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NOTES

Extraepitopic Compensatory Substitutions Partially Restore Fitness to Simian Immunodeficiency Virus Variants That Escape from an Immunodominant Cytotoxic-T-Lymphocyte Response

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Selection for escape mutant immunodeficiency viruses by cytotoxic T lymphocytes (CTL) has been well characterized and may be associated with disease progression. CTL epitopes accrue escape mutations at different rates in vivo. Interestingly, certain high-frequency CTL do not select for escape until the chronic phase of infection. Here we show that mutations conferring escape from immunodominant CTL directed against an epitope in the viral Gag protein are strongly associated with extraepitopic mutations in *gag* in vivo. The extraepitopic mutations partially restore in vitro replicative fitness of viruses bearing the escape mutations. Constraints on epitope sequences may therefore play a role in determining the rate of escape from CTL responses in vivo.

Cytotoxic-T-lymphocyte (CTL) responses are crucial to containment of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication (3, 8, 12, 13). Therefore, AIDS vaccine strategies currently being designed attempt to elicit CTL responses. Unfortunately, it is not clear how CTL responses elicited by vaccines should best be targeted. CTL commonly select for resistant viruses, both in HIV-infected humans and in SIV-infected macaques (1, 4–7, 9, 11, 14, 15, 17–19, 22). Indeed, strong CTL responses can select for escape variant viruses with extremely rapid kinetics, driving CTL-susceptible viral sequences to extinction within 4 weeks of infection (1, 17). However, some high-frequency CTL responses can persist throughout chronic infection but seldom, if ever, select for escape variants. This observation has led to the notion that frequency alone cannot accurately represent the strength of CTL responses, since only a subset of high-frequency CTL exert enough pressure to select for escape (10). Thus, efforts to develop CTL-based vaccines face a conundrum. It may be most beneficial to direct the CTL response against epitopes that escape slowly, since these epitopes are likely to persist in circulating virus strains. Conversely, rapid escape may be a biological indication of distinctly effective CTL responses.

A study of escape mutants selected in vitro has shown that CTL specific for an epitope in HIV-1 Nef, a nonstructural protein, selected for resistant viruses more rapidly than CTL directed against a Gag epitope (23). Moreover, a study of escape from CTL that recognized a different Gag epitope showed that escape mutations were selected for along with other mutations that did not contribute directly to immune evasion. The authors concluded that these mutations increased the fitness of escape variant viruses (9). However, while this is a likely explanation for selection for mutations that do not mediate escape, the authors did not test their hypothesis directly.

To understand more fully the constraints on viral escape from CTL responses, we analyzed evolution of the cloned, pathogenic virus SIVmac239 in rhesus macaques expressing the major histocompatibility complex (MHC) class I molecule Mamu-A*01 and tested the fitness of escape variants in an in vitro system. In the acute phase of SIVmac239 infection, high-frequency CTL are targeted toward two epitopes, $Tat_{28\text{-}35}$SL8 (STPESANL, Tat SL8) and $Gag_{181\text{-}189}$CM9 (CTPYDINQM, CM9), in Mamu-A*01-positive macaques (16). However, Tat SL8-specific CTL selected for escape during acute infection in all Mamu-A*01-positive animals ($n = 10$) in a previous study (1), while Gag CM9-specific CTL selected for escape in the chronic phase of infection in only 10 of 22 Mamu-A*01-positive animals whose viral sequences we analyzed for the present study (Fig. 1). We reasoned that one possibility for the lack of mutation in Gag CM9 epitope sequences was that the structural protein Gag was under greater functional constraints than the nonstructural protein Tat. While the structure of the SIV p27 capsid (CA) protein has not been solved, a previous

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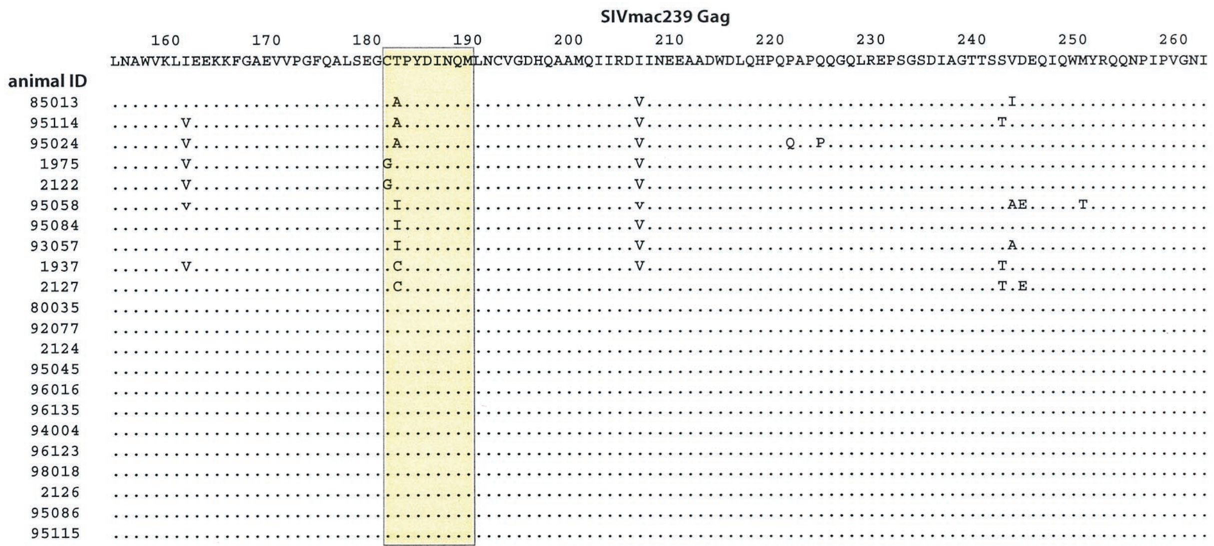

FIG. 1. Extraepitopic amino acid substitutions associated with escape from Gag CM9-specific CTL. vRNA was isolated from Mamu-A*01-positive macaques in the chronic phase of infection with SIVmac239. vRNA was reverse transcribed, and cDNA was amplified and directly sequenced. Predicted amino acid sequences are shown for a region of the open reading frame encoding Gag. Amino acid residue numbers are indicated above the reference sequence. The Gag CM9 epitope sequence is boxed in yellow. Lowercase letters indicate the amino acid encoded by the variant codon at sites of mixed-base nucleotide heterogeneity.

mutational analysis of the HIV-1 variant NL4-3 p24 CA protein showed that single amino acid replacements within the sequence analogous to SIVmac239 Gag CM9 greatly reduced or even eliminated infectivity (21). Another recently published report has shown that threonine 182 (residue 47 of p27 CA and position 2 of Gag CM9) is crucial for proper maturation of SIV virions, likely forming hydrogen bonds with aspartate 50 (20). These results strongly argue that the region of p27 CA that contains the Gag CM9 epitope is indeed under constraints and that escape from Gag CM9-specific CTL may require further mutations to compensate for the deleterious effects of escape on viral fitness.

Association of escape in Gag CM9 with extraepitopic mutations. To determine whether such compensatory substitutions were selected for in viruses escaping from immunodominant Gag CM9-specific CTL, we analyzed *gag* sequences of viruses isolated from Mamu-A*01-positive rhesus macaques during chronic infection with SIVmac239, as described previously (17). Strikingly, we found that two valine-for-isoleucine substitutions occurred at positions 161 and 206 of *gag* (I161V and I206V) only in animals that showed evidence of an escape mutation within the Gag CM9 epitope. The upstream substitution, I161V, was found in 6 of 10 macaques with mutated CM9 sequences, while the downstream substitution I206V was found in 9 of 10 (Fig. 1). Neither of these mutations was present in viruses isolated from 12 of 12 chronically infected macaques with intact Gag CM9 epitope sequences. Furthermore, several substitutions occurred in codons 242 to 250 in 6 of 10 animals with an escape variation in Gag CM9 but not in any of the animals whose viruses lacked an escape variation. The sequences surrounding Gag residues 161 and 206 do not contain known Mamu-A*01-restricted CTL epitopes or peptides that fit the described Mamu-A*01 binding motif. Moreover, the infected animals in this study all expressed Mamu-A*01 but are not known to share other MHC class I alleles. It is therefore unlikely that the observed extraepitopic mutations are selected for by CTL that recognize unidentified Mamu-A*01-restricted epitopes or novel epitopes bound by a single MHC class I molecule. However, all substitutions were significantly associated with Gag CM9 escape (I161V, $P < 0.005$; I206V, $P < 0.0001$; residues 242 to 250, $P < 0.005$; Fisher's exact test). Together, these data strongly suggested that the extraepitopic substitutions were selected for in association with Gag CM9 escape mutations.

Ontogeny of escape and associated mutations. It was possible to analyze viral sequences longitudinally for five animals with Gag CM9 escape virus. Strikingly, the pattern of viral evolution was recapitulated in each of the five animals. Mutations within the epitope emerged coincident with downstream mutations I206V or within codons 242 to 250, with the N-terminal I161V substitution appearing later (Fig. 2). In none of these samples could we detect Gag CM9 escape mutations without other changes in the Gag protein.

No growth in vitro of escape mutants without extraepitopic substitution I206V. To assess the contributions of extraepitopic substitutions to viral fitness, we constructed a panel of mutant SIVmac239 viruses bearing a common Gag CM9 escape mutation (alanine-for-threonine at codon 182, T182A) in various combinations with I161V and/or I206V. Since there was no consistent pattern of substitutions in codons 242 to 250 and because escape viruses from 4 of 10 animals had no mutations in this region, we did not target residues 242 to 250 in our mutagenesis strategy. Viruses were produced by transfection of Vero cells with plasmid DNA encoding wild-type SIVmac239 or epitope mutations. Viral stocks were amplified on CEMx174 cells and titrated for p27 antigen with a commercial enzyme-linked immunosorbent assay (Beckman-Coulter, Brea, Calif.). In repeated attempts, we failed to detect replication of

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                                              SIVmac239 Gag
                           160       170       180       190       200       210       220       230       240       250       260
animal ID  weeks p.i.  LNAWVKLIEEKKFGAEVVPGFQALSEGCTPYDINQMLNCVGDHQAAMQIIRDIINEEAADWDLQHPQPAPQQGQLREPSGSDIAGTTSSVDEQIQWMYRQQNPIPVGNI

95114      12 weeks    .............................................................................................................
           18 weeks    ............................A........................................................T....................
           28 weeks    ............................A.......................V...................................T....................
           53 weeks    .......V....................A.......................V...................................T....................

95024      35 weeks    .......v....................A.......................V..............q.........................................
           67 weeks    .......V....................A.......................V..............q.........................................
           85 weeks    .......V....................A.......................V..............Q..P......................................

95058      64 weeks    ............................I........................................................AE..................
           72 weeks    ............................I........................................................AE.....T............
           88 weeks    .......v....................I.......................v................................AE.....T............

1975       75 weeks    ...........................G........................v........................................................
           90 weeks    .......v...................G........................V........................................................
           100 weeks   .......V...................G........................V........................................................

1937       83 weeks    .............................................................................................................
           102 weeks   ............................S........................................................t....................
           137 weeks   ............................C.......................V...................................T....................
           167 weeks   .......V....................C.......................V...................................T....................
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FIG. 2. Ontogeny of Gag CM9 escape and associated substitutions. Predicted amino acid sequences are shown for the region surrounding the Gag CM9 epitope in five Mamu-A*01-positive animals infected with SIVmac239. Plasma virus RNA was isolated, reverse transcribed, and directly sequenced as described for Fig. 1. Lowercase letters indicate the amino acid encoded by the variant codon at sites of mixed-base nucleotide heterogeneity.

viruses containing the epitope substitution T182A alone or in combination with only the upstream substitution I161V after they had been in culture for more than 8 weeks. We did, however, produce stocks of escape mutant viruses harboring either both extraepitopic substitutions (I161V/T182A/I206V, SIV-VAV) or only the downstream substitution (T182A/I206V, SIV-AV). The mutant viruses did not induce CEMx174 cell syncytia until 3 weeks postinfection, whereas wild-type virus stocks caused peak cytopathic effects at 10 to 12 days postinfection. Genome sequencing of the mutant viruses showed that their *gag* genes harbored only the engineered substitutions. However, sites in other open reading frames displayed mixed base heterogeneity, two in SIV-AV (nucleotides 3,751 in *pol* and 9,916 in *nef*) and three in SIV-VAV (nucleotides 8,681 in *env* and 9,364 and 9,679 in *nef*). The latter virus also had one nucleotide replacement, at nucleotide 7,863 in *env*. While all the substitutions we detected were nonsynonymous, none introduced premature stop codons. Since the plasmids encoded only the desired mutations, it is likely that these substitutions represent stochastic changes that occurred during the amplification of the mutant virus stocks.

Extraepitopic substitution I206V increases fitness of Gag CM9 escape virus. For a direct comparison of viral replicative capacity, we infected 2 million phytohemagglutinin-stimulated primary macaque peripheral blood mononuclear cells with wild-type or mutant SIVmac239 alone or with mixtures of viruses totaling 10 ng of p27. Cultures were maintained in 25 cm^2 flasks and fed twice weekly. To assay viral replication, we sampled 1-ml aliquots of culture supernatant for accumulation of viral RNA (vRNA) by quantitative reverse-transcription (QRT)-PCR. We detected wild-type or mutant viruses in competition assays by using QRT-PCR with a sequence-specific primer directed against *gag* codon 182. Nonselective primers SIV-61F, 5′-CCACCTGCCATTAAGCCCGA-3′, and SIV-143R, 5′-CTGGCACTACTTCTGCTCCAAA-3′, were used to amplify total vRNA. Mutant virus sequences were detected specifically by using primer SIV-1680 3X short, 5′-ACATCTG ATTAATGTCATAGGGGGC-3′; the 3′-terminal nucleotide of this primer targets the alanine codon in mutants encoding T182A and does not amplify wild-type sequences in the cycling conditions used (data not shown).

In duplicate independent experiments, the wild-type virus outgrew the triple mutant SIV-VAV: after 7 days in culture, viral sequences were 95.5% wild type (average of two experiments) (Fig. 3). The wild-type virus also outgrew the double mutant SIV-AV, though to a lesser extent: sequences were 85.2% wild type at 7 days (average of two experiments). The results of these experiments were verified by direct sequencing of RT-PCR products derived from the same culture supernatants used in QRT-PCR (data not shown). We also used direct sequencing to track the composition of virus populations in cultures infected with mixed inocula containing equal amounts of the mutants SIV-VAV and SIV-AV, since a QRT-PCR assay to distinguish these two species was unavailable. After 7 days of infection, there appeared to be a nearly equal proportion of SIV-VAV and SIV-AV in duplicate experiments (data not shown). In infections of peripheral blood mononuclear cells with wild-type or mutant viruses alone, SIV-AV and SIV-VAV reached peak titers of 8 million copies/ml of supernatant, while wild-type SIVmac239 reached 13.6 million copies/ml. The mutant viruses also attained peak titers on day 5 (SIV-VAV) or 7 (SIV-AV) postinfection, while wild-type virus replication peaked at day 3. These results are a further indication that the replication capacity of the escape mutant viruses is reduced with respect to that of the wild type. We therefore conclude that, while replication capacity is restored to mutant viruses by extraepitopic substitutions, these mutants are still less fit than the wild type. Moreover, neither variant appears to have a clear fitness advantage over the other.

Our results suggest that evolution of CTL escape viruses

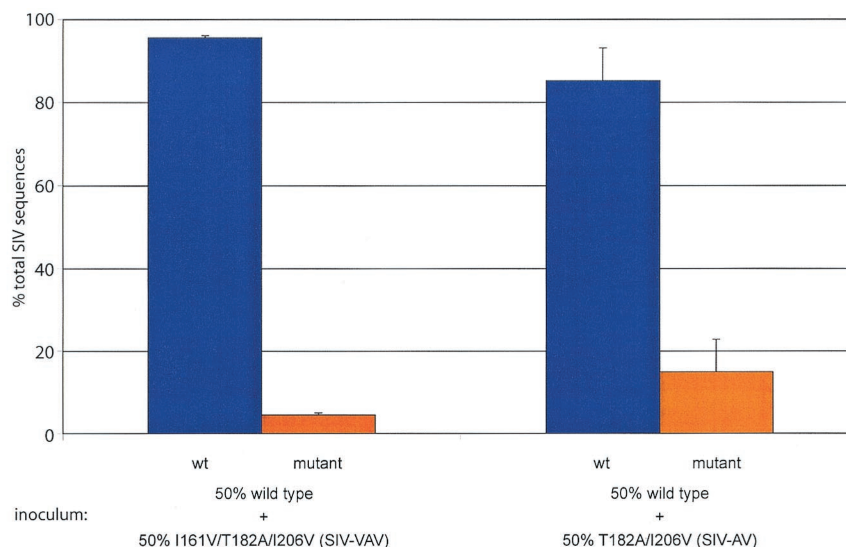

FIG. 3. Direct competition between wild-type SIV and variants reveals a fitness cost associated with escape from Gag CM9-specific CTL. Primary macaque lymphocytes were infected with mixtures of wild-type and epitope mutant SIV (5 ng of p27 for each virus). After the mixtures were cultured for 7 days, we quantified total vRNA and mutant vRNA in the culture supernatants by using QRT-PCR. The proportion of total viral sequences that were mutant could therefore be calculated for each sample (orange bars). The values shown are the averages of the results of duplicate independent experiments. Each error bar indicates the standard deviation from the average for each data point.

may be more complex than we have appreciated hitherto. Identification of mutations that compensate for a loss of fitness in escape variants is relatively straightforward when one can observe substitution of particular amino acids, in this case valine for isoleucine at two positions within the p27 CA protein, occurring reproducibly in multiple individuals in association with escape from an immunodominant CTL response. However, compensatory substitutions would be much harder to identify if several residues were tolerated at a compensatory site, if multiple sites could be involved in compensation, or if compensatory substitutions occurred in viral proteins other than the one recognized by selecting CTL. In our study, mutations in SIV Gag residues 242 to 250 are significantly associated with escape mutations within the Gag CM9 epitope, but there is no consistent pattern of substitutions that recurs in multiple animals. If these substitutions do in fact enhance the fitness of Gag CM9 escape viruses, they may represent the limit of our present ability to detect such compensatory mutations. There may also be other mutations that increase the fitness of viruses escaping from Gag CM9 CTL in vivo but are too difficult to detect by direct sequencing.

Furthermore, the requirement for extraepitopic substitutions to compensate for decreased fitness of SIV viruses escaping from Gag CM9-specific CTL highlights an emerging picture of the evolution of escape variants. It is now becoming clear that escape from many, if not most, CTL responses exacts a cost in viral fitness (23; Friedrich et al., submitted for publication). The outgrowth of viral escape mutants in vivo therefore likely depends on the balance of fitness benefits and costs associated with particular escape mutations. This cost-benefit relationship may help explain why even very-high-frequency CTL select for escape variants with differing kinetics.

It is likely that several factors play a role in determining the rate of viral escape from particular CTL responses. We have observed an association between high functional avidity of CTL and their ability to select for viral escape during acute infection (17). Others have suggested that CTL directed against epitopes derived from early proteins may be more effective at limiting the spread of infection, since they can recognize infected cells earlier in the viral life cycle (2). Indeed, the epitopes in which we have found evidence for escape during acute infection are clustered primarily in early and regulatory proteins (17). It is also possible that some epitope sequences are less tolerant of variation than others due to structural or functional constraints on the gene products of which they are a part. Mutations that confer the selective advantage of immune evasion may thus also come at a cost to viral replicative fitness. It therefore seems likely that the combination of factors affecting evolution of escape variants will be different for every CTL epitope. A detailed understanding of these mechanisms should help guide the conceptual design of CTL-based components of AIDS vaccines.

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REFERENCES

1. **Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins.** 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature **407:** 386–390.
2. **Baalen, C. A., C. Guillon, M. van Baalen, E. J. Verschuren, P. H. Boers, A. D. Osterhaus, and R. A. Gruters.** 2002. Impact of antigen expression kinetics on the effectiveness of HIV-specific cytotoxic T lymphocytes. Eur. J. Immunol. **32:**2644–2652.
3. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific $CD8^+$ cytotoxic T-lymphocyte activity associated with control

of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. **68:**6103–6110.

4. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. **3:**205–211.
5. **Evans, D. T., D. H. O'Connor, P. Jing, J. L. Dzuris, J. Sidney, J. da Silva, T. M. Allen, H. Horton, J. E. Venham, R. A. Rudersdorf, T. Vogel, C. D. Pauza, R. E. Bontrop, R. DeMars, A. Sette, A. L. Hughes, and D. I. Watkins.** 1999. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. Nat. Med. **5:**1270–1276.
6. **Goulder, P. J., C. Brander, Y. Tang, C. Tremblay, R. A. Colbert, M. M. Addo, E. S. Rosenberg, T. Nguyen, R. Allen, A. Trocha, M. Altfeld, S. He, M. Bunce, R. Funkhouser, S. I. Pelton, S. K. Burchett, K. McIntosh, B. T. Korber, and B. D. Walker.** 2001. Evolution and transmission of stable CTL escape mutations in HIV infection. Nature **412:**334–338.
7. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. **3:**212–217.
8. **Jin, X., D. E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J. Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho.** 1999. Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in simian immunodeficiency virus-infected macaques. J. Exp. Med. **189:**991–998.
9. **Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, S. Rowland-Jones, and R. E. Phillips.** 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. J. Exp. Med. **193:**375–386.
10. **Klenerman, P., Y. Wu, and R. Phillips.** 2002. HIV: current opinion in escapology. Curr. Opin. Microbiol. **5:**408–413.
11. **Koenig, S., A. J. Conley, Y. A. Brewah, G. M. Jones, S. Leath, L. J. Boots, V. Davey, G. Pantaleo, J. F. Demarest, C. Carter, et al.** 1995. Transfer of HIV-1-specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. Nat. Med. **1:**330–336.
12. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. **68:**4650–4655.
13. **Matano, T., R. Shibata, C. Siemon, M. Connors, H. C. Lane, and M. A. Martin.** 1998. Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J. Virol. **72:**164–169.
14. **McMichael, A.** 1998. T cell responses and viral escape. Cell **93:**673–676.
15. **Mortara, L., F. Letourneur, H. Gras-Masse, A. Venet, J. G. Guillet, and I. Bourgault-Villada.** 1998. Selection of virus variants and emergence of virus escape mutants after immunization with an epitope vaccine. J. Virol. **72:** 1403–1410.
16. **Mothe, B. R., H. Horton, D. K. Carter, T. M. Allen, M. E. Liebl, P. Skinner, T. U. Vogel, S. Fuenger, K. Vielhuber, W. Rehrauer, N. Wilson, G. Franchini, J. D. Altman, A. Haase, L. J. Picker, D. B. Allison, and D. I. Watkins.** 2002. Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. J. Virol. **76:**875–884.
17. **O'Connor, D. H., T. M. Allen, T. U. Vogel, P. Jing, I. P. DeSouza, E. Dodds, E. J. Dunphy, C. Melsaether, B. Mothe, H. Yamamoto, H. Horton, N. Wilson, A. L. Hughes, and D. I. Watkins.** 2002. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat. Med. **8:**493–499.
18. **Phillips, R. E., S. Rowland-Jones, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza, et al.** 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature **354:**453–459.
19. **Price, G. E., R. Ou, H. Jiang, L. Huang, and D. Moskophidis.** 2000. Viral escape by selection of cytotoxic T cell-resistant variants in influenza A virus pneumonia. J. Exp. Med. **191:**1853–1868.
20. **Rue, S. M., J. W. Roos, L. M. Amzel, J. E. Clements, and S. A. Barber.** 2003. Hydrogen bonding at a conserved threonine in lentivirus capsid is required for virus replication. J. Virol. **77:**8009–8018.
21. **von Schwedler, U. K., K. M. Stray, J. E. Garrus, and W. I. Sundquist.** 2003. Functional surfaces of the human immunodeficiency virus type 1 capsid protein. J. Virol. **77:**5439–5450.
22. **Wolinsky, S. M., B. T. Korber, A. U. Neumann, M. Daniels, K. J. Kunstman, A. J. Whetsell, M. R. Furtado, Y. Cao, D. D. Ho, and J. T. Safrit.** 1996. Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. Science **272:**537–542.
23. **Yang, O. O., P. T. Sarkis, A. Ali, J. D. Harlow, C. Brander, S. A. Kalams, and B. D. Walker.** 2003. Determinants of HIV-1 mutational escape from cytotoxic T lymphocytes. J. Exp. Med. **197:**1365–1375.