Germany). Reverse transcription was performed (ThermoScript RT-PCR System, Invitrogen, Tokyo, Japan) by using gene-specific reverse primers. PCR analysis was performed (Premix Ex*Taq* Hot-Start Version, Takara, Shiga, Japan) by using published sets of external primers (SRVenv1E and SRVenv2E) and nested primers (SRVenv3N and SRVenv4N).[9]

Results

SRV infection status of the 419 laboratory-bred breeders. Of the 419 (female, 364; male, 55) cynomolgus macaques evaluated, 22 were negative for both SRV-specific antibodies and RNA. Of the remaining 397 breeders, 340 were positive for SRV-specific antibodies but were not viremic, 29 were positive for both viral RNA and antibodies, and the remaining 28 monkeys had viremia without antibodies.

SRV infection status of 95 pairs of mothers and offspring at birth. RT-PCR and Western blotting of samples from 95 pairs of mothers and offspring at the time of birth revealed that the dams could be grouped into 1 of 3 categories based on the presence of SRV-specific antibodies and viremia.[2] Among the 95 dams, 76 developed SRV-specific Abs without viremia, 14 had both antibodies and viremia, and the remaining 5 were viremic without SRV-specific antibodies.

None of the offspring of the 76 dams that were antibody-positive but RNA-negative were viremic at birth. Eight infants (including 2 delivered by caesarian section) of the 14 dually positive dams were viremic at birth; the remaining 6 infants of dams in this group were viral RNA-negative. All 5 progeny (including 2 infants delivered by caesarian section) of viremic but antibody-negative dams were viremic at birth.

Plasma SRV-specific antibodies and RNA in viremic newborns during the first 6 mo. We then tested the SRV-specific antibody and RNA status of 3 representative viremic newborns at 1, 2, and 6 mo after birth (Table 1). All 3 of the dams exhibited SRV viremia at delivery, and 2 of them also were positive for SRV-specific antibodies. All 3 infants exhibited SRV-specific RNA at all time points, but none was antibody-positive at weaning.

Family analysis of two representative SRV-viremic dams. The SRV status of all 7 offspring born to 2 representative viremic mothers was verified in 2007. Dam 1319711082 and her 4 offspring (infant 1410311011, born 2003; infant 1420506016, born 2005; infant 1420608031, born 2006; and infant 1420709050, born 2007) all demonstrated SRV RNA in tests performed during 2007. In addition, this dam and her oldest infant (1410311011) were antibody-positive, unlike the 3 youngest siblings. Dam 1319710076 and her 3 offspring (infant 1410408017, born 2004; infant 1410508022, born 2005; and infant 1420701001, born 2007) were all RNA-positive but antibody-negative according to tests performed in 2007.

Discussion

In 2005, we reported that about 20% of the cynomolgus monkeys in the colony at our institution exhibited SRV-T viremia and that virus was present in saliva, urine, and feces from the viremic monkeys.[3-5] Because the virus secreted from these monkeys was a potential source of horizontal SRV-T infection, we performed the current large-scale survey of SRV infections in our laboratory-bred monkeys and assessed the transmission of SRV through the generations represented in the colony.

The present study validated our concerns about vertical and horizontal SRV infections in the colony, because more than 90% of the laboratory-born breeders were positive for SRV-specific antibodies or virus (or both). The rate of viremia in the present study (14%) was smaller than that (20%) in the earlier survey,[5] which involved 49 retired breeders. The rate of viremia in a colony may vary depending on the age distribution of animals and their countries of origin. In particular, we hypothesize that the 28 monkeys that exhibited SRV viremia without specific antibodies are immunotolerant to SRV because of being infected in utero, as is reported to occur in rhesus and pigtailed macaques.[7,12]

To evaluate transplacental maternal–infant transmission of SRV, we tested 95 pairs of mothers and newborns, including 4 infants delivered by caesarean section, by using SRV-specific RT-PCR. The results showed that all monkeys exhibiting SRV-specific antibodies without viremia produced newborns without viremia. However, the transplacental SRV infections observed in infants included 4 newborns delivered by cesarean section from viremic mothers. In pigtailed monkeys, SRV2 was detected in the tissues and amniotic fluid of fetuses and in the blood of newborns delivered from viremic mothers.[12] In other cynomolgus monkeys, SRV was transmitted through transfusion of blood from a viremic donor but not from a nonviremic donor.[13] These findings indicate that SRV viremia of the mother is essential to establishing transplacental infection of the fetus. However, the production of 6 SRV-negative newborns from 14 viremic dams with SRV-specific antibodies may indicate that these antibodies reduced the viral loads in the viremic mothers sufficiently to prevent transplacental infection with SRV. Further investigation to quantify SRV in blood and the occurrence of transplacental infections will resolve this question.

An important issue is whether SRV viremic newborns can convert to a nonviremic state after developing virus-specific antibodies. Three infants born from viremic mothers exhibited viremia, which was maintained at 1, 2, and 6 mo of age, with no antibodies at 6 mo of age. In addition, 7 offspring born from the representative 2 SRV-viremic mothers were all viremic, at ages of 6 mo to 4 y. Pigtailed monkey newborns infected transplacentally with SRV2 maintained a viremic state for 1 y without producing antibodies and harbored proviral DNA in many tissues.[11,12] A newborn rhesus monkey produced from a viremic mother was SRV1-positive within 24 h after birth and was antibody-negative for as long as 6 mo after birth.[7] These findings suggest that cynomolgus infants infected in utero with SRV and born from viremic mothers are immunologically tolerant to the virus and that they then become the source of SRV infection in the colony.

The cynomolgus monkey breeding colony at our institution has been maintained as SPF with regard to B virus, SVV, SIV, STLV1, and measles virus but not SRV. The cage system used during the first 25 y was a two-story type—monkeys were able to touch feces and urine of animals in adjacent cages. In addition, cages were washed with high-pressure water, perhaps helping to spread virus-contaminated waste and increasing the likelihood of horizontal infections. After redesigning the cage system to a single-story type that prevents monkeys from touching fecal and urine waste from another macaque, we anticipate that we will be able to establish an SRV-free colony by introducing SRV nonviremic monkeys into the breeding colony. Furthermore, elimination of viremic dams, which can become a source of transplacental infection, from the breeding colony is critical to establishing an

Table 1. SRV-specific antibodies and RNA in the plasma of viremic newborns during their first 6 mo

Infant ID	Method of delivery	Dam ID	Method of nursing	Status of dam at parturition		Status of infant at				
						0 d	1 mo	2 mo	Weaning (approximately 6 mo)	
				Antibodies	RNA	RNA	RNA	RNA	Antibodies	RNA
1310611144	Caesarean	1210003019	Artificial	+	+	+	+	+	–	+
1410508022	Natural	1319710076	Artificial	–	+	+	+	+	–	+
1420506016	Natural	1319711082	Maternal	+	+	+	+	+	–	+

Testing of infants for SRV-specific antibodies was delayed until weaning because transplacentally transferred maternal antibodies can persist at 2 mo of age.

SRV-free breeding colony. The establishment of an SRV-free cynomolgus breeding colony is paramount for supplying monkeys that are appropriate for many fields of investigation, including vaccine testing, gene therapeutics, organ transplantation, and infectious disease studies.

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Impaired Replication Capacity of Acute/Early Viruses in Persons Who Become HIV Controllers[▽]

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Human immunodeficiency virus type 1 (HIV-1) controllers maintain viremia at <2,000 RNA copies/ml without antiretroviral therapy. Viruses from controllers with chronic infection were shown to exhibit impaired replication capacities, in part associated with escape mutations from cytotoxic-T-lymphocyte (CTL) responses. In contrast, little is known about viruses during acute/early infection in individuals who subsequently become HIV controllers. Here, we examine the viral replication capacities, HLA types, and virus sequences from 18 HIV-1 controllers identified during primary infection. *gag-protease* chimeric viruses constructed using the earliest postinfection samples displayed significantly lower replication capacities than isolates from persons who failed to control viremia ($P = 0.0003$). Protective HLA class I alleles were not enriched in these early HIV controllers, but viral sequencing revealed a significantly higher prevalence of drug resistance mutations associated with impaired viral fitness in controllers than in noncontrollers (6/15 [40.0%] versus 10/80 [12.5%], $P = 0.018$). Moreover, of two HLA-B57-positive ($B57^+$) controllers identified, both harbored, at the earliest time point tested, signature escape mutations within Gag that likewise impair viral replication capacity. Only five controllers did not express "protective" alleles or harbor viruses with drug resistance mutations; intriguingly, two of them displayed typical B57 signature mutations (T242N), suggesting the acquisition of attenuated viruses from $B57^+$ donors. These data indicate that acute/early stage viruses from persons who become controllers have evidence of reduced replication capacity during the initial stages of infection which is likely associated with transmitted or acquired CTL escape mutations or transmitted drug resistance mutations. These data suggest that viral dynamics during acute infection have a major impact on HIV disease outcome.

Human immunodeficiency virus type I (HIV-1)-infected individuals who control viremia spontaneously without antiviral therapy have been termed HIV controllers (3, 18, 21, 48, 52). Unraveling the mechanisms associated with this phenotype should provide important insights regarding HIV pathogenesis and could contribute to vaccine development.

Host and viral genetics, as well as host innate and adaptive immune responses, influence the rate of disease progression in HIV-1 infection (reviewed in reference 18). Several studies have reported the correlation between *in vitro* HIV replication capacity and level of plasma virus loads or disease progression in individuals with chronic infection (6, 13, 35, 45, 50, 55). Studies of HIV-1 elite controllers (EC), who control viremia to below the limit of detection in commercial assays, have revealed the presence of replication-competent viruses in these individuals (7), although these viruses appear to be less fit based on studies of envelope (35) and Gag-protease (45). This

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fitness defect in the chronic phase of infection is due at least in part to fitness-impairing mutations induced by cytotoxic-T-lymphocyte (CTL) responses restricted by "protective" HLA class I alleles (46).

In contrast, little is known about viruses obtained from the acute/early phase of infection in persons who subsequently become HIV-1 controllers, largely due to the difficulty in enrolling such people during the acute/early phase of infection. The characterization of acute/early-phase viruses in individuals who subsequently achieve low set-point virus loads is of paramount importance to our understanding of the mechanisms of HIV-1 control.

In the present study, we analyzed acute/early-phase plasma HIV RNA sequences from 18 untreated individuals who were diagnosed during the acute/early phase and subsequently became controllers (<2,000 RNA copies/ml). We compared these to sequences from a group of HIV-1 noncontrollers enrolled similarly during acute/early infection. We also generated chimeric viruses carrying patient-derived *gag-protease* sequences from acute/early-phase infection and compared the viral replication capacities of the chimeric viruses from controllers and from noncontrollers.

We observed that the chimeric viruses derived from controllers have significantly reduced replicative capacities compared to those from noncontrollers. Moreover, we observed that at least 80% of these individuals who go on to become controllers featured transmission of attenuated drug-resistant viruses, transmission of HLA-B57-restricted CTL escape variants to HLA-mismatched recipients, selection of attenuated CTL escape variants in HLA-B57-positive ($B57^+$) recipients, or combinations of these factors. Taken together, these results indicate that the initial viral dynamics have a major influence on the subsequent course of disease.

MATERIALS AND METHODS

Study subjects and blood sample collection. All study participants were diagnosed during acute/early infection and remained untreated for a minimum of 1 year. Acute/early infection was defined according to published criteria (Acute Infection, Early Disease Research Program [AIEDRP] sponsored by NIAID) (28, 33). HIV controllers after primary infection (C-PI) were defined as HIV-1-infected subjects with acute/early infection who subsequently controlled viremia to less than 2,000 RNA copies/ml without antiviral treatment on at least 3 determinations over at least a 12-month period. Plasma and peripheral blood mononuclear cells (PBMC) from 18 C-PI were collected from multiple sites in the United States and Australia. Samples for 17 of these were obtained through the AIEDRP network. An additional subject (C-PI_13) was recruited at Massachusetts General Hospital. For individuals recruited through the AIEDRP network, the estimated date of infection (EDI) was calculated according to the AIEDRP criteria (37). For comparison, a group of 80 noncontrollers diagnosed during acute/early infection (noncontrollers after primary infection [NC-PI]) who failed to subsequently control viremia were drawn from AIEDRP and a previously described multicenter cohort with sites in the United States, Australia, and Germany (11). The participants from outside the AIEDRP network were subcategorized according to AIEDRP criteria as well. Written informed consent was obtained from all participants. The current study was approved by the appropriate institutional review boards, including that of Massachusetts General Hospital.

HLA typing. Human leukocyte antigen (HLA) class I typing of C-PI was determined using an in-house sequence-based method involving locus-specific nested PCR on extracted DNA from plasma or PBMC, followed by DNA sequence analysis and allele interpretation (12). HLA typing of NC-PI was performed as previously described (11).

Viral RNA isolation, reverse transcription-PCR, and sequencing. One to 3 ml of plasma from C-PI (or 0.5 ml for NC-PI) was centrifuged at 14,000 rpm (20,800 × *g*) for 2 h at 4°C, and viral RNA was extracted using a Qiagen viral RNA blood mini kit (Qiagen, Inc.). Reverse transcription-PCR and sequencing were performed as described previously (44). Briefly, nested reverse transcription-PCR was attempted for all HIV coding sequences from C-PI but only for *gag* and *pol* from NC-PI, such that comparisons between the groups were limited to these sequences. Bidirectional population ("bulk") DNA sequencing was performed on an ABI 3730xl automated DNA sequencer. Data were analyzed using Sequencher software (Gene Codes). If the secondary peak was observed in both the 5′ and 3′ direction readings and the height of the secondary peak exceeded 25% of the dominant peak height in at least one of the two sequences, the nucleotides were interpreted to exist in mixtures at that position. Multiple alignment was performed using ClustalW. Contamination and/or sample mix-ups were ruled out by constructing a maximum-likelihood phylogenic tree (DNAml incorporated in BioEdit). Viral subtypes were determined using entire *gag* sequences with REGA HIV-1 Subtyping Tool version 2.0, available through the Stanford HIV Drug Resistance Database (http://dbpartners.stanford.edu/RegaSubtyping/). Likewise, drug resistance mutations were examined using the HIVdb Program Sequence Analysis tool (http://hivdb.stanford.edu/pages/algs/sierra_sequence.html).

Generation of chimeric viruses. Generation of *gag-protease* chimeric NL4-3 viruses, viral titration, and the replication capacity assay were performed as described previously (45). Briefly, *gag-protease* reverse transcription-PCR amplicons were cotransfected with linearized Δ*gag-protease* pNL4-3 vector into a long terminal repeat (LTR)-driven green fluorescent protein (GFP) reporter T cell line of CEM origin (GXR cells) (10), and chimeric viruses were obtained by homologous recombination. The patient origins of the resulting chimeric virus stocks were verified by comparing their sequences to the original plasma HIV RNA sequences (not shown). Titration was performed by infecting GXR cells with the chimeric viruses and measuring GFP expression by flow cytometry. The titer data were used to adjust input viral volumes in the subsequent replication capacity assay to obtain a multiplicity of infection (MOI) of 0.002 on day 2. GFP expression was monitored daily for a week. Replication capacity was expressed as the slope of the natural log of the percentage of GFP-expressing cells between days 3 and 6. Replication capacity assays were performed in duplicate, examining all of the viruses at the same time, and the average of the relative value to that of wild-type NL4-3 virus for each subject was used for comparisons.

Statistical analysis. Statistical analyses of continuous variables were performed using the Mann-Whitney U test. Comparisons of categorical value were performed using Fisher's exact test. A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. Plasma viral sequences obtained in this study were submitted to GenBank under accession nmbers GU367395 to GU367447 and GU367449 to GU367592.

RESULTS

Kinetics of plasma viremia during primary infection in persons who become HIV controllers. Little is known about the levels and kinetics of viremia during acute/early infection among individuals who subsequently become HIV-1 controllers. To address this, we identified 18 subjects diagnosed at the time of primary infection who maintained viral loads of <2,000 RNA copies/ml for at least a year in the absence of therapy (HIV controllers after primary infection [C-PI]). For each of these subjects, longitudinal samples were available for analysis of the kinetics of plasma viral load following untreated primary infection.

As shown in Fig. 1A and B, these 18 subjects achieved HIV control within 200 days after the estimated date of infection (EDI); they were then followed for a median of 1,089 (range, 744 to 2,079) days. Six of the 18 C-PI subsequently achieved elite control (<50 RNA copies/ml for at least 1 year) during follow-up (Fig. 1A), and 12 of the C-PI controlled viremia at between 50 and 2,000 RNA copies/ml (Fig. 1B). For those who achieved elite control, the time to viral loads of less than 50 RNA copies/ml varied widely. One subject was diagnosed with early infection at 130 days post estimated date of infection (EDI), when the viral load was already less than 50 RNA

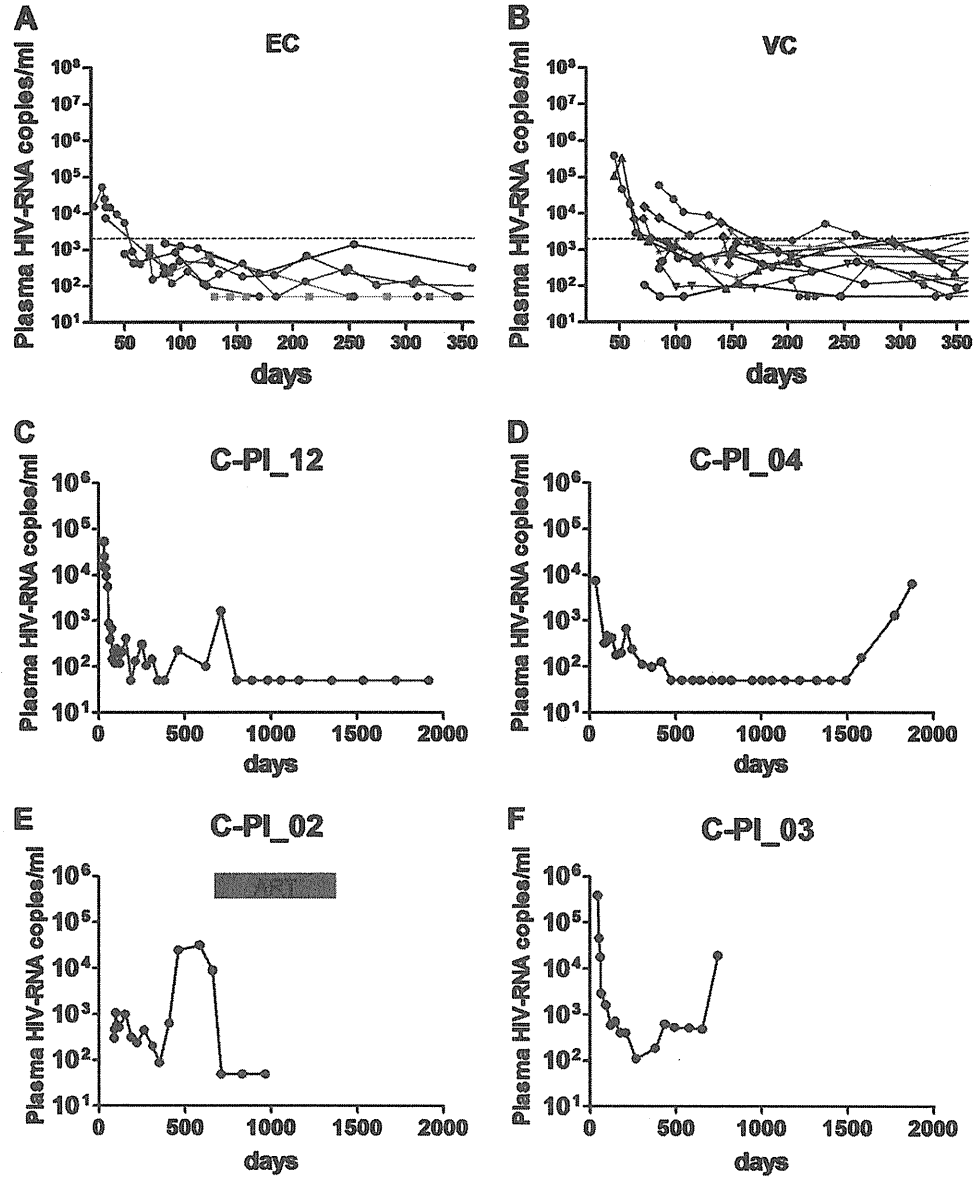

FIG. 1. Kinetics of plasma virus loads during acute/early phase of infection in individuals who subsequently achieve viremia control (<2,000 RNA copies/ml). (A) Data for six controllers after primary infection (C-PI) who achieved elite control (EC; <50 RNA copies/ml) during the follow-up period. Each line represents the plasma virus loads of an individual patient; the dashed line indicates 2,000 RNA copies/ml. The blue line shows data for C-PI_18, who achieved <50 RNA copies/ml after 600 days post-EDI and, therefore, displayed >50 RNA copies/ml during the period shown in the figure. (B) Data for twelve C-PI who remained viremic during the follow-up period. Each line represents the plasma virus loads of an individual patient; the dashed line indicates 2,000 RNA copies/ml. VC, viremic controllers. (C) Data for C-PI_12, who achieved elite control after 700 days post-EDI after experiencing a blip in viremia. (D) Data for C-PI_04, who achieved elite control after 470 days post-EDI and also experienced virologic escape after 1,500 days post-EDI. (E and F) Data for C-PI_02 and 03, who experienced virologic escape. ART, antiretroviral therapy.

copies/ml, and another achieved persistently undetectable viral loads at day 170, whereas another subject (C-PI_18) achieved elite control relatively slowly (604 days post-EDI; data not shown). One subject (C-PI_12) initially achieved a stable viremia of less than 2,000 RNA copies/ml until 710 days, at which point there was a blip in viremia and then a decline to below the limits of detection (<50 RNA copies/ml) (Fig. 1C). During the average-3-year follow-up, only 3 of the 18 subjects lost control, as evidenced by an increase in viral load to over 2,000 RNA copies/ml (Fig. 1D, E, and F), including one person initially meeting the criteria for elite control (C-PI_04) (Fig. 1D).

While the mean viral loads during acute infection have been reported to be in excess of 5×10^6 RNA copies/ml in other studies (31), the virus loads during acute phase in the HIV controllers studied here never exceeded 1×10^6 RNA copies/ml (Fig. 1A and B). Although it is important to note that the magnitude and timing of acute-phase peak viremia were likely not identified in most cases (as is also the case with other published studies), these observations nevertheless suggest

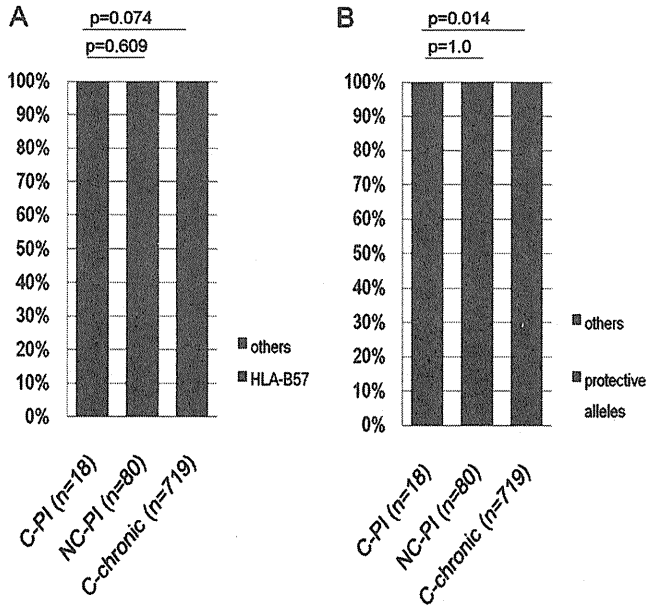

FIG. 2. Proportions of the individuals expressing protective HLA class I alleles. (A) Proportions of individuals expressing HLA-B57 among controllers after primary infection (C-PI), noncontrollers after primary infection (NC-PI), and chronic HIV controllers (C-chronic). (B) Proportions of individuals expressing protective HLA class I alleles (B13, B27, B51, and B57/B*1516/B*1517/B*5801) among C-PI, NC-PI, and C-chronic.

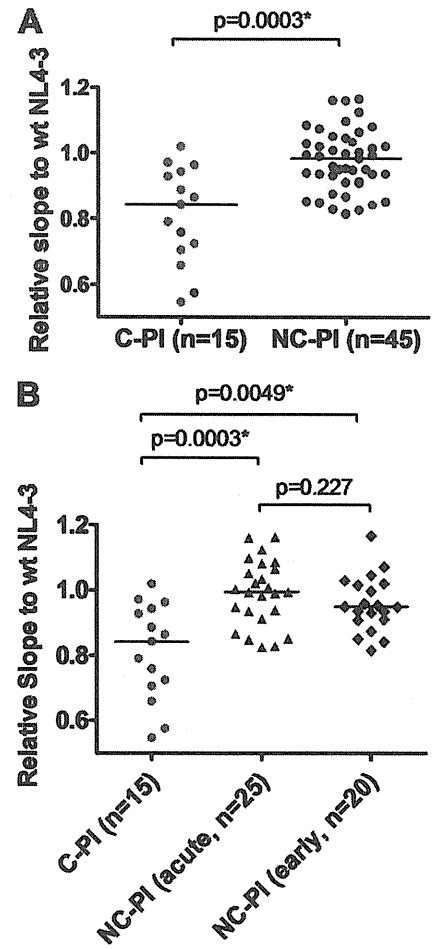

FIG. 3. Replication capacities of chimeric NL4-3 viruses carrying *gag-protease* derived from the acute phase of infection in controllers and noncontrollers. Replication capacities were compared between clade B-infected C-PI ($n = 15$) and NC-PI ($n = 45$). Average results of duplicate experiments for each virus normalized to the replication capacity of wild-type (wt) NL4-3 virus were plotted (expressed as the slope of the natural log of viral spread between days 3 and 6; see Materials and Methods). (A) Data for all of the chimeric viruses derived from individuals in acute/early phase of infection. The pink dots indicate viruses carrying protease inhibitor (PI) resistance mutations. (B) Data are the same as in the analysis shown in panel A, but the data for viruses derived from the NC-PI are stratified according to phase of infection (acute or early). Horizontal lines show medians of the results.

that persons who are able to control HIV spontaneously following primary infection may have lower levels of viremia during acute infection. These data indicate that viremia control can be initiated during the acute/early phase of infection and that when early control is achieved, it is largely maintained over the first few years of infection in persons who initially achieve this equilibrium. Moreover, they show that elite controller status may not be achieved until two or more years after primary infection.

HLA class I allele distribution in subjects who subsequently become HIV controllers. Among HIV controllers in the chronic phase of infection, there is an overrepresentation of "protective" HLA class I alleles, in particular, HLA-B57 and -B27 (4, 43, 48). To determine the potential role of host genetics in viral control in this cohort, we first performed high-resolution HLA typing. The frequency of individuals expressing HLA-B57 was the same in those who became HIV controllers (C-PI) as in those with primary infection who were defined as noncontrollers (NC-PI) (2/18 versus 5/80, $P = 0.6$) (Fig. 2A) but was lower than in an updated cohort of chronic HIV controllers (C-chronic [reference 48 and data not shown]) (224/719, $P = 0.074$). Likewise, the frequency of individuals expressing this and other so-called "protective" HLA class I alleles (defined as HLA-B13, -B27, -B51, and -B57/B*1516/B*1517/B*5801; see references 23, 25, 29, 32, and 47) among the C-PI did not differ from the frequency of such individuals among the NC-PI in the present study (5/18 versus 24/80, $P = 1.0$) (Fig. 2B) but, again, was significantly lower than in the chronic HIV controller cohort (5/18 versus 417/719, $P = 0.014$) (Fig. 2B).

Taken together, these data suggest that persons who achieve viral control following acute/early infection differ in host genetics from those HIV controllers classified using chronic-phase clinical and phenotypic definitions in that they are not enriched for the usual protective alleles associated with chronic HIV control (23, 25, 29, 32, 47).

Chimeric NL4-3 viruses carrying *gag-protease* derived from individuals who go on to become HIV controllers display reduced replication capacities. Our finding that protective HLA alleles were not enriched in this group suggested that factors other than host genetics might influence early control of viremia and led us to examine viral factors that might contribute to enhanced control. We recently reported that chimeric NL4-3 viruses carrying *gag-protease* derived from chronic elite controllers display reduced replication capacities compared to the

TABLE 1. HLA class I alleles, viral antiretroviral drug resistance mutations, and B57 footprint profiles in early controllers

C-PI	HLA class I alleles[c]	HLA-B57 footprint[d]	Viral drug resistance mutation(s) to:		
			PI	NRTI	NNRTI
01	A25/A68/B18/B57/Cw06/Cw12	T242N (I147L)	None	None	None
02	A02/A31/B08/B44/Cw05/Cw07	(I147L)	None	None	None
03	A01/A68/B08/B44/Cw05/Cw07	None	M46L, I54FL, I84V, N88D, L90 M	M41L, D67N, T69D, M184V, L210W, T215Y	A98G, K103N, Y318F
04	A26/A31/B07/B18/Cw07/Cw07	None	D30N, M46I, I84V, N88D, L90 M	M41L, E44D, D67N, K70R, L74I, V118I, L210Y, T215Y, K219Q	None
05	A01/A32/B35/B44/Cw02/Cw05	A146P (N252G)	None	None	None
06	A01/A01/B*1517/B49/Cw07/Cw12	G248A A146N (I147 M)	None	D67N, T215S, K219E, G333E	None
07	A02/A02/B07/B*5801/Cw07/Cw07	(G248E)	None	M41L	None
08	A03/A68/B14/B44/Cw07/Cw08	None	None	None	None
10	A23/A31/B39/B52/Cw07/Cw12	T242N A146P (I147L)	None	None	None
11	A01/A02/B44/B57/Cw05/Cw06	T242N G248A (I147L)	None	None	None
12	A01/A31/B37/B51/Cw01/Cw06	(I147L) G248A (M250I)	None	None	None
13[a]	A03/A31/B35/B35/Cw04/Cw04	N/D	None	None	None
14	A02/A32/B35/B44/Cw04/Cw05	None	None	M184V	G190A
15	A11/A31/B27/B35/Cw01/Cw01	(I147L)	None	None	None
16	A02/A29/B44/B49/Cw07/Cw16	NA	NA[b]	NA	NA
17	A02/A02/B44/B49/Cw07/Cw16	NA	NA	NA	NA
18	A02/A24/B*1501/B41/Cw03/Cw17	None	None	None	K103N P225H
20	A02/A03/B*1501/B56/Cw01/Cw03	T242N G248A (I147L)	None	None	None

[a] Unique recombinant form on clade A backbone.
[b] NA, not available.
[c] HLA-B57 and analogues are underlined.
[d] Mutations in parentheses are likely selected by HLA-B57.

replication capacities of those derived from chronic progressors (45). To determine the relative fitness of acute/early viruses in these subjects, we constructed chimeric *gag-protease* viruses from the earliest viral isolates from both C-PI and NC-PI subjects. In this analysis, *gag-protease* genes were amplified from plasma HIV RNA and chimeric viruses were generated for 15 subtype B-infected C-PI (C-PI_13 was infected with a unique recombinant form with a clade A backbone and, therefore, was excluded from the following analyses). As a control, we randomly chose 45 of the 80 NC-PI (median baseline virus load, 186,000 RNA copies/ml [interquartile range, 6,160 to 750,100]). As in previous studies (9, 45, 46, 53), replication capacity assays were performed and the slope of the natural log of viral spread in culture between days 3 and 6 was calculated. All viruses were assayed in the same experiment run in duplicate, and the results are presented as the values relative to the results for the wild-type NL4-3 virus.

As shown in Fig. 3A, chimeric viruses carrying *gag-protease* derived from individuals who go on to become controllers displayed significantly reduced replication capacities compared to those from individuals who became noncontrollers (C-PI versus NC-PI, 0.84 versus 0.98, $P = 0.0003$). Since many of the NC-PI were diagnosed during the acute phase of infection (AIEDRP group 1), whereas most of the C-PI were diagnosed during early infection (AIEDRP group 3), there was a concern that the observed difference might be a reflection of differences between acute and early infection. Therefore, we repeated the analysis, stratifying the 45 NC-PI into AIEDRP group 1 (acute, $n = 25$) or group 3 (early, $n = 20$), but found no statistically significant difference between them (0.95 versus 0.99, $P = 0.23$), whereas chimeric viruses carrying *gag-protease* from the C-PI displayed significantly reduced replication capacities compared to the replication capacities of viruses from acute and early NC-PI ($P = 0.005$ and 0.0003, respectively) (Fig. 3B). These data indicate that defects in viral fitness are strongly associated with the ability to control viremia following primary infection.

CTL escape mutations are present in viral sequences obtained from the acute/early phase in the persons who subsequently become HIV controllers. We recently reported that attenuation of the replication capacity of chimeric viruses carrying *gag-protease* derived from chronic elite controllers is associated with specific HLA class I alleles, indicating virus attenuation due to immune selection pressure (45). Since we observed the viruses in the C-PI to be attenuated, we next examined plasma viral sequences for evidence of CTL-driven escape mutations from the earliest available time point (median, 86 days, and range, 33 to 203 days post-EDI).

As shown in Tables 1 and 2, both C-PI expressing HLA-B57 (C-PI_01 and 11) harbored the B57-associated Gag T242N escape mutation within the TW10 (TSTLQEQIGW, Gag amino acids 240 to 249) epitope known to affect viral replication capacity (9, 22, 36, 40). One of the *gag* compensatory mutations, I223V, that is known to partially restore replication capacity impaired by the T242N mutation (9) was observed in C-PI_11, but none of these compensatory mutations was observed in C-PI_01 (Table 2). The relative replication capacities of chimeric NL4-3 viruses carrying *gag-protease* derived from these individuals were substantially reduced (0.864 and 0.724, respectively). Only one of the 45 NC-PI whose chimeric virus was tested for replication capacity expressed HLA-B57. The autologous viral sequence obtained at 59 days post-EDI exhibited the fitness-impairing T242N mutation but also the H219Q compensatory mutation, and the relative replication capacity

TABLE 2. Plasma viral Gag protein sequences in 15 clade B-infected early controllers[a]

C-PI	HLA-B57 analogue	Mutation(s) in indicated part of Gag sequence[a]	
		133 PIVQNLQGQMVHQAISPRTLNAWVK 157	213 DRLHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTNNPPI 256
01	B57	.....L.....	.....N.....
02	None	.....L.....	..T.....V....H....
03	None	.....	.....
04	None	..I.....	.....A.....S.....S....
05	None	.....I.....P.....	.....Q...V.....G....
06	B*1517	.....NM.....	.....Q...V....I.....A...H....
07	B*5801	.....	..V.....E...S..A.
08	None	.....	..M.....S....
10	None	.....PL.....	.....N.....
11	B57	.....L.....	.....V.....N.....A...S....
12	None	.....L.....	.....V.....A.I.....
14	None	.....M.....	.....
15	None	.....L.....	.....S....
18	None	.....M.....	..M.....H....
20	None	.....L.....	..T.....N.....A.....

[a] CTL epitopes restricted by HLA-B57 [IW9 (ISPRTLNAW) and TW10 (TSTLQEQIGW)] and major B57 footprint sites in sequences from the C-PI are underlined.

of the chimeric virus derived from this subject was slightly reduced (0.852). Although this subject was categorized as an NC-PI, the plasma virus load was only 3,680 RNA copies/ml on day 59 post-EDI, indicating that viremia had been relatively well controlled in this subject. Taken together, the replication capacities of chimeric viruses carrying *gag-protease* obtained during acute infection from HLA-B57-positive subjects appeared moderately reduced, which might be attributable to the typical fitness-impairing T242N escape mutation.

Evidence of transmitted CTL escape mutants from HLA-B57$^+$ donors. Recently, it was reported that individuals who acquired HIV from B57$^+$ donors have a better clinical course at least during the first year of infection (15), suggesting that the acquisition of attenuated viruses may benefit the recipient even in the absence of the restricting protective allele. Therefore, we investigated whether some C-PI might have acquired viruses from donors expressing protective HLA alleles. Examining Gag protein sequences in the C-PI, we observed the signature B57 escape mutation T242N that compromises replication capacity (9, 40) in two of the 5 C-PI who expressed no other protective alleles (C-PI_10 and 20) (Tables 1 and 2), and there was a trend of a higher frequency of the T242N mutation in the C-PI than in the NC-PI (2/5 versus 4/47). The viruses in C-PI_10 also exhibited the A146P/I147L signature escape mutations within and flanking the B57 ISW9 epitope (Gag amino acids 147 to 155) (20), adding further evidence that the viruses were transmitted from a B57$^+$ donor. The A146P/I147L mutations in conjunction with the T242N mutation have been shown to further reduce viral replication capacity (8). The relative replication capacity of the chimeric NL4-3 virus derived from C-PI_10 was extremely reduced (0.546), but the replication capacity of the virus derived from C-PI_20 was only minimally reduced (0.928). For C-PI_10, follow-up samples were available at 20 months after the EDI, but despite persistent low-level virus production *in vivo*, no reversions of B57-associated mutations (A146P/I147L/T242N) were observed (data not shown). These data suggest that transmission of viruses attenuated by CTL escape mutations in the HLA-B57$^+$ donors may account for some of the C-PI and indicate that transmitted CTL escape mutations may impair the replication capacity and pathogenicity of HIV following transmission.

High frequency of drug resistance mutations in plasma viral sequences in individuals who go on to become HIV controllers. Viral fitness can be impaired by defects in other HIV genes. In addition to *gag* sequences, we obtained plasma viral sequences for other HIV genes from the C-PI (16 *pol*, 14 *vif*, *vpr*, and *vpu*, 14 partial envelope, 11 *nef*, and 14 *rev* and *tat* sequences); however, similar to earlier studies of chronic elite controllers (44), virus sequencing in this cohort revealed no gross viral genetic defects (data not shown). Since selection pressures by antiretroviral drugs are known to impair viral replication capacity as well, we looked for the presence of "major" resistance mutations (as defined by the Stanford HIV Drug Resistance Database) in autologous viral sequences obtained during the acute phase of infection from the controllers and compared the frequency of these mutations to their frequency in sequences from noncontrollers.

Analysis of protease and reverse transcriptase (RT) sequences revealed a significantly higher prevalence of antiretroviral resistance mutations in C-PI (6/15 [40.0%]) than in NC-PI (10/80 [12.5%]) ($P = 0.018$) (Fig. 4A), some of which have been demonstrated to reduce viral replication capacity (M184V, L210W, T215Y, etc.) (26, 27, 39, 59). Among individuals who went on to become controllers, we observed two cases of protease inhibitor (PI) resistance mutations, 4 cases of nucleoside analogue reverse transcriptase inhibitor (NRTI) resistance mutations, and 3 cases of non-nucleoside analogue reverse transcriptase inhibitor (NNRTI) resistance mutations (Table 1). In addition, three of these cases exhibited multiclass drug resistance, which was also significantly more frequent than in NC-PI (3/15 versus 1/80, $P = 0.012$) (Fig. 4B and Table 1). Notably, excluding individuals expressing protective HLA alleles, the frequency of individuals infected with drug-resistant strains reached 60% among C-PI versus 8.5% in NC-PI (6/10 versus 4/47, $P = 0.0035$) (Fig. 4C), and the frequency of multiclass drug resistance strains reached 30% (Fig. 4D). Taking into consideration that resistance mutations can negatively impact viral replicative capacity (17, 39, 56, 58), these results

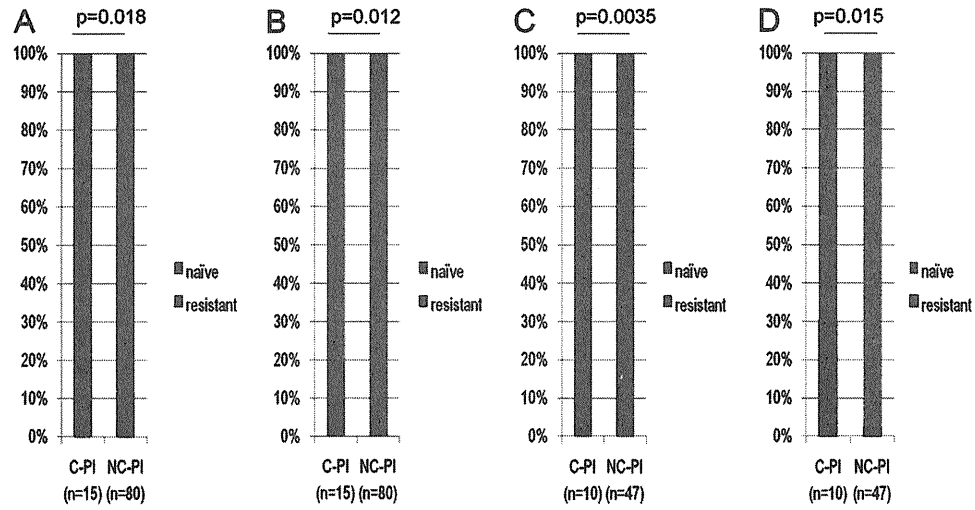

FIG. 4. Proportions of individuals diagnosed during acute/early phase and carrying viral strains with major drug resistance mutations (see Materials and Methods) are shown. (A) Data for individuals infected with strains with major PI, NRTI, and NNRTI resistance mutations. (B) Data for individuals infected with multiclass drug-resistant strains. (C) Data for individuals without protective HLA class I alleles infected with strains with major PI, NRTI, and NNRTI resistance mutations. (D) Data for individuals without protective HLA class I alleles infected with multiclass drug-resistant strains.

suggest that the transmission of drug-resistant variants contributes to viremia control during the early phase of infection in these individuals. It is important to note that in the present study, we analyzed replication capacity only for *gag-protease* chimeric viruses and, thus, that resistance mutations within RT were not evaluated. Among the total 60 viruses tested for replication capacity, five viruses (2 from C-PI and 3 from NC-PI) carried PI resistance mutations (Fig. 3A). Except for one virus derived from an NC-PI, all displayed moderately to severely impaired replicative capacities: the relative replication capacities of viruses derived from the two C-PI were 0.760 (C-PI_03) and 0.575 (C-PI_04), and viruses from two of the NC-PI carrying PI resistance mutations were moderately impaired (0.875 and 0.842).

Follow-up *pol* sequences were available 6 months later from two C-PI, both of whom became viremic controllers (50 to 2,000 RNA copies/ml). No change in resistance profile was observed in subject C-PI_06, whereas in subject C-PI_03, four of the 9 initially documented RT resistance mutations (M184V, L210F, and T215Y for NRTI and Y318F for NNRTI) (Table 1) reverted to the wild type (data not shown). However, plasma viral loads remained low in both individuals.

In addition, we were able to obtain the plasma *pol* sequence from subject C-PI_04, who became an elite controller despite lacking protective HLA alleles but experienced virologic escape after 4 years of infection (Fig. 1D). At that time, 2 of the NRTI resistance mutations (K70R and L74I) had reverted to the wild type, and the T215Y mutation partially reverted to T215C/S when the plasma viral load rose to 1,300 RNA copies/ml around 5 years post-EDI. Although the L74I and T215Y mutations are known to affect viral replicative fitness only moderately (39), the K70R mutation in conjunction with the M41L mutation strongly attenuates virus (30), suggesting that these reversions may have contributed to the virologic escape in this case.

DISCUSSION

Here, we demonstrate that viruses from persons who spontaneously control HIV following primary infection show impaired replicative capacity in the earliest stages of infection, indicating that characteristics of the infecting virus have a significant impact on viral set point and virus-host dynamics. By constructing chimeric viruses expressing the *gag-protease* region of patient isolates and inserting them into a laboratory strain backbone, we show that viruses derived from persons who are able to achieve viral loads of less than 2,000 RNA copies/ml are attenuated compared to contemporaneous viruses derived from persons who go on to develop progressive infection. Of subjects confirmed to be infected with clade B virus, 12/15 (80%) were recipients of potentially attenuated drug-resistant strains or viruses selected in $B57^+$ donors or expressed protective HLA alleles that selected for less fit viruses (summarized in Table 1). Remarkably, two cases had more than one factor: they expressed B57 analogues and were infected with NRTI-resistant strains. Together, these data indicate that viral dynamics in the earliest stages of HIV infection have a major impact on the course of disease.

In these persons who achieved viral control following primary infection, there was no enrichment of HLA alleles associated with long-term control found in chronic-controller cohorts. Instead, we saw enrichment for other factors that can attenuate virus replication, including transmission of drug-resistant strains and strains containing attenuating CTL escape mutations. The surprising finding that HLA-B57 was not enriched among controllers after primary infection may be related to the fact that persons expressing this allele are less symptomatic during acute infection and, thus, may have been less likely to be recognized and recruited in the acute phase of infection (1). Another important issue raised by this observation is the durability of control that is achieved early after acute

infection. Indeed, one C-PI who achieved elite control status but did progress later lacked protective HLA alleles. However, the majority of persons without protective alleles were able to persistently control viremia, in one case (C-PI_03) even after reversion of some of the drug resistance mutations, suggesting that early attenuation of virus may have sustained consequences. Additional follow-up will be required to answer this question.

The subjects in this study were largely selected from subjects enrolled in the Acute Infection, Early Disease Research Program, based on their ability to achieve controller status following documented primary infection. The criteria consisted of at least 1 year of viral loads of less than 2,000 RNA copies/ml, used to define HIV controllers because this is a level at which disease progression and transmission are much less likely (49). Of the 18 individuals who went on to become HIV controllers, 6 eventually met the criteria for elite control, with viral loads persisting at below 50 RNA copies/ml. Since one of the C-PI who achieved elite control (C-PI_04) and two C-PI who became viremic controllers (C-PI_02 and C-PI_03) later progressed, early control does not necessarily predict long-term control. Of note, none of them expressed protective HLA alleles, suggesting an important role of host genetics in eventual outcome.

Although we did not show any direct impact of individual mutations observed here on viral replicative capacity, there are several studies that demonstrate a fitness cost of PI resistance mutations in particular (41, 42, 60) and of CTL escape mutations within the Gag protein (9, 40, 53). Indeed, the viruses from two of the $B57^+$ C-PI were carrying the T242N escape mutation. In addition to these, we found putative B57 footprint mutations in the individuals expressing HLA-B*1517 (C-PI_06) and HLA-B*5801 (C-PI_07) that are known to cross-present B57 TW10 epitopes (23, 36). The autologous viral sequence for C-PI_06 did not have any of the typical signature B57 footprint mutations A146P/I147L/T242N but contained the G248A mutation that is associated with HLA-B57. Uncommon substitutions at the sites associated with HLA-B57 (A146N/I147M) were also observed (Tables 1 and 2) (20, 36). Likewise, none of the typical signature B57 mutations A146P/I147L/T242N were seen in C-PI_07, but the autologous viral sequence displayed a rare mutation (G248E) that has been reported to be selected in $B57^+$ subjects during primary infection (5). Although we do not have direct experimental evidence of a viral fitness cost due to these mutations, substitutions at these sites have been previously shown to impair viral replication capacity (8, 46). Indeed, the relative replication capacities of the chimeric NL4-3 viruses carrying *gag-protease* derived from these persons were reduced (0.887 and 0.658, respectively). Taken together, viruses in these 2 C-PI expressing HLA-B57 analogues likely harbored allele-specific mutations which might have contributed to compromised viral replication capacity.

We observed the typical HLA-B57 footprint mutation (T242N) in plasma viral sequences in two of the C-PI expressing none of the protective HLA alleles, supporting previous reports of better early clinical outcome of recipients who acquired viruses from B57-positive donors (15). By analyzing viral sequences in the rest of the C-PI, we found other mutations within and flanking the TW10 epitope that had likely been selected in $B57^+$ donors: autologous virus from C-PI_12 expressing the protective allele HLA-B51 had rare G248A/M250I mutations that were previously shown to be observed in $B57^+$ chronic elite controllers and to impair viral replication capacity *in vitro* (46), and the replication capacity of the *gag-protease* chimeric NL4-3 virus derived from this person was reduced (0.843). Another non-B57 analogue subject (C-PI_05) harbored the B57 signature mutation A146P and a rare N252G mutation in the flanking region of the TW10 epitope that had been observed in a B57 elite controller and demonstrated to affect viral replicative capacity (46). However, the replication capacity of the chimeric NL4-3 virus for this person was not appreciably reduced (0.943). These data suggest that there were additional individuals who potentially acquired viruses from B57-positive donors and went on to become controllers.

In the current study, we did not examine the function of the reverse transcriptase or the impact of individual resistance mutations on RT function, but there are a number of publications that also support the theory presented here (19, 26, 27, 30, 38, 39, 57, 59). For instance, the M184V mutation observed in C-PI_03 and 14 is selected by lamivudine and is known to strongly reduce viral replicative fitness (39, 59); the T215Y mutation observed in C-PI_03 and 04 has a similar effect, albeit moderately (26, 39), and in addition, the L210W mutation that was observed in C-PI_03 and remained at the later time point has been shown to have a strong negative impact on viral replication capacity (27). Moreover, there was a report that the number of NRTI resistance mutations inversely correlates with viral replicative capacity (51), further supporting the idea that viruses from the acute phase of infection carrying multiple resistance mutations in some of the individuals who go on to become controllers are attenuated.

While the difference in replication capacities of chimeric viruses derived from the acute/early phase of infection in controllers and noncontrollers was significant, chimeric viruses derived from some controllers displayed only minimal reductions in replication capacity, suggesting that a better early clinical course cannot be explained solely by attenuation of Gag-Protease function. Impairment of HIV replication by mutations in other genes, like *nef* deletions (16, 34), or by other mechanisms may also be important. Therefore, it will be warranted to investigate the function of other HIV proteins, including RT and envelope, in future studies.

The hypothesis presented here, that viral attenuation affects the course of disease following acute infection, has been proposed previously (2, 35, 50) and is supported by other studies showing that the transmission of escape mutations selected by protective alleles in the donor provides a clinical advantage to the recipient (15, 24). Moreover, a recent study of children born to $B57^+$ mothers revealed reduced viral loads in the $B57^+$ children despite the transmission of B57 CTL escape mutants (54). In addition, there is a report of an inverse relationship between plasma viral load and the number of genotypic resistance mutations during primary infection (14). These data strongly suggest that the transmission of less fit viruses due to attenuating antiretroviral resistance mutations and CTL escape mutations could result in a favorable clinical course in the recipients, at least during the early phase of infection. It should be noted that our findings might imply that the spread of drug-resistant variants will increase the proportion of individ-

uals who can control viremia in the early phase of infection; however, further investigations are necessary in order to clarify this point.

In conclusion, viruses obtained during the acute/early phase of infection from persons who subsequently become controllers were attenuated. This observation can be partly explained by the transmission of drug-resistant strains and/or CTL escape variants from $B57^+$ donors or by *de novo* CTL escape mutations in $B57^+$ recipients. During an average of 3 years of follow-up, the plasma virus load remained stable in the majority of subjects, even in one in whom apparent fitness-enhancing reversions were observed. These data suggest that viral attenuation in acute infection may have a long-term impact on control, but further longitudinal studies are warranted to reveal the role of viral fitness in long-term control of HIV-1 infection.

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Gag-Protease-Mediated Replication Capacity in HIV-1 Subtype C Chronic Infection: Associations with HLA Type and Clinical Parameters▽†

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The mechanisms underlying HIV-1 control by protective HLA class I alleles are not fully understood and could involve selection of escape mutations in functionally important Gag epitopes resulting in fitness costs. This study was undertaken to investigate, at the population level, the impact of HLA-mediated immune pressure in Gag on viral fitness and its influence on HIV-1 pathogenesis. Replication capacities of 406 recombinant viruses encoding plasma-derived Gag-protease from patients chronically infected with HIV-1 subtype C were assayed in an HIV-1-inducible green fluorescent protein reporter cell line. Viral replication capacities varied significantly with respect to the specific HLA-B alleles expressed by the patient, and protective HLA-B alleles, most notably HLA-B*81, were associated with lower replication capacities. HLA-associated mutations at low-entropy sites, especially the HLA-B*81-associated 186S mutation in the TL9 epitope, were associated with lower replication capacities. Most mutations linked to alterations in replication capacity in the conserved p24 region decreased replication capacity, while most in the highly variable p17 region increased replication capacity. Replication capacity also correlated positively with baseline viral load and negatively with baseline CD4 count but did not correlate with the subsequent rate of CD4 decline. In conclusion, there is evidence that protective HLA alleles, in particular HLA-B*81, significantly influence Gag-protease function by driving sequence changes in Gag and that conserved regions of Gag should be included in a vaccine aiming to drive HIV-1 toward a less fit state. However, the long-term clinical benefit of immune-driven fitness costs is uncertain given the lack of correlation with longitudinal markers of disease progression.

There is broad heterogeneity in the ability of HIV-infected individuals to control virus replication, ranging from elite controllers, who maintain undetectable viral loads without treatment, to rapid progressors, who progress to AIDS within 2 years of infection (9, 22, 32). Many interrelated factors, including host and viral genetic factors involved in antiviral immunity and the viral life cycle, may partially account for the differences in the course of disease progression (10, 11, 30, 41). The complex interplay between host genetic factors and viral factors is exemplified by human leukocyte antigen (HLA) class I-restricted cytotoxic T-lymphocyte (CTL) responses, which exert considerable immune pressure on the virus, resulting in escape mutations that affect the interaction of viral and host proteins, thereby influencing infection outcome.

The exact mechanisms by which some HLA class I alleles, such as HLA-B*57 and HLA-B*27, are associated with slower progression to AIDS, while others, such as B*5802 and B*18, are associated with accelerated disease progression (6, 20, 42), are unclear. The magnitude and/or breadth of HLA-restricted CTL responses to the conserved Gag protein has been correlated inversely with disease progression or markers of disease progression in several studies (12, 21, 28, 31, 35, 43, 46), although there are some exceptions (4, 16, 37), while preferential targeting of the highly variable envelope protein (as occurs in HLA-B*5802-positive individuals) correlates with higher viral loads (21, 29). Protective HLA alleles restrict CTL responses that impose a strong selection pressure on a few specific Gag p24 epitopes, resulting in escape mutations (14) for which fitness costs have been demonstrated either through site-directed mutations introduced into a reference strain background (2, 8, 25, 38) or through *in vivo* reversion of these mutations after transmission to an HLA-mismatched individ-

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ual (8, 24). Recent evidence suggests that Gag escape mutations with a fitness cost, particularly those in p24, are a significant determinant of disease progression: the transmitted number of HLA-B-associated polymorphisms in Gag was found to significantly impact the viral set point in recipients (although an associated fitness cost was not shown) (7, 15), and in a small number of infants, decreased fitness of the transmitted virus with HLA-B*5703/5801-selected mutations in Gag p24 epitopes resulted in slower disease progression (33, 39). Also, the number of reverting Gag mutations (thought to revert as a consequence of fitness costs) associated with individual HLA-B alleles was strongly correlated with the HLA-linked viral set point in chronically infected patients (26). A recent *in vitro* study showed that HLA-associated variation in Gag-protease, with resulting reduced replication capacity, may contribute to viral control in HIV-1 subtype B-infected elite controllers (27). Taken together, these studies suggest that CTL responses restricted by favorable HLA alleles select for escape mutations in conserved epitopes, particularly those in Gag, resulting in a fitness cost to HIV and therefore at least partly explaining the slower disease progression in individuals carrying these alleles.

To date, many of the studies investigating the fitness cost of Gag escape mutations and their clinical relevance have concentrated on escape mutations associated with protective HLA alleles, have not assessed fitness consequences in the natural sequence background (in the presence of other escape and compensatory mutations), and/or have focused on a limited number of patients. Most importantly, the majority of studies have focused on HIV-1 subtype B. The present study is the first to use a large population-based approach and clinically derived Gag-protease sequences to investigate comprehensively the relationships between immune-driven sequence variation in Gag, viral replication capacity, and markers of disease progression in chronic infection with HIV-1 subtype C, the most predominant subtype in the epidemic. We assayed the replication capacity of recombinant viruses encoding patient Gag-protease in an HIV-1-inducible green fluorescent protein (GFP) reporter cell line and found associations between lower replication capacities, protective HLA alleles, protective HLA-associated mutations, lower baseline viral loads, and higher baseline CD4 counts. However, Gag-protease replication capacity did not correlate with the subsequent rate of CD4 decline.

MATERIALS AND METHODS

Study subjects. The study subjects included 406 antiretroviral-naïve individuals chronically infected with HIV-1 subtype C from the Sinikithemba cohort in Durban, South Africa. These individuals were HLA typed to 4-digit resolution by molecular methods (20). Viral load (Roche Amplicor assay, version 1.5) and CD4 count (Trucount technology) measurements were obtained at study entry (baseline) for all participants and at 3-month intervals thereafter for 339 of the participants (20). At baseline, the median viral load of the cohort was 4.77 $\log_{10}$ HIV RNA copies/ml (interquartile range [IQR], 4.15 to 5.27 $\log_{10}$ HIV RNA copies/ml), and the median CD4 count was 340 cells/mm^3 (IQR, 238 to 477 cells/mm^3). Over the subsequent course of study follow-up (the mean follow-up time was 2.28 years per individual; IQR, 1.21 to 3.02 years), the median rate of CD4 decline was −30 cells/mm^3 per year (IQR, −73 to −3 cells/mm^3 per year). The median age of the study subjects at baseline was 31 years (IQR, 27 to 36 years), and 322 (79%) patients were female. Among the study participants, there was no significant association between age, gender, and baseline viral load or CD4 count as was reported previously (42), and therefore we did not control for these variables in analyses. Written informed consent was obtained from all study subjects, and the study protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.

Generation of Gag-protease NL4-3 recombinant virus stocks. Patient Gag-protease was isolated and inserted into an NL4-3 backbone to generate recombinant viruses. Protease was included to maintain the important interaction between Gag and protease, namely, cleavage of the Gag polyprotein by protease. Viral RNA was isolated from plasma by use of a QIAamp Viral RNA Mini kit from Qiagen (Valencia). Reverse transcription-PCR (RT-PCR) was performed as previously described (27), using a Superscript III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) and the following Gag-protease-specific primers: 5′ CAC TGC TTA AGC CTC AAT AAA GCT TGC C 3′ (HXB2 nucleotides 512 to 539) and 5′ TTT AAC CCT GCT GGG TGT GGT ATY CCT 3′ (nucleotides 2851 to 2825). A second round of PCR was performed with 100-mer forward and reverse primers that were exactly complementary to NL4-3 on either side of Gag-protease, using a TaKaRa Ex *Taq* HS enzyme kit (Takara, Shiga, Japan). Two 50-μl PCR mixtures were prepared for each sample, comprising 37 μl diethyl pyrocarbonate (DEPC)-treated water, 5 μl 10× Ex *Taq* buffer, 4 μl of deoxynucleoside triphosphates (dNTPs), 0.8 μl forward primer (10 μM), 0.8 μl reverse primer (10 μM), 0.25 μl Ex *Taq*, and 2 μl RT-PCR product. Thermocycler conditions were as follows: 94°C for 2 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min; and 72°C for 7 min. PCR products from two 50-μl reaction mixtures were pooled, and 10 μl was set aside for sequencing. The remainder was used in the generation of recombinant viruses. Gag-protease-deleted pNL4-3 plasmid was prepared as previously described (27), and large stocks of the plasmid were generated using a Plasmid Maxi kit (Qiagen, Valencia, CA). The plasmid was digested for 2 h at 60°C immediately prior to cotransfection of 2×10^6 Tat-inducible GFP reporter GXR T cells (3) in R10 medium (800 μl RPMI-1640 [Sigma, St. Louis, MO] supplemented with 10% fetal bovine serum [Gibco, NY], 2 mM L-glutamine [Sigma], 10 mM HEPES [Gibco], and 50 U/ml penicillin-streptomycin [Gibco]) with 10 μg digested plasmid and ≈85 μl Gag-protease PCR product via electroporation at 300 V and 500 μF (27). Following a 1-h incubation at room temperature, GXR cells were transferred to T25 flasks containing 4 ml of medium each. Five days later, 5 ml R10 medium was added to each flask. The percentage of infected cells was monitored from day 12 onwards by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Culture supernatants were harvested when approximately 30% of the GXR cells were infected and were stored in 1-ml aliquots at −80°C for use in subsequent titration and replication assays.

Titration and replication assays. Titration of virus stocks and replication assays were performed as previously described (2, 27, 38), using a multiplicity of infection (MOI) of 0.003. The mean slope of exponential growth from days 3 to 6 was calculated using the semilog method in Excel. This was divided by the slope of growth of the wild-type NL4-3 control included in each assay to generate a normalized measure of replication capacity. Replication assays were performed at least in duplicate, and results were averaged.

Sequencing of Gag-protease gene. The Gag-protease PCR product was diluted 1:15 in DEPC-treated water and population sequenced using Big Dye Terminator ready reaction mix V3 (Applied Biosystems, Foster City, CA) and the following sequencing primers: 5′ CTT GTC TAG GGC TTC CTT GGT 3′ (nucleotides 1098 to 1078), 5′ CTT CAG ACA GGA ACA GAG GA 3′ (nucleotides 991 to 1010), 5′ GGT TCT CTC ATC TGG CCT GG 3′ (nucleotides 1481 to 1462), 5′ CAA CAA GGT TTC TGT CAT CC 3′ (nucleotides 1755 to 1736), 5′ CCT TGC CAC AGT TGA AAC ATT T 3′ (nucleotides 1981 to 1960), 5′ TAG AAG AAA TGA TGA CAG 3′ (nucleotides 1817 to 1834), 5′ CAG CCA AGC TGA GTC AA 3′ (nucleotides 2536 to 2520), and 5′ GGA GCA GAT GAT ACA GTA TT 3′ (nucleotides 2331 to 2350). Sequences were analyzed on an ABI 3130xl genetic analyzer (Applied Biosystems) and were visualized and edited in Sequencher 4.8. Sequence data were aligned with the sequence of HIV-1 subtype B reference strain HXB2 (GenBank accession no. K03455), using a modified NAP algorithm (18), and insertions with respect to the HXB2 sequence were stripped from the sequences. A neighbor-joining tree was constructed from nucleotide sequences by using Paup 4.0 and was edited in Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Nucleotide differences between plasma and recombinant virus Gag-protease sequences were quantified using Highlighter (http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter.html). BioEdit 7.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to calculate the similarity of each sequence to the consensus subtype C Gag sequence from 2004.

Identification of HLA-associated polymorphisms. Statistical methods (described in detail in reference 5) that correct for phylogenetic relatedness between HIV sequences, amino acid covariation, and HLA linkage disequilibrium effects were used to identify HLA-associated polymorphisms. Briefly, a maximum like-

lihood phylogenetic tree was constructed for each gene, and a model of conditional adaptation was inferred for each observed amino acid at each codon. In this model, the amino acid is assumed to evolve independently down the phylogeny, until it reaches the observed sequences at the tree tips. In each host, the selection pressure arising from HLA-restricted CTL and covariation between HIV codons is modeled directly by a stochastic additive process. To identify which factors contribute to the observed sequences, a forward selection procedure is employed, in which the most significant association is iteratively added to the model, with P values computed using the likelihood ratio test. Each observed amino acid variant at each codon is evaluated in a binary fashion (presence versus absence thereof). Multiple tests are addressed using q values, the P value analogue of the false discovery rate (FDR), for each P value threshold (40). The FDR is the expected proportion of false-positive results among results deemed significant at a given threshold. For example, at a q value of ≤ 0.2, we would expect a false-positive proportion of 20% among identified associations.

Data analysis. Viral replication capacities were grouped according to the HLA class I alleles expressed by the host. Analysis of variance (ANOVA) was used to assess whether significant differences in replication capacities were observed within expressed HLA-A, -B, and -C alleles. Then, for each individual allele ($n \geq 5$), Student's t test (or the Mann-Whitney U test in cases where the assumptions of Student's t test were not met) was used to compare replication capacities of viruses generated from persons expressing versus not expressing the allele in question. The relationships between replication capacity and log viral load, CD4 count, rate of CD4 decline, number of HLA-associated polymorphisms, and sequence similarity to the HIV-1 subtype C Gag consensus were assessed using Pearson's correlation (for normally distributed variables) or Spearman's rank correlation (for non-normally distributed variables). Viruses were also categorized according to the 10th and 90th percentiles of the replication capacity data, into low- and high-replication-capacity groups, respectively. Clinical and sequence parameters were compared between these groups, using Student's t test (or the Mann-Whitney U test) or Fisher's exact test (in the case of proportion comparisons). The association between single amino acid residues in Gag-protease and the replication capacity was analyzed by Mann-Whitney U tests (univariate method) and linear regression with a forward selection process (multivariate method). q values were calculated in both cases to account for multiple comparisons (40). The significance cutoff for all analyses, unless indicated otherwise, was a P value of <0.05.

Nucleotide sequence accession numbers. Gag-protease sequences obtained in this study are available in the GenBank database under accession numbers HM593106 to HM593510.

RESULTS

Validation of generated Gag-protease NL4-3 recombinant viruses. Gag-protease NL4-3 recombinant virus stocks from 406 subjects were generated in a median of 27 days (IQR, 23 to 32 days) following cotransfection of HIV-inducible, GFP-expressing T cells with Gag-protease-deleted NL4-3 plasmid and clinically derived Gag-protease amplicons. To test whether recombinant viruses were representative of the original plasma quasispecies, Gag-protease was resequenced from 40 randomly selected recombinant viruses and compared with the original plasma HIV RNA sequences. The median number of total nucleotide differences between the recombinant virus sequence and the original plasma HIV RNA sequence (when mixtures were not included as differences) was 0 (IQR, 0 to 1.5), resulting in an average nucleotide similarity of 0.99% between pairs. The average number of nucleotide mixtures in recombinant virus sequences was 21 (standard deviation [SD] = 17), indicating somewhat reduced diversity (Student's t test; $P = 0.0002$) compared to that of the original plasma sequences (mean = 35; SD = 36). Recombinant virus Gag sequences closely clustered with respective plasma Gag sequences in a phylogenetic tree (see Fig. SA1 in the supplemental material). These data indicate that the Gag-protease recombinant viruses were representative of the original plasma quasispecies. All further analyses were based on the original plasma HIV sequences.

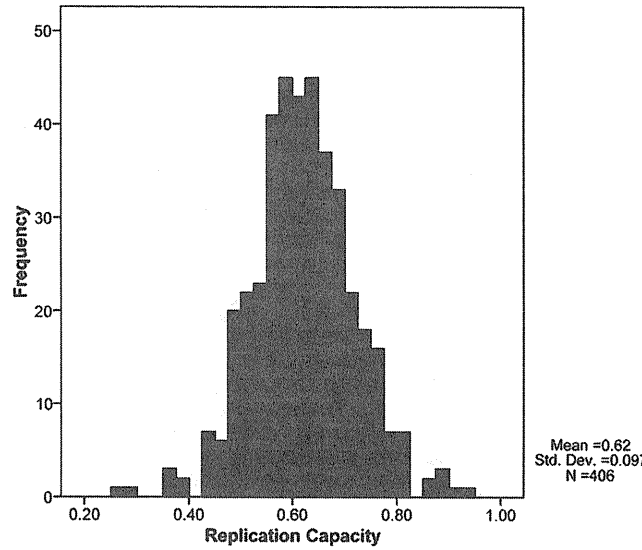

FIG. 1. Distribution of Gag-protease NL4-3 recombinant virus replication capacities, normalized to the growth of wild-type NL4-3.

Assay variability. The replication capacities of Gag-protease NL4-3 recombinant viruses were assayed in duplicate, independently, in an HIV-1-inducible GFP reporter cell line. Replication capacity was defined as the slope of the increase in the percentage of infected cells from days 3 to 6 following infection, normalized to wild-type NL4-3. Duplicate measurements were highly concordant (Pearson's correlation; $r = 0.88$ and $P < 0.0001$). Accuracy of recombinant viral titers was achieved: on day 3 of the assay, the mean % GFP-expressing cells was 0.65% (SD = 0.28%). Importantly, the observed variability in day 3 readings did not influence viral replication capacity measurements (Pearson's correlation; $r = 0.04$ and $P = 0.44$).

Distribution of replication capacities of recombinant viruses. The NL4-3-normalized replication capacities of the recombinant viruses generated from the 406 cohort participants approximated a normal distribution (mean = 0.62; SD = 0.1) (Fig. 1). The replication capacities of the recombinant HIV-1 subtype C Gag-protease sequences inserted into the NL4-3 backbone were considerably lower than those of the wild-type NL4-3 control and 25 subtype B Gag-protease NL4-3 recombinant viruses, whose mean replication capacity normalized to wild-type NL4-3 was 0.95 (SD = 0.13) (data not shown).

Association of recombinant virus replication capacities with specific HLA types. Replication capacities of recombinant viruses were grouped according to HLA alleles expressed by the host (Fig. 2). Overall, replication capacities varied significantly between the different HLA-B alleles (ANOVA; $P = 0.01$) but not between HLA-A or HLA-C alleles, suggesting that HLA-B alleles have the greatest impact on Gag-protease-mediated replication capacity. Relationships between specific HLA alleles and replication capacity were also observed, the strongest of which was the association of HLA-B*81 with lower replication capacities (Student's t test; $P < 0.0001$). P values presented are uncorrected for multiple comparisons. Only the association of HLA-B*81 with lower replication capacities

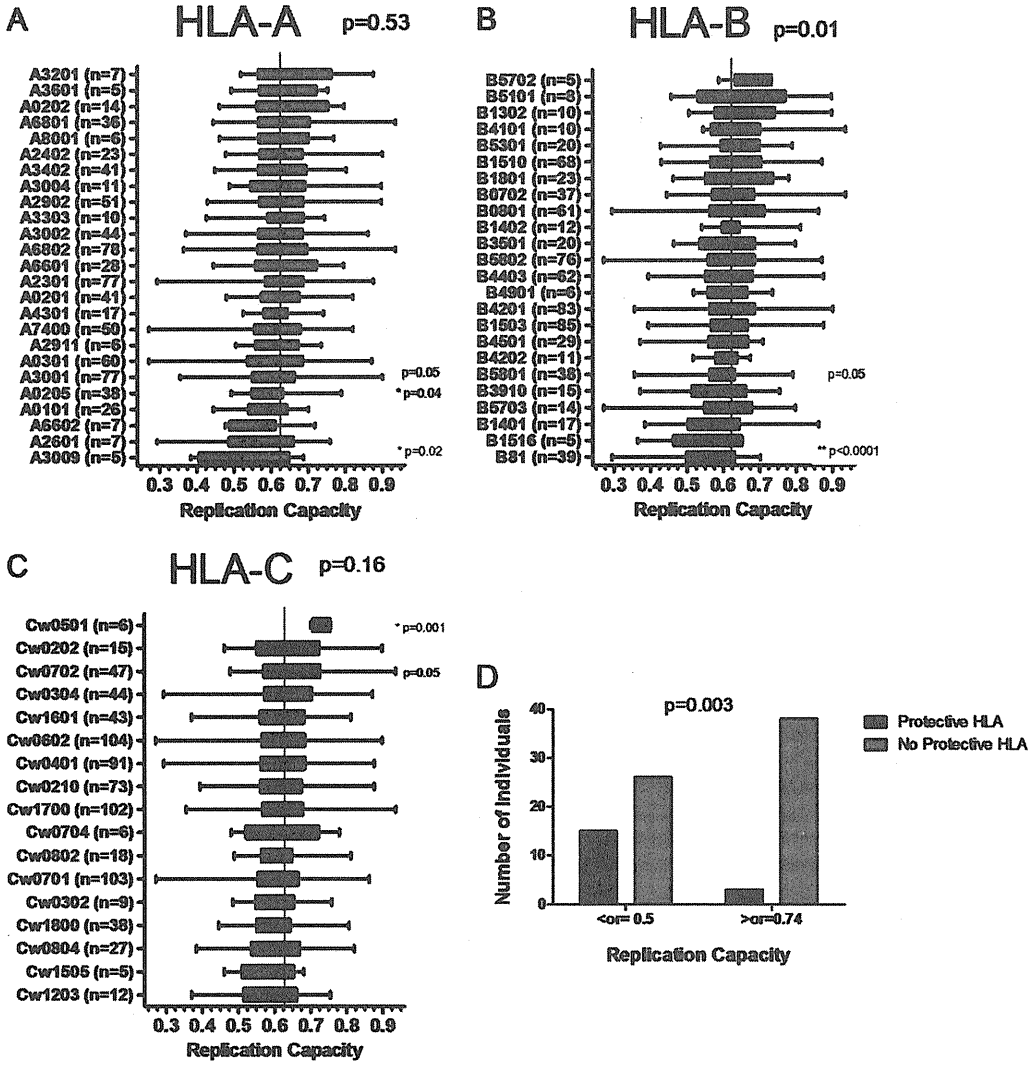

FIG. 2. Associations between HLA alleles and replication capacities of Gag-protease NL4-3 recombinant viruses. (A to C) Graphs show the mean (dot), median (vertical line), interquartile range (edges of boxes), and most extreme values (edges of whiskers) of replication capacities for each different HLA allele for which n was ≥ 5. *, individual significant ($P < 0.05$) associations (Student's t test); **, associations that survive Bonferroni correction for multiple comparisons. Overall P values (ANOVA) indicate the significance of differences in replication capacity between alleles of each group. (D) Graph showing a significantly greater proportion (Fisher's exact test) of individuals with protective HLA alleles (HLA-B*57, HLA-B*5801, and HLA-B*81) in the group with the least-fit viruses (replication capacity of ≤ 0.5; $n = 41$) than in the group with the fittest viruses (replication capacity of ≥ 0.74; $n = 41$).

would remain statistically significant following Bonferroni adjustment for multiple comparisons.

Besides HLA-B*81, other alleles that were associated with low-replication-capacity recombinant viruses were HLA-B*5801, HLA-A*0205 (Mann-Whitney U test; $P = 0.05$ and $P = 0.04$, respectively), HLA-A*3009, and HLA-A*3001 (Student's t test; $P = 0.02$ and $P = 0.05$, respectively). Due to tight linkage between HLA-B*5801 and HLA-A*0205 ($D' = 0.56$ [23]), the allele driving the effect could not be identified. Among 5 individuals with HLA-B*3009, 4 possessed HLA-B*81, which likely explains the association of HLA-B*3009 with lower replication capacities.

Alleles associated with higher replication capacities were HLA-Cw*0702 (Student's t test; $P = 0.05$) and HLA-Cw*0501 (Mann-Whitney U test; $P = 0.001$). Only 6 individuals in this study possessed HLA-Cw*0501, and all were linked to replication capacities above the 80th percentile of the data set (>0.7). HLA-Cw*0501 is in linkage disequilibrium with HLA-B*1801—5 of 6 individuals with HLA-Cw*0501 also carried HLA-B*1801—and could therefore partly contribute to the disadvantage associated with HLA-B*1801 in subtype C infection (20).

In an additional analysis, the HLA types of the individuals corresponding to the fittest recombinant viruses ($\geq$90th percentile of the data set, i.e., ≥ 0.74; $n = 41$) were compared to the HLA types of individuals with the least-fit recombinant viruses (10th percentile of the data set, i.e., ≤ 0.5; $n = 41$). Protective alleles were defined as those that were most strongly associated with lower viral loads in HIV-1 subtype C-infected individuals, namely, HLA-B*57, HLA-B*5801, and HLA-

B*8101 (20), and were also found later to be the most strongly associated with lower viral loads or higher CD4 counts in a cohort of over 1,000 HIV-1 subtype C-infected individuals (43). The proportion of individuals possessing a protective allele was significantly greater in the low-replication-capacity group than in the high-replication-capacity group (Fisher's exact test; $P = 0.003$) (Fig. 2D).

When HLA-A, -B, and -C alleles were ranked according to viral load and then according to replication capacity, the ranks correlated positively with one another for each group of HLA alleles, although not significantly for HLA-C alleles (Spearman's rank correlation; $r = 0.43$ and $P = 0.03$, $r = 0.42$ and $P = 0.04$, and $r = 0.47$ and $P = 0.06$, respectively), which indicates a relationship between viral load and Gag-protease replication capacity (data not shown).

Correlation between replication capacity and baseline log viral load or CD4 count. Replication capacities of recombinant viruses correlated positively with baseline log viral loads (Spearman's rank correlation; $r = 0.24$ and $P < 0.0001$) and negatively with baseline CD4 counts (Spearman's rank correlation; $r = -0.17$ and $P = 0.0004$) (Fig. 3A and B). These effects remained after removal of the protective alleles HLA-B*57, HLA-B*5801, and HLA-B*81 from analysis (Spearman's rank correlation; $r = 0.18$ and $P = 0.001$ for baseline log viral load and $r = -0.14$ and $P = 0.01$ for baseline CD4 count). Interestingly, analysis of the relationship between viral load or CD4 count and replication capacity among individuals expressing these protective alleles also revealed a significant positive correlation (Pearson's correlation; $r = 0.33$ and $P = 0.001$ for viral load and $r = -0.33$ and $P = 0.001$ for CD4 count).

Relationship between replication capacity and subsequent longitudinal rate of CD4 decline. An average of 2.28 years (SD = 1.3 years) of untreated follow-up was available for 339 Sinikithemba patients. For each study subject, linear regression was used to compute the rate of CD4 decline for the duration of untreated clinical follow-up. Spearman's correlation was then used to investigate the relationship between viral replication capacity and subsequent rate of CD4 decline. We observed no statistically significant relationship overall between replication capacity and CD4 decline (Spearman's rank correlation; $r = -0.01$ and $P = 0.79$). Stratification of the analysis by baseline CD4 counts ($\leq$200, $\geq$200, $\leq$350, and $\geq$350 cells/mm^3) also failed to reveal any significant correlations between replication capacity and the rate of CD4 decline (not shown). Figure 3C shows a lack of correlation between CD4 decline and Gag-protease-mediated replication capacity at baseline CD4 counts of $\geq$200 cells/mm^3 (Spearman's rank correlation; $r = -0.02$ and $P = 0.73$).

Association of sequence variability in Gag with replication capacity. (i) Overall variability. To investigate whether an increasing number of polymorphisms in Gag would tend to reduce replication capacity, the percent amino acid similarities of Gag sequences to the 2004 consensus subtype C Gag sequence were calculated using the sequence identity matrix function in BioEdit 7.0 and correlated with replication capacity. Unexpectedly, the calculated Gag percent similarity correlated negatively, although weakly, with replication capacity (Pearson's correlation; $r = -0.18$ and $P = 0.0004$), i.e., the fittest viruses were generally least like the consensus sequence (Fig. 4A). This analysis was repeated separately for each region

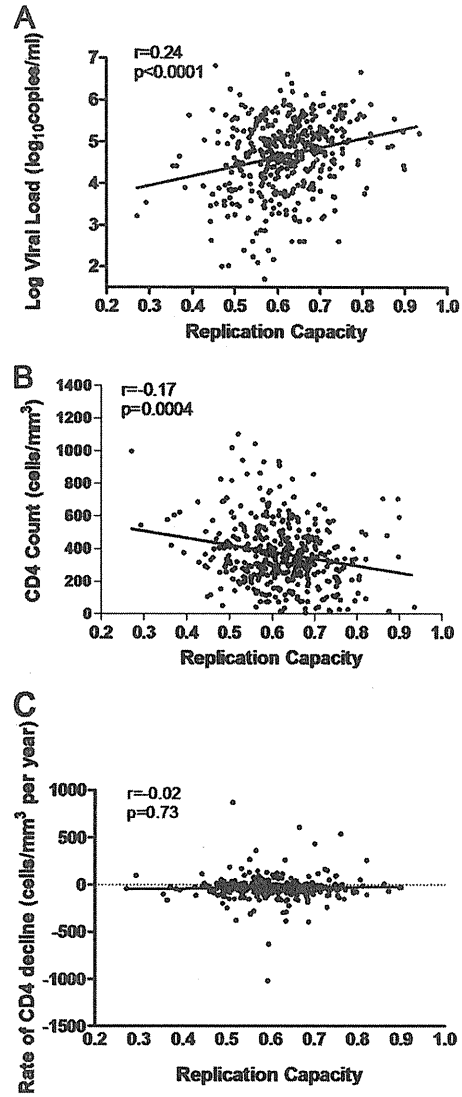

FIG. 3. Correlations between replication capacities of recombinant viruses encoding patient Gag-protease and markers of disease progression. The graphs show replication capacity versus baseline log viral load (A), baseline CD4 count (B), and rate of CD4 decline (C). Correlations were calculated with Spearman's rank correlation test.

of Gag, namely, p17, p24, p7, and p6, to see whether this relationship differed between regions. There remained an inverse relationship between percent similarity to consensus and replication capacity for every region of Gag except p24, although this was statistically significant only for p17 and p7 (Fig. 4B). There was no correlation between percent similarity to the subtype C Gag p24 consensus and replication capacity. In contrast, the majority of nonconsensus residues in p17/p7 increased replication capacity. It should be noted that divergence from the consensus subtype C sequence did not represent convergence to the consensus subtype B sequence, which would have indicated that divergence from the consensus subtype C sequence resulted in better compatibility with the subtype B NL4-3 backbone, and therefore in fitter viruses.

(ii) HLA-associated variability. HLA-associated polymorphisms—amino acids that are significantly more likely to occur

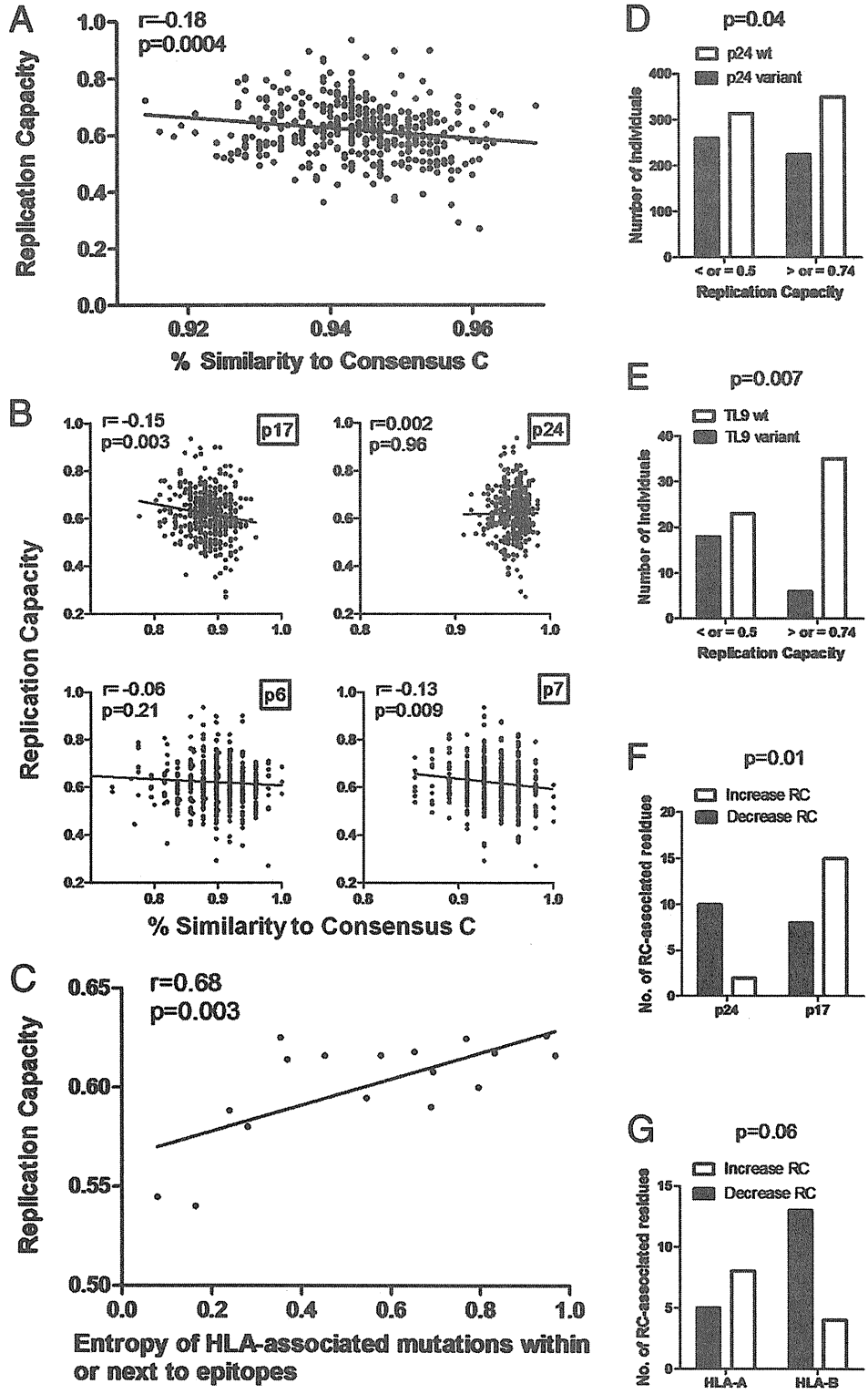

FIG. 4. Associations between sequence variability in Gag-protease and replication capacities of recombinant viruses encoding Gag-protease. (A) Significant negative correlation (Pearson's correlation; $n = 405$) between percent similarity of Gag sequences to the consensus subtype C Gag sequence and replication capacity. (B) Gag p17, p24, p6, and p7 percent similarity to consensus subtype C sequence versus replication capacity (Spearman's rank correlation; $n = 405$). (C) Significant positive correlation (Pearson's correlation; $n = 17$) between entropy of HLA-associated sites in or within five amino acids of Gag epitopes and the average replication capacity of viruses with mutations at these sites. (D and E) Significant differences (Fisher's exact test) in proportions of p24 variant epitopes and p24 consensus epitopes (D) and of TL9 variant epitopes and TL9 consensus epitopes (E) between the least-fit viruses (replication capacity of ≤ 0.5; $n = 41$) and the fittest viruses (replication capacity of ≥ 0.74; $n = 41$). (F and G) Significant differences (Fisher's exact test) in proportions of nonconsensus amino acids associated with decreased replication capacity (RC) in p24 versus p17 ($n = 12$ and $n = 23$) (F) and in HLA-A- versus HLA-B-restricted epitopes ($n = 13$ and $n = 17$) (G).

in the presence of a particular HLA allele—were identified in the current data set by use of methods that take into account the phylogenetic relatedness of sequences, amino acid covariation, and HLA linkage disequilibrium effects (5). Each sequence was then analyzed in the context of the patient's HLA class I profile, and the number of HLA-associated polymorphisms was computed. To further analyze the influence of HLA alleles on Gag-protease replication capacity, the computed polymorphisms were correlated with replication capacity. The numbers of HLA-A-, -B-, and -C-associated polymorphisms in each sequence did not correlate significantly with replication capacity overall. Likewise, no dose-dependent effects of polymorphisms on replication capacity were observed among polymorphisms associated with protective HLA types. Similarly, when the relationship between the number of HLA-associated polymorphisms and replication capacity was investigated irrespective of patient HLA class I profile, i.e., also taking into account inherited polymorphisms, no significant associations were found. Therefore, while some HLA-associated polymorphisms significantly impact replication capacity (8, 45), the sum of HLA-selected polymorphisms, irrespective of location in Gag, was not associated with replication capacity in this chronic infection cohort. There was, however, a weak trend (Spearman's rank correlation; $r = -0.09$ and $P = 0.08$) toward lower replication capacities with increasing numbers of HLA-associated polymorphisms in epitopes or within five amino acids of epitopes restricted by the selecting HLA allele (these polymorphisms are more likely to represent escape mutations, not secondarily arising compensatory mutations [15]). Previously, increasing numbers of HLA-B-associated polymorphisms in or within five amino acids of Gag epitopes were strongly associated with lower viral loads in early infection, and this was attributed to lower fitness levels of these viruses (15). The number of HLA-B-associated polymorphisms in or within five amino acids of Gag epitopes was negatively correlated with fitness (Spearman's rank correlation; $r = -0.11$ and $P = 0.03$), although not strongly so. The relatively weak relationship between the number of HLA-associated polymorphisms in Gag and replication capacity in the present chronic infection cohort might be explained by the accumulation of compensatory mutations during the course of infection. In fact, evidence has been found for a strong effect of HLA-mediated selection pressure in Gag on replication capacity in early infection and no such significant relationship in the very late chronic stage of infection, suggesting that this effect wanes over time, presumably due to the development of compensatory mutations (M. A. Brockman et al., submitted for publication).

Since there is some evidence that HLA-associated escape mutations occurring in conserved sites of HIV carry a greater fitness cost than those occurring in regions of high variability (45), we compared the average replication capacity of viruses possessing each HLA-associated polymorphism with the corresponding entropy at that position. A trend toward a significant correlation between these two parameters was found (Pearson's correlation; $r = 0.24$ and $P = 0.06$). When the analysis was restricted to those polymorphisms in epitopes or within five amino acids of epitopes restricted by the selecting HLA allele, the correlation was much stronger (Pearson's correlation; $r = 0.68$ and $P = 0.003$) (Fig. 4C). Thus, HLA-associated escape mutations at more conserved sites (with lower entropy) in Gag were associated with greater fitness costs.

(iii) Epitope variability: association of TL9 variant with lower replication capacity. Next, the relationship between sequence variability in specific HLA-restricted epitopes and replication capacity was examined. The proportion of variant Gag epitopes (i.e., nonconsensus) versus consensus epitopes was compared between the least-fit and fittest virus groups by Fisher's exact test, and no significant difference was found. However, there were marginally more variant Gag p24 epitopes in the sequences from the least-fit group (Fisher's exact test; $P = 0.04$) (Fig. 4D). This significant result was driven mainly by the greater proportion of variant HLA-B*81-restricted TL9 epitopes in the least-fit group (Fisher's exact test; $P = 0.007$) (Fig. 4E), although there were also significantly more variant HLA-B*57-restricted QW9 epitopes in viruses of lower fitness (Fisher's exact test; $P = 0.04$) (data not shown).

(iv) Single amino acid associations with replication capacity. In an exploratory analysis, the Mann-Whitney U test was used to identify specific amino acids in Gag-protease associated with increased or decreased replication capacity. Although none of the comparisons yielded Q values of ≤ 0.2, 58 associations with P values of <0.05 were found for Gag, and 9 were found for protease, when consensus-nonconsensus pairs were counted as a single association ($n \geq 5$ for both groups compared [see Table SA1 in the supplemental material]). Of the 58 associations in Gag, 23 occurred in p17, 12 in p24, 3 in the p2 linker peptide, 9 in p7, and 11 in p6.

Considering amino acids in Gag associated with alterations in viral replication capacity, most of the nonconsensus amino acids in p24 were associated with lower replication capacity (10/12 residues), while most of the nonconsensus residues in p17 were associated with increased replication capacity (15/23 residues). This difference was statistically significant (Fisher's exact test; $P = 0.01$) (Fig. 4F). Only 17 of 58 amino acids associated with replication capacity alterations corresponded to an HLA association at that position (not necessarily with the same amino acid), and 11 of these were HLA-B associated. Twenty-six associations occurred in published or previously predicted epitopes (13, 36), with 13 in HLA-A-restricted epitopes, 17 in HLA-B-restricted epitopes, and 6 in HLA-C-restricted epitopes (10 of these occurred in epitopes that were restricted by more than one HLA allele class and were thus considered under more than one category). Within HLA-B-restricted epitopes, 13 (8 of these were in p24) of 17 nonconsensus amino acids were associated with decreased replication capacity, while in the HLA-A-restricted epitopes, 8 of 13 nonconsensus amino acids (10 of these were in p17) were linked with increased replication capacity (Fisher's exact test; $P = 0.06$) (Fig. 4G). These results are suggestive of HLA-B-mediated selective pressure on Gag p24, with resulting lower replication capacity. It should also be noted that half of the amino acids associated with changes in Gag-protease-mediated replication capacity were neither HLA class I associated nor within known or predicted epitopes.

Multivariate analysis (linear regression with forward selection) was also undertaken. Seventeen of the 58 associations in Gag and 4 of the 9 associations in protease identified by univariate analysis also had P values of <0.05 (but Q values of >0.2) in the multivariate model, and the strongest of these