HIV and Cardiovascular Diseases Risk: Exploring the Interplay between T cell Activation, Coagulation, Monocyte Subsets and Lipid Subclass Alterations


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ABSTRACT

Although rollout of combined anti-retroviral treatment (cART) has blunted human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) onset, there is increased development of cardiovascular diseases (CVD) in HIV-infected individuals. While most HIV-infected individuals on cART achieve viral suppression, this may not necessarily result in complete immunological recovery. This study therefore evaluated T cell-mediated changes and coagulation markers in HIV-positive individuals to ascertain their potential to increase CVD risk. Eighty participants were recruited (Worcester, South Africa) and fasted blood collected to evaluate: a) immune activation (CD38 expression on CD4+ and CD8+ T cells) and thrombus formation (tissue factor [CD142]) on CD4+ and CD8+ T cells; b) monocyte subpopulations (non-classical, intermediate, classical) and; and c) classical regulatory T (Treg) cells with activation markers (glycoprotein A repetitions predominant [GARP], special AT-rich sequence-binding protein 1 [SATB-1]). High-and low-density lipoprotein subclasses (Lipoprint) were also determined. This study revealed four key findings for HIV-positive patients: a) co-expression of the CD142 coagulation marker together with immune activation on both CD4+ and CD8+ T cells during chronic infection stages; b) Treg cell activation and upregulated GARP and SATB-1 contributing to Treg dysfunction in chronic HIV; c) Pro-atherogenic monocyte subset expansion with significant correlation between T cell activation and macrophage activation (marker: CD163); and d) significant correlation between immune activation and lipid subclasses, revealing crucial changes that can be missed by traditional lipid marker assessments (LDL, HDL). These data also implicate lipopolysaccharide-binding protein (LBP) as a crucial link between immune activation, lipid alterations and increased CVD risk. (248 words).

Key words: HIV; cardiovascular diseases; immune activation; coagulation; lipid subclasses; Treg cells; monocyte subsets; lipopolysaccharide-binding protein
New & Noteworthy

With combined anti-retroviral treatment rollout, HIV-AIDS patients are increasingly associated with cardiovascular diseases onset. This study demonstrated the significant interplay between adaptive immune cell activation and monocyte/macrophage markers in especially HIV-positive individuals with virological failure and on second line treatment. Our data also show a unique link between immune activation and lipid subclass alterations, revealing important changes that can be missed by traditional lipid marker assessments (e.g. LDL and HDL).
1. INTRODUCTION

Although rollout of combined anti-retroviral treatment (cART) has been highly effective in controlling human immunodeficiency virus (HIV) and delaying acquired immunodeficiency syndrome (AIDS) onset, there is increased development of cardiovascular diseases (CVD) in HIV-infected individuals. While most HIV-infected individuals on cART achieve viral suppression, this may not necessarily result in complete immunological recovery (26). For example, significant CD4 T cell depletion from gut mucosa and subsequent microbial translocation are linked to systemic immune activation and permanent changes to host immune cells (34). Microbial translocation can in turn induce persistent macrophage activation that is unrelated to HIV viral load (34). The SMART Study was the first to show a strong association between inflammation, coagulation and CVD morbidity/mortality in HIV-infected individuals (20). Here D-dimer and interleukin-6 (IL-6) were crucial biomarkers for all-cause and CVD-related mortality. Tissue factor (TF) is also an important intracellular coagulation marker that can initiate extrinsic clotting pathways and thereby act as a link between inflammation and coagulation (23).

During immune activation, regulatory T (Treg) cells are major role players that mediate a response to HIV infection. Although there is evidence that Treg cells elicit beneficial effects by limiting immune activation, they may also trigger detrimental outcomes by suppressing the anti-viral immune response (18). T cell involvement in atherosclerosis is based on the notion that an imbalance between pathogenic T (Type 1 and 2 T helper) and Treg cells leads to the development and progression of atherosclerosis (24). Moreover, during HIV infection monocytes display activated phenotypes with expansion of the CD14+CD16+ subpopulation that is preferentially susceptible to HIV-1 infection (9, 13). The identification of such subpopulations during HIV infection is therefore important to better understand monocyte function and its links to immune activation and increased CVD risk.

Although immune activation and inflammation have emerged as potential contributors to HIV-mediated CVD onset, the underlying mechanisms driving this process require further elucidation. Furthermore, simultaneous evaluation of monocyte and T cell subset (CD4, CD8, Treg) alterations and their relationship to CVD risk markers have not been extensively investigated. For this study we
hypothesized that HIV-induced low-grade inflammation perturbs immune cell function/activation status together with lipid subclass alterations, contributing to increased CVD risk.

2. METHODS

2.1 Study population

This cross-sectional study forms part of a larger longitudinal investigation, the Cape Winelands HAART to HEART study (Worcester Community Day Centre, South Africa). This study complies with the Declaration of Helsinki and ethical approval was obtained from the Human Research Ethics Committee of Stellenbosch University and the Department of Health (Western Cape Government, South Africa). Prior to the study, all participants were informed about procedures and consent forms were signed by all. The inclusion criteria were male and female HIV-positive and HIV-negative participants aged 18-55 years, while HIV/tuberculosis infection patients and pregnant women were excluded. Subject recruits were age-matched and divided: HIV-negative (n= 13), HIV-positive cART naïve (n= 26), and HIV-positive on cART (total n= 41; first line n=21; second line n=20) (duration/median 60 months [range: 34-90 months]). For the first line treatment regimen, patients received: TDF (Tenofovir) + either 3TC (Lamivudine) or FTC (Emtricitabine) + EFV (Efavirenz). Some of patients received NVP (Nevirapine) instead of EFV. For second line treatment, all the patients received Aluvia (Lopinavir and Ritonavir [LPV/r]) + Lamzid (AZT [Zidovudine] + 3TC). All participants were recruited from the same community to ensure that they shared similar CVD risk and socio-economic backgrounds. We also employed a validated lifestyle questionnaire to document demographic and other baseline parameters, while vital sign assessments (pulse and blood pressure) were measured before blood collection.

2.2 Evaluation of systemic markers

After the completion of informed consent, fasted venous blood samples were obtained at the Worcester site and tested for CD4 counts, lipid profile (total cholesterol, triglycerides, low-density lipoprotein [LDL], high-density lipoprotein [HDL]) performed at the Worcester National Health Laboratory Service within 30 min. Plasma and serum were also immediately isolated and stored at -
80°C for analysis of viral load and various ELISA tests: D-dimer (marker for coagulation), lipopolysaccharide binding protein (LBP) (acute phase protein that can initiate a pro-inflammatory response), CD163 (marker for macrophage activation) (Abcam, Cambridge UK). The Lipoprint system (Quantimetrix, Redondo Beach CA) was employed for lipoprotein sub-fraction analysis: very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) 1-3, LDL 1-7 and HDL subclasses (17).

2.3 Assessment of immune activation markers and coagulation status by flow cytometric analysis

Cell preparation and staining
Blood samples were collected in EDTA tubes, mixed with BD FACS lysing solution (BD Biosciences, San Jose CA) for 10 min. Samples were then added to two separate tubes (100 µL/tube), i.e. the first one contained a monocyte and CD8+ T cell activation markers panel, while the second one consisted of a Treg marker panel – supplied by BD Biosciences (San Jose CA). The appropriate amount of antibody was then added (according to previously determined antibody panel titrations) to the respective tubes, followed by incubation for 30 min at room temperature in the dark, washed with 2 mL of PBS and centrifuged at 500 g for 5 min. Cells were washed twice with PBS and samples were processed using the BD FACS Aria flow cytometer (BD Biosciences, San Jose CA) and analyzed using FACS Diva software (BD Biosciences, San Jose CA). One hundred thousand events were collected per sample tube and expression for each marker determined based on total gated events.

Antibody panels
The following fluorochrome-conjugated antibody panels (BD Biosciences, San Jose CA) were employed for the immunological assessments performed by flow cytometry in this study:

a) For immune activation and coagulation analysis of T-lymphocytes and monocyte analysis:
   APC-H7 mouse anti-human CD3; BV421 mouse anti-human CD8; PE anti-CD38; APC anti-human CD142 (HTF); PE-CF594 mouse anti-human CD14; PE-CY7 mouse anti-human CD16.
b) For Treg cell analysis: APC-H7 mouse anti-human CD3; FITC mouse anti-human CD4; PE-CY7 mouse anti-human CD25; V450 mouse anti-human FOXP3; PE mouse anti-human glycoprotein A repetitions predominant [GARP]; Alexa fluor 647 mouse anti-human special AT-rich sequence-binding protein 1 [SATB-1]

Antibody titrations

The first titration experiment was performed with the highest recommended antibody volume (manufacturer instructions), where after it was titrated downwards (doubling dilution). Cells were washed and stained with the respective antibody dilutions and data were acquired using the BD FACS Aria (BD Biosciences, San Jose CA) followed by post-acquisition analysis using FACS Diva software (BD Biosciences, San Jose CA). A histogram was generated for each antibody titration showing both negative and positive populations for each antibody. Population statistics of the histogram were generated and the values were exported to Excel sheets (for each antibody) and used for generation of a standard curve. Additionally, the staining index (SI) value for each titration was calculated using the equation

\[ SI = \left( \frac{\text{MFI}_{\text{pos}} - \text{MFI}_{\text{neg}}}{\text{SD}_{\text{neg}}} \right) \]

(MFI= mean fluorescence intensity, SD= standard deviation).

Flow cytometry color compensation

BD Comp beads (BD Biosciences, San Jose CA) were used to perform color compensation. One full drop each of BD Comp beads (negative control) and BD Comp beads (positive control; anti-mouse Igκ) were added to 100 µL of staining buffer, followed by addition of the titrated antibody volume (for each marker) and incubation for 30 min in the dark at room temperature. Thereafter, 0.5 mL staining buffer was added to each tube and data acquired using the BD FACS Aria, followed by compensation.

Fluorescence minus one (FMO)

FMO was performed in order to differentiate between positive and negative events. This procedure involves the addition of all fluorescent reagents to a cell sample except for one. Eleven FMO tubes were prepared, i.e. each tube with all antibodies except the antibody that the respective tube was labeled with. After the appropriate antibodies were added to the respective tubes, 100 µL of FACS-
lysing blood was added and tubes gently mixed and incubated for 30 min in the dark at room temperature. Samples were stained with the appropriate antibodies and FMO analyses performed.

**Gating strategy for CD8+ activation markers**

One hundred thousand events were collected for each tube and cells were gated according to lymphocyte population. The side scatter (SSC) was plotted against forward scatter (FSC) to identify and differentiate lymphocytes. T cells were extracted from lymphocytes by plotting CD3 APC-H7 vs. SSC-A, while the doublets were excluded using forward scatter-height (FSC-H) vs. forward scatter-area (FSC-A). We then identified the CD3+CD8+ cells and determined the expression of CD38 and CD142 on CD8+ T cells.

**Gating strategy for monocyte sub-population and panel**

The identification of monocyte sub-populations was based on the expression of CD14 and CD16 markers. Furthermore, FMO for CD16 was used to differentiate between CD16+ and CD16- populations. CD14 vs. SSC were first analyzed to identify (separate) individual monocyte populations. The single cell monocyte (singlets) was generated using FSC-H and FSC-A. This was followed by CD14+CD3- vs. SSC to ensure that the CD3+ population was excluded from the monocyte population. Lastly, we performed CD16+ vs. SSC.

**Gating strategy for Treg cells**

The identification of Treg cells was based on the expression of classical Treg CD3+CD4+CD25++FOXP3+ and the identification of activated markers (GARP, SATB-1) on Treg cells. For this panel, the gating strategy was based on the expression of CD4+FOXP3+ cells and CD4+CD25++. We assessed both intra- and extracellular markers for Treg cell evaluation. Extracellular markers included APC-H7 mouse anti-human CD3, FITC mouse anti-human CD4, PE CY7 anti-human CD25 and PE mouse anti-human GARP. A cell permeabilization step was included for intracellular marker assessment (V450 mouse anti-human FOXP3 and Alexa fluor 647 mouse anti-humans STAB1). We started with SSC vs. FCS to gate the lymphocyte population, then CD3 single cell (singlet) using FCS-H vs. FCS-A, followed by CD3-APC-CY7 vs. FCS and CD4-FITC vs. FCS.
For Treg identification and differentiation, CD4+FOXP3+ (V450) was evaluated vs. CD25 ++ PE-CY7.

2.4 Statistical analysis

Statistical analyses were performed using Graph Pad Prism (Version 7, Graph Pad Software, San Diego CA). Comparisons included: control versus treatment-naïve versus cART-treated groups (first line & second line) in all patients, further we evaluated the same study groups with varying viral loads (VL) (VL<1,000) as well as groups with (VL >1,000). One-way ANOVA or Kruskal-Wallis analyses, with Tukey’s or Dunn’s post hoc tests were performed. Correlations between selected variables were performed using the Pearson’s or Spearman’s correlation, respectively, where appropriate. The statistical significance of results was accepted at p<0.05 (significant) and p<0.001 (very significant).

3. RESULTS

Demographics

A clinical evaluation of HIV-positive individuals using the World Health Organization staging classification of progression to symptomatic HIV disease (35), showed that 65% of the study population were categorized as clinical stage 1 (n=52), 11% as stage 2 (n=8), 15% as stage 3 (n=12), and 9% as stage 4 (n=7). No changes were observed between the four groups for systemic markers. However, all HIV-positive individuals (irrespective of treatment) displayed lower total cholesterol, LDL and triglyceride levels when compared to controls (data not shown). Of note, diastolic blood pressure was significantly lower in the HIV-positive second line-treated group versus first line-treated patients. There are several changes that are linked to second line treatment with virological failure and here the relatively long time interval since the initial diagnosis together with lower CD4+ counts likely plays a role in this process.

Increased expression of pro-coagulant TF (CD142) in CD4+ and CD8+ T cells in patients with virological failure and co-expression with immune activation marker (CD38)

Flow cytometry results revealed that the degree of immune activation (CD38 expression) on CD4+ and CD8+ T lymphocytes was significantly higher in all HIV-positive groups compared to controls.
(Figures 1 and 2 A, D and G). The highest CD142 expression was detected in the HIV-positive patients with virological failure on second line treatment (Figures 1 B and H and Figure 2 H). Our data also show co-expression of CD38 and CD142 on CD4+ and CD8+ T cells that was highly significant between the control group and HIV-positive participants on second line treatment (Figure 1 and 2 C, F, I)

**Increased expression of classical Treg cell marker (FOXP3) together with upregulation of anti-inflammatory marker (GARP) and pro-inflammatory SATB-1 with second line treatment**

Evaluation of Treg cells using flow cytometry showed an increased percentage of classical Treg cells (CD4+CD25++FOXP3+) during chronic HIV infection with second line treatment and with virological failure (Figure 3 G). Moreover, there was upregulation of both anti-inflammatory (GARP) and pro-inflammatory markers (SATB-1) on Treg cells during chronic HIV infection in the same group (Figure 3, H and I). D-dimer levels did not significantly change between the different studied groups (data not shown). However, our results revealed a robust correlation between activated Treg cell markers and a fibrin degradation product (D-dimer). The latter is a useful marker for all-cause and CVD-related mortality in especially HIV-infected persons (20) (Table 2).

**Expansion of pro-atherogenic monocyte subsets and activated macrophage marker CD163**

Flow cytometry evaluation of monocyte subsets showed that classical monocytes (CD14++ CD16-) are significantly decreased during HIV infection (cART-treated) in patients displaying virological failure (Figure 4 A and I). There is also a corresponding expansion of the intermediate (CD14++CD16+) monocyte subset with first line treatment (Figure 4 B and J), and also of the non-classical (CD14+ CD16++) subset particularly in chronic HIV-treated groups (Figure 4 C, G and K). There was also a significant increase of CD163, a marker of macrophage activation for all HIV-positive patients (Figure 4 D and L). Furthermore monocyte subsets strongly correlated with activated Treg cells (GARP and SATB-1) (Table 2), further supporting an interplay between adaptive Treg cells and monocytes.

**Correlation of T cell immune activation and coagulation markers with other immunological markers**
T cell immune activation and coagulation markers correlated positively with VL and negatively with CD4+ T cell count – individually and in combination (Table 3). Our findings also demonstrate strong correlations between the anti-inflammatory Treg cell marker GARP, and immune activation and coagulation. In parallel, there are consistent correlations between immune activation and coagulation (individually and in combination) and the non-classical monocyte subtype. There is also a strong correlation between the macrophage marker CD163 and immune activation on CD4+ T cells (Table 3).

**Lipid subclass analyses**

We found lower levels of the LDL-2 subclass in cART-treated HIV-positive individuals (Figure 5 C and G). The large HDL subclass for HIV-infected individuals was significantly higher in the HIV-positive group with virological failure and on second line treatment (Figure 5 I). This was associated with a decrease in small HDL levels in the same group (Figure 5 J). There was also a significant increase in LBP levels in HIV-infected individuals (Figure 5 D, H and L). We subsequently performed various correlation analyses and here there was a significantly strong negative correlation between large and small HDL fractions (Figure 6 A). These data also showed that LDL-2 displayed a negative association with large HDL (Figure 6 B), but a positive one with small HDL (Figure 6 C). In addition, there was a positive correlation between large HDL and immune activation in CD4+ T cells (Figure 6 D), while there was a negative correlation between immune activation and both small HDL and LDL-2, respectively (Figure 6 E and F).

**4. DISCUSSION**

For this study we hypothesized that HIV-induced low-grade inflammation can perturb immune cell function/activation together with lipid subclass alterations, thereby increasing the risk for CVD onset. Our data revealed a number of key findings for HIV-positive patients: a) elevated co-expression of a coagulation marker together with the immune activation marker CD38 on CD4+ and CD8+ T cells in HIV-positive individuals in the chronic stage, b) Treg cell activation together with upregulated anti-inflammatory GARP and pro-inflammatory SATB-1 may contribute to perturbed Treg cell function...
(in patients displaying virologic failure), c) Expansion of pro-atherogenic monocyte subsets; and d) lipid subclass alterations significantly correlated with immune activation markers.

Co-expression of coagulation marker and immune activation in CD4+ and CD8+ T cells

The current study showed – for the first time as far as we are aware – significant expression of TF (CD142) on CD8+ and non-CD8+ (including CD4+ T cells) during HIV infection, particularly in second line treatment with virological failure. TF also co-expressed with the immune activation marker CD38 thereby demonstrating the role of HIV per se in inducing inflammation and coagulation to increase CVD risk. In agreement, HIV-infected patients display a two- to-tenfold higher risk of developing thromboembolic events and a two-fold higher risk of an acute myocardial infarction (5).

The SMART Study also highlighted the role of inflammation and coagulation in the prediction of all-cause mortality and CVD events in HIV-positive individuals (20). Although TF is usually expressed on mononuclear cells (23), the disturbance of vascular integrity together with relatively high levels of pro-inflammatory cytokines (e.g. IL-6) can activate TF leading to stimulation of coagulation pathways (10).

Treg cell activation and upregulated GARP and SATB-1 contribute to Treg dysregulation

The data showed an expansion of FOXP3+ Treg cells (as percentage of total CD4+ T cells) with upregulation of GARP and SATB-1 during virological failure and second line treatment (chronic HIV infection). The enhanced Treg cell availability may be an indication of higher immune activation due to increased Treg activity/function (7). Here upregulated GARP can induce adaptive Treg cells by the inhibition of the effector function in naïve T cells, and by up regulation of FOXP3 and transforming growth factor-β which leads to increased suppressive activity (16). Together this would contribute to chronic viremia and the persistence of the HIV infection (18). The positive correlation found between GARP and immune activation/coagulation strongly indicates that the loss of the protective immune response likely contributes to persistent immune activation. Moreover, the upregulation of SATB-1 indicates a shift towards T effector cells together with the inhibition of Treg cell function. This may also show that there is skewing of the regulatory function towards the effector program and increasing...
pro-inflammatory pathways (3). The latter may help explain the correlation between SATB-1 and the relatively high levels of intermediate and non-classical monocyte subsets during chronic infection.

There is some controversy regarding the increase of GARP and SATB-1 expression on Treg cells during chronic HIV infection and that upregulation of the former is involved in the induction of adaptive regulatory T cells (16). Such upregulation can act as a form of immunomodulation by inhibition of effector function in naive T cells and by FOXP3 induction. Conversely, the upregulation of SATB-1 in Treg cells can indicate a weakened suppressive function and an increase of pro-inflammatory properties. It remains unclear whether the plasticity of Treg cells plays a role in this case and/or if there is an imbalance between Treg and T helper cells due to the immunodeficiency state. However, our data may also reflect an imbalance between anti- and pro-inflammatory cytokines during chronic HIV-infection. Further studies are required to gain additional insights into such intriguing questions.

**Pro-atherogenic monocyte subsets expansion (intermediate and non-classical monocyte) with HIV infection**

The monocyte data are consistent with previous work that detected a similar subset shift with HIV infection (particularly with HIV-treated groups) and emerging evidence shows that non-classical monocytes may be atherogenic (13). Non-classical monocytes correlated positively with immune activation and coagulation markers indicating an association with such pathogenic processes during HIV infection. In support, this is linked to inflammation and coagulation markers with chronic HIV infection (30). It is likely that as non-classical monocytes express relatively high levels of the fractalkine receptor (CX3CL1) that will attract circulating monocytes into atherosclerotic plaques (14). Non-classical monocytes also express relatively high TF levels during HIV infection, further supporting its important role to promote coagulation pathways (11). Intermediate monocytes may be an independent predictor of CVD events and that this could be linked to its ability to secrete inflammatory cytokines (27). As our monocyte subset profile also shows a positive correlation with Treg cell activation markers (SATB-1, GARP), this may help explain pro- and anti-inflammatory
roles for SATB-1 and GARP, respectively, and may indicate an imbalance in cytokine regulation during HIV infection.

**Significant lipid subclasses alteration and a significant correlation with immune activation markers**

Our findings revealed no real differences in lipid blood metabolite levels in HIV-infected individuals. Although there are studies demonstrating dyslipidemia in HIV-positive patients, varying lipid profiles were previously reported (29). Here treatment regimens can elicit fluctuating outcomes, e.g. TDF with FTC or 3TC were associated with lower total cholesterol, LDL, triglyceride and HDL levels versus other nucleoside reverse-transcriptase inhibitors (8). However, mechanisms responsible for such alterations remain unclear and genetic and environmental factors likely also play a role (29). The significance of relatively low levels remain unclear in terms of CVD risk for the patient and additional, longer-term studies are required to shed light on this matter. We propose that the lower levels here observed are most likely linked to the lipid subclass changes found and the latter linked to increased CVD risk. However, further studies are required to prove this link.

By contrast, the detailed lipid subclass analysis revealed interesting differences between the studied groups. Here there was an increase in the large HDL species and a corresponding decrease in small HDL levels for the HIV-positive group with virological failure on second line treatment (chronic HIV infection). Small HDL is the most protective lipoprotein in terms of atheroprotective effects and anti-inflammatory activities through inhibition of oxidized-LDL uptake and anti-thrombotic function (19). In support, some found that small HDL is inversely associated with IL-6 levels (2). Thus decreased small HDL availability would blunt its atheroprotective role, independently from other lipid parameters. The mechanisms underlying this HDL subclass shift require further investigation but our correlation data point to an intersection with immune dysregulation (Figure 6). The decrease in the larger anti-atherogenic LDL subclass (LDL-2) in the HIV-positive cART-treated group is consistent with recent work showing higher levels of small and dense LDL subclasses (e.g. LDL-4) and decreased LDL-2 in coronary artery disease and end-stage renal disease patients with atherosclerosis (6, 15). In support, increased levels of several pro-inflammatory lipid species (e.g. large HDL)
together with a decrease in protective lipid subclasses (LDL-2, small HDL) were observed in HIV-infected persons (12). It is our opinion that this phenomenon together with increased inflammatory monocytes and Treg dysfunction contribute to an increased risk for future CVD onset in especially HIV-positive individuals with virological failure and on second line treatment.

These data also demonstrate that there is potential cross-talk between the lipid subclasses, especially for small and large HDL, and LDL-2. Although the underlying mechanisms driving such changes remain unclear, we propose that LBP plays a contributory role as it is responsible for the binding and transport of lipopolysaccharide (LPS: marker of microbial translocation) within circulation (31). LPS is part of the outer membrane of gram-negative bacteria and triggers a pro-inflammatory response by activation of monocytes and endothelial cells (33). LBP-mediated transfer of LPS to macrophage receptors can also induce signaling pathways, triggering the release of pro-inflammatory cytokines (25). LBP also intersects with lipoprotein metabolism, e.g. LBP-mediated transfer of LPS to HDL can result in a decreased immune response (22). LPS-mediated effects can thus be attenuated by its incorporation into lipoproteins such as HDL, LDL and VLDL (22). This is postulated to result in HDL remodeling and may contribute to dyslipidemia observed in some HIV-infected individuals. Together these data strongly implicate LBP as a crucial link between immune activation, lipid alterations and increased CVD risk.

Limitations

Previous studies show that body composition measures, e.g. BMI can be increased by cART in association with hypertension (1, 4, 36). Furthermore, lipodystrophy is often also linked with metabolic and CVD risk in HIV-positive individuals treated with cART (21, 28). Our additional analyses revealed that we can exclude BMI, smoking and gender as confounding factors for immune activation. Here BMI and waist circumference were not affected in cART-treated groups, but were significantly lower in the cART naïve groups versus controls. The waist-to-hip ratio was unaffected in
all groups (data not shown). These measures all fall within the range for normal weight individuals in the HIV-positive groups.

We have also not completed any cardiac functional assessments and hence our findings would apply to the risk for future CVD onset for HIV-positive patients. However, our data revealed a robust negative correlation between immune activation and diastolic blood pressure ($r = -0.33; P=0.002$), and likewise for coagulation and diastolic blood pressure ($r= -0.22; P=0.04$). This is supported by a recent study showing that diastolic blood pressure (less than 60 mmHg) is associated with subclinical myocardial injury and immune activation (32). Thus we conclude that cART intervention in our study most likely elicits detrimental effects - including higher CVD risk - via dysregulated immune responses coupled with lower diastolic blood pressure and perturbations of lipoprotein subclasses to a greater extent than the traditional CVD risk factors.

5. Conclusion

In chronic HIV infection persistent T cell activation/inflammation can lead to pro-atherogenic effects such as increased coagulation, immune cell dysregulation (Treg dysfunction and monocyte subsets expansion) and lipid subclass alterations. This study demonstrated the significant interplay between adaptive immune cell activation and monocyte/macrophage markers, which further illustrates the pathogenic role of adaptive and innate immunity in terms of CVD onset. Furthermore, our findings show a unique link between immune activation and lipid subclass alterations, revealing important changes that can be missed by traditional lipid marker assessments (e.g. LDL and HDL).
GRANTS

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DISCLOSURES

None declared by the authors.

AUTHOR CONTRIBUTIONS

Conceived and designed research: M.F.E., E.T., R.H.G.


Drafted manuscript: E.T., D.E.J., M.F.E., R.H.G.


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**FIGURE LEGENDS**

**Figure 1:** Evaluation of immune activation and coagulation on CD4 T cells.

**A-C; complete study population:** (A) Immune activation (CD3<sup>+</sup>CD8<sup>-</sup>CD38<sup>+</sup>) (Control n=12; Treatment naïve n=17; First line cART-treated n=17; Second line cART-treated n=12); (B) Coagulation on (CD3<sup>+</sup>CD8<sup>-</sup>CD142<sup>+</sup>) (Control n=12; Treatment naïve n=17; First line cART-treated n=17; Second line cART-treated n=12); and (C) Co-expression of both immune activation and coagulation on (CD3<sup>+</sup>CD8<sup>-</sup>) (Control n=12; Treatment naïve n=17; First line cART-treated n=16; Second line cART-treated n=12). **D-F; patients with VL <1,000:** (D) Immune activation (CD3<sup>+</sup>CD8<sup>-</sup>CD38<sup>+</sup>) (Control n=12; Treatment naïve n=9; First line cART-treated n=4; Second line cART-treated n=10); (E) Coagulation on (CD3<sup>+</sup>CD8<sup>-</sup>CD142<sup>+</sup>) (Control n=12; Treatment naïve n=10; First line cART-treated n=9; Second line cART-treated n=4); (F) Co-expression of both immune activation and coagulation on CD3<sup>+</sup>CD8<sup>-</sup> (Control n=12; Treatment naïve n=10; First line cART-treated n=9; Second line cART-treated n=4). **G-I; patients with VL >1,000:** (G) Immune activation (CD3<sup>+</sup>CD8<sup>-</sup>CD38<sup>+</sup>) (Control n=12; Treatment naïve n=7; First line cART-treated n=8; Second line cART-treated n=8); (H) Coagulation on (CD3<sup>+</sup>CD8<sup>-</sup>CD142<sup>+</sup>) (Control n=12; Treatment naïve n=7; First line cART-treated n=6; Second line cART-treated n=8); (I) Co-expression of both immune activation and coagulation on CD3<sup>+</sup>CD8<sup>-</sup> (Control n=12; Treatment naïve n=6; First line cART-treated n=6; Second line cART-treated n=8). *P≤0.05, **P value between 0.05 and 0.001, ***P value between 0.001 and 0.0001, ****P<0.0001.

**Figure 2:** Evaluation of immune activation and coagulation on CD8 T cells.

**A-C; complete study population** (A) Immune activation (CD8<sup>+</sup>CD38<sup>+</sup>) (Control n=12; Treatment naïve n=26; First line cART-treated n=18; Second line cART-treated n=20); (B) Coagulation on CD8 (CD8<sup>+</sup>CD142<sup>+</sup>) (Control n=12; Treatment naïve n=26; First line cART-treated n=17; Second line cART-treated n=20); and (C) Co-expression of both immune activation and coagulation on CD8 (CD8<sup>+</sup>CD38<sup>+</sup>CD142<sup>+</sup>) (Control n=11; Treatment naïve n=22; First line cART-treated n=16; Second line cART-treated n=17). **D-F; patients with VL <1,000:** (D) Immune activation (CD8<sup>+</sup>CD38<sup>+</sup>) (Control n=12; Treatment naïve n=15; First line cART-treated n=8; Second line cART-treated n=8);
(E) Coagulation on CD8 (CD8+CD142+) (Control n=12; Treatment naïve n=15; First line cART-treated n=8; Second line cART-treated n=8); (F) Co-expression of both immune activation and coagulation on CD8 T cell (Control n=11; Treatment naïve n=13; First line cART-treated n=8; Second line cART-treated n=6). **G-I; patients with VL >1,000;** (G) Immune activation (CD8+CD38+) (Control n=12; Treatment naïve n=9; First line cART-treated n=8; Second line cART-treated n=11); (H) Coagulation on CD8 (CD8+CD142+) (Control n=12; Treatment naïve n=9; First line cART-treated n=7; Second line cART-treated n=11); (I) Co-expression of coagulation and immune activation markers (Control n=11; Treatment naïve n=8; First line cART-treated n=7; Second line cART-treated n=10). **P value between 0.05 and 0.001, ***P value between 0.001 and 0.0001, ****P<0.0001.

Figure 3: Evaluation of Treg cells with HIV infection. A-C; complete study population (A) FOXP3 expression in study groups (Control n=13; Treatment naïve n=26; First line cART-treated n=19; Second line cART-treated n=19); (B) SATB-1 expression on study groups (Control n=13; Treatment naïve n=25; First line cART-treated n=19; Second line cART-treated n=19); and (C) GARP expression on study groups (Control n=13; Treatment naïve n=26; First line cART-treated n=19; Second line cART-treated n=19). **D-F; HIV patients with VL <1,000;** (D) FOXP3 expression in study groups (Control n=13; Treatment naïve n=15; First line cART-treated n=8; Second line cART-treated n=8); (E) SATB-1 expression (Control n=13; Treatment naïve n=15; First line cART-treated n=9; Second line cART-treated n=8); and (F) GARP expression (Control n=13; Treatment naïve n=15; First line cART-treated n=9; Second line cART-treated n=7). **G-I; HIV patients with VL >1,000;** (G) FOXP3 expression in study groups (Control n=13; Treatment naïve n=9; First line cART-treated n=8; Second line cART-treated n=10); (H) SATB-1 expression in study groups (Control n=13; Treatment naïve n=9; First line cART-treated n=8; Second line cART-treated n=10); (I) GARP expression in HIV study groups (Control n=13; Treatment naïve n=9; First line cART-treated n=8; Second line cART-treated n=10). *P≤0.05, **P value between 0.05 and 0.001.
Figure 4: Evaluation of monocyte subsets and macrophage marker CD163: A-D; complete study population (A) Expression of classical monocyte in study groups (Control n=12; Treatment naïve n=25; First line cART-treated n=17; Second line cART-treated n=20); (B) Expression of intermediate monocyte in study groups (Control n=12; Treatment naïve n=25; First line cART-treated n=18; Second line cART-treated n=19); (C) Expression of non-classical monocyte (Control n=12; Treatment naïve n=25; First line cART-treated n=18; Second line cART-treated n=20); (D) expression of CD163 (Control n=13; Treatment naïve n=22; First line cART-treated n=14; Second line cART-treated n=17); E-H; HIV patients with VL < 1,000; (E) expression of classical monocyte (Control n=12; Treatment naïve n=15; First line cART-treated n=8; Second line cART-treated n=8); (F) Expression of intermediate monocyte in study groups (Control n=12; Treatment naïve n=15; First line cART-treated n=8; Second line cART-treated n=8); (G) Expression of non-classical monocyte (Control n=12; Treatment naïve n=15; First line cART-treated n=8; Second line cART-treated n=8); (H) expression of CD163 on study groups (Control n=13; Treatment naïve n=11; First line cART-treated n=8; Second line cART-treated n=6); I-L; HIV patients with VL > 1,000; (I) expression of classical monocyte (Control n=12; Treatment naïve n=9; First line cART-treated n=8; Second line cART-treated n=11); (J) expression of intermediate monocyte (Control n=12; Treatment naïve n=9; First line cART-treated n=8; Second line cART-treated n=11); (K) expression of non-classical monocyte (Control n=12; Treatment naïve n=9; First line cART-treated n=8; Second line cART-treated n=11); (L) expression of CD163 (Control n=13; Treatment naïve n=11; First line cART-treated n=5; Second line cART-treated n=10). *P≤0.05, **P value between 0.05 and 0.001, ***P value between 0.001 and 0.0001, ****P<0.0001.

Figure 5: Evaluation of lipid subclasses and LBP: A-D; complete study population (A) Expression of large HDL in study groups (Control n=11; Treatment naïve n=17; First line cART-treated n=12; Second line cART-treated n=15); (B) Expression of small HDL in study groups (Control n=11; Treatment naïve n=17; First line cART-treated n=12; Second line cART-treated n=15); (C) Expression of LDL-2 (Control n=12; Treatment naïve n=18; First line cART-treated n=15; Second line cART-treated n=17); (D) Expression of LBP in study groups (Control n=7; Treatment naïve n=23; First line cART-treated n=12; Second line cART-treated n=12); E-H; HIV patients with VL < 1,000; (E) Expression of large HDL (Control n=11; Treatment naïve n=9; First line cART-treated n=6; Second line cART-treated n=7); (F) expression of small HDL (Control n=11; Treatment naïve n=9; First line cART-treated n=6; Second line cART-treated n=7); (G) expression of LDL-2 (Control n=12; Treatment naïve n=9; First line cART-treated n=7; Second line cART-treated n=7); (H) expression of LBP in study groups (Control n=7; Treatment naïve n=13; First line cART-treated n=5; Second line cART-treated n=4); I-L; HIV patients with VL >1,000; (I) Expression of large HDL (Control n=11; Treatment naïve n=7; First line cART-treated n=5; Second line cART-treated...
(J) Expression of small HDL (Control n=11; Treatment naïve n=7; First line cART-treated n=5; Second line cART-treated n=7); (K) Expression of LDL-2 (Control n=12; Treatment naïve n=7; First line cART-treated n=6; Second line cART-treated n=10); (L) Expression of LBP in study groups (Control n=6; Treatment naïve n=8; First line cART-treated n=6; Second line cART-treated n=8).

*P≤0.05, **P value between 0.05 and 0.001

**Figure 6: Correlation between lipid subclasses and immune activation markers:** Spearman correlations between A) Small HDL and large HDL (n=56); B) LDL-2 and large HDL (n=56); (C) small HDL and LDL-2 (n=56); D) Large HDL and immune activation marker (CD38+) on CD4 T cells (n=42); E) Small HDL and immune activation marker (CD38+) expressed on CD4 T cells (n=43); F) LDL-2 and immune activation marker (CD38+) on CD8 T cells (n=62).

**Figure 7: Persistent immune activation in HIV-infected individuals and increased CVD risk.** Persistent immune activation starts relatively early during HIV infection and can remain despite cART. This phenomenon can elicit several downstream effects to increase the risk for CVD onset in HIV-positive patients. Here there is a shift to a pro-atherogenic monocyte subset that further increases inflammation and coagulation. It also results in CD4 and CD8 T cell activation together with increased expression of a coagulation marker (TF). In parallel, there is Treg cell dysfunction as indicated by the upregulation of both GARP (anti-inflammatory) and SATB-1 (pro-inflammatory). There is also a significant link between immune activation and lipid subclass remodeling that is likely mediated by LBP. Of note, most detrimental changes e.g. coagulation, Treg dysfunction and lipid alterations are linked to second line drug treatment. However, further studies are required to investigate this putative link. Together such changes contribute to an increased risk for future CVD onset in especially HIV-positive individuals with virological failure and on second line treatment.

cART: Combined anti-retroviral treatment (cART); GARP: Glycoprotein A repetitions predominant; HDL: High-density lipoprotein; LBP: Lipopolysaccharide-binding protein; LDL: Low-density lipoprotein; SATB-1: Special AT-rich sequence-binding protein 1; and TF: Tissue factor.
Table 1: Patient characterisation

<table>
<thead>
<tr>
<th></th>
<th>Control (n=13)</th>
<th>HIV positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment naïve (n=26)</td>
<td>First line cART treated (n=19)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>49 (29-54)</td>
<td>36 (29-42)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>6/7</td>
<td>13/13</td>
</tr>
<tr>
<td>CD4 count (cells/µl)</td>
<td>N/A</td>
<td>503 (297-654)</td>
</tr>
<tr>
<td>Viral load (C/ml)</td>
<td>N/A</td>
<td>558 (40-18850)</td>
</tr>
<tr>
<td>HIV/Dx (months)</td>
<td>N/A</td>
<td>36 (19-81)</td>
</tr>
<tr>
<td>T/cART (months)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Blood metabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>4.8 (4.6-5.6)</td>
<td>4.65 (4.3-5.15)</td>
</tr>
<tr>
<td>Plasma insulin (mU/L)</td>
<td>8.7 (5-33.1)</td>
<td>6.6 (3.8-10.4)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.3 (4.3-5.9)</td>
<td>4.1 (3.4-5.0)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4 (0.98-1.9)</td>
<td>0.88 (0.50-1.18)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.35 (1.9-3.7)</td>
<td>2.23 (1.7-3.3)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.22 (1.0-1.4)</td>
<td>1.04 (0.9-1.5)</td>
</tr>
<tr>
<td>Cardiovascular parameters</td>
<td></td>
<td></td>
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<tr>
<td>F/H CVD [n (%)]</td>
<td>6 (46)</td>
<td>16 (61)</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>120 (120-130)</td>
<td>120 (107-130)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80 (70-80)</td>
<td>75 (70-80)</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>80 (68-82)</td>
<td>80 (70-80)</td>
</tr>
</tbody>
</table>

Data presented as median (IQR), except gender. cART: combined anti-retroviral therapy; C/ml: copies per millilitre; F/H CVD: family history of cardiovascular disease; HDL: high density lipoprotein; HIV/Dx: time of HIV diagnosis; LDL: low density lipoprotein; T/cART: time since starting cART; N/A: not applicable; NS: not significant; *p < 0.05 vs. Control; #p < 0.05 vs. First line cART treated; ###p < 0.001 vs. First line cART treated.
Table 2: Correlation of CD4- and CD8-positive T-cells with immunological markers

<table>
<thead>
<tr>
<th></th>
<th>Immune activation (CD38+)</th>
<th>Coagulation (CD142+)</th>
<th>Immune activation &amp; coagulation (CD38+CD142+)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD4+ T-cells</td>
<td>CD8+ T-cells</td>
<td>CD4+ T-cells</td>
</tr>
<tr>
<td>CD4 count</td>
<td>r = -0.43, p = 0.002</td>
<td>r = -0.34, p = 0.005</td>
<td>r = -0.44, p = 0.002</td>
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<tr>
<td>Viral load</td>
<td>r = 0.35, p = 0.01</td>
<td>r = 0.27, p = 0.03</td>
<td>r = 0.37, p = 0.01</td>
</tr>
</tbody>
</table>

**Regulatory T-cells (Tregs)**

|                                |                           |                      |                                               |
|                                | Pro-inflammatory (CD25++SATB-1+) |                       |                                               |
|                                | NS                        | r = 0.22, p = 0.04   | NS                                             |
|                                |                            |                      |                                               |
|                                | Anti-inflammatory (CD25++GARP+) |                       |                                               |
|                                | r = 0.41, p = 0.001        | r = 0.41, p = 0.0001 | r = 0.44, p = 0.0005                         | r = 0.48, p < 0.0001 | r = 0.48, p < 0.0001 | r = 0.48, p < 0.0001 |

**Monocyte subclasses and CD163**

<p>| | | | |
|                                |                           |                      |                                               |
|                                | Classical monocytes (CD14++CD16-) |                       |                                               |
|                                | NS                        | NS                   | r = -0.23, p = 0.03                           | NS                        | NS                        | NS                        |
|                                |                            |                      |                                               |
|                                | Intermediate monocytes (CD14++CD16+) |                       |                                               |
|                                | NS                        | NS                   | NS                                             | NS                        | NS                        | NS                        |
|                                |                            |                      |                                               |
|                                | Non-classical monocytes (CD14+CD16++) |                       |                                               |
|                                | r = 0.37, p = 0.003        | r = 0.50, p &lt; 0.0001 | r = 0.40, p = 0.002                           | r = 0.30, p = 0.005       | r = 0.44, p = 0.0005       | r = 0.37, p = 0.001       |
|                                | r = 0.37, p = 0.0069       | NS                   | NS                                             | NS                        | NS                        | NS                        |
|                                |                            |                      |                                               |
|                                | CD163 (pg/ml)              |                      |                                               |
|                                |                            |                      |                                               |</p>
<table>
<thead>
<tr>
<th></th>
<th>CD25++GARP+ (%)</th>
<th>CD25++SATB1+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-dimer (µg/ml)</td>
<td>r = 0.39; p = 0.0007</td>
<td>r = 0.33; p = 0.005</td>
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<tr>
<td>CD14+CD16- (%)</td>
<td>r = -0.55; p &lt; 0.0001</td>
<td>r = -0.26; p = 0.02</td>
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<tr>
<td>CD14+CD16+ (%)</td>
<td>r = 0.49; p &lt; 0.0001</td>
<td>r = 0.25; p = 0.02</td>
</tr>
<tr>
<td>CD14+CD16++ (%)</td>
<td>r = 0.32; p = 0.003</td>
<td>r = 0.29; p = 0.009</td>
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</table>