Tertiary Mutations Stabilize CD8⁺ T Lymphocyte Escape-Associated Compensatory Mutations following Transmission of Simian Immunodeficiency Virus

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Compensatory mutations offset fitness defects resulting from CD8⁺ T lymphocyte (CD8TL) -mediated escape, but their impact on viral evolution following transmission to naive hosts remains unclear. Here, we investigated the reversion kinetics of Gag181-189CM9 CD8TL escape-associated compensatory mutations in simian immunodeficiency virus (SIV)-infected macaques. Preexisting compensatory mutations did not result in acute-phase escape of the SIVmac239 CD8TL epitope Gag181-189CM9 and instead required a tertiary mutation for stabilization in the absence of Gag181–189 CM9 escape mutations. Therefore, transmitted compensatory mutations do not necessarily predict rapid CD8TL escape.

Reversion of particular CD8⁺ T lymphocyte (CD8TL) escape mutations is well documented following human immunodeficiency virus (HIV) transmission between HLA-discordant individuals and is dependent upon the fitness cost of the escape mutation. Escape mutations that exact a high fitness cost often revert following HIV transmission to an HLA-discordant individual, while those that impose a low fitness cost do not (1–3). Two epitopes targeted by CD8TL responses associated with long-term HIV control, HLA-B*27-bound Gag263-272KK10 and HLA-B*57-bound Gag240–249TW10, lie within a conserved region of Gag, and escape mutations within these epitopes reduce viral fitness. However, while the codon 242 threonine-to-asparagine (T242N) escape mutation within Gag240–249TW10 reverts readily following transmission to an HLA-disparate host, the R264K escape mutation within Gag263–272KK10 does not (3, 4). This finding is surprising given that the R264K mutation reduces viral replicative fitness to a significantly greater extent than the T242N mutation (5). Compensatory mutations that maintain viral fitness while allowing for intraepitopic variation have been described for both Gag263–272KK10 and Gag240–249TW10, and the difference in reversion kinetics between these epitopes is likely dependent on these compensatory mutations (4, 6, 7).

In simian immunodeficiency virus mac239 (SIVmac239)-infected rhesus macaques, the Mamu-A1*001:01-bound Gag181–189 escape epitope Gag181-189CM9 is analogous to Gag263–272KK10 and Gag240–249TW10 in that it lies within a conserved region of Gag and has associated compensatory mutations that increase the fitness of viral variants containing Gag181–189CM9 escape mutations. We, and others, have previously shown that the canonical Gag181–189CM9 escape mutation T182A is frequently linked with the flanking downstream mutation I161V and reverts following transmission to Mamu-A1*001:01-negative rhesus macaques (8–10). Additionally, the I206V compensatory mutation is consistently associated with a subsequent upstream mutation, I161V. This mutation is not necessary for the emergence of viruses with the T182A escape mutation, but it is believed to further increase viral fitness following mutations at both T182 and I206.

We hypothesized that preexisting I161V and I206V compensatory mutations would facilitate rapid, acute-phase escape within the conserved Gag181–189CM9 CD8TL epitope upon transmission to Mamu-A1*001:01-positive rhesus macaques. To test this hypothesis, we engineered an SIVmac239 variant harboring the I161V and I206V compensatory mutations associated with Gag181–189CM9 escape (SIVmac239-2V) by site-directed mutagenesis of SIVmac239 plasmid DNA using the QuikChange XL kit (Stratagene, La Jolla, CA) and generated a virus stock as previously described (2). Following intravenous infection of a Mamu-A1*001:01-positive rhesus macaque, r98003, with 100 ng Gag p27CA, SIVmac239-2V replicated efficiently, peaking at 3.96 × 10⁷ viral RNA (vRNA) copies/ml plasma at 10 days postinfection (d.p.i.), as measured by quantitative reverse transcription-PCR (RT-PCR) using previously described techniques (11) (Fig. 1A). This level of viral replication is consistent with a large body of data collected from wild-type SIVmac239-infected Mamu-A1*001:01-positive rhesus macaques (12, 13). Additionally, major histocompatibility complex (MHC) class I tetramer staining of freshly isolated peripheral blood mononuclear cells (PBMC) revealed that SIVmac239-2V elicited a strong CD8TL response against Gag181–189CM9, with 3.5% of all circulating CD8TL tar-
Subsequently, we detected two Gag181-189CM9 escape mutations, are shown. Reverted almost entirely to wild type, with I206V present in only 1.7% of all pyrosequencing reads (Fig. 1B). The C181G mutation is rare but is a previously described Gag181-189CM9 escape mutation targeting this epitope at 14 d.p.i. (Fig. 1A). Importantly, this Gag181-189CM9-specific CD8$^+$ T-cell response remained at high frequency in r98003 through 15 weeks p.i. (w.p.i.).

Next, we monitored viral evolution within gag by performing Roche/454 pyrosequencing on plasma samples as described previously (8). Unexpectedly, instead of observing rapid, acute-phase escape of Gag$_{181-189}$ CM9, we detected high-frequency reversions of both the I$_{161}V$ and I$_{206}V$ compensatory mutations (Fig. 1B). The frequency of revertant viruses in plasma increased through 8.5 w.p.i., when 98.3% of plasma virus sequences were wild-type SIVmac239 at I$_{161}$ and 84% were wild-type SIVmac239 at I$_{206}$. Subsequently, we detected two Gag$_{181-189}$CM9 escape mutations, C$_{181}G$ and T$_{182}A$, only after the I$_{206}V$ compensatory mutation had arisen (data not shown). While we failed to observe rapid acute-phase CD8$^+$ escape, r98003 did Roche/454 pyrosequencing (8) (Fig. 1C). The Gag$_{181-189}$CM9 escape mutations of nine Mamu-A1*001:01-positive rhesus macaques (filled circles) and animal r98003 (open circle). The means and standard deviations of times to Gag$_{181-189}$CM9 escape are shown.

The SIV compensatory mutations Gag I$_{161}V$ and I$_{206}V$ do not always revert following transmission to Mamu-A1*001:01-negative rhesus macaques. Given the surprising reversion of the compensatory mutations following infection of a naive host, we next investigated additional animals infected with SIVmac239 harboring the preengineered I$_{161}V$ and I$_{206}V$ mutations. Friedrich
et al. previously described three rhesus macaques infected with 3XSIVmac239, a virus encoding the Gag181–189CM9 escape mutation T182A and the two compensatory substitutions I161V and I206V (2). We Roche/454 pyrosequenced virus from plasma samples obtained from these three animals, spanning the first 8 months of infection, to make direct comparisons with virus sequences from r98003 and to glean additional insight into why SIVmac239-2V reverted at positions I161V and I206V before Gag181–189CM9 escape. Two of these rhesus macaques were Mamu-A1*001:01 negative (r98020, r96115), and one was Mamu-A1*001:01 positive (r97089). The 3XSIVmac239 circulating in r97089 retained all three (I161V, T182A, and I206V) preengineered mutations, most likely due to low-frequency Mamu-A1*001:01 restricted, Gag181–189CM9-specific CD8+TIL (14) (Fig. 2A). In con-

![FIG 2 Consensus sequences of the Gag181–189CM9 region in rhesus macaques infected with 3XSIVmac239. The Gag181–189CM9 regions from three rhesus macaques infected with 3XSIVmac239 were Roche/454 pyrosequenced at the indicated w.p.i. (A) r97089 (Mamu-A1*001:01 positive); (B) r96115 (Mamu-A1*001:01 negative); (C) r98020 (Mamu-A1*001:01 negative). Each consensus sequence indicates all mutations present in 1% or more of total sequence reads (limit of detection). Amino acid substitutions are shown above the reference sequence; the frequencies of the mutations are shown both as percentages and as shaded boxes according to prevalence, as indicated by the key at the bottom right. Boxed amino acids within the reference sequence comprise the Gag181–189CM9 epitope.](http://jvi.asm.org/content/jvi/82/10/3600.full.pdf)
The Gag A312P mutation stabilizes the compensatory mutations Gag I161V and I206V. Consensus Sanger sequences spanning Gag I161 through I206 and the tertiary mutation site Gag A312 at the indicated w.p.i. are shown. (A) Eighteen SIVmac239-infected rhesus macaques (seven Mamu-A1*001:01 positive and eleven Mamu-A1*001:01 negative). *, r03035 was infected with the SIVmac239 variant B*008:01 8 Mamu-A1*001:01-negative animals. The 3XSIVmac239 circulating in r97089, and r98020, at various time points postinfection (11 chronically SIV-infected rhesus macaques, including r98003, test this hypothesis, we analyzed Gag sequences from a cohort of Gag181–189CM9 escape mutation.

The divergent viral evolution described (15). We found that viral sequences from five of seven SIVmac239-infected, Mamu-A1*001:01-positive animals contained mutations I161V, T182A/C, I206V, and a downstream mutation, Gag A312P (Fig. 3A). Virus from the two remaining Mamu-A1*001:01-positive, SIVmac239-2V-infected rhesus macaque. (D) One Mamu-A1*001:01-negative rhesus macaque infected by transfer of plasma and PBMC from rh1937 at 166 w.p.i. (see the arrow). Reference sequences for respective infecting SIV strains are shown across the top.

The downstream Gag mutation A312P stabilizes SIV containing compensatory mutations I161V and I206V in the absence of a Gag181–189CM9 escape mutation. The divergent viral evolution between r98003, r96115, and r98202 raised the possibility that a tertiary mutation was maintaining viral fitness while allowing for the retention of the I161V and I206V compensatory mutations. To test this hypothesis, we analyzed Gag sequences from a cohort of 25 chronically SIV-infected rhesus macaques, including r98003, r97089, and r98202, at various time points postinfection (11 Mamu-A1*001:01-positive, 14 Mamu-A1*001:01-negative macaques) using consensus Sanger sequencing as previously described. We found that viral sequences from five of seven SIVmac239-infected, Mamu-A1*001:01-positive animals contained mutations I161V, T182A/C, I206V, and a downstream mutation, Gag A312P (Fig. 3A). Virus from the two remaining Mamu-A1*001:01-positive animals (r98001 and r99084) harbored the A312P mutation alone, suggesting that this mutation can precede the I161V, T182A/C, and I206V mutations. In contrast, none of these mutations were observed in Mamu-A1*001:01-negative animals (Fig. 3A). Thus, it appeared that A312P was associated with Gag181–189CM9 escape. Next, we examined the association of A312P with the maintenance of the I161V and I206V compensatory mutations following transmission of our engineered viruses to naive hosts. In two out of the three Mamu-A1*001:01-positive animals infected with 3XSIVmac239 (r97089 and r97035), the A312P mutation arose following infection, which again suggested that A312P stabilizes the Gag181–189CM9 escape-associated compensatory mutations (Fig. 3B). In the two Mamu-A1*001:01-negative animals infected with 3XSIVmac239 (r98020 and r96115), A312P was present only in r98020, where the compensatory mutations persisted in the absence of Gag181–189CM9 escape. In r98003,

![Reversion of Compensatory Mutations](http://jvi.asm.org/)

**FIG 3** The Gag A312P mutation stabilizes the compensatory mutations Gag I161V and I206V. Consensus Sanger sequences spanning Gag I161 through I206 and the tertiary mutation site Gag A312 at the indicated w.p.i. are shown. (A) Eighteen SIVmac239-infected rhesus macaques (seven Mamu-A1*001:01 positive and eleven Mamu-A1*001:01 negative). *, r03035 was infected with the SIVmac239 variant B*008:01 8 Mamu-A1*001:01-negative animals. The 3XSIVmac239 circulating in r97089, and r98020, at various time points postinfection (11 chronically SIV-infected rhesus macaques, including r98003, test this hypothesis, we analyzed Gag sequences from a cohort of Gag181–189CM9 escape mutation.

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the Mamu-A1*001:01-positive animal infected with SIVmac239-2V, the lack of A312P was associated with reversion of the compensatory mutations to wild type (Fig. 3C).

To further test if the tertiary mutation A312P would stabilize the I161V and I206V compensatory mutations during reversion of Gag181–189CM9 epitope mutations, we infected a Mamu-A1*001:01-negative animal, r00033, with virus from rh1937 that contained the T182C Gag181–189CM9 escape mutation, the I161V and I206V compensatory mutations, and the tertiary A312P mutation (Fig. 3A and D). In agreement with our previous observations, the A312P mutation stabilized the I161V and I206V compensatory mutations, while the T182C escape mutation reverted to wild type (Fig. 3D). Cumulatively, these data suggest that A312P is necessary for the maintenance of I161V and I206V compensatory mutations in the absence of Gag181–189CM9 escape.

Gag A312P is a bona fide compensatory mutation. We observed that A312P consistently associated with the Gag181–189CM9 escape-associated compensatory mutations, but we wanted to further assess the compensatory characteristics of the A312P mutation. We first investigated the possibility that A312P could be a CD8TL escape mutation but found no evidence of T cells targeting this region of Gag. Using in silico binding algorithms (http://www.cbs.dtu.dk/services/NetMHC/) we found that no 8-, 9-, or 10-mer peptides between SIVmac239 Gag residues 301 and 323 are predicted to bind Mamu-A1*001:01 with physiologically relevant affinity (50% inhibitory concentration [IC50] ≤ 500 nM) (16). In addition, we detected no CD8TL responses to the A312 region by a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) in 7 Mamu-A1*001:01-positive rhesus macaques with detectable Gag181–189CM9-specific CD8TL responses, 4 of

FIG 4 Gag A312P is a tertiary compensatory mutation stabilizing SIVmac239 CA expressing I206V. (A) Seven Mamu-A1*001:01-positive rhesus macaques infected with SIV were screened for T cell responses against three overlapping 15-mer peptides spanning the A312 region by IFN-γ ELISpot. Animals harboring SIV with the A312P mutation are represented with open red symbols. SFC, spot-forming cells. The dashed line at 50 SFC is the limit of detection for this assay. (B) Frequency of Gag p27 CD4 + T cell targets infected with engineered mutant viruses in vitro. Values were normalized to the frequency of Gag p27 targets infected with SIVmac239. (C) Table showing Sanger sequencing results from vRNA taken at day 14 of the in vitro replication assay. (D) Structural modeling of SIVmac239 capsid, showing positions of the I161, T182, I206, and A312 amino acid residues. (Top) Top view of the SIVmac239 CA hexamer, with one monomer colored light blue and a second monomer colored dark blue. (Bottom) Magnified side view of the potential interaction between A312 (within the light-blue monomer) and I206 (within the dark-blue monomer). (E) Representative Gag p27-staining histogram, showing the mean fluorescent intensity of intracellular Gag p27-fluorescein isothiocyanate (FITC) staining for each virus at day 21 of our in vitro replication assay. Results are indicative of separate assays performed with two different Gag p27-specific antibodies.

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which also had circulating virus harboring the A312P mutation (Fig. 4A, red symbols). These results, in combination, support the conclusion that CD8\(_{\text{T}}\) pressure does not drive the emergence of A312P in *Mamu-A1*^*001:01*-positive animals.

Next, we performed an *in vitro* SIVmac239 replication assay to compare the growth kinetics and levels of stability of viruses containing the I161V, I206V, and A312P mutations. We engineered two SIVmac239 mutants (in addition to SIVmac239-2V) containing the I161V, I206V, and A312P mutations together (SIVmac239-2V-P) or the A312P mutation alone (SIVmac239-P). We infected separate cultures of staphylococcal-enterotoxin B-stimulated rhesus macaque CD4\(^+\) T cells with 2 \times 10\(^5\) virus particles of either SIVmac239 or a mutant virus in complete medium containing 100 IU interleukin 2 (IL-2)/ml and cultured them for 7 days. On days 7 and 14, virus-containing cell supernatant was diluted 1:4 with medium and used to infect additional activated CD4\(^+\) T cells. We compared the frequencies of infected targets for each mutant virus on days 14 and 21 by intracellular Gag p27 antibody staining, normalizing to the frequency of SIVmac239-infected targets (Fig. 4B). Mutant viruses showed replication kinetics similar to that of SIVmac239 over the first 14 days but exhibited a higher replicative capacity by day 21. We Sanger sequenced the mutant viruses from our replication assay at day 14 and found that SIVmac239-2V, but not SIVmac239-2V-P, showed a prominent double peak indicative of reversion of the I206V mutation (Fig. 4C).

Finally, we generated structural models of the SIVmac239 capsid (CA) hexamer as previously described (17) and assessed locations and potential interactions of the I161, T182, I206, and A312 amino acid residues (Fig. 4D, top). The I161 residue is found in helix 1 of CA, and its side chain protrudes into helix 4, where the I206 residue is located, supporting the observed linkage between the I161V and I206V mutations. Importantly, the A312 residue lies within CA helix 9, near the I206-containing helix 4 of the adjacent CA monomer (Fig. 4D, bottom). Thus, SIVmac239 CA structural data suggest that the A312P mutation may influence the intermolecular interactions and stability of the CA hexamer. In support of this hypothesis, we consistently detected higher levels of intracellular Gag p27 CA in cells infected with viruses containing the A312P mutations (Fig. 4E). Taken together, our structural and *in vitro* sequencing data strongly support our conclusion that A312P is a tertiary compensatory mutation that stabilizes SIVmac239 expressing the Gag\(_{181-189}\)CM9-associated compensatory mutation.

The association of A312P with the I161V and I206V compensatory mutations has not previously been described in studies of Gag, nor has the association of A312P with the I161V and I206V mutations in transmitted viruses. Therefore, further study of additional compensatory mutations, in terms of their frequency in circulating HIV strains and their effect on posttransmission CD8\(_{\text{T}}\) responses, is warranted to fully understand their impact on the adaptation of HIV to HLA molecules present at high frequency within the human population.

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