Original Article

Latent and Active Tuberculosis Infection Increase Immune Activation in Individuals Co-Infected with HIV[☆],[☆☆],[★]

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ABSTRACT

In recent years, chronic immune activation and systemic inflammation have emerged as hallmarks of HIV disease progression and mortality. Several studies indicate that soluble inflammatory biomarkers (sCD14, IL-6, IL-8, CRP and hyaluronic acid), as well as surface markers of T-cell activation (CD38, HLA-DR) independently predict progression to AIDS and mortality in HIV-infected individuals. While co-infections have been shown to contribute to immune activation, the impact of latent tuberculosis infection (LTBI), which is widely endemic in the areas most affected by the global AIDS epidemic, has not been evaluated. We hypothesized that both active and latent states of *Mycobacterium tuberculosis* co-infection contribute to elevated immune activation as measured by these markers. In HIV-infected individuals with active, but not latent TB, we found elevated levels of soluble markers associated with monocyte activation. Interestingly, T-cell activation was elevated individuals with both latent and active TB. These results suggest that in the highly TB- and HIV-endemic settings of southern Africa, latent TB-associated T-cell activation may contribute to HIV disease progression and exacerbate the HIV epidemic. In addition, our findings indicate that aggressive campaigns to treat LTBI in HIV-infected individuals in high-burden countries will not only impact TB rates, but may also slow HIV progression.

Significance: Latent tuberculosis, which affects an estimated 1/3 of the world's population, has long been thought to be a relatively benign, quiescent state of *M. tuberculosis* infection. While HIV co-infection is known to exacerbate *M. tuberculosis* infection and increase the risk of developing active TB, little is known about the potential effect of latent TB infection on HIV disease. This study shows that HIV-infected individuals with both active and latent TB have elevated levels of inflammation and immune activation, biomarkers of HIV disease progression and elevated risk of mortality. These results suggest that, in the context of HIV, latent TB infection may be associated with increased risk of progression to AIDS and mortality.

1. Introduction

Although the incidence of HIV has peaked in much of sub-Saharan Africa, the tuberculosis (TB) epidemic continues unabated with TB remaining the leading cause of death in those living with HIV (World Health Organization, 2014). While many studies have investigated the impact of HIV infection on anti-TB immunity, the interaction between *Mycobacterium tuberculosis* (*M. tb*) infection and HIV disease progression is less well understood. Previous work suggests that in the absence of combination antiretroviral therapy (cART), the progression of HIV infection to AIDS and mortality among HIV-infected people in sub-Saharan Africa may be higher than in Western populations (Morgan et al.,

1997). While this is likely multifactorial, the different burdens of chronic co-infections in these settings may play a role.

Recent studies have demonstrated that elevated levels of certain soluble markers of inflammation or hyperactivation of the innate immune system can independently predict progression to AIDS, immune reconstitution inflammatory syndrome (IRIS), and death in HIV-infected patients (Boulware et al., 2011; Sandler et al., 2011; Marchetti et al., 2013). Specifically, soluble markers of monocyte turnover, inflammation, and fibrosis, including soluble CD14 (sCD14), lipopolysaccharide, interleukin (IL)-6, IL-8, interferon gamma-induced protein 10 (IP-10), hyaluronic acid, and C-reactive protein (CRP) have been shown to be associated with increased risk of progression to AIDS and death (Boulware et al., 2011; Hasegawa et al., 2009; Neuhaus et al., 2010; French et al., 2009).

Markers of lymphocyte activation have also been shown to predict disease progression in HIV-infected patients. Immune activation, as measured by the elevated expression of CD38 or co-expression of CD38 and HLA-DR on T-cells has been shown to be associated with shorter survival, transient low level viremia, drop in CD4+ T-cell count, and mortality upon initiation of cART (Giorgi et al., 1993, 1999; Hunt et al., 2003, 2011a; Deeks et al., 2004; Sousa et al., 2002; Vujkovic-Cvijin et al., 2013; Zhang et al., 2013; Taiwo et al., 2013; Dillon et al., 2014). Certain co-infections such as cytomegalovirus (CMV), hepatitis C virus (HCV), and hepatitis B virus (HBV) have been shown to contribute to immune activation in HIV-infected individuals (Hunt et al., 2011b; Beltran et al., 2014; Crane et al., 2014; Marchetti et al., 2014).

The impact of *M. tb* co-infection on immune activation during HIV infection has not been fully characterized. Active TB has been shown to contribute to immune activation and inflammation in the absence of HIV infection (Bloom et al., 2012, 2013). Additionally, active TB has been implicated in elevated plasma sCD14 and increased T-cell activation in HIV co-infected individuals (Toossi et al., 2013; Lawn et al., 2000; Mahan et al., 2010). However, the impact of latent TB infection, which affects an estimated 1/3 of people worldwide and an estimated 77–88% of adults in South Africa (Barry et al., 2009; Hanifa et al., 2009; Wood et al., 2010), on either soluble markers of inflammation or lymphocyte activation in HIV-patients has not been assessed.

We hypothesized that markers of deleterious inflammation and immune activation would be elevated in individuals with both latent and active TB infection. We sought to determine whether HIV-infected persons in KwaZulu-Natal, South Africa showed increased levels of soluble and cellular inflammatory biomarkers based on their TB infection status. To this end, we measured the plasma levels of sCD14, CRP, IL-6, IL-8, IP-10, and hyaluronic acid, and the lymphocyte expression of CD38 and HLA-DR in HIV-infected individuals with no evidence of TB infection, latent TB infection (LTBI), and active TB disease.

2. Materials and Methods

2.1. Patient Selection

80 HIV-positive individuals with well-defined states of TB infection were included in this study. All patients were participants in the iThimba (n = 64), Sinikithemba (n = 12), or the "Diagnosis of smear negative HIV associated pulmonary tuberculosis using a novel intra-esophageal device" (TB String Study, n = 4) cohorts based in KwaZulu-Natal, South Africa. Ethical approval was obtained and written informed consent was obtained from all patients. All subjects were HIV-positive and were categorized into one of three states of TB co-infection: (1) active TB infection, (2) latent TB infection, or (3) no evidence of TB infection. All subjects were ART, TB therapy, and isoniazid prophylaxis therapy (IPT) — naïve at the time of assessment. BCG vaccination history was not documented, but all subjects were likely vaccinated in infancy per South African standards. Subjects with active TB (AT) were defined as having symptoms of pulmonary TB and a culture-

positive sputum (spontaneous or induced) for *M. tb*. Subjects from the iThimba cohort with latent TB (LTBI) were defined as having no TB symptoms, having a tuberculin skin test (TST) with induration greater than 5 mm, a positive ESAT-6 and/or CFP-10 (RD-1) specific IFN-γ ELISpot, an induced sputum that was culture-negative for *M. tb*, and normal lung parenchyma on chest-X ray (CXR). Subjects from the iThimba cohort with no evidence of TB infection (no TB) were defined as having no TB symptoms, a negative TST, a negative RD-1 ELISpot, an induced sputum that was culture-negative for *M. tb* and normal lung parenchyma on CXR.

Because there were differences in the availability between cryopreserved plasma and peripheral blood mononuclear cells (PBMCs), two distinct but overlapping groupings of subjects were utilized in this study. The number of subjects in each group and their characteristics are detailed in Tables 1 and 2. Sample sizes for each comparison were calculated to provide 80% power to detect effect sizes drawn from previous studies at a Bonferroni-corrected level of significance ranging from 0.025–0.008.

2.2. Soluble Biomarker Analysis

Cryopreserved plasma from a subset of subjects from whom plasma was available was assayed for concentration of the following biomarkers using a multiplexed bead-based immunoassay: IL-6, IL-8, and IP-10 (all Invitrogen; Carlsbad, CA). sCD14 (R&D Systems; Minneapolis, MN) and CRP (Invitrogen) were also measured by bead-based immunoassays. All plasma was thawed once, aliquoted, and thawed once more for use in this analysis. Assays were performed according to the manufacturer's instructions and analyzed on a BioPlex-200 Luminex system (Bio-Rad; Hercules, CA). Plasma concentrations of hyaluronic acid were assayed by ELISA (Corgenix; Broomfield, CO) according to the manufacturer's instructions.

2.3. Flow Cytometric Analysis

Cryopreserved peripheral blood mononuclear cells (PBMCs) from a subset of subjects from whom PBMCs were available were thawed and rested overnight at 37 °C in order to minimize the effect of cellular activation due to thawing. All antibodies were pre-titrated in order to determine appropriate working concentrations. Rested PBMCs were then stained with the following antibodies: aqua viability dye (Invitrogen), CD3-PECF594 (BD; Franklin Lakes, NJ; clone UCHT1), CD4-PerCP-Cy5.5 (Biolegend; San Diego, CA; clone RPA-T4), CD8-APC-H7 (BD, clone SK1), CD38-PECy7 (BD, clone HIT2), and HLA-DR-eFluor605 (eBioscience; San Diego, CA; clone LN3). All stains were performed at 4 °C, and cells were fixed with 4% para-formaldehyde after staining. Stained PBMCs were subsequently analyzed on an LSR Fortessa cytometer (BD), using Rainbow Fluorescent Particles (BD) and application settings in BDFACSDiva7 to correct for day-to-day variations in instrument performance. Data were analyzed in FlowJo10 (Treestar; Ashland, OR). Cells were gated on aqua viability dye negative (live), lymphocytes determined by forward scatter vs. side scatter, and subsequently gated on CD3+CD4+ or CD3+CD8+ cells. Levels of activation markers (CD38 and HLA-DR) were measured by percentage positivity on CD4+ and CD8+ cells based on gates created using fluorescence minus one (FMO) controls.

2.4. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA). P-values reported are for Mann Whitney U or Kruskal–Wallis tests, in the case of non-parametric data, or Student's t-test or one-way analysis of variance (ANOVA) for parametric data. All correlations reported were tested by Spearman's rank. p values < 0.05 were considered significant and in cases of multiple comparisons the Bonferroni correction was applied.

Table 1

Clinical and demographic characteristics of subjects compared in the analysis of soluble inflammatory markers.

Characteristic	HIV + No TB n = 13	HIV + LTBI n = 21	HIV + AT n = 28	p-Value
Age, median years (IQR)	35 (31–47)	35 (29–50)	30 (26–37)	0.09
Female sex, no. (%)	11 (85)	17 (81)	17 (63)	0.14
CD4 T-cell count, median cells/μL (IQR)	425 (322–498)	426 (358–542)	167 (30–392)	<0.01
HIV RNA, median $\log_{10}$ copies/mL (IQR)	3.7 (3.1–4.1)	4.1 (3.2–4.7)	4.8 (3.8–5.3)	0.03
Comorbidities, no. (%)	2 (15.38)	4 (19.05)	3 (10.71)	0.72

Includes HIV-infected individuals with no evidence of *M. tb* infection (no TB), HIV-infected individuals with latent TB infection (LTBI), and HIV-infected individuals with active TB (AT). Comorbidities included arthritis (n = 2), asthma (n = 3), hypertension (n = 5), connective tissue disease (n = 1), liver disease (n = 1), and lung disease (n = 1). All subjects described here were members of the iThimba cohort. p-Values reported for Kruskal–Wallis one-way analysis of variance.

3. Results

3.1. Plasma sCD14, CRP, IL-6, and IP-10 are Elevated in HIV-infected Subjects With Active TB Infection

Our first aim was to assess the effect of latent and active TB infection on soluble inflammatory biomarkers that have been shown to predict poor outcomes in HIV-infected individuals. We measured plasma levels of sCD14, CRP, IL-6, IL-8, IP-10, and hyaluronic acid in HIV-infected subjects with no evidence of TB infection (no TB), latent TB infection (LTBI), and active TB disease (AT, Table 1). We found no significant differences between levels of these plasma biomarkers for individuals with no TB versus those with LTBI (Fig. 1). Individuals with active TB had higher levels of sCD14 (p = 0.0076), CRP (p = 0.022), IL-6 (p = 0.0040) and IP-10 (p = 0.0185, not significant after Bonferroni correction). Similarly, individuals with active TB had higher levels of sCD14 (p = 0.0012), CRP (p < 0.0001), IP-10 (p = 0.003), IL-6 (p < 0.0001) and IL-8 (p = 0.0130, not significant after Bonferroni correction) than those with no evidence of TB. Levels of hyaluronic acid did not vary significantly between the three groups.

We found most of the markers of interest to be significantly correlated with CD4 cell count (Supplementary Fig. 1). The patients with no evidence of TB and those with LTBI were well matched for CD4 cell count. However, because CD4 cell count was significantly lower in the active TB group compared to the other two groups, we were concerned that differences in CD4 count confounded the relationship between soluble inflammatory markers and active TB. To address this, we used samples from an additional cohort (Sinikithemba) to create CD4-matched groups and repeated our analysis (Supplementary Table 1). Again, we found elevated levels of sCD14 (p = 0.004), CRP (p = 0.0187), IL-6 (p = 0.0012), and IP-10 (p = 0.0366, not significant after Bonferroni correction), in individuals with active TB as compared to those without active TB (Supplementary Fig. 2). Importantly, the addition of subjects from the Sinikithemba cohort limited our ability to determine which of the healthy, asymptomatic patients had LTBI by our stringent definition, so in this analysis active TB patients were compared to HIV-positive patients with no evidence of active TB.

Together, these data indicate that soluble markers of immune activation are elevated in HIV-positive individuals with active TB compared to those with either latent TB or no evidence of TB infection. We found no difference in soluble markers of immune activation between HIV-positive patients with no evidence of TB infection and those with latent TB infection.

3.2. Lymphocyte Activation is Elevated in HIV-infected Individuals With Latent and Active TB Infection

After finding elevated levels of plasma biomarkers in individuals with active, but not latent TB, we next assessed the level of lymphocyte activation in our groups of interest. To this end, we measured levels of CD38 and HLA-DR on CD4 + and CD8 + T-cells in HIV-infected subjects with no evidence of TB infection (no TB), latent TB infection (LTBI), and active TB disease (AT, Fig. 2A). Due to availability of samples, the three groups used for this analysis were overlapping but distinct from those used for the plasma analysis. Definitions of TB co-infection status were consistent. In this instance there were no statistical differences in CD4 count or viral load between the three groups (Table 2).

As expected, subjects with active TB had higher levels of T-cell activation than those with latent TB or no TB (Fig. 2). CD38 expression on CD4 + and CD8 + T-cells was higher in those with active TB compared to those with no TB and those with LTBI (p = (Fig. 2B and C). Co-expression of CD38 and HLA-DR on CD4 + and CD8 + T-cells was also higher in those with active TB compared to those with no TB and those with LTBI (Fig. 2D and E). Interestingly, subjects with latent TB had elevated CD38 expression on both CD4 + and CD8 + T-cells compared to subjects with no evidence of TB (Fig. 2B and C). These results were consistent when CD38 expression was evaluated by median fluorescence intensity (MFI) (data not shown).

Table 2

Clinical and demographic characteristics of subjects compared in the analysis of lymphocyte activation markers.

Characteristic	HIV + no TB n = 13	HIV + LTBI n = 21	HIV + AT n = 8	p-Value
Age, median years (IQR)	35 (31–47)	35 (29–50)	33 (27–49)	0.95
Female sex, no. (%)	11 (85)	17 (81)	4 (50)	0.16
CD4 T-cell count, median cells/μL (IQR)	425 (322–498)	426 (358–542)	286 (254–492)	0.33
HIV RNA, median $\log_{10}$ copies/mL (IQR)	3.7 (3.1–4.1)	4.1 (3.2–4.7)	4.4 (3.5–5)	0.4
Comorbidities, no. (%)	2 (15.38)	4 (19.05)	1 (12.5)	0.91

Includes HIV-infected individuals with no evidence *of M. tb* infection (no TB), HIV-infected individuals with latent TB (LTBI) and HIV-infected individuals with active pulmonary TB (AT). Comorbidities included arthritis (n = 2), hypertension (n = 5), asthma (n = 2), connective tissue disease (n = 1), and liver disease (n = 1). All subjects described here were members of the iThimba or TB String Study cohorts. p-Values reported for Kruskal–Wallis one-way analysis of variance.
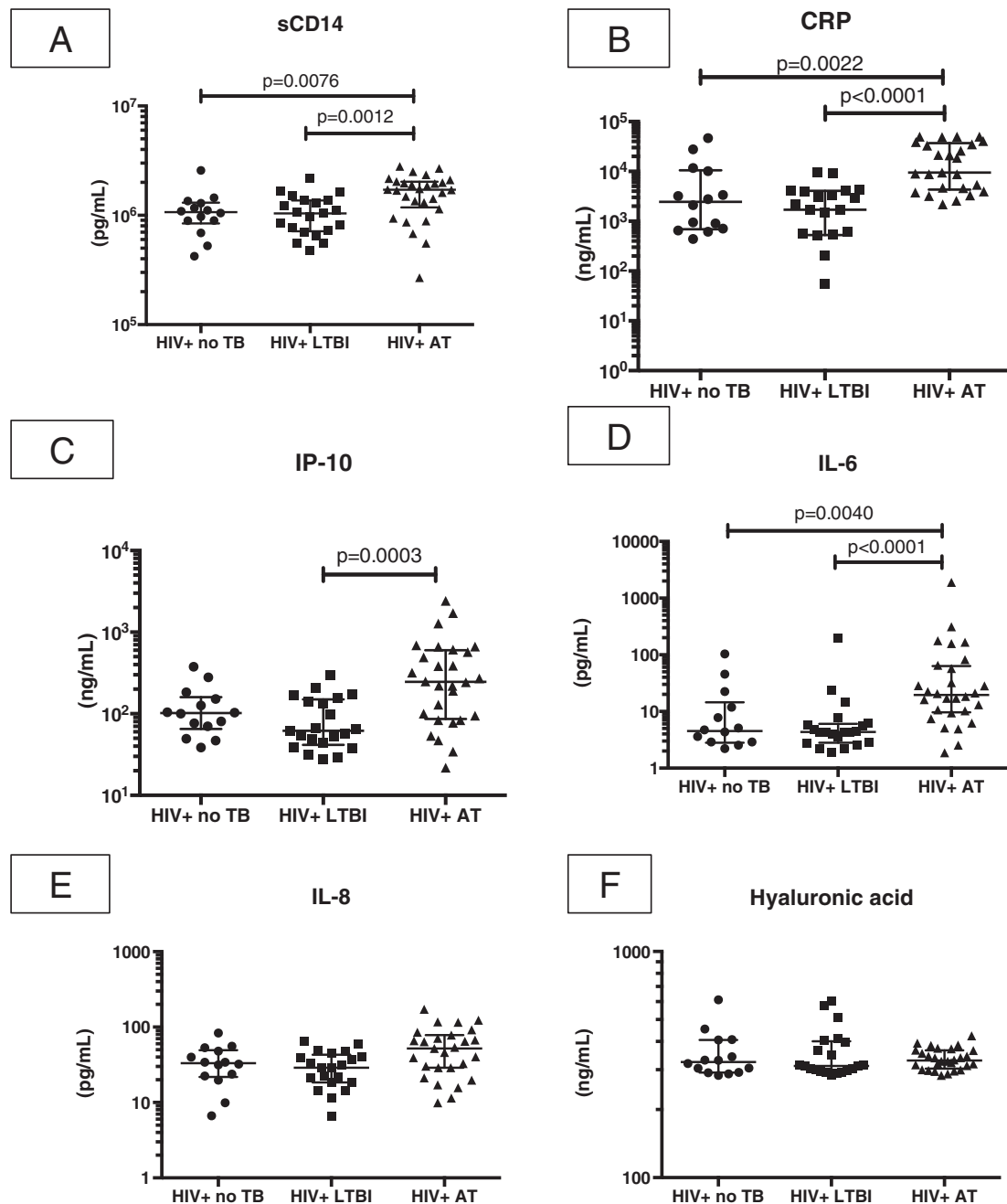
Fig. 1. Plasma concentrations of A) soluble CD14 (sCD14), B) C-reactive protein (CRP), C) IL-6, D) IL-8, E) interferon gamma-induced protein 10 (IP-10), and F) hyaluronic acid in HIV-infected individuals with no evidence *of M. tb* infection (no TB), latent TB (LTBI), and active TB (AT). Subjects represented here were members of the iThimba cohort. p-Values reported for Mann Whitney U test, with a threshold for significance of 0.0083 after Bonferroni correction. p-Values greater than 0.0083 not displayed. Data displayed as median with interquartile range.

4. Discussion

We have shown that in HIV-infected individuals, both active and latent TB co-infections are associated with biomarkers of immune activation that are known to correlate with more rapid HIV disease progression and mortality (Giorgi et al., 1993, 1999; Hunt et al., 2003, 2011a; Deeks et al., 2004; Sousa et al., 2002; Vujkovic-Cvijin et al., 2013; Zhang et al., 2013; Taiwo et al., 2013; Dillon et al., 2014). Soluble markers of inflammation were elevated only in those with active TB. In contrast, T-cell activation, as measured by CD38 and HLA-DR expression on CD4 + and CD8 + T-lymphocytes, was elevated in both latent and active TB. Though other studies have shown that active TB contributes to systemic inflammation in HIV-infected and uninfected individuals, the finding that individuals latently co-infected with TB also display elevated T-cell activation has not been previously described, and may be important for understanding the interplay between HIV and TB in individuals and in populations.

These results are particularly relevant in the context of South Africa and other TB-endemic settings, as widespread latent TB infection and the resultant elevated T-cell activation we have observed may provide an explanation for more rapid progression of HIV to AIDS and increased mortality in this setting. Additionally, repeated episodes of active TB are often seen among HIV-infected individuals in this setting (Wood et al., 2011), and the observed increase in inflammatory cytokines during these episodes may also drive an accelerated course of HIV disease. Our finding of increased T-cell activation may also provide additional support for the treatment of latent TB infection with isoniazid in HIV-infected individuals. While recent guidelines have recommended
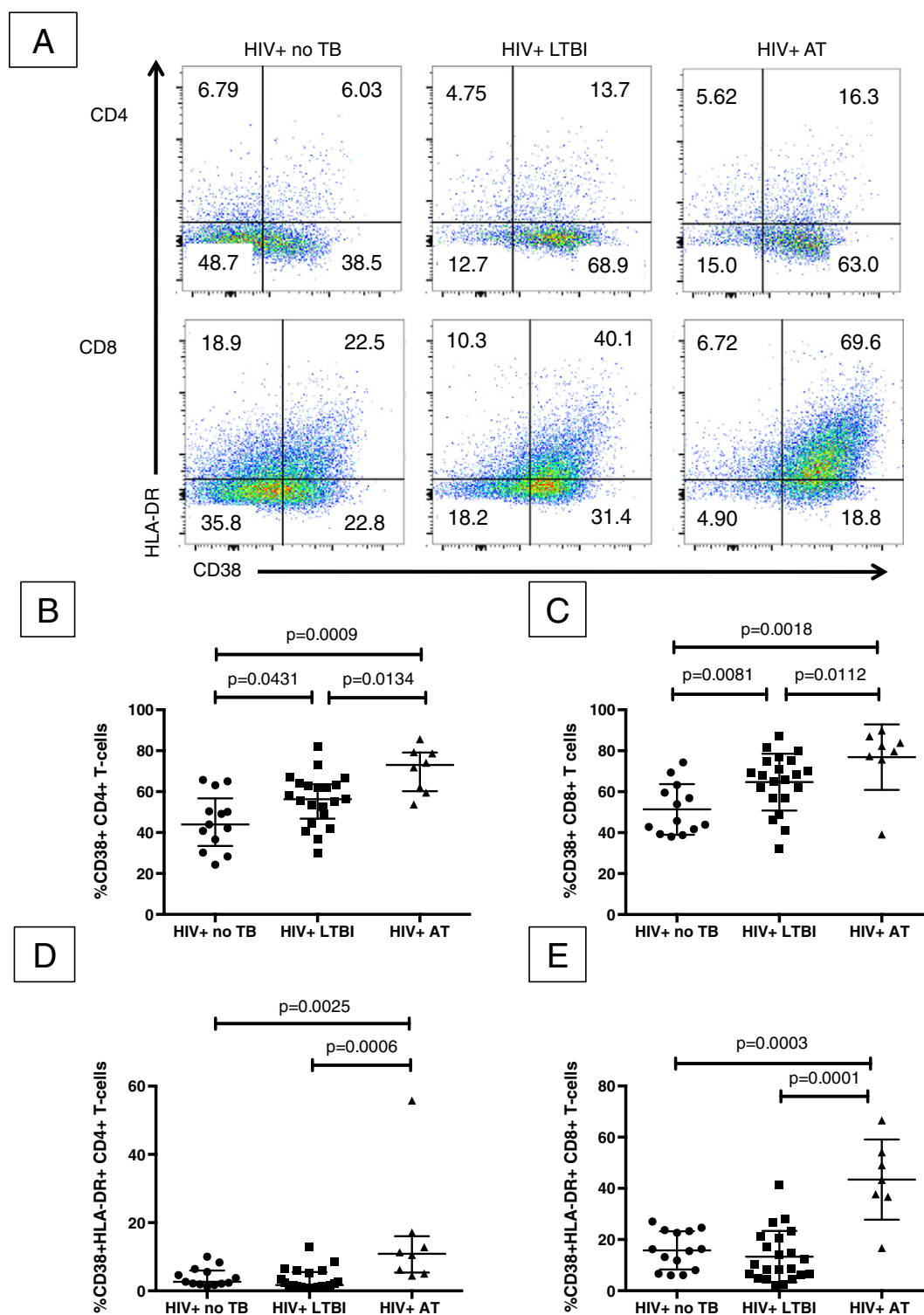
Fig. 2. A) representative flow cytometry plots of CD38 and HLA-DR expression on CD8 + (top panel) and CD4 + (bottom panel) T-cells from HIV-infected individuals with no evidence of *M. tb* infection (no TB), latent TB (LTBI), or active TB (AT). Percentage of CD8 + (B) and CD4 + (D) T-cells expressing CD38 in no TB, LTBI, or AT individuals. Percentage of CD8 + (C) and CD4 + (E) T-cells co-expressing CD38 and HLA-DR in no TB, LTBI, or AT individuals. Subjects represented here were matched for CD4 + T-cell count and HIV viral load, and were members of the iThimba or TB String Study cohorts. p-Values reported for Mann Whitney U test, with p-values greater than 0.05 not displayed. Data displayed as median with interquartile range.

widespread treatment of LTBI (diagnosed by TST or *M. tb*-specific interferon gamma release assay (IGRA)) among those co-infected with HIV, actual coverage rates remain low (Young et al., 2009; Lawn et al., 2010). Our work suggests that by driving immune activation, latent TB may contribute to progression to AIDS and mortality, thereby potentially adding to the reasons for more widespread use of isoniazid preventative therapy

(IPT) for those co-infected with LTBI and HIV. The ability of IPT to decrease T-cell activation associated with LTBI should be tested.

A strength of this study is our use of stringent criteria for defining each patient group. To exclude the possibility of active TB in asymptomatic individuals, we rigorously investigated subjects in the no TB group and LTBI group by chest X-ray and induced sputum. Though we cannot

fully exclude the possibility of subclinical infection or undetected extrapulmonary TB, we have used all tools available to rule out these possibilities. We also cannot exclude the possibility that other undetected comorbidities or co-infections may have been present in our study subjects. However, we have no evidence to suggest that these would have been biased towards any particular study group. This study is based on a relatively small sample size, and this factor may have limited the extent of our findings. While our sample size was powered to detect a modest to large effects in most markers, small changes would not be identified. For example, we were unable to detect differences in plasma concentrations of soluble biomarkers in individuals with latent TB compared to those with no TB. While this may be due to a lack of difference, it is also possible that with a larger sample size these two populations might show inflammatory profile differences. Our study population was limited to South African subjects, which limits its extrapolation to other populations with less extensive HIV epidemics and TB endemicity. This highlights the need for further studies of HIV-associated immune activation in settings with varying burdens of co-infection. A comparative analysis of immune activation in HIV/TB co-infected individuals from Western and non-Western settings would be an important complement to our findings.

Our findings may reflect differences in the character of systemic inflammation experienced by HIV-infected individuals living with varying degrees of TB co-infection. Latently infected individuals in this study showed lymphocyte activation but an absence of the soluble markers of monocyte-associated activation, while those with active TB showed significantly elevated markers of both monocyte-associated and lymphocyte activation. These data may suggest that in HIV-infected individuals, TB latency reflects a bacterial burden sufficient to activate lymphocytes, but not to cause significant changes in plasma biomarkers of monocyte activation. Future work to characterize the functional capacity and activation state of TB-specific lymphocytes and monocytes/macrophages in the context of HIV and LTBI will help to clarify the interaction between control of bacterial burden and immune activation.

The different mechanisms by which immune activation may be affected by latent and active states of TB infection remain unclear, but recent work suggests that replicating and nonreplicating bacteria may elicit different immune responses. Mariotti et al. report that while replicating *M. tuberculosis* is able to induce IL-1b, modulate the macrophage inflammasome, and activate T-cells, nonreplicating bacteria are able to trigger T-cell activation, but not any other inflammatory processes (Mariotti et al., 2013). This observation may partially explain why latently infected individuals display lymphocyte markers of activation, while individuals with active TB disease harboring large numbers of replicating mycobacteria have measurable inflammation and activation in both the innate and adaptive branches. Further in vitro and translational work is required to test this hypothesis, as well as to better define the metabolic activity of bacteria in HIV-infected individuals with latent TB co-infection.

Because previous studies of the effect of latent or active TB on immune activation have primarily characterized HIV-uninfected individuals, and because we were specifically interested in immune activation markers implicated in HIV pathogenesis, we only assessed these markers in HIV co-infected subjects. While previous studies have found no evidence for elevated immune activation (of either bulk T-cells or *M. tb*-specific T-cells) in latently-infected, HIV-negative individuals (Rodrigues et al., 2002; Wergeland et al., 2011; de Almeida et al., 2012; Adekambi et al., 2012; Hodapp et al., 2012), our data suggest that this is not the case for HIV-infected individuals who also harbor latent TB infection. This surprising finding challenges the notion of latency as a fully dormant state of TB infection, and suggests that HIV co-infection may skew this heterogeneous state towards a state that immunologically resembles more active infection. Despite the rigorous definitions used to characterize individuals as latently infected, it appears that, like active TB, latent TB may be a fundamentally different immunological phenomenon in the context of HIV co-infection. In a 2009 review, Barry et al. propose a redefinition of

the different stages of *M. tb* infection, with five different categories of disease rather than the canonical latent vs. active dichotomy (Barry et al., 2009). Taken into the context of this spectrum, the HIV + LTBI group that we describe in this study while clearly not having "clinical disease" may fall between the categories of "quiescent" and "active" infection (Barry et al., 2009). Importantly, evidence shows that integration of isoniazid preventive therapy (IPT) with ART is crucial for successful treatment of latent TB in HIV-infected individuals, further suggesting that HIV co-infection may fundamentally alter latent TB and its susceptibility to treatment (Houben et al., 2014). The development of improved diagnostic tools and imaging techniques, such as positron emission tomography-computed tomography (PET-CT) for determining an individual's placement along the spectrum of TB disease will allow for a more nuanced picture of TB disease, and will also facilitate a deeper understanding of the role played by HIV at different stages of TB infection (Ghesani et al., 2014).

Our results provide evidence that latent TB co-infection may be driving elevated levels of immune activation in HIV-infected individuals living in parts of the world where TB is endemic. The differing contributions of latent and active TB infection to activation and inflammation are indicative of the complexity of the relationship between HIV and TB in these settings and the need for larger scale and more mechanistic investigations into these questions.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.03.005.

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