High-avidity, high-IFNγ-producing CD8 T-cell responses following immune selection during HIV-1 infection

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HIV-1 mutations, which reduce or abolish CTL responses against virus-infected cells, are frequently selected in acute and chronic HIV infection. Among population HIV-1 sequences, immune selection is evident as human leukocyte antigen (HLA) allele-associated substitutions of amino acids within or near CD8 T-cell epitopes. In these cases, the non-adapted epitope is susceptible to immune recognition until an escape mutation renders the epitope less immunogenic. However, several population-based studies have independently identified HLA-associated viral changes, which lead to the formation of a new T-cell epitope, suggesting that the immune responses that these variants or ‘neo-epitopes’ elicit provide an evolutionary advantage to the virus rather than the host. Here, we examined the functional characteristics of eight CD8 T-cell responses that result from viral adaptation in 125 HLA-genotyped individuals with chronic HIV-1 infection. Neo-epitopes included well-characterized immunodominant epitopes restricted by common HLA alleles, and in most cases the T-cell responses against the neo-epitope showed significantly greater functional avidity and higher IFNγ production than T cells for non-adapted epitopes, but were not more cytotoxic. Neo-epitope formation and emergence of cognate T-cell response coincident with a rise in viral load was then observed in vivo in an acutely infected individual. These findings show that HIV-1 adaptation not only abolishes the immune recognition of early targeted epitopes, but may also increase immune recognition to other epitopes, which elicit immunodominant but non-protective T-cell responses. These data have implications for immunodominance associated with polyvalent vaccines based on the diversity of chronic HIV-1 sequences.

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Cytotoxic T-lymphocyte (CTL) response is associated with marked reduction of viraemia during acute HIV-1 infection in most individuals, but ongoing replicative infection in the presence of partially suppressive responses rapidly selects for mutations that allow viral escape from these responses. The emergence of HIV-specific CD8 T-cell responses commensurate with falling plasma HIV-1 RNA concentration and positive selection of mutations within the targeted T-cell epitopes have been directly observed 14 days after HIV p24 antigen detection and preceding HIV-1 antibody seroconversion. Studies of HIV-1 seropositive individuals have found evidence of CTL escape mutations in viral sequences as early as 4 weeks after the peak viraemia of acute infection, as well as at later time points in chronic infection. Loss or reduction of human leukocyte antigen (HLA)–viral epitope binding, reduction in T-cell receptor (TCR) recognition of the HLA/epitope complex or disruption of intracellular processing of viral epitopes are all well-documented mechanisms by which viral mutation leads to evasion from T-cell recognition and cytotoxic clearance of virus-infected cells.

Once a virus escapes from initial immunodominant CTL responses, new responses to alternative subdominant epitopes may be generated and select further mutation networks in the viral genome, such that there is often a hierarchy of immunodominant and subdominant CTL responses. At the population level, this intrahost viral evolution is manifest as inter-host viral diversity that correlates with HLA class-I alleles. Population-based studies of HLA–HIV polymorphism associations have shown a greater diversity of HLA alleles and viral epitope targets putatively associated with immune selection in vivo. Several examples of experimentally characterized HIV-1 mutations known to cause CTL escape have been

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consistently apparent as HLA allele-associated HIV-1 polymorphisms at the population level. For example, a mutational network involving Gag residues 210, 242, 248 and others associated with escape from HLA-B57-restricted T-cell responses targeting the Gag TW10 (240–249) epitope has been detected consistently as HLA-B57-associated polymorphisms in a number of population-based studies.6,20,25 Some-what unexpectedly however, this genetics-based approach has also shown instances where a viral epitope, which is more susceptible to CTL cell recognition in T-cell assays, is enriched in individuals with carriage of the restricting HLA allele.16,19,25 For example, HLA-A*0301 was associated with an arginine (R)-to-lysine (K) substitution at position 3 of a known HLA-A*0301-restricted CD8 T-cell p7 epitope LR9; however the HLA-A*0301-adapted form of the epitope (LAKNCRAP) elicited higher magnitude and more functionally avid responses than the non-adapted variant in some tested individuals.16 Similarly, a well-characterized HLA-B*0702-restricted Gag epitope GL9, which elicited interferon-γ (IFNγ) responses, was relatively enriched in those with carriage of HLA-B*0702 in a subtype-C population-based association study.13 In both these cases, the epitope, which was ‘CTL-adapted’ in genetic analyses, was also immunogenic by standard immunological measures, in contrast to the classical description of viral adaptation causing loss of immunogenicity. Whereas de novo CD8 T-cell responses to epitope variants25 and cross-reactive T-cell responses25 have been described in chronic HIV-1 infection, these have not been explored as a product of viral adaptation and therefore offering strategic advantages for the virus rather than the host. A population-wide association between enrichment of a particular viral epitope sequence and an HLA allele suggests that maintenance of the epitope and cognate epitope-specific T-cell response is adaptive for the virus in some way. Furthermore, according to an evolutionary argument, the immune responses that are evaded by HIV-1 may be qualitatively different from those immune responses that are tolerated by the virus and do not select further changes. Understanding the differences between these responses may therefore uncover key aspects of TCR–epitope/HLA interactions and functional T-cell immunity, which should be either harnessed or avoided by HIV vaccines.

A study of associations between HLA alleles and HIV-1 polymorphisms in a large population of predominantly HIV-1 subtype-B-infected individuals in the United States of America and Australia detected over 800 HLA class-I allele-specific polymorphisms across the HIV-1 genome.25 Scanning for known or predicted CD8 T-cell epitopes around these polymorphic sites in the study cohort sequences identified 97 HLA-viral polymorphism associations in which an amino-acid substitution led to the creation rather than abolition of an epitope with the same HLA restriction overlapping or adjacent to the original epitope. There were nine HLA–epitope combinations in particular in which the HLA-adapted epitope sequence corresponded to a published epitope for which CD8 T-cell recognition had been well established in the scientific literature (see Supplementary Table).13,28–38 For example, HLA-B*0702 was associated with a serine (S)-to-glycine (G) change at position 357 of Gag, creating the previously mentioned HLA-B*0702-restricted Gag epitope GL9 (GPGHKARVL). Rather than the restricting HLA allele driving a departure from this sequence in keeping with loss of the epitope, HLA-B*0702 was associated with creation of the epitope, making the immune-susceptible epitope also HLA-adapted, rather than non-adapted or ‘wild-type’. Other such adapted ‘neo-epitopes’ included the extensively studied immunodominant HLA-A*0201-restricted Gag 77–83 SL9 (SLYNVTAVL) epitope and Vpr 59–67 AL9 (AIIRLQQL); HLA-A*0301-restricted Pol 424–432 QR9 (QLYPGIKVR); HLA-B*0702 and HLA-B*4201-restricted Nef 128–137 TL10 (TPGPGVRYPL); HLA-B*1503 Nef 183–191 WF9 (WRFDSRLAF) and HLA-B*4402-restricted Pol 724–734 QW11 (QEEHEKHSNW) and the HLA-C*0702-restricted Nef 105–115 KY11 (KROEILDLWYY) epitope, all of which have been detected as one of few commonly detected responses in chronically infected individuals.19 (see Supplementary Table). Independent HLA association studies and observational studies of HIV sequence evolution conducted in geographically distinct populations have also shown the same associations and direction of substitutions for four of these cases: HLA-A*03 Pol Q9R,17,18,24 HLA-B*0702 Gag GL9,13,19,18 HLA-A*0201 Vpr AL9,17,19 and HLA-B*1503 WF9.11 We chose to focus on these nine HLA–epitope combinations in detail as they were all well-characterized, published optimal epitopes restricted by prevalent HLA alleles and shown by others to elicit measureable and usually immunodominant CD8 T-cell responses in chronically infected subjects (http://www.hiv.lanl.gov/content/immunology-tables/optimal_ctl_summary.html).39 The concordance in significance level and direction of the specific amino-acid substitution in four of these cases from independently conducted studies reduces the likelihood that the non-adapted and adapted epitope sequences had been falsely assigned in this study.25 The functional characteristics of the memory CD8 T-cell response against the non-adapted and adapted epitopes in 125 chronic HIV-infected individuals in the Western Australian HIV Cohort Study (WAHCS), the relevant HLA genotypes and sufficient cryopreserved peripheral blood mononuclear cells (PBMCs) were examined. In addition, we serially tracked and characterized viral adaptation and neo-epitope formation over time in an individual in whom primary HIV-1 infection was diagnosed 5 days after an epidemiologically proven transmission event and preceding seroconversion. This individual expressed two of the HLA alleles associated with viral adaptation to neo-epitopes and one HLA allele associated with a more classically described CTL escape.

RESULTS
We performed assays of HIV-1 epitope-specific responses on 216 PBMC samples from 125 individuals. The medians and ranges for CD4 and CD8 T-cell counts were 540 cells μl−1 (16–1591) and 932 cells μl−1 (110–3519), respectively. Eight-seven percent of the individuals tested had some anti-retroviral therapy experience, with a median HIV RNA viral load of 50 copies μl−1 (range <50 to >1 million copies per millilitre). Fifty-seven percent of the individuals had an undetectable viral load (<50 copies ml−1) and 69% had viral loads less than 1000 copies ml−1.

Memory CD8 T-cell responses against neo-epitopes
ELISpot assay was initially used to screen for memory IFNγ responses after ex vivo stimulation with peptides representing both non-adapted and adapted neo-epitopes in individuals carrying HLA-A*0201 (n=36), HLA-A*0301 (n=19), HLA-B*0702 (n=56), HLA-B*1503 (n=16), HLA-B*4402 (n=32) and HLA-C*0702 (n=57). Positive IFNγ responses to both non-adapted and adapted epitopes were detected in 21% (n=46) of the samples on screening. The median and range of the values for non-adapted and adapted responses were 450 (53–2225) and 575 (53–3700) spot-forming units (SFUs), respectively (n=46, P=0.09, mixed models). These 46 IFNγ responses included six against the HLA-A*0201-restricted Gag SL9 epitope, two against the HLA-A*0201 Vpr AL9 epitope, six against the HLA-B*0702-restricted Gag GL9 epitope, 15 against the HLA-B*0702-restricted Nef TL10 epitope, one against the HLA-B*1503-restricted Nef WF9 epitope, three against the HLA-B*4402-restricted Pol QW11
epitope and 13 against the HLA-C*0702-restricted Nef KY11 epitope. In the subset of samples with detectable viral load and detectable IFNγ responses to both non-adapted and adapted epitopes (n=22), responses to adapted epitopes were marginally greater than responses to non-adapted epitopes (792 (55–3700) versus 462 (58–1982) SFUs for adapted and non-adapted responses, respectively), although this was not statistically significant in this sample set (Figure 1). No responses were detected to the A*0301-restricted Pol QR9 peptides in the 19 samples with carriage of HLA-A*0301. Of note 63% (12 of 19) of HLA-A*0301 samples had undetectable viral loads.

Forty-six of the 216 samples tested were from individuals who had previously been severely immunodeficient with a nadir CD4 T-cell count <200 cells µL−1. There was no significant overall correlation between nadir CD4 T-cell counts and magnitude of IFNγ responses; however, individuals with nadir CD4 T-cell counts less than 50 cells µL−1 had lower rates of detectable IFNγ responses against non-adapted and adapted epitopes (P<0.007, for non-adapted, P=0.014 for adapted epitopes, n=215, mixed-model logistic regression) compared with individuals with nadir CD4 T-cell counts >50 cells µL−1.

HLA-adapted neo-epitopes stimulate higher-avidity memory CD8 T-cell responses in contrast to epitopes associated with ‘classical’ CTL escape

The tendency of equivalent or in some cases even increased IFNγ responses to the HLA-adapted forms of these epitopes on screening assays with excess peptide concentrations suggested that the avidity of these responses may be higher than anti-non-adapted responses. This contrasts with previous demonstrations of intra-patient mutational escape from CTLs, in which immune recognition of variant epitopes is reduced or completely abrogated.40 Therefore, we formally compared the functional avidity of responses against non-adapted and adapted neo-epitopes in those individuals with positive responses to both peptides on screening using serial dilutions of peptides and IFNγ production as the functional readout (Figure 2). This included seven epitopes after exclusion of HLA-A*0301 Pol QR9. To illustrate the contrasting patterns with ‘classically’ described CTL escape mutations, we also tested in parallel the comparative avidity of two well-described CTL escape variants. These were variants of HLA-B*0801-restricted Nef 90–97 FL8 (FLKEKGGL) in which lysine (K)-to-glutamine (Q), -glutamic acid (E) or -asparagine (N) substitutions at position 5 mediates functional viral CTL escape,24 (Figure 2a) as well as variants of the HLA-B*5701-restricted Integrase 128–132 SW10 (STTVKAACWW) epitope, in which mutations at amino-acid positions 2 and 3 abrogate CTL responses.38 (Figure 2b). Combined data from these HLA-B*0801 FL8 and HLA-B*57 SW10 responses (n=14 samples from 11 individuals) measured at 2 µg mL−1 peptide concentration showed the expected pattern of significantly reduced IFNγ responses to the adapted epitopes, accounting for repeated measures in individuals (raw median 203 SFUs for non-adapted versus 65 SFUs for adapted, P=0.03).

By contrast, responses to the seven adapted neo-epitopes and their non-adapted epitope pair frequently showed the opposite pattern of avidity differences, with adapted neo-epitopes inducing greater IFNγ production at lower peptide concentrations compared with the non-adapted epitopes (Figures 2c–f). The pattern of equivalent or increased IFNγ production induced by adapted epitope was observed across multiple individuals for most of the epitopes studied (8 subjects for Nef KY11, 6 subjects for Nef TL10 epitope, 5 subjects for Gag GL9, 4 subjects Gag SL9 and 2 subjects for Pol QW11) and in one subject each for Nef WF9 and Vpr AL9. In the subset of 46 individuals with measureable responses to both non-adapted and adapted epitopes on screening, the combined data of all HLA–epitope pairs measured at 2 µg mL−1 peptide concentration showed no significant decline in IFNγ production in response to the variant epitopes (449 SFUs for non-adapted epitopes compared with 572 SFUs for adapted epitopes, n=46, P=0.6, mixed-model sign test, P=0.09 based on the square roots of absolute differences) (Figure 3), although this subset would tend to select out the more equivalent responses. In the larger data set of 216 screening assays where low-level or no responses to either peptide were included, there was a trend towards increased IFNγ responses to the adapted peptide relative to the non-adapted peptide (median 14.4 SFUs versus 12.5 SFUs, P=0.06 for adapted and non-adapted peptide responses). Peptide binding scores using SFYEPITHI, IMAS or IEDB for epitope pairs that could be compared across the three software programs were not consistent. Three of the five peptide pairs showed similar binding scores, whereas the remaining two epitope pairs showed differences and thus peptide binding scores did not completely explain the observed patterns of avidity (see Supplementary Figure). Differences in avidity may therefore be attributed to peptide HLA interaction with TCR. Only three of 15 individuals with HLA-B*0702 expressed HLA-B*0702 or HLA-B*44, which have an overlapping restriction to KY11, and two of the 21 HLA-B*0702 cases expressed HLA-B*5301, which could potentially bind to the B*0702-restricted TL10 epitope, indicating that, for the majority of cases tested, the avidity differences could not be explained by alternative HLA alleles with overlapping peptide restriction.

GL9, TL10 and KY11 neo-epitope-stimulated CD8 T-cell responses are not associated with differences in the production of IL-2, dual IFNγ/IL-2 or cytotoxicity

HLA-B*0702-expressing PBMCs from two individuals were cultured with the HLA-B*0702-restricted Gag (GL9) and Nef (TL10) non-adapted and adapted epitopes. In addition HLA-C*0702-expressing PBMCs from seven individuals were cultured with HLA-C*0702-restricted Nef (KY11) adapted and non-adapted epitopes to generate epitope-specific CD8 T-cell lines. PBMCs were cultured for a median of 12 days (range 9–17 days) after which the frequency of single interleukin-2 (IL-2)- and IFNγ-producing cells, and dual IL-2/IFNγ-producing cells, in effector memory and central memory populations were assessed by flow cytometry after overnight peptide re-stimulation. Central memory and effector memory CD8 T cells were
determined by the expression levels of CCR7 and CD45RA. Across all three epitope pairs, there were overall greater proportions of effector memory CD8 T cells compared with central memory CD8 T cells among all epitope-specific cell lines, with no significant difference in these proportions between T cells cultured with either non-adapted or adapted epitopes (20(3–35) versus 14(3–29)% median(range) of CCR7+CD45RA−/CCR7−CD45RA− central memory CD8 T cells and 58 (25–78) versus 61 (21–82)% CCR7−CD45RA− effector memory CD8 T cells for adapted and non-adapted stimulated cultures, respectively (n=11); data not shown). This was also observed in overnight stimulations of T-cell lines with media alone, or PHA/SEB (data not shown), suggesting that there was peptide-driven differentiation of central memory cells to effector cells, in addition to the proliferation of effector memory cells during the 12-day culture.

For all three epitope pairs associated with HLA-B*0702 and HLA-C*0702, the frequencies of IFNγ responses were not significantly different whether directed against non-adapted or adapted epitopes (Figure 4), although central memory populations in general were associated with increased numbers of IL-2-producing cells compared with effector

**Figure 2** Comparative functional avidity of IFNγ responses against non-adapted and adapted epitopes associated with ‘classical escape’ from (a) HLA-B*0801-restricted responses against FLKEKGL and (b) HLA-B57-restricted response against STTVKACW. In both examples, responses are abrogated by the HLA-associated substitution. By contrast, CTL responses against neo-epitopes have higher functional avidity than their non-adapted epitopes (c-f). Representative plots of peptide-stimulated IFNγ responses to HLA-C*0702-restricted KRG2DLDDLWV (c), HLA-B*4402-restricted QEEHE(R/K)THSNNW (d), HLA-B*0702-restricted TPGPG(W)RYPL (e) and HLA-A*0201-restricted SLNTV(W)ATL. Peptide concentrations (x-axis) from 2μg/ml followed by 1:2 dilution, and thereafter 1:10 dilutions depending on cell availability. Non-adapted epitopes are shown as triangles and neo-epitopes (adapted epitopes) are squares in all plots. CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen; IFN, interferon.

**Figure 3** Patterns of immunoreactivity associated with neo-epitopes in which IFNγ responses show equivalent or marginally enhanced IFNγ responses to the HLA-adapted variant epitope, in contrast to the ‘classical escape’ in which there is typically abrogation of IFNγ responses to escape variants. HLA, human leukocyte antigen; IFN, interferon.
memory populations (1.4 (0.4–2.8) versus 0.2 (0.1–0.6))% IL2-producing central memory and effector memory cells for adapted cells (P<0.001), and (2.1 (0.2–6.5) versus 0.1 (0.05–2.8))% of IL2-producing central memory and effector memory cells for adapted cells (P=0.002), in keeping with a central memory functional phenotype.31 In the case of single IFNy-producing cells, adapted (peptide-stimulated) cultures were associated with higher frequencies of single IFNy-producing central memory and effector memory cells compared with non-adapted, peptide-stimulated cultures (30 (6–67) and 37 (10–66)% IFNy-producing central memory and effector memory CD8 T cells, respectively, from adapted cultures compared with 7 (1–55) versus 10 (1–58)% IFNy-producing central memory and effector memory CD8 T cells for non-adapted cultures), in keeping with the high IFNy levels observed in the ELISPOT screening assays and functional avidity profiles of adapted, peptide-stimulated responses observed in PBMCs, although the values did not reach significance for central memory (P=0.11) or effector memory (P=0.09) cultures. To assess differences in functional cytotoxicity, chromium-release assays were performed on CD8 T-cell lines cultured from one individual expressing HLA-C*0702. The co-expressed HLA class-I alleles were HLA-A*03, A*23, B*0702, B*4001 and C*0304 in this case. As expected, B-cell targets pulsed with non-adapted Nef KY11 (KQDIDLWVY) were killed more readily by non-adapted KY11-specific T cells compared with targets pulsed with adapted Nef KY11 (KQKQDLWVY) (18 versus 12% lysis). However, the reverse was observed for adapted KY11-specific T cells, which killed fewer numbers of adapted, KY11-pulsed targets compared with non-adapted, KY11-pulsed target cells (33 versus 43% lysis n=1). That is, although adapted neo-epitope-specific T cells showed demonstrable cytotoxicity, this was more evident for non-adapted, peptide-pulsed targets (33 versus 12%) than for adapted, peptide-pulsed target and 42 versus 18% for non-adapted, peptide-pulsed targets n=1), which would predict the outgrowth of the adapted virus over the non-adapted species in the presence of adapted CTLs.

An alternative target cell line (expressing HLA-A*01, A*02, B*0702, B*4001, C*0304 and C*04) was used to determine the cytotoxicity for the HLA-B*0702-restricted CTLs derived from two individuals with the HLA-B*0702 allele common to the HLA class-I genotypes of the patients and the target cell line. Non-adapted and adapted HLA-B*0702-restricted CTLs did not show differential killing of the target cells (n=4 assays).

TCR Vβ-gene family expression levels were similar between adapted and non-adapted CTLs

TCR Vβ-chain family diversity was assessed in HLA-C*0702- and HLA-B*0702-restricted, adapted and non-adapted peptide-specific CTLs. We found no differences in TCR Vβ family repertoire between the adapted and non-adapted CTLs examined in the three samples tested (data not shown). Vβ 13.1 was the most common Vβ family for the HLA-C*0702-restricted CTLs, with more than 50% of the CD8 IFNy-producing cells expressing Vβ 13.1. The predominant Vβ family for the HLA-B*0702 Gag-specific CTLs was Vβ 12, contributing to more than 30% of CD8 IFNy-producing cells. Greater than 55% of the HLA-B*0702 Nef-restricted CTLs expressed Vβ 23. As expected, CTLs directed against Gag and Nef epitopes from the same patient expressing HLA-B*0702 showed different Vβ family hierarchies, with Vβ 12 predominating among the Gag CTLs and Vβ 23 more commonly expressed by the Nef CTLs, thus reflecting distinct clonotypes for different HIV epitopes.

Directly observed neo-epitope formation during acute HIV infection

We were able to examine neo-epitope selection on a longitudinal basis in an individual who was diagnosed with acute subtype-A HIV-1 infection days after an epidemiologically proven sexual transmission event. At first testing HIV-1 p24 antigen was detected in the absence of a p24 antibody, indicating the patient was Fiebig stage-II.42 Repeat testing on day 15 after transmission indicated detectable p24 antigen with reactive EIA screen and indeterminate group-4 western blotting using antibodies directed against p24, gp160 and equivocally against gp120 (Fiebig stage-IV). At this time the HIV RNA concentration (viral load) was >10^6 copies ml^{-1}. By day 98 the western blot was fully reactive and the viral load had declined without antiretroviral medication to 125 893 HIV RNA copies per millilitre (Fiebig stage-VI). The HLA class-I genotype of the individual was HLA-A*23, HLA-A*3004, HLA-B*08, HLA-B*15, HLA-C*04 and HLA-C*07, which included two HLA class-I alleles, HLA-B*15 and HLA-C*07, associated with the neo-epitopes studied here; HLA-B*1503-restricted Nef (183–191) WRFEDSRALF and HLA-C*0702-restricted Nef (105–115) KQKQDLWVY, as well as the HLA-B*08 allele, associated with the ‘classical’ escape in Nef FL8 as described previously. Nine consecutive PBMC samples were collected and cryopreserved from days 37 to 658 after transmission. IFNy responses to 28 HIV epitopes were evaluated in the ELISPOT assay. These epitopes included the HLA-B*15 and HLA-C*07 adapted and non-adapted epitopes, and the HLA-B*08-restricted Nef FL8 epitope with three of its adapted variants, in addition to 23 other well characterized CD8 T-cell epitopes restricted to the individual’s HLA class-I alleles.39 Eight plasma samples were also collected from day 15 to 469 after transmission, seven of which were collected prior to commencement of antiretroviral therapy.

The IFNy responses to HLA-B*0801-restricted Nef FLKEKGGL (3735 SFUs) and its variant FLKENGGGL (1298 SFUs) were among
the three responses detected in the first available time point for immunological testing 37 days after transmission; the other being the response to HLA-B*1503-restricted Pol RKAKIIRDY (230 SFUs). The FL8 epitope was in the non-adapted form in the patient's viral sequence from the earliest plasma samples (day 15 after transmission) and remained unchanged despite detection of high-magnitude IFNγ responses. Ex vivo responses to the FLKEQGGL-adapted variant were reduced at all nine time points tested. The HLA-C*0702-restricted KY11 epitope remained invariant at all time points; however, the position associated with HLA-C*0702 selection in the subtype-B population analysis within this epitope is known to be a site of subtype-specific variation, with E (KROEILDIVY) present in subtype-A reference sequences and therefore likely to be present in the transmitted founder sequence. Indeed, the E was also present in the donor sequence, suggesting this was the case. Low-level responses to the KY11 epitope were detected at days 98 (62 SFUs) and 385 (60 SFUs) after transmission. In Nef WF9, the non-adapted epitope WKFDRLAF was present in the individual's sequence on day 15. A mixture of the adapted WRFDSLAF and the non-adapted WKFDRLAF sequence was detected in the patient sample by day 266, and the adapted sequence was detected in subsequent patient samples (days 385 and 469) in keeping with the specific substitution predicted by population-based HLA association studies. IFNγ responses to the non-adapted WF9 epitope were detected on day 98 (216 SFU) and day 266 (178 SFU). Responses then decreased to 88 SFUs by day 385 after the autologous viral adaptation was detected. Responses further declined to 30 SFUs on day 469 and were not detected thereafter. Low-level responses to the adapted WF9 neo-epitope reached 70 SFUs by day 385, having been below the positive cut-off previously (36 SFUs on day 98 and 38 SFUs on day 266) (Figure 5). The patient commenced therapy thereafter and no further responses were detected to the adapted peptide. The change in WF9 following strong WF9 T-cell responses coincided with a rise in viral load over 3 months from an apparent set-point of 70 795 copies ml⁻¹ to 245 471 HIV RNA copies per millilitre (Figure 5), coincident with a decline in CD4 T-cell count from 572 to 357 cells μl⁻¹ over the same time points. There was a broadening of CD8 T-cell responses overall, peaking at 20 epitope-specific responses against HIV epitopes in Gag, Pol, Env and Nef at this time point. The patient subsequently commenced antiretroviral therapy. Overall, T-cell responses were detected to 21 of the 28 peptides evaluated, with a median of 7 (inclusive of responses to the five variants) and ranging from 3 to 20 responses over 9 time points (Figures 6a and b). All Nef variants examined in the study were included in the analysis and are shown separately.

DISCUSSION

Studies of HIV-1 in natural infection, after vaccination, during transmission between hosts and on exposure to antiretroviral drugs have shown that patterns of genetic variations of HIV-1 in all these scenarios are strongly shaped by the virus' evolutionary drive to preserve and optimize replicative fitness. HIV mutation, which reduces or abolishes CTL recognition of single epitopes, is a pre-eminent example of this. Mutation, which leads to persistent and immunodominant CD8 T-cell responses against epitopes, which then remain invariant, implies that these responses either do not hinder, or possibly even enhance, viral fitness. HIV exploiting immunodominance by actively promoting particular non-suppressive responses would effectively block alternative epitope-specific T cells from which the virus would have to accommodate further mutation networks with potential compromises in replicative capacity. Several population-based studies have independently generated HLA-HIV
polymorphism associations, which match established cases of ‘classical’ T-cell escape,15–25 and these cases have in part been used to provide experimental validation of the statistical associations and the association approach in general. In the same studies, including the population-based study used here, associations generated by the same methods and stratified by the same statistical criteria show that HIV-1 adaptation serves to create epitopes as well. If the former examples of ‘classical’ CTL escape are accepted as validating observations, then these associations showing creation of epitopes cannot be rejected a priori. Many neo-epitopes are commonly presented, and are among the relatively narrow repertoire of immunodominant responses documented in several studies of chronic HIV-1 infection.

We have systematically studied a group of these epitopes selected on the basis of genetic signals and pre-existing immunological observations. We show that the HLA allele-specific selection pressures on single codons, which create these epitopes in vivo, cause a significant enhancement of the functional avidity of the memory responses elicited to them, compared with the non-adapted epitopes. These responses did not show a more ‘poly-functional’ cytokine profile as determined by either increased IL-2 secretion or dual IL-2/IFNγ secretion. Instead neo-epitope-specific responses had a high-IFNγ-producing memory and central memory phenotype, and an inconsistent profile of cytotoxicity. In one of the five cases tested, the cytotoxicity of the adapted epitope-specific T-cell response was lower against the adapted epitope than the non-adapted epitope, predicting the persistence and selection of viruses bearing the adapted epitope in vivo. We did not prove that T-cell populations reacting to adapted and non-adapted sequences were distinct T-cell clonotypes using Vβ-chain family usage, although a clear signal of selection away from one response and towards the other was evident in the viral sequences (Supplementary Table). Finally, in an acutely infected individual we directly observed the early presence of the HLA-B*1503-non-adapted Nef WP9 epitope sequence and cognate CD8 T-cell IFNγ response, followed by viral adaptation within the epitope and emergence of the neo-epitope-specific CD8 T-cell response, confirming that the genetic analyses had correctly identified the direction of amino-acid substitution typically associated with HLA-B*1503-restricted selection pressure, and that the functional characteristics of the neo-epitope CD8 T-cell response could not be explained by the ‘original antigenic sin’ of the transmitted sequence in this case.47 The emergence of HLA-B*1503-driven selection coincided temporally with the first increase in the viral load after a decreasing viral load associated with the other early CD8 T-cell responses. Taken together, these data may provide evidence of HIV-1 adaptation having a broader influence on the immunodominance patterns of chronic infection beyond abrogation of the viral inhibition of individual epitope-specific CTLs. In particular, induction of high-avidity CD8 T-cell responses in this context appears the result, rather than the cause of, immune selection, with the effect being facilitation of immunodominance of high-IFNγ-producing responses (even within the central memory compartment) rather than improvement of cytotoxic viral control.

High functional avidity or antigen sensitivity, measured as the recognition of peptide–major histocompatibility complex at low densities on target cell surface, has been generally considered a favourable qualitative property of antiviral CTLs, associated with greater elimination of target cells and viral clearance.8–52 In the setting of HIV and SIV infection, high-avidity CD8 T cells are associated with the intense selection pressure of acute infection53 and greater antiviral control in established chronic infections.54,55 Furthermore, the functional profile of HIV-specific CD8 T-cell responses is strongly determined by levels of antigen concentration and TCR engagement.56,57 For example, highly avid HLA B*2705-restricted T-cell clones and PBMC-derived T cells against HIV-1 p24 Gag KK10 showed greater IFNγ, tumour necrosis factor-α and CD107a mobilization per cell compared with less avid T cells.58 However, the rapid lymphoproliferation triggered by T-cell activation at low antigen concentrations represents a potential ‘double-edged sword’ in which high-avidity responses can preferentially expand and dominate the antigen-specific response,59 but also progress to a state of replicative and functional senescence more rapidly in the presence of ongoing antigen stimulation.60 While the multiplicity of general (non-epitope-specific) immune defects in HIV infection affecting the circulating naive T-cell pool, CD4 T-cell help, thymic output and haematopoiesis all contribute to the failure of clonotype renewal in the initial wave of early immunodominant CTL responses, our data suggest that there can be replacement by high-avidity variant-specific clonotypes, but this is not necessarily useful for immune containment if the targeted variants are HLA-adapted.

These data have further implications for understanding the complex interactions between T-cell avidity, viral sequence evolution and cell function. Data from the influenza field suggest that high T-cell avidity may in fact be inversely correlated with cytotoxicity. Among the influenza-A virus-specific CTLs recovered from the infected lung of B6 mice, T cells with lower epitope avidity associated with a short-lived ‘kiss-and-run’ engagement with the HLA–peptide complex lysed more target cells than high-avidity, high-IFNγ-producing T cells.62 In a study of CD8 T-cell responses against HIV-1, cytomegalovirus, EBV and influenza, greater IL-2 production and antigen-specific proliferation of more protective HLA-B-restricted T-cell responses were associated with lower-avidity TCRs compared with less favourable IFNγ-secreting, effector-only responses expressing the T-cell exhaustion marker PD-1 and high-avidity receptors.63 HLA-restricted T-cell responses to mutated epitopes in Gag restricted by HLA A*11 and B*57, and in Pol, Nef and Env restricted by HLA-B*35, have also shown decreased proliferative capacity associated with variant-specific responses.26,64,65 A number of studies of chronic HIV-1 infection have also documented high-avidity CD8 T cells associated with high viraemia66 and a lack of a simple correlation between magnitude or breadth of IFNγ responses alone and viral load.67 Whereas this reflects the likely importance of qualitative markers aside of IFNγ, our data suggest that epitope specificity is also important. Despite the degeneracy of TCR recognition, single viral polymorphisms can facilitate particular epitope-specific responses over others once infection is established. Here, we have not obtained clear evidence of a superior functional profile such as IL-2 production associated with the non-adapted epitope responses; however, such profiles are presumably rare when considering memory responses in chronically infected individuals. Our observations support a study by Karlsson et al.,13 who found that HIV-1 escapes temporally effective, acute immune responses, but then broadens to target epitope variants, which match a consensus subtype-B sequence. Such broadening was associated with a higher viral load during the first year of infection. The authors argue that T-cell breadth in this context was not a favourable host response to HIV variation, but the ‘immunological footprint’ of viral adaptation, which helps to maintain some elements of the consensus viral sequence in a population. Here we show that this phenomenon is evident at the population level. By escaping from early, strongly cytotoxic CD8 T-cell responses and then creating neo-epitopes, which elicit highly avid but exhausted effector phenotype T-cell responses, HIV is effectively exploiting immunodominance to shift to non-inhibitory responses.
Aside of the particular epitopes examined here, there were a large number of overlapping putative epitopes/neo-epitopes associated with HLA-associated polymorphism in population-based studies, suggesting that this could be applied to many more of the measurable responses in HIV-infected individuals. This model would reconcile conflicting arguments about the utility of CD8 T-cell immunity in HIV infection. The findings that high-frequency, HIV-specific CTLs are detectable in individuals with AIDS, and the adverse interference between Env and accessory protein responses with Gag-specific responses would all be expected if HIV can itself promote certain non-suppressive responses over suppressive ones as an adaptive strategy. Several mechanisms of viral enhancement are possible—‘decaying’ from other better epitopes, direct effects of epitope variation on T cells to IFNγ-driven exhaustion or activatory recruitment of CD4 T-cell targets. Regardless of the mechanism, the possibility that some epitopes serve the virus and not the host has implications for vaccine design. Polyvalent vaccines designed to combat HIV strain diversity by incorporating as much of HIV-1 variation as possible may increase the breadth of CD8 T-cell reactivity, but this may not be desirable if this recapitulates the immunodominance hierarchies that such variation facilitates in nature. Diversity coverage strategies based on very early acute or founder sequences may circumvent this issue; however, such sequences may also include adaptive changes made in the donor host that have not yet reverted or are slow to revert in the recipient host because of minimal or enhancing effects on viral fitness. We were limited in this study to assay for memory CD8 T-cell responses in a cohort of chronically infected individuals, many of whom with highly suppressed HIV viral load on antiretroviral medication. This probably accounted for the high rate of CD8 T-cell non-responders generally in the study, as previous studies have shown reduced HIV-specific CTL responses in patients on antiretroviral therapy, including absence of responses against the HLA-A*0301-restricted responses against Pol QR9. Furthermore, we have not taken account of other factors, which do modulate T-cell avidity in vivo, such as dendritic cell function, and it is also possible that the functional avidity apparent in the ELISpot assay does not reflect in vivo reactivity with natural antigen presentation mechanisms. Nevertheless, we have in each case compared the relative functional avidity profiles and immunological characteristics of two epitope variants—non-adapted and adapted—under equivalent assay conditions, and the fact that the differences between the profiles are consistent across seven different epitope pairs and 125 individuals tested, and the consistent direction of amino-acid substitution associated with the creation of neo-epitopes in independent genetic studies, suggests this is a robust observation. In the absence of detailed immunological characterization of the large number of prevalent or possible HLA-restricted CD8 T-cell responses in naturally infected or vaccinated individuals, population-based genetic signals as used here can serve to delineate empirically which HLA-restricted responses are likely to mediate relative suppression, non-suppression or enhancement of viral replication based on the evolutionary solutions of HIV to evade or exploit them.

**METHODS**

**Study cohort**

The 125 subjects used for all cellular studies were enrolled into the WAHCS, which is a prospective, observational study of HIV-infected individuals established in 1983 and approved by the Royal Perth Hospital Ethics Committee, and conforms to the provisions of the Declaration of Helsinki. All subjects gave informed consent to these studies. Longitudinal CD4, CD8 T-cell counts, viral load measurements and antiretroviral medication history were available on all subjects.

**HIV peptides**

Eight HIV-1 9- to 11-mer peptide pairs were synthesized by Invitrogen (Melbourne, VIC, Australia); HLA-A*0201-restricted Vpr (59–67) A\_GDPGRKVRPL and A\_GDPGRKVRPL; HLA-B*0702- and HLA-B*4201-restricted Nef (128–137) TPGPGVRYP and TPGPGVRYP; HLA-B*1503-restricted Nef (183–191) WFRDSSLAF and WFRDSSLAF; HLA-C*0702-restricted Nef (105–115) KBQIELILDQVY and KBQIELILDQVY; HLA-A*0301-restricted Pol (424–432) QIYPGKVR and QIYPGKVR; HLA-A*0201-restricted Gag (77–85) SLYNTVATL and SLYNTVATL epitope; and HLA-B*4402-restricted Pol 724–734 QEEHEKYSHNW and QEEHEKYSHNW. Peptides were reconstituted in dimethyl sulphoxide to 10 mg ml\(^{-1}\) and stored at minus 80 °C prior to dilution for use in assays.

**IFNγ ELISpot assay**

Cryopreserved PBMCs were thawed and left to settle overnight at 37 °C. Viable lymphocytes were enumerated by trypan blue exclusion using a Neubauer haemacytometer. A total of 100 000 cells resuspended in culture medium (10% fetal calf serum/RPMI-1640) were dispensed per well using a modified version of the previously described IFNγ ELISpot assay.76 Sterile 96-well nitrocellulose-backed plates (MAIP S4510; Millipore, Bedford, MA, USA) were coated with 2 μg ml\(^{-1}\) IFNγ-coating antibody (Mabtech; Naka Strand, Sweden) overnight at 4 °C in sterile phosphate-buffered saline. The plates were washed with sterile phosphate-buffered saline and blocked with culture medium (10% fetal calf serum/RPMI-1640, 30 min, room temperature, after which cells and stimulants (synthetic HIV peptides dispensed in triplicate at limiting dilution (2 μg ml\(^{-1}\), 1 μg ml\(^{-1}\), then 10-fold dilutions, dependent on cell numbers), with positive (anti-CD3 antibody; Mabtech, Melbourne, VIC, Australia) and negative (culture media alone) controls) were dispensed for overnight incubation under CO2 at 37 °C. The plates were then washed with sterile phosphate-buffered saline. Biotinylated IFNγ (Mabtech, VIC, Australia) was added (2 h, room temperature), after which plates were washed and streptavidin horseradish peroxidase (Mabtech, VIC, Australia) was added (1 h, room temperature). The plates were washed and developed with 100 μl well\(^{-1}\) tetramethylbenzidine substrate (Mabtech, VIC, Australia) for 10 min at room temperature. The plates were washed extensively with MilliQ H2O and left to dry prior to analysis with the AID iSpot reader (AID, Starsburg, Germany) using the AID software (5.0 B7337). Responses were determined by subtracting the mean of the negative control wells from the mean of triplicate stimulated wells. Results are presented as SFUs per 10⁶ cells. Responses were considered positive if they were > 50 SFUs based on the distribution of the negative controls and test data (see section Statistical analysis).

**EBV-transformed B cells**

EBV-transformed B-lymphoblastoid cell lines were generated in-house at the Department of Clinical Immunology (DCI), Royal Perth Hospital, Western Australia. The HLA-homozygous B-cell line (workshop HHKB) R86 12350C, expressing HLA-A*0301, B*0702 and C*0702, was used to present the HLA-C*0702-restricted Nef KY11 peptide and a non-adapted variant to CTLs from the HLA-A*0301-expressing patient. The R04 0244215 (HLA-A*01, HLA-A*02, HLA-B*0702, HLA-B*4001, HLA-C*0304, HLA-C*0704) cell line was used to present HLA-B*0702-restricted Gag GL9 and Nef TL10 peptides, and variants. All cells were maintained in culture media for a minimum period of 2 weeks prior to use in cytotoxicity assays.

**CD8 T-cell lines**

HLA-restricted, peptide-specific CD8 T-cell lines were generated from cryopreserved PBMCs. PBMCs were thawed and left to settle overnight in culture medium and counted. A total of 2–5×10⁶ cells were incubated with 20 μg of an HIV-specific peptide to give a final concentration of 20 μg peptide in a total volume of 1 ml, and incubated for 2 h at 37 °C in a CO2 incubator. The cells were then washed and resuspended in 1 ml of T-stim media (15 ml—T-cell growth media harvested from a T lymphoblastoid cell line (donated by Professor James McCluskey, University of Melbourne, Australia), 25 ml—culture media, 7.5 ml—fetal calf serum) supplemented with 30 IU of IL-2 (Chiron, Emeryville, CA, USA) in a single well of a 24-well plate and left for 3 days at 37 °C in a
CO₂ incubator. On day 4, 1 ml of T-stim media, supplemented with IL-2 at final concentration of 100 IU ml⁻¹, was added to the culture. The cultures were fed or split as required every 2–3 days up to day 14. On day 14–16, peptide-specific CTLs were confirmed by flow cytometry. The cytotoxicity of peptide-positive CTLs was determined using the standard ⁴⁶ chromium-release assay.⁷⁷

T-cell immunophenotyping
CD3+/CD8+ T-cell effector and central memory cell phenotypes were differentiated by surface expression ofCCR7 and CD45RA by flow cytometry using the BD Biosciences (San Jose, CA, USA) FACSCanto II instrument. Results were analysed using the FlowJo Software (TreeStar, Ashland, OR, USA). A total of 0.5 × 10⁶ CTLs per millilitre were re-stimulated with specific HLA-restricted peptides and appropriate positive (Staphylococcus enterotoxin-B/phthoaeamaglutinin) or negative (culture media alone) controls in overnight cultures. A 10-µl volume each (from 1:10 stock dilutions) of anti-CD49 and anti-CD28 (BD Biosciences) was also added to the cultures. Brefeldin-A (Sigma, St Louis, MO, USA) was added after 2 h (final concentration 5 µg ml⁻¹). After overnight re-stimulation, CTLs were washed with flow buffer (1% fetal calf serum/phosphate-buffered saline), centrifuged, counted and 0.5 million cells were dispensed per tube for membrane and intracellular cytokine staining. A cocktail of fluorescence-labelled membrane antibodies were added first to each tube (CD3 APC Cy7, CD8 PerCP PCy5.5, CCR7 PeCy7, CD45RA APC). After a 15-min incubation period, cells were washed, fixed (Intraprep kit; Beckman Coulter, Immunotech, Marseille, France) and washed again prior to permeabilization (Intraprep kit). Intracellular cytokine antibodies (IL-2 phycoerythrin and IFNγ Alexa Fluor-488) were then added for 15 min at room temperature. The cells were washed, resuspended in flow buffer and analysed using the FACSCanto flow cytometer.

CTLs were prepared, as stated above for overnight peptide re-stimulation, for Vβ TCR repertoire staining. CD3 APC Cy7 and CD8 PerCP PCy5.5 were then added from the previously mentioned cocktail of surface membrane antibodies and phycoerythrin- and FITC-labelled Vβ antibodies (Beckman Coulter), replacing the remaining membrane antibodies. Cells were then fixed and permeabilized, after which IFNγ conjugated-APC-A (BD Biosciences) was added to each tube. The cells were washed, resuspended in flow buffer and analysed using the flow cytometer.

Cytotoxicity assays
Cytotoxicity assay was performed using the standard ⁴¹ Cr-release assay.⁷⁷ Target cells (EBV-transformed B cells expressing the HLA allele of interest) were labelled with sodium ⁴¹ Cr-labelled chromate (Perkin-Elmer, Melbourne, VIC, Australia) and pulsed with 2 µg ml⁻¹ HIV-specific peptide overnight at 37 °C. After thorough washing, the B-cell targets were admixed with peptide-specific CTLs at specific effector:target ratios (2:1, 10:1, 20:1) in triplicate wells of 96-well round-bottom plates. The supernatants were harvested after 4 h, transferred to counting tubes and counted. Chromium labelled B cells not pulsed with peptide were used as negative controls. 100% maximal lysis was determined by addition of 100 µl of 0.5% Triton X-100 (Sigma). Specific percent lysis was calculated using the following formula: 
\[
\text{Percent lysis} = \left( \frac{\text{c.p.m. spontaneous} - \text{c.p.m. specific}}{\text{c.p.m. spontaneous}} \right) \times 100.
\]
Peptide-specific CTLs added to unpulsed target cells controlled for nonspecific CTL lysis (median=0, range 0–7% for unpulsed target cells). Where possible, two B-cell lines were used to determine cytotoxicity.

HIV sequencing
Viral sequencing was performed on a single patient studied longitudinally. HIV-1 RNA was extracted from plasma samples and quantitated using the AmplicPrep/COBAS TaqMan HIV-1 version 1.5 according to the manufacturer’s instructions. Samples were analysed using the COBAS TaqMan 48 analyser using a linear dynamic range of 40–1 × 10⁴ copies ml⁻¹. Standard bulk sequencing of HIV-1 Nef was based on a nested PCR of a first-round 6-kb fragment, followed by shorter-fragment-amplification of a region containing Nef. Direct sequencing was performed on PCR products using the ABI 3130XL analyser (Applied Biosystems, CA, USA) and electropherograms were analysed and edited using Sequencher software (GeneCodes, Per, WA, Australia). The viral sequences in the longitudinal patient were aligned with a reference subtype-A strain A-KE-Q23-17 (GenBank accession number AF004885.1)

HLA class-I typing
Genomic DNA was isolated from all study participants. PCR amplification of HLA-A, B and C genes was performed using sequence-specific primers. The products were resolved to a two- or four-digit-level resolution based on exon 2–3 sequence using standard sequence-based typing (SBT) by DGI, Royal Perth Hospital.

Statistical analysis
Summary data are typically presented as the mean and standard deviation, or median and range of the sample values as appropriate. These summaries do not take account of possible correlations induced by common host cells. However, paired differences for adapted versus non-adapted responses were analysed by mixed models accommodating repeated measures on the same individuals. The threshold for defining a ‘positive’ response was chosen approximately as the third standard deviation value based on fitting a half mean-zero-normal distribution to the negative responses relative to background, under the assumption that these represent only a random non-response.

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