we extended these studies to a TB-endemic setting in Kenya and determined that AIM assays can be used to detect CFP-10/ESAT-6–specific CD4 T cells at similar frequencies in HIV-uninfected and HIV-infected Kenyan adults. Whereas AIM assays with CFP-10/ESAT-6 peptide pool clearly differentiated IGRA$^-$ and IGRA$^+$ individuals, AIM assays with *M. tuberculosis* lysate enable detection of a broader mycobacteria-specific CD4 T cell response in our cohorts in Kenya, a high-TB burden setting in which infant BCG vaccination is routine (29), and there is high exposure to nontuberculous mycobacteria (36). Despite lower quantitative *M. tuberculosis*–specific IFN-γ responses in HIV-infected individuals as measured by QFT, we did not find significant differences in the frequency of *M. tuberculosis- specific* AIM+ CD4

T cells by HIV infection status, although there is a trend toward a lower frequency of *M. tuberculosis*–specific AIM[+] CD4 T cells in HIV-infected individuals. These data provide additional evidence that AIM assays facilitate detection of *M. tuberculosis*–specific CD4 T cells encompassing more comprehensive functions than would be detected by IFN-γ production alone.

Important considerations in analysis of Ag-specific CD4 T cells in HIV-infected individuals include the potential for high viral load and/or low CD4 count to modify T cell phenotypes and dampen effector functions. Although the frequency of HCMV-specific AIM[+] CD4 T cells correlated inversely with HIV viral load, we did not find any direct relationship between the frequency of *M. tuberculosis*–specific AIM[+] CD4 T cells and either HIV viral load or CD4 count. These data indicate that AIM assays facilitate robust identification of *M. tuberculosis*–specific CD4 T cells in HIV-infected individuals across a spectrum of HIV disease states. However, it is important to acknowledge that the HIV-infected participants in our cohort all had CD4 counts >200 cells/μl. It is possible that performance of *M. tuberculosis*–specific AIM assays will be more compromised in HIV-infected individuals with more severe immunosuppression.

A notable advantage of the AIM assay over traditional intracellular cytokine staining assays is the lack of a requirement for Golgi transport inhibitors during the Ag stimulation period, thus enabling analysis of soluble molecules secreted by Ag-specific cells as well as isolation of live cells for other downstream applications such as single-cell RNA sequencing. We initially leveraged this aspect of the AIM assay to evaluate soluble cytokine production by Luminex in the AIM assay supernatants. Our findings of reduced CFP-10/ESAT-6–specific IL-2 production in HIV-infected IGRA[+] individuals are consistent with previous reports of decreased *M. tuberculosis*–specific IL-2 production capacity in HIV-infected individuals (8, 9, 37, 38). All of the individuals in our study were evaluated prior to initiation of antiretroviral therapy, provision of which is associated with a decline in the incidence of TB disease (39). Future longitudinal studies of *M. tuberculosis*–specific CD4 T cells in HIV-infected individuals are warranted to determine if antiretroviral therapy restores IL-2 production capacity and to determine if loss of IL-2 production predicts progression to active TB disease.

By analyzing the frequency of AIM[+] CD4 T cells with cytokine production profiles, we determined that CFP-10/ESAT-6–specific AIM[+] CD4 T cells correlated positively with the Th1 cytokines IFN-γ,TNF-α, and IL-2 inboth HIV-infected and HIV-uninfected individuals. Consistent with previous reports of low frequencies of CFP-10/ESAT–specific IL-17^{+} CD4 T cells in LTBI (40, 41), IL-17 was produced at low-to-undetectable levels by Luminex following stimulation with CFP-10/ESAT-6 peptides in both HIV-uninfected and HIV-infected individuals (Fig. 4). We found no significant correlations between CFP-10/ESAT-6–induced IL-17 production and the frequency of CD4 T cells expressing any combination of one or more activation markers (Fig. 5 and data not shown).

Although the frequency of CFP-10/ESAT-6–specific AIM[+] CD4 T cells correlated positively with IL-22 production in HIV-uninfected individuals, this relationship was absent in HIV-infected individuals. To determine if CFP-10/ESAT-6–specific IL-22 production correlated

with AIM subsets other than CD25+OX40+ and CD69+CD40L+ cells in HIV-infected individuals, we correlated CFP-10/ESAT-6–specific IL-22 production with all possible subsets expressing one or more activation marker in HIV-infected individuals and found no significant correlations (data not shown).

Although we did not observe a significant difference in the concentration of IL-22 in CFP-10/ESAT-6–stimulated PBMCs between the two groups, IL-22 production was significantly reduced in HIV-infected individuals following stimulation with *M. tuberculosis* lysate compared with HIV-uninfected individuals, consistent with previous reports of dampened *M. tuberculosis*–specific IL-22 production in HIV-infected individuals (42). Increasing evidence from animal models and human studies indicates an important role of IL-22 in the immune response to *M. tuberculosis* (43). IL-22, a member of the IL-10 family of cytokines (44), was identified as a surrogate of protection against bovine TB in BCG-vaccinated cattle (45) and as a mediator of protective immunity in mice infected with *M. tuberculosis* HN878 (46). In humans, patients with active pulmonary TB disease have decreased frequencies of mycobacteria-specific IL-22+ CD4 T cells compared with healthy adults (41). In a Chinese cohort study, a single nuclear polymorphism in the IL-22 promoter region was associated with TB susceptibility (47).

Whereas a positive correlation was found between CFP-10/ESAT-6–specific IL-22 production and AIM+ CD4 T cells in HIV-uninfected individuals only, a positive correlation between CFP-10/ESAT-6–specific IL-10 production and AIM+ CD4 T cells was found in HIV-infected individuals only. Elevated plasma levels of IL-10 have been described in HIV-infected individuals (48), and in vitro blockade of IL-10R signaling enhances *M. tuberculosis*–specific T cell function in HIV+ individuals with LTBI (49). IL-10–deficient mice infected with *M. tuberculosis* have decreased bacterial loads in the lung (50,51), suggesting IL-10 may have a role in modulating immune control of *M. tuberculosis*. Future studies are warranted to sort *M. tuberculosis*–specific AIM+ CD4 T cells from HIV-infected and HIV-uninfected individuals for transcriptional and epigenetic profiling to define pathways, such as cytokines in the IL-10 family, that may be dysregulated in HIV-infected individuals and identify potential targets that are amenable to therapeutic intervention to enhance *M. tuberculosis*–specific CD4 T cell function in people living with HIV who are at highest risk for developing active TB disease.

This study included evaluation of *M. tuberculosis*–specific AIM assays with PBMCs from asymptomatic IGRA− and IGRA+ individuals. Currently available tests for LTBI include IGRAs and the TST, both of which have the limitation of relying on detection of cellular immune responses to *M. tuberculosis* and do not detect the presence of the bacteria itself. Although we used IGRA positivity as an indication of *M. tuberculosis* infection (52), it is possible that some individuals who are IGRA− are infected with *M. tuberculosis* and either do not have CFP-10/ESAT-6–specific T cells in peripheral blood or have low frequencies of these cells that are below the threshold for IGRA positivity. We also did not evaluate AIM assays in individuals with active TB disease; thus, it is possible that identification of *M. tuberculosis*–specific CD4 T cells on the basis of surface expression of Ag-induced activation markers will be less sensitive in patients with active TB, particularly given the inflammatory nature of TB disease. Future studies evaluating the performance of AIM

assays in HIV-infected and HIV-uninfected patients with TB disease will be necessary to determine if AIM assays are compromised in the setting of active disease. An additional limitation of this study is the evaluation of a limited range of soluble cytokines in PBMC supernatants, which could be expanded to include additional cytokines in future studies. A general limitation of the AIM assay is that it captures the overall Ag-specific CD4 T cell response, based on surface upregulation of activation markers, which could include multiple subsets of Ag-specific T cells with distinct junctional profiles. As such, the degree of phenotypic and functional heterogeneity of Ag-specific T cell responses could be masked by AIM assays. However, AIM assays provide a platform for sorting live, Ag-specific CD4 T cells for downstream transcriptional profiling that will further define distinct T cell populations contributing to the overall Ag-specific CD4 T cell response.

In summary, our results demonstrate the potential for AIM assays to identify novel populations of *M. tuberculosis*–specific CD4 T cells in HIV-infected and HIV-uninfected individuals that may otherwise not be detected by IGRAs or traditional intracellular cytokine staining assays. The AIM assay is thus a promising approach for sorting *M. tuberculosis*–specific CD4 T cell populations for downstream applications such as single-cell RNA sequencing, TCR sequencing, and T cell cloning, which will greatly facilitate further definition of T cell immune correlates that mediate durable control of *M. tuberculosis* infection and prevent progression to TB disease, particularly in highly vulnerable populations including people living with HIV.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Abbreviations used in this article:

AIM	activation-induced marker
BCG	bacille Calmette-Guérin
COMPASS	Combinatorial Polyfunctionality Analysis of Single Cells
HCMV	human CMV
IGRA	IFN-γ release assay
KEMRI	Kenya Medical Research Institute
LTBI	latent *M. tuberculosis* infection

QFT	QuantiFERON-TB Gold test
SEB	staphylococcal enterotoxin B
TB	tuberculosis

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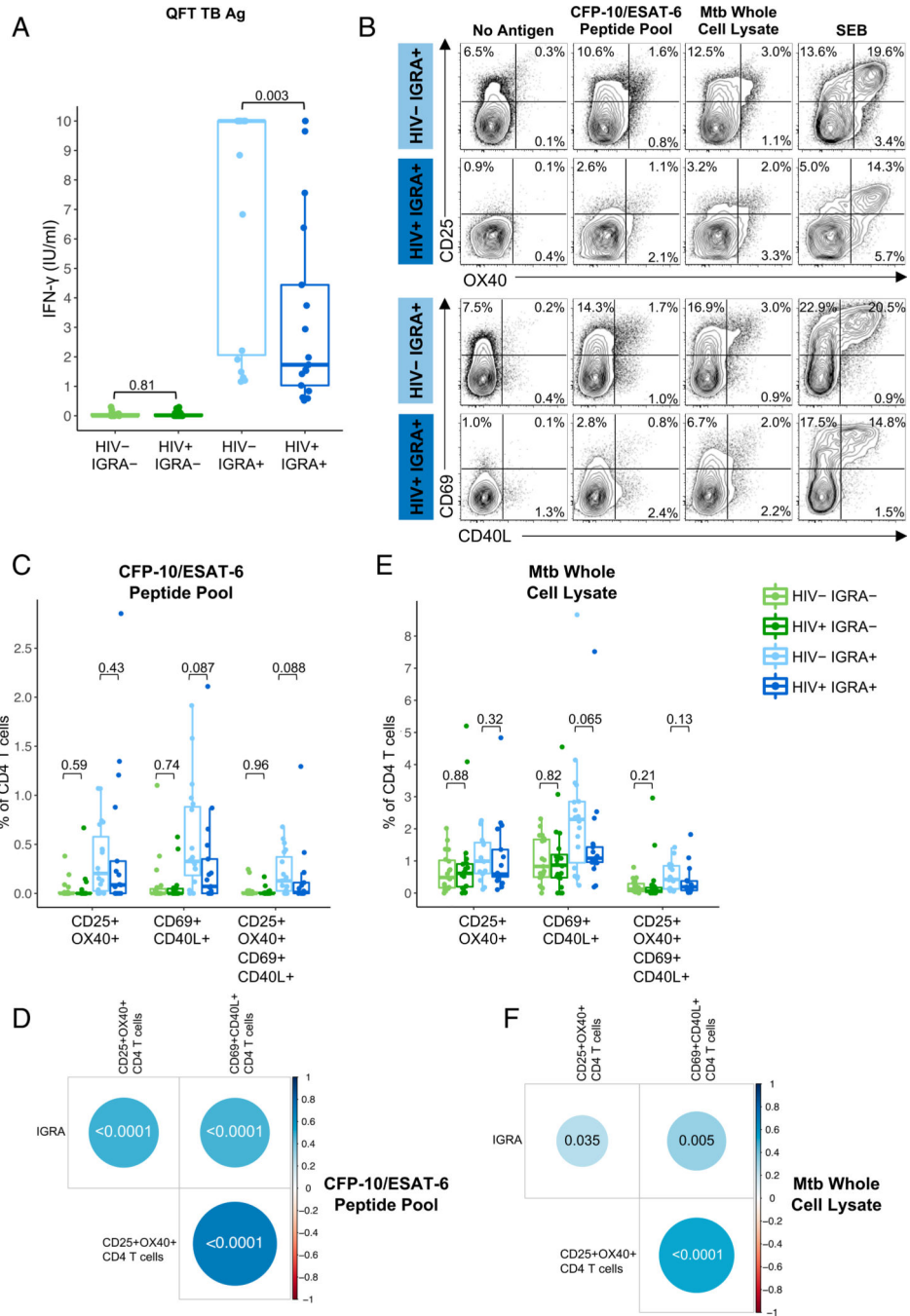
FIGURE 1. Differential upregulation of activation markers on _M. tuberculosis_–specific CD4 T cells from IGRA⁻ and IGRA⁺ individuals.

Study participants were evaluated by IGRA to determine _M. tuberculosis_ infection status (n = 75, see Table I). Cryopreserved PBMCs from each participant were used in the AIM assay. (**A**) Composite data of the TB Ag response in the QFT assay. Data are shown after subtraction of background IFN-γ in the QFT Nil tube. Horizontal lines represent the median. (**B**) Representative flow cytometry data from the AIM assay indicating Ag-induced expression of CD25⁺OX40⁺ and CD69⁺CD40L⁺ CD4 T cells from individuals in the HIV⁻

IGRA$^+$ and HIV$^+$ IGRA$^+$ groups. Plots are shown gated on live CD3$^+$CD4$^+$ T cells. (**C**) Composite data of CD4 T cell expression of the indicated activation markers following stimulation with CFP-10/ESAT-6 peptide pool. (**D**) Correlogram analysis between IGRA responses (TB Ag-Nil) and CFP-10/ESAT-6–induced AIM expression on CD4 T cells from all study participants ($n = 75$). (**E**) Composite data of CD4 T cell expression for the indicated markers following stimulation with *M. tuberculosis* whole cell lysate. (**F**) Correlogram analysis between IGRA responses (TB Ag-Nil) and *M. tuberculosis* lysate-induced AIM expression on CD4 T cells from all study participants ($n = 75$). Data in (C) and (E) are shown after subtraction of background AIM expression on CD4 T cells incubated in media alone. Boxes in (C) and (E) represent the median and interquartile ranges; whiskers represent $1.5 \times$ interquartile range. Differences in the frequencies of AIM$^+$ CD4 T cells between IGRA$^-$ and IGRA$^+$ groups and between HIV$^-$ and HIV$^+$ groups were assessed using the Mann–Whitney U test. Correlations in (D) and (F) were evaluated using a nonparametric Spearman rank correlation, with p values indicated in each circle. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients.
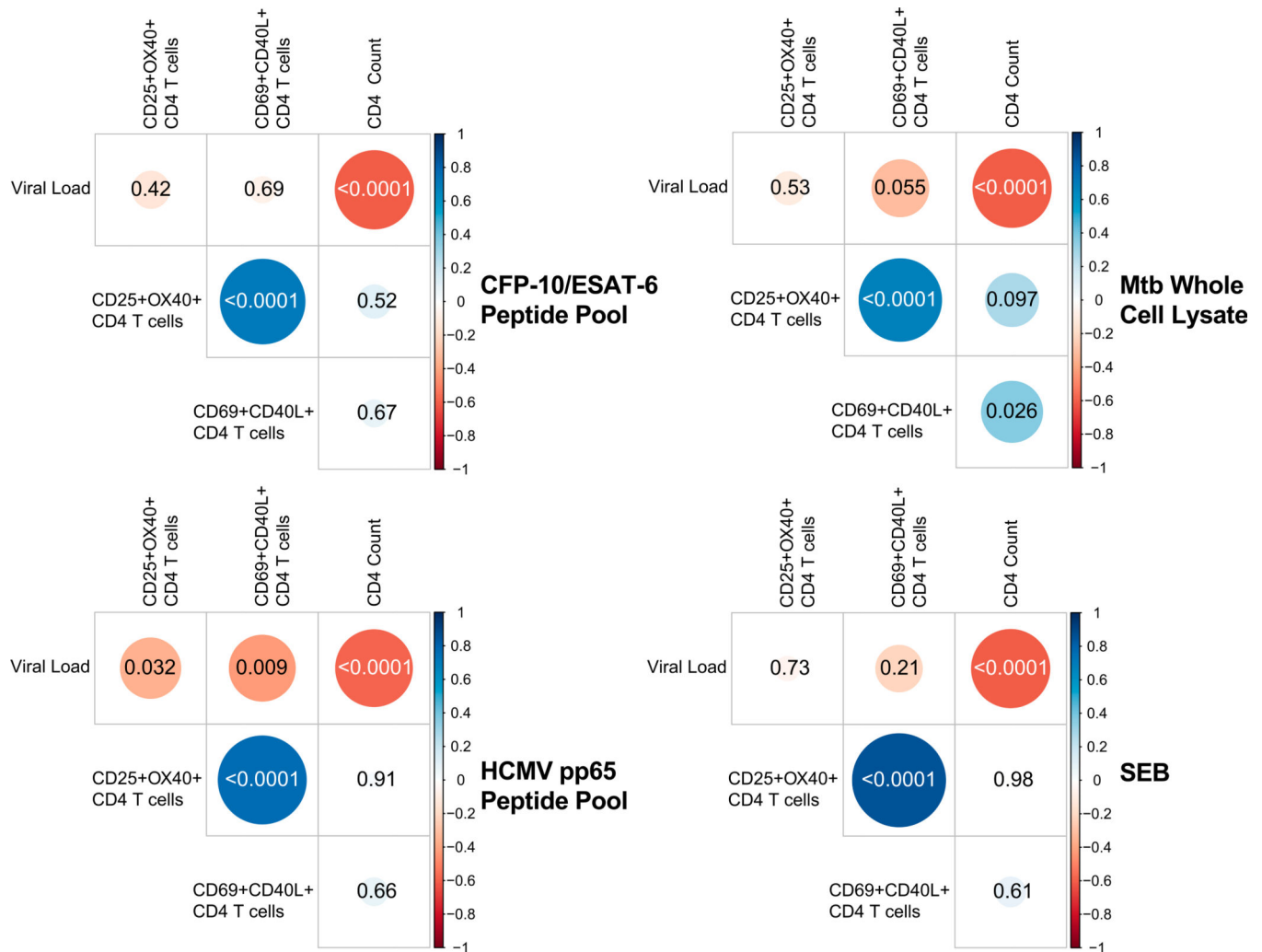
FIGURE 2. Frequencies of CFP-10/ESAT-6–specific AIM+ CD4 T cells do not correlate directly with HIV viral load or CD4 count.

Correlograms were generated to determine the relationship between HIV viral load, CD4 count, and the frequencies of Ag-specific AIM+ CD4 T cells. Data from 37 HIV+ individuals were included in this analysis ($n = 20$ IGRA−; $n = 17$ IGRA+). Correlations were evaluated using a nonparametric Spearman rank correlation, with p values indicated in each circle. Positive correlations are displayed in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients.
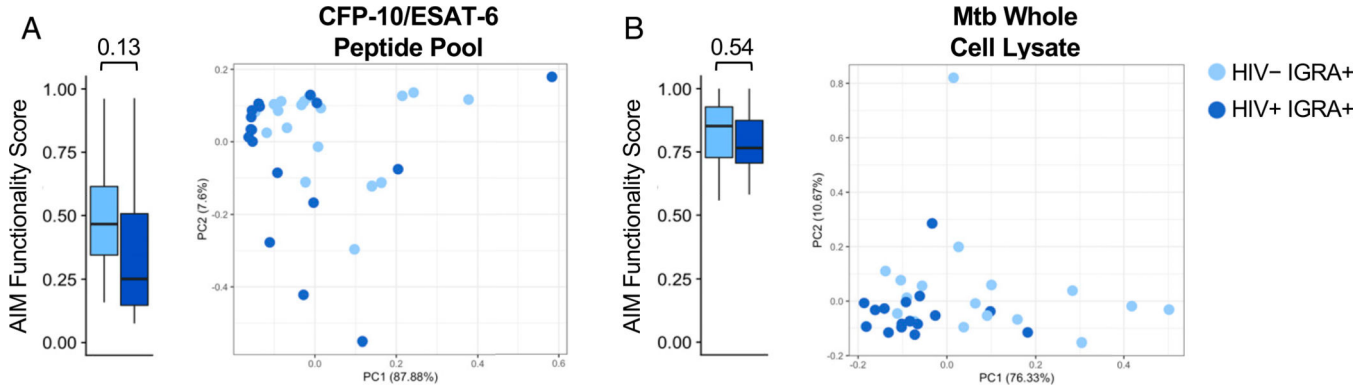
FIGURE 3. *M. tuberculosis*–specific CD4 T cell AIM assay functionality scores in HIV-infected and HIV-uninfected individuals.
Combinations of AIM markers (CD25, OX40, CD69, and CD40L) expressed on CD4 T cells from HIV-uninfected and from HIV-infected IGRA[+] individuals were analyzed by flow cytometry as described in Fig. 1. Boolean analysis was used to define subsets of CD4 T cells expressing all possible combinations of the four AIM markers. COMPASS was then used to summarize the Ag-specific AIM marker upregulation profile of each group, generating an AIM assay functionality score for CFP-10/ESAT-6–stimulated CD4 T cells (**A**) and *M. tuberculosis* whole cell lysate-stimulated CD4 T cells (**B**). The functionality score ranges from zero to one and indicates the proportion of Ag-specific AIM subsets expressed for a given individual among all possible subsets expressing one or more activation marker. Principal component analysis plots of the expression profiles of activation markers are shown in the right panel for each Ag. Differences in the functionality scores of CD4 T cells expressing activation markers between HIV-uninfected and HIV-infected individuals were assessed using the Mann-Whitney *U* test.
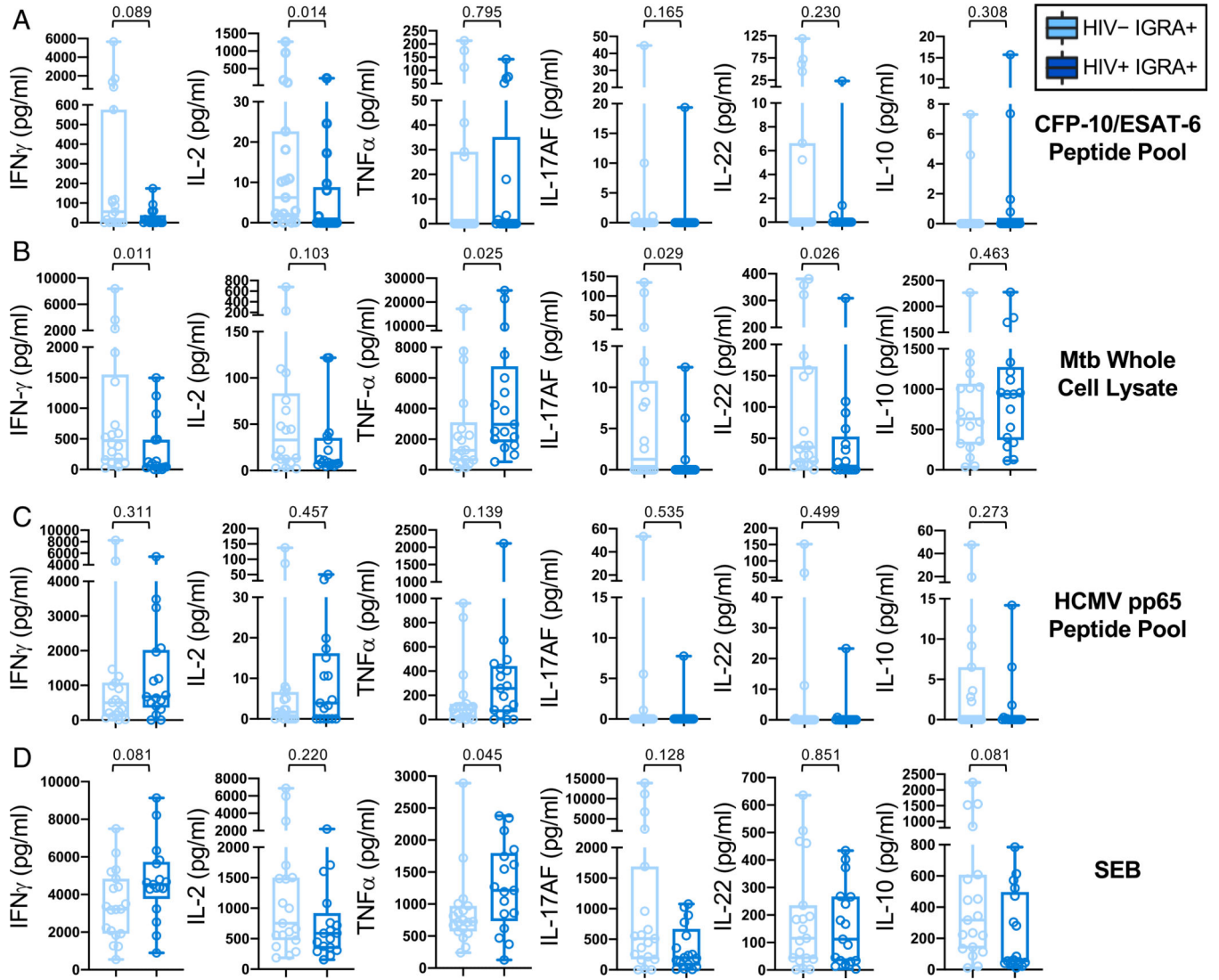
FIGURE 4. HIV coinfection modifies *M. tuberculosis*–specific cytokine production profiles in individuals with LTBI.

PBMCs from HIV⁻ (*n* = 19) and HIV⁺ (*n* = 17) IGRA⁺ individuals were stimulated with Ags as described in Fig. 1. After 16 h, cell supernatants were harvested, and cytokine levels were quantified by Luminex bead assay. Concentrations of IFN-γ, IL-2, TNF-α, IL-17AF, IL-22, and IL-10 are shown following stimulation of PBMCs with CFP-10/ESAT-6 peptide pool (**A**), *M. tuberculosis* whole cell lysate (**B**), HCMV pp65 peptide pool (**C**), and SEB (**D**). Boxes represent the median and interquartile ranges; whiskers represent the minimum and maximum values. Differences in cytokine concentration between HIV⁻ and HIV⁺ individuals were assessed using the Mann-Whitney *U* test.
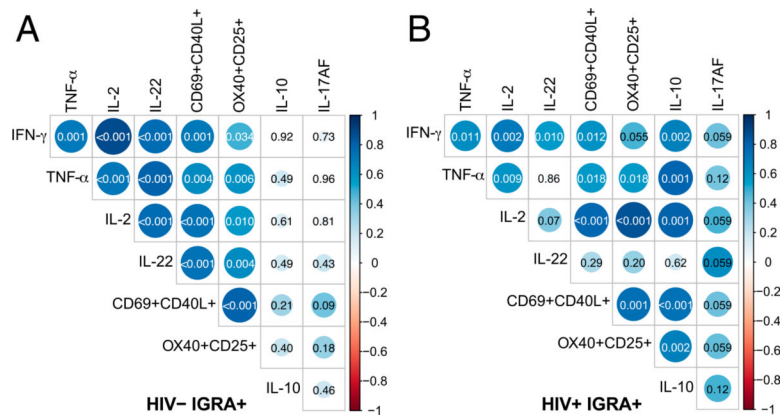
FIGURE 5. Coinfection with HIV skews the relationship between *M. tuberculosis*–specific AIM+ CD4 T cells and cytokine production profiles.

Correlogram analysis between CFP-10/ESAT-6–specific cytokine production and surface AIM expression by CD4 T cells from HIV− IGRA+ individuals (**A**) and HIV+ IGRA+ individuals (**B**). Correlations were calculated by Spearman rank-order correlation, with *p* values indicated in each circle. The size and color intensity of each circle are proportional to the strength and direction (blue: positive; red: negative) of each correlation coefficient. Correlograms are ordered by the angular order of the eigenvectors of HIV− individuals; correlogram data from HIV+ individuals are ordered according to the angular order of the eigenvectors of HIV− individuals.

TABLE I.

Characteristics of study participants

	HIV⁻ IGRA⁻	HIV⁺ IGRA⁻	HIV⁻ IGRA⁺	HIV⁺ IGRA⁺
n	19	20	19	17
Age, y [a] (IQR)	23 (19–28)	29 (25–36) [b]	30 (20–54)	32 (24–37)
Sex (% male)	21	45	26	59
IGRA, [c] IFN-γ IU/ml [d] (IQR)	0 (0–0.07)	0 (0–0.08)	10 (1.91–10)	1.73 [e] (0.94–5.41)
CD4 count, cells/μl [d] (IQR)	N/A	515 (320–758)	N/A	590 (432–838)
HIV viral load, copies RNA/ml plasma [d] (IQR)	N/A	85,488 (13,441–537,421)	N/A	17,550 (3,873–141,188)

[a] Value denotes median age in years.

[b] $p < 0.05$ compared with HIV⁻ IGRA⁻.

[c] TB Ag minus Nil from the QFT assay.

[d] Values denote median.

[e] $p < 0.01$ compared with HIV⁻ IGRA⁺.

IQR, interquartile range, N/A, not applicable.