blood

2011 118: 2138-2149
Prepublished online July 6, 2011;
doi:10.1182/blood-2011-01-328781

Escape from highly effective public CD8$^+$ T-cell clonotypes by HIV

Maria Candela Iglesias, Jorge R. Almeida, Solène Fastenackels, David J. van Bockel, Masao Hashimoto, Vanessa Venturi, Emma Gostick, Alejandra Urrutia, Linda Wooldridge, Mathew Clement, Stéphanie Gras, Pascal G. Wilmann, Brigitte Autran, Arnaud Moris, Jamie Rossjohn, Miles P. Davenport, Masafumi Takiguchi, Christian Brander, Daniel C. Douek, Anthony D. Kelleher, David A. Price and Victor Appay

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Escape from highly effective public CD8+ T-cell clonotypes by HIV

*Maria Candela Iglesias,[1] *Jorge R. Almeida,[1,2] Solène Fastenackels,[1] David J. van Bockel,[3] Masao Hashimoto,[4] Vanessa Venturi,[5] Emma Gostick,[6] Alejandra Urrutia,[1] Linda Wooldridge,[6] Mathew Clement,[6] Stéphanie Gras,[7] Pascal G. Wilmann,[7] Brigitte Autran,[1,8] Arnaud Moris,[1] Jamie Rossjohn,[7] Miles P. Davenport,[9] Masafumi Takiguchi,[4] Christian Brander,[10,11] Daniel C. Douek,[2] Anthony D. Kelleher,[3] David A. Price,[2,6] and Victor Appay[1,8]

[1]Inserm UMR S 945, Infections and Immunity, Avenir Group, Université Pierre et Marie Curie-Paris 6, Hôpital Pitié-Salpêtrière, Paris, France; [2]Human Immunology Section, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD; [3]St Vincent's Centre for Applied Medical Research and the Kirby Institute, University of New South Wales, Darlinghurst, Australia; [4]Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan; [5]Computational Biology Group, Centre for Vascular Research, University of New South Wales, Kensington, Australia; [6]Department of Infection, Immunity and Biochemistry, Cardiff University School of Medicine, Cardiff, United Kingdom; [7]Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Victoria, Australia; [8]AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Laboratoire d'Immunologie Cellulaire et Tissulaire, Paris, France; [9]Complex Systems in Biology Group, Centre for Vascular Research, University of New South Wales, Kensington, Australia; [10]AIDS Research Institute IrsiCaixa–HIVACAT, Hospital Universitari Germans Trias i Pujol Ctra del Canyet, Barcelona, Spain; and [11]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

Mapping the precise determinants of T-cell efficacy against viruses in humans is a public health priority with crucial implications for vaccine design. To inform this effort, we performed a comprehensive analysis of the effective CD8+ T-cell clonotypes that constitute responses specific for the HIV p24 Gag-derived KK10 epitope (KRWIILGLNK; residues 263-272) restricted by HLA-B*2705, which are known to confer superior control of viral replication in HIV-infected individuals. Particular KK10-specific CD8+ T-cell clonotypes, characterized by *TRBV4-3/TRBJ1-3* gene rearrangements, were found to be preferentially selected in vivo and shared between individuals. These "public" clonotypes exhibit high levels of TCR avidity and Ag sensitivity, which impart functional advantages and enable effective suppression of HIV replication. The early $L_{268}M$ mutation at position 6 of the KK10 epitope enables the virus to avoid recognition by these highly effective CD8+ T-cell clonotypes. However, alternative clonotypes with variant reactivity provide flexibility within the overall KK10-specific response. These findings provide refined mechanistic insights into the workings of an effective CD8+ T-cell response against HIV. (*Blood.* 2011;118(8):2138-2149)

Introduction

The importance of Ag-specific CD8+ T cells in the control of viral infections is well established. However, the parameters that allow for an effective CD8+ T-cell response have been difficult to elucidate in humans. The magnitude and targeting breadth of antiviral CD8+ T-cell responses in vivo correlate poorly, if at all, with the control of viral replication, thereby demonstrating that not all CD8+ T cells with specificity for a given virus are equally efficacious. Consequently, qualitative rather than quantitative attributes of antiviral CD8+ T cells have received greater attention in recent years, with several studies unveiling functional correlates of protection.[1] Technological advances have fostered ever more precise studies of Ag-specific CD8+ T-cell immunity and recent findings highlight the need to integrate fine analyses of individual clonotypes, defined on the basis of specific TCR expression, into our understanding of antiviral CD8+ T-cell efficacy.[2] Thus, each Ag-specific T-cell population is constituted from several different clonotypes, which can be considered as the fundamental units of T-cell reactivity. Collectively, the nature of these individual clonotypes determines the qualitative attributes of a given T-cell population. For example, the Ag sensitivity (AgS) of CD8+ T-cell populations, which may be important for antiviral efficacy,[3] is likely governed primarily by the structural and biophysical properties of individual TCR interactions with cognate peptide–MHC class I (pMHCI) molecules. Moreover, particular interest surrounds the nature and functional relevance of public clonotypes, which bear Ag-specific TCRs shared between individuals.[4,5] Despite the vanishingly small probability of TCR sharing between individuals given the vast potential for combinatorial diversity during the process of V(D)J gene rearrangement, public clonotypes can be identified in the majority of Ag-specific T-cell populations[6]; furthermore, their presence can be associated with distinct biologic outcomes.[7-9]

In this study, we aimed to unravel the forces that dictate the selection and maintenance of virus-specific CD8+ T-cell clonotypes associated with effective control of HIV replication in vivo. To this end, we performed detailed parallel ex vivo and in vitro analyses of CD8+ T cells specific for the p24 Gag-derived KK10 epitope (KRWIILGLNK; residues 263-272) restricted by HLA-B*2705. The KK10-specific CD8+ T-cell response is immunodominant in HLA-B*2705+ individuals infected with HIV clade B and linked with slower disease progression rates.[10,11] Moreover, the emergence of viral escape mutations in this epitope during late

Submitted January 4, 2011; accepted June 20, 2011. Prepublished online as *Blood* First Edition paper, July 6, 2011; DOI 10.1182/blood-2011-01-328781.

*M.C.I. and J.R.A. contributed equally to this work.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

infection has been associated with progression to AIDS.[12-14] Here, we report that KK10-specific clonotypes with *TRBV4-3/TRBJ1-3* gene rearrangements exhibit high levels of AgS, suppress HIV replication effectively, and tend to be public. Despite such functional advantages, however, these cells were typically subdominant in vivo, a phenomenon that could be linked to their inability to recognize the early $L_{268}M$ mutation that frequently occurs within the KK10 epitope.

Methods

Patients

Samples were obtained from untreated HIV-1–infected HLA-B*2705$^+$ patients enrolled in cohorts in France, Australia, and Spain. All patients were asymptomatic with CD4$^+$ T-cell counts > 300 cells/mm^3 and viral loads ranging from undetectable to 3.5×10^5 copies HIV-1 RNA/mL plasma. The study was approved by the institutional review board and local ethics committee of the Hospital Pitié Salpêtrière. Informed consent was obtained in compliance with the Declaration of Helsinki. PBMCs were separated from citrate anticoagulated blood and cryopreserved for subsequent studies. HIV-1 *gag* DNA sequencing was performed on whole cellular DNA extracted from PBMCs as described previously.[11]

Tetramers, Abs, CD8$^+$ T-cell clones, and viruses

Soluble biotinylated KK10/HLA-B*2705 monomers and variants thereof were generated and tetramerized as described previously.[15] The D227K/T228A compound mutation was introduced into the α3 domain of HLA-B*2705 to generate CD8-null monomers based on extrapolation from studies with HLA-A*0201.[16,17] Loss of soluble CD8 binding and maintenance of TCR docking integrity were verified for these novel reagents using surface plasmon resonance (SPR) as described previously with minor modifications[16] (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). mAbs were obtained from the following vendors: (1) αCD4-APCCy7, αCD107a-Cy5PE, αIL-2-APC, αIFNγ-Alexa 700, and αTNFα-PECy7 (BD Biosciences); (2) αCD8-Alexa405 (Caltag Laboratories); (3) αMIP-1β-FITC (LIVE DEAD Aqua Systems); and (4) αp24-PE (Beckman Coulter). The viability dye LIVE DEAD Aqua (Molecular Probes) was used to eliminate dead cells from the analysis. HIV-specific CD8$^+$ T-cell clones were isolated from PBMC samples obtained from 3 different HIV-1–infected HLA-B*2705$^+$ patients and characterized as detailed previously.[18] The HIV strains used for infection of HLA-B*2705$^+$ CD4$^+$ T cells were HIV_{NL4-3} and HIV_{NL4-3} Δ*Nef*, and a strain of HIV_{NL4-3} with a point mutation creating the $L_{268}M$ substitution.

Clonotypic analysis

Molecular analysis of *TRB* gene expression in KK10-specific CD8$^+$ T-cell populations isolated directly ex vivo by flow cytometry was conducted using a template switch anchored RT-PCR as described previously.[7,19] A similar approach was used to characterize *TRB* and *TRA* gene expression in KK10-specific CD8$^+$ T-cell clones. In all cases, TCR nomenclature was directly translated from the IMGT database using web-based alignment of molecular TRB or TRA transcripts (IMGT, The ImMunoGeneTics information system; http://imgt.cines.fr). The germline alignments for the TRBV4-3/TRBV1-3 clonotypes shown in supplemental Figure 2 were determined independently of this program to allow for both potential P additions from the *TRBD* genes and the determination of multiple alignments.

Assessment of Ag sensitivity and TCR avidity

The AgS of CD8$^+$ T cells was assessed by measuring the peptide concentration required to induce half-maximal responses (EC_{50}) in cytolytic Cr^{51} release (for clones) or IFN-γ ELISPOT assays (for CD8$^+$ T cells within PBMCs), as described previously.[11,18] TCR avidity was measured using tetramer dilution assays. CD8$^+$ T-cell clones were incubated with a range of KK10/HLA-B*2705 (standard or CD8-null) tetramer concentrations (30 μg/mL to 0.013 μg/mL in 1/3 dilutions) for 30 minutes at 4°C, and then stained for CD8 expression before fixation. The median fluorescence intensity (MFI) values for tetramer staining and the percentage of tetramer$^+$ CD8$^+$ T cells were evaluated by flow cytometry.

HIV suppression assay

Primary HLA-B*2705$^+$ CD4$^+$ T cells were purified from thawed PBMCs by positive magnetic bead selection, stimulated for 2 days with PHA (1 μg/mL), and then cultured with 100 U/mL rhIL-2. Seven days later, 10^5 cells/well were infected with virus by spinoculation[20] and mixed with CD8$^+$ T-cell clones at different CD8$^+$/CD4$^+$ ratios. To compare HIV_{NL4-3} versus HIV_{NL4-3} Δ*Nef* suppression activity, titrated amounts of each virus were used to generate similar levels of infectivity (ie, equivalent intracellular p24 expression 3 days after infection) in the absence of CD8$^+$ T-cell clones, thereby compensating for the potentially attenuated replication of HIV_{NL4-3} Δ*Nef* compared with HIV_{NL4-3} in our assays. Typically, we infected with 20 ng and 90 ng of p24/mL for HIV_{NL4-3} and HIV_{NL4-3} Δ*Nef*, respectively. Cells were harvested at day 3 postinfection, and stained intracellularly for CD4 and p24 to evaluate the elimination of HIV-infected targets.

Proliferation and polyfunctional assays

For proliferation, CD8$^+$ T-cell clones were stained with CFSE (Molecular Probes, Invitrogen) at 5μM for 10 minutes, then washed and stimulated with HLA-B*2705$^+$ EBV-transformed B-cell lines pulsed with cognate peptide at the indicated concentrations. Five days later, the percentage of CFSE-low cells was evaluated by flow cytometry. For polyfunctional profiling, CD8$^+$ T-cell clones were incubated for 1 hour at 37°C in the presence of αCD107a mAb and HLA-B*2705$^+$ CD4$^+$ T cells infected 3 days earlier with titrated levels of HIV_{NL4-3} or HIV_{NL4-3} Δ*Nef* virus; monensin (2.5 μg/mL; Sigma-Aldrich) and brefeldin A (5μg/mL; Sigma-Aldrich) were added for a further 5 hours. Staining for intracellular markers and data analysis were performed as described previously.[18]

Statistics

Group medians and distributions were compared using the Mann-Whitney *U* test or the Wilcoxon signed-rank test. Associations between variables were determined using the nonparametric Spearman rank correlation test. The χ^2 test was used for evaluation of *TRAV/TRBV* gene usage associations. *P* values $< .05$ were considered significant. Bonferroni correction was used for multiple comparisons, with *P* values $< .05$/(number of comparisons) considered significant.

Results

KK10-specific TRBV4-3/TRBJ1-3 clonotypes are highly selected in vivo

Clonotypic analyses of KK10-specific CD8$^+$ T-cell populations sorted directly ex vivo from 19 HLA-B*2705$^+$ individuals with HIV infection have generated more than 1200 TCRβ sequences comprising nearly 200 distinct clonotypes. Examination of these sequences led to the striking observation that 3 TRBV4-3/TRBJ1-3 clonotypes are public (each found in 2 patients, ie, 6 of 19 patients; Table 1). Furthermore, these public KK10-specific TRBV4-3/TRBJ1-3 clonotypes display closely related CDR3 sequences with a conserved pattern of amino acid usage (Figure 1A). The preferential representation of these clonotypes in KK10-specific CD8$^+$ T-cell populations suggests that they are readily recruited and expand efficiently in vivo. In 2 cases (of the total 19 HLA-B*2705$^+$ donors screened), patients 02.011 and L8146, these clonotypes were even found to be numerically dominant. On this basis, we sought to gain further insight into the public nature of

Table 1. Clonotypic analysis of KK10-specific CD8+ T-cell populations harboring public TRBV4-3/TRBJ1-3 sequences

Patient/TRBV	CDR3 sequence, aa	TRBJ	Frequency, %
02.011, CD4c = 1227, pVL = 1880			
4-3*†	**CASSPGQLGNTIY*†**	**1-3*†**	**54**
7-6	CASRLLGGGQETQY	2-5	35
4-3	CASSPGVFGVTQY	2-3	11
01.01 DOF, CD4c = 716, pVL = 4400			
4-3	CASSQGQYGNEQF	2-1	37
20-1	CSARREANYGYT	1-2	22
4-3	CASSEGANAYEQY	2-7	9
3-1	CASSQDGVYSNQPQH	1-5	6
4-3	CASSMGQNSNEQY	2-7	4
4-3	CASSQGLSSNEQF	2-1	3
18	CASSLGGLDIEQY	2-7	3
27	CATSGVTGELF	2-2	3
4-3*†	**CASSPGQLGNTIY*†**	**1-3*†**	**1**
4-3*	**CASSPGVLGNTIY***	**1-3***	**1**
20-1	CARREANYGYT	1-2	1
27	CASAMTGEGYGYT	1-2	1
27	CASSGGAHTEAF	1-1	1
27	CASSSKTGELF	2-2	1
27	CASSSRTGELF	2-2	1
7-2	CASSSTRGTEAF	1-1	1
L8146, CD4c = 332, pVL = 202590			
4-3*‡	**CASSQGQLGNTIY*‡**	**1-3*‡**	**40**
7-6	CASSLGGTNHGYT	1-2	13
7-2	CASSLVLAIEQY	2-7	7
9	CASSPGTGKNIQY	2-4	7
4-1	CASSQEGVNTEAF	1-1	4
4-3	CASSQAQGLSNSPLH	1-6	4
5-1	CASSRTQGPNTDTQY	2-3	4
7-6	CASSLDHLAGVNNEQF	2-1	4
6-1	CASSGQLLEAF	1-1	2
7-8	CASSLEGSRNTEAF	1-1	2
11-2	CASSQPPDRGYGYT	1-2	2
12-4	CASSLVGSYNEQF	2-1	2
12-4	CASSTTSGRYEQY	2-7	2
18	CASSFGQGAIEQY	2-7	2
27	CASSLSSDEHGYT	1-2	2
11.007, CD4c = 716, pVL = 4400			
20-1	CSARDLGLAGDTDTQY	2-3	84
7-9	CASSLDSYEQY	2-7	13
4-3*‡	**CASSQGQLGNTIY*‡**	**1-3*‡**	**2**
28	CASSLGIPGTAQWVYGYT	1-2	2
LTS 12, CD4c = 462, pVL = 17254			
28	CASSLRGGNTDTQY	2-3	61
24-1	CATSETGELF	2-2	29
4-3*§	**CASSPGQFGNTIY*§**	**1-3*§**	**9**
30	CAWSLTGMNQPQH	1-5	1
LTS 57, CD4c = 1050, pVL = 1900			
27	CASMGGANTEAF	1-1	88
27	CASSPTTYGYT	1-2	3
4-3*§	**CASSPGQFGNTIY*§**	**1-3*§**	**3**
10-3	CAISEYGNAASPLH	1-6	2
9	CASSVLGTSGGAEQF	2-1	2
20-1	CSARDWASGLSSYEQY	2-7	2

Data are shown for 6 of 19 KK10-specific CD8+ T-cell populations sort-purified directly ex vivo from HLA-B*2705+ patients infected with HIV-1. CD4+ T-cell counts (CD4c) and plasma viral load (pVL) are indicated at time of sample.

*Bold values represent all TRBV4-3/TRBJ1-3 clonotypes.

†First of 3 public TRBV4-3/TRBJ1-3 clonotypes.

‡Second of 3 public TRBV4-3/TRBJ1-3 clonotypes.

§Third of 3 public TRBV4-3/TRBJ1-3 clonotypes.

these particular clonotypes and their relevance for the control of HIV replication.

For a TCR to be public, it must first be present in the naive repertoire of more than one individual. To satisfy this prerequisite, the TCR in question should be produced efficiently during genetic rearrangement from the enormous array of combinatorial possibilities, and survive thymic selection. Recent research has indicated that interindividual TCR sharing within Ag-specific memory CD8+

A

TRBV/TRBJ usage	CDR3 sequence (aa)	Number of n-additions	Public TCR	Name of *in vitro* isolated clones
4-3/1-3	C A S S P G Q L G N T I Y	4 to 10	Yes	C2A / E3A
4-3/1-3	C A S S Q G Q L G N T I Y	2 to 5	Yes	A11C / F11C / G11C
4-3/1-3	C A S S P G Q F G N T I Y	2 to 4	Yes	B9A
4-3/1-3	C A S S P G V L G N T I Y	4	-	H9F
4-3/1-3	C A S S P G T L A N T I Y	6 to 8	-	F10C

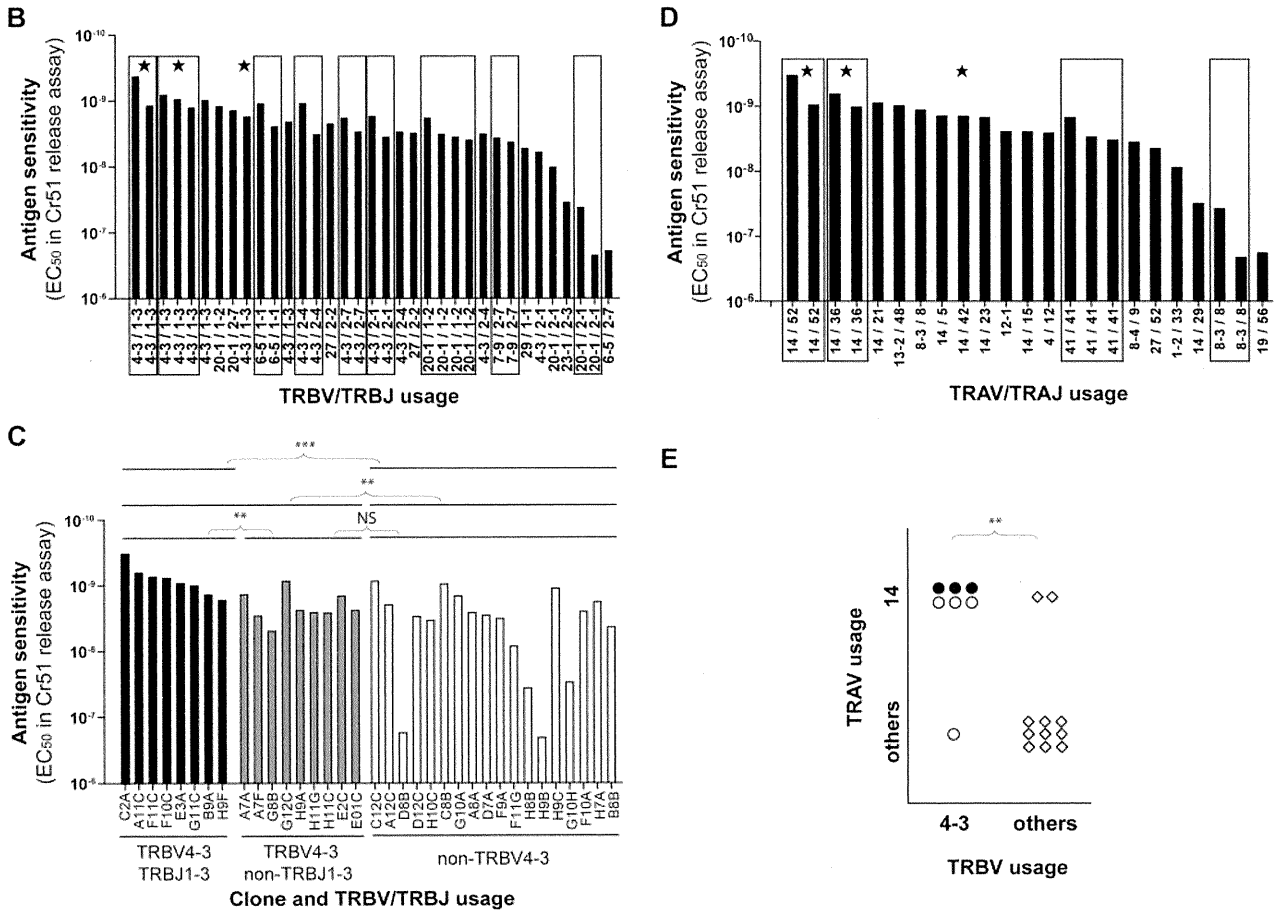

Figure 1. KK10-specific clonotypes with high levels of Ag sensitivity express TRBV4-3/TRBJ1-3 TCRs. (A) Alignment of observed TCRβ amino acid sequences for TRBV4-3/TRBJ1-3 clonotypes; 3 public and 2 private sequences are shown. Amino acid residues that differ between clonotypes are highlighted in gray. The numbers of n additions required to produce each observed nucleotide sequence and the isolated clone assignments are indicated for each clonotype. (B) KK10-specific $CD8^+$ T-cell clones (n = 35) isolated from 3 patients are grouped by TCRβ sequence, indicated by the box frames, and classified according to mean cognate Ag sensitivity (EC_{50} for Cr^{51} release). Public clonotypes are highlighted with a star. (C) Classification of KK10-specific $CD8^+$ T-cell clones according to cognate Ag sensitivity (EC_{50} for Cr^{51} release) and TRBV/TRBJ usage. Each bar represents one clone: TRBV4-3/TRBJ1-3 clones are in black, TRBV4-3/non-TRBJ1-3 clones are in gray, and non-TRBV4-3 clones are in white. The clone reference is indicated on the x-axis, and the last letter of the code corresponds to the patient from whom the clone was obtained (A and F for patient 01.01 DOF, B and G for patient 04.064, and C and H for patient 11.007). Statistical analyses were conducted using the Mann-Whitney *U* test with Bonferroni correction for multiple comparisons ($P < .0125$ was considered significant). (D) Isolated KK10-specific $CD8^+$ T-cell clones (n = 23) are grouped by TCRα sequence, indicated by the box frames, and classified according to mean cognate Ag sensitivity (EC_{50} for Cr^{51} release). Public clonotypes are highlighted with a star. (E) Association between *TRAV14* and *TRBV4-3* gene usage in KK10-specific $CD8^+$ T-cell clones. Each symbol represents one clone: ●, TRBV4-3/TRBJ1-3 clones; ○, TRBV4-3/non-TRBJ1-3 clones; and ◇, non-TRBV4-3 clones. The χ^2 test was used to assess statistical significance. $^{**}P < .01$ and $^{***}P < .001$, respectively.

T-cell populations can be predicted on the basis of relative production frequencies regardless of the selection processes that guide recruitment of individual clonotypes from the naive pool in response to Ag.[5] The process by which random V(D)J recombination generates a frequency spectrum of TCR nucleotide and amino acid sequences has been termed convergent recombination[21]; this process operates in all Ag-specific $CD8^+$ T-cell repertoires examined to date[21-23] and also shapes clonotypic prevalence in the naive T-cell repertoire, both within and between individuals.[24,25] Thus, we assessed the likelihood that public KK10-specific TRBV4-3/TRBJ1-3 clonotypes are produced efficiently during V(D)J recombination. Indicators of the efficiency with which TCRs can be produced by V(D)J recombination include the extent of germline encoding, the number of recombination events that can produce each nucleotide sequence and the variety of nucleotide sequences that can encode each amino acid sequence.[5] Analyses of the multiple nucleotide sequences encoding each observed TRBV4-3/TRBJ1-3 clonotype showed that the conserved amino acids in the CASSXGXXXNTIY motif are predominantly germline-encoded (see supplemental Figure 2). Two of the public TRBV4-3/TRBJ1-3 clonotypes could be made with as few as 2 nucleotide additions and 1 required a minimum of 4 nucleotide additions; this compares with a median of 8 nucleotide additions across all observed KK10-specific sequences (data not shown). Moreover, each of the public

TRBV4-3/TRBJ1-3 clonotypes was encoded by at least 3 different nucleotide sequences, several of which could have been produced by different recombination events (ie, different contributions from the germline genes and different nt additions; supplemental Figure 2). Overall, these data suggest that public TRBV4-3/TRBJ1-3 clonotypes can be produced with reasonable efficiency.

KK10-specific TRBV4-3/TRBJ1-3 clonotypes exhibit high levels of Ag sensitivity

In addition to genetic considerations, a public TCR must be efficiently recruited from the naive T-cell pool to be detected in the Ag-specific memory T-cell pool of multiple individuals. To assess the functional attributes of TRBV4-3/TRBJ1-3 clonotypes that might impinge on Ag-driven selection processes, we used a bank of KK10-specific CD8$^+$ T-cell clones generated in vitro from HLA-B*2705$^+$ patients infected with HIV. A total of 35 individual clones (from 3 individuals) were derived and characterized. Of note, we were able to isolate and study distinct clones bearing each of the 3 different public TRBV4-3/TRBJ1-3 sequences, as well as 2 clones with private TRBV4-3/TRBJ1-3 sequences (Figure 1A). Functional measurements using target cells loaded with a gradient of KK10 peptide concentrations revealed a broad range of AgS profiles across the bank of clones.[18] However, clones with identical TCR sequences displayed very similar levels of AgS (Figure 1B), thereby supporting the premise that the primary determinants of AgS are TCR-dependent. Notably, high levels of AgS were apparent for clones bearing public TRBV4-3/TRBJ1-3 sequences. Further analysis revealed that TRBV4-3 usage (n = 17) conferred significantly higher AgS (P = .004) relative to clones with other TRBV segments (n = 18). Moreover, concomitant TRBJ1-3 usage was largely responsible for this difference (P = .005 for TRBV4-3/TRBJ1-3 [n = 8] vs TRBV4-3/non-TRBJ1-3 [n = 9]); indeed, the TRBV4-3/non-TRBJ1-3 clones did not differ significantly from the non-TRBV4-3 clones with respect to AgS (Figure 1C, supplemental Figure 3A). These differences were not conditional on the presence of weakly sensitive clones among the non-TRBV4-3 group (supplemental Figure 3B). Thus, TRBV4-3/TRBJ1-3 TCRs seem to furnish KK10-specific CD8$^+$ T cells with high levels of AgS. A variety of TCRα sequences were also expressed among these KK10-specific clones, and TRAV14 usage was particularly common (Figure 1D). Of note, we found a significant association between TRAV14 and TRBV4-3 usage (Figure 1E); indeed, all TRBV4-3/TRBJ1-3 clones also expressed rearranged *TRAV14* gene products.

We hypothesized that the high AgS levels displayed by KK10-specific TRBV4-3/TRBJ1-3 clonotypes could confer the ability to proliferate vigorously in response to cognate Ag encounter. To test this possibility, we measured clonal proliferation in response to stimulation with exogenous KK10 peptide at a concentration of 10^{-8}M, which reflects KK10 epitope densities on the surface of HIV-infected cells in vitro as inferred from functional comparisons conducted with 2 different CD8$^+$ T-cell clones.[18] Clones with higher levels of AgS divided more readily than clones with lower levels of AgS (Figure 2A). The extent of proliferation, measured as the percentage of cells undergoing division over a period of 5 days, correlated with AgS (Figure 2B). This correlation was maintained across different peptide concentrations. The peptide concentrations required to trigger proliferation were substantially higher (> 100-fold in some cases) for clones with low AgS levels compared with clones with high AgS levels (Figure 2C). Thus, clones with high AgS levels likely proliferate more effectively under physiologic conditions, thereby providing these cells with an expansion advantage. These data support the preferential selection of TRBV4-3/TRBJ1-3 clonotypes in vivo.

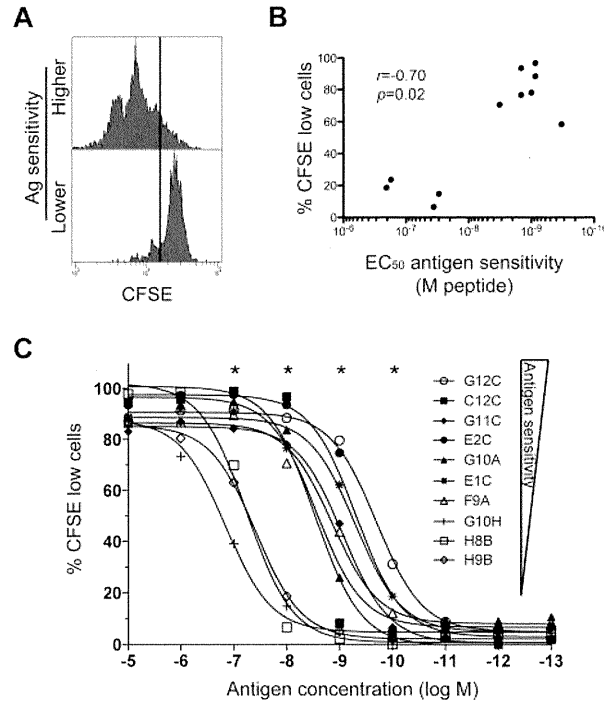

Figure 2. Differential induction of CD8$^+$ T-cell proliferation according to Ag sensitivity. (A) Representative examples of CD8$^+$ T-cell proliferation measured by dilution of CFSE fluorescence at 10^{-8}M peptide for clones with higher (C2A) and lower (D8B) AgS levels. Cells were labeled with CFSE and then stimulated with KK10 peptide-loaded EBV-transformed HLA-B*2705$^+$ B cells for 5 days. (B) Proliferation induced by 10^{-8}M cognate peptide is plotted as a function of AgS (EC_{50} for Cr^{51} release). Each dot represents a distinct clone. Minimal proliferation was observed in the absence of exogenous cognate peptide. The correlation was determined using the Spearman rank test. (C) Proliferation (% of cells with diluted CFSE fluorescence) across a gradient of peptide concentrations for 10 different KK10-specific CD8$^+$ T-cell clones with different AgS levels. *A significant correlation between AgS and proliferation at a given concentration of peptide.

TCR-pMHCI interactions determine Ag sensitivity and HIV-suppressive capacity

We have recently shown that AgS is a major determinant of the qualitative CD8$^+$ T-cell attributes associated with effective control of HIV replication (ie, polyfunctionality and HIV suppressive capacity) and may be considered a robust correlate of CD8$^+$ T-cell efficacy.[18] However, the factors that determine acquisition of high AgS levels are not fully defined. The results reported above suggest a strong relationship between specific TCR sequences and high levels of AgS. We therefore performed tetramer dilution assays, which focus specifically on the TCR-pMHCI interaction, to measure TCR avidity for a series of KK10-specific CD8$^+$ T-cell clones with a range of AgS levels. For this purpose, we used both standard and CD8-null tetramers. These latter reagents, which incorporate a compound D227K/T228A mutation in the α3 domain that abrogates CD8 coreceptor binding without affecting the fidelity of TCR docking (supplemental Figure 1), were produced to enable the assessment of intrinsic TCR avidity in the absence of CD8 compensation.[15,17] The percentage of tetramer$^+$ cells and median fluorescence intensity (MFI) were quantified by flow cytometry as a function of decreasing tetramer concentrations[26-28] for different clones (Figure 3). Marked differences between CD8$^+$ T-cell clones were observed according to their AgS. Increases in

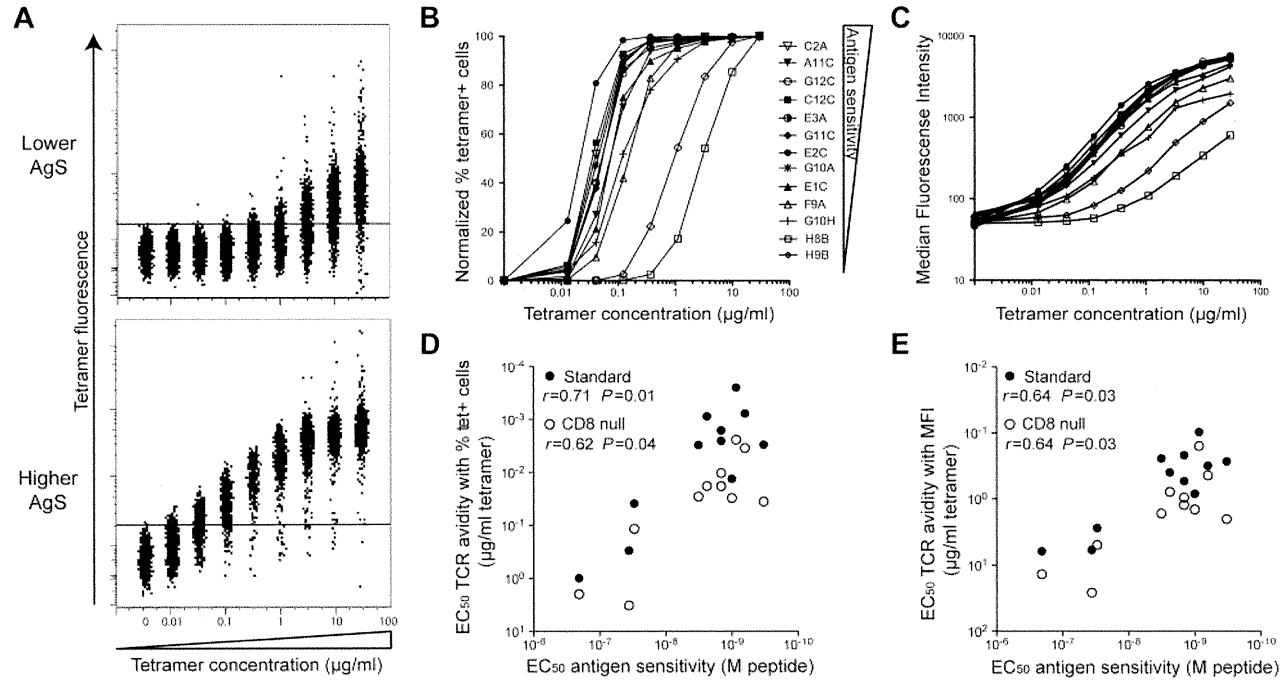

Figure 3. Ag sensitivity correlates with TCR avidity. Clones with different AgS levels were labeled with KK10/HLA-B*2705 tetramer across a range of concentrations. (A) Representative tetramer titrations for 1 clone with lower (H8B) and 1 clone with higher (C2A) levels of AgS. (B-C) Representative tetramer titration curves for several clones displayed as a percentage (B) or MFI (C) of tetramer$^+$ cells. The symbol key shown in panel B applies to both panels. (D-E) Correlation between AgS (EC_{50} for Cr^{51} release) and TCR avidity displayed as tetramer concentration EC_{50} for percentage (D) or MFI (E) of tetramer$^+$ cells. Data are shown for both standard (●) and CD8-null (○) tetramer titration assays. Correlations were determined using the Spearman rank test.

tetramer MFI were apparent for clones with high AgS levels at the most diluted tetramer concentrations used (down to 1.3×10^{-2} μg/mL). In contrast, substantially higher (> 10-fold) tetramer concentrations were required to observe MFI increases in clones with lower AgS levels; these clones also required very high tetramer concentrations to attain 100% staining (Figure 3A). Normalized percentages of tetramer$^+$ cells (Figure 3B) and MFI (Figure 3C) were plotted as a function of tetramer concentration, and the tetramer concentrations required to obtain half maximal values (EC_{50}) were calculated to quantify TCR avidity. EC_{50} values were generally higher with CD8-null tetramers compared with standard tetramers, consistent with the role of CD8 in stabilization of the TCR-pMHCI interaction.[29] However, regardless of the tetramers used (ie, standard or CD8-null), this calculated measure of clonal TCR avidity correlated with the AgS of KK10-specific CD8$^+$ T cells (Figure 3D-E). These data indicate that TCR avidity, determined by the TCR-pMHCI interaction, is a principal determinant of AgS in this set of clones.

By extension, we reasoned that if TCR-mediated avidity for Ag is a primary determinant of AgS, then clones with high levels of TCR avidity should require fewer TCR-pMHCI ligations to kill infected cells and suppress HIV replication. To evaluate this, we compared the ability of CD8$^+$ T-cell clones with high or low levels of AgS to suppress HIV replication in HLA-B*2705$^+$ CD4$^+$ T cells infected either with wild-type (wt) or Nef-deleted viruses. The HIV Nef protein is known to down-regulate HLA class I molecule expression on the surface of infected cells,[30] thus altering the number of cognate pMHCI molecules available for TCR ligation. Indeed, CD4$^+$ T cells infected with a wt HIV strain exhibited significant, although not complete, down-regulation of HLA-B*27 expression on the cell surface; in contrast, CD4$^+$ T cells infected with a ΔNef virus displayed cell surface HLA-B*27 levels similar to those observed on the surface of uninfected cells (Figure 4A). Suppression of HIV replication, calculated as the fold reduction in the percentage of p24 Gag Ag-expressing CD4$^+$ T cells detected by flow cytometry, was evaluated after 3 days of infected CD4$^+$ T-cell coculture with different CD8$^+$ T-cell clones. In line with our previous findings,[18] clones with high levels of TCR avidity eliminated infected CD4$^+$ T cells more efficiently than clones with low levels of TCR avidity. Regardless of TCR avidity, the ability to suppress HIV replication was more pronounced for ΔNef compared with wt virus (Figure 4B-C). This suggests stronger CD8$^+$ T-cell activation, likely because of higher levels of HLA class I expression on the surface of CD4$^+$ T cells infected with the ΔNef virus. Consistent with this possibility, representative high- and low-avidity clones displayed marginally more polyfunctional profiles when tested in the presence of CD4$^+$ T cells infected with ΔNef compared with wt virus (supplemental Figure 4). Overall, TCR avidity emerges as a major determinant of AgS and HIV suppressive capacity in CD8$^+$ T cells. Taken together, these results indicate that TCRs constructed with the TRBV4-3/TRBJ1-3 pairing confer KK10-specific CD8$^+$ T cells with functional properties that enable preferential control of HIV replication.

HIV escapes recognition by public TRBV4-3/TRBJ1-3 clonotypes through the $L_{268}M$ mutation

Although public TRBV4-3/TRBJ1-3 clonotypes could proliferate vigorously, they were found to dominate the KK10-specific CD8$^+$ T-cell population in only 2 of 6 patients (02.011 and L8146) in whom they were detected (Table 1). This may reflect the replacement of senescent clonotypes, as we suggested previously[11]; however, the most noticeable feature that was shared between these 2 individuals emerged at the level of the dominant autologous viral sequence. Specifically, the prevalent viral species in patients with dominant TRBV4-3/TRBJ1-3

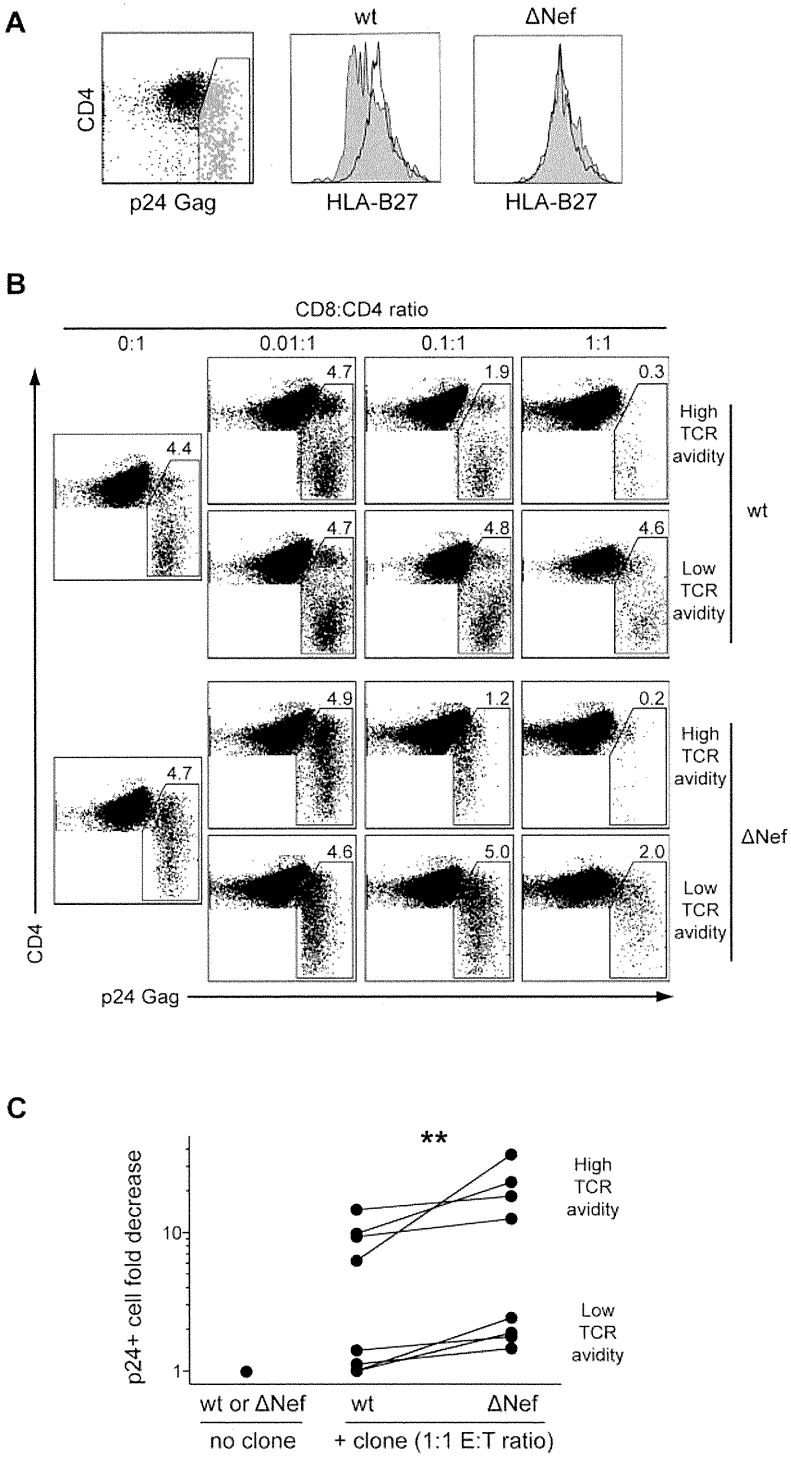

Figure 4. TCR avidity determines the efficacy of HIV suppression by CD8$^+$ T cells. (A) HLA-B*2705$^+$ CD4$^+$ T cells infected with wt (HIV_{NL4-3}) or ΔNef ($HIV_{NL4-3\Delta Nef}$) viruses were assayed for HLA-B*27 expression by flow cytometry. Surface expression of HLA-B*27 is shown for infected p24$^+$ cells (gray) and uninfected p24$^-$ cells (black). (B) Representative flow cytometry plots showing p24 expression in CD4$^+$ T cells infected with titrated amounts of wt or ΔNef viruses after 3 days of coculture with high- or low-avidity CD8$^+$ T-cell clones. Percentages of p24$^+$ cells and E:T ratios are indicated. (C) Fold decrease in the percentage of p24$^+$ CD4$^+$ T cells infected with titrated amounts of wt or ΔNef viruses after 3 days of coculture with higher (n = 4) or lower (n = 4) avidity CD8$^+$ T-cell clones at E:T ratios of 1:1. The Wilcoxon signed-rank test was used to compare suppression of wt versus ΔNef viruses. $^{**}P < .01$.

clonotypes contained wt KK10 epitope sequences. In contrast, individuals with subdominant TRBV4-3/TRBJ1-3 clonotypes harbored viruses with KK10 epitope mutations, in particular the common $L_{268}M$ substitution (Figure 5A). We reasoned that these public clonotypes, although apparently highly efficient, might actually be narrowly constrained and unable to recognize the $L_{268}M$ variant effectively, thereby mitigating their selection advantage in vivo in the presence of mutant viruses.

To confirm this hypothesis, we evaluated the AgS of the 3 public TRBV4-3/TRBJ1-3 CD8$^+$ T-cell clones for both the wt and $L_{268}M$ mutant KK10 epitopes in Cr^{51} cytotoxicity assays. The AgS of these clones for the $L_{268}M$ mutant was found to be at least 2 orders of magnitude lower than that observed for the wt KK10 epitope (Figure 5B representative results are displayed in the left panel). HIV suppression experiments using HLA-B*2705$^+$ CD4$^+$ T cells infected with either wt virus or a strain presenting the $L_{268}M$ substitution corroborated these results. A public TRBV4-3/TRBJ1-3 clone efficiently eliminated CD4$^+$ cells infected with wt virus but was almost completely ineffective against the $L_{268}M$ variant strain (Figure 5C left panel). For comparison, 2 private non-TRBV4-3/TRBJ1-3 clones (with low or high AgS levels for both the wt KK10 epitope and the $L_{268}M$ variant) were also studied

A

CDR3 sequence (aa)	Patient	Dominance / frequence (%)	KK10 sequence
CASSPGQLGNTIY	02.011	Yes / 54	KRWIILGLNK
	01.01DOF	No / 1	KRWII**M**GLNK
CASSQGQLGNTIY	L8146	Yes / 42	KRWIILGLNK
	11.007	No / 2	KRWII**M**GLNK
CASSPGQFGNTIY	LTS 12	No / 9	KRWIILGL$^{N}_{H}$K*
	LTS 57	No / 3	KRWII**M**GLNK

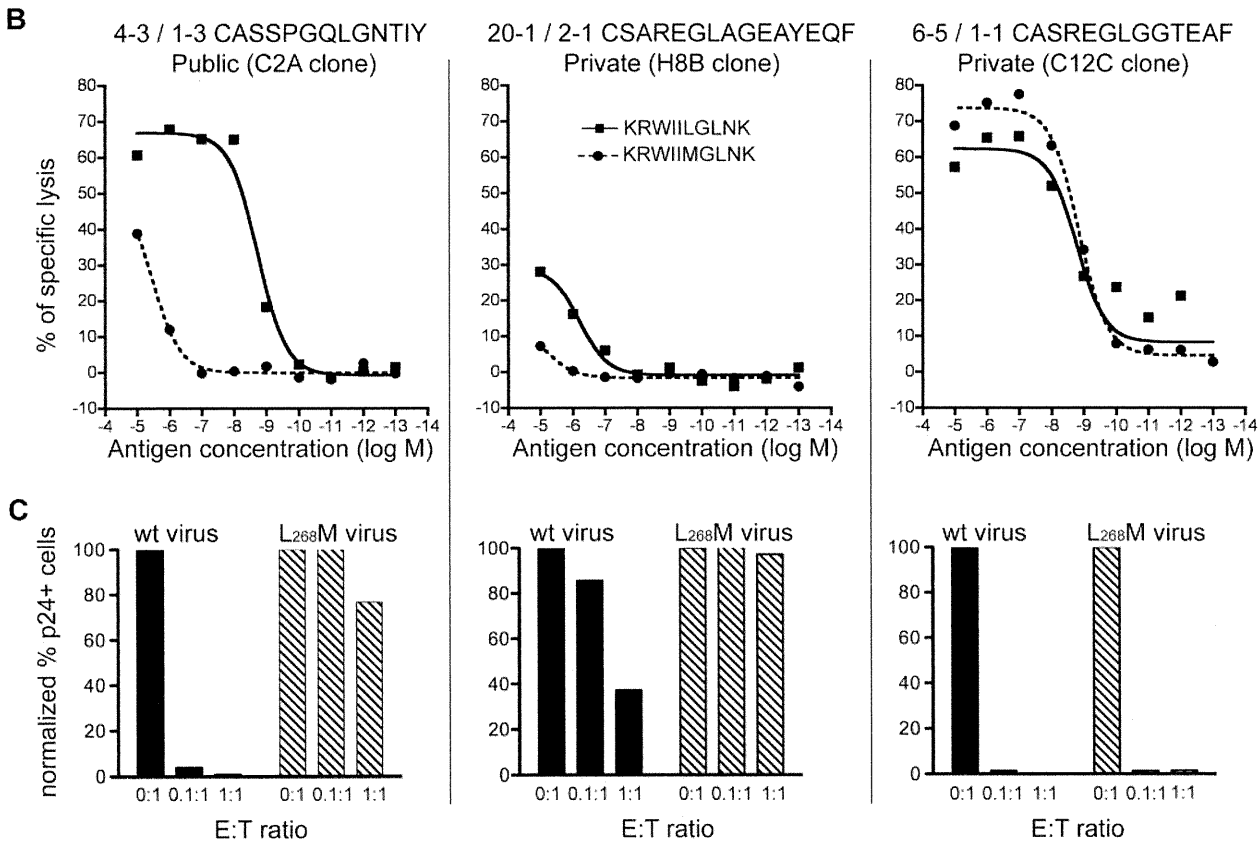

Figure 5. Inefficient recognition of the $L_{268}M$ mutant by public TRBV4-3/TRBJ1-3 clonotypes. (A) In vivo dominance of public TRBV4-3/TRBJ1-3 clonotypes and prevalent viral variants at the time of sampling. *Codominant viral KK10 sequences. (B) Ag sensitivity of representative $CD8^+$ T-cell clones for the wt KK10 and $L_{268}M$ mutant epitopes was measured in Cr^{51} cytotoxicity assays using EBV-transformed HLA-B*2705$^+$ B cells pulsed with the corresponding peptides across a range of concentrations. (C) Suppression of HIV replication in HLA-B*2705$^+$ $CD4^+$ T cells infected with wt (HIV_{NL4-3}) virus (■) or a mutant virus encoding the $L_{268}M$ epitope mutation (▨) by representative $CD8^+$ T-cell clones at 3 different E:T ratios. The percentage of $p24^+$ $CD4^+$ T cells was measured by flow cytometry at day 3 postinfection.

(Figure 5B-C middle and right panels). These results demonstrate that the public TRBV4-3/TRBJ1-3 clonotypes, although very effective at recognizing the wt KK10 epitope, are functionally disabled (ie, consigned to low AgS) by the $L_{268}M$ mutant. Thus, the $L_{268}M$ mutation enables HIV to escape recognition and suppression by otherwise highly effective TRBV4-3/TRBJ1-3 clonotypes. Several other KK10-specific $CD8^+$ T-cell clones also displayed weaker sensitivity for the $L_{268}M$ variant compared with the wt peptide (supplemental Figure 5). Although this may reflect the use of wt KK10/HLA-B*2705 tetramers to isolate these cells for cloning, it does indicate that HIV $L_{263}M$ escape is likely not exclusive to TRBV4-3/TRBJ1-3 clonotypes.

Nonetheless, in contrast to the $R_{264}K$ mutation, the emergence of the $L_{268}M$ mutation in vivo is usually not associated with loss of viral control and HIV disease progression,[31] thereby suggesting that KK10-specific $CD8^+$ T-cell responses remain effective in the presence of this variant. Direct ex vivo assessment of KK10 reactivity revealed that $CD8^+$ T cells from 3 patients (11.007, 05.005, and 01.225) harboring a predominant $L_{268}M$ virus were able to recognize this variant efficiently. In contrast, cells from patient 02.011, in whom the circulating virus carried the wt KK10 epitope and the cognate $CD8^+$ T-cell population was dominated by a public TRBV4-3/TRBJ1-3 clonotype, recognized the wt KK10 epitope with higher levels of AgS and greater overall magnitude compared with the $L_{268}M$ variant (Figure 6A-C). These observations may reflect adaptation of the immune response to the appearance of $L_{268}M$ mutants, characterized by the replacement of highly effective but wt epitope-constrained cells, such as those with

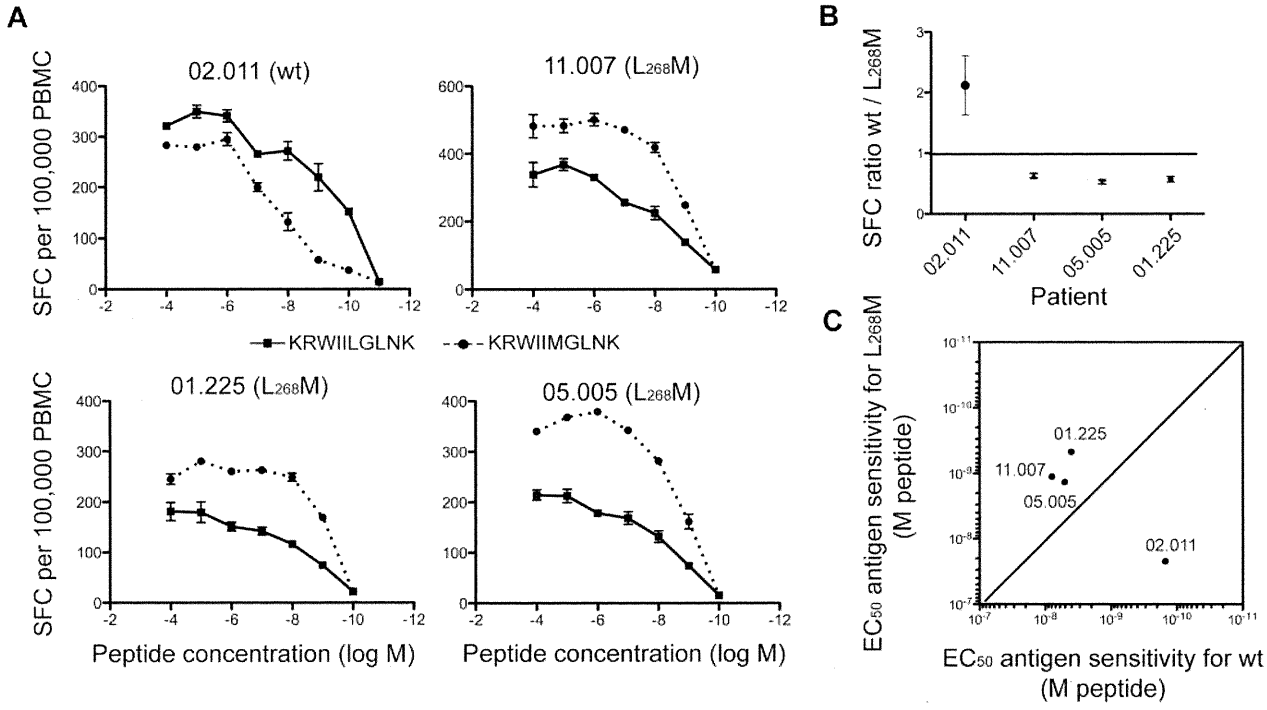

Figure 6. Reactivity of KK10-specific CD8+ T-cell populations to emerging $L_{268}M$ mutants. (A) Ag sensitivity of KK10-specific CD8+ T-cell populations from 4 HLA-B*2705+ individuals infected with HIV-1 was measured directly ex vivo using IFN-γ ELISPOT analysis across a range of wt KK10 and mutant $L_{268}M$ peptide concentrations. The patients harbored predominantly either wt (02.011) or $L_{268}M$ (11.007, 01.225, and 05.005) viruses at the time point studied. (B) SFC ratio of wt/$L_{268}M$-specific responses are plotted for each patient. Ratios were calculated for each peptide concentration and are shown as the mean ± SEM for all peptide concentrations. (C) Ag sensitivity (EC_{50} values) for the wt KK10 and mutant $L_{268}M$ peptides in each individual. SFC indicates spot-forming cell.

TRBV4-3/TRBJ1-3 TCRs, by $L_{268}M$-reactive cells. This process could ensure the maintenance of viral control despite the emergence of $L_{268}M$ escape variants.

Discussion

The Ag-mobilized TCR repertoire is a likely determinant of many critical qualitative parameters that contribute to CD8+ T-cell efficacy. Thus, individual clonotypes specific for a given pMHCI structure within an Ag-specific CD8+ T-cell population can potentially exhibit diverse properties, which in sum define the composite nature of the overall response. To dissect this complexity in the setting of a protective HIV-specific response, we performed extensive TCR repertoire analyses together with functional assessments of isolated CD8+ T-cell clones specific for the p24 Gag KK10 epitope restricted by HLA-B*2705. Thus, we were able to assess the role of specific TCRs in relation to CD8+ T-cell efficacy without the confounding variables of differential epitope targeting and HLA restriction.

Initially, we observed that 3 closely related TRBV4-3/TRBJ1-3 clonotypes within the KK10-specific CD8+ T-cell population were public, that is, shared in vivo between different individuals. Several theories have been put forward to explain the existence of public clonotypes,[5] which appear to be a pervasive feature of Ag-specific T-cell responses despite the extremely low theoretical probability of recurrence.[6] Sequence-based explanations propose that public TCRs are produced by near-germline recombination of V(D)J segments, with some deletion of nucleotides but no or minimal nucleotide additions.[32-34] We evaluated this possibility in our public TRBV4-3/TRBJ1-3 clonotypes and found that a minimum of 2-4 nucleotide additions was required to produce each of the observed TCRβ sequences. Nonetheless, these TCRβ amino acid sequences were also encoded by different nucleotide sequences, many of which required greater numbers of nucleotide additions. Thus, as shown for other public TCRs,[21,35,36] germline likeness in isolation cannot explain the shared nature of such sequences. Recently, "convergent recombination" has been proposed as a mechanism that could explain the existence of public TCRs.[5] In brief, it is known that many different V(D)J recombination events can converge to produce identical nucleotide sequences and that many different nucleotide sequences can converge to encode identical amino acid sequences or motifs. Thus, the number of different ways in which any given TCR amino acid sequence can be constructed will contribute to its public or private nature. The observation that multiple nucleotide sequences can encode each of the public TRBV4-3/TRBJ1-3 amino acid sequences, some of which could be produced by different V(D)J recombination events, suggests that convergent recombination plays a role in the interindividual sharing of these clonotypes. Moreover, in our in vitro experiments, all KK10-specific CD8+ T-cell clones bearing TCRβ amino acid sequences constructed from *TRBV4-3/TRBJ1-3* gene segments displayed high levels of cognate AgS. Such TRBV4-3/TRBJ1-3 clones were also characterized by TRAV14 usage, in contrast to the majority of non-TRBV4-3 clones. Thus, these specific *TRA* and *TRB* gene segments appear to generate a TCR structure that is particularly Ag-reactive. Given that such high levels of AgS also confer a proliferative advantage, it seems likely that avidity-based selection of TRBV4-3/TRBJ1-3 clonotypes in vivo also contributes to the observed sharing of such TCR sequences within the KK10-specific memory CD8+ T-cell pools of HIV-1 infected HLA-B*2705+ individuals.

The high levels of AgS displayed by TRBV4-3/TRBJ1-3 clonotypes conferred not only extensive proliferative capacity, but also potent HIV-suppressive activity. These findings suggest that TRBV4-3/TRBJ1-3 clonotypes could be major players in the KK10-specific CD8$^+$ T-cell response, which is a characteristic immunologic feature of HLA-B*2705$^+$ individuals who control HIV replication in vivo. In line with this possibility, our data indicate that the virus can mutate specifically to escape recognition by these highly effective clonotypes. Indeed, subdominance of TRBV4-3/TRBJ1-3 clonotypes in vivo, which was observed in 4 of 6 patients in whom these TCR sequences were detected (of 19 patients in total), was associated with the preponderance of circulating viral strains carrying the $L_{268}M$ mutant epitope. The $L_{268}M$ mutation is known to occur early during the course of HIV infection. It is generally not considered a "true" escape mutation because no increase in viral load occurs after its appearance. Instead, it has been proposed that the $L_{268}M$ substitution is a compensatory change required for the appearance, late in infection, of the $R_{264}K$ mutation, which results in increased viral loads and clinical progression.[12-14] The $R_{264}K$ mutation dramatically decreases peptide binding to HLA-B*27; in contrast, the $L_{268}M$ mutation does not coincide with an anchor residue and appears to have little or no impact on HLA-B*27 binding.[13,14,37] However, the emergence of $L_{268}M$ variants is associated with TCR repertoire changes within KK10-specific CD8$^+$ T-cell populations, suggesting a differential impact on clonotypic recognition.[31] Consistent with this possibility, we observed that AgS was reduced by at least 2 orders of magnitude when TRBV4-3/TRBJ1-3 clonotypes were confronted with the $L_{268}M$ variant; furthermore, the HIV-suppressive capacity of these clonotypes was almost entirely abrogated, rendering them ineffective in the face of an $L_{268}M$ variant virus. Thus, it is likely that the $L_{268}M$ mutation enables the virus to escape from the intense immune pressure exerted by such highly efficacious clonotypes early in HIV infection with no apparent fitness cost.[38] The appearance of this variant is likely followed by the expansion of $L_{268}M$-specific clonotypes that supersede the intially mobilized repertoire and thus maintain control of HIV replication.[39] Nonetheless, because reversion to the wt epitope rarely occurs in vivo,[40] we hypothesize that the $L_{268}M$ variant may ultimately favor the virus in the tight equilibrium between HIV replication and immune control. For instance, newly generated clonotypes may not attain the levels of AgS and anti-HIV efficacy displayed by TRBV4-3/TRBJ1-3 clonotypes for wt virus.

In addition to reinforcing the importance of AgS for CD8$^+$ T-cell selection and efficacy against HIV, our data provide further insights into the determinants of AgS. Several factors contribute to AgS, effectively making it a composite parameter. These factors include the density and topography of HLA class I molecules, the quality of Ag presentation, costimulatory, and coinhibitory receptors on the CD8$^+$ T-cell and target cell, pMHCI decay kinetics, membrane flexibility and TCR-dependent parameters such as monomeric affinity for Ag.[29,41-46] The present work highlights the central role of TCR avidity as a determinant of AgS. TCR avidity, measured in the current study by pMHCI tetramer titrations to eliminate the confounding influences of other molecular interactions that can occur in bi-membrane domains, takes into account the intrinsic binding strength (affinity) of the TCR and the role of the CD8 coreceptor, as well as the density, topography, and coordinate relationship of these Ag-binding receptors within the constraints of cell-surface mobility. There was no relationship between AgS and CD8 density, as measured by flow cytometry at the time of assay (data not shown), on the surface of the KK10-specific CD8$^+$ T-cell clones used in this study. A dominant role for the CD8 coreceptor was also excluded by the measurement of intrinsic TCR avidity using CD8-null pMHCI tetramer titrations. Furthermore, studies in mice have shown that the relationship between TCR density and the response to Ag is nonexponential.[47] Thus, it seems unlikely that differential cell-surface densities of TCR and CD8 determine the range of clonal Ag avidities observed in this study. This is consistent with the cosegregation of TCR avidities and AgS levels with distinct TCR sequences. Altogether, these data highlight the importance of TCR-pMHCI interactions for CD8$^+$ T-cell efficacy against HIV. This is further exemplified by the increased HIV-suppressive activity of CD8$^+$ T-cell clones in assays using a ΔNef virus, which does not down-regulate HLA class I molecules from the surface of infected target cells. This effect may contribute, at least in part, to the establishment of long-term nonprogressive disease status in patients infected with viruses carrying deletions in the *Nef*/LTR region, as described previously in the Sydney Blood Bank cohort.[48,49] In addition to the attenuated fitness of such viruses, control of HIV may be more easily achieved as lesser constraints are placed on the selection of CD8$^+$ T-cell clonotypes with high levels of AgS.

In summary, the present study of a protective HIV-specific CD8$^+$ T-cell response at the clonotypic level illustrates the importance of TCR avidity as a determinant of AgS and emphasizes that the selection of cognate clonotypes with high AgS levels may be critical for CD8$^+$ T-cell efficacy. Controlling this selection process may be a key consideration for the rational design of effective T cell–based vaccines,[50] although further studies with other HIV epitope-specific CD8$^+$ T-cell populations are required to confirm and extend our findings. While the present observations may only represent one of several potential scenarios that could occur during the T-cell response to HIV, the demonstration herein that HIV can escape from specific clonotypes with high levels of AgS highlights the intricacy of the host-pathogen equilibrium and the level of complexity that may be necessary to design an effective T cell–based vaccine.

Acknowledgments

The authors are very grateful to the staff and patients who participated in this study, to the Agence Nationale de la Recherche sur le SIDA (ANRS) Cohorts Asymptomatique à Long Terme group and to the National Centre in HIV Epidemiology and Clinical Research long-term nonprogressor cohort. They thank Catherine Blanc for sorting viable infected cells at the Pitié-Salpêtrière Flow Cytometry Platform. They are indebted to Zaïna Aït Arkoub and Henri Agut for help with HIV-1 *gag* DNA sequencing.

This work was supported by the ANR (project ANR-09-JCJC-0114-01), Sidaction, the Inserm AVENIR grant, the French ANRS, the National Institutes of Health via the Intramural Program of the Vaccine Research Center (National Institute of Allergy and Infectious Diseases [NIAID]) and R01 067077 (C.B.), the Australian Research Council (ARC), the Australian National Health and Medical Research Council (NHMRC), and the UK Medical Research Council (MRC). M.C.I. is supported by a Sidaction Fellowship. J.R.A. is supported by a Fundação para a Ciência e Tecnologia Fellowship.

V.V. is an ARC Future Fellow. P.G.W. is an NHMRC C. J. Martin Fellow. J.R. is an ARC Federation Fellow. M.P.D. is a Sylvia and Charles Viertel Senior Medical Research Fellow. D.A.P. is an MRC (UK) Senior Clinical Fellow.

Authorship

Contribution: M.C.I. designed the study, performed research, analyzed data, and wrote the manuscript; J.R.A. designed the study, performed research, and analyzed data; S.F. performed research; D.J.v.B. performed research and analyzed data; M.H. performed research; V.V. analyzed data and wrote the paper; E.G. performed research and contributed vital new reagents; A.U. performed research; L.W. performed research; M.C. performed research; S.G. performed research; P.G.W. performed research; B.A. contributed vital reagents; A.M. contributed vital reagents; J.R. contributed vital analytical tools; M.P.D. contributed vital analytical tools; M.T. contributed vital new reagents; C.B. performed research and analyzed data; D.C.D. designed the study and contributed vital analytical tools; A.D.K. designed the study and analyzed data; D.A.P. designed the study, contributed vital new reagents, and wrote the manuscript; and V.A. designed the study, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Victor Appay, Inserm UMR S 945, Infections and Immunity, Avenir Group, Université Pierre et Marie Curie-Paris6, Hôpital Pitié-Salpêtrière, Paris, France; e-mail: victor.appay@upmc.fr.

References

1. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol.* 2008;8(4):247-258.
2. Davenport MP, Price DA, McMichael AJ. The T cell repertoire in infection and vaccination: implications for control of persistent viruses. *Curr Opin Immunol.* 2007;19(3):294-300.
3. Appay V, Douek DC, Price DA. CD8+ T cell efficacy in vaccination and disease. *Nat Med.* 2008; 14(6):623-628.
4. Turner SJ, Doherty PC, McCluskey J, Rossjohn J. Structural determinants of T-cell receptor bias in immunity. *Nat Rev Immunol.* 2006; 6(12):883-894.
5. Venturi V, Price DA, Douek DC, Davenport MP. The molecular basis for public T-cell responses? *Nat Rev Immunol.* 2008;8(3):231-238.
6. Miles JJ, Douek DC, Price DA. Bias in the αβ T cell repertoire: implications for disease pathogenesis and vaccination. *Immunol Cell Biol.* 2011; 89(3):375-387.
7. Price DA, West SM, Betts MR, et al. T cell receptor recognition motifs govern immune escape patterns in acute SIV infection. *Immunity.* 2004; 21(6):793-803.
8. Menezes JS, van den Elzen P, Thornes J, et al. A public T cell clonotype within a heterogeneous autoreactive repertoire is dominant in driving EAE. *J Clin Invest.* 2007;117(8):2176-2185.
9. Price DA, Asher TE, Wilson NA, et al. Public clonotype usage identifies protective Gag-specific CD8+ T cell responses in SIV infection. *J Exp Med.* 2009;206(4):923-936.
10. Kaslow RA, Carrington M, Apple R, et al. Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nat Med.* 1996;2(4):405-411.
11. Almeida JR, Price DA, Papagno L, et al. Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. *J Exp Med.* 2007;204(10):2473-2485.
12. Feeney ME, Tang Y, Roosevelt KA, et al. Immune escape precedes breakthrough human immunodeficiency virus type 1 viremia and broadening of the cytotoxic T-lymphocyte response in an HLA-B27-positive long-term-nonprogressing child. *J Virol.* 2004;78(16):8927-8930.
13. Goulder PJ, Phillips RE, Colbert RA, et al. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. *Nat Med.* 1997;3(2):212-217.
14. Kelleher AD, Long C, Holmes EC, et al. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. *J Exp Med.* 2001;193(3): 375-386.
15. Price DA, Brenchley JM, Ruff LE, et al. Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. *J Exp Med.* 2005;202(10):1349-1361.
16. Purbhoo MA, Boulter JM, Price DA, et al. The human CD8 coreceptor effects cytotoxic T cell activation and antigen sensitivity primarily by mediating complete phosphorylation of the T cell receptor zeta chain. *J Biol Chem.* 2001;276(35): 32786-32792.
17. Wooldridge L, Lissina A, Cole DK, van den Berg HA, Price DA, Sewell AK. Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology.* 2009;126(2):147-164.
18. Almeida JR, Sauce D, Price DA, et al. Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. *Blood.* 2009;113(25):6351-6360.
19. Douek DC, Betts MR, Brenchley JM, et al. A novel approach to the analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape. *J Immunol.* 2002;168(6): 3099-3104.
20. Saez-Cirion A, Lacabaratz C, Lambotte O, et al. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. *Proc Natl Acad Sci U S A.* 2007;104(16):6776-6781.
21. Venturi V, Kedzierska K, Price DA, et al. Sharing of T cell receptors in antigen-specific responses is driven by convergent recombination. *Proc Natl Acad Sci U S A.* 2006;103(49):18691-18696.
22. Venturi V, Chin HY, Asher TE, et al. TCR beta-chain sharing in human CD8+ T cell responses to cytomegalovirus and EBV. *J Immunol.* 2008; 181(11):7853-7862.
23. Venturi V, Chin HY, Price DA, Douek DC, Davenport MP. The role of production frequency in the sharing of simian immunodeficiency virus-specific CD8+ TCRs between macaques. *J Immunol.* 2008;181(4):2597-2609.
24. Quigley MF, Greenaway HY, Venturi V, et al. Convergent recombination shapes the clonotypic landscape of the naive T-cell repertoire. *Proc Natl Acad Sci U S A.* 2010;107(45):19414-19419.
25. Robins HS, Srivastava SK, Campregher PV, et al. Overlap and effective size of the human CD8+ T cell receptor repertoire. *Sci Transl Med.* 2010; 2(47):47ra64.
26. Yee C, Savage PA, Lee PP, Davis MM, Greenberg PD. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *J Immunol.* 1999;162(4):2227-2234.
27. Vingert B, Perez-Patrigeon S, Jeannin P, et al. HIV controller CD4+ T cells respond to minimal amounts of Gag antigen due to high TCR avidity. *PLoS Pathog.* 2010;6(2):e1000780.
28. Williams MA, Ravkov EV, Bevan MJ. Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory. *Immunity.* 2008;28(4): 533-545.
29. Laugel B, van den Berg HA, Gostick E, et al. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. *J Biol Chem.* 2007;282(33):23799-23810.
30. Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med.* 1996;2(3):338-342.
31. Lichterfeld M, Kavanagh DG, Williams KL, et al. A viral CTL escape mutation leading to immunoglobulin-like transcript 4-mediated functional inhibition of myelomonocytic cells. *J Exp Med.* 2007; 204(12):2813-2824.
32. Argaet VP, Schmidt CW, Burrows SR, et al. Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. *J Exp Med.* 1994;180(6):2335-2340.
33. Cibotti R, Cabaniols JP, Pannetier C, et al. Public and private V beta T cell receptor repertoires against hen egg white lysozyme (HEL) in non-transgenic versus HEL transgenic mice. *J Exp Med.* 1994;180(3):861-872.
34. Fazilleau N, Cabaniols JP, Lemaitre F, Motta I, Kourilsky P, Kanellopoulos JM. Valpha and Vbeta public repertoires are highly conserved in terminal deoxynucleotidyl transferase-deficient mice. *J Immunol.* 2005;174(1):345-355.
35. Lim A, Trautmann L, Peyrat MA, et al. Frequent contribution of T cell clonotypes with public TCR features to the chronic response against a dominant EBV-derived epitope: application to direct detection of their molecular imprint on the human peripheral T cell repertoire. *J Immunol.* 2000; 165(4):2001-2011.
36. Trautmann L, Rimbert M, Echasserieau K, et al. Selection of T cell clones expressing high-affinity public TCRs within human cytomegalovirus-specific CD8 T cell responses. *J Immunol.* 2005; 175(9):6123-6132.
37. Goulder PJ, Brander C, Tang Y, et al. Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature.* 2001;412(6844):334-338.
38. Schneidewind A, Brockman MA, Yang R, et al. Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *J Virol.* 2007;81(22):12382-12393.
39. van Bockel DJ, Price DA, Munier ML, et al. Persistent survival of prevalent clonotypes within an immunodominant HIV Gag-specific CD8+ T-cell response. *J Immunol.* 2011;186(1):359-371.
40. Schneidewind A, Brumme ZL, Brumme CJ, et al. Transmission and long-term stability of compensated CD8 escape mutations. *J Virol.* 2009;83(8): 3993-3997.
41. Cawthon AG, Kroger CJ, Alexander-Miller MA. High avidity CD8+ T cells generated from CD28-deficient or wildtype mice exhibit a differential dependence on lipid raft integrity for activation. *Cell Immunol.* 2004;227(2):148-155.

42. Cawthon AG, Lu H, Alexander-Miller MA. Peptide requirement for CTL activation reflects the sensitivity to CD3 engagement: correlation with CD8alphabeta versus CD8alphaalpha expression. *J Immunol.* 2001;167(5):2577-2584.

43. Motozono C, Yanaka S, Tsumoto K, Takiguchi M, Ueno T. Impact of intrinsic cooperative thermodynamics of peptide-MHC complexes on antiviral activity of HIV-specific CTL. *J Immunol.* 2009; 182(9):5528-5536.

44. Park JH, Adoro S, Lucas PJ, et al. 'Coreceptor tuning': cytokine signals transcriptionally tailor CD8 coreceptor expression to the self-specificity of the TCR. *Nat Immunol.* 2007;8(10):1049-1059.

45. Schamel WW, Arechaga I, Risueno RM, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J Exp Med.* 2005;202(4):493-503.

46. Casartelli N, Guivel-Benhassine F, Bouziat R, Brandler S, Schwartz O, Moris A. The antiviral factor APOBEC3G improves CTL recognition of cultured HIV-infected T cells. *J Exp Med.* 2010; 207(1):39-49.

47. Labrecque N, Whitfield LS, Obst R, Waltzinger C, Benoist C, Mathis D. How much TCR does a T cell need? *Immunity.* 2001;15(1):71-82.

48. Learmont J, Tindall B, Evans L, et al. Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. *Lancet.* 1992; 340(8824):863-867.

49. Dyer WB, Ogg GS, Demoitie MA, et al. Strong human immunodeficiency virus (HIV)-specific cytotoxic T- lymphocyte activity in Sydney Blood Bank Cohort patients infected with nef-defective HIV type 1. *J Virol.* 1999;73(1):436-443.

50. Appay V, Iglesias MC. Antigen sensitivity and T-cell receptor avidity as critical determinants of HIV control. *Curr Opin HIV AIDS.* 2011;6(3):157-162.

Mwimanzi et al. Retrovirology 2011, 8:50
http://www.retrovirology.com/content/8/1/50

RETROVIROLOGY

SHORT REPORT Open Access

Effects of naturally-arising HIV Nef mutations on cytotoxic T lymphocyte recognition and Nef's functionality in primary macrophages

Philip Mwimanzi, Zafrul Hasan, Ranya Hassan, Shinya Suzu, Masafumi Takiguchi and Takamasa Ueno*

Abstract

Background: Although HIV can infect several cellular subsets, such as $CD4^+$ T lymphocytes and macrophages, it remains unclear whether an HIV infection in macrophages supports cytotoxic T lymphocyte (CTL) escape. Here, we tested two naturally-arising mutations located in the well-conserved polyproline region of Nef for their effects on CTL recognition, Nef's functionality, and viral replication capacity in macrophages. These mutations were selected because they are known to cause CTL escape in the context of T lymphocytes.

Findings: Monocyte-derived macrophages (MDMs) infected with the wild-type virus, but not with variant viruses, were efficiently killed by CTL clones targeting Nef epitopes, VY8 (VPLRPMTY) and RY11 (RPQVPLRPMTY). The CTL-escape mutation, $Arg^{75}Thr$, or $Arg^{75}Thr/Tyr^{85}Phe$ double mutation, reduced the HLA class I down-regulation activity and, interestingly, increased the susceptibility of virus-infected MDMs to recognition by CTLs targeting a different epitope. The same mutations reduced the CCR5, but not CD4, down-regulation activity. Moreover, the Nef variants were impaired for Hck activation and enhancement of viral replication in MDMs.

Conclusions: These results suggest that HIV-infected MDMs are killed by CTLs targeting Nef epitopes, contributing to selection and adaptation of CTL-escape viral variants.

Findings

Several different cellular subsets such as $CD4^+$ T lymphocytes, macrophages, and dendritic cells can be targets for an HIV infection; although they differentially support HIV replication and persistence *in vivo* [1-3]. Macrophages may be the early target of HIV, but are highly resistant to the cytopathic effects of an HIV infection and continuously produce infectious virions for a long period of time [4,5]. It is thought that the differences in fitness of viral replication among the different cellular environments could influence the selection and adaptation of viral quasispecies in these cells. The HLA class I-restricted $CD8^+$ cytotoxic T lymphocyte (CTL) response is thought to play an important role in controlling HIV replication [6-8] and to mediate a major selective force for the emergence of viral variants [9,10]. Certain CTL escape mutations, in well-conserved regions of Gag and Nef, have been reported to impose functional constraints on these proteins and to modulate viral replication in the context of T lymphocytes [11-13]. However, in the context of macrophages, the selection of CTL escape variants and functional adaptation of viral proteins are not yet fully understood. We previously showed that the HLA-B35-restricted CTL responses toward a well-conserved proline-rich region in Nef results in the emergence of a CTL escape mutation, either Arg75Thr or Tyr85Phe, from phylogenetically different viral quasispecies even within an HIV-infected host [13]. These mutations constrain some of the important Nef functions in $CD4^+$ T cells [13]. Here we tested whether an HIV-1 infection in macrophages would have any influence on CTL recognition and escape as well as Nef's functionality and adaptation in the infected macrophages.

Susceptibility of HIV-infected macrophages to recognition by the cognate CTLs

We previously reported that in HIV-infected patients with HLA-B35, the Nef protein elicits dominant CD8 T

* Correspondence: uenotaka@kumamoto-u.ac.jp
Center for AIDS Research, Kumamoto University, Kumamoto, Japan

cell responses [14], with the short epitope VY8 (Nef_{78-85}; VPLRPMTY) being the early epitope, which subsequently shifts to the amino terminal-extended longer epitope RY11 (Nef_{75-85}; RPQVPLRPMTY) [13]. Autologous virus sequence analysis revealed that the mutations Tyr85 to Phe (85F) and Arg75 to Thr (75T) are associated with the early and chronic phase of an HIV infection, respectively, in HIV-infected individuals with *HLA-B35* but that these 85F and 75T mutations are derived from phylogenetically different lineages [13].

We first examined CTL activity toward macrophages infected with HIV-1 strain JRFL, in which *nef* gene had been replaced with that of strain SF2 (referred as JRFL-SF2nef) and its variants. In this JRFL-SF2nef, we had created unique restriction sites, *Cla* I and *Not* I adjacent to the ends of the *nef* open reading frame [15] and confirmed that the resultant viruses, prepared by transfecting 293 T cells with JRFL and JRFL-SF2nef, had comparable replication capacity in primary monocyte-derived macrophages (MDMs) (data not shown). To prepare mature MDMs, $CD14^+$ cells were isolated from PBMCs of HIV-negative donors, in accordance with the human experimentation guidelines of Kumamoto University, and cultured for 7 days in the presence of 100 ng/ml of macrophage colony-stimulating factor (Peprotech GmbH, Germany). Previously established CTLs, specific for VY8 and RY11, [13,14] were highly cytotoxic toward MDMs infected with wild-type (wt) HIV-1, suggesting that HIV-infected MDMs were a preferable target for CTLs. The VY8-specific CTLs showed higher cytotoxicity toward wt virus-infected MDMs than did the RY11-specific CTLs (Figure 1), in good agreement with the observation obtained with HIV-infected $CD4^+$ T cells [13,16]. In contrast, VY8- and RY11-specific CTLs failed to kill primary MDMs infected with 85F and 75T viruses, respectively (Figure 1), indicating that the 85F and 75T single mutations conferred escape from CTLs specific for VY8 and RY11, respectively, but not simultaneously. In contrast, the TF virus could escape from both types of CTLs (Figure 1). It should be noted that Western blot analysis of Nef proteins in virus-producing cells showed a comparable level of Nef expression among wt and all variant viruses except for ΔNef (data not shown).

Effects of the Nef mutations on Hck activation

Nef is known to associate via its PxxP motif with the SH3 domain of several different cellular kinases including Hck [17,18]. We tested whether the CTL-escape variants in the PxxP region would affect the Hck activation by Nef by using the *in vitro* Hck activation assay as described earlier [19] (Figure 2A). Expectedly, the wild-type Nef showed robust Hck activation; whereas the AxxA variant Nef (Pro76Ala and Pro79Ala) did not show substantial activation (Figure 2B). The 85F variant Nef did not affect Hck activation, whereas the Hck activation was substantially reduced by the 75T and TF variants of Nef (Figure 2B). These results suggest that CTL-escape variants in the PxxP motif affect Hck activation in macrophages.

Effects of the Nef mutations on HLA class I down-regulation

Because Nef helps HIV-infected cells to evade CTL lysis by down-modulating cell-surface HLA-I and the PxxP motif is critical for this activity [13,20,21], we examined the HLA-I down-regulation activity by Nef in MDMs infected with wt and variant viruses by flow cytometry (Figure 3A). The surface levels of HLA-I within $p24^+$ subsets in wt virus-infected MDMs were much reduced compared with those in uninfected cells (Figure 3B) and that no HLA-I down-regulation was observed in ΔNef virus-infected MDMs (Figure 3B). In contrast, both the 75T and the TF variant viruses showed substantially diminished down-regulation activity; whereas the 85F variant virus showed down-regulation activity comparable to that of the wt (Figure 3B).

Susceptibility of HIV-infected MDMs to recognition by CTLs of another specificity

We postulated that the impaired Nef-mediated down-regulation activity of HLA-I in MDMs could influence the susceptibility to killing of HIV-infected MDMs by CTLs. To test this, we first created the variant virus having M20A or P82A (numbering based on the SF2 strain) because these mutations have been shown to completely disrupt the Nef-mediated HLA-I down-regulation activity [22,23]. We then assessed the cytolytic activity of CTL clones specific for another Nef epitope presented by HLA-A24 ($Nef_{138-147}$: RYPLTFGWCF) toward MDMs infected with wt, M20A, or P82A viruses. Although the amino-acid sequences in the epitope region of A24-Nef were the same among the wt and these variant viruses tested, the CTL-mediated killing activity toward MDMs infected with M20A and P82A variant viruses was much increased compared to those infected with the wt virus (Figure 4).

Next, we also determined CTL cytotoxic activity toward MDMs infected with 75T, 85F, and TF variant viruses. The A24-Nef CTLs showed the most potent activity toward MDMs infected with either the 75T or TF variant viruses; whereas their cytotoxic activity was less potent toward MDMs infected with either the wt or the 85F mutant virus (Figure 4). These data suggest that the diminished HLA-I down-regulation (i.e., increased level of cell-surface HLA-I) in MDMs infected with the 75T and the TF mutant viruses (Figure 3) resulted in increased susceptibility to killing by CTLs of another specificity (Figure 4), leading to a possible

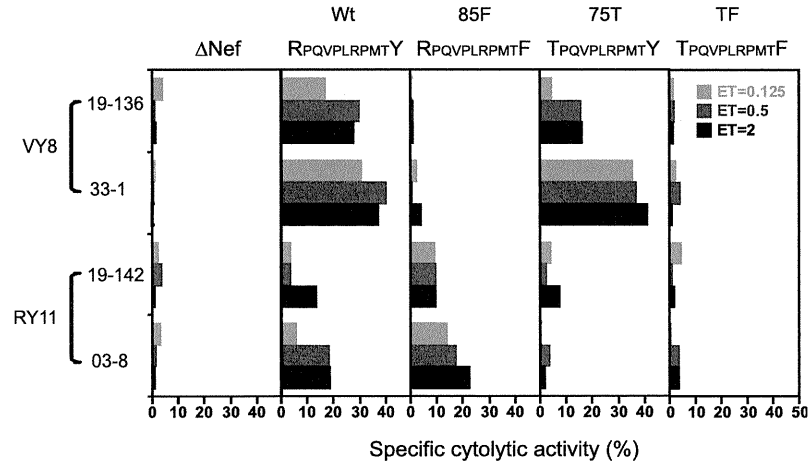

Figure 1 Susceptibility of HIV-infected MDMs to recognition by the cognate CTLs. Cytotoxic activity of HLA-B35-restricted CTL clones specific for VY8 (VPLRPMTY) and RY11 (RPQVPLRPMTY) epitopes in Nef toward HIV-infected MDMs is shown. MDMs were isolated from an HIV-negative donor (*HLA-B*35:01*$^{+}$) and infected with wild-type or one of the variant viruses indicated. The resultant HIV-infected MDMs (2×10^3/well) were then mixed with CTL clones at various effector-to-target cell ratios (E/T) for 6 hr at 37°C after having been labeled with ^{51}Cr. The frequency of HIV-infected cells among target cells as determined by intracellular p24 Ag expression was 48.7, 55.4, 51.0, and 48.8% for wt, 85F, 75T, and TF variants, respectively. CTL 19-136 and 19-142 were derived from the same HIV-infected donor (019), and CTL 33-1 and 03-8 were derived from different donors, 033 and 03, respectively. CTL activity toward uninfected cells was deducted from the data as background. An additional experiment showed similar results.

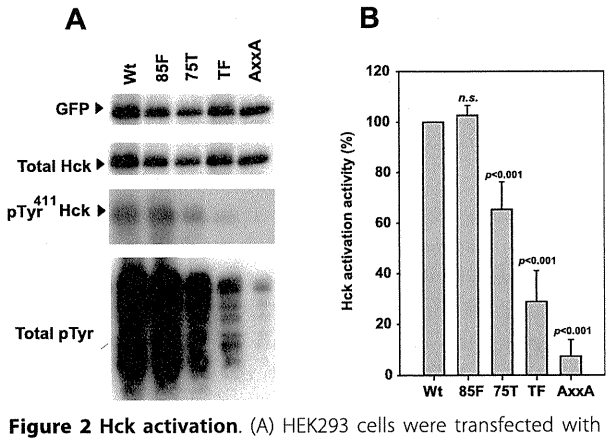

Figure 2 Hck activation. (A) HEK293 cells were transfected with cDNA encoding GFP alone or Nef-GFP fusion proteins in the presence of Hck plasmid, and analyzed by Western blotting with anti-GFP (FL; Santa Cruz), anti-Hck (clone 18; Transduction Laboratories, Lexington, KY), anti-Hck phosphorylated at tyrosine 411 (Hck-pTyr411; Santa Cruz) and anti-phosphotyrosine (PY99; Santa Cruz) as indicated. The Nef variants tested are indicated in the figure. A representative datum set of 3 independent experiments is shown. (B) Quantification of Hck activation by Nef. The indicated values represent the Hck activation activity after the level of phosphorylated Hck had been normalized to the amounts of total Hck. The values presented were calculated from the data shown in panel A, and are relative to the wild-type control arbitrarily set to 100%. Data represent the means ± SD of 3 independent experiments, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

selective disadvantage for the variant viruses under anti-HIV CTL responses.

Effects of the Nef mutations on down-regulation of viral receptors

We also examined whether Nef's down-regulation activity of viral receptors, i.e., CD4 and CCR5, could be influenced by the mutations in HIV-infected MDMs (Figure 5A). The cell-surface expression of CCR5 was substantially reduced in wt virus-infected MDMs but not affected in the ΔNef variant virus-infected ones (Figure 5B). Interestingly, the 85F variant virus showed CCR5 down-regulation activity comparable to that of the wt virus; whereas the 75T and TF variant were substantially impaired in this activity in MDMs (Figure 5B). In contrast, CD4 down-regulation activity was not affected for all of the viruses with mutated Nefs except for ΔNef (Figure 5C), consistent with the observation that CD4 down-regulation activity is mediated by a specific region in Nef other than the PxxP motif [21].

Effects of the Nef mutations on viral replication

We finally examined whether the mutations would differently affect the enhancement of viral replication in MDMs. In MDMs from 2 HIV-negative donors, the wt HIV-1 showed the highest replication among the various viruses tested; whereas the ΔNef variant showed much decreased replication (Figure 6A), consistent with the

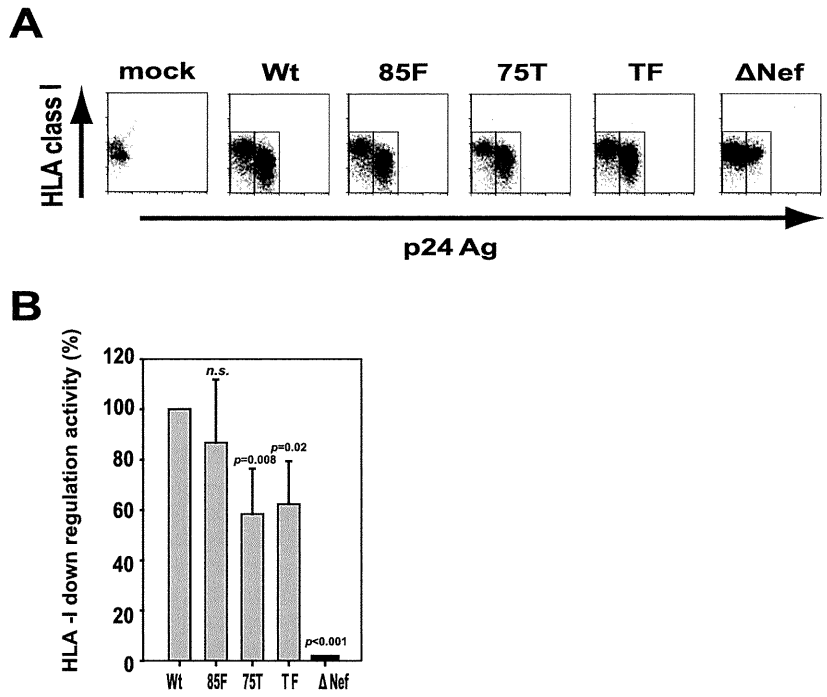

Figure 3 HLA class I down-regulation in HIV-infected MDMs. (A) MDMs prepared from an HIV-negative donor were infected with wild-type or variant viruses as indicated. Cells were stained with 7-amino-actinomycin D (7-AAD; BD Biosciences, CA) and anti-HLA class I allotype antibody SFR8-B6 followed by intracellular staining with FITC-labeled anti-p24 Gag mAb (KC-57; Beckman Coulter, CA) as described before [13]. In flow cytometric analysis (FACS Canto II, BD Biosciences), cells negative for 7-AAD were gated and analyzed for their fluorescence intensity for HLA class I and p24 Gag. (B) The same experiment as above was done by using 3 additional HIV-negative donors. The HLA class I allotype-specific antibodies used were either SFR8-B6 or A11,1 M as appropriate for each donor. The relative down-regulation activity of HLA class I by wt Nef and its variants is presented relative to that of the wild-type Nef activity set to 100%. Data represent the means ± SD of all 4 donors, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

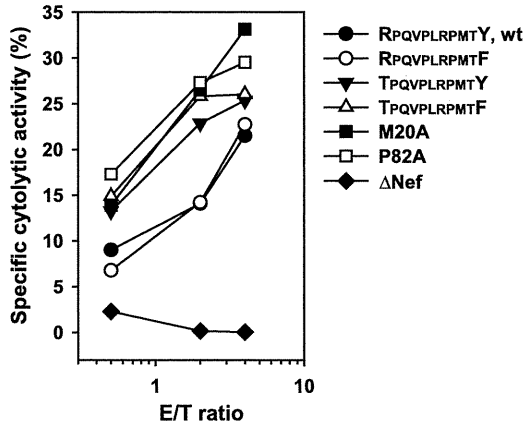

Figure 4 Susceptibility of HIV-infected MDMs to recognition by CTLs of another specificity. MDMs prepared from an HIV-negative donor (*HLA-A*24:02*$^+$) infected with the indicated viruses were used as target cells for cytolysis by an HLA-A24-restricted CTL clone specific for the Nef epitope ($Nef_{138-147}$: RYPLTFGWCF). The frequency of HIV-infected cells among the target cells, as determined by intracellular p24 Ag expression, was 41.4, 48.3, 44.5, 40.8, 40.0, and 45.0% for wt, 85F, 75T, TF, M20A, and P82A variants, respectively. CTL activity toward uninfected cells was deducted from the data as background. An additional experiment showed similar results.

previous observation [24]. The replication of the 85F variant virus was partially impaired in MDMs from one of the donors and was comparable to that of the wt virus in MDMs from the other donor (Figure 6A). In contrast, the replication of the 75T and TF variant viruses was impaired in MDMs from both donors (Figure 6A). To account for this donor variability, we summarized the results from a total of 5 donors in Figure 6B. Because the peak of the virus replication was between 6 to 12 days after infection, depending on the donor and the virus, the peak p24 Ag values of each of the viruses are presented and were used for statistical analysis (Figure 6B). The 75T and the TF variant viruses showed significantly diminished capacity for viral replication compared with the wt; whereas the 85F virus did not show much difference in replication capacity (Figure 6B).

Discussion and Conclusions

Although the Nef protein is thought to have very high mutational plasticity, we showed here that the naturally-arising CTL escape variants in the well-conserved PxxP region in Nef alone or in combination can modulate some pathogenic functions of Nef in the

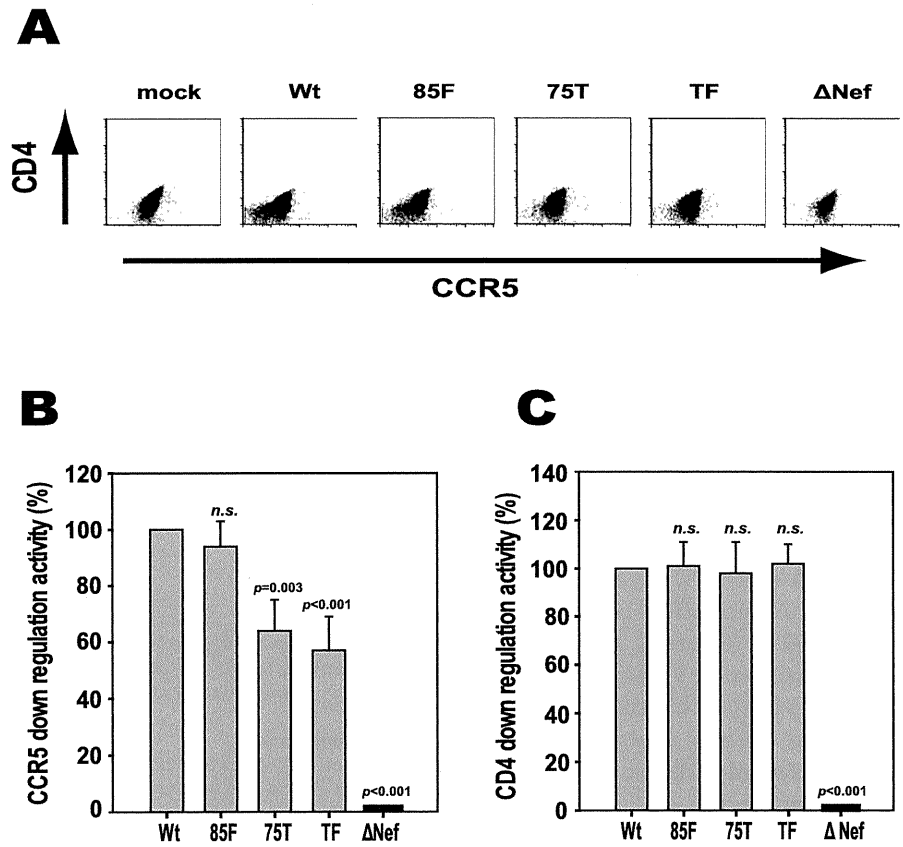

Figure 5 Viral receptor down-regulation in HIV-infected MDMs. (A) MDMs prepared from an HIV-negative donor were infected with wt or variant viruses as indicated. The cells were stained with 7-AAD and allophycocyanin-Cy7 anti-human CD4 mAb (Biolegend, CA) and phycoerythrin-Cy7-conjugated anti-human CCR5 mAb (BD Biosciences) followed by intracellular staining with antibody against p24 Gag. In flow cytometric analysis, cells negative for 7-AAD and positive for p24 Gag were gated and analyzed for their fluorescence intensity for CD4 and CCR5. (B, C) The same experiment as above was done by using 3 additional HIV-negative donors. The relative down-regulation activity of wt Nef and its various variants toward CCR5 (panel B) and CD4 (panel C) is presented, with the wt Nef activity set to 100%. Data represent the means ± SD of all 4 donors, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

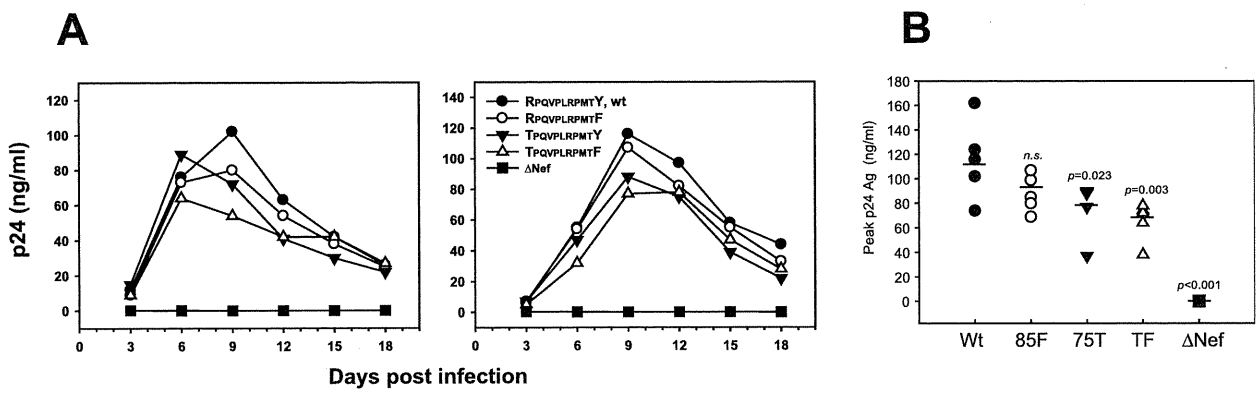

Figure 6 HIV replication in MDMs. (A) MDMs prepared from 2 HIV-negative donors were incubated at 3 × 10^5 cells/well in a 96-flat bottomed well plate, infected for 6 hr with wt or variant HIV-1 at 10 ng of p24 antigens, and then continuously cultured at 37°C in fresh culture medium for an additional 18 days. Culture supernatants were collected and replaced with fresh medium every 3 days. To monitor viral replication, the concentration of p24 Gag in the culture supernatant was quantified by ELISA for their p24 Gag concentration (ZeptoMetrix Corp. Buffalo, NY). (B) The same experiment was done by using MDMs prepared from 3 additional HIV-negative donors. The peak p24 Ag values were plotted and statistically analyzed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant. Horizontal bars represent the means of data obtained from all 5 donors

context of human primary macrophages infected with a CCR5-tropic virus. There are 2 different aspects of CTL-mediated functional constraints on the PxxP-dependent Nef activities in MDMs reported here, one through immune evasion activity (HLA-I down-regulation activity) and the other acting on the intrinsic capacity to boost viral replication and persistence (Hck activation, viral co-receptor down-regulation activity, and enhancement of viral replication). In particular, one of the single mutants, 75T, impaired these Nef activities in MDMs infected with a CCR5-tropic virus. This is in line with the previous report showing that 75T mutation modulated Nef-stimulated viral replication in immature dendritic cell/T cell cocultures infected with a CCR5-tropic virus [25] although this mutation alone had virtually no influence on the same Nef activities in primary $CD4^+$ T cells infected with a CXCR4-tropic virus in the previous study [13]. In addition, the 75T mutation, located outside the VY8 epitope, reduced the cytolytic activity of VY8-specific CTLs in the context of $CD4^+$ T cells [13], but did not affect their cytolytic activity in the context of MDMs (Figure 1), suggesting the differential intracellular processing of the VY8 peptide between $CD4^+$ T cells and MDMs. This observation is in line with the previous report showing a substantial difference in intracellular processing of antigenic HIV peptides between monocytes and lymphocytes [26]. Overall, these results suggest that an antigenic variation of viruses can differentially influence viral replication and persistence between cellular subsets because of their different effects on the intracellular antigen-processing machinery, the susceptibility to CTL killing, as well as the fitness cost to viral replication.

Of particular interest are the data showing that the CTL-escape Nef mutation, 75T, impaired HLA-I down-regulation activity by Nef and rendered the HIV-infected MDMs more susceptible to killing by CTLs with another specificity. Such phenomenon was also observed in the context of $CD4^+$ T cells in our previous study [13]. However, wt-virus-infected cells, regardless of $CD4^+$ T cells or MDMs, could be killed to some extent by CTLs, suggesting that the Nef-mediated HLA-I down-regulation is insufficient for HIV to escape from CTL recognition and that, CTL-escape variant viruses are selected and emerged. Conversely, Swigut *et al.*, [27] reported that monkeys infected with SIV containing *nef* mutations that selectively eliminated MHC down-regulation activity exhibited higher level of SIV-specific CD8 T cell responses. In any event, an important question remains to be addressed which is how significant is Nef-mediated HLA-I down-regulation activity for HIV replication and persistence in HIV-infected humans.

Although HLA-B*35-restricted CTLs targeting PxxP region of Nef can impose functional constraints in viral replication in this study, we did not find any beneficial effects on clinical parameters (such as CD4 count and viral load) in HIV-infected patients with HLA-B*35 as well as those with HLA-B*35 and HLA-A*24 in our cohort to date (data not shown). Functional impairment in Nef induced by CTL-escape variants could be compensated later by mutations at secondary sites in Nef. For example, an inverse dose-response relationship has been observed between the number of CTL-escape mutations in Nef and CD4 counts in patients in a large population study [28]. Therefore, only some CTL-escape variants may play a role in modulating Nef functions *in vivo*, such as in the case of $HLA\text{-}B57^+$ elite suppressors [29]. Further studies using a large number of clinically-isolated *nef* alleles are needed to extend this observation, such as how Nef-specific CTL responses, Nef functions, and clinical outcome of HIV-infected individuals are related to each other at the population level.

Acknowledgements
We thank Dr. M Fujiwara and Ms. S. Doki for their great help. This research was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan and by a grant-in-aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan.

Authors' contributions
PM, MT, and TU designed the study. PM, ZH, RH, SS, and TU conducted the experiments. PM, SS, and TU wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 11 March 2011 Accepted: 22 June 2011
Published: 22 June 2011

References

1. Eckstein DA, Penn ML, Korin YD, Scripture-Adams DD, Zack JA, Kreisberg JF, Roederer M, Sherman MP, Chin PS, Goldsmith MA: **HIV-1 actively replicates in naive CD4+ T cells residing within human lymphoid tissues.** *Immunity* 2001, **15**:671-682.
2. Keele BF, Tazi L, Gartner S, Liu Y, Burgon TB, Estes JD, Thacker TC, Crandall KA, McArthur JC, Burton GF: **Characterization of the follicular dendritic cell reservoir of human immunodeficiency virus type 1.** *J Virol* 2008, **82**:5548-5561.
3. Zhu T, Muthui D, Holte S, Nickle D, Feng F, Brodie S, Hwangbo Y, Mullins JI, Corey L: **Evidence for human immunodeficiency virus type 1 replication in vivo in CD14+ monocytes and its potential role as a source of virus in patients on highly active antiretroviral therapy.** *J Virol* 2002, **76**:707-716.
4. Aquaro S, Bagnarelli P, Guenci T, De Luca A, Clementi M, Balestra E, Caliò R, Perno CF: **Long-term survival and virus production in human primary macrophages infected by human immunodeficiency virus.** *J Med Virol* 2002, **68**:479-488.
5. Brown A, Zhang H, Lopez P, Pardo CA, Gartner S: **In vitro modeling of the HIV-macrophage reservoir.** *J Leu Biol* 2006, **80**:1127-1135.
6. Borrow PH, Lewicki BH, Hahn GM, Shaw MB, Oldstone : **Virus-specific $CD8^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.** *J Virol* 1994, **68**:6103-6110.
7. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD: **Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.** *J Virol* 1994, **68**:4650-4655.
8. Ogg GS, Jin X, Bonhoeffer S, Dunbar PR, Nowak MA, Monard S, Segal JP, Cao Y, Rowland-Jones SL, Cerundolo V, *et al*: **Quantitation of HIV-1-specific**

cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 1998, **279**:2103.
9. Goulder PJR, Watkins DI: **HIV and SIV CTL escape: implications for vaccine design.** *Nat Rev Immunol* 2004, **4**:630-640.
10. Motozono C, Mwimanzi P, Ueno T: **Dynamic interplay between viral adaptation and immune recognition during HIV-1 infection.** *Protein & Cell* 2010, **1**:514-519.
11. Brockman MA, Schneidewind A, Lahaie M, Schmidt A, Miura T, DeSouza I, Ryvkin F, Derdeyn CA, Allen S, Hunter E, *et al*: **Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A.** *J Virol* 2007, **81**:12608-12618.
12. Schneidewind A, Brockman MA, Sidney J, Wang YE, Chen H, Suscovich TJ, Li B, Adam RI, Allgaier RL, Mothe BR, *et al*: **Structural and functional constraints limit options for cytotoxic T-lymphocyte escape in the immunodominant HLA-B27-restricted epitope in human immunodeficiency virus type 1 capsid.** *J Virol* 2008, **82**:5594-5605.
13. Ueno T, Motozono C, Dohki S, Mwimanzi P, Rauch S, Fackler OT, Oka S, Takiguchi M: **CTL-mediated selective pressure influences dynamic evolution and pathogenic functions of HIV-1 Nef.** *J Immunol* 2008, **180**:1107-1116.
14. Ueno T, Idegami Y, Motozono C, Oka S, Takiguchi M: **Altering effects of antigenic variations in HIV-1 on antiviral effectiveness of HIV-specific CTLs.** *J Immunol* 2007, **178**:5513-5523.
15. Fujiwara M, Takiguchi M: **HIV-1-specific CTLs effectively suppress replication of HIV-1 in HIV-1-infected macrophages.** *Blood* 2007, **109**:4832-4838.
16. Motozono C, Yanaka S, Tsumoto K, Takiguchi M, Ueno T: **Impact of intrinsic cooperative thermodynamics of peptide-MHC complexes on antiviral activity of HIV-specific CTL.** *J Immunol* 2009, **182**:5528-5536.
17. Briggs SD, Sharkey M, Stevenson M, Smithgall TE: **SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1.** *J Biol Chem* 1997, **272**:17899-17902.
18. Trible RP, Emert-Sedlak L, Smithgall TE: **HIV-1 Nef selectively activates Src family kinases Hck, Lyn, and c-Src through direct SH3 domain interaction.** *J Biol Chem* 2006, **281**:27029-27038.
19. Hassan R, Suzu S, Hiyoshi M, Takahashi-Makise N, Ueno T, Agatsuma T, Akari H, Komano J, Takebe Y, Motoyoshi K, Okada S: **Dys-regulated activation of a Src tyrosine kinase Hck at the Golgi disturbs N-glycosylation of a cytokine receptor Fms.** *J Cell Physiol* 2009, **221**:458-468.
20. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D: **HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes.** *Nature* 1998, **391**:397-401.
21. Saksela K, Cheng G, Baltimore D: **Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4.** *Embo J* 1995, **14**:484-491.
22. Akari H, Arold S, Fukumori T, Okazaki T, Strebel K, Adachi A: **Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation.** *J Virol* 2000, **74**:2907-2912.
23. Yamada T, Kaji N, Odawara T, Chiba J, Iwamoto A, Kitamura Y: **Proline 78 is crucial for human immunodeficiency virus type 1 Nef to down-regulate class I human leukocyte antigen.** *J Virol* 2003, **77**:1589-1594.
24. Miller MD, Warmerdam MT, Gaston I, Greene WC, Feinberg MB: **The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages.** *J Exp Med* 1994, **179**:101-113.
25. Fackler OT, Wolf D, Weber HO, Laffert B, D'Aloja P, Schuler-Thurner B, Geffin R, Saksela K, Geyer M, Peterlin BM: **A natural variability in the proline-rich motif of Nef modulates HIV-1 replication in primary T cells.** *Current Biology* 2001, **11**:1294-1299.
26. Lazaro E, Godfrey SB, Stamegna P, Ogbechie T, Kerrigan C, Zhang M, Walker BD, Le Gall S: **Differential HIV epitope processing in monocytes and CD4 T cells affects cytotoxic T lymphocyte recognition.** *J Infect Dis* 2009, **200**:236-243.
27. Swigut T, Alexander L, Morgan J, Lifson J, Mansfield KG, Lang S, Johnson RP, Skowronski J, Desrosiers R: **Impact of Nef-mediated downregulation of major histocompatibility complex class I on immune response to simian immunodeficiency virus.** *J Virol* 2004, **78**:13335-13344.
28. Brumme ZL, Brumme CJ, Heckerman D, Korber BT, Daniels M, Carlson J, Kadie C, Bhattacharya T, Chui C, Szinger J, *et al*: **Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1.** *PLoS Pathogens* 2007, **3**:e94.
29. Bailey JR, Brennan TP, O'Connell KA, Siliciano RF, Blankson JN: **Evidence of CD8+ T-cell-mediated selective pressure on human immunodeficiency virus type 1 nef in HLA-B*57+ elite suppressors.** *J Virol* 2009, **83**:88-97.

doi:10.1186/1742-4690-8-50
Cite this article as: Mwimanzi *et al.*: Effects of naturally-arising HIV Nef mutations on cytotoxic T lymphocyte recognition and Nef's functionality in primary macrophages. *Retrovirology* 2011 **8**:50.