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Title page

- Generation of a replication-competent simian–human immunodeficiency virus, the neutralisation sensitivity of which can be enhanced in the presence of a small molecule CD4 mimic
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Summary

Simian-human immunodeficiency virus (SHIV) carrying the envelope from the clade B clinical HIV-1 isolate HIV-1 MNA, designated SHIV MNA, was generated through intracellular homologous recombination. SHIV MNA inherited biological properties from the parental HIV-1, including CCR5 co-receptor preference, resistance to neutralisation by the anti-V3 loop monoclonal antibody KD-247, and loss of resistance in the presence of the CD4-mimic small molecule YYA-021. SHIV MNA showed productive replication in rhesus macaque peripheral blood mononuclear cells.

Experimental infection of a rhesus macaque with SHIV MNA caused a transient but high titre of plasma viral RNA and a moderate antibody response. Immunoglobulin in the plasma at 24 weeks post-infection was capable of neutralising SHIV MNA in the presence but not in the absence of YYA-021.

SHIV MNA could serve a model for development of novel therapeutic interventions based on CD4-mimic-mediated conversion of Env susceptible to antibody neutralisation.

Text

Control of primate lentiviral infection by antibodies directed against viral envelope protein is theoretically feasible. This was confirmed by the successful protection of macaque monkeys from challenge inoculation with simian-human immunodeficiency virus (SHIV) carrying an envelope protein (Env). Env was derived from a laboratory strain of human immunodeficiency virus type 1 (HIV-1) through the passive immunisation of neutralising monoclonal antibodies directed against HIV-1 (Mascola *et al.*, 2000; Nishimura *et al.*, 2003). This neutralisation is consistent with the results normally seen in cell culture systems.

Clinical isolates of HIV-1, which have not been subjected to extensive passage in T-cell lines, on the other hand, are generally resistant to antibody-mediated neutralisation (Moore *et al.*, 1995). It has been shown that virus in infected individuals is under selective pressure to develop a variety of means to evade attack by neutralising antibodies, including sequence variation, glycosylation, tertiary structural shielding formed by the Env trimer, and the rapid kinetics of conformational changes of Env, which affect fusion between the viral envelope and the plasma membrane of target cells

(Kong & Sattentau, 2012). Although four major neutralising epitopes have been identified in the HIV-1 Env; *i.e.* the V1/V2 loop, the glycan-V3 site and CD4-binding site of gp120, and the membrane-proximal external region (MPER) of gp41, few reports of antibodies directed against these epitopes capable of neutralising a broad range of isolates have been published, for reasons that are as yet unclear (Kwong & Mascola, 2012). High titres of antibodies directed against the V3 loop are elicited in individuals during the early phase of HIV-1 infection, but these are incapable of neutralising the virus because the epitope in functional Env trimer is likely shielded from the antibody (Davis *et al.*, 2009b). Therefore, it is necessary to develop a means of rendering these epitopes accessible to the antibodies, to make antibody-mediated suppression of HIV-1 a valid therapeutic option.

It has been reported that neutralisation mediated by antibodies directed against the V3 loop (Lusso *et al.*, 2005) or CD4-induced epitope (CD4i) (Thali *et al.*, 1993) can be enhanced in the presence of soluble CD4 (sCD4). It is known that the interaction of Env with sCD4 drives a conformational change of the viral protein and makes the cryptic/occult epitopes accessible to these antibodies (Wyatt *et al.*, 1998). Small

molecules that emulate sCD4 for its interaction and subsequent induction of conformational change of Env may be employed to intensify antibody-mediated interventions against HIV-1 infection. Compounds with the above-mentioned properties; *i.e.* NBD-556 and NBD-557, have been reported previously (Zhao *et al.*, 2005). NBD-556 has been shown in cell culture to interact with the CD4-binding pocket to induce a conformational change in gp120 (Madani *et al.*, 2008) and enhance exposure of the Env of primary HIV-1 isolates to neutralising epitopes (Yoshimura *et al.*, 2010).

The present study was performed to evaluate small molecule CD4-mimic-based enhancement of antibody-mediated virus neutralisation, in the context of virus infection *in vivo*. The simian-human immunodeficiency virus (SHIV)/macaque monkey model of AIDS is particularly suitable for such studies, as SHIV carries the HIV-1 Env and the neutralisation sensitivity of SHIV is comparable to that of the parental HIV-1 (Shibata & Adachi, 1992).

As NBD-556, unlike sCD4, inhibits infection with select HIV-1 isolates (Yoshimura *et al.*, 2010), we generated a new SHIV strain carrying Env, the sensitivity of which to antibody-mediated neutralisation is enhanced in the presence of a CD4 mimic. An

95 HIV-1 isolate, MNA, previously designated primary isolate HIV-1 Pt.3, was used as the
96 source of Env, as the viral protein has been reported to interact with NBD-556
97 (Yoshimura *et al.*, 2010). While the virus belongs to a distinct subset of HIV-1 isolates,
98 as mentioned above, it has also been reported to utilise the CCR5 molecule to gain entry
99 into target cells, a property that is shared by the majority of HIV-1 strains (Yoshimura
100 *et al.*, 2010). A monoclonal antibody directed against the tip of the V3 loop (GPGR),
101 KD-247 (Eda *et al.*, 2006), was employed to assess this concept, as HIV-1 MNA was
102 resistant to KD-247-mediated neutralisation, despite carrying the exact epitope
103 sequence in the tip of V3 loop, and was converted to being sensitive to the antibody by
104 NBD-556 in a dose-dependent manner (Yoshimura *et al.*, 2010).

105 First, we reproduced the results of Yoshimura *et al.* using a neutralisation assay
106 employing TZM-bl cells (Platt *et al.*, 1998), obtained from the NIH AIDS Reagent
107 program (Fig. S1). The virus was resistant to KD-247, as described previously, and
108 required almost 50 µg/mL of the antibody to achieve 50% neutralisation in our assay.
109 The observed resistance was abrogated in the presence of 2 µM of NBD-556. However,
110 50% neutralisation was achieved in the presence of ~0.1 µg/mL of KD-247,

corresponding to 1/500 of the amount of the antibody to achieve the same degree of neutralisation in the absence of the CD4 mimic.

With reproduction of the properties of HIV-1 MNA Env, we generated an SHIV strain carrying Env through intracellular homologous recombination, as described previously (Fujita *et al.*, 2013) with minor modifications (Fig. S2). DNA fragments representing the 5' and 3' ends of the SHIV genome (fragments I and II, respectively) were amplified by PCR from the proviral DNA plasmid SHIV KS661. A DNA fragment containing *env* (fragment III) was amplified from complementary DNA (cDNA) of the HIV-1 MNA genome, which was prepared from virus particles (virion-associated RNA) in the culture supernatant of PM1/CCR5 cells (Yusa *et al.*, 2005) infected with the virus. The PCR primers used are listed in Table S1. Using a FuGENE HD transfection reagent, lipofection was performed on the C8166-CCR5 cells (Shimizu *et al.*, 2006) to co-transfect them with 0.2 µg of DNA. A cytopathic effect presumably caused by the emerged recombinant virus was observed on day 13 post-transfection. The emerged virus, designated SHIV MNA, carried the entire gp120 and three quarters of gp41 from HIV-1 MNA Env (Fig. 1a). The rest of Env was from

127 SHIV KS661, the Env of which was derived from HIV-1 89.6 (Shinohara *et al.*, 1999).
128 The CD4 binding site, and the regions and elements that reportedly interact with
129 NBD-556 (Madani *et al.*, 2008; Yoshimura *et al.*, 2010), are preserved in SHIV MNA
130 Env (Fig. S3). The virus was replication-competent in PM1/CCR5 cells (data not
131 shown).

132 As HIV-1 MNA was suggested to be a CCR5-utilising virus, we were intrigued
133 whether SHIV MNA inherited the trait from the parental virus. We subjected SHIV
134 MNA and the parental HIV-1 MNA to co-receptor usage assay as described previously
135 (Nishimura *et al.*, 2010), with minor modifications (Fig. S4). As expected, SHIV MNA
136 was shown to utilise CCR5 as an entry co-receptor.

137 We next assessed the neutralisation profiles of SHIV MNA in comparison with the
138 parental HIV-1 MNA, as described previously (Li *et al.*, 2005; Wei *et al.*, 2002). Both
139 SHIV MNA and HIV-1 MNA showed essentially no neutralisation by KD-247 up to 25
140 µg/mL and 50% neutralisation was achieved at 50 µg/mL (Fig. 1b). As the CD4 mimic,
141 we employed YYA-021, a compound generated and characterised through studies
142 concerning the structure-activity relationships of small molecules (Narumi *et al.*, 2013;

143 Narumi *et al.*, 2011; Narumi *et al.*, 2010; Yamada *et al.*, 2010). The compound was
144 shown to be slightly less potent but to exhibit substantially lower toxicity than
145 NBD-556, and was therefore a suitable choice for our purposes in future studies in
146 animal models. SHIV MNA was resistant to neutralisation by YYA-021 at all
147 concentrations examined, except 25 and 50 μ M, and showed a neutralisation profile
148 almost identical to that of HIV-1 MNA (Fig. 1c). To further characterise the biological
149 properties of SHIV MNA Env, a set of entry assays was conducted (Fig. S5). The *env*
150 genes cloned from SHIV MNA and HIV-1 MNA, were utilised to generate
151 pseudo-typed viruses. These pseudotypes were inoculated into TZM-bl cells in the
152 presence of increasing amounts of NBD-556, YYA-021 or soluble CD4. A control
153 group was derived from another virus preparation pseudotyped with A-MLV Env
154 (Landau *et al.*, 1991). When the efficiency of entry was defined by intracellular
155 luciferase activities, virtually no difference was observed between Envs of SHIV MNA
156 and the parental HIV-1. Thus SHIV MNA Env replicated in C8166-CCR5 cells retained
157 sensitivity to small molecule CD4 mimics and soluble CD4 comparable to that of
158 HIV-1 MNA.

We next examined whether the synergistic neutralisation of HIV-1 MNA by KD-247 antibody in the presence of NBD-556 (Yoshimura *et al.*, 2010) would be reproduced when CD4 mimic was substituted by YYA-021. The synergistic neutralisation effect of KD-247 and YYA-021 was reproduced in our experiments (Fig. 1d). At 50 $\mu\text{g/mL}$, KD-247 barely achieved 50% neutralisation of HIV-1 MNA but resulted in 50% neutralisation at $< 0.05 \mu\text{g/mL}$ in the presence of 20 μM of YYA-021.

Finally, to examine whether these two agents neutralise SHIV MNA in the same manner as the parental HIV-1, we conducted a neutralisation assay with KD-247 in the presence of increasing amounts of YYA-021 (0, 5, 10, 20 and 40 μM) (Fig. 1e). The neutralisation curve of KD-247 against SHIV MNA showed an upward shift as the concentration of YYA-021 increased (Fig. 1e), similar to the observations with HIV-1 (Fig. 1d), indicating augmentation of neutralisation, and complete neutralisation of both viruses was achieved at 20 μM YYA-021 (Fig. 1d and e). Based on these results, we concluded that the neutralisation profile of SHIV MNA was comparable to that of HIV-1 MNA.

Reproduction of the neutralisation characteristics of HIV-1 MNA in the newly

generated SHIV prompted us to assess the ability of SHIV MNA to replicate in monkey cells. SHIV MNA, along with SIV239 and SHIV KS661, were normalised with infectious titres and inoculated into rhesus macaque peripheral blood mononuclear cell (PBMC) preparations from four animals, as described previously (Fujita *et al.*, 2013) (Fig. 2a). SHIV KS661, a CXCR4-utilising virus, replicated to the highest titres of all the viruses in all PBMC preparations. Compared to SHIV KS661, SIV239 replicated to lower titres. Under these experimental conditions, SHIV MNA showed productive replication in the cells with similar replication kinetics and peak titres to SIV239. Based on these results, we concluded that SHIV MNA was replication-competent in primary monkey lymphocytes.

Productive replication of SHIV MNA in monkey PBMC justified experimental infection of the virus *in vivo*. We inoculated 1.75×10^5 TCID₅₀ of SHIV MNA intravenously into a rhesus macaque and monitored plasma viral RNA burden and circulating CD4⁺ T-lymphocyte levels. Plasma viral RNA burden reached a peak of 5.6×10^6 copies/mL at 1 week post-infection (wpi), and declined rapidly thereafter reaching low levels of detection at 7 wpi (around 2.8×10^2 copies/mL). Circulating CD4⁺

191 T-cell numbers showed a transient decrease around 1 wpi, rebounded around 3 wpi and
192 stabilised around 70% of the pre-infection level from 4 wpi. During the period of
193 observation, the animal developed no obvious clinical manifestations related to
194 lentivirus infection.

195 As SHIV MNA replicated *in vivo* without depleting helper T-cells, it was expected
196 that the animal mounted an anti-viral immune reaction. The production of antibody
197 directed against Env was assessed by western immunoblotting, as described previously
198 (Igarashi *et al.*, 1999). Purified Env protein (Advanced Biotechnologies Inc. Md.
199 U.S.A.) was used as the antigen (Fig. 3a). Anti-Env antibody was detected at 3 wpi and
200 the level of antibody—judged by the intensity of the band—increased gradually with
201 time.

202 We next examined whether the animal generated neutralising antibodies against
203 SHIV MNA. Because plasma samples from this specimen exhibited high background
204 activity, immunoglobulin G (IgG) was purified from these samples collected on day 0
205 and in week 24 post-infection using protein G spin columns (GE healthcare Japan.
206 Tokyo. Japan). While the IgG from day 0 exhibited no neutralising activity (Fig. 3b), as

207 expected, the immunoglobulin collected at 24 wpi neutralised SHIV MNA, although a
208 concentration > 100 µg/mL was required to suppress replication of 100 TCID₅₀ of the
209 input virus (Fig. 3c).

210 We examined whether the observed marginal neutralisation by the antibody could be
211 enhanced by the presence of YYA-021. Upon addition of YYA-021 in the assay system,
212 SHIV MNA became sensitive to IgG obtained at 24 wpi (Fig. 3c), while no
213 enhancement was identified from day 0 (Fig. 3b).

214 In this study, we generated a replication-competent SHIV MNA strain carrying an
215 Env resistant to the monoclonal neutralising antibody KD-247 but conditionally
216 sensitive in the presence of the CD4 mimic YYA-021. As the observed neutralisation
217 characteristics were identical to those of HIV-1 MNA, which contributed the majority
218 of the Env sequence to the chimera, the utility of the CD4 mimic as a means of
219 enhancing antibody-mediated virus neutralisation should be assessed in the context of
220 infection *in vivo*. This concept could be tested during the acute phase of SHIV MNA
221 infection, during which the virus undergoes substantial replication. To examine the
222 feasibility of CD4-mimic-mediated enhancement of virus neutralisation in the context

223 of chronic infection, the conditions under which this type of intervention should be
224 applied to HIV-1-infected patients in a clinical setting, the virus must be modified to
225 sustain productive replication for a longer period. SHIV MNA in the present form does
226 not fulfil this requirement. It is possible that animal-to-animal passage could increase
227 the fitness of the virus in monkeys.

228 This study demonstrated that a CD4 mimic could modulate viral Env protein to be
229 more susceptible to neutralisation by less potent antibodies generated in the context of
230 infection. During the early phase of infection, patients mount high titres of
231 non-neutralising antibodies directed against the V3 loop (Davis *et al.*, 2009a). Patients
232 with HIV-1 clade C generate anti-Env antibodies, including anti-CD4i antibodies, with
233 poor neutralising activity against recent infection (Gray *et al.*, 2007). It is possible that
234 the CD4 mimic YYA-021 causes a conformational change in SHIV MNA Env, which
235 renders sequestered epitope(s) accessible to potentially neutralising IgG, such as the V3
236 loop and CD4i.

237 The current study extended the previous study by Yoshimura *et al.* and used HIV-1
238 MNA belonging to clade B to generate a new SHIV strain carrying Env. The

neutralisation sensitivity of this strain is characteristically augmented in the presence of a small molecule CD4 mimic. Similar observations by Decker *et al.* show that infections of a wide range of HIV-1 strains of multiple clades or circulating recombinant forms elicits high titres of anti-CD4i antibodies, These anti-CD4i antibodies neutralise viruses as divergent as HIV-2 in the presence of soluble CD4 (Decker *et al.*, 2005). Taking these observations into account, small molecule CD4 mimics such as YYA-021 could potentially enhance the neutralisation activity of the antibodies directed against autologous viruses belonging not only to clade B but also to multiple HIV-1 strains of various clades, and possibly even HIV-2. Our results pave the way for a novel therapeutic intervention based on administration of CD4 mimics to patients with HIV to facilitate control of the virus by their own antibodies.

250

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Figure legends

Figure 1. Genomic organisation (a) and neutralisation sensitivity (b – e) of SHIV MNA.

(a) Grey boxes represent genes derived from SIV239, open boxes those from HIV-1 89.6 and filled dark grey boxes those from HIV-1 MNA. (b – e) Percentage neutralisation was calculated as follows: % neutralisation = $100 \times \{1 - (\text{RLU.N} - \text{RLU.B})/(\text{RLU.V} - \text{RLU.B})\}$. RLU, relative luciferase units; RLU.N, RLU in wells with cells, virus and KD-247 and/or YYA-021; RLU.V, RLU in wells with cells and virus; RLU.B, RLU in wells with cells.

Figure 2. Replication of SHIV MNA in rhesus macaque PBMCs (a) and *in vivo* (b). (a) Multiplicity of infection was adjusted to 0.01 (TCID₅₀/cell). (b) Experimental infection of a rhesus macaque with SHIV MNA. SHIV MNA (1.75×10^5 TCID₅₀) was intravenously inoculated into a rhesus macaque, and the plasma viral RNA burden (filled circles) and circulating CD4⁺ T-lymphocytes (open triangles) were monitored.

Figure 3. Antibody induced against SHIV MNA.

423 (a) The anti-HIV-1 gp120 antibody response was assessed by immunoblotting with
424 plasma samples collected at the indicated times. An anti-HIV-1 V3 monoclonal
425 antibody 4G10 ascites (1:100) (von Brunn *et al.*, 1993), obtained from the NIH AIDS
426 Reagent program, was used as a positive control (lane anti-V3). (b and c) Neutralisation
427 of SHIV MNA with IgG purified from plasma of the infected rhesus macaque (day 0
428 and 24 wpi) with/without YYA-021 (20 μ M).

1 Table S1. PCR primers.

Primer	Sequence	Position (nt [†])
Fragment I		
SIVU3Not-F	5'-atgcggccgctggaagggatttattacagtgaag-3'	1 – 25 [*]
Preenv-R	5'-aaagagcagaagacgagtggcaa-3'	6204 – 6226 [#]
Fragment II		
SHenv5.5F	5'-tcataatgatagtaggaggc-3'	8278 – 8297 [#]
SIVU5Eco-R	5'-tgcagaattctgctagggattttcctgcttcggtt-3'	10255 – 10279 [*]
Fragment III		
HIV-1vpr-F	5'-agatggaacaagccccagaaga-3'	5557 – 5578 [#]
SHenv6R	5'-gctgaagaggcacaggctccgc-3'	8525 – 8504 [#]

2 [†], Nucleotide positions of PCR primers were numbered relative to the SIV239 (*,

3 GenBank Accession No. M33262) or HXB2 (#, GenBank Accession No. K03455)

4 genome sequences.

Figure S1. Enhanced neutralisation of HIV-1 MNA by KD-247 in the presence of NBD-556.

100 TCID₅₀ of HIV-1 MNA was pre-incubated with increasing amounts of KD-247 with/without 2 µM of NBD-556 at 37°C for 60 min, followed by inoculation into 5×10³ TZM-bl cells. The cells were lysed at 48 h post-infection and luciferase activity was measured. The percentage of neutralisation was measured as RLU reduction relative to virus control wells.

Figure S2. Genomic organisation of SHIV KS661 and HIV-1 MNA and PCR fragments employed for preparation of DNA fragments for generation of SHIV MNA.

Colour-coded boxes represent genes derived from the following viruses: grey boxes, SIV239; open boxes, HIV-1 89.6; grey boxes, HIV-1 MNA. SHIV KS661 carries *tat*, *rev*, *vpu*, and *env* genes from subtype B HIV-1 89.6. Broad lines represent PCR fragments that were amplified using the primers indicated by arrows (A – F).

20 Figure S3. Deduced amino acid sequence alignment of Env from HIV-1 MNA, SHIV
21 MNA, and SHIV KS661.
22 (*) = Amino acids that form part of the CD4 binding site.
23 (†) = Regions/elements that are reported to interact with NBD-556 (Madani *et al.*, 2008;
24 Yoshimura *et al.*, 2010).
25 Parts of SHIV MNA Env, that were putatively derived from HIV-1 MNA or SHIV
26 KS661 are respectively color-coded as grey or black.
27
28 Figure S4. Co-receptor preference of SHIV MNA.
29 SHIV MNA, along with controls for CCR5-tropic (SIV239) and CXCR4-tropic (HIV-1
30 NL4-3) and the parental HIV-1 MNA, were inoculated into TZM-bl cells in the
31 presence of increasing amounts of AD101 (Trkola *et al.*, 2002), provided by Dr. J.
32 Strizki, Schering Plough Research Institute, Kenilworth, NJ, and/or AMD3100
33 (Sigma-Aldrich, St. Louis, MO) (Donzella *et al.*, 1998).
34

35 Figure S5. Sensitivity of Env proteins from HIV-1 MNA and SHIV MNA to soluble
36 CD4 and small-molecule CD4 mimics.
37 Pseudotyped viruses carrying Env from SHIV MNA or HIV-1 MNA were normalised
38 by infectious titre at 100 TCID₅₀ and inoculated to TZM-bl cells in the presence of
39 increasing amounts of NBD-556, YYA-021 or soluble CD4. A pseudotyped virus
40 bearing A-MLV Env is acting as the negative control.

Figure S1

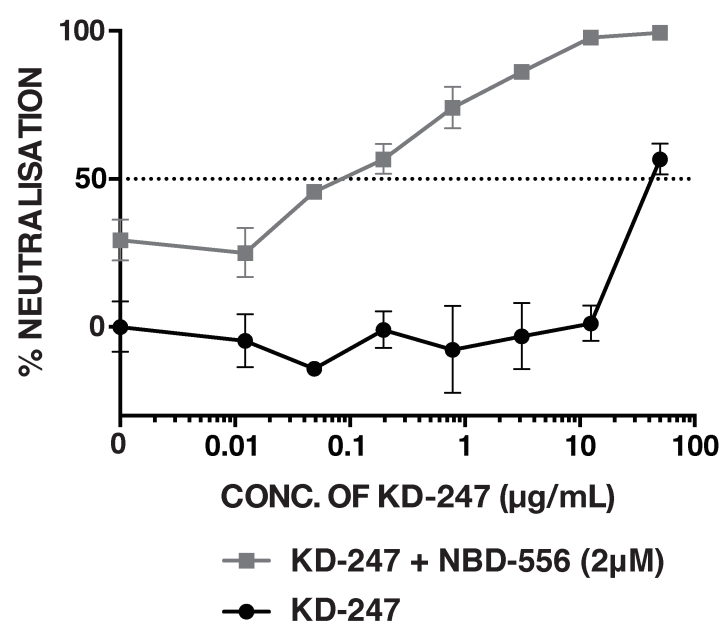


Figure S2

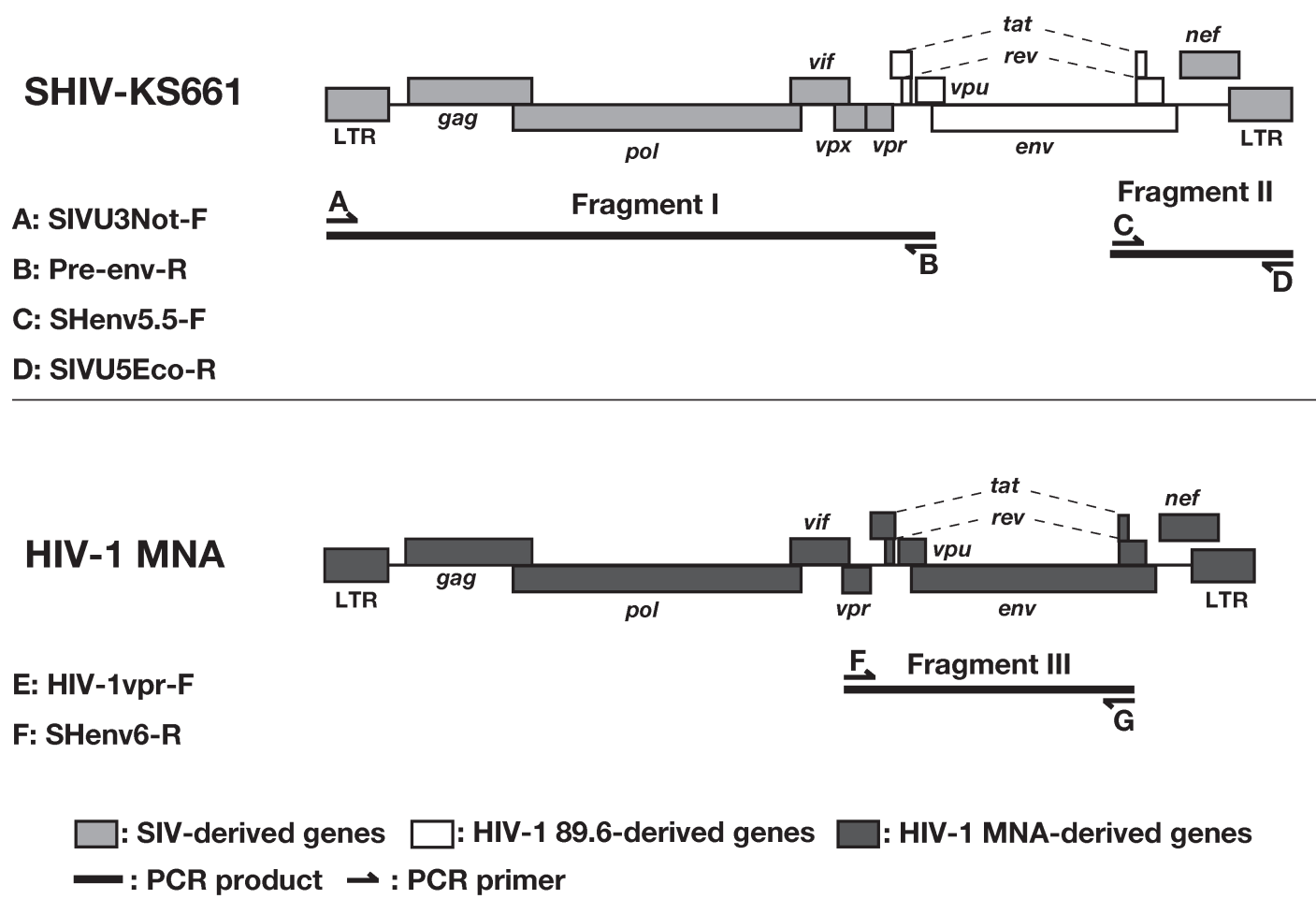


Figure S3

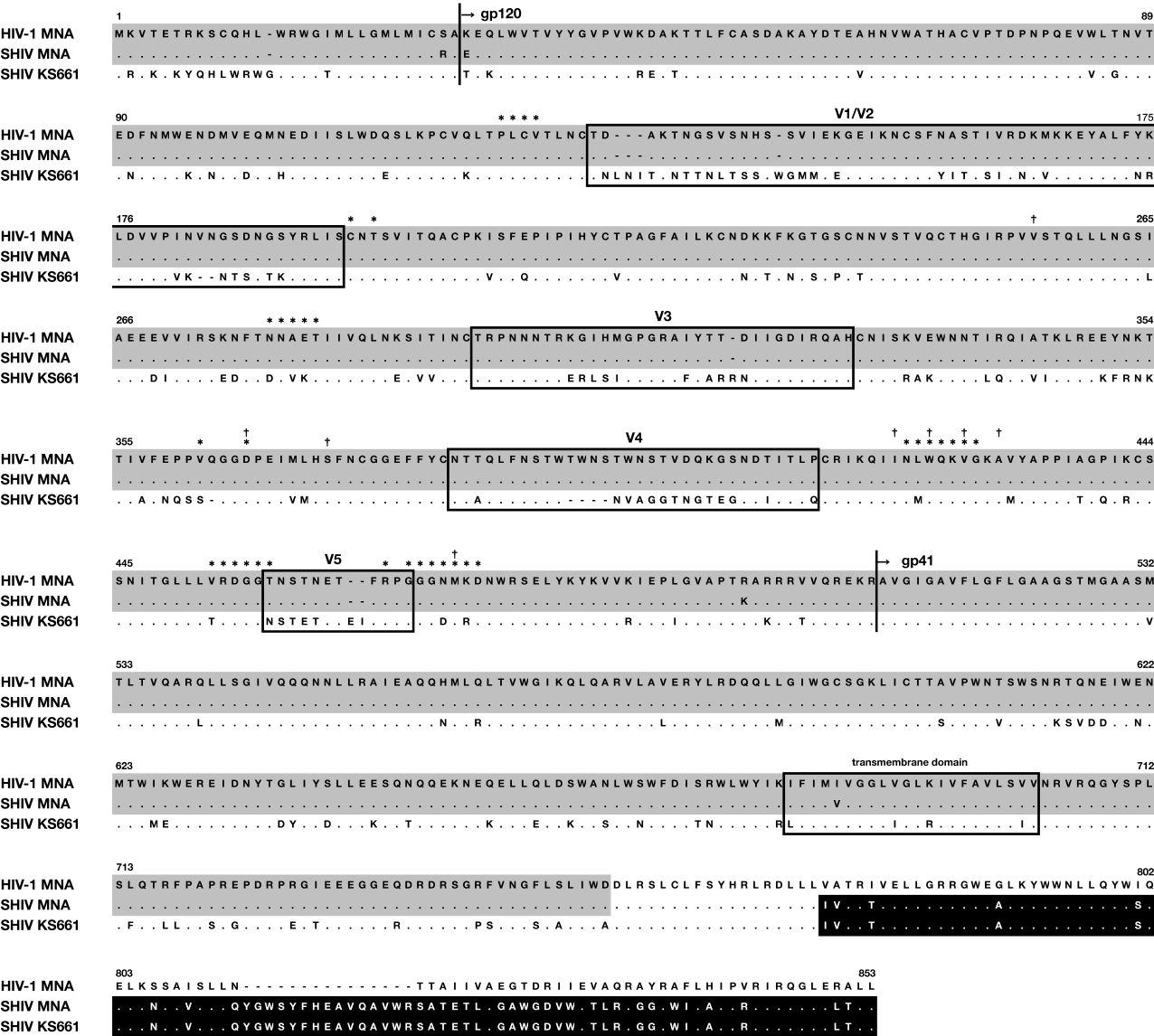


Figure S4

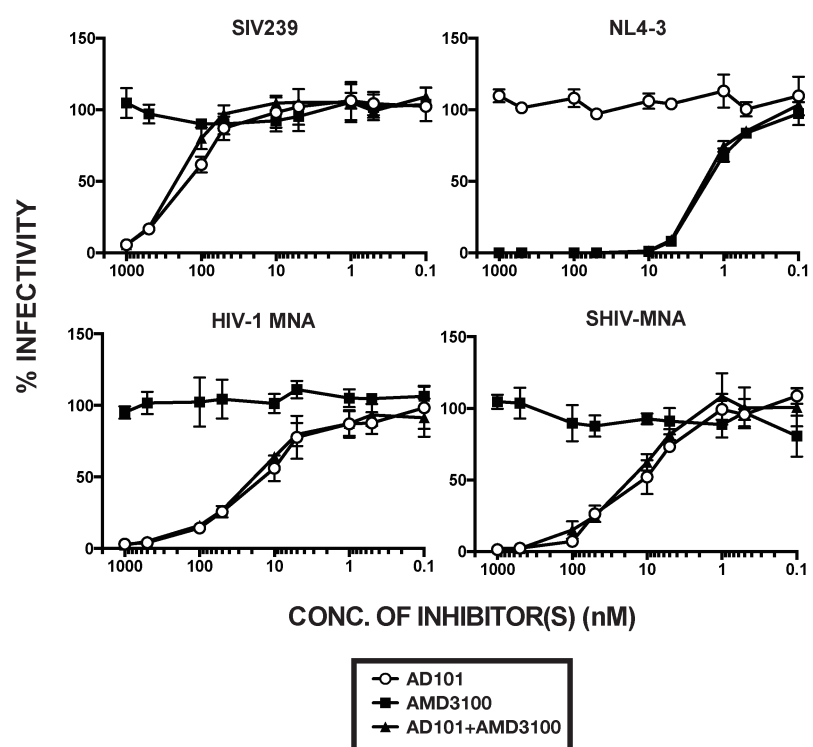


Figure S5

