associations was the consensus T at position 186, with increased replication capacity (see Table SA1 in the supplemental material).

(v) HLA-B*81-associated T186S mutation is linked to lower replication capacity. Since the consensus residue 186T was strongly associated with an increased replication capacity, changes away from consensus at this position were compared between the least-fit and fittest viruses. In the least-fit group, 9 sequences had an S and 1 had an A at position 186, while only 1 had an S at this position in the fittest group (Fisher's exact test; $P = 0.01$) (Fig. 5A). With the exception of 1 sequence with 186A and another sequence which had a mixture of T and S at this position, the nonconsensus amino acid at codon 186 was S. Overall, 186S was associated with a decrease in replication capacity compared with that for the 186T consensus (Student's t test; $P = 0.006$; $n = 403$) (Fig. 5B). This polymorphism is associated with HLA-B*81 and occurs in the HLA-B*81-restricted epitope TL9. The difference in numbers of variant TL9 epitopes between the low- and high-fitness groups could be attributed largely to variability at position 186. However, when only HLA-B*81-positive individuals were considered, the replication capacities of viruses with 186S and 186T were both below average and were not significantly different from one another (data not shown), indicating that other mutations are also responsible for the lower fitness of viruses from these individuals. The lack of difference in replication capacity between viruses with 186S and 186T from individuals with HLA-B*81 may also suggest that the fitness cost of 186S was compensated for in some cases.

(vi) Residues covarying with 186S. Codon covariation lists were generated from the current data set as previously described (5). Amino acids positively associated with 186S and/or negatively associated with 186T included 177D, 182S, 190A, 190I, 256I, and 343I ($P < 0.05$; $Q < 0.2$). Amino acids negatively associated with 186S and/or positively associated with 186T included 65Q, 177E, 190T, 256V, and 343L ($P < 0.05$; $Q < 0.2$). Replication capacities of viruses with 186S and various numbers of associated residues (Q65X, E177X, Q182S, T190X, V256X, and L343X) were compared to assess whether these might function as compensatory mutations. The number of covarying residues present correlated positively but not significantly with replication capacity (Pearson's correlation; $r = 0.26$ and $P = 0.19$). However, on closer examination of sequences with 186S, a greater occurrence of mutations at positions 182 and 190 (but not at other covarying positions) was noted in the fitter viruses (Fig. 5C). This was statistically significant (Student's t test; $P = 0.006$), suggesting that 190X and 182S, which occur parallel to and on either side of residue 186 in a helix structure, might indeed be compensatory mutations.

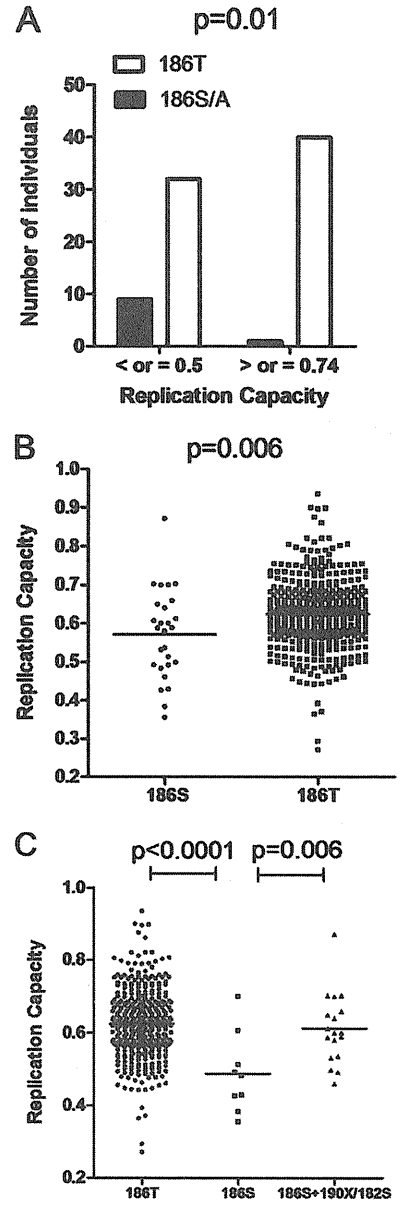

FIG. 5. Single Gag amino acid associations with altered replication capacities of Gag-protease NL4-3 recombinant viruses. (A) Greater proportion (Fisher's exact test) of 186S/A residues in the least-fit viruses (replication capacity of ≤0.5; $n = 41$) than in the fittest viruses (replication capacity of ≥0.742; $n = 41$). (B) Lower replication capacity of viruses with 186S ($n = 27$) than that of viruses with the consensus 186T ($n = 376$; Student's t test). (C) Significantly higher replication capacities of viruses with 186T ($n = 376$) and with 186S with putative compensatory mutations 190X and 182S ($n = 18$) than those of viruses with 186S alone ($n = 9$; Student's t test).

DISCUSSION

The mechanisms underlying HIV-1 control by protective HLA alleles are not fully understood and could involve targeting of functionally important epitopes in Gag, resulting in selection of escape mutations with a fitness cost. Therefore, this study was undertaken to investigate, at the population level, the impact of HLA-mediated immune pressure in Gag on viral fitness and its impact on HIV-1 pathogenesis.

Our results showed an association between protective HLA alleles (HLA-B*57, HLA-B*5801, and HLA-B*81) and lower Gag-protease replication capacities. Since (i) protective HLA alleles were associated with lower viral loads, (ii) Gag-protease replication capacity correlated with viral loads even on removal of protective HLA alleles from the analysis and within individuals with protective alleles, and (iii) replication capacity ranked according to HLA-A, -B, and -C alleles correlated significantly with the ranks according to viral load, the possi-

bility that HLA alleles and replication capacity are indirectly related to each other through association with viral load cannot be excluded. However, since mutations in Gag selected by the protective HLA alleles B*5703 and B*5801 were shown to significantly decrease the overall replication capacity of isolates and to confer benefits on infant and adult recipients (7, 8, 15, 33, 39), except in the presence of compensatory mutations (39), it seems very likely that a direct relationship exists between HLA alleles and Gag-protease replication capacity. Gag-protease replication capacity varied significantly between the different HLA-B but not HLA-A or HLA-C alleles, consistent with the idea that HLA alleles influence Gag-protease replication capacity through selecting mutations, as HLA-B alleles have the greatest selection pressure (20). Moreover, increasing numbers of HLA-B-associated mutations in or flanking epitopes (likely HLA-selected escape mutations) correlated with decreased HIV replication capacities. In further support of a direct relationship between protective HLA alleles and replication capacity, HLA-B*81 was by far the allele most strongly associated with lower replication capacity, even though HLA-B*5703-positive individuals had a lower average viral load than HLA-B*81-positive individuals, and 186S present in the HLA-B*81-restricted epitope TL9 (positions 180 to 188) was the mutation most strongly associated with lowered replication capacity, thereby providing a possible mechanism for the influence of HLA-B*81 on replication capacity. TL9 was previously described as one of the key Gag epitopes under strong selection pressure by a beneficial HLA allele, with variance mainly at residues 182 and 186 (both with changes predominantly to serine) (14). Interestingly, in a recent study, the number of public T-cell clonotypes specific for simian immunodeficiency virus (SIV) Gag CM9 (residues 181 to 189), which occurs in nearly the exact same position as TL9 in HIV, correlated strongly and negatively ($r^2 = -0.71$) with the viral set point in rhesus macaques (34). Residue 186 in HIV Gag has also been classified as a site where mutations revert upon transmission to a host lacking the HLA allele that selected them, presumably due to a fitness cost (26). It should be noted, though, that differences in fitness associated with variability at position 186 did not translate into viral load differences in this chronic infection cohort (data not shown), which could suggest that the fitness cost of the 186S mutation may be compensated in some cases, and therefore not of lasting benefit, and that the balance between the fitness cost of 186S and an effective CTL response to TL9 may be important in determining the outcome. However, taking the results together, it seems likely that protective HLA alleles, in particular HLA-B*81, influence Gag-protease replication capacity through CTL selection pressure and that this may partly contribute to their protective effect. From the present data, this seems likely to be a more prominent mechanism of protection for HLA-B*81 than for HLA-B*57 and HLA-B*5801 in subtype C infection.

Given our observation that lower Gag-protease replication capacities were related to protective HLA types, lower baseline viral loads, and higher baseline CD4 counts, we wished to investigate whether viral replication capacity may also correlate with the subsequent rate of CD4 decline during chronic infection. However, such a correlation was not observed in the present study. This may be explained partly by the balance that exists between Gag CTL responses and replication capacity in influencing clinical outcomes. Accumulation of escape mutations in HIV carries a fitness cost to the virus, but the disadvantage to the virus is offset by the advantage of escaping effective CTL responses that were holding replication in check, resulting in increased viral loads and accelerated disease progression despite a replication-deficient virus (8, 19). Another consideration is that replication capacity is not static and compensatory mutations may have developed at a time point later than that measured, influencing the subsequent rate of CD4 decline. Data from the present study and previous studies suggest that mutations with a fitness cost are readily compensated. The T186S mutation was most strongly associated with decreased replication capacity, yet in the presence of covarying mutations at positions 182 and 190, the mean replication capacity was not significantly different from the mean for the entire cohort, suggesting that the possible fitness cost of this mutation was compensated in these cases. Therefore, although there may be a benefit to decreased replication capacity (as supported by cross-sectional correlations with viral loads and CD4 counts), the data do not support an enduring benefit or a lasting significant impact of Gag-protease replication capacity on the rate of disease progression, at least once the chronic infection stage has been reached. The results of Brockman et al. (submitted) are consistent with this notion. However, acute infection studies and/or longitudinal analysis of replication capacity and sequence changes, together with CTL responses, may be necessary to better assess the relative impact of each on disease progression. Site-directed mutagenesis experiments would also be necessary to confirm the suspected fitness costs and compensatory roles of some of the mutations described above.

The data support the hypothesis that mutations at conserved residues/regions, in particular in conserved Gag p24 as opposed to the less-conserved Gag p17, are more likely to result in a fitness cost: HLA-associated escape mutations at conserved sites were associated with lower replication capacities, there were significantly more variant p24 epitopes in the least-fit viruses than in the fittest viruses, and most of the mutations significantly associated with altered replication capacities in p24 decreased replication capacity, while most in p17 increased replication capacity. In agreement with these data, beneficial HLA alleles in an African cohort were associated with strong selection at key epitopes which occurred mostly in Gag p24 (14), and there is recent evidence that HLA-B*57 mediates its protective effect mainly through attenuating mutations in Gag p24 (39). Furthermore, the breadth of Gag p24, but not p17 or p15, CD8 T-cell responses in HLA-B*13-positive individuals was significantly associated with decreasing viral loads (17). Taken together, the data generally support the inclusion of conserved regions such as Gag p24 in a vaccine that is aimed at driving HIV toward a less-fit state.

Interestingly, a larger number of amino acid differences from the consensus subtype C Gag sequence were weakly but significantly associated with increasing viral fitness. The percent amino acid similarity to the consensus subtype C Gag sequence also correlated negatively with viral load and positively with CD4 count (data not shown), suggesting that more changes from consensus and increased fitness of viruses may occur with disease progression. In fact, the fitness

of HIV isolates was previously shown to increase with disease progression (44). Consensus amino acids could, in some instances, be escape mutations in response to common HLA alleles, but we speculate that they represent the nonescape form in the majority of cases and that nonconsensus residues represent escape and compensatory mutations in response to CTL and non-CTL immune pressure, although they could also represent random mutations. Based on this conjecture, we suggest that more changes away from consensus likely indicate more compensation, and therefore fitter viruses. Another explanation is that the majority of mutations introduced into HIV are likely to have no or little fitness cost or to actually increase fitness. Consistent with this idea, p17 and p7 were significantly more divergent from the consensus than p24 was, i.e., significantly more mutations occurred in p17 and p7 than in p24, and the percent similarity to consensus for both p17 and p7 was negatively correlated overall with fitness, while there was no correlation for p24. The direct relationship between replication capacity and the entropy of mutated sites in the present study, as well as the recent finding that escape mutations in conserved Gag p24 carry significant fitness costs while most of the escape mutations in the highly variable *env* gene are fitness neutral or increase fitness (45), lends further support to this argument.

Another interesting finding was that most of the mutations in Gag associated with altered replication capacity were not HLA associated (71%). It should be noted, however, that a limitation of this study was the insertion of subtype C Gag-protease into a subtype B backbone, and therefore some Gag-protease mutations associated with altered replication capacity might represent those that interact with other components of the backbone. A significantly lower replication capacity of subtype C/B recombinants than that of subtype B recombinants was observed, which could suggest that mixing of subtypes results in suboptimal replication. Alternatively, this finding could mean that Gag-protease function is inferior in subtype C versus subtype B viruses, which may partly explain previously described fitness differences between subtypes (1). Further experiments are required to discriminate between these possibilities. Supporting the latter rather than the former possibility, convergence of subtype C Gag sequences to the consensus subtype B sequence was not associated with fitter recombinant viruses. Furthermore, the findings of the present study are in agreement with those of Brockman et al. (submitted), which show that subtype B Gag-protease NL4-3 recombinant viruses correlate with cross-sectional viral load and CD4 count data as well as with specific HLA types, strongly supporting the hypothesis that the current assay system is clinically and biologically relevant.

In summary, there is evidence that protective HLA alleles, especially HLA-B*81, influence subtype C HIV replication capacity through selection of mutations in Gag that incur a fitness cost. Moreover, mutations in conserved rather than more-variable regions of Gag are more likely to carry a fitness cost, suggesting that conserved regions such as Gag p24 should be included in a vaccine aiming to drive HIV toward a less-fit state. However, the long-term clinical impact of immune-driven fitness costs requires further investigation, given the evidence for compensation and the observation that replication capacity does not correlate with the subsequent rate of CD4 decline in chronic infection.

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REFERENCES

1. **Abraha, A., I. L. Nankya, R. Gibson, K. Demers, D. M. Tebit, E. Johnston, D. Katzenstein, A. Siddiqui, C. Herrera, L. Fischetti, R. J. Shattock, and E. J. Arts.** 2009. CCR5- and CXCR4-tropic subtype C human immunodeficiency virus type 1 isolates have a lower level of pathogenic fitness than other dominant group M subtypes: implications for the epidemic. J. Virol. **83:** 5592–5605.

1a. **Brockman, M. A., Z. L. Brumme, C. J. Brumme, T. Miura, J. Sela, P. C. Rosato, C. M. Kadie, J. M. Carlson, T. J. Markle, H. Streeck, A. D. Kelleher, M. Markowitz, H. Jessen, E. Rosenberg, M. Altfeld, P. R. Harrigan, D. Heckerman, B. D. Walker, and T. M. Allen.** Early selection in Gag by protective HLA alleles contributes to reduced HIV-1 replication capacity that may be largely compensated for in chronic infection. J. Virol., in press.

2. **Brockman, M. A., A. Schneidewind, M. Lahaie, A. Schmidt, T. Miura, I. DeSouza, F. Ryvkin, C. A. Derdeyn, S. Allen, E. Hunter, J. Mulenga, P. A. Goepfert, B. D. Walker, and T. M. Allen.** 2007. Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. J. Virol. **81:**12608–12618.

3. **Brockman, M. A., G. O. Tanzi, B. D. Walker, and T. M. Allen.** 2006. Use of a novel GFP reporter cell line to examine replication capacity of CXCR4- and CCR5-tropic HIV-1 by flow cytometry. J. Virol. Methods **131:**134–142.

4. **Brumme, Z., B. Wang, K. Nair, C. Brumme, C. de Pierres, S. Reddy, B. Julg, E. Moodley, C. Thobakgale, Z. Lu, M. van der Stok, K. Bishop, Z. Mncube, F. Chonco, Y. Yuki, N. Frahm, C. Brander, M. Carrington, M. Freedberg, P. Kiepiela, P. Goulder, B. Walker, T. Ndung'u, and E. Losina.** 2009. Impact of select immunologic and virologic biomarkers on CD4 cell count decrease in chronic HIV-1 subtype C infection: results from Sinikithemba Cohort, Durban, South Africa. Clin. Infect. Dis. **49:** 956–964.

5. **Carlson, J. M., Z. L. Brumme, C. M. Rousseau, C. J. Brumme, P. Matthews, C. Kadie, J. I. Mullins, B. D. Walker, P. R. Harrigan, P. J. R. Goulder, and D. Heckerman.** 2008. Phylogenetic dependency networks: inferring patterns of CTL escape and codon covariation in HIV-1 Gag. PLoS Comput. Biol. **4:**1–23.

6. **Carrington, M., and S. J. O'Brien.** 2003. The influence of HLA genotype on AIDS. Annu. Rev. Med. **54:**535–551.

7. **Chopera, D. R., Z. Woodman, K. Mlisana, M. Mlotshwa, D. P. Martin, C. Seoighe, F. Treurnicht, D. Assis de Rosa, W. Hide, S. A. Karim, C. M. Gray, C. Williamson, and C. S. Team.** 2008. Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage. PLoS Pathog. **4:**1–12.

8. **Crawford, H., W. Lumm, A. Leslie, M. Schaefer, D. Boeras, J. G. Prado, J. Tang, P. Farmer, T. Ndung'u, S. Lakhi, J. Gilmour, P. Goepfert, B. D. Walker, R. Kaslow, J. Mulenga, S. Allen, P. J. R. Goulder, and E. Hunter.** 2009. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. J. Exp. Med. **206:**909–919.

9. **Deeks, S. G., and B. D. Walker.** 2007. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. Immunity **27:**406–416.

10. **Derdeyn, C. A., and G. Silvestri.** 2005. Viral and host factors in the pathogenesis of HIV infection. Curr. Opin. Immunol. **17:**366–373.

11. **Donfack, J., F. J. Buchinsky, J. C. Post, and G. D. Ehrlich.** 2006. Human susceptibility to viral infection: the search for HIV-protective alleles among Africans by means of genome-wide studies. AIDS Res. Hum. Retroviruses **22:**925–930.
12. **Edwards, B. H., A. Bansal, S. Sabbaj, J. Bakari, M. J. Mulligan, and P. A. Goepfert.** 2002. Magnitude of functional $CD8^+$ T-cell responses to the Gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. J. Virol. **76:**2298–2305.
13. **Frahm, N., B. Baker, and C. Brander.** 2008. Identification and optimal definition of HIV-derived cytotoxic T lymphocyte (CTL) epitopes for the study of CTL escape, functional avidity and viral evolution, p. 3–24. *In* B. T. M. Korber, C. Brander, B. F. Haynes, R. Koup, J. P. Moore, B. D. Walker, and D. I. Watkins (ed.), HIV molecular immunology 2008. Los Alamos National Laboratory, Theoretical Biology and Biophysics, Los Alamos, NM.
14. **Frater, A. J., H. Brown, A. Oxenius, H. F. Günthard, B. Hirschel, N. Robinson, A. J. Leslie, R. Payne, H. Crawford, A. Prendergast, C. Brander, P. Kiepiela, B. D. Walker, P. J. R. Goulder, A. McLean, R. E. Phillips, et al.** 2007. Effective T-cell responses select human immunodeficiency virus mutants and slow disease progression. J. Virol. **81:**6742–6751.
15. **Goepfert, P. A., W. Lumm, P. Farmer, P. Matthews, A. Prendergast, J. M. Carlson, C. A. Derdeyn, J. Tang, R. A. Kaslow, A. Bansal, K. Yusim, D. Heckerman, J. Mulenga, S. Allen, P. J. R. Goulder, and E. Hunter.** 2008. Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. J. Exp. Med. **205:**1009–1017.
16. **Gray, C. M., M. Mlotshwa, C. Riou, T. Mathebula, D. de Assis Rosa, T. Mashishi, C. Seoighe, N. Ngandu, F. van Loggerenberg, L. Morris, K. Mlisana, C. Williamson, and S. A. Karim.** 2009. Human immunodeficiency virus-specific gamma interferon enzyme-linked immunospot assay responses targeting specific regions of the proteome during primary subtype C infection are poor predictors of the course of viremia and set point. J. Virol. **83:**470–478.
17. **Honeyborne, I., A. Prendergast, F. Pereyra, A. Leslie, H. Crawford, R. Payne, S. Reddy, K. Bishop, E. Moodley, K. Nair, M. van der Stok, N. McCarthy, C. M. Rousseau, M. Addo, J. I. Mullins, C. Brander, P. Kiepiela, B. D. Walker, and P. J. R. Goulder.** 2007. Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple Gag-specific $CD8^+$ T-cell epitopes. J. Virol. **81:**3667–3672.
18. **Huang, X., and J. Zhang.** 1996. Methods for comparing a DNA sequence with a protein sequence. Comput. Appl. Biosci. **12:**497–506.
19. **Kawada, M., H. Igarashi, A. Takeda, T. Tsukamoto, H. Yamamoto, S. Dohki, M. Takiguchi, and T. Matano.** 2006. Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. J. Virol. **80:**1949–1958.
20. **Kiepiela, P., A. J. Leslie, I. Honeyborne, D. Ramduth, C. Thobakgale, S. Chetty, P. Rathnavalu, C. Moore, K. J. Pfafferott, L. Hilton, P. Zimbwa, S. Moore, T. Allen, C. Brander, M. M. Addo, M. Altfeld, I. James, S. Mallal, M. Bunce, L. D. Barber, J. Szinger, C. Day, P. Klenerman, J. Mullins, B. Korber, H. M. Coovadia, B. D. Walker, and P. J. R. Goulder.** 2004. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature **432:**769–774.
21. **Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker, and P. Goulder.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. **13:**46–53.
22. **Le Clerc, S., S. Limou, C. Coulonges, W. Carpentier, C. Dina, L. Taing, O. Delaneau, T. Labib, R. Sladek, A. G. Group, C. Deveau, H. Guillemain, R. Ratsimandresy, M. Montes, J. L. Spadoni, A. Therwath, F. Schächter, F. Matsuda, I. Gut, J. D. Lelièvre, Y. Lévy, P. Froguel, J. F. Delfraissy, S. Hercberg, and J. F. Zagury.** 2009. Genomewide association study of a rapid progression cohort identifies new susceptibility alleles for AIDS (ANRS Genomewide Association Study 03). J. Infect. Dis. **200:**1194–1201.
23. **Leslie, A., P. C. Matthews, J. Listgarten, J. M. Carslon, C. Kadie, T. Ndung'u, C. Brander, H. Coovadia, B. D. Walker, D. Heckerman, and P. J. R. Goulder.** 2010. Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J. Virol. **84:**9879–9888.
24. **Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St. John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. R. Goulder.** 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat. Med. **10:**282–288.
25. **Martinez-Picado, J., J. G. Prado, E. E. Fry, K. Pfafferott, A. Leslie, S. Chetty, C. Thobakgale, I. Honeyborne, H. Crawford, P. Matthews, T. Pillay, C. Rousseau, J. I. Mullins, C. Brander, B. D. Walker, D. I. Stuart, P. Kiepiela, and P. Goulder.** 2006. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J. Virol. **80:**3617–3623.
26. **Matthews, P. C., A. Prendergast, A. Leslie, H. Crawford, R. Payne, C. Rousseau, M. Rolland, I. Honeyborne, J. Carlson, C. Kadie, C. Brander, K. Bishop, N. Mlotshwa, J. I. Mullins, H. Coovadia, T. Ndung'u, B. D. Walker, D. Heckerman, and P. J. R. Goulder.** 2008. Central role of reverting mutations in HLA associations with human immunodeficiency virus set point. J. Virol. **82:**8548–8559.
27. **Miura, T., M. A. Brockman, Z. L. Brumme, C. J. Brumme, F. Pereyra, A. Trocha, B. L. Block, A. Schneidewind, T. M. Allen, D. Heckerman, and B. D. Walker.** 2009. HLA-associated alterations in replication capacity of chimeric NL4-3 viruses carrying *gag-protease* from elite controllers of human immunodeficiency virus type 1. J. Virol. **83:**140–149.
28. **Ndongala, M. L., Y. Peretz, S. Boulet, M. Doroudchi, B. Yassine-Diab, M. R. Boulassel, D. Rouleau, C. Tremblay, R. LeBlanc, J. P. Routy, R. P. Sékaly, and N. F. Bernard.** 2009. HIV Gag p24 specific responses secreting IFN-gamma and/or IL-2 in treatment-naïve individuals in acute infection early disease (AIED) are associated with low viral load. J. Clin. Immunol. **131:**277–287.
29. **Ngumbela, K. C., C. L. Day, Z. Mncube, K. Nair, D. Ramduth, C. Thobakgale, E. Moodley, S. Reddy, C. De Pierres, N. Mkhwanazi, K. Bishop, M. van der Stok, N. Ismail, I. Honeyborne, H. Crawford, D. G. Kavanagh, C. Rousseau, D. Nickle, J. Mullins, D. Heckerman, B. Korber, H. Coovadia, P. Kiepiela, P. J. R. Goulder, and B. D. Walker.** 2008. Targeting of a CD8 T cell Env epitope presented by HLA-B*5802 is associated with markers of HIV disease progression and lack of selection pressure. AIDS Res. Hum. Retroviruses **24:**72–82.
30. **O'Brien, S. J., and G. W. Nelson.** 2004. Human genes that limit AIDS. Nat. Genet. **36:**565–574.
31. **Patke, D. S., S. J. Langan, L. M. Carruth, S. M. Keating, B. P. Sabundayo, J. B. Margolick, T. C. Quinn, and R. C. Bollinger.** 2002. Association of Gag-specific T lymphocyte responses during the early phase of human immunodeficiency virus type 1 infection and lower virus load set point. J. Infect. Dis. **186:**1177–1180.
32. **Pereyra, F., S. Palmer, T. Miura, B. L. Block, A. Wiegand, A. C. Rothchild, B. Baker, R. Rosenberg, E. Cutrell, M. S. Seaman, J. M. Coffin, and B. D. Walker.** 2009. Persistent low-level viremia in HIV-1 elite controllers and relationship to immunologic parameters. J. Infect. Dis. **200:**984–990.
33. **Prado, J. G., A. Prendergast, C. Thobakgale, C. Molina, G. Tudor-Williams, T. Ndung'u, B. D. Walker, and P. Goulder.** 2010. Replicative capacity of human immunodeficiency virus type 1 transmitted from mother to child is associated with pediatric HIV-1 disease progression rate. J. Virol. **84:**492–502.
34. **Price, D. A., T. E. Asher, N. A. Wilson, M. C. Nason, J. M. Brenchley, I. S. Metzler, V. Venturi, E. Gostick, P. K. Chattopadhyay, M. Roederer, M. P. Davenport, D. I. Watkins, and D. C. Douek.** 2009. Public clonotype usage identifies protective Gag-specific CD8+ T cell responses in SIV infection. J. Exp. Med. **206:**923–936.
35. **Rivière, Y., M. B. McChesney, F. Porrot, F. Tanneau-Salvadori, P. Sansonetti, O. Lopez, G. Pialoux, V. Feuillie, M. Mollereau, and S. E. A. Chamaret.** 1995. Gag-specific cytotoxic responses to HIV type 1 are associated with a decreased risk of progression to AIDS-related complex or AIDS. AIDS Res. Hum. Retroviruses **11:**903–907.
36. **Rousseau, C. M., M. G. Daniels, J. M. Carlson, C. Kadie, H. Crawford, A. Prendergast, P. Matthews, R. Payne, M. Rolland, D. N. Raugi, B. S. Maust, G. H. Learn, D. C. Nickle, H. Coovadia, T. Ndung'u, N. Frahm, C. Brander, B. D. Walker, P. J. R. Goulder, T. Bhattacharya, D. E. Heckerman, B. T. Korber, and J. I. Mullins.** 2008. HLA class I-driven evolution of human immunodeficiency virus type 1 subtype C proteome: immune escape and viral load. J. Virol. **82:**6434–6446.
37. **Schellens, I. M. M., J. A. M. Borghans, C. A. Jansen, I. M. De Cuyper, R. B. Geskus, D. van Baarle, and F. Miedema.** 2008. Abundance of early functional HIV-specific CD8+ T cells does not predict AIDS-free survival time. PLoS One **3:**e2745.
38. **Schneidewind, A., M. A. Brockman, R. Yang, I. Adam, B. Li, S. Le Gall, C. R. Rinaldo, S. L. Craggs, R. L. Allgaier, K. A. Power, T. Kuntzen, C. S. Tung, M. X. LaBute, S. M. Mueller, T. Harrer, A. J. McMichael, P. J. Goulder, C. Aiken, C. Brander, A. D. Kelleher, and T. M. Allen.** 2007. Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. J. Virol. **81:**12382–12393.
39. **Schneidewind, A., Y. Tang, M. A. Brockman, E. G. Ryland, J. Dunkley-Thompson, J. C. Steel-Duncan, M. A. St. John, J. A. Conrad, S. A. Kalams, F. Noel, T. M. Allen, C. D. Christie, and M. E. Feeney.** 2009. Maternal transmission of human immunodeficiency virus escape mutations subverts HLA-B57 immunodominance but facilitates viral control in the haploidentical infant. J. Virol. **83:**8616–8627.
40. **Storey, J. D., and R. Tibshirani.** 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. U. S. A. **100:**9440–9445.
41. **Takeuchi, H., and T. Matano.** 2008. Host factors involved in resistance to retroviral infection. Microbiol. Immunol. **52:**318–325.
42. **Tang, J., S. Tang, E. Lobashevsky, A. D. Myracle, U. Fideli, G. Aldrovandi,**

S. Allen, R. Musonda, R. A. Kaslow, et al. 2002. Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human immunodeficiency virus type 1. J. Virol. **76**:8276–8284.

43. **Thobakgale, C. F., A. Prendergast, H. Crawford, N. Mkhwanazi, D. Ramduth, S. Reddy, C. Molina, Z. Mncube, A. Leslie, J. Prado, F. Chonco, W. Mphatshwe, G. Tudor-Williams, P. Jeena, N. Blanckenberg, K. Dong, P. Kiepiela, H. Coovadia, T. Ndung'u, B. D. Walker, and P. J. R. Goulder.** 2009. Impact of HLA in mother and child on disease progression of pediatric human immunodeficiency virus type 1 infection. J. Virol. **83**:1–11.
44. **Troyer, R. M., K. R. Collins, A. Abraha, E. Fraundorf, D. M. Moore, R. W. Krizan, Z. Toossi, R. L. Colebunders, M. A. Jensen, J. I. Mullins, G. Vanham, and E. J. Arts.** 2005. Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. J. Virol. **79**:9006–9018.
45. **Troyer, R. M., J. McNevin, Y. Liu, S. C. Zhang, R. W. Krizan, A. Abraha, D. M. Tebit, H. Zhao, S. Avila, M. A. Lobritz, M. J. McElrath, S. Le Gall, J. I. Mullins, and E. J. Arts.** 2009. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. PLoS Pathog. **5**:1–13.
46. **Zuniga, R., A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C. H. Linde, H. S. Hewitt, W. Hildebrand, M. Altfeld, T. M. Allen, B. D. Walker, B. T. Korber, T. Leitner, J. Sanchez, and C. Brander.** 2006. Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J. Virol. **80**:3122–3125.

Infrequent Recovery of HIV from but Robust Exogenous Infection of Activated CD4+ T Cells in HIV Elite Controllers

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(See the editorial commentary by Saag and Deeks, on pages 239–241.)

Background. Human immunodeficiency virus (HIV) elite controllers are able to control infection with HIV-1 spontaneously to undetectable levels in the absence of antiretroviral therapy, but the mechanisms leading to this phenotype are poorly understood. Although low frequencies of HIV-infected peripheral CD4+ T cells have been reported in this group, it remains unclear to what extent these are due to viral attenuation, active immune containment, or intracellular host factors that restrict virus replication.

Methods. We assessed proviral DNA levels, autologous viral growth from and infectability of in vitro activated, CD8+ T cell–depleted CD4+ T cells from HIV elite controllers (mean viral load, <50 copies/mL), viremic controllers (mean viral load, <2000 copies/mL), chronic progressors, and individuals receiving highly active antiretroviral therapy.

Results. Although we successfully detected autologous virus production in *ex vivo* activated CD4+ T cells from all chronic progressors and from most of the viremic controllers, we were able to measure robust autologous viral replication in only 2 of 14 elite controllers subjected to the same protocol. In vitro activated autologous CD4+ T cells from elite controllers, however, supported infection with both X4 and R5 tropic HIV strains at comparable levels to those in CD4+ T cells from HIV-uninfected subjects. Proviral DNA levels were the lowest in elite controllers, suggesting that extremely low frequencies of infected cells contribute to difficulty in isolation of virus.

Conclusions. These data indicate that elite control is not due to inability of activated CD4+ T cells to support HIV infection, but the relative contributions of host and viral factors that account for maintenance of low-level infection remain to be determined.

A small proportion of human immunodeficiency virus type 1 (HIV-1)–infected individuals, called elite and viremic controllers, spontaneously control plasma HIV RNA levels to undetectable (elite controller) or <2000 copies/mL (viremic controller) in the absence of antiretroviral therapy. Some have postulated that elite controllers exhibit control predominantly as a consequence of infection with replication-defective virus, resulting in poor viral outgrowth [1–3]. Although impaired in vitro viral replication capacity has been documented in some elite controllers [4], studies thus far have shown that replication-competent viruses can be isolated from these individuals as well, suggesting that not all elite controllers are infected with defective viruses [5, 6]. Host genetic analyses suggest that immune mechanisms, associated with the major-histocompatibility complex, contribute to the extraordinary control of viral replication observed in this unique patient population [7–12]. The overrepresentation of certain HLA class I alleles [10, 13], CD8+ T cell–depletion studies [14], evidence of selection for cytotoxic T lymphocyte epitope mutations [12, 15], and in vitro studies showing strong antiviral activity of CD8+ T cells [11, 16] suggest that cellular immune mechanisms are involved in this remarkable antiviral control.

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Interestingly, long-term nonprogressors not only exhibit reduced levels of plasma viral replication but also have lower numbers of $CD4^+$ T cells with integrated HIV provirus, compared with individuals with fast-progressive disease and AIDS [17, 18]. This finding suggests that (a) these individuals have a unique capacity to control viral replication actively over time, (b) they are infected with viruses that do not replicate, and/or (c) these individuals possess $CD4^+$ T cells with a unique ability to resist HIV infection. To begin to explore the 2 latter possibilities, we sought to determine whether elite controllers are infected with replication-competent virus, compared with a group of normal HIV-infected progressors, and whether their $CD4^+$ T cells, after vigorous in vitro activation, are differentially susceptible to HIV-1 infection in vitro, compared with those of seronegative control subjects.

MATERIALS AND METHODS

Study subjects. A total of 25 HIV elite controllers with plasma HIV RNA levels <50 copies/mL were randomly selected from the International HIV Controllers Study. Also included were 10 viremic controllers with plasma HIV RNA levels of 50–2000 copies/mL (mean plasma HIV RNA, 1256 copies/mL). Both groups were antiretroviral therapy naive, and a minimum of 3 qualifying determinations of plasma HIV RNA levels spanning at least a 12-month period was required for inclusion in the study. Eleven untreated viremic progressors with plasma HIV RNA levels >10,000 copies/mL (mean plasma HIV RNA, 125.158 copies/mL) and 9 subjects receiving successful highly active antiretroviral therapy (HAART) (mean plasma HIV RNA, <75 copies/mL) were recruited from outpatient clinics at local Boston hospitals. HAART was defined as treatment with ⩾3 antiretroviral drugs, including 2 nucleoside reverse-transcriptase inhibitors and a nonnucleoside reverse-transcriptase inhibitor or a protease inhibitor. Additionally, we obtained blood from 12 healthy control subjects. All subjects gave written informed consent.

Assessment of autologous virus production. To detect autologous virus growth, $CD4^+$ cells were purified from freshly isolated peripheral blood mononuclear cells (PBMCs) by negative selection using the Rosette Sep $CD4^+$ cell enrichment cocktail (Stemcell Technologies) depleting $CD8^+$ T cells, natural killer cells, B cells, macrophages, monocytes, and dendritic cells. $CD4^+$ cells were then stimulated in interleukin 2 (50 units/mL) containing T cell medium in the presence of a bispecific anti-CD3:anti-CD8 monoclonal antibody, which selectively activates $CD4^+$ T lymphocytes while simultaneously depleting all remaining $CD8^+$ T cells [19]. $CD4^+$ T cell blasts, generated from HIV-uninfected donors, were added every 7 days to maintain the cultures and to provide additional targets for viral outgrowth.

In parallel, an aliquot of $CD4^+$ T cells from elite controllers was infected with a clinical HIV-1 (X4) isolate at multiplicity of infection of 0.01 as described previously [20] and was maintained under the same culture conditions. Every 2–3 days, p24 was measured in culture supernatants using a p24-based enzyme-linked immunosorbent assay (ELISA; PerkinElmer Life Sciences) in accordance with the manufacturer's protocol.

Infectability assays. To determine in vitro infectability of $CD4^+$ T cells and their intrinsic ability to support viral replication, comparing elite controllers with HIV-uninfected individuals, whole PBMCs were depleted from $CD8^+$ T cells using anti-CD8 magnetic beads (DYNAL) in accordance with the manufacturer's protocol, and $CD4^+$ T cell blasts were generated using the bispecific anti-CD3:anti-CD8 antibody in the presence of interleukin 2 (50 units/mL) for 3 days. $CD4^+$ T cells were then infected with X4 and R5 HIV laboratory strains (NL4-3 and JRCSF) at a multiplicity of infection of 0.01 for 4 h. Cells were washed and then cultured in interleukin 2 (50 units/mL) containing medium for 7 days without the addition of further CD4 blasts, and p24 was measured in culture supernatants every 2–3 days.

Real-time PCR–based quantification of proviral HIV-1 DNA. HIV-1 DNA purification from 10^7 PBMCs was performed with a standard protocol (QIAamp DNA Blood Kit; Qiagen). A single-step real-time polymerase chain reaction (PCR) was used to quantify proviral HIV-1 DNA in a 50-μL PCR reaction mix containing 25 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 20 μL of HIV-1 DNA, and primers and probe that anneal to the 5′ and 3′ ends of the R and U5 region of the long terminal repeat (LTR), respectively, as has been described previously [21], using forward primer 5′-GG CTA ACT AGG GAA CCC ACT G-3′ and reverse primer 5′-GCT AGA GAT TTT CCA CAC TGA CTA A-3′. The fluorescence TaqMan probe was 5′-GGA TCT CTA GTT ACC AGA GTC A-3′. Amplification reactions were performed with an Applied Biosystems 7000 real-time PCR system. The thermocycling conditions were 95°C for 10 min, 50 cycles at 95°C for 15 s and 60°C for 1 min, and a final cycle at 72°C for 5 min. Copy-number estimation of proviral HIV-1 DNA was performed in duplicate and was determined by extrapolation from a standard curve generated with a plasmid that harbors the sequence of the HIV-1 LTR and *CCR5* gene. Proviral HIV-1 DNA copy number was calculated relative to *CCR5* gene copy number previously quantified with the standard curve.

HLA typing. HLA class I typing was performed as described previously [22].

Statistical analyses. Values throughout the text are expressed as mean ± standard deviation. *P* values were calculated using the Kruskal-Wallis test followed by Dunn's method for multiple comparisons and Wilcoxon rank-sum tests for pairwise comparisons.

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RESULTS

Autologous virus production. We first evaluated whether *ex vivo* activation of $CD4^+$ T cells from elite controllers and viremic controllers resulted in outgrowth of autologous virus, comparing these results with those obtained from chronically HIV-infected patient subpopulations. PBMCs were stimulated with a bispecific monoclonal antibody that results in the selective expansion of $CD4^+$ T cells and the elimination of $CD8^+$ T cells [19], and uninfected activated donor $CD4^+$ cells were added to the cultures weekly, such that viral outgrowth was being examined in non-autologous cells. Of 14 elite controllers examined, virus was detected in vitro in only 3, and in 1 subject, detection by p24 ELISA was transient and could not be confirmed by repeated real-time PCR. In contrast, 9 of 9 untreated HIV progressors and 3 of 4 HAART-treated subjects demonstrated p24 antigen production in the stimulated cultures (Figure 1*A*). Although virus was significantly less frequently detected in the elite controllers, in both of the individuals in whom sustained virus production was observed, $\log_{10}$ p24 levels of 4.08–4.82 $\log_{10}$ pg/mL could be detected, comparable to the p24 antigen levels observed in HIV progressor cultures (3.91 ± 0.5 $\log_{10}$ pg/mL), suggesting that the viruses that did grow were as replication competent as were the viruses infecting the progressors (Figure 1*B*). Viral replication was not detectable in $CD4^+$ cultures from the remaining 11 elite controllers, despite that these cultures were maintained for a median of 37 ± 12 days and some for as long as 78 days. Interestingly, 8 of 10 HIV viremic controllers displayed robust viral replication in vitro after 20 days despite their low plasma HIV RNA loads in vivo, with levels lower but comparable to those observed in progressors ($\log_{10}$ p24 levels, 3.4 ± 1.3 $\log_{10}$ pg/mL in viremic controllers and 3.91 ± 0.5 $\log_{10}$ pg/mL in progressors) (Figure 1*B*). Of the remaining 2 viremic controllers, one showed a delayed viral growth (first virus detected after >30 days), whereas no autologous virus was detected in the other subject's cultures for up to 50 days. Interestingly, both individuals were homozygous for protective HLA B alleles (B2705/B5701 and B5701/B5703, respectively), which suggests that a strong immune response may have led to the reduced viral reservoir in these individuals, suggesting the possible role of active immune containment of viral replication (Figure 1*B*). These data indicate furthermore that replication-competent HIV can be isolated from a minority of elite controllers, suggesting either that the majority of elite controllers are infected with replication-

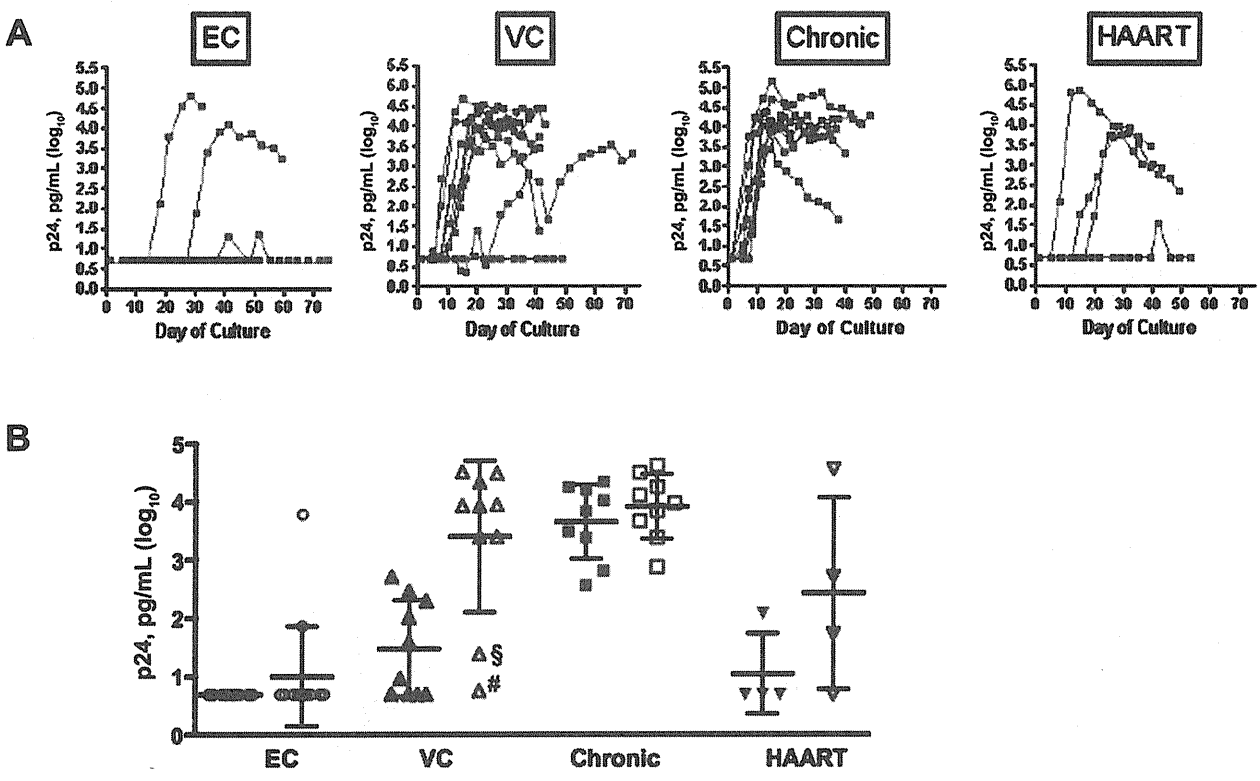

Figure 1. Autologous virus replication in activated autologous $CD4^+$ T cells. *A,* Kinetics of autologous virus replication ($\log_{10}$ p24 levels in pg/mL) among elite controllers (EC), viremic controllers (VC), chronic progressors, and individuals receiving highly active antiretroviral therapy (HAART). *B,* Mean $\log_{10}$ p24 levels (in pg/mL) after 10 days *(filled symbols)* and 20 days *(open symbols)* among EC, VC (§, HLA B5701/B5703; #, HLA B2705/B5701), chronic progressors, and individuals receiving HAART.

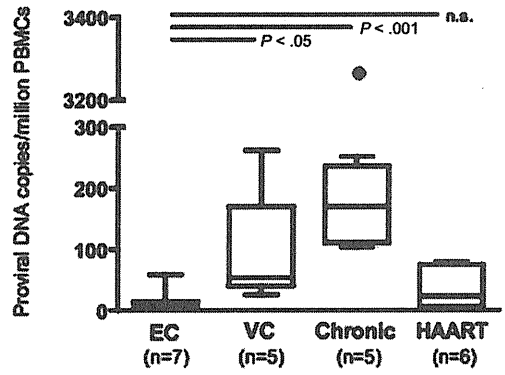

Figure 2. Proviral loads (DNA copies/10^6 peripheral blood mononuclear cells [PBMCs]) among elite controllers (EC), viremic controllers (VC), chronic progressors, and individuals receiving highly active antiretroviral therapy (HAART). n.s., not significant.

defective virus or that the frequency of HIV-1–infected $CD4^+$ T cells is extremely low in the peripheral blood of elite controllers, resulting in a reduced chance of capturing an infected cell in a given PBMC sample.

Proviral HIV-1 DNA. To test the hypothesis that the reduced outgrowth of autologous virus from in vitro activated HIV controller $CD4^+$ T cells is the result of the low frequency of infected $CD4^+$ cells in the peripheral blood, we measured HIV proviral DNA levels in total PBMCs of 7 elite controllers and 5 viremic controllers and compared these with the levels of 5 chronic progressor patients and 6 virally suppressed individuals receiving HAART. Elite controllers showed significantly lower levels of provirus, compared with viremic controllers and chronic progressors (12.79 ± 20.92 proviral DNA copies/10^6 PBMCs for elite controllers vs. 94.80 ± 95.47 proviral DNA copies/10^6 PBMCs for viremic controllers and 792.0 ± 1383 proviral DNA copies/10^6 PBMCs for chronic progressors; $P < .05$ and $P < .001$, respectively). In contrast, we did not detect any significant difference between elite controllers and patients receiving HAART (34.76 ± 32.74 proviral DNA copies/10^6 PBMCs; not significant) (Figure 2). This observation suggests that the low viral reservoir in the elite controller $CD4^+$ compartment, rather than general replication incompetence of their infecting virus, might lead to limited outgrowth of autologous virus in the elite controller. This assumption is supported by the observation that the elite controller who demonstrated the strongest outgrowth of autologous virus in the long-term cultures (peak $\log_{10}$ p24 levels, 4.82 $\log_{10}$ pg/mL at day 28) showed the highest proviral DNA loads of the elite controller group (58.33 proviral DNA copies/10^6 PBMCs).

Susceptibility to HIV infection. The reduced outgrowth of autologous virus from in vitro activated $CD4^+$ T cells in elite controllers could also indicate that these cells may be less permissive to HIV virus replication. To test this hypothesis, we determined whether the same elite controller $CD4^+$ T cells used for the outgrowth cultures were susceptible to in vitro infection with an X4 tropic HIV-1 clinical isolate (F716). We therefore infected $CD4^+$ T cells with exogenous virus in parallel while setting up the outgrowth cultures. $CD4^+$ T cells from all elite controllers were readily infectable and exhibited peak $\log_{10}$ p24 levels of 4.29 ± 0.3 $\log_{10}$ pg/mL after 10 days (Figure 3).

We next ascertained whether there were differences in the level of superinfecting viral production in in vitro stimulated $CD4^+$ T cells between elite controllers and non–elite controller control subjects. Because of the confounding issue of the outgrowth of autologous virus in cells from viremic individuals, we compared $CD4^+$ T cell infectability of elite controllers with that of a group of matched HIV-seronegative controls. By infecting with X4 and R5 tropic (NL4-3 and JRCSF) HIV-1 strains, we were able to observe peak levels of viral replication in $CD4^+$ T cells from elite controllers on day 7, reaching a mean $\log_{10}$ p24 level of 4.98 ± 0.45 $\log_{10}$ pg/mL for NL4-3 infection and 5.28 ± 0.49 pg/mL for JRCSF infection (Figure 4*A* and 4*B*). Similar levels and kinetics of viral replication were seen in $CD4^+$ cell cultures from HIV-uninfected individuals ($\log_{10}$ p24 level, 4.88 ± 0.59 $\log_{10}$ pg/mL for NL4-3 infection and 5.26 ± 0.42 $\log_{10}$ pg/mL for JRCSF infection, respectively). These data suggest that $CD4^+$ T cells from elite controllers, after exogenous activation, are not resistant to HIV-1 in vitro infection.

DISCUSSION

In this study, we investigated whether low plasma HIV RNA levels in elite and viremic controllers are associated with infection by replication-incompetent viruses or whether their $CD4^+$ T cells are not able to produce virus or become infected. To address these possibilities, purified $CD4^+$ T cells from elite controllers and viremic controllers were isolated and were stimulated in vitro, and outgrowth of autologous virus was assessed. Among 14 elite controllers tested, autologous virus grew ro-

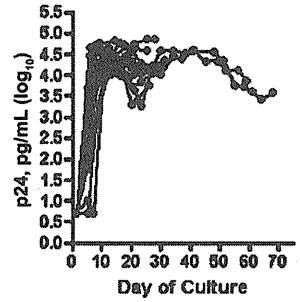

Figure 3. Replication of a human immunodeficiency virus type 1 clinical isolate in activated $CD4^+$ T cells of 14 elite controllers.

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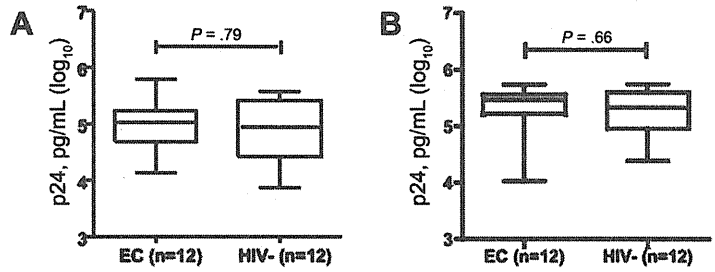

Figure 4. Replication of the X4 tropic human immunodeficiency virus type 1 (HIV-1) strain NL4-3 *(A)* and the R5 tropic HIV-1 strain JRCSF *(B)* in activated $CD4^+$ T cells of 12 elite controllers and 12 HIV-uninfected (HIV−) individuals (multiplicity of infection, 0.01). Shown are mean $\log_{10}$ p24 levels (in pg/mL) at day 7.

bustly from samples derived from only 2 individuals' $CD4^+$ T cell cultures. In contrast, in vitro activated $CD4^+$ T cells from all elite controllers were readily infectable with exogenous virus following in vitro stimulation, suggesting that the elite control observed is not associated with an inability of activated $CD4^+$ cells to support HIV replication. However, we cannot exclude that a distinct interaction between elite controller $CD4^+$ T cells and their infecting viral strain might exist that would not allow viral outgrowth in these individuals. Because of the limitation in retrieving viral strains from elite controllers, we were unable to test this hypothesis.

Our results are consistent with those of recent reports, in which difficulty in growing autologous virus was observed in the vast majority of elite controllers [5, 6]. However, because all elite controller $CD4^+$ T cells were infectable and produced large quantities of virus after superinfection with laboratory or primary HIV strains, our data suggest that low levels of virally infected cells in the peripheral circulation, rather than an intrinsic inability of their activated $CD4^+$ T cells to support robust virus replication, may explain the difficulty in isolating autologous virus in vitro. This was supported by the reduced levels of HIV proviral DNA we found in the elite controllers, compared with those in chronic progressors and even viremic controllers or individuals receiving suppressive antiretroviral therapy. Consistent with this observation, we detected the strongest outgrowth of autologous virus in the elite controller with the highest proviral DNA levels.

Since autologous virus replicated readily in most of the viremic controllers despite their low plasma viral loads, the exclusive explanation of HIV control by a defective infecting virus is questionable. Ours and other groups have shown that elite control is associated with persistent low but fluctuating levels of viremia, suggesting individual differences in host immune responses [23, 24]. Our observation that the 2 viremic controllers who showed either a delayed or no autologous viral outgrowth express the protective HLA B alleles B27 and B57 implies that the reduction of the viral reservoir in the peripheral $CD4^+$ compartment may be mediated by a potent and persistent immune response. These findings are supported by a recent report from Saez-Cirion et al [25], who demonstrated that, among elite controllers with weak antiviral $CD8^+$ T cell responses, highly in vitro replicative viruses were detectable.

It has been postulated that $CD4^+$ T cells from elite controllers may be less susceptible to infection, as has been shown for individuals who are highly exposed to HIV-1 and yet remain uninfected [26, 27]. However, detectable low level viremia in the majority of elite controllers [24] points to a small persistent cellular reservoir for ongoing viral replication. Here, we artificially activated $CD4^+$ T cells from all donors, and after maximal activation, superinfected the cells with either an X4 or an R5 laboratory strain. In this setting, we did not observe any differences among the elite controllers or HIV-uninfected control subjects in their ability to support viral replication. This observation argues against but does not disprove the hypothesis that host factors may render activated $CD4^+$ T cells less infectable as a cause for the reduced frequencies of infected peripheral $CD4^+$ T cells in HIV controllers. However, it is still plausible that other factors, such as potential in vivo differences in $CD4^+$ T cell activation levels or more subtle differences in the ability to support autologous virus replication, may differentiate the infectability of $CD4^+$ cells of elite controllers, compared with those of other individuals. This said, it is still plausible that added intrinsic differences in activation potentials among $CD4^+$ T cells from elite controllers and controls may account for differences in susceptibility to HIV-1 infection or in the burst size of viruses produced per infected cell.

Overall, these data suggest that in vitro activated $CD4^+$ cells from elite controllers can readily accommodate HIV replication and that elite controllers can harbor replication-competent HIV. The poor autologous virus outgrowth from elite controllers is likely related to the low frequency of HIV-infected cells in the peripheral circulation of elite controllers. Low levels of HIV-infected $CD4^+$ T cells in vivo are likely due to highly antiviral immune pressure unique to HIV controllers, but fur-

ther studies examining subtle differences in innate effector mechanisms that might be detectable with more sensitive assays are warranted.

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Potential conflicts of interest. All authors: no conflicts.

References

1. Alexander L, Weiskopf E, Greenough TC, et al. Unusual polymorphisms in human immunodeficiency virus type 1 associated with nonprogressive infection. J Virol **2000**; 74:4361–76.
2. Deacon NJ, Tsykin A, Solomon A, et al. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **1995**; 270:988–91.
3. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med **1995**; 332:228–32.
4. Miura T, Brockman MA, Brumme ZL, et al. HLA-associated alterations in replication capacity of chimeric NL4-3 viruses carrying gag-protease from elite controllers of human immunodeficiency virus type 1. J Virol **2009**; 83:140–9.
5. Lamine A, Caumont-Sarcos A, Chaix ML, et al. Replication-competent HIV strains infect HIV controllers despite undetectable viremia (ANRS EP36 study). AIDS **2007**; 21:1043–5.
6. Blankson JN, Bailey JR, Thayil S, et al. Isolation and characterization of replication-competent human immunodeficiency virus type 1 from a subset of elite suppressors. J Virol **2007**; 81:2508–18.
7. Cao Y, Qin L, Zhang L, Safrit J, Ho DD. Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N Engl J Med **1995**; 332:201–8.
8. Bailey JR, Williams TM, Siliciano RF, Blankson JN. Maintenance of viral suppression in HIV-1–infected HLA-B*57$^+$ elite suppressors despite CTL escape mutations. J Exp Med **2006**; 203:1357–69.
9. Migueles SA, Osborne CM, Royce C, et al. Lytic granule loading of CD8$^+$ T cells is required for HIV-infected cell elimination associated with immune control. Immunity **2008**; 29:1009–21.
10. Pereyra F, Addo MM, Kaufmann DE, et al. Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. J Infect Dis **2008**; 197:563–71.
11. Saez-Cirion A, Lacabaratz C, Lambotte O, et al. HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proc Natl Acad Sci U S A **2007**; 104:6776–81.
12. Miura T, Brumme CJ, Brockman MA, et al. HLA-associated viral mutations are common in human immunodeficiency virus type 1 elite controllers. J Virol **2009**; 83:3407–12.
13. Deeks SG, Walker BD. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. Immunity **2007**; 27:406–16.
14. Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by CD8$^+$ lymphocytes. Science **1999**; 283:857–60.
15. Miura T, Brockman MA, Schneidewind A, et al. HLA-B57/B*5801 HIV-1 elite controllers select for rare Gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. J Virol **2009**; 83:2743–55.
16. Yang OO, Kalams SA, Trocha A, et al. Suppression of human immunodeficiency virus type 1 replication by CD8$^+$ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. J Virol **1997**; 71:3120–8.
17. Garbuglia AR, Salvi R, Di Caro A, et al. Peripheral lymphocytes of clinically non-progressor patients harbor inactive and uninducible HIV proviruses. J Med Virol **1995**; 46:116–21.
18. Lambotte O, Boufassa F, Madec Y, et al. HIV controllers: a homogeneous group of HIV-1–infected patients with spontaneous control of viral replication. Clin Infect Dis **2005**; 41:1053–6.
19. Wilson CC, Wong JT, Girard DD, et al. Ex vivo expansion of CD4 lymphocytes from human immunodeficiency virus type 1–infected persons in the presence of combination antiretroviral agents. J Infect Dis **1995**; 172:88–96.
20. Chen H, Piechocka-Trocha A, Miura T, et al. Differential neutralization of HIV replication in autologous CD4 T cells by HIV-specific CTL. J Virol **2009**; 83:3138–49.
21. Brussel A, Sonigo P. Analysis of early human immunodeficiency virus type 1 DNA synthesis by use of a new sensitive assay for quantifying integrated provirus. J Virol **2003**; 77:10119–24.
22. Frahm N, Korber BT, Adams CM, et al. Consistent cytotoxic-T-lymphocyte targeting of immunodominant regions in human immunodeficiency virus across multiple ethnicities. J Virol **2004**; 78:2187–200.
23. Dinoso JB, Kim SY, Siliciano RF, Blankson JN. A comparison of viral loads between HIV-1–infected elite suppressors and individuals who receive suppressive highly active antiretroviral therapy. Clin Infect Dis **2008**; 47:102–4.
24. Pereyra F, Palmer S, Miura T, et al. Persistent low-level viremia in HIV-1 elite controllers and relationship to immunologic parameters. J Infect Dis **2009**; 200:984–90.
25. Saez-Cirion A, Sinet M, Shin SY, et al. Heterogeneity in HIV suppression by CD8 T cells from HIV controllers: association with Gag-specific CD8 T cell responses. J Immunol **2009**; 182:7828–37.
26. Paxton WA, Martin SR, Tse D, et al. Relative resistance to HIV-1 infection of CD4 lymphocytes from persons who remain uninfected despite multiple high-risk sexual exposure. Nat Med **1996**; 2:412–7.
27. Saez-Cirion A, Versmisse P, Truong LX, et al. Persistent resistance to HIV-1 infection in CD4 T cells from exposed uninfected Vietnamese individuals is mediated by entry and post-entry blocks. Retrovirology **2006**; 3:81.

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Early Selection in Gag by Protective HLA Alleles Contributes to Reduced HIV-1 Replication Capacity That May Be Largely Compensated for in Chronic Infection▽†

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Mutations that allow escape from CD8 T-cell responses are common in HIV-1 and may attenuate pathogenesis by reducing viral fitness. While this has been demonstrated for individual cases, a systematic investigation of the consequence of HLA class I-mediated selection on HIV-1 *in vitro* replication capacity (RC) has not been undertaken. We examined this question by generating recombinant viruses expressing plasma HIV-1 RNA-derived Gag-Protease sequences from 66 acute/early and 803 chronic untreated subtype B-infected individuals in an NL4-3 background and measuring their RCs using a green fluorescent protein (GFP) reporter CD4 T-cell assay. In acute/early infection, viruses derived from individuals expressing the protective alleles HLA-B*57, -B*5801, and/or -B*13 displayed significantly lower RCs than did viruses from individuals lacking these alleles ($P < 0.05$). Furthermore, acute/early RC inversely correlated with the presence of HLA-B-associated Gag polymorphisms ($R = -0.27$; $P = 0.03$), suggesting a cumulative effect of primary escape mutations on fitness during the first months of infection. At the chronic stage of infection, no strong correlations were observed between RC and protective HLA-B alleles or with the presence of HLA-B-associated polymorphisms restricted by protective alleles despite increased statistical power to detect these associations. However, RC correlated positively with the presence of known compensatory mutations in chronic viruses from B*57-expressing individuals harboring the Gag T242N mutation ($n = 50$; $R = 0.36$; $P = 0.01$), suggesting that the rescue of fitness defects occurred through mutations at secondary sites. Additional mutations in Gag that may modulate the impact of the T242N mutation on RC were identified. A modest inverse correlation was observed between RC and CD4 cell count in chronic infection ($R = -0.17$; $P < 0.0001$), suggesting that Gag-Protease RC could increase over the disease course. Notably, this association was stronger for individuals who expressed B*57, B*58, or B*13 ($R = -0.27$; $P = 0.004$). Taken together, these data indicate that certain protective HLA alleles contribute to early defects in HIV-1 fitness through the selection of detrimental mutations in Gag; however, these effects wane as compensatory mutations accumulate in chronic infection. The long-term control of HIV-1 in some persons who express protective alleles suggests that early fitness hits may provide lasting benefits.

The host immune response elicited by $CD8^+$ cytotoxic T lymphocytes (CTLs) is a major contributor to viral control following human immunodeficiency virus type 1 (HIV-1) infection (6, 39), but antiviral pressure exerted by CTLs is diminished by the selection of escape mutations in targeted regions throughout the viral proteome (7, 18, 29, 35, 41, 45, 57). A comprehensive identification of HLA-associated viral polymorphisms has recently been achieved through population-based analyses of HIV-1 sequences and HLA class I types from different cohorts worldwide (3, 8, 13–15, 34, 43, 50, 56, 63). However, despite improved characterization of the sites and pathways of immune escape, effective ways to incorporate these findings into immunogen design remain an area of debate. A better understanding of the impact of escape mutations on viral fitness may provide novel directions for HIV-1 vaccines that are designed to attenuate pathogenesis.

The development of innovative vaccine strategies that can

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overcome the extreme diversity of HIV is a key priority (4). One proposed approach is to target the most conserved T-cell epitopes, which presumably cannot escape from CTL pressure easily due to structural or functional constraints on the viral protein (55). Complementary approaches include the design of polyvalent and/or mosaic immunogens that incorporate commonly observed viral diversity (4, 38) or the specific targeting of vulnerable regions of the viral proteome that do escape but only at a substantial cost to viral replication capacity (RC) (1, 40). A chief target of such vaccine approaches is the major HIV-1 structural protein Gag, which is known to be highly immunogenic and to elicit CTL responses that correlate with the natural control of infection (22, 36, 66). Indeed, several lines of evidence support a relationship between the selection of CTL escape mutations and reduced HIV-1 fitness. These include the reversion of escape mutations following transmission to an HLA-mismatched recipient who cannot target the epitope (19, 24, 41) as well as reduced plasma viral load (pVL) set point following the transmission of certain escape variants from donors who expressed protective HLA alleles (17, 27). Notably, these *in vivo* observations have been made most often for variations within Gag that are attributed to CTL responses restricted by the protective alleles HLA-B*57 and -B*5801 (17, 19, 27, 41). Most recently, reduced *in vitro* RCs of clinical isolates and/or engineered strains encoding single or multiple escape mutations in Gag selected in the context of certain protective HLA alleles, including B*57, B*5801, B*27, and B*13, have been demonstrated (9, 10, 42, 53, 59, 62). Despite these efforts, the goal of a T-cell vaccine that targets highly conserved and attenuation-inducing sites is hampered by a lack of knowledge concerning the contribution of most escape mutations to HIV-1 fitness as well as a poor understanding of the relative influence of HLA on the viral RC at different stages of infection.

The mutability of HIV-1 permits the generation of progeny viruses encoding compensatory mutations that restore normal protein function and/or viral fitness. Detailed studies have demonstrated that the *in vitro* RC of escape variants in human and primate immunodeficiency viruses can be enhanced by the addition of secondary mutations outside the targeted epitope (10, 20, 52, 59, 65). Thus, vaccine strategies aimed at attenuating HIV-1 must also consider, among other factors, the frequency, time course, and extent to which compensation might overcome attenuation mediated by CTL-induced escape. Despite its anticipated utility for HIV-1 vaccine design, systematic studies to examine the consequences of naturally occurring CTL escape and compensatory mutations on viral RC have not been undertaken.

We have described previously an *in vitro* recombinant viral assay to examine the impact of Gag-Protease mutations on HIV-1 RC (47, 49). Gag and protease have been included in each virus to minimize the impact of sequence polymorphisms at Gag cleavage sites, which coevolve with changes in protease (5, 37). Using this approach, we have demonstrated that viruses derived from HIV-1 controllers replicated significantly less well than those derived from noncontrollers and that these differences were detectable at both the acute/early (49) and chronic (47) stages. Escape mutations in Gag associated with the protective HLA-B*57 allele, as well as putative compensatory mutations outside known CTL epitopes, contributed to this difference in RC (47). However, substantial variability was observed for viruses from controllers and noncontrollers, indicating that additional factors were likely to be involved. Benefits of this assay include its relatively high-throughput capacity as well as the fact that clinically derived HIV-1 sequences are used in their entirety. Thus, it is possible to examine a large number of "real-world" Gag-Protease sequences, to define an RC value for each one, and to identify sequences within the population of recombinant strains that are responsible for RC differences.

Here, we use this recombinant virus approach to examine the contribution of HLA-associated immune pressure on Gag-Protease RC during acute/early (n = 66) and chronic (n = 803) infections in the context of naturally occurring HIV-1 subtype B isolates from untreated individuals. In a recent report (64), we employed this system to examine the Gag-Protease RC in a similar cohort of chronic HIV-1 subtype C-infected individuals. The results of these studies provide important insights into the roles of immune pressure and fitness constraints on HIV-1 evolution that may contribute to the rational design of an effective vaccine.

MATERIALS AND METHODS

Patients and samples. The acute/early cohort was comprised of 66 antiretroviral-naïve individuals (median time postinfection of 61 days [interquartile range {IQR}, 37 to 74 days]; median pVL of 5.5 $\log_{10}$ RNA copies/ml [IQR, 4.0 to 5.9 $\log_{10}$ RNA copies/ml]; median CD4 count of 483 cells/mm^3 [IQR, 401 to 654 cells/mm^3]) from Acute Infection and Early Disease Research Program (AIEDRP) network sites in the United States and Australia as well as a private medical clinic in Germany (11). Thirty-nine patients were identified during acute infection as defined by documented positive HIV RNA (>5,000 copies/ml) or detectable serum p24 antigen and either a negative HIV-1 enzyme immunoassay (EIA) result or a positive EIA result but a negative or indeterminate Western blot result. The time frame for acute infection as defined here ranges up to 6 weeks following infection, and sample distribution is comparable to Fiebig stages 1 to 2 (n = 11) and 3, 4, and 5 (n = 28) (23). The remaining 27 individuals were identified during early HIV infection, as defined by a negative EIA result during the previous 6 months or a positive EIA result but a negative detuned HIV-1 EIA result (Vironostika-LS EIA; bioMérieux, Raleigh, NC) (33) at enrollment. These samples are comparable to Fiebig stage 6. The date of HIV infection was estimated using clinical history (where available), by subtracting 4 weeks from the baseline in cases of a negative EIA result, by subtracting 6 weeks from the baseline in cases of a positive EIA result, by calculating the midpoint between the last negative and the first positive EIA result, or by subtracting 4 months from the date of a negative detuned EIA result (11).

The chronic cohort was comprised of 803 individuals (median pVL of 5.1 $\log_{10}$ RNA copies/ml [IQR, 4.7 to 5.5 $\log_{10}$ RNA copies/ml] and median CD4 cell count of 273 cells/mm^3 [IQR, 130 to 420 cells/mm^3]). Of these individuals, 762 (94.9%) represented a baseline (antiretroviral-naïve) cross-section of the British Columbia HOMER cohort (12–14). The remaining 41 (5.1%) individuals represented chronically infected individuals recruited from Massachusetts General Hospital who were untreated at the time of sample collection (47). Although time since infection was unknown for these individuals, the relatively low median CD4 cell count indicates that this cohort was relatively progressed and thus may not be entirely representative of earlier stages of chronic infection. HLA class I typing was performed by using sequence-based methods. Ethical approval was obtained through the relevant institutional review boards.

Generation of recombinant Gag-Protease viruses. Recombinant viruses were generated on an NL4-3 background as described previously (47, 49). NL4-3 was chosen because this strain is commonly used for *in vitro* mutagenesis studies of HIV-1 replication, and its Gag sequence displays greater similarity to consensus subtype B than to other available molecular clones (13 amino acid differences from consensus subtype B, 2004). Briefly, the Gag-Protease region was amplified by reverse transcription (RT)-PCR from plasma HIV-1 RNA using sequence-specific primers. Second-round PCR was performed by using PAGE-purified recombination primers designed to match the NL4-3 sequence directly upstream of Gag (forward primer GACTC GGCTT GCTGA AGCGC GCACG GCAAG

TABLE 1. Acute/early cohort information

Parameter	Value for acute/early cohort			*P* value[b]
	Total	Protective HLA allele[a]	No protective HLA allele	
No. of individuals	66	20	46	
Median $\log_{10}$ plasma viral load (IQR[c])	5.47 (4.05–5.88)	4.44 (3.76–5.61)	5.59 (4.97–5.97)	0.02
Median CD4 cell count (IQR)	483 (401–654)	526 (428–676)	465 (377–621)	0.31
Median estimated days postinfection (IQR)	59 (36–72)	60 (40–72)	51 (32–96)	0.75

[a] Protective HLA alleles defined as B*13, B*27, B*57, and B*5801.
[b] *P* values determined by a Mann-Whitney U test.
[c] IQR, interquartile range.

AGGCG AGGGG CGGCG ACTGG TGAGT ACGCC AAAAA TTTTG ACTAG CGGAG GCTAG AAGGA GAGAG ATGGG) and downstream of protease (reverse primer GGCCC AATTT TTGAA ATTTT TCCTT CCTTT TCCAT TTCTG TACAA ATTTC TACTA ATGCT TTTAT TTTTT CTTCT GTCAA TGGCC ATTGT TTAAC TTTTG).

Plasmid pNL4-3ΔGag-Protease was developed by inserting unique BstEII restriction sites at the 5′ end of Gag and the 3′ end of the protease by using the QuikChange XL kit (Stratagene), followed by the deletion of the Gag-Protease region by BstEII digestion (New England Biolabs). This plasmid was maintained by using *Escherichia coli* Stbl3 cells (Invitrogen). To generate recombinant viruses, 10 μg of BstEII-linearized plasmid plus 50 μl of the second-round amplicon (approximately 5 μg) were mixed with 2.0×10^6 cells of a Tat-driven green fluorescent protein (GFP) reporter T-cell line (GXR 25 cells [41]) in 800 μl of $R10^+$ medium (RPMI 1640 medium containing 10% fetal calf serum [FCS], 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) and transfected by electroporation using a Bio-Rad GenePulser II instrument (exponential protocol of 300 V and 500 μF). Following transfection, cells were rested for 45 min at room temperature, transferred into 25-mm^3 flasks in 5 ml of $R10^+$ medium, and fed with 5 ml $R10^+$ medium on day 5. GFP expression was monitored by flow cytometry (FACSCalibur; BD Biosciences), and once GFP-positive (GFP^+) expression reached >15% among viable cells, supernatants containing the recombinant viruses were harvested, and aliquots were stored at −80°C.

Replication capacity assays. Virus titers and replication assays were performed as described previously (47, 49). Replication assays were initiated at a multiplicity of infection (MOI) of 0.003 and included six negative (uninfected cells only) and six NL4-3 infection controls. For each virus, the natural-log slope of the percentage of GFP^+ cells was calculated during the exponential phase of viral spread (days 3 to 6). This value was divided by the mean rate of spread of wild-type (WT) NL4-3 to generate a normalized, quantitative measure of the replication capacity (RC). An RC value of 1.0 indicates a rate of viral spread that was equal to that of NL4-3, while RC values of <1.0 and >1.0 indicate rates of spread that were higher than or lower than those of NL4-3, respectively. Duplicate or triplicate assays were performed in independent experiments, and average replication rates are reported. Quality control experiments were done to test assay reproducibility and to assess the potential impact of recombination on the NL4-3 RC. RC values determined using bulk products were highly concordant for a given patient sample. Furthermore, the reintroduction of cloned NL4-3 sequences into the NL4-3 backbone did not significantly alter viral fitness.

Viral sequencing and sequence analysis. Bulk plasma HIV-1 RNA sequences were previously collected for all individuals in the acute/early (11) and chronic (14) infection cohorts. Recombinant viral stocks for all acute/early infections and the majority (528 of 803; 65.7%) of chronic viruses were sequenced to confirm patient origin and to assess diversity. Bulk HIV-1 RNA was extracted from viral culture supernatants by using the QIAamp viral RNA kit (Qiagen), amplified by nested RT-PCR using sequence-specific primers, sequenced bidirectionally on an ABI 3730xl sequencer (Applied Biosystems), and analyzed by using Sequencher 4.9 software (Gene Codes). Nucleotide mixtures were called if the secondary-peak height exceeded 25% of the dominant-peak height. All viruses were confirmed as subtype B by comparison to reference sequences (http://www.hiv.lanl.gov). Nucleotide alignments were performed by using a modified NAP algorithm (32), and maximum likelihood phylogenetic trees were generated by using PHYmL (30). Trees were visualized by using Figtree v.1.2.2 (http://tree.bio.ed.ac.uk/software/figtree). Chronic HIV-1 sequences were previously deposited in GenBank (14, 47).

Statistical analysis. All statistical analyses are identified in the text. An unpaired *t* test was used to compare differences in replication capacity between groups (e.g., presence versus absence of HLA alleles). The relationship between the number of HLA-associated escape mutations and RC was assessed by using Pearson's correlation, while Spearman's correlation was used to investigate the relationship between HIV-1 clinical parameters (CD4 cell count and pVL) and RC. In an exploratory analysis, pairwise Mann-Whitney U tests were used to identify specific amino acids in Gag and protease associated with RC. Multiple tests were addressed by using a *q* value approach (60).

Nucleotide sequence accession numbers. Data for acute/early viral sequences have been deposited in the GenBank database under accession numbers GU390464 to GU390529.

RESULTS

We have utilized a high-throughput recombinant virus assay to examine the impact of HLA-associated mutations in the Gag and protease proteins on viral replication capacity (RC) in order to elucidate the consequences of immune-mediated pressure on HIV-1 fitness during the natural course of infection.

Construction of Gag-Protease recombinant viruses from acute/early and chronic infections. Recombinant viruses were generated from 66 acute/early-infected individuals and 803 untreated individuals at a relatively late stage of chronic infection (Tables 1 and 2). The Gag regions of all acute/early viruses and the majority of chronic viruses were resequenced to confirm patient origin and to assess viral diversity. As expected, limited diversity was observed in the acute/early virus bulk

TABLE 2. Chronic cohort information

Parameter	Value for chronic cohort			*P* value[b]
	Total	Protective HLA allele[a]	No protective HLA allele	
No. of individuals	803	165	638	
Median $\log_{10}$ plasma viral load (IQR[c])	5.08 (4.65–5.46)	4.96 (4.42–5.43)	5.11 (4.72–5.47)	0.02
Median CD4 cell count (IQR)	273 (130–420)	290 (120–430)	270 (130–420)	0.91

[a] Protective HLA alleles defined as B*13, B*27, B*57, and B*5801.
[b] *P* values determined by a Mann-Whitney U test.
[c] IQR, interquartile range.

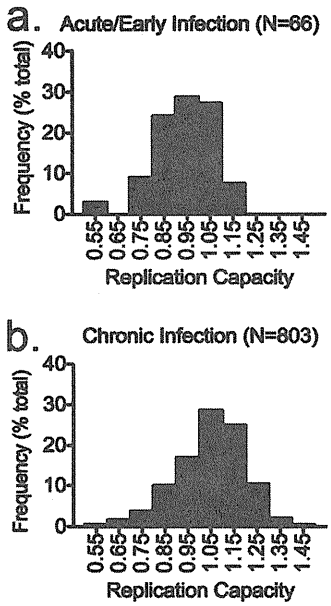

FIG. 1. *In vitro* RCs of recombinant NL4-3-derived viruses encoding patient-derived Gag-Protease sequences. The distribution of viral RC is shown for samples collected during acute/early infection (n = 66) (a) and chronic infection (n = 803) (b). Viruses were generated and RCs were measured by using a GFP reporter T-cell line, as described in Materials and Methods. The average RC was determined for each recombinant virus using data from two (acute/early infection) or three (chronic infection) independent assays. Results were normalized to mean RCs of WT NL4-3 controls assessed in parallel such that values greater than or less than 1.0 indicate viruses that replicated faster or slower than NL4-3, respectively. Gag-Protease RC values were not significantly different between cohorts (the median RC was 1.01 for both cohorts) and were distributed normally around means of 0.98 (±0.12) for acute/early infection samples and 1.00 (±0.15) for chronic infection samples.

plasma HIV-1 RNA as well as recombinant virus sequences (~55% of plasma and recombinant viruses were clonal at the amino acid level, while the remainder exhibited at least one amino acid mixture). However, a comparison of chronic plasma versus recombinant viral sequences revealed reduced viral diversity in the latter compared to that of the former: 6% of plasma versus 40% of recombinant sequences were clonal at the amino acid level ($P < 0.0001$), which is indicative of an *in vitro* genetic bottleneck, likely at the homologous recombination stage. Despite reduced diversity, recombinant viral sequences were highly concordant with the original bulk plasma sequences: the median number of full amino acid differences observed between plasma and recombinant virus was 1 out of 500 codons in Gag (<0.2% [IQR, 0% to 2%]).

In vitro RC was measured for each Gag-Protease recombinant virus as described previously (47, 49). The NL4-3 normalized median RC was highly concordant between cohorts (1.01 [IQR, 0.91 to 1.07] for acute/early viruses and 1.01 [IQR, 0.91 to 1.10] for chronic viruses; $P = 0.29$) (Fig. 1) and indicated that, on average, the *in vitro* function of patient-derived Gag-Protease sequences was comparable to that of NL4-3. To investigate the relationship between recombinant virus quasispecies diversity and RC, we compared the RC of acute/early viruses encoding clonal Gag sequences (n = 37) to those containing at least one amino acid mixture (n = 29), and we observed no significant difference in RCs between the two (P = 0.51). A similar analysis undertaken on the chronic virus data set revealed a slight growth advantage for viruses containing amino acid mixtures: the median RC of chronic recombinant viruses with clonal Gag sequences (n = 210) versus that for viruses with at least one amino acid mixture in Gag (n = 318) was 0.98 versus 1.01, respectively (P = 0.01). Taken together, these data suggest that recombinant stocks containing more than one viral species replicated marginally better in our cell culture system; however, the magnitude of this effect (0.03 units, equivalent to 20% of 1 standard deviation in the data set) was relatively minor.

Reduced RC of acute/early viruses from individuals expressing certain protective HLA alleles. Based on the results of previous studies (9, 10, 42, 46, 53, 59), we hypothesized that if viral escape mutations in Gag were to significantly reduce HIV-1 fitness, this would most likely occur early following infection and in the context of immune pressure elicited by protective HLA class I alleles. To assess this, we divided acute/early infection samples into two groups based upon host expression of protective HLA alleles that are known to target epitopes in Gag (B*13, B*27, B*57, and B*5801) (16, 31). The time of sample collection postinfection and the proportion of recombinant viruses exhibiting clonal sequences were similar between groups (Table 1 and not shown). However, pVL was significantly lower for acute/early-infected individuals who expressed a protective HLA allele than for those who did not (Table 1). Notably, we observed that recombinant Gag-Protease viruses derived from individuals who expressed at least one of these protective HLA alleles demonstrated significantly lower *in vitro* RCs than those who did not ($P < 0.0001$) (Fig. 2a). In an analysis stratified by the individual HLA-B alleles expressed, viruses derived from HLA-B*57, -B*5801, and -B*13 displayed the lowest RCs overall (Fig. 2b). Note that one individual expressed both B*5801 and B*13, but similar results were observed after the removal of this sample (not shown). Stratification by expressed HLA-A and HLA-C alleles revealed no significant associations with Gag-Protease RC (not shown).

Evidence for early immune selection as a correlate of reduced RC. We hypothesized that alterations in the RC in acute/early infection were due to the rapid selection of immune escape mutations by host HLA alleles. We assessed this hypothesis in three ways. First, we investigated the presence of HLA-associated escape mutations previously associated with reduced fitness in the four viruses with the lowest overall RC values in the cohort (which were derived from individuals expressing B*5701 [n = 2], B*1302, and B*5801) and compared these viruses to viruses derived from the remaining individuals who expressed B*57/B*5801 (n = 4) or B*13 (n = 4) (9, 10, 42, 53). All four viruses exhibiting the lowest RC values harbored mutations previously associated with reduced fitness, including mutations at Gag residues 147 (2 of 3 viruses from B*57- or B*5801-expressing individuals), 242 (3 of 3 viruses), 248 (2 of 3 viruses), and 340 (1 of 3 viruses). An HLA B*57/B*5801-associated Gag mutation at codon 173 was also seen for all three of these viruses; however, to our knowledge, the consequence of this mutation on fitness has not been evaluated. Furthermore, the recombinant virus derived from the B*13-

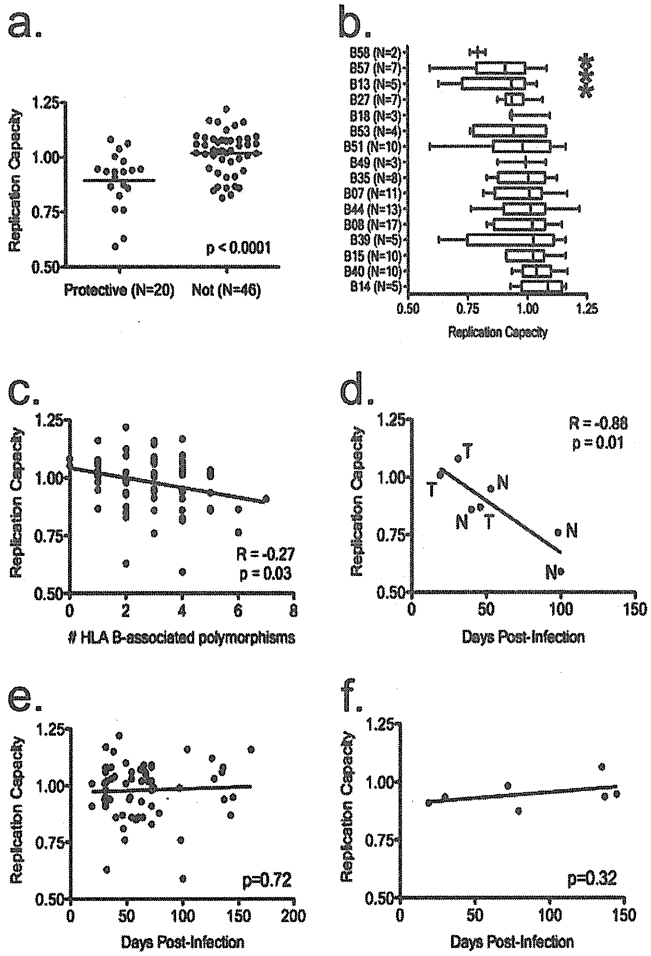

FIG. 2. Host HLA-B expression is associated with Gag-Protease RC in acute/early infection. (a) Significantly lower RCs were observed among viruses derived from individuals who expressed HLA-B*13, -B*27, -B*57, and/or -B*5801 ("protective") ($n = 20$) than among viruses derived from individuals who lacked these HLA alleles ("non-protective") ($n = 46$) ($P < 0.0001$ by a t test). The median RC for each group is indicated by a horizontal line. (b) Host expression of HLA-B*13, -B*57, and -B*5801 contributed to lower RCs ($P = 0.04$, $P = 0.01$, and $P = 0.02$, respectively, by a t test) (indicated by asterisks), while the expression of B*27 did not ($P = 0.50$). Results for individual alleles were not significant after correction for multiple comparisons (all $q > 0.2$). Median RC values (lines), interquartile ranges (boxes), and extreme values (whiskers) are indicated for each group. (c) Total number of Gag polymorphisms associated with host HLA-B alleles inversely correlated with the RC ($R = -0.27$; $P = 0.03$). (d) Among seven acute/early viruses derived from HLA-B*57-expressing hosts, a significant inverse correlation was observed between RC and the estimated time postinfection when the sample was collected ($R = -0.88$; $P = 0.01$). The viral sequence at residue 242 (T or N) is indicated as a reference. (e and f) No similar association with time was observed among viruses for the entire cohort (e) or among viruses derived from HLA-B*27-expressing hosts (f). The slope of each association is indicated by a solid line.

expressing individual harbored a known escape mutation at Gag codon 437 within the B*13-RI9 ($Gag_{428-437}$) epitope. In contrast, these polymorphisms were observed less frequently in the more-fit recombinant viruses derived from the remaining individuals expressing these alleles, including those at codon 147 (1 one 4 viruses), 173 (1 of 4 viruses), 242 (1 of 4 viruses), 248 (2 of 4 viruses), or 340 (0 of 4) in the presence of B*57/B*5801 and at codon 437 (0 of 4 viruses) in the presence of B*13. These data are consistent with the *de novo* selection of escape mutations by host HLA-restricted CTL responses in each of these cases.

Second, we used a previously reported list of HLA-associated polymorphisms derived from statistical analyses of an international cohort of more than 1,500 antiretroviral-naïve, chronically infected individuals, which incorporated a correction for HLA linkage disequilibrium and HIV codon covariation (14), to explore the relationship between HLA-associated Gag polymorphisms and viral RC in a more systematic way. We observed a significant inverse correlation between the total number of host HLA-associated polymorphisms in acute/early Gag sequences and RC ($R = -0.30$; $P = 0.01$) (not shown). This result appeared to be driven largely by polymorphisms selected in the presence of the host's HLA-B alleles ($R = -0.27$; $P = 0.03$) (Fig. 2c). Significant correlations between RC and HLA-A- or HLA-C-associated Gag polymorphisms were not observed (not shown).

Finally, we hypothesized that CTL-mediated selection of escape mutations will occur gradually and therefore that changes in viral RC would become more apparent over time following infection. We therefore correlated RC with the estimated time postinfection when each sample was collected. Despite the cross-sectional nature of this analysis and limited data, we observed a significant inverse correlation between RC and days postinfection for the seven viruses derived from HLA B*57-expressing individuals ($R = -0.88$; $P = 0.01$) (Fig. 2d). This result is consistent with an effect of B*57-mediated immune pressure on Gag-Protease that results in the selection of escape mutations that attenuate viral fitness during the first months following infection. Indeed, viruses derived from earlier samples (collected at 19, 31, and 46 days postinfection) encoded the wild-type T at position 242, while those derived from later samples (days 40, 53, 98, and 100) contained the T242N escape mutation within the B*57-TW10 ($Gag_{240-249}$) epitope. In contrast to B*57-expressing individuals, no association between RC and time since infection was observed when we examined the entire cohort (Fig. 2e) or when we assessed viruses derived from individuals expressing B*27 ($n = 7$) (Fig. 2f), suggesting that the early attenuation of HIV-1 may be limited to a relatively small subset of HLA alleles. Altogether, these data support a dominant influence of some host HLA-B alleles, particularly B*57, on Gag-Protease fitness in early infection.

No evidence for transmitted escape mutations as a correlate of reduced RC. The acquisition of HIV-1 harboring Gag polymorphisms restricted by protective HLA alleles has been shown to affect acute-phase viral load (17, 27), presumably through alterations in viral fitness. We therefore investigated the relationship between transmitted mutations in Gag-Protease and viral RC. Using the same list of HLA-associated polymorphisms described above, we identified mutations in each virus that were likely to be selected in the previous host. However, we observed no significant correlations between acute/early viral RC and the total number of Gag polymorphisms associated with these putative transmitted escape mutations (not shown).

Identification of specific mutations associated with acute/early virus RC. To identify specific amino acids in Gag-Protease associated with acute/early virus RC, we performed a systematic pairwise analysis of all observed amino acid variants and viral RCs. This analysis revealed correlations between RC and 18 polymorphisms located at 14 codons in Gag ($P < 0.05$), although none reached statistical significance after correction for multiple comparisons (all $q > 0.2$) (see Table S1 in the supplemental material). A lower median RC was observed for strains encoding the Gag V7I, S53T, S67A, E260D, S342X, Y484X, or L486X mutation as well as those encoding a deletion at residue T371. Notably, several consensus subtype B residues in Gag were also seen to be significantly associated with a lower RC, including L34, Q55, L75, V218, I479, and L498, which may help to explain the observation that many patient-derived viruses displayed higher RCs than did the NL4-3 control. Polymorphisms at a number of these sites were associated previously with HLA-mediated selection in Gag (14), and well-documented CTL epitopes overlap many of these regions (see the HIV molecular immunology database at www.hiv.lanl.gov). Despite its association with reduced RCs, no HLA B*57-associated polymorphisms were observed at a P value of <0.05, although trends were seen for Gag polymorphisms at several known B*57-targeted residues, including the T242N (present in 6 of 66 viruses; $P = 0.06$) and I147L (present in 16 of 66 viruses; $P = 0.11$) mutations.

Contribution of host HLA alleles to viral RC in chronic infection. In light of the observed effects of host HLA on the Gag-Protease RC during acute/early infection, we wished to investigate whether HLA-associated viral attenuation persisted into later stages of infection. To do this, we constructed and evaluated a large panel of recombinant viruses ($n = 803$) from untreated individuals with relatively advanced chronic infection (Table 2). In contrast to acute/early infection results, no significant association was observed between host expression of protective HLA alleles (B*13, B*27, B*57, and B*5801) and chronic viral RC ($P = 0.28$) (Fig. 3a) despite a greatly increased power to detect such differences. Furthermore, the stratification of data by individual HLA alleles also failed to reveal associations between most of these alleles and RC (Fig. 3c). Of interest, the stratification of the RC by individual HLA alleles revealed a relatively broad range of RC values for HLA-B (from medians of 0.89 for B*56 to 1.06 for B*37) (Fig. 3c) but narrower ranges for HLA-A and -C (Fig. 3b and d), supporting a greater influence of HLA-B on chronic viral fitness. HLA alleles A*26, A*31, B*48, B*53, and B*5801 were independently associated with lower viral RCs in chronic infection (all $q < 0.2$).

Impact of the number of HLA-associated polymorphisms on viral RC in chronic infection. In contrast to acute/early infection, no significant relationship was observed between the overall total number of HLA-A-, HLA-B-, or HLA-C-associated polymorphisms and viral RC in chronic infection (not shown). Due to the substantially increased power in the chronic cohort, it was additionally possible to undertake these analyses at the individual HLA allele level. Of interest, we observed significant inverse correlations between RC and the number of HLA-specific polymorphisms restricted by A*25 ($n = 30$) ($R = -0.37$; $P = 0.04$), A*26 ($n = 46$) ($R = -0.35$; $P = 0.02$), B*14 ($n = 50$) ($R = -0.30$; $P = 0.04$), B*41 ($n = 12$)

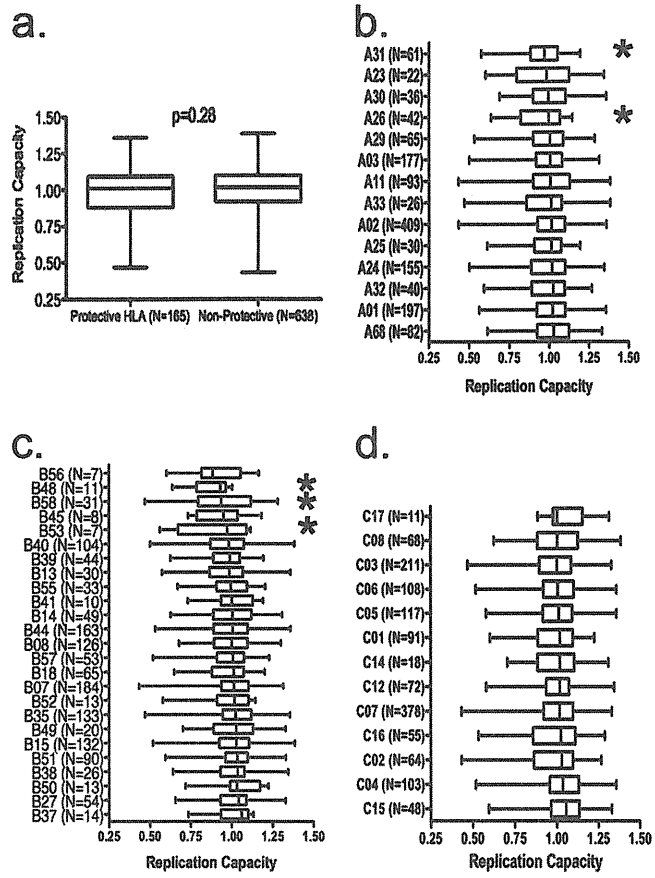

FIG. 3. Lack of strong associations between protective HLA alleles and RC in chronic infection. (a) The RC of viruses derived from chronically infected individuals who expressed the protective HLA alleles B*13, B*27, B*57, and/or B*5801 was not significantly different from those of viruses derived from individuals who did not express these alleles. (b and c) When host HLA alleles were analyzed individually, several alleles were associated with significantly lower Gag-Protease viral RCs after correction for multiple comparisons ($q < 0.2$) (indicated by asterisks), namely, A*26 and A*31 (b) as well as B*48, B*53, and B*58 (c). (d) No associations with chronic infection RC were observed for HLA-C alleles. Median RC values (lines), interquartile ranges (boxes), and extreme values (whiskers) are indicated for each group.

($R = -0.89$; $P < 0.0001$), and B*55 ($n = 33$) ($R = -0.53$; $P = 0.002$) for individuals expressing these alleles (Fig. 4). Associations for HLA-A*26, -B*41, and -B*55 remained significant after correction for multiple comparisons ($q < 0.2$). These results suggest a potential dose-dependent relationship between the induction of escape mutations by these HLA alleles and reduced RCs in some individuals. Notably, A*26 was also associated with a lower overall RC (Fig. 3c). The finding that these other alleles are not broadly associated with reduced fitness suggests that their effects may be driven by Gag responses that are poorly elicited in many cases or are due to atypical escape mutations that result in greater viral attenuation.

Since the transmission of HLA-associated Gag mutations may alter viral fitness, the potential impact of polymorphisms that could not be attributed to the current host's HLA profile (i.e., escape mutations acquired at transmission) was also ex-

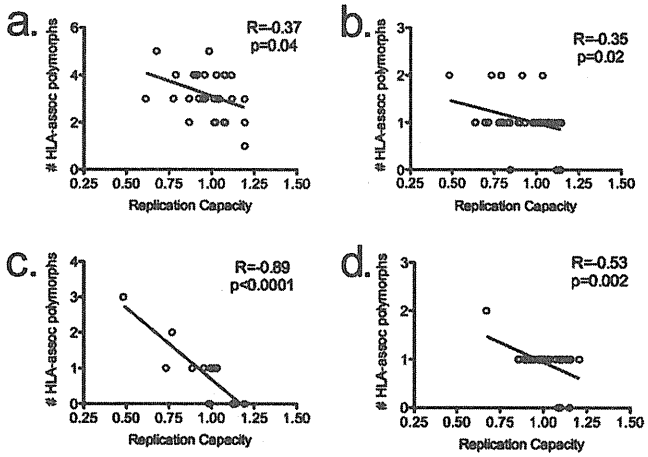

FIG. 4. Cumulative effects of HLA-associated polymorphisms on viral RC during chronic infection. RC measurements were compared to the total number of Gag mutations present in each virus that could be attributed to the host's HLA, as defined previously (14). Significant associations were observed between the number of HLA-associated polymorphisms specific for A*25 (Pearson $R = -0.37$; $P = 0.04$) (a), A*26 ($R = -0.35$; $P = 0.02$) (b), B*41 ($R = -0.89$; $P < 0.0001$) (c), and B*55 ($R = -0.53$; $P = 0.002$) (d) and lower RCs in individuals who expressed these alleles, suggesting a dose-dependent impact of new Gag polymorphisms selected for by these HLA alleles. Associations for HLA-A*26, -B*41, and -B*55 remained significant after correction for multiple comparisons ($q < 0.2$). The slope of each association is indicated by a solid line.

amined. Consistent with results from acute/early infection studies, no associations were observed between the viral RC and the total number of HLA-A-, HLA-B-, or HLA-C-associated polymorphisms for alleles that were not expressed by the host (not shown). This result suggests that putative transmitted mutations could be observed for chronic viruses but that these polymorphisms were not a major determinant of RC for this cohort. However, the possibility that fitness-reducing mutations were transmitted and subsequently reverted cannot be ruled out.

Associations between Gag sequence polymorphisms and viral RC in chronic infection. A systematic analysis of viral amino acids associated with the RC in chronic infection was also undertaken. A total of 63 polymorphisms located at 40 codons in Gag and two polymorphisms located at codon 61 in protease that were associated with viral RC at a q value of <0.2 were identified (see Table S2 in the supplemental material). Of the Gag codons associated with viral RC, 22 (55%) are under selection pressure by at least one HLA allele (14). Polymorphisms correlating with lower viral RCs in chronic infection included Gag E12Q (associated with B*49), K28T (A*03, A*24, and C*17), R30K (B*15), R58G (B*49), S67A (C*03), T122I (A*33), A146P (B*13, B*39, B*57, and C*08), S148T (B*53, C*02, C*06), V218Q (B*40), V223L (A*25, B*55, B*56), F383T (A*31 and B*27), T389I (B*13, B*27, B*39, B*42, and B*44), T427P (A*26, B*40, and B*58), and E482D (B*40 and B*78). Of note, changes at Gag codons 67, 218, 479, and 486 were identified as being associated with RC in both the acute/early and chronic infection data sets.

Impact of secondary mutations in Gag on the B*57-associated T242N mutation in chronic infection. In contrast to acute/early infection, B*57-derived recombinant viruses did not display significantly reduced RCs in chronic infection. In order to further investigate this observation, we examined chronic viral sequences for the presence of HLA B*57-associated polymorphisms. As expected, we observed a significant enrichment of known Gag CTL escape mutations, A146P, I147L, T242N, and others, in chronically infected B*57-expressing individuals; however, we failed to observe a significant correlation between the overall number of B*57-associated polymorphisms in these sequences and viral RC (not shown). Taken together, these observations indicate that during chronic infection, the relationship between viral sequence and RC is more complex than the raw number of primary escape mutations.

We therefore hypothesized that the lack of a B*57 association with RC at the chronic stage was due to the accumulation of compensatory mutations during the natural course of infection. To assess the potential relationship between compensatory mutations and viral RC in chronic infection, we examined the sequences of 50 viruses derived from B*57-expressing individuals harboring the T242N mutation for evidence of secondary mutations at Gag H219Q, I223V, M228I, and G248A. These mutations have been shown to restore the *in vitro* RC of T242N variants, presumably by altering the ability of capsid to interact with the host cyclophilin A protein (10, 41, 42). In viruses encoding T242N, we observed a significant positive correlation between RC and the number of compensatory mutations in the recombinant viral sequence ($R = 0.36$; $P = 0.01$) (Fig. 5a). Similar results were obtained if all T242N mutation-containing viruses (regardless of the host HLA type) were analyzed ($n = 72$; $R = 0.33$; $P = 0.004$) (not shown). Together, these data indicate that the effects of the T242N escape mutation on RC may be restored by secondary mutations in a cumulative manner.

Limited experimental data exist to show the consequence of secondary mutations following CTL escape on HIV-1 fitness. We hypothesized that other polymorphisms in Gag might alter the impact of the T242N mutation, and therefore, we examined our chronic data set for evidence of additional secondary mutations that were associated with either an enhanced or reduced RC in context of T242N. Polymorphisms at Gag codons 12 ($P = 0.027$), 127 ($P = 0.027$), 146 ($P = 0.003$), 315 ($P = 0.015$), 483 ($P = 0.015$), and 488 ($P = 0.016$) were associated with lower RCs, indicating a deleterious effect of these mutations in combination with T242N (Fig. 5b). Polymorphisms at residues 146 and 315 are consistent with escape from HLA B*57-restricted CTL responses to the IW9 ($Gag_{147\text{-}155}$) and QW9 ($Gag_{308\text{-}316}$) epitopes, but only Gag codon 146 remained significant after correction for multiple comparisons ($q < 0.2$). These results support previous work indicating an additive effect of B*57-mediated escape mutations on viral fitness (9, 19).

In addition, we observed that polymorphisms at Gag codons 84 ($P = 0.005$), 132 ($P = 0.008$), 218 ($P = 0.030$), 219 ($P = 0.015$), 228 ($P = 0.0006$), 248 ($P = 0.002$), 370 ($P = 0.031$), and 487 ($P = 0.014$) were associated with higher RCs in T242N mutation-containing viruses (Fig. 5c). Of these polymorphisms, those present at codons 219, 228, and 248 were described previously to function as compensatory mutations for the T242N mutation (10, 41, 42). Note that only codons 228 and 248 remained significant after correction for multiple compar-

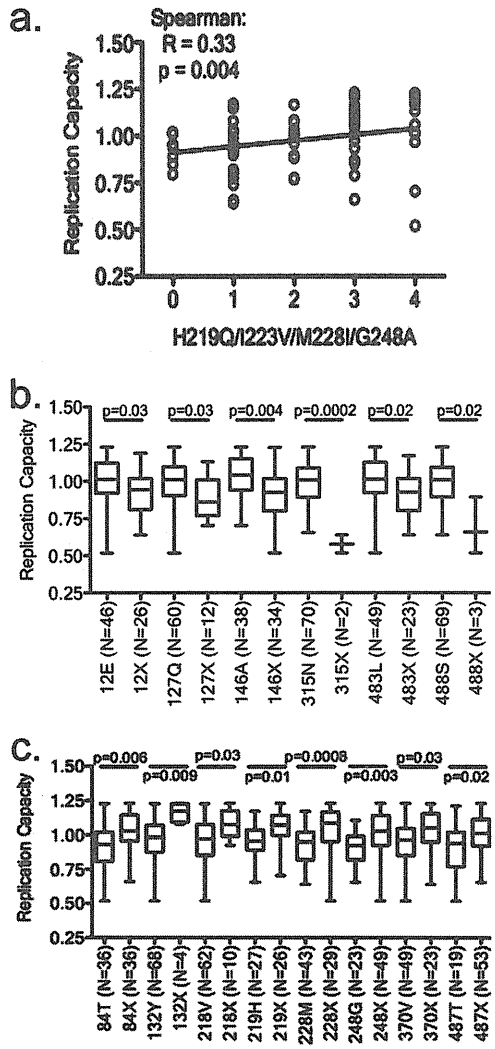

FIG. 5. Impact of secondary mutations in Gag on RC of chronic viruses carrying the T242N mutation. The presence of Gag H219Q, I223V, M228I, and G248A mutations was examined for recombinant viruses derived from B*57-expressing hosts that carried the T242N mutation ($n = 50$). (a) A significant correlation was observed between the RC and the total number of sites harboring previously described compensatory mutations (Pearson $R = 0.36$; $P = 0.01$). The slope of the association is indicated by a solid line. To identify novel secondary mutations that might alter the fitness of T242N mutation-carrying viruses, pairwise Mann-Whitney U tests were conducted to compare the RCs of 72 chronic viruses from all patients that harbored the T242N mutation with all variable residues in Gag. (b and c) Results indicated significant associations between mutations at Gag codons 12, 127, 146, 315, 483, and 488 with lower RCs (b) and between mutations at Gag codons 84, 132, 218, 219, 228, 248, 370, and 487 with higher RCs (c) (all $P < 0.05$). Associations at codons 146, 228, and 248 remained significant after correction for multiple comparisons ($q < 0.2$). Median RC values (lines), interquartile ranges (boxes), and extreme values (whiskers) are indicated for each group.

isons ($q < 0.2$). Altogether, our results confirm and extend the list of secondary mutations in Gag that might enhance or compensate for fitness costs associated with the T242N mutation.

Clinical associations with viral RC in chronic infection. Finally, we wished to examine associations between Gag-Protease RC and clinical parameters (Fig. 6). A modest positive correlation was observed between RC and plasma viral load (pVL) ($R = 0.12$; $P = 0.0007$), while an inverse correlation was observed between RC and the CD4 cell count ($R = -0.17$; $P < 0.0001$) in chronic infection. Restricting this analysis to individuals expressing protective HLA alleles associated with lower acute/early fitness in our study (B*13, B*57, or B*5801) ($n = 110$) revealed stronger associations between the RC and pVL ($R = 0.27$; $P = 0.005$) and CD4 cell counts ($R = -0.33$; $P = 0.0005$). Although these analyses are derived from cross-sectional data, results suggest that Gag fitness may increase over the course of chronic infection. The stronger Rho obtained from the analysis of protective alleles suggests that the increase in viral RC over the infection course may be greater for these alleles than for others, which is consistent with our observation of reduced RCs in acute/early infection with recombinant viruses derived from individuals expressing protective alleles.

DISCUSSION

An effective AIDS vaccine must overcome the extreme genetic diversity of HIV-1 (4, 26, 61). Targeting of the most conserved regions of the viral proteome has been proposed as a means to elicit robust, long-lasting CD8 T-cell responses. Indeed, highly conserved HIV-1 epitopes that escape very slowly during natural infection have been identified, for example, the B*57-restricted KF11 ($Gag_{162-172}$) (20) and B*27-restricted KK10 ($Gag_{263-272}$) (25, 29) epitopes. Structural constraints on these regions of the viral capsid likely require compensatory mutations to occur concurrently in order for viruses encoding these altered epitopes to remain viable (20, 58, 59, 65). A vaccine approach that targets these and other regions of Gag may also force HIV-1 to adopt an attenuated phenotype through the selection of detrimental CTL escape mutations. This seems plausible, since some conserved epitopes escape relatively rapidly in the presence of CTL pressure but revert to the wild-type sequence upon transmission to an HLA-mismatched recipient. The B*57-restricted TW10 ($Gag_{240-249}$) epitope is an example of one such case where escape mutations reduced viral fitness; however, this mutation also appears to be restored through the subsequent acquisition of compensatory mutations (8, 10, 42). The functional consequences of escape and potential pathways for compensation have been described for only a limited number of selected HIV-1 mutations, and data remain biased toward certain protective HLA alleles. Therefore, it is not known whether escape mutations selected in the presence of other alleles will display similar functional limitations that might be exploited for vaccine design.

Here, we have examined clinical Gag and protease sequences from individuals during acute/early infection and relatively advanced chronic infection to investigate the impact of HLA-associated immune selection pressures on HIV-1 fitness. We observed that Gag-Protease function, as indicated by the *in vitro* RC, was significantly lower for acute/early viruses derived from individuals who expressed a protective HLA class I allele, either B*13, B*57, or B*5801. This was associated with the presence of known HLA-associated polymorphisms in Gag that were expected in the context of each host's HLA class I

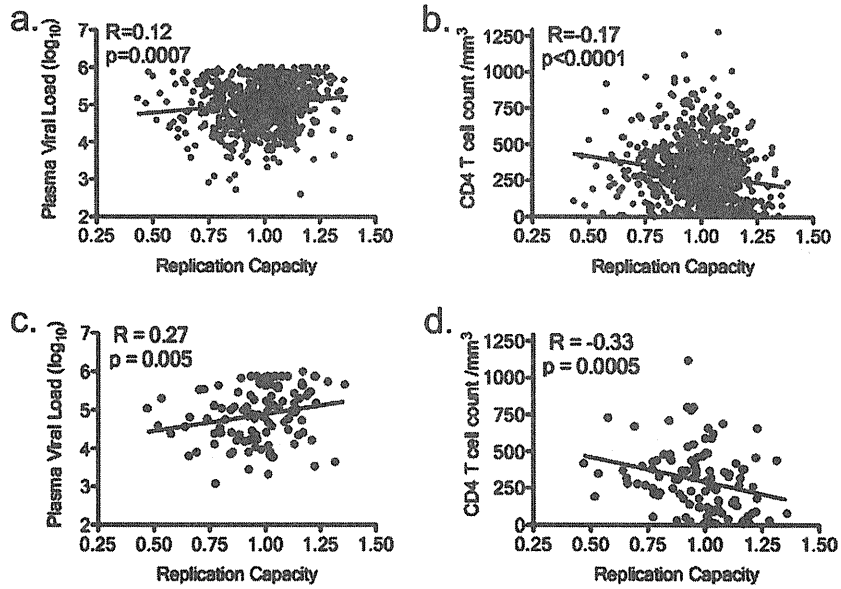

FIG. 6. Clinical associations with Gag-Protease RC in chronic infection. RC was compared to clinical pVL and peripheral blood CD4 T-cell counts at the time of collection for all chronic samples (a and b) and for the subset of samples collected from individuals who expressed a protective HLA allele, either B*13, B*57, or B*5801 (n = 110) (c and d). (a and b) Among all subjects, modest statistically significant correlations were observed between RC and pVL (a) (Pearson $R = 0.14$; $P < 0.001$) and CD4 cell count (b) ($R = -0.21$; $P < 0.0001$). (c and d) For individuals who expressed protective HLA alleles, we observed stronger associations between RC and pVL (c) ($R = 0.27$; $P = 0.005$) as well as CD4 cell count (d) ($R = -0.33$; $P = 0.0005$). The slope of each association is indicated by a solid line.

genotype. Notably, for viruses derived from acute/early-infected, B*57-expressing individuals, we observed that the RC correlated inversely with the number of days elapsed since the estimated infection date. Although based on a cross-sectional analysis, this result is consistent with the appearance of escape mutations in Gag early following infection (2, 7, 11, 21, 28, 44, 54) and their continued selection over time (29), leading to cumulative reductions in the viral replication capacity during the early stages of HIV-1 infection (8, 19). Our observation that the RC of viruses from acute/early infection correlated inversely with the total number of host HLA-B-associated polymorphisms present in Gag also supports this model. Taken together, these results suggest that an accumulation of primary escape mutations, selected predominantly by HLA-B alleles, contributes to reduced Gag function in a cumulative manner during acute/early infection. More detailed longitudinal analyses of Gag protein function will be necessary to evaluate this association further.

In an exploratory analysis of acute/early viruses, we identified polymorphisms at 14 codons that were significantly associated with viral RC (see Table S1 in the supplemental material). A majority of these sites (8 of 14 sites) were associated previously with HLA pressure (14), and all of them lay within known CTL epitopes. However, we were surprised to observe an overall lack of significant associations between RC and specific escape mutations restricted by protective HLA alleles. Trends were observed for several epitopes targeted by HLA-B*57/*5801, suggesting that the effect of these HLA alleles on RC may require multiple mutations to act in concert. The significant inverse association identified between the RC and the total number of host-specific HLA-B polymorphisms in Gag further suggests that the observed functional defects result from an additive effect of mutations, perhaps selected by more than one HLA-B allele during acute/early infection. It should be noted that our data set of 66 acute/early viruses was insufficiently powered to comprehensively identify specific amino acids associated with viral RC and to fully investigate potential correlations between RC, HLA, and clinical parameters.

Using a panel of 803 recombinant viruses generated from patients with chronic infection, we failed to observe an association between these same protective HLA alleles and Gag-Protease RC. However, a number of significant associations between RC and host HLA alleles were apparent. Our results indicated that host expression of HLA-A*26, -A*31, -B*48, -B*53, and -B*5801 may be associated with lower Gag function during chronic HIV-1 infection. Of these alleles, A*26, A*31, and B*5801 have been associated with a lower relative hazard for progression to AIDS in natural-history studies (51). In this study, only the host expression of HLA B*5801 correlated with a lower Gag-Protease RC in both acute/early infection and chronic infection.

The lack of a major association between HLA-B*57 and viral fitness in our chronic cohort may be due to the relatively late-stage infection of these individuals. In a previous study, we observed a modest association between HLA-B*57/B*5801 and a reduced Gag-Protease RC in a smaller cohort of chronically infected individuals with higher CD4 cell counts (48). Indeed, it is possible that the functional impact of early immune pressure by protective HLA class I alleles on Gag wanes over the disease course. This outcome is consistent with the ability of HIV-1 to evolve and to develop compensatory mutations, which appeared to be particularly relevant for the chronic infection samples analyzed here. Notably, we observed that nearly all viruses derived from chronically infected B*57-