143 not evident in the passage control but became dominant at later passages in the presence

144 of higher concentrations of KD-247 (Figs. 1 and 2). To examine whether the insertion

145 of a PNGS in the V2 region existed among the baseline HIV-1_{BaL} variants, we

146 sequenced a total of 61 env clones from HIV-1_{BaL} before selection. No PNGS insertion

147 was observed in the V2 region among the baseline clones (Fig. 1). This result indicates

148 that the virus with the PNGS insertion in V2 either did not exist, or existed at a very low

149 level within the baseline variants.

150 Neutralization sensitivities of pseudoviruses that have a mutated env gene

151 To determine which substitutions were responsible for KD-247 resistance, we

152 constructed chimeric viruses, which contained the representative envelopes of HIV-1_{BaL}

153 (200) p5 and HIV-1_{BaL} (1000) p13 and were designated BaL-STA and BaL-PNGS/SKL,

154 respectively (Fig. 3). Chimeric envelopes were constructed by replacing wild-type

155 sequences with mutated envelope-encoding sequences for V2, C2 and V3 in the

156 HIV-1_{BaL} wild-type (BaL-WT) virus with the resulting viruses designated BaL-PNGS

157 and BaL-SKL. Sensitivity was compared between the BaL-WT and mutant viruses

158 using a single round neutralization assay. As shown in Figure 4A, the V3 mutated

159 pseudoviruses, BaL-SKL and BaL-PNGS/SKL were highly resistant to KD-247 (> 2500

160 and > 5500-fold, respectively) compared with wild-type virus, whereas the C2 and V3

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

168 To determine the effect of these KD-247-induced mutations in gp120 to other entry
169 inhibitors, we examined the sensitivities of these chimeric pseudotyped viruses to
170 rsCD4, 2D7 and maraviroc, a CCR5 inhibitor. The IC_{50} values for rsCD4, 2D7 and
171 maraviroc to the chimeric viruses were comparable to those of wild-type virus (Fig.
172 4B–D).

173 Analyses of replication kinetics of infectious molecular clones with mutant Env

174 In order to clarify the role of the PNGS insertion during the process of neutralization
175 evasion, we constructed replication-competent viruses with the PNGS in the V2 region
176 and/or mutations in C2 and V3 of gp120 using pWT/BaL proviral plasmid (Fig. 3; they
177 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.

214 Sensitivity of infectious molecular clones containing mutated Env protein to KD-247

215 and a CCR5 inhibitor

216 To compare the neutralization sensitivity of the infectious molecular clones the IC_{50}

217 values were determined for KD-247 using TZM-bl cells (Table 2). The neutralization

218 sensitivities of clones containing only the PNGS in V2, T240S in C2 and F317L in V3

219 were virtually the same as the wild-type. Whereas the HX-BaL-PNGS/K demonstrated

220 a high level of resistance to KD-247 when compared with HX-BaL-K containing the

221 R315K mutation alone ($p = 0.006$). We constructed a mutant containing an amino acid

222 insertion in V2 without the addition of a glycosylation site and with the R315K

223 mutation (designated HX-BaL-Q/K). The sensitivity of this mutant was comparable to

224 that of the HX-BaL-K mutant ($p = 0.89$). These results taken together suggest that the

225 variant with the PNGS in addition to the R315K mutation in V3 had the highest

226 resistance phenotype to KD-247. We also evaluated sensitivities of these clones to the

227 CCR5 inhibitor, maraviroc. No significant difference was detected for the IC_{50} values of

228 the wild-type and mutant variants to maraviroc.

229 We then investigated whether the mutations that conferred KD-247 resistance, which

230 were related to a loss in replication efficiency, reverted back to the baseline after several

231 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

240 **Discussion**

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

254 In this study, we obtained neutralization evading mutants using HIV-1_{BaL} by *in vitro*
255 selection with the anti-V3 MAb, KD-247, and analysed the functional role of the
256 mutations *in vitro*. In the presence of low concentrations of KD-247, viruses with three
257 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 $HIV\text{-}1_{BaL}$
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.

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

336

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 $\times$ the 50% tissue culture infective dose ($TCID_{50}$) of HIV-1_{BaL} pre-incubated with

354 KD-247 for 30 min at 37 °C. After incubation for 5 h, cells were centrifuged,
355 resuspended in RPMI 1640 medium supplemented with 10% FCS without KD-247. The
356 culture supernatant was harvested on day 6 and used to infect fresh PM1/CCR5 cells for
357 the next round of culture in the presence of increasing concentrations of KD-247. When
358 the virus began to propagate rapidly in the presence of KD-247, the MAb concentration
359 was further increased. After the virus had been passaged in the presence of up to 2,000
360 μg ml^{-1} of KD-247, the KD-247-resistant virus, HIV-1$_{BaL}$ (2000) p16, was recovered
361 from the cell culture supernatant. After 16 passages with KD-247, we continued
362 culturing the virus for a further 14 passages without KD-247. HIV-1$_{BaL}$ virus was also
363 passaged for the same period in PM1/CCR5 cells in the absence of KD-247, and the
364 resulting virus was designated HIV-1$_{BaL}$ (-) p16. Proviral DNA from infected cells at
365 various passages was subjected to DNA sequencing.

366 Amplification of proviral DNA (pDNA) and nucleotide sequencing

367 Proviral DNA was extracted and nested PCR was performed to amplify the gp120 C1 to
368 C4 coding region as described previously (Wang *et al.*, 2002). The primers used were as
369 follows: for the first-step PCR, 1B (5'-AGA AAG AGC AGA AGA CAG TGG CAA
370 TGA-3') and H (5'-TAG TGC TTC CTG CTG CTC CCA AGA ACC C-3'); for the
371 second-step PCR, 2B (5'-AGC AGA AGA CAG TGG CAA TGA GAG TGA-3') and F

372 (5'-ATA TAA TTC ACT TCT CCA ATT GTC CCT CAT-3'). The PCR products were

373 inserted into a TA vector (Invitrogen) and sequenced.

374 The neutralization-sensitivity assay.

375 The neutralization-sensitivity of each passaged HIV-1_{BaL} virus to KD-247 was

376 determined using TZM-bl cells. Briefly, a virus concentration of 300 $TCID_{50}$ was

377 incubated with various dilutions of KD-247 in duplicate for 30 min at 37 °C in a 96-well

378 flat-bottom culture plate (Corning-Costar). Freshly trypsinized cells (2×10^4 cells in 50

379 μl of 10% FCS/DMEM containing 10 μg ml^{-1} DEAE-dextran) were added to each well.

380 After incubation for 2 days at 37 °C β-galactosidase activity in each well was measured

381 using Galacto-Star substrate (Applied Biosystems).

382 Construction of mutant envelope expression vectors

383 Proviral DNA isolated from the infected cells at various passages was cloned into

384 envelope expression vectors as previously described (Li *et al.*, 2005; Shibata *et al.*,

385 2007). Briefly, we amplified the full-length gp160 regions from the most frequent

386 clones at the baseline, passage 5 and passage 13 using LA *Taq* (Takara) with primers

387 ENVA (5'-GGC TTA GGC ATC TCC TAT GGC AGG AAG AA-3') and ENVN

388 (5'-CTG CCA ATC AGG GAA GTA GCC TTG TGT-3'), and the PCR products were

389 inserted into the pCR-XL-TOPO vector (Invitrogen) and designated pCR-XL-BaL-WT,

390 pCR-XL-BaL-p5 and pCR-XL-BaL-p13, respectively. Chimeric vectors were generated

391 based on the pCR-XL-BaL-WT by replacing the fragments from pCR-XL-BaL-p5 and

392 pCR-XL-BaL-p13 digested at the restriction enzyme sites indicated below. The

393 *Nde*I-*Sca*I fragment for the pCR-XL-BaL-p5 env gene was subcloned into

394 pCR-XL-BaL-WT, designated pCR-XL-BaL-STA. The *Nde*I-*Sca*I, *Nde*I-*Stu*I and

395 *Stu*I-*Sca*I fragments for the pCR-XL-BaL-p13 env gene were subcloned into the

396 pCR-XL-BaL-WT, designated pCR-XL-BaL-PNGS/SKL, pCR-XL-BaL-PNGS and

397 pCR-XL-BaL-SKL, respectively. Each *EcoR*I fragment of these vectors was ligated into

398 pCXN2 to give pCXN-BaL-WT, pCXN-BaL-STA, pCXN-BaL-PNGS/SKL,

399 pCXN-BaL-PNGS and pCXN-BaL-SKL.

400 <u>Pseudovirus preparation</u>

401 Approximately 5 μg of pSG3$^{\Delta env}$ (Wei *et al.*, 2002) and 0.5 μg of pRSV-Rev (Hope *et*

402 *al.*, 1990), supplied by the ARRRP, and 4.5 μg of the HIV-1_{BaL} env-expressing $pCXN_2$

403 were co-transfected into 293T cells. At 24 h after transfection the

404 pseudovirus-containing supernatants were harvested, filtered and stored at -150 °C.

405 <u>A single-round assay for measuring neutralization of the pseudoviruses</u>

406 A single-round infectivity assay was used to measure the neutralization of HIV-1_{BaL}

407 pseudoviruses as described previously (Li *et al.*, 2005). Briefly, reagents including an

408 entry inhibitor, MAbs or rsCD4 at various concentrations and a pseudovirus suspension

409 corresponding to 300 $TCID_{50}$ were pre-incubated for 30 min at 37 °C. The

410 virus-compound mixtures were added to TZM-bl cells in a 96-well plate (2×10^4 cells

411 $well^{-1}$). After incubation for 2 days at 37 °C the β-galactosidase activity in each well

412 was measured as described above. The reduction in infectivity was determined by

413 comparing the relative light units in the presence and absence of each compound and

414 was expressed as the percentage of neutralization.

415 <u>Construction of chimeric pWT10/BaL env proviruses</u>

416 Chimeric proviruses were constructed from the pWT/BaL proviral plasmid (from the

417 ARRRP) (Hwang *et al.*, 1991) by replacing the region encoding the envelope gp160.

418 Briefly, the env genes obtained from escaped HIV-1_{BaL} variants or induced by

419 site-directed mutagenesis were substituted into the pWT/BaL vectors after digestion at

420 the restriction enzyme sites *Sal*I and *BamH*I. The resulting replication-competent

421 viruses were designated HX-BaL-X (e.g., HX-BaL-WT, HX-BaL-PNGS/SKL etc.).

422 <u>Preparation of infectious clones and viral replication assays in PM1/CCR5 cells</u>

423 Approximately 5 μg of the plasmids from the env mutants were transfected into 293T

424 cells using the Effectene transfection reagent (QIAGEN). At 48 h after transfection, the

425 virus-containing supernatants were harvested, filtered and frozen in aliquots at -150 °C.

426 Viral yields were quantified using the HIV-1 p24 antigen ELISA (ZeptoMetrix).

427 PM1/CCR5 cells (3×10^4) were exposed to pWT/BaL env chimeric viruses

428 corresponding to 2 ng or 10 ng of p24 for 4 h at 37 °C. Following incubation, cells were

429 centrifuged and resuspended in RPMI 1640 medium supplemented with 10% FCS and

430 cultured for 6 days. Viral replication was monitored by measuring concentration of p24

431 antigen in culture supernatants.

432 Statistical analysis

433 Statistical correlations were analysed using Student's t test. *P* values < 0.05 were

434 considered statistically significant.

435 Nucleotide sequence accession numbers

436 The sequence data of env expression vectors from passaged samples have been

437 deposited in the DNA Data Bank of Japan under accession numbers

438 AB521136–AB521148.

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