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Title:	Rapid Selection of Escape Mutants by the First CD8 T cell Responses in Acute HIV-1 Infection.
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Title: Rapid selection of escape mutants by the first CD8 T cell responses in acute HIV-1 infection

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Abstract:

The recent failure of a vaccine that primes T cell responses to control primary HIV-1 infection has raised doubts about the role of CD8+ T cells in early HIV-1 infection. We studied four patients who were identified shortly after HIV-1 infection and before seroconversion. In each patient there was very rapid selection of multiple HIV-1 escape mutants in the transmitted virus by CD8 T cells, including examples of complete fixation of non-synonymous substitutions within 2 weeks. Sequencing by single genome amplification suggested that the high rate of virus replication in acute infection gave a selective advantage to virus molecules that contained simultaneous and gained sequential T cell escape mutations. These observations show that whilst early HIV-1 specific CD8 T cells can act against virus, rapid escape means that these T cell responses are unlikely to benefit the patient and may in part explain why current HIV-1 T cell vaccines may not be protective.

Introduction (references formatted for Nature Immunology)

HIV-1 is most commonly transmitted by sexual contact where it first infects memory CD4 T and Langerhans cells in the genital mucosa [1]. The virus replicates locally and then disseminates to draining lymph nodes and to the gut associated lymphoid tissue (GALT). In the GALT, HIV-1 replicates exponentially, infecting and significantly depleting CD4 T cells [2, 3]. Between 5-18 days following infection, HIV-1 first becomes detectable in the blood by standard diagnostic assays [4]; shortly after which patients can present with flu-like symptoms that broaden with increased viral load in some but not all patients [5, 6].Plasma viremia peaks within 1-3 weeks of its first appearance [4], and is most likely curbed by exhaustion of GALT CD4+ cell targets for infection [3 7] and the host immune response . In the majority of infections, virus load declines from peak viremia stabilizing between 3 to 6 months after infection to a level known as 'viral setpoint'. Here, patients enter a phase of chronic infection where there is a relative balance between virus replication and the host immune response. Even so, the immune system is progressively weakened and in the absence of antiretroviral drug treatment, viral load increases and the patient presents with AIDS-defining illnesses usually within 5-15 years [8]. Viral setpoint varies greatly between patients and correlates to the rate of HIV-1 related disease progression [9]. Understanding to what extent

the host immune response controls the acute viremia and determines setpoint is critical to the design of vaccines aimed at controlling HIV-1 infection.

HIV-1 is highly diverse. In its envelope glycoprotein (Env), differences between clades in the chronic stage of infection are as high as 20 -30% whilst intra-clade variation ranges from 10-15% (reviewed in [10]). The diversity of HIV-1 is generated by a lack of proofreading activity of its reverse transcriptase producing a misincorporation, deletion, insertion or duplication in every $10^4 - 10^5$ nucleotides [11], together with a high replication rate that generates $>10^9$ virions per day in acute infection. Non-synonymous changes alter amino acids to provide the major immune evasion or 'escape' mechanism of this virus, whether from primary or vaccine-induced immune responses.

CD8 T cells appear central to successful host immunity to HIV-1. Studies of chronic HIV-1 infection have described a strong and broad HIV-1 specific CD8 T cell response in patients, although neither the breadth nor magnitude of the CD8 T cell response to peptides matching consensus clade sequences correlates with setpoint- [12, 13]. In contrast, in acute HIV-1 infection, CD8 T cells can be first detected prior to peak viremia and continue to expand coincidentally with the post-peak viral load decline suggesting primary T cells contribute to the early control of the virus [14, 15 16]. From acute through to chronic HIV-1 infection there is evidence for the selection of mutants that escape recognition by CD8 T cells, -which implies continuous immune pressure [17-19]. Certain HLA alleles, most significantly B57 and B27, that govern the sequence- of -antigenic peptides and the context in which they are presented to CD8 T cells, correlate with lower viral setpoint [20] and delayed progression to AIDS defining illness [21]. Further evidence of a protective role for CTL against HIV comes from studies in macaques challenged with simian immunodeficiency virus (SIV) or chimeric simian-human immunodeficiency virus (SHIV). Depletion of CD8 T cells in acute and chronic SIV infection resulted in increased virus loads [22, 23]. Furthermore, macaques immunized with vaccines that induce CD8 T cells specific for the challenge virus had significantly lower viral loads when compared to non-vaccinated controls following challenge with some SIV and SHIV hybrid viruses [24]; and protection was lost when viral escape occurred from an immundominant CD8 T cell response [25]. These data have been central to the rationale for vaccines designed to stimulate HIV-1-specific CD8+ T cells ahead of acute infection, which in theory, -should lower virus setpoint and decrease transmission rates. However in a recent clinical trial, the Merck recombinant gag, pol and nef adenovirus vaccine induced CD8+ T cell responses to a median of three HIV-1 epitopes in vaccinees, but failed to reduce virus load when participants subsequently became infected (http://www.hvtn.org/science/1107.html). At first sight this result suggests that CD8 T cell responses fail to offer any protection attains HIV-1. However information is needed -on the role CD8 T cells play in the control of the early, highly damaging viremia in acute HIV-1 infection, before abandoning this approach to vaccines.

Recently Keele et al [26], using modeling and phylogenetic tree analysis of Env sequences derived by single genome amplification (SGA), found that 76% of

HIV-1 transmissions result from infection with a single HIV-1 virion or virus-infected cell. The remaining patients were infected with 2-5 viruses [26]. Subsequent serial virus sequencing of the Env protein from plasmas detected substitutions in the weeks following transmission that became fixed (found in all SGA sequences) within 6-10 weeks suggesting selective pressure by the early immune response. In all subjects studied, Env diversification was first detected around the time of peak viremia and several of the changes occurred within motifs predicted to be epitopes recognized by CD8 T cells appropriate to the HLA of the patient, suggesting that T cells exert immune pressure very early in acute HIV-1 infection. Similar observations were made by Bernadin et al who reported that non-synonymous mutations sequenced in acute infection corresponded to previously described CTL epitopes [27]; although no T cell data were reported. In this study, we extend these and other earlier studies [18, 19, 28] presenting full length virus sequence data alongside cellular data to confirm that the transmitted HIV-1 virus begins to vary as soon as specific CD8 T cell responses first appear around the time of peak viremia. We show that the majority of primary HIV-1-specific CD8 T cell responses select escape mutations and that virus escape can be unexpectedly rapid with complete virus sequence change (fixation) occurring over a two-week period, sometimes in more than one epitope on the same viral genome in the same short time frame. The rate of fixation of these CD8 T cell escape mutants in acute HIV-1 infection is comparable to selection of mutants by single antiretroviral drug treatment, implying that the anti-HIV effect of CD8 T cells is greater than previously thought. These observations give a clear message for vaccine design.

Materials and Methods:

Patients and samples: Patients CH40, CH58 and CH77 gave full informed consent to enroll in to the acute infection arm of the CHAVI 001 cohort. Briefly, 'acute infection' was defined as positive for HIV-1 vRNA in plasma but negative or discordant for HIV-1 serology at a screening visit. Patients were bled using at multiple timepoints over 2 years. See Table 1 for the demographical and clinical data of each patient. Patient SUMA was detected in symptomatic acute HIV-1 infection as described in Graziosi et al[29].
Patient peripheral blood mononucleocytes (PBMC) were isolated from blood following centrifugation over a ficoll gradient. Cells were washed and counted then cryopreserved in liquid N2 in aliquots (~ 10^7 cells/vial). Plasma was obtained following centrifugation of blood collected in EDTA tubes or from ficoll gradients of blood described above.

Sequencing and alignments: RNA extraction, cDNA synthesis and single genome amplification (SGA) is described in Salasar-Gonzalez et, al[30]. SGA involves dilution of cDNA to the point that only 1 amplifiable cDNA template exists in a well 80% of the time. The benefit of this approach is to prevent PCR-based recombination events that can occur using other methodologies. SGA produced, depending on the PCR primers used, the full- or part-length sequences of individual viruses.

HLA typing: HLA typing was performed by T. Rostrum (WIMM, Oxford UK) using that Sequence-Specific Primer (SSP) method that uses allele-specific primer combinationa in PCR amplification to provide absolute HLA resolution to 2-digits and high probability resolution to 4-digits.

Exvivo ELISpot assays: 18mer peptides overlapping by 10 were synthesized (Sigma-Genosys, US or MRC Human Immunology Unit, WIMM, Oxford UK) to match the transmitted sequence of CH40, CH58 and CH77. .Approximately 400 peptides for each patient were arranged into pools in a matrix format using 'Peptide Portal' programme (SCHARP) adapting codes from the 'Deconvolute this!' programme [31]. Each peptide was repeated 4 times in the matrix peptide plate. Cryopreserved PBMC samples were thawed and rested for 2 hours before being placed in the ELISpot plates at 1x 10^5 cells/well. Antigen in the peptide plates were mixed with 1:1 with PBMC in the ELISpot plate to a final concentration of peptide 2 µg/ml and incubated for 20hours at 37°C, 5% CO_2. Coating, development and reading of ELISpot plates has been described previouslyl[32]. Putative positive peptide-specific T cell responses were confirmed in triplicate in a follow-up IFN-γ ELISpot with 1x 10^5 cells/well and a peptide concentration of 2 µg/ml. IFN-γ ELISpots performed at other time-points were performed similarly to the deconvolution assays though in some cases when cells were limiting 0.5x10^4 cells/well were used against 9mer peptides. For all assays 6 negative control wells (media only) and at least 1 positive control well (10 µg/ml PHA) were used.

Flow Cytometry: Cryopreserved PBMC samples were thawed and rested overnight. PBMC were stimulated with 2 µg/ml peptide with anti-CD28 mAb (1 mg/ml; L293; BD Biosciences), anti-CD49d mAb (1 mg/ml; L25; BD Biosciences) in the presence of anti-CD107a-Alexa 680 (H4A3; BD Biosciences), 5 µg/ml Brefeldin A (Sigma) and I µg/ml Golgi Stop (Pharmingen) for 5.5 hours at 37°C, 5% CO_2. After washing the PBMCs were surfaced stained with anti-CD14-Cascade Blue (M5E2), anti-CD19-Cascade Blue (HIB 19), anti-CD4-Cy5.5-phycoerythrin (PE) (M-T477), anti-CD8-QD705 (RPA-T8), anti-CD27-Cy5-PE (M-T271), anti-CD57-QD605) (NK-1), anti-CD45RO-PE-Texas Red (all BD Biosciences) for 20 mins at room temperature. PBMC were then fixed and permeabilized with Cytofix/Cytoperm and Perm/WashBuffer (both BD Biosciences). PBMC were stained with anti-CD3-Cy7-APC (SK7), anti-IFN-g-fluorescein isothiocyanate (FITC) (B27), anti-IL-2- Allophycocyanin (APC) (MQ1-17H12), anti-TNF-a-Cy7-PE (MAb11) and anti-MIP-1b-PE (D21-1351) (all BD Biosciences) for 1 hour at 4°C. After washing and fixation samples were run on a custom built LSRII (BD Bioscences). A minimum of 300 000 total events were acquired and data analysis was performed with FlowJo (Tree Star) with Pestle and Spice (Dr. M. Roederer).

Results:

Detection of a CD8 T cell escape mutant in acute HIV-1 infection

The extent and kinetics of viral mutation to escape from the CD8 T cell response induced in early HIV infection was investigated previously [19] by mapping the epitopes recognized by the primary HIV-specific T cell response and performing population-based sequencing of the autologous virus quasispecies over time. This approach revealed that within a given patient there can be escape from multiple epitope-specific T cell responses within the first few months of infection, with some mutations becoming fixed in the viral quasispecies by as early as 2 months following the onset of symptoms (FOSx), close to the time of peak viremia. In one patient (Figure 1a), this approach was re-refined using the novel approach of single genome amplification (SGA) sequencing which enabled identification of previously undetected early and rapid mutations within an CD8 T cell epitope in Rev (Figure 1b). Because there were several variants occurring within the peptide, the population sequencing approach may have missed these changes. A single non-synonymous substitution was detected at D20FOSx and at D34FOSx mutations were detected in 22/28 SGA sequences (Figure 1b). By D70FOSx, the transmitted or 'wildtype' sequence was no longer detectable (supplementary data). CD8 T cell selection was confirmed using ex vivo ELISpot showing a T cell response to the earliest sequence and a loss in magnitude to peptides representing virus escape mutants (Figure 1c). This observation suggested that T cell escape mutations could be selected far more rapidly than has been previously appreciated. To explore this finding in more detail, we studied the evolution of the viral quasispecies in response to CD8 T cell immune selection in 3 additional subjects.

Mapping of primary T cell responses induced in response to the transmitted HIV-1 virus.

Three patients were recruited in early acute HIV-1 infection (AHI) and bled at multiple time-points over the subsequent 6 months (Table 1). Throughout this report, visits are indicated as days (d) from screening for CH40, CH58 and CH77. When first screened, all three patients were seronegative but had high plasma virus suggesting that sampling occurred close to peak viremia (Figure 2 a-c). No patient received anti-retroviral drugs during the period of the study. Patient CH40 was a typical progressor with a set point viral load (VL) > 10000 copies/ml (Figure 2a), whereas CH58 and CH77, who are both HLA-B*5701 positive, controlled virus load to < 300 and 2000 copies /ml respectively (Figure 2 b,c). CD4 T cell counts remained over 350 cells/ul for all 3 patients over the course of the study (Figure 2 a-c). Single genome amplification (SGA) of the full-length viral genome was performed on the earliest available plasma sample, taken at screening. Analysis of multiple SGA Envelope (Env) sequences showed that each patient had been infected with a single virus (or virus-infected cell) consistent with transmission through sexual contact [26].

For patients, CH40, CH58 and CH77, overlapping peptides, matched to and spanning the whole transmitted virus sequence were synthesized and arrayed in a matrix format. In the first assay, PBMC taken 145-181d after screening were tested against this matrix of autologous peptide pools in IFN-γ *ex vivo* ELISpot assays. A low threshold of positivity (30 SFU /10^6 PBMC, > 3x background) was applied to peptide pools to maximize sensitivity. Data from positive pools were then deconvoluted [31] to identify candidate epitope peptides. These individual peptides were tested in triplicate and the positive T cell responses (≥50 SFU/ million, > 4x background) were determined for each patient: 8 in CH40, 3 in CH58 and 18 in CH77 (Figure 2e-f). All three, T cell responses for CH58 contained known HLA-B5701-restricted epitopes. In CH77, the immunodominant T cell response was to a known HLA-B5701-restricted epitope. (Figure 2)

Rapid fixation of non-synonymous viral mutations at sites corresponding to CTL epitopes or predicted motifs

The initial analyses, shown in Figure 2, were designed to detect the full breadth of the T cell response induced by the transmitted virus over the first 6 months of infection, expecting that memory T cells irrespective of virus escape would persist at levels detectable by ex vivo assays. , However, to ensure that no important earlier responses were missed additional sequencing of virus genomes was performed at multiple timepoints between screening and setpoint to detect potential T cell escapes, followed by testing T cells against appropriate peptides. SGA sequencing techniques were again used, sequencing each genome in 2 parts (5' and 3' halves), at multiple timepoints between screening and setpoint for CH40 and CH77. The 5' half sequenced the region spanning Gag and Pol whilst the remaining proteins Vif, Vpr, Tat, Rev, Vpu, Env, and Nef were sequenced in the 3' half.

In patients CH40 and CH77, 19 and 22 sites were defined where the percentage of the transmitted or 'wildtype' virus was < 100% approximately 6 months after infection (Figure 3a,b). It was striking that in CH40 and CH77, 50% fixation of non-synonymous substitutions was reached within 50d of screening at several sites within the genome (CH40 6 sites, CH77 4 sites) (Figure 3 a, b) . These mutations, that were both early and rapid, occurred across the proteome in 7 different HIV-1 proteins. SGA sequence was generated for CH58 for the 2 earliest timepoints in acute infection, but was not available at later timepoints because of the very good virus control preventing successful full length amplification. Even so, a Y586H substitution in the Env gene was found in 10/49 SGA sequences 9 days after screening (Figure 3c).

The Los Alamos National Laboratory HIV-1 databases that support CTL epitope scans and motif predictions (http://www.hiv.lanl.gov/content/immunology/) were used to determine which of these varying sites corresponded to known HIV-1 epitopes or predicted CTL motifs matched to the patients' HLA alleles. These analyses showed

that majority of rapid sequence changes occurred within known or predicted CD8 T cell epitopes (data not shown / supplementary data), appropriate to the HLA type of the patient and included the early and rapid substitutions that achieved 50% fixation by 50d (Table 2). In addition, one mutation that became fixed by d111 in CH40, corresponded to an antibody epitope escape in the V1 region of envelope (Figure 3a). In some sites, no known or predicted CD8 T cell epitopes or antibody epitope motifs were identified. Such sites could represent epitopes with atypical motifs, processing or compensating mutations associate with another epitope or sites selected by some other process .

T cell assays were focused on confirming whether the earliest and most rapid virus escapes (<50% within 50d of screening) in CH40 and CH77 were indeed CD8 T cell-induced. 18'mer and optimal CD8 T cell peptides and the corresponding putative escape peptides were synthesized and tested against the PBMC taken during and immediately after fixation of the sequence change (Figures X and Y). In CH40, 2 of 6 putative CTL-induced very early escapes were confirmed, with a T cell response to the wildtype sequence and no, or a lower, response to the variant peptide (Table 2). Similarly in CH77, 3 of the 4 very rapid virus escapes were confirmed as CTL-induced with no response in IFN-γ ELISpot to the variant peptides (Table 2). In all these cases, the optimal epitope was defined, and there was little or no IFN-γ response to its mutated short peptide. Not surprisingly, the magnitude of the T cell response to the optimal epitope was 4-8 fold greater than the response to its parent 18'mer (Table 2). For CH58, a flow cytometry assay was used to measure production of IFN-γ, IL-2, MIP-1β, TNF-α and CD107a by $CD3^+CD8^+$ T lymphocytes. A strong cytokine response by 3.59% of all CD3/CD8 T cells was detected following stimulation with the wild-type peptide that was 12-fold higher than the T cell response to the Y586H mutant peptide. Together these data demonstrate that these virus escapes were selected by the T cell responses during peak viremia and within weeks, became fixed in the virus population.

Kinetics of T cell responses in relation to virus escape

The kinetics of the T cell responses that selected virus escapes were examined in patient CH40 using a combination of ELISpot and multi-parameter flow cytometry assays. In CH40, the CD8 T cells specific for the HLA A*3101-restricted SLAFRHVAR (Nef187-196) peptide selected the emergence of S188N and R196Q mutations (Table 2). Sixteen days from screening, half of all the SGA sequences contained one of these mutations and by 45d, all 14 sequences contained either of these mutations (supplementary data). Two overlapping 18-mer peptides containing the transmitted HLA-A*3101-restricted SLAFRHVAR epitope were tested in ELISpot at 16, 45, 111 and 181 d following screening. Both peptides produced detectable T cell responses at 16d after screening but declined and were no longer detectable by 111d. These data are presented in Figure 4 together with the kinetic data for other T cell responses defined for CH40 that selected escape at different stages within the first 6 months of infection (Figure 4a).

The Gag I401L mutation emerged slightly later than the Nef substitutions just described (Figure4b). In contrast to the nef response, the T cell response to 18'mer peptide containing the HLA-A*3101-restricted epitope, CGKEGHIAR was maintained, albeit at low levels until at least 412d. For the the Pol the (73-90) peptide, in which a K76R substituion was selected between days 45 and 412,), the ex vivo ELISpot to result was below the 50SFU/10^6 threshold used to define a positive T cell response . However, at 412d the 18'mer peptide was again tested and a positive T cell response was seen (Figure 4c); intermediate time points were not tested but the sequence data suggest a continuous selective pressure duing the year. The emergence of K122E substitution in the Vif protein was also relatively slow, with the proportion of SGA sequences containing the wild-type virus not falling below 50% until 181d (Figure 4d). Here, the corresponding T cell response to the Vif(161-178) peptide declined coincident with the escape within peptide motif . In Rev, a S61N substitution was detected in around 10% of SGA sequences at 111d from screening which was coincident with the decline in the T cell response detected against the 18mer peptide spanning the mutation (Figure 4e). Four other T cell responses were detected in CH40 that did not select virus escape over the first 400d from screening (Figure 4g-j).

In patient CH77, K82Q and A83G substitutions occurred within the KAALDSHF epitope in Nef, which were found either together or alternatively in all SGA sequences only 14 days after screening (Table 3). A strong T cell response was detected at 14d (Figure 3k, Table 2) that was maintained at low levels until at least 159d. In contrast, multiple non-synonymous substitutions occurred within another Nef T cell epitope that was clearly positive at 14d (Table 2) but no longer detectable by 32d (data not shown). A third T cell escape mutant was detected in Env, also by 14 days after screening. An R35K substitution in the HLA-Cw*0401-restricted QFRNKTIVF motif, that was found in 44/80 SGA sequences 14d from screening, increased and was present in all 11 SGA sequences, 32d post screening (supplementary data). A T cell response in IFN-γ ELISpot was detected to the overlapping 18'mer peptides containing the QFRNKTIVF epitope 14d after screening (Figure 3k, inset) but was not detected at 159d (data not shown). No ELISpot data are available for the intervening timepoints. However, a positive multi-functional T cell response in flow cytometry was observed at 32d, then declined markedly but remained detectable at day 159 (Figure 3k). These data suggest that when complete escape occurs in acute HIV-1 infection the primary T cell response often rapidly declines and in some cases the T cell response to the 18'mer peptide was lost within weeks of fixation.

<u>The CTL response induced by the transmitted HIV-1 virus broadens over time</u>

Figure 5 displays the overlayed T cell kinetic data for CH40 and shows that multiple T cell responses emerge and peak at different times following infection and that the T cell response to the transmitted virus broadens over time. As described above, the Nef–specific T cell response (SLAFRHVAR) that selects early escape was clearly positive at 16d but then declined rapidly as the virus escapes, suggesting this was the earliest T cell response induced in response to infection. Other T cell responses

peaked around 45d after screening declining slowly around 6 months post-infection then again increasing at 1 year coincident with a 5-fold decrease in total viral load. Both the Gag (389-406) and Vif (113-130) CD8 T cell responses that selected escape more slowly were detected at within weeks of infection, increased around 100 days and then declined but remained detectable at 400d following screening. It was striking that a T cell response to the Pol(825-842) peptide, VKTITDNGSNFTSTTVK, was first weakly detectable at 45d, but became very immunodominant at around 1 year. No escape mutants were detected within this eptiope (Figure 4j). Some other T cell responses were observed in the first year that showed expansion and contraction profiles but did not select escape mutants (Figure 3f-i), suggesting factors other than antigen load may contribute to their distinct kinetic profiles [33].

Virus escape to CTL pressure occurs simultaneously and /or sequentially on the same molecule

SGA provides long sequence data for single virus molecules without the risk of artefactual PCR recombination. As described above, we confirmed the emergence of CD8 T cell-induced escape mutants at multiple sites along the HIV-1 genome, at different times and rates over the first 6 months of HIV-1 infection. The 5' and 3' sequences generated at multiple time-points during the first 6 months of infection were examined to understand the evolution of T cell escape mutants on individual viruses.

Within the 5' SGA half for CH40 simultaneous non-synonymous substitutions in Gag and Pol both of which enabled T cell escape were detected at 45d from screening (Table 3). At 45d, all SGA sequences (9/12) that contained the K76R Pol substitution also contained the Gag R403K mutation], indicating that as early as 45d from screening that viruses that contained double T cell escape mutants were dominant in the virus quasispecies. By 111d, a second mutation, I401L, was also observed in 4/9 SGA sequences within the Gag epitope, but none of these sequences contained the K76R substitution in Pol, indicating that Pol mutation was linked to the R142K but not the I401L mutation in Gag. At 159 d, the Pol K76R was also found on the same viruses containing the I140L Gag mutation, suggesting that recombination occurred and was then selected as the optimal escape virus.

The 3' SGA sequences of CH40 also show sequential escape occurring on the same viruses. Multiple substitutions or wobbles occurred within the Nef SLAFRHVAR epitope in all SGA sequences at 45d following screening (Table 4). At 111d, non-synonymous substitutions in both the Vif and Rev epitopes were detected (see also Figure 4). All SGA sequences in which either Vif or Rev substitutions occurred also contained mutations in Nef. By 412d, 9/12 sequences contain mutations in all 3 CD8 T cell epitopes, whilst the remaining 3 sequences containing mutations within 2 epitopes.

A more remarkable observation was made even earlier in acute infection in CH77 (Table 5). In this patient, complete T cell escape occurred the REQFRNK epitope in

Env only 14d after screening (Table 5). Eighteen days later further mutations had been selected in two confirmed T cell epitopes in nef 17-34 and 73-90 (KAALDLSF). Either these fixations occurred sequentially within nine days each or more likely simultaneously. Thus, within 32 days of peak viremia T cells had selected a triple escape mutation in this patient.

Discussion:

This study was designed to examine the evolution of the first T cell responses in acute HIV-1 infection, being part of a larger ongoing study of the whole immune response in early HIV infection. The patients were identified when they were virus positive by PCR, yet still seronegative and were then bled at frequent intervals over a six-month period. Virus sequence was determined by single genome analyses on the full-length genome at the first bleed, before any detectable immune response, and changes in sequence were monitored over time. Peptides based on the first determined virus sequence were made so that all T cell responses could be measured against transmitted virus. Sequence changes showed that some T cells had selected escape mutants and then declined and were no longer detectable at 6 months. We therefore tested both the transmitted and mutant peptides in which sequence changes were found within the first 50d of infection. These studies revealed multiple early virus escapes from T cell responses across the four patients studied.

A total of 7/12 putative sequence 'escapes' that occurred within the first 2 months of infection were confirmed using peptides in T cell assays, but some that became fixed were not confirmed in T cell assays. There are several possibilities that may explain this. Some of the changes could have been reversions; that is mutations selected in the infecting sexual partner that have fitness costs and revert to the fittest variant when the T cell force is removed in a person of a different HLA type. Most reversions reported to date have been slower than these though more recently, Fernandez et al[34] demonstrated reversion in pigtail macaques within 2 weeks of SHIV infection. Also it was not uncommon to find more than one sequence being selected within a single epitope, which is much more suggestive or T cell selection than reversion to a single most fit sequence. Therefore, we think it more likely there are T cell responses involved but they have been missed either for technical (eg T cells that do not produce IFN-γ) or for other reasons. The latter could include mutations that affect antigen processing of a nearby epitope or mutations that compensate for a more distant escape mutation and T cell responses that are localized to anatomic sites such as the GALT rather than blood. It is also possible that some mutations might reflect selection by other immune responses. Where the mutations are not in envelope this would not be neutralizing antibody but antibody involved in ADCC responses, and peptides involved in NK cell recognition of infected cells might be possibilities. The virus escapes from CD8 T cell responses occurred as soon as the T cell responses were detectable and in several cases the escapes were rapid, changing from 100% wild type to 100% mutant in two to three weeks. Often a variety of epitope mutations appeared before a single variant was fixed as the escape mutation. Further, it was found that two or more mutants can be selected during the same period and long sequence analysis showed that these changes were occurring at two sites sometime simultaneously and sometimes sequentially on one viral genome. These escapes are selected as rapidly as nevirapine driven escape, implying that the anti-viral properties of the T cells were as potent as reverse

transcriptase inhibitors [35] even though CTL cannot prevent virus infecting cells. The rapid escape must be aided by the extremely, high level of virus replication during acute viremia [36] when every single point mutation is generated every day (Figure 6). There is a good chance that enough double mutants could be generated to give simultaneous double escape seen for patient CH77. Furthermore there could be recombination events between single mutant genomes, as implied in patient CH40, [37]. This rapid escape from T cell immune pressure contrasts with some earlier studies, which described slower fixation, and by implication weak suppressive pressure, by the T cells, mostly studied during later stages of acute infection or during chronic infection [38, 39]. The data shown here with natural HIV-1 infection are consistent with the data in macaques acutely infected intravenously with SIV of known virus sequence [17, 40].

T cell selected escapes were described in the first 2 months following screening when virus load was declining from peak viremia. These data suggest some T cells can have strong suppressive effects on the virus but rapid escape may negate any sustained benefit for the patient. This is comparable to the effects of neutralizing antibody in ongoing HIV infection; the virus simply evades the antiviral effect. However, where T cell responses were not lost following escape, it is possible that there is a fitness cost to the virus and the T cells are maintained because the virus tends to revert. Such responses may be more beneficial to the patient. Thus, despite HIV-1 escape to primary T cell responses within weeks of infection, in all patients studied total virus load continued to decline over the following few months reaching low setpoints in CH58 and CH77. This decline was also coincident with a broadening of the T cell response and the detection of HLA B*5701 restricted epitopes within the immunodominant T cell responses in CH58 and CH77 at setpoint. This suggests that even in the face of early escape some T cell responses may have a still contribute to virus control and establishment of setpoint. Detailed examination of the T cell response in CH40, CH77 and CH58 identified some T cell responses that did not select escape mutations or escapes that occurred later in infection (Figure 3 f-i). 'Lack of escape' within T cell epitopes could be explained if the T cell targeting that site was poorly suppressive of HIV-1. Alternatively, specific T cells may recognize some regions of the virus that could not vary without major fitness cost and therefore no escape occurred or escape was delayed as has been described for some B*5701 epitopes[41]. The latter would be the most important in real control of the virus and a major contributor to acute control and decline in viremia.

These results have implications for the design of vaccines aimed at stimulating CD8+ T cells to control HIV infection. The recombinant adenovirus-5 vaccine used in the recent STEP trial stimulated a median of three CD8 T cell responses (Casmiro D and McElrath J, http://www.hvtn.org/science/step_roberts.html). The data shown here imply that around half of CD8 T cell responses specific for HIV-1 may be useless because they can rapidly select virus escape mutants. This could be compounded by sequence differences between infecting virus compared to the vaccine insert which could reduce the number of initially matched T cell responses by half [42]. These together could negate any beneficial effect of vaccination in a

substantial proportion of vaccine recipients. Although in a vaccinated person, memory T cells would be present before infection, it is clear from studies in macaques with SIV and in mice with other virus infections that whilst memory T cells can rapidly produce cytokines in response to antigen, there is a delay before they can proliferate[43, 44]. By this time in HIV-1 infection, the virus may already be replicating rapidly and the expanding memory T cells may already have to chase virus mutants.

It will be important therefore for vaccines to generate much broader T cell responses, to stimulate responses to common virus variants and to focus vaccine induced T cell responses on conserved regions of HIV-1.

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Figure Legends (no more 250 words / description):

Figure 1: SGA viral sequencing methods can detect early virus mutations. Virus loads (log10) and CD4 counts over time for patient, SUMA (a). SGA sequence data spanning the REVepitope show sequences contained non-synonymous substitutions at D20FOSx that increased to 22/28 SGA sequences by D34FOSx (b). Exvivo IFN-γ ELISpot assay analysis of T cell response to the transmitted and escape peptides was performed at D59DOSx (c). Data are expressed as the mean spot forming units per million PBMC (SFU/10^6) +/- SEM.

Figure 2: Mapped T cell responses against the transmitted HIV-1 virus 6 months following screening. The 4-digit HLA type and the virus load and CD4 counts are shown over the first 400 days from screening months of infection for CH40(a) and CH58(b) and 200 days for CH77(c). The sequence of the transmitted virus for each subject was defined and overlapping peptides spanning the entire proteome were synthesized. Patient PBMC from 181d, 145d and 159d following screening for CH40, CH58 and CH77 respectively ,were tested against autologous peptides were pooled in matrix format and tested in IFN-γ ELISpot. T cell responses detected against pools were deconvoluted then tested individually in triplicate in exvivo IFN-γ ELISpot. The peptides-specific T cell responses are shown for CH40 (d) CH58 (e) CH77 (f). The peptide sequence in (e) and (f) highlighted in blue identifies B*5701-restricted T cell epitopes. ELISpot data are expressed as the mean spot forming units per million PBMC (SFU/10^6) +/- SEM.

Figure 3: Virus escape emerges early and rapidly at multiple sites over the first year 12 months of infection. Full length SGA sequencing was performed at screening (0d) for CH40, CH58 and CH77. Sequencing at multiple timepoints subsequent to screening used SGA techniques but was derived from separate sequencing of the 5' (Gag, Pol) and 3' (Vif, Vpr, Tat, Rev, Vpu, Env, Nef) regions of the virus genome. All sites where non-synonymous substitutions were detected before 100d from screening are shown for CH40 (a) and CH77 (b). The positions of the amino acid substitutions are shown in parentheses. Text that is highlighted in red, denote early and rapid mutations that achieved 50% fixation by 50d from screening. Text that is highlighted in blue, denote virus escape at a site previously defined as a V1 antibody epitope. SGA sequences for CH58 were available only from screening and the enrolment visit (+9days). Within this limited timeframe, a non-synonymous substitution was detected (c).

Figure 4: HIV-1 specific T cells induced in primary infection display both antigen dependant and antigen independent kinetics. Each panel shows the T cell response to the 18'mer peptide described in the title. Exvivo IFN-γ ELISpots were performed on PBMC from patient CH40 (a-j) , CH77(k) at the days indicated using peptides matching the transmitted virus. Data are expressed as the mean spot forming units per million PBMC (SFU/10^6) +/- SEM. Data from both patients are graphed against % wild-type virus which reflects the proportion of SGA sequences produced at each timepoint that contain the transmitted virus sequence spanning that 18'mer peptide, The sum of the background-subtracted IFN-γ, TNF-α, MIP-1β, IL-2 and CD107α production in memory CD8 T cells is shown for CH77 (l). The inset shows the mean (+/-SEM) IFN-γ ELISpot response to ENV(350-368) at 14d

Figure 5: The primary T cell response to the transmitted HIV-1 virus is both dynamic and complex. Ex vivo IFN-γ ELISpots were performed in CH40 over the first 6 months on infection. T cell responses that were both >50 SFU and 4x background at least 1 time point are shown. Data are expressed as the mean spot forming units per million PBMC (SFU/10^6) +/- SEM. Line colour is used to group T cell responses that peaked at different times during the first year on HIV-1

Figure 6: HIV-1 molecules that contain more than 1 mutation escape CTL selection at a higher rate. CTL of different specificities can recognize and suppress HIV-1 virus in infected cells (a). When a non-synonymous mutation 'A' occurs through random mutation, recognition by CTLA A is lost. CTL A can still recognize infected cells containing mutation 'B'. Similarly CTLB can recognize an suppress virus in infected cells harbouring mutation A (b). Random mutation in high viremia also produces virus genomes containing 2 mutations in A and B. Neither CTLA or CTLB can recognize and suppress these cells. This virus genome replicates and rapidly becomes fixed within the patient.

Table 1: Demographical and clinical data of study volunteers

Patient	Gender	Age at screening	Ethnicity	CD4+ Count median[1]	Enrolment criteria at screening
CH40[2]	Male	56	Caucasian	990 cells/ul	NAT positve[3], seronegative
CH58[2]	Male	23	African American	858 cells/ul	NAT positve, seronegative
CH77[2]	Male	23	African American	827 cells/ul	NAT positve, seronegative
SUMA	Male	23	Caucasian	952 cells/ul	Symptomatic, seronegative

[1]Median calculated from available data from visits over the first 6-7 months from infection
[2]Patient self reported being a MSM and no history of intravenous drug use
[3]NAT positive = nucleaic acid test positive

Table 2

See separate file

Table 3: CH40 5' SGA direct sequencing spanning CTL epitopes

DFS	SGA SEQUENCE	GAG POSITION	POL POSITION
0	B.US.06.KBE000 BP17_WGA1	IVKCFNCGKEGHIARNCR[1,2]	QLKEALLDTGADDTVLEEMNLPGRW
45	CH40_wk4_I12	.....K...	..R.....
	CH40_wk4_I14	.....K...	..R.....
	CH40_wk4_I10	.....K...	..R.....
	CH40_wk4_I11	.....K...	.....
	CH40_wk4_I13	.....K...	.....
	CH40_wk4_I15	.....K...	.....
	CH40_wk4_I16	.....K...	..R.....
	CH40_wk4_I3	.....K...	.....
	CH40_wk4_I4	.....K...	..R.....
	CH40_wk4_I6	.....K...	.....
	CH40_wk4_I7	.....K...	..R.....
	CH40_wk4_I9	.....K...	..R.....
	CH40_wk12_G1	.....K...	.....
	CH40_wk12_G3	.....K...	..R.....
111	CH40_wk12_G4	.....K...	..R.....
	CH40_wk12_G7	.....K...	.....
	CH40_wk12_G8	....L.....	.....
	CH40_wk12_H2	.....K...	..R.....
	CH40_wk12_I1	.....K...	.....
	CH40_wk12_I2	....L.....	.....
	CH40_wk24_G3	.....K...	..R.....
	CH40_wk24_H1	....L.....	.....
	CH40_wk24_H2	....L.....	.....
181	CH40_wk24_H3	.....K...	..R.....
	CH40_wk24_H4	.....K...	..R.....
	CH40_wk24_H6	.....K...	..R.....
	CH40_wk24_H7	.....K...	.....
	CH40_wk24_I1	.....K...	..R.....
	CH40_wk24_I2	.....K...	..R.....
	CH40_wk24_I5	.....K...	..R.....
	CH40_wk24_I6	.....K...	..R.....
412	CH40_wk60_G3	.....K...	..R.....
	CH40_wk60_G4	.....K...	..R.....
	CH40_wk60_G5	....L.....	..R.....
	CH40_wk60_G7	....L.....	..R.....
	CH40_wk60_G9	....L.....	..R.....
	CH40_wk60_H3	....L.....	.....
	CH40_wk60_H15	....L.....	..R.....
	CH40_wk60_H2	.....K...	..R.....
	CH40_wk60_H7	.....K...	..R.....
	CH40_wk60_H9	.....K...	..R.....
	CH40_wk60_I11	.....K...	..R.....

[1] red = non-synonymous mutation occurring 2 or more times
[2] underline = confirmed optimal T cell epitope

Table 4: CH40 3' SGA direct sequencing spanning CTL epitopes

DAY	SGA SEQUENCE	NEF POSITION 187-203	VIF POSITION 84-104	REV 58-65
0	B.US.06.KBE000 BP17_WGA1	SSLAFRHVARELHPEYY[1,2]	DCFS-ESAIRKAILGRIVS	QRQIRSISERILSTYLER
45	CH40_wk4_D18	.....Q.....	....-.....	.....
	CH40_wk4_D2	.....Q.....	....-.....	.....
	CH40_wk4_D7	.....Q.....	....-.....	.....
	CH40_wk4_A3	.....Q.....	....-.....	.....
	CH40_wk4_B2	.....Q.....	....-.....	.....
	CH40_wk4_D16	.....Q.....	....-.....	.....
	CH40_wk4_A2	.N.....	....-.....	.....
	CH40_wk4_D17	.N.....	....-.....	.....
	CH40_wk4_A1	.....Q.....	....-.....	.....
	CH40_wk4_C7	.....Q.....	....-.....	.....
	CH40_wk4_D14	.....Q.....	....-.....	.....
	CH40_wk4_B1	.....Q.....	....-.....	.....
	CH40_wk4_C1	.....X...X.....	....-.....	.....
	CH40_wk4_C2	.....Q.....	....-.....	.....
111	CH40_wk12_C1	.N.....	....-.....	.....F.....
	CH40_wk12_D1	.....H.....	....-.....	.....
	CH40_wk12_D4	.....H.....	....-.....L.....	.K.....
	CH40_wk12_D3	.....H.....	....-.....	.....
	CH40_wk12_D5	.....H.....	....-.....	.....
	CH40_wk12_C1	.....Q.....	....-.....	.....
	CH40_wk12_D2	.....Q.....	....-.....	.....
	CH40_wk12_D7	.....Q.....	....-.....	.....
	CH40_wk12_D6	.....Q.....	....-.....	.....
185	CH40_wk24_D7	.....Q.....	....-.....T.....	.....
	CH40_wk24_D8	.....Q.....	...F#.....T.....	.....
	CH40_wk24_D10	.....Q.....	....-.....T.....	.....
	CH40_wk24_D9	.....Q.....	....-.....T.....	.....
	CH40_wk24_D5	.....Q.....	....-.....	.....
	CH40_wk24_D1	.....Q.....	....-.....	.....F.....
	CH40_wk24_B2	.....Q.....	....-.....V.....	.....
412	CH40_wk60_D12	.....Q.....	....-.....	.....N.....
	CH40_wk60_C3	.....Q.....	....-.....E.....	.....N.....
	CH40_wk60_D18	.....Q.....	....-.....E.....	.....N.....
	CH40_wk60_D8	.....Q.....	....-.....E.....	.....N.....
	CH40_wk60_D3	.....Q.....	....-.....E.....	.....N.....
	CH40_wk60_C1	.....Q.....	....-.....E.....	.....F.....
	CH40_wk60_D7	.N.....	....-.....E.....	.....N.....
	CH40_wk60_D10	.N.....	....-.....E.....	.....N.....
	CH40_wk60_D19	.N.....	....-.....E.....	.....N.....
	CH40_wk60_D5	.N.....	....-.....E.....	.....N.....
	CH40_wk60_D13	.N.....	....-.....	.....N.....
	CH40_wk60_D4	.....Q.....	....-.....	.....N.....

[1] red = non-synonymous mutation occurring 2 or more times
[2] underline = confirmed optimal T cell epitope

Table 5: CH77 5' SGA direct sequencing spanning CTL epitopes

DAY	SGA SEQUENCE CLONE	ENV POSITION (250-270)	NEF POSITION (17-34)	NEF POSITION (80-94)
0	CONSENSUS	SHVVDKLREQFRNKTIVFNH	RERRRRDEPAAVGVGPAS	KAALDLSHFLKEK
14	CH77_WGA_B3	..........K.....	.....	.....
	CH77_WGA_C7	..........K.....	.....	.....
	CH77_WGA_C9	..........K.....	.....	.....
	CH77_WGA_C4	..........K.....	.....	.....
	CH77_WGA_D5	....G.....K.....	.....	.....
	CH77_WGA_C3	..........K.....	.....	.....
32	CH77_WK03_3_B1	..........K.....	....G.....	Q.....
	CH77_WK03_3_D1	..........K.....	.....S.....	.....I.....
	CH77_WK03_3_D2	..........K.....	.....E.....	Q.....
	CH77_WK03_3_D3	..........K.....	.....K.....	Q.....
	CH77_WK03_3_TB1	..........K.....	.....K.....	Q.....
	CH77_WK03_3_TB2	..........K.....	.....E.....	T.....
	CH77_WK03_3_TB4	..........K.....	.....S.....	...H.....
	CH77_WK03_3_TB5	..........K.....	.....N.....	Q.....
	CH77_WK03_3_TB6	..........K.....	..GA.ERR.....	Q.....
	CH77_WK03_3_TB7	..........K.....	.....K.....	Q.....
	CH77_WK03_3_TB8	..........K.....	.....E.....	T.....
	CH77_WK03_3_TC1	..........K.....	.....N.....	Q.....
102	CH77_WK12_3_E1	.Y.....K.....	.....N.....	Q.....
	CH77_WK12_3_TA1	..........K.....	.....K.....	Q.....
	CH77_WK12_3_TA2	..........K.....	.....T.....	Q.....
	CH77_WK12_3_TB1	..........K.....	....G.....	.G.....
	CH77_WK12_3_TB2	..........K.....	.....G.....	.G.....
159	CH77_WK24_3_TA1	..........K.....	K.....E.....	.G.....#..
	CH77_WK24_3_TA2	....N.....K.....	.....G.....T..	Q.....
	CH77_WK24_3_TA3	..........K.....	.....G.....	Q.....
	CH77_WK24_3_TA4	..........K.....	.....N.....S..	Q.....
	CH77_WK24_3_TA6	....N.....K.....	.....E.....	.G.....
	CH77_WK24_3_TB1	....N.....K.....	.....G.....	Q.....#..
	CH77_WK24_3_TB2	N.....K.....	.....T.....	Q.....
	CH77_WK24_3_TB3	....N.....K.....	.....K.....V.	Q.....
	CH77_WK24_3_TB4	..........K.....	.....K.....V.	Q.....
	CH77_WK24_3_TB5	G.....K.....	.....T.....	.G.....
	CH77_WK24_3_TB6	....N.....K.....	.....E.....V.	.G.....
	CH77_WK24_3_TB7	....N.....K.....	.....G.....	.G.....

[1] red = non-synonymous mutation occurring 2 or more times
[2] underline = confirmed optimal T cell epitope

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