HLA-Associated Alterations in Replication Capacity of Chimeric NL4-3 Viruses Carrying *gag-protease* from Elite Controllers of Human Immunodeficiency Virus Type 1[▽]

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Human immunodeficiency virus type 1 (HIV-1)-infected persons who maintain plasma viral loads of <50 copies RNA/ml without treatment have been termed elite controllers (EC). Factors contributing to durable control of HIV in EC are unknown, but an HLA-dependent mechanism is suggested by overrepresentation of “protective” class I alleles, such as B*27, B*51, and B*57. Here we investigated the relative replication capacity of viruses (VRC) obtained from EC ($n = 54$) compared to those from chronic progressors (CP; $n = 41$) by constructing chimeric viruses using patient-derived *gag-protease* sequences amplified from plasma HIV RNA and inserted into an NL4-3 backbone. The chimeric viruses generated from EC displayed lower VRC than did viruses from CP ($P < 0.0001$). HLA-B*57 was associated with lower VRC ($P = 0.0002$) than were other alleles in both EC and CP groups. Chimeric viruses from B*57⁺ EC ($n = 18$) demonstrated lower VRC than did viruses from B*57⁺ CP ($n = 8$, $P = 0.0245$). Differences in VRC between EC and CP were also observed for viruses obtained from individuals expressing no described “protective” alleles ($P = 0.0065$). Intriguingly, two common HLA alleles, A*02 and B*07, were associated with higher VRC ($P = 0.0140$ and 0.0097 , respectively), and there was no difference in VRC between EC and CP sharing these common HLA alleles. These findings indicate that cytotoxic T-lymphocyte (CTL) selection pressure on *gag-protease* alters VRC, and HIV-specific CTLs inducing escape mutations with fitness costs in this region may be important for strict viremia control in EC of HIV.

Human immunodeficiency virus type 1 (HIV-1)-infected individuals who control viremia to below the limit of detection (<50 RNA copies/ml plasma) without antiviral therapy have been termed elite controllers (EC) (16). Unraveling the mechanisms associated with this rare and remarkable phenotype, which has been documented in some persons infected for 30 years and counting, should provide important insights regarding our understanding of HIV pathogenesis and could provide important insights for vaccine development.

Host genetics, host innate and adaptive immune responses, and viral sequence variation have all been suggested to influence the rate of disease progression in HIV-1 infection (reviewed in reference 16). Recently, we reported that viruses sequenced from plasma and peripheral blood mononuclear cells from EC do not share a common ancestor, do not contain shared amino acid changes that are specific to this phenotype, and do not typically harbor gross viral genetic defects (36). However, such studies do not address functional properties of the encoded proteins, such as viral replication capacity (VRC). Reductions in VRC have been observed following exposure to antiretroviral drug selection pressure (17, 25, 31, 34) and therefore may also occur in response to immune selection pressure.

Focused studies of VRC in long-term nonprogressors/long-term survivors with detectable viremia have suggested that their viruses have reduced VRC compared to those of chronic progressors (CP) (7, 37, 39).

Due to the difficulties in isolating viruses from EC, investigating VRC in this rare group has been very challenging. Blankson et al. reported isolation of replication-competent viruses from 4 of 10 EC studied (8). On the other hand, the same group reported a case of a B*27/57 EC whose virus had reduced replication capacity compared to those of laboratory reference strains and the virus in the transmitting donor (4). However, to date, there have been no studies comparing VRC between EC and CP.

Factors that influence VRC are beginning to be defined. Several studies have concluded that the envelope protein is a major determinant for overall fitness of HIV-1 (6, 32, 40, 44). However, little is known about the influence on viral replication of the Gag protein, whose gene sequence is much more conserved than that of envelope. Not only is the Gag protein essential for viral replication, but it is known to be a preferred target for HIV-specific cytotoxic T lymphocytes (CTL) and is subject to mutations driven by CTL selection pressure (9, 21, 28, 35, 38, 45). Although it has been firmly established that in vivo polymorphisms in the conserved *pol* gene selected under antiretroviral drug therapy can markedly impair viral replication (25, 34), the consequences of immune-driven mutations for VRC remain incompletely understood.

Recent studies have indicated that escape mutations in Gag

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CTL epitopes restricted by HLA B*27 or B*57, each of which is a known protective HLA class I allele, result in a fitness cost (10, 15, 30, 33, 41, 42). Moreover, CTL targeting epitopes with escape mutations were associated with low plasma viral load (28, 35). Furthermore, relative viremia control was observed in the early phase of infection in persons who acquired viruses from donors with protective HLA alleles that target multiple epitopes within Gag (14, 22), suggesting fitness costs incurred as a result of CTL escape in the donor. These considerations motivated us to examine the influence of the *gag* gene on VRC.

In the present study, we generated *gag-protease* chimeric viruses on an HIV-1 NL4-3 virus backbone using PCR products amplified from plasma of 54 EC and 41 CP and compared VRC by using an infectible cell line with a green fluorescent protein (GFP) reporter to monitor viral replication in vitro. Since there are multiple protease cleavage sites within the Gag protein, the patient-derived *protease* gene was included in the chimeric constructs to preserve Gag processing by the autologous protease. We observed marked differences in VRC when comparing EC and CP and a differential impact on VRC by certain HLA class I alleles. This is the first study to compare VRC between EC and CP and also the first study to investigate associations between HLA alleles and in vitro VRC of HIV-1.

MATERIALS AND METHODS

Study subjects and plasma collection. Fifty-four untreated EC (<50 copies/ml plasma) and 41 untreated CP (median viral load, 79,900 [interquartile range, 33,600 to 225,000] copies/ml) were included in the present study. Three of the EC subsequently blipped during clinical follow-up (on average, 813, 183, and 498 RNA copies/ml, respectively) but unless otherwise indicated are grouped with the EC in this analysis. This cohort has been described in detail elsewhere (38). Informed consent was obtained from all participants. Plasma was obtained by standard procedures and stored at -80°C until use.

Viral RNA isolation and nested RT-PCR amplification of *gag-protease* from plasma. For the majority of EC, the first-round reverse transcription (RT)-PCR products from plasma containing the entire *gag* and *protease* genes were obtained in the context of a previous study (36), and additionally some EC were added to the current study by using exactly the same methods. The primers used to amplify the first-round products were as follows: forward, AAATCTCTAGCAGTGGC GCCGAACAG (HXB2 nucleotides 623 to 649), and reverse, TAACCCTGC GGGATGTGGTATTC (2849 to 2826). For CP, viral RNA was isolated from 0.5 ml of plasma following the same procedures as those used for EC for generation of first-round PCR products. Second-round PCR was performed using 100-mer primers that completely matched the pNL4-3 sequence using Takara EX Taq DNA polymerase, Hot Start version (catalog no. RR006; Takara Bio Inc., Shiga, Japan). One hundred microliters of reaction mixture was composed of 10 μl of 10 $\times$ EX Taq buffer, 4 μl of deoxynucleoside triphosphate mix (2.5 mM each), 6 μl of 10 μM forward primer (GAC TCG GCT TGC TGA AGC GCG CAC GGC AAG AGG CGA GGG GCG GCG ACT GGT GAG TAC GCC AAA AAT TTT GAC TAG CGG AGG CTA GAA GGA GAG AGA TGG G, 695 to 794) and reverse primer (ATG CTT TTA TTT TTT CTT CTG TCA ATG GCC ATT GTT TAA CTT TTG GGC CAT CCA TTC CTG GCT TTA ATT TTA CTG GTA CAG TCT CAA TAG GAC TAA TGG G, 2646 to 2547), 0.5 μl of enzyme, and 2 μl of first-round PCR product and water. The forward primer overlapped the *gag* coding sequence by five bases (underlined), and the reverse primer ended just one base downstream of the *protease* gene. Thermal cycler conditions were as follows: 95°C for 2 min, followed by 40 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min and then followed by 7 min of 72°C . PCR products were purified with the Purelink PCR purification kit (catalog no. K3100-002; Invitrogen, California) and eluted in 50 μl of DNase-RNase-free water.

Generation of chimeric viruses. *gag-protease*-deleted pNL4-3 was developed by inserting the unique restriction enzyme site BstEII at the 5' end of the *gag* gene and then 45 bases downstream from the 3' end of the *protease* gene of pNL4-3 by site-directed mutagenesis using QuikChange II (Stratagene, La Jolla, CA). The *gag-protease* gene was then deleted by BstEII digestion (New England Biolabs, Ipswich, MA), resulting in self-ligation of the remaining plasmid. A large amount

of vector was prepared using the Hispeed plasmid maxi kit (catalog no. 12663; Qiagen, Maryland), eluted in 1 ml Tris-EDTA buffer, and stored at -20°C until use. This vector was then linearized by BstEII digestion at 60°C for 2 h and purified (Purelink PCR purification kit). Ten micrograms of linearized vector and 5 to 10 μg of purified PCR product were cotransfected into 2.5×10^6 cells of the tat-driven GFP reporter T-cell line (GXR cells, CEM origin [11]) in 800 μl of R10 Plus medium (RPMI medium with 10% fetal calf serum containing penicillin and streptomycin) by electroporation (exponential protocol, 300 V, 500 μF). Cells were incubated for 45 min at room temperature and transferred to T25 flasks in 10 ml of R10 Plus medium. GFP expression was monitored by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) every 1 to 2 days after day 5. Supernatants were harvested after GFP expression reached 15% among the viable cells and were stored at -80°C as viral stocks for subsequent replication capacity experiments. Virus titration and VRC assays were performed as described previously (10, 36, 41) at a multiplicity of infection (MOI) of 0.002. Replication capacity assays were performed in duplicate, using gating strategies as previously described (10, 11, 36, 41, 42). The slope of the natural log of percent GFP-expressing cells was calculated between days 2 and 6. The natural log was used for the slope calculation, as appropriate for an exponential growth curve. The MOI of 0.002 was chosen based on initial experiments with this cell line and the chimeric viruses showing that, at this MOI, a robust and clear exponential curve could be plotted before reaching a plateau level of 30 to 40% at 7 to 8 days postinfection, which represents the saturation level in this system. In addition, for each viral stock, viral RNA was extracted from 30 μl of stock supernatant using the ChargeSwitch EasyPlex viral kit (catalog no. CS12281-04m; Invitrogen, California) following the manufacturer's instructions, followed by nested RT-PCR, as described above, to provide product for sequencing.

Sequencing and phylogenetic analysis. Plasma and viral stock sequencing was performed as described previously (36). The origin of *gag-protease* genes from chimeric NL4-3 was confirmed by the maximum-likelihood method (DNAm1, PHYLIP). Protein amino acid sequence trees were drawn by the maximum-likelihood method as well (Prom1, PHYLIP).

Statistical analysis. VRC was compared between the grouped pairs using the Mann-Whitney U test. The association between (i) VRC and the presence or absence of HLA alleles and (ii) VRC and the presence or absence of individual viral amino acid residues was analyzed by Mann-Whitney U test. To account for multiple tests, *q* values were computed, with cutoffs for significance as indicated elsewhere (43).

Nucleotide sequence accession numbers. Viral sequences which had not been reported before were submitted to GenBank (accession numbers EU864042-114 and EU873002-005).

RESULTS

Generation and validation of *gag-protease* chimeric NL4-3 viruses. Cotransfection of the amplified patient-derived *gag-protease* genes with the linearized Δ *gag-protease* NL4-3 plasmid into a GFP reporter cell line resulted in production of infectious progeny viruses, with expression of GFP in at least 15% of the cells within a median of 14 days (range, 7 to 28 days) in all 54 EC and 41 CP. The entire *gag* region of the chimeric viruses was then sequenced from the resultant chimeric viral stocks and compared to the original source plasma (Fig. 1). A median of 0.598% (interquartile range, 0.199 to 1.397%) difference at the protein level was observed between plasma HIV RNA and chimeric virus, and there was a significant difference between EC and CP viruses (0.299% [0.150 to 1.199] versus 0.796% [0.399 to 1.796], $P = 0.003$). However, the vast majority of the differences were observed at a codon in which we detected mixtures in the sequences, and as expected these were more frequent in persons with higher plasma viral loads. If amino acid mixtures were excluded from analysis, there was no difference between EC and CP (0.0% [0.0 to 0.120] versus 0.0% [0.0 to 0.120], $P = 0.887$). These data indicated that although there may have been some selection of chimeric viruses during recombination and/or culture, which was particularly evident for CP, probably due to higher intraindividual

FIG. 1. Validation of obtained chimeric NL4-3 sequences by phylogenetic analysis. The tree was drawn by the maximum-likelihood method (Proml, PHYLIP) using entire Gag protein sequences. Blue taxa indicate sequences from chimeric viral stocks. Red taxa indicate the original plasma viral sequence. Note that all viral stocks and plasma viral sequences from the same subjects shared the same branches.

diversity than that for EC, Gag-Protease of chimeric viruses represented the dominant form of plasma viruses in both EC and CP, and selection during culture was unlikely to affect the overall analysis. For subsequent analyses, sequences derived directly from the chimeric viruses were used.

Comparison of VRCs of chimeric NL4-3 between EC and CP. In order to quantitatively compare the *in vitro* VRCs of chimeric viruses from EC and CP, we infected the GFP reporter cell line with the chimeric viral stocks at an MOI of

0.002 and measured VRC by performing flow cytometric quantitation of the percentage of cells expressing GFP from days 2 to 6 after infection (Fig. 2A). *gag-protease* chimeric viruses derived from EC displayed significantly reduced replication capacity compared to those derived from CP. All but two EC were infected with clade B viruses; significant differences remained ($P < 0.0001$) even after removal of these two non-clade B viruses (Fig. 2B). Three of the EC were subsequently determined to have had blips in viral load at the time at which the

FIG. 2. Comparison of VRCs of *gag-protease* chimeric viruses between EC and CP. VRCs of chimeric NL4-3 were compared among 54 EC and 41 CP. (A) Kinetics of viral replication; red and black lines indicate EC and CP, respectively. Representative data from duplicate experiments are shown. (B) Comparison of the slope of the natural log between day 2 and day 6. Data shown here are the averages of duplicate experiments. Dashed line indicates VRC of wild-type NL4-3.

gag-protease gene was amplified (viral loads of 812, 183, and 498 RNA copies/ml); removing these from the analysis also did not affect the results (data not shown). We observed a weak positive correlation between VRC and plasma viral loads of CP (Pearson $r = 0.29$, $P = 0.065$, data not shown), supporting the finding that chimeric viruses derived from the subjects with lower plasma viral loads had reduced in vitro replication capacity.

Association between specific HLA alleles and VRC of chimeric NL4-3 viruses. Having shown that in vitro VRC varies significantly among viruses obtained from HIV-infected persons, we next examined whether the particular HLA alleles expressed affected this parameter. In this analysis, we included 90 clade B virus-infected subjects in whom HLA class I types were available and excluded the two subjects with non-B-clade virus infection. Again, using the slope of percent GFP infection between days 2 and 6 as a measure of VRC, we found four class I HLA alleles significantly associated with differences in VRC: B*57, Cw*18, A*02, and B*07 (all $P < 0.05$ and $q < 0.2$ [Table 1]). B*57 showed the strongest negative impact on VRC (0.777 in B*57⁺ versus 0.919 in B*57⁻, $q < 0.0001$), which supported previous reports of fitness costs by B*57 CTL escape

mutations in Gag (10, 30, 33). Cw*18 also showed a weaker negative impact; however, this is likely due to the strong linkage disequilibrium between B*5703 and Cw*18, rather than a true negative impact of Cw*18 on VRC. In contrast, A*02 and B*07 were associated with higher VRC (0.948 versus 0.816 for A*02 and 0.987 versus 0.831, $q = 0.175$, respectively [Table 1]). The fact that these alleles are both common in the U.S. population (A*02 frequency, 28.3% for Caucasians and 18.7% for African Americans; B*07 frequency, 11.3% for Caucasians and 9.8% for African Americans) (12) suggests the possibility that these are not actively increasing VRC but rather that the virus may have become most adapted to these populations, rendering them neutral in comparison to a large group of less-frequent HLA alleles, which may still be impacting viral load (1, 20, 29).

Since homozygosity at HLA class I loci has been linked to more-rapid HIV disease progression (13), we also examined the impact of homozygosity for HLA A, B, or C alleles on VRC. However, no association with VRC of the chimeric viruses was observed (data not shown). Also of interest, we did not see a negative impact on *gag-protease* function by HLA B*27 and B*51, despite these alleles being enriched in EC (38) (Table 1). However, these results could be due to limited statistical power; analysis of larger data sets will be necessary to further evaluate this.

Stratification of VRC by controller status and particular HLA alleles. Given the above results showing that VRC is associated with EC status and with expression of particular HLA alleles, we next compared the relative VRC within the EC group in relation to specific HLA alleles and did the same for the CP group. As shown in Fig. 3A, chimeric viruses from EC expressing HLA-B*57 showed significantly lower VRC than did those from non-B*57 EC (0.707 versus 0.827, $P = 0.0074$). Likewise, chimeric viruses from B*57 CP displayed significantly reduced VRC compared to those from non-B*57 CP (0.850 versus 1.005, $P = 0.0489$). Notably, there was a significant difference in VRC between not only B*57 EC and B*57 CP ($P = 0.0245$) but also non-B*57 EC and non-B*57 CP ($P = 0.0002$), indicating that the reduced VRC of EC was not due just to biased distribution of B*57 in EC (Fig. 3A). The difference between B*57 EC and B*57 CP is consistent with a previous study in which accumulation of compensatory mutations led to recovery of fitness loss induced by escape mutations arising within CTL epitopes in B*57 progressors (10). There was still a significant difference between EC and CP when the three alleles most strongly associated with better outcome (B*27, B*51, and B*57) were removed (0.831 versus 1.004, $P = 0.0065$ [Fig. 3B]), indicating that the observed differences in VRC were not solely mediated by the most protective HLA alleles.

We next performed a subanalysis of the two alleles, A*02 and B*07, associated with higher VRC. Chimeric viruses from A*02-expressing EC showed significantly higher VRC than did those from non-A*02-expressing EC (0.893 versus 0.786, $P = 0.0087$). In contrast, no difference in VRC was observed between A*02-expressing and non-A*02-expressing CP (0.9733 versus 1.018, $P = 0.6597$ [Fig. 3C]). Intriguingly, there were no significant differences in VRC between A*02-expressing EC and A*02-expressing CP, although a trend toward lower VRC in A*02-expressing EC than in A*02-expressing CP was ob-

TABLE 1. VRC of *gag-protease* chimeric NL4-3 by HLA alleles^a

HLA allele	Negative	Positive	Median VRC		<i>P</i>	<i>q</i>
			Negative	Positive		
B57	64	26	0.918744	0.776763	0.00017	0
C18	85	5	0.894651	0.683553	0.00286	0.1
B07	76	14	0.831421	0.98701	0.00966	0.166667
A02	50	40	0.81622	0.947621	0.01395	0.175
C06	69	21	0.91498	0.81304	0.03648	0.3
A30	74	16	0.899372	0.801353	0.08055	0.65
B18	85	5	0.863755	0.996052	0.11408	0.68
A23	84	6	0.892988	0.790454	0.16033	0.68
B51	76	14	0.85955	0.960374	0.16265	0.68
C08	79	11	0.874475	0.963308	0.17641	0.68
A11	78	12	0.872829	0.928979	0.19625	0.689474
C01	82	8	0.890879	0.777692	0.21866	0.689474
A26	82	8	0.892988	0.786078	0.22567	0.695
C07	54	36	0.827049	0.916617	0.25348	0.714286
B35	77	13	0.874475	0.895588	0.30665	0.754167
B08	82	8	0.892988	0.811218	0.33788	0.773077
C02	82	8	0.878189	0.957642	0.33907	0.773077
A01	64	26	0.895119	0.820042	0.39111	0.817241
B40	83	7	0.874475	0.904317	0.40789	0.817241
C03	71	19	0.874475	0.894651	0.41269	0.817241
C15	85	5	0.881904	0.95744	0.51352	0.864706
C16	80	10	0.878189	0.931695	0.54464	0.883333
A68	82	8	0.886168	0.847137	0.55424	0.883333
A24	81	9	0.881904	0.918255	0.59584	0.907895
C05	84	6	0.878189	0.963358	0.59611	0.907895
B58	85	5	0.890432	0.827043	0.66985	0.917778
A32	85	5	0.890432	0.748514	0.68176	0.917778
B44	72	18	0.869115	0.928518	0.69403	0.917778
C04	68	22	0.886168	0.87754	0.70478	0.917778
A03	68	22	0.892988	0.825664	0.70622	0.917778
B49	85	5	0.881904	0.890432	0.73285	0.926087
B13	85	5	0.890432	0.833029	0.83725	0.947059
C12	81	9	0.890432	0.844625	0.8744	0.956604
B14	82	8	0.886168	0.851265	0.91648	0.975926
B15	77	13	0.890432	0.814578	0.93781	0.982143
B27	80	10	0.890879	0.844526	0.99515	0.984483

^a HLA alleles seen in more than four EC are shown. *q* values of <0.2 are shown in bold.

served (Fig. 3C). Similar results were obtained for B*07 (Fig. 3D). Since B*57 showed the strongest negative impact on VRC of chimeric viruses and there was biased distribution of B*57 in EC, we repeated this analysis excluding this allele. After B*57-expressing subjects were removed, a trend toward reduced VRC in non-A*02 EC compared to A*02 EC remained (0.815 versus 0.896, $P = 0.0650$ [Fig. 3E]). The difference between A*02 EC and A*02 CP was further weakened ($P = 0.2823$) despite the fact that the difference between non-A*02 EC and non-A*02 CP was maintained ($P = 0.0020$ [Fig. 3E]). Combining A*02 and B*07, we obtained a similar result and the difference between A*02/B*07 EC and other EC reached significance ($P = 0.0390$ [Fig. 3F]). These results further support the hypothesis that A*02 and B*07 alleles themselves have little impact on VRC on *gag-protease* chimeric viruses compared to other HLA alleles, but non-A*02/B*07 alleles enriched in EC have a greater negative impact on VRC, albeit much weaker than that of B*57. In addition, the data suggest that reduced VRC of *gag-protease* chimeric viruses from EC is likely driven by HLA-mediated mutations that include other alleles besides HLA B*57, rather than transmission of viruses with attenuated *gag-protease* genes.

Association between VRC and specific amino acid residues.

Having sequenced all of the *gag-protease* genes from the persons included in this study, we next examined the association between specific amino acid residues and VRC. Only clade B virus infections ($n = 92$) were included in this analysis. Table 2 shows the list of codons in Gag associated with lower VRC based on a *q* value cutoff of <0.2 (see Materials and Methods). There was no significant association between codons in *gag-protease* and VRC (data not shown), but four positions in Gag (84, 207, 242, and 483) were statistically associated with differences. Gag 84T was associated with lower VRC, and interestingly T is the current consensus amino acid for clade B at this residue. Of EC, 88.5% had 84T but only 68.3% of CP were carrying T ($P = 0.029$). In the Los Alamos National Laboratory database, 87.5% (28/32) of clade B virus sequences sampled before 1995 in the United States included 84T, but after 1995, only 63.6% (14/22) did so ($P = 0.05$). These percentages correspond well to the differences observed at this residue in EC and CP and are likely explained by older strain infection in EC than in CP, as previously reported (36). T84V was reported as an escape mutation of A*02 (26), which is the most common HLA class I allele in the United States. Therefore, accumula-

FIG. 3. Comparison of VRC between EC and CP by HLA alleles. VRC of chimeric NL4-3 was compared between EC and CP in the context of the designated class I HLA alleles. (A) B*57 versus non-B*57; (B) protective (B*27, B*51, and B*57) alleles versus others; (C) A*02 versus others; (D) B*07 versus others; (E) A*02 versus others after removal of B*57; (F) A*02 or B*07 versus others after removal of B*57.

TABLE 2. Association between Gag codons and viral replication capacity^a

Position on HXB2	AA	No. of subjects		VRC		P	q
		AA ⁻	AA ⁺	AA ⁻	AA ⁺		
84	T	19	74	1.0153	0.8234	0.0000	0.0000
84	V	74	19	0.8234	1.0153	0.0000	0.0000
483	Q	88	4	0.8909	0.5795	0.0010	0.1000
207	D	91	2	0.8904	0.4253	0.0017	0.1500
242	N	69	24	0.9043	0.7768	0.0018	0.1500
207	E	2	91	0.4253	0.8904	0.0018	0.1500
242	T	25	68	0.7780	0.9042	0.0029	0.2000

^a AA, amino acid(s). Only q values of ≤0.2 are shown.

tion of T84V in the population over time can be supported by this study. Alternatively, it may be explained that limited viral evolution in EC prevents emergence of the T84V escape mutation in A*02⁺ EC, since this Gag epitope (SLYNTVATL) is typically first targeted during chronic infection after set point viral loads are achieved (26) and results in a higher frequency of 84T in EC. In any case, the association between 84T and lower VRC is unlikely to reflect a true causal relationship but rather infection by older strains in EC and/or maintenance of the wild-type A*02 epitope in EC.

Another significant association between VRC and specific sequence differences was the Gag T242N mutation, a widely recognized escape mutation from CTL restricted by B*57, which has a negative impact on VRC (10, 33). Therefore, an association between VRC and the T242N mutation would be expected. However, this does not necessarily mean a causal relationship. The other two Gag mutations associated with reduced VRC, 207D and 483Q, were present in only two EC and four EC, respectively. Interestingly, 207D was present in two of the three EC with the lowest VRC of the entire cohort (0.46 and 0.39). The final significant association between sequence usage and diminished VRC was the 483Q mutation, which was seen in an EC with the lowest VRC recorded, 0.39. Codon 207 did not correspond to any predicted CTL epitopes for the two EC with 207D. Codon 483 corresponded to predicted CTL epitopes in three of the four EC (http://www.hiv.lanl.gov/content/immunology/tables/ctl_summary.html). However, those predicted epitopes were defined for non-clade B virus sequences, and there are substantial mismatches within this region between clade B and non-clade B sequences. Therefore, it is unclear whether this region acts as a CTL epitope for these subjects infected with clade B virus. Larger studies and mutagenesis approaches will be necessary to examine whether these rare mutations are truly associated with EC and affect VRC or not. In summary, except for T242N, a known escape mutation from B*57-restricted TW10-specific CTL, none of the common amino acid changes could be causally linked to reduced VRC of *gag-protease* chimeric NL4-3.

DISCUSSION

In this study, we assessed VRC of chimeric NL4-3 viruses expressing the *gag-protease* gene derived from a well-characterized cohort of EC of HIV infection and compared these to chimeric viruses derived from persons with progressive HIV

infection. This is the first study to directly compare VRC between large numbers of EC and CP. Our results indicate that *gag-protease* genes from EC are attenuated in replication capacity compared to those from CP. Moreover, our data indicate that there are differential impacts on VRC by HLA class I alleles, which contribute to differences observed in VRC between EC and CP. Notably, the allele most associated with a protective effect in HIV-infected persons, HLA-B*57, displayed the most profound impact on VRC.

Increasing evidence indicates that VRC can impact HIV pathogenesis (10, 15, 22, 33, 41). It has, however, been challenging to amplify target HIV-1 genes and to isolate primary viruses from EC due to the extremely low plasma viral loads. Recently, we and others have successfully amplified HIV-1 genes from plasma viral RNA of EC, which likely represent actively circulating viruses in vivo (3, 5, 36). The availability of these PCR products allowed us to generate chimeric viruses carrying HIV-1 genes from EC plasma. Combining the generation of chimeric NL4-3 with a recently developed GFP reporter T-cell line (11) enabled us to examine a large number of subjects in the context of diverse HLA alleles and to use flow cytometry to assess viral replication kinetics precisely. Our results provide evidence of a link between HLA class I alleles and VRC, suggesting an important mechanism by which CTL may contribute to viral containment.

Comparison of VRC of primary isolates between progressors and long-term nonprogressors/long-term survivors has been reported (7, 37, 39), but the current study adds important information to these earlier studies. Most reported studies of VRC have used primary CD4⁺ T cells as a source of autologous viruses, which do not necessarily represent actively replicating viruses in vivo. Given observed discrepancies between plasma viral and proviral sequences in subjects with extremely low viremia (reference 5 and our unpublished data), namely, that cellular proviruses maintain more wild-type sequences in EC subjects than do circulating plasma viruses in the same subjects, we reasoned it wise to avoid using primary isolates derived from autologous CD4⁺ T cells for comparison between controllers and progressors and instead focused on viruses derived from plasma. Moreover, previous studies have indicated that the envelope gene is the major determinant of overall replication capacity of HIV-1; that intraindividual envelope diversity positively correlates with in vitro VRC; and that as disease progresses and plasma viral load increases, VRC also increases (2, 39, 44). Since envelope sequences in EC are less evolved than those in CP (3, 36) and contain fewer mixtures than those in CP (our unpublished observations), indicating less envelope sequence diversity in EC, comparison of VRC of primary isolates between EC and CP could merely represent different degrees of diversity of envelope genes, something which has been observed when comparing acute/early- and late-phase HIV infection in a given subject (44). In other words, the difference in VRC of primary isolates between EC and CP might be just the result of limited envelope sequence diversity in EC due to host factors including immunity.

Here we were able to extend studies of VRC beyond the *env* gene. Given a growing body of evidence suggesting an important role for fitness costs by CTL escape mutations within Gag in controlling viremia (10, 28, 33, 35), we focused on this

protein. Since it is not known whether the diversity of the Gag protein sequence correlates with its function, there remains the possibility that the observed differences in VRC between EC and CP might reflect distinct interindividual diversity of the Gag protein. We also note that there is still considerable overlap in VRC between EC and CP (Fig. 2B). Therefore, the observed reduction of Gag-Protease function in EC is not the sole factor to explain elite control but rather one of the factors contributing to suppression of viremia.

The novelty of the present study is not only in scale or use of EC samples but also in showing a differential impact on viral replication by different HLA class I alleles. Although only B*57 showed a significant negative impact on VRC, indirect evidence that common HLA alleles like A*02 and/or B*07 had little impact on VRC suggests that other HLA class I alleles likely had some negative impact on VRC, albeit to a lesser extent than B*57. This finding and additional observations of significantly reduced VRC in non-A*02/B*07/B*57 EC compared to non-A*02/B*07/B*57 CP indicate that reduced VRC in non-A*02/B*07/B*57 EC is likely mediated by HLA class I alleles enriched in EC. These findings further support recent studies suggesting the importance of CTL targeting Gag epitopes and inducing escape mutations with fitness costs (22, 28, 35).

Although this study implies a contribution of altered VRC to viral control in persons with HLA-B*57, it does not link other well-known protective HLA alleles like B*27 and B*51 to altered VRC. Since there are no known B*51 CTL epitopes within the Gag-protease (19), it is perhaps not surprising that we did not see any impact by this allele. On the other hand, B*27 is known to contain an immunodominant CTL epitope within Gag protein, and escape from this CTL response is frequently observed (18, 23, 27) and has been demonstrated to compromise VRC (41, 42). However, these mutations typically do not arise until late-stage infection (24, 41), and it has been demonstrated that the dominant CTL escape form R264K Gag requires an upstream mutation (S173A) which fully compensates the fitness cost and is required prior to complete escape (41). Thus, the lack of an effect of B*27 on *gag-protease* chimeric virus replication capacity in the present study does not contradict previous findings. These results indicate more heterogeneous mechanisms of elite control even among HIV-specific CTL responses.

Another important finding of this study is the observation that B*57 EC showed significantly reduced VRC compared to B*57 CP. This might be explained by an additional effect of other protective HLA alleles in B*57 EC that may not be present in B*57 CP. However, as reported before, accumulation of compensatory mutations that recover reduced VRC by B*57 CTL escape mutations also is a feasible explanation (10, 15). However, since we observed rare escape variants in many of the B*57 EC (unpublished data), the mechanism of attenuation of viruses in B*57 EC seems more complex. Additional studies are required to reveal the role of such rare mutations in VRC in B*57 EC. Likewise, the difference between non-A*02/B*07/B*57 EC and non-A*02/B*07/B*57 CP might be due to accumulation of compensatory mutations that recover fitness costs by escape mutations, or unique mutations in EC, rather than different HLA allele distribution between EC and CP. Larger data sets may allow this question to be answered.

It has been suggested that EC might acquire viruses with reduced replication capacity at the time of transmission, rather than having these arise in vivo. However, this would not explain the HLA-associated differences in VRC observed in the present study. Therefore, we conclude that the diminished VRC of EC viruses is achieved after transmission rather than transmitted from donors. However, very recently, it was suggested that the subjects considered to have obtained viruses from donors with protective HLA alleles could maintain lower levels of viremia, at least temporarily, after infection (22). It is quite possible that controllers who have strong immune responses obtain further benefit when an attenuated virus is transmitted, and this may further contribute to elite control. In order to clarify this point, longitudinal studies of large numbers of persons in acute infection with diverse set point viral loads will be necessary.

Although *gag-protease* was the focus of the current study, there remains the possibility that there is dysfunction of other HIV-1 genes that may be associated with elite control. Enrichment in EC of B*51, which contains no Gag CTL epitopes, is a potential example of an allele that modifies VRC via immune-selected mutations in another viral gene. Evaluation of function of other HIV-1 genes is warranted to further understand the mechanisms of elite control.

In conclusion, we observed significantly reduced VRC of chimeric NL4-3 viruses carrying *gag-protease* genes from HIV EC and found this to be associated with particular HLA class I allele expression. HLA-B*57 displayed the most profound effect on VRC, whereas the more common HLA alleles A*02 and B*07 had the least impact. Non-A*02/B*07 HLA alleles enriched in EC also appear to be able to attenuate Gag-protease function. This differential impact by HLA alleles on VRC of chimeric viruses also indicates that attenuation of *gag-protease* genes is achieved following transmission. It will be important to generate longitudinal data on acute infection and expand these data sets to further define the role of fitness defects in disease outcome and the potential contribution of class I-restricted selection pressure on other coding regions to viral fitness, which has potential important implications for vaccine design.

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