

RESEARCH ARTICLE

Terminal differentiation of T cells is strongly associated with CMV infection and increased in HIV-positive individuals on ART and lifestyle matched controls

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Abstract

HIV-1-positive individuals on successful antiretroviral therapy (ART) are reported to have higher rates of age-associated non-communicable comorbidities (AANCCs). HIV-associated immune dysfunction has been suggested to contribute to increased AANCC risk. Here we performed a cross-sectional immune phenotype analysis of T cells in ART-treated HIV-1-positive individuals with undetectable viremia (HIV-positives) and HIV-1-negative individuals (HIV-negatives) over 45 years of age. In addition, two control groups were studied: HIV negative adults selected based on lifestyle and demographic factors (Co-morBidity in Relation to AIDS, or COBRA) and unselected age-matched donors from a blood bank. Despite long-term ART (median of 12.2 years), HIV-infected adults had lower CD4⁺ T-cell counts and higher CD8⁺ T-cell counts compared to well-matched HIV-negative COBRA participants. The proportion of CD38⁺HLA-DR⁺ and PD-1⁺ CD4⁺ T-cells was higher in HIV-positive cohort compared to the two HIV-negative cohorts. The proportion CD57⁺ and CD27⁻CD28⁻ cells of both CD4⁺ and CD8⁺ T-cells in HIV-positives was higher compared to unselected adults (blood bank) as reported before but this difference was not apparent in comparison with well-matched HIV-negative COBRA participants. Multiple regression analysis showed that the presence of an increased proportion of terminally differentiated T cells was strongly associated with CMV infection. Compared to appropriately selected HIV-negative controls, HIV-positive individuals on ART with long-term suppressed viraemia exhibited incomplete immune recovery and increased immune activation/exhaustion. CMV infection rather than

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treated HIV infection appears to have more consistent effects on measures of terminal differentiation of T cells.

Introduction

Antiretroviral therapy (ART) for human immunodeficiency virus type 1 (HIV-1) infection has dramatically reduced AIDS-associated morbidity and mortality [1–3]. However, HIV-1-positive individuals on successful ART are reported to have higher rates of age-associated noncommunicable comorbidities (AANCCs) than the general population [3–7]. Several contributing factors have been implicated, including ART toxicity, chronic immune activation, immune dysfunction and a higher prevalence of traditional risk factors [8, 9]. Interestingly, immunological alterations observed during treated HIV-1 infection reflect those observed in the general population during aging [7, 10–12]. These include high levels of soluble inflammatory and coagulation related proteins, high levels of T cell activation, high levels of T cell exhaustion, low levels of naïve T cells and an extensive proliferative history of CD8 T cells [13–18]. Together, these age-associated immunological alterations are referred to as immune senescence [19, 20], although the precise definition and clinical significance of this syndrome remains controversial [4, 21, 22].

The immune senescent phenotype was first reported in a group of elderly adults who progressed more rapidly [23]. Subsequent work suggested that chronic viral infections such as cytomegalovirus (CMV), hepatitis B virus (HBV), hepatitis C virus (HCV) and HIV, contribute to the development of this phenotype [8, 13, 15, 24–27]. Indeed, CMV infection is also associated with low CD4:CD8 ratios, increased systemic inflammation and increased expansion of terminally differentiated and senescent T cells [13, 15, 28, 29]. CMV prevalence is extremely high in HIV-positive individuals and therefore CMV infection may well contribute to the immune senescent phenotype observed in HIV-positive individuals. Recently, we demonstrated that levels of terminal differentiation of T cells and immune senescence did not differ between HIV-positive individuals on ART and HIV-negative controls with comparable age, lifestyle and demographic characteristics [30], findings which contrasted with those from other studies [13–16]. To increase our understanding of these findings, we analysed the effect of HIV-1 and CMV infection on T cell activation, exhaustion and terminal differentiation of T cells in HIV-1-positive individuals with suppressed viraemia on ART (HIV-positive), HIV-1-negative individuals (HIV-negative) comparable regarding most lifestyle and demographic factors derived from the Co-morBidity in Relation to AIDS (COBRA) cohort, and age-matched unselected blood bank donors (referred in the tables as BBD). Notably, these blood bank donors are at lower risk of acquiring blood borne infections than the general population but often used as a control group for comparative studies.

Materials and methods

Subjects

Study subjects other than the bloodbank donors participated in an ongoing European Commission-funded project known as COBRA (Co-morBidity in Relation to AIDS). COBRA is a detailed, prospective evaluation of the burden of AANCC among 134 HIV-1-positive patients on ART and 79 appropriately chosen non-infected controls who have comparable socio-demographic and behavioral (risk) factors. COBRA aims to provide a robust estimate of the effect of treated HIV infection on the prevalence, incidence and age of onset of AANCC. Furthermore,

COBRA aims to clarify the pathogenic mechanisms underlying this causative link, including the possible induction of an inflammation-associated accelerated ageing phenotype. Exclusion criteria were: age under 45 years, and self-reported current intravenous drug use (in the past six months), daily use of recreational drugs (with the exception of cannabis), and excess alcohol intake (>48 units per week). All HIV-positive participants were required to be on ART and to have had undetectable plasma HIV RNA (<50 copies/mL) for ≥ 12 months prior to enrolment. Participants were recruited from two study sites: London and Amsterdam. For this immunological study, 40 HIV-positive and 40 HIV-negative participants were randomly selected with equal numbers in each of the following age-groups (45–50, 51–55, 56–60, 61–65, 66–70, and 70+), except for the oldest age category in which only a few individuals were available. Materials from blood bank donors were obtained from the Dutch national blood bank in Amsterdam, the Netherlands (www.sanquin.nl). Blood bank donors (median: 58, IQR: 52.0–65.0) were matched for age with the HIV-positive (Median: 58.5, IQR: 53.5–63.5) and HIV-negative (median: 59.0, IQR: 53.0–64.5) COBRA participants and selected in such a way that the different age categories in the COBRA, except for the category of 70+, were equally represented. Blood bank donors from the Netherlands are actively screened for HIV, Hepatitis B, Hepatitis C, syphilis, and HTLV infection. Individuals aged above 70 years or individuals that display high risk behavior for blood born infections are excluded from blood donation. Of note, blood bank donors infected with HBV but who cleared the infection documented by an anti-HBs antibody titer of at least 200 IU/L or blood bank donors that have been vaccinated against HBV are not excluded from blood donation.

Ethics statement

This study has been conducted in accordance with the ethical principles set out in the declaration of Helsinki and was approved by the institutional review board of the Academic Medical Center (AMC) (NL 30802.018.09), the London (Stanmore) Research Ethics Committee (REC) (13/LO/0584), and the Ethics Advisory Body of the Sanquin Blood Supply Foundation in Amsterdam. Written informed consent was obtained from all participants.

T cell phenotyping and flow cytometry

Cryopreserved PBMC were thawed and cell viability was analysed by trypan blue staining and for FACS analysis cell viability was required to be >75%. PBMC were stained with monoclonal antibodies for 30min at 4°C in the dark, to determine expression levels of different T-cell surface molecules. T cell differentiation was defined as the proportion of naïve ($CD45RA^+ CD27^+ CCR7^+$), central memory ($CD45RA^- CCR7^+ CD27^+$), transitional memory ($CD45RA^- CCR7^- CD27^+$), effector memory ($CD45RA^- CCR7^- CD27^-$), and terminally differentiated effector memory (TEMRA; $CD45RA^+ CCR7^- CD27^-$) within the total CD4 or CD8 T cell population. T cell activation was defined as the proportion of cells that were positive for both CD38 and HLA-DR within the total CD4 or CD8 T cell population. T cell exhaustion was defined as the proportion of PD1 positive cells within the total CD4 or CD8 T cell population. Terminally differentiated T cells were defined as proportion of CD57 positive cells within the total CD4 or CD8 T cells population, the proportion of cells negative for both CD27 and CD28 within the total CD4 or CD8 T-cells population, or the proportion of CD57 positive within the $CD28^- CD4^+$ or $CD28^- CD8^+$ T cell populations. The following directly conjugated monoclonal antibodies were used for cell surface marker staining: CD3 V500, CD4 PE-Cy7, HLA-DR Fitc, CD38 PE, CD28 PerCP Cy5.5, CD45RA PE-Cy7, CD8 Pacific Blue, CD57 APC, CCR7 PE, CD27 PerCP Cy5.5 (BD Biosciences, San Jose, CA, USA), CD27 APCeFluor780, CD4 APC eFluor780, and PD-1 PE (eBioscience, San Diego, CA, USA). Fluorescence was measured with

the FACS Canto II (BD Biosciences). The proportion of cells expressing each marker were determined using FlowJo 7.6 (TreeStar, Ashland, OR, USA). The gating strategy is displayed in the supplementary information (S1 Fig).

CMV antibody titers

CMV specific IgG levels are believed to be a surrogate marker of CMV reactivation and concurrent immune response to control infection [31]. Thus, increased CMV total antibody and high avidity titers indicate recurrent CMV antigen exposure, whereas CMV high avidity titers are also indicative of increased antibody maturation. CMV total antibody titers and high avidity antibody titers were measured by ELISA-VIDITEST anti-CMV-IgG and IgG avidity (VIDIA, Praha, Czech republic) according to the manufacturer's instruction. For quantification a standard curve was prepared by serial dilution of plasma from a known CMV seropositive individual.

Statistical analysis

Differences in subject characteristics between HIV-positive, HIV-negative and blood bank donors were assessed using Student's T test, Mann-Whitney U test, or Pearson Chi-square test, as appropriate. Associations of immunological markers with study group (HIV-positive, HIV-negative, blood bank donors) and CMV serostatus were evaluated using multivariable linear regression adjusted for age and gender. In the sub-group of participants who were CMV seropositive, we then considered the association of each immunological marker with group and i) CMV total IgG and ii) CMV high avidity IgG, with the same adjustments. Some outcomes were transformed to attain normality as indicated in the table. Unstandardized coefficient (B) indicates the difference in the outcome (immunological markers) with respect to the variables (study group, CMV infection), and are given in the table. Uncorrected p values are given in the tables and the required p value for significance after Bonferroni adjustment for multiple testing is indicated. Analyses were performed in IBM SPSS Statistics for Windows v.23 (IBM, Armonk, NY, USA) and GraphPad Prims 6 (GraphPad, La Jolla, CA, USA).

Results

Baseline characteristics of COBRA participants and blood bank donors

HIV-positive individuals had a median (IQR) age of 58.5 (53.5–63.5) years and HIV-negative controls had a median (IQR) age of 59 (53–63.5) years. The groups were representative of the main COBRA cohort study (HIV-positive individuals: 93.3% male, 86.6% MSM, 12.0% of African descent; HIV-negative controls: 92.4% male, 79.8% MSM, 2.6% of African descent). HIV-positive individuals had been diagnosed with HIV for a mean (standard deviation) of 13.9 (4.8) years ago, had been on ART for a mean (standard deviation) of 12.2 (4.7) years and had spent a median (IQR) of 8.0 (5.3–10.9) years with an undetectable plasma viral load (Table 1). Whilst the percentage of males did not differ significantly between the HIV-positive and HIV-negative COBRA participants, it was significantly lower in the group of blood bank donors. HIV-positive and HIV-negative COBRA participants were more often (co-)infected with CMV and HCV as compared to the blood bank donors, whereas HIV-positive COBRA participants were more often infected with CMV, HBV and HCV when compared to HIV-negative COBRA participants (Table 1). HIV-positive COBRA participants, despite long-term suppression of HIV-replication by ART, exhibited incomplete CD4⁺ T-cell restoration and elevated CD8⁺ T-cell counts compared to HIV-negative COBRA participants, as reflected by lower CD4 counts, higher CD8 counts and an inverted CD4:CD8 T-cell ratio (Table 1).

Table 1. Baseline characteristics HIV-positive and HIV-negative COBRA participants and blood bank donors.

	HIV-positive n = 40	HIV-negative n = 40	HIV-positive vs HIV- negative	Blood bank donors n = 35	HIV-positive vs BBD	HIV-negative vs BBD
	n (%) or median (IQR)	n (%) or median (IQR)	p value ¹	n (%) or median (IQR)	p value ¹	p value ¹
Age (Years)	58.5 (53.5–63.5)	59.0 (53.0–64.5)	0.9	58 (52.0–65.0)	0.5	0.5
Male sex	36 (90.0%)	37 (92.5%)	0.7	18 (51.4%)	< .001	< .001
African descent	5 (12.5%)	1 (2.5%)	0.09	n.a.		
MSM	32 (80.0%)	30 (75.0%)	0.8	n.a.		
CMV	38 (95.0%)	31 (77.5%)	0.02	8 (22.9%)	< .001	< .001
anti-CMV IgG	50.9 (23.5–108.6)	23.9 (13.8–87.8)	0.03	11.3 (10.2–16.8)	0.002	0.09
High avidity anti-CMV IgG	30.7 (13.0–57.0)	13.3 (8.2–39.7)	0.048	10.7 (10.0–13.2)	0.046	0.4
HBV	21 (52.5%)	7 (17.5%)	0.001	n.a.		
Cleared	18 (45.0%)	7 (17.5%)	0.008	n.a.		
Chronic	3 (7.5%)	0 (0%)	0.08	0 (0%)	0.1	n.d.
HCV (chronic and acute)	6 (15%)	0 (0%)	0.01	0 (0%)	0.02	n.d.
CD4 counts, cells/μl	589 (470–800)	961 (759–1233)	< .001	n.a.		
CD8 counts, cells/μl	762 (636–1029)	488 (364–621)	< .001	n.a.		
CD4:CD8 ratio	0.80 (0.61–1.13)	1.95 (1.33–2.83)	< .001	n.a.		
CD4 nadir, cells/μl	180 (60–180)					
Years since HIV diagnosis	13.9 (9.1–18.7)					
Years since ART	12.2 (7.9–16.9)					
Years undetectable plasma viral load (<200 c/ml) ²	8.0 (5.3–10.9)					

¹ p value calculated using Student's t-test, Mann-Whitney U test or Chi-Square test, or Wilcoxon's rank-sum test where applicable.

² The threshold was set at 200 c/ml to exclude incidental viral blips from the period in which plasma viral load was detectable.

Abbreviations: HIV, human immunodeficiency virus; BBD, blood bank donors n.a., not available; n.d., not determined; IQR, interquartile range; MSM, men who have sex with men; CMV, Cytomegalovirus; HBV, hepatitis B virus; HCV, hepatitis C virus; ART, antiretroviral therapy.

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T cell activation and exhaustion in COBRA participants and blood bank donors

The percentages of activated (HLA-DR⁺CD38⁺) CD4 T cells and exhausted (PD1⁺) CD4 T cells were higher in ART-treated HIV-positive COBRA participants compared to both cohorts of HIV-negative participants (Table 2, Fig 1A and 1B). Interestingly, the percentage of PD1⁺ expressing CD4 T cells was higher in both the HIV-positive and HIV-negative COBRA participants when compared to the blood bank donors (Fig 1B and Table 2). The percentage of activated (HLA-DR⁺CD38⁺) CD8 T cells was higher in the HIV-positive COBRA participants but not in the HIV-negative COBRA participants when compared to the blood bank donors (Fig 1C and Table 2). No differences were observed in the percentage of exhausted (PD1⁺) CD8 T cells between the three groups (Fig 1D and Table 2).

T cell differentiation in COBRA participants and blood bank donors

In the CD4 compartment, the percentage of naïve cells (CD45RA⁺CD27⁺CCR7⁺) was lower in the HIV-positive COBRA participants compared to the HIV-negative COBRA participants, but no differences were observed in the percentage of central memory (CD45RA⁻CCR7⁺CD27⁺), transitional memory (CD45RA⁻CCR7⁻CD27⁺), effector memory (CD45RA⁻CCR7⁻CD27⁻) and TEMRA cells (CD45RA⁺CCR7⁻CD27⁻) (Table 2). When compared to the blood bank

Table 2. T cell activation, exhaustion and differentiation in HIV-positive and HIV-negative COBRA participants and blood bank donors.

Markers	HIV-positive median (IQR)	HIV-negative median (IQR)	HIV-pos vs HIV-neg p value ¹	BBD median (IQR)	BBD vs HIV-pos p value ¹	BBD vs HIV-neg p value ¹
CD4⁺ T cells						
% HLA-DR ⁺ CD38 ⁺ of CD4 ⁺ T cells ²	2.58 (1.73–3.96)	1.56 (1.03–2.57)	0.001	2.09 (1.41–2.65)	0.06	0.06
% PD1 ⁺ of CD4 ⁺ T cells ²	6.77 (5.16–10.69)	6.2 (3.38–8.36)	0.015	4.2 (2.73–5.03)	2.0E-06	0.03
% CD57 ⁺ of CD4 ⁺ T cells ²	12.14 (6.32–18.43)	12.21 (7.01–19.31)	0.8	4.73 (2.91–8.47)	3.2E-04	0.001
% CD27 ⁺ CD28 ⁻ of CD4 ⁺ T cells ²	4.76 (1.14–8.49)	6.03 (1.46–12.25)	0.8	0.19 (0.05–0.57)	1.0E-06	3.0E-06
% CD57 ⁺ of CD4 ⁺ CD28 ⁻ T cells	58.88 (37.64–81.46)	79.59 (45.93–85.51)	0.07	32.23 (15.29–63.46)	0.015	2.9E-04
% CD45RA ⁺ CCR7 ⁺ CD27 ⁺ of CD4 ⁺ T cells ⁴	11.4 (4.9–20.01)	18.54 (9.62–29.43)	0.017	23.37 (13.06–40.8)	9.4E-05	0.1
% CD45RA ⁻ CCR7 ⁺ CD27 ⁺ of CD4 ⁺ T cells ⁴	16.6 (11.33–23.1)	15.99 (10.74–22.9)	0.5	19.4 (11.44–22.44)	0.5	0.4
% CD45RA ⁻ CCR7 ⁻ CD27 ⁺ of CD4 ⁺ T cells ²	23.99 (21.17–33.27)	23.87 (18.75–27.1)	0.3	27.16 (19.81–33.86)	0.8	0.1
% CD45RA ⁻ CCR7 ⁻ CD27 ⁻ of CD4 ⁺ T cells ²	11.85 (7.57–24.88)	10.55 (6.57–18.93)	0.4	6.97 (3.87–9.37)	.001	0.046
% CD45RA ⁺ CCR7 ⁻ CD27 ⁻ of CD4 ⁺ T cells ²	0.93 (0.55–2.52)	2.05 (1.06–4.41)	0.1	0.74 (0.3–1.33)	0.2	0.039
CD8⁺ T cells						
% HLA-DR ⁺ CD38 ⁺ of CD8 ⁺ T cells ²	7.46 (4.54–10.8)	5.6 (3.29–9.65)	0.2	4.05 (2.66–5.68)	9.7E-05	0.051
% PD1 ⁺ of CD8 ⁺ T cells ²	19.58 (14.06–23.91)	16.08 (11.56–24.02)	0.2	15.79 (12.42–20.41)	0.4	0.9
% CD57 ⁺ of CD8 ⁺ T cells	50.6 (36.81–57.2)	45.08 (35.57–57.44)	0.8	30.4 (14.91–42.4)	.009	.031
% CD27 ⁺ CD28 ⁻ of CD8 ⁺ T cells	38.1 (24.8–46.4)	36.4 (22.55–50.05)	0.7	12.2 (5.56–20.7)	4.5E-06	3.4E-04
% CD57 ⁺ of CD8 ⁺ CD28 ⁻ T cells ³	75.75 (67.06–80.98)	79.31 (74.03–85.81)	0.045	61.4 (49.4–83.37)	0.6	0.037
% CD45RA ⁺ CCR7 ⁺ CD27 ⁺ of CD8 ⁺ T cells ⁴	6.31 (3.67–10.12)	8.36 (4.19–15.23)	0.08	18.46 (9.85–30.48)	1.3E-05	0.025
% CD45RA ⁻ CCR7 ⁺ CD27 ⁺ of CD8 ⁺ T cells ²	3.71 (2.81–5.69)	4.47 (1.87–7.61)	1.0	5.34 (3.08–8.79)	0.8	1.0
% CD45RA ⁻ CCR7 ⁻ CD27 ⁺ of CD8 ⁺ T cells ⁴	26.8 (19.12–33.19)	20.72 (15.22–27.11)	0.06	34.49 (29.81–42.38)	0.2	0.003
% CD45RA ⁻ CCR7 ⁻ CD27 ⁻ of CD8 ⁺ T cells ²	19.18 (14.37–29.89)	12.9 (9.13–19.46)	0.006	9.23 (5.79–15.22)	0.001	0.3
% CD45RA ⁺ CCR7 ⁻ CD27 ⁻ of CD8 ⁺ T cells ⁴	19.17 (12.96–24.8)	19.18 (11.26–36.78)	0.3	8.88 (5.44–20.4)	0.1	0.047

¹ Multivariable linear regression, corrected for age and gender. Uncorrected p values are given. Bonferroni adjustment for multiple testing required a p value of <0.0025 for significance.

² LOG transformed to obtain normality.

³ arcsine transformed to obtain normality.

⁴ square root transformed to obtain normality

Abbreviations: HIV, human immunodeficiency virus; BBD, blood bank donors; IQR, interquartile range.

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donors, we observed that HIV-positive and HIV-negative COBRA participants had a higher percentage of effector memory cells (CD45RA⁻CCR7⁻CD27⁻), whereas an additional increase in the percentage of TEMRA cells (CD45RA⁺CCR7⁻CD27⁻) was observed in the HIV-negative COBRA participants (Table 2).

Within the CD8 compartment, the percentage of effector memory cells (CD45RA⁻CCR7⁻CD27⁻) was higher in HIV-positive COBRA participants compared to the HIV-negative COBRA participants, whereas no differences were observed in the other populations (Table 2). When compared to the blood bank donors, HIV-positive and HIV-negative COBRA participants had a lower percentage of naïve CD8 cells (CD45RA⁺CD27⁺CCR7⁺). The percentage of effector memory cells (CD45RA⁻CCR7⁻CD27⁻) was higher in HIV-positive, while the percentage of transitional memory (CD45RA⁻CCR7⁻CD27⁺) was lower and TEMRA cells

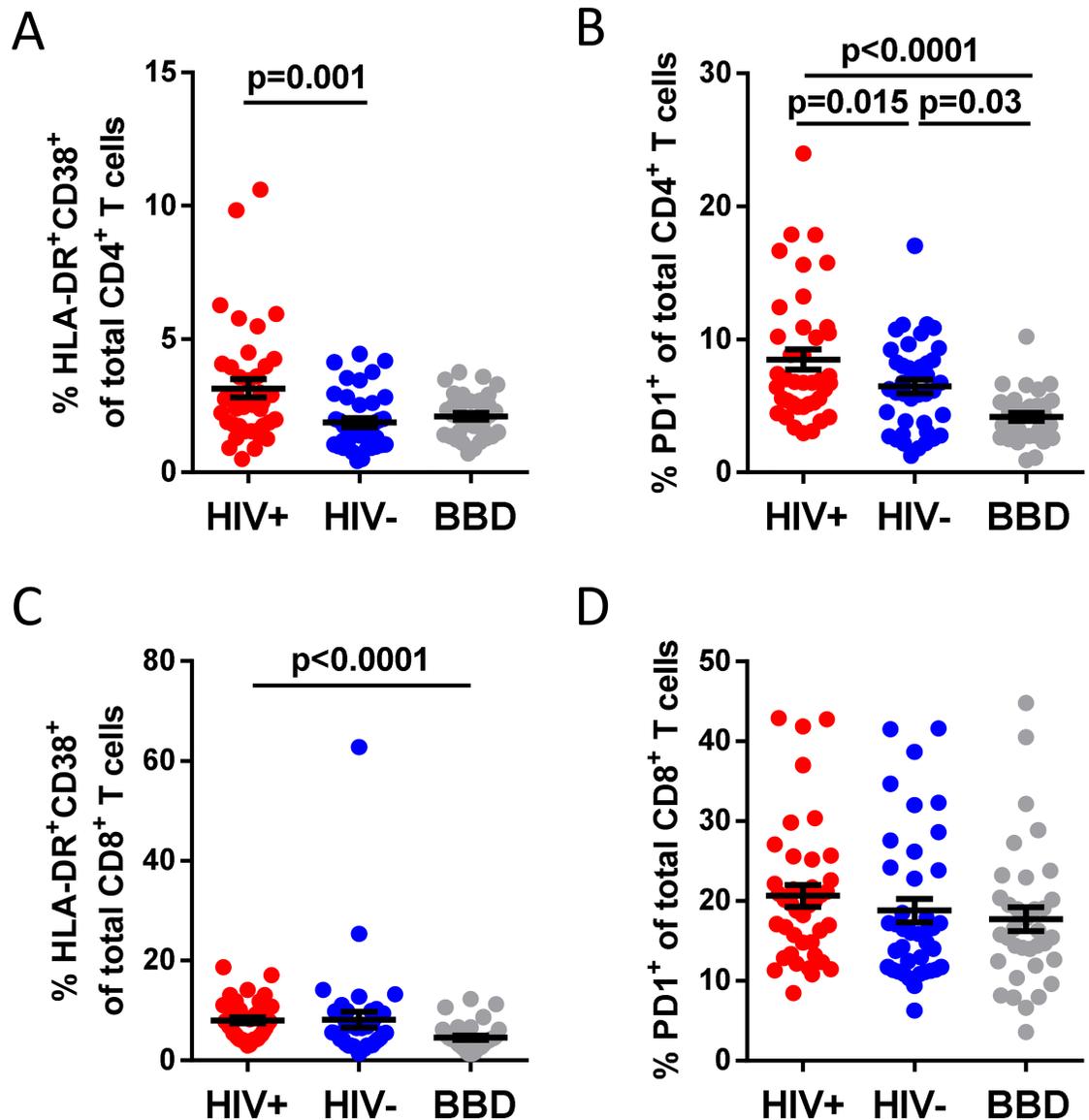


Fig 1. CD4 and CD8 T cell activation and exhaustion in HIV-positive and HIV-negative COBRA participants, and blood bank donors. (a) The percentage of activated (HLA-DR⁺CD38⁺) and (b) exhausted (PD-1⁺) CD4⁺ T cells within the CD4⁺ T-cell population. (c) The percentage of activated (HLA-DR⁺CD38⁺) and (d) exhausted (PD-1⁺) T cells within the CD8⁺ T-cell population. Significance was assessed with multivariable linear regression, corrected for age and gender. HIV+, HIV-positive; HIV-, HIV-negative; BBD, blood bank donors.

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(CD45RA⁺CCR7⁻CD27⁻) higher in HIV-negative COBRA participants as compared to the blood bank donors (Table 2).

Percentage of terminally differentiated T cells is increased in COBRA participants compared to blood bank donors

No significant differences in the percentages of terminally differentiated T cells between the HIV-positive and HIV-negative COBRA participants were observed (Fig 2A and 2B and

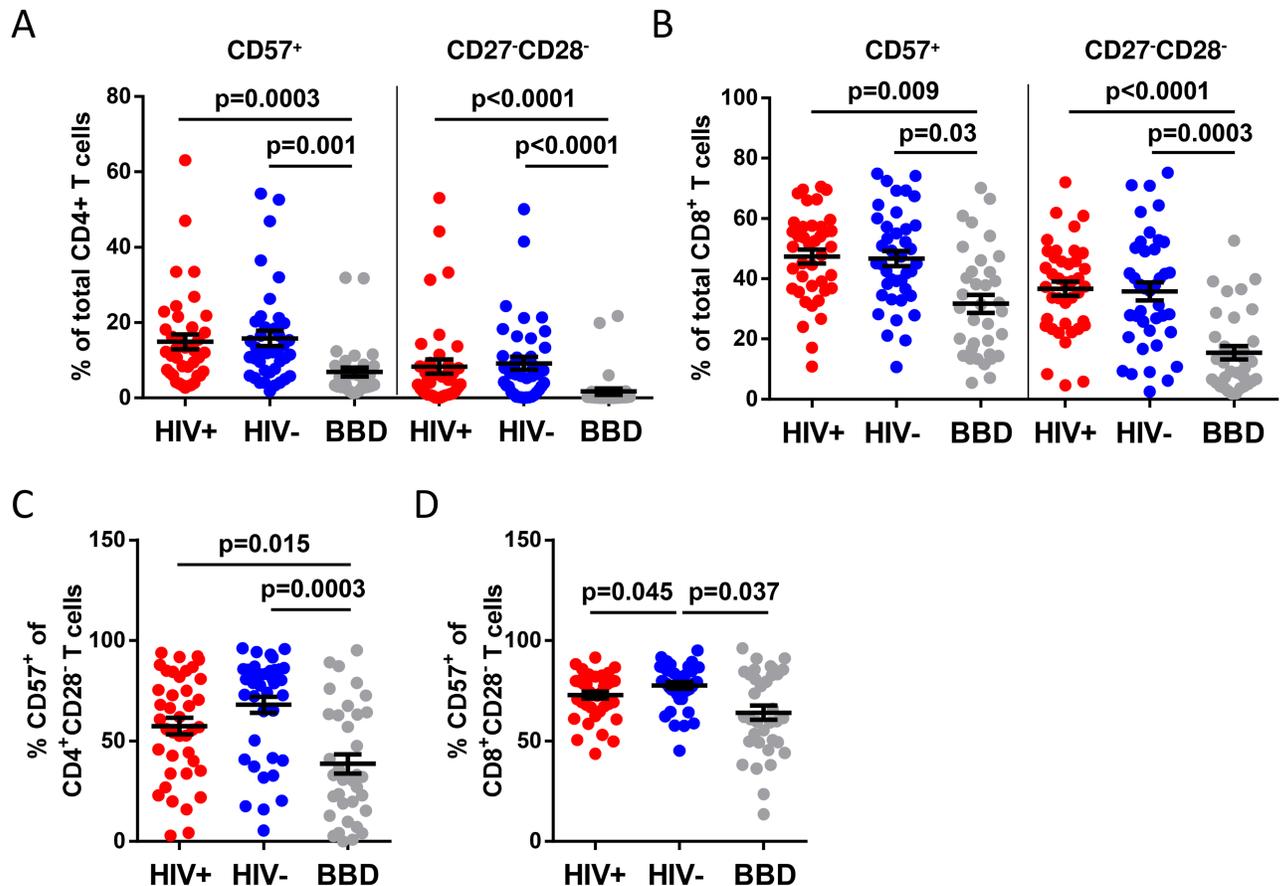


Fig 2. Terminally differentiated CD4 and CD8 T cells in HIV-positive and HIV-negative COBRA participants, and blood bank donors. (a) The percentage of CD57⁺ and CD27⁻CD28⁻ T cells within the CD4⁺ T cell population. (b) The percentage of CD57⁺ and CD27⁻CD28⁻ T cells within the CD8⁺ T cell population. (c) The percentage of CD57⁺ cells in the CD4⁺CD28⁻ and (d) CD8⁺CD28⁻ cell population. Significance was assessed with multivariable linear regression, corrected for age and gender. HIV+, HIV-positive; HIV-, HIV-negative; BBD, blood bank donors.

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Table 2). Strikingly, a strong increase in the percentages of both terminally differentiated CD4 and CD8 T cells (CD57⁺ and CD27⁻/CD28⁻) was observed both when HIV-positive or HIV-negative COBRA participants were compared to blood bank donors (Fig 2A and 2B and Table 2). In addition, we analysed the percentage of CD57 expressing cells within the CD28⁻CD8⁺ T-cells or CD28⁻CD4⁺ T cells as these cells have been shown to accumulate upon chronic antigenic stimulation by viral pathogens [25]. The percentage of CD57 expressing cells within the CD28⁻CD4⁺ T cell population was not different between HIV-positive and HIV-negative COBRA participants (Fig 2C and Table 2). However when compared to the blood bank donors, the percentage of CD57 expressing cells within the CD28⁻CD4⁺ T cells was higher in both the HIV-positive and HIV-negative COBRA participants (Fig 2C and Table 2). The percentage of CD57 expressing cells within the CD28⁻CD8⁺ T cells was lower in HIV-positive COBRA participants as compared to HIV-negative COBRA participants (Fig 2D and Table 2). Blood bank donors have lower levels of CD57 expressing cells within the CD28⁻CD8⁺ T cells when compared to HIV-negative COBRA participants, but not when compared to HIV-positive COBRA participants (Table 2 and Fig 2D).

HIV-1 and CMV infection are both associated with T cell activation, exhaustion and terminal differentiation

Multivariable linear regression analysis was performed to determine whether HIV-1 infection and CMV infection (CMV serostatus) were independently associated with T cell activation, exhaustion, and terminal differentiation of T cells, after adjusting for age and gender. Within the CD4 compartment, both T cell activation (HLA-DR⁺CD38⁺) and T cell exhaustion (PD1⁺) were higher in HIV-positive participants compared to HIV-negative COBRA participants, whereas T cell exhaustion was lower in blood bank donors as compared to HIV-negative COBRA participants (Table 3). Within the CD8 compartment, HIV infection was associated with higher T cell exhaustion while CMV infection was independently associated with lower CD8 T cell exhaustion (PD1⁺) (Table 3).

When we analysed markers of terminally differentiated T cells (CD57⁺ and CD27⁻CD28⁻) we observed that the percentage of CD27⁻CD28⁻ cells within CD4⁺ T cells was lower in both HIV-positive COBRA participants and blood bank donors when compared to HIV-negative COBRA participants (Table 3). Moreover, a strong association between a higher proportion of terminally differentiated (CD57⁺ and CD27⁻CD28⁻) CD4 and CD8 T cells and CMV infection was observed (Table 3). The percentages of CD57 expressing cells within the CD28⁻CD8⁺ T-cells and CD28⁻CD4⁺ T cells were lower in HIV-positive COBRA participants, while a positive association with CMV infection was observed.

CMV IgG titers are associated with terminal differentiation of T cells and CD8 T cell exhaustion

Anti-CMV IgG antibodies were positive in 95% of the HIV-positive COBRA participants, 77.5% of the HIV-negative COBRA participants, and 22.9% of the blood bank donors. HIV-positive COBRA participants had higher levels of total and high avidity CMV-IgG antibody titers as compared to both the HIV-negative COBRA participants and the blood bank donors (Table 1), confirming previous observations [30, 32, 33]. Increased CMV IgG levels are suggestive of increased stimulation by CMV antigens, which may further contribute to T cell activation, exhaustion and terminal differentiation. Total CMV IgG was associated with higher CD4 T cell activation (HLA-DR⁺CD38⁺), and terminally differentiated (CD57⁺ and CD27⁻CD28⁻) CD4 and CD8 T cells in CMV-positive individuals irrespective of HIV-1 infection (Table 3). In this model, CD4 T cell activation (HLA-DR⁺CD38⁺), CD4 T cell exhaustion (PD1⁺) and CD8 T cell exhaustion (PD1⁺) were still higher in HIV-positive participants, whereas the percentage of CD4⁺CD27⁻CD28⁻ T cells, the percentage of CD57 expressing cells within the CD28⁻CD8⁺ T-cells and CD28⁻CD4⁺ T cells were lower in HIV-positive COBRA participants when compared to HIV-negative COBRA participants (Table 3). Similarly, CMV high avidity IgG levels were associated with higher CD4 T cell activation, terminally differentiated (CD57⁺ and CD27⁻CD28⁻) CD8 T cells, the percentage of CD57⁺ cells within CD8⁺CD28⁻ cells, but not terminally differentiated (CD57⁺ and CD27⁻CD28⁻) CD4 T cells (Table 3).

Discussion

Despite effective ART, HIV-positive individuals are reported to have higher rates of age-associated non-communicable diseases and a shorter average life expectancy compared to uninfected persons of the same age [3, 5, 7]. The increased morbidity and mortality in the HIV-1 infected population may be the result of several contributing factors such as ART toxicity, chronic immune activation and immune dysfunction as well as a higher prevalence of traditional risk factors for these AANCC [8, 9]. Here we analysed markers of T cell activation,

Table 3. Multivariable analyses of the association between HIV-1 infection, CMV serostatus, as well as CMV total and high avidity IgG titers, and T cell activation, exhaustion and terminal differentiation in HIV-positive and HIV-negative COBRA participants and blood bank donors.

Activation and Exhaustion			Complete population adjusted for CMV serostatus ¹		CMV positive participants adjusted for CMV Total IgG ²		CMV positive participants adjusted for CMV High Avidity IgG ²	
			B (95% CI) ⁵	p value	B (95% CI) ⁵	p value	B (95% CI) ⁵	p value
% HLA-DR+CD38+ of CD4+ T cells ³	Group	HIV-neg (ref)						
		HIV-pos	0.21 (0.10–0.32)	0.0003	0.18 (0.06–0.31)	0.005	0.19 (0.06–0.31)	0.003
		BBD	0.10 (-0.038–0.24)	0.2	0.07 (-0.15–0.30)	0.5	0.07 (-0.15–0.29)	0.5
		CMV	0.01 (-0.12–0.13)	0.9	0.001 (0.0003–0.002)	0.006	0.002 (0.0007–0.003)	0.003
% PD1+ of CD4+ T cells ³	Group	HIV-neg (ref)						
		HIV-pos	0.13 (0.02–0.24)	0.02	0.15 (0.04–0.26)	0.01	0.16 (0.04–0.27)	0.007
		BBD	-0.14 (-0.27–0.01)	0.04	-0.06 (-0.26–0.14)	0.6	-0.06 (-0.26–0.14)	0.5
		CMV	-0.002 (-0.12–0.12)	1.0	0.0003 (-0.0003–0.001)	0.3	0.0002 (-0.0009–0.001)	0.7
% HLA-DR+CD38+ of CD8+ T cells ³	Group	HIV-neg (ref)						
		HIV-pos	0.07 (-0.05–0.19)	0.3	0.03 (-0.11–0.17)	0.7	0.03 (-0.10–0.17)	0.6
		BBD	-0.11 (-0.26–0.03)	0.1	-0.23 (-0.47–0.01)	0.06	-0.23 (-0.48–0.009)	0.06
		CMV	0.07 (-0.07–0.21)	0.3	0.00005 (-0.0003–0.001)	0.2	0.0008 (-0.0005–0.002)	0.2
% PD1+ of CD8+ T cells ³	Group	HIV-neg (ref)						
		HIV-pos	0.08 (-0.008–0.17)	0.07	0.11 (0.03–0.19)	0.008	0.11 (0.03–0.19)	0.007
		BBD	-0.07 (-0.18–0.04)	0.2	0.01 (-0.13–0.15)	0.9	0.01 (-0.13–0.15)	0.9
		CMV	-0.14 (-0.24–0.04)	0.006	-0.0006 (-0.0005–0.0004)	0.8	-0.0002 (-0.001–0.0006)	0.7
Terminal differentiation								
% CD57+ of CD4+ T cells ³	Group	HIV-neg (ref)						
		HIV-pos	-0.09 (-0.23–0.05)	0.2	-0.14 (-0.30–0.02)	0.08	-0.14 (-0.29–0.02)	0.09
		BBD	-0.13 (-0.30–0.04)	0.1	-0.16 (-0.44–0.13)	0.3	-0.16 (-0.45–0.12)	0.3
		CMV	0.36 (0.20–0.51)	1.3E-05	0.001 (0.00003–0.002)	0.04	0.001 (-0.0001–0.003)	0.07
% CD27-CD28- of CD4+ T cells ³	Group	HIV-neg (ref)						
		HIV-pos	-0.28 (-0.54–0.03)	0.03	-0.33 (-0.62–0.05)	0.02	-0.32 (-0.61–0.03)	0.03
		BBD	-0.35 (-0.66–0.04)	0.03	-0.49 (-1.00–0.03)	0.06	-0.49 (-1.01–0.02)	0.06
		CMV	1.32 (1.03–1.61)	5.2E-15	0.002 (0.00005–0.003)	0.04	0.003 (-0.0002–0.006)	0.06
% CD57+ of CD8+ T cells	Group	HIV-neg (ref)						
		HIV-pos	-1.89 (-8.07–4.28)	0.5	-3.77 (-10.52–2.99)	0.3	-3.63 (-10.25–2.99)	0.3
		BBD	-1.13 (-8.82–6.56)	0.7	-5.91 (-17.90–6.08)	0.3	-6.03 (-17.87–5.81)	0.3
		CMV	16.02 (8.97–23.07)	1.6E-05	0.05 (0.016–0.09)	0.006	0.10 (0.04–0.17)	0.003
% CD27-CD28- of CD8+ T cells	Group	HIV-neg (ref)						
		HIV-pos	-2.64 (-8.65–3.37)	0.4	-6.07 (-12.98–0.85)	0.09	-5.72 (-12.59–1.15)	0.1
		BBD	-5.22 (-12.70–2.27)	0.2	-15.21 (-27.49–2.94)	0.02	-15.40 (-27.68–3.12)	0.02
		CMV	21.38 (14.51–28.23)	1.2E-08	0.05 (0.02–0.09)	0.007	0.09 (0.03–0.16)	0.008
% CD57+ of CD4+CD28- T cells	Group	HIV-neg (ref)						
		HIV-pos	-17.67 (-27.73–7.61)	0.001	-18.31 (-29.31–7.32)	0.001	-18.67 (-29.45–7.88)	0.001
		BBD	-7.59 (-20.13–4.95)	0.2	-7.09 (-26.61–12.42)	0.5	-7.05 (-26.335–12.24)	0.5
		CMV	39.74 (28.25–51.24)	4.5E-10	0.04 (-0.03–0.10)	0.2	0.10 (-0.01–0.20)	0.08
% CD57+ of CD8+CD28- T cells ⁴	Group	HIV-neg (ref)						
		HIV-pos	-0.10 (-0.18–0.01)	0.03	-0.10 (-0.19–0.02)	0.02	-0.10 (-0.19–0.02)	0.02
		BBD	-0.05 (-0.16–0.06)	0.3	-0.07 (-0.23–0.08)	0.3	-0.07 (-0.23–0.08)	0.3
		CMV	0.13 (0.03–0.23)	0.01	0.0004 (-0.0001–0.0009)	0.1	0.001 (0.00007–0.002)	0.04

¹ Multivariable linear regression adjusted for age and gender in HIV-positive (n = 40), HIV-negative (n = 40) and BBD (n = 35). Uncorrected p values are given. Bonferroni adjustment for multiple testing required a p value of <0.005 for significance.

² Multivariable linear regression adjusted for age and gender in HIV-positive (n = 38), HIV-negative (n = 31) and BBD (n = 8) infected with CMV. Uncorrected p values are given. Bonferroni adjustment for multiple testing required a p value of <0.005 for significance.

³ LOG transformed to obtain normality.

⁴ arcsine transformed to obtain normality.

⁵ Unstandardized coefficient (B) indicates the difference in the outcome (immunological markers) with respect to the variables (study group, CMV infection). For example, B is the increase in LOG transformed % of HLA-DR+CD38+ of CD4+ T cells in a HIV-positive individual as compared to a HIV-negative participant.

Abbreviations: HIV, human immunodeficiency virus; BBD, blood bank donors; B, unstandardized regression coefficient; CI, confidence interval; CMV, Cytomegalovirus; IgG, Immunoglobulin G

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exhaustion, and differentiation in a cohort of HIV-positive adults who were on apparently effective ART and compared these to data from adults who were comparable for age, lifestyle and demographic factors (COBRA) and a cohort age matched blood bank donors. HIV-positive COBRA participants showed incomplete immune recovery as reflected by lower CD4 T cell counts and higher CD8 T cell counts than observed in either well-matched or just age-matched uninfected controls. Furthermore, a higher percentage of activated and exhausted CD4 cells, a lower percentage of CD4 naïve cells and higher percentage of CD8 effector memory cells were observed when compared to HIV-negative COBRA participants which is broadly consistent with previous studies [30, 34–40]. In contrast, measures of terminally differentiated T cells (CD57⁺ and CD27⁻ CD28⁻), did not differ between the treated HIV-positive and HIV-negative COBRA participants, confirming previous observations [17, 30], but contradicting other studies [8, 13, 25, 27]. Collectively, these data provide strong support for the concept that certain HIV-mediated immunologic perturbations (e.g., low CD4/CD8 ratio, shift towards more differentiated memory cell types, high PD-1 expression) do indeed persist indefinitely during ART, while other markers more typically associated with terminal differentiation and senescence may normalize, at least compared to levels observed in well-matched persons who lack HIV but have other risk factors, particularly CMV infection.

Strikingly, the percentages of activated CD8 T cells, exhausted CD4 T cells and terminally differentiated CD4 and CD8 T cells were higher in both HIV-positive and HIV-negative COBRA participants when compared to the general populations (e.g., the blood bank donors). HIV-positive and negative COBRA participants are more often infected with CMV when compared to the blood bank donors which may be due to the high number of men-who-have-sex-with-men in the COBRA participants. Multivariable analysis showed that HIV-1 infection is independently associated with CD4 T cell activation and T cell exhaustion but not with terminally differentiated T cells (CD57⁺ and CD27⁻ CD28⁻). CMV infection was strongly associated with increased proportion of terminally differentiated T cells and may at least partially explain the higher levels of terminally differentiated T cells in the HIV-positive and HIV-negative COBRA participants. This is in line with previous publications in which CMV infection was strongly associated with increased terminal differentiation of T cells and CD8 T cell exhaustion [13, 15, 17, 28, 29]. However, no association of CMV infection with T cell activation was observed which is in contrast with previous studies [41–43]. HIV-positive individuals in our study are considered to be successfully treated, which is reflected in the high CD4 counts and relatively high CD4:CD8 ratios. Furthermore, they have a relative low % of activated CD8 cells (median 7.5% CD8⁺HLA-DR⁺CD38⁺) as compared to the other studies (15–20% CD8⁺HLA-DR⁺CD38⁺) [41–43], and this might at least in part explain the differences between the studies. We did however observe that higher CMV IgG titers were associated with higher CD4 T cell activation in the CMV-positive participants.

HIV-positive COBRA participants had increased levels of CMV specific IgG when compared to HIV-negative COBRA participants and blood bank donors, confirming previous observations [30, 32, 33]. CMV specific IgG levels are considered a surrogate marker of CMV reactivation and concurrent immune response to control infection, and high levels are associated with ageing and increased morbidity and mortality in both the general population and HIV-positive individuals [30–33, 44–46]. Total CMV IgG was independently associated with the presence of a higher proportion of terminally differentiated T cells and CD4 T cell activation, indicating that reactivation of CMV infection contributes substantially to terminal differentiation of T cells and CD4 activation in treated HIV-positive individuals. This could suggest that the high CMV prevalence combined with increased CMV reactivation may contribute to the increased comorbidity burden that has been reported in treated HIV-positive individuals [7]. Ageing and CMV infection are associated with terminal differentiation and proliferation

of CD8⁺CD28⁻CD57⁺ effector memory cells, however HIV-1 has been shown to inhibit the terminal differentiation of these cells which is reflected in a decreased percentage of CD28⁻CD8⁺ T-cells expressing CD57 [17, 18]. In line with these findings we observed that HIV-1 infection was independently associated with a decrease in the percentage of CD57 expressing cells within the CD28⁻CD8⁺ T cells, whereas CMV infection was independently associated with an increased percentage of these cells.

Cigarette smoking is known to have an impact on different immune functions, with both proinflammatory and immune suppressive effects having been described [47, 48]. For example, increased levels of markers of inflammation (CRP, IL-6, D-dimer, sCD14), T cells activation (% HLA-DR⁺CD38⁺ of CD4 and CD8 cells), and decreased T cell function (% PD1⁺ CD4 and CD8 T cells) have been associated with smoking in HIV-infected individuals as well as in the general population [49–52]. In the present study, data about cigarette smoking was only available for the COBRA participants (HIV-positive and HIV-negative), and in this group no association between smoking and T cell activation, T cell exhaustion, and terminal differentiation of CD4 and CD8 cells was observed (data not shown). However, we cannot exclude that the limited number of individuals analysed and the lack of data about cigarette smoking in the BBD group might explain why previously reported associations between cigarette smoking and T cell activation and function could not be confirmed [49, 51].

Due to its observational nature, our study does suffer from several limitations. Firstly, although we attempted to identify independent effects of HIV and CMV, CMV infection was highly prevalent in both groups of COBRA participants, reflecting the high numbers of MSM in each group (for example, only 2 of the 40 HIV-positive participants were CMV-negative). Thus our ability to differentiate the independent effects of the two viruses is limited. For this reason, analyses of CMV IgG may provide greater discriminative ability. Secondly, we cannot rule out the possibility that the effects seen were a consequence of other unmeasured differences between the groups rather than HIV and CMV infection per se. Finally, the small numbers in some of our groups, and the low prevalence of coinfection with HBV and HCV meant that we were unable to assess the effects of coinfection with these viruses.

In conclusion, HIV-positive individuals on ART with long-term suppressed viraemia exhibited incomplete immune recovery and increased immune activation/exhaustion compared to HIV-negative controls matched for age, lifestyle and demographic factors. However, no evidence for increased immune senescence as determined by the level of terminally differentiated T cells, was observed when HIV-positive individuals on suppressive ART were compared to appropriately selected controls matched for age, lifestyle and demographic factors. If only blood donors or perhaps someone poorly matched convenience sample had been used as controls one would have falsely concluded that HIV was independently associated with increased proportion of terminally differentiated T cells. This illustrates the importance of recruiting and utilising appropriate control populations for such studies.

Supporting information

S1 Fig. Gating strategy flow cytometry. **A.** Gating of CD4⁺ and CD8⁺ T cells. **B.** T cell differentiation was defined as the proportion of naïve (N; CD45RA⁺CD27⁺CCR7⁺), central memory (CM; CD45RA⁻CCR7⁺CD27⁺), transitional memory (TM; CD45RA⁻CCR7⁻CD27⁺), effector memory (EM; CD45RA⁻CCR7⁻CD27⁻), and terminally differentiated effector memory (TEMRA; CD45RA⁺CCR7⁻CD27⁻) within the total CD4 or CD8 T cell population. **C.** T cell activation was defined as the proportion of cells that were positive for both CD38 and HLA-DR within the total CD4 or CD8 T cell population. T cell exhaustion was defined as the proportion of PD1 positive cells within the total CD4 or CD8 T cell population. Terminally

differentiated T cells were defined as proportion of CD57 positive cells within the total CD4 or CD8 T cells population, the proportion of cells negative for both CD27 and CD28 within the total CD4 or CD8 T-cells population, or the proportion of CD57 positive within the CD28⁻CD4⁺ or CD28⁻CD8⁺ T cell population.
(PDF)

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