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# Viral Replication Capacity as a Correlate of HLA B57/B5801-Associated Nonprogressive HIV-1 Infection[1]

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**HLA B57 and the closely related HLA B5801 are over-represented among HIV-1 infected long-term nonprogressors (LTNPs). It has been suggested that this association between HLA B57/5801 and asymptomatic survival is a consequence of strong CTL responses against epitopes in the viral Gag protein. Moreover, CTL escape mutations in Gag would coincide with viral attenuation, resulting in low viral load despite evasion from immune control. In this study we compared HLA B57/5801 HIV-1 infected progressors and LTNPs for sequence variation in four dominant epitopes in Gag and their ability to generate CTL responses against these epitopes and the autologous escape variants. Prevalence and appearance of escape mutations in Gag epitopes and potential compensatory mutations were similar in HLA B57/5801 LTNPs and progressors. Both groups were also indistinguishable in the magnitude of $CD8^+$ IFN-$\gamma$ responses directed against the wild-type or autologous escape mutant Gag epitopes in IFN-$\gamma$ ELISPOT analysis. Interestingly, HIV-1 variants from HLA B57/5801 LTNPs had much lower replication capacity than the viruses from HLA B57/5801 progressors, which did not correlate with specific mutations in Gag. In conclusion, the different clinical course of HLA B57/5801 LTNPs and progressors was not associated with differences in CTL escape mutations or CTL activity against epitopes in Gag but rather with differences in HIV-1 replication capacity.** *The Journal of Immunology,* **2007, 179: 3133–3143.**

It has been convincingly demonstrated in the rhesus macaque model of SIV infection and in correlative studies of HIV-1 infection in humans that $CD8^+$ T cells play a major role in restricting lentiviral replication, thereby controlling the clinical course of infection (1–6). Indeed, there is a temporal relationship between the development of $CD8^+$ T cell responses and the decline in viremia after acute infection (3, 7). Moreover, depletion of $CD8^+$ T cells in SIV-infected macaques led to higher viral loads and more rapid disease progression (1, 4, 5).

Despite immune pressure from CTLs, most HIV-1-infected individuals experience high levels of ongoing virus replication. This lack of control can be attributed to escape mutations in CTL epitopes that commonly develop during infection with HIV-1 (8–17) and SIV (18–23). These mutations either abolish presentation by the restricting HLA molecule or recognition by the appropriate TCR. In some cases, escape mutations correlated directly with the loss of viral suppression and disease progression (14, 23, 24). Taken together, these studies imply that viral escape from CTL is an important mechanism of immune evasion, allowing ongoing viral replication and progressive loss of $CD4^+$ T cells in most individuals infected with HIV-1. However, several studies have suggested that at least certain escape mutations can come at a fitness cost to the virus (25–27). In this situation, continuous immune pressure on the wild-type virus variant will favor the outgrowth of the attenuated escape variant that is unable to give rise to high viral load.

A potential loss of viral fitness has been most clearly demonstrated for escape mutations in epitopes that are restricted by HLA-B27 and HLA-B57/5801, for instance the T242N mutation in the HLA B57/5801-restricted TW10 epitope (25, 26). This loss of fitness has been used to explain the protective effect of these HLA types and a long-term nonprogressive clinical course of HIV infection (28–30). There is indeed an overrepresentation of HLA B57 and HLA B27 in long-term nonprogressors (LTNPs)[3] (28). However, HLA B57 is also present in up to 11% of HIV infected patients with progressive disease, which is similar to the frequency in the Caucasian population (28).

A previous study showed that all HLA B57 progressors but only some HLA B57 LTNPs had the T242N mutation in the HLA B57-restricted TW10 epitope, which would suggest that CTL escape mediated by this mutation has a more dramatic negative effect on the clinical course of infection than the viral attenuation associated with it. However, the frequencies of CTL against the wild-type peptides and the escape variants were comparable in both groups (31).

To gain further insight in the association between HLA B57/5801 and the clinical course of HIV-1 infection, we performed a longitudinal analysis on the dynamics of CTL escape mutations in HLA B57/5801-restricted epitopes in Gag of clonal HIV-1 variants that were isolated from HLA B57/5801 LTNPs and progressors

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[3] Abbreviations used in this paper: LTNP, long-term nonprogressor; ACH, Amsterdam cohort; L (with number), LTNP participant; P (with number), progressor participant; SC, seroconversion.

Table I. *Patient characteristics*

| Patient | HLA A Allele | HLA A Allele | HLA B Allele | HLA B Allele | CCR5 Genotype | Seroconversion (S) or Entry (E) |
|---|---|---|---|---|---|---|
| P1 | A*33 | A*33 | B*5801 | B*14 | Wild type | 24-10-1984 (E) |
| P2 | A*01 | A*03 | B*5701 | B*5101 | Wild type | 25-10-1984 (E) |
| P3 | A*01 | A*24 | B*5701 | B*3502 | Wild type | 20-05-1992 (S) |
| P4 | A*03 | A*6801 | B*5701 | B*3501 | Wild type | 11-01-1988 (S) |
| P5 | A*02 | A*3201 | B*5701 | B*1401 | Wild type | 04-09-1991 (S) |
| P6 | A*01 | A*32 | B*57 | B*1501 | Wild type | 11-04-1990 (S) |
| P7 | A*0205 | A*33 | B*5801 | B*1503 | Wild type | 02-01-1989 (E) |
| P8 | A*02 | A*24 | B*5801 | B*1501 | Wild type | 20-02-1991 (E) |
| P9 | A2 | A3 | B*5701 | B*0702 | Wild type | 20-01-1985 (E) |
| P10 | A*02 | A*26 | B*5701 | B*4102 | Wild type | 01-01-1984 (E) |
| P11 | A1 | A11 | B*5701 | B*0801 | Wild type | 15-05-1990 (S) |
| P12 | A*01 | A*26 | B*5701 | B*3801 | Wild type | 24-08-1992 (E) |
| P13 | A*01 | A*01 | B*5701 | B*0801 | Wild type | 22-01-1985 (E) |
| L1 | A*01 | A*02 | B*5701 | B*0702 | Heterozygous | 22-01-1985 (E) |
| L2 | A*01 | A*24 | B*5701 | B*1501 | Wild type | 18-02-1985 (E) |
| L3 | A*02 | A*03 | B*5801 | B7 | Wild type | 24-06-1986 (S) |
| L4 | A*01 | A*03 | B*57 | B*07 | Wild type | 09-05-1989 (E) |
| L5 | A*26 | A*6801 | B*57 | B*0702 | Wild type | 06-11-1984 (E) |
| L6 | A3 | A11 | B*5801 | B7 | Heterozygous | 09-02-1988 (E) |
| L7 | A*01 | A*02 | B*5701 | B*0801 | Heterozygous | 03-12-1984 (E) |
| L8 | A*02 | A*02 | B*5701 | B*3701 | Wild type | 06-02-1990 (S) |
| L9 | A*01 | A*6802 | B*5701 | B*1402 | Wild type | 04-10-1985 (S) |

and we measured patient CTL reactivity against these autologous and wild-type Gag peptides. Finally, we also analyzed the replication capacity of HIV-1 variants in relation to the presence of potential compensatory mutations in Gag.

## Materials and Methods

### *Patient characteristics*

All participants from the Amsterdam Cohort (ACH) studies on HIV infection who carried the HLA B57 or HLA B5801 allele ($n = 22$) were selected for this study (Table I). Fourteen individuals were HIV-1 infected (seroprevalent) at the moment of entry in the cohort studies and eight individuals seroconverted for HIV Abs during active follow-up. All cohort participants had routine three monthly visits for blood donation and physical examination. After being selected for this study on the basis of HLA B57/5801 expression, the participants' HLA B type was first confirmed using high resolution HLA typing (32). Individuals who developed AIDS or who started with antiviral therapy within 9 years after seroconversion (SC) or seroprevalent entry in the ACH studies were called progressors (P; $n = 13$: ACH19985 (P1), ACH19980 (P2), ACH19901 (P3), ACH19629 (P4), ACH19372 (P5), ACH18763 (P6), ACH13968 (P7), ACH13918 (P8), ACH19567 (P9), ACH18932 (P10), ACH18887 (P11]), ACH13879 (P12), and ACH11679 (P13)). LTNPs (L; $n = 9$) were those participants who had stable CD4 counts that were still >400 cells/$\mu$l blood in the 10th year after SC or seroprevalent entry in the ACH studies or who had a decline of $<40 \times 10^9$ CD4 cells/L per year over a period of at least 10 years (ACH19552 (L1), ACH19417 (L2), ACH19285 (L3), ACH15991 (L4), ACH19933 (L5), ACH19922 (L6), ACH19789 (L7), ACH19784 (L8), ACH19291 (L9)).

Five progressors (P9, P10, P11, P12, and P13) and five LTNP (L5, L6, L7, L8, and L9) were analyzed longitudinally. Three participants (L6, L7, and L1) were heterozygous for the 32-bp deletion within CCR5, whereas all other participants had a CCR5 WT genotype. Typing of the HLA A and B alleles of all participants (Table I) did not reveal other HLA types that were associated with a difference in disease progression, with the exception of P3 who was typed HLA B*3502. This allele is known to accelerate the clinical course of infection (29, 33–35).

### *Cells*

PBMCs were isolated from buffy coats by Ficoll density centrifugation and all PBMCs used originated from healthy donors who were homozygous for the CCR5 wild-type allele. For stimulation, $5 \times 10^6$ cells/ml were cultured for three days in IMDM supplemented with 10% FBS (Perbio), 100 U/ml penicillin (Invitrogen Life Technologies), 100 $\mu$g/ml streptomycin (Invitrogen Life Technologies), 5 $\mu$g/ml Ciproxin (Bayer), and 1 $\mu$g/ml PHA (Remel Europe). Subsequently, PBMCs ($10^6$/ml) were grown in the absence of PHA in medium supplemented with 10 U/ml rIL-2 (Cetus) and 5 $\mu$g/ml Polybrene (hexadimethrine bromide; Sigma-Aldrich).

### *Viruses*

From the longitudinally studied cohort participants, clonal HIV-1 variants were isolated from patient PBMCs that were obtained early after SC or entry in the cohort studies at a time point as late as possible in the course of infection before the start of therapy and from PBMCs that had been isolated from an in-between time point. For some participants, virus isolation from PBMCs that were obtained early in the course of infection failed because of low viral loads at these time points. From all other PBMC samples at least two biological virus clones were isolated per time point. Cryopreserved PBMCs from infected individuals were thawed and cocultivated under limiting diluting conditions to obtain clonal virus variants as described previously (31). In brief, increasing numbers of patient PBMCs were cocultivated with $10^5$ 3-day PHA-stimulated healthy donor PBMCs per well in 96-well microtiter plates with four parallel microcultures per patient cell number. Every week, culture supernatants were tested for the presence of p24 Ag by an in-house p24 Ag capture enzyme-linked immunosorbent assay (31). At the same time, one-third of the culture volume was transferred to a new 96-well plate and fresh PHA-stimulated healthy-donor PBMCs were added to propagate the culture. From the positive wells, virus stocks were grown and stored at −70°C until use. All participants were therapy naive at the time of virus isolation.

### *DNA isolation, PCR amplification, and sequencing*

DNA was isolated from PBMCs that were infected with one clonal HIV-1 variant by using the L6 isolation method (36). Gag DNA was amplified by a nested PCR with outer primers, Gag forward (5′-CGACGCAG GACTCGGCTTGCTG-3′) and Gag reverse (5′-GCCTGTCTCTCAG TAC-3′), and two different inner PCR primer combinations, Gag *Bss*HII forward (5′-TGCTGAAGCGCCCGCACGGC-3′) or Gag *Cla*I forward (5′-GGGAGAATTAGATCGATGGG-3′) in combination with Gag p17 reverse (5′-CAAAACTCTTGCCTTATGG)-3′) and Gag p17 forward (5′-TGCTAAACACAGTGGGGGGACAT-3′) in combination with Gag *Apa*I reverse (5′-TTCCTAGGGGCCCTGCAA-3′). The use of biologically clonal HIV-1 allowed us to use two independent Gag primer sets to amplify the *gag* gene without the risk of amplifying *gag* fragments from different virus variants.

PCR products were purified and sequenced with the ABI Prism BigDye Terminator sequencing kit (Applied Biosystems) on an ABI 3130 XL DNA sequencer according to the manufacturer's protocol using the same PCR primers that were used for the nested PCR. DNA sequences were analyzed

with SeqMan (Lasergene software package; DNAStar). The nucleotide sequences of the Gag region were translated and edited with the BioEdit program (version 7.0.5; T. Hall, Ibis Therapeutics). For identification of HLA B57/5801-restricted epitopes, the Los Alamos database was used (http://www.hiv.lanl.gov/).

### *IFN-γ ELISPOT assay*

IFN-γ-producing Ag-specific $CD8^+$ T cells were measured using the IFN-γ ELISPOT assay using multiscreen, 96-well, membrane-bottom plates (MSIPN4550; Millipore) and IFN-γ-specific mAbs (Mabtech). Cryopreserved PBMCs were thawed and suspended in RPMI 1640 medium containing 10% FCS and incubated at a final concentration of $10^5$ cells per well in triplicate in the presence or absence of 20 μg/ml peptide for 20–24 h at 37°C. Responses were tested against the following peptides in Gag (substituted amino acids are set in boldfaced type): KF11 (KAFSPEVIPMF, positions 162–172), IW9 (ISPRTLNAW and mutants **M**SPRTLNAW, **LT**PRTLNAW, **L**SPRTLNAW; positions 147–155), TW10 (TSTLQEQIGW and mutants TS**N**LQEQIGW, TSTL QEQI**A**W, TS**N**LQEQI**T**W, TS**N**LQEQI**A**W; position 240–249), and QW9 (QASQEVKNW and mutant peptides QA**T**QEVKNW and QASQ **D**VKNW; positions 308–316).

All peptides were synthesized by the peptide facility at the Netherlands Cancer Institute (Amsterdam, The Netherlands). PHA stimulation served as a positive control to test the capacity of PBMCs to produce IFN-γ, and medium without peptide or PHA served as a negative control. IFN-γ-producing cells were detected as dark spots and counted using an Eli.Scan ELISPOT scanner with Eli.Analyse software, version 4 (A.EL. VIS). The number of IFN-γ producing cells was calculated by subtracting the negative control value and reported as the number of spot-forming units per $10^6$ PBMCs. Samples with >100 spots per million PBMCs after subtraction of the negative control values were considered positive.

### *In vitro characterization of virus replication rate*

From each participant three clonal HIV-1 variants per time point of virus isolation were analyzed for their replication capacity, and if coexisting R5 and X4 variants were present, three variants of each were analyzed. All viruses were analyzed in a single experiment for which a single pool of healthy donor PBMCs was used. The PBMC pool originated from buffy coats from 10 healthy blood donors from which PBMCs were isolated, pooled, aliquoted, and cryopreserved. Three days before inoculation, cryopreserved, pooled PBMCs were thawed and stimulated with PHA. First, the titer of the virus stocks was quantified by determination of the 50% tissue culture infectious dose in these PHA-stimulated PBMC. Subsequently, additional PBMC from the same pool were thawed and stimulated with PHA. PHA-stimulated PBMC were split in $2 \times 10^6$ aliquots and inoculated with 100 50% tissue culture infectious doses per virus variant. Cells were incubated for 2 h at 37°C in a shaking water bath in 15-ml conical tubes in a 1.5-ml volume. PBMC were then washed twice with 5 ml of culture medium, resuspended in 2 ml of culture medium containing 20 U/ml rIL-2 (Proleukin; Chiron Benelux), and cultured for 18 days in 1 well of a 6-well plate. After 5, 8, and 11 days, $10^6$ fresh PHA-stimulated PBMC from the same pool of buffy coats were added in 1 ml of culture medium containing rIL-2. Samples for the determination of Gag p24 Ag production (50 μl) were harvested every day after inoculation for 18 days and stored at −70°C. All samples were tested for Gag p24 Ag production simultaneously at the end of the experiment in an in-house p24 ELISA (37). On every 96-well ELISA plate a standard curve with a known p24 content was included. Variation between standard curves in different ELISA plates was <10%, thus allowing comparison of data from different ELISA plates. The p24 production per milliliter of supernatant was calculated and corrected for the amount of culture supernatant that was removed and the amount of fresh culture medium that was added during the experiment between the moments of sampling.

### *Statistical analysis*

For statistical analysis of the association of specific residues in the Gag 219–252 region with the clinical course of infection, the Fisher's Exact Test (SPSS 13.0 software) was used. Associations between the clinical course of infection and compensatory mutations were analyzed by comparing the prevalence of any single mutation or combinations of mutations in virus variants obtained from LTNPs and progressors in a cross table. The first day of detection of HIV-1 variants in the replication assay was determined by linear regression with a detection limit of 25 ng of p24 per milliliter of culture supernatant. The *t* test (SPSS) was used to determine the statistical significance of differences on the first day of detection in the viral replication assay and that of differences in the number of mutations in the Gag 219–252 region of HIV-1 variants from progressors and LTNPs. The statistical significance of differences in the magnitude of $CD8^+$ T cell IFN-γ responses between progressors and LTNPs was calculated with the Mann-Whitney *U* test (SPSS) using the highest response against either the wild-type peptide or the autologous escape variant. $p < 0.05$ was considered significant.

## Results

### *Potential escape mutations in CTL epitopes in HIV-1 Gag of HLA B57/5801 LTNPs and progressors*

Gag sequences were successfully PCR amplified from clonal virus variants that were isolated from therapy naive LTNPs ($n = 9$) and progressors ($n = 13$) and studied for changes in four previously described HLA B57/5801 epitopes (31) (IW9, KF11, TW10, and QW9). IW9 showed an I147L or I147M substitution in seven of nine LTNPs and in nine of 13 progressors. The A146P substitution, which is known to alter Ag processing (38), was found in the viruses of 11 of 22 individuals but was not associated with the clinical course of infection. At the anchor residue position, a S148T substitution was found only in viruses from five of 13 progressors. The KF11 epitope did not reveal sequence variation in virus variants of any of the patients. In agreement with a previous study (25) but not with others (31), a T242N substitution was observed in the TW10 epitope in HIV-1 variants from all study participants. A G248A or G248T substitution in the same epitope was observed in viruses from nine of 13 progressors and in six of nine LTNPs.

The QW9 epitope harbored a S310T substitution in viruses from three progressors and two LTNPs. An E312D substitution in the same epitope was seen in viruses from three of eight progressors and one of four LTNPs.

To gain more insight in the dynamics by which these CTL escape mutations emerged in the course of infection, we performed clonal virus isolation from at least two time points during the course of infection of five progressors (P9–P13) and five LTNPs (L5–L9) (Tables II and III, respectively). Despite several attempts, we were unable to isolate clonal virus variants from early PBMC samples from individuals L5, L8, L9, and P10 because of low viral loads early in infection. The earliest time points at which virus could be isolated from these individuals were 89, 70, 42, and 69 mo after SC or entry in the cohort studies, respectively.

In most individuals, sequence variations in the HLA B57/5801-restricted epitopes IW9 and TW10 in Gag were observed in viruses from the earliest time points onwards. Viruses obtained at the early time point from participant L6 lacked the I148L substitution, whereas the early viruses from P13 lacked the T242N substitution in the TW10 epitope. The G248X substitution in the same epitope was ultimately present in viruses from seven of 10 individuals and was observed at the earliest time point studied in only three of these seven participants.

In this longitudinal analysis, again no sequence variation was observed in the KF11 epitope despite a follow-up time of up to 14 years.

### *$CD8^+$ T cell IFN-γ responses directed against HLA B57/5801-restricted Gag epitopes*

Next we studied the $CD8^+$ T cell IFN-γ responses directed against HLA B57/5801-restricted Gag epitopes. Using IFN-γ ELISPOT assays, $CD8^+$ T cell responses to the well-characterized HLA B57/5801-restricted epitopes IW9, TW10, QW9, and KF11 in Gag were assessed in the 10 individuals who were studied longitudinally. In addition, $CD8^+$ T cell IFN-γ responses directed against

Table II. *HIV-1 Gag epitope sequences of HLA B57/5801 progressors*[a]

| Patient | Time after SC or Entry in the Cohort (mo) | CD4 Count (per μl) | No. Sequences per Total | IW9 147–155 AISPRTLNAW | KAF11 162–172 KAFSPEVIPMF | TW10 240–249 TSTLQEQIGW | QW9 308–316 QASQEVKNW |
|---|---|---|---|---|---|---|---|
| P1 | 7 | 1490 | 1/5 | --T------- | ---------- | --N-----A- | --T------- |
| | | | 1/5 | --T------- | ---------- | --N-----A- | --------- |
| | | | 1/5 | ---------- | ---------- | --N-----A- | --T------- |
| | | | 2/5 | ---------- | ---------- | -------MA- | --------- |
| P2 | 85 | NA | 5/5 | -L-------- | ---------- | --N-----T- | ----D---- |
| P3[b] | 36 | 260 | 3/5 | P--------- | ---------- | --N-----A- | --T------- |
| | | | 1/5 | P-T------- | ---------- | --N-----A- | --T------- |
| | | | 1/5 | PLT------- | ---------- | --N-----A- | --T------- |
| P4[b] | 79 | 340 | 3/5 | -M-------- | ---------- | --N-----A- | --------- |
| | | | 1/5 | PM-------- | ---------- | --N-----A- | --------- |
| | | | 1/5 | P-T------- | ---------- | --N-----A- | --------- |
| P5[b] | 63 | 340 | 4/4 | P--------- | ---------- | --N------- | --------- |
| P6[b] | 83 | 240 | 1/1 | P--------- | ---------- | --N------- | --------- |
| P7 | 60 | 210 | 1/1 | -L-------- | ---------- | --N-----A- | ----D---- |
| P8 | 31 | 350 | 4/4 | ---------- | ---------- | --N------- | --------- |
| P9 | 9 | 680 | 8/8 | PMT------- | ---------- | --N-----A- | --------- |
| | 78 | 310 | 3/3 | -L-------- | ---------- | --N-----A- | --------- |
| P10 | 69 | 610 | 5/5 | -L-------- | ---------- | --N------- | ----D---- |
| | 84 | 450 | 2/6 | -L-------- | ---------- | --N------- | ----D---- |
| | | | 4/6 | -L-------- | ---------- | --N-----T- | ----D---- |
| | 123 | 20 | 6/6 | -L-------- | ---------- | --N-----T- | ----D---- |
| P11[b] | 3 | 1060 | 1/2 | ---------- | ---------- | --N------- | --------- |
| | | | 1/2 | P--------- | ---------- | --N-----A- | --------- |
| | 25 | 870 | 1/9 | PM-------- | ---------- | --N------- | --------- |
| | | | 1/9 | P--------- | ---------- | --N------- | --------- |
| | | | 3/9 | PLT------- | ---------- | --N------- | --------- |
| | | | 4/9 | PL-------- | ---------- | --N------- | --------- |
| | 32 | 820 | 1/23 | ---------- | ---------- | --N------- | --------- |
| | | | 2/23 | PM-------- | ---------- | --N-----A- | ----D---- |
| | | | 2/23 | PL-------- | ---------- | --N-----A- | --------- |
| | | | 4/23 | PLT------- | ---------- | --N------- | --------- |
| | | | 4/23 | PM-------- | ---------- | --N-----A- | --------- |
| | | | 1/23 | -L-------- | ---------- | --N------- | --------- |
| | | | 9/23 | PL-------- | ---------- | --N------- | --------- |
| | 69 | 490 | 1/12 | PM-------- | ---------- | --N-----A- | ----D---- |
| | | | 1/12 | PL-------- | ---------- | --N-----A- | --------- |
| | | | 10/12 | -L-------- | ---------- | --N-----A- | --------- |
| P12 | 0–4 | 380 | 1/5 | -L-------- | ---------- | --N------- | --T-D---- |
| | | | 2/5 | -L-------- | ---------- | --N------- | --------- |
| | | | 2/5 | -L-------- | ---------- | --N------- | ----D---- |
| | 48 | 210 | 1/11 | -L-------- | ---------- | --N------- | ----D---- |
| | | | 4/11 | SL-------- | ---------- | --N------- | ----D---- |
| | | | 2/11 | -L-------- | ---------- | --N------- | --T-D---- |
| | | | 4/11 | SL-------- | ---------- | --N------- | --T-D---- |
| | 74 | NA | 4/12 | SL-------- | ---------- | --N------- | --T-D---- |
| | | | 2/12 | SL-------- | ---------- | --N------- | ----D---- |
| | | | 6/12 | SL-------- | ---------- | --N------- | ----D---- |
| P13 | 3 | NA | 1/6 | -L-------- | ---------- | ---------- | --------- |
| | | | 5/6 | -M-------- | ---------- | ---------- | --------- |
| | 46 | 420 | 1/6 | -M-------- | ---------- | ---------- | -------S- |
| | | | 5/6 | -M-------- | ---------- | ---------- | --------- |
| | 88 | 20 | 3/6 | -M-------- | ---------- | --N-----A- | --------- |
| | | | 3/6 | -M-------- | ---------- | --N-----A- | ----D---- |

[a] Consensus sequences of four known HLA B57/5801 restricted epitopes in Gag (IW9, KF11, TW10, and QW9) and aligned predicted amino acid sequences of 13 progressors (P). P1–P8 were studied cross-sectionally and P9–P13 were studied longitudinally. Only amino acid substitutions relative to the consensus sequence are shown.

[b] Individuals who seroconverted for HIV-1 antibodies during active follow-up in the cohort studies. NA, Not analyzed.

peptides that corresponded to the autologous escape variants of these epitopes in HIV-1 were studied (Fig. 1).

$CD8^+$ T cell IFN-γ responses against wild-type peptides of the IW9, TW10, QW9, and KF11 epitopes in Gag and the autologous mutant peptides were mostly high and sustained during follow-up. The magnitude of $CD8^+$ T cell IFN-γ responses against wild-type and autologous mutant peptides of the IW9, TW10, and QW9 epitopes were similar (Fig. 1, *A–C*). Sequence variation was absent in the KF11 epitope in all 10 individuals. Therefore, only $CD8^+$ T cell IFN-γ responses against the wild-type KF11 epitope sequence were measured (Fig. 1*D*) and were significantly better in progressors than in LTNPs ($p = 0.027$).

### *Replication capacity of longitudinally obtained clonal HIV-1 variants in relation to CTL escape and potential compensatory mutations in Gag*

It has previously been suggested that the T242N mutation is associated with a severe loss of viral fitness. Additional mutations in the Gag 219–252 region have been suggested to compensate for

Table III. *HIV-1 Gag epitope sequences of HLA B57/5801 LTNP*[a]

| Patient | Time after SC or Entry in the Cohort (mo) | CD4 Count (per μl) | No. Sequences per Total | IW9 147–155 AISPRTLNAW | KAF11 162–172 KAFSPEVIPMF | TW10 240–249 TSTLQEQIGW | QW9 308–316 QASQEVKNW |
|---|---|---|---|---|---|---|---|
| L1 | 141 | 300 | 2/2 | -L--------- | ---------- | --N-----A- | --------- |
| L2 | 21 | 530 | 1/5 | PL--------- | ---------- | --N------- | ----D---- |
| | | | 1/5 | P--------- | ---------- | --N------- | ----D---- |
| | | | 3/5 | ---------- | ---------- | --N------- | ----D---- |
| L3[b] | 115 | 330 | 5/5 | P--------- | ---------- | --N-----A- | --------- |
| L4 | 132 | 230 | 5/5 | -L--------- | ---------- | --N-----A- | --T------ |
| L5 | 89 | 660 | 6/6 | PL--------- | ---------- | --N------- | --------- |
| | 177 | 320 | 6/6 | PL--------- | ---------- | --N------- | --------- |
| L6 | 17 | 750 | 1/5 | ---------- | ---------- | --------A- | --------- |
| | | | 4/5 | ---------- | ---------- | --N------- | --------- |
| | 114 | 380 | 1/15 | ---------- | ---------- | --N------- | --------- |
| | | | 2/15 | ---------- | ---------- | --------A- | --------- |
| | | | 4/15 | ---------- | ---------- | --N-----T- | --------- |
| | | | 8/15 | -L--------- | ---------- | --N------- | --------- |
| L7 | 26 | 940 | 1/5 | -L--------- | ---------- | --N-----A- | --T------ |
| | | | 4/5 | -L--------- | ---------- | --N-----A- | ----D---- |
| | 78 | 770 | 2/6 | -L--------- | ---------- | --N-----A- | ----D---- |
| | | | 4/6 | -L--------- | ---------- | --N-----A- | --T------ |
| | 102 | 460 | 1/8 | -L--------- | ---------- | --N-----A- | ----D---- |
| | | | 1/8 | ---------- | ---------- | --N-----A- | --T------ |
| | | | 1/8 | ---------- | ---------- | --N-----A- | ----D---- |
| | | | 5/8 | -L--------- | ---------- | --N-----A- | --T------ |
| | 136 | 270 | 1/12 | -L--------- | ---------- | --N-----A- | --T-D---- |
| | | | 1/12 | -L--------- | ---------- | --N-----A- | --------- |
| | | | 10/12 | -L--------- | ---------- | --N-----A- | ----D---- |
| L8[b] | 70 | 840 | 1/1 | -L--------- | ---------- | --N------- | ----D---- |
| | 91 | 630 | 1/7 | -L--------- | ---------- | --S-----A- | --------- |
| | | | 6/7 | -L--------- | ---------- | --N-----A- | --------- |
| | 137 | 290 | 2/5 | -L--------- | ---------- | --N------- | ----D---- |
| | | | 3/5 | -L--------- | ---------- | --N-----A- | ----D---- |
| L9[b] | 42 | 610 | 1/1 | P--------- | ---------- | --N------- | --------- |
| | 59 | 740 | 1/1 | P--------- | ---------- | --N------- | --------- |
| | 77 | 770 | 3/3 | P--------- | ---------- | --N------- | --------- |

[a] See notes to Table II. The LTNPs L1–L4 were studied cross-sectionally and L5–L9 were studied longitudinally.
[b] Individuals who seroconverted for HIV-1 Abs during active follow-up in the cohort studies.

this loss in viral replication capacity, specifically H219Q, I223V, M228I, N252H, and G248X, which is present in the TW10 epitope (T. Allen, unpublished observations). We analyzed clonal HIV-1 variants isolated from P9–P13 and L5–L9 for these mutations in the Gag 219–252 region and found no association between the presence of any single one of these five potential compensatory mutations and the course of infection (data not shown). However, any combination of two or more of these compensatory mutations was significantly associated with disease progression ($p = 0.031$). No difference in the number of mutations in the Gag 219–252 region between viruses isolated relatively early and late from LTNPs was observed. However, there was a trend toward an increased number of mutations in the Gag 219–252 region of progressor viruses isolated late in infection as compared with early virus variants, although this was not significant ($p = 0.075$). In addition, the number of mutations in this region in late progressor viruses was significantly higher than in late LTNP viruses ($p = 0.042$).

Next, we determined the replication kinetics of virus variants isolated from these same patients (P9–P13 and L5–L9) (Fig. 2). We analyzed at least three clonal HIV-1 variants per time point in the course of infection from each individual with the exception of patient L9, from whom we had only one or two clonal virus variants per time point.

All viruses isolated from progressors within the first 9 mo after SC or entry into the cohort studies were slowly replicating (Fig. 2*A*). The earliest viruses from patient P10 were obtained 69 mo after entry, which is relatively late, and replicated relatively rapidly with no further increase in replication capacity (Fig. 2*A*). The replication kinetics of viruses from all other progressors increased in the course of infection as revealed by a significantly earlier first day of detection in the virus replication assay of late stage viruses vs virus variants isolated early in infection ($p = 0.003$) (Fig. 3). In three progressors (P10, P11, and P12) and one LTNP (L7), X4 variants emerged during the course of infection. Coexisting R5 and X4 virus variants did not differ in their replication kinetics and the first day of detection in the replication assay was not significantly different between coexisting X4 and R5 HIV-1 variants (data not shown).

The replication kinetics of virus variants from LTNPs were relatively constant throughout the course of infection (Fig. 2*B*). No significant differences were observed on the first day of detection in the replication assay between viruses isolated at the early time points compared with the late time points in the clinical course of LTNP infection (Fig. 3). L7 was the only LTNP that harbored slowly replicating virus variants early in infection and fast replicating virus variants late in infection. However, replication kinetics and the first day of detection of all virus variants from patient L7 isolated before the emergence of X4 variants after 102 mo were comparable to those of the virus variants of other LTNPs (Fig. 2*B*). The first day of detection of p24 production in PBMC cultures infected with virus variants isolated early in infection from either progressors or LTNPs was similar. However, this first day of detection was significantly earlier for virus variants isolated late in infection from progressors as stated above, but also when compared with early ($p = 0.021$) and late LNTP viruses ($p = 0.029$) (Fig. 3).

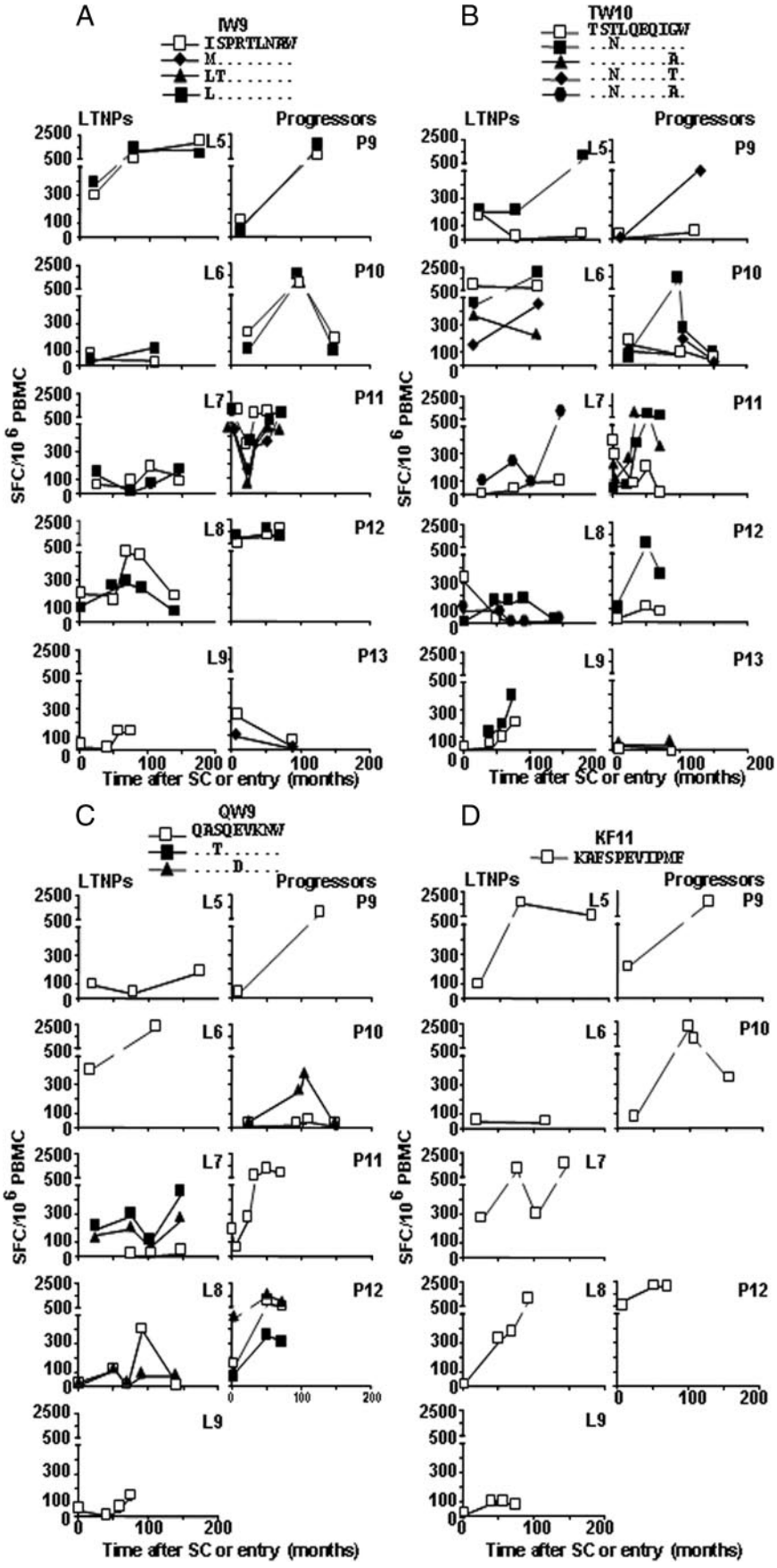


**FIGURE 1.** $CD8^+$ T cells producing IFN-γ in response to stimulation with wild-type and autologous mutant epitopes in Gag. *A*, Responses against IW9. *B*, Responses against TW10. *C*, Responses against QW9. *D*, Responses against KF11. $CD8^+$ T cell responses of LTNP (*left panels*) and progressors (*right panels*) are given as spot forming cells (SFC) per $10^6$ PBMC (*y*-axis) and were measured at different time points in the course of infection (months after SC or entry; *x*-axis). Responses against different mutant peptides are indicated with symbols that are presented at the top of each panel.

Next, we analyzed whether viral replication kinetics were directly related to the presence of the potential compensatory mutations in the virus variants. In three of five progressors (P11, P12, and P13), the increase in viral replication capacity was associated with an increase in the number of potential compensatory mutations. In the other two progressors (P9 and P10) this association

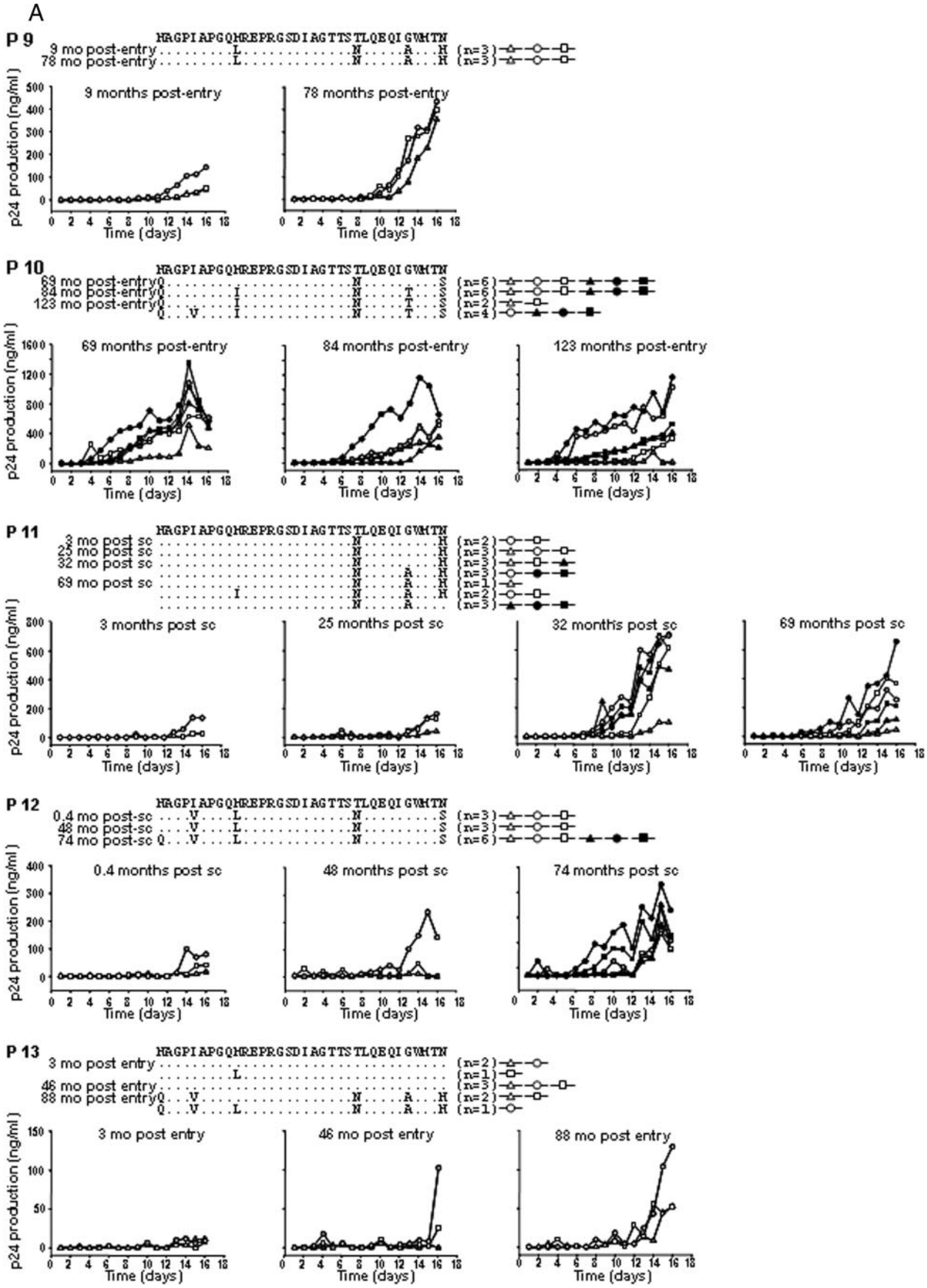


**FIGURE 2.** Replication kinetics of HIV-1 variants that were isolated at different time points in the course of infection from five progressors (P9–P13) (*A*) and five LTNPs (L5–L9) (*B*). p24 production was measured in the culture supernatant every day after inoculation for 16 days. From each virus variant that was tested for replication capacity the amino acid sequence (positions 219–252) of the Gag region is shown (corresponding symbols). When applicable, replication kinetics of R5 variants are shown with open symbols and of those of X4 variants with closed symbols.

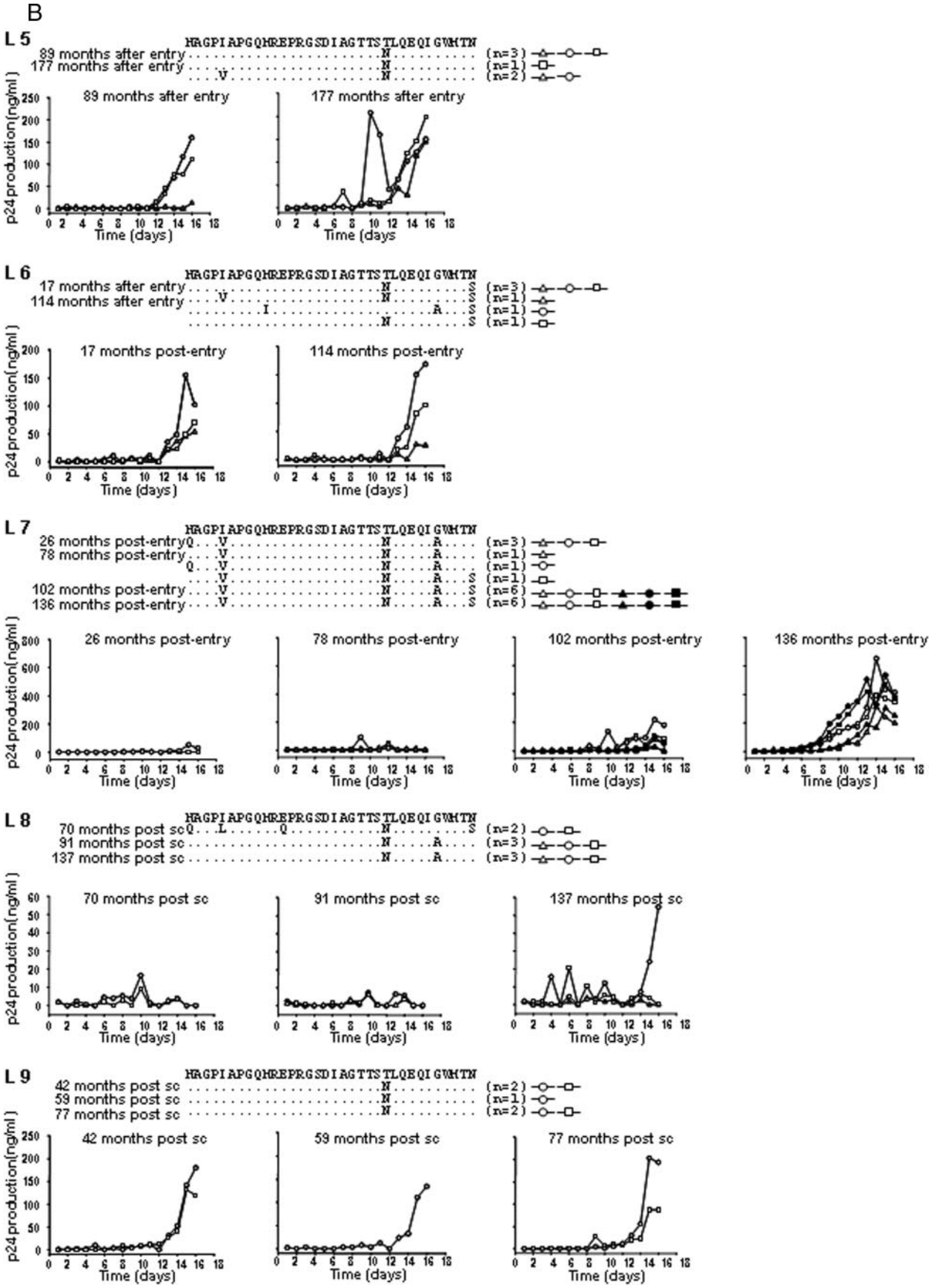


**FIGURE 2.** (*continued*)

was absent. Replication kinetics of HIV-1 variants from P9 increased over time without any sequence changes in the Gag 219–252 region, whereas HIV-1 variants isolated between 69 and 123 mo after entry in the cohort studies from P10 showed an increase in the number of mutations while no changes in replication capacity were observed.

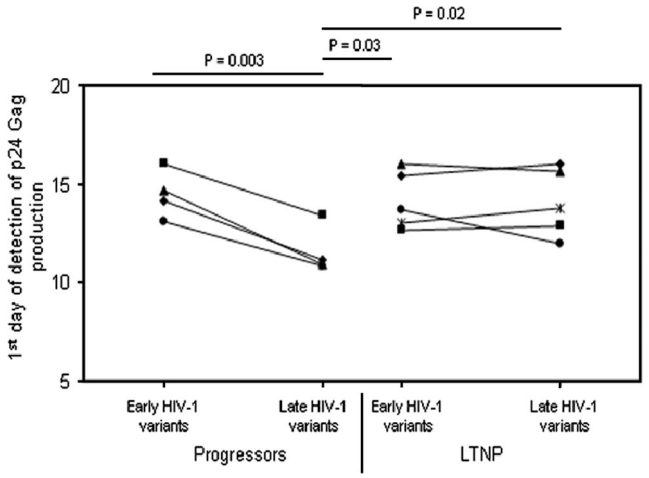


**FIGURE 3.** First day of detection of p24 production in the replication assay with virus variants isolated from the PBMC sample at the earliest available time point in the course of infection and virus variants isolated from the PBMC sample obtained at the end of the study period from either progressors or LTNPs.

## Discussion

Multiple studies have indicated an association between HLA B57 and a more benign clinical course of infection (28, 39–41). The underlying mechanism for this protective effect is still unknown. It has been hypothesized that HLA B57/5801 restricted CTL target epitopes in highly conserved regions of the virus. Escape mutations in these epitopes would support evasion from the immune system but come at such a fitness loss to the virus that the net effect would still be a reduced viral burden. The reversal of the T242N substitution in the HLA B57/5801-restricted TW10 epitope upon transmission from an HLA B57/5801$^+$ to an HLA B57/5801$^-$ individual supported this idea (25). In addition, viruses with the T242N substitution in Gag had lower in vitro replication fitness as compared with viruses with the wild-type TW10 sequence (26). If this mutation would be relevant for the more benign clinical course in HLA B57/5801-typed individuals, one would expect its absence in HLA B57/5801 progressors. In our present study we compared participants from the ACH studies on HIV infection and AIDS (www.amsterdamcohortstudies.org/) who were all were HLA B57/5801 but showed a highly variable disease course. Although we observed an overrepresentation of HLA B57/5801 among cohort participants who fulfilled the definition of LTNP, a normal frequency of the HLA B57/5801 allele was present among individuals with a more progressive disease course. Interestingly, HLA B57/5801$^+$ progressors were indistinguishable from progressors with other HLA types with respect to the set point viral load in plasma and the rate of CD4$^+$ T cell decline in the course of infection (data not shown), confirming previous observations by others (31).

Our comparison of HLA B57/5801 LTNP and progressors did not reveal a difference in the prevalence of the T242N substitution in the TW10 epitope in Gag between patient groups. In agreement, Migueles et al. (31) also demonstrated the presence of this escape mutation in HLA B57 progressors. This suggests that the attenuating effect on the virus of this substitution may be limited, considering the high viral loads in the progressors. Alternatively, HIV-1 variants in progressors may have accumulated compensatory mutations.

Our study also did not reveal any differences in escape mutations in the QW9 epitope in Gag, which contrasts the previously reported overrepresentation of the E312D substitution in this epitope in HLA B57 progressors as compared with LTNPs (31). It cannot be excluded that this difference is due to a difference in patient groups between studies. Taken together, our study did not reveal differences in the prevalence of CTL escape mutations in any of the four HLA B57/5801-restricted epitopes in Gag that could potentially relate to the differential disease course in our HLA B57/5801 LTNPs and progressors.

One could argue that the ability of the host to generate an effective CTL response against the wild-type sequence and, subsequently, the escape variants will contribute to the control of viral replication. Recent studies have shown that the immune system is able to respond to viral escape by generating new CTL responses that are directed against the escape variants (42, 43). Due to a limited number of patient cells we were only able to test a single peptide concentration of 20 $\mu$g/ml. However, we could confirm here (43) that de novo CTL responses restricted by HLA B57/5801 can be generated against HIV-1 CTL escape variants harboring amino acid substitutions at positions that determine TCR binding. Indeed, HLA B57/5801 progressors and LTNPs in our study were not only similar in the magnitude of the CD8$^+$ IFN-$\gamma$ responses specific for the wild-type IW9, TW10, and QW9 Gag epitopes, they also had excellent reactivity against escape variants in Gag. Our results extend previous conclusions that differences between HLA B57/5801 LTNPs and progressors are not due to the loss of immune system recognition of autologous Gag sequences of the virus (31). However, differences in other functional or qualitative aspects of T cell responses may exist between LTNPs and progressors that could contribute to their differential clinical courses (44).

It was previously demonstrated that a virus clone containing the T242N mutation was outgrown by the wild-type virus in a direct competition assay, suggesting attenuation associated with this mutation (26). Several mutations (H219Q, I223V, M228I, N252H, and G248X) in the same Gag 219–252 region may compensate for this loss in viral fitness (T. Allen, unpublished observations). Interestingly, we observed these exact mutations in our patient group, showing that sequence variation may follow specific patterns although the number of mutations varied per patient. We did not, however, find an association between any of these five mutations and disease progression within the HLA B57/5801 group. However, any combination of two of these potential compensatory mutations was associated with disease progression in the HLA B57/5801 individuals.

From three (P10, L5, and L8) of the 10 individuals who were studied longitudinally we failed to isolate HIV-1 variants early after SC or entry. This was associated with low viral loads in vivo tjat could reflect optimal CTL pressure and an initial absence of CTL escape.

Our sequence analysis was performed on biologically cloned HIV-1 variants and one could argue that these viruses may not be fully representative of the total replication-competent viral quasispecies in plasma. However, there is evidence that the viral quasispecies in plasma and the replication-competent HIV-1 variants in productively infected cells are closely related, if not the same viral compartments. Indeed, the kinetics of viral load changes and the emergence of drug resistance mutations in plasma/serum and productively infected cells are highly correlated (45–47). Moreover, an interesting aspect of our approach is that it allowed us to directly compare replication capacity in relation to sequence diversity.

In agreement with a previous study in which we compared the replication rates of early and late viruses from progressors and LTNPs (48), we observed increasing replication rates of viruses isolated in the course of infection from HLA B57/5801 progressors

and only slowly replicating virus variants throughout the course of infection in LTNPs. It should be noted that in our assay of replication capacity, cell-free, primary passaged virus isolates were inoculated on donor PBMCs in the complete absence of autologous $CD8^+$ T cells with potential antiviral activity. Viral replication is thus not determined in any sense by B57/5801-restricted CTLs in the current study.

Despite the fact that late progressor viruses had significantly more mutations in the Gag 219–252 region than LTNP viruses irrespective of the time point of virus isolation, we could not find an association between viral replication rates and Gag sequences. This may imply that the high number of mutations in late progressor viruses was a consequence rather than a cause of increased viral replication and that other viral factors regulate HIV-1 replication (49). Indeed, viruses from individual L9 harbored a four-amino acid deletion in Nef (data not shown) that may be causally related to the slow replication rates of his virus variants (48).

Overall, we have shown that differences in the clinical course of infection within a group of HLA B57/5801 individuals is related neither to the differences in escape mutations in Gag epitopes nor to differences in CTL activity against epitopes in Gag but rather to differences in viral replication kinetics. How these differences in viral replication are determined remains to be elucidated.

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## Disclosures

The authors have no financial conflict of interest.

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