The proportion of CD57+ cells among effector CD8+ T cells is lower in HIV controllers compared with antiretroviral therapy-treated patients

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Spontaneous HIV control is associated with a high proportion of non-CD57-expressing effector CD8$^+$ T cells.

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Abstract

Background:

HIV infection has often been linked to faster immune aging. We sought to determine whether or not treatment-naïve spontaneous HIV-1 controllers (HICs) and ART-exposed patients differ with regard to the expression of cell senescence markers.

Methods:

Eighty-two chronically infected HICs and ART-exposed patients (median time since infection: 15 years) with an undetectable plasma HIV RNA load (for at least the previous two years) were included. We used flow cytometry to measure immunosenescence markers (KLRG-1 and CD57) expression in fresh blood samples collected from patients and healthy donors (HDs).

Results:

For the $CD8^+$ T cell population as a whole, the ART-exposed patients and the HICs exhibited a much higher proportion of KLRG-1^+ $CD8^+$ T cells (but not $CD57^+$ $CD8^+$ T cells) than HDs. For the CD8 T cell subsets, HICs had a lower proportion of $CD57^+$ effector $CD8^+$ T cells than ART patients or HDs, whereas the proportions of KLRG-1^+ effector were similar. A similar trend was observed for terminal effectors. Expression of CD57 was correlated with the CD8 count. The cytotoxic granule content was greater in the $CD57^+$ fraction than in the $CD57^-$ fraction in both the HIC and ART groups. However, when considering the $CD57^-$ effector memory and effector subsets, the cytotoxic granule content was greater in HICs than in the ART-exposed patients.

Conclusion:

The proportion of $CD57^+$ effector $CD8^+$ T cells is lower in HICs than in ART-exposed patients. This difference might boost the CD8 response and/or reflect limited senescence of the effector fraction.

Keywords: CD8 effector function, CD57, senescence, terminal differentiation, cytotoxic function, replicative senescence, aging.

Introduction

Immune dysregulation (e.g. immunosenescence and chronic inflammation) is a biological process that has often been linked to HIV infection; it is attracting increasing attention as the HIV-infected population ages. Accelerated aging may indeed contribute to cardiovascular diseases, non-AIDS cancers, diabetes, and cirrhosis [1,2]. Immunosenescence of T cells is characterized by decrease in the CD4/CD8 ratio and the percentage of naive T cells, and by an increase in the percentage of terminally differentiated $CD28^-$ T cells [3]. Along with assays of T cell differentiation markers, the various memory cell subpopulations are usually evaluated further by measuring the CD57 and killer cell lectin-like receptor-1 (KLRG-1) marker [3,4].

CD57 is a marker routinely used to explore senescence, although its exact relevance in this context is subject to debate [5]. The marker has been linked to replicative senescence: $CD57^+CD8^+$ T cells display a low proliferative capacity and elevated susceptibility to antigen-induced apoptosis [6]. Expression of CD57 also identifies terminally differentiated T cells, i.e. those able to produce cytokines and which have a high cytotoxic potential [7,8]. Furthermore, expression of CD57 is associated with specific migratory potencies [7]. *In vitro*, CD57 expression on $CD8^+$ T cells has also been linked to immunosuppressive function [9–11] - suggesting that the functional activity of $CD57^+$ $CD8^+$ T cells may be more complex than initially thought. Lastly, a recent study of $CD45RA^+$ terminal effector (TE) $CD8^+$ T cells demonstrated that the $CD57^-$ TE fraction exhibited greater functional plasticity than their $CD57^+$ counterparts [12]. This finding indicates that a high proportion of $CD57^+$ cells may be associated with a poor immune response. Overall, $CD57^+CD8^+$ T cells appear to have several functional properties. Nevertheless, $CD57^+$ cells have been predominantly associated with senescence [13]. Elevated percentages of $CD57^+$ cells have been described in the setting of CMV infection and in older adults [14]. It has been found that $CD8^+$ T cells from various subgroups of HIV-infected people (ART-naïve patients, ART-exposed patients, viremic patients, and HIV controllers [HICs, i.e. the very few patients who spontaneously control HIV replication]) express higher levels of CD57 than cells from non-infected people [7,14–17]. Other studies have reported that the proportion of $CD57^+$ T cells in the $CD28^-CD8^+$ subset [16-18] is low during HIV infection but rises following ART. Furthermore, a low proportion of $CD57^+CD28^-CD8^+$ T cells was a predictive marker of mortality among ART-exposed, HIV-

infected patients with advanced disease [18]. Overall, the results appear to differ as a function of the variable studied (the proportion or absolute count of $CD57^+CD28^-CD8^+$ T cells [19], or the frequency of $CD57^+$ cells in the $CD28^-CD8^+$ T population [20]) and the infectious status [17]. However, it is generally accepted that the $CD57^+$ fraction increases in chronically HIV-infected patients [13,21]. Data on immunosenescence in HICs are scarce. Bansal et al. [22] found that the results in HICs varied as a function of the percentage of $CD4^+$ T cells. Ruiz-Mateo et al. studied immunosenescence profiles for T cells among "elite" HICs [23,24], and reported a higher proportion of terminally differentiated $CD4^+$ T cells (but not $CD8^+$ T cells) in these patients.

The cadherin receptor KLRG-1 is another marker of immunosenescence expressed on $CD8^+$ T cells: the proportion of KLRG1-expressing $CD8^+$ T cells rises dramatically with age [25,26]; this is partly due to the age-related increase in the frequencies of memory cells and highly differentiated end-stage cells, which are predominantly $KLRG\text{-}1^+$. Much as with CD57, KLRG1 is probably more than a senescence marker (for a review, see Henson et al. [27]). KLRG1 can also be considered as an activation marker because it is also used to distinguish between short-lived effector (E) $CD8^+$ T cells ($KLRG1^{high}$) and memory precursor E $CD8^+$ T cells ($KLRG1^{low}$) during acute viral responses in mice [28] or in a clinical setting [29]. Lastly, KLRG1 has a direct inhibitory effect on T cell activation *in vitro* [26]. In line with the divergent functions attributed to CD57 and KLRG1, the proteins' respective expression profiles on T cells do not overlap fully [30]. $KLRG1^+CD57^-$ subsets reportedly retain the ability to proliferate and secrete IFNγ, whereas $CD57^+KLRG1^+$ subsets fail to proliferate and display only traces of IFNγ [30].

The objective of the present study was to investigate the senescence phenotype in chronically HIV-infected, ART-responsive patients and in HICs with equivalent $CD4^+$ T cell counts.

Materials and methods

Ethic statement

All subjects provided their written, informed consent to participation. The study was approved by the local independent ethics committees consulted by the cohorts (*CPP Ile de France III* and *VII*, Paris, France).

Study participants

Samples were collected from 82 chronically HIV-infected patients: 44 HICs enrolled in the ANRS CO21 CODEX cohort (no exposure to ART, and the last 5 plasma HIV RNA values below 400 copies/mL), and 38 ART-exposed, HIV-infected patients enrolled in the ANRS CO06 PRIMO cohort (ART for more than 4 years, plasma HIV RNA <40 copies/mL in the last 2 years, and no HBV or HCV co-infections). In the present study, long-term HIV infection was defined as a diagnosis more than 5 years previously. Samples from non-infected healthy blood donors (HDs, n=22) were obtained from the Etablissement Français du Sang (Saint Louis Hospital, Paris, France). Serologic status for CMV was determined using a Liaison XL CMV IgG assay (Diasorin, Sallugia, Italy). An additional 92 samples from HIV-infected patients studied at primo-infection, ART-exposed patients at m24 post-infection (ANRS 147 OPTIPRIM trial) [31] and from HICs were used to study the impact of blood sample processing on CD57 expression by $CD8^+$ T cells.

Blood sample processing and staining for flow cytometry

Fresh whole blood samples were incubated with human Fc block for 20 min at room temperature and then with labeled antibodies against surface markers. After incubation, red blood cells were lysed using BD FACS™ Lysing Solution (BD Biosciences, Franklin Lakes, NJ, USA), washed in PBS 1X, and fixed with 1% PFA. The following antibodies were used: PD-1 (EH12-2H7), CD38 (HIT2), CD3 (SK7), HLA-DR (G46-6), CD45RO (UCHL1), CD27 (L128), CD28 (CD28.2), CD57 (NK-1), CD4 (OKT4), CCR7 (GO25H7), CD8 (SK1), KLRG-1 (REA261), and TIGIT (MBSA43). For a small number of samples, cytotoxic granule content was evaluated via intracellular staining for perforin (D48) and granzyme B (GB11) after fixation/permeabilization, using a Foxp3 transcription factor fix/perm kit (eBioscience/ThermoFisherScientific, Waltham, MA, USA).

Cryopreserved peripheral blood mononuclear cells (PBMCs) were also used to compare patient samples prior to flow cytometry. The PBMCs were isolated from EDTA-anticoagulated blood using Ficoll density gradient centrifugation. Cells were cryopreserved in fetal bovine serum supplemented with 10% dimethyl sulfoxide. Samples were acquired on an LSR Fortessa cell

analyzer (BD Biosciences), and the resulting data were analyzed using FlowJo V10 software (BD Biosciences).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 8, GraphPad Software, San Diego, CA, USA). Intergroup comparisons were performed using non-parametric, unpaired Kruskal-Wallis or Mann-Whitney tests. Non-parametric, paired Wilcoxon tests were used for paired data (e.g. a comparison of CD57+ and CD57- fractions in the same sample). Spearman's coefficient was calculated in correlation analyses. The threshold for statistical significance was set to $p<0.05$, and p values were labelled as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; and ****, $p<0.0001$).

Results

Study participants

Forty-four HICs and 38 chronically infected, ART-exposed patients (infected for more than 5 years, and with a controlled viral load for more than 2 years) were analyzed and compared with 22 uninfected HDs (Table 1). The HIC and ART groups did not differ significantly in terms of the male/female ratio, the CD4 and CD8 counts, and the proportion of $CD8^+$ T cells. The proportion of $CD4^+$ T cells and the CD4/CD8 ratio were higher in the HIC group than in the ART group. The percentage of CMV-immunized individuals was significantly higher in two HIV groups than in the HDs (50% for HDs, 89% for HIC, and 92% for ART patients; $p<0.0001$).

CD57 expression by E and TE $CD8^+$ T cells is lower in HICs than in HDs

Given that immunosenescence has been associated with greater terminal differentiation, we analyzed the distribution of $CD8^+$ T cells subsets on the basis on the CD45RO, CCR7, CD28 and CD27 expression [32]. The percentage of naïve (N) $CD8^+$ T cells was significantly lower in HICs and ART patients than in HDs (16.9±13.0, 14.7±14.9 and 25.3±10.4, respectively; $p=0.02$ for HDs vs. HICs, and $p=0.001$ for HDs vs. ART patients). The proportion of central memory (CM) $CD8^+$ T cells was low in all groups [33] but was significantly lower in ART patients than in HICs and HDs

(2.0±1.6%, 3.3±2.3% and 3.5±1.8%, respectively; p=0.004 for ART patients vs. HDs, and p=0.02 for ART patients vs. HICs). We did not observe any intergroup differences in the distribution profiles of effector memory (EM) or effector (E) $CD8^+$ T cells. The end-differentiated TE $CD8^+$ T cells subset was more abundant in both HICs and ART patients, relative to HDs (24.3±14.5%, 26.4±17.2%, and 12.1±10.8% respectively; p=0.001 for HICs vs. HDs, and p=0.0006 for ART patients vs. HDs). We next evaluated intergroup differences in KLRG-1 and CD57 expression for total $CD8^+$ T cells or the $CD8^+$ T cell subsets (Fig 1B and C). KLRG-1 expression on total $CD8^+$ T cells was higher in HICs and ART patients than in HDs (64.3±17.0%, 63.4±21.0%, and 50.4±17.1%, respectively; p=0.02 for HICs vs. HDs, and p=0.02 for ART patients vs. HDs), and increased progressively from the CM subset to the EM, E and TE subsets in that order. There were no intergroup differences in KLRG-1 expression on $CD8^+$ T cells subpopulations with the exception of the N subset, for which the proportion of KLRG-1-expressing cells was slightly but significantly higher in HICs than in HDs (7.3±5.0% and 3.6±2.5%, respectively; p=0.007). A different picture emerged when considering CD57 expression. Although the proportions of CD57-expressing total $CD8^+$ T cells were similar in the three groups, HICs displayed significantly lower proportions of $CD57^+$ E and TE $CD8^+$ T cells (45.8±25.3% for E and 52.8±24.8% for TE) than HDs (77.3±19.5% for E and 76.9±18.4% for TE; p<0.0001 for E, and p=0.0002 for TE). HICs also displayed a lower proportion of $CD57^+$ E $CD8^+$ T cells than ART patients (63.5±21.7% for E, p=0.01). These results reveal that E and TE fractions in HICs have a peculiar phenotype, with lower CD57 expression than in HDs and (for E $CD8^+$ T cells) ART patients but no differences in KLRG-1.

Discrepancy between measurements of CD57 expression in fresh vs. frozen-thawed blood samples

The lower proportion of CD57-expression on $CD8^+$ T cells in HICs than in ART patients was not consistent with previous results (including those from our laboratory) [16]. Our two studies differed with regard to the blood sample processing and the patients' characteristics (the time since diagnosis). All the analyses were performed on fresh whole blood in the present study and on cryopreserved PBMCs in the previous study. Hence, in order to investigate the potential influence

of blood sample preparation on our results, we compared the percentage of $CD57^+$ $CD8^+$ T cells in 92 samples from HICs and ART patients prepared with the two approaches in parallel (i.e. fresh whole blood and cryopreserved PBMCs; Fig. 2A). We observed a significantly higher proportion of CD57-expressing cells in the cryopreserved samples than in the fresh blood samples (32.3±14.2% vs. 24.1±14.2%, respectively; $p<0.0001$). Moreover, the relative increase in CD57 expression after cryopreservation was heterogeneous: the proportion CD57-expressing cells was even more greater when basal CD57 expression in fresh blood was low (with a 12.0±9.2% increase in CD57 expression for low levels of CD57 expression, and a 4.4±13.5% increase for high levels of CD57 expression, $p=0.0003$) (Fig 2B). Our results highlighted the harmful impact of PBMC isolation and/or cryopreservation on the analysis of CD57 expression.

CD57 expression on $CD8^+$ T cells is associated with CMV status in HDs, and with CD8 counts in ART-exposed patients and HICs

We evaluated the impact of CMV infection on CD57 expression in all groups of participants. Whereas CMV directly impacted CD57 expression in total $CD8^+$ T cells in HDs (data not shown), these analyses were not available in HIV-infected patients who were predominantly immunized against CMV. However, the impact of CMV status on the total $CD8^+$ T cell population in HDs was not observed for the $CD8^+$ T cell subsets - suggesting that the association between CMV status and CD57 expression primarily reflects a change in the proportions of cell subsets. Importantly, the difference in CD57 expression by E $CD8^+$ T cells between HICs and ART-exposed patients was also observed when considering CMV-immunized participants only. We next looked at whether CD57 expression was associated with the CD4/CD8 ratio and the CD8 count, i.e. standard markers of immunosenescence: (Fig 3A). We did not observe any correlation between CD57 expression and CD4/CD8 ratio in HICs or in ART-exposed patients; this was presumably because the direct influence of HIV infection on the CD4/CD8 ratio had overcome the senescence-driven change in the CD4/CD8 ratio. However, the percentage of $CD57^+CD8^+$ T cells was positively correlated with the CD8 count in HICs ($p=0.02$, $r=0.35$) and even more so in ART-exposed patients ($p=0.005$, $r=0.44$).

Non-CD57-expressing CD8$^+$ T cells exhibit a sizeable proportion of cells with cytotoxic potential, which are more numerous in HICs than in ART-exposed patients

CD57 can also be considered as a marker of full E cell differentiation. As such, low CD57 expression might correspond to limited cytotoxic potential among CD8$^+$ T cells in HICs. To address this aspect, we evaluated the cytotoxic potential of CD57$^+$ and CD57$^-$ fractions among EM, E and TE subsets (the three subsets with the potential for direct cytotoxic activity *ex vivo*) in HICs and ART-exposed patients by detecting perforin (Perf) and granzyme B (GrzB) contents (Fig 3B). The low numbers of CD57$^-$ cells in EM, E and TE subsets in HDs prevented us from comparing the latter group with HICs and ART-exposed patients. In the EM CD8$^+$ T cell fraction, the proportion of cells containing GrzB-Perf was higher among CD57$^+$ cells than among their CD57$^-$ counterparts for both HICs (46.0±20.4% in CD57$^+$ cells and 32.8±16.9% in CD57$^-$ cells; p=0.001) and in the ART group (26.4±11.7% in CD57$^+$ cells and 12.6±8.1% in CD57$^-$ cells; p=0.002). However, both the CD57$^+$ and CD57$^-$ EM fractions displayed significantly greater direct cytotoxic potential in HICs than in ART-exposed patients (p=0.016 and p=0.006 for the CD57$^+$ and CD57$^-$ fractions, respectively). In the E CD8$^+$ T cell fraction, CD57$^+$ fractions also contained more Perf and GrzB than their CD57$^-$ counterparts in both HICs (65.3±21.8% in CD57$^+$ cells and 49.9±22.9% in CD57$^-$ cells; p=0.001) and ART-exposed patients (47.4±15.5% in CD57$^+$ cells and 30.2±16.0% in CD57$^-$ cells; p=0.002); this finding is in line with the literature data on an association between CD57 expression and greater cytotoxic contents. Importantly, CD57$^+$ and CD57$^-$ E CD8$^+$ T cell fractions had a significantly higher intracellular GrzB-Perf content *ex vivo* in HICs than in ART-exposed patients (p=0.036 and p=0.025 for CD57$^+$ and CD57$^-$ cells, respectively). In the most differentiated fraction (TE CD8$^+$ T cells), CD57$^+$ cells still contained more Perf and GrzB than their CD57$^-$ counterparts (67.5±19.1% vs. 51.5±19.9% in HICs; p=0.003; 55.8±19.1% vs. 37.4±18.9% in the ART group; p=0.004). The CD57$^-$ CD8$^+$ T cell fraction also had a greater intracellular GrzB-Perf content *ex vivo* in HICs than in ART-exposed patients, although the difference was not statistically significant.

Taken as a whole, we thus confirmed the presence of higher levels of cytotoxic contents *ex vivo* in CD57-expressing cells than in CD57$^-$ counterparts, and showed that such this difference in cytotoxic potential affected the all subsets considered (EM, E and TE CD8$^+$ T cells). We also

observed a sizeable proportion of $CD57^-$ cells with high levels of cytotoxic content. Importantly, the proportion of cells exhibiting high cytotoxic content in the $CD57^-$ EM, and E subsets of $CD8^+$ T cells (and, to a lesser extent, the TE $CD8^+$ T cells) was significantly higher in HICs than in ART-exposed patients.

Discussion

In the present study, we assessed immunosenescence in chronically HIV-infected HICs and ART-exposed patients (median time since diagnosis: around 15 years) by measuring the frequency of CD57 and KLRG1 expression. Expression of KLRG-1 (but not CD57) on total $CD8^+$ T cells was more frequent in both groups of HIV-infected patients than in HDs. A different picture emerged when considering the differentiation status of $CD8^+$ T cells. In line with the literature data, CD57 expression was strongly associated with E and TE fractions. Whereas KLRG1 was strongly expressed in E $CD8^+$ T cell fractions from all groups (and thus was not discriminative), HICs had a lower frequency of CD57-expressing E and TE $CD8^+$ T cell than HDs and (for the E $CD8^+$ T cells) the ART-exposed patients. This observation was unexpected because it contrasted with previous reports (including those from our group) [7,14–16]. Here, we demonstrated that measurements of CD57 expression were sensitive to Ficoll isolation and freeze-thawing; our results therefore strongly suggest that CD57 staining of fresh, whole blood samples should always be used to accurately assess CD57 expression. We next ruled out bias due to CMV status: the difference in CD57 expression on E $CD8^+$ T cells between HICs and ART-exposed patients was still observed when comparing CMV-immunized participants only. The functional relevance of low CD57 expression among E $CD8^+$ T cells is difficult to assess because of CD57's multiple roles. Indeed, CD57 expression has been linked to proliferative senescence, terminal differentiation, migration towards non-lymphoid sites and even an immunosuppressive function in some reports. These conflicting observation also reflect our poor knowledge of CD57 expression. The protein is not expressed in macaques or mice, which thus precludes a detailed characterization of its regulation. When considering the association between CD57 expression with E $CD8^+$ T cell differentiation, we found that the proportions of E and TE fractions were similar in HICs and ART-exposed patients; this finding suggests that E $CD8^+$ T cell differentiation occurs in both groups. A recent study

demonstrated that $CD57^+$ TE cells may have low functional plasticity [12] and may thus impact the quality of immune responses. HICs may have a more balanced CD57 expression profile among their E and TE $CD8^+$ T cell fractions. Regarding the diversity of the HIV reservoir (present in both lymphoid and non-lymphoid tissues) and persistent mucosal alterations, preservation of both the $CD57^-$ fraction (with its migratory potential) and the $CD57^+$ fraction may allow the development of a consistent immune responses at all reservoir sites. However, it is important to note that these analyses were performed on total $CD8^+$ T cells and did not enables firm conclusion to be drawn concerning the differentiation profile of HIV-specific $CD8^+$ T cells. In fact, these analyses probably reflected the impact of long-term, controlled HIV infection on total $CD8^+$ T cell differentiation. Regardless of the HIV-specific $CD8^+$ T cells' responses, our results demonstrate that the modification of this differentiation profile in HICs does not remove the $CD8^+$ T compartment's cytotoxic potential. The GrzB-Perf content in the $CD57^-$ fraction from HICs was similar to that observed in the $CD57^+$ fraction of ART-exposed patients. Taken as a whole, our results suggest that $CD8^+$ T differentiation involves different pathways in long term ART-exposed patients and HICs. In ART-exposed patients (but not HICs) $CD8^+$ T cell differentiation is terminal and predominantly leads to the generation of $CD57^+$ TE cells - presumably indicating the development of senescence. The mechanisms underlying this difference remain to be determined but might be related to the cumulative viral load, cumulative inflammation, and/or the intrinsic properties of $CD8^+$ T cells. Furthermore, we observed an association between CD57 expression in $CD8^+$ T cells and the $CD8^+$ T cell count (a standard marker of senescence) - suggesting that CD57 expression in this context may also be a marker of cell senescence. The lack of an association between the CD4/CD8 ratio and CD57 expression may reflect the fact that the CD4/CD8 ratio is obviously greatly influenced by the level and duration of HIV replication (during both the acute and chronic phases) and the duration of ART, which may thus blur the influence of aging on this parameter.

In conclusion, HICs exhibited a more balanced CD57 expression profile among E and TE $CD8^+$ T cell fractions than ART-exposed patients did. Our present results showed that $CD57^-$ EM, E (and, to a lesser extent, TE) $CD8^+$ T cells display non-negligible cytotoxic potential, especially in HICs. The $CD57^-$ cell fraction may be more plastic (in terms of proliferative, migratory and cytotoxic activity) than the terminally differentiated $CD57^+$ fraction. Given that CD57 expression is also

associated with senescence, it is tempting to speculate that $CD8^+$ T cells from HICs may be less affected by senescence; limited differentiation towards the $CD57^+$ fraction might preserve the plasticity of CD8 T cells.

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References

1 Marcus JL, Chao CR, Leyden WA, Xu L, Quesenberry CP, Klein DB, *et al.* Narrowing the Gap in Life Expectancy Between HIV-Infected and HIV-Uninfected Individuals With Access to Care. *JAIDS J Acquir Immune Defic Syndr* 2016; **73**:39–46.

2 Ghosn J, Taiwo B, Seedat S, Autran B, Katlama C. HIV. *Lancet (London, England)* 2018; **392**:685–697.

3 Xu W, Larbi A. Markers of T Cell Senescence in Humans. *Int J Mol Sci* 2017; **18**:1742.

4 Larbi A, Fulop T. From "truly naïve" to "exhausted senescent" T cells: When markers predict functionality. *Cytom Part A* 2014; **85**:25–35.

5 Kared H, Martelli S, Ng TP, Pender SLF, Larbi A. CD57 in human natural killer cells and T-lymphocytes. *Cancer Immunol Immunother* 2016; **65**:441–452.

6 Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, *et al.* Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 2003; **101**:2711–2720.

7 Le Priol Y, Puthier D, Lecureuil C, Combadiere C, Debre P, Nguyen C, *et al.* High cytotoxic and specific migratory potencies of senescent CD8+ CD57+ cells in HIV-infected and uninfected individuals. *J Immunol* 2006; **177**:5145–5154.

8 Chattopadhyay PK, Betts MR, Price DA, Gostick E, Horton H, Roederer M, *et al.* The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. *J Leukoc Biol* 2009; **85**:88–97.

9 Mollet L, Sadat-Sowti B, Duntze J, Leblond V, Bergeron F, Calvez V, *et al.* CD8hi+CD57+ T lymphocytes are enriched in antigen-specific T cells capable of down-modulating cytotoxic activity. *Int Immunol* 1998; **10**:311–23.

10 Chong LK, Aicheler RJ, Llewellyn-Lacey S, Tomasec P, Brennan P, Wang ECY. Proliferation and interleukin 5 production by CD8hiCD57+ T cells. *Eur J Immunol* 2008; **38**:995–1000.

11 Sadat-Sowti B, Debré P, Mollet L, Quint L, Hadida F, Leblond V, *et al.* An inhibitor of

cytotoxic functions produced by CD8+CD57+ T lymphocytes from patients suffering from AIDS and immunosuppressed bone marrow recipients. *Eur J Immunol* 1994; **24**:2882–2888.

12 Verma K, Ogonek J, Varanasi PR, Luther S, Bünting I, Thomay K, *et al.* Human CD8+ CD57- TEMRA cells: Too young to be called "old" *PLoS One* 2017; **12**:e0177405.

13 Appay V, Almeida JR, Sauce D, Autran B, Papagno L. Accelerated immune senescence and HIV-1 infection. *Exp Gerontol* 2007; **42**:432–7.

14 Appay V, Fastenackels S, Katlama C, Ait-Mohand H, Schneider L, Guihot A, *et al.* Old age and anti-cytomegalovirus immunity are associated with altered T-cell reconstitution in HIV-1-infected patients. *AIDS* 2011; **25**:1813–22.

15 Ladell K, Hellerstein MK, Cesar D, Busch R, Boban D, McCune JM. Central memory CD8+ T cells appear to have a shorter lifespan and reduced abundance as a function of HIV disease progression. *J Immunol* 2008; **180**:7907–18.

16 Simonetta F, Hua S, Lécuroux C, Gérard S, Boufassa F, Sáez-Cirión A, *et al.* High eomesodermin expression among CD57+ CD8+ T cells identifies a CD8+ T cell subset associated with viral control during chronic human immunodeficiency virus infection. *J Virol* 2014; **88**:11861–71.

17 Palmer CD, Romero-Tejeda M, Scully EP, Lockhart A, Seaman MS, Goldenthal A, *et al.* Increased frequencies of $CD8^+$ $CD57^+$ T cells are associated with antibody neutralization breadth against HIV in viraemic controllers. *J Int AIDS Soc* 2016; **19**:21136.

18 Lee SA, Sinclair E, Jain V, Huang Y, Epling L, Van Natta M, *et al.* Low Proportions of CD28- CD8+ T cells Expressing CD57 Can Be Reversed by Early ART Initiation and Predict Mortality in Treated HIV Infection. *J Infect Dis* 2014; **210**. doi:10.1093/infdis/jiu109

19 Lee SA, Sinclair E, Hatano H, Hsue PY, Epling L, Hecht FM, *et al.* Impact of HIV on CD8+ T Cell CD57 Expression Is Distinct from That of CMV and Aging. *PLoS One* 2014; **9**:e89444.

20 Cobos Jiménez V, Wit FWNM, Joerink M, Maurer I, Harskamp AM, Schouten J, *et al.* T-Cell Activation Independently Associates With Immune Senescence in HIV-Infected

Recipients of Long-term Antiretroviral Treatment. *J Infect Dis* 2016; **214**:216–25.

21 Ongrádi J, Kövesdi V, Aspinall R, Giudice G Del, Effros R, Grubeck-Loebenstein B, *et al.* Factors that may impact on immunosenescence: an appraisal. *Immun Ageing* 2010; **7**:7.

22 Bansal A, Sterrett S, Erdmann N, Westfall AO, Dionne-Odom J, Overton ET, *et al.* Normal T-cell activation in elite controllers with preserved CD4+ T-cell counts. *AIDS* 2015; **29**:2245–54.

23 Ruiz-Mateos E, Ferrando-Martinez S, Machmach K, Viciana P, Pacheco YM, Nogales N, *et al.* High levels of CD57+CD28- T-cells, low T-cell proliferation and preferential expansion of terminally differentiated CD4+ T-cells in HIV-elite controllers. *Curr HIV Res* 2010; **8**:471–81.

24 Ferrando-Martinez S, Casazza JP, Leal M, Machmach K, Munoz-Fernandez MA, Viciana P, *et al.* Differential Gag-Specific Polyfunctional T Cell Maturation Patterns in HIV-1 Elite Controllers. *J Virol* 2012; **86**:3667–3674.

25 Ouyang Q, Wagner WM, Voehringer D, Wikby A, Klatt T, Walter S, *et al.* Age-associated accumulation of CMV-specific CD8+ T cells expressing the inhibitory killer cell lectin-like receptor G1 (KLRG1). *Exp Gerontol* 2003; **38**:911–20.

26 Henson SM, Franzese O, Macaulay R, Libri V, Azevedo RI, Kiani-Alikhan S, *et al.* KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells. *Blood* 2009; **113**:6619–28.

27 Henson SM, Akbar AN. KLRG1--more than a marker for T cell senescence. *Age (Dordr)* 2009; **31**:285–91.

28 Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, *et al.* Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 2007; **27**:281–295.

29 Bengsch B, Spangenberg HC, Kersting N, Neumann-Haefelin C, Panther E, von Weizsacker F, *et al.* Analysis of CD127 and KLRG1 Expression on Hepatitis C Virus-Specific CD8+ T Cells Reveals the Existence of Different Memory T-Cell Subsets in the Peripheral Blood and Liver. *J Virol* 2007; **81**:945–953.

30 Ibegbu CC, Xu Y-X, Harris W, Maggio D, Miller JD, Kourtis AP. Expression of killer cell

lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. *J Immunol* 2005; **174**:6088–94.

31 Chéret A, Nembot G, Mélard A, Lascoux C, Slama L, Miailhes P, *et al.* Intensive five-drug antiretroviral therapy regimen versus standard triple-drug therapy during primary HIV-1 infection (OPTIPRIM-ANRS 147): a randomised, open-label, phase 3 trial. *Lancet Infect Dis* 2015; **15**:387–396.

32 Tomiyama H, Matsuda T, Takiguchi M. Differentiation of human CD8(+) T cells from a memory to memory/effector phenotype. *J Immunol* 2002; **168**:5538–50.

33 Ghiglione Y, Falivene J, Ruiz MJ, Laufer N, Socías ME, Cahn P, *et al.* Early skewed distribution of total and HIV-specific CD8+ T-cell memory phenotypes during primary HIV infection is related to reduced antiviral activity and faster disease progression. 2014; **9**:e104235.

Table 1. Characteristics of the study population

	HDs	HICs	ART-exposed	p
n	22	44	38	
Median [interquartile range] age (years)	43 [35-55]	52 [44-56]	51 [45-58]	0.042 #
CMV$^+$ serostatus (%)	50	89	92	<0.0001 §
Males (%)	68	48	58	ns
Time since infection (years)		14 [12-24]*	16 [10-25]	ns
Treatment duration (years)		*not applicable*	12 [7-18]	
HIV RNA (copies/mL)		<400	<40	
CD4 T cell count (/µL)		719 [534-907]	726 [485-926]	ns
CD4 T cells (%)		40 [32-46]	32 [23-43]	0.006
CD8 T cell count (/µL)		673 [526-936]	846 [506-1234]	ns
CD8 T cells (%)		35 [30-43]	42 [30-50]	ns
CD4/CD8 ratio		1.2 [0.8-1.5]	0.8 [0.4-1.4]	0.019

analysis of variance for the three groups: p=0.046 for HDs vs. ART-exposed patients

§ Chi-squared test for the three groups. Fisher's exact test : p=0.0015 for HDs vs. HICs, and p=0.0004 for HDs vs. ART-exposed patients

* Defined as the estimated time since diagnosis

Legends

Figure 1. Lower CD57 expression on effector and terminal effector $CD8^+$ T cells in long-term HICs than in ART-exposed patients

(A) Distributions of naive (N), central memory (CM), effector memory (EM), effector (E), and terminal effector (TE) $CD8^+$ T cell subsets in HIV- HDs (stars), HICs (open circles) and ART-exposed patients (grey circles). The subsets were defined as follows: naive (N) $CD45RO^-CCR7^+CD28+CD27^+$, central memory (CM) $CD45RO^+CCR7^+CD28^+CD27^+$, effector memory (EM) $CD45RO^{+/-}CCR7^-CD28^{+/-}CD27^+$, E $CD45RO^+CCR7^-CD28^-CD27^-$ and TE $CD45RO^-CCR7^-CD28^-CD27^-$. (B), (C) Percentages of KLRG-1- and CD57-expressing cells among total $CD8^+$ T cells and $CD8^+$T cell subsets. Staining was performed on fresh, whole blood samples collected from 22 HDs, 43 HICs and 38 (for CD57) or 33 (for KLRG-1) ART-exposed patients. Intergroup comparisons between groups were performed using non-parametric, unpaired Kruskal-Wallis tests. Graphs show the median values and p values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

Figure 2. CD57 expression as a function of the blood sample processing

Comparison of the percentages CD57-expressing $CD8^+$ T cells measured in fresh whole blood or with cryopreserved PBMCs. The two methods were used on 92 samples collected from HIV-infected populations (HICs and ART-exposed patients). Left graph: CD57 expression measured on fresh blood samples or on freeze-thawed PBMCs. Right graph: impact of Ficoll separation and cryopreservation on CD57 expression as a function of the level of CD57 expression in fresh whole blood. Samples were separated on the basis of below-median or above-median CD57 expression measured in fresh, whole blood. Differences were assessed in a Wilcoxon test for paired samples.

Figure 3. Association of CD57 expression with immunosenescence markers and cytotoxic granule contents

(A) Graphs showing the percentage of CD57-expressing total $CD8^+$ T cells as a function of the CD4/CD8 ratio (left), and the blood $CD8^+$ T cell count (right) among HICs (open circles) and ART-exposed patients (grey circles). (B) Analyses of cytolytic granule contents in EM (left), E

(middle) and ET (right) $CD8^+$ T cell fractions from HICs and ART-exposed patients. The graphs show the percentage of $perforin^+$ $granzyme\ B^+$ cells among the $CD57^+$ (black circles) and $CD57^-$ (open circles) $CD8^+$ T cell fractions in 11 HICs and 10 ART-exposed patients.

Figure 1

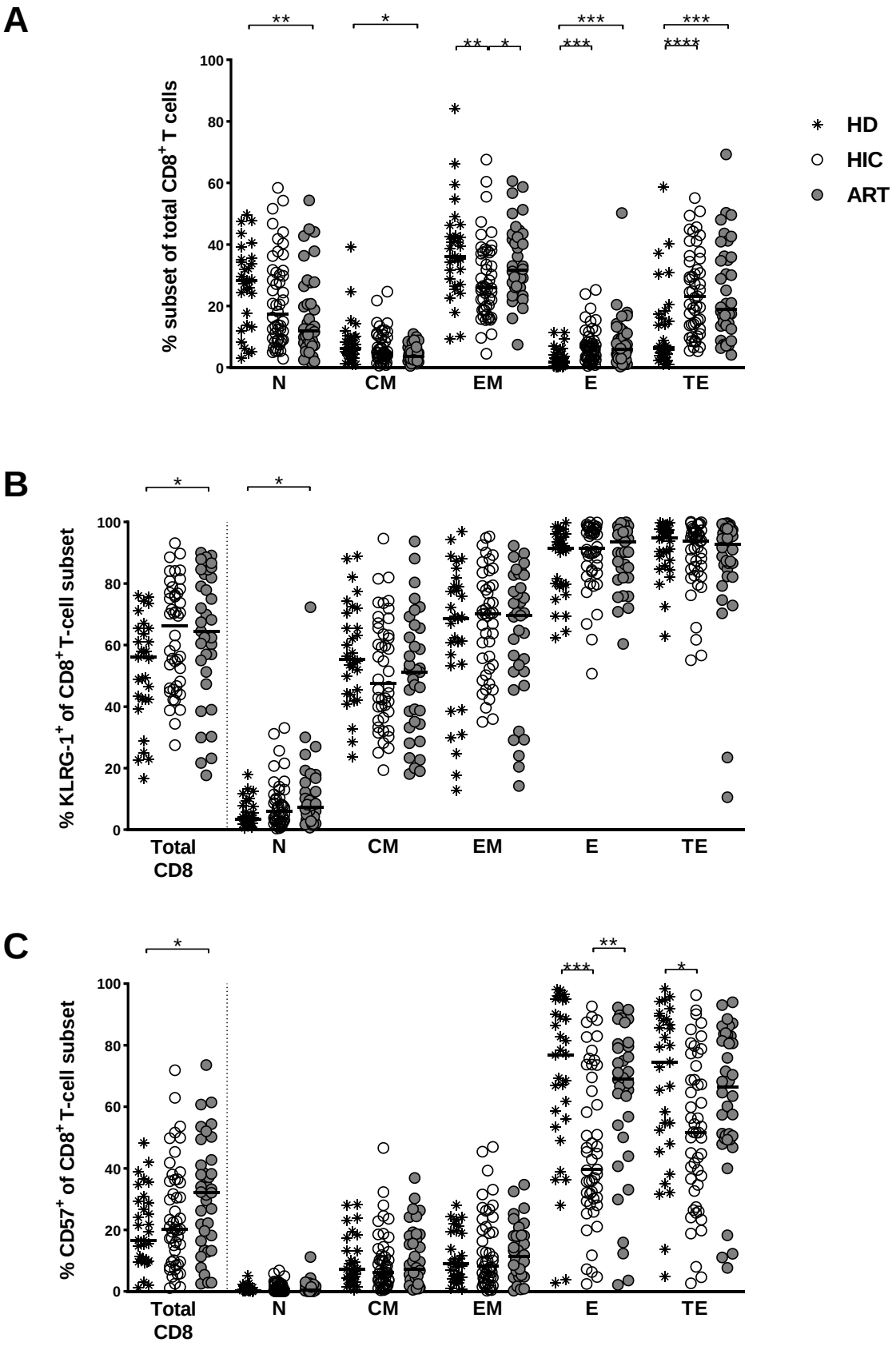

A
B
C
HD
HIC
ART
% subset of total CD8+ T cells
% KLRG-1+ of CD8+T-cell subset
% CD57+ of CD8+T-cell subset
Total CD8
N
CM
EM
E
TE

Figure 2

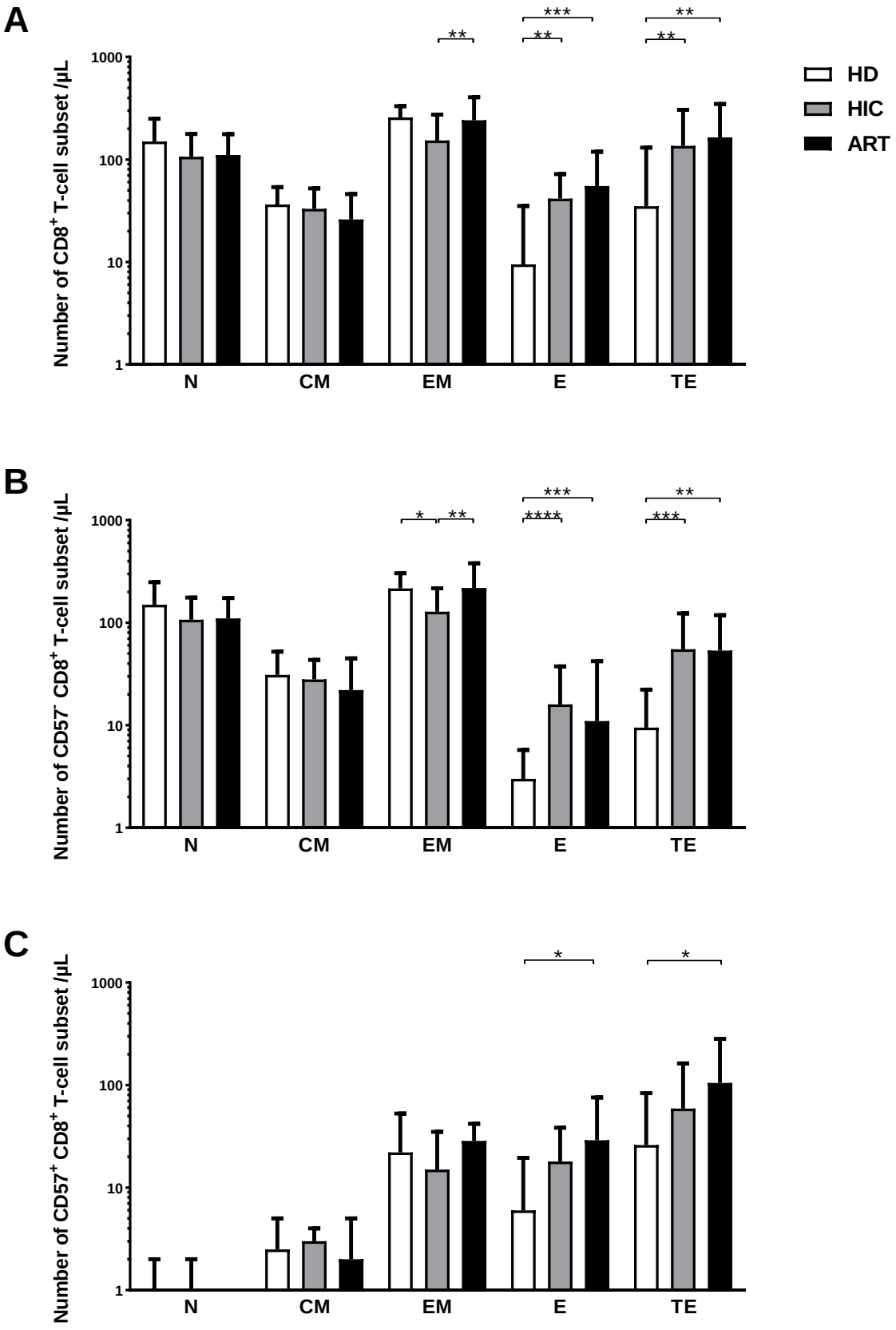
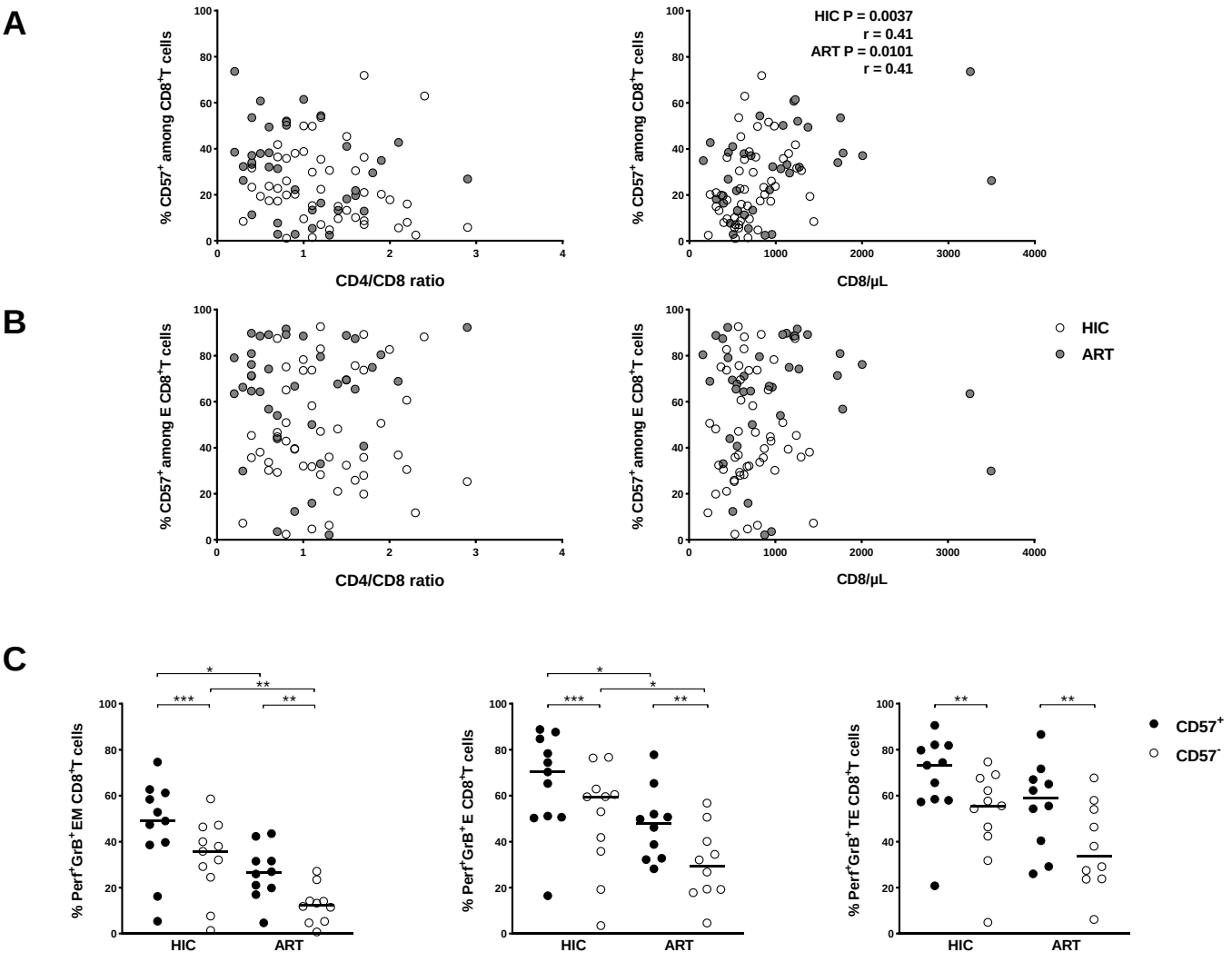

A
B
C
HD
HIC
ART
Number of CD8+ T-cell subset /µL
Number of CD57- CD8+ T-cell subset /µL
Number of CD57+ CD8+ T-cell subset /µL
1000
100
10
1
N
CM
EM
E
TE
*
**
***
****

Figure 3


A
% CD57+ among CD8+T cells
CD4/CD8 ratio
CD8/µL
HIC P = 0.0037
r = 0.41
ART P = 0.0101
r = 0.41
B
% CD57+ among E CD8+T cells
CD4/CD8 ratio
CD8/µL
HIC
ART
C
% Perf+GrB+EM CD8+T cells
% Perf+GrB+E CD8+T cells
% Perf+GrB+TE CD8+T cells
HIC
ART
CD57+
CD57-

Supplemental Table 1

	HIC	
n	50	
Follow-up duration (years)	14 [12-22]	
Subjects with VL<50 copies/mL at the time of analysis	34 (68%)	
VL in subjects with VL>50 copies/mL at the time of analysis (copies/mL)	107 [73-203]	
Elite Controllers (subjects who never experienced VL>50 copies/mL during the follow-up)	13 (26%)	
Blippers (subjects who experienced VL≥50 copies/mL during the follow-up)	37 (74%)	
Subjects with protective HLA-B alleles (HLA-B*27, HLA-B*57 and/or HLA-B*58)	24 (48%)	
CD8 T cell response (SFC/10^6 PBMC)	796 [130-4155]	n=47
Capacity of viral replication inhibibition (Log P24 decrease)	0.46 [0.07-2.45]	n=49
Strong Responders (subjects with Log P24 decrease>2)	14 (29%)	n=49
Weak Responders (subjects with Log P24 decrease<2)	35 (71%)	n=49

Supplemental Figure 1

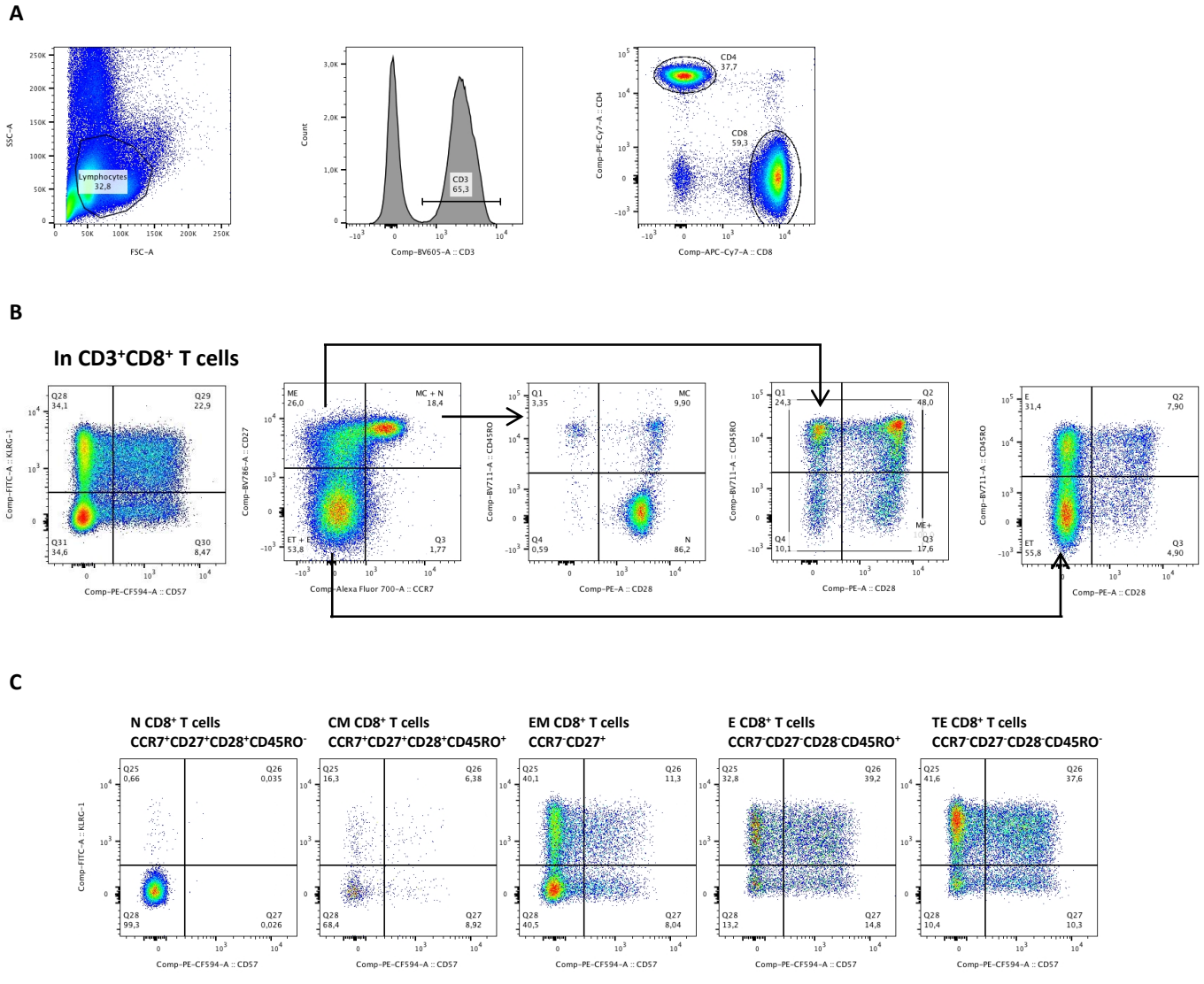


Figure S1. Gating strategy and antibodies references

Fresh blood samples were analyzed for the expression of 11 markers allowing characterization of T cell differentiation, activation and senescence profile. The gating strategy is shown for (A) CD4+ and CD8+ selection among CD3+ cells; (B) naive (N), central memory (CM), effector memory (EM), effector (E) and terminal effector (TE) gating among CD8+ T cells and (C) for the identification of CD57+ and KLRG1+ cells in total CD8+ T cell or each specific subset.

The characteristics of the antibody used are as follows: CD38-V500 (HIT2, BD); CD3-BV605 (SK7, BD); HLA-DR-BV650 (G46-6, BD); CD45RO-BV711 (UCHL1, BD); CD27-BV786 (L128, BD); KLRG-1-FITC (REA261, Miltenyi); CD28-PE (CD28.2, BD); CD57-PECF594 (NK-1, BD); CD4-PE-Cy7 (OKT4, BD); CCR7-AF700 (GO25H7, BD); CD8-APCH7 (SK1,BD)). The gating strategy for the expression of 11 markers

Supplemental Figure 2

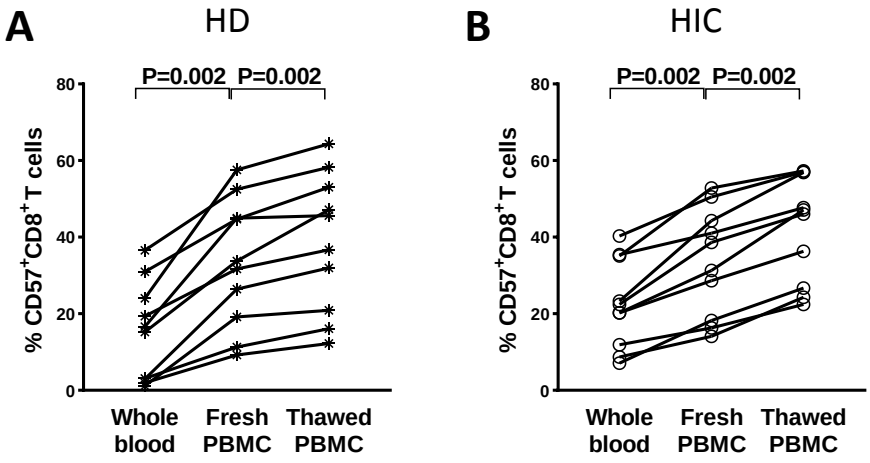


Figure S2. Impact of blood sample treatment on CD57 expression
Comparison of the percentage of CD57+ among CD8 T cells analyzed from fresh whole blood, fresh PBMC or cryopreserved PBMCs from 10 healthy donors (HD) and 10 HIC.
Statistical differences evaluated by Wilcoxon paired t- test.

Supplemental Figure 3

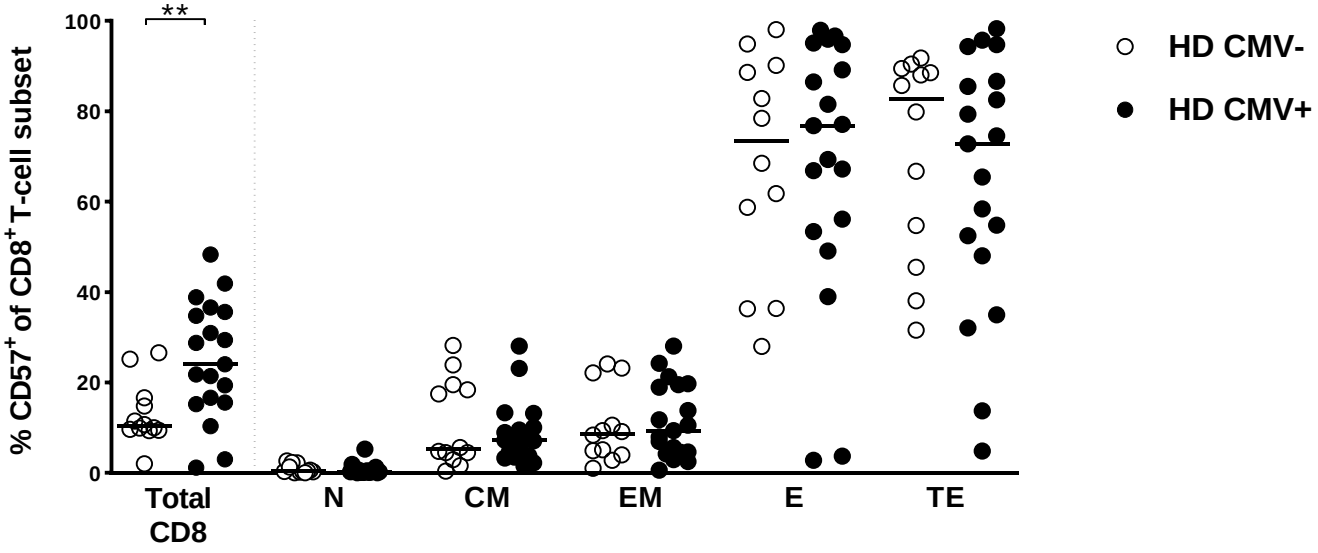


Figure S3. Impact of CMV status on CD57 expression

Subgrouping of HD based on CMV+ or CMV- seropositivity. Comparison of the percentage of CD57+ cells either in total CD8 T cells or in the various T cell subsets (N, CM, EM, E, TE). Statistical differences evaluated by Mann-Whitney test.

Supplemental Figure 4

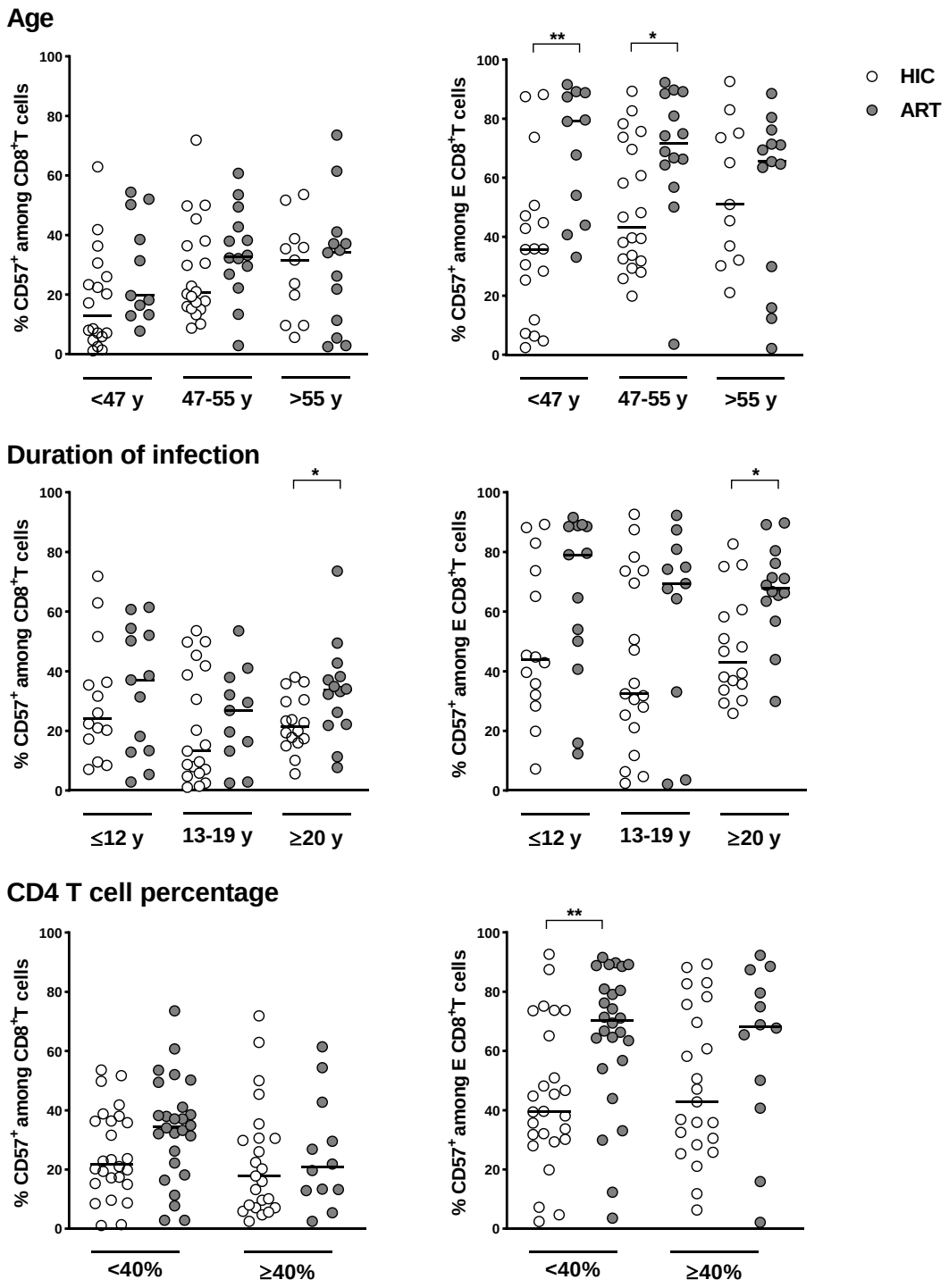


Figure S4. Impact of age, the duration of infection and CD4 T cell percentage on the percentage of CD57+ among total and effector CD8 T cells

Subgrouping of the HIV-infected patients presented in Fig 1. Subgrouping for age and the duration of infection was performed to obtain three groups of equal size. Subgrouping for CD4 T cell percentage was based on strategy already described in the literature. Statistical differences evaluated by Mann-Whitney test (for CD4 T cell percentage) or Kruskal-Wallis test (for age and duration of infection).

Supplemental Figure 5

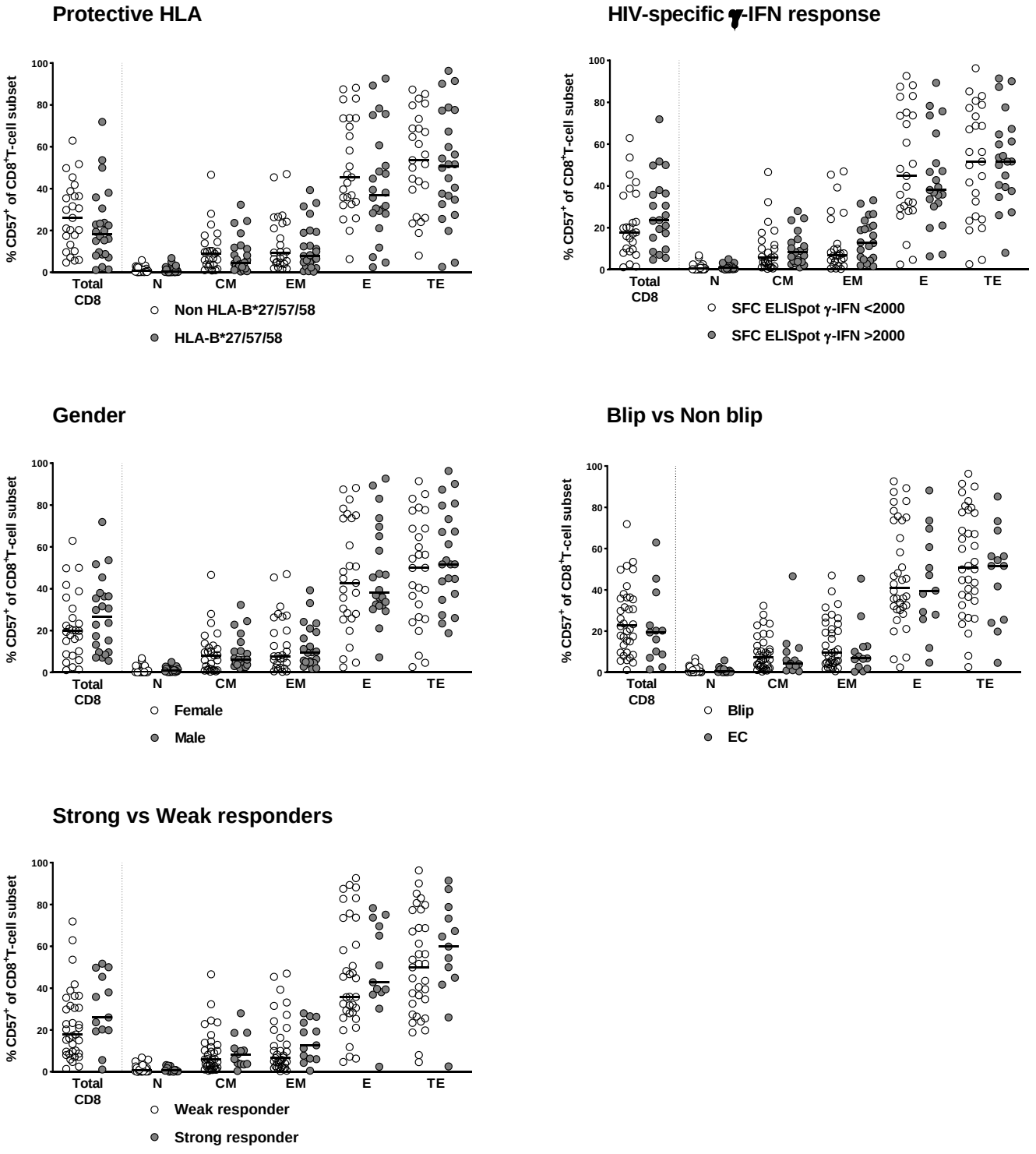


Figure S5. Impact of various parameters involved in HIV control on the percentage of CD57+ among total and effector CD8 T cells

Subgrouping of the HIC patients presented in Fig 1. Subgrouping for protective HLA, gender, HIV specific γ-IFN responses, blip vs non blip, strong vs weak responders. Statistical differences evaluated by Mann-Whitney test.