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Disruption of Env Tyrosine-Dependent Sorting Signal Does Not Affect Susceptibility of HIV-1 to Cytotoxic T Lymphocytes

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Infections with live-attenuated viruses within SIV models of HIV-1 pathogenesis have offered the best evidence that a vaccine could generate protective immunity. Desrosiers and colleagues [1] first demonstrated this principle using *nef*-defective SIV, which established a chronic low-grade asymptomatic infection in macaques associated with protection against subsequent challenge with wild-type SIV. At least in part, this phenomenon appeared to be due to attenuation of viral replication.

More recently, Shacklett *et al* [2] and Hoxie and *et al* [3] have examined attenuated SIV infection via mutations in the transmembrane domain of gp41, which also subsequently protects from challenge by wild-type SIV. Shacklett *et al* found that SIV containing multiple (stop and point) mutations disrupting this domain reduced viral replicative capacity *in vitro*, likely due to effects on gp41 membrane trafficking in infected cells [4,5]. Hoxie and colleagues more specifically created mutations in the tyrosine-based sorting motif (Y712xxϕ) in the membrane-proximal cytoplasmic domain of gp41 [3]. Despite the role this motif in gp41 trafficking [4,5], the mutations appeared to have minimal impact on viral fitness, reflected by normal peak viremia during acute infection *in vivo* and growth kinetics *in vitro*. Thus the mechanism of attenuation and subsequent immune protection is unclear, but probably not due to markedly reduced viral replication capacity. Interestingly, *in vivo* CD8 depletion experiments have suggested that $CD8^+$ T lymphocytes (CTLs) may contribute to the protective immunity [6].

To assess whether analogous mutations in HIV-1 might affect viral susceptibility to CTLs, mutations in the Y712xxϕ motif were constructed in HIV-1 NL4-3 [7] by point mutagenesis (QuikChange, Stratagene). These included EnvY712I, EnvY712S and EnvΔGY mutations in the cytoplasmic domain of gp41 (Figure 1A), engineered into the whole genome context of NL4-3.1 containing the clade B consensus sequence at Gag 77-85 (HXB2 a.a. numbering) [8]. These viruses were examined for their ability to replicate in T1 cells [9] (Figure 1B). The EnvY712I and EnvY712S mutants had growth kinetics similar to wild-type NL4-3 (EnvY712),

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similar to SIVmac239 [3]. In contrast, the EnvΔGY mutant was replication incompetent, suggesting that this mutation more severely impaired HIV-1 than SIV.

To assess the susceptibility of these mutants to CTLs, these viruses were tested in virus suppression assays [10,11] using HIV-1-specific CTL clones: S1-SL9-3.23T recognizing the HLA A*02-restricted epitope SLYNTVATL in Gag p17 (a.a. 77-85) [12], S31-IV9-10.11T recognizing the HLA A*02-restricted epitope ILKEPVHGV in RT (a.a. 309-317), and S58-PL10-10.8 recognizing the HLA A*02-restricted epitope PLTFGWCYKL in Nef (a.a. 136-145). The HIV-1-permissive target cells were A*02-expressing T1 cells [10]. Comparisons of these viruses showed similar degrees of suppression of the mutant and wild-type viruses by the three CTL clones (Figure 1C and D). Similar results were noted in independent experiments using two other CTL clones recognizing other epitopes (not shown). Overall, these results suggested that directly increased susceptibility to CTL inhibition is not the mechanism of *in vivo* attenuation of infection with viruses containing these mutations.

Because attenuated SIV infection has been the most robust example of protective vaccination in the SIV-macaque model [1,6,13–15], understanding how viral attenuation affects antiviral immunity is clearly an important goal. The observation that SIV mutated in the Y712xxϕ motif yields infections with typical high peak viremia followed by chronic low viremia in macaques subsequently protected from wild-type virus challenge [2,3,6] suggests that viral replication is not markedly affected by the mutations, but that replication is suppressed during chronic infection after development of CTL responses, which are the major determinant of set-point viremia [16–18]. Furthermore, preliminary data suggests that the low set-point viremia of macaques infected with Y712xxϕ-disrupted SIV may be related to the CTL response [6]. Thus, a simple explanation could be that disruption of this motif somehow renders SIV directly more susceptible to CTL.

However, our results suggest that this is not the case for HIV-1; disruption of the Y712xxϕ motif did not appear to increase susceptibility of HIV-1 to CTLs directly. While our *in vitro* assay may not necessarily predict the interaction of virus and CTLs *in vivo*, it seems likely that the mechanism of attenuation is either not mediated by CTLs, or indirectly increases the antiviral activity of CTLs. Interestingly, it has been suggested that the HIV-1 Env may facilitate viral escape from CTLs in lymph nodes [19,20]. Although our data do not address this issue directly, they are compatible with a mechanism whereby reduced levels of Env could reduce viral escape from CTLs. Further work would be required to explore this possibility.

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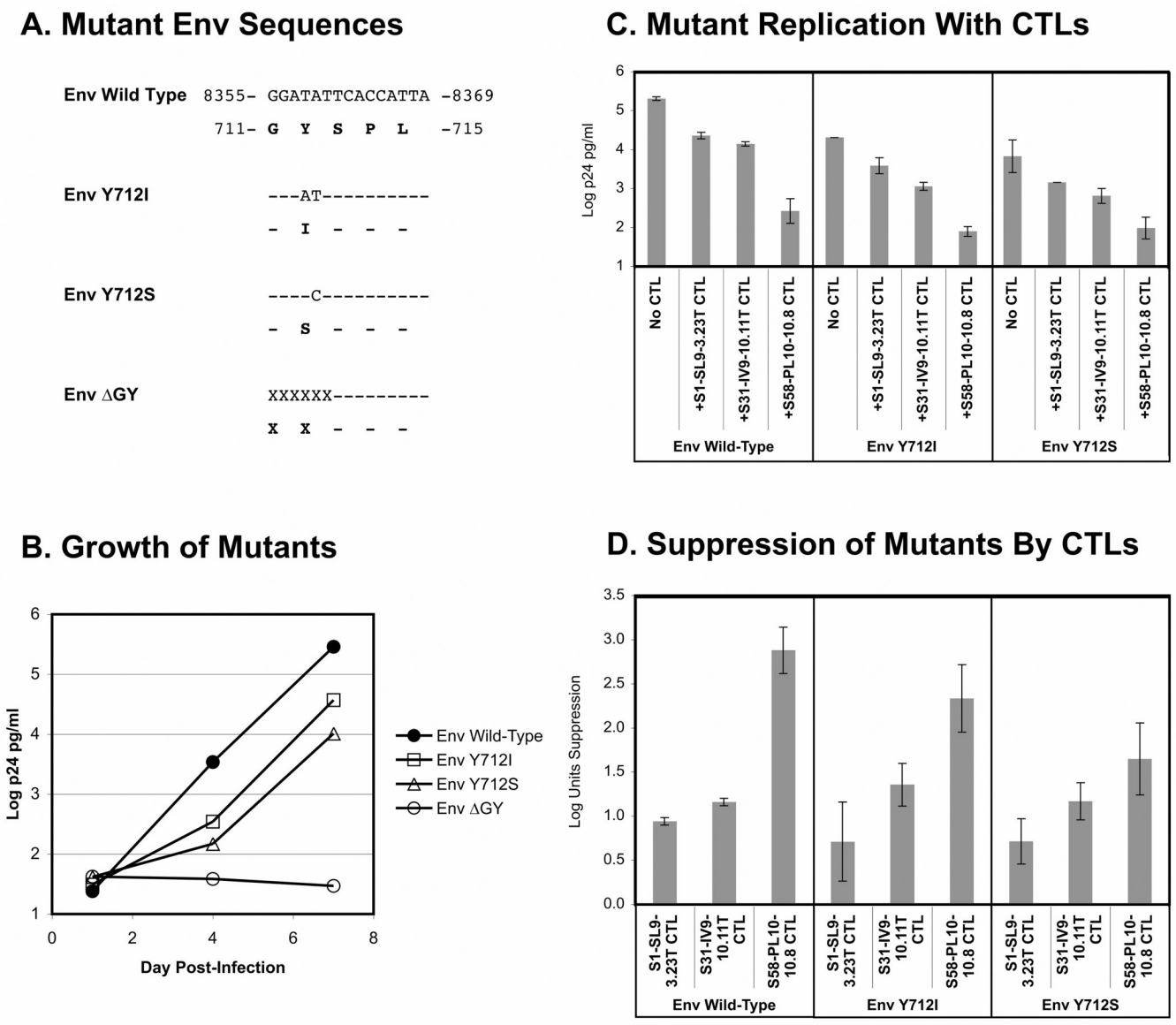

Figure 1. Susceptibility of Env Y712xxϕ motif mutant viruses to HIV-1-specific CTL

A. Nucleotide (NL4-3 residues 8355-8369) and amino acid (NL4-3 Env residues 711-715) sequences are given for the wild type NL4-3.1 virus and generated mutants. "-" indicates the same nucleotide or amino acid as wild type, and "X" indicates a deletion of a nucleotide or amino acid compared to wild type. B. $1x10^6$ HIV-1 permissive T1 cells were infected with the indicated mutant or wild type virus at an input of 1000pg p24 antigen and cultured in a 24-well tissue culture plate. Viral replication was measured by quantitative p24 ELISA (Perkin Elmer). These results are representative of 4 independent experiments. C. T1 cells were infected with 500 pg p24/10^6 cells and cultured in triplicate in 96-well tissue culture plates with the indicated CTL clones ($1.25x10^4$ CTLs with $5x10^4$ target cells). Viral replication was measured by quantitative p24 ELISA (Perkin Elmer) on day 4 after infection. D. Inhibition was calculated by comparing replication in cells with or without CTLs. The plotted data indicate means of triplicates (error bars indicate 1 SD) for 1 experiment, and the results are representative of 2 independent experiments with these clones. Similar results were seen using a B*57-restricted

CTL clone recognizing the epitope TSTLQEQIGW in Gag p24, and an A*02-restricted CTL clone recognizing the epitope AIIRILQQL in Vpr in other experiments (data not shown).