# A Nonprogressive Clinical Course in HIV-Infected Individuals Expressing Human Leukocyte Antigen B57/5801 Is Associated with Preserved CD8+ T Lymphocyte Responsiveness to the HW9 Epitope in Nef

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**The human leukocyte antigen (HLA) B57 allele and the closely related HLA-B5801 allele are overrepresented among human immunodeficiency virus type 1 (HIV-1)–infected individuals with a long-term nonprogressive clinical course of disease (known as "long-term nonprogressors" [LTNPs]). These alleles are, however, also present among individuals with normal disease progression (known as "progressors"). In a comparison of HLA-B57/5801–expressing progressors and LTNPs, we observed a similar prevalence of escape mutations in 4 Nef epitopes and a similar reactivity of CD8+ T cells against 3 of 4 of these epitopes and their autologous escape variants. However, LTNPs tended to have frequent and preserved CD8+ T cell interferon-γ responses against the wild-type HW9 Nef epitope, whereas progressors did not maintain a specific CD8+ T cell response. This finding is in line with the findings of a more exhausted phenotype of CD8+ T cells in progressors, as is demonstrated by their enhanced level of expression of inhibitory receptor "programmed death 1" (PD-1). The results of the present study suggest that preservation of HW9-specific T cell responses is associated with a more benign clinical course of infection.**

Certain HLA types have been associated with the clinical course of HIV-1 infection. The prevalence of HLA-B57 and HLA-B5801 alleles is higher among individuals with a long-term nonprogressive clinical course of disease (known as "long-term nonprogressors" [LTNPs]), and the presence of the HLA-B57/5801 allele has been shown to be predictive of prolonged asymptomatic survival [1, 2]. The underlying mechanism for this protective effect has not yet been elucidated; however, considering the role of HLA molecules in the presentation of viral antigens to T cells, a role for cytotoxic T lymphocytes (CTLs) seems to be likely.

Several lines of evidence point to a central role of CTLs in the control of viral replication during asymptomatic HIV-1 infection [3–7]. CTL pressure, however, may select for escape variants of HIV-1 that are capable of evading antigen presentation to T cells or CTL recognition. Escape of HIV-1 from CTLs has been observed in humans [7–16] as well as in animal models [17–21] and is generally associated with increased viral replication and progressive loss of CD4+ T cells.

Several studies have implied that at least certain CTL escape mutations can come at a fitness cost to the virus [22–24]. One of the most prominent examples of loss of viral fitness has been associated with the T242N mutation in the HLA-B57–restricted TW10 epitope in Gag,

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**Table 1. HLA typing and CD4 counts of HLA-B57/5801–expressing individuals with a progressive disease course (progressors) and individuals with a long-term nonprogressive clinical course of disease (LTNPs).**

| Patient | HLA-A allele | HLA-B allele | CD4 cell count, $\times 10^6$ cells/L | |
|---|---|---|---|---|
| | | | At the end of follow-up[a] | After 9 years of follow-up |
| Progressor | | | | |
| P1 | A*33/A*33 | B*5801/B*14 | 480 (25) | NA |
| P2 | A*01/A*03 | B*5701/B*5101 | 490 (45) | NA |
| P3 | A*01/A*24 | B*5701/B*3502 | 150 (41) | NA |
| P4 | A*03/A*6801 | B*5701/B*3501 | 130 (94) | NA |
| P5 | A*02/A*3201 | B*5701/B*1401 | 360 (65) | NA |
| P6 | A*01/A*32 | B*57/B*1501 | 120 (79) | NA |
| P7 | A*0205/A*33 | B*5801/B*1503 | 200 (63) | NA |
| P8 | A*02/A*24 | B*5801/B*1501 | 230 (40) | NA |
| P9 | A2/A3 | B*5701/B*0702 | 190 (79) | NA |
| P10 | A*02/A*26 | B*5701/B*4102 | 10 (135) | 40 |
| P11 | A1/A11 | B*5701/B*0801 | 220 (76) | NA |
| P12 | A*01/A*26 | B*5701/B*3801 | 240 (71) | NA |
| P13 | A*01/A*01 | B*5701/B*0801 | 30 (89) | NA |
| LTNP | | | | |
| L1 | A*01/A*02 | B*5701/B*0702 | 300 (147) | 380 |
| L2 | A*01/A*24 | B*5701/B*1501 | 490 (149) | 660 |
| L3 | A*02/A*03 | B*5801/B7 | 440 (120) | 440 |
| L4 | A*01/A*03 | B*57/B*07 | 120 (137) | 370 |
| L5 | A*26/A*6801 | B*57/B*0702 | 310 (196) | 560 |
| L6 | A3/A11 | B*5801/B7 | 330 (123) | 460 |
| L7 | A*01/A*02 | B*5701/B*0801 | 140 (140) | 860 |
| L8 | A*02/A*02 | B*5701/B*3701 | 250 (143) | 590 |
| L9 | A*01/A*6802 | B*5701/B*1402 | 500 (120) | 590 |

**NOTE.** NA, not applicable, because, at this time point, patients had died of AIDS or were excluded from the study because they started receiving antiviral therapy.

[a] Data in parentheses denote the month of follow-up.

and this loss of fitness has been used as one of the explanations for the association between HLA-B57 and a long-term nonprogressive clinical course of HIV infection [22].

Despite the clear association between HLA-B57/5801 and long-term nonprogressive HIV infection, a substantial group of HIV-infected individuals expressing the HLA-B57/5801 allele has demonstrated relatively rapid progression to AIDS [25]. In a study from our group published elsewhere [26], the T242N mutation in the HLA-B57/5801–restricted TW10 Gag epitope in HIV-1 was present in all viruses from both individuals with disease progression (known as "progressors") and LTNPs expressing HLA-B57/5801 from the Amsterdam Cohort Studies on HIV Infection and AIDS (hereafter referred to as the "Amsterdam Cohort Studies"). In addition, the frequencies of CTLs directed against wild-type sequences and autologous CTL escape variants of the 4 known HLA-B57/5801 epitopes in Gag were similar in progressors and LTNPs. Thus, this finding could not explain the observed differences in the clinical course of disease.

In the present study, we focused on Nef-specific CTLs and sequence dynamics in 4 previously described CTL epitopes in Nef, to test whether they are associated with the differential clinical course of infection in our group of LTNPs and progressors expressing HLA-B57/5801.

## METHODS

***Study subjects.*** All participants from the Amsterdam Cohort Studies who carried the HLA-B57 or HLA-B5801 allele ($n = 22$) were selected for inclusion in the present study (table 1). Fourteen individuals were HIV-1 infected (had HIV-1 seroprevalence) at the moment of entry in the cohort studies, and 8 individuals demonstrated seroconversion for HIV antibodies during active follow-up. All cohort participants made routine visits to donate blood and undergo physical examination every 3 months. After being selected for this study on the basis of HLA-B57/5801 expression, participants had their HLA-B type confirmed using high-resolution HLA typing [27]. Individuals who

developed AIDS or who started receiving antiviral therapy within 9 years after seroconversion or seroprevalent entry into the Amsterdam Cohort Studies were called "progressors" ($n = 13$: patients 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" ($n = 9$; ACH19552 [L1], ACH19417 [L2], ACH19285 [L3], ACHD15991 [L4], ACH19933 [L5], ACH19922 [L6], ACH19789 [L7], ACH19784 [L8], and ACH19291 [L9]) were study participants who had stable CD4 cell counts that remained at the level of >400 cells/$\mu$L of blood in year 10 after seroconversion or seroprevalent entry in the Amsterdam Cohort Studies or who had a decrease of <40 CD4 cells/$\mu$L per year over a period of ≥10 years.

Five progressors (P9–P13) and 5 LTNPs (L5–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 wild-type genotype. All study participants were therapy naive during the study period.

Typing of the HLA-A and -B alleles of all participants did not reveal other HLA types that were associated with a difference in disease progression, with the exception of P3, whose HLA-B type was HLA-B*3502. This allele is known to accelerate the clinical course of infection.

***Isolation of biological virus clones and sequence analysis.*** Clonal HIV-1 variants were obtained as described elsewhere [28]. In short, DNA was isolated from HIV-negative donor peripheral blood mononuclear cells (PBMCs) infected with clonal HIV-1 variants, by use of the L6 method [29]. Nef DNA was amplified using a nested polymerase chain reaction (PCR) with the following outer primers—Nef-1 forward (fw) primer AGCCATAGCAGTAGCTGAGG and Nef-1 reverse (rev) primer GCTTATATGCAGGATCTGAGG—and the following inner primers—Nef-2 fw primer AGCTTGTAGAGCTATTCGCCACA and Nef-2 rev primer AGCAAGCTCGATGTCAGCAG. PCR products were purified and sequenced using the ABI prism BigDye Terminator sequencing kit (Perkin Elmer) on an ABI 3130 XL DNA sequencer, according to the manufacturer's protocol.

***Interferon (IFN)–$\gamma$ enzyme-linked immunospot (ELISPOT) assay.*** IFN-$\gamma$–producing antigen-specific $CD8^+$ T cells were measured using the IFN-$\gamma$ ELISPOT assay with the use of multiscreen, 96-well, membrane-bottomed plates (MSIPN4550; Millipore) and IFN-$\gamma$–specific monoclonal antibodies (Mabtech). Cryopreserved PBMCs were thawed and suspended in RPMI 1640 medium containing 10% fetal calf serum (FCS) and were incubated at a final concentration of $10^5$ cells/well in triplicate. Responses were measured against an excess concentration of 20 $\mu$g/mL of the following peptides: HTQGYFPDWQ, **N**TQGYFPDWQ, KGALDLSHF, K**A**ALDLSHF, K**A**A**V**DLSHF, GPGIRYPLTFGWCF, GPG**V**RYPLTFGWCY, and YFDPWQNYT. Responses against the HTQGYFPDWQ and **N**TQGYFPDWQ peptides were tested at additional concentrations of 0.1, 1, and 10 $\mu$g/mL, to investigate potential differences in the ability to respond to low peptide concentrations.

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

***Analysis of the expression of inhibitory receptor "programmed death 1" (PD-1).*** PBMCs were resuspended in RPMI 1640 medium containing 10% FCS. Cells were incubated with $\alpha$CD8-peridinin chlorophyll protein (PerCP) and $\alpha$-programmed death fluorescein isothiocyanate ($\alpha$PD-1-FITC) for 20 min at 4°C (Becton Dickinson). Cells were fixed in cellfix (Becton Dickinson), and flow cytometry was performed. At least 300,000 events were acquired using the LSRII flow cytometer (Becton Dickinson). Lymphocytes were gated by forward and sideward scatter. PD-1 expression on total $CD8^+$ T cells was analyzed using Diva software (Becton Dickinson).

## RESULTS

***Sequence dynamics in 4 HLA-B57/5801–restricted CTL epitopes in Nef of HIV-1 variants isolated from LTNPs and progressors.*** We performed a longitudinal analysis of the dynamics of CTL escape mutations in 4 well-defined HLA-B57/5801–restricted epitopes in Nef of clonal HIV-1 variants isolated from 5 HLA-B57/5801 progressors (table 2) and 5 HLA-B57/5801 LTNPs (table 3). CTL escape mutations from 12 additional HLA-B57/5801 participants (8 progressors and 4 LTNPs) were analyzed at a single time point during infection.

In the HLA-B57/5801–restricted Nef epitope YT9 (YFPDWQNYT; amino acid positions 120–128), sequence variation was found only in viruses from L8 that were isolated 137 months after seroconversion. Various potential CTL escape mutations occurred in HLA-B57/5801–restricted Nef epitopes KF9 (KGALDLSHF; amino acid positions 82–90) and GF14 (GPGIRYPLTFGWCF; amino acid positions 130–143) of HIV-1 variants isolated from both LTNPs and progressors. These are, therefore, not likely to influence the clinical course of infection. HIV-1 variants isolated from 12 progressors and 7 LTNPs harbored a H116N substitution in HLA-B57/5801–restricted Nef epitope HW9 (HTQGYFPDW; amino acid positions 116–124), whereas viruses isolated from 3 individuals (L6, L9 and P4)

**Table 2. Sequence analysis of HLA-B57/5801–restricted cytotoxic T lymphocyte (CTL) epitopes in Nef in HIV-1 isolated from individuals with a progressive disease course (progressors).**

| Analysis, patient | Time after SC or entry into study cohort, months | CD4 cell count, cells/$\mu$L | RNA load, copies/mL | Frequency, no. of clones bearing this sequence/ total no. of clones | Consensus sequence, by known HLA-B57/5801–restricted epitope in Nef (amino acid position) and amino acid sequence | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | KF9 (82–90) KGALDLSHF | HW10 (116–124) HTQGYFPDW | YT9 (120–128) YFPDWQNYT | GF14 (130–143) GPGIRYPLTFGWCF |
| Cross-sectional | | | | | | | | |
| P1 | 7 | 1490 | NA | 1/5 | ...F..... | ......... | ......... | ...V..... |
| | | | | 4/5 | ...H..... | N........ | ......... | ...V..... |
| P2 | 85 | NA | NA | 4/4 | ......... | N........ | ......... | .....C..... |
| P3[a] | 36 | 260 | 31,000 | 1/1 | .A.H.F... | N........ | ......... | ...V.F..... |
| P4[a] | 79 | 340 | 130,000 | 2/2 | ...V..... | ......... | ......... | ...V..... |
| P5[a] | 63 | 340 | 200,000 | 1/1 | .A.I.M... | N........ | ......... | ..... |
| P6[a] | 83 | 240 | NA | 1/1 | ...F...F. | N........ | ......... | ..... |
| P7 | 60 | 210 | NA | 2/2 | ......... | N........ | ......... | ..... |
| P8 | 31 | 350 | NA | 1/3 | ......... | ......... | ......... | ...V..... |
| | | | | 2/3 | ......... | N........ | ......... | ...T.F..... |
| Longitudinal | | | | | | | | |
| P9 | 9 | 680 | 46,000 | 5/5 | .A.F..... | N........ | ......... | ...V..... |
| | 78 | 310 | 5800 | 1/3 | ......... | N........ | ......... | ...V.....Y |
| | | | | 2/3 | ......... | N........ | ......... | ..... |
| P10 | 69 | 610 | NA | 2/4 | ......... | N........ | ......... | ...V..... |
| | | | | 2/4 | ...V..... | N........ | ......... | ...V..... |
| | 84 | 450 | 15,000 | 1/3 | .A..... | N........ | ......... | ..... |
| | | | | 2/3 | ......... | N........ | ......... | ...V..... |
| | 123 | 20 | 54,000 | 1/3 | ......... | N........ | ......... | ...V..... |
| | | | | 2/3 | RA.V..... | N........ | ......... | ...V..... |
| P11[a] | 3 | 1060 | 8800 | 2/2 | .A..... | ......... | ......... | ..... |
| | 25 | 870 | 28,000 | 3/9 | ......... | N........ | ......... | ..... |
| | | | | 6/9 | .A..... | N........ | ......... | ..... |
| | 32 | 820 | 33,000 | 1/13 | .A..... | N........ | ......... | ..... |
| | | | | 12/13 | ......... | N........ | ......... | ..... |
| | 69 | 490 | 57,000 | 9/9 | ......... | N........ | ......... | ..... |
| P12 | 0.4 | 380 | <1000[b] | 2/5 | .A..... | N........ | ......... | ..... |
| | | | | 3/5 | ......... | N........ | ......... | ..... |
| | 48 | 210 | 5200 | 2/5 | .A..... | N........ | ......... | ..... |
| | | | | 3/5 | ......... | N........ | ......... | ..... |
| | 74 | NA | NA | 6/6 | ......... | N........ | ......... | ..... |
| P13 | 3 | NA | NA | 6/6 | ......... | N........ | ......... | ..... |
| | 46 | 420 | NA | 1/10 | ......... | ......... | ......... | ..... |
| | | | | 2/10 | ...V..... | N........ | ......... | ..... |
| | | | | 7/10 | ......... | N........ | ......... | ..... |
| | 88 | 20 | 250,000 | 1/6 | .A..... | N........ | ......... | ..... |
| | | | | 1/6 | ..GVVDIS. | N........ | ......... | ..... |
| | | | | 4/6 | ...V..... | N........ | ......... | ..... |

**NOTE.** Consensus sequences of 4 known HLA-B57/5801–restricted epitopes in Nef (KF9, HW9, YT9, and GF14) and aligned predicted amino acid sequences of HIV variants from 13 progressors (denoted by "P" in column 1). Only sequence differences are shown. SC, seroconversion.

[a] Individuals who demonstrated seroconversion for HIV-1 antibodies during active follow-up in the cohort studies.

[b] Lower limit of detection of the assay.

lacked this mutation. Of the 5 progressors and 5 LTNPs from whom HIV-1 variants were isolated longitudinally, the substitution never appeared in 2 individuals (L6 and L9); however, it appeared during the course of infection in 2 individuals (L5 and P11), and it was already present in virus variants obtained at the earliest time points in the remaining 6 participants. The early selection of the H116N substitution indicates that this mutation might have a positive effect in an individual with HLA-B57/5801

**Table 3. Sequence analysis of HLA-B57/5801–restricted cytotoxic T lymphocyte epitopes in Nef in HIV-1 isolates recovered from individuals with a long-term nonprogressive clinical course of disease (LTNPs).**

| Analysis, patient | Time after SC or entry into the study cohort, months | CD4 cell count, cells/μL | RNA load, copies/mL | Frequency, no. of clones bearing this sequence/ total no. of clones | Consensus sequence, by known HLA-B57/5801–restricted epitope in Nef (amino acid position) and amino acid sequence | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | KF9 (82–90) KGALDLSHF | HW10 (116–124) HTQGYFPDW | YT9 (120–128) YFPDWQNYT | GF14 (130–143) GPGIRYPLTFGWCF |
| Cross-sectional | | | | | | | | |
| L1 | 141 | 300 | <1000[a] | 2/2 | .A..... | N..... | ..... | ..... |
| L2 | 21 | 530 | <1000[a] | 1/6 | ..... | N..... | ..... | ...V.H..C..... |
| | | | | 1/6 | ..... | N..... | ..... | ..... |
| | | | | 4/6 | ..... | N..... | ..... | ...V....C..... |
| L3[b] | 115 | 330 | <1000[a] | 4/4 | ..... | N..... | ..... | ...V..... |
| L4 | 132 | 230 | 1600 | 2/2 | SA.V..... | N..... | ..... | ...V..... |
| Longitudinal | | | | | | | | |
| L5 | 89 | 660 | <1000[a] | 1/1 | ..... | ..... | ..... | ..... |
| | 177 | 320 | 5800 | 3/3 | ..... | N..... | ..... | ..... |
| L6 | 17 | 750 | <1000[a] | 2/8 | ....N.... | ..... | ..... | ..... |
| | | | | 6/8 | ..... | ..... | ..... | ..... |
| | 114 | 380 | 5000 | 12/12 | ..... | ..... | ..... | ..... |
| L7 | 26 | 940 | 26,000 | 9/9 | ..... | N..... | ..... | ..... |
| | 78 | 770 | <1000[a] | 1/7 | .A.V..... | ..... | ..... | ...V.....Y |
| | | | | 1/7 | ...V..... | N..... | ..... | ..... |
| | | | | 5/7 | ..... | N..... | ..... | ..... |
| | 102 | 460 | 3200 | 1/8 | .A.V..... | ..... | ..... | ...V.....Y |
| | | | | 1/8 | ....N.... | N..... | ..... | ..... |
| | | | | 2/8 | .A.V..... | ..... | ..... | ...V..... |
| | | | | 4/8 | ..... | N..... | ..... | ..... |
| | 136 | 270 | 54,000 | 1/7 | .A.V..... | ..... | ..... | ...V..... |
| | | | | 1/7 | .A.V..... | N..... | ..... | ..... |
| | | | | 5/7 | ..... | N..... | ..... | ..... |
| L8[b] | 70 | 840 | <1000[a] | 2/2 | .A.V..... | N..... | ..... | .....V..... |
| | 91 | 630 | 9800 | 5/5 | .A..... | N..... | ..... | .....V..... |
| | 137 | 290 | 16,470 | 1/5 | .A..... | N..... | .....H... | ..... |
| | | | | 4/5 | .A..... | N..... | ..... | .....V..... |
| L9[b] | 42 | 610 | <1000[a] | 1/1 | .A..... | ..... | ..... | ..... |
| | 59 | 740 | <1000[a] | 1/1 | .A.F..... | ..... | ..... | ..... |
| | 77 | 770 | <1000[a] | 1/2 | .A..... | ..... | ..... | ..... |
| | | | | 1/2 | .A.MF.... | ..... | ..... | ..... |

**NOTE.** Consensus sequences of 4 known HLA-B57/5801–restricted epitopes in Nef (KF9, HW9, YT9, and GF14) and aligned predicted amino acid sequences of HIV variants from 9 LTNPs (denoted by an "L" in column 1). Only sequence differences are shown. SC, seroconversion.

[a] Lower limit of detection of the assay.

[b] Individuals who demonstrated seroconversion for HIV-1 antibodies during active follow-up in the cohort studies.

expression. Of interest, peptide prediction programs based on major histocompatibility complex binding, proteasomal cleavage, and transport of a transporter associated with antigen processing (epitope prediction and analysis tools are presented on the IEDB Analysis Resource Web site; available at: http://tools.immuneepitope.org) did not reveal a difference in the processing and/or presentation of the H116N mutant epitope, compared with the wild-type sequence.

***HW9-specific CD8+ T cell responses in LTNPs and progressors.*** To study whether both wild-type and virus variant epitopes were recognized, we measured $CD8^+$ T cell IFN-γ responses against these 4 HLA-B57/5801–restricted Nef epitopes by use of the IFN-γ ELISpot assay for 10 individuals (P9-P13 and L5-L9) at a relatively late time point during HIV infection (between 20 and 88 months after seroconversion or at study entry for progressors and between 40 and 170 months after seroconversion or at study entry for LTNPs). With the exception of LTNP L6, none of the individuals showed a $CD8^+$ T cell response against either the wild type or the autologous mutants of the KF9 and GF14 epitopes.

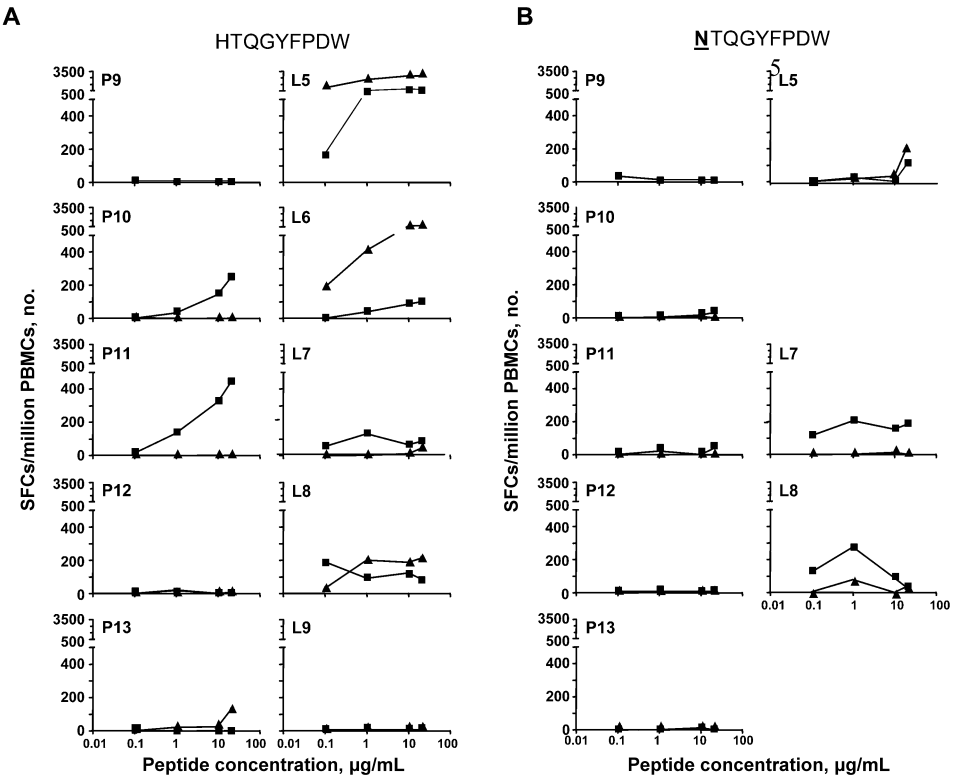


**Figure 1.** Stronger CD8+ T cell interferon (IFN)–γ responses to the HW9 epitope in Nef in individuals with a long-term nonprogressive clinical course of disease ("long-term nonprogressors" [LTNPs]). CD8+ T cell responses after stimulation with the wild-type HW9 epitope *(A)* or the H116N mutant epitope *(B)* of individuals with disease progression ("progressors") *(left panel)* and LTNPs *(right panel)* are expressed as the no. of spot-forming cells (SFCs) per $10^6$ peripheral blood mononuclear cells (*y*-axis) and were measured at 2 different time points during infection. Measurements at the earliest and latest time points during infection are denoted by squares and triangles, respectively. Peptides used for stimulation were tested at increasing concentrations (*x*-axis).

CD8+ T cell IFN-γ responses against the YT9 (YFPDWQNYT) peptide were observed in 3 progressors (P10–P12) and in 1 LTNP (L6) (data not shown). At the same time points, 3 of 5 LTNPs showed CD8+ IFN-γ T cell responses against the wild-type HW9 epitope, even at low peptide concentrations, whereas none of the progressors showed a significant response to the wild-type HW9 epitope (figure 1*A*).

CD8+ T cell reactivity against the H116N mutant epitope was tested only for those patients who actually had developed the mutant HIV-1 variant, with L6 and L9 excluded from the analysis. After stimulation with the H116N mutant epitope, IFN-γ production was observed for CD8+ T cells from only 1 individual (L5), and it was noted only when the highest peptide concentration was used (figure 1*B*).

To study whether the absence of CTL responses against the wild-type HW9 epitope and the H116N mutant could be due to exhaustion at this relatively late time point during infection, we analyzed T cell responses at an earlier time point (between 6 and 35 months after seroconversion or at the time of study entry for progressors and between 11 and 42 months after seroconversion or at the time of study entry for LTNPs). As depicted in figure 1*A*, 4 of 5 LTNPs and 2 of 5 progressors showed a clear response to the HW9 epitope at the earlier time point. T cells from LTNPs in whom HIV-1 variants with the H116N escape mutation were present also showed T cell reactivity against the mutant peptide, whereas no responses were observed after stimulation of progressor CD8+ T cells with the H116N mutant epitope (figure 1*B*). These results show that, although functional HW9-specific CD8+ T cell responses were present early during infection in at least some progressors, they were apparently lost late during infection.

***Up-regulation of PD-1 expression during chronic HIV-1 infection.*** Several studies have recently shown that expression of PD-1 is up-regulated on the surface of exhausted T cells in mice [30], and PD-1 up-regulation has been implicated as a mechanism for failing CD8+ T cell responses in individuals with progressive HIV infection [31, 32]. To investigate the expression of this marker on the total CD8+ T cell population in our group of HLA-B57/5801–positive progressors and LTNPs, we measured, early and late during infection, PD-1 expression on gated CD8+ T cells from 9 individuals who were studied longitudinally. A representative dot-plot analysis of PD-1 staining in an LTNP (L6) and a progressor (P11) is shown in figure 2*A*. P9 was excluded from this analysis because of a paucity of PBMCs at the

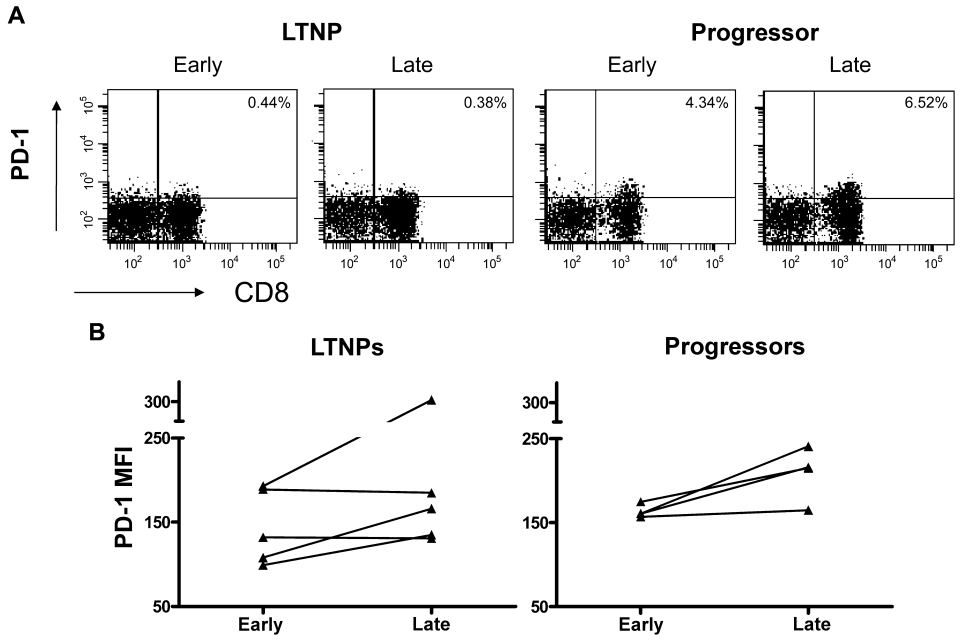


**Figure 2.** Up-regulation of inhibitory receptor "programmed death 1" (PD-1) during disease progression. *A,* Representative flow cytometry plots of PD-1 staining for both an individual with a long-term nonprogressive clinical course of disease (a "long-term nonprogressor" [LTNP]) and an individual with disease progression (a "progressor") at 2 different time points during infection. *B,* Mean fluorescence intensity (MFI) of PD-1 expression on total $CD8^+$ T cells in 5 LTNPs and 4 progressors at 2 different time points. P9 was excluded because too few cells from the earliest time point were available for this analysis. PD-1 expression was up-regulated at the late time point in both progressors and LTNPs (for comparison of early vs. late time points during infection: for the whole study population, $P = .02$; for progressors, $P = .068$; and $P = .225$ for LTNPs, by Wilcoxon signed rank test).

earliest time point. As shown in figure 2*B*, PD-1 expression was up-regulated at a late time point in both progressors and LTNPs (figure 2*B*) (for the comparison of early vs. late time points during infection in the whole study population, $P = .02$, by Wilcoxon signed rank test). Up-regulation of PD-1 during chronic HIV-1 infection was somewhat more pronounced in progressors ($P = .068$, by Wilcoxon signed rank test) than in LTNPs ($P = .225$, by Wilcoxon signed rank test). Furthermore, already early after infection, PD-1 expression tended to be higher in progressors than in LTNPs, albeit not significantly higher.

## DISCUSSION

It has been hypothesized that the less progressive clinical course of HIV-1 infection in individuals expressing HLA-B57/B5801 may be explained by targeting HLA-B57/5801–restricted CTLs to epitopes in highly constrained regions of the virus [23, 24, 33]. Escape mutations in these epitopes would support evasion of HIV-1 from the immune system but would come at such a high 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–restricted TW10 epitope at the time of transmission from an HLA-B57–positive to an HLA-B57–negative individual [33] supports this idea.

Despite the association between HLA-B57/5801 and slow progression of HIV disease, HLA-B57/5801 is also present among HIV-1–infected patients with progressive disease. A previous study showed that all progressors expressing HLA-B57—but only some LTNPs expressing HLA-B57—had the T242N mutation in the TW10 epitope in Gag [25]. Frequencies of CTLs against the wild type and the escape variants were comparable in progressors and LTNPs with both wild-type and mutant viruses. This finding suggests that the CTL escape mediated by the T242N mutation has a more dramatic negative effect on the clinical course of infection than does the viral attenuation associated with it [25]. We found that the T242N substitution in the HLA-B57/5801–restricted TW10 Gag epitope was present both in progressors and in LTNPs, and that there was no difference in $CD8^+$ IFN-$\gamma$ responses against Gag consensus peptides or their autologous escape variants [26].

In the present study, we focused on the Nef gene, because several reports have demonstrated a great influence of Nef on the course of disease. Even small deletions in Nef have been shown to have tremendous beneficial effects on the clinical course of infection, both in macaques [34, 35] and in humans [36–38]. Moreover, CTLs directed against viral gene products that are expressed early in the viral life cycle may be more effective, because these would optimally curtail production of new virions from a newly infected cell [39, 40].

Only in HIV-1 variants from LTNP L9 did we observe a single amino acid deletion in Nef (data not shown), which may help to

explain the low viral load in this patient. Sequence diversity in the 4 HLA-B57/5801–restricted epitopes KF9, GF14, YT9, and HW9 was similar for HIV-1 variants from progressors and LTNPs. The H116N mutation in Nef, which has been described as an HLA-B57 escape variant [41, 42] and which is present in 25.3% of all Nef sequences in the Los Alamos database, was present in HIV-1 variants from all but 2 HLA-B57/5801 LTNPs and 1 progressor. This mutation, on its own, thus did not seem to be associated with the clinical course of infection.

Although HW9-specific $CD8^+$ T cell responses were present early during infection in at least some of the progressors and LTNPs; later during infection, T cells from progressors had lost the ability to produce IFN-$\gamma$ after in vitro stimulation with the HW9 peptide, whereas these responses were preserved in 3 of 5 LTNPs. Because the exact moment of seroconversion is not known for some of the patients, the early time point used for analysis in our study already may, in fact, be occurring relatively late during the course of infection. Thus, the absence of a response against HW9 at the earliest time point in some of the progressors does not exclude the possibility that a response had been present even earlier during infection. The presence of HIV-1–specific $CD8^+$ T cell responses at the earliest time point in 2 progressors suggests that the absence of T cell reactivity later during infection is not due to a complete inability to respond to the HW9 peptide but, rather, to an inability to preserve this immune response.

It has previously been shown that HIV-specific $CD8^+$ T cell responses get exhausted in the face of a high viral burden [31, 32]. Indeed, in our study, LTNPs had better preserved $CD8^+$ T cell responses against wild-type and mutant Nef peptides than did progressors [43, 44]. Moreover, up-regulation of PD-1 during chronic infection tended to be more pronounced in progressors than in LTNPs, a finding indicative of exhaustion of their $CD8^+$ T cells. This finding is in agreement with observations in a larger group of progressors and LTNPs with a wider HLA background [45], although we did not see a significant correlation between viral load and PD-1 expression, as reported elsewhere [32]. This lack of a signficant correlation may have been due to the low number of subjects in our study. It was recently reported that high-avidity T cells are deleted as HIV infection progresses [46]. In line with this finding, we observed that T cells from LTNPs were responsive to lower peptide concentrations than were T cells from progressors, which suggests that the specific $CD8^+$ T cells in LTNPs had a higher avidity for their antigen [47, 48]. The exact underlying mechanism for exhaustion of the immune system is not known, although continuous immune activation driving T cell turnover has been implicated as a factor in this process [49].

In the present study, we used the ability to produce IFN-$\gamma$ after in vitro stimulation as a marker of functional HIV-specific $CD8^+$ T cells. Although it has been shown that IFN-$\gamma$ production is the last function to be lost during chronic viral infection [50], it is thought that the presence of polyfunctional T cells is an immunological correlate of protection against progression [44]. Possible differences in the presence of polyfunctional T cells between LTNPs and progressors may further contribute to the observed difference in clinical course.

In conclusion, we have shown here that the differences between the clinical course of HLA-B57/5801–expressing progressors and LTNPs do not relate to differences in CTL escape mutations in Nef epitopes but, rather, to a difference in preservation of CTL activity against the HW9 epitope in Nef. Although we cannot rule out the possibility that the maintenance of CTL activity against HW9 is a consequence rather than a cause of slow progression of disease, it is tempting to conclude that the ability to maintain CTL activity against such epitopes as the HW9 Nef epitope is contributing to a more benign course of HIV-1 infection.

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## References

1. Migueles SA, Sabbaghian MS, Shupert WL, et al. HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc Natl Acad Sci USA **2000**; 97: 2709–14.
2. Gao X, Bashirova A, Iversen AK, et al. AIDS restriction HLA allotypes target distinct intervals of HIV-1 pathogenesis. Nat Med **2005**; 11:1290–2.
3. Jin X, Bauer DE, Tuttleton SE, et al. Dramatic rise in plasma viremia after $CD8^+$ T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med **1999**; 189:991–8.
4. Klein MR, Van Baalen CA, Holwerda AM, et al. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. J Exp Med **1995**; 181:1365–72.
5. Koup RA, Safrit JT, Cao Y, et al. Temporal associations of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol **1994**; 68:4650–5.
6. Schmitz JE, Kuroda MJ, Santra S, et al. Control of viremia in simian immunodeficiency virus infection by $CD8^+$ lymphocytes. Science 1999; 283:857–60.
7. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MBA. Virus-specific $CD8^+$ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol **1994**; 68:6103–10.
8. Couillin I, Culmann-Penciolelli B, Gomard E, et al. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus type 1 NEF protein. J Exp Med **1994**; 180:1129–34.
9. Goulder PJR, Brander C, Tang Y, et al. Evolution and transmission of stable CTL escape mutants in HIV infection. Nature **2001**; 412:334–8.
10. Goulder PJR, Phillips RE, Colbert RA, et al. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med **1997**; 3:212–7.

11. Jones NA, Wei X, Flower DR, et al. Determinants of human immunodeficiency virus type 1 escape from the primary CD8$^+$ cytotoxic T lymphocyte response. J Exp Med **2004**; 200:1243–56.
12. Kelleher AD, Long C, Holmes EC, et al. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. J Exp Med **2001**; 193:375–86.
13. Koenig S, Conley AJ, Brewah YA, et al. Transfer of HIV-1 specific cytotoxic T lymphocytes to an AIDS patient leads to selection for mutant HIV variants and subsequent disease progression. Nat Med **1995**; 1:330–6.
14. Phillips RE, Rowland-Jones S, Nixon DF, et al. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature **1991**; 354:453–9.
15. Price DA, Goulder PJ, Klenerman P, et al. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc Natl Acad Sci USA **1997**; 94:1890–5.
16. Feeney ME, Tang Y, Pfafferott K, et al. HIV-1 viral escape in infancy followed by emergence of a variant-specific CTL response. J Immunol **2005**; 174:7524–30.
17. Allen TM, O'Connor DH, Jing P, et al. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature **2000**; 407:386–90.
18. Barouch DH, Kunstman J, Kuroda MJ, et al. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 2002; 415:335–9.
19. Chen ZW, Craiu A, Shen L, et al. Simian immunodeficiency virus evades a dominant epitope-specific cytotoxic T lymphocyte response through a mutation resulting in the accelerated dissociation of viral peptide and MHC class I. J Immunol **2000**; 164:6474–9.
20. O'Connor DH, Allen TM, Vogel TU, et al. Acute phase cytotoxic T lymphocyte escape is a hallmark of simian immunodeficiency virus infection. Nat Med **2002**; 8:493–9.
21. Peyerl FW, Barouch DH, Yeh WW, et al. Simian-human immunodeficiency virus escape from cytotoxic T-lymphocyte recognition at a structurally constrained epitope. J Virol **2003**; 77:12572–8.
22. Leslie A, Kavanagh D, Honeyborne I, et al. Transmission and accumulation of CTL escape variants drive negative associations between HIV polymorphisms and HLA. J Exp Med **2005**; 201:891–902.
23. Martinez-Picado J, Prado JG, Fry EE, et al. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J Virol **2006**; 80:3617–23.
24. Peyerl FW, Bazick HS, Newberg MH, Barouch DH, Sodroski J, Letvin NL. Fitness costs limit viral escape from cytotoxic T lymphocytes at a structurally constrained epitope. J Virol **2004**; 78:13901–10.
25. Migueles SA, Laborico AC, Imamichi H, et al. The differential ability of HLA B*5701$^+$ long-term nonprogressors and progressors to restrict human immunodeficiency virus replication is not caused by loss of recognition of autologous viral gag sequences. J Virol **2003**; 77:6889–98.
26. Navis M, Schellens I, van Baarle D, et al. Viral replication capacity as a correlate of HLA B57/B5801-associated nonprogressive HIV-1 infection. J Immunol **2007**; 179:3133–43.
27. Wu J, Bassinger S, Griffith BB, Williams TM. Analysis of HLA class I alleles via direct sequencing of PCR products. Kansas: Lenexa, **2000**:7.1–7.9.
28. Schuitemaker H, Koot M, Kootstra NA, et al. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus populations. J Virol **1992**; 66:1354–60.
29. Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, Van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol **1991**; 28:495–503.
30. Barber DL, Wherry EJ, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature **2006**; 439:682–7.
31. Day CL, Kaufmann DE, Kiepiela P, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature **2006**; 443:350–4.
32. Trautmann L, Janbazian L, Chomont N, et al. Upregulation of PD-1 expression on HIV-specific CD8$^+$ T cells leads to reversible immune dysfunction. Nat Med **2006**; 12:1198–202.
33. Leslie AJ, Pfafferott KJ, Chetty P, et al. HIV evolution: CTL escape mutation and reversion after transmission. Nat Med **2004**; 10:282–9.
34. Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, Desrosiers RC. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science **1992**; 258:1938–41.
35. Kestler HW 3rd, Ringler DJ, Mori K, et al. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **1991**; 65:651–62.
36. Deacon NJ, Tsykin A, Solomon A, et al. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **1995**; 270:988–91.
37. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med **1995**; 332:228–32.
38. Learmont JC, Geczy AF, Mills J, et al. Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1: a report from the Sydney Blood Bank Cohort. N Engl J Med **1999**; 340:1756–7.
39. Gruters RA, Van Baalen CA, Osterhaus AD. The advantage of early recognition of HIV-infected cells by cytotoxic T-lymphocytes. Vaccine **2002**; 20:2011–5.
40. Van Baalen CA, Pontesilli O, Huisman RC, et al. Human immunodeficiency virus type 1 Rev- and Tat-specific cytotoxic T lymphocyte frequencies inversely correlate with rapid progression to AIDS. J Gen Virol **1997**; 78( Pt 8):1913–8.
41. Feeney ME, Tang Y, Pfafferott K, et al. HIV-1 viral escape in infancy followed by emergence of a variant-specific CTL response. J Immunol **2005**; 174:7524–30.
42. Pillay T, Zhang HT, Drijfhout JW, et al. Unique acquisition of cytotoxic T-lymphocyte escape mutants in infant human immunodeficiency virus type 1 infection. J Virol **2005**; 79:12100–5.
43. Kostense S, Vandenberghe K, Joling J, et al. Persistent numbers of tetramer$^+$ CD8$^+$ T cells, but loss of interferon-$\gamma^+$ HIV-specific T cells during progression to AIDS. Blood **2002**; 99:2505–11.
44. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8$^+$ T cells. Blood **2006**; 107:4781–9.
45. Zhang JY, Zhang Z, Wang X, et al. PD-1 up-regulation is correlated with HIV-specific memory CD8$^+$ T-cell exhaustion in typical progressors but not in long-term nonprogressors. Blood **2007**; 109:4671–8.
46. Lichterfeld M, Yu XG, Mui SK, et al. Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8$^+$ T cells after early HIV-1 infection. J Virol **2007**; 81:4199–214.
47. Derby M, Alexander-Miller M, Tse R, Berzofsky J. High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL. J Immunol **2001**; 166:1690–7.
48. Sedlik C, Dadaglio G, Saron MF, et al. In vivo induction of a high-avidity, high-frequency cytotoxic T-lymphocyte response is associated with antiviral protective immunity. J Virol **2000**; 74:5769–75.
49. Jansen CA, van Baarle D, Miedema F. HIV-specific CD4$^+$ T cells and viremia: who's in control? Trends Immunol **2006**; 27:119–24.
50. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol **2003**; 77:4911–27.