Monitoring Circulating Immune Checkpoint Proteins as Predictors of Non-AIDS Morbid Events in People with HIV Initiating Antiretroviral Therapy

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ABSTRACT

Background. Although cell surface immune checkpoint proteins (ICPs) such as PD-1 expressed on T cells are associated with T cell exhaustion, HIV disease progression, and AIDS events, they have shown limited utility in predicting non-AIDS morbidity. Given that ICPs also exist in soluble forms and are elevated in ART-treated HIV infection, we tested the hypothesis whether soluble ICPs are predictive of non-AIDS events in adults initiating ART.

Methods. Utilizing a nested case-control study from the AIDS Clinical Trials Group ALLRT cohort we measured plasma levels of 15 soluble inhibitory and activating ICPs by Luminex. Participants (134 cases, 292 matched controls) were evaluated pre-ART, a year post-ART, and immediately preceding a non-AIDS event, which included myocardial infarction (MI)/stroke, malignancy, serious bacterial infection, and non-accidental death.

Results. Conditional logistic regression analysis determined that higher levels of soluble CD27 were associated with increased risk of non-AIDS events at all time points. Higher levels of CD40 at baseline and pre-event, and CD80 at pre-event were associated with increased risk of non-AIDS events. Examining specific non-AIDS events, multiple ICPs were associated with malignancy at baseline and pre-event whereas only higher CD27 levels were associated with increased risk of MI/stroke at year 1 and pre-event.

Conclusions. While select soluble ICPs were associated with non-AIDS events, CD27 emerged as a consistent marker irrespective of ART. Our data may offer guidance on new targets for early clinical monitoring in people with HIV who remain at greater risk of specific non-AIDS events.

Keywords. HIV; non-AIDS event; ART; morbidity; immune checkpoint proteins
INTRODUCTION

Despite the success of effective antiretroviral therapy (ART), people living with HIV (PLWH) disproportionately suffer from comorbidities, such as cardiovascular disease and non-AIDS-associated malignancies, compared to the general population. Compromised immune function, chronic activation of immune cells, and inflammation remains hallmarks of ART-treated HIV infection, and these factors are often thought to drive the occurrence of non-AIDS morbidities. Multiple mechanisms likely contribute to the increased morbidity risk and immune dysfunction in PLWH, including, but not limited to, the direct effects of HIV persistence, gut microbial translocation, increased circulation of inflammatory lipids, and co-infection persistence. While various biomarkers associated with inflammation, immune activation, and microbial translocation have been linked to non-AIDS events, immune regulatory pathways involved in the pathogenesis of these complications are not fully understood. Further elucidating these associated pathways could facilitate the development of ideal comprehensive clinical trial endpoints and the discovery of predictors for the use in the clinical management of PLWH on ART.

The persistent activation, exhaustion, and senescence of CD4+ and CD8+ T cells, natural killer (NK) cells, and B cells have been associated with HIV immunopathogenesis and AIDS-related complications; however, few studies have examined whether lymphocyte dysfunction characteristics are informative for non-AIDS morbid outcomes in PLWH on ART. The co-activation and dysfunction of T, B, and NK lymphocytes can be regulated by several immune checkpoint costimulatory and inhibitory receptor/ligand interactions, and the overexpression of these proteins, such as PD-1 (programmed cell death protein 1) and TIM-3 (T cell immunoglobulin and mucin-domain containing-3), have been shown to contribute to lymphocyte dysfunction and viral persistence in HIV. Immune checkpoint proteins (ICPs) also exist in soluble forms at normal physiological conditions, originating from the alternative splicing of mRNA or through the protease-mediated cleavage of their membrane-bound form. There is ongoing and increasing interest in measuring soluble ICPs as many have been elevated in the plasma of individuals with cancer, HIV, or other inflammatory diseases, suggesting they could serve as promising predictive biomarkers and may be involved in disease pathology. Our group and others have identified soluble ICPs, such as TIM-3 and CD27, associated with HIV disease progression and immune activation, yet the utility of ICPs as early biomarkers of non-AIDS events remains unclear.
Given the increased incidence of cardiovascular events and cancer in PLWH and the established link between ICPs and these comorbid outcomes in the general population, we aimed to measure and identify soluble ICPs in plasma as biomarkers of the onset of non-AIDS events. To investigate this relationship, plasma levels of soluble costimulatory and inhibitory checkpoint proteins were measured prior and post-ART initiation in PLWH who did or did not experience a non-AIDS event. We tested the predictive efficacy of these ICPs for total non-AIDS events and specific events, including mortality, cardiovascular events (myocardial infarction [MI] or stroke), and non-AIDS-defining malignancy. Ultimately, further understanding the link between these novel biomarkers of persistent lymphocyte activation and dysfunction and comorbid outcomes in HIV could uncover potential targets for therapeutic intervention and improve the clinical monitoring of PLWH virologically suppressed on ART.

METHODS

Study population. NWCS 411 is an ALLRT-nested case-control study of PLWH enrolled from 2001 to 2009, which examined potential predictive biomarkers and their relationships with non-AIDS events and death. This study builds off a previous case-control study, which examined a multitude of inflammatory and immune activation markers and found associations between several markers of immune activation and non-AIDS events and death, as previously described. Cases were defined as participants who experienced a MI or a stroke, a non-AIDS-defining malignancy, serious bacterial infection, or died from a nonaccidental non-AIDS-related event. For each case, 1-3 participants (controls) with an event-free follow-up equal or greater than the relevant case and matched for age and sex at time of visit, pre-ART CD4+ T cell count, and ART regimen at year 1 were evaluated.

All participants were ART-naive when enrolled in their original ACTG study and had plasma HIV RNA <400 copies/mL one-year post-ART initiation. Most study participants maintained their plasma HIV RNA <400 copies/mL after year 1; participants with values >400 copies/mL were included if preceding and subsequent HIV RNA values were <400 copies/mL without a change in ART regimen. For the events, the data were reported by sites following standardized ACTG diagnosis criteria. Cancer, MI, and serious bacterial infection events in the ALLRT analysis datasets were reviewed and confirmed by the ALLRT team. The non-accidental death designation was based on thorough review of reported cause of death by statisticians and study chairs. The team (ALLRT/NWCS) did not have direct access to medical records or death registries.
Soluble Immune Checkpoint Quantification. Stored plasma at the time before ART initiation (pre-ART), 1 year (48-64 weeks) after ART initiation, and the time (visit) proximal to/immediately preceding the non-AIDS event (and corresponding time point in controls) were tested for the following soluble co-stimulatory and inhibitory proteins using a multiplex immunoassay (Milliplex): CD27 (cluster of differentiation 27), CD28, CD40, CD80 (B7-1), CD86 (B7-2), GITR (glucocorticoid-induced TNFR-related protein), GITRL (GITR ligand), HVEM (herpesvirus entry mediator), BTLA (B- and T-lymphocyte attenuator), ICOS (inducible T cell co-stimulator), CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), LAG-3 (lymphocyte-activation gene 3), PD-1 (programmed cell death protein 1), PD-L1 (programmed death-ligand 1), and TIM-3 (T cell immunoglobulin and mucin-domain containing-3). Data was acquired on a Luminex 200™ analyzer (Luminex) and analyzed using MILLIPLEX® Analyst software (Millipore). All samples were analyzed in duplicate.

Statistics. Demographic and clinical characteristics are presented using median (IQR) for continuous variables and frequency for categorical variables. Soluble markers were $\log_{10}$ transformed prior to all analyses. Spearman rank correlations were used to assess the associations among ICPs, with pertinent clinical assessments and previously assessed biomarkers at each timepoint among the controls. Differences in paired data from baseline to year 1 were summarized with Hodges-Lehmann estimates and 95% confidence intervals (CIs). Conditional logistic regression analysis assessed associations of soluble ICPs with non-AIDS events. Models were adjusted for pertinent covariates at each timepoint, including the following potential confounders: HIV disease measures (concurrent $\log_{10}$ HIV RNA levels at baseline, CD4$^+$ T cell counts at year 1 and pre-event), chronic hepatitis B or C, intravenous drug use, waist-to-hip ratio, clinician-diagnosed diabetes, clinician-diagnosed hypertension, use of anti-hypertensive or lipid lowering agents, smoking status, and family history of MI. Additional models considered associations with adjustment for biomarkers previously linked to non-AIDS events. All analyses were performed in SAS version 9.4 (SAS Institute).

RESULTS

Description of Study Population

For our analysis we included three timepoints: baseline (pre-ART; 66 cases, 97 controls), one-year post-ART (112 cases, 211 controls), and immediately preceding an event (89 cases, 162 controls).
Case and control groups were comparable in age and sex at time of visit, CD4+ T cell counts at baseline (pre-ART) and ART regimen at week 48 (whether it contained a protease inhibitor or abacavir), and parent ACTG study (Table 1). At baseline, participants were predominately male (84%), with a median (Q1-Q3) age of 45 (39-51) years, 213 (79-334) cells/μL CD4+ T cell count, and 4.8 (4.4-5.4) log_{10} copies/mL plasma HIV RNA. CD4+ T cell counts at year 1 were 404 (269-561) cells/μL for controls and 347 (229-479) cells/μL for cases. Among cases, non-AIDS events occurred at a median of 2.8 years (Q1-Q3: 1.7-4.6) after ART initiation and 10.5 (6, 19) weeks from the pre-event timepoint, and included non-accidental deaths (13.4%), MI/stroke (28.4%), malignancy (37.3%), and serious bacterial infection (26.9%).

Co-stimulatory/Inhibitory Immune Checkpoint Protein Distributions and Correlates

Distributions of soluble co-stimulatory and inhibitory ICPs at each timepoint for cases and controls are illustrated in Figure 1. Of note, ICP levels of CD27 and TIM-3 decreased from baseline to one year after ART initiation in both cases and controls, while CD28, BTLA, and CD80 increased (Supplementary Table 1). We next assessed potential associations among ICPs and correlations with biomarkers of inflammation, immune activation, coagulopathy, and microbial translocation as well as pre-ART factors. ICPs were highly associated with one another at all timepoints (r≥0.60; Supplementary Figure 1A), apart from weak correlations with CD27, HVEM, LAG-3 and TIM-3. Overall, ICPs did not correlate with previous biomarkers associated with NAEs (Supplementary Figure 1B), except for HVEM, TIM-3, and CD27. HVEM and TIM-3 correlated with suPAR and TNFRI/II at baseline and pre-event (r≥0.33), and TNFRI/II at year 1 (r≥0.38); CD27 correlated with TNFRII at baseline (r=0.39). Among pre-ART factors, baseline ICPs HVEM and TIM-3 inversely correlated with CD4+ T cell count (r<0.22; Supplementary Figure 1C), TIM-3 correlated with HIV RNA level (r=0.36), and CD27, CD40, HVEM, and TIM-3 correlated with age (r≥0.22).

Pre-ART Soluble Immune Checkpoint Proteins Levels and Non-AIDS Events

Higher baseline plasma levels of CD27 (odds ratio (OR) per 1 IQR = 2.1; 95%CI, 1.2-3.6; p=0.008) and CD40 (OR=1.8; 95%CI, 1.1-2.8; p=0.019) were associated with increased risk of non-AIDS events in unadjusted conditional logistic regression analysis (Figure 2); however, associations were not observed with other ICPs at baseline. CD27 and CD40 associations with non-AIDS events remained significant after adjustment for HIV RNA levels and other potential confounders (Supplementary Tables 2-3), except for when adjusted for waist-hip ratio. When adjusted for biomarkers previously
shown to be predictive of non-AIDS events, associations were slightly attenuated for CD27 when adjusted for suPAR; and CD40 with adjustments for suPAR, EBV DNA, and CMV DNA (Supplementary Tables 4-5). Examining specific non-AIDS events, higher levels of CD27 were associated with increased risks of death (n=9 events; OR=4.8; 95%CI, 0.8-28.1) and MI/Stroke (n=15 events; OR=5.3; 95%CI, 0.8-35.4), and higher levels of CD40 were associated with increased risk of malignancy (n=28 events; OR=2.4; 95%CI, 0.9-6.9). Other IPCs were associated with increased risk of specific non-AIDS events, and include BTLA, GITRL, and CD80, CTLA4, and PD-L1, which associated with increased risk of malignancy (OR=2.2-3.7).

**Year 1 Soluble Immune Checkpoint Proteins Levels and Non-AIDS Events**

Conditional logistic regression results are detailed in Figure 3. Higher levels of CD27 were associated with increased risk of non-AIDS events at year 1 (OR=1.6; 95%CI, 1.2-2.2; p=0.001), while no significant associations were found with other soluble co-stimulatory proteins. This association with CD27 remained after adjustments for CD4+ T cell counts, other pertinent confounders and biomarkers previous shown to be predictive of non-AIDS events (Supplementary Table 6-7). Inhibitory checkpoints marker levels at year 1 were also not associated with increased risk of having a non-AIDS event. Regarding specific non-AIDS events, higher levels of CD27 and TIM-3 were associated with increased risk of MI/stroke (n=32 events; OR=2.3-2.9).

**Pre-Event Soluble Immune Checkpoint Protein Levels and Non-AIDS Events**

Figure 4 illustrates the associations between pre-event levels of soluble co-stimulatory proteins and non-AIDS events. Higher pre-event levels of CD27 (OR=2.1; 95%CI, 1.4-3.3; p<0.001), CD40 (OR=1.7; 95%CI, 1.2-2.5; p=0.008), and CD80 (OR=1.6; 95%CI, 1.1-2.4; p=0.017) were associated with increased risk of non-AIDS events. CD27 associations remained after adjustments for CD4+ T cell counts, confounders, and other biomarkers, while CD40 and CD80 associations were attenuated when adjusting for Hep B/C, smoking, and IL-6 (Supplementary Tables 8-13). The only inhibitory checkpoint marker associated with non-AIDS events was PD-1 (OR=1.5; 95%CI, 1.0-2.2; p=0.045); however, this association was attenuated when adjusting for CD4+ T cell count. Examining specific non-AIDS events, higher CD27 associated with an increased risk of MI/stroke (n=23 events; OR=4.1; 95%CI, 1.3-12.8), while CD40, CD80, GITRL, LAG-3, PD-1, and PD-L1 were all associated with increased risk of malignancy (n=35 events; OR=2.1-4.1).
DISCUSSION

Lymphocyte immune perturbations can be mediated through the immune checkpoint axis, many belonging to the CD28 and the TNFR superfamilies \(^{16,17}\). While phenotypic analysis expression of these checkpoints on specific cellular subsets has been extensively studied in HIV, these ICPs also exist in soluble forms and may give a proxy of overall lymphocyte activation and exhaustion status. Here we report that of 15 soluble ICPs, plasma CD27, CD40, and CD80 were associated with non-AIDS events overall. We demonstrate that many ICPs, particularly GITRL, CD80, and PD-L1, were associate with malignancy before and after ART initiation. However, soluble CD28, ICOS, HVEM, or CD86 showed no association with total or specific non-AIDS events. These findings reveal a previously unappreciated role for soluble ICPs in PLWH that may assist in predicting non-AIDS events.

A major outcome of this study was identifying circulating levels of CD27 as a predictor of non-AIDS event outcomes throughout the course of untreated and treated chronic HIV infection. Cell-surface CD27 is expressed on T cells and activated B cells and through binding to its ligand CD70, provides costimulatory signals to enhance proliferation and increase effector function \(^{18}\). However, tonic CD27-CD70 interaction is thought to cause immune dysregulation \(^{19}\). In PLWH, CD27 on effector-memory CD8\(^+\) CTLs promotes their long-term survival while an upregulation on CD4\(^+\) T cells is associated with poor proliferative responses \(^{20,21}\). Soluble CD27 can be derived from the shedding by activated lymphocytes \(^{22}\). Elevated plasma levels of CD27 are associated with HIV disease progression and have been suggested as a surrogate marker of immune activation during HIV infection \(^{15,23,24}\). Higher pre-ART levels of CD27 have also been associated with AIDS-defining malignancies and mortality that occurred after the initiation of suppressive treatment \(^{25,26}\). Further understanding its significance throughout the course of HIV would reveal its potential as a clinical indicator for total comorbid complications during durable and suppressive ART.

To our knowledge, the soluble forms of CD40 and CD80 have not previously been associated with HIV severe outcomes. Cell-bound CD40 is mainly regulated to antigen presenting cells (APCs) and upon binding to its respective ligand, CD40L, induces their activation. CD80 is expressed on activated APCs, monocytes, and B cells, and binds to CD28 and CTLA-4 to activate or inhibit T cell responses, respectively \(^{17}\). Levels of plasma CD40, as well as CD27, were higher in individuals prior to death due
to alcoholic hepatitis compared to those that survived, with CD40 being able to predict a 90-day mortality risk. While not observed in our study, CD40 in blood has been associated with organ damage, particularly stroke, in those with hypertension outside the context of HIV infection.

Interestingly, in our study most soluble ICPs did not correlate with biomarkers associated with inflammatory, myeloid activation, and microbial translocation mechanisms, indicating these are possibly independent pathways linked to non-AIDS events and revealing an underappreciated role for these markers in the context of HIV infection. ICPs were also highly intercorrelated, illustrating these additional immune activation and exhaustion pathways could be intertwined with one another. Many of the inhibitory immune checkpoints become expressed after activation to TCR and BCR mechanism. Whether levels we find in circulation represent the activation or exhaustive state of peripheral or tissue-resident lymphocytes should be determined.

Another important finding of our study is that we identified many soluble costimulatory and inhibitory ICPs associated specifically with the onset of cancer, particularly at pre-ART and pre-event timepoints. ICPs have been heavily investigated in cancer as potential prognostic markers. Plasma CD40 was previously identified as a potential prognostic marker of malignant pleural effusion with non-small cell lung cancer. Elevated levels of CD80 are shown to be associated with the incidence of various hematologic malignancies with malignant cells, in addition to APCs, suggested as potential sources. Additionally, soluble forms of PD-1, PD-L1, LAG-3 are thought to have predictive and prognostic significance in various cancers as well as directly correlating with anti-tumor immunity.

ICPs are shown to be functional as their soluble forms in vitro by acting as antagonist decoys that competitively regulate their membrane-bound counterparts. For instance, CD27 cleaved from T cells is shown to bind membrane-associated CD70 on APCs, facilitating competition between T cells for access to MHC:peptide complexes, while soluble CD40 can bind membranous CD154 and inhibit B cell activity. Soluble ICPs can also directly stimulate activation and/or inhibition checkpoint pathways. Soluble CD27 has been shown to directly contribute to the activation of T cells and B cells leading to a state of hyper-activation. Soluble CD80 on the other hand can enhance anti-tumor responses by promoting tumor-infiltrating lymphocytes by simultaneously inhibiting PD-1 and providing co-stimulation of anti-tumor T cell activity. Immunotherapeutic strategies targeting ICP
pathways are ongoing in clinical trials for cancer and are being investigated as HIV curative approaches by enhancing anti-HIV T cell responses \(^{36,37}\). Whether fluctuating levels of the soluble forms of ICPs play a role in altering the efficacy of these strategies targeting membrane-bound immune checkpoint receptors or ligands should be determined.

Our study has several limitations. Case and control numbers varied across time points due to limitations on specimen availability, which limits the ability to evaluate longitudinal changes and could explain some of our inconsistent findings. ICPs were found to be highly correlated with one another. However, these intercorrelations could be driven by the multiplex due to a systemic artifact that renders these markers close together. Further studies will need to determine if these associations might represent the debris that remains in circulation after cellular turnover or death. Our analysis only consisted of adjustments for single variables. This was to check the consistency of the results when considering potential confounders rather than to create a predictive model. Of note, the prevalence of Hep B/C was rather dissimilar between cases and controls. Although we adjusted for Hep B/C in our analyses, these differences between the two groups could still potentially influence their relative ICP expression. Many study participants were on ART regimens that have been phased out and no longer recommended for initial treatment of HIV infection, including D4T that can cause serious side effects such as peripheral neuropathy and lipoatrophy \(^{38}\). However, only 55 (17%) of participants were on D4T at year 1 and case and controls were matched by ART regimen. Furthermore, as current first line regimens, such as integrase strand transfer inhibitors, were not represented in the cohort, it would be essential to determine whether these findings could be extrapolated to a contemporary PLWH population who now start modern ART much sooner after HIV diagnosis and achieve virologic suppression at a faster rate. Finally, HIV participants within our cohort had relatively lower CD4\(^+\) T cell counts as compared to those who receive modern ART regimens immediately upon diagnosis and women were under-represented, of importance as there are sex-related differences in the risk of select non-AIDS comorbidities \(^{39}\).

While our findings of soluble ICPs as potential predictors for severe outcomes are compelling, the next logical step would be to confirm that these associations are consistent using a HIV cohort with a modern and early initiated ART regimen. Also, larger prospective studies evaluating a broader assessment of non-AIDS events will allow the confirmation of these markers at various stages of disease and ART exposure. Of practical interest, their potential use as surrogate markers for end...
points in clinical trials targeting inflammation and immune dysfunction aimed to alleviate morbidity and mortality in PLWH on ART should be evaluated. These studies should focus on whether individual ICPs or their inclusion in composite panels can serve as optimal predictors and monitoring tools. Furthermore, subsequent studies should determine if there is a causative link between soluble ICPs, perturbations in lymphocyte function, and co-morbid events, which may facilitate uncovering the molecular mechanisms soluble ICPs may be involved in during untreated and treated HIV infection and lay groundwork for potential therapeutic targets.

Patient Consent Statement

Participants (or, for minors, their parent or legal guardian) provided written informed consent, and institutional review board approval for ALLRT was obtained by each ACTG site.

Availability of data and materials

Individual participant data and a data dictionary defining each field in the set will be made available to investigators on a case-by-case basis via request to the AIDS Clinical Trials Group (ACTG) via the link: https://submit.actgnetwork.org/. Completion of an ACTG Data Use Agreement may be required.

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Potential Conflicts of interest

LCN has served on an advisory board for Abbvie, ViiV, and Cytodyn for work unrelated to this project. MH has received project funding from Gilead, Pfizer, and Astellas. MML has consulted for Lilly and has received competitive grant funding from Gilead for unrelated work. All other authors declare no competing interests.
Author contributions
TAP and LCN wrote the manuscript; TAP conducted soluble marker acquisition and data interpretation; CBM and AM contributed to data analysis and interpretation; STY, APSP, MJL, MH, EIL, MML, and ALL provided critical review of the manuscript. LCN, SG, and CBM contributed to study design and concept. All authors reviewed the manuscript.

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Figure Legends

Figure 1. Distribution of soluble co-stimulatory and inhibitory checkpoints. Levels among cases (blue) and controls (black) at pre-ART initiation (baseline), a year after ART initiation (year 1), and visit immediately preceding a non-AIDS event (pre-event). Jitter plots including median and interquartile range are displayed.

Figure 2. Soluble co-stimulatory and inhibitory checkpoint protein levels and odds ratios (ORs) of having a non-AIDS event at baseline (pre-ART). Adjusted analyses controlled for concurrent HIV viral load. $^dP<0.05$, $^eP<0.1>0.05$. MI=myocardial infarction.

Figure 3. Soluble co-stimulatory and inhibitory checkpoint protein levels and odds ratios (ORs) of having a non-AIDS event at year 1. Adjusted analyses controlled for concurrent CD4 count. $^cP<0.01$, $^dP<0.05$, $^eP<0.1>0.05$. MI=myocardial infarction.

Figure 4. Soluble co-stimulatory and inhibitory checkpoint protein levels and odds ratios (ORs) of having a non-AIDS event at pre-event. Adjusted analyses controlled for concurrent CD4 count. $^dP<0.05$. MI=myocardial infarction.
Table 1: Cohort demographic and clinical characteristics at baseline

| Characteristic                                      | Case (N=134) | Control (N=292) | Total (N=426) |
|-----------------------------------------------------|--------------|-----------------|---------------|
| **Age at parent study entry (years)**               | 47 (40-53)   | 44 (39-50)      | 45 (39-51)    |
| **Regimens evaluated, by parent study**             |              |                 |               |
| ACTG 384: (AZT + 3TC vs d4T + ddI) + (EFV vs NFV vs NFV + EFV) | 40 (30%) | 85 (29%) | 125 (29%) |
| ACTG 388: (AZT + 3TC vs d4T + 3TC) + (IDV vs NFV vs IDV + NFV) |            |                 |               |
| A5014: NVP + [LPV/r vs (ABC + 3TC + d4T)]/          |              |                 |               |
| A5095: AZT/3TC + (ABC vs EFV vs ABC + EFV)/         | 62 (46%)     | 144 (49%)       | 206 (48%)     |
| A5142: (EFV + AZT/d4T + 3TC) vs (LPV/r + AZT/d4T + 3TC) vs (EFV + LPV/r) | | | |
| A5202: (ABC/3TC vs TFV/FTC) + (ATV/r vs EFV)        | 32 (24%)     | 63 (22%)        | 95 (22%)      |
| **Sex**                                             |              |                 |               |
| Male                                                | 112 (84%)    | 247 (85%)       | 359 (84%)     |
| Female                                              | 22 (16%)     | 45 (15%)        | 67 (16%)      |
| **Race/ethnicity**                                  |              |                 |               |
| White Non-Hispanic                                  | 70 (52%)     | 138 (47%)       | 208 (49%)     |
| Black Non-Hispanic                                  | 48 (36%)     | 82 (28%)        | 130 (31%)     |
| Hispanic (regardless of race)                       | 15 (11%)     | 61 (21%)        | 76 (18%)      |
| Other                                               | 1 (1%)       | 11 (4%)         | 12 (3%)       |
| **Baseline CD4+ T cell count (cells/µl)**           | 207 (87-334) | 220 (76-332)    | 213 (79-334)  |
| **Baseline log10 HIV-1 RNA (copies/mL)**            | 4.8 (4.4-5.3)| 4.8 (4.4-5.4)   | 4.8 (4.4-5.4) |
|                                |       |       |       |
|--------------------------------|-------|-------|-------|
| Chronic hepatitis B/C status   | 33 (25%) | 29 (10%) | 62 (15%) |
| Current or previous injection drug use | 17 (13%) | 26 (9%) | 43 (10%) |
| Waist-to-hip ratio             | 0.92 (0.89-0.96) | 0.92 (0.88-0.97) | 0.92 (0.89-0.97) |
| History of clinician-diagnosed diabetes | 11 (8%) | 15 (5%) | 26 (6%) |
| History of hypertension        | 43 (32%) | 55 (19%) | 98 (23%) |
| Use of antihypertensive or lipid lowering agents | 30 (22%) | 40 (14%) | 70 (16%) |
| Current or past smoker         | 100 (75%) | 159 (54%) | 259 (61%) |
| Family history of myocardial infarction | 28 (21%) | 43 (15%) | 71 (17%) |

Categorical variables are represented as frequency and continuous variables as median (Q1-Q3). Abbreviations: 3TC (lamivudine), ABC (abacavir), ATZ/r (ritonavir-boosted atazanavir), AZT (zidovudine), d4T ( stavudine), ddI (didanosine), EFV (efavirenz), FTC (emtricitabine), IDV (indinavir), LPV/r (ritonavir-boosted lopinavir), NFV (nelfinavir), NVP (nevirapine), TFV (tenofovir)
Figure 1
| Soluble Costimulatory Checkpoints | OR (95% CI) per one IQR | P-value | OR | OR | OR |
|----------------------------------|--------------------------|---------|----|----|----|
|                                  |                          |         | Death | Cancer | MI/Stroke |
| CD28                            | Unadjusted               | 1.3 (0.8, 2.1) | 0.302 | 0.6 | 2.3* | 0.8 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.3 (0.8, 2.2) | 0.270 |         |         |     |
| CD27                            | Unadjusted               | 2.1 (1.2, 3.8) | 0.008 | 4.8* | 1.8 | 5.3* |
|                                 | Adjusted for HIV-1 RNA (log 10) | 2.3 (1.3, 3.8) | 0.006 |         |         |     |
| CD40                            | Unadjusted               | 1.8 (1.1, 2.8) | 0.019 | 13.5 | 2.4* | 1.3 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.8 (1.1, 3.0) | 0.015 |         |         |     |
| ICOS                            | Unadjusted               | 1.4 (0.9, 2.2) | 0.160 | 0.6 | 2.1* | 0.8 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.4 (0.9, 2.3) | 0.120 |         |         |     |
| GITR                            | Unadjusted               | 1.2 (0.8, 1.9) | 0.334 | 0.7 | 2.0* | 0.6 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.2 (0.8, 1.9) | 0.335 |         |         |     |
| GITRL                           | Unadjusted               | 1.2 (0.9, 2.1) | 0.164 | 0.6 | 2.2* | 0.9 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.4 (0.9, 2.2) | 0.140 |         |         |     |
| HVEM                            | Unadjusted               | 1.3 (0.9, 2.3) | 0.200 | 2.5 | 1.2 | 1.5 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.3 (0.9, 2.0) | 0.170 |         |         |     |
| BTLA                            | Unadjusted               | 1.3 (0.8, 2.1) | 0.227 | 0.6 | 2.4* | 0.8 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.4 (0.9, 2.2) | 0.169 |         |         |     |
| CD60                            | Unadjusted               | 1.2 (0.8, 1.8) | 0.304 | 0.5 | 2.3* | 0.8 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.2 (0.8, 1.9) | 0.305 |         |         |     |
| CD66                            | Unadjusted               | 1.2 (0.8, 1.9) | 0.345 | 0.6 | 1.9 | 0.8 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.3 (0.8, 2.0) | 0.263 |         |         |     |

| Soluble Inhibitory Checkpoints  | OR (95% CI) per one IQR | P-value | OR | OR | OR |
|----------------------------------|--------------------------|---------|----|----|----|
|                                  |                          |         | Death | Cancer | MI/Stroke |
|                                 |                          |         |        |        |        |
| CTLA4                           | Unadjusted               | 1.6 (1.0, 2.7) | 0.069 | 0.9 | 2.9* | 1.0 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.7 (1.0, 2.9) | 0.057 |         |         |     |
| LAG-3                            | Unadjusted               | 1.1 (0.7, 1.7) | 0.800 | 0.9 | 1.2 | 0.7 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.1 (0.7, 1.7) | 0.790 |         |         |     |
| PD-1                             | Unadjusted               | 1.4 (0.8, 2.3) | 0.220 | 0.8 | 2.7* | 0.8 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.4 (0.8, 2.4) | 0.187 |         |         |     |
| PD-L1                            | Unadjusted               | 1.4 (0.9, 2.2) | 0.186 | 0.5 | 3.7* | 0.8 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.4 (0.9, 2.3) | 0.161 |         |         |     |
| TIM-3                            | Unadjusted               | 1.1 (0.7, 1.8) | 0.568 | 1.3 | 1.6 | 1.3 |
|                                 | Adjusted for HIV-1 RNA (log 10) | 1.1 (0.7, 1.8) | 0.556 |         |         |     |
### Soluble Costimulatory Checkpoints

| Checkpoint | Unadjusted | Adjusted for CD4 count |
|------------|------------|------------------------|
| CD28       | 1.0 (0.7, 1.3) | 0.931 (0.7, 1.4) |
| CD7        | 1.6 (1.2, 2.2) | 0.001 (1.2, 2.2) |
| CD40       | 1.2 (0.9, 1.6) | 0.268 (0.9, 1.6) |
| ICOS       | 1.0 (0.7, 1.3) | 0.854 (0.7, 1.3) |
| GITR       | 0.9 (0.7, 1.2) | 0.507 (0.7, 1.2) |
| GITRL      | 1.1 (0.8, 1.4) | 0.694 (0.8, 1.4) |
| HVEM       | 1.1 (0.9, 1.4) | 0.479 (0.9, 1.4) |
| BTLA       | 1.0 (0.7, 1.3) | 0.876 (0.7, 1.3) |
| CD80       | 1.2 (0.8, 1.8) | 0.334 (0.8, 1.8) |
| CD86       | 0.9 (0.7, 1.2) | 0.614 (0.7, 1.2) |

### Soluble Inhibitory Checkpoints

| Checkpoint | Unadjusted | Adjusted for CD4 count |
|------------|------------|------------------------|
| CTLA4      | 1.0 (0.8, 1.4) | 0.844 (0.8, 1.4) |
| LAG-3      | 0.9 (0.7, 1.2) | 0.445 (0.7, 1.2) |
| PD-1       | 1.0 (0.8, 1.4) | 0.628 (0.8, 1.4) |
| PD-L1      | 1.0 (0.8, 1.4) | 0.873 (0.8, 1.4) |
| TIM-3      | 1.1 (0.8, 1.4) | 0.560 (0.8, 1.4) |

*OR (95% CI) per one IQR*
| Soluble Costimulatory Checkpoints | OR (95% CI) per one IQR | P-value | OR Death | OR Cancer | OR MI/Stroke |
|----------------------------------|---------------------------|---------|----------|-----------|-------------|
| CD28                            |                           |         |          |           |             |
| Unadjusted                      | 1.4 (0.9, 2.1)            | 0.106   | 1.1      | 1.3       | 1.7         |
| Adjusted for CD4 count           | 1.3 (0.9, 2.0)            | 0.180   |          |           |             |
| CD27                            |                           |         |          |           |             |
| Unadjusted                      | 2.1 (1.4, 3.3)            | <.001   | 2.1      | 1.7       | 4.1<sup>4</sup> |
| Adjusted for CD4 count           | 2.1 (1.4, 3.3)            | <.001   |          |           |             |
| CD40                            |                           |         |          |           |             |
| Unadjusted                      | 1.7 (1.2, 2.5)            | 0.008   | 1.5      | 2.4<sup>4</sup> | 2.1         |
| Adjusted for CD4 count           | 1.7 (1.1, 2.5)            | 0.013   |          |           |             |
| ICOS                            |                           |         |          |           |             |
| Unadjusted                      | 1.3 (0.9, 1.9)            | 0.126   | 1.1      | 1.7       | 1.2         |
| Adjusted for CD4 count           | 1.3 (0.9, 1.8)            | 0.218   |          |           |             |
| GITR                            |                           |         |          |           |             |
| Unadjusted                      | 1.3 (0.9, 1.7)            | 0.149   | 1.2      | 1.1       | 1.2         |
| Adjusted for CD4 count           | 1.2 (0.9, 1.6)            | 0.294   |          |           |             |
| GITRL                           |                           |         |          |           |             |
| Unadjusted                      | 1.6 (1.0, 2.4)            | 0.034   | 1.3      | 2.5<sup>4</sup> | 1.4         |
| Adjusted for CD4 count           | 1.5 (1.0, 2.3)            | 0.066   |          |           |             |
| HVEM                            |                           |         |          |           |             |
| Unadjusted                      | 1.4 (1.0, 1.9)            | 0.089   | 0.8      | 1.2       | 1.7         |
| Adjusted for CD4 count           | 1.3 (0.9, 1.8)            | 0.175   |          |           |             |
| BTLA                            |                           |         |          |           |             |
| Unadjusted                      | 1.4 (0.9, 2.0)            | 0.111   | 1.6      | 1.5       | 1.3         |
| Adjusted for CD4 count           | 1.3 (0.9, 1.9)            | 0.152   |          |           |             |
| CD86                            |                           |         |          |           |             |
| Unadjusted                      | 1.6 (1.1, 2.4)            | 0.017   | 1.3      | 2.3<sup>4</sup> | 1.7         |
| Adjusted for CD4 count           | 1.5 (1.0, 2.3)            | 0.035   |          |           |             |
| Soluble Inhibitory Checkpoints   |                           |         |          |           |             |
| CTLA-4                          |                           |         |          |           |             |
| Unadjusted                      | 1.5 (1.0, 2.2)            | 0.066   | 1.5      | 1.3       | 1.5         |
| Adjusted for CD4 count           | 1.4 (0.9, 2.1)            | 0.124   |          |           |             |
| LAG-3                           |                           |         |          |           |             |
| Unadjusted                      | 1.1 (0.8, 1.6)            | 0.619   | 1.5      | 2.3<sup>4</sup> | 0.8         |
| Adjusted for CD4 count           | 1.0 (0.7, 1.5)            | 0.961   |          |           |             |
| PD-1                            |                           |         |          |           |             |
| Unadjusted                      | 1.5 (1.0, 2.2)            | 0.045   | 1.4      | 2.1<sup>4</sup> | 1.4         |
| Adjusted for CD4 count           | 1.4 (1.0, 2.1)            | 0.079   |          |           |             |
| PD-L1                           |                           |         |          |           |             |
| Unadjusted                      | 1.5 (1.0, 2.2)            | 0.051   | 1.2      | 2.2<sup>4</sup> | 1.3         |
| Adjusted for CD4 count           | 1.4 (0.9, 2.1)            | 0.007   |          |           |             |
| TIM-3                           |                           |         |          |           |             |
| Unadjusted                      | 1.2 (0.9, 1.7)            | 0.177   | 1.3      | 1.0       | 1.7         |
| Adjusted for CD4 count           | 1.2 (0.9, 1.7)            | 0.157   |          |           |             |