Changes in HIV-1 Capsid Stability Induced by Common Cytotoxic-T-Lymphocyte-Driven Viral Sequence Mutations

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ABSTRACT

HIV-1-infected individuals with protective HLA class I alleles exhibit better control of viremia and slower disease progression. Virus control in these individuals has been associated with strong and potent HIV-1-specific cytotoxic-T-lymphocyte (CTL) responses restricted by protective HLA alleles, but control of viremia also occurs in the presence of selected CTL escape mutations. CTL escape mutations restricted by protective HLA class I molecules are frequently located in the conserved p24 Gag sequence of HIV-1 that encodes the conical capsid core and have been suggested to reduce viral replication capacity. In this study, the consequences of well-described CTL-associated p24 Gag sequence mutations for HIV-1 capsid stability were assessed using a cyclosporine (CsA) washout assay. The frequently occurring HLA-B57- and HLA-B27-associated CTL escape mutations T242N and R264K resulted in delayed capsid uncoating, suggesting modulation of capsid stability. The described compensatory mutations L268M and S173A observed in R264K viruses reconstituted the capsid-uncoating half-time. Interestingly, capsid stability was correlated with infectivity. Taken together, these data demonstrate that CTL-driven escape mutations within p24 Gag restricted by protective HLA class I alleles have a significant impact on capsid stability that might contribute to the persistent control of viral replication observed despite viral escape from CTL responses.

IMPORTANCE

Sequence mutations within p24 Gag selected by CTL responses restricted by protective HLA class I alleles have been associated with reduced viral fitness. However, the precise mechanisms underlying the reduced viral replication capacity and lower viral loads associated with these mutations remain unclear. Here, we demonstrate that dominant HLA-B27-associated CTL escape mutations within HIV-1 capsid lead to enhanced capsid rigidity, providing a possible mechanism for the reduced viral fitness of these variants.

Some HIV-1-infected individuals with specific HLA class I alleles, including B27 and B57, exhibit better control of viremia and slower HIV-1 disease progression (1, 2). This has been associated with strong and highly potent HIV-1-specific cytotoxic-T-lymphocyte (CTL) responses restricted by these HLA class I molecules, forcing the virus to evade immune pressure through the selection of CTL escape mutations (3, 4). However, once mutated viruses are transmitted to HLA-B27/57-negative individuals, the viruses revert to wild-type (WT) sequences, indicating a replicative fitness cost of these CTL escape mutations (2–4). Importantly, several of the HLA-B27- and HLA-B57-associated mutations are located in areas that have been suggested to be critical for capsid structure (2, 4, 5), and *in vitro* experiments have shown that some mutations can reduce viral replication capacity (5, 6). However, the precise mechanisms underlying the reduced viral replication capacity and lower viral loads associated with these mutations remain unknown.

HLA-B27- and HLA-B57-restricted CTL escape mutations with *in vitro* impact on viral fitness are located within the HIV-1 p24 Gag sequence that encodes the conical capsid core consisting of viral capsid (CA) protein, which houses the viral genome (7). After fusion of the HIV-1 particle with human cells, the capsid is released into the cytoplasm, and a process referred to as “uncoating” is initiated. Early biochemical data supported a model suggesting that core disassembly occurred immediately after capsid is

released into the cytoplasm (8, 9). However, more recent data favor a model in which capsid shields the viral genome during cytoplasmic travel toward the nuclear pore complex (NUC) (10–12), and some data even suggest that capsid docks to the NUC before the preintegration complex (PIC) containing the viral cDNA is released (13, 14). Taken together, these data suggest that HIV-1 capsid can both provide an enclosed environment for reverse transcription of HIV-1 RNA and shield the viral genome during this process from innate immune sensing by cytoplasmic host restriction factors (13, 15–17).

Modeling studies have suggested that some of the CTL-re-

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stricted mutations in p24 might have a critical impact on the interactions of the CA protein (18) and thus could also have an influence on the overall stability of HIV-1 capsid (7). To assess the functional impact of the dominant p24 Gag mutations observed in HLA-B27⁺ and HLA-B57⁺ individuals on HIV-1 capsid stability, we employed a recently adapted cyclosporine (CsA) washout assay (19, 20) and observed an impact of the HLA-B57-associated T242N and HLA-B27-associated R264K mutation on capsid stability and infectivity.

MATERIALS AND METHODS

Cells and pharmaceuticals used. All cells were maintained at 37°C and 5% CO₂. HEK 293T cells were obtained from the ATCC (Manassas, VA, USA) and were kept in Dulbecco's modified Eagle medium (Sigma-Aldrich, Munich, Germany), 10% inactivated fetal bovine serum (Biochrom, Berlin, Germany), 1% penicillin-streptomycin (Sigma-Aldrich, Munich, Germany), and 1% L-glutamine (Sigma-Aldrich, Munich, Germany) (DMEM10 plus 2). Owl monkey kidney (OMK) cells were provided by the Heinrich Pette Institute (Hamburg, Germany) and were kept in DMEM10 plus 2. For passaging, OMK cells were detached using trypsin-EDTA (0.25%; ThermoFisher Scientific, Carlsbad, CA, USA). CsA (Sigma-Aldrich, Munich, Germany) was prepared in ethanol (EtOH) at 5 mM and used at a final concentration of 2.5 μM. Peripheral blood was obtained from healthy donors of the University Medical Center Hamburg-Eppendorf (UKE), Hamburg, Germany, cohort of healthy donors (HHCH) after informed consent was obtained, and peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll technique. The PBMCs were then kept in RPMI 1640 medium (Gibco, Carlsbad, CA, USA), 10% fetal bovine serum (Biochrom, Berlin, Germany), and 1% penicillin-streptomycin (Sigma-Aldrich, Munich, Germany) (RPMI10 plus 1). On the same day, PBMCs were activated with Dynabeads Human T-Activator CD3/CD28 (ThermoFisher Scientific, Carlsbad, CA, USA) at a ratio of 1:1, and interleukin 2 (IL-2) was added (PepRoTech, Rocky Hill, NJ, USA; 100 U per 1 × 10⁶ PBMCs). Polybrene (hexadimethrine bromide; Sigma-Aldrich, Munich, Germany) was prepared in double-distilled water (ddH₂O).

Construction of viruses containing single or combined sequence polymorphisms within HIV-1 capsid. A plasmid encoding an HIV-1 NL4-3 ΔEnv strain expressing green fluorescent protein (GFP) in place of Nef (HIV-GFP) was used. The HIV-GFP plasmid (21) was modified using the QuikChange II XL site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) to encode the Gag protein mutations T242N (TN), R264K (RK), L268M (LM), G221V (GV), R264K plus L268M (RK plus LM), and S173A plus R264K plus L268M (SA plus RK plus LM). Mutagenesis was performed using the following 5' oligonucleotide primers: T242N-Fw (Gag nucleotide [Gnt] c725a) (CTATTTGTTCTCCTGAAGGTTACTA GTAGTTCCTGCTATGTCAC), T242N-Rv (Gnt c725a) (GTGACATAG CAGGAAGTACTAGTAACCTTCAGGAACAAATAG), R264K-Fw (Gnt g791a) (CCCAGGATTATCCATTTTTTATAGATTCTCCTACTGGGATAGG), R264K-Rv (Gnt g791a) (CCTATCCCAGTAGGAGAAATCT ATAAAAATGGATAATCCTGGG), L268M-Fw (Gnt c802a) (CTTACT ATTTTATTTAATCCCATGATTATCCATCTTTATAGATTCTCCT ACTG), L268M-Rv (Gnt c802a) (CAGTAGGAGAAATCTATAAAAGAT GGATAATCATGGGATTAATAAAATAGTAAG), G221V-Fw (Gnt g662t) (CCTGGTGCAATAGGCACTGCATGCACTGGAT), G221V-Rv (Gnt g662t) (ATCCAGTGCATGCAGTGCCTATTGCACCAGG), R264K in L268M-Fw (Gnt g791a) (CCCATGATTATCCATTTTTTATAGATT CTCTACTGGGATAGG), R264K in L268M-Rv (Gnt g791a) (CCTATC CCAGTAGGAGAAATCTATAAAATGGATAATCATGGG), S173A-Fw (Gnt t517g) (CTTCTGATAATGCTGCAACATGGGTATTACTTCTG GGC), and S173A-Rv (Gnt t517g) (GCCCAGAAGTAATACCCATGTTT GCAGCATTATCAGAAG) (all from Integrated DNA Technologies, Coralville, USA). The primer positions of the mutations are according to the wild-type NL4-3 sequence (GenBank accession no. AF324493.2). After mutagenesis, several colonies of XL10-Gold-Ultracompetent cells (Agi-

lent, Santa Clara, CA, USA) were picked, and plasmid stocks were generated with a QIAprep Spin Miniprep kit (Qiagen, Venlo, Netherlands). The plasmids were sequenced using the primers G00, G50, G100, G01, and G85 (22) (Seqlab, Goettingen, Germany) in order to ensure they harbored the desired mutations and lacked any unwanted mutations. Maxipreps were performed for selected plasmids encoding only the desired mutations using the PureLink HiPure Plasmid Maxiprep kit (Invitrogen, Carlsbad, CA, USA), and the plasmids were stored at -80°C.

Virus production. Vesicular stomatitis virus G (VSV-G)-pseudotyped HIV-GFP was produced by cotransfecting HEK293T cells with 22 μg of wild-type pHIV-GFP (and the respective mutations; 7 plasmids in total) and 2 μg pVSV-G using Lipofectamine 2000 (ThermoFisher Scientific, Carlsbad, CA, USA) in Opti-MEM medium (ThermoFisher Scientific, Carlsbad, CA, USA). After 4 h, the medium was changed to DMEM10 plus 2, and at 48 h posttransfection, supernatants were harvested and filtered, and aliquots of viral stocks were frozen at -80°C. All transfections were performed using the same split of HEK293T cells on the same day. The p24 concentration of the viral stocks was quantified by p24 enzyme-linked immunosorbent assay (ELISA) (Genscreen HIV-1 Ag assay; Bio-Rad, Munich, Germany). Since the point mutations in p24 Gag that were investigated in this study might affect the detection of p24 by ELISA, the viral stocks were additionally analyzed by real-time PCR. HIV-RNA in cell culture supernatants was quantified using the fully automated Cobas 6800 system (Cobas HIV-1; Roche Molecular Diagnostics, Pleasanton, CA, USA). The HIV-1 PCR assay quantifies between 20 copies/ml and 10,000,000 copies/ml by targeting two highly conserved regions in the viral long terminal repeat (LTR) and gag region to overcome possible mutations in one primer target site. The results of the measurement of copies per milliliter by PCR and p24 (picograms per milliliter) as quantified by ELISA in the seven viral stocks that were used in this study showed a strong and significant correlation ($r = 0.93$; $P = 0.0025$). p24 ELISA was subsequently used for normalization of viral stocks.

Assessment of infectivity. PBMCs were isolated and activated as described above. Forty-eight hours after activation, 1 × 10⁵ PBMCs were plated in a 96-well plate and kept in RPMI10 plus 1; 450 ng of p24, as determined by ELISA (see above), from each viral stock was added to the appropriate wells following spinoculation for 90 min (1,200 × g; 16°C). After spinoculation, the plates were incubated for 30 min at 37°C before the supernatant was discarded and 200 μl of new RPMI10 plus 1 was added. Forty-eight hours later, the cells were stained with phycoerythrin (PE)/Cy5 anti-human CD3 antibody, allophycocyanin (APC) anti-human CD4 antibody, and Brilliant Violet 605 anti-human CD8a antibody (all from BioLegend, San Diego, CA, USA). After fixation in phosphate-buffered saline (PBS) (Sigma-Aldrich, Munich, Germany) with 4% paraformaldehyde (Sigma-Aldrich, Munich, Germany), the percentage of GFP⁺ cells, as a marker for infectivity, was determined using fluorescence-activated cell sorter (FACS) analysis on the BD LSR Fortessa (BD, Franklin Lakes, USA). The assay was performed with PBMCs from three different healthy donors. For each donor, the viral variants were tested on one 96-well plate in quintuplicate. As the distribution across each quintuplicate was symmetric, the mean of each quintuplicate was used for further calculations. The results of each experiment were then calculated as the fold change over the wild type for each individual experiment. In an initial experiment, activated PBMCs were infected in the same way as described above with increasing amounts of wild-type HIV-GFP (from 20 ng to 4,000 ng). A plateau of the infection rate was observed around 20 to 25% GFP⁺ PBMCs beginning from approximately 1,000 ng of virus (data not shown). Thus, 450 ng was chosen as the final amount of virus for the subsequent experiments in order to detect differences between the viral variants and to avoid saturation with virus.

Cyclosporine washout assay. The CsA washout assay was performed as described previously (19, 23). In short, OMK cells were plated on a 96-well plate 1 day prior to starting the CsA washout assay. On day 2, wild-type HIV-GFP or HIV-GFP containing the indicated point mutations, together with CsA and Polybrene, was added to OMK cells follow-

FIG 1 GFP expression in CD4⁺ T cells following infection with viruses containing escape mutations and compensatory mutations. Shown is an assessment of the infectivity of escape mutations (T242N mutation in the TW10 epitope [TN] and R264K in the KK10 epitope [RK] in p24 Gag) and compensatory mutations of RK (LM, RK plus LM, and SA plus RK plus LM) in p24 Gag. PBMCs were infected with a standardized amount of p24 of HIV-GFP. (A) Dot plots showing GFP expression of CD3⁺ CD4⁺ CD8⁺ lymphocytes. GFP positivity of the cells indicates infected cells. The numbers show the percentages of GFP⁺/infected cells. SSC, side scatter. (B) Fold change of infected cells over WT, which was set to 1 ($n = 3$). ***, $P < 0.0001$ versus WT; **, $P < 0.01$ versus WT; *, $P < 0.05$ versus WT. The error bars indicate ranges.

ing spinoculation (90 min; $1,200 \times g$; 16°C). After spinoculation, medium containing HIV-GFP, CsA, and Polybrene was replaced by warmed DMEM10 plus 2 with CsA. At time points 0, 15, 30, and 45 min and 1, 2, 3, 4, and 5 h, the medium was replaced by DMEM10 plus 2 that did not contain CsA, and thus, CsA was washed out of the cell culture. Additionally, an aliquot of medium with HIV-GFP, CsA, and Polybrene was left after spinoculation as a positive control. Two days after infection, cells were harvested with trypsin, and the percentage of GFP⁺ cells was determined by FACS analysis. As an increasing number of capsids uncoated over time and uncoated particles were not inhibited any longer by the endogenous Trim-CypA of OMK cells, increasing amounts of GFP⁺ OMK cells were observed over time when cells were harvested and analyzed after 2 days. At each time point, the CsA washout assay was performed in triplicate for each virus, and two viruses were directly compared on a single plate, resulting in 60 wells used per plate. All experiments comparing 2 specific viral strains were performed using the same passage of cells to minimize any differences in infectibility. Each 96-well plate was considered an individual experiment, and each comparison was tested in multiple experiments (96-well plates). The number of experiments performed per comparison of 2 viruses was as stated (see the legend to Fig. 3) and ranged from six to nine. Data were normalized by defining the percentage of GFP⁺ cells at 4 h or 5 h (as indicated) after infection as 100%. In order to determine the half-life of capsid uncoating, the best-fit line was drawn between the 2 time points within which 50% of the cells were infected.

Statistical analysis. Flow data were analyzed using FlowJo (FlowJo, Ashland, OR, USA). All data are presented as median and range. All analyses were performed using Prism 6 (GraphPad Software, La Jolla, CA, USA). Differences between 2 uncoating half-times were analyzed using the Mann-Whitney U test. The one-sample Student t test was used to compare the relative infectivity rate and the uncoating half-times to the reference value of 1. The comparisons to the reference value were performed for each viral variant, with a Bonferroni correction of the P values to adjust for test multiplicity (due to the number of viral variants tested).

All statistical tests were two tailed, and statistical significance was set at a P value of <0.05 .

RESULTS

Impact of escape mutations within HIV-1 capsid on infectivity in primary cells. Sequence mutations within p24 Gag/capsid might have an effect on the kinetics by which new viral proteins are produced by impacting the kinetics of viral RNA/cDNA shuttling through the cytoplasm to the nucleus (14, 21, 24) or by af-

fecting the kinetics and location of viral cDNA integration in the host cell genome (12). We therefore initially assessed the impact of p24 Gag CTL escape mutations observed in HIV-1-infected HLA-B27⁺ and HLA-57⁺ individuals on the production of new virus-encoded proteins using a VSV-G-pseudotyped NL4-3 ΔEnv HIV-1 encoding GFP. Use of the VSV-G-pseudotyped virus ensured that GFP production was affected only by viral replication steps following standardized VSV-G-mediated fusion (25), including uncoating of the capsid, transport of the preintegration complex (PIC) to the nuclear pore, PIC release, and HIV-1 cDNA integration into the host genome. Furthermore, the ΔEnv feature of the HIV-GFP strain used ensured a single-round infection and eliminated any alteration by postintegration steps, such as virus assembly or extracellular maturation. The characteristics of the virus strain used in the assay, therefore, linked the amount of GFP⁺ cells infected by different viral strains directly to differences of cytoplasmic travel to the nucleus and PIC release and integration, features that are at least partially dependent on capsid function.

Compared to virus strains containing the wild-type HIV-1 NL4-3 sequence, viruses containing the HLA-B27-associated RK mutation in p24 Gag showed a significant (82.8%) reduction of GFP⁺ CD4⁺ T cells ($P = 0.001$) (Fig. 1B). A modest but nonsignificant reduction in GFP⁺ CD4⁺ T cells was also observed with viruses containing the HLA-B57-associated TN mutation (10.5%; not significant [n.s.]), while no difference was observed with the LM mutation (2.5%; n.s.) (Fig. 1B). The observed significant decrease in GFP⁺ CD4⁺ T cells using the VSV-G-pseudotyped NL4-3 ΔEnv HIV-1 containing the R264K mutation, the merely modest decrease in the presence of the T242N mutation, and the lack of any effect in the presence of the L268M mutation observed here are in line with previous data from other groups using different systems, such as reporter cell lines (5, 6, 26). To ensure that the differences in infectivity of the investigated viral strains were not due to differences in particle-to-infectivity ratios, we performed an analysis of infectivity over a range of different p24 concentrations. Therefore, we repeated the assay described above in PBMCs with different amounts of p24 (1,800 ng, 900 ng, 450 ng, 225 ng, and 112.5 ng) (Fig. 2). The results showed that the amount of p24

FIG 2 GFP expression in CD4⁺ T cells following infection with different amounts of p24 of viruses containing escape mutations and compensatory mutations. The infectivities of the escape mutations (TN and RK in p24 Gag) and compensatory mutations of RK (LM, RK plus LM, and SA plus RK plus LM) in p24 Gag were assessed with the use of different amounts of p24 (1,800 ng, 900 ng, 450 ng, 225 ng, and 112.5 ng). The patterns of the infectivities of the different viral strains remained similar over a wide range of p24 input (PBMCs from one healthy donor; three wells per virus for each dose of p24). The error bars indicate ranges.

input did not alter the observed differences between the mutated virus strains.

In addition, we assessed the impact of the G221V mutation in p24 Gag, which alters the catalytic center of the CypA-binding site and thereby inhibits CypA binding (12), on the percentage of GFP⁺ cells. Interestingly, with a 97.8% decrease in GFP⁺ CD4⁺ T cells, the G221V mutation showed the strongest alteration compared to wild-type virus ($P = 0.003$), indicating the necessity for CypA binding during capsid trafficking and viral cDNA integration in primary human cells (Fig. 1). Taken together, these data show that some mutations within p24 Gag, and in particular the HLA-B27-associated R264K mutation, as well as the G221V mutation at the catalytic center of the CypA-binding site, can have a strong impact on the viral life cycle.

The p24 Gag L268M and S173A mutations restore the infectivity of the R264K virus. After observing a significant reduction in GFP⁺ CD4⁺ T cells in the context of the HLA-B27-associated R264K mutation, we next determined whether the described compensatory mutations of the R264K mutation (6) were able to restore GFP expression. It was previously shown that the L268M mutation in p24 Gag appeared before the development of the R264K mutation in HLA-B27⁺ HIV-1-infected individuals (6). Subsequently, a third mutation (S173A) developed and almost completely restored viral fitness to levels similar to those observed using wild-type sequences (6). Therefore, VSV-G-pseudotyped NL4-3 Δ Env HIV-1 encoding both the R264K and L268M mutations (referred to as RK plus LM) or all three mutations (S173A, R264K, and L268M, referred to as SA plus RK plus LM) were constructed. Viruses containing the RK plus LM mutations showed a 74.9% reduction of infectivity compared to the wild type ($P = 0.011$), already slightly recovering the infectivity of the RK mutation as described above. Furthermore, with a modest increase of 3.8% in GFP⁺ cells compared to the wild-type virus (n.s.), viruses containing the SA plus RK plus LM variants were able to fully restore the viral fitness of the R264K variant, as previously reported (6). Overall, these data show that the compensatory mutations of R264K in HIV-1 p24 Gag were able to restore GFP expression in infected CD4⁺ T cells to wild-type levels, as previously reported using reporter cell lines (6, 26).

Modulation of the kinetics of HIV-1 capsid uncoating by HLA-B27- and HLA-B57-associated sequence mutations. The above-

mentioned data demonstrate a significant impact of the HLA-B27-associated CTL escape mutation R264K in p24 Gag on the ability of GFP-encoding viruses to infect CD4⁺ T cells and to express GFP and also demonstrate that compensatory mutations can rescue this effect. However, it remains unclear precisely which steps involved in viral cDNA trafficking to the nucleus, cDNA integration, and subsequent GFP production were affected by these mutations. As modeling studies have suggested that HLA-B27- and HLA-B57-associated mutations in p24 Gag might affect sites critical for capsid formation and stability, we subsequently employed a kinetic functional CsA washout assay to determine the consequences of mutations within p24 Gag for capsid stability.

Using the CsA washout assay, viruses containing the HLA-B27- and HLA-B57-associated CTL escape mutations T242N, L268M, and R264K were compared to the NL4-3 WT virus. The assay uses cyclosporine to inhibit Trim-CypA of OMK cells, which restricts viral particles with an intact capsid. Thus, by washing out CsA in a time-dependent manner, increasing amounts of uncoated particles are not restricted by Trim-CypA any longer and can infect the OMK cell. The final readout is the quantification of GFP-positive OMK cells via flow cytometry (Fig. 3A). The GV mutation inhibits Trim-CypA binding, and thus, viruses containing the GV mutation resulted in high percentages of GFP-positive OMK cells at any time point independent of CsA washout (Fig. 3B). Raw mean data demonstrating the percentage of GFP-positive cells at each time point following CsA washout (normalized to the highest percentage of GFP⁺ cells, defined as 100%) are shown in Fig. 3C to E. The median capsid-uncoating half-time was longer than that of the HIV-GFP WT for viruses containing the mutations T242N (WT, 110 min [range {RNG}, 93 to 137 min] versus T242N, 133 min [RNG, 106 to 151 min]; $P = 0.05$) and R264K (WT, 118 min [RNG, 97 to 153 min] versus R264K, 166 min [RNG, 143 to 179 min]; $P < 0.001$). A modestly but not significantly longer uncoating half-time was observed for viruses containing the L268M mutation (WT, 105 min [RNG, 76 to 122 min] versus L268M, 113 min [RNG, 91 to 126 min]; n.s.). For a better evaluation of the differences between WT virus and viruses containing the respective escape mutations, Fig. 4A shows each pair of comparative assays with the results of the WT virus normalized to 1. These data demonstrate that the HLA-B57- and HLA-B27-as-

FIG 3 GFP⁺ OMK cells following infection with viruses containing escape mutations and compensatory mutations after CsA washout in a time-dependent manner. (A) Dot plots showing original data from the CsA washout assay. (Top row) CsA washout of HIV-GFP WT with increasing amounts of GFP⁺ OMK cells over time. (Middle row) Same assay with HIV-GFP WT but with EtOH instead of CsA as a control. Since EtOH does not inhibit the HIV-1 restriction factor Trim-Cyp, expressed in OMK cells, only very few infectious cells could be detected, and there was no time-related effect. (Bottom row) CsA washout assay with the HIV-GFP R264K mutation. The amount of GFP⁺ OMK cells also increased over time, as seen with WT virus, but a substantial delay could be observed, especially in the first 2 h. (B) CsA and EtOH washout assay of HIV-GFP WT and HIV-GFP G221V. The amount of GFP⁺ cells increased over time in the CsA washout assay using HIV-GFP WT, whereas this effect was not observed in the EtOH washout, as described for panel A. Neither CsA nor EtOH washout showed a time-dependent effect using the HIV-GFP G221V mutant, since the G221V mutation altered the CypA catalytic binding site, preventing Trim-CypA from binding to HIV-1 capsid. (C to E) CsA washout assays comparing the TN ($n = 9$), LM ($n = 8$), and RK ($n = 8$) mutations, respectively, to the HIV-GFP WT. (F and G) CsA washout assays comparing compensatory mutations RK plus LM and SA plus RK plus LM, respectively, to HIV-GFP RK (both $n = 6$). The error bars indicate ranges.

sociated point mutations T242N and R264K extended the capsid-uncoating half-time, favoring a model of enhanced rigidity of the capsid structure in these mutated viral strains.

Since the above-described compensatory mutations RK plus

LM and SA plus RK plus LM were able to restore the fitness of viruses containing the R264K mutation, we next investigated whether the combined mutations RK plus LM and SA plus RK plus LM were also able to restore the longer uncoating half-time

FIG 4 Half-life of uncoating of viruses containing escape mutations and compensatory mutations as calculated from the CsA washout assay. The uncoating half-time was calculated from original data from the CsA washout assay, as described in Materials and Methods. (A) The WT was set to 1, and the escape mutations were calculated as fold change over the WT. While viruses containing the LM and TN mutations showed only a moderately longer uncoating half-time, with increases of 13% and 8%, respectively, viruses containing the RK mutation showed the most impaired uncoating, with an increase of 35%. Only viruses containing the mutations T242N ($n = 9$) and R264K ($n = 8$) proved to have a significant impact on the capsid-uncoating half-time compared to the WT ($P = 0.007$ and $P = 0.001$, respectively). (B) RK was set to 1, and the compensatory escape mutations were calculated as the fold change over RK. RK plus LM showed an ameliorated (13% faster; n.s.; $n = 6$) and SA plus RK plus LM showed a significantly shorter (27%; $P = 0.011$; $n = 6$) uncoating time compared to RK. The error bars indicate ranges.

observed for the R264K-containing strain. Therefore, each of these compensatory mutations was directly compared to the R264K mutation using the CsA washout assay. The raw mean data for each time point of the washout assay of R264K versus RK plus LM ($n = 6$) and R264K versus SA plus RK plus LM ($n = 6$) are shown in Fig. 3F and G. A slightly shorter but not significantly different capsid-uncoating half-time compared to the R264K mutation was observed for the RK plus LM variant (R264K, 137 min [RNG, 109 to 150 min] versus RK plus LM, 128 min [RNG, 102 to 134 min]; n.s.). A significantly shorter uncoating half-time was seen with the SA plus RK plus LM variant compared to R264K (R264K, 153 min [RNG, 109 to 175 min] versus SA plus RK plus LM, 104 min [RNG, 95 to 138 min]; $P = 0.01$). For a better evaluation of the comparisons between the R264K mutations alone and in the presence of the compensatory mutations, Fig. 4B shows each pair with the R264K variant set to 1. Taken together, the described compensatory mutations for the HLA-B27-associated R264K mutation also restored HIV-1 capsid-uncoating half-times.

FIG 5 Correlation of capsid-uncoating half-time and viral GFP expression in CD4⁺ T cells. (A) The fold changes in the uncoating half-time and viral fitness of viruses containing the escape mutations over those of the WT were plotted against each other and showed a significant correlation (Pearson $r = -0.95$; $P = 0.048$). (B) The fold changes in the uncoating half-time and viral fitness of the compensatory mutations over the R264K mutation in the KK10 epitope of p24 Gag (RK) were plotted against each other and showed a correlation that did not reach statistical significance (Pearson $r = -0.97$; n.s.).

Correlation between HIV capsid-uncoating half-time and viral GFP production in CD4⁺ T cells. The viral replication assay using PBMCs and the CsA washout assay using OMK cells both measure the impact of p24 Gag sequence mutations on viral replication steps following viral entry and prior to *de novo* assembly of viruses using different approaches. We were therefore interested in assessing whether the results of the two assays showed any correlation. The half-time of capsid uncoating of the WT virus was set to 1, and the fold changes of average capsid-uncoating half-times in OMK cells and the fold changes of GFP⁺ CD4⁺ T cells of the respective viral variants were correlated. A correlation was observed for the initial viral escape mutations detectable in HLA-B27/57⁺ HIV-infected individuals (Pearson $r = -0.95$; $P = 0.048$) (Fig. 5A). The same analysis was performed for the R264K variant and its compensatory mutations. The results from the CsA assay and GFP⁺ CD4⁺ T cells again showed a correlation; however, no statistical significance was reached, likely due to the limited data points available (Pearson $r = -0.97$; $P = 0.16$) (Fig. 5B). These correlations between the results from the two assays suggest that the impact of CTL-induced viral sequence mutations within HIV-1 capsid on GFP expression is directly linked to the capsid stability of the respective strains.

DISCUSSION

Large genome-wide association studies (GWAS) performed in cohorts of HIV-1-infected individuals with spontaneous control of HIV-1 viremia (HIV elite controllers) demonstrated that all single nucleotide polymorphisms (SNPs) associated with viral control were located in HLA class I genes (27, 28). Multiple studies have identified HLA-B57 and HLA-B27 as the two HLA class I alleles most strongly associated with HIV-1 control (27). While immunodominant virus-specific CTLs in HLA-B57⁺ individuals infected with HIV-1 are directed against the TW10 (TSTLQEQ IGW) epitope within p24 Gag, CTLs in HLA-B27⁺ individuals are directed against the KK10 (KRWILGLNK) p24 Gag epitope, resulting in frequent CTL escape mutations within these epitopes (B57⁺, T242N; B27⁺, R264K and L268M) (2, 6, 26, 29). Additional studies linked the low HIV-1 viremia observed in HLA-B27/57⁺ individuals to reduced viral fitness of HIV-1 strains containing the selected CTL escape mutations (5, 6, 29). The significant impact of single point mutations within the p24 Gag region on viral replicative fitness is believed to be due to the high sequence conservation of the region (18), with sequence mutations leading to structural changes (30). Additionally, it has been suggested that some of these point mutations in p24 Gag can destabilize specific helices of the CA protein (4), which might lead to an alteration of the stability of the HIV-1 capsid structure. Here, we investigated the impact of escape, as well as compensatory, mutations seen in HIV-1-infected HLA-B57/27⁺ individuals on viral fitness and the contribution of capsid stability to this process, using a functional CsA washout assay.

Several studies have assessed the impact of point mutations on viral replication fitness using assays that are based on reporter cell lines (5, 6, 26). In line with findings from these studies but using human primary PBMCs, we observed that the HLA-B27-restricted escape mutation R264K in the KK10 epitope, and to a lesser extent the HLA-B57-restricted T242N mutation in the TW10 epitope, in p24 Gag resulted in a reduction of GFP⁺ CD4⁺ T cells and that the described compensatory mutations for the R264K mutation were able to partially reconstitute this effect.

In the current study, we used a VSV-G-pseudotyped HIV-1 NL4-3 Δ Env strain expressing GFP in place of Nef, enabling flow cytometric quantification of infected cells producing new viral proteins by measuring GFP⁺ cells. As VSV pseudotyping was used for all the virus strains tested, viral infection of cells was standardized, and changes in GFP⁺ cells were a result of postfusion differences, including capsid uncoating, reverse transcription, forming of the PIC, integration of cDNA into the host genome, and GFP transcription and translation. VSV-G pseudotyping was employed for all the viruses that were used in this study, since the CsA washout assay is based on the use of OMK cells, which lack a CD4 receptor. In order to correlate capsid uncoating and infectivity, VSV-G-pseudotyped viral strains were used in all the assays, even though VSV-G pseudotyping has been reported to alter entry pathways compared to viruses with a native HIV-1 envelope (31, 32). Viruses containing the HLA-B27-associated R264K mutation, and also the G221V mutation affecting the catalytic center of the CypA-binding site, showed significantly fewer GFP⁺ cells following infection of PBMCs than the wild-type virus, suggesting that viruses containing these mutations had reduced infectivity (12).

Given that the HLA-B27- and HLA-B57-associated sequence

mutations used in this study were all located in HIV-1 p24 Gag, which encodes capsid, we hypothesized that the impact of these mutations on infectivity was affected by the process of capsid uncoating following infection. Indeed, we observed a correlation between the percentage of GFP⁺ cells and the half-time of capsid uncoating as measured using the CsA washout assay. The R264K mutation had the strongest negative effect on the percentage of GFP⁺ cells compared to NL4-3 wild-type sequences and was also the mutation that resulted in the most prolonged capsid-uncoating half-time. Furthermore, the compensatory mutations described for R264K, RK plus LM and SA plus RK plus LM, were able to both shorten the half-time of capsid uncoating and enhance the percentage of GFP⁺ PBMCs compared to viruses containing the R264K mutation alone. While other steps of the virus life cycle following capsid uncoating can also contribute to reduced infectivity, including integration of cDNA into the host genome, transcription, and translation, these data demonstrate that modulated capsid stability is a major determinant of the reduced infectivity of HIV-1 variants containing frequent HLA-B27- and HLA-B57-associated sequence mutations within HIV-1 capsid.

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