Recombination-mediated escape from primary CD8+ T cells in acute HIV-1 infection

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Abstract

Background: A major immune evasion mechanism of HIV-1 is the accumulation of non-synonymous mutations in and around T cell epitopes, resulting in loss of T cell recognition and virus escape.

Results: Here we analyze primary CD8+ T cell responses and virus escape in a HLA B*81 expressing subject who was infected with two T/F viruses from a single donor. In addition to classic escape through non-synonymous mutation/s, we also observed rapid selection of multiple recombinant viruses that conferred escape from T cells specific for two epitopes in Nef.

Conclusions: Our study shows that recombination between multiple T/F viruses provide greater options for acute escape from CD8+ T cell responses than seen in cases of single T/F virus infection. This process may contribute to the rapid disease progression in patients infected by multiple T/F viruses.

Keywords: HIV-1, T cell, Multiple infection, Recombination, Immunodominance, Acute infection

Background

HIV-1 specific CD8+ T cells are first detected prior to peak viremia and expand concomitantly with decline of acute plasma virus load (pVL) [1,2]. HIV-1 peptide epitopes are presented to CD8+ T cells in complex with polymorphic human leukocyte antigen (HLA)-I molecules. Certain HLA allotypes are associated with lower pVL setpoints and better clinical outcomes, suggesting an important role for CD8+ T cells in control of HIV-1 replication [3-6]. HIV-1 specific CD8+ T cells are also a major selective force in viral evolution in vivo [7,8] and can select non-synonymous virus escape mutants in and around the reactive epitope, that wholly or partially ablate T cell reactivity, within weeks of infection [9,10]. The timing of escape for each epitope is not random and is heavily impacted by the relative immunodominance of an individual CD8+ T cell response and the Shannon entropy, or population variability, of the targeted epitope [10,11].

HIV-1 infection with a single transmitted/founder (T/F) virus occurs in around 80% of heterosexual infections [12-14]. The proportion of multiple T/F viruses initiating infection increases in other groups, such as men who have sex with men and intravenous drug users. Infection with multiple T/F viruses is linked to factors that are known to increase overall transmission rates, such as higher risk sex acts and other concurrent sexually transmitted infections [12,15-19]. Several studies have associated infection with multiple HIV-1 T/F viruses, multiple subtypes, and/or a diverse virus population, with higher pVL setpoint, faster CD4+ T cell decline, earlier need for anti-retroviral therapy and a worse prognosis for the infected individual [14,20-24].

The emergence of recombinant viruses results from infection of a cell with two or more different viruses [25]. HIV-1 is highly recombinogenic [26] and HIV-1 recombination has been observed in patients infected with multiple viruses within weeks-months of infection [12,14,15,17]. Although none of these acute-phase studies have experimentally linked the emergence of recombinants to immune responses, several mathematical models have suggested that recombination may impact escape from CD8+ T cell responses [27,28]. Such associations have been suggested in one study of superinfection during the chronic stage of HIV-1 infection [29].

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Here we report on a subject infected with two T/F viruses. We find that differential T cell targeting of the two T/F viruses drives accelerated recombination-mediated escape in acute infection.

**Results**

**Acute HIV-1 replication in subject CH078**

Subject CH078 was detected in acute HIV-1 infection stage Fiebig I/II (seronegative, pVL = 3 748 087 copies/ml), near peak viremia [30,31]. Genital ulcer disease, which has been associated with higher risk of HIV-1 transmission [32], was diagnosed at enrolment, 3 weeks later. From peak viremia, his pVL declined rapidly by ~2 log within the first 28 days from Fiebig I-II, then stabilized, even increasing slightly over the next 7 weeks (days 28–77). This was followed by a period of slower pVL decline of ~1 log over several months to establish a setpoint of 3,520 copies/ml around 6 months post-screening (Figure 1). CD4+ cell counts increased from a nadir of 251 cells/µl, 21 days post-screening and remained >300 cells/µl over the rest of the study period (441 days total) (Figure 1). His HLA type (A*01, A*30, B*42, B*81, Cw*17, Cw*18) included the protective HLA B*81 allele. In accordance with local clinical practice guidelines applicable at the time, he was not initiated on antiretroviral therapy during the course of this study.

**Patient CH078 was infected with two T/F viruses**

Single genome amplification (SGA) and sequencing of overlapping 5′ and 3′ halves of HIV-1 genomes from subject plasma were performed at nine time points from screening to 441 days post-screening (Figure 1). This approach [13], allowed for analysis of recombination events. Fifty, 3′-half genome sequences were analyzed at screening (Fiebig I-II) giving >90% confidence to detect virus variants at the 5% level [12]. Analysis identified (Additional file 1: Figure S1), a ‘major’ (96%) predominating virus with the other T/F ‘minor’ accounting for the remainder of the viral populations. These viruses were highly related (1.2% nucleotide differences in env) suggesting transmission from a single donor. The greatest variability was observed in Nef which differed by 10.7% at the amino acid (aa) level (Table 1). Two viral lineages were also detected in the 5′ side of the virus; however the presumed minor population (2 of 11 sequences) was not detected until 28 days post-screening (Additional file 1: Figure S1). Therefore, the 5′ half of the genome sequence for the minor T/F virus could not be reliably inferred and differences between the major and minor T/F viruses in the 3′ half genome were used to track changes in relative viral frequencies and recombination.

The first recombinant viruses were detected at day 35 from Fiebig I/II, as pVL initially stabilized (day 28–77). Recombinants then rapidly increased in frequency, reaching 44% of all sequences at day 42 and representing 100% of all sequences at day 77 and all subsequent timepoints (Additional file 1: Figure S1).

**Primary HIV-1 specific CD8+ T cell responses target both viruses**

We examined whether acute T cell responses were associated with virus escape by mutation and recombination. HIV-1 specific T cell responses were comprehensively mapped in IFN-γ ELISpot assays using overlapping
peptides that matched and spanned both the major and minor T/F viruses, and optimal epitopes subsequently defined experimentally. All T cell responses were CD8+ restricted and dominated by a central memory CD45RO+CD27+ phenotype (Additional file 2: Figure S2).

Five primary HIV-1 specific T cell responses were detected when pVL was rapidly declining in acute infection (Figures 1 and 2A) of which four (Gag180-188, Pol426-434, Vif73-81, Rev12-20) targeted identical epitopes in the major and minor viruses (shared epitopes). Variable escape kinetics were observed following the accumulation of non-synonymous mutations within the targeted epitopes (Figure 3). The fifth T cell response targeted a 9-mer epitope (Nef68-76) that varied between the two T/F viruses by 1 aa at position 71 (variable epitope).

As infection progressed, three new HIV-1 specific T cell responses emerged, two targeting shared epitopes (Gag2534, Env209-217) (Figure 2). The third targeted a variable 9-mer epitope (Nef19-27), which differed between the two T/F viruses at 2 aa residues at positions 25 and 26.

### Escape from T cell responses by virus recombination

The CD8+ T cell response that targeted the variable Nef68-76 epitope recognized the major T/F epitope with greater magnitude (>10-fold), higher functional avidity (Figure 4A and B), and an increased proportion of reactive cells producing 3 or more cytokines when compared with the minor virus epitope (Additional file 3: Figure S3).

Within 50aa upstream of the Nef68-76 reactive epitope, a second Nef specific T cell response, Nef19-27, also recognized an epitope that was variable. In contrast to the Nef68-76 T cell response, the Nef19-27 response detected the minor virus and not the corresponding major virus epitope (Figure 4C). The minor virus existed at only 4% at Fiebig I-II and therefore the level of presentation...
of this epitope would have been very low relative to the corresponding major epitope. By day 35, the minor Nef19-27 epitope was found in 15% of sequences but no T cell response was detectable in ex vivo assays. However, at the next timepoint (day 49) when cells were available, this T cell response was detected strongly at > 500 SFU/10^6 cells, suggesting this T cell response emerged between day 35 and 49.

Given that these Nef-reactive T cell responses each targeted the major and minor T/F viruses in a reciprocal manner, we explored whether HIV-1 could evade these two T cell responses through recombination. Amino acid variation between the two T/F viruses was also found in the region (114aa) surrounding these epitopes (Figure 5), which allowed us to unequivocally distinguish the recombinants from the T/F viruses and identify recombination breakpoints. No recombination was detected before day 35 (Additional file 4: Figure S4). At day 35, 2 out of 25 (8%) of sequences were recombinants, and both of these shared identical breakpoints (Figure 5, Additional file 4: Figure S4). Recombinants increased to 44% and 65% at days 42 and 49 respectively, with different breakpoints identified in different sequences (Additional file 4: Figure S4). From days 35 to 49, a total of 32 sequences had a recombination breakpoint between the two epitopes, and in all cases were found to be carrying the epitopes least targeted by T cell responses (Figure 5). Five distinct breakpoints were identified in this population of 32 sequences (Figure 5). This suggests that these viruses became dually resistant to the Nef-reactive CD8+ T cell

**Figure 3** CD8+ T cells targeting shared epitopes escaped via accumulation of variants in the reactive epitope. The epitopes Rev12-20 (A), Pol426-434 (B), Vif73-81 (C), and Gag25-34 (D) were shared between the major and minor T/F viruses and showed escape. The top graph in each figure represents the changing frequencies of each epitope as measured by SGA sequencing over time. The bottom graph in each figure represents the absolute magnitude of the T cell responses targeting all tested variants of the epitope, measured using ex vivo IFN-γ ELISpots. Each figure has a different scale on the Y axis for the bottom graph. Data for the T/F epitope are shown using squares, data relating to the escape variants tested in T cell assays are shown as triangles, while in the top graph only, other epitope variants (neither T/F nor tested escape variants) are shown as gray circles (others). Screening corresponds to Fiebig I/II.
responses through independent, recombination events and the recombinants were selected because they afforded a replication advantage under dual T cell selection pressure.

Classical T cell escape of variable epitope occurred following recombination

As infection progressed, initial, partial escape from the T cell responses targeting two Nef epitopes through recombination was followed by classic escape, i.e. the emergence of de novo mutations (not present in either T/F virus and not derived through recombination) within the targeted epitopes, conferring complete escape. The R71T change at position 4 in the Nef68-76 epitope, which was first detected at day 134 and was present in 100% of SGA sequences at day 441, abrogated all detectable T cell recognition (Figure 4A). In the Nef 19–27 epitope, additional intra-epitope R19K and R21K changes also independently ablated T cell recognition (Figure 4C).

Discussion

Two major determinants of virus escape patterns are immunodominance and epitope entropy (population level sequence variability) [10,11]. In CH078, the early immunodominant Rev-specific T cell response which targeted a high entropy epitope escaped within weeks (Figure 3). Escape in this epitope occurred classically through the accumulation of non-synonymous mutations within the epitope. Conversely, the acute CD8+ T cell response targeting the Gag TL9 epitope which is very low entropy did not escape over the study window, despite subsequently gaining immunodominance. Slow escape in the TL9 epitope has been described elsewhere [33].

By contrast, the Nef68–76 CD8+ T cell response, which was subdominant and targeted a low entropy epitope, both parameters associated with slow virus escape [10], escaped rapidly, within weeks of infection. Here, recombination provided an accelerated escape in acute HIV-1 infection. The efficiency of recombination as a rapid mode of escape from T cell immune pressure in acute HIV-1 infection was highlighted by selection of recombinants that simultaneously excised two Nef epitopes located 50aa apart. Five different recombination patterns were selected within the same short period, strongly implying that direct selective forces were acting on this region as opposed to selection occurring elsewhere in the genome and recombinant strains becoming dominant through linkage between two epitopes with escape mutations.
These observations of rapid recombination and CD8+ T cell escape in acute infection are novel. They add to, but are distinct from, two previous reports that describe superinfection in the chronic stage of HIV-1 infection. Those studies described the selection of the superinfecting strain because it contained mutations that conferred escape from circulating T cell responses [29,34]. In one of these studies, recombination was detected in a patient two months after the superinfection event, and was associated with T cell pressure [29]. Here, our more detailed sampling clearly shows that primary HIV-1 specific T cell responses can select recombination between distinct T/F viruses even more rapidly, within weeks of infection when pVL is stabilizing prior to the establishment of setpoint. These observations provide empirical evidence of a mechanism beyond stochastic events to explain the emergence of recombination reported in acute infection [12,14,15,17]. A striking feature of the results presented here are the five independent recombinations observed in a very short time period and the fact that two separate T cell responses, neither immunodominant, combined to select the recombinations.

Very interestingly, as infection progressed the early recombination mediated escape in the Nef epitopes was followed by the emergence of non-synonymous mutations within the targeted epitopes that themselves more strongly ablated CD8 T cell recognition. This is consistent with recombination offering not only an alternate route of escape but in some cases more rapid escape.

Our observations are also consistent with others [35,36], that show T cell escape is a dynamic process. In addition to the ongoing emergence of HIV-1 variants through either recombination or mutation, we also observed changing recognition of T cell escape variants...
over time (Figure 3, Additional file 5: Supplementary Text). This is best explained by the emergence of discrete circulating T cell clonotypes that differentially recognized epitopes as HIV-1 infection progressed [33]. These data underscore the complexity of interpreting T cell selection pressure in chronic HIV-1 infection.

Although it seems likely that the selection of recombinants in some patients may have negative outcomes for the host, this did not occur in this patient, at least in the study window observed. Other host responses, including the subject’s more dominant T cell responses which exhibited consistent oligofunctionality, could have contributed to the virus control observed; particularly as this subject expressed the HLA B*81 allele which is overrepresented in viremic controllers [37]. Other viremic factors, variation in Nef-mediated immune evasion [38,39] or Gag-Protease replicative capacity [40] could have resulted in emergence of less fit and or less pathogenic viruses resulting in the lower setpoint. Given the number of variables in this, a single patient study, we cannot draw firm conclusions regarding the HIV-1 control observed.

Conclusions
This in depth case study of a patient infected with two T/F HIV-1 viruses has demonstrated that the appearance of recombinants known to occur during acute infection [12,14,15,17] can be driven by the selective action of CD8+ T cell responses.

These observations have clinical implications. Whilst we show that recombination mediated escape can occur within weeks of infection, the generation of escape mutants will be generally slower in regions of sequence conservation. Vaccine-induced T cell responses that dominantly target conserved regions of HIV-1 will therefore also be less subject to rapid escape by recombination suggesting a conserved immunogen design for prophylactic T cell vaccines could also retain benefit in individuals infected with > 1 HIV-1 T/F virus. Functional cure of HIV-1 is an emerging field with a role for HIV-1 specific CD8 T cells to detect and inhibit HIV-1 reservoirs recently highlighted [41]. Our observations suggest that following reactivation of the HIV-1 reservoir, recombination between proviruses, even in individuals who received very early antiretroviral therapy, could mediate very rapid escape from T cell responses.

Methods
Study subject
CHAVI patient 078 is a black South African male aged 22 at the time of screening. Transmission occurred via heterosexual sex from a known HIV-1+ patient. The subject remained antiretroviral naive over the subsequent study period of 442 days. At screening, approximately 22 days post transmission, samples were collected for viral identification and serology only. Twenty-one days post-screening, the patient was enrolled into the acute infection arm of the Center for HIV/AIDS Vaccine Immunology (CHAVI) 001 study. At enrolment and all subsequent visits, blood was drawn, PBMCs isolated, and these cells used for subsequent T cell studies. Plasma and sera were also isolated for viral sequencing at these visits.

Amplification of near full-length viral genome by SGA
Viral RNA was extracted from longitudinal plasma samples using the PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA). cDNA was synthesized using the SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with the primers 07Rev8 5′- CCTARTGGGATGTG TGTACCTTCTGAACCTTT-3′ (nt5193-5219 in HXB2) for the 5′ half genome, or primer 1.R3.B3R 5′- ACTTCTGTTTTCCAT-3′ (nt9611-9642) for the 3′ half genome. Single genome amplification (SGA) was performed to obtain the 5′ half, 3′ half or near full-length HIV-1 genome as described previously [13,42]. For the 5′ half genome amplification, the first round PCR was carried out using the primers 1.U5.B1F 5′-CCTTGAAGTGCTTCAAGTAGTG TGCGGCGTCGTG T-3′ (nt538-571) and 07Rev8 5′- CCTARTGGGATGTGTACTTCTGAACTTT-3′ (nt5193-5219), and the second round PCR with primers Upper1A 5′- AGTGGCCGCCCAGACAGG-3′ (nt634-650) and Rev11 5′- ATCATCACTGCGCATCTGTTTTCCAT-3′ (nt5041-5066). To amplify the 3′ half genome, the first round PCR was performed using the primers 07For7 5′- CAAATTAYAAAAATTCCAAATTTCGGGTTTTATCATG-3′ (nt4875-4912) and 2.R3.B6R 5′- TGAAGCAGCATAAGGCAAGCTTATGGGTTCCAT-3′ (nt9636-9607), and the second round PCR with primers VIF1 5′- GGGTATTACACGAGGAGCAGCAG-3′ (nt4900-4923) and Low2c 5′- TGAGGCTTAAGGAGTGTTCC-3′ (nt9591-9612).

Sequence analysis
The SGA amplicons were directly sequenced by the cycle sequencing and dye terminator methods on an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, CA). Individual sequences were assembled and edited using Sequencher 4.7 (Gene Codes, Ann Arbor, MI). The sequences were aligned using CLUSTAL W [43] and the manual adjustment for optimal alignment was performed using Seaview. The Neighbor-joining (NJ) tree was constructed using the Kimura 2-parameter model. The highlighter plot was generated using the Highlighter tool at the Los Alamos HIV sequence database (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html). Genbank accession numbers: 3′ sequences KC149035-149139, 5′ sequences KC148775-149034.
Recombination analysis

All sequences from different time points were compared to the parental T/F sequences. Recombination breakpoints were identified by RDP3 followed by manually inspection of the Highlighter plot. The aligned sequences were analyzed for recombination signals using BOOTSCAN, GENECONV, MAXCHI, CHIMAERA, SISCAN and 3SEQ in the program RDP3 with a window size of 20 nucleotides [44]. The T/F sequences were used as the parental sequences.

T cell response analysis

IFN-γ ELISpot mapping. Shannon entropy calculations [45] and time to T cell escape were performed as previously described [10]. For mapping, the entire proteome of both T/F viruses were tested using PBMCs from days 28 and 187, while responses against both nef proteomes were additionally tested for at days 42 and 105. To investigate whether ‘novel’ T cell responses were induced in response to viral diversification, variant peptides that matched non-synonymous ‘stripes’ that emerged over the course of the study window were tested. No de novo T cell responses were detected.

Once all optimal epitopes had been identified, these were tested at 1 μM concentrations as outlined across multiple visits using ex vivo IFN-γ ELISpots and flow cytometry. Avidity of responses was tested at some timepoints using peptide dilutions. Peptides are identified by alignment to HXB2 proteins.

Flow cytometry

Following thawing and O/N resting, PBMCs were stimulated with peptide or control (0.45% DMSO) for 6 hrs in the presence of Costim™ (BD Biosciences) plus Brefeldin A and Monensin. Anti-CD107a fluorochrome-labelled antibody was also added to assay for degranulation. Cells were then washed with PBS and incubated with LIVE/DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) were then washed with PBS and incubated with LIVE/DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were then washed with PBS and incubated with LIVE/DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixed with DEAD Aqua viability dye (Invitrogen Life Technologies) and permeabilised using Cytofix. CD4, CD8, CD19, CD45RO and CD27. Antibody was also added to assay for degranulation. Cells were fixe...
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