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Incomplete APOBEC3G/F Neutralization by HIV-1 Vif Mutants Facilitates the Genetic Evolution from CCR5 to CXCR4 Usage

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Incomplete APOBEC3G/F neutralization by a defective HIV-1Vif protein can promote genetic diversification by inducing G-to-A mutations in the HIV-1 genome. The HIV-1 Env V3 loop, critical for coreceptor usage, contains several putative APOBEC3G/F target sites. Here, we determined if APOBEC3G/F, in the presence of Vif-defective HIV-1 virus, can induce G-to-A mutations at V3 positions critical to modulation of CXCR4 usage. Peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) from 2 HIV-1-negative donors were infected with CCR5-using 81.A-Vif$_{WT}$ virus (i.e., with wild-type [WT] Vif protein), 81.A-Vif$_{E45G}$, or 81.A-Vif$_{K22E}$ (known to incompletely/partially neutralize APOBEC3G/F). The rate of G-toA mutations was zero or extremely low in 81.A-Vif$_{WT}$- and 81.A-Vif$_{E45G}$-infected PBMC from both donors. Conversely, G-to-A enrichment was detected in 81.A-Vif$_{K22E}$-infected PBMC (prevalence ranging from 2.18% at 7 days postinfection [dpi] to 3.07% at 21 dpi in donor 1 and from 10.49% at 7 dpi to 8.69% at 21 dpi in donor 2). A similar scenario was found in MDM. G-to-A mutations occurred at 8 V3 positions, resulting in nonsynonymous amino acid substitutions. Of them, G24E and E25K strongly correlated with phenotypically/genotypically defined CXCR4-using viruses ($P = 0.04$ and 5.5e−7, respectively) and increased the CXCR4 N-terminal binding affinity for V3 (WT, −40.1 kcal/mol; G24E, −510 kcal/mol; E25K, −522 kcal/mol). The analysis of paired V3 and Vif DNA sequences from 84 HIV-1-infected patients showed that the presence of a Vif-defective virus correlated with CXCR4 usage in proviral DNA ($P = 0.04$). In conclusion, incomplete APOBEC3G/F neutralization by a single Vif amino acid substitution seeds a CXCR4-using proviral reservoir. This can have implications for the success of CCR5 antagonist-based therapy, as well as for the risk of disease progression.

APOBEC3 is an important component of the cellular innate immunity against exogenous and endogenous retroviruses (1). Several members of the APOBEC3 family (A to H) show different degrees of antiviral potency, with APOBEC3G and APOBEC3F generally regarded as exerting the strongest HIV-1 restriction in peripheral blood mononuclear cells (PBMC) and in monocyte-derived-macrophages (MDM), respectively (2–5).

APOBEC3G/F proteins are packaged into HIV-1 virions and associate with the reverse transcription (RT) complex. There, they deaminate cytosine residues to uracil in the single-stranded DNA minus strand, resulting in proviral guanosine-to-adenosine (G-to-A) nucleotide mutations in the plus-strand DNA (6, 7). These mutations predominantly occur in a GG or GA dinucleotide context, which are the preferential (but not exclusive) target sites for APOBEC3G and APOBEC3F, respectively (2, 6). Previous studies have shown that the frequency of G-to-A nucleotide mutations increases over the genome in the 5′-to-3′ direction, with the lowest frequency in the 5′ long terminal repeat and the highest in the 5′ portion of the *nef* gene sequence (6). Beyond G-to-A mutations, C-to-T nucleotide mutations in plus-strand DNA have also been described (6).

The activity of APOBEC3G/F is generally counteracted by the HIV-1 regulatory protein Vif by promoting proteasome-dependent APOBEC3G/F degradation (8) and inhibiting packaging in the producer cell (9). However, the activity of Vif against APOBEC3G/F is not absolute. Indeed, some Vif mutants fail (to different extents) to neutralize these enzymes (2, 10–13). For instance, previous *in vitro* studies showed that in the presence of the Vif amino acid substitution K22E, Vif completely loses the ability to neutralize APOBEC3G (implying APOBEC3G editing of the viral genome) and retains partial activity against APOBEC3F (implying less editing of APOBEC3F than APOBEC3G). In the presence of the Vif amino acid substitution E45G, Vif retained weak activity against APOBEC3G (implying a low rate of APOBEC3G editing) and completely neutralized APOBEC3F activity (implying no or limited APOBEC3F editing) (2).

Previous studies have shown that these two Vif amino acid substitutions can facilitate the emergence of the G-to-A mutation

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81.A V3 loop SEQUENCE					
Number of Codons	1	2	3	4	5
Nucleotides	TGT	ACA	AGA	CCC	AAC
Amino Acids	C	T	R	P	N
Number of Codons	6	7	8	9	10
Nucleotides	AAC	AAT	ACA	AGA	AAA
Amino Acids	N	N	T	R	K
Number of Codons	11	12	13	14	15
Nucleotides	AGT	ATA	CAT	ATA	GGA
Amino Acids	S	I	H	I	G
Number of Codons	16	17	18	19	20
Nucleotides	CCA	GGC	AGA	GCA	TTT
Amino Acids	P	G	R	A	F
Number of Codons	21	22	23	24	25
Nucleotides	TAT	ACA	ACA	GGA	GAA
Amino Acids	Y	T	T	G	E
Number of Codons	26	27	28	29	30
Nucleotides	ATA	ATA	GGA	GAT	ATA
Amino Acids	I	I	G	D	I
Number of Codons	31	32	33	34	35
Nucleotides	AGA	CAA	GCA	CAT	TGT
Amino Acids	R	Q	A	H	C

FIG 1 Nucleotide and amino acid sequences of the V3 loop of the 81.A virus. GG or GA motifs are underlined. V3 positions 11, 24, and 25 (known to impact HIV-1 coreceptor usage) are shown in gray boxes.

at the HIV-1 RT position 184, which is associated with lamivudine resistance (M184I) (14). It has been postulated that the predominant effect of APOBEC3G-driven rather than APOBEC3F-driven cytidine deamination reflects the impairment of these Vif mutants (K22E and E45G) to efficiently neutralize the deamination activity of APOBEC3G rather than APOBEC3F (14).

In the presence of other HIV-1 variants with suboptimal Vif activity, the limited APOBEC3G/F-driven editing may also contribute to genetic diversity and thus benefit the virus by facilitating escape from other antiretroviral drugs (such as rilpivirine or thymidine analogues) and also cellular immunity (11, 12, 15–19). HIV-1 sequences carrying the genetic footprints of past deamination events have been detected in treated and untreated chronically infected patients (20, 21), vertically infected infants (22), and long-term nonprogressors (23), suggesting that variations in Vif activity against APOBEC3G/F can occur in many different clinical settings.

Among the different domains of HIV-1 gp120, the V3 loop is recognized as the primary determinant for HIV-1 coreceptor usage (24–26). The V3 loop contains several putative APOBEC3G/F target sites, including positions 24 and 25, which are known to be critical for coreceptor usage (Fig. 1) (27–35). Recent studies supposed a role of APOBEC3 proteins in inducing G-to-A nucleotide mutations in the V3 region (3, 31). However, those studies were limited to *in silico* observations or to investigation of members of the APOBEC3 family other than APOBEC3G/F (such as APOBEC3D).

Based on ultradeep 454 pyrosequencing (UDPS), we recently suggested a role for APOBEC3G in inducing G-to-A nucleotide mutations in the V3 region of a CXCR4-using strain (NL4.3) with mutations at Vif positions 22 and 45 (36). These results provided the rationale to investigate whether K22E and E45G in Vif can affect the burden of G-to-A nucleotide mutations at V3 positions critical for CXCR4 usage in a pure CCR5-using virus, such as 81.A, thus facilitating the genetic evolution from CCR5 to CXCR4 coreceptor usage. In order to also support this hypothesis *in vivo*, we investigated the correlation of Vif variability with coreceptor usage in a set of 84 matched Vif/V3 sequences from HIV-1 subtype B-infected patients.

MATERIALS AND METHODS

Cells. PBMC and MDM were obtained from the blood of healthy seronegative donors by separation over a Ficoll-Hypaque gradient. PBMC were seeded in a T25 flask (Costar) at a density of 1.0×10^6 cells/ml in RPMI 1640 supplemented with 100 U/ml penicillin plus 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum (FBS) in addition to 2 μg/ml of phytohemagglutinin (PHA; Sigma) and 50 U/ml recombinant interleukin-2 (IL-2; Peprotech). In order to prepare MDM, PBMC were resuspended in RPMI 1640 medium supplemented with 20% FBS and then seeded into 48-well plates (1.8×10^6 cells/well). After 5 days, nonadherent cells were carefully removed by repeated washings. The number of MDM was estimated to be 10^5/well at the time of infection. Adherent cells obtained using this technique generally consisted of pure differentiated MDM (upon cytofluorimetric analysis, more than 95% of cells were $CD14^+$ $CD4^+$ $CD3^-$). HEK-293T cells, generously donated by M. Zazzi, were grown in a 37°C humidified atmosphere containing 5% CO_2, using complete Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum.

Plasmids and transfection. The CCR5-using HIV-1 p81.A plasmid (obtained from Bruce Chesebro [37–40] and gently provided by A. Cara) was used in all of the experiments. The characteristics and genomic sequence of this strain have been previously described (41). HIV-1 Vif amino acid substitutions K22E and E45G, known to reduce Vif activity against APOBEC3G/F (2, 14), were introduced into the p81.A wild-type backbone by site-directed mutagenesis (QuikChange II XL site-directed mutagenesis kit, Agilent) according to the manufacturer's instructions. Plasmid DNA was further isolated by using the Qiaprep plasmid midiprep kit (Qiagen). HEK-293T cells were plated in 6-well plates (200,000 cells/well) 24 h before transfection. On the day of transfection, cell confluence was approximately 70 to 80%. The different plasmids used for the study were transfected using the Xtremegene 9 reagent (Roche) according to the manufacturer. At day 3 posttransfection, the viruses were harvested and filtered, and aliquots were stored at −80°C. In order to quantify the viral particle amounts, supernatants were analyzed for p24 production by using a commercial enzyme-linked immunosorbent assay kit (Ag-Genscreen HIV-1 assay; Bio-Rad) according to the manufacturer's instructions.

Virus infection in primary cells. In order to evaluate the editing activity of APOBEC3G/F in human primary cells, stimulated PBMC and MDM were infected with 10,000 pg/ml of p24 for 2 h of adsorption. Then, cells were extensively washed to remove any residual viral particles, resuspended in complete medium (1 ml), and cultured on 48-well plates for 21 days (PBMC) or 28 days (MDM). In the case of PBMC, samples were cocultivated every 7 days with new stimulated PBMC (500,000 cells/well) from different donors. Every 7 days, both cells and supernatants were harvested and stored at −20°C and −80°C. Virus growth was monitored at all times postinfection by measuring supernatant p24 levels, as described above. Sequencing of the proviral Vif region at the end of each infection revealed maintenance of the specific Vif mutations.

In order to extract viral nucleic acids from both PBMC and MDM, infected cells were harvested, and after gently washing with warm phosphate-buffered saline they were counted, dry pelleted, and stored at −20°C. Experiments were performed at least in triplicate, using two different HIV-seronegative donors.

Ultradeep V3 pyrosequencing. (i) Nucleic acid extraction. HIV-1 DNA was extracted from each cellular pellet by using the QIAamp blood DNA minikit (Qiagen, Courtaboeuf, France).

(ii) Pyrosequencing. UDPS was performed using a 454 GS Junior pyrosequencing system. A 367-nucleotide (nt) fragment that included the V3 *env* region (nt 901 to 1,005 of the HIV-1 p81.A gp120 *env* gene, corresponding to V3 amino acids [aa] 1 to 32) was generated by nested PCR using a bar code-modified primer after a first amplification under the following conditions: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 35 s, and a final extension at 72°C for 7 min. For this first amplification we used the forward primer V3S10, 5′-CCAATTC CCATACATTATTGT-3′ (nt 649 to 669 of the HIV-1 p81.A gp120 *env* gene), and the reverse primer V3AS5, 5′-CTTCTCCAATTGTCCCTC A-3′ (nt 1421 to 1439).

For UDPS, a nested PCR was performed with the following primers: inner forward primer V3S5, 5′-GTTAAATGGCAGTCTAGCAG-3′ (nt 790 to 814), and reverse primer V3AS1, 5′-GAAAAATTCCCCTCCACA ATT-3′ (nt 1138 to 1158). Nested PCR was performed with the Fast Start HiFi PCR system (Roche Diagnostics, Mannheim, Germany) under the following conditions: 1 cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 67°C for 30 s, and 72°C for 35 s, and a final extension at 72°C for 7 min. The amplified products were purified using Agencourt AMPure PCR purification beads (Beckman Coulter, Brea, CA) and quantified with the Quant-iT PicoGreen double-stranded DNA assay kit (Life Technologies, Eugene, OR, USA) on a GloMax multidetection system (Promega, Madison, WI, USA). Pooled PCR products were clonally amplified on captured beads in water-oil emulsion microreactors. A total of 500,000 DNA-enriched samples were deposited in the wells of a full GS Junior Titanium PicoTiterPlate device and pyrosequenced in both the forward and reverse directions. The 200 nucleotide cycles were performed in a 10-h sequencing run. Phylogenetic analyses excluded any possibly of sample contamination (data not shown). For each run, a maximum of 5 samples (including a plasmid control) were loaded to ensure adequate coverage of sequences.

(iii) Bioinformatic analyses of V3 sequences. The V3 *env* region sequences obtained after 454 pyrosequencing were demultiplexed and quantified using the SFF tool (Roche). By using a home-made Perl script that utilizes a BLAST algorithm, all reads were checked for homology with reference strains (>80%), and the orientation was discerned (42). After this filtering, SHORAH package 0.5.1 (43) was used to correct sequences for homopolymeric region-associated errors. Subsequently, the sequences were clustered, taking into account read redundancy and forward/reverse directions, using the Exonerate 2.2.0 package, and aligned against the plasmid reference using the Needleman-Wunsch algorithm (EMBOSS package) (44–46). The final alignments were manually checked for insertions or deletions in the homopolymeric region that would result in a frameshift. Nucleotidic variants were evaluated and quantified with a home-made Perl script.

(iv) Sensitivity of UDPS for detecting minor V3 *env* variants. The frequency of errors resulting from V3 amplification and deep sequencing was assessed for the 5 plasmid controls used. The mismatch nucleotide error rate was very low after sequencing correction (<0.006% for each plasmid). Furthermore, considering only the variants covered in both orientations in more than 5 reads, we did not find a mismatch in the plasmid controls examined; thus, only variants with these characteristics were considered reliable for the analyses. To evaluate the linkage of variants in each sequence, only reads covering the entire HIV-1 V3 *env* region were considered.

HIV-1 tropism determination of V3 sequences obtained by UDPS. **(i) *In silico* analyses.** V3 sequences with at least one amino acid substitution induced by a G-to-A deamination event (obtained in previous experiments) were first submitted to the geno2pheno algorithm (http://coreceptor.geno2pheno.org/) to evaluate the false-positive rate (FPR) and the related HIV coreceptor usage. The G-to-A-induced amino acid substitutions, conferring a reduction of the FPR compared to 81.A-Vif$_{WT}$, were further investigated for their association with a dual/pure CXCR4 tropism *in vivo* by using an independent data set of 251 HIV-1 subtype B RNA samples obtained for routine clinical practice. For each sample, coreceptor usage was determined both phenotypically (by using the enhanced sensitivity version of Trofile) and genotypically (by V3 sequencing followed by using the geno2pheno prediction set at an FPR of 10%) (47). The prevalence of G-to-A-induced amino acid substitutions was determined for 192 V3 sequences reported to be CCR5 tropic by both genotypic and phenotypic tests and 59 V3 sequences reported to be pure CXCR4 tropic or dual/mixed tropic by the enhanced sensitivity version of Trofile and confirmed CXCR4 tropic by a genotypic tropism test. Chi-square tests of independence were performed to identify statistically significant differences between the 2 groups of samples.

Finally, a structural analysis was also performed to evaluate the impact of these G-to-A-induced amino acid substitutions on CCR5 or CXCR4 recognition, according to methods described elsewhere (36, 48). Detailed information about our methodology is available in Text S1 in the supplemental material.

(ii) *In vitro* analysis. In the *in vitro* experiments, we focused on V3 amino acid substitutions conferring an FPR of <10% (which was used as a threshold to infer CXCR4 coreceptor usage). These amino acid substitutions were first introduced in the p81.A wild-type backbone by site-directed mutagenesis, as reported in the previous section. Viral stocks obtained after transfection were used to infect the PM1 cell line, which predominantly expresses the CCR5 coreceptor, and the C8166 cell line, which exclusively expresses the CXCR4 coreceptor (49). Wild-type 81.A and NL4.3 viruses were used as controls.

PM1 and C8166 were infected with 20,000 pg/ml of HIV-1 p24 with an overnight adsorption step. Then, cells were extensively washed to remove any residual viral particles, resuspended in complete medium (1 ml), and cultured on 48-well plates for 10 days in triplicate. HIV-1 replication was estimated 10 days postinfection by measuring the p24 released into culture supernatants. NL4.3 and 81.A were used as controls for C8166 and PM1 cell infection, respectively.

***In vivo* correlation between the presence of Vif-defective isolates and non-CCR5 usage in proviral DNA.** The correlation between the presence of a defective Vif and CXCR4 usage was assessed by analyzing paired Vif and V3 proviral DNA sequences obtained from PBMC of 84 HIV-1 subtype B-infected patients under suppressive, highly active antiretroviral therapy (HAART; patients with a viral load of <50 copies/ml). In accordance with the European Guidelines, an FPR set at 20% by the geno2pheno algorithm was used to infer HIV tropism (47). The protocol for V3 population sequencing has been previously described (50), while the protocol for Vif population sequencing is reported in Text S2 in our supplemental material. A Vif protein was considered defective if it contained any stop codon or at least one substitution at the following amino acid positions described in the literature as impairing Vif activity: 21, 22, 32, 38, 40, 45, 69, 108, 115, 138, 139, 150, 114, and 133 (2, 11, 14, 51, 52). HIV-1 DNA quantification was performed according to the methodology described in reference 50. The correlation between the presence of a defective Vif and CXCR4 usage was analyzed with the chi-square test of independence and a two-by-two contingency table. The correlation between the presence of a defective Vif and the amount of HIV-1 DNA (expressed in copies per 10^6 cells) was analyzed by using the Mann-Whitney test.

RESULTS

Viral replication of 81.A strains with Vif variants in human primary cells. We first evaluated the replication capacity of the CCR5-using 81.A-Vif$_{WT}$, 81.A-Vif$_{K22E}$, and 81.A-Vif$_{E45G}$ viruses by measuring the amount of p24 released in culture supernatants at different time points postinfection. 81.A-Vif$_{WT}$ replicated with mean (± standard deviation [SD]) p24 production ranging from 12,296 ± 4,748 pg/ml at 7 days to 7,849,353 ± 5,767,237 pg/ml at 21 days postinfection in PBMC from donor 1, and from 85,371 ± 20,722 pg/ml at 7 days to 294,968 ± 14,088 pg/ml at 21 days postinfection in PBMC from donor 2 (Fig. 2A and B). Similar

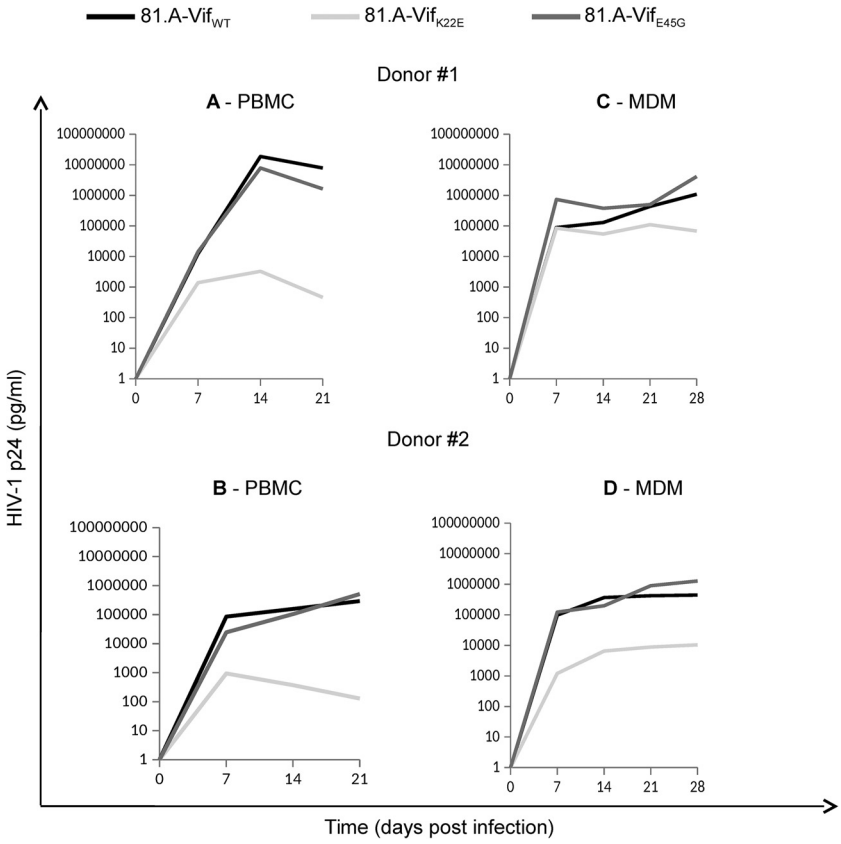

FIG 2 Replication capacities of 81.A-Vif$_{WT}$, 81.A-Vif$_{K22E}$, and 81.A-Vif$_{E45G}$ in PBMC (A and B) and MDM (C and D) obtained from 2 different donors. PBMC and MDM were infected with 10,000 pg p24 per 10^6 cells. Donor 1 and donor 2 are the two different donors of the study. Mean values for at least 3 wells for each point are shown. Error bars indicate intraexperimental standard deviations.

results were obtained for MDM, with a mean ± SD p24 production ranging from 88,459 ± 3,522 pg/ml at 7 days to 1,090,044 ± 809,661 pg/ml at 28 days postinfection from donor 1 and from 97,867 ± 6,879 pg/ml at 7 days to 442,188 ± 148,422 pg/ml at 28 days postinfection from donor 2 (Fig. 2C and D).

While the replication characteristics of 81.A-Vif$_{E45G}$ were comparable to those observed for 81.A-Vif$_{WT}$, a remarkable decrease in HIV replication capacity was observed in PBMC infected with 81.A-Vif$_{K22E}$. Indeed, 81.A-Vif$_{K22E}$ replicated with a mean p24 production ranging from 1,387 ± 238 pg/ml at 7 days to 457.5 ± 72 pg/ml at 21 days postinfection from donor 1 and from 938 ± 405 pg/ml at 7 days to 129 ± 29 pg/ml at 21 days postinfection from donor 2 (Fig. 2A and B). An attenuated defect in viral replication capacity was observed in MDM infected with 81.A-Vif$_{K22E}$, with mean p24 production ranging from 84,217 ± 2,999 pg/ml at 7 days to 68,286 ± 58,147 pg/ml at 28 days postinfection from donor 1 and from 1,201 ± 157 pg/ml at 7 days to 10,419 ± 833 pg/ml at 28 days postinfection from donor 2 (Fig. 2C and D). The replication profiles of Vif mutants 81.A-Vif$_{K22E}$ and 81.A-Vif$_{E45G}$ were superimposable to those observed by Mulder et al. (14).

The expression levels of APOBEC3G and APOBEC3F were confirmed by Western blotting in both PBMC and MDM (see Fig. S1 in the supplemental material). Overall, these data suggested that single-nucleotide mutations affecting Vif activity can interfere with viral fitness at endogenous APOBEC3G/F expression levels in both PBMC and MDM.

HIV-1 Vif mutants and overall sequence diversity in V3. By using UDPS, the mean (± SD) number of V3 sequences obtained after infection was 16,641 ± 1,164 for PBMC and 12,614 ± 723 for MDM.

Overall, the median number of G-to-A nucleotide mutations detected in V3 sequences by UDPS was 1 (range, 0 to 2). In particular, no V3 nucleotide substitutions were detected in HIV-1 DNA from 81.A-Vif$_{WT}$-infected PBMC (carrying a functional Vif gene) for either donor (Table 1). Similarly, an extremely low rate of nucleotide variations (exclusively due to non-APOBEC3G-related mutations) was detected in 81.A-Vif$_{E45G}$-infected PBMC (Table 1). A different scenario was observed in PBMC infected with 81.A-Vif$_{K22E}$ (which fails to completely neutralize APOBEC3G and, partially, APOBEC3F). In particular, V3 sequences with at least one G-to-A nucleotide mutation were detected at each time point postinfection, with a prevalence ranging from 2.07% to 3.07% in donor 1 and from 6.37% to 10.49% in donor 2. These mutations accounted for 95.96% of V3 mutated sequences.

Similarly, V3 sequences with nucleotide substitutions were not detected in 81.A-Vif$_{WT}$-infected MDM and with a very low prevalence in 81.A-Vif$_{E45G}$-infected MDM (all corresponding to non-APOBEC3-related mutations) (Table 1). Conversely, in 81.A-Vif$_{K22E}$-infected MDM, V3 sequences with at least one G-to-A nucleotide mutation were detected at 14 and 21 days postinfection, with prevalence rates of 5.95% and 5.24% for donor 1 and

TABLE 1 Number and type of V3 sequences obtained by UDPS at different times postinfection in viruses with WT or a variant Vif

Cell type and source	Virus	Day postinfection	Total no. of V3 sequences	No. (%) of V3 sequences with at least one change of[a]: G to A (overall)	G to A (in GG motif)	G to A (in GA motif)	C to T	Other mutations
PBMC								
Donor 1	81.A-Vif$_{WT}$	7	14,622	0	0	0	0	0
		14	20,024	0	0	0	0	0
		21	14,351	0	0	0	0	0
	81.A-Vif$_{K22E}$	7	16,720	363 (2.18)	282 (77.69)	81 (22.32)	0	0
		14	10,672	220 (2.07)	79 (35.91)	141 (64.1)	0	0
		21	10,364	318 (3.07)	318 (100)	7 (2.21)	0	0
	81.A-Vif$_{E45G}$	7	20,024	0	0	0	0	0
		14	11,719	0	0	0	0	0
		21	11,324	0	0	0	0	138 (1.22)
Donor 2	81.A-Vif$_{WT}$	7	27,466	0	0	0	0	0
		14	22,248	0	0	0	0	0
		21	23,046	0	0	0	0	0
	81.A-Vif$_{K22E}$	7	19,323	2,026 (10.49)	2,026 (100)	242 (11.95)	0	0
		14	23,345	1,485 (6.37)	1,485 (100)	124[b] (8.36)	0	139 (0.6)
		21	24,896	2,161 (8.69)	1,811 (83.81)	51 (2.37)	0	0
	81.A-Vif$_{E45G}$	7	20,363	0	0	0	0	0
		14	17,943	0	0	0	0	0
		21	15,730	0	0	0	0	0
Total			324,180	6,573 (2.03)	600 (91.3)	646 (9.83)	0	277 (0.09)
MDM								
Donor 1	81.A-Vif$_{WT}$	14	12,229	0	0	0	0	0
		21	11,056	0	0	0	0	209 (1.9)
		28	4,972	0	0	0	0	0
	81.A-Vif$_{K22E}$	14	13,872	824 (5.95)	824 (100)	0	0	0
		21	10,279	538 (5.24)	538 (100)	0	1,290[c] (12.55)	15 (0.15)
		28	5,481	0	0	0	0	0
	81.A-Vif$_{E45G}$	14	13,690	0	0	0	0	0
		21	11,861	*0*	0	0	0	0
		28	14,433	0	0	0	0	0
Donor 2	81.A-Vif$_{WT}$	14	13,202	0	0	0	0	19 (0.15)
		21	15,847	0	0	0	0	7 (0.05)
		28	14,358	0	0	0	0	0
	81.A-Vif$_{K22E}$	14	12,181	116 (0.96)	116 (100)	0	0	0
		21	16,494	7 (0.05)	7 (100)	0	0	0
		28	14,546	0	0	0	0	0
	81.A-Vif$_{E45G}$	14	11,869	0	0	0	0	7 (0.06)
		21	11,394	0	0	0	0	0
		28	14,299	0	0	0	0	138 (0.97)
Total			222,063	1,485 (0.67)	1,485 (100)	0	1,290 (0.59)	395 (0.18)

[a] The number and prevalence of V3 sequences with at least one G-to-A mutation, with at least one C-to-T mutation, and with any other nucleotide mutations are shown. The numbers of G-to-A V3 mutated sequences that were found in a GG or GA dinucleotide context were also determined.
[b] All of these G-to-A mutations in the GA context were present in the same haplotype containing G-to-A mutations in the GG context.
[c] A total of 97 of these C-to-T mutations were present in the same haplotype containing G-to-A mutations.

0.96% and 0.05% for donor 2 (Table 1). It is noteworthy that V3 sequences with at least one C-to-T nucleotide mutation (also related to APOBEC3 deamination events [6]) were detected in 81.A-Vif$_{K22E}$-infected MDM from donor 1 at 21 days postinfection, at a prevalence of 12.55%.

The increased genetic diversity in conjunction with attenuated replication capacity observed with the 81.A-Vif$_{K22E}$ mutant strain was in line with previous studies that showed a lower plasma viremia in HIV-1-infected patients with G-to-A-enriched proviruses (21).

In PBMC and MDM, 91.3% and 100% of V3 sequences with at least one G-to-A nucleotide mutation occurred in a GG context, known to be the preferential target site for APOBEC3G (Table 1).

Genotypic characterization of V3 HIV-1 DNA derived from 81.A-Vif$_{K22E}$. The G-to-A nucleotide mutations occurring in HIV-1 DNA from 81.A-Vif$_{K22E}$-infected PBMC encode amino acid substitutions at four V3 positions in donor 1 (aa positions 15, 24, 25, and 28) and at six V3 positions in donor 2 (aa positions 15, 17, 19, 24, 28, and 29) (Fig. 3). In both donors, the G-to-A nucleotide mutations generated G15R, G24E, and G28R, which were detected at prevalence rates reaching 2.73%, 1.18%, and 0.49% in donor 1 and 6.24%, 0.53%, and 5.62% in donor 2, respectively.

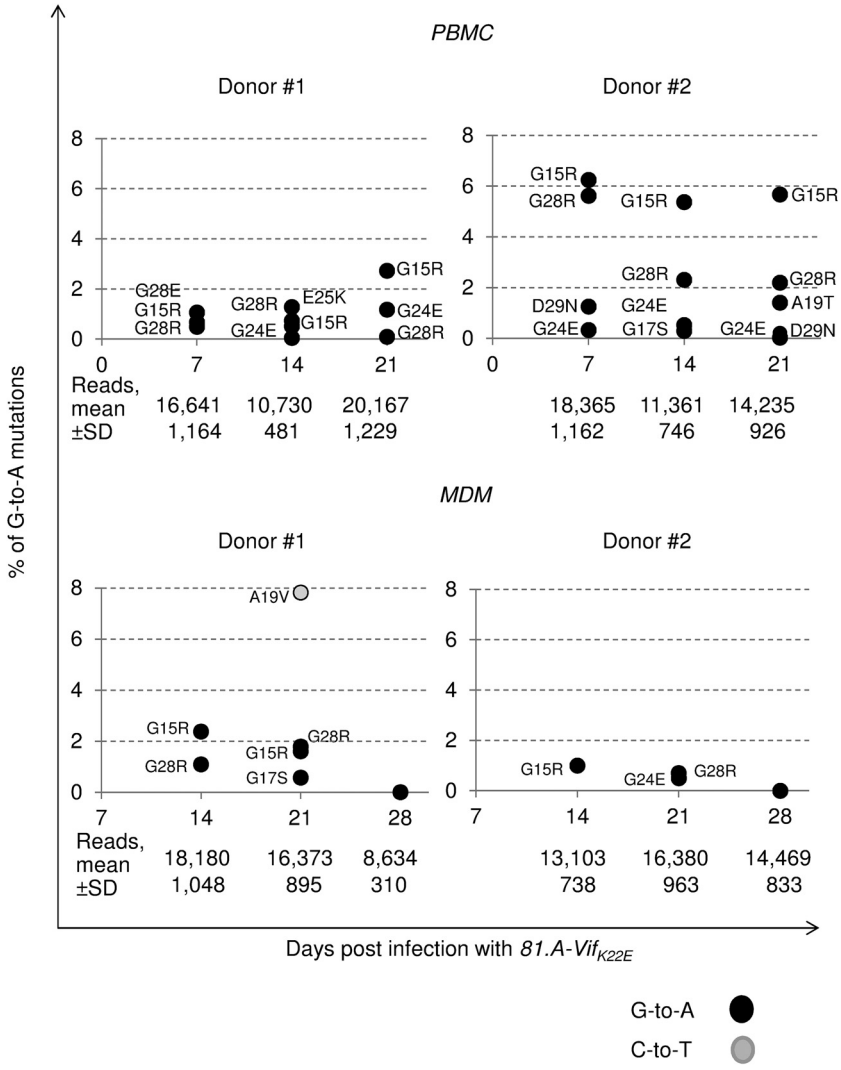

FIG 3 Prevalence of the single G-to-A and C-to-T mutations detected in HIV DNA extracted from 81.A$_{K22E}$-infected PBMC at 7, 14, and 21 days postinfection and from 81.A$_{K22E}$-infected MDM at 14, 21, and 28 days postinfection. Mutations present in more than 4 reads with both forward and reverse primers or in more than 50 reads in only one sense were retained for the analysis.

Other amino acid substitutions induced by G-to-A mutations were exclusively selected in donor 1 or donor 2, such as E25K (prevalence, 1.28%) or G17S and A19T (prevalence, 0.3% and 1.4%, respectively). These prevalence rates are similar to those observed in other *in vitro* studies that analyzed the emergence of G-to-A nucleotide mutations in other regions of the HIV-1 genome (11, 12, 14, 16, 18, 19).

Similarly, in MDM, 81.A-Vif$_{K22E}$ generated a pool of G-to-A nucleotide mutations encoding G15R, G17S, G24E, and G28R (Fig. 3), with a prevalence ranging from 0.58% to 2.38% in donor 1 and from 0.07% to 0.9% in donor 2. As reported in the previous paragraph, C-to-T mutations also occurred at 21 days postinfection of MDM from donor 1 and resulted in the nonsynonymous substitution A19V.

By haplotype analysis, the V3 amino acid substitutions G15R and G28R frequently occurred in combination in 81.A-Vif$_{K22E}$-infected PBMC, suggesting the occurrence of >1 deamination event along the V3 region (Table 2).

V3 amino acid substitutions induced by G-to-A nucleotide mutations and their association with CCR5 or CXCR4 usage. (i) *In silico* analyses. As a next step of this study, we evaluated the impact of each single V3 amino acid substitution, detected in the previous analysis, on genotypic predictions of HIV-1 tropism by using the geno2pheno algorithm (Table 2). In this analysis, three amino acid substitutions (A19V, G24E, and E25K) determined a reduction of the FPR compared to that observed for 81.A-Vif$_{WT}$ virus (FPR, 27.70% for 81.A, 13.8% for A19V, 6.8% for G24E, and 5.0% for E25K). Among them, G24E and E25K are associated with an FPR of <10% (classically used as a threshold to define non-CCR5 usage) (47).

The role of A19V, G24E, and E25K (conferring a reduction in the FPR compared to 81.A-Vif$_{WT}$) in modulating coreceptor usage was then investigated by using 251 HIV-1 subtype B RNA samples obtained from a routine clinical practice (192 samples reported to be CCR5 tropic and 59 samples reported to be CXCR4 tropic or dual/mixed tropic by both genotypic tropism test [FPR

TABLE 2 V3 sequences with G-to-A mutations demonstrated via UDPS

Cell type and donor no.	Virus	Day postinfection	No. of V3 sequences[a]	V3 amino acid sequence[b]	G-to-A or C-to-T mutation(s)	Corresponding amino acid substitution(s) in V3 motif	FPR (%)[c]
PBMC							
Donor 1	81.A-Vif$_{K22E}$	7	176	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5
			106	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
			81	CTRPNNNTRKSIHIGPGRAFYTTGEII**E**DIRQAHC	GGA28GAA	G28E in GG	54.4
		14	136	CTRPNNNTRKSIHIGPGRAFYTTG**K**IIGDIRQAHC	GAA25AAA	E25K in GA	**5**
			56	CTRPNNNTRKSIHI**R**PGRAFYTTGEII**R**DIRQAHC	GGA15AGA, GGA28AGA	G15R in GG, G28R in GG	65.4
			23	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5
			5	CTRPNNNTRKSIHIGPGRAFYTT**E**EIIGDIRQAHC	GGA24GAA	G24E in GG	**6.8**
		21	258	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
			53	CTRPNNNTRKSIHI**R**PGRAFYTTGEII**R**DIRQAHC	GGA15AGA, GGA28AGA	G15R in GG, G28R in GG	65.4
			7	CTRPNNNTRKSIHIGPGRAFYTT**E**EII**R**DIRQAHC	GGA24GAA, GGA28AGA	G24E in GG, G28R in GG	**9.6**
Donor 2	81.A-VifK$_{22E}$	7	1,052	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
			732	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5
			215	CTRPNNNTRKSIHIGPGRAFYTTGEII**RN**IRQAHC	GGA28AGA, GAT29AAT	G28R in GG, D29N in GG	46.7
			17	CTRPNNNTRKSIHI**R**PGRAFYTT**E**EIIGDIRQAHC	GGA15AGA, GGA24GAA	G15R in GG, G24E in GG	**9.6**
			10	CTRPNNNTRKSIHI**R**PGRAFYTTGEII**R**DIRQAHC	GGA15AGA, GGA28AGA	G15R in GG, G28R in GG	65.4
		14	774	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
			409	CTRPNNNTRKSIHI**R**PGRAFYTTGEII**R**DIRQAHC	GGA15AGA, GGA28AGA	G15R in GG, G28R in GG	65.4
			124	CTRPNNNTRKSIHIGPGRAFYTT**E**EII**R**DIRQAHC	GGA24GAA, GGA28AGA	G24E in GG, G28R in GG	**9.6**
			109	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5
			69	CTRPNNNTRKSIHI**RPS**RAFYTTGEIIGDIRQAHC	GGA15AGA, GGC17AGC	G15R in GG, G17S in GG	68.6
		21	1,265	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
			350	CTRPNNNTRKSIHIGPGR**T**FYTTGEIIGDIRQAHC	GCA19ACA	A19T in GC	58.6
			346	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5
			149	CTRPNNNTRKSIHI**R**PGRAFYTTGEII**R**DIRQAHC	GGA15AGA, GGA28AGA	G15R in GG, G28R in GG	65.4
			51	CTRPNNNTRKSIHIGPGRAFYTTGEII**RN**IRQAHC	GGA28AGA, GAT29AAT	G28R in GG, D29N in GG	46.7
MDM							
Donor 1	81.A-Vif$_{K22E}$	14	566	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
			253	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5
			5	CTRPNNNTRKSIHI**R**PGRAFYTTGEII**R**DIRQAHC	GGA15AGA, GGA28AGA	G15R in GG, G28R in GG	65.4
		21	1,193	CTRPNNNTRKSIHIGPGR**V**FYTTGEIIGDIRQAHC	GCA19GTA	A19V in GT	13.8
			181	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
			97	CTRPNNNTRKSIHIGPGR**V**FYTTGEII**R**DIRQAHC	GCA19GTA, GGA28AGA	A19V in GT, G28R in GG	23.6
			94	CTRPNNNTRKSIHIGP**S**RAFYTTGEIIGDIRQAHC	GGC17AGC	G17S in GG	46.7
			88	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5
			78	CTRPNNNTRKSIHI**R**PGRAFYTTGEII**R**DIRQAHC	GGA15AGA, GGA28AGA	G15R in GG, G28R in GG	65.4
Donor 2	81.A-Vif$_{K22E}$	14	116	CTRPNNNTRKSIHI**R**PGRAFYTTGEIIGDIRQAHC	GGA15AGA	G15R in GG	44.1
	81.A-Vif$_{K22E}$	21	7	CTRPNNNTRKSIHIGPGRAFYTTGEII**R**DIRQAHC	GGA28AGA	G28R in GG	43.5

[a] Number of sequences obtained by UDPS.
[b] Amino acids shown in boldface indicate substitutions induced by the G-to-A or C-to-T mutations.
[c] FPRs below the cutoff of 10% are shown in bold.

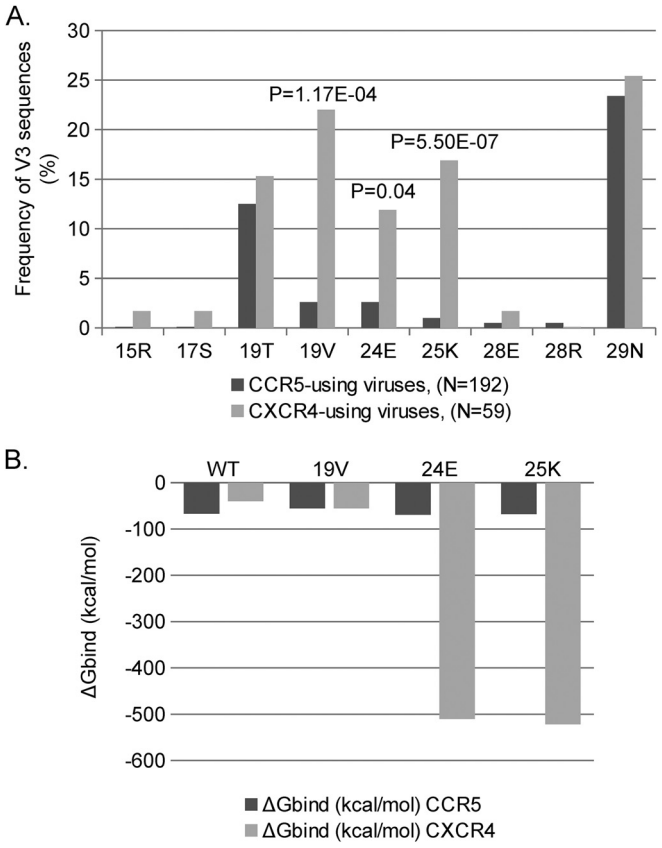

FIG 4 (A) Combined phenotypic and genotypic tropism predictions of HIV-1 strains containing V3 amino acid substitutions induced by G-to-A nucleotide mutations. The analysis was performed on 192 samples reported R5 tropic by both genotypic and phenotypic tests and on 59 samples reported X4/DM tropic by Trofile HS and X4 tropic by genotypic test (FPR set at 10%). Statistically significant differences were assessed by chi-square tests of independence. *P* values in bold were confirmed also after correction for multiple comparisons by the Benjamini and Hochberg method (70), using a false-positive rate of 0.05. (B) Evaluation of the free energy of complexation, calculated for all gp120 optimized complexes in the presence of both coreceptors, CCR5 and CXCR4. ΔG_{bind}, free energy of complexation for all gp120 optimized complexes in the presence of both coreceptors CCR5 and CXCR4.

set at 10%] and the enhanced sensitivity version of the Trofile assay). In this analysis, G24E and E25K occurred in ≤2% of V3 sequences from CCR5-using viruses and in >10% of V3 sequences from dual/pure CXCR4-tropic viruses ($P = 0.04$ and 5.5e−7, respectively) (Fig. 4A). Interestingly, the amino acid substitution A19V, characterized by an FPR of 13.8%, was also significantly associated with a dual/pure CXCR4 tropism ($P = 1.7e{-}4$) (Fig. 4A).

Finally, we conducted a docking analysis to investigate the effects of G24E, E25K, and A19V on the binding affinity between gp120 and the CXCR4 or CCR5 coreceptor. We observed a remarkable increase in CXCR4 affinity for gp120 in the presence of either G24E or E25K (ΔG_{bind}, −510.0 and −522.0, respectively) compared to WT (ΔG_{bind}, −40.1 kcal/mol) (Fig. 4B). Accordingly to its FPR of 13.8%, A19V was related to a slight CCR5 thermodynamic disadvantage and with a CXCR4 thermodynamic advantage compared with the WT (CCR5, −67.2 kcal/mol for WT and −55.4 kcal/mol for A19V; CXCR4, −40.1 kcal/mol for WT and −55.2 kcal/mol for A19V) (Fig. 4B).

(ii) *In vitro* analysis. For *in vitro* experiments, we focused on G24E and E25K, both of which confer an FPR of <10%. We introduced these amino acid substitutions in the pure CCR5-using virus 81.A by site-directed mutagenesis and then we evaluated their ability to replicate in PM1 cells, which predominantly express the CCR5 coreceptor, and C8166 cells, which exclusively express the CXCR4 coreceptor (47). 81.A and NL4.3 replicated in PM1 and C8166 cells with a mean p24 production of 20,732.4 ± 714 and 638,583 ± 49,166 pg/ml at 10 days postinfection, respectively. Although no p24 production was observed in C8166 cells infected by G24E and E25K, a strong reduction (>90%) of HIV-1 replication was observed in PM1 at 10 days postinfection for both amino acid substitutions (124 ± 7.3 and 1,585 ± 620 pg/ml, respectively). These results suggested that G24E and E25K can seriously affect the ability of a pure CCR5-using virus to bind the CCR5 coreceptor, posing the genetic bases for evolution toward a dual or a pure CXCR4-using virus and supporting the need for multiple amino acid substitutions in the tropism switch.

***In vivo* correlation between the presence of Vif-defective isolates and CXCR4 usage in proviral DNA.** To validate the *in vitro* and structural results, we evaluated the correlation between the presence of a partially defective Vif and the presence of CXCR4-using proviruses in 84 HIV-1-infected patients under suppressive HAART.

Overall, 41 Vif sequences (48.8%) carried markers of a defective Vif (stop codon and/or amino acid substitution known to affect Vif activity) (2, 11, 14, 51, 52). In particular, at least one stop codon was detected in 6 (7.1%) Vif sequences (at positions 90, 98, 120, 153, 170, and 174), while the following substitutions known to affect Vif activity were detected: K22N ($n = 20$; prevalence, 22.6%), K22H ($n = 9$; 10.7%), E45D ($n = 7$; 9.5%), E45Q ($n = 1$; 1.2%), W38G ($n = 1$; 1.2%), C114G ($n = 1$; 1.2%), and G138R ($n = 1$; 1.2%).

The analysis of paired V3 and Vif DNA sequences showed that proviral CXCR4-using strains have a higher probability of having a partially defective Vif protein; in particular, a partially defective Vif protein was observed in 61.8% of CXCR4-tropic viruses (21/34), versus 40.0% of CCR5-tropic viruses (20/50; $P = 0.04$) (Fig. 5A).

In addition, the presence of a defective Vif correlated with a level of total HIV-1 DNA lower than that observed in the wild type (median [interquartile range], 1,183 [292 to 2,307] versus 2,367 [558 to 8,232]; $P = 0.04$), in line with a defect in viral replication capacity (Fig. 5B).

DISCUSSION

In this study, we described an increased number of V3 sequences that had at least one G-to-A nucleotide mutation in the presence of suboptimal Vif activity. Some of these G-to-A nucleotide mutations in V3 corresponded to amino acid substitutions (such as G24E and E25K) that are associated with the acquisition of a genotypically and phenotypically defined dual/pure CXCR4 tropism and with an increased affinity for the CXCR4 N terminus. Their role in conferring CXCR4 tropism is well known and was established in several *in vitro* and *in vivo* studies (25–30, 48, 53, 54). Our *in vitro* model (based on PM1 and C8166 cells) also suggests that G24E and E25K can seriously affect the ability of pure CCR5-using 81.A to replicate in cells predominantly expressing the CCR5 coreceptor. These amino acid substitutions might thus pose the genetic bases for the evolution toward a dual/pure CXCR4 tropism.

A.

	CCR5-using virus (N=50)	CXCR4-using virus (N=34)	P-value
Defective VIF[§]	20 (40.0)	21 (61.8)	0.04

B.

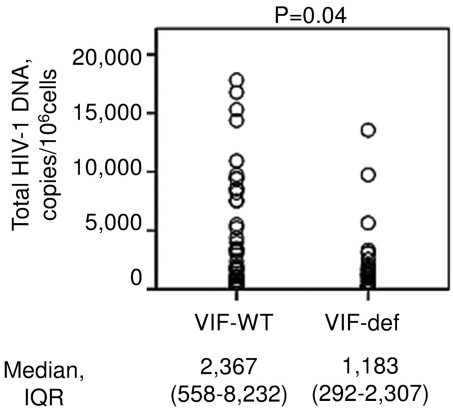

FIG 5 (A) Correlation between a defective Vif gene and CXCR4 coreceptor usage. (B) Quantitative HIV-1 DNA (expressed in copies per 10^6 cells) in patients infected with HIV-1 subtype B with defective Vif (Vif-def) or nondefective Vif (Vif-WT). IQR, interquartile range.

It is conceivable that multiple amino acid substitutions are necessary to confer a pure CXCR4 tropism, supporting the complex phenomenon underlying the tropism switch.

The *in vitro* results were supported by the findings of an *in vivo* analysis based on 84 paired V3 and Vif sequences from proviral DNA. In this analysis, we found a significant correlation between the presence of amino acid substitutions known to hamper Vif activity and CXCR4 usage.

By analyzing the overall variability of the V3 region, 395 nucleotide substitutions, not accounting for G-to-A or C-to-T mutations, were detected in a total of 222,063 V3 sequences, with a base substitution rate of 1.8 per 10^5 bases. This datum is only slightly lower than the base substitution error rate estimated for HIV-1 (3 nucleotide substitutions per 10^5 bases) (55). The nucleotide mutations increased to 3,170 when we also considered G-to-A and C-to-T events, with the base mutation rate increasing to 14.9 per 10^5 bases. These findings support the role of APOBEC3G/F-driven mutagenesis in increasing the extent of V3 genetic variability and in potentially facilitating viral evolution toward a dual/pure CXCR4 tropism.

Our results are in line with previous studies that showed that, in the presence of amino acid substitutions that hamper Vif activity, APOBEC3G/F can contribute to HIV-1 genetic diversification (2, 10–15, 19, 56, 57). Thus, natural variability in Vif should be taken into account in clinical studies aimed at evaluating the possible use of pharmacological modifiers of APOBEC3G/F expression as an alternative strategy to increase the natural protection against HIV replication (58).

In our study, V3 G-to-A nucleotide mutations were detected in the presence of the Vif amino acid substitution K22E and not in the presence of E45G. This may reflect the different impacts of K22E and E45G on the ability of Vif to neutralize APOBEC3G and APOBEC3F (2). Indeed (and as also reported in the introduction), in the presence of K22E, Vif completely loses the ability to neutralize APOBEC3G (implying APOBEC3G editing of the viral genome) and retains partial activity against APOBEC3F (implying less editing by APOBEC3F than APOBEC3G). Conversely, in the presence of E45G, Vif retained weak activity against APOBEC3G but completely neutralized APOBEC3F activity (2).

A smaller number of G-to-A nucleotide mutations were detected in MDM than in PBMC. This can be explained by the fact that K22E completely abrogates Vif's ability to neutralize APOBEC3G (predominantly expressed in PBMC) and partially affects Vif activity to neutralize APOBEC3F (predominantly expressed in MDM) (2, 4, 18). This result also can be explained by the fact that MDM are nondividing, terminally differentiated cells with a smaller pool of endogenous nucleotides that in turn can slow down the reverse transcriptase activity and, consequently, the incorporation of G-to-A mutations by APOBEC3G (59).

Furthermore, it should be stressed that in this study we evaluated the rate of deamination events in the presence of endogenous expression levels of APOBEC3G/F. This allowed us to determine differences in the amount of these nucleotide mutations between donor 1 and donor 2.

However, the detection of G-to-A mutations in proviral DNA from MDM may play a relevant pathogenic role, considering that MDM are widely distributed in all tissues and organs and can fuse with $CD4^+$ lymphocytes by transferring the virus to these cells (60–62).

Understanding the driving forces leading to the emergence of CXCR4-using strains is of lively interest. CXCR4-using viruses generally emerge during the late phase of HIV infection (63, 64). However, they have been found even during primary infection, with a prevalence estimated at 3% to 6.4% by population-based phenotyping and up to 17.2% by population-based sequencing, and they have been to be associated with more rapid disease progression, even in the setting of primary infection (65–67).

The transmission of CXCR4-using viruses may be favored by a

defect in the expression of CCR5 at the cell surface, such as a 32-bp deletion in the CCR5 gene (68). Other studies suggest that CCR5-using viruses are selectively transmitted and that CXCR4-using viruses emerge later through the acquisition of mutations in the gp120 gene (69). All these hypotheses are not mutually exclusive, and together they provide explanations for the time profile of HIV-1 tropism during an infection. Our study suggests that the APOBEC3G-driven mutagenesis can also play a role in this phenomenon. This hypothesis is also consistent with recent *in vivo* and *in vitro* studies (3, 31). By analyzing 26 longitudinal samples from 10 patients, Heger et al. described an *in vivo* increase of G-to-A mutations (all occurring in a GG or GA dinucleotide context) coding for substitutions at positions 22, 24, and 25, which are associated with CXCR4 usage (3). Recently, Sato et al. revealed that APOBEC3D/F can promote the emergence of E25K, and in turn the ability to bind the CXCR4 coreceptor (31). The emergence of E25K was also observed in our study, mainly via an APOBEC3G deamination event. Our overall findings support the role of these enzymes in the evolution from CCR5 toward CXCR4 tropism. This may also have implications for the success of CCR5 antagonist-based therapy, as well as for the risk of disease progression.

Interestingly, Pido-Lopez et al. (69) found that the stimulation of the CCR5 receptor with CCL3, one of its natural ligands, increases the mRNA levels of APOBEC3G. It is possible that HIV binding to the CCR5 coreceptor induces the expression of APOBEC3G proteins, thus facilitating mutations in the coreceptor-relevant sites. This concept deserves further investigation.

A limitation of this study is that we have not determined if the viruses resulting from the activity of APOBEC3G/F editing are replication competent. We can speculate that the stable frequency over time of G-to-A mutations detected in PBMC, cocultured every 7 days with new PBMC from a healthy donor, can be consistent with continuous replication (at a low level) of these viral strains. Interestingly, by analyzing the gp120 regions flanking the V3 nucleotide sequence, we detected the presence of a stop codon at 81.A gp120 position 342 in only 4.8% and 20.5% of G-to-A mutated sequences from PBMC and MDM infections, respectively, resulting from the TGG-to-TAG mutation at the tryptophan position. In the remaining 95.2% and 79.5% of G-to-A mutated sequences, no stop codons were found, suggesting that these HIV-1 DNA sequences may be capable of infecting new cells. In addition, as proposed by Mulder et al. (14), it is possible that these G-to-A mutations have a role for replication-competent viruses in the context of viral recombination, thus allowing for the selection of replication-competent viruses with G-to-A mutations.

In conclusion, this study showed that APOBEC3G/F incomplete neutralization by single Vif mutations may seed a proviral reservoir characterized by the presence of genetic signatures in V3 that can modulate HIV-1 tropism toward a dual/pure CXCR4 usage. These genetic signatures occur at positions known to be critical for coreceptor usage, such as positions 24 and 25. Although further studies are necessary to evaluate the replication competence of these proviruses, this study may have implications for the success of CCR5 antagonist-based therapy, as well as for the risk of disease progression.

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