Immune-Driven Recombination and Loss of Control after HIV Superinfection

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Accessibility
Immune-driven recombination and loss of control after HIV superinfection

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After acute HIV infection, CD8+ T cells are able to control viral replication to a set point. This control is often lost after superinfection, although the mechanism behind this remains unclear. In this study, we illustrate in an HLA-B27+ subject that loss of viral control after HIV superinfection coincides with rapid recombination events within two narrow regions of Gag and Env. Screening for CD8+ T cell responses revealed that each of these recombination sites (~50 aa) encompassed distinct regions containing two immunodominant CD8 epitopes (B27–KK10 in Gag and Cw1–CL9 in Env). Viral escape and the subsequent development of variant-specific de novo CD8+ T cell responses against both epitopes were illustrative of the significant immune selection pressures exerted by both responses. Comprehensive analysis of the kinetics of CD8 responses and viral evolution indicated that the recombination events quickly facilitated viral escape from both dominant WT- and variant-specific responses. These data suggest that the ability of a superinfecting strain of HIV to overcome preexisting immune control may be related to its ability to rapidly recombine in critical regions under immune selection pressure. These data also support a role for cellular immune pressures in driving the selection of new recombinant forms of HIV.

The strong antiviral activity of KK10-specific CD8 responses might be caused by their ability to effectively recognize early viral escape variants. Viral escape typically develops rapidly in the KK10 epitope through a position six L268M escape mutation (7). However, de novo variant-specific CD8 responses against the L268M escape variant are commonly mounted (8, 9), which eventually leads to the subsequent selection of the more potent position 2 escape mutation (R264K) that is associated with the eventual loss of viral control late in chronic infection (7, 10, 11). Therefore, variant-specific responses may play an important role in the control of HIV, enabling prolonged recognition of escaped viruses (12, 13). Unfortunately, the vast diversity of HIV-specific CD8 responses in infected subjects, and the progressive viral escape from these responses, has made it difficult to determine the
The relative importance of particular responses and escape mutations, either singly or collectively, on viral containment and disease progression.

The ability of the immune system to contain viral replication is also substantially impacted after HIV superinfection. Numerous cases of superinfection have been identified, usually on the basis of a sudden increase in viral loads (14–17). A dramatic shift in the immunodominance patterns of CD8 responses before and after superinfection has also been observed (14); this shift may be related to the transmission of mutations within targeted CD8 epitopes. Although new CD8 responses arise after superinfection, control over viral replication is often lost (14–16), and the factors contributing to the inability of preexisting immune responses to contain the superinfecting strain have as yet not been identified.

HIV superinfection may also enable recombination between two different strains (17), which could facilitate evasion of host immune responses. Recent data suggest that circulating recombinant forms (CRFs) of HIV may be far more common than previously observed (18). New CRFs may have a critical impact on vaccine design as they continue to expand the extensive global diversity of HIV (19). Equally problematic is that there appears to be little or no pattern to the selection of recombination sites within CRFs (15, 20) and the forces governing recombination (15, 21).

In this study, we provide a comprehensive analysis of the forces dictating HIV recombination after superinfection, which rapidly lead to the dramatic loss of viral containment in a subject expressing the otherwise protective MHC class I allele HLA-B27.

RESULTS AND DISCUSSION

Loss of control of viral replication in the setting of HLA-B27

Control of HIV in the presence of HLA-B27 (6, 7, 11, 22) has been attributed to the early and immunodominant targeting of a highly conserved CD8 epitope (KK10; KRWIIILGLNK) in Gag. The HLA-B27+ subject AC160, identified during primary HIV infection, rapidly controlled viral replication after a peak viremia of 468,000 copies/ml (Fig. 1). As early as day 22 after presentation, the dominant CD8 response was directed against the KK10 epitope and its variants, as measured by IFN-γ ELISpot assay (Table I). Only six other CD8 responses were detected by day 419, with the KK10 response remaining the most immunodominant (Table I). Viral sequencing revealed escape in this epitope through the stereotypic CTL escape mutation L268M, which first developed at day 419 (T3) coincident with a decline in the KK10 WT-specific response (Fig. 2 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080281/DC1). Viral escape coincided with an initial rise of viral loads to 14,000 copies/ml (Fig. 1), suggesting that loss of this key CD8 response may have partially impaired early viral containment. However, longitudinal testing revealed the subsequent development of an L268M variant-specific response, which expanded substantially by day 419 (Fig. 2 A). These data support not only an important contribution of the B27-KK10 response to early control of HIV, but also a role for variant-specific CD8 responses in limiting the impact of this early escape mutation (13).

Functionality of WT and variant–specific CD8 responses

Various studies suggest that the polyfunctionality of a CD8 response may be a critical indicator of vital effector functions and the ability of CD8 responses to actively impair HIV replication (23). Moreover, Almeida et al. have correlated the superior control of HIV replication in subjects expressing HLA-B27 with the polyfunctional capacity of the KK10–specific response (24). To further determine the role of the WT- and L268M-specific B27-KK10 CD8 responses, we examined the polyfunctional capacity of these responses by assessing five effector functions, including IFN-γ, IL-2, TNF-α, MIP-1β, and CD107a expression by multimer flow cytometry.

Table I. CD8+ T cell responses after superinfection and recombination

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Protein</th>
<th>Sequence</th>
<th>22 d (SFC/Mio.)</th>
<th>419 d (SFC/Mio.)</th>
<th>1,160 d a (SFC/Mio.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B27-KK10</td>
<td>p24</td>
<td>KRWIIILGLNK</td>
<td>85</td>
<td>830</td>
<td>250</td>
</tr>
<tr>
<td>B27-VL9</td>
<td>Vpr</td>
<td>VRHFPRiWL</td>
<td>-</td>
<td>550</td>
<td>920</td>
</tr>
<tr>
<td>B7-IL9</td>
<td>gp41</td>
<td>IPRRIRQGL</td>
<td>-</td>
<td>480</td>
<td>-</td>
</tr>
<tr>
<td>A2-AL9</td>
<td>Vpr</td>
<td>AIIRILQQL</td>
<td>-</td>
<td>310</td>
<td>-</td>
</tr>
<tr>
<td>Cw1-CL9</td>
<td>gp120</td>
<td>CAPAGFAIL</td>
<td>-</td>
<td>240</td>
<td>180</td>
</tr>
<tr>
<td>B27-IK9</td>
<td>p17</td>
<td>IRLPQGKK</td>
<td>-</td>
<td>230</td>
<td>960</td>
</tr>
<tr>
<td>Cw7-RY11</td>
<td>Nef</td>
<td>RR(D)LDDLWY</td>
<td>-</td>
<td>160</td>
<td>70</td>
</tr>
<tr>
<td>A1-YT9</td>
<td>Nef</td>
<td>YFPDQNYT</td>
<td>-</td>
<td>-</td>
<td>1,440</td>
</tr>
<tr>
<td>B7-RL9</td>
<td>Nef</td>
<td>RPMTYKAA</td>
<td>-</td>
<td>-</td>
<td>1,330</td>
</tr>
<tr>
<td>Cw1-VL8</td>
<td>p24</td>
<td>VIPFMSAL</td>
<td>-</td>
<td>-</td>
<td>1,200</td>
</tr>
<tr>
<td>B7-TL10</td>
<td>Nef</td>
<td>TP(D)GVRY</td>
<td>-</td>
<td>-</td>
<td>1,070</td>
</tr>
<tr>
<td>B7-RV9</td>
<td>Nef</td>
<td>RPMTYKAA</td>
<td>-</td>
<td>-</td>
<td>1,040</td>
</tr>
<tr>
<td>A2-SAV10</td>
<td>gp41</td>
<td>SLI(NATA/A</td>
<td>-</td>
<td>-</td>
<td>980</td>
</tr>
</tbody>
</table>

*6 of the strongest responses, out of a total of 16 new responses developing after superinfection, are shown.
After the L_{268}M-specific CD8 response was first detectable (day 363), it substantially increased in polyfunctional capacity, reaching a similar polyfunctional profile compared with the WT KK10 response (day 545). Both the WT and variant-specific responses exhibited comparable polyfunctional capacities over time (Fig. 2 B), which were not significantly statistically different from one another. To exclude possible cross recognition between the WT and L_{268}M-specific responses, we assessed their antigen-specificity in a dual-tetramer staining experiment (Fig. 2 C), confirming two distinct responses with no detectable cross-reactivity.

The cell surface marker for exhaustion programmed death-1 (PD-1) is up-regulated in CD8+ T cells under high antigen load, but is significantly down-regulated in subjects exhibiting low viral loads or those under antiretroviral therapy (25, 26). Therefore, because it may also be interpreted as an indirect marker of antigen recognition, we assessed the expression of PD-1 on tetramer-specific CD8 cells for the WT KK10 and L_{268}M-specific KK10 responses. Interestingly, both responses revealed similar levels of T cell exhaustion (median fluorescent intensity, WT KK10 530 vs. L_{268}M 518; unpublished data). Collectively, these data support that both the WT and L_{268}M-specific CD8 responses are actively recognizing infected cells, and, in concert, exhibiting immune selection pressures against both forms of the B27-KK10 epitope. More importantly, they suggest that functional variant-specific...
responses may be contributing to the control of viral replication by tempering the impact of early CTL escape mutations (13).

**Superinfection is associated with loss of viral control**

Development of the R264K escape mutation in B27-KK10 nearly completely abrogates binding of the epitope to HLA-B27 (11) and has been correlated to a dramatic loss of control over viral replication (6, 7, 11). However, when viral loads rapidly increased to 380,000 copies/ml at day 503 (T4), this specific mutation was notably absent. Rather, a phylogenetic analysis revealed that the virus present at day 503, in fact, represented a distinct strain of HIV (Fig. 3 A and Fig. S1), which is indicative of superinfection with a second strain of HIV. The second strain then dominated over the nearly 3 yr of follow up, with no subsequent detection of the original infecting strain (unpublished data). The shift within the immunodominance pattern of CD8 responses over this same time frame supported this change in the circulating virus (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20080281/DC1). Remarkably, five of the seven CD8 responses present before superinfection subsequently declined, whereas other novel CD8 responses became immunodominant. A comparison of viral sequences revealed that the superinfecting strain transmitted mutations in five of the seven initially targeted epitopes (Table S1). Similar changes in the patterns of CD8 responses after superinfection have been previously described (14), suggestive of a

Figure 3. Superinfection and recombination. (A) HIV gag sequences from subject AC160 and 19 other HIV chronic-infected subjects were compared using a neighbor-joining phylogenetic tree. Sequences from subject AC160 derived from the first year of infection (days 22, 83, and 419) cluster independently from sequences derived later in infection (days 503 and 545). Scale bar indicates the genetic distance along the branches, and bootstrap values >60 are shown. (B) SimPlot recombination analysis of a full-length HIV sequence derived from day 545 compared with viruses derived from pre-superinfection (day 83, green; day 419, red) and the superinfecting strain (day 503, blue) using a window of 100 bp and a step size of 10 bp. Two regions with double recombination breakpoints were observed in Gag and Env and Yates-corrected /H9273 values, and P values were calculated for each putative breakpoint. Breakpoints in gag were detected around positions 690 (/H9273 = 5.8; P = 0.0165) and 822 (/H9273 = 50.1; P < 0.0001), and breakpoints in env were detected around positions 6,047 (/H9273 = 50.1; P < 0.0001) and 6,164 (/H9273 = 30.1; P < 0.0001). By day 860 (T8), the breakpoints in gag were no longer detectable, supportive of a possible second recombination event in the recombinant strain that restored this region of the original superinfecting strain. (C) Longitudinal amino acid alignment of Gag sequences in AC160. Sequences derived from the primary infecting strain (black), the superinfecting strain (red), and the new recombinant form (blue) are aligned to clade B consensus. Early viral escape was observed in the B27-KK10 epitope boxed in red. Flanking sequences are deleted (//) to illustrate sequence diversity between the two strains. Sequences suggested to be involved in the first recombination event are shaded gray, whereas those involved in the second recombination event are shaded yellow. (D) Longitudinal amino acid alignment of Env sequences containing the Cw1-CL9 epitope are boxed in red. Sequences suggested to be involved in the third recombination event in Env are shaded gray.
potentially critical role for transmitted mutations in the eva-
sion of CD8 responses and enabling outgrowth of the new
incoming strain.

Viral recombination in regions under strong immune
selection pressure
Despite the documented importance of the KK10-specific
CD8 response, unexpectedly, there was no sequence evolution
observed in the KK10 epitope at the time of superinfection.
Rather the same preexisting L\textsubscript{268}M escape mutation was
present in the superinfecting strain. Surprisingly, however,
only 2 mo after superinfection (day 345), we observed an un-
characteristic reversion of the L\textsubscript{268}M mutation to the WT se-
quence in addition to other sequence changes surrounding
KK10 (Fig. S1). Strikingly, the comparison of longitudinal
sequences revealed a short recombination event in Gag be-
tween the original and superinfecting strain at day 545 (T5;
Fig. 3 B), which resulted in substitution of a narrow region of
Gag from the original strain between amino acid residues 220
and 274 within the superinfecting strain (Fig. 3 C). This short
region overlapped with the B27-KK10 epitope and resulted in
an unusual replacement of the L\textsubscript{268}M mutation with the
WT form present during acute infection. To evaluate whether
immune selection pressures might have influenced this re-
combination event, we examined the WT and variant-specific
KK10 CD8 responses at the time of superinfection. Longitu-
dinal analysis of IFN-γ ELISpot responses revealed that at
the time of superinfection the L\textsubscript{268}M-specific response was
actually substantially stronger (1528 SFC/Mio.), and also of
higher avidity (IC\textsubscript{50} \sim 0.26 μg/ml; not depicted), than the WT-
specific response (531 SFC/Mio.; IC\textsubscript{50} \sim 0.88 μg/ml; Fig. 2 A).
Therefore, in the setting of a substantially stronger L\textsubscript{268}M-
specific response at day 503, substitution of this region con-
taining the L\textsubscript{268}M mutation with the WT form of the epitope
would have facilitated evasion from a stronger L\textsubscript{268}M-specific response. Supportive of this hypothesis, levels of the L\textsubscript{268}M-
specific response subsequently declined by as early as day 664
(T6; Fig. 2 A). These data suggest that a recombination event
in a narrow region of Gag within 2 mo of superinfection
facilitated rapid escape from the dominant variant-specific
KK10 response.

Transient regain of viral control is lost through development
of a second recombination event in Gag
After recombination to the WT sequence, viral loads de-
clined under the presence of a WT KK10-specific CD8 re-
sponse to 20,000 copies/ml by day 762, but then rebounded
again to 139,000 copies/ml at day 860 (T8; Fig. 1). Viral se-
quences in the KK10 epitope at this later time point illus-
trated development of the R\textsubscript{264}K escape mutation (Fig. 3 C).
Moreover, these sequences revealed the likely occurrence of
a second recombination event having again substituted a short
sequence surrounding KK10 and facilitating development of
the R\textsubscript{264}K mutation (Fig. 3 C), although the narrow length of
this second recombination event precluded a critical analysis
of breakpoints. The R\textsubscript{264}K mutation has been shown to sub-
stantially impair the recognition of both the WT- and L\textsubscript{268}M-
specific responses (6, 7) and normally requires the specific
compensatory mutation S\textsubscript{173}A (11). In subject AC160, how-
ever, the S\textsubscript{173}A mutation did not arise, but rather two other
distinct mutations (T\textsubscript{239}V and N\textsubscript{252}S) accompanied R\textsubscript{264}K.
As we have previously observed the development of R\textsubscript{264}K in
conjunction with other rare mutations in its proximity (22),
it is likely that one or both of these mutations serve to com-
pensate for the fitness defect of R\textsubscript{264}K. Therefore, develop-
ment of R\textsubscript{264}K through recombination was likely facilitated
by suitable compensatory mutations developing within the
backbone sequence of the superinfecting strain rather than
within the original infecting strain.

This development of R\textsubscript{264}K resulted in the impaired recog-
nition of both WT and variant-specific responses as indicated
by their decline by day 1,034 (T9; Fig. 2 A). Interestingly,
although the L\textsubscript{268}M-specific response was dominant and of
higher functional avidity than the WT KK10 response before
the first recombination event, both the WT and L\textsubscript{268}M re-
ponses were co-dominant and of similar functional avidity at
day 664 (T6) before this second recombination event (1,334
SFC/Mio. and logIC\textsubscript{50} \sim 0.04 vs. 1,450 SFC/Mio. and logIC\textsubscript{50}
\sim 0.04, respectively; Fig. 2 A). Therefore, at the time the
R\textsubscript{264}K mutation developed in conjunction with a likely second
recombination event, both the WT and the variant-specific
responses were eliciting similar immune selection pressure.

Recombination in envelope
To determine whether recombination at sites of strong im-
mune selection pressure might represent a more common
phenomenon to escape potent CD8 responses after superin-
fection, full-length viral genome sequences from nine time
points over the course of infection were compared. Only one
other recombination event of \sim 40 aa in length was detected
across the whole viral genome. This small recombination
event between aa 220 and 260 of envelope arose at day 545
(T5), at the same time as the first recombination event in
KK10 (Fig. 3 B). This region resides outside of the V3 loop
of the virus, and no switch in the co-receptor tropism was
detected using the geno2pheno method (27). Notably, this
short region of recombination contained one of the other
six CD8 epitopes targeted before superinfection, namely the
Cw1-CL9 epitope CAPAGFAIL (Table I and Fig. 4). Similar to
the KK10 response, this Cw1-restricted response exerted
immune selection pressure against the virus as indicated by
early viral escape through an A\textsubscript{217}T mutation developing at
day 419 (T3) that was shown to impair T cell recognition
(Fig. 3 D and not depicted). As superinfection had resulted
in transmission of the WT form of the Cw1-CL9 epitope,
the subsequent recombination event at day 545 (T5) rapidly
replaced this region with a sequence containing the A\textsubscript{217}T
escape mutation derived from the virus circulating at day 419
(T3; Fig. 3 D). Thus, viral escape in this epitope after super-
infection was also accomplished through recombination
rather than through a single amino acid substitution. Simi-
lar to the KK10 epitope, an A\textsubscript{217}T variant-specific response
The current study indicates that specific selection forces, in this case cellular immune pressures, can strongly influence recombination. The diversity of HLA class I alleles in the population, and the variety of CD8 responses, would explain the complex array of recombination breakpoints between different HIV strains observed to date (19) and the inability to ascribe a strong predictor of recombination hotspots. In conclusion, these data suggest that the ability of a superinfecting strain to overcome immune control may be related to its ability to rapidly recombine in critical regions under immune selection pressure. These data also support a critical role for cellular immune pressures in driving the selection of recombination break points, thus contributing to the selection of new circulat- ing recombinant forms of HIV.

MATERIALS AND METHODS

Subjects. The HLA-B27+ subject AC160, identified during primary HIV infection, and 19 other chronically infected control subjects, were enrolled at Massachusetts General Hospital in Boston. The study was approved by the institutional review board of the Massachusetts General Hospital.

Sequencing of autologous virus. Population sequences of autologous full-length viral genomes were derived from proviral DNA, as previously described (13). In brief, genomic DNA extracted from 5 million PBMCs using the QIAamp DNA Blood Mini kit (QIAGEN). Nested PCR protocols with limiting dilution used to amplify full-length HIV genomes using EXL DNA Polymerase (Stratagene). The sequences of primary forward and reverse PCR primers, respectively, are 5'-AAATCTCTAGAGTGGGCCCAGCAG-3' and 5'-TCAGGGATCTCTAGTTACACAGTG-3', whereas the nested forward and reverse primers are 5'-GGCGGAGCG- TAGAAAGGAGAGAGGTCG-3' and 5'-GCACCTAGGCAAGCTTTAGTTATG-3'. PCR cycling conditions were as follows: 92°C for 2 min; 10 cycles of 10 s at 92°C, 30 s at 56°C, or 10 min at 68°C; and 20 cycles of 10 s at 92°C, 30 s at 60°C, 10 min at 68°C, and a final extension of 10 min at 68°C. Five independent PCR products of each sample were pooled and purified using the QIAquick PCR Purification kit (QIAGEN) and population sequenced bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems) using 70 clade B consensus sequencing primers, as previously described (14).

Autologous clonal gag and env sequences were derived from plasma RNA, as previously described (13). Viral RNA was isolated from plasma, and nested PCR was conducted using a set of described primers specific for HIV (14). First round PCR cycling conditions were as follows: 94°C for 2 min, 35–50 cycles of 30 s at 94°C, 30 s at 56°C, 2 min at 72°C, and a final extension of 68°C for 20 min, and nested PCR reactions were shortened to a 1-min extension time. PCR fragments were then gel purified and sequenced directly or cloned (TOPO TA Cloning kit; Invitrogen). Plasmid DNA was isolated by miniprep (QIAprep Turbo Miniprep) and sequenced bi-directionally. Sequence data were manually edited using Sequencher 4.6 (Gene Codes Corporation). In regions where secondary peaks were observed, the dominant base was called. Nucleotide sequences were conceptually translated and aligned using MacVector 7.2.3 (Accelrys). The clade B HIV consensus sequence (2002) from Los Alamos National Laboratory HIV Sequence Database was used as the reference sequence to compare with our sequencing data. All sequence data were deposited in GenBank under accession nos. EU616639–EU616649.

Phylogenetic analysis. Gag sequences from subject AC160 were aligned and compared with sequences derived from chronically infected subjects within a chronic infection cohort in Boston using Phylib3.6 for constructing neighbor-joining phylogenetic tree.

Recombination. Recombination break points were determined using SimPlot (see Supplemental materials and methods and Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20080281/DC1). Full-length HIV sequences were analyzed using a 100-bp window and a 10-bp step size, and Yates-corrected χ2 values and P values were calculated for each putative breakpoint.

ELISpot. Baseline HIV-specific CD8+ T cell responses were quantified on freshly isolated PBMCs by IFN-γ ELISpot assay (4) using 410 overlapping
peptides, varying from 15–20 aa in length and overlapping by 10 aa, which spanned the entire clade B consensus sequence 2001, as previously described (4). In addition, described optimal peptides corresponding to HLA-matched epitopes were used to detect responses from frozen cells. Peptides were synthesized commercially (Research Genetics) or at the Massachusetts General Hospital Peptide Synthesis Core Facility. PBMCs were plated at 150,000 cells/well with peptides at a final concentration of 14 μg/ml in 96-well plates and processed as previously described (4). PBMCs were incubated with medium alone (negative control) and PHA (positive control). The number of specific IFN-γ-secreting T cells were counted using an automated ELISpot reader (AID), calculated by subtracting the average negative control value and expressed as SFCs per 10^9 input cells. Negative controls were always ≤ 30 SFCs per 10^6 input cells. A response was considered positive if ≥ 5 SFCs per 10^6 and at least 3 times greater than mean background activity. Functional avidity curves for the comparison of recognition of epitope variants were performed by ELISpot assay using serial dilutions of truncated peptides, as previously described (4, 14).

Assessment of CD8+ T cell polyfunctionality. Cryopreserved PBMCs were thawed, resuspended to 1–2 × 10^6 cells/ml in R10 media (RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 5.5 ml Hepes buffer), and rested for 1–2 h at 37°C, 5% CO₂. PBMCs were examined for viability by trypan blue exclusion (typically 80–90% viable) and adjusted to 10^6 cells/ml. Co-stimulatory antibodies (CD28 and 49d; 1 μg/ml; BD Biosciences) and CD107a-PE-Cy5 (BD Biosciences) were added, and the cells were aliquoted at 1 ml to each tube containing 2 μg/ml of each peptide. An unstimulated (R10 only) and a positive control (2 μL of PMA 1 μg/ml and 1 μL of ionomycin 1 μg/ml; AG scientific®) were included in each assay. Cells were incubated for 30 min at 37°C, 5% CO₂, and monensin (0.7 μl/ml GolgiStop; BD Biosciences) were added. After a total incubation of 6 h, the cells were washed with PBS and stained for intracellular amine groups to discriminate between live and dead cells (blue viability dye; Invitrogen). Cells were washed again and stained with a panel of anti-CD3-Pacific Blue (BD Biosciences) and anti-CD8-APC-Cy7 (BD Biosciences). Cells were then fixed in 1% paraformaldehyde, washed with PBS, and permeabilized using Fix Perm A and Fix Perm B solution (Caltag Laboratories). Cells were intracellularly stained using a panel of IL-2-FITC (BD Biosciences), IFN-γ-PE-Cy7 (BD Biosciences), TNF-α-Alexa700 (BD Biosciences), and MIP-1β-PE (BD Biosciences). Between 150,000 and 500,000 events were collected per sample. All data were analyzed using FlowJo 8.3.3 software (Tree Star, Inc.). Initial gating was on the lymphocyte population, and then used a forward scatter width (FSC-W) versus height (FSC-H) plot to remove doublets. Subsequently, the events were gated through a side scatter (SSC) versus blue viability dye (UV) and sequentially gated on CD3+ and CD8+ events. After identification of CD8+ T cells, a gate was made for each respective function using combinations that provided optimal separation. After the gates for each function were created, we used the Boolean gate platform to create the full array of possible combinations, equating to 32 response patterns when testing 5 functions. Data are reported after background correction, and the percent of epitope-specific CD8+ T cell responses had to be more than twofold higher than background for individual cytokines or CD107a to be considered as a positive response. Data were then analyzed using SPICE software (provided by M. Roederer, National Institutes of Health) for WT variant of KK10 and a PE-labeled pentamer (ProImmune) for L→M variant of KK10 for 20 min at room temperature. After an additional wash, cells were stained for surface antibodies against CD3, CD4, CD8 (BD Biosciences), and PD-1 (provided by G. Freeman, Harvard Medical School, Boston, MA) and incubated for 20 min at room temperature. Cells were fixed with 1% paraformaldehyde, and events were acquired on a LSRII (BD Biosciences). Data were analyzed using FlowJo software 8.3.3.

Online supplemental material. Fig. S1 shows longitudinal amino acid alignment of Gag sequences. Fig. S2 shows that superinfection and recombination is paralleled by a disintegration of the CD8+ T cell immunodominance patterns. Fig. S3 shows the detection of superinfection using the reversible (GTR) model of nucleotide substitution. Table S1 lists the viral sequences of the targeted CD8+ T cell responses before and after superinfection and recombination, with supplemental materials and methods providing a description of the analysis of recombination using clonal gag sequences. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20080261/DC1.

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