21 **Summary**

22 It has been reported that the addition of a potential N-linked glycosylation site (PNGS)
23 to the gp120 HIV-1 envelope glycoprotein provides protection against neutralizing
24 antibodies (NAbs) by acting as a 'glycan shield'. In this study, we induced insertion of a
25 PNGS in the V2 region of HIV-1_{BaL} with the KD-247 anti-V3 neutralizing monoclonal
26 antibody. In the presence of KD-247 (200 μg ml^{-1}) at passage five, viruses with three
27 amino acid mutations in the C2 (T240S and I283T) and V3 (T319A) regions expanded
28 from pre-existing variants. After six passages with KD-247 (> 300 μg ml^{-1}), a PNGS
29 emerged in the V2 region in addition to C2 (T240S) and V3 mutations (R315K and
30 F317L). A variant with a PNGS insertion in V2 but no V3 mutations was sensitive to
31 KD-247, whereas a clone with a V2 PNGS insertion and mutations in V3 demonstrated
32 a high level of resistance to KD-247. Replication kinetics analysis revealed that the
33 F317L mutation in V3 played a compensatory role for fitness-loss caused by the PNGS
34 insertion in V2. The evading HIV-1 variant did not revert back to the wild-type virus
35 after 14 passages without KD-247. These findings demonstrate that the virus with
36 fitness-loss mutations can replicate equally as well as the wild-type virus to acquire
37 some key mutations in the V3 stem and the C2 region, and the compensated variants
38 containing PNGS do not revert back to the ancestral virus even in the absence of NAb.

Introduction

A neutralizing antibody (NAb) against human immunodeficiency virus type 1 (HIV-1) is an essential component of a protective vaccine. However, primary isolates of HIV-1 are relatively resistant to neutralization compared with variants selected for growth in permanent cell lines (Moore *et al.*, 1995; Pugach *et al.*, 2004). Studies addressing differences between neutralization-sensitive and -resistant variants have revealed several mechanisms that are responsible for neutralization resistance in primary isolates. These mechanisms include the occlusion of epitopes within the envelope glycoprotein (Env) oligomer and the extensive glycosylation and extension of variable loops from the surface of the complex leading to steric and conformational blocking of receptor binding sites (Kwong *et al.*, 2002; McCaffrey *et al.*, 2004; Pinter *et al.*, 2004; Saunders *et al.*, 2005). The structural features of one envelope glycoprotein, Env gp120, means that it can tolerate a vast array of mutations permitting the selection of neutralization evading variants, as has been previously demonstrated in culture assays, animal models and infected individuals (Johnson & Desrosiers, 2002).

Although there is ample data showing that NAbs can protect against HIV-1 infection *in vitro* and *in vivo*, their activity in infected humans remains controversial (Cao *et al.*, 1995; Deeks *et al.*, 2006; Montefiori *et al.*, 2001; Sullivan *et al.*, 1993). Passive transfer

of a combination of broadly neutralizing monoclonal antibodies (MAbs) 2G12, 2F5 and 4E10 in patients during a structured treatment interruption resulted in a significant delay in viral rebound in some patients compared with viral rebound in the absence of these antibodies (Trkola *et al.*, 2005). This would indicate that viral suppression was because of the antiviral activity of the administered antibodies. Subsequent studies addressing the pharmacokinetics of each MAb (Joos *et al.*, 2006), neutralization-resistance mutations (Manrique *et al.*, 2007) and protective neutralization titres *in vivo* (Trkola *et al.*, 2008) using samples from the study further supported the protective effects of NAb *in vivo*.

Clinical studies examining NAbs in primary infections have suggested that the majority of recently infected individuals mount a vigorous antibody response against autologous virus. However, the rapid evolution of HIV in the presence of NAb results in the emergence of evading mutants. As a consequence, at any time during the early stages of HIV infection, NAbs are more likely to recognize earlier form of the viruses as opposed to recent variants. Despite evidence of phenotypic resistance, the genetic basis of the mechanism allowing primary viruses to evade NAbs is poorly understood.

Wei *et al.* found that glycosylation of Env plays an important role in evading neutralization. The evolving 'glycan shield' can sterically block antibody binding

without mutation at the antibody-binding site (Wei *et al.*, 2003). Also, insertion of potential N-linked glycosylation sites (PNGSs) along with other mutations has been associated with viral evasion of NAbs (Bunnik *et al.*, 2008; Wei *et al.*, 2003). Conversely, Frost *et al.* reported that viral evasion of NAb correlates to the rate of amino acid substitution rather than changes in glycosylation and insertions or deletions in Env (Frost *et al.*, 2005). This would suggest that the individual contribution of PNGSs to the neutralization sensitivity of HIV-1 depends on the presence of other mutations in the Env sequence. However, the relationship between PNGSs and mutations of NAb resistance has not been investigated because of technical difficulties resulting from the polyclonal nature of NAbs and the primary isolates used in previous clinical studies. To clarify the genetic mechanisms responsible for evading neutralization, it is important to analyse individual mutations resulting from neutralization evasion of NAbs in an *in vitro* culture system.

Neutralization evasion from anti-V3 MAbs has been reported and associated with amino acid substitution within the epitope of the V3 loop and outside V3 (Gorny *et al.*, 2004; Masuda *et al.*, 1990; Park *et al.*, 1998; Pinter *et al.*, 2004; Shibata *et al.*, 2007; Yoshimura *et al.*, 2006; Zolla-Pazner, 2004). However, the role(s) of PNGSs in

92 resistance to neutralization is not clear because the induction of PNGSs under
93 neutralizing MAbs pressure *in vitro* has not been reported.

94 In this study, we obtained evasion mutants harbouring PNGSs in the V2 region and
95 mutations in the C2 and V3 regions, during induction of neutralization evasion mutants
96 from anti-V3 MAb KD-247 in HIV-1_{BaL}. KD-247 is a humanized MAb that
97 demonstrates cross-neutralizing activity against HIV-1 isolates in clade B. The epitope
98 of KD-247 was mapped to six amino acids, IGPGRA, at the tip of the V3 loop (Eda *et*
99 *al.*, 2006). A series of analyses using viral clones that have corresponding mutations
100 present in evading viruses revealed a mutant that has both a PNGS-insertion in V2 and
101 mutations in V3 along with a highly resistant phenotype to the NAb. However, the
102 mutant requires further mutation to compensate for reduced replication ability. Studies
103 to elucidate replication kinetics indicated that the F317L mutation in V3 and the T240S
104 mutation in C2 play a key role in maintaining resistant mutations in V2 and V3, which
105 were related to the fitness-loss. Our study partially explains the complex nature of the
106 development of neutralization resistance observed in previous clinical studies.
107

Results

Selection of anti-V3 MAb KD-247 evasion mutants from HIV-1_{BaL}

To select a HIV-1 variant able to evade neutralization by KD-247 *in vitro*, we exposed PM1/CCR5 cells to HIV-1_{BaL} and serially passaged the virus in the presence of increasing concentrations of KD-247. PM1/CCR5 cells were highly sensitive to both X4 and R5 HIV-1 infection, displaying prominent syncytia (Yusa *et al.*, 2005). As a control, HIV-1_{BaL} was passaged under the same conditions without MAb to monitor spontaneous changes that occurred in the virus during prolonged PM1/CCR5 cell passage (denoted as passage control). Because HIV-1_{BaL} was sensitive to neutralization by KD-247 with an IC_{50} of 3.8 μg ml^{-1} as determined by MTT assay (data not shown), the selected virus was initially propagated in the presence of 5 μg ml^{-1} KD-247. During the course of the selection procedure, the MAb concentration was increased to 2,000 μg ml^{-1}. Following five rounds of passage (p5), a viral variant designated HIV-1_{BaL} (200) p5, arose that replicated in the presence of 200 μg ml^{-1} KD-247. After passage 16, a viral variant designated HIV-1_{BaL} (2000) p16, arose that infected PM1/CCR5 cells efficiently in the presence of 2,000 μg ml^{-1} KD-247. We harvested viruses at six passages (p2, p5, p6, p7, p10 and p16) as well as a baseline virus, HIV-1_{BaL} (0) p0, and a passage control designated HIV-1_{BaL} (0) p10. These viruses were evaluated for their sensitivity to

126 KD-247 using TZM-bl as target cells (Table 1). The IC_{50} values of KD-247 against
127 HIV-1_{BaL} (0) p0, HIV-1_{BaL} (200) p5, HIV-1_{BaL} (300) p6 and HIV-1_{BaL} (2000) p16 were
128 0.32 ± 0.2 μg ml^{-1}, 5.68 ± 1.48 μg ml^{-1}, > 100 μg ml^{-1} and > 100 μg ml^{-1} respectively,
129 indicating that HIV-1_{BaL} acquired a resistant phenotype against KD-247 during *in vitro*
130 selection. At passage 5, HIV-1_{BaL} acquired a moderately resistant phenotype and after
131 passage 6, the virus had developed a highly resistant phenotype.

132 DNA sequence of the envelope region of evasion mutants

133 To determine the genetic basis of resistance in the variant HIV-1_{BaL} strains, the C1 to
134 C4 region of the env gene was amplified from genomic DNA extracted from the
135 infected cells, cloned and sequenced (Fig. 1).

136 At passage 5, moderately resistant variants with T240S, I283T and T319A mutations
137 were in the majority. However, the proportion of variants decreased gradually as the
138 KD-247 concentration was increased (Fig. 1B and Fig. 2E). This observation suggests
139 that at low concentrations of KD-247, the variants with moderate resistance to the
140 anti-V3 MAb are selected from the pre-existing variants.

141 Insertion of a PNGS in the V2 region and an amino acid substitution at the V3 tip
142 (R315K) were observed at passages 5 and 6, respectively. Both of these alterations were

not evident in the passage control but became dominant at later passages in the presence of higher concentrations of KD-247 (Figs. 1 and 2). To examine whether the insertion of a PNGS in the V2 region existed among the baseline HIV-1_{BaL} variants, we sequenced a total of 61 env clones from HIV-1_{BaL} before selection. No PNGS insertion was observed in the V2 region among the baseline clones (Fig. 1). This result indicates that the virus with the PNGS insertion in V2 either did not exist, or existed at a very low level within the baseline variants.

Neutralization sensitivities of pseudoviruses that have a mutated env gene

To determine which substitutions were responsible for KD-247 resistance, we constructed chimeric viruses, which contained the representative envelopes of HIV-1_{BaL} (200) p5 and HIV-1_{BaL} (1000) p13 and were designated BaL-STA and BaL-PNGS/SKL, respectively (Fig. 3). Chimeric envelopes were constructed by replacing wild-type sequences with mutated envelope-encoding sequences for V2, C2 and V3 in the HIV-1_{BaL} wild-type (BaL-WT) virus with the resulting viruses designated BaL-PNGS and BaL-SKL. Sensitivity was compared between the BaL-WT and mutant viruses using a single round neutralization assay. As shown in Figure 4A, the V3 mutated pseudoviruses, BaL-SKL and BaL-PNGS/SKL were highly resistant to KD-247 (> 2500 and > 5500-fold, respectively) compared with wild-type virus, whereas the C2 and V3

mutated virus at passage 5, BaL-STA, was partially resistant (25-fold). The susceptibility of the clone with the PNGS insertion in the V2 region alone (BaL-PNGS) to KD-247 was highly comparable to that for BaL-WT, indicating that this variant is sensitive to KD-247. In contrast, the clone with both the PNGS-insertion in V2 and mutations in V3 had a highly resistant phenotype to the MAb. The IC_{50} value for BaL-PNGS/SKL was slightly higher than for BaL-SKL in three independent experiments although there was no significant difference between these two clones.

To determine the effect of these KD-247-induced mutations in gp120 to other entry inhibitors, we examined the sensitivities of these chimeric pseudotyped viruses to rsCD4, 2D7 and maraviroc, a CCR5 inhibitor. The IC_{50} values for rsCD4, 2D7 and maraviroc to the chimeric viruses were comparable to those of wild-type virus (Fig. 4B–D).

Analyses of replication kinetics of infectious molecular clones with mutant Env

In order to clarify the role of the PNGS insertion during the process of neutralization evasion, we constructed replication-competent viruses with the PNGS in the V2 region and/or mutations in C2 and V3 of gp120 using pWT/BaL proviral plasmid (Fig. 3; they were designated HX-BaL-X). Using these competent viruses with Env mutations we

178 compared the replication kinetics in the absence of KD-247. As shown in Fig. 5A,
179 HX-BaL-PNGS containing a PNGS in V2 had low levels of p24 antigen compared with
180 the wild-type clone (HX-BaL-WT). Whereas, HX-BaL-PNGS/SKL containing a PNGS
181 and mutations in the C2 and V3 regions exhibited a replication rate equivalent to the
182 wild-type and HX-BaL-SKL. We also compared their replication kinetics in the
183 presence of low, moderate and high concentrations of KD-247 (Fig. 5B). High
184 concentrations of KD-247 (2,000 μg ml^{-1}) resulted in HX-BaL-PNGS/SKL replicating
185 slightly faster than HX-BaL-SKL. HX-BaL-PNGS demonstrated slightly more efficient
186 replication kinetics than the wild-type in the presence of a low concentration of KD-247
187 (5 μg ml^{-1}), while there was no difference between the two viruses when KD-247 was
188 used at 50 μg ml^{-1}. These results indicated that viruses harbouring a PNGS in V2 were
189 selected for at low concentrations of KD-247 but at high concentrations of the MAb,
190 variants harbouring the additional V3 mutations outgrew the former variants.

191 To elucidate which amino acid mutation would compensate for the fitness-loss induced
192 by insertion of a PNGS, we compared the replication kinetics of the infectious clones
193 with individual mutations in addition to the PNGS insertion (Fig. 5C). The variants with
194 the T240S, R315K and F317L mutations (HX-BaL-PNGS/SKL) and F317L mutation
195 (HX-BaL-PNGS/L) exhibited a higher replication rate than HX-BaL-PNGS. On the

196 other hand, viruses containing the T240S or R315K mutation in addition to the PNGS
197 insertion (HX-BaL-PNGS/S or HX-BaL-PNGS/K, respectively) replicated as well as or
198 less efficiently than HX-BaL-PNGS. These results suggest that the replication
199 deficiency of the HIV-1_{BaL} variant with the PNGS in the V2 region was compensated by
200 the F317L mutation in the V3 region. An improvement in replication capacity of
201 HX-BaL-PNGS/K was observed in variants with the additional T240S mutation (Fig.
202 5C).
203 Both the T240S (41/61 clones, 67.2%) and F317L (18/61, 29.5%) substitutions were
204 observed in the HIV-1_{BaL} sequence of the baseline viruses. The ratio of threonines and
205 serines at position 240 in gp120 of the passage control remained unchanged at passage
206 16 (8/13, 61.5%). The F317L variant in the passage control increased gradually and
207 became more prevalent in the viral population at passage 16 (12/13, 92.3%) (Fig. 2).
208 These findings suggest that the serine substitution at position 240 in the C2 region of
209 gp120 was required to compensate for inefficient replication rates caused by the R315K
210 and T319A substitutions in the V3 region. Furthermore, the F317L substitution in V3
211 might play a role in adaptations to survive in PM1/CCR5 cells *in vitro* and also
212 compensate for fitness-loss by the PNGS-insertion in the V2 region induced by KD-247
213 selection.

Sensitivity of infectious molecular clones containing mutated Env protein to KD-247 and a CCR5 inhibitor

To compare the neutralization sensitivity of the infectious molecular clones the IC_{50} values were determined for KD-247 using TZM-bl cells (Table 2). The neutralization sensitivities of clones containing only the PNGS in V2, T240S in C2 and F317L in V3 were virtually the same as the wild-type. Whereas the HX-BaL-PNGS/K demonstrated a high level of resistance to KD-247 when compared with HX-BaL-K containing the R315K mutation alone ($p = 0.006$). We constructed a mutant containing an amino acid insertion in V2 without the addition of a glycosylation site and with the R315K mutation (designated HX-BaL-Q/K). The sensitivity of this mutant was comparable to that of the HX-BaL-K mutant ($p = 0.89$). These results taken together suggest that the variant with the PNGS in addition to the R315K mutation in V3 had the highest resistance phenotype to KD-247. We also evaluated sensitivities of these clones to the CCR5 inhibitor, maraviroc. No significant difference was detected for the IC_{50} values of the wild-type and mutant variants to maraviroc.

We then investigated whether the mutations that conferred KD-247 resistance, which were related to a loss in replication efficiency, reverted back to the baseline after several passages in the absence of the anti-V3 MAb. Two mutations, F317L and R315K in the

232 V3 region, the PNGS in the V2 and the T240S mutation in C2 remained dominant in the
233 population of viruses that were adept at evasion after culturing for 16 passages in the
234 presence of KD-247 and an additional 14 passages without KD-247 (Fig. 6). The T319A
235 mutation was observed in 90% of clones sequenced at passage 19. However, the
236 frequency of this mutation gradually decreased thereafter (Fig. 6E). These findings
237 show that after acquisition of the mutations in order to compensate for fitness-loss the
238 HIV-1 variants did not revert back to the wild-type after 14 passages without KD-247.
239

Discussion

HIV-1 evolution in relation to evasion of humoral immunity has been observed in the early stages of HIV-1 infection and has been suggested to act as a driving force for the establishment of viral quasispecies *in vivo* (Bunnik *et al.*, 2008; Frost *et al.*, 2005; Mahalanabis *et al.*, 2009; Richman *et al.*, 2003; Wei *et al.*, 2003). In response to NAb pressure, the numbers and/or positions of surface-expressed carbohydrates can evolve to create a continuously changing glycan shield on the surface of the Env protein (Wei *et al.*, 2003). Large sequence variation in the variable loops, including large insertions and deletions, and changes in the number of PNGS in these regions has also been associated with evasion of NAbs (Sagar *et al.*, 2006; Saunders *et al.*, 2005). An especially strong influence of a mutation in the V1/V2 domain on neutralization activity has been reported by a number of investigators (Krachmarov *et al.*, 2005; Pinter *et al.*, 2005; Shibata *et al.*, 2007). However, insertion of a PNGS in the V2 region of the R5 virus during *in vitro* selection using an anti-V3 MAb has not previously been reported.

In this study, we obtained neutralization evading mutants using HIV-1_{BaL} by *in vitro* selection with the anti-V3 MAb, KD-247, and analysed the functional role of the mutations *in vitro*. In the presence of low concentrations of KD-247, viruses with three amino acid mutations in C2 (T240S and I283T) and V3 (T319A) expanded from

258 pre-existing variants in the baseline population. In the presence of high concentrations
259 of KD-247, an increase in the number of mutants that had a PNGS in the V2 region and
260 containing C2 (T240S) and V3 (R315K and F317L) mutations was observed. To
261 identify how each of these mutations effects resistance to KD-247 and the replication
262 kinetics of the virus, we constructed pseudoviruses and infectious clones containing
263 each mutation and combinations of these mutations and compared the IC_{50} and
264 replication kinetics with the wild-type virus. Our results showed that the HIV-1_{BaL}
265 variant with the PNGS in the V2 region and no V3 mutations was sensitive to KD-247.
266 The clone with both the V2 PNGS and mutations in V3 had a high level of resistance to
267 KD-247 and was more resistant than variants with the V3 mutation alone (Table 2). In
268 replication kinetics analyses, the F317L mutation in V3 and the T240S mutation in C2
269 played a compensatory role for a fitness-loss caused by the V2 PNGS-insertion and
270 R315K mutation in the V3-tip. Our data indicate that the virus with fitness-loss
271 mutations can replicate as well as the wild type virus to acquire some key mutations in
272 the V3 stem and the C2 region of gp120 with or without exposure to KD-247.

273 In order to estimate the mechanism of neutralization resistance conferred by the T319A
274 mutation observed at passage 5, we simulated structures of the V3 region using
275 Swiss-PdbViewer software (Supplemental Fig. S1) (Guex *et al.*, 1999). According to

276 the simulation data the side chain of arginine at position 315 on the V3 loop in the 315R,
277 317F and 319A variant at passage 5 was bent to the C terminal side of the protein
278 compared with its position in the 315R, 317F and 319T variant. It is possible that
279 alanine at position 319 may contribute to KD-247 resistance by altering the three
280 dimensional conformation of the V3 loop.

281 Our initial analysis using pseudoviruses suggested that the variant with the PNGS alone
282 was sensitive to KD-247 (Fig. 4A). Moreover, the difference in neutralization
283 sensitivity between the viruses with the V3 mutations alone or those with the V2
284 modifications was not clear in this single round assay (Fig 4A). We then constructed a
285 panel of replication competent viruses to compare resistance. As shown in Table 2
286 HX-BaL-PNGS/K containing the R315K mutation in V3 and the PNGS demonstrated a
287 high level of resistance to KD-247 when compared to HX-BaL-K containing the R315K
288 mutation alone. These data suggest that the contribution of the PNGS in neutralization
289 resistance was moderate in a single cycle of viral replication but played a significant
290 role in multiple rounds of infection. It is also possible that the existence of the other
291 mutations such as T240S and F317L in the pseudovirus might have some influence on
292 neutralization sensitivity.

293 Although we did not examine whether the PNGS in V2 at position 186 was actually
294 glycosylated or not, previous studies describing the assignment of glycosylation sites
295 for IIIB and SF2 gp120 show that it is glycosylated (Cutalo *et al.*, 2004; Zhu *et al.*,
296 2000). The difference in neutralization resistance of HX-BaL-PNGS/K which has
297 PNGS-insertion in V2 with the counterpart HX-BaL-Q/K of amino acid insertion
298 without glycosylation further supports glycosylation of the site in HIV-1_{BaL} Env (Table
299 2).HX-BaL-PNGS, which contained the PNGS alone, demonstrated low levels of p24
300 production compared with the wild-type (Fig. 5A). Moreover, the variant with the
301 R315K mutation in addition to the PNGS further hindered the replication capacity of the
302 variant (Fig. 5C). Interestingly, HX-PNGS-SKL, which contained the additional T240S
303 mutation in the C2 and F317L in the V3 regions, could replicate at the same level as the
304 wild-type clone. These observations indicate the role of the PNGS and the V3 mutation
305 in neutralization resistance, together with the contribution of two other mutations which
306 compensate for loss in replication ability induced by the first mutations in the process of
307 neutralization evasion.

308 The interaction of V1/V2 with V3, including the influence on V2 glycosylation, has
309 been reported not only for the neutralization sensitivity but also coreceptor usage
310 (Bontjer *et al.*, 2009; Nabatov *et al.*, 2004). Additonally, mutations associated with

311 resistance to CCR5 antagonists have been mapped to the V3 loop of gp120 and in some
312 cases, to the outside of V3 including the N-terminus of gp41 (Anastassopoulou *et al.*,
313 2009; Baba *et al.*, 2007; Berro *et al.*, 2009; Kuhmann *et al.*, 2004; Marozsan *et al.*,
314 2005; Ogert *et al.*, 2008; Westby *et al.*, 2007). We then evaluated pseudoviruses and
315 infectious clones derived from the evasion mutants for their sensitivities to maraviroc
316 and a MAb to CCR5 (2D7). As shown in figures 4C, 4D and Table 2, no change in
317 co-receptor usage or sensitivity to maraviroc was observed in the evasion mutants.

318 To elucidate the stability of these evasion variants, we cultured HIV-1_{BaL} (2000) p16
319 viruses in the absence of KD-247 (Fig. 6). Despite culturing these viruses for 14
320 passages, no decrease in the proportion of the population containing the PNGS or the
321 R315K, T240S and F317L mutations was observed. The T319A mutation appeared to
322 subsided at passage 30. Bunnik *et al.* reported that reversion of NAb-induced changes in
323 amino acid usage in Env was observed at a late stage of infection in the face of
324 declining neutralizing immunity, suggesting a negative effect of these changes on viral
325 fitness (Bunnik *et al.*, 2008). In contrast, our results suggest that the addition of
326 compensatory amino acid changes can stabilise the replication capacity of the evasion
327 variants with the PNGS and R315K mutation.

In conclusion, we induced highly resistant viral variants against anti-V3 MAb, KD-247, harbouring a PNGS in the V2 region and a V3 mutation, together with mutations that compensate for replication deficiencies induced by the resistant mutations. The mutations associated with viral evasion may play a role in multiple different mechanisms which contribute towards evasion of antibody neutralization. The elucidation of the interplay between these mutations which results in neutralization evasion of the virus has important implications for the development of effective vaccines against HIV-1.

337 **Materials and Methods**

338 Cells, culture conditions, reagents and viruses

339 PM1/CCR5 cells (Yusa *et al.*, 2005) were maintained in RPMI 1640 medium (Sigma)
340 supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone), 50 U ml^{-1} of
341 penicillin, 50 mg ml^{-1} of streptomycin and 100 μg ml^{-1} of the antibiotic G418 (Nacalai).
342 TZM-bl cells (Platt *et al.*, 1998; Wei *et al.*, 2002) obtained from the AIDS Research and
343 Reference Reagent Program (ARRRP) and 293T cells were maintained in Dulbecco's
344 modified Eagle medium (DMEM; Sigma) supplemented with 10% FCS. KD-247 was
345 provided by the Chemo-Sero-Therapeutic Research Institute (Eda *et al.*, 2006). The
346 MAb, 2D7, was purchased from BD Biosciences Pharmingen. Human recombinant
347 soluble CD4 (rsCD4) was purchased from R&D Systems. Maraviroc, a CCR5 inhibitor,
348 was a gift from Pfizer Inc. The R5 isolate, HIV-1_{BaL} (Gartner *et al.*, 1986) was passaged
349 in PM1/CCR5 and the culture supernatant was stored at -150 °C prior to use.

350 Isolation of a KD-247-resistant mutant from HIV-1_{BaL} *in vitro*.

351 The selection of KD-247 evasion variants from HIV-1_{BaL} was performed as previously
352 described (Yoshimura *et al.*, 2006). Briefly, PM1/CCR5 cells (4×10^4) were exposed to
353 500 × the 50% tissue culture infective dose ($TCID_{50}$) of HIV-1_{BaL} pre-incubated with