had V2 mutations and a resistant phenotype against KD-247 were selected under pressure from relatively low concentrations of KD-247 (10-200 μg/ml), and that evolution of fully resistant variants with mutation in the V3-tip was observed under pressure from high concentrations of the antibody.

We then determined whether the KD-247 escape variants remained sensitive to other neutralizing antibodies (447-52D and 17b), rsCD4, anti-CCR5 antibody (2D7), anti-CD4 antibody (RPA-T4) and the small molecule CCR5 inhibitor (TAK-779) (Fig. 4 and Table 1). The KD-247 escape variants with the P313L mutation, MOKW-KNL/V3m and MOKW-RDP/V3m, were also resistant to another anti-V3 MAb, 447-52D, and V2 mutated viruses without V3 mutation, MOKW-KNL/C3m and MOKW-KNL, were partially resistant (the same as for KD-247). In contrast, the V2 mutated viruses, MOKW-KNL/C3m, MOKW-KNL/V3m and MOKW-KNL, showed resistance to rsCD4 and 17b (a MAb to the CD4-induced epitope; CD4i) compared with the pseudoviruses without V2 mutations, MOKW-RDP, MOKW-RDP/C3m and MOKW-RDP/V3m. Moreover, the pseudoviruses with V3-tip mutations, MOKW-KNL/V3m and MOKW-RDP/V3m, became significantly more sensitive to TAK-779 and 2D7 as compared with pseudoviruses without the P313L mutation (Fig. 4 and Table 1). No significant differences with respect to sensitivity to RPA-T4 were observed between any pseudoviruses (Table 1). These data indicate that V3-tip and V2 mutations confer neutralization resistance against anti-V3 antibodies, and that these mutations affect viral sensitivity to neutralizing antibodies recognizing different epitopes and anti-CCR5 antibody/agents.

Binding affinity of neutralizing antibodies to MOKW Envs on the cell surface.

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1 To elucidate the mechanism by which escape virus variants with V3-tip and V2 mutations
2 become less sensitive to neutralizing antibodies, MOKW Env-expressing 293T cells were
3 established by transfection with each Env expression plasmid, and then stained with the
4 MAbs. Binding of KD-247, 447-52D and 17b to the surface-expressed Env proteins was
5 assayed using fluorescence-activated cell sorter analysis. As shown in Fig. 5A, the mean
6 fluorescence intensities (MFI) of KD-247 binding to the Envs without either V2 or V3
7 mutations, in the MOKW-RDP and MOKW-RDP/C3m cells, were 30.13 and 29.20
8 respectively. However, the corresponding values for the V3-tip mutated Env-expressing
9 cells, MOKW-KNL/V3m and MOKW-RDP/V3m, were almost the same as negative
10 controls (6.90 and 6.66, respectively). The MFI of the V2-mutated Env-expressing cells,
11 MOKW-KNL/C3m and MOKW-KNL, indicated a lower binding affinity (17.89 and 19.18,
12 respectively) than for Envs without V2 and V3 mutations. The binding pattern of 447-52D
13 to these Env-expressing cells was similar to that of KD-247 (Fig. 5B). However, reduction
14 in the binding of 17b was observed for strains with V2-mutated Envs, MOKW-KNL/C3m,
15 MOKW-KNL/V3m and MOKW-KNL, whereas no difference in 17b binding was noted for
16 the V3-mutants without V2 mutations (Fig. 5B). These findings are consistent with the
17 results of a single-round neutralization assay (Fig. 4). Taken together, these data suggest
18 that the mutations in V2 have a significant influence on access by antibodies to V3 as well
19 as to the CD4i epitope. This is because access by antibodies to the epitopes of the
20 functional envelope is related to neutralization sensitivity/resistance.
21
22 **Identification of the V2 region site responsible for the neutralization-resistant
23 phenotype by site-directed mutagenesis of specific residues.** Because KD-247

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recognizes an epitope containing the IGPGR amino acid sequence in the V3-tip, the MAb could not bind the V3-tip of mutated Envs. Consequently, KD-247 does not neutralize V3-tip mutated virus strains (67). However, the mechanism of neutralization resistance associated with V2 mutations is not known. To clarify the responsible mutation in the V2 region that confers the escape phenotype with respect to KD-247, we introduced V2 amino acid changes individually and in combination into the MOKW-RDP Env expression vector (Fig. 3), and measured the sensitivities of pseudoviruses with these envelopes to KD-247. As shown in Fig. 7, the R166K/D167N double mutant, MOKW-KNP, showed almost the same neutralization sensitivity as MOKW-RDP against KD-247. Surprisingly, a single aa change (P175L in MOKW-RDL) was sufficient to confer >10000-fold resistance upon MOKW-RDP, with an IC_{50} of >100 μg/ml. R166K/P175L (MOKW-KDL) mutations also conferred resistance. Both MOKW-RDL and MOKW-KDL were much more resistant than the fully V2 mutated virus, MOKW-KNL (>100-fold and >10-fold resistance, respectively) (Fig. 6). The D167N/P175L (MOKW-RNL) mutant was more resistant than MOKW-KNL (10-fold), but less resistant than MOKW-RDL and MOKW-KDL. We also constructed a V2-mutatnt of JR-FL and confirmed that JR-FL with aa substitution of Leu to Pro in 175th residue became highly sensitive to KD-247 compared with JR-FL with Leu at 175th aa in Env (data not shown). These results suggest that the 175th residue (Pro or Leu) is the crucial amino acid for determining neutralization sensitivity against KD-247, and that the phenotypic influence of the R166K and D167N changes is strictly context dependent, requiring the presence of Leu at residue 175.

We then determined whether these pseudoviruses with various V2 mutations remained sensitive to other neutralizing antibodies (447-52D and IgGb12), rsCD4, 2D7,

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RPA-T4 and TAK-779 (Fig. 6). MOKW-RDL and MOKW-KDL were also resistant to another anti-V3 MAb, 447-52D, CD4 binding site MAb, IgGb12, and rsCD4. MOKW-RNL was partially resistant compared with MOKW-KNL, but less resistant than MOKW-RDL and MOKW-KDL against 447-52D, IgGb12 and rsCD4. These results were similar to those for KD-247. All V2 mutated clones were sensitive to TAK-779 and 2D7, as was MOKW-RDP (Fig. 6 and not shown). However, the anti-CD4 MAb RPA-T4 neutralized both MOKW-RDL and MOKW-KDL at an approximately 3-fold lower concentration compared with other viruses (Fig. 6). These results suggest that the 166th and 167th amino acids (RD and KN) may help compensate for any reduced fitness of viruses with Leu at the 175th aa. On the other hand, Pro at position 175 in MOKW-RDP might be accumulated because it confers better fitness to replicate on PM1/CCR5 cells in the absence of KD-247 pressure.

Binding affinity of MAbs against monomeric or cell-surface expressed gp120 with mutations in V2. To determine the difference in binding of MAbs to monomeric gp120 of MOKW Env with V2 mutations relative to that of MOKW-RDP, we performed MAb binding assays. Monomeric gp120 was prepared from pseudoviruses that had a series of V2 mutations and was captured on an ELISA plate followed by detection by MAbs. No difference was noted in the binding activity of KD-247 or 447-52D to monomeric gp120 from V2-mutated and MOKW-RDP envelopes (Fig. 7). These results suggest that V2-mutated Envs retain the neutralizing epitope at least in monomeric gp120.

In contrast to the monomeric form, gp120 expressed on the cell surface contained, to a certain degree, functional envelope oligomers that were directly related to the

infectivity and neutralization sensitivity of the virus. To compare the binding activity of MAbs for the surface-expressed Env with the results obtained for monomeric gp120, 293T cells transfected with MOKW-RDP and MOKW-KNP, a V2-mutant strain, were subjected to FACS analysis. As shown in Fig. 8A and 8B, the relative binding of KD-247, 447-52D and IgGb12 to Env expressed on the cell surface was no different than for MOKW-RDP and MOKW-KNP.

Consistent with the results of the single round neutralization assay shown in Fig. 6, MOKW-RDL had the lowest binding affinity for all tested MAbs. To determine which mutations (166K or 167N) further influence binding affinity, in addition to the MOKW-RDL background, we constructed MOKW-KDL and MOKW-RNL Envs and measured the binding affinity by FACS. The MOKW-KDL Env was found to have a slightly greater binding affinity for KD-247, 447-52D and IgGb12 than MOKW-RDL. But cell surface binding of all tested MAbs to MOKW-RNL was better than for MOKW-KDL. The strain with fully V2-mutated Env, MOKW-KNL, had a binding profile that was intermediate between single- or double-mutated Envs and non-mutated Env, but in the case of IgGb12, the binding affinity of MAbs for MOKW-KNL was comparable to that for MOKW-RDP. These data were consistent with the results obtained from the neutralizing assay using a high concentration of each MAb (Fig. 6).

Comparison of replication kinetics between NL-MOKW-RDL and NL-MOKW-KNL. Although the MOKW-RDL variant was much more resistant against KD-247 than the MOKW-KNL variant (Fig. 6) and the RD sequence was more prevalent than KN at positions 166 and 167 in the V2 region before selection (Fig. 2), the MOKW

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variants with the 166K/167N/175L were selected and outgrown under KD-247 pressure (Fig. 2). It was possible that the KN sequences at positions 166 and 167 are necessary to compensate for the fitness of the variants with 175L in PM1/CCR5 cells as shown in Fig. 6. To clarify the role of KN at positions 166 and 167 in replication, we constructed replication competent viruses with MOKW Env with RD or KN in addition to 175L (NL-MOKW-RDL and NL-MOKW-KNL) and compared their replication kinetics. As shown in Fig. 9, NL-MOKW-KNL replicated faster than NL-MOKW-RDL in PM1/CCR5 cells. These data suggested that KN sequences at positions 166 and 167 with 175L variant confer replication advantage in PM1/CCR5 cells. Therefore, the intermediate resistant variant MOKW with the KNL sequence in the V2 region might replicate more rapidly than the highly resistant variant MOKW with RDL against KD-247 in the course of selection.

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Discussion

Although an attack from the humoral immune response, especially anti-V3 NAb, is lasting against HIV-1 in vivo, it is not clear why the V3-tip sequence is conserved in the course of the infection. In the present study, by using a genetically heterogeneous HIV-1 primary R5 isolate, MOKW we found that V2- and C3-mutated virus expanded in conditions with a relatively low concentration of KD-247. Further, we found that the V3-tip mutated virus was induced only in conditions with a high concentration of MAb (more than 500 μg/ml). Using region-swapping analysis, it was found that both V2 and V3-tip mutations can cause an escape phenotype against anti-V3 antibody. Neutralization escape variants with V2 mutations could be selected from quasi-species existing in the primary isolate at relatively low antibody pressures. On the other hand, highly resistant variants with amino acid substitutions in the V3 epitope emerged via evolution of the virus in the presence of a high concentration of the MAb.

The V1/V2 region of gp120 is highly diverse, not only in respect to virus subtypes but also in respect to intra-species diversity in the same patient (16, 61, 66). The primary isolate, MOKW, also displayed diversity in the V2 region (Fig. 2) and the 1st passaged virus already harbored mutations in the V2 region. Many researchers have reported that the V1/V2 domain strongly influences neutralization of the anti-V3 MAbs, MAbs to the other epitopes and rsCD4 (12, 13, 25, 27, 3044, 49, 50). Moreover, structural models of the Env trimer have been proposed that place the base of the V1/V2 loop of one subunit in proximity to the V3 loop of a neighboring subunit (32, 34). In the present study we observed a reduction in the binding of anti-V3 MAbs to V2-mutated Env expressed on the cell surface, whereas mutations in V2 did not have an effect on the binding of the MAbs to

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1 monomeric gp120. These results suggest the association of V2 mutations with anti-V3
2 antibody accessibility in the context of the oligomeric conformation of the functional
3 envelope. It has been proposed that the gp120 of TCLA strains forms a relatively open
4 conformation and that the primary isolate trimeric complex has a more closed conformation
5 (2, 51). These findings suggest that antibody-induced V2 mutations may affect envelope
6 oligomers on the viral surface so that they form a more closed conformation; thus
7 neutralization epitopes become less accessible to antibodies. The essential amino acid
8 residues responsible for neutralization resistance located at the center of the V2 region may
9 have a role in the interaction with the V3-loop of neighboring gp120 molecules.

10 We previously described the in vitro selection and characterization of a
11 KD-247-escape mutant of JR-FL (67). The amino acid substitution that was critical for the
12 resistant phenotype was Gly to Glu at residue 314 (G314E) in the V3-tip region. The
13 genetically engineered mutant was completely resistant to neutralization by KD-247. Other
14 researchers have also reported the induction of V3-mutated viruses by strain-specific
15 anti-V3 MAb in in vitro culture systems (8, 37, 65). In earlier studies, combinations of
16 genetically cloned viruses and highly potent NAb were used for in vitro selection (8, 37,
17 65). The escape mutants were induced in the presence of high concentrations of MAbs to
18 acquire V3-tip mutation(s). In contrast to these observations in vitro, the Gly-Pro-Gly
19 amino acid sequence in the V3-tip varies to a negligible extent in clinical isolates from
20 HIV-1-infected patients (35). The important role of the V3-tip in forming the β-turn of the
21 V3 loop and in interacting with chemokine receptors may partly explain the discrepancy
22 between in vivo and in vitro studies (19). In addition to V3 loop, variation in or near the
23 V1/V2 region is known to contribute to coreceptor usage of HIV-1 (17, 21, 27, 28, 47, 56,

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64, 66). However, it is possible that HIV-1 suffers critical damage with respect to replication and infectivity through mutation in the V3 region, especially in the tip region, because the V3 loop plays a major role in the interaction of gp120 with coreceptors. Thus, mutations in the V2 region may be important, not only to avoid anti-V3 pressure but also to maintain replication efficiency at a suitable level.

In the present study, which used a MOKW primary virus for selection, the virus underwent acquisition of resistance via V2 mutations and then V2 plus V3 mutations in response to increases in the concentration of MAb. By contrast, no V2 mutations were selected in JR-FL by KD-247 pressure in our previous study (67). Because the primary isolate, MOKW, was a few passaged viruses that contained quasi-species of related but distinct viruses, relatively resistant variants with mutations in V2 were easily selected for replication. Pinter et al. found that inherent neutralization resistance in JR-FL is mediated by the V1/V2 domain (50). It is therefore possible that the V1/V2 sequence (or the conformation of this sequence) in JR-FL already had a resistant phenotype against anti-V3 antibodies, because the escape variant underwent mutation directly in the V3-tip of the KD-247-reacting epitope (67).

In the in vitro selection process, the 175th amino acid (Pro or Leu) in the V2 region of MOKW played a crucial role in dramatically changing the oligomeric state of the envelopes. However, MOKW-RDP obtained by prolonged culture in vitro without KD-247 became neutralization-sensitive compared with MOKW. 175P was the amino acid responsible for the change to the neutralization-sensitive phenotype, whereas viruses with 175L became highly resistant to the MAbs and rsCD4. The same phenomenon was observed in the relatively resistant strain JR-FL. 175L is highly conserved among HIV-1

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strains, and is located at the center of the V2 loop (35), and the V2 region also mediates gp41-independent intersubunit contact (5). It is therefore possible that the V2 region, including the 175th residue, by mediating changes in the conformation of the gp120 oligomer, contributes to resistance to neutralization by limiting the exposure of epitopes.

Although the MOKW-RDL virus had highly resistant phenotype against KD-247, MOKW with R166K/D167N and P175L in the V2 region and with the C3 mutations, which were less neutralization-resistant than MOKW-RDL, were expanded in vitro selection. Substitutions at residues 165-167 during the adaptation of various HIV-1 strains to replication in vitro have been reported; the adaptation is associated with an increase of the positive charge of this amino acid motif (1, 13, 39, 52, 57, 63). In our present study, the amino acid change at 166/167 in the V2 region in passaged MOKWs with KD-247 was RD to KN, again increasing the positive charge. MOKW-RDL was partially sensitive to anti-CD4 MAb (RPA-T4) compared with MOKWs with 175P, MOKW-RDP, and other 166/167-mutated MOKWs. Pugach et al. also showed that charged aa at 165-167 with 175L in the V2 region emerged during in vitro replication and that those viruses also had their sensitivity to rsCD4 and resistance to the anti-CD4 antibody slightly changed by the 165-167 aa charge (52). It is therefore possible that the 166th/167th amino acid mutations are necessary to compensate variants with 175L for interactions with CD4 molecules on the target cell membrane. As shown in Fig. 9, KN sequences at positions 166 and 167 with the 175L variant confer replication advantage in PM1/CCR5 cells. Therefore, 166/167th aa may help compensate for the reduced fitness of the viruses with Leu as the 175th aa in PM1/CCR5 cells. C3 mutations may also be involved in a minor compensation effect in replication cycles under moderate selective pressure from KD-247 (45, 60).

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The neutralization resistance of primary HIV-1 variants is considered instrumental for HIV-1 persistence in the presence of NAbs in vivo. Various immunological pressures always induce escape variants by eliciting appropriate mutation(s) (14, 60, 62). In the present study, we found that HIV-1 could escape from the broadly reactive anti-V3 MAb, KD-247, by stepwise mutation in the V2 and V3 regions. These observations strongly support the idea that the major problem facing the development of V3-based immunogens is not sequence variation within V3 but, rather, that access of most V3-directed antibodies to their epitopes in functional Env complexes is blocked, often by the V1/V2 domain (29, 50).

Our observations support the hypothesis that neutralization-escape in a primary isolate is mainly mediated by amino acid substitutions in the V2 region in vivo, because only a moderate selective pressure by neutralizing antibodies against autologous viruses has been reported for infected individuals (9, 14, 26, 50, 62). The large sequence diversity observed for V2 in quasi-species existing in patients may represent the accumulation of escape mutants early in HIV-1 infection in response to NAb pressure. Our observations may also explain why the V3 sequence in quasi-species existing in patients is relatively conserved in the face of a vigorous antibody response, especially in early HIV-1 infection. A recent study by Deeks et al. has important implications for understanding the NAb response against autologous virus (9): although NAb responses against contemporaneous autologous viruses are absent in early HIV infection, they can be detected at low levels in some patients with chronic infection. These data suggest the existence of an NAb response that overcomes the emergence of escape mutants. Further characterization of the response in humans who have potent and broadly neutralizing activities not affected by V1/V2

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blocking effects may allow the identification of additional neutralization sites in HIV-1 Env, which might allow new targets to be identified for vaccine development.

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Acknowledgement

We are grateful to S. Zolla-Pazner of the New York University School of Medicine for providing MAb 447-52D, and Yasuhiro Kou for technical support. We also thank Yoko Kawanami for excellent technical assistance.

This work was supported in part by the Ministry of Health, Labor and Welfare of Japan (H-16-AIDS-001 and -012), a Grant-in aid for Scientific Research (C-18591119) from the Ministry of Education, Science and Culture of Japan, and the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Re-emerging Infectious Diseases.

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1 **Figure legends**

2 Fig. 1. Selection of neutralization-resistant virus variants against KD-247. (A) The selection
3 was carried out in PM1/CCR5 cells, as described in Materials and Methods. (B) Sensitivity
4 of MOKW5p(200) and MOKW9p(2000) to KD-247 as determined by MTT assay.
5 PM1/CCR5 cells ($2x10^3$) were exposed to 100 $TCID_{50}$ of MOKW9p(-), MOKW5p(200) or
6 MOKW9p(2000), and were cultured in the presence of various concentrations of KD-247.
7 The IC_{50} values were determined by MTT assay on day 7 of culture. The assay was
8 conducted in duplicate. The values shown are representative of three separate experiments.

9

10 Fig. 2. Amino acid sequences of gp120 from the supernatants of MOKW-infected
11 PM1/CCR5 cells passaged in the presence or absence of KD-247. Viral RNA from the cell
12 culture supernatants at several concentrations of KD-247 was reverse-transcribed. After
13 subjecting the obtained cDNAs to PCR amplification and cloning, the *env* regions in the
14 viruses passaged in the presence or absence of KD-247 were sequenced. The V2, V3 and
15 C3 regions are denoted. The top amino acid sequence represents one of major sequences
16 from supernatants of MOKW-infected PBMCs. The locations and numbers of specific
17 amino acids, based on the HXB2 sequence, are shown above the consensus line. The
18 numbers show the number of clones with the listed sequence among the total number of
19 clones tested. For each set of clones, the deduced amino acid sequence of the gp120 was
20 aligned by the single amino acid code. Dots denote sequence identity.

21

22 Fig. 3. Schematic representation of recombinant MOKW *env* genes used for analysis of the
23 genetic basis for resistance to KD-247. MOKW-RDP, MOKW-KNL/C3m and

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MOKW-KNL/V3m *env* genes were amplified from passaged MOKW-infected PM1/CCR5 cells in the absence or presence of KD-247. MOKW-KNL, MOKW-RDP/V3m and MOKW-RDP/C3m *env* genes were constructed by replacing each region of MOKW-RDP with the corresponding MOKW-KNL/C3m or MOKW-KNL/V3m sequence. MOKW-KNP, MOKW-RDL, MOKW-KDL and MOKW-RNL were constructed by site-directed mutagenesis. Construction of the clones and mutagenesis procedures are described in the Materials and Methods. The locations and numbers of specific amino acids, based on the HXB2 sequence, are shown above the MOKW-RDP sequence.

Fig. 4. Neutralization sensitivities of pseudoviruses with *env* genes from passaged MOKW viruses to MAbs, rsCD4 and CCR5 inhibitors. Pseudoviruses that have envelope sequences listed in Fig. 4 were prepared as described in the Materials and Methods. KD-247, 447-52D, rsCD4 and 17b were preincubated with 100 $TCID_{50}$ of each MOKW pseudotype virus for 15 min, followed by addition of the mixtures to the target cells (GHOST-hi5). The target cells were treated with TAK-779 and 2D7 for 15 min, followed by inoculation of the pseudotype clones. The inhibitory effects were determined by measuring the luciferase activities on day 2 of culture.

Fig. 5. Comparison of antibody binding to cell surface-expressed MOKW Envs. (A) 293T cells transfected with MOKW Env-expression vectors were harvested at 24 h post-transfection and stained with KD-247. Flow cytometry data for binding of the KD-247 (black lines) to cell surface MOKW Envs are shown among GFP-gated 293T cells along with the control antibody (normal human IgG; dotted lines). The number at the top right of

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1 each graph shows the mean fluorescence intensity (MFI). (B) Each bar indicates the relative
2 binding of KD-247, 447-52D and 17b to MOKW Env-expressing cell surfaces. Data were
3 normalized to each antibody's MFI for MOKW-RDP.
4
5 Fig. 6. Neutralization sensitivities of pseudoviruses with *env* genes from MOKW9C(-) with
6 selected V2 mutations to MAbs, rsCD4 and CCR5 inhibitors. Pseudoviruses that have
7 envelope sequences with the selected V2 mutations listed in Fig. 4 were prepared as
8 described in Materials and Methods. KD-247, 447-52D, rsCD4 and IgGb12 were
9 preincubated with 100 $TCID_{50}$ of each MOKW pseudotype virus for 15 min, followed by
10 addition of the mixtures to the target cells (GHOST-hi5). Target cells were treated with
11 TAK-779 and RPA-T4 for 15 min, followed by inoculation of the pseudotype clones.
12 Inhibitory effects were determined by measuring the luciferase activities on day 2 of
13 culture.
14
15 Fig. 7. Binding affinity of anti-V3 MAbs to monomeric gp120. Viral lysates for each
16 MOKW pseudovirus were used. Gp120 was captured onto microtiter wells using a sheep
17 polyclonal antibody specific for the C terminus of gp120. Serial dilutions of KD-247 or
18 447-52D were tested for binding by ELISA. Because of differences in the amount of bound
19 gp120, optical density at 405 nm (OD_{405}) values were normalized to saturating levels of
20 antibody (5 μg/ml) for comparison.
21
22 Fig. 8. Comparison of antibody binding to cell surface-expressed MOKW Envs with V2
23 mutations. (A) 293T cells transfected with MOKW Env-expression vectors were harvested

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at 24 h post-transfection and stained with KD-247. Flow cytometry data for binding of the KD-247 (black lines) to cell surface MOKW Envs are shown for GFP-gated 293T cells along with data for the control antibody (normal human IgG; dotted lines). The number at the top right of each graph shows the mean fluorescence intensity (MFI). (B) Each bar indicates relative binding of KD-247, 447-52D and IgGb12 to MOKW Env-expressing cell surfaces. Data were normalized to each antibody's MFI for MOKW-RDP.

Fig. 9. Replication kinetics of infectious molecular clone NL-MOKW-RDL and NL-MOKW-KNL. PM1/CCR5 cells were exposed to NL-MOKW-RDL (open square) or the NL-MOKW-KNL (filled square) and cultured for 10 days. Virus replication was monitored by measuring the amounts of p24 gag protein produced in the culture supernatants. The data is representative of the results from two independent experiments.

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