Activation profile of *Mycobacterium tuberculosis*-specific CD4+ T cells reflects disease activity, irrespective of HIV status.

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Research Letter to the Editor

To the Editor:

The diagnosis of pulmonary tuberculosis in HIV-infected individuals is particularly challenging, as HIV-induced alterations of the immune system lead to reduced cavitations, limiting the sensitivity of sputum-based assays (1). Thus, alternate markers are needed to distinguish between latent (LTBI) and active TB (aTB) in this high-risk group. Several attributes of Mtb-specific CD4+ T cells have been shown to efficiently delineate LTBI and aTB in HIV-uninfected individuals, including their polyfunctional or memory profiles (2-4). Moreover, Adekambi *et al.* recently demonstrated that the activation profile of Mtb-specific CD4+ T cells accurately discriminates between LTBI and aTB (5). As chronic HIV infection is characterized by persistent systemic immune activation (6), it is plausible that these blood-based markers may not be relevant for HIV-infected individuals.

We therefore compared the potential of the activation and polyfunctional profiles of Mtb-specific CD4+T cells to distinguish between LTBI and aTB in HIV-uninfected and HIV-infected individuals. We analyzed 76 participants divided in four groups according to their TB and HIV status (Table S1): LTBI/HIV- (n=17), aTB/HIV- (n=17), LTBI/HIV+ (n=21, median CD4 count: 316 cells/mm^3, IQR: 231-543) and aTB/HIV+ (n=21, CD4 count: 250 cells/mm^3, IQR: 155-295). LTBI was defined as TST positive, IGRA positive, sputum culture negative and normal CXR. aTB was diagnosed based on symptoms suggestive of tuberculosis and Mtb positive smear and/or sputum culture, as previously described (7). All HIV-infected participants were ART-naïve. UCT ethics committee approved the study and written consent was obtained from participants. Cryopreserved PBMCs were stimulated for 16 hours with ESAT-6/CFP-10 peptide pool and intracellular staining using a live/dead marker and antibodies towards CD3, CD4, CD8, HLA-DR, Ki67, CD38, IFN-γ, TNF-α and IL-2 was performed. Positive ESAT-6/CFP-10 responses (defined as twice the background) were detectable in 16 subjects in the LTBI/HIV- and LTBI/HIV+ groups; and in 15 and 18 individuals in the aTB/HIV- and aTB/HIV+ groups, respectively. No significant differences

were observed in the overall magnitude of IFNγ+ responses between the four groups (data not shown).

We first compared the activation profile of IFNγ+ Mtb-specific CD4+T cells between the four groups (**Figure 1A**). As previously shown (5), in HIV-uninfected persons, HLA-DR, Ki67 and CD38 expression on IFNγ+ Mtb-specific CD4+T cells were significantly higher in aTB participants when compared to LTBI (**Figure 1B**). Interestingly, while HLA-DR expression on Mtb-specific CD4+T cells in the LTBI/HIV+ group (median 41.7%, IQR: 25.7-54.6) was significantly higher when compared to the LTBI/HIV- group (13.7%, IQR: 8.9-27.5), HLA-DR expression on these cells was significantly further increased in HIV-infected individuals with aTB (84%, IQR: 73.7-87.9) (**Figure 1B**). Additional analyses showed that in LTBI/HIV+ individuals, HLA-DR expression on Mtb-specific CD4+T cells mirrors HLA-DR expression in the whole CD4 compartment ($p=0.02$, $r=0.56$), but this association was not apparent in aTB/HIV+ individuals (data not shown). Unlike HLA-DR, Ki67 and CD38 expression levels were comparable between HIV-uninfected and HIV-infected individuals with LTBI. In HIV-infected persons with aTB, Ki67 expression on IFNγ+ Mtb-specific CD4+T cells was significantly higher ($p<0.0001$) when compared to LTBI, while the up-regulation of CD38 was more modest between these two groups ($p=0.03$). Of note, in the aTB/HIV+ group, the expression of CD38 was significantly higher in individuals with a positive smear when compared to smear negative ($p=0.01$, data not shown), suggesting that CD38 expression could reflect bacterial load. To assess the accuracy of these markers to discriminate between LTBI and aTB status, ROC curves and cross-over plots were performed. **Figure 1C** shows the data for HLA-DR, AUC and *p*-values reflect that HLA-DR expression on IFNγ+ Mtb-specific CD4+T cells distinguishes LTBI and aTB in both the HIV- and HIV+ groups (AUC=0.98, $p<0.0001$; AUC=0.9, $p<0.0001$, respectively). However, the optimum cutoff values discriminating LTBI from aTB were distinct for HIV-uninfected (40%) and HIV-infected individuals (70%). In our experimental setting, the expression of Ki67 and

CD38 were less robust to differentiate TB status in HIV-uninfected (AUC=0.896, p=0.00017, cutoff=1.4% and AUC=0.858, p=0.0007, cutoff=4%, respectively) and in HIV-infected individuals (AUC=0.89, p=0.0002, cutoff=2.4% and AUC=0.72, p=0.026, cutoff=5%, respectively) (data not shown). Our data were comparable to (5), despite disparity in the cutoff value for these markers, which could be explained by flow-cytometry technical differences.

The polyfunctional profile of Mtb-specific CD4+T cells has also been shown to discriminate between LTBI and aTB in HIV-uninfected individuals (2, 3), but conflicting data exists for HIV-infected persons (8-10). Thus, we compared the profile of ESAT-6/CFP-10-specific CD4+T cells, based on their capacity to secrete IFN-γ, TNF-α and/or IL-2, between the four groups (**Figure 2A**). HIV-uninfected individuals with LTBI were characterized by a predominant proportion of IFNγ+IL2+TNFα+ cells (median: 44%, IQR: 35-49), a subset that was significantly lower in individuals with HIV (20%, IQR: 15-32), aTB (16%, IQR: 4-19) or both (9%, IQR: 2.6-22) (**Figure 2B**). In participants with HIV and/or aTB, IFNγ+ IL2-TNFα+ cells counterweighed the reduction of triple positive cells. Of note, unlike previously reported (2), no differences in the proportion of TNF-α single positive Mtb-specific CD4+ T cells were observed, these differences could arise from significant disparities in the age, ethnicity and TB diagnosis in the study cohorts. ROC curve analyses (**Figure 2C**) show that the proportion of IFNγ+IL2+TNFα+ or IFNγ+IL2-TNFα+ Mtb-specific CD4+T cells allowed the distinction between LTBI and aTB in HIV-uninfected individuals (AUC=0.97, p<0.0001 and AUC=0.92, p<0.0001, respectively) but not in HIV-infected persons.

In summary, these data show that HLA-DR expression level on IFNγ+ Mtb-specific CD4+T cells represents a robust marker to distinguish between LTBI and aTB in both HIV-uninfected and ART naïve HIV-infected individuals. This suggests that despite HIV-induced systemic immune activation, active bacterial replication promotes further up-regulation of HLA-DR on Mtb-specific CD4+T cells. On the contrary, the polyfunctional profile of Mtb-

specific CD4+T cells associated with TB status solely in HIV-uninfected individuals, suggesting that HIV infection may alter the secretion potential and/or localization of Mtb-specific CD4+T cells even in the absence of bacterial replication. One main limitation of such assays, requiring cell stimulation to identify Mtb-specific CD4+T cells, is that the analysis is restricted to individuals with detectable Mtb responses. Inclusion of additional immunodominant Mtb antigens could improve the "coverage" of Mtb responders. Further experiments will be needed to confirm these data in larger study including HIV-infected participants on antiretroviral treatment. Nevertheless, this study confirms that HLA-DR expression could represent an important alternate tool to assess TB status in HIV-uninfected individuals and expand this finding to HIV-infected subjects.

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158 **LEGENDS:**

159 **Figure 1.** Comparison of the activation profile of IFNγ+ ESAT-6/CFP-10-specific CD4+ T
160 cells between HIV-uninfected and HIV-infected individuals with latent or active TB disease.
161 *(A)* Representative overlay plots of HLA-DR, CD38 and Ki-67 expression in total CD4+ T
162 cells (grey) and IFNγ+ Mtb-specific CD4+ T cells (red). *(B)* Expression of HLA-DR, Ki67
163 and CD38 on IFNγ+ Mtb-specific CD4+ T cells in LTBI/HIV- (n=16), aTB/HIV- (n=15),
164 LTBI /HIV+ (n=16) and aTB/HIV+ (n=18) participants. Open circles (○) depict LTBI
165 individuals, closed circles (●) represent smear positive aTB patients and crosses (X)
166 correspond to smear negative and culture positive individuals with aTB. Horizontal lines
167 indicate the median. Statistical comparisons were performed using a non-parametric Mann-
168 Whitney U test. *(C)* Receiver operator characteristics (ROC) curves and specificity/sensitivity
169 cross-over plots for HLA-DR expression level in IFNγ+ Mtb-specific CD4+ T cells to
170 discriminate between LTBI or aTB in HIV-uninfected and HIV-infected individuals. The
171 area-under-the-curve (AUC), *p*-value and confidence intervals (CI) are shown. The dotted line
172 depicts an AUC of 0.5, representing a random test. The vertical line on the cross-over plots
173 represents the optimal threshold to distinguish LTBI and aTB individuals.
174
175 **Figure 2.** Comparison of the polyfunctional profile of ESAT-6/CFP-10-specific CD4+ T cells
176 between HIV-uninfected and HIV-infected individuals with latent or active TB disease. *(A)*
177 Representative dot plots of IFN-γ, TNF-α and IL-2 production in response to ESAT-6/CFP-10
178 peptide pool in one LTBI/HIV- individual. NS: No Stimulation. Numbers represent the
179 frequencies of cytokine-producing cells expressed as a percentage of the total CD4+ T cell
180 population. *(B)* Proportion of Mtb-specific CD4+ T cells producing any possible
181 combinations of IFN-γ, TNF-α or IL-2. Horizontal bars represent the median values and IQR.
182 Statistical analysis was performed using Mann-Whitney test and significant differences are
183 indicated by asterisks (***: $p<0.001$, **: $p<0.01$, *: $p<0.05$). Each slice of the pie corresponds

to a distinct combination of cytokine. A key to colors used in the pie charts is shown at the bottom of the graph. *(C)* Receiver operator characteristics (ROC) curves and specificity/sensitivity cross-over plots for the proportion of IFNγ+IL2+TNFα+ (top panel) and IFNγ+IL2-TNFα+ (bottom panel) Mtb-specific CD4+ T cells to discriminate between LTBI or aTB in HIV-uninfected and HIV-infected individuals.

Table S1:

Clinical characteristics of the study cohort.

Groups	**n**	**Female**	**Age (years)** Median [IQR]	**CD4 count (cells/mm^3)** Median [IQR]	**Previous TB**	**Smear positive**	**Culture positive**
LTBI/HIV-	17	8/17 (47%)	22 [20-24]	nd	1/17 (6%)	nd	nd
aTB/HIV-	17	5/17 (29%)	27 [21-33]	nd	3/16 (19%)	11/14 (79%)	13/14 (93%)
LTBI/HIV+	21	14/21 (67%)	29 [27-34]	316 [231-543]	0/20 (0%)	0/17 (0%)	0/17 (0%)
aTB/HIV+	21	12/21 (57%)	35 [29-40]	250 [155-295]	7/21 (33%)	8/21 (38%)	20/21 (95%)

IQR: Interquatrile range, nd: not determined.

Figure 1

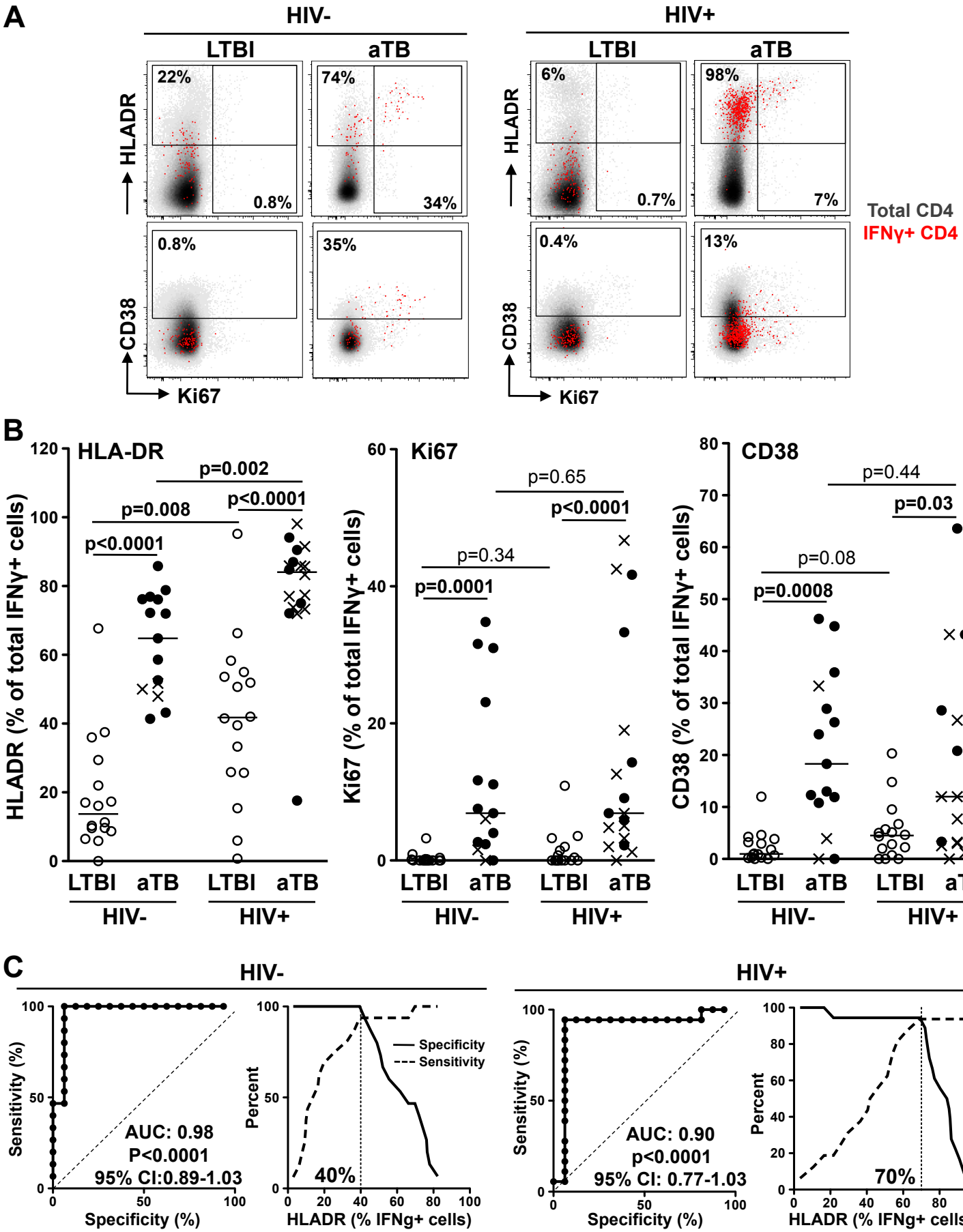
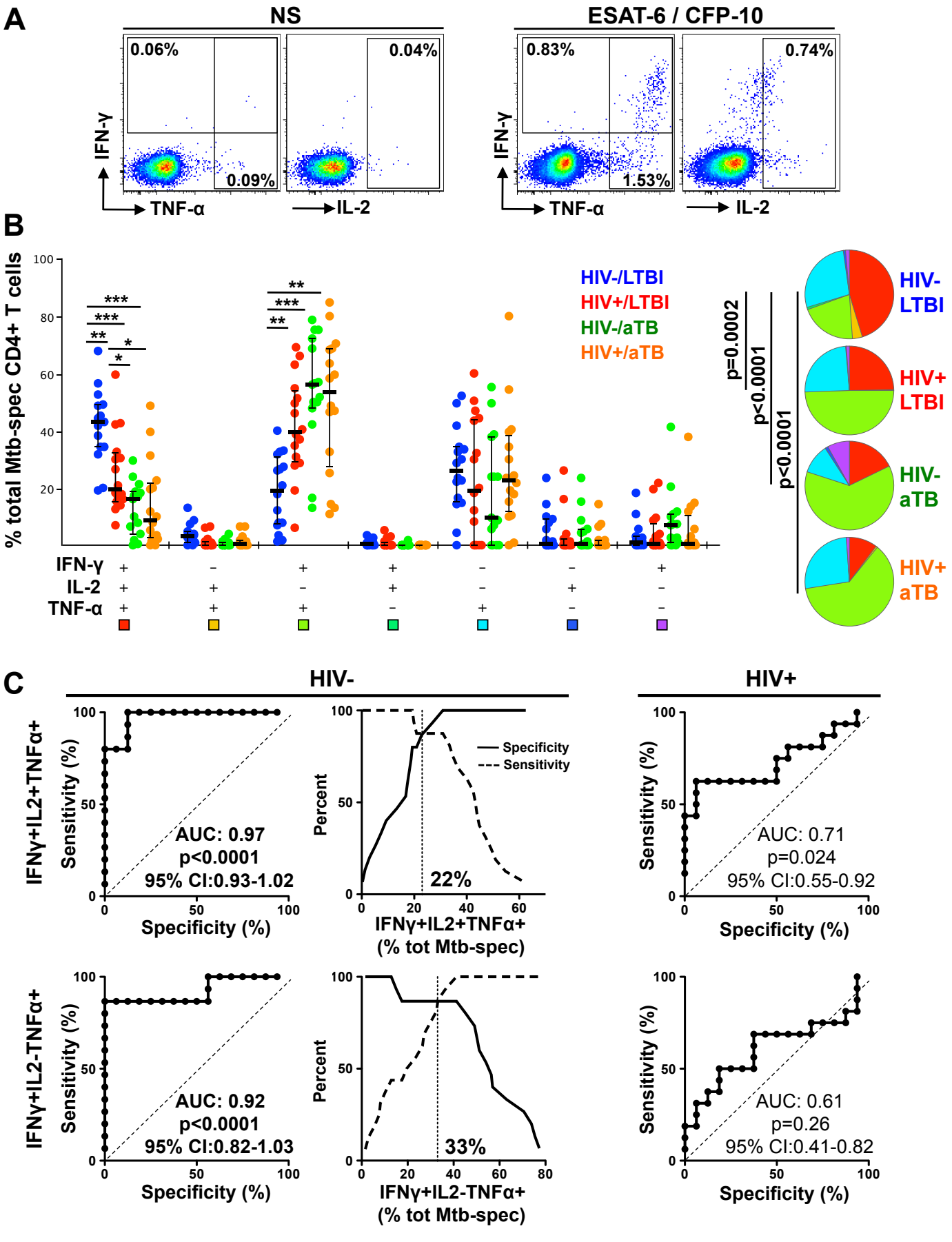

A
HIV-
HIV+
LTBI
aTB
HLADR
CD38
Ki67
22%
0.8%
74%
34%
0.8%
35%
6%
0.7%
98%
7%
0.4%
13%
Total CD4
IFNγ+ CD4
B
HLA-DR
HLADR (% of total IFNγ+ cells)
p=0.002
p=0.008
p<0.0001
p<0.0001
Ki67
Ki67 (% of total IFNγ+ cells)
p=0.65
p<0.0001
p=0.34
p=0.0001
CD38
CD38 (% of total IFNγ+ cells)
p=0.44
p=0.03
p=0.08
p=0.0008
C
HIV-
HIV+
Sensitivity (%)
Specificity (%)
AUC: 0.98
P<0.0001
95% CI:0.89-1.03
Percent
HLADR (% IFNg+ cells)
Specificity
Sensitivity
40%
AUC: 0.90
p<0.0001
95% CI: 0.77-1.03
70%

Figure 2


A
NS
ESAT-6 / CFP-10
0.06%
0.09%
0.04%
0.83%
1.53%
0.74%
IFN-γ
TNF-α
IL-2
B
% total Mtb-spec CD4+ T cells
HIV-/LTBI
HIV+/LTBI
HIV-/aTB
HIV+/aTB
p=0.0002
p<0.0001
p<0.0001
HIV-LTBI
HIV+LTBI
HIV-aTB
HIV+aTB
IFN-γ
IL-2
TNF-α
C
HIV-
HIV+
IFNγ+IL2+TNFα+
Sensitivity (%)
Specificity (%)
AUC: 0.97
p<0.0001
95% CI:0.93-1.02
Specificity
Sensitivity
Percent
22%
IFNγ+IL2+TNFα+
(% tot Mtb-spec)
AUC: 0.71
p=0.024
95% CI:0.55-0.92
IFNγ+IL2-TNFα+
AUC: 0.92
p<0.0001
95% CI:0.82-1.03
33%
AUC: 0.61
p=0.26
95% CI:0.41-0.82