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Mutational escape from the CTL response represents a major driving force for viral diversification in HIV-1-infected adults, but escape during infancy has not been described previously. We studied the immune response of perinatally infected children to an epitope (B57-TW10) that is targeted early during acute HIV-1 infection in adults expressing HLA-B57 and rapidly mutates under selection pressure. Viral sequencing revealed the universal presence of escape mutations within TW10 among B57- and B5801-positive children. Mutations in TW10 and other B57-restricted epitopes arose early following perinatal infection of B57-positive children born to B57-negative mothers. Surprisingly, the majority of B57/5801-positive children exhibited a robust response to the TW10 escape variant while recognizing the wild-type epitope weakly or not at all. These data demonstrate that children, even during the first years of life, are able to mount functional immune responses of sufficient potency to drive immune escape. Moreover, our data suggest that the consequences of immune escape may differ during infancy because most children mount a strong variant-specific immune response following escape, which is rarely seen in adults. Taken together, these findings indicate that the developing immune system of children may exhibit greater plasticity in responding to a continually evolving chronic viral infection.


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epitope to T cells. Other mutations, such as those involving TCR contact residues, may allow the variant peptide to be processed and presented but compromise its recognition by T cells. A variant epitope of this latter variety could theoretically lead to priming of a second, variant-specific immune response. Although some cross-recognition of epitope variants is common (8), superior recognition of a mutated epitope following CTL escape has not been demonstrated.

Escape within targeted viral epitopes can provide strong evidence of effective immune-mediated selection pressure; indeed, the marked heterogeneity of HIV-1 is attributable in large part to the class I HLA-restricted CTL response (9). While CTL escape is common among HIV-infected adults, escape during infancy has not been described previously. Although HIV-specific CTL clones have been isolated from cord blood (10), several studies suggest that in general HIV-specific CTLs are later to emerge and lower in frequency among infants than adults (10–12). Moreover, HIV-specific CTL responses primed during infancy appear to be less durable in the absence of sustained antigenic exposure, because early antiviral therapy leads to the disappearance of these responses among infants but not adults (11). It remains to be determined whether infants are able to mount fully functional HIV-specific CTL responses that are capable of exerting immune selection pressure.

To determine the extent and consequences of viral escape during early childhood, we examined the immune response to a HIV-1 epitope (TW10-Gag; TSTLQEQIGW) that is known to be targeted very early by adult subjects expressing HLA-B57 (13), an allele associated with exceptionally low HIV-1 viral loads and delayed progression to AIDS (14). Escape from TW10 and other B57-restricted HIV-1 epitopes was observed to occur early during the course of perinatal HIV-1 infection, indicating that CTL responses

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generated during infancy are sufficiently potent to drive viral evolution. Moreover, we present evidence that the maturing immune system of children demonstrates greater plasticity in immune recognition and is able to mount a strong response to a variant epitope following CTL escape, which is not observed among adults in whom these same variants arise.

Materials and Methods

Subjects
Perinatally HIV-1-infected children expressing HLA-B57 or the closely related B5801 allele were recruited at the Boston Medical Center and Children’s Hospital (Boston, MA), the National Cancer Institute (Bethesda, MD), and the Queen Elizabeth Hospital (Bridgetown, Barbados). Biological parents, when available, were also approached to consent for blood sampling. Additional HIV-1-infected adult control subjects were recruited at Massachusetts General Hospital and the Royal Perth Hospital. Additional plasma samples were obtained for viral sequencing from HIV-infected children in the Hemophilia Growth and Development Study (CH-15 to CH-20). This study was approved by the Institutional Review Board at all participating institutions, and all subjects and/or legal guardians signed written informed consent before participation.

Class I HLA typing
Class I HLA typing was performed by DNA PCR with sequence-specific primers (Dynal Biotech). HIV-1 peptides were synthesized on an automated peptide synthesizer using Fmoc chemistry at the Peptide Core Facility at the Massachusetts General Hospital.

ELISPOT assays
Fresh PBMC were plated in R10 medium at 100,000 cells/well with peptides at concentrations ranging from 10^{-4} to 10^{-10} M. Plates were incubated overnight and processed by standard methods (15). Individual IFN-γ-secreting cells were counted using the AID Elispot Reader System (Cell Technology). Results were calculated as the number of spot-forming cells per million input cells after subtraction of background.

Sequencing of HIV-1 RNA
Sequencing of HIV-1 RNA was performed from plasma following amplification of gag and nef by nested PCR (primers and conditions previously described) (15). PCR products of the secondary reaction were purified and sequenced directly on an ABI 3100 DNA Analyzer from Applied Biosystems. In addition, clonal sequence analysis was performed on longitudinal plasma samples from mother-child pairs CH-05M-05 and CH-06/M-06 using the Topo-TA vector system (Invitrogen Life Technologies).

Peptide-specific clones
Peptide-specific clones were generated as described previously (16). Briefly, PBMC were plated at limiting dilution with allogeneic feeder cells and the anti-CD3 Ab 12F6 in the presence of IL-2. Wells were screened for specific recognition of HLA-matched, peptide-pulsed. 51Cr-labeled B cell targets after 21 days in culture, and cells showing high specific recognition of the relevant peptide were then transferred to 24-well plates and restimulated with 10^6 feeder cells, 12F6, and IL-2. Expanded wells were retested for lytic activity after 14 days by 51Cr release assay using HLA-matched B cell line targets.

Statistical analysis
Statistical analyses were performed using Stata Statistical Software, Release 8.0 (StataCorp). Categorical variables were compared using Fisher’s exact test. All tests were two-tailed, with p < 0.05 considered significant.

Results

Differential recognition of Gag epitope TW10 by adults and children
Among B57^- adult children with primary HIV-1 infection, the Gag epitope TSTLQEQIGW (TW10) dominates the early CTL response (13), resulting in nearly universal escape during the early stages of infection (17). To determine whether similar immune selection pressure is present in perinatally HIV-1-infected children, we assessed recognition of TW10 and other B57^- restricted HIV-1 epitopes in an IFN-γ ELISPOT assay among 14 children and 34 adults expressing HLA-B57 or the closely related B5801 allele, which presents many of the same epitopes, including TW10 (18). The TW10 epitope was recognized by 83% of acutely HIV-1-infected adults (n = 12) and 50% of chronically infected adults (n = 22), consistent with a waning response following escape during the acute phase of infection. However, recognition of this epitope was much less frequent among pediatric subjects, with only 7% of children (n = 14), demonstrating an IFN-γ response (Fig. 1; p = 0.0005). Recognition of the other B57^-restricted HIV-1 epitopes listed in the Los Alamos HIV Immunology Database (www.hiv.lanl.gov) did not statistically differ between children and adults in this cohort (Fig. 2).

Universal presence of escape mutations within TW10 among children expressing HLA-B57 or B5801
To determine whether sequence variation within TW10 contributes to its infrequent recognition by children, we sequenced viral isolates from the 14 pediatric subjects referenced above and 7 additional B57/5801-positive children from whom plasma was available. All 21 children demonstrated mutations in TW10, principally at positions 3 and 9 within the epitope (Gag HXB2 residues 242 and 248), where sequence variation has been shown to be strongly associated with expression of HLA-B57 or 5801 (17) (Table I). In particular, the threonine-to-asparagine substitution at position 3 (T242N), present in 18 of 21 children, is exceedingly rare among chronically HIV-1-infected adults who lack these alleles (0 of 187 subjects) (17). Therefore, the frequent presence of these mutations in our pediatric cohort provides strong evidence of B57^-mediated immune selection pressure.
Because the presence of B57-associated escape mutations in perinatally HIV-1-infected children could result either from selection pressure mediated by the child’s own immune system or the transmission of an escape mutation selected for in a B57/5801-positive mother (19), HLA typing was performed on available parents to determine whether the transmitting mother also expressed HLA-B57 or B5801 (Table I). In 6 of the 11 subjects for whom HLA inheritance could be determined, the B57/5801 allele was inherited paternally. Plasma from five of the six B57/5801-negative mothers was available for HIV-1 sequencing; no escape mutations within the TW10 epitope were observed in any of these mothers. The sixth B57/5801-negative mother was deceased, but in this case, the HLA-B57 allele was inherited from a father who was HIV-negative, and it is therefore highly unlikely that B57-associated mutations would have been transmitted to the child either directly or indirectly. Therefore, it can be inferred that the TW10 mutations among children who inherited B57 paternally arose subsequent to vertical transmission because of selection pressure exerted by the child’s own immune response.

**Frequent early escape from B57-restricted epitopes during infancy**

Although an ex vivo response to TW10 could be demonstrated in only one child, the universal presence of TW10 escape mutations in children born to B57/5801-negative mothers suggested that a CTL response to this epitope was present in these children at an earlier point in time and drove viral escape. To establish the existence of a memory response to the original TW10 epitope in one such child, we sought CTL clones specific for the wild-type TW10 epitope in the 4-year-old subject CH-05, who developed two escape mutations (T242N and G248A) within TW10 before 24 mo of age, following vertical transmission of the wild-type epitope from his B57-negative mother. We derived seven CTL clones from this child that were specific for the wild-type TW10 epitope and only weakly cross-reactive against the autologous N3A9 variant (Fig. 3A). Of note, PBMC obtained from this child at the same time point failed to recognize the wild-type TW10 epitope, but a response to the autologous N3A9 variant was readily demonstrable ex vivo (Fig. 3B). The successful derivation of CTL clones specific for the wild-type TW10 epitope in this subject demonstrates that a memory CTL population specific for the original wild-type TW10 epitope persists at low frequency despite the occurrence of CTL escape.

**FIGURE 3.** Memory response to wild-type TW10 epitope demonstrated by T cell cloning. **A**, A CTL clone derived from CH-05 at 4 years of age preferentially recognizes the wild-type TW10 epitope over the autologous N3A9 variant in a $^{51}$Cr release assay. This titration curve is representative of seven clones specific for the wild-type epitope. **B**, PBMC obtained at the same time point from this subject recognize the autologous TW10 variant N3A9, but no response to the wild-type TW10 epitope is demonstrable ex vivo (bars indicate SEM).
To determine the extent and timing of CTL escape from B57 epitopes during early childhood, we performed longitudinal sequencing of HIV-1 RNA from the two youngest members of our cohort (CH-05 and CH-06), both of whom were born to B57-negative mothers and HIV-negative fathers (Table II). Sequence variation within TW10 and two other B57-restricted epitopes, ISW9-Gag and HW9-Nef, had emerged in both children by 24 mo of age. Viral isolates from the mothers of both children demonstrated wild-type sequence in all three of these epitopes, confirming that the mutations arose under selection pressure in the children following vertical transmission. These data offer compelling evidence of an effective CTL immune response that is sufficient to drive viral escape during infancy among children expressing HLA-B57.

**Recognition of variant epitopes following HIV-1 escape in adults and children**

To follow up on the unexpected observation of an ex vivo CTL response to the autologous N3A9 variant following TW10 escape in subject CH-05 (Fig. 2), we assessed the ability of CTL from other B57/5801-positive children to recognize TW10 epitope variants following escape. Recognition of the wild-type TW10 epitope, the autologous escape variant, and other commonly occurring variants were compared in an IFN-γ ELISPOT assay in all children from whom sufficient cells were available (subjects CH-01 to CH-13). Surprisingly, most children (9 of 13) demonstrated a robust response to the autologous variant epitope while recognizing the wild-type TW10 epitope weakly or not at all (Fig. 4). In several cases these variant-specific T cells were of very high frequency, representing one of the two immunodominant B57- or B5801-restricted responses in subjects CH-04, -06, -09, and -13. In all but the youngest child, the autologous variant was recognized with higher avidity than all other TW10 variants tested. This child (CH-06) instead preferentially recognized the variant bearing a single position 9 substitution (G248A) that arose at 14 mo of age, although he has since developed an additional mutation at position 3 (T242N). All four subjects who lacked a variant-specific response also failed to recognize the wild-type TW10 epitope; two of these subjects had nondetectable viremia on HAART3 and weak overall HIV-1-specific CTL activity. Of note, strong recognition of the autologous TW10 variant was observed in the majority of children who inherited the B57/5801 allele paternally (four of six), as well as those who inherited the allele maternally. Therefore, strong variant-specific responses are not merely due to priming by maternally transmitted escape variants but are also generated in children who were infected originally with the wild-type TW10 epitope and subsequently escaped.

To determine whether frequent recognition of escape variants is characteristic of all B57/5801-positive individuals or unique to children, we performed a similar comparison of wild-type and variant TW10 epitope recognition among 22 chronically HIV-1-infected adults expressing these alleles. Mutations within TW10 were present in all subjects from whom the gag sequence could be determined (n = 17). Among these 22 adults, 10 had no response to either TW10 or the autologous variant, whereas 10 recognized the wild-type TW10 epitope exclusively or more strongly than the variant peptides (Fig. 5). These wild-type responses were generally of low magnitude (median 210 spot-forming cells per million input PBMC), and their avidity was comparable to that of the variant-specific responses seen in children. Only 2 of the 22 adult subjects showed superior recognition of the autologous TW10 variant. Therefore, while recognition of escape variants is not unique to children, pediatric subjects appear to be preferentially able to mount such responses compared with adults (73 vs 9.1%, p = 0.0004).

**Higher frequency of multiple escape mutations within TW10 among children**

The spectrum of TW10 escape mutations observed among adults subjects resembled that of our pediatric cohort, with T242N and/or G248A representing the most common variants. However, a higher degree of sequence variation was observed within TW10 in children compared with adults, based on an expanded analysis of 103 published (17, 20) and unpublished sequences from B57/5801-positive adults infected with clade B HIV-1 (Fig. 6). Selection has driven more than two nonsynonymous nucleotide substitutions in a significantly higher proportion of children (6 of 21) than adults (10 of 103; p = 0.03), resulting in either triple amino acid substitutions or more complex substitutions such as G248T and G248Q, which require multiple nucleotide changes. The higher degree of escape within the TW10 epitope in pediatric infection is consistent with the observation that variant-specific CTL are generated in children, which are themselves capable of driving further escape.

**Discussion**

The universal presence of TW10 escape mutations among B57/5801-positive children in this study confirms the existence of strong immune selection pressure on this epitope. There are two possible scenarios by which these mutations may have arisen, both of which are likely to have occurred in our pediatric cohort. First, in those cases where the child’s mother also expressed HLA-B57/5801, variant TW10 epitopes are likely to have been transmitted vertically at birth, given the extremely high prevalence of these mutations in B57-positive adults (17). In this subset of children, the TW10 variants represent “neo-epitopes” arising in response to maternal selection pressure and subsequently recognized at high

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3 Abbreviation used in this paper: HAART, highly active antiretroviral therapy.
frequency by their offspring (21). Such neo-epitopes may undergo additional CTL-driven escape following transmission, which could explain the greater degree of TW10 nucleotide variation observed in children relative to adults. Given the high rate of HIV-1 viral evolution, such neo-epitopes may be arising with great frequency in the circulating HIV-1 population, although studies in the SIV/macaque model suggest that many transmitted escape mutations are not immunogenic (21). Among the remaining children who inherited B57/5801 paternally, the wild-type TW10 epitope was transmitted at birth and escaped during infancy. The presence of strong CTL responses to the autologous TW10 variant in such subjects indicates that, unlike adults, most children are able to mount an immune response to the viral variant following escape. Taken together, these data indicate that children are not only able to mount functional immune responses of sufficient potency to exert selection pressure on the virus and drive immune escape, but they also exhibit greater plasticity in immune recognition of the evolving virus.

Mutational escape from an established CTL response is of lasting advantage to the virus only if the host is subsequently unable to mount an efficient immune response to the variant epitope. In this regard, it is important to consider why adult subjects are generally unable to respond to variant epitopes following escape, despite the proven immunogenicity of these variants in children. One possible explanation is that a phenomenon akin to “original antigenic sin,” originally described in the humoral response to influenza, may be at play in the cellular immune response (22, 23). Extension of the original antigenic sin paradigm to CTLs was first suggested in studies of lymphocytic choriomeningitis virus-infected mice, in which an existing CTL response appeared to limit the ability of the immune system to recognize a closely related variant epitope (22). More recently, a similar phenomenon was described in the response to recurrent dengue viral infection of humans (23). If human CTLs are indeed constrained in their ability to recognize emerging viral variants, this would have important implications for the immune response to a continually evolving chronic viral infection such as HIV-1. Although the mechanism of original antigenic sin in the CTL response is not clear, it has been suggested that the relative proportion of naive and memory cells may play a role (24) because the likelihood of a HLA-peptide complex encountering a naive T cell of appropriate specificity may be

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**FIGURE 4.** Children preferentially recognize the autologous TW10 variant. PBMC from three representative pediatric subjects recognize the autologous TW10 variant (solid line) with higher avidity than any other epitope variant (dotted lines), including the wild-type TW10 epitope (blue, recognized only by CH-09).

**FIGURE 5.** Recognition of escape variants by children and adults. Recognition of the wild-type TW10 epitope and the autologous viral variant were compared in 13 children and 22 adults expressing HLA-B57/5801. Preferential recognition of the autologous TW10 escape variant was observed in most children but was rare in adults.

**FIGURE 6.** B clade consensus TW10 epitope sequence and nonsynonymous nucleotide changes resulting in observed TW10 epitope variants. A greater proportion of children than adults was observed to have more than two nonsynonymous nucleotide changes within the TW10 epitope (6 of 21 vs 10 of 103; p = 0.03, Fisher’s exact test).
lower than the likelihood of encountering a weakly cross-reactive memory T cell. If this is the case, then original antigenic sin may be less apt to occur in children, who possess a relative abundance of naïve T cells. It should be noted that in our study, children were not only more likely than adults to recognize the escape variant but also less likely to demonstrate a persistent ex vivo response to wild-type epitope following escape. Nearly half of adult subjects continued to recognize the wild-type TW10 epitope after its disappearance from the plasma. It is possible that the escape variants act as altered peptide ligands, boosting the response to the original epitope via cross-recognition by wild-type TW10-specific CTLs, yet fail to sensitize cells for efficient lysis by CTL or even antagonize the wild-type-specific T cells (25). Such cross-reactive boosting may be fundamental to the mechanism of original antigenic sin (24).

The failure of TW10 escape mutations to back-revert in the presence of a variant-specific CTL response poses an interesting paradox. After the CTL population, which originally drove selection of the escape mutants, has declined to the extent that it is no longer detectable ex vivo and has been eclipsed by a variant-specific CTL response of much higher frequency, one might expect that it would be advantageous for the virus to revert to its original sequence. The factors governing the likelihood of reversion of escape mutations are likely to be multiple and complex (17) and include the impact on viral replicative fitness and the presence of compensatory mutations. A more direct explanation for the lack of reversion in the present case is suggested by the persistence in memory of a low-frequency T cell population specific for the wild-type epitope, confirmed by our ability to grow out a wild-type-specific CTL clone more than 2 years following escape. This memory CTL population might conduct surveillance and lyse revertant virus with high efficiency, despite the fact that it is of such low frequency that a response is not detectable ex vivo (21). In support of this hypothesis, recent studies in the macaque model have demonstrated that although engineered CTL escape mutants revert following transmission to a MHC-mismatched host, reversion fails to occur after inoculation into an animal who shares the restricting MHC allele, even though no response to the epitope can be demonstrated ex vivo (26). The recent demonstration that mutations within TW10 revert to wild-type upon transmission to a B57/B801-negative host (17) suggests that a similar force, presumably a low-frequency wild-type-specific CTL response, may prevent reversion of TW10 in these B57-positive children.

The potential implications of these data for vaccine design are mixed. On the positive side, these observations suggest that a strategy of dual vaccination with both an epitope and its common variants may mitigate the consequences of viral escape. Our data indicate that such a strategy may be more efficacious in children than adults. Moreover, therapeutic vaccination of HIV-infected children may be effective in inducing a variant-specific response that could avert positive selection of escape variants as they arise. On the negative side, the low degree of variant-specific recognition among adult subjects underscores the sobering possibility that successful induction of vaccine-specific responses to variable epitopes may do more harm than good if the vaccine-specific response interferes with recognition of variant epitopes present in circulating viral strains.

In conclusion, the present data demonstrate that escape from the CTL response is frequent during infancy among subjects expressing the HLA-B57 allele. Moreover, the developing immune system may display greater plasticity in recognizing viral variants as they arise. Such adaptability of the immune response may present a considerable advantage in the containment of a continually evolving chronic viral infection such as HIV-1. These observations may open the door to vaccine strategies involving simultaneous vaccination with both optimal HIV-1 epitopes and predicted escape variants, because original antigenic sin may be less of a barrier to this approach in children than in adults.

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Disclosures

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