Distinct differentiation profiles of HIV-Gag and Nef-specific central memory CD8$^+$ T cells associated with HLA-B57/5801 and virus control

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Objectives: A superior capacity of controlling HIV has been attributed to CD8$^+$ T cells directed against HIV-Gag compared to Nef, particularly in the context of some protective human leukocyte antigen (HLA) alleles. To further elucidate this protective effect, we compared the multifunctional and differentiation characteristics of CD8$^+$ T cells specific for HIV-Gag and Nef in HLA-B57/5801-positive and negative nonprogressors.

Methods: A head-to-head comparison of CD8$^+$ T cells specific for HIV-Gag and Nef frequencies, cytokine production and differentiation was conducted, in 11 HLA-B57/5801$^+$ and 11 HLA-B57/5801$^-$ HIV-infected individuals selected from a cohort of 53 nonprogressors by using IFN-γ-ELISpot assay and flow cytometry analysis of intracellular cytokine production and differentiation profile. Correlations with HIV parameters were studied.

Results: Frequencies of Gag-specific but not of Nef-specific CD8$^+$ T cells correlated with peripheral blood mononuclear cell (PBMC)-associated HIV-DNA. The HIV-Gag and Nef-specific CD8$^+$ T cells did not differ for IL-2 production in either HLA-B57/5801$^+$ or HLA-B57/5801$^-$ individuals. The IFN-γ-producing Gag-specific CD8$^+$ T cells in HLA-B57/5801$^+$ individuals significantly differed from their Nef-specific counterparts by displaying higher proportions of central memory CD45RA$^-$CCR7$^+$ cells positive for CD27. This differentiation pattern was not observed in HLA-B57/5801$^-$ individuals. Only these HLA-B57/5801-positive Gag-specific CD27$^+$ central memory CD8$^+$ T cells, but not their Nef-specific counterparts, negatively correlated with cell-associated HIV-DNA.

Conclusion: HLA-B57/5801 drives a preferential CD27$^+$ differentiation of central memory CD8$^+$ T cells directed against HIV-Gag but not Nef that may contribute to the ability of Gag-specific CD8$^+$ T cells to better control HIV in HLA-B57/5801$^+$ nonprogressors.

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Introduction

Efforts to define immune correlates of protection against HIV propose a key role to some major histocompatibility complex (MHC) class I alleles and to qualitative parameters of HIV-specific $CD8^+$ T cells [1–6]. The human leukocyte antigen (HLA)-B27, B57, or the closely related B5801 (HLA-B57/5801), alleles dictate robust HIV-specific $CD8^+$ T cells that are associated with HIV control [7–12]. Antigen specificity of $CD8^+$ T cells also appears to be critical with a stronger protection conferred by Gag-specific T cells [13–18], but mechanisms involved appear to differ and are not yet fully elucidated. HLA-B27 drives the superior antiviral efficacy of Gag-specific $CD8^+$ T cells in long-term nonprogressors by allowing higher cell avidity, polyfunctionality and clonal turnover [19]. In contrast to HLA-B27, which presents to $CD8^+$ T cells an immunodominant epitope in Gag but not in Nef, HLA-B57/5801 present several Gag and Nef epitopes [20,21]. In HLA-B57/5801$^+$ individuals Gag-specific T cells appear to mediate a superior antiviral control than Nef ones [13], although the latter was shown to be preserved in HLA-B57/5801$^+$ nonprogressors [22]. However, little attention has been paid thus far to the differentiation triggered by specific HIV antigens in HLA-B57/5801$^+$ nonprogressors and their association with disease protection. Therefore deciphering the relative properties of HIV-specific $CD8^+$ T cells against the Gag and Nef epitopes in HLA-B57/5801$^+$ nonprogressors should help further understand the protective effect of HIV-specific $CD8^+$ T cells.

In order to identify attributes of the $CD8^+$ T-cell-mediated protection against HIV in HLA-B57/5801$^+$ nonprogressors, we investigated in HLA-B57/5801-positive and negative individuals, whether antigen specificity and HLA restriction trigger distinct patterns of cell differentiation that could explain the association between HLA-B57/5801 and virus control.

Materials and methods

Patients

Samples were obtained from 53 French ALT-ANRS-CO15 cohort patients (Supplemental Table 1, http://links.lww.com/QAD/A70). As described [23,24], inclusion criteria in the cohort were: HIV seropositivity for at least 8 years, CD4 cell counts above 600/μl for the last 5 years without symptoms or antiretroviral therapy. The median length of HIV seropositivity was 9 years at entry into the cohort [23]. The study was approved by the institutional review board at the Pitié-Salpêtrière Hospital, and all patients provided written informed consent.

HLA genotyping

HLA genotyping was performed by amplification refractory mutation system PCR, with sequence-specific primers [25].

Parameters of HIV production

Plasma HIV-RNA were quantified as described [23,24]. The HIV-DNA in PBMCs was quantified with a modified Amplicor Monitor assay (Roche Laboratories) with a cut-off value of 5 copies of HIV-DNA/10^6 PBMC [24].

Synthetic peptides

Synthetic 15-mer peptides overlapping by 11 amino acids (Neosystem, France) and spanning the HIV-HxB2 Gag and Nef sequences were combined into 11 and 3 pools, respectively. Eight $CD8^+$ T-cell epitopes derived from HIV-HxB2 Gag or Nef were synthesized according to the Los Alamos HIV database. The B57-restricted peptides were: ISPRTLNAW (Gag147-155, IW9), KAFSPEVIPMF (Gag162-172, KF11), TSTLQEQIGW (Gag240-249, TW10), and HTQGYFPDW (Nef116-124, HW9). The A3-restricted peptides were: KIRLRPGGK (Gag18-26, KK9), and QVPLRPMTYK (Nef73-82, QK10). The A26-restricted peptide was EVIPMFSAL (Gag167-175, EL9). Lastly, the B35-restricted peptide was TPGPGVRYPL (Nef128-137, TL10).

IFN-γ enzyme-linked immunospot assay

PBMCs were incubated with phytohaemagglutinin (positive control), medium alone (negative control), Gag and Nef peptide pools as described [24]. Spots were considered positive if above 50 SFC/10^6 PBMCs after subtracting background. Functional avidity was assessed by testing serial 10-fold dilutions ranging from 100 to 0.0001 μg/ml of optimal peptides and determining the peptide concentration inducing half-maximal responses in enzyme-linked immunospot (ELISpot) assays [19].

Intracellular cytokine staining, phenotyping and tetramer staining

PBMCs were incubated overnight with staphylococcal enterotoxin β (positive control), medium (negative control), Gag and Nef pools found to be positive in the ELISpot assay. Brefeldin-A was added as described [26]. Cells were stained with anti-CD8-PE-Cy7, anti-CD45RA-ECD (Beckman-Coulter), anti-CCR7-PE (R&D System), anti-CD27-FITC and anti-IFN-γ-APC and/or anti-IL-2-PE (Becton Dickinson). Cells were analyzed on a Beckman Coulter FC500 flow cytometer using the CXP Analysis (Beckman Coulter) software. The $CD3^+CD8^+CD45RA^-CD27^{+/-}CCR7^{+/-}$ IFN-γ^+ cells accounted for up to 99% of the $CD8^+$ $CD45RA^-CD27^{+/-}CCR7^{+/-}$IFN-$\gamma^+$ cells as analyzed in parallel on a FACSCanto-I flow cytometer (Becton Dickinson), thus assessing the T-cell nature of the IFN-γ-producing $CD8^+$ cells. CD27 expression on $CD8^+$ T cells binding the B57/KF11 tetramer (Beckman-Coulter)

was also analyzed in combination with anti-CCR7-PE-Cy7, anti-CD27-APC, anti-CD8-APC-Cy7 (Becton Dickinson), and anti-CD45RA-FITC (Beckman-Coulter) by a FACSCanto-I flow cytometer and the FlowJo (version 8.0, TreeStar) software on three HLA-B57/5801$^+$ individuals.

Statistical analysis

Statistics were conducted with SPSS 13.0 software (SSPS Inc., Chicago, Illinois, USA). The Wilcoxon rank sum test, Mann–Whitney *U* test and Spearman's rank correlation were used. The Benjamini–Holberg procedure was used to correct for the multiplicity of tests [27] and mentioned as 'corrected *P*' value.

Results

Frequencies of Gag-specific CD8$^+$ T cells but not Nef-specific ones correlates negatively with cell-associated HIV-DNA

In this group of 53 nonprogressors, the five-fold higher numbers of IFN-γ-producing Gag-specific T cells than their Nef-specific counterparts (median: 2527 vs. 480 SFC/10^6 PBMC, corrected $P < 0.0001$; Supplemental Table 1, http://links.lww.com/QAD/A70 and Fig. 1a), negatively correlated with cell-associated HIV-DNA loads ($r = -0.395$, corrected $P = 0.004$; Fig. 1b), whereas the Nef-specific ones did not ($r = -0.151$, $P = 0.294$; Fig. 1c). Despite HIV-DNA loads highly correlated with plasma HIV-RNA loads ($r = 0.801$, corrected $P < 0.0001$; Fig. 1f), HIV-specific cell frequencies did not correlate with plasma HIV-RNA levels (Fig. 1d and e).

HLA-B57/5801 molecules are frequent in nonprogressors and associated with virus control. They restrict CD8$^+$ T-cell recognition of epitopes in both Gag and Nef. We further investigated whether such differences could be triggered by HLA-B57/5801. We therefore performed a head-to-head comparison of Gag and Nef-specific CD8$^+$ T cells in samples from 11 HLA-B57/5801$^+$ individuals and 11 HLA-B57/5801$^-$ individuals with simultaneous responses to Gag and Nef peptide pools containing the HLA-B57/5801-restricted epitopes (Supplemental Table 1, http://links.lww.com/QAD/A70 and Supplemental Fig. 1, http://links.lww.com/QAD/A70).

Recognition of the corresponding Gag and Nef epitopes restricted by HLA-B57/5801 and other HLA types (Supplemental Fig. 2, http://links.lww.com/QAD/A70) confirmed differences in magnitude between these sets of cells. A multifunctional flow cytometry analysis showed few of the Gag and Nef-specific CD8$^+$ T cells producing IL-2 (Supplemental Fig. 3, http://links.lww.com/QAD/A70), regardless of HLA.

HLA-B57/5801 dictates a preferential CD27 expression of Gag-specific central memory CD8$^+$ T cells compared to Nef ones

The superior magnitude of Gag-specific IFN-γ$^+$CD8$^+$ T cells compared to Nef-specific ones did not reflect a distinct repartition of the classical central and effector memory cells [central memory T cells (TCM) and effector memory T cells (TEM), respectively] in either HLA group (Supplemental Fig. 4a, http://links.lww.com/QAD/A70). However, the HLA-B57/5801$^+$ Gag-specific IFN-γ$^+$IL-2$^-$CD8$^+$ T cells differed from their Nef-specific counterparts by a higher proportion of IFN-γ$^+$CD45RA$^-$CD8$^+$ cells displaying CD27 (38 vs. 26%, corrected $P = 0.007$; Fig. 2a and Supplemental Fig. 4b, http://links.lww.com/QAD/A70). This difference was maintained in HLA-B57/5801$^+$ individuals between CD27 expression on 39% [interquartile range (IQR) 31–56%] of Gag-specific CD8$^+$ TCM cells and 33% (IQR 16–42%) of Nef-specific ones (corrected $P = 0.007$; Fig. 2c), but not in the HLA-B57/5801$^-$ population (Fig. 2b and d). The preferential CD27 expression of HLA-B57/5801$^+$ TCM was observed as well on HLA-B57-tetramer binding Gag-specific CD8$^+$ TCM (Supplemental Fig. 5, http://links.lww.com/QAD/A70). A similar but not significant trend was observed on the Gag-specific TEM whatever the HLA (Fig. 2c and d). In contrast effector Gag and Nef-specific cells displayed similar CD27 expression (data not shown).

The preferential CD27 expression on HLA-B57/5801$^+$ Gag-specific CD8$^+$ TCM correlates with virus control

The CD27$^+$ Gag-specific IFN-γ$^+$CD8$^+$ TCM negatively correlated with both plasma HIV-RNA ($r = -0.502$, corrected $P = 0.024$) and HIV-DNA ($r = -0.567$, corrected $P = 0.018$; Fig. 2e) in the total group. This negative correlation was maintained in the HLA-B57/5801$^+$ ($r = -0.683$, corrected $P = 0.042$; Fig. 2e) but not in the HLA-B57/5801$^-$ group. The Nef-specific counterpart was not correlated with either the HIV-DNA (Fig. 2f) or the plasma HIV-RNA loads, regardless of HLA. In addition, no significant correlation was found between CD27$^+$ Gag or Nef-specific IFN-γ$^+$ TEM and HIV burden in either HLA group (Supplemental Table 2, http://links.lww.com/QAD/A70).

These results show that a 37.5% Gag-specific CD27$^+$ TCM are required to be associated with a 1 log reduction in HIV-DNA load in the whole group. In the HLA-B57/5801$^+$ group, however, a 29% Gag-specific CD27$^+$ TCM is sufficient.

Discussion

Our findings demonstrate HLA-B57/5801 confer HIV-Gag-specific T cells an advantage to control HIV by

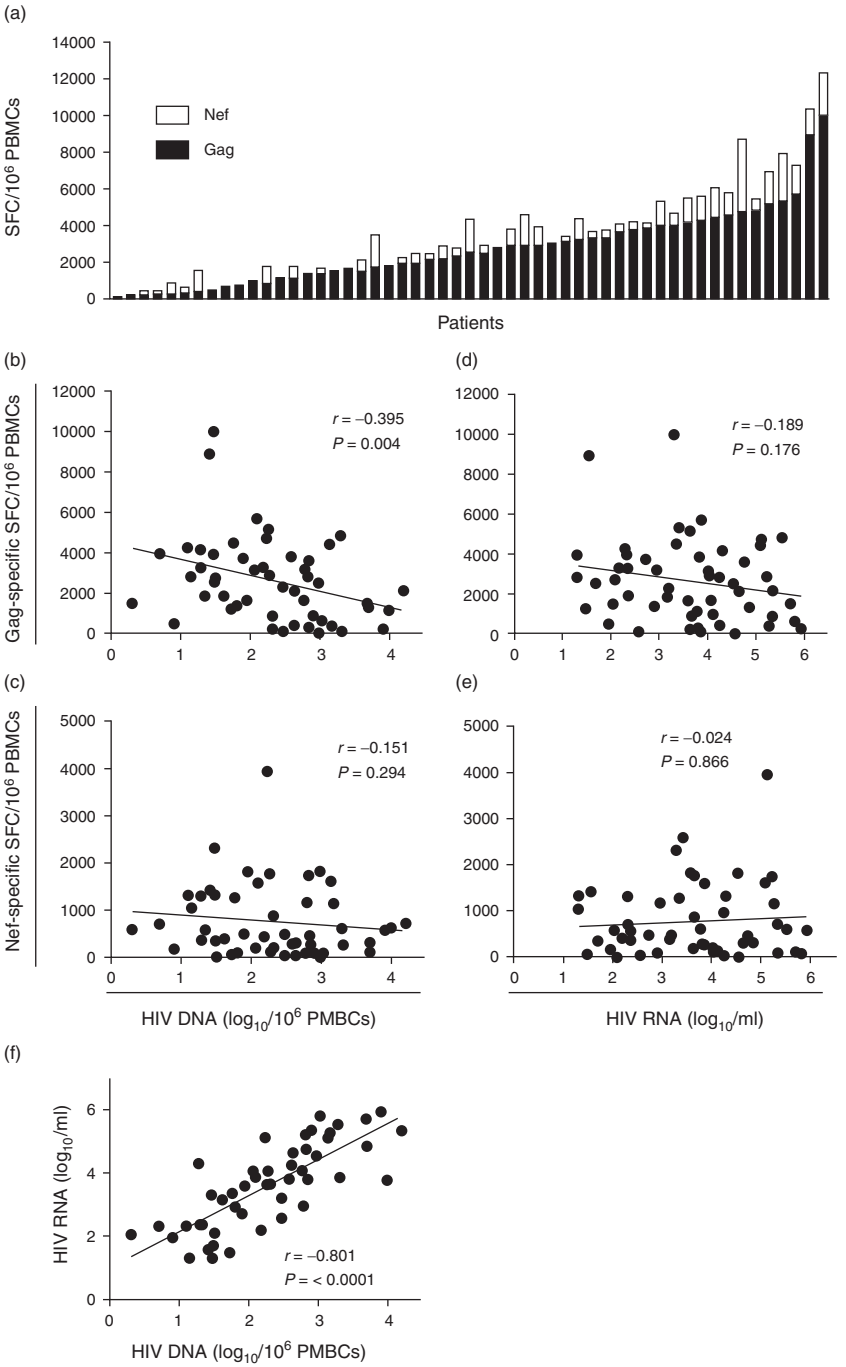

Fig. 1. Frequencies of IFN-γ-producing CD8$^+$ T cells specific for HIV-Gag but not Nef negatively correlate with cell-associated HIV-DNA loads. (a) Frequencies of CD8$^+$ T cells specific for Gag or Nef from 53 HIV-infected nonprogressors determined by IFN-γ ELISpot assays. (b, d) Correlation between frequencies of Gag-specific CD8$^+$ T cells and cell-associated HIV-DNA load (b), and plasma HIV-RNA (d), respectively. (c, e) Lack of correlation between frequencies of CD8$^+$ T cells specific for Nef and HIV-DNA load (c) or plasma HIV-RNA (e), respectively. (f) Correlation between cell-associated HIV-DNA and plasma HIV-RNA.

allowing higher numbers of Gag-specific central memory CD8$^+$ T cells to display CD27 compared to Nef-specific ones. These results provide new insights into the mechanisms by which protective HLA-B57/5801 alleles support superior control exerted by Gag-specific compared to Nef-specific T cells.

In addition, we provide novel evidence that the cell-associated HIV-DNA load is a better surrogate marker for cell-mediated virus control than the plasma HIV-RNA load [28]. Indeed the negative correlation observed in the whole group of nonprogressors between total Gag-specific CD8$^+$ T cells and cell-associated

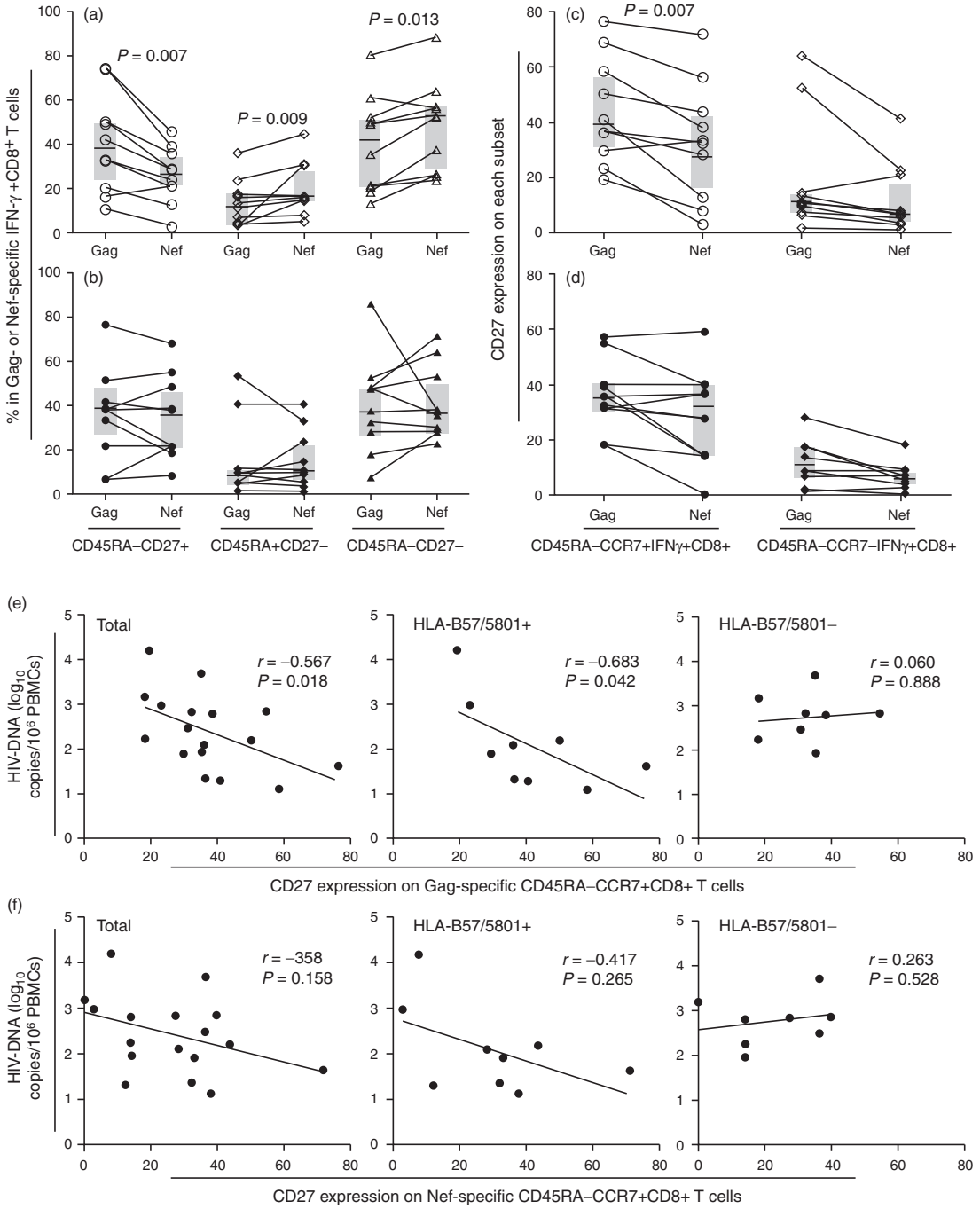

Fig. 2. HLA-B57/5801 dictates preferential CD27 expression on Gag-specific IFN-γ$^+$CD8$^+$ T cells. (a, b) Differentiation of IFN-γ-producing CD8$^+$ T cells against HIV-Gag and Nef in 20 nonprogressors including 10 HLA-B57/5801$^+$ (a) and 10 HLA-B57/5801$^-$ (b) individuals. (c, d) Patterns of CD27 expression on central memory and effector memory CD8$^+$ T cells producing IFN-γ against HIV-Gag and Nef in 20 nonprogressors including 10 HLA-B57/5801$^+$ (c) and 10 HLA-B57/5801$^-$ (d) individuals. (e, f) Correlation between CD27 expression on Gag-specific (e) or Nef-specific (f) IFN-γ$^+$ central memory CD8$^+$ T cells and cell-associated HIV-DNA load.

HIV-DNA is re-inforced in HLA-B57/5801$^+$ individuals in whom Gag-specific CD27$^+$ TCM correlated with cell-associated HIV-DNA load. As HIV-specific CD8$^+$ T cells detect infected cells producing HIV antigens but not free virus particles, and assuming the number of HIV-DNA copies per million cells roughly represents the numbers of infected cells, the cell-associated HIV-DNA more accurately reflects the infected cell targets of CD8$^+$ T cells than the plasma HIV load. It remains unclear yet whether the HIV-DNA load is determined by the CD8$^+$ T-cell phenotype or *vice versa*.

The new characteristics we demonstrate here for HLA-B57/5801$^+$ Gag-specific CD8$^+$ T cells contrasts with previous study suggesting persistent CD27 expression explain the failure of HIV-specific CD8$^+$ T cells to control the virus [29]. The known property of CD27 to promote cell survival and proliferation required for the generation and long-term maintenance of antigen-specific T-cell immunity [30] fits, however, with a better control of HIV mediated by these Gag-specific CD8$^+$ TCM. Alternatively, stronger CD27 expression on HLA-B57/5801$^+$ Gag-specific CD8$^+$ TCM might simply reflect preferential survival in the context of a low antigen burden, although both HLA groups were comparable for CD4 cell counts and plasma viral loads.

Those distinct stages of differentiation reached by HLA-B57/5801$^+$ Gag and Nef-specific cells are reminiscent of a distinct differentiation pattern reported for CD8$^+$ T cells specific for lytic and latent Epstein-Barr virus (EBV) proteins[31], suggesting modalities of antigen stimulation and kinetics of antigen expression may account for differences in the differentiation profiles. HIV-Nef indeed differs from Gag since expressed only in virus-producing cells, whereas the abundantly released capsid antigens can be cross-presented by professional antigen-presenting cells. Alternatively, more frequent viral escape from the Nef-specific immune response than against the Gag-specific one might account for such differences [32–34]. More differentiated T cells, as observed for Nef-specific cells, might exert a stronger selective pressure and facilitate emergence of variants. On the contrary, structural constraints on Gag epitopes might allow specific T cells to persist in a more quiescent central-memory status.

Altogether our findings provide new insight into the immune correlates of HIV control and the mechanisms of protection conferred by HLA-B57/5801 and confirm the need for closer attention to the nature of the HIV antigens included in vaccines against HIV.

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J.X. performed the research, analyzed data, and wrote the manuscript. W.L. performed the research and participated in writing the manuscript. A.S. participated in data management and coordination of the French ALT cohort. D.C. provided supervision for statistical analysis. A.S., B.S., C.B. and M.A. participated in performing the research. I.T. oversaw the HLA genotyping. C.R. supervised the viral load quantification. B.A. managed and designed the research, coordinated the French ALT cohort, and provided critical review of the manuscript.

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