Combination antiretroviral therapy (cART) has significantly reduced morbidity and mortality of HIV-infected patients, yet their life expectancy remains reduced compared with the general population. Most HIV-infected patients receiving cART have some persistent immune dysfunction characterized by chronic immune activation and premature aging of the immune system. Here we review biomarkers of T-cell activation (CD4+,-25 and -38, HLA-DR, and soluble CD26 and -30); generalized immune activation (C-reactive protein, IL-6 and D-dimer); microbial translocation (lipopolysaccharide, 16S rDNA, lipopolysaccharide-binding protein and soluble CD14); and immune dysfunction of specific cellular subsets (T cells, natural killer cells and monocytes) in HIV-infected patients on cART and their relationship to adverse clinical outcomes including impaired CD4 T-cell recovery, as well as non-AIDS clinical events, such as cardiovascular disease.

KEYWORDS: antiretroviral therapy • HIV • immune activation • immune dysfunction • immune senescence • microbial translocation • non-AIDS illness

A large number of biomarkers have been evaluated in HIV-infected patients and have largely been used to predict clinical outcome, including AIDS and death. Given the widespread availability of combination antiretroviral therapy (cART) and the ability of cART to reduce morbidity and mortality, this article primarily focuses on biomarkers of interest in HIV-infected patients receiving cART, with an emphasis on the effect of microbial translocation and immune activation. Following cART, many patients continue to demonstrate ongoing immune abnormalities and patients are at an increased risk of non-AIDS events including cardiovascular disease (CVD) and malignancy. Some of these biomarkers are in early clinical development while others have been evaluated in larger clinical trials and cohorts to determine whether they can predict adverse clinical outcomes in patients on cART. Most of these biomarkers are not yet used in routine clinical practice but are currently being evaluated to understand the pathogenesis of immune dysfunction in patients on cART, identify patients at highest risk of immune dysfunction and non-AIDS events, such as CVD and malignancy, and develop novel immunotherapeutic approaches that could be used in addition to cART.

HIV pathogenesis & response to cART

The physiological hallmark of HIV infection is the destruction of CD4+ T cells and persistent immune activation. HIV predominantly infects activated CD4+ T cells, resulting in productive viral infection [1], and a rapid and profound depletion of GI tract-associated CD4+ T cells, leading to loss of integrity of the intestinal mucosa [2,3]. The depletion of GI tract CD4+ T cells has been associated with increased translocation of microbial products from the intestinal lumen into the bloodstream, which can trigger the innate immune system to produce proinflammatory cytokines and chronic immune activation [3]. However, the pathogenesis of immune activation is complex and multifactorial. In addition to the translocation of microbial products, immune activation is believed to be driven by direct infection of CD4+ T cells and release of cytokines, a combination of innate and adaptive immune responses against viral antigens and/or secondary infections, and an imbalance in the immune response caused by the loss of T regulatory and central memory T cells (reviewed in [4]).

Combination antiretroviral therapy has led to a dramatic reduction in mortality and morbidity in HIV-infected patients [5,6]. Despite long-term suppression of HIV RNA and recovery of the total number of CD4+ T cells in most patients following cART, full life expectancy of patients is restored in some [7], but not all, patients [6,8]. The chance of an HIV-infected patient reaching the age of 70 years is still approximately 50% that of HIV-uninfected controls [8]. HIV-infected patients experience higher rates of CVD, non-AIDS malignancies, dementia, liver
disease, renal diseases and bone disorders [9,10]. Factors that drive ongoing morbidity include a combination of immune activation, aging of the immune system and cART toxicity (reviewed in [11]).

**Biomarkers in HIV-infected patients & the effect of cART**

**Immune activation**

A detailed overview of markers of persistent immune activation in HIV-infected patients has recently been published [12]. Here we will focus only on measures of immune activation in patients receiving cART (summarized in Table 1) with a focus on larger prospective studies that have aimed to identify associations between the relevant biomarker and clinical outcomes.

**T-cell activation: cellular markers CD69, -25 & -38, & HLA-DR**

T-cell activation can be monitored using either cell-associated or soluble markers. T-cell activation is identified by increased expression of CD69, -25 and -38, and HLA-DR. CD69, a transmembrane C-type lectin, is an early marker of activation [13] and is followed by increased expression of CD25, the α-chain of the IL-2 receptor [14]. The MHC class II antigen, HLA-DR, is expressed in later phases of T-cell activation, while CD38 (an extracellular ADP-ribose cyclase) is expressed constitutively in activated T cells [15].

CD69 expression is a useful tool to monitor T-cell activation in vitro [16]; however, expression of CD69 from freshly isolated blood is low and is similar in HIV-infected and HIV-uninfected patients [16,17], potentially because of the transient expression of these markers or sequestration of CD69+ T cells in lymph nodes and other organs. CD25, although a marker of T-cell activation and used as such in vitro, is also expressed on regulatory T cells [18], often in combination with other intracellular markers such as FoxP3 [19]. Most studies characterizing T-cell activation during HIV infection in vivo have therefore focused on expression of CD38 and HLA-DR [20–22].

Increased expression of CD38 and HLA-DR on CD4+ and CD8+ T cells in untreated HIV infection has been associated with shorter survival and faster disease progression [23–25]. However, expression of CD38 on CD8+ T cells before the initiation of cART was not predictive for clinical outcomes in patients receiving cART [26]. Following cART, expression of CD38 and HLA-DR on both CD4+ and CD8+ T cells declines significantly but remains higher than in uninfected controls [27]. Persistent elevation of CD38 and HLA-DR on both CD4+ and CD8+ T cells has been associated with impaired magnitude and speed of CD4+ T-cell recovery [27–29], and higher levels of expression of CD38 on CD8+ T cells was observed in patients on cART at the time of an opportunistic infection or death [26]. T-cell activation markers were significantly higher in HIV-infected patients from Africa compared with the USA and were associated with poorer CD4+ T-cell recovery and increased mortality following cART in this Ugandan-based prospective study [30].

**T-cell activation: soluble markers**

Plasma markers for T-cell activation are of potential interest, as these can be more easily measured than cell-associated markers in clinical practice. Activated T cells express a number of proteins on their surface that are shed, including CD26, -27 and -30, and the ligand of the CD40 receptor, leading to increased levels of their soluble forms in plasma.

Th1 cells predominantly express and release CD26, while Th2 cells express and release CD30. Therefore, these plasma markers have been investigated in conditions that are defined by either a Th1 [31] or a Th2 environment [32,33]. While soluble (s)CD30 is directly quantified in plasma using a standard ELISA, sCD26 is usually indirectly detected through its enzymatic activity [34].

A decrease of enzymatic activity of sCD26 had been inversely correlated with HIV RNA in untreated HIV-infected patients [35]. In patients receiving cART, there was no significant elevation in sCD26 compared with uninfected individuals, regardless of whether patients had detectable HIV RNA [34]. Despite the potential use of sCD26 as a diagnostic or prognostic marker in other diseases [36], its value as a marker in the context of HIV infection has not been established and, therefore, requires further investigation.

Plasma levels of sCD30 are elevated during the early phase of HIV infection and decrease following seroconversion, correlating with detectable HIV p24 antigen [37], and were associated with faster progression to AIDS in untreated HIV-infected patients [38,39]. Data regarding changes in sCD30 in untreated HIV-infected patients are inconsistent, with some studies reporting higher levels [40,41] or levels similar to uninfected subjects [34]. However, a recent large retrospective cohort study (n = 290)
demonstrated a significant decrease of sCD30 following initiation of cART, even in the absence of virological control. sCD30 also correlated with hypergammaglobulinemia, an indicator of B-cell activation [42]. In a separate cohort study of patients receiving cART (n = 276), higher baseline levels of sCD30 prior to cART initiation were associated with higher HIV RNA [43]. In patients who achieved virological suppression (<500 copies/ml), a faster decrease of sCD30 in the first 6 months predicted a higher risk of subsequent viral rebound [43]. To date, no studies have evaluated the relationship between sCD30 and clinical outcome on cART.

Receptors of the TNF receptor (TNFR) superfamily include CD27 expressed on T cells, CD40 expressed on antigen presenting cells and TNFR-1, which is widely expressed across cell types. The TNFR superfamily are involved in pathways regulating a wide array of cellular functions, such as cell proliferation, differentiation and survival, and are often shed following receptor engagement. The CD40 ligand (CD40L) is expressed and shed by activated T cells. Plasma levels of sCD27 [44,45] and sCD40L are elevated in untreated HIV infection [46].

Combination antiretroviral therapy leads to a significant reduction of plasma levels of sCD27 [42], while increased levels of sCD40L do not appear to be affected by cART [47].

In a recent case–control study of treatment-naive patients initiating cART, pretreatment levels of sTNFR-1, sCD27 and sCD40L were each significantly associated with the risk of an AIDS-defining malignancy or death [26]. These clinical events presented a median of 51 weeks after cART initiation, by which time the majority of subjects had over 200 circulating CD4 lymphocytes/mm³ and had suppressed plasma viremia to less than 50 copies/ml. Therefore, these markers may potentially identify patients at the highest risk of an AIDS-defining malignancy or death on cART [26].

Generalized immune activation & thrombotic risk: C-reactive protein, IL-6 & D-dimer

C-reactive protein (CRP), IL-6 and D-dimer have been extensively studied as biomarkers of persistent immune activation and increased thrombotic risk in patients on cART [12]. The interest in these biomarkers started following the Strategic Management of Antiretroviral Therapy (SMART) study. The SMART study was a randomized controlled trial comparing the effect of intermittent CD4-guided cART versus continuous cART on morbidity and mortality [48]. SMART was one of the first studies to identify a relationship between viral replication, immune activation and non-AIDS events, and several substudies of SMART have identified multiple biomarkers that are associated with non-AIDS events [49–53] and mortality [54].

C-reactive protein, generally measured using a high-sensitivity assay and, therefore, referred to as highly sensitive (hs)CRP, is an acute-phase protein produced by hepatocytes in response to IL-6 and is involved in complement formation [55]. Elevated CRP has been associated with CVD in over 30 prospective cohort and case–control studies in previously healthy middle-aged and elderly populations [56,57]. In the setting of HIV infection, levels of hsCRP are higher than age- and sex-matched HIV-negative controls [52], and increased levels of CRP have been demonstrated to be associated with a greater risk of development of opportunistic infection [50,58], and cardiovascular or all-cause mortality [49,59]; although the association with cardiovascular risk was not reported in all studies [49,59,60]. Following initiation of cART, most studies have reported little or no change in hsCRP, although one study demonstrated a significant reduction in hsCRP [42,51,61–63]. However, increased levels of hsCRP in plasma of patients receiving cART was associated with increased carotid intima media thickness, a predictor for CVD [64].

IL-6 is a proinflammatory cytokine produced mainly by monocytes, endothelial cells and lymphocytes, [65]. IL-6 levels increase with age in the general population and have been associated with increased CVD and mortality in the elderly [57,66,67]. Compared with the general population, IL-6 levels are significantly increased in HIV infection [52]. In untreated HIV-infected patients, IL-6 levels are elevated and associated with markers of vascular dysfunction (reduced small artery elasticity) and some plasma markers of endothelial function, including E-selectin and soluble ICAM-1 [52,68]. As with the observations for hsCRP, cART did not lead to a significant reduction in IL-6 [51], not even after 3 years of continuous cART [42]. However, when cART was interrupted, IL-6 levels significantly increased, and the magnitude of the increase correlated with levels of HIV RNA [49]. Increased baseline levels of IL-6, before the initiation of cART, was the strongest predictor of all-cause mortality in the SMART study [49] and was also associated with the development of opportunistic disease [26,50].
### Table 1. Biomarkers commonly used to assess HIV-infected patients on combination antiretroviral therapy and their relationship with clinical outcomes.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Assay type</th>
<th>Specimen</th>
<th>HIV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T-cell activation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD38/HLA-DR</td>
<td>Flow cytometry</td>
<td>WB/PBMC</td>
<td>Elevated on CD4+ and CD8+ T cells; Reduced but slow/no normalization</td>
</tr>
<tr>
<td>sCD26</td>
<td>Enzymatic activity assay</td>
<td>Plasma</td>
<td>Reduced but slow/no normalization</td>
</tr>
<tr>
<td>sCD27</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Reduced</td>
</tr>
<tr>
<td>sCD30</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Reduced/elevated in IRD</td>
</tr>
<tr>
<td>sCD40L</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Elevated</td>
</tr>
<tr>
<td>sTNFR-1</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Reduced</td>
</tr>
<tr>
<td><strong>Inflammation/immune activation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Reduced in some studies</td>
</tr>
<tr>
<td>IL-6</td>
<td>ELISA</td>
<td>Plasma</td>
<td>No change</td>
</tr>
<tr>
<td>D-dimer</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Reduced</td>
</tr>
<tr>
<td><strong>Microbial translocation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>Enzymatic activity assay</td>
<td>Plasma</td>
<td>Reduced but slow/no normalization</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>PCR</td>
<td>Plasma</td>
<td>Reduced</td>
</tr>
<tr>
<td><strong>Immune response to bacterial products</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBP</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Elevated</td>
</tr>
<tr>
<td>sCD14</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Reduced but slow/no normalization</td>
</tr>
<tr>
<td>EndoCAb</td>
<td>ELISA</td>
<td>Plasma</td>
<td>Reduced</td>
</tr>
<tr>
<td><strong>T-cell dysfunction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD28/CD57</td>
<td>Senescence</td>
<td>Flow cytometry</td>
<td>WB/PBMC</td>
</tr>
<tr>
<td><strong>Antigen-specific dysfunction</strong></td>
<td>Abnormal immune restoration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Against CMV</td>
<td>Functional assay</td>
<td>WB/PBMC</td>
<td>Elevated</td>
</tr>
<tr>
<td>Against Mtb</td>
<td>Functional assay</td>
<td>WB/PBMC</td>
<td>Reduced</td>
</tr>
<tr>
<td>Against HBV</td>
<td>Functional assay</td>
<td>WB/PBMC</td>
<td>Reduced</td>
</tr>
<tr>
<td><strong>NK cell dysfunction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-DR on NK</td>
<td>Immune activation</td>
<td>Flow cytometry</td>
<td>WB/PBMC</td>
</tr>
</tbody>
</table>

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All associations are positive correlations unless otherwise specified.

† Incubation with specific antigen or peptides and cytokine production measured by enzyme-linked immunospot (ELISpot) or intracellular cytokine staining.

‡ Functional assay requires ex vivo stimulation and intracellular staining.

§ CD107a expression elevated if measured by surface expression ex vivo but reduced if measured in response to experimental stimulus.

¶ Cell bound TF measured by flow cytometry. sTF requires an Enzymatic Activity Assay.

ADCC: Antibody-dependent cell cytotoxicity; BMD: Bone mineral density; cART: Combination antiretroviral therapy; CMV: Cytomegalovirus; CRP: C-reactive protein; CVD: Cardiovascular disease; EndoCAb: Endotoxin core antibody; HBV: Hepatitis B virus; HCV: Hepatitis C virus; IRD: Immune restoration disease; LBP: Lipopolysaccharide-binding protein; LPS: Lipopolysaccharide; Mtb: Mycobacterium tuberculosis; NHL: Non-Hodgkin’s lymphoma; NK: Natural killer; PBMC: Peripheral blood mononuclear cell; s: Soluble; TF: Tissue factor; TNFR: TNF receptor; WB: Whole blood.
### Association with other biomarkers

<table>
<thead>
<tr>
<th>Association with other biomarkers</th>
<th>Association with clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Naive</strong></td>
<td><strong>On cART</strong></td>
</tr>
<tr>
<td>LPS, 16S rDNA, sCD14, inverse correlation with CD28/CD57, TF</td>
<td>—</td>
</tr>
<tr>
<td>Inverse correlation with HIV RNA</td>
<td>—</td>
</tr>
<tr>
<td>sTNFR-1, IL-6, CD4 count</td>
<td>—</td>
</tr>
<tr>
<td>HIV RNA</td>
<td>—</td>
</tr>
<tr>
<td>sTNFR-1</td>
<td>—</td>
</tr>
<tr>
<td>sCD27, IL-6, HIV RNA inverse correlation with CD4 count</td>
<td>—</td>
</tr>
</tbody>
</table>

| **HIV RNA, CD4 count** | — | — | Vascular dysfunction, endothelial dysfunction | Mortality, opportunistic disease | CVD, mortality | CVD, BMD | [42,49,50,52,56–64] |
| | — | — | — | — | — | — | [26,42,49–52,57,6–68] |
| **TF** | HIV RNA, TF | — | Mortality, opportunistic disease | CVD, mortality | — | — | [49–52,69,71–73] |

### Association with clinical outcome

| **16S rDNA, LBP, sCD14, CD8+ T-cell activation** | 16S rDNA, sCD14, CD8+ T-cell activation | Opportunistic infection, dementia, liver disease progression (HCV) | Inverse correlation with CD4+ T-cell recovery | — | [3,77,80–89,91,101] |
| **HIV RNA, CD8+ T-cell activation** | LPS, CD8+ T-cell activation | — | Inverse correlation with CD4+ T-cell recovery | — | [77] |

| **LPS, sCD14** | — | — | Faster CD4+ T-cell recovery | — | [3,91] |
| **LPS, CD8+ T-cell activation, NK activation** | LPS | Faster disease progression, dementia | Mortality | — | [3,48,54,78,83,89–91,94,95] |
| **Inverse correlation with LPS** | — | — | — | — | [3,54] |

| **Inverse correlation with HLA-DR on CD4+ T-cells** | — | Increase of CD28+ with AIDS | Lower arterial distensibility | Aging | [105,106,111–114,160] |
| — | — | — | — | — |
| — | — | — | Carotid intima thickness, CVD | CVD | [121–125] |
| — | — | — | Severe Mtb disease | — | [118,119] |
| — | — | — | — | — | [117] |

| **sCD14** | — | — | — | — | [Lichtfuss GF, Lewin SR, Jaworski A, Crowe SM, Unpublished Data] |
Microbial translocation & associated immune reponse

With the emergence of the hypothesis that microbial translocation through the GI tract might be a driving factor for immune activation in HIV-infected patients, soluble markers of microbial translocation have recently been studied as novel biomarkers in HIV infection [74–76]. Microbial translocation can be measured in plasma by direct quantification of bacterial products, including lipopolysaccharide (LPS), a major component of the Gram-negative bacterial cell wall [3], bacterial DNA fragments using PCR-based assays [77] or other microbe-specific compounds (e.g., peptidoglycan, a component of the Gram-positive bacterial cell wall) [78]. 

In vivo, LPS binds to LPS-binding protein (LBP), which is expressed at high concentrations by numerous cell types during the acute phase of the innate immune response to bacterial infection [79]. The LPS/LBP complex is then bound by CD14 and the Toll-like receptor 4 on monocytes. This leads to monocyte activation and release of sCD14 into the circulation. Therefore, measurement of sCD14 and LBP can also indirectly quantify the effects of microbial translocation.

Table 1. Biomarkers commonly used to assess HIV-infected patients on combination antiretroviral therapy and their relationship with clinical outcomes (cont.)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Assay type</th>
<th>Specimen</th>
<th>HIV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD107a on NK</td>
<td>NK cell activity</td>
<td>Flow cytometry¹</td>
<td>WB/PBMC</td>
</tr>
<tr>
<td>CD16 on NK</td>
<td>NK cell ADCC capacity</td>
<td>Flow cytometry</td>
<td>WB/PBMC</td>
</tr>
</tbody>
</table>

All associations are positive correlations unless otherwise specified.

¹Incubation with specific antigen or peptides and cytokine production measured by enzyme-linked immunospot (ELISpot) or intracellular cytokine staining.

²Functional assay requires ex vivo stimulation and intracellular staining.

CD14, CD16++ monocytes

Flow cytometry

WB/PBMC

Elevated

Normalized

Monocyte activation

CD38 on monocytes

Flow cytometry

WB/PBMC

Elevated

Reduced/no normalization

TF

Immune response to bacterial products

Flow cytometry³

WB/PBMC/plasma

Elevated

–

D-dimer is a fibrin degradation product, which has been associated with CVD and death in the general population and is elevated in untreated HIV-infected patients [69]. D-dimer has also been investigated as a marker for predicting the development of opportunistic disease and mortality in patients receiving cART [70]. Following initiation of cART, there was a significant reduction in plasma levels of D-dimer [51,69,71]. Increased baseline plasma levels of D-dimer before initiating cART and elevated levels immediately preceding an event were associated with the development of an opportunistic infection [50,52]. In the SMART trial, increased baseline levels of D-dimer were also associated with a higher risk of CVD and all-cause mortality [72]. The magnitude of change in D-dimer levels following interruption of cART in the SMART study correlated with changes in HIV RNA [49], suggesting that viral replication may drive the production of D-dimer. D-dimer levels have also been correlated with levels of tissue factor (TF) expressed on activated monocytes, which were associated with both the level of HIV RNA and markers of microbial translocation [73].
poor reproducibility of the assay with reported interassay variability ranging from 25 to 30% \([77,83]\). Therefore, multiple levels of quality control need to be in place when utilizing the assay for clinical studies.

Plasma LPS is elevated in HIV-infected patients not receiving cART \([3,83,84]\). Following cART, LPS levels significantly reduce but do not return to levels of healthy controls \([83–85]\). In HIV-infected patients living in Africa, the relationship between elevated levels of LPS and HIV disease progression is less clear. Some studies have reported elevated levels of LPS \([85–87]\) and sCD14 \([85]\), while another study has demonstrated no change in either of these markers over time in untreated HIV infection \([88]\). In this latter study, although LPS and sCD14 levels were not significantly elevated compared with normal controls, LBP levels were raised and correlated (albeit weakly) with the increase in proinflammatory cytokines IL-6 and TNF-α. It is unknown why the relationship between LPS and clinical outcome might be different in this African cohort to other studies from the USA, but one possible explanation might be that the higher prevalence of co-infections may drive immune activation and, therefore, obscure the associations usually seen between LPS and disease progression. In addition, differences in markers of microbial translocation may also be confounded by ethnic differences in the patient population \([88,89]\) and should be taken into account during analysis.

The 16S genomic rDNA can also be quantified in plasma using PCR-based techniques. This is a technically challenging assay and persistent DNA translocation may also be affected by B-cell dysfunction in HIV-infected patients compared with healthy controls \([3,54]\). Unpublished Data\[Kramski M, Purcell DFJ, Unpublished Data] \([77]\). Elevated levels of 16S rDNA have also been associated with higher HIV RNA and poor CD4+ T-cell recovery \([77]\).

### Indirect measures of microbial translocation: endotoxin-specific immune response

Plasma LBP levels may measure exposure to LPS more reliably than direct measurement of LPS \([90]\). LBP levels are increased in untreated HIV-infected patients \([3]\) and patients with AIDS \([91]\). However, to our knowledge, the effect of cART on LBP has not been studied so far. Endotoxin core antibodies (EndoCAb) are produced by B cells in response to LPS and can bind to LPS to neutralize its activity \([92]\). EndoCAb titers are significantly reduced in HIV-infected patients compared with healthy controls \([3,54]\). Levels of EndoCAb were significantly lower in patients receiving cART compared with untreated patients \([54]\). Although levels of EndoCAb have been found to be inversely correlated with LPS \([3]\), the levels of EndoCAb may also be affected by B-cell dysfunction in HIV \([93]\) and, therefore, may not be a reliable indirect measure of LPS function.

<table>
<thead>
<tr>
<th>Association with other biomarkers</th>
<th>Association with clinical outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Naive</strong></td>
<td><strong>On cART</strong></td>
<td><strong>Naive (HIV-)</strong></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>sCD14</td>
<td>–</td>
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<tr>
<td>–</td>
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<tr>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HIV RNA, sCD14, CD8+ T-cell activation, D-dimer</td>
<td>–</td>
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</tr>
</tbody>
</table>
Plasma levels of sCD14 are measured by ELISA. Therefore, unlike the LPS and 16S rDNA assays, which are both technically challenging, quantification of sCD14 is highly reproducible and could potentially be reliably used in large-scale clinical studies. sCD14 levels are elevated in untreated HIV-infected patients, and are elevated in patients with faster progression to AIDS [3,94]. Similar to LPS, cART leads to a significant reduction in sCD14, but levels remain significantly elevated compared with healthy controls [83,95]. Using detailed longitudinal assessment of LPS and sCD14 in patients on cART together with mathematical modeling, we recently demonstrated that both markers will eventually normalize in patients on long-term cART [83].

Relationship of markers of microbial translocation to each other
A direct correlation between all markers for microbial translocation would be expected; however, the relationship between LPS and the immunological response, involving membrane-bound CD14, sCD14, LBP and EndoCAb, are complex, and the interaction among these components may differ in patients who are on and off cART [96,97]. Studies have demonstrated a significant correlation between 16S DNA and LPS [77]; LPS and LBP [91]; and LPS and sCD14 [3,83,91]. LBP itself also correlates with sCD14 [3]. In addition, 16S rDNA, LPS and sCD14 have all been demonstrated to correlate with T-cell activation (defined as expression of CD38·HLA-DR+ on CD8+ T cells) [Lichtfuss GF, Lewin SR, Jaworwski A, Crowe SM, Unpublished Data] [3,77].

Clinical relevance of microbial translocation
The effects of microbial translocation on reconstitution of CD4+ T cells following cART has been examined in several studies [3,77,83,98,99]. Some of these studies demonstrated an inverse association between CD4+ T-cell increase and the amount of circulating microbial products as measured by levels of LPS [3] or 16S rDNA [77]. We recently examined the relationship between LPS, sCD14 and long-term CD4 recovery in a cohort of 96 patients initiating cART, and used multivariate analysis to determine factors associated with the speed of CD4+ T-cell recovery to over 500 cells/µl [83]. Faster restoration to CD4+ T-cell counts of over 500 cells/µl was associated with, in addition to other factors, higher baseline CD4+ T-cell counts, lower baseline LPS levels and paradoxically higher baseline sCD14 levels, highlighting the complexity of LPS and sCD14 interactions, which can be both pro- and anti-inflammatory [100]. In addition, we did not find that the level of LPS or sCD14 in patients on cART was associated with the speed of CD4+ T-cell recovery [83].

Increased microbial translocation has also been associated with several adverse HIV-related clinical outcomes, including HIV disease progression, dementia, liver disease and mortality. In cART-naïve patients, development of an opportunistic infection was associated with elevated LPS [85]. Higher plasma levels of LPS and sCD14 were also associated with HIV-associated dementia in patients who had either failed or never received cART [91].

In patients with HIV–hepatitis C virus (HCV) co-infection, elevated LPS was associated with more rapid progression of liver disease [101]. Patients with an LPS in the upper quartile (>42 pg/ml) had a 19-fold greater risk of liver disease progression (p = 0.018). By contrast, in a small cross-sectional study of patients with HIV–hepatitis B virus (HBV) co-infection (n = 55), we found that LPS was not associated with an increased risk of liver disease (measured by liver biopsy) [102]. Whether elevated LPS in the setting of HIV–HCV co-infection is a cause or a consequence of liver disease remains uncertain, but it appears that the impact of HCV–HBV co-infection is different.

Finally, a recent prospective substudy of patients enrolled in the SMART study demonstrated that subjects with sCD14 levels in the highest quartile had a sixfold higher risk of death than those in the lowest quartile (95% CI: 2.2–16.1; p = 0.0004), with minimal change after adjustment for inflammatory markers, CD4+ T-cell count and HIV RNA [54].

Immune dysfunction
T cells
HIV not only significantly reduces the number of CD4+ T cells and causes the expansion of CD8+ T cells, but also has a profound effect on T-cell function. While the total number of CD4+ T cells is often normalized following cART, the composition of the T-cell populations and their function are not.

In patients receiving cART, naïve CD4+ T cells often do not return to normal levels [103,104] and the number of activated and senescent cells is elevated [105]. Senescent cells have shortened telomere length and are defined as CD28-CD57+, which are both established biomarkers for immunological aging in a healthy aging population [106].
The activating co-receptor, CD28, expressed on CD4+ and CD8+ T cells, promotes cell survival, IL-2 production, metabolic activity and clonal expansion [107–110]. Therefore, loss of CD28 is accompanied by phenotypic changes that inhibit T-cell function and the accumulation of CD28+ T cells is a hallmark of immunologic aging [111]. In untreated HIV-infected patients, an increase in CD8+CD28+ T cells and a decrease in CD4+CD28+ and CD8+CD28+ T cells was associated with progression to AIDS [112], and loss of CD28 inversely correlated with an increase in HLA-DR expression on CD4+ T cells [113]. Patients receiving suppressive cART showed increased proportions of senescent T cells, similar to levels found in the elderly (defined as CD8+CD57+CD28+) [105,106], and higher numbers of CD57+CD8+ senescent T cells were associated with lower arterial distensibility [114].

Recovery of antigen-specific T-cell responses can also be impaired or dysregulated following cART, especially in patients who initiate cART at low CD4+ T-cell counts [115,116]. In HIV–HBV co-infected patients we recently described little or no recovery in HBV-specific CD8+ T cells despite HBV-active cART, which successfully suppressed both HIV and HBV [117]. In HIV-infected patients co-infected with Mycobacterium tuberculosis, recovery of M. tuberculosis-specific cells is variable and can be associated with an excessive T-cell-mediated immune response against M. tuberculosis antigens, often leading to severe clinical disease [118,119].

In HIV-infected patients, cytomegalovirus (CMV)-specific T cells account for close to 10% of all antigen-specific CD4+ and CD8+ T cells [120], and there has been recent interest in the role of these cells in immune activation and non-AIDS events. The number of CMV-specific T cells was significantly higher in HIV-infected patients on cART compared with uninfected controls [121,122] and in patients naive to cART [123]. The percentage of CMV-specific IFN-γ-producing CD8+ T cells was also positively correlated with carotid intima thickness in both HIV patients and uninfected control subjects, suggesting that an increased inflammatory response to CMV may be contributing to CVD [124]. Interventions that reduce CMV replication, such as valganciclovir, have recently been demonstrated to reduce CD8+ T-cell activation in HIV-infected patients receiving cART [125]. This novel strategy requires further evaluation but may have some role in the future to reduce inflammation and potentially reduce the risk of CVD.

In conclusion, not all T-cell subsets recover equally following cART and this imbalance may contribute to ongoing immune dysfunction, even in patients with a normal total CD4+ T-cell count.

**Natural killer cells**

Natural killer (NK) cells are traditionally defined as CD3 CD56亮 cells, and are further differentiated by the level of expression of CD56 and whether there is expression of the Fc receptor, CD16, also known as the receptor for antibody-dependent cell cytotoxicity. Therefore, NK cells can be grouped into the smaller regulating CD3 CD56亮CD16%， the larger, predominantly cytolytic CD3 CD56亮CD16% [126] and the functionally inert CD3 CD56 CD16% subsets [127]. Activity of NK cells can also be assessed by expression of HLA-DR as a marker of activation [128] and CD107a as a marker of active degranulation [129–131].

Early studies in treatment-naive HIV-infected patients demonstrated a significant reduction in the total number of NK cells compared with healthy controls [131–135]. However, recent studies have also demonstrated an altered distribution of NK cell subsets, with an increase in CD3 CD56亮CD16%, CD56亮CD16% NK cells compared with uninfected controls [136]. In addition, in treatment-naive HIV-infected patients, NK cells had reduced expression of activating surface receptors, and reduced cytotoxicity and cytokine production with little improvement following cART (reviewed in [137]). Following cART, the expanded functionally inert CD3 CD56 CD16% NK cells persist [138] and restoration of CD56% NK cell numbers occurs, although at a slower rate than CD4+ T cells [139–141].

We and others have observed that NK cells in HIV-infected patients naive to cART express high levels of CD107a consistent with active degranulation, a marker for high cytolytic activity [130,131,142]. Following cART, we observed a reduction of CD107a expression while the co-expression of CD38 and HLA-DR remained elevated, consistent with persistent NK cell activation [Lichtfuss GF, Lewin SR, Jaworwski A, Crowe SM, Unpublished Data]. In addition, expression of CD16 on NK cells was low in patients with HIV [143] and did not return to normal levels following cART [142,144], possibly as a direct result of NK cell activation [144,145]. The relationship between persistent NK cell dysfunction on cART and long-term clinical outcomes has not been determined but is an area of interest for future research.
Monocytes

Monocytes in peripheral blood are characterized by the combination of CD14 and -16 surface antigen expression. The majority of monocytes express CD14 without detectable expression of CD16 (CD14++CD16 monoocytes). A minor subset representing 5–15% of monocytes expresses CD16 and variable levels of CD14, and these cells are phenotypically described as CD14+CD16++. HIV preferentially infects these CD14+CD16++ monocytes. Although this subset was reported to be expanded in HIV-infected individuals in the pre-cART era, our more recent data show that the CD14+CD16++ monocyte population was not significantly increased in HIV-infected individuals in the setting of cART.

Hallmarks of monocyte activation include the shedding of CD14, production of IL-6 and catalysis of D-dimer, and have been discussed in the previous sections. Similar to T-cell activation markers, monocyte activation continues for significant periods of time following suppression of HIV RNA with cART. One measure of monocyte activation, the surface expression of CD38, declines following cART, although levels remain significantly higher than in uninfected controls. Recent evidence suggests that persistence of markers of monocyte activation may be independent of persistent T-cell activation. In the presence of LPS and other Toll-like receptor ligands, the surface expression of TF (thromboplastin and CD142) is elevated in untreated HIV-infected patients and correlates with the activation of the monocyte coagulation cascade, eventually leading to the cleavage of fibrinogen into D-dimer.

Chronic monocyte activation may directly contribute to the elevated risk of CVD, as heightened expression of TF has been linked to myocardial infarction and angina in HIV-uninfected patients. Activated monocytes are crucial cells in the pathogenesis of atherosclerosis: they are the precursors of fat-laden foam cells, which are the major cells found within the atheromatous plaque, potentially contributing to the increased risk of CVD in HIV-infected patients, even in those with virologic suppression (reviewed in [152,153]). Studies to determine the precise role of monocytes in the pathogenesis of non-AIDS morbidity and mortality in HIV-infected patients with virologic suppression on cART are ongoing.

Biomarkers & clinical disease in HIV-infected patients on cART

Biomarkers of inflammation and coagulation have been most extensively studied regarding their relationship with overall mortality, CVD and cardiovascular mortality. There are far fewer studies examining the relationship between persistent immune dysfunction and inflammation, and other non-AIDS clinical diseases, including malignancy. There is a clear relationship between lower total CD4+ T-cell counts and rates of malignancy (both HIV related and non-HIV related), and the cytokines, IL6, IL-10 and sCD30, have all been associated with an increased risk of non-Hodgkin’s lymphoma.

HIV-infected adults have a higher prevalence of low bone mineral density (BMD; 40–83%) compared with HIV-uninfected subjects, and HIV-infected patients receiving cART have a lower BMD than HIV-infected patients naive to cART. Baseline levels of hsCRP and IL-6, as well as change in IL-6, are associated with lower BMD in the general population, and also associated with greater risk of fragility fracture. There are currently no data evaluating levels of inflammatory cytokines or immune activation on the prevalence of osteopenia or osteoporosis, or change in BMD in HIV patients; given the importance of this complication of cART, further studies are warranted.

Conclusion

Despite control of viral replication and CD4+ T-cell reconstitution following cART, HIV-infected patients experience high rates of non-AIDS events, including CVD, malignancy and premature death. Current research on biomarkers in HIV infection is focusing on the potential links between persisting immune activation and dysfunction, and adverse clinical outcomes following cART. While a number of biomarkers have been evaluated in large clinical prospective studies, none of these biomarkers are used in routine clinical practise. Further work is still needed to define the cause and effect of the relationship between the relevant biomarker and clinical outcome, and whether reduction of a specific biomarker following a therapeutic intervention leads to improved clinical outcomes.

Future perspective

After years of success in the use of cART to manage HIV infection, clinicians now face the challenge of reducing non-AIDS events in patients on cART. Unfortunately, CD4+ T-cell count and HIV RNA as sole biomarkers are unable to fully predict health outcomes in HIV-infected patients on cART. The recent clinical trials of IL-2 clearly demonstrated that an increase in total CD4+ T-cell count did not correlate with improved health outcomes for patients receiving IL-2 in addition to...
cART [359]. In addition to conventional biomarkers used in HIV-uninfected patients that predict diseases such as CVD, novel biomarkers that are highly reproducible will be needed to monitor ongoing immune activation and dysfunction in patients on cART. Larger longitudinal clinical studies are necessary to convincingly demonstrate clear associations between biomarkers and clinical outcomes. In addition, continuing efforts in fundamental immunology research are needed to understand the mechanistic links between biomarkers and clinical disease. These studies will also potentially enable the development of therapeutic interventions beyond cART to reduce the incidence of non-AIDS events.

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### Executive summary

#### HIV-infected patients receiving combination antiretroviral treatment continue to experience worse health outcomes compared with the uninfected population

- Combination antiretroviral treatment (cART) successfully suppresses HIV RNA and restores the total number of CD4+ T cells to normal levels in most patients.
- HIV-infected patients on long-term cART experience higher rates of cardiovascular disease (CVD), non-AIDS malignancy, neurocognitive impairment, and liver, kidney and bone disease.
- HIV-infected patients on long-term cART have a shorter life expectancy compared with the general population.

#### cART fails to normalize generalized immune activation of HIV patients

- Cellular markers for immune activation (e.g., CD38/HLA-DR coexpression on CD4+ and CD8+ T cells) are elevated in patients on cART and has been linked to impaired CD4+ T-cell recovery and increased mortality.
- Plasma markers for inflammation (e.g. C-reactive protein, IL-6 and D-dimer) are elevated in patients on cART, and are associated with increased CVD, opportunistic disease and all-cause mortality.
- HIV is associated with elevated circulating microbial products.
- In HIV-infected patients on or off cART, microbial products (e.g., lipopolysaccharide or bacterial 16S rDNA in plasma) are significantly elevated, and these correlate with markers of T-cell activation.
- Elevated levels of microbial products are associated with the development of opportunistic disease, slower T-cell recovery and faster progression of liver disease in patients co-infected with hepatitis C virus.
- The immunological response specific for microbial products can be measured (e.g., soluble CD14, lipopolysaccharide-binding protein or endotoxin core antibody), and in HIV-infected patients receiving cART, elevated soluble CD14 been associated with an increased risk of mortality.

#### cART fails to normalize the composition of the T-cell compartment

- Overall, total CD4+ T-cell numbers return to normal following cART, but there is skewed recovery of some CD4+ T-cell subpopulations.
- HIV-infected patients receiving cART have increased proportions of CD28-CD57+ T cells, a sign of immunological senescence which is associated with increased CVD.
- The repertoire of antigen-specific T cells is altered in some patients receiving cART and is skewed towards particular common pathogens (e.g., cytomegalovirus).

#### cART fails to normalize phenotype & function of other immune cells

- Natural killer cells, which are important for antiviral immune response and tumor surveillance, are altered by HIV infection and are characterized by increased activation and a large functionally inert subpopulation that is only slowly restored following cART.
- Monocytes, the source of several plasma markers of immune activation (e.g., sCD14), show increased expression of tissue factor, which mediates the catalysis of D-dimer, which can enhance coagulation.
- The role of natural killer cells and monocytes in the pathogenesis of disease in cART patients is currently being investigated, and they are potentially linked to enhanced CVD.

### Conclusion

- Increased morbidity and mortality in HIV patients receiving cART is associated with incomplete restoration of immune function.
- Biomarkers for immune activation and immune dysfunction are useful research tools to understand pathogenesis of and increased risk for non-AIDS events.
- Further research of these biomarkers is required to reliably predict adverse health outcomes and to develop novel interventions to be used in addition to cART for the management of HIV infection.
et al. Large prospective cohort study on life

Key paper that was the first to establish a


Large prospective cohort study on life expectancy of HIV-infected patients on combination antiretroviral therapy (cART).


Concise and detailed overview of current complications of cART.


Detailed review of current biomarkers in HIV.


First study to clearly demonstrate persistent T-cell activation in HIV-infected patients receiving cART.


Biomarkers of immune dysfunction following combination antiretroviral therapy

- Pizzolo G, Vinante F, Morosato L et al.: High serum level of the soluble form of CD30 molecule in the early phase of HIV-1 infection as an independent predictor of progression to AIDS. AIDS 8(6), 741–745 (1994).
- Provided the main body of evidence for biomarkers of immune activation and non-AIDS events in HIV-infected patients receiving cART.
- Important substudy of Strategies for the Management of Antiretroviral Therapy (SMART) that investigated the relationship of C-reactive protein, IL-6 and D-dimer as biomarkers in HIV-infected patients receiving cART.
- Important substudy of the SMART study that established an association between elevated soluble CD14 and increased mortality in HIV-infected patients receiving ART.
Important study that investigated the association between microbial translocation, immune activation and cardiovascular disease in HIV-infected patients receiving cART.


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