

RESEARCH ARTICLE

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Immunosenescence of the CD8⁺ T cell compartment is associated with HIV-infection, but only weakly reflects age-related processes of adipose tissue, metabolism, and muscle in antiretroviral therapy-treated HIV-infected patients and controls

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Abstract

Background: Despite effective antiretroviral therapy (ART), HIV-infected patients exhibit systemic inflammation, early onset of age-related diseases, and features of immunosenescence. The role of inflammation in the development of age-related diseases is widely recognized. However, the role of immunosenescence is not well established. Studying immunosenescence in HIV-infection could give insight into its role in ageing processes. In this cross-sectional study, we aimed to investigate whether ART-treated HIV-infected patients exhibit immunosenescence; and whether immunosenescence is associated with age-related processes of inflammation, metabolism, adipose tissue, and muscle. T cell immunosenescence and exhaustion were assessed by flow cytometry analysis of CD8⁺ cells from 43 ART-treated HIV-infected patients (HIV⁺) and ten Controls using markers of differentiation: CD27/CD28; maturation: CD27/CD45RA; senescence: killer cell lectin-like receptor G1 (KLRG1); and exhaustion: programmed death-1 (PD-1). Relationships between CD8⁺ T cell immunosenescence, exhaustion, and age-related processes were assessed using linear regressions.

Results: HIV-infection was strongly associated with more highly differentiated and mature CD8⁺ T cell phenotypes. PD-1 and KLRG1 expression did not differ between HIV⁺ and Controls, but depended on differentiation and maturation stages of the cells. CD8⁺ T cell maturation was associated with age. KLRG1 expression was associated with age, metabolic syndrome, visceral adipose tissue, and high muscle mass. PD-1 expression was not associated with age-related parameters.

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Conclusions: HIV-infection strongly affected CD8⁺ T cell differentiation and maturation, whereas age-related processes were only weakly associated with immune parameters. Our findings suggest that, in contrast to inflammation, immunosenescence appears to be highly dependent on HIV-infection and is only to a small extent associated with age-related parameters in well-treated HIV-infection.

Keywords: Immunosenescence, Ageing, HIV, PD-1, KLRG1

Background

The world's population is ageing rapidly. The population of individuals aged 60 or over is the fastest growing, and it is estimated to represent over 20 % of the world's population by 2050 [1]. As a result, the prevalence of age-related diseases—such as cardiovascular diseases, metabolic syndrome and diabetes, loss of muscle mass, and cancer—is increasing. It is now evident that ageing is associated with a state of chronic low-grade inflammation known as “inflammaging”, and that most age-related diseases are—in part—caused by inflammaging. Inflammaging is characterized by elevated levels of pro-inflammatory biomarkers such as interleukin-6 (IL-6), tumour necrosis factor alpha, and C-reactive protein. However, it is not well known what causes the increased inflammation [2, 3].

As immune cells are major producers of inflammatory proteins, inflammaging could originate from the changes that occur to immune cells during ageing, termed immunosenescence [4]. Immunosenescence is characterized by decreased output of naïve T cells following thymic involution, and accumulation of highly differentiated memory CD8⁺ T cells as a result of repeated antigenic stimulation and chronic viral infections such as cytomegalovirus (CMV)-infection [5]. This results in an inversion of the CD4:CD8 T cell ratio [5, 6]. The highly differentiated T cells are senescent in that they lose proliferative capacity, rendering them inefficient against pathogens. However, they are able to produce elevated amounts of pro-inflammatory cytokines [7–9].

Antiretroviral therapy (ART)-treated HIV-infected patients are characterised by earlier onset of age-associated diseases like cardiovascular diseases; features of immunosenescence; inflammaging; and loss of T cell effector functions, referred to as T cell exhaustion [10–14]. Moreover, ART is associated with development of lipodystrophy, a syndrome of adipose tissue redistribution characterized by loss of subcutaneous adipose tissue and gain of visceral adipose tissue (VAT), and which resembles the changes in body composition that occur with age [15, 16].

While immunosenescence is well-documented with age, and is increasingly reported in treated HIV-infection, it is not known whether these immunological changes are involved in age-associated disease processes.

Studying the associations between immunosenescence and clinical age-related processes in HIV-infected patients could therefore yield insight into the role of immunosenescence in disease development. In this study we therefore wanted to investigate whether ART-treated HIV-infected patients exhibit immunosenescence; and whether this immunosenescence is associated with age-related processes of inflammation, metabolism, adipose tissue, and muscle.

Methods

Study design and participants

Between November 2010 and October 2012, 75 participants were included in this cross-sectional study: 60 patients with HIV-infection (HIV⁺) and 15 healthy men of similar age (Controls). HIV⁺ were recruited from the Department of Infectious Diseases, and Controls were recruited by advertisement at Copenhagen University Hospital, Hvidovre, Denmark. Inclusion criteria were: male sex; white ethnicity; > 18 years old; testing negative for hepatitis B and C; no intravenous drug use; and no current immunomodulating, lipid-lowering, anti-diabetic, or endocrinologic treatment. HIV⁺ were also required to have plasma HIV-RNA <400 copies/mL; ART for at least 12 months; and CD4⁺ T cell counts ≥200 cells/μL. The relationships between inflammatory biomarkers, adipose tissue distribution and low muscle mass in this cohort have been investigated [17]. Moreover, microdialysis results from 18 of the study participants have been published in a methodological study [18]. The study was approved by the local ethics committee of the Capital Region of Denmark (H-4-2010-045), the Danish Data protection agency (2010-41-4952), and was conducted in accordance with the Declaration of Helsinki. All participants gave written informed consent.

Plasma inflammation markers and blood sample measurements

Plasma levels of the inflammation markers soluble urokinase plasminogen activator receptor (suPAR) and IL-6 were measured by enzyme-linked immunosorbent assays: suPARnostic (ViroGates A/S, Birkerød, Denmark) and Quantikine HS-IL-6 (R&D Systems, Minneapolis, MN). CD4⁺, CD8⁺ T cell counts, and HIV-RNA were routine measurements by the Department of Clinical

Biochemistry, Copenhagen University Hospital, Hvidovre, Denmark. Peripheral blood mononuclear cells were isolated by centrifugation using BD Vacutainer cell preparation tubes (BD Biosciences, San Jose, CA) and cryopreserved.

Flow cytometric analysis of CD8⁺ T cells

Cryopreserved peripheral blood mononuclear cells from all participants were thawed and washed in complete RPMI 1640 media supplemented with GlutaMAX (Life Technologies, Grand Island, NY), 10 % fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. 1.5 million cells were resuspended in 100 µL PBS and stained with the Zombie NIR Fixable Viability Kit (BioLegend, San Diego, CA) for 30 min. Cells were washed and resuspended in 10 % heat inactivated human serum in PBS for 20 min for Fc receptor blocking. Cells were incubated for 30 min with titrated anti-CD3-FITC, anti-CD8-PerCp-Cy5.5, anti-CD27-APC, anti-CD28-PE-Cy7 or anti-CD45RA-PE-Cy7 (BD Biosciences), and anti-PD-1-PE or anti-KLRG1-PE (BioLegend) antibodies and washed in FACS buffer (2 mM EDTA (Life Technologies) and 0.5 % bovine serum albumin (Miltenyi Biotec, Cologne, Germany) in PBS). Matching isotype and unstained controls were included for each participant. Compensations were generated for each fluorochrome using CompBeads (Anti-Mouse Ig, κ) (BD Biosciences). For each sample, 50 000 to 100 000 CD8⁺ events were recorded on a FACSCanto II flow cytometer (BD Biosciences). HIV- and lipodystrophy-status of the participants were concealed until after data analysis. Data were analysed using Cytobank [19].

CD8⁺ T cells could be subtyped into four maturation subsets based on CD27 and CD45RA expression: naïve (T_N : CD27⁺CD45RA⁺); central memory (T_{CM} : CD27⁺CD45RA⁻); effector memory (T_{EM} : CD27⁻CD45RA⁻); or effector memory re-expressing CD45RA (T_{EMRA} : CD27⁻CD45RA⁺) [20, 21]. By evaluating the expression of CD27 and CD28, CD8⁺ T cells could also be subtyped into four differentiation subsets: early differentiated (T_{ED} : CD27⁺CD28⁺); intermediate differentiated (T_{ID} : CD27⁺CD28⁻); late differentiated (T_{LD} : CD27⁻CD28⁻); or CD27⁻CD28⁺ [8]. The expression of the exhaustion marker PD-1 and of the senescence marker KLRG1 was evaluated in each of the maturation and differentiation subsets and in the total CD8⁺ T cell population [22].

Assessment of body composition

Anthropometric measurements comprised body weight, height, and waist and hip circumferences, measured as in [15]. Abdominal VAT mass at the level of L4 was derived from computed tomography scans (Somatom Sensation 10, Siemens, Germany). Body composition was determined by dual energy X-ray absorptiometry (Norland XR-36, Gammatec A/S, Værløse, Denmark).

Fat mass index (FMI) was calculated as: total fat mass/height² (kg/m²). Leg lean mass index (LLMI) was calculated as: leg lean mass/height² (kg/m²). Lean mass was used as a surrogate marker for muscle mass as lean mass is principally made of skeletal muscle. This is especially true for lean mass in the extremities [23].

Assessment of metabolism

Fasting glucose levels were routine measurements by the Department of Clinical Biochemistry. Fasting plasma levels of insulin were measured using Immulite 2000 System (Siemens, NY, USA) and insulin resistance was calculated using the homeostasis model assessment (HOMA-IR) [24]. Metabolic syndrome was defined according to the 2009 consensus definition for high risk populations [25]. The assessment of metabolic syndrome was based on the presence of at least three of the five following criteria: elevated waist circumference (≥94 cm, as recommended for high risk populations); elevated triglycerides (≥1.7 mmol/L); reduced HDL-C (<1.0 mmol/L); elevated blood pressure (systolic ≥130 or diastolic ≥85 mm Hg, or anti-hypertensive treatment); elevated fasting glucose (≥100 mg/dL).

Assessment of lipodystrophy

Lipodystrophy status and the type of lipodystrophy were evaluated by clinical evaluation of the adipose tissue distribution of several subcutaneous regions (i.e. face, retroauricular and dorsocervical regions, upper arms, thighs, buttocks, and abdomen) and of the visceral region, as in [15]. HIV⁺ were characterized as lipodystrophic or non-lipodystrophic. Patients with lipodystrophy were further characterized as lipohypertrophic, lipoatrophic, or having mixed type lipodystrophy.

Statistical analysis

Comparisons between HIV⁺ and Controls were performed using unpaired Student's *t*-test or Wilcoxon two-sample test where appropriate. For categorical parameters, Chi-squared test or Fisher's exact test were used. To investigate the effect of HIV-infection on CD8⁺ T cell phenotypes, we used unpaired Student's *t*-test or Wilcoxon two-sample test and linear regression analyses. The associations between HIV- and ART-related variables and CD8⁺ T cell phenotypes were investigated in the HIV⁺ group using multiple linear regression analyses adjusted for age. The associations between age, inflammation, metabolism, body composition, and CD8⁺ T cell phenotypes were investigated in the HIV⁺ group using simple and multiple linear regression analyses adjusted for age, HIV-, ART-duration, and current ART. The T_{CM} , T_{EM} , and T_{EMRA} subsets were grouped into one Memory group. Thus CD8⁺ T cell maturation was defined as either an antigen-inexperienced naïve (T_N) or an

antigen-experienced Memory phenotype. CD8⁺ T cell differentiation was defined as either an undifferentiated CD28⁺ phenotype composed of T_{ED} and CD27⁻CD28⁺ cells, or a highly differentiated CD28⁻ phenotype composed of T_{ID} and T_{LD} cells. The loss of CD28 expression is considered to identify a group of cells characterized by functional changes and replicative senescence [9]. By grouping the individual subsets according to relevant biological and functional characteristics, we could reduce the number of analyses and simplify the interpretation. Nonetheless, data on the individual subsets are available as Additional files. Due to the compositional nature of the data, only the estimates of the Memory and CD28⁻ cells are presented in analyses for group sizes. The estimates for T_N cells are the opposite of the estimates for Memory cells since their proportions add up to 100 %, and the same is true for CD28⁺ and CD28⁻ cells. If the residuals were not normally distributed, the parameter was log-transformed to fulfil model requirements using log₂(x). Beta estimates (β) for log-transformed parameters were back transformed using $(2^{\beta} - 1) \times 100$, and thereby shown as percent change in outcome per unit increase of the covariate.

Statistical significance was defined as a *P*-value < 0.05. Analyses were performed using Statistical Analysis Systems (SAS) version 9.4 (SAS Institute, Cary, NC, USA). Graphs were made with GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

Results

Viable PBMCs were available from 53 of the 75 participants, and only these participants were included in the study (Fig. 1). Flow cytometry analysis of maturation and differentiation markers, and PD-1, was done for all 53 participants, whereas KLRG1 analyses was done for only 39 participants since not enough cells were available for all participants.

General characteristics of the study participants are summarized in Table 1. HIV⁺ had significantly higher CD8⁺ T cell numbers (*P* < 0.0001) and lower CD4:CD8 ratio (*P* < 0.0001) than Controls. Smoking prevalence (*P* = 0.05), HOMA-IR index (*P* = 0.05), and suPAR levels (*P* = 0.02) were significantly higher in HIV⁺ compared to Controls. Age and body composition did not differ between HIV⁺ and Controls.

CD8⁺ T cell compartment re-modulation in HIV-infection

Lipodystrophy was not associated with changes in CD8⁺ T cell phenotype (Additional file 1 and Additional file 2). Therefore, patients with and without lipodystrophy were grouped (HIV⁺) in further analyses. CD8⁺ T cell maturation phenotypes: naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), or effector memory re-expressing CD45RA

(T_{EMRA}), were determined based on CD27 and CD45RA expression. HIV⁺ had significantly lower proportions of T_N cells (*P* < 0.001) and significantly higher proportions of T_{EM} cells (*P* = 0.002) than Controls (Fig. 2a). CD8⁺ T cell differentiation phenotypes: early (T_{ED}), intermediate (T_{ID}), late (T_{LD}) differentiated, or CD27⁻CD28⁺, were determined based on CD27 and CD28 expression. HIV⁺ had significantly lower proportions of T_{ED} cells (*P* < 0.001) and significantly higher proportions of T_{LD} cells (*P* < 0.001) than Controls (Fig. 2b). We investigated whether these changes were mediated by differences in age or age-related processes using multiple regression analyses. The differences in CD8⁺ T cell subset distribution between HIV⁺ and Controls were primarily related to HIV or ART. Adjusting for age, inflammation, metabolism, or body composition, hardly affected the associations between HIV-infection and CD8⁺ T cell maturation and differentiation (Additional file 3).

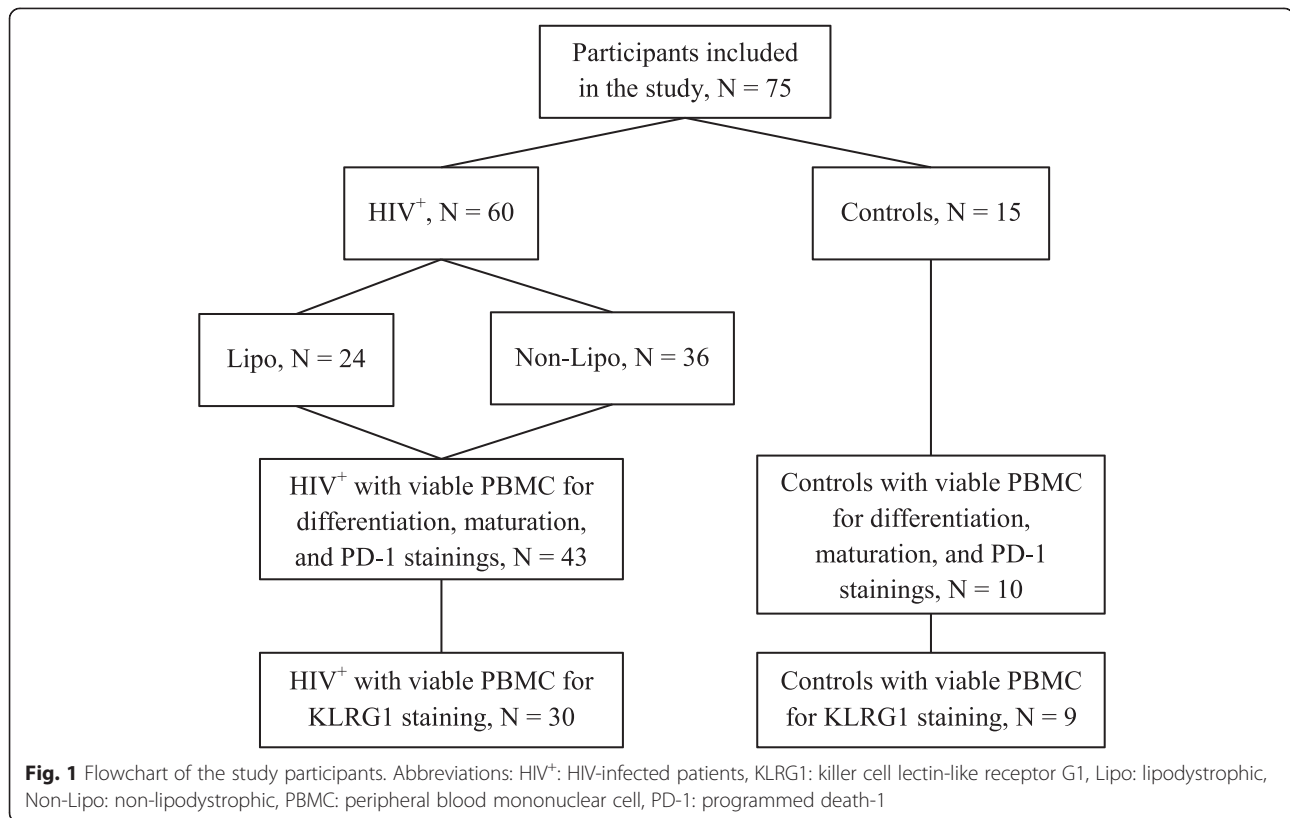
Moreover, we investigated whether HIV-, ART-duration or current ART was associated with differences in CD8⁺ T cell subset distribution in HIV-infected patients using linear regressions adjusted for age. Current use of protease inhibitors was significantly associated with higher proportions of T_{EM} (*P* = 0.002) and T_{LD} (*P* = 0.002), and lower proportions of T_N (*P* = 0.007) and T_{ED} (*P* = 0.04) CD8⁺ T cells. Current use of non-nucleoside reverse-transcriptase inhibitors was associated with higher proportions of T_{ID} (*P* = 0.01). HIV- (*P* = 0.36–0.99) or ART-duration (*P* = 0.07–0.94) did not have an effect on CD8⁺ T cell subset sizes.

PD-1 and KLRG1 expression in HIV-infection

CD8⁺ T cell senescence and exhaustion were assessed on the basis of KLRG1 and PD-1 expression, respectively. HIV-infection did not have a significant effect on PD-1 and KLRG1 expression, neither in the total CD8⁺ T cell population or in the subsets (*P* = 0.06–0.94) (Fig. 3). Instead, PD-1 and KLRG1 expression depended on the maturation and differentiation stages of the cells. PD-1 expression was highest in intermediate subsets (Fig. 3a). KLRG1 expression was highest in highly differentiated subsets (Fig. 3b). Moreover, there was a significant association between proportions of PD-1⁺ and KLRG1⁺ CD8⁺ T cells across both groups, the proportion of KLRG1⁺CD8⁺ T cells was multiplied by 2.7 % for each 1 % increase in the proportion of PD-1⁺CD8⁺ T cells (Estimate = 2.72 %; *P* = 0.02, 95 % CI = 0.45–5.04). We did not find any association between HIV-, ART- duration, or ART and changes in KLRG1 or PD-1 expression (*P* = 0.06–0.89).

Associations between CD8⁺ T cell subsets, age, and age-related parameters

The aim of the study was to investigate changes within the CD8⁺ T cell compartment in well-treated



HIV-infected patients, and further to investigate whether these changes were associated with age-related parameters. In contrast to the virus, which is thought to affect subsets with specific effector functions, we expected the ageing process to have a more general effect on the CD8⁺ T cell population. Therefore, we grouped T_{CM}, T_{EM}, and T_{EMRA} subsets into one Memory group; T_{ED} and CD27⁻CD28⁺ cells into one CD28⁺ group; and T_{ID} and T_{LD} into one CD28⁻ group. When grouping the subsets, HIV-infection remained associated with lower proportions of T_N and CD28⁺, and higher proportions of Memory ($P = 0.0002$) and CD28⁻ ($P = 0.0003$) CD8⁺ T cells. We investigated the associations between CD8⁺ T cell distribution, age, and age-related parameters in the HIV⁺ group in simple and adjusted analyses (Fig. 4). Analyses for the individual subsets are available in Additional file 4. HOMA-IR index was significantly associated with higher proportions of Memory (Unadjusted $P = 0.02$, adjusted $P = 0.03$), mainly T_{EM} cells (Unadjusted $P = 0.02$, adjusted $P = 0.003$), and lower proportions of T_N cells (Unadjusted $P = 0.02$, adjusted $P = 0.03$) (Fig. 4 and Additional file 4). We found no significant associations between memory or CD28⁻ CD8⁺ T cell group sizes with age, inflammation or VAT overall (Fig. 4). However, at the individual subset level, IL-6 levels were associated with changes in the proportions of T_{ID}, and VAT was

associated with lower proportions of T_{EMRA} CD8⁺ T cells (Additional file 4).

Associations between PD-1 and KLRG1 expression, age and age-related parameters

We found PD-1 and KLRG1 expression to vary according to subset differentiation and maturation, but not to HIV-infection (Fig. 3). Thus, we wanted to investigate whether PD-1 and KLRG1 expression in total CD8⁺ T cells were associated with age and age-related processes in the HIV⁺ group in simple and adjusted regression analyses. When grouping the subsets, HIV-infection was not associated with changes in KLRG1 or PD-1 expression ($P = 0.14-0.87$). There were no associations between KLRG1 or PD-1 expression in total CD8⁺ T cells and age-related parameters (Fig. 5).

Within each CD8⁺ T cell subset, there were still large variations in the number of KLRG1⁺ and PD-1⁺ cells. We therefore also investigated whether age and age-related processes were associated with PD-1 and KLRG1 expression in specific CD8⁺ T cell groups: T_N and Memory, and CD28⁺ and CD28⁻ cells. In participants with metabolic syndrome, the proportion of KLRG1⁺CD28⁻ cells increased by 14 % in unadjusted analyses, and by 18 % in adjusted analyses (Unadjusted $P = 0.05$, adjusted $P = 0.04$), compared to those without metabolic syndrome (Table 2). ILMI was associated

Table 1 General characteristics of the study participants

	Controls		HIV ⁺	
	N = 10		N = 43	
	Median or n	IQR or %	Median or n	IQR or %
Demography and lifestyle				
Age (years)	48.1	41.9–62.3	49.8	43.3–55.2
Smoking	0	0 %	14*	32.6 %
Body composition				
Lipodystrophy	—	—	14	32.6 %
BMI (kg/m ²)	25.4	24.7–28.6	25.1	23.0–27.7
FMI (kg/m ²)	5.2	4.6–7.5	4.6	3.4–6.4
VAT (cm ²)	111.6	93.5–157.5	150.8	115.0–212.2
ILMI (kg/m ²)	6.4	6.2–6.8	6.1	5.7–6.6
Metabolic parameters				
HOMA-IR	0.5	0.5–1.3	1.2*	0.6–2.4
Metabolic syndrome	1	10 %	20	46.5 %
Biomarkers of inflammation				
IL-6 (pg/mL)	1.1	0.7–2.4	1.6	1.1–2.6
suPAR (ng/mL)	1.8	1.5–2.1	2.2*	1.8–2.8
HIV- and immune-related parameters				
HIV duration (years)	—	—	14	6.6–21.0
ART duration (years)	—	—	10.2	4.4–14.8
Current PI treatment	—	—	13	30.2 %
Current NRTI treatment	—	—	43	100 %
Current NNRTI treatment	—	—	28	65.1 %
Other current ART	—	—	4	9.30 %
HIV RNA ≤20 copies/mL	—	—	39	90.7 %
CD4 ⁺ T cell count (cells/μL)	729	674–880	580	403–820
CD8 ⁺ T cell count (cells/μL)	373	281–440	828***	618–1120
CD4:CD8 ratio	2.0	1.8–3.0	0.7***	0.5–1.0

Continuous measures are listed as: median and interquartile range. Categorical variables are listed as: number of participants and percentage. Abbreviations: *BMI* body mass index, *ART* antiretroviral therapy, *FMI* fat mass index, *HOMA-IR* homeostatic model assessment of insulin resistance, *IL-6* interleukin-6, *IQR* interquartile range, *ILMI* leg lean mass index, *NRTI* nucleoside reverse-transcriptase inhibitors, *NNRTI* non-nucleoside reverse-transcriptase inhibitor, *PI* protease inhibitor, *suPAR* soluble urokinase plasminogen activator receptor, *VAT* visceral adipose tissue. The bold values are statistically significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

with an increase in the proportions of KLRG1⁺T_N cells ($P = 0.02$). VAT was associated with an increase in the expression of KLRG1 in CD28⁺ (Unadjusted $P = 0.02$, adjusted $P = 0.03$) and CD28⁻ cells (Adjusted $P = 0.04$). Associations of KLRG1 expression and metabolic syndrome, VAT and ILMI were also observed in the individual subsets (Additional file 5).

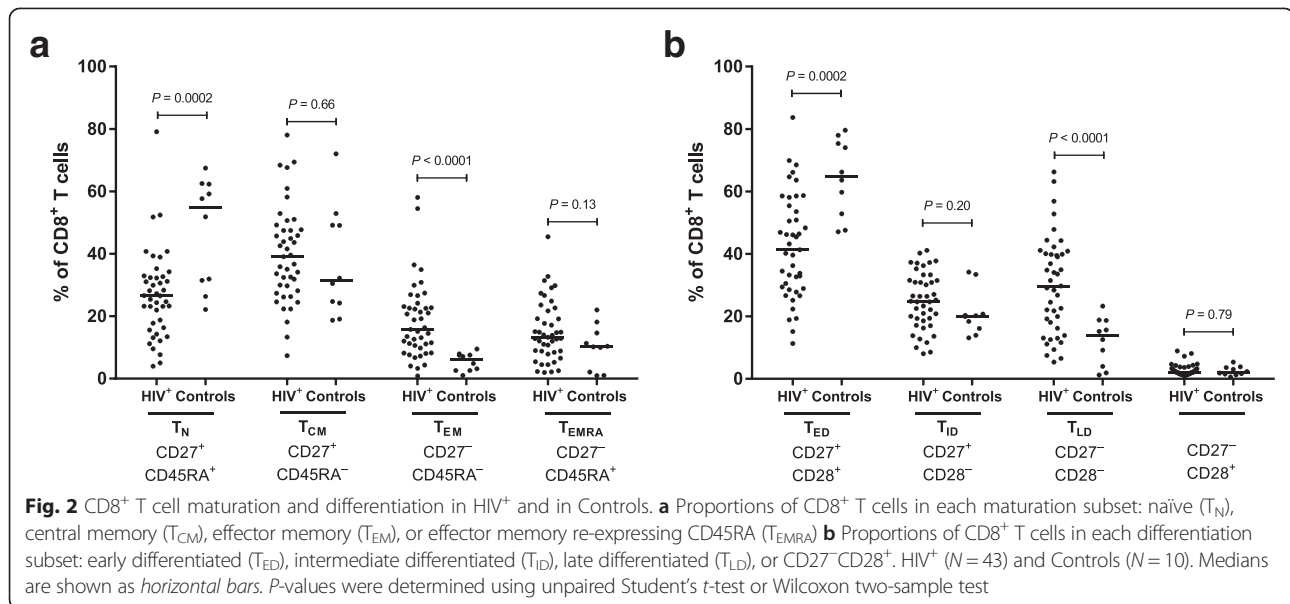
We found no associations between PD-1 expression and age-related parameters (Additional file 6 and Additional file 7).

Discussion

We investigated whether CD8⁺ T cell maturation, differentiation, senescence, and exhaustion differed between

well-treated HIV-infected patients and Controls; and whether these immunological changes were associated with age and age-related processes of inflammation, metabolism, adipose tissue, or muscle. HIV⁺ had significantly higher levels of CD8⁺ T cells with mature and highly differentiated phenotypes, but not higher levels of CD8⁺ T cells expressing KLRG1 or PD-1 compared to Controls. Instead, KLRG1 and PD-1 expression were highly dependent on CD8⁺ T cell maturation and differentiation. In contrast, age-related processes were only weakly and inconsistently associated with CD8⁺ T cell phenotypes.

In agreement with previous studies, HIV-infection was strongly associated with lower proportions of relatively



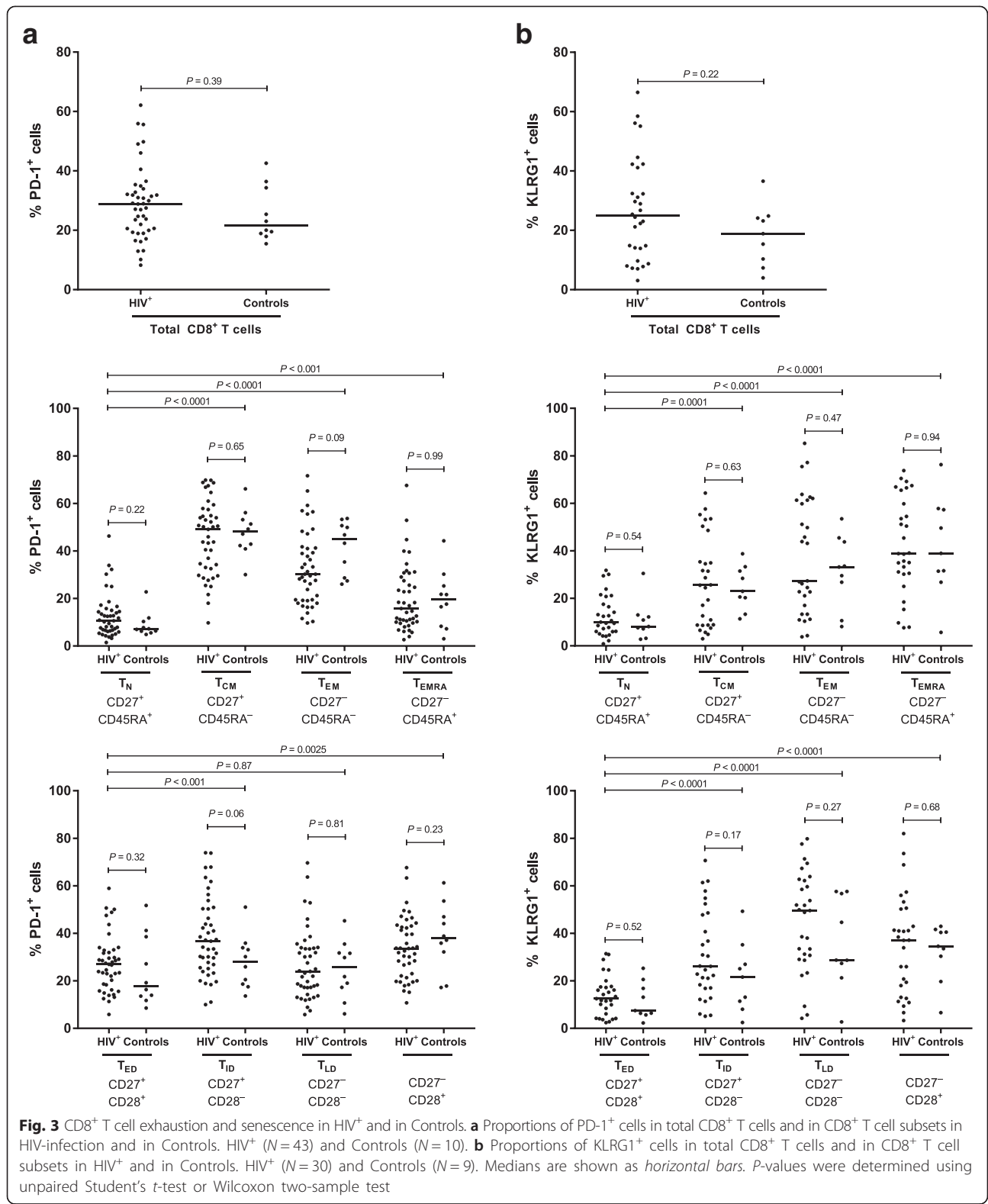
undifferentiated and naïve CD8⁺ T cells, higher proportions of highly differentiated and mature CD8⁺ T cells, and an inverted CD4:CD8 T cell ratio (<1) [12, 26]. These features are characteristic of the immunosenescence phenotype previously described in healthy elderly individuals [4]. However, while HIV-infection is characterized by increased proportions of intermediate T_{EM} CD8⁺ T cells, ageing is characterized by increased proportions of more highly differentiated T_{EMRA} CD8⁺ T cells, often attributed to CMV infection [21, 26–28]. This indicates that there are subtle differences in the way CD8⁺ T cell subsets are affected by HIV-infection and age.

HIV⁺ patients did not normalize their CD8⁺ maturation and differentiation pattern despite successful ART, indicating that HIV viral load is not the only driver of CD8⁺ T cell phenotypic abnormalities. Other explanations include disturbed thymic function [29, 30], ongoing replication in HIV-reservoirs [31], co-infections by other chronic viruses, and bacterial translocation in the gut [32]. The effect of HIV-infection on CD8⁺ T cell maturation and differentiation did not appear to be mediated by age-related processes, since the estimates for the associations between HIV-infection and T cell phenotypes were unchanged when adjusted.

Previous studies have investigated the association between lymphocytes phenotypes and age-associated clinical outcomes, but with varying results. Highly differentiated CD28⁻CD8⁺ T cells were associated with frailty in older women [33]. Others have found associations of CD8⁺ T cell differentiation and maturation with obesity in children [34]. CD8⁺ T cell maturation was associated with HOMA-IR index. However, we found HIV-infection to be associated with both higher

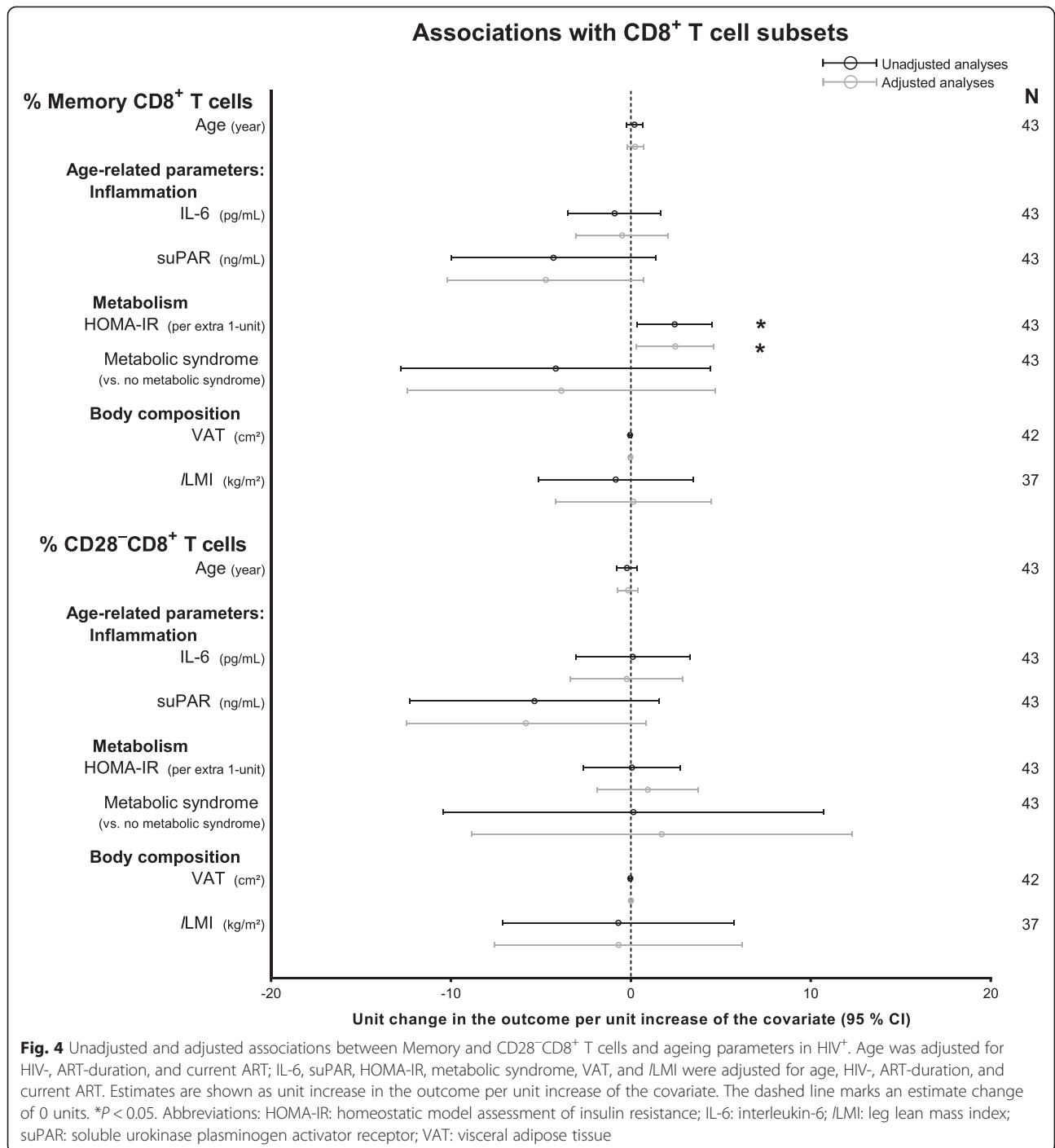
proportions of memory CD8⁺ T cells, and with elevated HOMA-IR index. Thus HIV-infection could be a confounder of this association. We did not observe robust associations between CD8⁺ T cell maturation and differentiation and body composition. Our results are in agreement with Erlandson et al. who reported an association of low physical function with inflammation, but not with highly differentiated CD28⁻ T cells, in HIV-infected patients, [35]. Moreover, Wallet et al. reported neither elevated inflammation nor higher proportions of senescent CD57⁺ CD4⁺ and CD8⁺ T cells to be associated with physical function in older HIV-infected patients [36].

HIV-infection was not associated with higher PD-1 or KLRG1 expression in CD8⁺ T cells. However, PD-1 and KLRG1 expression depended on differentiation and maturation stages of the cells. Consistent with previous studies, PD-1 expression was highest in intermediately differentiated and mature subsets, and KLRG1 expression was highest in highly differentiated and mature subsets [37–39]. PD-1 expression has been reported to be dependent on HIV viral load [39]. In our study, the majority of HIV⁺ had undetectable viral loads, which may explain why PD-1 expression was not increased in these patients. It is unclear whether KLRG1 expression is also dependent on the viral load, and this could not be investigated in our study due to the low number of patients with detectable viral loads. These observations suggest that CD8⁺ T cells from treated HIV-infected patients appear to be functional despite the skewed differentiation and maturation. However, due to the limited number of viable cells and FACS lasers, we could not investigate the functionality directly by assessing functional markers like CD56; the co-expression of PD-1



and KLRG1, and co-expression with other inhibitory receptors like TIM-3. But we did find a positive association between KLRG1 and PD-1 expression. Investigating

CD56 in the subsets could have yielded insight into the functionality of CD8⁺ T cells by assessing cytotoxicity [40]. Moreover, assessing TIM-3 expression as a marker of



exhaustion could have yielded insight into the exhaustion of CD8⁺ T cells with cytotoxic effects (CD56⁺) as in Poonia et al. [40]. Co-expression of several inhibitory receptors may be necessary to affect cellular functions, and may be a prominent feature in chronic viral infections [41, 42]. However, the aim of this study was to assess the effect of immunosenescence and exhaustion in CD8⁺ T cells on age and age-related parameters,

rather than CD8⁺ T cell functions. We therefore investigated KLRG1 and PD-1, as these have been shown to reflect CD8⁺ T cell senescence and exhaustion [8, 14].

KLRG1 expression in the subsets, but not in total CD8⁺ T cells, was influenced to a minor degree by age-related processes of metabolism, adipose tissue, and muscle. VAT and metabolic syndrome were associated with higher KLRG1 expression in CD28⁺ and

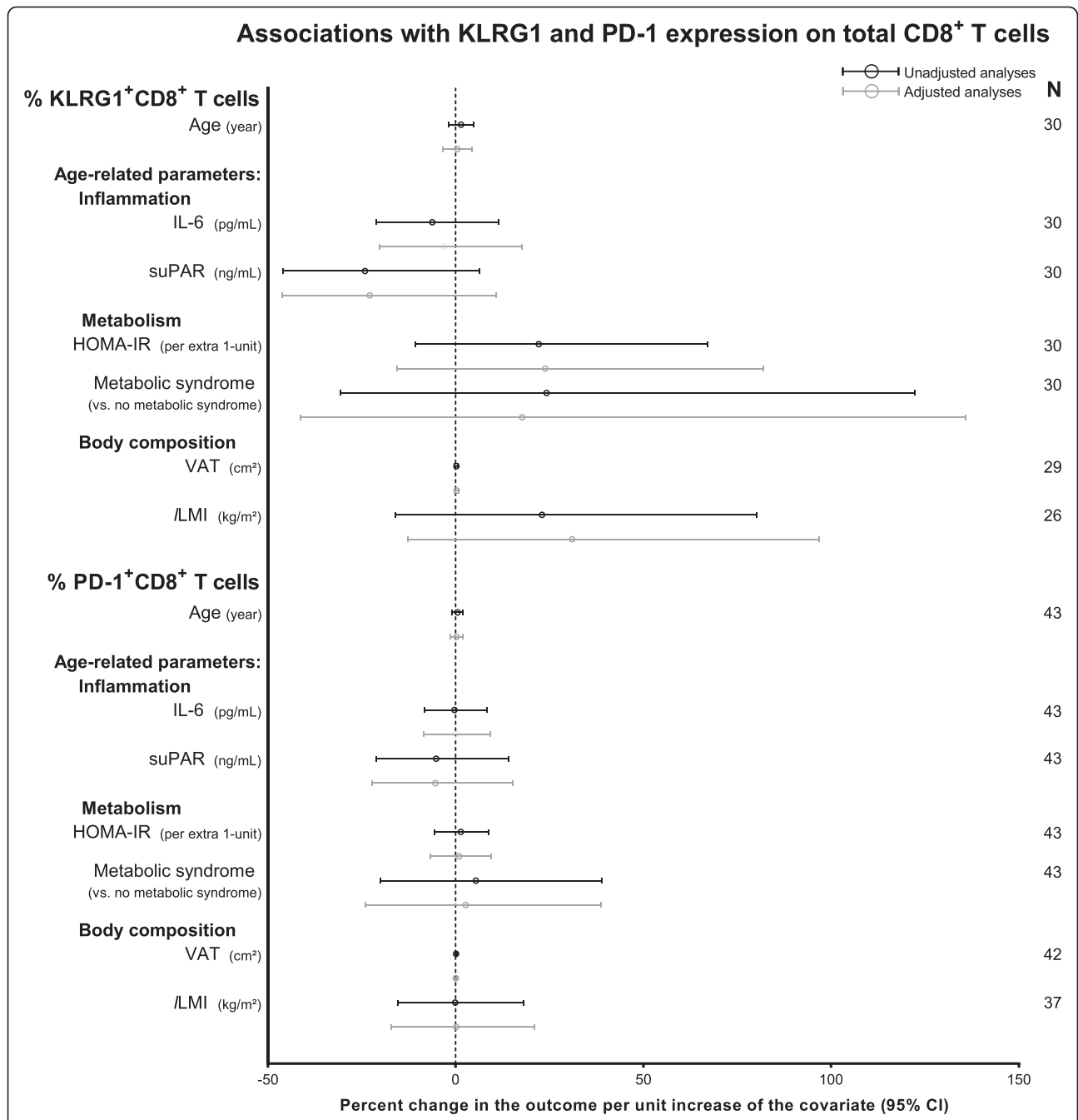


Fig. 5 Unadjusted and adjusted associations between KLRG1⁺ and PD-1⁺CD8⁺ T cells and ageing parameters in HIV⁺. Age was adjusted for HIV-, ART-duration, and current ART; IL-6, suPAR, HOMA-IR, metabolic syndrome, VAT, and lLMI were adjusted for age, HIV-, ART-duration, and current ART. Parameters were transformed using log₂(x). Estimates and 95 % CI are back transformed using (2^B-1) × 100 and shown as percent change in the outcome per unit increase of the covariate. The dashed line marks an estimate change of 0 %. Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance; IL-6: interleukin-6; KLRG1: killer cell lectin-like receptor G1; lLMI: leg lean mass index; PD-1: programmed death-1; suPAR: soluble urokinase plasminogen activator receptor; VAT: visceral adipose tissue

CD28⁻ cells. KLRG1 expression in T_N cells was associated with high muscle mass. However, due to the wide confidence intervals for the associations with metabolic syndrome and lLMI, and to the small estimate of the association with VAT, further investigations are

required to determine whether these associations are true, or whether they are artefacts. PD-1 expression was not influenced by age-related parameters. Moreover, we did not find obvious associations between CD8⁺ T cell phenotypes and inflammation, suggesting

Table 2 Unadjusted and adjusted associations between KLRG1⁺CD8⁺ T cell groups and ageing parameters in HIV⁺

	Maturation - CD27/CD45RA				Differentiation - CD27/CD28			
	% KLRG1 ⁺ T _N ^a		% KLRG1 ⁺ Memory ^a		% KLRG1 ⁺ CD28 ⁺ ^a		% KLRG1 ⁺ CD28 ⁻	
	Estimate	95 % CI	Estimate	95 % CI	Estimate	95 % CI	Estimate	95 % CI
Age (per year extra)	-0.84	-4.32 - 2.77	1.12	-2.19 - 4.53	1.54	-1.73 - 4.93	0.05	-0.81 - 0.92
	-0.16	-4.22 - 4.07	0.32	-3.53 - 4.32	0.49	-3.16 - 4.28	-0.24	-1.24 - 0.76
IL-6 (per extra pg/mL)	-0.24	-17.69 - 20.92	-8.07	-22.94 - 9.68	3.27	-13.80 - 23.71	-0.84	-5.53 - 3.85
	-2.98	-21.71 - 20.23	-7.08	-23.90 - 13.47	9.10	-9.84 - 32.03	0.11	-5.16 - 5.38
suPAR (per extra ng/mL)	-13.66	-40.90 - 26.14	-21.72	-44.54 - 10.50	-18.83	-42.12 - 13.82	-5.62	-14.38 - 3.14
	-15.63	-43.65 - 26.30	-20.25	-45.09 - 15.84	-17.17	-41.55 - 17.38	-4.90	-14.36 - 4.56
HOMA-IR (per extra 1-unit)	16.39	-17.36 - 63.93	18.91	-13.42 - 63.30	27.27	-6.34 - 72.93	6.02	-1.96 - 14.01
	35.74	-9.23 - 103.01	22.91	-16.71 - 81.38	27.45	-10.90 - 82.29	6.98	-2.65 - 16.16
Metabolic syndrome (vs. no metabolic syndrome)	23.25	-34.76 - 132.83	46.43	-17.97 - 161.42	70.88	-1.41 - 196.17	14.31*	0.10 - 28.53
	48.94	-29.02 - 212.55	41.31	-29.88 - 184.78	61.77	-15.73 - 210.55	17.59*	0.73 - 34.45
VAT (per extra cm ²)	-0.10	-0.55 - 0.35	0.28	-0.12 - 0.70	0.45*	0.06 - 0.84	0.10	-0.00 - 0.20
	0.14	-0.54 - 0.82	0.28	-0.34 - 0.90	0.62*	0.07 - 1.16	0.16*	0.01 - 0.31
ILMI (per extra kg/m ²)	66.47*	10.22 - 151.43	36.88	-5.52 - 98.30	22.54	-20.06 - 87.86	7.92	-2.88 - 18.71
	59.19	-0.55 - 154.80	45.52	-1.35 - 114.65	33.83	-13.85 - 107.92	10.09	-1.22 - 21.39

Adjusted analyses are in grey. Age was adjusted for HIV-duration, ART-duration, and current ART; IL-6, suPAR, HOMA-IR, metabolic syndrome, VAT, and ILMI were adjusted for age, HIV-duration, ART-duration, and current ART

Abbreviations: *HOMA-IR* homeostatic model assessment of insulin resistance, *IL-6* interleukin-6, *KLRG1* killer cell lectin-like receptor G1, *ILMI* leg lean mass index, *suPAR* soluble urokinase plasminogen activator receptor, *T_N* naïve T cell, *VAT* visceral adipose tissue

The bold values are statistically significant at **P* < 0.05

^aParameters were transformed using log₂(*x*). Estimates and confidence intervals are back transformed using (2^{*B*}-1) × 100, and shown as percent change in the outcome per unit increase of the covariate

that highly differentiated, mature, senescent, or exhausted CD8⁺ T cells may not be major contributors to systemic IL-6 and suPAR levels.

In participants from this study, IL-6 and suPAR were associated with FMI, VAT and low ILMI [17]. These observations suggest that inflammation reflects age-related processes of adipose tissue redistribution and low muscle mass. In contrast, we did not find definite associations of immunosenescence and T cell exhaustion with age-related parameters or inflammation. This indicates that CD8⁺ T cell immunosenescence and exhaustion may not play major roles in inflammaging and age-related processes in HIV-infected patients.

This study has some limitations. Its cross-sectional nature does not allow for causal interpretation of the associations. Since only ART-treated patients were included, we could not determine whether our findings were a result of HIV-infection, or ART, or a combination. Moreover, due to difficulties in including treatment naïve patients, we were not able to include elite controllers for comparison. By restricting the inclusion criteria to men we aimed to limit variation in the immune and inflammatory variables due to menopause and menstrual cycle-related hormonal fluctuations [43]. Therefore, our findings are only representative for men. The lack of data on CMV-seropositivity of the participants may also be a limitation to the interpretation

of the results. CMV seroprevalence is higher in HIV-infection (>75 %) than in the general population (40–50 %) [44]. Thus, CMV-seropositivity could also contribute to the skewed distribution of CD8⁺ T cells we observed in HIV⁺. Another limitation to the study is the likelihood of type I error due to multiple testing. We limited the number of analyses by grouping the CD8⁺ T cell subsets into larger groups based on relevant biological and functional features. Moreover, we based the interpretation of our data on other findings, and did not make definite conclusions from our findings that did not reach high statistical significance. The study population was relatively small due to a number of non-viable samples. We therefore did not have sufficient power to assess ageing parameters in the healthy Controls.

Conclusions

We showed that despite effective treatment, HIV-infection is associated with a skewed distribution of CD8⁺ T cells towards more differentiated and mature phenotypes, suggestive of accelerated immune ageing. However, HIV-infection was not associated with higher KLRG1 or PD-1 expression on CD8⁺ T cells. Instead, the pattern of KLRG1 and PD-1 expression followed subset distribution—and to a minor degree, age. We did not find obvious relationships between CD8⁺ T cell phenotypes and age-related processes of

inflammation, metabolism, adipose tissue and muscle. These findings suggest that in contrast to inflammation, immunosenescence appears to be highly dependent on HIV-infection and is only to a smaller extent associated with age-related parameters in well-treated HIV-infected patients. Moreover, CD8⁺ T cell differentiation, maturation, senescence, or exhaustion did not appear to be major causes of inflammaging in HIV-infected patients.

Additional files

Additional file 1: Figure S1. CD8⁺ T cell maturation and differentiation in HIV⁺ with and without lipodystrophy, and Controls. (a) Proportions of CD8⁺ T cells in each maturation subset: naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), or effector memory re-expressing CD45RA (T_{EMRA}) (b) Proportions of CD8⁺ T cells in each differentiation subset: early differentiated (T_{ED}), intermediate differentiated (T_{ID}), late differentiated (T_{LD}), or CD27⁺CD28⁺. HIV⁺ with lipodystrophy (Lipo) (N = 14), HIV⁺ without lipodystrophy (Non-lipo) (N = 29), and Controls (N = 10). Medians are shown as horizontal bars. P-values were determined using one-way ANOVA. (PDF 64 kb)

Additional file 2: Figure S2. CD8⁺ T cell exhaustion and senescence in HIV⁺ with and without lipodystrophy, and Controls. (a) Proportions of PD-1⁺ cells in CD8⁺ T cell subsets and total CD8⁺ T cells in HIV⁺ with and without lipodystrophy, and Controls. HIV⁺ with lipodystrophy (Lipo) (N = 14), HIV⁺ without lipodystrophy (Non-lipo) (N = 29), and Controls (N = 10). (b) Proportions of KLRG1⁺ cells in CD8⁺ T cell subsets and total CD8⁺ T cells in HIV⁺ with and without lipodystrophy, and Controls. HIV⁺ with lipodystrophy (Lipo) (N = 8), HIV⁺ without lipodystrophy (Non-lipo) (N = 22), and Controls (N = 9). Medians are shown as horizontal bars. P-values were determined using one-way ANOVA. (PDF 98 kb)

Additional file 3: Table S1. Linear regression analyses for the effect of HIV-infection on CD8⁺ T cell maturation and differentiation. †Parameter was transformed using log₂(x). Estimates and confidence intervals are back transformed using (2^β-1) × 100, and shown as percent change in the outcome per unit increase of the covariate. The bold values are statistically significant at *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance, IL-6: interleukin-6, /LMI: leg lean mass index, suPAR: soluble urokinase plasminogen activator receptor, T_{CM}: central memory T cell, T_{ED}: early differentiated T cell, T_{EM}: effector memory T cell, T_{EMRA}: effector memory T cell re-expressing CD45RA, T_{ID}: intermediate differentiated T cell, T_{LD}: late differentiated T cell, T_N: naïve T cell, VAT: visceral adipose tissue. (XLSX 14 kb)

Additional file 4: Table S2. Unadjusted and adjusted associations between CD8⁺ T cell subset sizes and ageing parameters in HIV⁺. Adjusted analyses are in grey. Age was adjusted for HIV-duration, ART-duration, and current ART; IL-6, suPAR, HOMA-IR, metabolic syndrome, VAT, and /LMI were adjusted for age, HIV-duration, ART-duration, and current ART. †Parameters were transformed using log₂(x). Estimates and confidence intervals are back transformed using (2^β-1) × 100, and shown as percent change in the outcome per unit increase of the covariate. The bold values are statistically significant at *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance, IL-6: interleukin-6, /LMI: leg lean mass index, suPAR: soluble urokinase plasminogen activator receptor, T_{CM}: central memory T cell, T_{ED}: early differentiated T cell, T_{EM}: effector memory T cell, T_{EMRA}: effector memory T cell re-expressing CD45RA, T_{ID}: intermediate differentiated T cell, T_{LD}: late differentiated T cell, T_N: naïve T cell, VAT: visceral adipose tissue. (XLSX 16 kb)

Additional file 5: Table S3. Unadjusted and adjusted associations between KLRG1⁺CD8⁺ T cell subsets and ageing parameters in HIV⁺. Adjusted analyses are in grey. Age was adjusted for HIV-duration, ART-duration, and current ART; IL-6, suPAR, HOMA-IR, metabolic syndrome, VAT, and /LMI were adjusted for age, HIV-duration, ART-duration, and current ART. †Parameters were transformed using log₂(x). Estimates and confidence intervals are back transformed using (2^β-1) × 100, and shown as percent change in the outcome per unit increase of the covariate. The bold values are statistically significant at

*P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance, IL-6: interleukin-6, KLRG1: killer cell lectin-like receptor G1, /LMI: leg lean mass index, suPAR: soluble urokinase plasminogen activator receptor, T_{CM}: central memory T cell, T_{ED}: early differentiated T cell, T_{EM}: effector memory T cell, T_{EMRA}: effector memory T cell re-expressing CD45RA, T_{ID}: intermediate differentiated T cell, T_{LD}: late differentiated T cell, T_N: naïve T cell, VAT: visceral adipose tissue. (XLSX 16 kb)

Additional file 6: Table S4. Unadjusted and adjusted associations between PD-1⁺CD8⁺ T cell groups and ageing parameters in HIV⁺. Adjusted analyses are in grey. Age was adjusted for HIV-duration, ART-duration, and current ART; IL-6, suPAR, HOMA-IR, metabolic syndrome, VAT, and /LMI were adjusted for age, HIV-duration, ART-duration, and current ART. †Parameters were transformed using log₂(x). Estimates and confidence intervals are back transformed using (2^β-1) × 100, and shown as percent change in the outcome per unit increase of the covariate. Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance, IL-6: interleukin-6, PD-1: programmed death-1, /LMI: leg lean mass index, suPAR: soluble urokinase plasminogen activator receptor, T_{CM}: central memory T cell, T_{ED}: early differentiated T cell, T_{EM}: effector memory T cell, T_{EMRA}: effector memory T cell re-expressing CD45RA, T_{ID}: intermediate differentiated T cell, T_{LD}: late differentiated T cell, T_N: naïve T cell, VAT: visceral adipose tissue. (XLSX 15 kb)

Additional file 7: Table S5. Unadjusted and adjusted associations between PD-1⁺CD8⁺ T cell subsets and ageing parameters in HIV⁺. Adjusted analyses are in grey. Age was adjusted for HIV-duration, ART-duration, and current ART; IL-6, suPAR, HOMA-IR, metabolic syndrome, VAT, and /LMI were adjusted for age, HIV-duration, ART-duration, and current ART. †Parameters were transformed using log₂(x). Estimates and confidence intervals are back transformed using (2^β-1) × 100, and shown as percent change in the outcome per unit increase of the covariate. Abbreviations: HOMA-IR: homeostatic model assessment of insulin resistance, IL-6: interleukin-6, PD-1: programmed death-1, /LMI: leg lean mass index, suPAR: soluble urokinase plasminogen activator receptor, T_{CM}: central memory T cell, T_{ED}: early differentiated T cell, T_{EM}: effector memory T cell, T_{EMRA}: effector memory T cell re-expressing CD45RA, T_{ID}: intermediate differentiated T cell, T_{LD}: late differentiated T cell, T_N: naïve T cell, VAT: visceral adipose tissue. (XLSX 17 kb)

Abbreviations

ART: antiretroviral therapy; BMI: body mass index; CMV: cytomegalovirus; FMI: fat mass index; HIV⁺: HIV-infected patient; HOMA-IR: homeostatic model assessment of insulin resistance; IL-6: interleukin-6; KLRG1: killer cell lectin-like receptor G1; /LMI: leg lean mass index; PD-1: programmed death-1; suPAR: soluble urokinase plasminogen activator receptor; T_{CM}: central memory; T_{ED}: early differentiated; T_{EM}: effector memory; T_{EMRA}: effector memory re-expressing CD45RA; T_{ID}: intermediate differentiated; T_{LD}: late differentiated; T_N: naïve; VAT: visceral adipose tissue.

Competing interests

ViroGates A/S donated the suPARnostic® kits for suPAR measurements, but did not have any influence on the design of the study. OA is inventor of the patent on suPAR and risk. Hvidovre Hospital, Denmark, owns the patent which is licensed to ViroGates A/S. JT, AL, THH, JHH, FKJ, and JP declare no conflicts of interest.

Authors' contributions

AL, JP, and OA designed the study. JT and AL carried out the laboratory measurements. JHH participated in the coordination of the DXA scans. FKJ evaluated the CT scans. JT, AL, THH, and JP participated in the statistical analyses. JT wrote the manuscript with participation from AL, THH, JHH, JP, and OA. All authors read and approved the final manuscript.

Acknowledgments

We thank all the individuals of the study for their participation. We are also grateful to the staff at the Department of Infectious diseases, Copenhagen University Hospital, Hvidovre, for assisting in patient recruitment. We are very thankful to Michele Weber, Kristian Zobbe, and Henrik Hedegaard Klausen for technical support and assistance at examinations. This study was supported by Aase and Ejnar Danielsens Fond and Arvid Nilssons Fond to AL.

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Received: 6 August 2015 Accepted: 20 November 2015

Published online: 26 November 2015

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