Constraints on HIV-1 evolution and immunodominance revealed in monozygotic adult twins infected with the same virus

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The predictability of virus–host interactions and disease progression in rapidly evolving human viral infections has been difficult to assess because of host and genetic viral diversity. Here we examined adaptive HIV-specific cellular and humoral immune responses and viral evolution in adult monozygotic twins simultaneously infected with the same virus. CD4 T cell counts and viral loads followed similar trajectories over three years of follow up. The initial CD8 T cell response targeted 17 epitopes, 15 of which were identical in each twin, including two immunodominant responses. By 36 months after infection, 14 of 15 initial responses were still detectable in both, whereas all new responses were subdominant and remained so. Of four responses that declined in both twins, three demonstrated mutations at the same residue. In addition, the evolving antibody responses cross-neutralized the other twin's virus, with similar changes in the pattern of evolution in the envelope gene. These results reveal considerable concordance of adaptive cellular and humoral immune responses and HIV evolution in the same genetic environment, suggesting constraints on mutational pathways to HIV immune escape.

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Abbreviations used: BLCL, B lymphoblastoid cell line; SFC, spot-forming cell.

Understanding HIV evolution, adaptive immunity, and disease pathogenesis is complicated by genetic diversity among infecting viruses. HIV is defined by different clades, which vary from one another in some regions by 30% or more; even within a single clade, the overall amino acid variability may be 20% in their envelope proteins (1). Given the high replication rate of this retrovirus, and the inherent errors in reverse transcription, it is estimated that a mutation arises within every amino acid position of each HIV-1 protein every day, although structural and functional constraints likely limit the outgrowth of many variants (2–6). The potential impact of viral variability on disease pathogenesis is suggested by studies of viral infections in genetically identical mice, where it has been shown that as few as two amino acid changes in the entire viral genome can result in the difference between an infection that is successfully contained by the immune system versus one that leads to chronic viremia and death (7).

Although the macaque model of AIDS virus infection has been able to control for the infecting virus, no studies have been able to address HIV evolution after acute infection in the identical host genetic context. In humans, examination of immune responses in genetically related individuals presumably infected with the same virus has shown discordance in targeted epitopes and viral variants that evolve (8), but no studies have examined these issues early after infection in monozygotic twins. In mouse models of chronic viral infection, such as lymphocytic choriomeningitis virus, the dominant and subdominant T cell responses are highly predictable (9), but such studies have not been possible in rapidly evolving human virus infections.

Here, we evaluate the unique situation of monozygotic twins that were simultaneously infected with the same HIV-1 strain at the age

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of 21 yr. Over a period of 30 mo of follow up, the development of cellular and humoral immune responses and viral evolution were studied in this setting that controls for initial host and viral diversity. The results, compared with a third brother who was infected with the twins' virus ~13 mo later, indicate a striking degree of concordance in immune selection pressure and viral evolution in HIV infection, suggesting that HIV is constrained by restricted pathways to immune evasion, which has important implications for vaccine design.

RESULTS

HIV-1 disease course in monozygotic adult twins infected with the same virus

TW1 and TW2 are monozygotic twins diagnosed with primary HIV infection at age 21, based on routine serologic screening done as a result of the risk factor of injection drug use. A brother (BR3) who also used injection drugs seroconverted 13 mo later. Because the twins reported only a single episode of needle sharing with others, and they also shared needles with their brother, *pol* and *env* genes from all three brothers were sequenced to determine if they might be infected with the same virus.

When compared with other viral isolates, all the sequences from viruses infecting TW1, TW2, and BR3 clustered together with a mean genetic diversity of the *pol* gene of 0.3 ± 0.1% and a maximum distance of 1% (Fig. 1 A). In contrast, the mean genetic diversity among randomly selected sequences from local epidemiologically unrelated HIV-infected individuals included in the analysis was 4.4 ± 0.3%. Similar phylogenetic analysis on the *env* gene estimated a mean genetic diversity of 3.2 ± 0.2% among the siblings' viruses over the course of infection. The high degree of genetic similarity among viral isolates obtained from all three siblings indicates that they were infected by the same viral strain during needle sharing.

Longitudinal analysis of plasma RNA and CD4 cell counts revealed similar trajectories in both twins during the first 3 yr of infection, at which point TW1 died from an injection drug overdose (Fig. 1 B). Similar analyses in BR3, who shared a single class I A, B, and C haplotype with the twins, likewise revealed a very similar viral load, but a more rapid decline in CD4 count after infection. These data show similarity in disease course as defined by viral load and CD4 cell count in persons of identical genetic background infected with the same strain of virus.

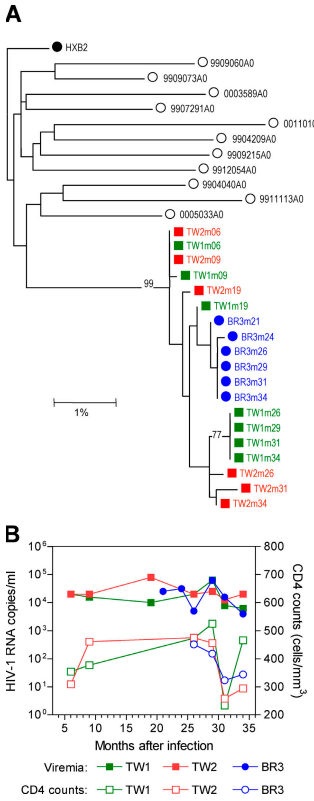

Figure 1. The three siblings shared the same infecting virus and had similar clinical evolution. (A) Maximum likelihood tree reconstructed from HIV-1 *pol* sequences (corresponding to protease and first 235 amino acids of reverse transcriptase) in the three brothers' viruses over the study period compared with other contemporary viral isolates of treatment-naive subjects in the same city (open circles) and the reference strain HXB2 (closed circle), used as outgroup. The HKY85 with gamma distribution (HKY+I+G) model was used, which accounts for a transition/transversion bias, variable base frequency data, and variable substitution rates at different nucleotide positions. Scale bar represents 1% genetic distance (0.01 substitutions/site). Bootstrap values are shown at nodes with >70% support. (B) Plasma viremia and CD4 cell counts over time for all three siblings.

Evolution of virus-specific T cell responses

The only studies evaluating HIV-specific CD8 T cell responses in persons with the same HLA class I alleles revealed differences in these responses years after infection (8, 10), but no studies have examined these events in the critical initial period of acute infection. We screened both HIV-infected twins independently in an IFN-γ Elispot assay for detection of antigen-specific T cell responses using a set of overlapping 15–19 amino acid peptides spanning all expressed HIV proteins (11, 12). At the first time point available (6 mo after infection), 16 responses directed at five viral proteins (Gag, Pol, Vpr, Env, and Nef) were detectable in each of the twins, 15 of which were identical (Fig. 2 and Fig. 3 A). Moreover, the two strongest responses targeted in the early phase of infection were the same, both of which were determined to be B*4001-restricted CD8 T cell responses: IL9 in Pol (RT) and KL9 in Nef.

Conversely, each of the twins had a single subdominant response that could not be detected in the other at the earliest time point, TW1 in Nef and TW2 in Env (Fig. 2 and Fig. 3 B); both remained minor responses or disappeared over prolonged follow up. Although the magnitude of responses in TW2 was weaker than in TW1, comparison of all responses at the earliest time point revealed a strikingly significant correlation ($R^2 = 0.52$, $P = 0.0011$; Fig. 4 A), which is in contrast to the CD8 T cell responses in acutely infected individuals reported to date (13).

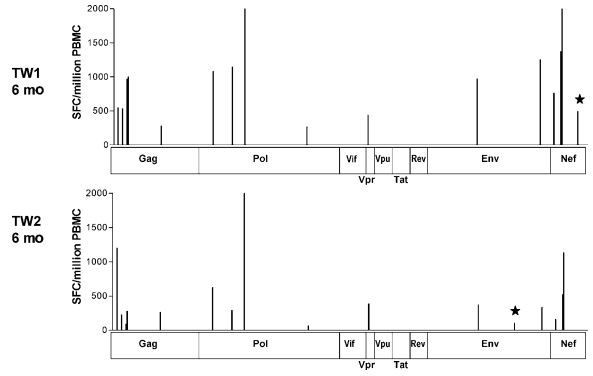

Figure 2. Similar distribution and magnitude of antigen-specific T cell responses in both twins early after infection. IFN-γ T cell responses in TW1 and TW2 at the first available time point (6 mo) after infection expressed as SFCs per million PBMCs. The x axis represents all 410 overlapping peptides spanning all HIV proteins. Each bar represents one response. Stars indicate responses not shared by the twins.

These initial T cell responses were followed using longitudinally obtained cryopreserved PBMCs. By 34 mo after infection, 14 of the 15 concordant initial responses were still detectable (Fig. 3 A). The only response that was completely lost was the same in both subjects, a B*4001-restricted response in Gag. Longitudinal quantitation of the magnitude of responses by IFN-γ Elispot revealed that 11 of the 15 concordant initial responses (including both of the early immunodominant responses) remained similar in both twins: 7 persisted in both, and 4 declined in both.

Over time, new responses became detectable to six epitopes in TW1 and five in TW2, four of these were targeted by both (Fig. 3 C), and all were subdominant responses. After 3 yr of persistent viremia, TW1 and TW2 both targeted 22 epitopes; 20 of the epitopes were targeted by both twins. Although the distribution was less even, the correlation remained statistically significant ($R^2 = 0.30$, $P = 0.007$; Fig. 4 B).

Immune selection pressure within targeted CD8 T cell epitopes

We next evaluated the impact of persistent T cell responses on viral evolution by sequencing the targeted regions of the virus from both subjects. We initially concentrated on those responses that declined in both twins because the declining CD8 T cell responses have been associated with effective immune escape (14, 15). The response to the initially dominant B*4001-restricted Pol epitope declined in both subjects, with similar kinetics (Fig. 5 A), and was associated in both subjects with a conversion from wild-type sequence present 6 mo after infection to the same Q→E mutation at position 6 in the epitope later in infection (Fig. 5 B), which was no longer recognized (Fig. 5 C). TW1 developed an additional I→V mutation at position 1 of the epitope at 34 mo after infection, which was likewise not recognized (unpublished data). In contrast, the immunodominant B*4001 Nef epitope that was

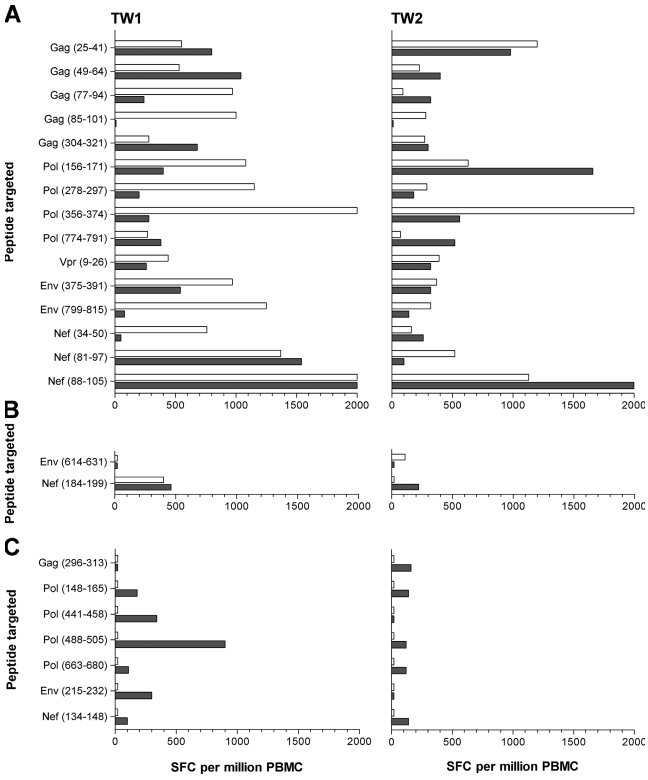

Figure 3. T cell responses during the course of the study. Left panels correspond to TW1 and right panels correspond to TW2. Open bars show the responses at 6 mo after infection and closed bars show the responses at 34 mo after infection. Responses targeted by both twins at the first study time point (A), responses targeted by only one of the twins at the first study time point (B), and new responses arising during the disease course in one or both of the twins (C). Details on epitope sequences can be found in Tables S1–S3 (available at http://www.jem.org/cgi/content/full/jem.20052116/DC1).

persistently targeted remained unchanged in both subjects (unpublished data).

Similar results were found for the other epitopes that were initially targeted and subsequently declined in both

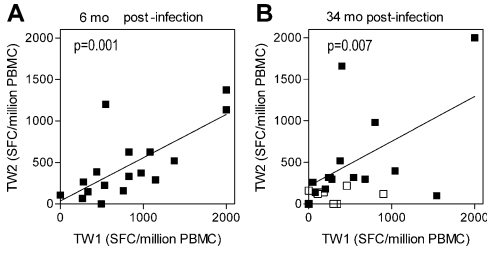

Figure 4. Comparison of the magnitude of T cell responses between twins. Each square represents one T cell response. The x axis represents the magnitude of the response for TW1, and the y axis represents the magnitude of the response for TW2. (A) The responses at 6 mo after infection. (B) The same analysis at 34 mo after infection. Identical initial responses are as shown as closed squares and different responses or responses coming up later in the disease course are shown as open squares.

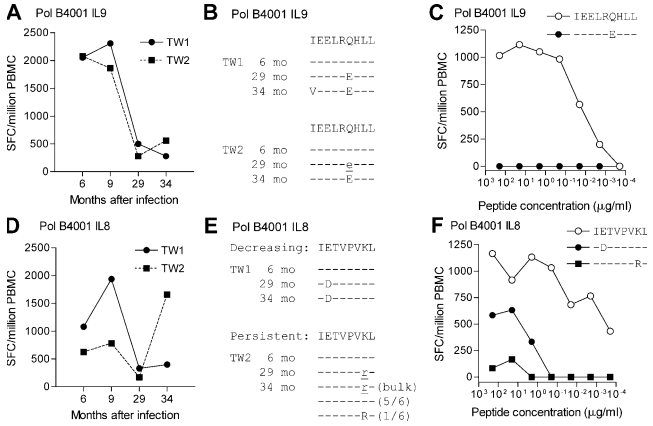

Figure 5. Concordant and discordant antigen-specific T cell responses and viral evolution in both twins. Concordant (A–C) and discordant (D–F) CD8 T cell responses and viral evolution. (A) Magnitude of the CD8 T cell response toward the B*4001-restricted epitope Pol IL9 in TW1 and TW2 over the study period. (B) Sequence data of the autologous virus of TW1 and TW2 for this epitope at designated time points. Sequences are shown as bulk sequences. The Q→E substitution at position 6 corresponds to the same nucleotide change, CAA→GAA. Lowercase underlined letters indicate the dominant amino acid in population-based sequences showing mixtures of nucleotides. (C) Peptide titration assays using the wild-type peptide and the mutated peptide as antigen in Elispot assay. Frozen PBMCs were used as effectors. (D) Magnitude of the CD8 T cell response toward the epitope IETVPVKL in TW1 and TW2 over the study period. (E) Sequence data of the autologous virus of TW1 and TW2 for this epitope at designated time points. Sequences are shown as bulk sequences. Lowercase underlined letters indicate the dominant amino acid in population-based sequences showing mixtures of nucleotides. In addition, clonal data are shown for TW2 at 34 mo. (F) Peptide titration assays using the wild-type peptide and the mutated peptides as antigen in Elispot assay. Frozen PBMCs were used as effectors.

twins (Table I). For three of four concordant declining responses, mutations arose at the identical amino acid positions. For the Gag IL10 response, viral evolution within the epitope was identical, with the same amino acid change (D; codon: GAC in both) occurring at a similar time point. The Env QL10 epitope was associated with a mixture at position 5 of the wild-type amino acid N and the substitution K in both twins, which additionally developed substitution S at later time points. Both mutations (K and S) led to loss of recognition. The fourth concordant declining response was to the A2 restricted epitope YV9 in Pol. TW1 developed a V→I mutation at position 9 of the epitope that did not influence peptide recognition. TW2 did not develop any mutations within the epitope. There were also no additional mutations in the immediate flanking region of this epitope detectable (unpublished data), thus the cause of decline of the response remains unclear.

Viral evolution within discordant CD8 T cell responses

We next examined the four subdominant CD8 T cell responses in which the response decreased in one twin and persisted in the other. Table II summarizes the sequence data for these responses. In three cases, TW1 lost the response. In one case, the response was only lost in TW2. For all four epitopes, viral evolution was different, providing an explanation for the differential effect on established cellular responses.

The first example is the epitope IL8 in Pol (Fig. 5 D), which fits the B*4001 motif but for which there were insufficient cells to formally demonstrate HLA restriction. The response to this epitope decreased in TW1, whereas it persisted in TW2. TW1 developed an E→D mutation at position 2, which became dominant; in contrast, TW2 developed a mutation at position 7 (K→R), but this remained a minor species (Fig. 5 E). Both mutations led to a loss of recognition of this epitope in peptide titration assays (Fig. 5 F) and indicate that there was immune selection pressure being applied to this epitope in both twins, but only the virus in TW1 had successfully escaped recognition, as indicated by the declining CD8 T cell response.

TW1 developed the T→V mutation in A2 SL9 that was just recently described as an escape mutation (16), whereas the wild-type epitope persisted, as did the response, in TW2. For the initially strong Cw3 restricted epitope AL9 in Nef, TW1 showed changes at position 5, whereas TW2 developed a position 1 mutation. The latter mutation was associated with a drop in CD8 T cell responses in TW2 in vivo, but neither mutant affected in vitro recognition, suggesting antigen processing changes, as recently described (17, 18). The fourth discordant response was to epitope LS9 in Nef, which was shown to be presented by both B*4001 and B*50 (unpublished data). Both subjects developed a mutation at different positions of the epitope: the E→D in TW1 led to complete loss of recognition. The I→V peptide is still recognized only two logs less well than the wild-type epitope, which may explain why the response persisted in TW2.

Thus, among the discordant CD8 T cell responses that evolved over time, all occurred within initially subdominant epitopes and there was heterogeneity in terms of the mutations that arose. These data suggest that there are differences in the kinetics of escape within the subdominant epitopes and are consistent with there being limited mutations leading to functional CD8 T cell escape (defined by a declining CD8 T cell response) within a given epitope.

Relationship between Shannon entropy scores and mutational escape

To further examine potential structural limitations affecting escape mutations, we examined the relationship between emerging escape mutations and population sequence diversity reported at each of these amino acid residues among reported clade B isolates in the Los Alamos National Laboratory HIV Sequence Database (www.hiv.lanl.gov). Quantification of sequence diversity for each residue in the escaping mutations was determined using normalized Shannon entropy scores (19). 9 of 12 escaping residues from the epitopes presented in Tables I and II arose at the most variable residue in each epitope (Table S4, available at http://www.jem.org/cgi/content/full/jem.20052116/DC1). The remaining three

Table I. Autologous sequences of concordant decreasing CD8 T cell responses

Epitope (HXB2 aa position)	Time after infection	Sequence TW1	Magnitude TW1	Sequence TW2	Magnitude TW2	Impact on peptide recognition
	mo					
Pol	6	IEELRQHLL		IEELRQHLL		-----E---↓
B4001 IL9 (357–365)	34	V----E---	↓	-----E---	↓	V----E---↓
Gag	6	IdIKDTKEAL		IdIKDTKEAL		-D--------↓
B4001 IL10 (92–101)	34	-D--------	↓	-D--------	↓	
Env	6	QELKnSAVSL[a]		QELKnSAVSL[a]		----S-----↓
B4001 QL10 (805–814)	34	----k-----[b]	↓		↓	----K-----↓
Pol	6	YTAFTIPSV		YTAFTIPSV		--------I↔
A2 YV9 (282–290)	34	--------I	↓	---------	↓	

Lowercase underlined letters indicate the dominant amino acid in sequences showing mixtures of nucleotides.
[a]Clonal analysis showed mixtures of amino acids N and K.
[b]Clonal analysis showed mixtures of amino acids N, K, and S after 34 mo of infection.
Magnitude, magnitude of CD8 T cell response; ↓, decreasing; ↔, no difference.

mutations still occurred at highly variable residues, but in the context of other more highly variable residues in each epitope. These results indicate that the escape mutations are preferentially occurring at residues that are more prone to sequence variation, and support the conclusion that there are constraints to the allowable mutations that can occur within CD8 T cell epitopes.

Immodominance and HIV evolution of the twins' virus in a partially HLA-matched environment

In this study, we also had the opportunity to evaluate the predictability of our findings in a third brother who shared half the HLA class I alleles with the twins and who became infected through needle sharing with them 13 mo after they became infected. He shared HLA-B*4001 with the twins, and again the B*4001 KL9 Nef epitope was the immunodominant response in BR3 at the earliest time point. However, there was no detectable response to the B*4001-restricted epitope IL10 in Gag (Fig. 6 A). Sequence data in BR3 within this epitope showed that all viral clones contained the E→D mutation at position 2 that arose in the twins before infection of BR3. We were unable to detect a reversion of this mutation during 20 mo of follow up which was the last sample we could obtain in this subject (Fig. 6 B). In addition, we were unable to demonstrate a memory response to this epitope after in vitro stimulation with the B*4001-restricted peptide IL10 (Fig. 6 C). Together, these data suggest that BR3 was infected with an escape mutant in the B*4001 epitope IL10 and never mounted a response to this region. Moreover, the longitudinal data suggest that this epitope mutation is stable and does not readily revert.

Evolution of neutralizing antibody responses

The similarity in adaptive immune responses and viral evolution demonstrated by examination of CD8 T cell responses prompted us to evaluate the evolution of virus-specific humoral immune responses in each subject and compare the phenotypic ability of the viruses to escape autologous and heterologous neutralizing responses.

All viruses collected from the twins over the first 34 mo of infection were determined to use CCR5 for viral entry (unpublished data). Based on the neutralization of virus envelope pools by autologous plasma samples, antibody titers were low (inhibitory concentration of <100-fold dilution) for contemporaneous plasma and were higher against viruses from preceding bleeds and increased over time. Detectable

Table II. Autologous sequences of discordant CD8 T cell responses (persistent vs. decreasing)

Epitope (HXB2 aa position)	Time after infection	Sequence TW1	Magnitude TW1	Sequence TW2	Magnitude TW2	Impact on peptide recognition
	mo					
Pol	6	IETVPVKL		IETVPVKL		-D------↓
B4001 IL8 (160–167)	34	-D------	↓	------r-	↔	------R-↓
GagA2	6	SLYNTVATL		SLYNTVATL		-------V-↓
SL9 (77–85)	34	-------V-	↓	---------	↔	
Nef	6	AALDMSHFL		AALDLSHFL		----M----↔
Cw3 AL9 (83–91)	34	----I----	↔	G--------	↓	----I----↔
Nef	6	LEKHGAITS		LEKHGAITS		-D-------↓
B50/B4001 LS9 (37–45)	34	-D-------	↓	------V--	↔	------V--↓

Lowercase underlined letters indicate the dominant amino acid sequences showing mixtures of nucleotides. Magnitude, magnitude of CD8 T cell response; ↓, decreasing; ↔, persistent/no difference.

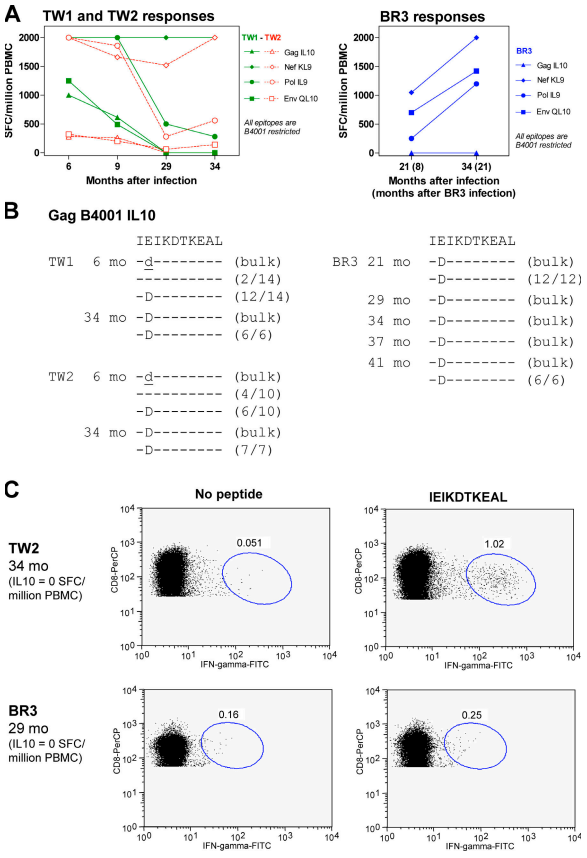

Figure 6. Comparative antigen-specific T cell responses and viral evolution between the twins and their brother. (A) Magnitude of the B*4001-restricted CD8 T cell responses over time. (left) Solid line, TW1; dotted line, TW2; (right) BR3. (B) Bulk and clonal sequence data of the autologous virus in all three brothers at designated time points for the Gag B4001 IL10 epitope. Lowercase underlined letters indicate the dominant amino acid in population-based sequences showing mixtures of nucleotides. (C) Intracellular cytokine staining using in vitro–grown, peptide-stimulated CD8 T cell lines and a synthetic peptide as antigen. For TW2, the CD8 line was grown from frozen PBMCs at month 34. For BR3, the CD8 line was grown from frozen PBMCs at month 29 (16 mo after infection). The magnitude of the ex vivo Gag B4001 IL10 response was 0 SFCs/million PBMCs for both at the mentioned time point.

levels of neutralizing antibody to the early autologous virus were found at 6 mo after infection for the three individuals. Neutralization antibody titers to the early autologous virus reached a peak (inhibitory concentration of >1,000-fold dilution) 34 mo after infection (Fig. 7) and were highest with the first virus and decreased with each subsequent virus. Both twins had a similar profile of neutralization to their own viruses at every time point tested. Notably, cross-neutralization assays revealed the twin's plasma to have a similar profile of neutralization to each other's viruses, in marked contrast to the low cross-neutralization between closely related viral strains in studies reported to date (20, 21). Moreover, BR3 did not develop potent cross-neutralization antibodies to twins' isolates or to control viruses.

It has been recently shown that neutralizing antibody responses play a pivotal role in the diversification of HIV-1 envelope during early stages of infection (22). Therefore, we compared the patterns of clonal evolution of the *env* gene between the three siblings. We estimated average pairwise *dN* and *dS* between the viruses isolated at the first available sample and subsequent viral samples (Fig. 8). The rate of nonsynonymous divergence averaged over the study period was higher than the synonymous divergence rate in the three patients (1.31 ± 0.24, 1.24 ± 0.16, and 1.54 ± 0.38 for TW1, TW2, and BR3, respectively). The amino acid sites subject to substantial selective pressure were clustered in the five variable regions of gp120 and at few positions in gp41, specifically two positions in membrane-proximal regions, and four positions in the cytoplasmic tail (unpublished data). The patterns of glycosylation for the different variable regions of the viral envelope were relatively stable during the first two years of infection and similar among the three siblings (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20052116/DC1). Finally, we looked for changes in the length of the variable regions in *env*. Although the twins showed similar patterns of deletions over time in V1 and V4 and insertions in V2 and V5, most of them happening earlier in TW2, BR3 showed little length variation over time (Fig. S2). These data indicate that both viral evolution and humoral immune response evolution are strikingly similar within the same host genetic background, suggesting that there are restrictions governing adaptive neutralizing immune responses and pathways to immune escape from these responses.

DISCUSSION

We present evidence that despite extensive HIV sequence variation within and among infected persons, there is predictability not only in the viral epitopes targeted but also in subsequent HIV evolution under immune selection pressure in the same genetic background after acute infection. These monozygotic twins infected simultaneously with the same virus experienced a similar clinical course, as well as marked similarity in breadth and magnitude of CD8 T cell responses and neutralizing antibody responses. Moreover, viral evolution within regions targeted by both cellular and humoral immune responses demonstrated a striking degree of similarity. Together these data infer substantial constraints on viral evolution under adaptive humoral and cellular immune selection pressure.

The similarity in early viral evolution under adaptive immune selection pressure was observed for both T cell and antibody responses. For each twin, 15 of 16 initially detected immune responses were identical. The early immunodominant T cell responses were also identical, and for the four initial CD8 T cell responses that declined, indicating effective immune escape, three occurred with mutation at the same residue and two because of the same amino acid substitution. Early neutralizing antibody responses likewise revealed similar patterns of evolution of the *env* gene and

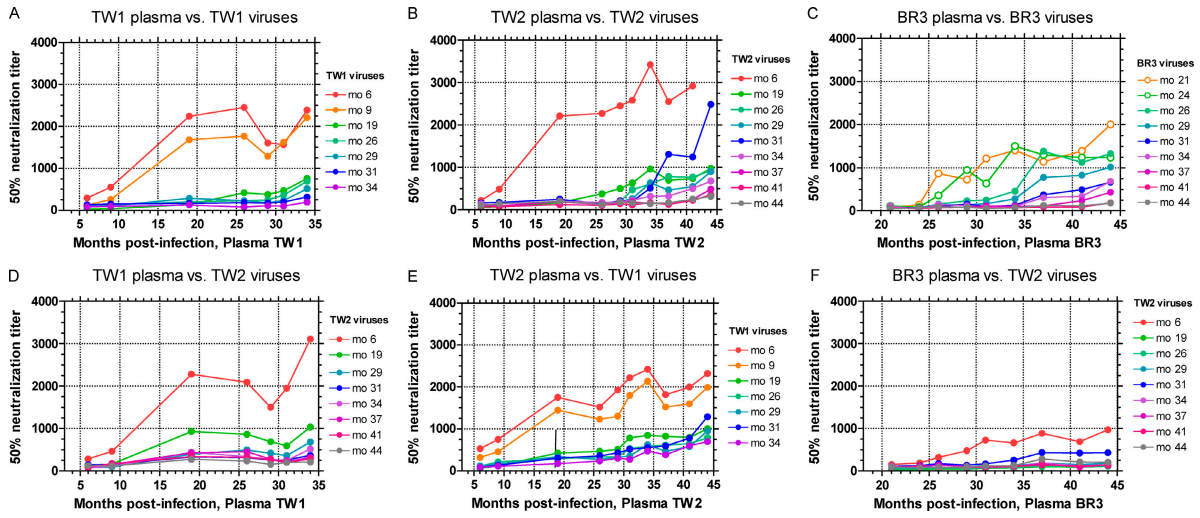

Figure 7. Antibody-neutralizing titers of TW1, TW2, and BR3 plasma against autologous and heterologous viruses. The autologous neutralizing antibody responses are displayed for TW1 (A), TW2 (B), and BR3 (C). The heterologous neutralizing antibody responses are displayed for TW1 plasma samples against TW2 viruses (D), for TW2 plasma samples against TW1 viruses (E), and for BR3 plasma against TW2 viruses (F). Antibody-neutralizing titers of BR3 plasma against TW1 viruses were similar to TW2 viruses. All time points refer to the twins infection; BR3 was infected with the twins' virus ~13 mo later. (C) Open circles indicate samples that were available only for BR3.

occurred similarly in both twins' viruses (21, 23). The continuous ongoing accumulation of amino acid substitutions was probably the main component driving diversification of envelope to allow escape from neutralizing antibodies (22). However, changes in the length of variable loops that bind antibodies and glycosylation patterns may also play a secondary role. Thus, even for the highly variable *env* gene, the data suggest that there are substantial constraints on evolution under immune selection pressure.

Although there have been other instances of HIV infection occurring in siblings, data have been lacking that have simultaneously examined immune responses and viral evolution in the context of initially identical host and viral genetics early in infection. HLA identical triplets infected by the same blood pack on the first day of their lives (24) were shown to exhibit remarkably uniform clinical manifestations with a very similar decline in CD4 counts and the same opportunistic infections occurring at approximately the same age; unfortunately, adaptive immune responses were not characterized. The importance of examining events after acute infection is suggested by the discordant responses observed 10 yr after infection in a study of selected CD8 T cell responses in hemophiliac brothers (2 yr apart in age) with the same class I A, B, and C alleles who were presumably infected by the same batch of HIV-contaminated factor VIII concentrate (8) and in identical twins infected by a blood pack within the first days of their lives whose CTL responses were studied 16 yr after infection using expanded CD8 lines (10). In both of those later stage studies, CD8 T cell responses differed substantially. In contrast, the current study showed remarkable similarity at the earliest time point after acute infection, but more discordance over time. Using the most comprehensive methodological approach available to date, we demonstrate that the overall CTL response in acute infection is almost the same. This is true not only for the immunodominant but also for the subdominant epitopes. We therefore conclude that CTL responses are determined by HLA and viral sequence despite the fact that the rearrangement of the TCR is a stochastically determined event. Moreover, the present data show concordance not only for the CD8 T cell response but also for the antibody response.

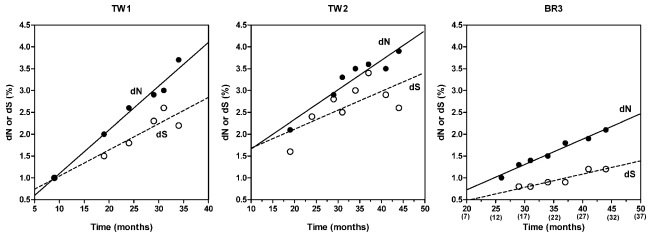

Figure 8. Accumulation of amino acid substitutions drives diversification of envelope to allow escape from neutralizing antibodies. Nonsynonymous (*dN*) and synonymous (*dS*) divergence from baseline virus (in percent) over time, averaged over the whole envelope sequence. Numbers in brackets refer to the estimated time of infection of BR3.

The current study also provides insights into immunodominance in the setting of the same genetic background. Both twins showed the same pattern of immunodominance, and in neither twin did a late arising response ever become dominant. That there is something intrinsic to the particular epitope and HLA allele that confers immunodominance is suggested by the data from BR3 (who shares half the genetic background and half of the HLA alleles with the twins), in whom HLA-B*4001 KL9 was the immunodominant epitope and two additional B*4001 epitopes were the same as those

recognized by the twins. Reasons for immunodominance of specific epitopes remain to be defined, but our data are in concordance with studies in SIV infection in macaques and acute HIV infection in humans (25–28). The existing HLA class I allele is an important predictor for the T cell response and immunodominance. Although immunodominance per se was not examined with respect to the neutralizing antibody responses, the fact that the twins were able to cross-neutralize each other's viruses and that sequence evolution was similar suggest that there may also be predictability to humoral immunodominance.

Partial immune selection pressure leading to predictable amino acid substitutions is perhaps not unanticipated given what has been reported in humans as a consequence of partial antiviral drug selection pressure with certain antiviral drugs. The predictability of immune escape is also suggested by studies showing allele-specific polymorphisms in chronic infection (29, 30). The development of identical escape mutations in the same epitope has been described, e.g., the HLA-B57 restricted epitope ISPRTLNAW (31, 32) and the HLA-A24 restricted epitope KYKLKHIVW (17). On the other hand, there are clearly some epitopes that have multiple pathways to immune escape. Examples are the Tat epitope STPESANL in SIV infection, which escapes very early in infection (25), and the Gag epitopes KRWIIMGLMK (33, 34), SLYNVATL (16, 17), and TSTLQEQIAW in HIV infection (31, 35).

If any randomly selected mutation can confer a selective advantage to the virus, such as nonrecognition by a CTL response, the corresponding quasispecies will overtake the others, following a Darwinian selection process. The rapidity of this process depends on the level of the selective advantage conferred by the mutation, the prevalence of the mutant within the virus population, and the operating pressure of the host immune response. This can therefore explain why an escape mutation arises earlier in one twin and later in the other.

This study also provides additional evidence for transmission of viruses containing CD8 T cell escape mutations, in this case into an HLA-similar recipient without reversion and the failure of the recipient to mount a CD8 T cell response toward this epitope, as previously reported for transmission of an escape mutation in an HLA-B27 restricted epitope from the mother to the child (36). Clearly there are differences in stability of escape mutations: a specifically engineered SIV strain containing escape mutations for three dominant epitopes was preserved when HLA-similar animals were infected (37), whereas mutation in a dominant A3 restricted HIV epitope transmitted into an HLA-A3–positive recipient reverted back beginning 482 d after infection. In this case, the subject mounted a response toward the A3 epitope after the wild-type sequence reappeared (38). We were unable to detect reversion in BR3, but our follow-up period consisted of only 20 mo. It is likely that escape mutations will need to be studied individually to define those with a propensity for reversion and those that become stable, both of which have implications for HIV immunogen design.

In conclusion, monozygotic twins infected with the same virus showed considerable concordance of humoral and cellular immune responses and HIV evolution during the critical early phase of infection. The results, compared with a third brother who was acutely infected with the twins' virus ~13 mo later, indicate a striking degree of concordance in immune selection pressure and viral evolution in HIV infection. The definition of preferred pathways to immune escape under dominant immune selection pressure provides important information for immunogen design that may allow one to immunize not only against the prevalent strains, but also against what HIV will evolve to become under immune selection pressure.

MATERIALS AND METHODS

Study subjects. Subjects TW1 and TW2 are identical twins 21 yr old at the time of HIV infection through injection drug use. Both had a negative HIV-1 ELISA in December 1998 and a seropositive result in September 1999. Both shared needles with the same HIV+ person at the same time and no other persons. BR3 is a third brother, 3 yr older than the twins, who shared needles with them. His HIV-1 ELISA was negative in December 1999 and positive in November 2000. All three were hepatitis C virus coinfected. None was treated with antiretroviral therapy and none experienced an opportunistic infection during the study period. All three gave informed consent for participation in this study. This study was approved by the Hospital Universitari Germans Trias i Pujol review board on biomedical research. Pertinent laboratory data are outlined in Fig. 1 B.

Zygosity assay and HLA tissue typing. Zygotic identity of the twins was assessed by analysis of polymorphic short tandem repeat loci by using the AmpFlSTR profiler PCR amplification kit (Applied Biosystems; reference 39). Analysis of the PCR products was performed on the genetic analyzer. Fragment size determination was done with GeneScan v3.7 (Applied Biosystems). Genotyper v3.7 was used for allele designation with respect to an allelic ladder. HLA tissue typing was performed by high resolution sequencing for HLA-A and -B. HLA-C was typed by serology. The twins' HLA class I genotype was as follows: A0201/2402; B4001/5001; Cw03/04. BR3's HLA class I genotype (A0201/0201; B4001/44; Cw03/05) matched at a single allele at each of the A, B, and C loci.

Isolation of PBMCs and generation of CD8 lines and BLCLs. Antigen-specific CD8 T cell lines were generated from cryopreserved PBMCs stimulated with 40 μg of synthetic peptide in the presence of 20 million irradiated feeder PBMCs in R10 medium (RPMI 1640 [Sigma-Aldrich] supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM Hepes, and 10% heat-inactivated FCS [Sigma-Aldrich]). The following day, 50 U/ml of recombinant interleukin-2 was added. EBV-transformed B lymphoblastoid cell lines (BLCLs) were established as described previously (40).

Peptides. 410 peptides (15−19 amino acids long and 10–amino acid overlap; consensus sequence clade B 2001 [http://hiv-web.lanl.gov]) spanning all expressed HIV proteins were synthesized on an automated peptide synthesizer (MBS 396; Advanced Chemtech) using fluorenylmethoxycarbonyl chemistry. Peptide truncations for epitope mapping and peptides corresponding to the known optimal epitopes for the subjects' HLA alleles were also synthesized. Peptides were used at 14 μg/ml for screening purposes and 140 to 0.00014 μg/ml for titration assays.

Elispot assay. HIV-1–specific CD8 T cell responses were quantified by IFN-γ Elispot assay using fresh or frozen PBMCs ($0.5–1 \times 10^5$ per well), as described previously (41). The incubation period was 14 to 16 h. IFN-γ–producing cells were counted by direct visualization and are expressed as spot-forming cells (SFCs) per 10^6 PBMCs. Negative controls were always

<30 SFCs per 10^6 input cells. The positive controls consisted of incubation of PBMCs with phytohemagglutinin. Wells were counted as positive if they were at least 50 SFCs/10^6 PBMCs and at least three times above background (12). We chose an upper cut-off of 200 spots per well, i.e., 2,000 SFCs/10^6 PBMCs when adding 10^5 PBMCs per well and 4,000 SFCs/10^6 PBMCs when adding 10^5 PBMCs per well. Responses to adjacent overlapping peptides were counted as responses to one epitopic region because some T cell epitopes can be located in the overlapping region of two adjacent peptides, resulting in responses to both overlapping peptides (42).

Fine mapping of epitopes by Elispot assay was performed as described (43), using peptide truncations. In brief, $0.5–1 \times 10^5$ effectors/well were incubated with concentrations of peptides ranging from 140 to 0.00014 μg/ml overnight on the Elispot plate. All assays were run in duplicate. The optimal peptide was defined as the peptide that induced 50% maximal specific IFN-γ production of T cells at the lowest peptide concentration.

Intracellular cytokine staining for IFN-γ production. For determination of HLA class I restriction of CD8 T cell responses, intracellular cytokine staining assays using autologous or HLA partly matched BLCLs were performed as described elsewhere with minor modifications (43). In brief, BLCLs were pulsed with 2 μg of peptide for 1 h and washed three times. They were then incubated with effector cells (5×10^5 cells of CD8 line and 5×10^4 BLCLs; E/T = 10:1) in FACS tubes and 1 μg/ml of the costimulatory mAbs anti-CD28 and anti-CD49d (BD Biosciences) at 37°C and 5% CO_2 for 1 h before adding 10 μg/ml Brefeldin A (Sigma-Aldrich). After a 6-h incubation, the cells were washed with PBS/1% FCS and stained with surface antibodies anti–CD8-PerCP and anti–CD4-APC (BD Biosciences) at 4°C for 30 min. After washing again, PBMCs were fixed using Fixation/Permeabilization kit A (Caltag Laboratories) and placed at 4°C overnight. Cells were permeabilized using Fixation/Permeabilization kit B (Caltag Laboratories) and anti–IFN-γ–FITC (BD Biosciences) was added. Cells were analyzed on a FACS Calibur Flow Cytometer (BD Biosciences). For negative controls, cells were incubated with BLCLs without peptide, but were treated otherwise identically. A positive response was defined as threefold above background.

Neutralizing antibody responses. Neutralizing antibody titers were determined using the ViroLogic Phenosense Entry Assay that measures inhibition of a recombinant virus in a single replication cycle assay (20). Coreceptor tropism was determined by measuring the ability of recombinant viruses to infect U87 CD4/CXCR4 or CD4/CCR5 cell lines and verified by inhibition of infection by specific coreceptor antagonists.

Sequencing of viral RNA or proviral DNA. Viral RNA was extracted from the subjects' plasma using the QIAamp RNA viral mini kit (QIAGEN) or viral DNA was extracted from PBMCs using the Puregene DNA isolation kit (Gentra) according to the manufacturer's protocol. Viral RNA was transcribed into cDNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and the sequence-specific primers as described (44). DNA was amplified using nested PCR and primers as described (44). PCR cycling conditions were as follows: 94°C for 2 min, 35–40 cycles of 30 s at 94°C, 30 s at 56°C, 1.5 min at 72°C, and a final extension of 68°C for 20 min. In the nested PCR reaction, the extension time was shortened to 1 min. Regions of interest were cloned: PCR fragments were gel purified (QIAquick gel extraction kit; QIAGEN) and cloned using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was isolated using QiaPrep Turbo Miniprep (QIAGEN). PCR products or plasmid DNA were sequenced bidirectionally by the Sequencing Core Facility at Massachusetts General Hospital (Boston, MA). Sequencher 4.1 (Gene Codes Corporation) was used to edit and align sequences.

Sequence analysis. HIV-1 RNA was extracted from plasma at different time points during follow up. Each subject's virus isolates were processed in totally independent batches to avoid cross-contamination. *Pol* (protease and the first 235 codons of the reverse transcriptase) and *env* (C2 to V5 regions) (45) fragments were reverse transcribed, PCR-amplified, and bidirectionally sequenced on a 3100 automatic sequencer (Applied Biosystems). In addition, a total of 61 molecular clones encompassing the *env* gene were characterized for the three siblings at different time points. Sequence alignments were obtained using ClustalW (46) and manually edited in the regions of variable length in particular to suppress the gaps within *N*-glycosylation sites (identified using an NXYX motif where X is any amino acid except proline and Y is either serine or threonine). Maximum likelihood phylogenetic trees of each subject's *pol* and *env* sequences were constructed using PAUP* version 4.0b10 (47) under Modeltest 3.0 (48). The reliability of clustering in phylograms was assessed by bootstrapping analyses (49). Genetic distances and evolutionary rates were computed using a Kimura 2 parameter model in MEGA (50). The nonsynonymous and synonymous substitution rates were calculated using the modified method of Nei and Gojobori (51) that considers a transition/transversion ratio of 2, as implemented in MEGA (50).

Statistical analysis. Correlation analysis was done using linear regression analysis with Graph Pad Prism, Version 4.0.

Online supplemental material. The online supplemental material shows the T cell responses at the first and last study time points (Tables S1–S3), the residue conservation within escaping CD8 epitopes in the twins' viruses using clade B sequences (Table S4), the number of *N*-glycosylation sites over time for the variable loops of gp120, measured by using multiple clonal sequences (Fig. S1), and the length of the variable regions of the viral envelope (in amino acids) over time, measured by using multiple clonal sequences (Fig. S2). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20052116/DC1.

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