Figure 3. Assay sensitivities for minor populations. pRE11-NL4-3 (X4) and pRE11-BaL (R5) were mixed at indicated ratios. The total plasmid concentration in the mixture was adjusted to 100 ng/µl. The mixture was transfected to 293FT cells and fused to the indicator cells. (a) RL activities. Black columns show the RL activities on X4 indicator (N4X4-DSP$_{1-7}$), while white columns on R5-indicator (N4R5-DSP$_{1-7}$). Small bars at the top of each column indicate the mean RLU $\pm$ SD from three independent experiments. (b) GFP activities. Left panel shows activities of the mixture with indicated ratio on X4 indicator (N4X4-DSP$_{1-7}$), while right panel on R5-indicator (N4R5-DSP$_{1-7}$).

(Figure 2a) and GFP signals (Figure 2b). The absence of RL activities on N4-DSP$_{1-7}$ confirmed the importance of co-receptors for the fusion.

Assay detection thresholds and sensitivity for minor populations

To evaluate assay sensitivity in identifying minor variants within a single sample, we mixed pRE11-NL4-3 (X4) and pRE-BaL (R5) in varying ratios and measured RL activities and GFP signals (Figure 3a and b). Both methods of detection identified X4 viruses more readily than R5 viruses. Based on luciferase activity, the presence of approximately 0.3% X4 viruses gave values significantly higher than background

(0% X4), while R5 viruses had to comprise approximately 5% of the mixture for the signal to be detectable over background (Figure 3a). Similarly, based on GFP signals, X4 viruses comprising as little as 0.1% of the mixture could be detected, while detection of R5 viruses had a minimum threshold of approximately 1% (Figure 3b).

Validation of the chemokine receptor specificity using the CXCR4 inhibitor AMD3100 and CCR5 inhibitor maraviroc

293FT cells expressing *env* from reference strains NL4-3 (X4) or BaL (R5) were co-cultured with N4X4-DSP$_{1-7}$ or N4R5-DSP$_{1-7}$ cells in the absence or presence of AMD3100 or maraviroc (Figure 4a and b). In the absence of inhibitors, RL activities of

the matched co-culture were high (Figure 4a). In the presence of AMD3100, the RL activity of the co-culture of 293FT cells expressing NL4-3-derived *env* with N4X4-DSP$_{1-7}$ cells was reduced by 83%. The RL activity of the co-culture of 293FT cells expressing BaL-derived *env* with N4X4-DSP$_{1-7}$ cells was low in the absence of AMD3100 and was not affected significantly by its presence. The RL activity of the co-culture of 293FT cells expressing BaL-derived *env* with N4R5- DSP$_{1-7}$ was reduced by 81% in the presence of maraviroc. The RL activity of the co-culture of 293FT cells expressing NL4-3-derived *env* with N4R5- DSP$_{1-7}$ was low regardless of the presence or absence of maraviroc. The results indicated that DSP-Pheno could be used as an assay for entry inhibitors.

Cell-fusion assay of clinical samples

To evaluate assay performance using clinical samples, we selected plasma samples from 101 treatment-naïve, HIV-1-positive patients, whose infection with clade B viruses had been confirmed (data not shown). The patient population was classified into two groups based on CD4 T cell count. The low CD4 group consisted of 57 patients with CD4 T cell counts <350 cells/μl; median 228 (range 2–350) cells/μl, and median viral load was 4.77 (range 2.97–6.62) log 10 copies/ml (Figure 5a and b). The high CD4 group consisted of 44 patients with CD4 cell counts >350 cells/μl; median 442 (range 351–843) cells/μl, and median viral load was 4.04 (range 1.60–5.41) log 10 copies/ml. The viral load differences
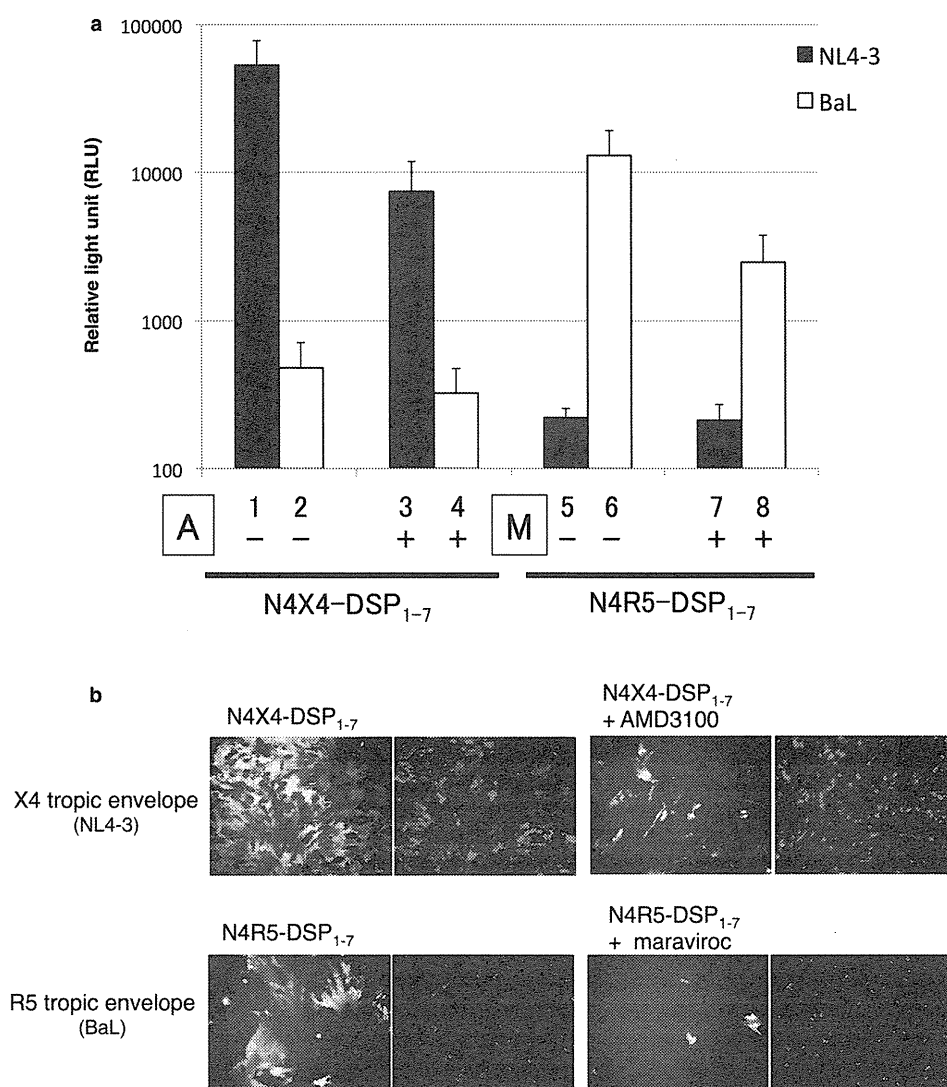

Figure 4. Inhibition of cell fusions by entry inhibitors. Two μM/well of CXCR4 inhibitor AMD3100 or CCR5 inhibitor maraviroc were added into N4X4-DSP$_{1-7}$ and N4R5-DSP$_{1-7}$ cells 90 minutes prior to cell-fusion assay using env derived from reference strains.(a) RL activities. Columns show the mean RLU±SD from 5 independent experiments. Black columns, RL activities of env derived from X4 reference strain (NL4-3); white columns, RL activities of env derived from R5 reference strain (BaL). Results from X4-indicator (N4X4-DSP$_{1-7}$) (lanes 1–4) and R5-indicator (N4R5-DSP$_{1-7}$) (lanes 5–8). A, AMD3100; M, maraviroc. Presence or absence of inhibitor indicated by + or −, respectively. (b) GFP activities. Green fluorescence in the left panel of each pair shows successful cell fusions; red spots in the right panels show the successful transfection. Reference strains, indicator cells and inhibitors used are shown in the figure.
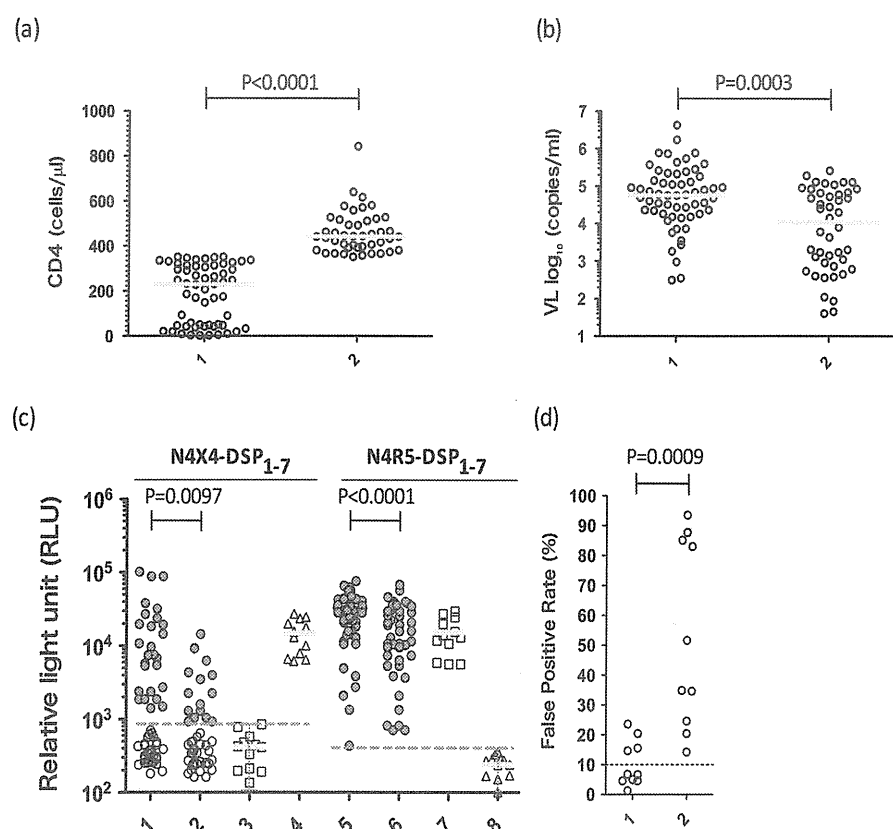
Figure 5. DSP-Pheno and Geno2Pheno on clinical samples. Patients were assigned to one of the two groups based on CD4+ T cell counts. Horizontal green bars indicate the median value. (a) CD4 counts of the patients. Lane 1, Fifty-seven patients with CD4 <350 cells/μl, median =228 (range 2–350) cells/μl. Lane 2, Forty-four patients with CD4 >350 cells/μl, median 442 (range 351–843) cells/μl. (b) Viral load of each group. Lane 1, CD4 <350 group, median viral load =4.77 (range 2.97–6.62) log10 copies/ml. Lane 2, CD4 <350 group, median viral load =4.04 (range 1.60–5.41) log 10 copies/ml. (c) Mean luciferase activities of the patients' plasma samples. Lanes 1 and 5, CD4 <350 group; lanes 2 and 6, CD4 >350 group; lanes 3 and 7, R5 controls (BaL); lanes 4 and 8, X4 controls (NL4-3). Dashed red lines are the cut-off value, that is, the mean value+2SD based on 3 three determinations in 12 independent experiments for each combination of negative control and indicator cell. (d) Geno2Pheno [co-receptor] analysis of representative samples. Lane 1, 10 samples from dual/X4 [N4X4-DSP$_{1-7}$ (+), N4R5-DSP$_{1-7}$ (+)] group by DSP-Pheno; Lane 2, 10 samples from R5 [N4X4-DSP$_{1-7}$ (−), N4R5-DSP$_{1-7}$ (+)] group by DSP-Pheno. For dual/X4 and R5 group, five patients each from CD4 < 350 and CD4 >350 groups were chosen. Dashed line indicates the cut-off value as 10% of FPR.

between the two groups were statistically significant by the Mann–Whitney U test (p <0.001). Aliquots of viral envelope DNA from each plasma sample were used to construct pRE11-envbulk for transfection into 293FT cells. The plasma viral load necessary for the assay was roughly 3.00 log 10 copies/ml for subtype B viruses, although we could amplify the env gene in a patient with 1.60 log 10 copies/ml.

We used the laboratory strain, BaL as the R5 control and NL4-3 as the X4 control to define the cut-off values. We examined BaL on N4X4-DSP$_{1-7}$ cells and NL4-3 on N4R5-DSP$_{1-7}$ cells. We defined the cut-off value tentatively as the mean value+2SD based on 3 determinations in 12 independent experiments for each combination of negative control and indicator cell (red dashed line in Figure 5c). As expected, both combinations showed stably low RL activities, with cut-off values of 876 for N4X4-DSP$_{1-7}$ cells and 397 RLU for N4R5-DSP$_{1-7}$ cells.

Samples from all patients gave positive RL signals on R5 indicator cells (N4R5-DSP$_{1-7}$) in the fusion assay, which suggested that the bulk of virus in each patient was able to

use CCR5 as the co-receptor (Figure 5c, lanes 5 and 6). Median RLU value of the low CD4 group was significantly higher than that of the high CD4 group on R5 indicator cells (p <0.0001). Median RLU value of the low CD4 group was also higher significantly on X4 indicator cells (p =0.0097) and 26/57 (46%) of low CD4 cases versus 15/44 (34%) of high CD4 cases gave positive RL signals (Figure 5c, lanes 1 and 2). Higher fusion activities on both indicator cells are compatible with higher viral loads in patients with lower CD4 T cell counts and may suggest more dual or X4 tropic (dual/X4) viruses in this group of patients.

To compare the result with conventional GTA, we selected 10 samples each from dual/X4 [N4X4-DSP$_{1-7}$ (+), N4R5-DSP$_{1-7}$ (+)] and R5 [N4X4-DSP$_{1-7}$ (−), N4R5-DSP$_{1-7}$ (+)] cases. Env V3 nucleotide sequences from pRE11-envbulk plasmids were subjected to the Geno2Pheno [co-receptor]. R5-representative samples showed significantly higher FPR than dual/X4-representative samples (p =0.0009) (Figure 5d). DSP-Pheno and Geno2Pheno gave concordant results in 10/10 R5 and 6/10 dual/X4 samples (Figure 5d).

Although there were four samples with discordant result in dual/X4 samples, FPR of these samples were low (range: 14.7–23.6%).

Discussion

We developed a quick, safe and sensitive HIV-1 PTA utilizing double split proteins (DSP-Pheno) and validated the specificity of the assay using laboratory strains with known co-receptor usage. We recognize several limitations of this preliminary study, but the results nevertheless are promising. We assayed bulk envelope genes amplified from plasma from HIV-1-infected patients, rather than cloned envelope genes, and our sample only included subtype B HIV-1. Future studies are necessary to demonstrate the usefulness of the DSP-Pheno.

One caveat of the DSP-Pheno assay is that it is a cell-fusion system, and cell–cell fusion may differ in significant details from virus–cell fusion. For example, recent studies have shown that HIV-1 virions carry fewer surface glycoproteins than previously assumed [24]. The DSP-Pheno assay uses neuroglyoma cell-derived NP-2 cell lines with overexpressed CD4 and co-receptors. Although these NP-2-derived cell lines have been characterized extensively [16,17], some unknown cell surface molecules may be involved in the fusion process. The DSP-Pheno assay is a gag-free system and requires only the assembly of reporter proteins pre-formed in the fusion partner, but infection by a retrovirus requires that the entire gag particle pass through the fusion pore. Careful comparison between DSP-Pheno and in-house pseudoviral assay or GTA using clonal clinical isolates is under way.

GFP portion is necessary as a module of DSP to compensate weak self-association of split RL [15]. Although RL would be more suitable for quantitative assay, GFP may prove single clear positive fusion in the sample with very low RL readout. This feature of DSP-Pheno incorporating two different assays may be useful for certain scientific purposes.

Although several issues remain to be clarified, DSP-Pheno has multiple advantages over the conventional pseudoviral PTA: (i) the turnaround time for DSP-Pheno is short, with results available in as few as 5 days, starting from patients' plasma; (ii) DSP-Pheno is a virus-free assay that does not require a special biosafety facility, making it particularly appealing for in-house use; and (iii) the RL assay in DSP-Pheno has high sensitivity and specificity and compares favourably with the best pseudoviral PTA published in the detection of minor X4 populations using laboratory strains. Trofile™ (Monogram Biosciences Inc., CA, USA) is currently the only commercially available PTA approved for clinical use, and the latest version, "Enhanced Trofile™," detects X4 minor populations present in concentrations as low as 0.3% [25]. A pseudoviral PTA described by Soda and colleagues had 1% detection threshold for X4 viruses [16]. Although the RL assay in DSP-Pheno could detect X4 laboratory strains present in concentrations as low as 0.3%, further studies are needed to apply the assay for the clinical use. DSP-Pheno may also be useful for the comparison of with GTA to improve the algorithm for the co-receptor usage of non-B subtypes.

Conclusions

We described a new cell-fusion-based, high-throughput PTA for HIV-1, which would be suitable for in-house studies. Equipped with a two-way reporter system, RL and GFP, DSP-Pheno is sensitive and offers a short turnaround time. Although maintenance of cell lines and laboratory equipment for the assay is necessary, it provides a safe assay system without infectious viruses. With further validation against other conventional analysis, DSP-Pheno may prove to be a useful laboratory tool. The assay may be useful especially for the research on non-B subtype HIV-1 whose co-receptor usage has not been studied much.

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Significant Reductions in Gag-Protease-Mediated HIV-1 Replication Capacity during the Course of the Epidemic in Japan

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Human immunodeficiency virus type 1 (HIV-1) evolves rapidly in response to host immune selection pressures. As a result, the functional properties of HIV-1 isolates from earlier in the epidemic may differ from those of isolates from later stages. However, few studies have investigated alterations in viral replication capacity (RC) over the epidemic. In the present study, we compare Gag-Protease-associated RC between early and late isolates in Japan (1994 to 2009). HIV-1 subtype B sequences from 156 antiretroviral-naïve Japanese with chronic asymptomatic infection were used to construct a chimeric NL4-3 strain encoding plasma-derived *gag-protease*. Viral replication capacity was examined by infecting a long terminal repeat-driven green fluorescent protein-reporter T cell line. We observed a reduction in the RC of chimeric NL4-3 over the epidemic, which remained significant after adjusting for the CD4$^+$ T cell count and plasma virus load. The same outcome was seen when limiting the analysis to a single large cluster of related sequences, indicating that our results are not due to shifts in the molecular epidemiology of the epidemic in Japan. Moreover, the change in RC was independent of genetic distance between patient-derived sequences and wild-type NL4-3, thus ruling out potential temporal bias due to genetic similarity between patient and historic viral backbone sequences. Collectively, these data indicate that Gag-Protease-associated HIV-1 replication capacity has decreased over the epidemic in Japan. Larger studies from multiple geographical regions will be required to confirm this phenomenon.

I t has been almost 30 years since the discovery of human immunodeficiency virus type 1 (HIV-1) (1), a pathogen that first infected human populations approximately 100 years ago (2, 3). Over the course of the pandemic, substantial and various selection pressures have been exerted on HIV-1 by its human host, possibly resulting in alterations in viral replication capacity (RC), virulence, and/or other properties (4). However, few studies to date have examined population-level alterations in HIV-1 replication capacity over the epidemic's course (5, 6), and none have investigated the potential role of immune escape mutations selected by cellular immune responses in modulating this phenomenon.

Cytotoxic T lymphocytes (CTLs) play a major role in controlling viremia and disease progression in HIV-1 infection (7–11). However, the selection of escape mutations within or near CTL epitopes facilitates viral immune evasion (12–16) and represents a major challenge for HIV vaccine design. Since CTL responses are restricted by human leukocyte antigen (HLA) class I alleles, CTL escape mutations emerge in an HLA-specific manner. Many CTL escape mutations have been identified experimentally (15, 17–20); moreover, statistical analyses of large population-level data sets have yielded HLA-associated mutation maps of HIV-1 protein sequences, thereby identifying putative CTL escape sites (21–27). Importantly, these escape variants may be transmitted both vertically and horizontally (12, 28, 29). Furthermore, CTL escape variants selected by common HLA class I alleles may have been accumulating at the population level over the course of the epidemic in some regions, most notably, Japan (30, 31). If this is the case, active CTL epitopes restricted by common HLA class I alleles may be lost through mutational escape as the epidemic matures, pos-

sibly leading to increased viral virulence through enhanced immune evasion in these populations.

Although CTL escape mutations allow HIV to evade immune detection, they can also reduce viral replication capacity (28, 32–38). Furthermore, while certain virus-attenuating escape mutations revert upon transmission to recipients lacking the relevant HLA class I allele (20, 28, 36), this is not always the case (39, 40). Indeed, a recent study suggested that fixation of viruses carrying such attenuating escape mutations is increasing in an allele frequency-dependent manner in certain populations (30). These observations have led to the hypothesis that the *in vitro* replication capacity of HIV-1 may have been decreasing over the epidemic's course in certain populations, at the expense of the loss of active CTL epitopes at the population level through mutational escape.

In the present study, we generated chimeric HIV-1 isolates by inserting plasma HIV RNA-derived *gag-protease* sequences from 156 asymptomatic, chronically infected treatment-naïve Japanese patients dating from 1994 to 2009 into a laboratory strain backbone (HIV-1 NL4-3) and examined their replication capacity using published methods (33, 41–44). We specifically focused on the

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TABLE 1 Demographic characteristics of the participants[a]

Characteristic	Value
Median (range) age (yr)	31 (18–73)
No. (%) of participants by gender	
Male	167 (94)
Female	10 (5.6)
Median (IQR) CD4^{+} T cell count (no. of cells μl^{-1})	339 (269–452)
Median (IQR) pVL (no. of RNA copies/ml)	23,000 (5,700–46,000)
No. (%) of participants by route of transmission[b]	
Men who have sex with men	147 (83)
Heterosexual	25 (14)
Unknown	5 (2.8)

[a] Data are for 177 participants.
[b] Hemophilia patients were excluded from this study.

Gag protein, as it is likely to be the most important target of HLA-restricted CTLs (45) and because numerous fitness-reducing HLA-associated escape mutations have been described therein (28, 32–38). As such, Gag is ideal for investigating the potential effects of immune-mediated HIV attenuation over time. Overall, we have observed a significant reduction in Gag-Protease-mediated HIV-1 replication capacity as the epidemic has matured in Japan.

MATERIALS AND METHODS

Study participants. A total of 177 antiretroviral-naïve Japanese individuals with asymptomatic chronic HIV-1 infection who visited the Research Hospital of the University of Tokyo from April 1992 through March 2009 were enrolled. Individuals with acute HIV infection, chronically infected individuals with a history of AIDS-defining illnesses, and hemophilia patients were excluded (hemophilia patients were excluded because Japanese hemophiliacs acquired HIV-1 from imported blood products in the mid-1980s [46, 47], a fact which could confound our analyses). The sociodemographic characteristics of the participants are shown in Table 1. Blood collected at the earliest available time point during the asymptomatic chronic phase of infection (median, 173 days after diagnosis; interquartile range [IQR], 56 to 525 days after diagnosis; range, 0 to 4,313 days after diagnosis) was studied. Plasma and peripheral blood mononuclear cells (PBMCs) were separated by standard procedures and stored at −80°C and in liquid nitrogen, respectively, until use. The study was approved by the Institutional Review Board of the Institute of Medical Science, University of Tokyo. Written informed consent was obtained from all participants.

HLA class I typing. Genomic DNA was extracted from PBMCs using a QIAamp DNA blood minikit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. High-resolution HLA class I typing was performed using a WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan) and a Luminex multianalyte profiling system (Luminex Corporation, Austin, TX).

Viral RNA isolation. Plasma (500 μl) was quickly spun down to remove cell debris. The resulting clarified plasma was then centrifuged at 14,000 rpm (20,000 × g) for 120 min to pellet the virions. After centrifugation, 360 μl of the supernatant was discarded, leaving 140 μl plasma for which viral RNA was extracted using a QIAamp viral RNA minikit (Qiagen Inc., Valencia, CA). Extracted viral RNA was eluted in 80 μl of elution buffer and stored at −80°C until use.

Plasma virus sequencing. The HIV-1 gag-protease region was amplified from extracted plasma HIV RNA as described previously, with some modifications (48). We included protease since disruption of the autologous combination of Gag and Protease may negatively affect Protease-mediated cleavage of Gag protein products, thus compromising the RC of the recombinant viruses. Briefly, reverse transcriptase PCR (RT-PCR) was

performed using a Superscript III one-step RT-PCR system with Platinum Taq DNA polymerase with high fidelity (Invitrogen, Carlsbad, CA). Each 50-μl reaction mixture was composed of 4 μl of viral RNA, 25 μl of 2× reaction mix, 200 nM forward and reverse outer primers, 1 μl of enzyme mix, and water. RT-PCR primer sequences were AAATCTCTAGCAGTG GCGCCCGAACAG (strain HXB2 nucleotide numbering, positions 623 to 649) for the forward primer and TAACCCTGCGGGATGTGGTA TTCC (positions 2849 to 2826) for the reverse primer. Thermal cycling conditions for the RT-PCR were 50°C for 30 min and 94°C for 2 min, followed by 35 cycles of 94°C for 15 s, 56°C for 30 s, and 68°C for 2 min. Second-round DNA PCR was performed using TaKaRa Ex Taq DNA polymerase Hot Start enzyme (TaKaRa Bio Inc., Shiga, Japan). Each reaction mixture contained 2 μl of the PCR product from the RT-PCR. PCR primer sequences were GCGGCGACTGGTGAGTACGCC (positions 734 to 754) for the forward primer and TCCTTTAGTTGCCCCCCTATC for the reverse primer (positions 2314 to 2294). Thermal cycling conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min and, finally, 10 min at 68°C. To examine protease sequences, the gag-protease region was reamplified from existing 1st-round RT-PCR products using forward primer GCTAGAAGGAGAGAG ATGGG (positions 775 to 794 on HXB2) and reverse primer CAGTCTC AATAGGACTAATGGG (positions 2550 to 2571) with the same thermal cycler conditions described above. PCR amplifications were confirmed by agarose gel electrophoresis, and successful amplicons were purified using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Population sequences were obtained by bidirectional reading using an ABI Prism BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems 3130xl genetic analyzer. Chromatograms were edited using Sequencher software (Gene Codes Corporation, Ann Arbor, MI); nucleotide mixtures were called if the height of the secondary peak exceeded 25% of the dominant peak height. Multiple alignments were constructed using the ClustalW program. Maximum-likelihood phylogenetic trees were drawn from nucleotide alignments using DNAml of the PHYLIP program integrated into the BioEdit software package. HIV-1 subtypes were determined by the REGA HIV subtyping tool (http://hivdb .stanford.edu/). Recombinant viruses were detected using the recombination identification program (RIP; available at http://www.hiv.lanl.gov /content/sequence/RIP/RIP.html) and the jpHMM program (GOBICS; University of Göttingen). Pairwise genetic distances between individual gag sequences and the HIV-1 reference strain NL4-3 were calculated using DNAdist of the PHYLIP program integrated into BioEdit software.

Generation of chimeric viruses. Chimeric NL4-3 viruses were generated as previously described (33, 49). Briefly, the gag-protease region was reamplified from the 1st-round RT-PCR products using 100-bp-long primers homologous to the NL4-3 reference strain (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 [positions 695 to 794 on HXB2]; 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 [positions 2649 to 2550]). Note that the forward primer overlapped the gag-coding sequence by five bases (underlined), and the reverse primer ended one base downstream of the protease gene. The PCR was undertaken in a final volume of 100 μl, consisting of 4 μl of 10 μM forward and reverse primers, 90 μl of Invitrogen Platinum PCR SuperMix high fidelity (Invitrogen, Carlsbad, CA), and 2 μl of the RT-PCR product. Thermal cycling conditions were 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min and a 7-min extension of 72°C. PCR products were purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and eluted in 50 μl of elution buffer. pNL4-3 with a gag-protease deletion (33) and 5 to 10 μg of purified PCR product were cotransfected into 2.5 × 10⁶ cells of a long terminal repeat (LTR)-driven green fluorescent protein (GFP)-reporter T cell
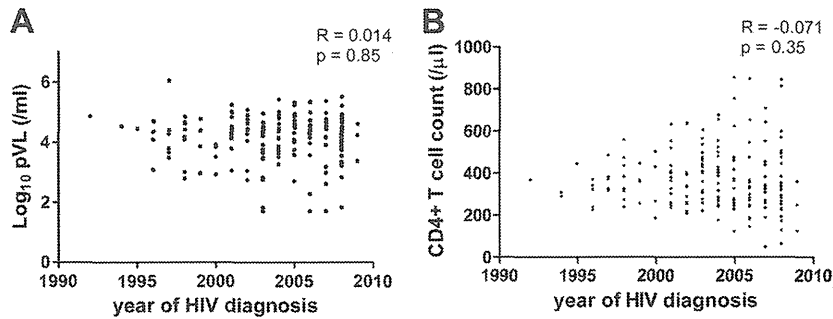
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FIG 1 Correlation between plasma virus load, CD4$^+$ T cell count, and year of HIV-1 diagnosis. No significant correlation between plasma HIV-1 load and the year of diagnosis (A) or the CD4$^+$ T cell count and the year of diagnosis (B) was observed. Plasma virus loads and CD4$^+$ T cell counts are based upon a single time point (date of blood sampling). Each dot represents a single individual ($n = 177$).

line (GXR cells, CEM origin [50]) in 800 μl of R10+ 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, subsequently transferred to T25 flasks in 10 ml of R10+ medium, and incubated at 37°C with 5% CO$_2$. GFP expression was monitored by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) every 1 to 2 days after day 5. Supernatants containing recombinant virus stocks were harvested after GFP expression reached 15% among viable cells, and 1-ml aliquots were stored at −80°C until use. To verify the patient origin of each recombinant virus stock and rule out contamination or sample mix-up, viral RNA was extracted from 30 μl of virus stock using a ChargeSwitch EasyPlex viral kit (Invitrogen, Carlsbad, CA). *gag* sequences were obtained using the same method used for plasma virus sequencing described earlier, and maximum-likelihood phylogenetic trees incorporating the original bulk and recombinant virus sequences were constructed using DNAml.

Determination of recombinant virus titers and RC assay. Determination of the titers of recombinant virus stocks and viral RC assays were performed as described previously (32, 35, 48, 50, 51). For determination of viral titer, 400-μl viral stocks were mixed with 1.0×10^6 GXR cells in 100 μl of R10+ medium in a 24-well plate and incubated at 37°C with 5% CO$_2$. After 24 h, 1 ml R10+ medium was added to each culture. After 48 h, the titers of virus stocks were determined by measuring the percentage of GFP-positive (GFP$^+$) cells using flow cytometry. In the subsequent replication capacity assay, virus stocks were used to infect 1.0×10^6 GXR cells at a multiplicity of infection of 0.002 in 500 μl of R10+ medium in a 24-well plate. All assays included a positive (wild-type NL4-3) and a negative (cells-only) control. Assay mixtures were incubated overnight at 37°C with 5% CO$_2$, and 1 ml of R10+ medium was added on the following day (day 1). The percentage of GFP$^+$ cells was then measured by flow cytometry every other day for the following week (days 2 to 8). For each virus, the natural log slope of the percentage of GFP$^+$ cells was calculated during the exponential phase of viral spread (days 2 to 6). This value was divided by the mean rate of spread of the wild-type NL4-3 control to generate a normalized, quantitative measure of RC. An RC value of 1.0 indicates a rate of viral growth that was equal to that of NL4-3, while RC values of <1.0 and >1.0 indicate rates of spread that were lower and higher than the rate for wild-type NL4-3, respectively. All viruses were tested in a single experiment by a single operator; this experiment was performed in triplicate using fresh viral stocks for each one. Final RC values therefore represent the averages of wild-type NL4-3-normalized triplicate measurements.

Statistical analysis. Statistical comparisons between independent groups were performed using the Mann-Whitney U test. Univariate correlation analysis was performed using Spearman's correlation. Multiple-regression analyses were performed using standard least-squares methods. For these analyses, a P value of <0.05 was considered significant.

Analyses were performed in GraphPad Prism (version 5.03) software (GraphPad Software, La Jolla, CA).

Published phylogenetically informed methods were used to identify amino acids in Gag and Protease significantly associated with HLA class I alleles expressed in our data set (52, 53). Associations between viral RC and specific amino acid residues within Gag and Protease observed with a minimum frequency of 3 were identified using the Mann-Whitney U test. In these analyses, multiple comparisons were addressed using q values, the P value analogue of the false discovery rate (FDR) (54). The FDR is the expected proportion of false positives among results deemed significant at a given P-value threshold; for example, at a q value of ≤0.2, we expect 20% of identified associations to be false positives.

Nucleotide sequence accession numbers. *gag* and *protease* sequences have been submitted to GenBank (accession numbers JX264247 to JX264562).

RESULTS

No significant temporal changes in CD4$^+$ T cell count or pVLs by year of HIV-1 diagnosis. A total of 177 asymptomatic, chronically HIV-1-infected Japanese individuals were enrolled. Since the replicative capacity of viruses within an individual's quasispecies tends to increase over the infection course (55–58) and immune-driven selection in Gag by protective HLA alleles predominantly affects viral RC in acute/early infection (44), individuals with acute infection and those with a history of AIDS-defining illnesses were excluded. Clinical markers of HIV infection (CD4$^+$ T cell count and plasma virus loads [pVLs] at the time of sampling) were comparable among subjects, despite differences in year of HIV diagnosis (Fig. 1). Furthermore, although the timing of blood sampling varied substantially between subjects, no correlation between diagnosis year and the duration from diagnosis to blood sampling was observed (data not shown; $R = 0.0084, P = 0.92$).

Relationship between viral replication capacity and clinical markers of HIV infection. Of the 177 enrolled patients, full-length amplification of *gag-protease* was successful for 168 of these (94.9%). Eight individuals infected with non-clade B viruses (6 infected with CRF01_AE, 1 with CRF02_AG, and 1 with A1) and two individuals with intersubtype clade B *gag* recombinants were excluded from study. After construction of chimeric viral stocks and confirmation of their patient origin (Fig. 2), a further two samples were excluded due to suspected contamination. Viral RC was thus assessed for the remaining 156 recombinant viruses. Each recombinant virus was used to infect a GFP reporter T cell line, and its *in vitro* RC was examined over a 7-day period. Since
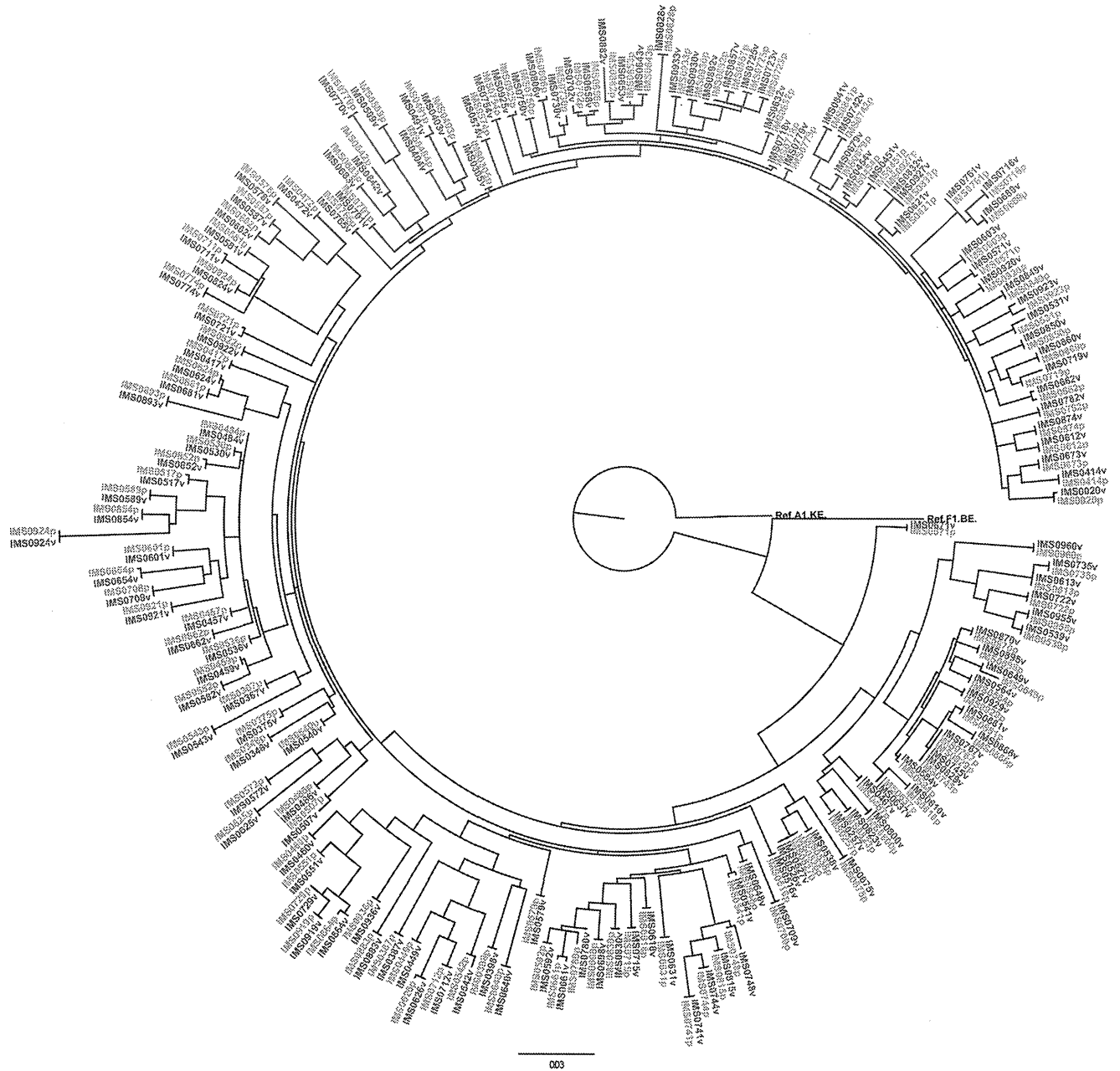
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FIG 2 Validation of the origin of the *gag-protease* region in chimeric NL4-3 viruses. To verify the patient origin of each chimeric virus, a maximum-likelihood phylogenetic tree was constructed using plasma (red) and chimeric (blue) *gag* sequences. This tree includes 156 validated chimeric viruses that clustered with their original bulk sequences (two viruses were removed due to suspected contamination). The tree is rooted using the HIV-1 subtype A1 reference strain with GenBank accession number AF004885.

the set-point viral load has been associated with the *in vitro* replication of chimeric viruses (33, 42, 43, 55–59), we first examined the potential correlation between plasma viral load, CD4$^+$ T cell count, and RC of chimeric NL4-3 strains. A significant positive correlation between RC and plasma virus load was observed ($R = 0.21, P = 0.0072$; Fig. 3A), consistent with previous reports of the reduced RC of chimeric NL4-3 derived from HIV-1 controllers (33, 41, 42). No significant correlation between CD4$^+$ T cell count and RC was observed ($R = -0.042, P = 0.60$; Fig. 3B), possibly due to exclusion of individuals with advanced disease from the present study.

Change in Gag-Protease-associated viral replication capacity over the epidemic in Japan. In order to investigate temporal changes in viral RC over the epidemic in Japan, the correlation between RC and year of HIV diagnosis was analyzed, revealing a significant inverse correlation ($R = -0.27, P = 0.0006$; Fig. 4A). This observation remained statistically significant in a multivariate linear regression model adjusting for CD4$^+$ T cell count and plasma virus load ($P = 0.0008$; partial regression coefficients, -0.0064; 95% confidence interval [CI], -0.0101 to -0.0027). Consistent results were obtained when the original analysis was stratified by CD4$^+$ T cell count at blood sampling (for CD4 T cell
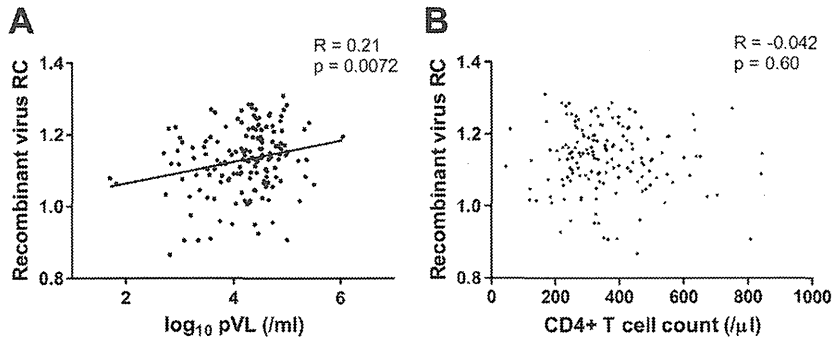
FIG 3 Correlation between replication capacity of chimeric NL4-3 and clinical markers of HIV infection. The correlation of replication capacity of chimeric NL4-3 with pVL (A) and CD4 T cell count (B) at the time of blood sampling ($n = 156$) is shown. A statistically significant positive correlation between RC and pVL was observed ($R = 0.21$, $P = 0.0072$).

counts of >200, $R = -0.28$ and $P = 0.0009$; for CD4 T cell counts of >300, $R = -0.25$ and $P = 0.013$; for CD4 T cell counts of >500, $R = -0.39$ and $P = 0.080$; Fig. 4B to D). Taken together, these results support a decline in Gag-Protease-mediated RC in HIV-1 over the course of the Japanese epidemic which is independent of differences in pVLs and CD4 T cell counts in the studied population. Since the duration from HIV diagnosis to blood sampling varied among the subjects, the analysis was repeated and was lim-
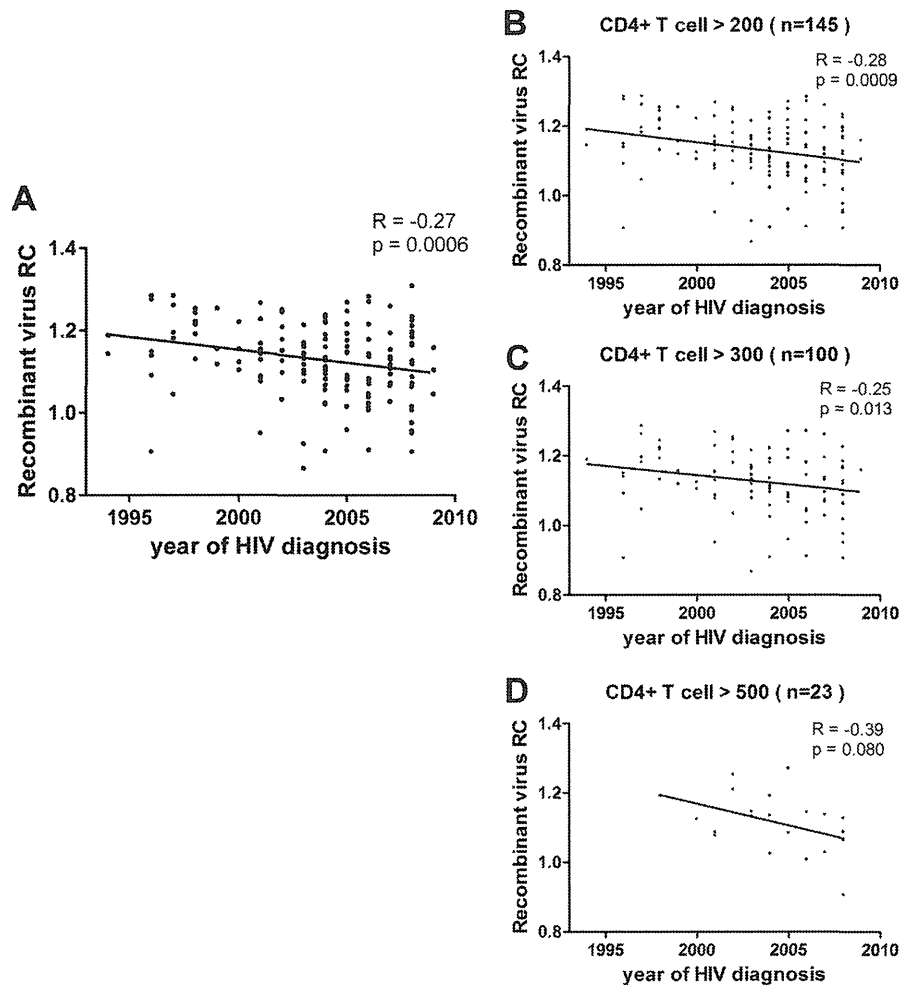

FIG 4 Change in Gag-Protease-associated viral replication capacity over the epidemic in Japan. A statistically significant inverse correlation between year of diagnosis and replication capacity was observed in all subjects regardless of CD4 T cell count ($n = 156$) (A), only in subjects with a CD4$^+$ T cell count of >200/μl ($n = 145$) (B), and only in subjects with a CD4$^+$ T cell count of >300/μl ($n = 100$) (C). A similar tendency was observed when the analysis was limited to subjects with a CD4$^+$ T cell count of >500/μl ($n = 23$) (D).
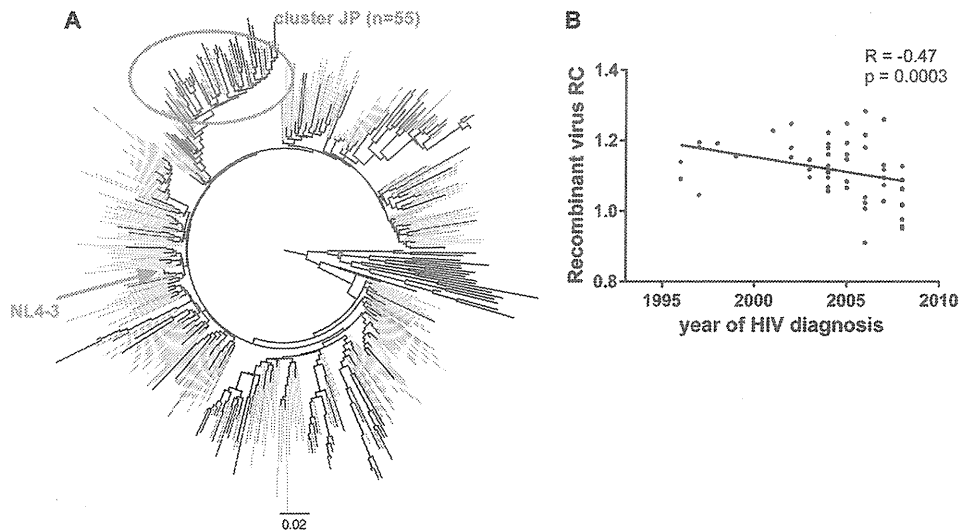
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FIG 5 Temporal change in Gag-Protease-associated viral replication capacity of 55 sequences from a phylogenetic cluster. (A) Maximum-likelihood phylogenetic tree constructed using *gag* sequences from 156 Japanese individuals obtained in the present study and 263 individuals from other countries (randomly selected from the Los Alamos National Laboratory HIV sequence database). Purple, light blue, and blue branches, Japanese sequences with HIV diagnoses of 1999 or earlier, 2000 to 2004, and 2005 or later, respectively; green branches, sequences from the United States and Canada; yellow branches, sequences from other countries (Argentina, Australia, Brazil, China, Cuba, Cyprus, Denmark, France, Germany, Hong Kong, India, Italy, Jamaica, Myanmar, Netherlands, Russia, South Africa, South Korea, Spain, Taiwan, Thailand, and the United Kingdom). Reference strains (which include two reference sequences for each of the HIV-1 group M subtypes, as well as inferred ancestral sequences of the A, B, and C subtypes [obtained from the Los Alamos National Laboratory database]) are shown in black. NL4-3 is shown as red. The tree is rooted using the HIV-1 subtype A1 reference strain with GenBank accession number AF004885. A large cluster of Japanese sequences ($n = 55$) is indicated by the large red circle. (B) A significant inverse correlation between the replication capacity of chimeric viruses and year of HIV diagnosis for the viruses within this large Japanese cluster ($n = 55$).

ited to the subjects whose blood collection had been performed within a year of diagnosis of HIV infection; however, the inverse correlation remained significant ($n = 105, R = -0.25, P = 0.0080$; data not shown).

A potential confounder in such analyses is the molecular epidemiology of the epidemic itself. Theoretically, if distinct subtype B lineages with differential replication capacities were introduced into Japan at different times during the study period, this could influence our results. In order to exclude this possibility, we constructed a phylogenetic tree featuring global HIV-1 subtype B sequences (retrieved from the HIV sequence database at Los Alamos National Laboratory). Multiple clusters of Japanese clade B sequences were interspersed throughout the tree; however, all clusters contained sequences spanning the entire study period, indicating that there have been no major intraclade shifts within Japan in the past 2 decades (Fig. 5A). To further address this issue, we restricted our analysis to one particularly large cluster containing 55 sequences sampled over the study period (Fig. 5A) and found that the significant inverse correlation between RC and the year of diagnosis remained highly statistically significant in this cluster ($R = -0.47, P = 0.0003$; Fig. 5B). Taken together, these results support a decline in Gag-Protease-mediated RC in HIV-1 over the course of the Japanese epidemic which is not likely explained by shifts in the molecular epidemiology of HIV-1 over the period studied.

No correlation between replication capacity of chimeric viruses and their genetic distance from wild-type strain NL4-3. Chimeric NL4-3 virus carrying *gag-protease* derived from subtype C isolates (42, 43) displayed reduced RC compared to viruses derived from subtype B, likely due in part to the substantial genetic distance between insert and backbone. It is therefore conceivable

that subtype B sequences sampled from the early part of the epidemic may be more similar to the NL4-3 backbone sequence (first characterized in 1986 [60]) than those sampled later and that this may influence RC. To investigate this potential confounder, we calculated the genetic distance between each patient isolate and the wild-type NL4-3 *gag* sequence, and we examined the correlation between genetic distance and viral RC. No such relationship was observed ($R = 0.0015$, $P = 0.98$; Fig. 6). Moreover, RC of chimeric viruses remained inversely correlated with the year of HIV diagnosis in multivariate analyses controlling for genetic distance from NL4-3 ($P = 0.0001$; partial regression coefficients, -0.0086; 95% CI, -0.0129 to -0.0044). It is therefore reasonable
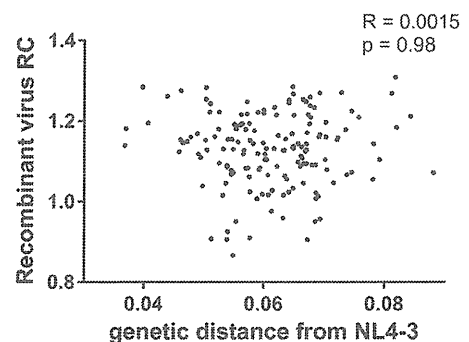
FIG 6 Chimeric virus replication capacity is not related to genetic distance between the insert and backbone. Pairwise genetic distances between the *gag* nucleotide sequence of each insert (clinical isolate sequence) and backbone (wild-type NL4-3) were calculated as described in Materials and Methods. No statistically significant correlation between the genetic distance and replication capacity of chimeric NL4-3 was observed.

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to conclude that the decline in Gag-Protease-associated viral RC observed over the epidemic in Japan is unlikely to be explained by gross genetic incompatibility between NL4-3 and later clinical sequences circulating in the Japanese population.

Accumulation of PI resistance-associated mutations over time? Twelve major protease inhibitor (PI) resistance-associated mutations have been reported in the HIV Drug Resistance Database at Stanford University (http://hivdb.stanford.edu/), and some of these reduce viral replication capacity (61–67). To address the temporal accumulation of PI resistance mutations as a potential confounder, HIV-1 *protease* sequences were examined for 140 subjects for whom chimeric viruses were generated (for the remaining 15 patients, PCR or sequencing was unsuccessful). PI resistance-associated mutations were observed in 6 subjects, none of whom carried more than one PI resistance-associated mutation (4 were M46L and 2 were N88S), and all but 1 were enrolled after 2005. The median RC of chimeric NL4-3 derived from subjects with these PI resistance-associated mutations ($n = 6$) was higher than that of the others ($n = 134$) (1.21 versus 1.13; $P = 0.013$). Moreover, after excluding viruses derived from these 6 subjects from the original analysis, the temporal decline in chimeric virus RC remained significant ($R = -0.33$, $P < 0.0001$). Collectively, these data indicate that accumulation of PI resistance-associated variants in the population does not explain the change in viral RC over time in Japan.

Relationship between HLA class I alleles and temporal change in replication capacities of chimeric viruses. Chimeric NL4-3 virus derived from recent patient sequences displayed reduced *in vitro* RC compared to earlier isolates. To investigate whether immune selection pressure by specific HLA class I alleles could have contributed to this relative attenuation, the RCs of chimeric viruses were compared with respect to the presence versus absence of particular HLA-A, -B, and -C alleles in their host (note that analyses were limited to HLA alleles expressed in >20 individuals). In a cross-sectional analysis undertaken on the whole cohort regardless of sampling date, no significant associations between HLA class I expression and viral RC were observed (data not shown). However, when recombinant viruses were stratified on the basis of the year of HIV diagnosis (2002 or earlier versus 2003 or later), significantly lower median RC values were observed among A*24-expressing persons in early stages (for A*24-positive [A*24$^+$] versus A*24-negative [A*24$^-$] persons, 1.14 versus 1.21; $P = 0.024$) but not later stages (for A*24$^+$ versus A*24$^-$ persons, 1.12 versus 1.11; $P = 0.20$) of the epidemic (Fig. 7A), suggesting that the viral RC in A*24$^-$ persons in the early stage might have declined to levels comparable to those of A*24$^+$ persons in the late stage. Such a phenomenon was not observed for other HLA class I alleles (the results for A*02, B*40, and C*03 are shown in Fig. 7B to D, respectively). Nearly 70% of Japanese express HLA-A*24, making it the most common class I allele in this population. Our finding raises the intriguing hypothesis that A*24-associated escape mutations, alone or in combination, reduce viral RC to a modest extent and that these A*24-attenuated viruses have increased in prevalence at the population level over the course of the Japanese epidemic via transmission to and persistence in non-A*24-expressing persons.

Due to the relative rarity of this allele in Caucasians and Africans, HLA-A*24-restricted CTL epitopes have not been studied extensively; nevertheless, two optimal A*24 CTL epitopes within Gag and Protease have been reported: KW9 in p17 (Gag positions 28 to 36) (68) and RL11 in p24 (Gag positions 294 to 304) (69). However, we observed no accumulation of particular mutations within either of these known epitopes (data not shown). A published *in silico* analysis undertaken on >1,500 subtype B-infected individuals from Canada, the United States, and Australia reported a putative escape association between HLA-A*24 and the K30R substitution in p17^{Gag} (21). However, the accumulation of K30R was not observed in the present study (data not shown), and the RCs of chimeric viruses with this substitution were not statistically different from the RCs of those without it. In addition, a phylogenetically corrected analysis of HLA-associated polymorphisms in 156 Japanese viral sequences from the present study identified Gag V362I to be significantly associated with HLA-A*24 (present in 17% of A*24$^+$ patients versus 4.1% of A*24$^-$ patients; $P < 0.001$, $q < 0.1$). However, as this substitution was observed in only 3 of 74 A*24-negative individuals in the present study, it was not possible to demonstrate its accumulation over the study period.

Accumulation of mutations associated with reduced replication capacity. Lastly, we conducted an exploratory analysis to identify specific amino acids in Gag and Protease associated with viral RC in our data set. Although no associations were observed at a q value of <0.2, 34 amino acids within Gag and 5 within Protease were identified as being associated with a lower RC at a P value of <0.05 (all q values were <0.4; not shown). We then investigated whether the frequencies of viruses carrying these Gag or Protease mutations increased over the course of the epidemic. Of the 34 polymorphisms identified in Gag, 6 significantly increased over the study period (V46L, L64I, D121G, A224P, T470A, I479L), while of the 5 mutations identified in Protease, 1 (R41K) significantly increased over the study period. However, with the exception of Gag L64I (reported to be associated with A*68) (21), none of these polymorphisms are known to be HLA associated.

DISCUSSION

In the present study, we observed a significant reduction in Gag-Protease-associated HIV-1 replication capacity over the past 15 years of the HIV-1 epidemic in Japan.

Our analyses addressed a number of potential confounders. First, viral RC is known to change over the course of HIV infection (70, 71). Examining the RCs of viruses isolated from acutely infected subjects is therefore ideal; however, the availability of such historic panels of specimens from a particular geographical area is extremely limited. Therefore, to rule out infection stage as a potential confounder, we undertook multivariate analyses adjusting for CD4 count and pVL as surrogate markers of disease stage. Second, to exclude the possibility that the molecular epidemiology of HIV in Japan differed in the early and late phases of the epidemic, we performed a subanalysis limited to a particularly large cluster composed of only Japanese clade B sequences sampled over the study period. Lastly, to address a concern about incompatibility between backbone NL4-3 and *gag-protease* from recent clinical isolates, we demonstrated that the year of HIV diagnosis correlated with RC independently of the genetic distance between patient-derived sequences and the wild-type NL4-3 sequence. In all cases, our original findings still held after controlling for these potential confounders. Furthermore, temporal trends in RC did not appear to be driven by protease inhibitor resistance mutations (which were infrequent in the studied population). Nevertheless, we cannot rule out the possibility that the introduction of increas-
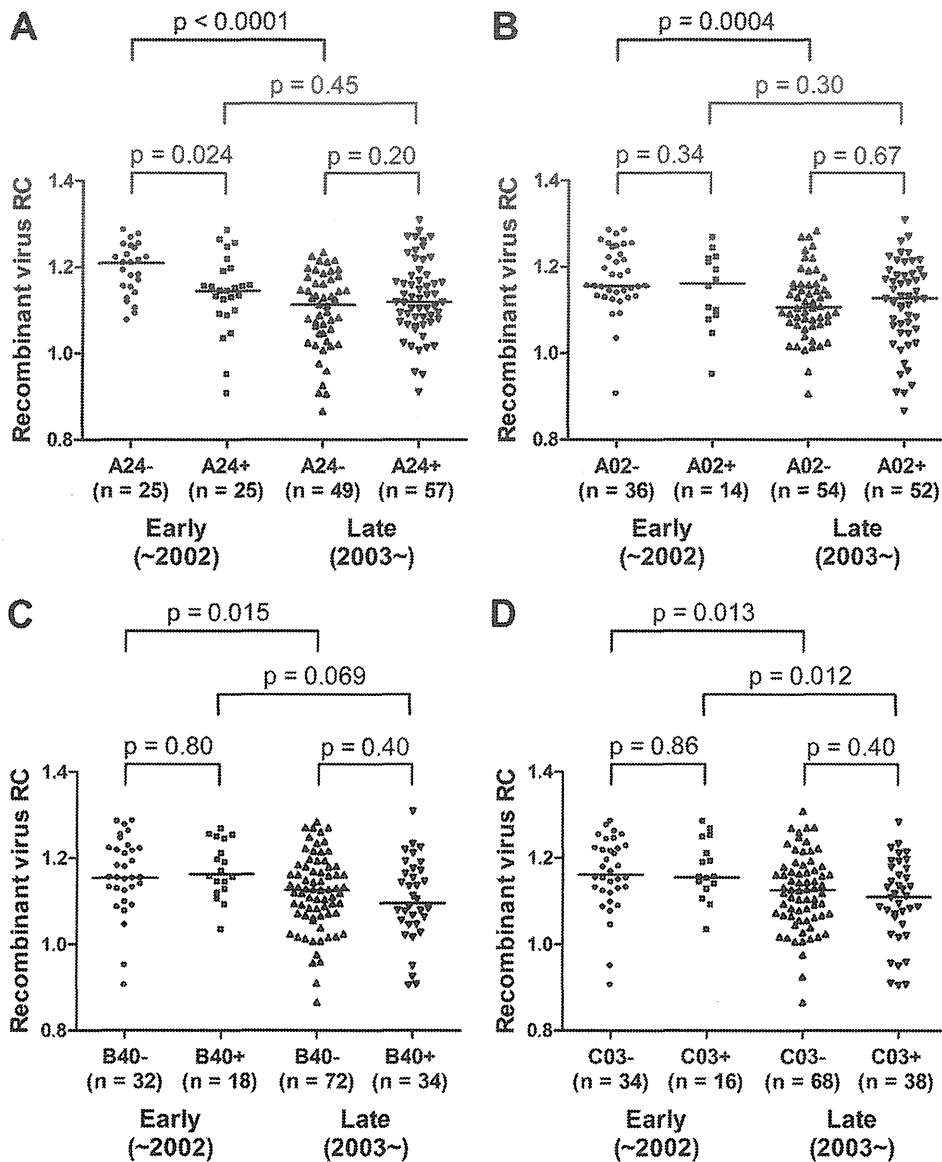
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FIG 7 Relationship between common HLA alleles and chimeric virus replication capacity during early and late epidemic periods. To examine the potential impact of selection pressures by common HLA alleles in the Japanese population on the change in viral replication capacity, chimeric viruses were grouped according to year of HIV diagnosis (early [2002 or earlier] and late [2003 or later]), and associations between replication capacity and expression of particular HLA class I alleles were examined. Recombinant viruses from HLA-A*24-expressing hosts exhibited reduced RC before 2002 but not thereafter (A). However, such a phenomenon was not observed for other alleles investigated: A*02, B*40, and C*03 (B to D). Horizontal bars indicate the median values.

ingly more potent protease inhibitors over time is driving the selection of novel secondary drug resistance-associated polymorphisms that compromise RC and that such polymorphisms may be increasing in frequency in the general population.

Attempts have been made to study population-level changes in plasma HIV loads, CD4 T cell counts, and rates of disease progression over time as indirect evidence for altered HIV virulence over the epidemic's course. However, the lack of historic data, inherent limitations in conducting observational studies, and changes in the technologies used to measure clinical parameters have made this an extremely difficult issue to address. Despite this, a pattern appears to be emerging. While studies undertaken prior to the mid-1990s yielded conflicting results (72–76), more recent re-

ports support the observation that HIV may be increasing in virulence as the epidemic progresses (77–85).

At first, published reports of increased virulence over time may appear to be inconsistent with our findings of reduced in vitro viral RC over the course of the Japanese epidemic. However, it is important to note that in vitro RC does not necessarily equate with viral virulence, as the former assesses the ability of a recombinant virus to replicate in a controlled in vitro environment devoid of host or other selection pressures, while the latter reflects the far more complex capacity of the virus to cause disease in its host. Indeed, while certain immune escape mutations reduce in vitro viral RC (28, 32–38), we must also consider that mutants with such escape mutations are highly adapted to their in vivo environ-

ment within a host expressing the relevant HLA class I allele. Indeed, while RC may be somewhat compromised compared to that of wild type, escape mutant viruses able to fully or partially evade CTL detection *in vivo* are almost certainly more virulent in the HLA-matched host environment, leading to increased virion production and thereby enhanced pathogenesis. The context dependency of viral fitness is similarly illustrated by antiretroviral resistance mutations such as M184V within reverse transcriptase (86–91): viruses harboring this mutation display relative *in vitro* replicative defects but are certainly more fit than their wild-type counterparts in the presence of lamivudine both *in vivo* and *in vitro*.

It is notable that viremia remained relatively unchanged over the study period. One potential explanation for this observation is the existence of two conflicting processes that offset each other: on the one hand, an increase in virus production (manifested as pVL) as a result of viral adaptation to its host and, on the other, a concomitant decrease in RC as a result of the fitness costs of this adaptation. To investigate this hypothesis, we conducted a multivariate analysis to examine the relationship between pVL and the year of diagnosis when conditioned on RC; however, no apparent trend was observed (data not shown); larger-scale studies will therefore be necessary to further investigate the interplay between these factors.

It was surprising to see such a clear decline in viral RC over the relatively short study period, especially in a country where HIV incidence and prevalence are low (HIV prevalence in Japan is <0.1%; [HIV and AIDS Data Hub for Asia-Pacific, http://www .aidsdatahub.org/]). However, Japan is relatively ethnically homogeneous and its population exhibits a far narrower HLA frequency spectrum than the populations of Western countries (92). This more limited HLA diversity may facilitate the rapid accumulation of CTL escape mutations in circulating HIV-1 sequences (93), most notably, those restricted by common Japanese HLA class I alleles (30, 31).

Intriguingly, lower viral RC was observed in HLA-A*24$^+$ patients than HLA-A*24$^-$ patients in earlier but not later periods of the Japanese epidemic. We propose the following interpretation for this observation: regardless of epidemic stage, viruses from HLA-A*24-positive patients carry A*24 escape mutations; therefore, no differences in RC are observed between early and late isolates from A*24-positive individuals. However, if single (or combinations of) A*24 escape mutants, some of which carry modest replicative costs, are transmitted to A*24-negative individuals and if some of these escape mutants persist in A*24-negative individuals, despite these modest replicative costs, it is possible that such mutations will increase in frequency in the general population over the course of the epidemic. If so, it is conceivable that the RC in A*24-negative individuals will concomitantly decline to levels similar to those in A*24-positive individuals over time. Although we did not observe any differences in the prevalence of particular amino acids within known A*24-restricted CTL epitopes in Gag and Protease in early versus later sequences, accumulation of A*24 CTL escape mutations within Nef in circulating viruses in Japan has been reported (31), suggesting that a similar phenomenon could be occurring in Gag. The link between A*24-associated immune pressure and temporal reductions in RC therefore remains speculative; future studies will be required to define CTL escape mutations for HLA alleles frequently observed

in Japanese populations and demonstrate the accumulation of such mutations during the HIV epidemic.

Despite the limited statistical power of the present study to detect such associations, we performed an exploratory analysis to identify specific amino acid residues that could explain observed reductions in viral RC. Although we identified a number of putative amino acids that were associated with lower RCs at a *P* value of <0.05 and whose frequency appeared to increase over the study period, these associations did not remain significant after correction for multiple comparisons, and thus, our results should be interpreted with caution. Larger studies aimed at identifying amino acids associated with temporal alterations in RC are therefore warranted.

Another caveat is that only Gag-Protease-associated viral RC was evaluated in the present study; as such, our results may not be representative of RC of whole virus isolates. The reasons for specifically investigating Gag-Protease-associated RC are 2-fold. First, our primary purpose was to investigate temporal changes in viral RC potentially attributable to HLA-associated immune pressures in Gag, a critical target of CTL responses. Whole-virus assays would have been confounded by the autologous envelope sequence, which is a major determinant of viral fitness (57) but is highly sensitive to infection stage due to shifts in coreceptor usage (94–96). Second, the generation and evaluation of large numbers of viruses require higher-throughput methods. Whole-virus isolates are traditionally replicated in primary CD4$^+$ T cells, but the laborious and costly nature of these assays precludes their application to large sample panels such as that used in the present study.

Despite these limitations, the present study sheds light on the replicative costs of HIV-1 adaptation to its human host. Additional, larger studies spanning greater durations of the HIV epidemic undertaken in different geographic areas and host populations, as well as studies elucidating the clinical implications of alterations in viral RC, are warranted, to determine whether this is a local or global phenomenon.

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Control of Simian Immunodeficiency Virus Replication by Vaccine-Induced Gag- and Vif-Specific CD8+ T Cells

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For development of an effective T cell-based AIDS vaccine, it is critical to define the antigens that elicit the most potent responses. Recent studies have suggested that Gag-specific and possibly Vif/Nef-specific CD8+ T cells can be important in control of the AIDS virus. Here, we tested whether induction of these CD8+ T cells by prophylactic vaccination can result in control of simian immunodeficiency virus (SIV) replication in Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-010-Ie* associated with dominant Nef-specific CD8+ T-cell responses. In the first group vaccinated with Gag-expressing vectors (*n* = 5 animals), three animals that showed efficient Gag-specific CD8+ T-cell responses in the acute phase postchallenge controlled SIV replication. In the second group vaccinated with Vif- and Nef-expressing vectors (*n* = 6 animals), three animals that elicited Vif-specific CD8+ T-cell responses in the acute phase showed SIV control, whereas the remaining three with Nef-specific but not Vif-specific CD8+ T-cell responses failed to control SIV replication. Analysis of 18 animals, consisting of seven unvaccinated noncontrollers and the 11 vaccinees described above, revealed that the sum of Gag- and Vif-specific CD8+ T-cell frequencies in the acute phase was inversely correlated with plasma viral loads in the chronic phase. Our results suggest that replication of the AIDS virus can be controlled by vaccine-induced subdominant Gag/Vif epitope-specific CD8+ T cells, providing a rationale for the induction of Gag- and/or Vif-specific CD8+ T-cell responses by prophylactic AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces persistent viral replication, leading to AIDS onset in humans. Virus-specific CD8+ T-cell responses play a central role in the resolution of acute peak viremia (1–4) but mostly fail to contain viral replication in HIV infection. Prophylactic vaccination resulting in more effective CD8+ T-cell responses postexposure than those in natural HIV infections might contribute to HIV control. Current trials in macaque AIDS models have shown that vaccine induction of T-cell responses can result in control of postchallenge viral replication (5–10). It is now critical to define the antigens that elicit the most potent responses for development of an effective T-cell-based AIDS vaccine.

Recent studies have implicated Gag-specific CD8+ T cells in the control of HIV and simian immunodeficiency virus (SIV) replication (11–16). Several HLA or major histocompatibility complex class I (MHC-I) alleles have been shown to be associated with lower viral loads (17–25). Virus control associated with some of these protective MHC-I alleles is attributed to Gag epitope-specific CD8+ T-cell responses (26–29). For instance, CD8+ T-cell responses specific for the HLA-B*57-restricted Gag$_{240-249}$ TW10 and HLA-B*27-restricted Gag$_{263-272}$ KK10 epitopes exert strong suppressive pressure on HIV replication and frequently select for escape mutations with viral fitness costs, leading to lower viral loads (27, 30–33). Thus, certain individuals possessing MHC-I alleles associated with dominant Gag-specific CD8+ T-cell responses could have a greater chance to control HIV replication than those without these alleles. For those individuals that do not express these MHC-I alleles, the question arises as to whether prophylactic vaccination inducing Gag epitope-specific CD8+ T-cell responses might contribute to HIV control. Furthermore, recent studies have shown that CD8+ T-cell responses targeting SIV

antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, exert strong suppressive pressure on SIV replication (10, 34, 35).

We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (36). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (5, 37). Unvaccinated animals possessing *90-120-Ia* dominantly elicited CD8+ T-cell responses specific for the Gag$_{206-216}$ (IINEE AADWDL) and the Gag$_{241-249}$ (SSVDEQIQW) epitopes after SIV challenge (38, 39). DNA/SeV-Gag-vaccinated *90-120-Ia*-positive macaques showed enhanced Gag$_{206-216}$-specific and Gag$_{241-249}$-specific CD8+ T-cell responses in the acute phase after SIV challenge, resulting in viremia control (37). This implies virus control by vaccine-based enhancement of Gag-specific CD8+ T-cell responses in animals possessing MHC-I alleles associated with dominant Gag CD8+ T-cell epitopes. However, we have not defined the efficacy of prophylactic vaccination inducing Gag-specific CD8+ T-cell responses against HIV/SIV infection in the hosts pos-

sessing MHC-I alleles not associated with dominant Gag CD8$^+$ T-cell epitopes.

In the present study, we first examined efficacy of prophylactic vaccination inducing Gag-specific CD8$^+$ T-cell responses against SIVmac239 challenge in a group of macaques that possess the *90-010-Ie* MHC-I haplotype (referred to as E) associated with dominant Nef-specific CD8$^+$ T-cell responses (39, 40). Furthermore, we examined the efficacy of prophylactic vaccination inducing Vif/Nef-specific CD8$^+$ T-cell responses in these E$^+$ macaques. Our results show SIV control in those vaccinees that mounted efficient Gag- or Vif-specific CD8$^+$ T-cell responses in the acute phase postchallenge.

MATERIALS AND METHODS

Animal experiments. Animal experiments were carried out in Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBP), with the help of the Corporation for Production and Research of Laboratory Primates after approval by the Committee on the Ethics of Animal Experiments of NIBP (permission number DS21-28 and DS23-19) under the guideline for animal experiments at NIBP and National Institute of Infectious Diseases, which is in accordance with the Guidelines for Proper Conduct of Animal Experiments established by Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia.

We used Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-010-Ie* (E) (39, 40). The determination of MHC-I haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis of *Mamu-A* and *Mamu-B* genes and detection of major *Mamu-A* and *Mamu-B* alleles by cloning the reverse transcription (RT)-PCR products as described previously (39–41). Confirmed MHC-I alleles consisting of the MHC-I haplotype E are *Mamu-A1*066:01*, *Mamu-B*005:02*, and *Mamu-B*015:04*. Unvaccinated R01-011, R05-007, R08-003, R08-007, R09-011, and R06-038 and Gag-vaccinated R01-010 and R01-008 used in our previous experiments (39, 42) are included in the present study. At week 1, unvaccinated macaque R06-038 was intravenously infused with 300 mg of nonspecific immunoglobulin G purified from uninfected rhesus macaques as described before (43). All animals were intravenously challenged with 1,000 50% tissue culture infective doses (TCID$_{50}$) of SIVmac239 (44).

Macaques R01-010, R05-010, R01-008, R08-002, and R08-006 received prophylactic DNA prime/SeV-Gag boost vaccination (referred to as Gag vaccination) (5). The DNA used for the vaccination, cytomegalovirus (CMV)-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian-human immunodeficiency virus SHIVMD14YE (45) molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6 × 10^9 cell infectious units (CIU) of F-deleted replication-defective Sendai virus (SeV) expressing SIVmac239 Gag (SeV-Gag) (46).

Macaques R08-012, R10-012, R10-013, R10-010, R10-011, and R10-014 received prophylactic DNA prime/SeV-VifNef boost vaccination (referred to as Vif/Nef vaccination). The Vif-expressing DNA used for the vaccination, pcDNA-SIVvif-opt, was constructed by introducing an optimized SIVmac239 Vif cDNA (GenScript) into pcDNA3.1. The Nef-expressing DNA used for the vaccination, pcDNA-SIVnef-G2A, has an SIVmac239 Nef cDNA with a mutation resulting in glycine (G) to alanine (A) at the 2nd amino acid (aa) in Nef. Animals intramuscularly received 3 mg of Vif-expressing DNA at the first DNA vaccination and 3 mg of Vif-expressing DNA and 3 mg of Nef-expressing DNA at the second DNA vaccination. Six weeks after the first DNA prime, animals received a single boost intranasally with 1 × 10^9 CIU of F-deleted SeV expressing Vif-opt

(SeV-Vif) and 1 × 10^9 CIU of F-deleted SeV expressing Nef-G2A (SeV-Nef) (47).

Analysis of antigen-specific CD8$^+$ T-cell responses. We measured virus-specific CD8$^+$ T-cell frequencies by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (48, 49). Autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) were pulsed with each peptide (at a final concentration of 1 μM) or peptide pools (at a final concentration of 1 to 2 μM for each peptide) using panels of overlapping peptides spanning the entire SIVmac239 Gag, Vif, and Nef amino acid sequences (Sigma-Aldrich Japan) for 1 h. Peripheral blood mononuclear cells (PBMCs) were cocultured with these pulsed B-LCLs in the presence of GolgiStop (monensin; BD) for 6 h. Intracellular IFN-γ staining was performed with a Cytofix/Cytoperm kit (BD) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ monoclonal antibodies (BioLegend). In the flow cytometric analysis, PBMCs were gated in forward scatter-side scatter dot plots, and B-LCLs were excluded in this step. Specific T-cell frequencies were calculated by subtracting nonspecific IFN-γ T-cell frequencies (less than 100 per million PBMCs) from those after peptide-specific stimulation. Specific T-cell frequencies lower than 100 per million PBMCs were considered negative.

Sequencing analysis of plasma viral genomes. Viral RNAs were extracted using the high pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from macaque plasma obtained around 1 year after challenge. Fragments of cDNAs encoding SIVmac239 Gag, Vif, and Nef were amplified by nested RT-PCR (25 cycles at the first RT-PCR using the PrimeScript one-step RT-PCR kit, version 2 [TaKaRa] and 30 cycles at the second PCR using KOD-Plus, version 2 [Toyobo]) from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan) as described before (39). Predominant nonsynonymous mutations were determined.

Statistical analysis. Statistical analysis was performed with Prism software version 4.03, with significance levels set at a P value of <0.050 (GraphPad Software, Inc.). Antigen-specific CD8$^+$ T-cell frequencies were compared by the nonparametric Mann-Whitney U test. Correlation was analyzed by the Pearson test.

RESULTS

Plasma viral loads after SIVmac239 challenge. We used a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-010-Ie* (E). In our previous study (39), unvaccinated E$^+$ macaques consistently showed persistent viremia after SIVmac239 challenge. CD4$^+$ T-cell percentage in PBMCs declined to less than 20% in a year. In the present study, we compared viral loads in vaccinated animals with those in these unvaccinated animals.

The first vaccine group of five E$^+$ macaques received a DNA prime and an SeV-Gag boost vaccination, followed by an SIVmac239 challenge. Two of these Gag-vaccinated animals failed to control viral replication, but the remaining three showed SIV control (Fig. 1). In the latter controllers, plasma viremia became undetectable in a few months. Macaques R01-008 and R08-006 rapidly controlled SIV replication and maintained high CD4 levels (Fig. 1).

The second group of six E$^+$ macaques received a DNA prime and an SeV-Vif/Nef boost vaccination, followed by an SIVmac239 challenge. The vaccine protocol first delivered Vif-expressing DNA, with the second vaccination consisting of Vif-expressing and Nef-expressing DNAs, and the third with Vif-expressing and Nef-expressing SeVs (SeV-Vif and SeV-Nef) with intervals of 3 weeks. After SIV challenge, three of these Vif/Nef-vaccinated an-
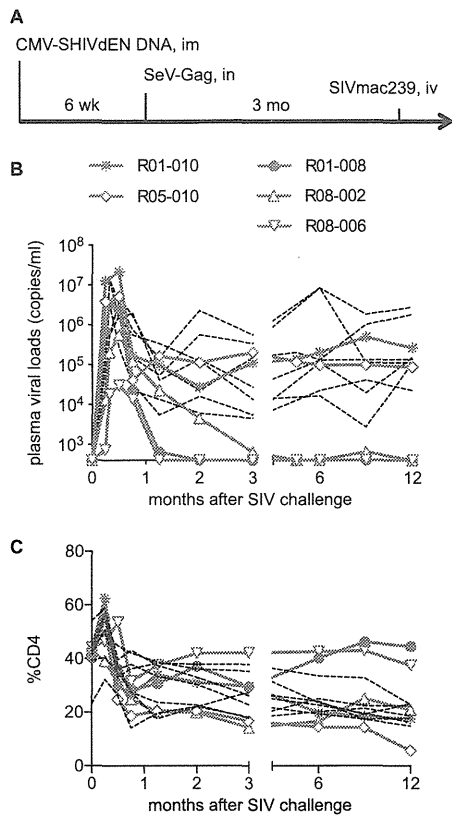
A

CMV-SHIVdEN DNA, im

FIG 1 Viral loads and percentages of CD4 in Gag-vaccinated animals after SIVmac239 challenge. (A) Protocol of Gag vaccination and SIVmac239 challenge. (B) Plasma viral loads (SIV *gag* RNA copies/ml plasma) determined as described previously (5). The lower limit of detection is approximately 4×10^2 copies/ml. (C) Percentages of CD4$^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals ($n = 7$) are shown by dotted lines for comparison. Data on six unvaccinated (39) and two Gag-vaccinated (R01-010 and R01-008) (42) animals used in our previous studies are included.
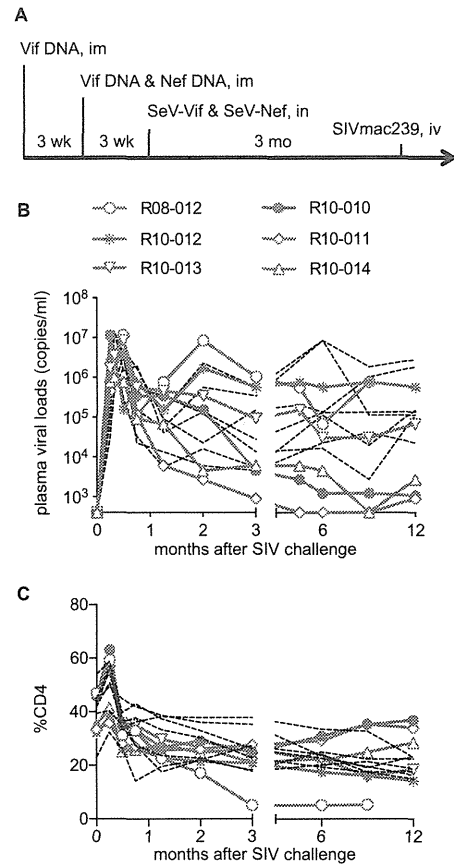

FIG 2 Viral loads and percentages of CD4 in Vif/Nef-vaccinated animals after SIVmac239 challenge. (A) Protocol of Vif/Nef vaccination and SIVmac239 challenge; (B) plasma viral loads; (C) percentages of CD4$^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals are shown by dotted lines for comparison.

imals failed to control viral replication and had high levels of set-point viral loads equivalent to those in unvaccinated macaques, but the remaining three showed SIV control with low levels of set-point viral loads (geometric mean of viral loads from 6 months to 1 year in each controller, $<2.0 \times 10^3$ copies/ml) and maintained higher CD4 levels (Fig. 2). Indeed, these six SIV controllers, consisting of three Gag-vaccinated and three Vif/Nef-vaccinated animals, showed significantly higher percentages of CD4 at 1 year than those in the remaining noncontrollers (see Fig. S1 in the supplemental material).

Gag-, Vif-, and Nef-specific CD8$^+$ T-cell responses in unvaccinated and vaccinated animals. We examined Gag-, Vif-, and Nef-specific CD8$^+$ T-cell responses in these animals. Unvaccinated macaques showed SIV-specific CD8$^+$ T-cell responses equivalent to those observed in Indian rhesus macaques (8) (Fig. 3). All of these E$^+$ unvaccinated macaques elicited immunodominant Nef-specific CD8$^+$ T-cell responses, consistent with our previous study analyzing other E$^+$ macaques (50). Gag-specific and Vif-specific CD8$^+$ T-cell responses were detected but were not immunodominant in these animals.

In contrast, all Gag-vaccinated E$^+$ macaques showed Gag-specific CD8$^+$ T-cell responses after the SeV-Gag boost and in the early phase after SIV challenge (Fig. 3). In these animals, Nef-

specific CD8$^+$ T-cell responses mostly became immunodominant in the later phase. Importantly, all three animals that controlled SIV replication showed efficient Gag-specific CD8$^+$ T-cell responses in the acute phase postchallenge, suggesting a significant contribution of these Gag-specific CD8$^+$ T-cell responses to SIV control.

In the second group of Vif/Nef-vaccinated E$^+$ animals, analysis of Gag-specific, Vif-specific, and Nef-specific CD8$^+$ T-cell responses showed different patterns of responses between SIV controllers and noncontrollers (Fig. 3). In the acute phase after SIV challenge, the noncontrollers (R08-012, R10-012, and R10-013) elicited immunodominant Nef-specific CD8$^+$ T-cell responses, whereas the controllers (R10-010, R10-011, and R10-014) showed immunodominant Vif-specific CD8$^+$ T-cell responses. This suggests that the Vif-specific CD8$^+$ T-cell responses contributed to primary SIV control. In the chronic phase, Nef-specific CD8$^+$ T-cell responses were immunodominant except for one noncontroller, R10-012.

Thus, among 18 E$^+$ animals, consisting of seven unvaccinated, five Gag-vaccinated, and six Vif/Nef-vaccinated animals, three Gag-vaccinated and three Vif/Nef-vaccinated animals controlled SIV replication. Comparison between these six SIV controllers and the remaining 12 noncontrollers showed no significant difference in the sum of Gag-, Vif-, and Nef-specific CD8$^+$ T-cell fre-

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