学　位　論　文

Doctoral Thesis

Effect of maraviroc-resistant mutations on sensitivity to anti-HIV-1 antibody-mediated neutralization

（マラビロク耐性変異が抗HIV−1抗体による中和感受性に及ぼす影響）

ブンチャワリト　サマッチャヤ

Boonchawalit Samatchaya

熊本大学大学院医学教育部博士課程医学専攻

エイズ先端研究者育成コース

指導教員

吉村　和久　客員准教授

熊本大学大学院医学教育部博士課程医学専攻エイズ学 XII

松下　修三　教授

熊本大学大学院医学教育部博士課程医学専攻エイズ学 II

２０１５年９月

学　位　論　文

Doctoral Thesis

Effect of maraviroc-resistant mutations on sensitivity to anti-HIV-1 antibody-mediated neutralization

（マラビロク耐性変異が抗HIV−1抗体による中和感受性に及ぼす影響）

ブンチャワリト　サマッチャヤ

Boonchawalit Samatchaya

指導教員名：熊本大学大学院医学教育部博士課程医学専攻エイズ学 XII

吉村　和久

熊本大学大学院医学教育部博士課程医学専攻エイズ学 II

松下　修三

審査委員名　　エイズ学 III 担当教授　　岡田　誠治

免疫識別学担当教授　　西村　泰治

感染防御学担当准教授　　前田　洋助

エイズ学 VII 担当准教授　　鈴　伸也

2015 年 9 月

CONTENTS

CONTENTS (Cont.)

SUMMARY

Background and Purpose: A maraviroc (MVC) resistant virus generated using *in vitro* selection exhibited high sensitivity to several neutralizing monoclonal antibodies (NMAbs) compared to the passage control (PC) virus. We tried to identify that MVC resistant mutations, especially four mutations; T297I, M434I, V200I and K305R, which appeared sequentially in the Env region of the MVC resistant virus had a role in changing the neutralization susceptibility to NMAbs.

Methods: MVC resistant and PC infectious clones carrying MVC resistant mutation(s) were constructed using NL4-3 virus as a backbone. The sensitivity of chimeric viruses to anti-V3 (447-52D and KD-247), anti-CD4 binding site (b12 and 0.5δ), soluble CD4 (sCD4) and anti-CD4 induced epitope (4E9C) NMAbs, autologous plasma IgGs and heterologous sera was determined. Constructed virus and PM1/CCR5 cell were added to serially diluted NMAbs or sera and incubated for 6 days. The WST-8 assay was performed to determine cell viability.

Results: The MVC resistant virus became more sensitive to almost all NMAbs and autologous plasma IgGs after the first mutation, T297I, appeared in Env and the sensitivity increased following accumulation of the M434I and V200I mutations. However, the sensitivity to those antibodies reduced after the virus acquired K305R mutation. The M434I mutation conferred the greatest neutralizing sensitivity among four MVC-resistant mutations, and the single M434I mutation was sufficient for the enhanced neutralization by NMAbs, autologous plasma IgGs and heterologous sera relative to the parental virus. V65K mutation in C1 region and amino acid changes in gp41 of MVC resistant virus are also involved in changing the sensitivity to NMAbs.

Conclusions: Taken together, the Env mutations induced under MVC pressure increased neutralization sensitivity to diverse NMAbs and plasmas or sera from HIV-1 infected patients. Those mutations apparently affect the envelope conformation and expose epitopes for NMAbs.

LIST OF REFERENCE ARTICLE

1. Boonchawalit S, Harada S, Shirai S, Gatanaga H, Oka S, Matsushita S, Yoshimura K. Impact of maraviroc-resistant mutation M434I in the C4 region of HIV-1 gp120 on sensitivity to antibody-mediated neutralization. **Jpn J Infect Dis**, 2015..
2. Yoshimura K, Harada S, Boonchawalit S, Kawanami Y, Matsushita S. Impact of Maraviroc-resistant and Low CCR5-1 adapted Mutations Induced by in vitro Passage on Sensitivity to Anti-Env Neutralizing Antibodies**.** J Gen Virol., 95, 2014
3. Harada S, Yoshimura K, Yamaguchi A, Boonchawalit S, Yusa K, Matsushita S., Impact of antiretroviral pressure on selection of primary human immunodeficiency virus type 1 envelope sequences in vitro, J Gen Virol., 94, 2013

ACKNOWLEDGEMENTS

I would like to expose the deepest appreciation to my supervisor, Associate Professor Kazuhisa Yoshimura, for his great support and assistance in the pursuit of this study and in the preparation of this thesis. I am also very grateful to my colleagues, Dr. Shigeyoshi Harada and Miss Mai Goryo for their guidance and support my experiments. This thesis had been finished with the advice, suggestion and helps from all of them.

In addition, I express my sincerely thanks to Professor Shuzo Matsushita for kindly give the advice and accepted me as a temporary member in his laboratory. It was my precious time to join in his group and I wish I could have more time to stay with them. I also thanks to Dr. Tetsuro Matano, the director of AIDS Research Center, National institute of Infectious Diseases, for the opportunity to accomplish this research in the institute. I'd like to give special thanks to Dr. Mark de Souza for not only English proofreading of almost every article I have written during the Ph.D. course, but also his supports in every important moments during my staying in Tokyo.

I give many thanks for all of spiritual supporters, my family and friends, for always beside me and let me be myself.

Finally, I would like to express my gratitude to thank the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) for a financial support. Without the scholarship from MEXT, I would never have the opportunity to be a foreign student in Japan.

LIST OF ABBREVIATIONS

ARV : Antiretroviral

R5 : CCR5-tropic

Env : Envelope

HR-1/-2 : Heptad repeat-1/-2

MVC : Maraviroc

ART : Antiretroviral therapy

MPI : Maximum per cent inhibition

IC_{50} : Half maximal inhibitory concentration

NAb : Neutralizing antibody

BNAb : Broad neutralizing antibody

CD4bs : CD4 binding site

CD4i : CD4 induced

PC : Passage control

NMAb : Neutralizing monoclonal antibody

sCD4 : Soluble CD4

1. BACKGROUND AND OBJECTIVE OF STUDY

1.1 Background

HIV/AIDS is a global pandemic. As in 2013, approximately 35 million people are living with HIV/AIDS and 1.5 million people died from AIDS related illnesses. The number of newly infection at the end of 2013 was 2.1 million people. Sub-Saharan Africa was the most critical area due to about 25 million HIV infected people resided there (WHO, 2014). UNAIDS reported that there were 14,706 HIV infected and 6,719 AIDS cases in Japan at the end of 2012. The number of HIV cases in Japan continued to increase since HIV was discovered however, it has been slightly decreased from 2008. There were 1,002 newly infected and 447 AIDS cases in the year 2012. Sexual contact was the main route of transmission which 72% was homosexual and only 18% through heterosexual contact (UNAIDS, 2015).

About 13 million people living with HIV were receiving ARV drug that is only 36% of globally HIV infected patient (WHO, 2014). The development of HIV drug started soon after HIV was discovered. The successful of a combination therapy called HAART decreased HIV morbidity and mortality rate. However, there is still a great demand for the developments of ARV drug as a reason of many patients are suffer from side effects and importantly, many of them develops drug resistance. The emerging of drug resistant HIV variants obstructs the successful treatment by those drugs. Recently, there are more than 20 approved ARV drugs in 6 classes licensed for the treatment of HIV-1(FDA, 2014). The patients with a history of failure or suffer from the side effect of some ARV drug can be successfully treat by other drugs. However, the development of ARV will always be needed due to the virus continues to escape from the drug effect. HIV-1 has a rapid turnover (Arts and Hazuda, 2012; Ho et al., 1995), about $\sim 10^{10}$/day. Together with the errors made during viral replication, HIV-1 genome is highly mutated. It is possible that 1-10 mutations occur in every replication cycle (Arts and Hazuda, 2012).

HIV-1 infects the target cell using the viral envelope gp120 region binds to its receptor, CD4, on the surface of macrophages or T-helper lymphocytes (Kuritzkes, 2009).The binding to CD4 changes the conformation of gp120 then, exposes the structure elements that involves in binding to chemokine receptors, CCR5 or CXCR4, on the target cell. Consequently, the fusion peptide of gp41 ectodomain inserts into the target cell membrane. The two helically coiled heptad repeats (HR-1 and HR-2) in gp41 form a six-helix bundle leads to the virus membrane come close to target cell, resulting in fusion (Chan et al., 1997) (Fig. 1). Two interactions between CCR5 co-receptor and gp120 domain are elucidated. The V3 loop of HIV-1 envelope bind to extracellular loop 2 (ECL2) of CCR5 co-receptor and a site on gp120 that involves the 4-stranded bridging sheet region and the base of V3 interacts with CCR5 N-terminus (Berro et al., 2009; Cormier and Dragic, 2002; Huang et al., 2005; Rizzuto et al., 1998; Roche et al., 2013).

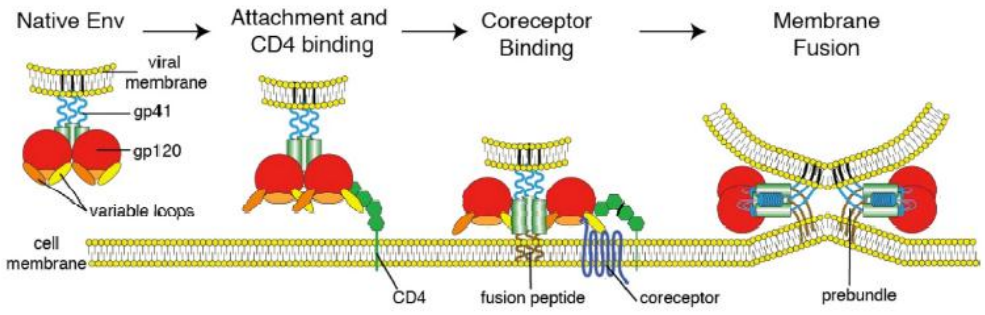


Figure 1. **A model for HIV entry.** The picture is modified from Didigu, C.A. and Doms, R.W, "Novel Approaches to Inhibit HIV Entry," 2012 (Didigu and Doms, 2012). HIV-1 Env (red) binds to CD4 molecule (green) on host cell then triggers changes of the structural of the gp120 variable loops (orange and yellow) and exposes the area for binding to co-receptor (blue). Then, the fusion peptide (brown) of gp41 ectodomain inserts into the target cell membrane. The six-helix bundle is formed leads to the virus membrane come close to target cell, resulting in fusion.

The recent ARV drugs targets on HIV-1 life cycle, mainly on inhibit the replication cycle and cell entry. The CCR5 inhibitor is a class of ARV recently represented only by maraviroc (MVC) which was approved by U.S. FDA in 2007 to treat CCR5-tropic (R5) HIV-1 infected patient (FDA, 2014). MVC binds to a hydrophobic pocket in CCR5 transmembrane domain (Fig. 2B) leads to alter the conformation of the second extracellular loop of CCR5 co-receptor and inhibits the binding with the V3 loop of HIV-1 gp120 subsequently, virus entry is inhibited (Fig.2) (Berro et al., 2009; Dragic et al., 2000; Kondru et al., 2008; Pugach et al., 2008; Tsamis et al., 2003).

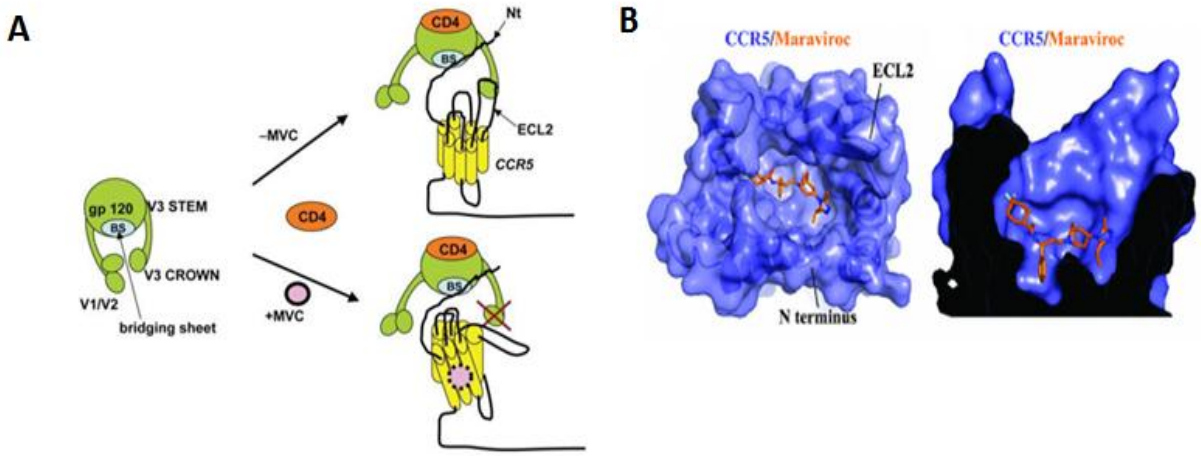


Figure 2. **A model of action of MVC.** The picture is modified from Soriano V , "Optimal use of MVC in clinical practice", 2008 (Soriano et al., 2008) (A) and Qiuxiang T, "Structure of the CCR5 Chemokine Receptor–HIV Entry Inhibitor Maraviroc Complex", 2013 (Tan et al., 2013) (B). HIV-1 gp120 (green) bind to CD4 molecule (orange) leads to exposes the co-receptor binding site on HIV Env. V3 loop and base of V3 of HIV Env binds to a second extracellular loop (ECL2) and N-terminal of CCR5 co-receptor, respectively. MVC (pink) binds to a hydrophobic pocket in CCR5 transmembrane domain (yellow) then, the structural ECL2 have changed so that the viral entry is inhibited (A). MVC (orange stick) properly binds to a hydrophobic pocket of CCR5 co receptor (blue) (B).

Although MVC and another CCR5 inhibitor, vicriviroc (VCV), can efficiently suppress HIV-1 replication, resistant variants can arise both *in vitro* and *in vivo* (Asin-Milan et al., 2013; Berro et al., 2009; Delobel et al., 2010; Kuhmann et al., 2004; Marozsan et al., 2005; Ogert et al., 2009; Ogert et al., 2010;

Ratcliff, Shi, and Arts, 2013; Roche et al., 2011; Roche et al., 2013; Swenson et al., 2013; Tilton et al., 2010; Tsibris et al., 2008; Westby et al., 2007; Yuan et al., 2011; Yuan et al., 2013; Yusa et al., 2005). MVC resistant virus gain the ability to enter cells via the inhibitor-CCR5 complex (non-competitive binding) while retaining the use of free CCR5 (Berro et al., 2012; Pugach et al., 2007). The reducing of percent maximum inhibitor (MPI) is a characteristic of MVC resistant virus rather than the increasing of percent inhibitory concentration (IC_{50}) (Asin-Milan et al., 2013; Pugach et al., 2007; Roche et al., 2013; Westby et al., 2007). The presence of MVC resistant variant may due to the outgrowth of pre-existing CXCR4-tropic viruses (Fätkenheuer et al., 2008; Westby et al., 2006) or the selection of MVC resistant CCR5 mutants (Swenson et al., 2013; Yuan et al., 2013). MVC resistant mutations are commonly detected in the gp120 V3 loop and are strain specific (Asin-Milan et al., 2013; Berro et al., 2012; Berro et al., 2009; Swenson et al., 2013; Yoshimura et al., 2014; Yuan et al., 2011).

Neutralizing antibody (NAb) against HIV-1 Env appears relatively slow after the onset of infection. HIV-1 antibody in patient's plasma is detected ~2-5 weeks after transmission (Tomaras et al., 2008) and the autologous NAb arises more than one month after detection of HIV-specific antibodies (Wei et al., 2003). About 10-20% of HIV-1 patients generated potent cross-reactive NAbs (Mascola and Haynes, 2013). However, only a few individuals develop broadly cross-NAb (BNAb) which typically takes ~2 to 3 years to develop (Gray et al., 2011; Mikell et al., 2011).

The antibody response to the virus stimulates a viral escape, leads to the mutant virus resistant to autologous plasma (Liao et al., 2013; Wei et al., 2003). The virus can evade this autologous antibody response by several means, including generation of epitope-specific mutations, decoration with a dense glycan shield, hypervariable loops that mask conserved features and high intrinsic conformational dynamics that render it a poorly defined antigen (Pantophlet and Burton, 2006).

The envelope variable loop, CD4 binding site (CD4bs), chemokine coreceptor binding site, N-linked glycan and membrane proximal gp41 domains are known to be epitopes for neutralization targets (Decker et al., 2005). The critical neutralizing epitopes such as V3 domain, CD4bs and chemokine coreceptor binding site are transiently or not exposed on the trimer form (Poignard et al., 2001; Zwick et al., 2001). Interestingly, the increasing of the accessibility to epitopes of some neutralizing monoclonal antibodies (NMAbs) has been reported for entry inhibitor resistant variants. Other CCR5 inhibitors, AD101 or vicriviroc escape viruses were more sensitive to neutralizing antibodies and some of sera from HIV-1 infected patients compared to the parental virus (Pugach et al., 2008). Moreover, the MVC resistant virus obtained from the patient who experienced on virologic failure on treatment regimen containing MVC showed slightly more neutralization sensitive to anti-CD4bs b12 and gp41 2F5 NMAbs and HIV-IgG (Tilton et al., 2010). These previous studies imply that mutation(s) in Env that confer resistance to the entry inhibitors may increase the accessibility to epitopes of neutralizing antibodies.

In our recent study, we induced *in vitro* selected-MVC resistant virus using the PM1/CCR5 cell line. Four accumulated MVC-resistant mutations, T297I, M434I, V200I and K305R, were detected sequentially. The MVC resistant virus became greater sensitive to Env NMAbs: anti-V3, CD4 induced epitope (CD4i) and CD4bs and autologous plasma IgGs compared to the passage control (PC) virus (Yoshimura et al., 2014). However, it is still not clear which single or combination of mutation(s) affect the accessibility of and autologous plasma IgGs to the epitopes in Env.

In this study, we assessed which mutation(s) confers the greatest enhanced neutralization sensitivity of the MVC resistant variant. We constructed several infectious clones with single or a combination of the four MVC resistant mutations, V200I, T297I, K305R and M434I, and determined the effect on the neutralization sensitivity to three different kinds of NMAb, autologous plasma IgGs and heterologous sera from HIV-1 infected subjects.

1.2 Objective

We determined the effect of MVC resistant mutations on the neutralization sensitivity to many kinds of NMAbs and patient sera. Our previous study showed that the *in vitro* selected MVC resistant virus markedly increased the sensitivity to difference types of NMAbs and autologous plasma IgGs. We hypothesized that four MVC resistant mutations that located in gp120 domain of Env might involve in changing the sensitivity to those antibodies. Moreover, we also aimed to determine mutation(s) among those four mutations that confer highly sensitivity to anti-Env antibodies. Revealing the relationship between drug resistant mutation and the sensitivity to neutralizing antibody might suggests the possibility of using a combination of MVC and neutralizing antibody for HIV-1 infected patient.

2. MATERIALS AND METHODS

2.1 Viruses

The *in vitro* selected-maraviroc resistant and PC infectious viruses were obtained from the previous study (Yoshimura et al., 2014). Briefly, HIV-1 KP-5 was isolated from HIV-1 subtype B infected patient and passaged in PHA-activated PBMCs. Infected PBMCs were co-cultured for 5 days with PM1/CCR5 cells and the culture supernatants were store at -150^{o}C (Yoshimura et al., 2010). The virus was isolated prior to MVC therapy. MVC resistant virus was generated by culturing KP-5 in PM1/CCR5 cell with the increased concentration of MVC (0-10,000 nM) for 48 passages. The KP-5 was also cultured in PM1/CCR5 cell without MVC for 48 passages to obtain the PC virus. *Env* of viruses obtained from passage 48 were amplified by nested PCR then, clone into the cloning vector. The gp160 regions of selected clones were introduced into pNL4-3 backbone to generate the MVC resistant and PC infectious clones (Harada et al., 2013; Hatada et al., 2010; Yoshimura et al., 2014).

2.2 Cells and culture conditions

The CD4-positive T-cell line PM1 expressing high CCR5 co-receptor, PM1/CCR5, was kindly provided by Dr. Yosuke Maeda (Maeda, Yusa, and Harada, 2008; Yoshimura et al., 2014). PM1/CCR5 cells were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, South Logan, UT) and 0.1 mg/ml of G418 (Nakarai Tesque, Kyoto, Japan). 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum.

2.3 Antibodies and inhibitors

Anti-V3 NMAbs: KD-247 (Eda et al., 2006; Yoshimura et al., 2006) and 447-52D (Zolla-Pazner et al., 1999), anti-CD4bs: 0.5∂ (3D6) (Ramirez Valdez et al., 2014; Yoshimura et al., 2010) and b12 (Kessler et al., 1997) and anti- CD4i: 4E9C (Yoshimura et al., 2010) were used for neutralization susceptibility assays. KD-247 was kindly provided by The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). 0.5∂ and 4E9C were established in the Center for AIDS Research, Kumamoto University. The b12 and 447-52D were purchased from Polymun Scientific Immunbiologische Forschung GmbH (Klosterneuburg, Austria). The CCR5 inhibitor MVC was kindly provides by Pfizer (Groton, CT). sCD4 was purchased from R&D Systems, Inc. (Minneapolis, MN).

2.4 Patient plasma IgGs and sera

Autologous plasma samples were collected and purified using Protien A-Sepharose (Affi-gel Protein A; Bio-Rad,Hercules, CA) (Kimura et al., 2002; Yoshimura et al., 2014; Yoshimura et al., 2010). The purified plasma IgGs were designated KP-5-IgG-1, -2, -5 and -7.

Sera from HIV-1 infected patients were provided by the National Center for Global Health and Medicine（Tokyo, Japan）. The sera from two HIV-1 subtype B infected patient, ACC-B1 and ACC-B2, were collected before and during the treatment with MVC. ACC-B1-1 and ACC-B2-1 sera were collected from patients 1 week before treatment with MVC. Sera ACC-B1-2 and ACC-B2-2 were collected 2 and 5 weeks after starting treatment with MVC. Sera ACC-B1-3 and ACC-B2-3 were collected 8 weeks after starting treatment with MVC. Other subtype-B and non-B sera were obtained from antiretroviral therapy (ART) naïve patients. Phlebotomy was performed following signed informed consent in accordance with the study protocol. The study was reviewed and approved by the Ethics committee for clinical research & advanced medical technology at the Kumamoto University Medical School (Kumamoto, Japan) and the National Center for Global Health and Medicine (Tokyo, Japan).

2.5 Construction of Env/NL4-3 chimeric infectious clones

2.5.1 Construction of wild type and mutated KP-5 MVC resistant and PC infectious clones

The gp160 region of the *in vitro* selected MVC resistant, and PC were amplified using primers EnvFv (5-AGCAGAAGACAGTGGCAATGAGAGCGAAG-3) and EnvR (5-AGCAGAAGACAGTGGCAATGAGAGCGAAG-3). Each point mutation was introduced into the MVC resistant and PC gp160 region by site-directed mutagenesis, using PrimeSTAR mutagenesis Basal Kit (TAKARA Bio Inc, Shiga, Japan) and the specific mutagenesis primer sets as shown in Table 1.

Table 1. List of primers for site-direct mutagenesis

Infectious clone	DNA template	Forward primer	(5' – 3')	Reverse primer	(5' – 3')
MVC-R (4m-)	MVC-R(T297I)	YTA_I297T_F	ATTGTACAAGACCCAACAACAATACT	YTA_I297T_R2	GGGTCTTGTACAATTAATGTACACAGG
MVC-R (T297I)	MVC-R(T297I/M434I)	YTA_I434M_F2	AAAGCAATGTATGCCCCTCCCATCAGT	YTA_I434M_R2	GGCATACATTGCTTTTCCTACTTCTTG
MVC-R (T297I/M434I)	MVC-R(V200I/T297I/M434I)	YTA_I210V_F	ACCTCAGTCATAACACAGGCCTGTCCA	YTA_I210V_R2	TGTAATGACTGAGGTGTTACAACTTGT
MVC-R (T297I/M434T/V200I)	MVC-R(T297I/M434I/V200I/K305R)	YTA_R305K_F	ACAAGAAAAGGTATACATATAGGACCA	YTA_R305K_R	TATACCTTTTCTTGTATTGTTGTTGGG
MVC-R (M434I)	MVC-R	YTA_M434I_F	AAAGCAATATATACCCCTCCCATCAGT	YTA_M434I_R	GGTATATATTGCTTTTCCTACTTCTTG
MVC-R (V200I)	MVC-R	YTA_V200I_F	ACCTCAATCATTACACAGGCCTGTCCA	YTA_V200I_R	TGTAATGATTGAGGTGTTACAACTTGT
MVC-R (K305R)	MVC-R	YTA_K305R_F	ACAAGAAGAGGTATACATATAGGACCA	YTA_K305R_R	TATACCTCTTCTTGTATTGTTGTTGGG
PC (V65K)	PC	YTA_V65K_F	ACAGAGAAGCATAATGTTTGGGCCACA	YTA_V65K_R	ATTATGCTTCTCTGTATCATATGCTTT
PC (V65M)	PC	YTA-2-40_V65M_F	ACAGAGATGCATAATGTTTGGGCTACA	YTA-2-40_V65M_R	ATTATGCATCTCTGTATCATATGCTTT
MVC-R (K65V/T297I/M434I/V200I/K305R)	MVC-R(T297I/M434I/V200I/K305R)	YTA_K65V_F	ACAGAGGTGCATAATGTTTGGGCCACA	YTA_K65V_R	ATTATGCACCTCTGTATCATATGCTTT
MVC-R (K65M/T297I/M434I/V200I/K305R)	MVC-R(T297I/M434I/V200I/K305R)	YTA_K/V65M_F	ACAGAGATGCATAATGTTTGGGCCACA	YTA_K/V65M_R	ATTATGCATCACTGTATCATATGCTTT
MVC-R (T297I/V200I/K305R)	MVC-R(T297I/M434I/V200I/K305R)	YTA_I434M_F	AAAGCAATGTATGCCCCTCCCATCAGT	YTA_I434M_R	GGTATATATTGGTTTTCCTACTTCTTG
MVC-R (4m-/K65V)	MVC-R	YTA_K65V_F	ACAGAGGTGCATAATGTTTGGGCCACA	YTA_K65V_R	ATTATGCACCTCTGTATCATATGCTTT
MVC-R (K65V/T297I/V200I/K305R)	MVC-R(K65VT297I/M434I/K305R)	YTA_I434M_F	AAAGCAATGTATGCCCCTCCCATCAGT	YTA_I434M_R	GGTATATATTGCTTTTCCTACTTCTTG
JRFL (M434I)	JRFL	JRFL_M434I_F	AAAGCAATATATGCCCCTCCCATCAGA	JRFL_M434I_R	GGCATATATTGCTTTTCCTACTTCCTG
JRFL (V65K)	JRFL	YTA_V65K_F	ACAGAGAAGCATAATGTTTGGGCCACA	YTA_V65K_R	ATTATGCTTCTCTGTATCATATGCTTT
JRFL (V65M)	JRFL	YTA_K/V65M_F	ACAGAGATGCATAATGTTTGGGCCACA	YTA_K/V65M_R	ATTATGCATCACTGTATCATATGCTTT
YU2 (M434I)	YU2	JRFL_M434I_F	AAAGCAATATATGCCCCTCCCATCAGA	JRFL_M434I_R	GGCATATATTGCTTTTCCTACTTCCTG
YU2 (V65K)	YU2	YTA_V65K_F	ACAGAGAAGCATAATGTTTGGGCCACA	YTA_V65K_R	ATTATGCTTCTCTGTATCATATGCTTT
YU2 (V65M)	YU2	YTA_K/V65M_F	ACAGAGATGCATAATGTTTGGGCCACA	YTA_K/V65M_R	ATTATGCATCACTGTATCATATGCTTT

Chimeric wild type and mutated KP-5 Env/NL4-3 infectious viruses were constructed from the pNL4-3 proviral plasmid by overlapping PCR as described previously (Shibata et al., 2007). Briefly, fractions of the NL4-3 provirus containing *Eco*RI restriction site (nucleotides 5284 to 6232) and *Xho*I restriction site (nucleotides 8779 to 9045) were amplified with specific primers set; NL(5284)F (5'-GGTCAGGGAGTCTCCATAGAATGGAGG-3') and NL(6232)Rv (5'-CTTCGCTCTCATTGCCACTGTCTTCTGCT-3') for *Eco*RI fragment and NL(8779)F (5'-GCTATAAGATGGGTGGCAAGTGGTCAAAA-3') and NL(9045)R (5'-GATCTACAGCTGCCTTGTAAGTCATTGGTC-3') for *Xho*I fragment. Overlapping PCR was used to join the mutated/wide type gp160 coding sequence to the *Eco*RI and *Xho*I fragments that had been amplified from pNL4-3 (Fig. 3). The mutated/wide type KP-5 gp160 containing *Eco*RI and *Xho*I enzyme restriction sites was digested with *Eco*RI and *Xho*I restriction enzymes (Takara Bio Inc, Shiga, Japan) then cloned into *Eco*RI/*Xho*I digested NL4-3 proviral plasmid to generate the recombinant KP-5 Env/NL4-3 proviral DNA. The Env region from each constructed infectious clone was sequenced using a 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA).

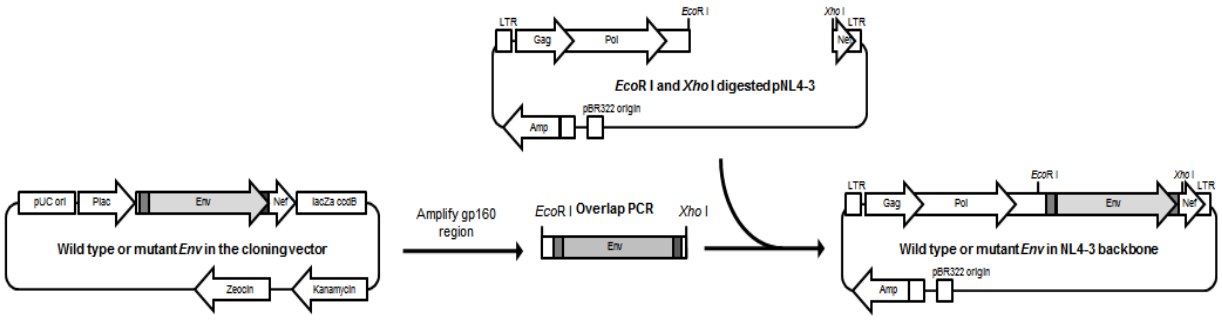


Figure 3. Diagram briefly shows the construction of wild type or mutated KP-5 *Env*/NL4-3 chimeric infectious clones.

2.5.2 Construction of wild type and mutated JRFL and YU2 infectious clones

Chimeric wild type JRFL and YU2 Env/NL4-3 infectious viruses were constructed using slightly difference method. The wild type JRFL and YU2 Env were amplified using EcoRIF (5'-GTGGAAGCCATAATAAGAATTCTGCAACAACTGCTG-3') and NL(9045)R primers then, *Xho*I and *Eco*RI fragments were added at N- and C- terminal respectively using overlapping PCR. The gp160 regions were digested with *Eco*RI and *Xho*I restriction enzymes then cloned into *Eco*RI/*Xho*I digested NL4-3 proviral plasmid to generate the recombinant JRFL or YU2 Env/NL4-3 proviral DNA

Chimeric mutated JRFL and YU2 Env/NL4-3 infectious viruses were constructed using a method as shows briefly in figure 4. The mutated JRFL and YU2 Env were amplified into two fragments from JRFL/NL4-3 and YU2/NL4-3 plasmid which successfully constructed as previous described. The fragment-1 was amplified using EcoRIF and the reverse primer for site-direct mutagenesis as shown in Table 1. The DNA fragment-2 was amplified from the mutagenesis forward primer as shown in Table 1 and NL(9045)R. Both fragments were combined using overlapping PCR with EcoRIF and NL(9045)R primers. Combined DNA was digested with *Eco*RI and *Xho*I restriction enzymes subsequently cloned into *Eco*RI/*Xho*I digested NL4-3 proviral plasmid.

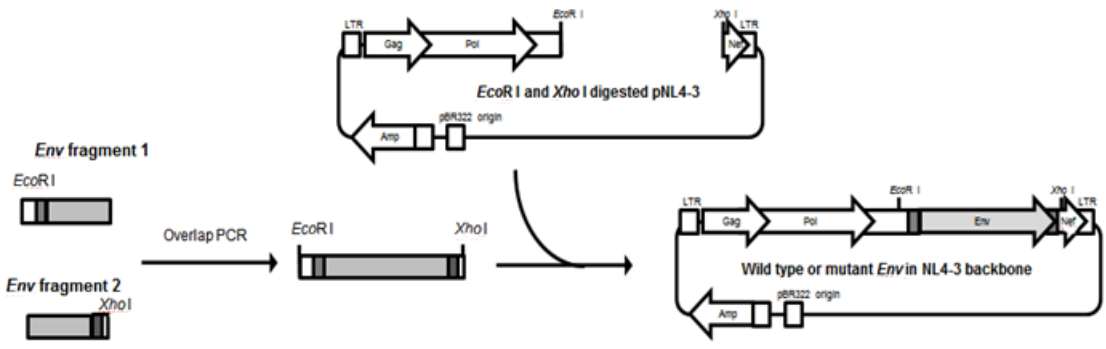


Figure 4. Diagram briefly shows the construction of JRFL and YU2 mutant Env/NL4-3 chimeric infectious clones.

2.5.3 Construction of KP-5 gp120/gp41 chimeric infectious clone

The gp120/gp41 chimeric env were also constructed using overlapping PCR using a method as shows briefly in figure 5. The gp120 region of MVC resistant and PC env was amplified using EnvFv and H primers (5'–TAGTGCTTCCTGCTGCTCCCAAGAACCC-3') and the gp41 region was amplified using KFw (5'-GGGTTCTTGGGAGCAGCAGGAAGCACTA-3') and EnvR primers. The overlapping PCR was used to connect gp120 and gp41 regions to make the chimeric gp120 and gp41 env designated as MVC-R/PC and PC/MVC-R env. The *Eco*RI and *Xho*I sites were attached at the N- and C- terminal of chimeric env respectively using overlapping PCR. The chimeric env was cloned into *Eco*RI/*Xho*I digested NL4-3 proviral plasmid.

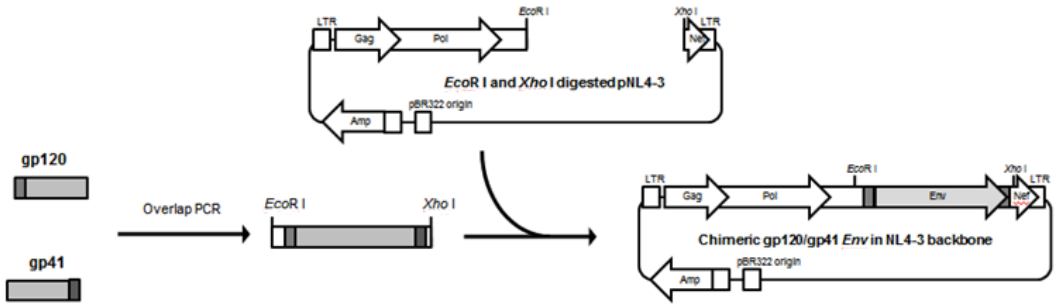


Figure 5. Diagram shows the construction of chimeric gp120/gp41 *Env*/NL4-3 chimeric infectious clones.

The chimeric Env/NL4-3 was transformed to One-shot Stbl3 chemically competent *E*.coli (Invitrogen, Carlsbad, CA). Individual colonies were picked and cultured in Fast-media Amp LB medium (Invivogen, San Diego, CA). The extracted plasmid was sequenced using a 3500xL Genetic Analyzer.

2.6 Preparation of multi replicated-chimeric *Env*/NL4-3 viruses

293T cells were transfected with chimeric Env/NL4-3 proviral DNA using Lipofectamine™ 2000 transfection reagent (invitrogen, Carlsbad, CA). The supernatant was collected 48 hours after transfection and stored at -80°C until use. Viral titer was determined using the Cell Counting Kit-8 assay (WST-8 assay, Dojindo Laboratories, Kumamoto, Japan).

2.7 Neutralization susceptibility assay

The sensitivity of the chimeric infectious viruses to MVC and anti-HIV-1 Env antibodies was determined using PM1/CCR5 cells (Yoshimura et al., 2014). Briefly, $2x10^3$ PM1/CCR5 cells were infected with $100TCID_{50}$ chimeric Env/NL4-3 infectious clones in U-bottom 96-well microplates in the presence of various concentrations of MVC, NMAbs, autologous plasma IgGs or heterologous sera. NMAbs and autologous plasma IgGs were 1log serial dilution and patient' sera were 1log serially diluted from 1:100 to 1:1,000,000. The plate was incubated at 37°C for 7 days. The half maximal inhibitory concentration (IC_{50}) value was determined using the Cell Counting Kit-8 assay. A reciprocal neutralization titer of >100 was used to define serum sensitivity.

2.8 Crystal structure of gp120

The cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer was retrieved from the PDB (entry 3J5M). The side chains of the mutated residues that appeared during MVC are shown in red. Segmentation and color coding is as follows: gp120 (blue), gp41 (orange), V1/V2 (purple), and V3 (green).

2.9 Statistical analysis

Neutralizing antibody titers were summarized using geometric mean titers and statistical comparisons were performed using Student's t test. A p value <0.05 were considered statistically significant. Data were analyzed using Prism 6.05 for Windows, Graphpad Software Inc. (San Diego, CA).

3. RESULTS

3.1 Construction and characterization of MVC resistant mutants

Previously, we induced MVC resistance by *in vitro* selection using the HIV-1 subtype B primary isolate (KP-5) as a parental virus (Yoshimura et al., 2014). This MVC resistant virus contains four MVC resistant mutations; T297I, M434I, V200I and K305R in the gp120 coding region (Fig. 6 and 7A), which appeared sequentially during culture of KP-5 in the presence of increasing concentrations of MVC. The T297I mutation appeared earliest at passage 17. The M434I mutation accumulated at passage 21 followed by the V200I mutation at passage 34. The last mutation, K305R, appeared at passage 41. Moreover, the MVC selected clone became highly sensitive to anti-envelope NMAbs and autologous plasma IgGs (Yoshimura et al., 2014). We concluded that the four MVC-resistant mutations required for entry using MVC-bound CCR5 resulted in a conformational change of envelope protein that is associated with a phenotype sensitive to anti-envelope NMAbs. To examine the role of the four mutations in sensitivity to NMAbs, we constructed several infectious clones containing single or various combinations of mutations within the gp120 backbone of PC or MVC resistant virus (Fig. 7B). The MVC resistant infectious clone harboring the first MVC resistant mutation T297I, MVC-R (T297I), exhibited a lower MPI compared to the MVC resistant infectious clone without the four mutations, MVC-R (4m-): 93% and 102%, respectively. The MPI gradually decreased with the accumulation of all four mutations (from 102% to 86%) (Fig. 7C). The IC_{50} values between the 8 mutant MVC resistant infectious clones were similar (range: 27 to 55 nM), whereas the PC infectious clone showed a low IC_{50} value (4.8 nM) to MVC (Fig. 7C). Among all the mutated infectious clones, the MVC-R (T297I/M434I/V200I/K305R) clone and the clone harboring the K305R single mutation, MVC-R (K305R), showed high resistance to MVC (IC_{50}; 55 and 47 nM and MPI; 86% and 84%, respectively) (Fig. 7C). This result supported our previous report that the MVC resistant virus arose following the accumulation of four mutations and K305R mutation had a major impact on MVC resistance (Yoshimura et al., 2014).

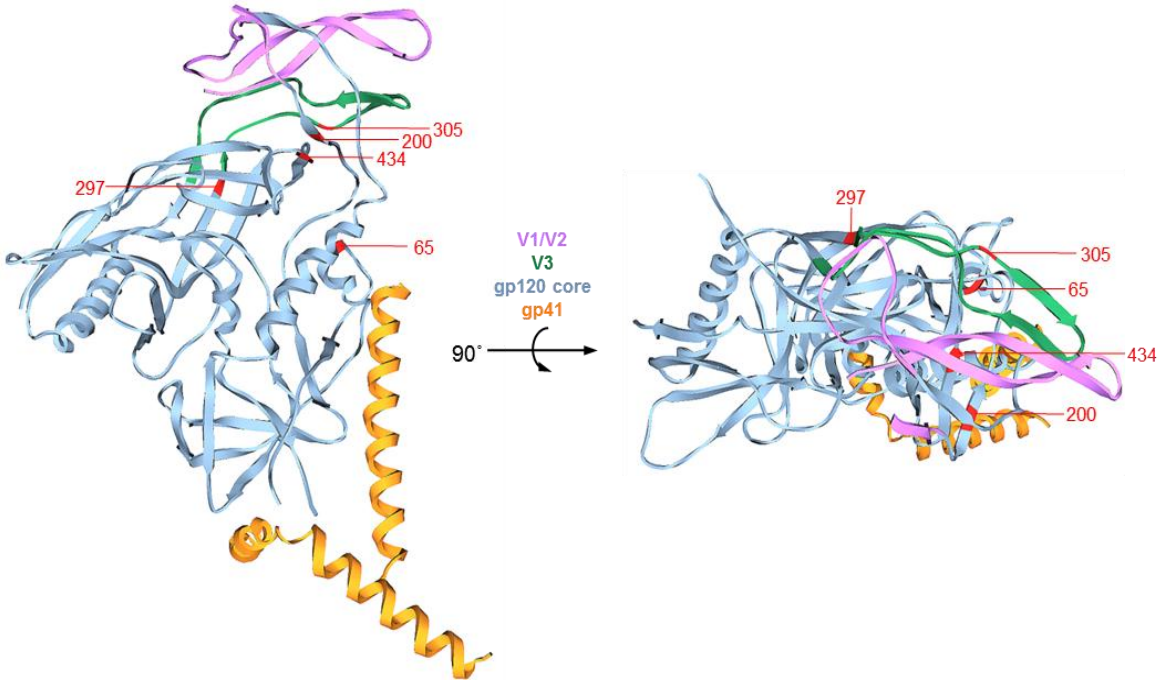
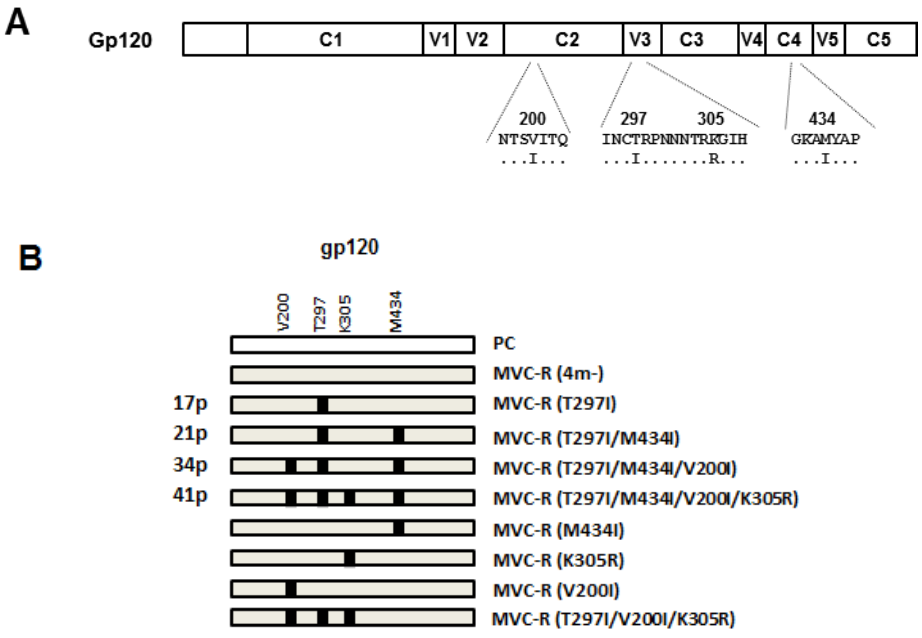
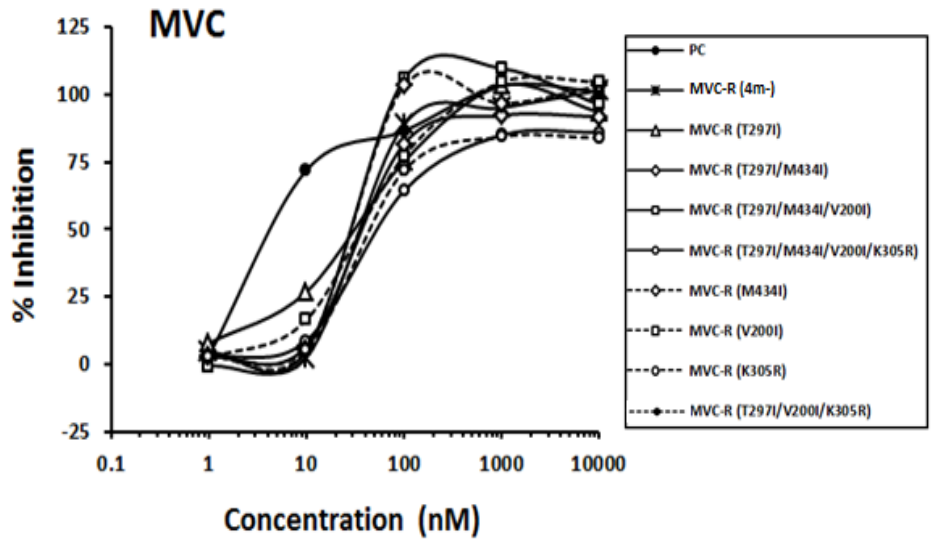


Figure 6. Locations of substitutions in the gp120 induced by *in vitro* selection with MVC. The cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope was retrieved from the PDB (entry 3J5M). Segmentation and color coding is as follows: gp120 (blue), gp41 (yellow), V1/V2 (purple), and V3 (green).



C



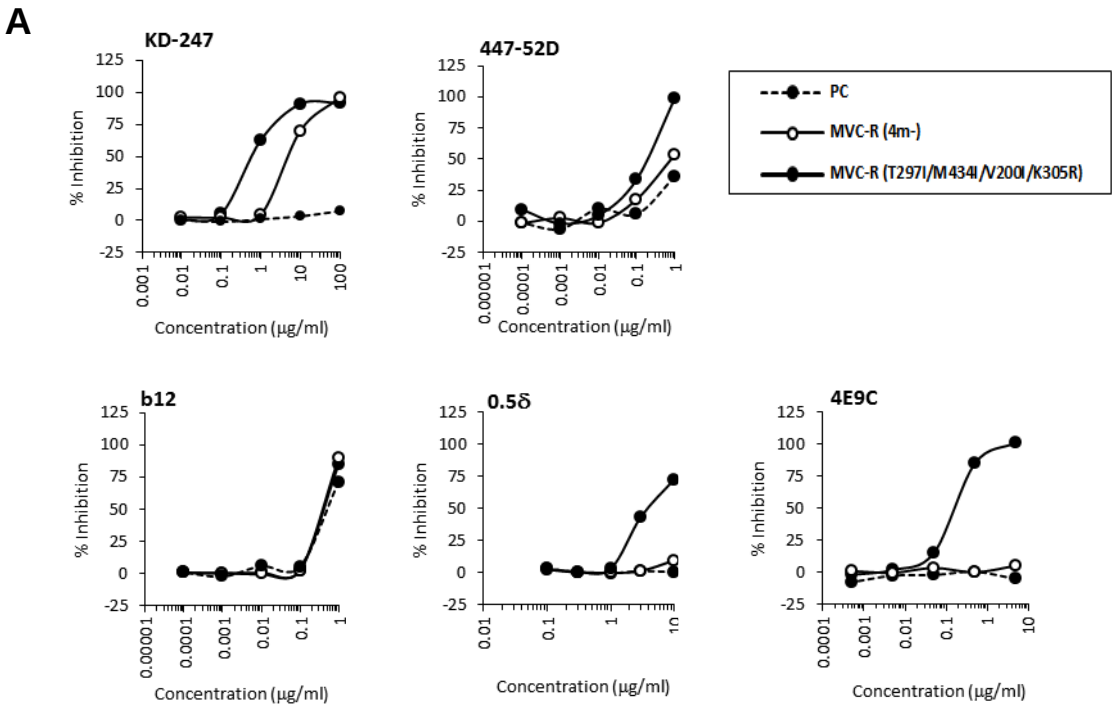
D

Infectious virus	MPI (%)	IC_{50} (nM)
PC	101	4.8
MVC-R (4m-)	102	35
MVC-R (T297I)	93	30
MVC-R (T297I/M434I)	91	38
MVC-R (T297I/M434T/V200I)	96	27
MVC-R (T297I/M434I/V200I/K305R)	86	55
MVC-R (M434I)	103	29
MVC-R (V200I)	105	36
MVC-R (K305R)	84	47
MVC-R (T297I/V200I/K305R)	96	37

Figure 7. Schematic representation of KP-5 *env* gp120 region with four MVC resistant mutations and sensitivity of infectious clones to MVC. The location of the four MVC resistant mutations in the gp120 region (A). Site-direst mutagenesis was used to generate MVC resistant Env carrying combinations of four MVC resistant mutations. MVC resistant Env clones carrying M434I, V200I or K305R single mutations were also constructed (B). Sensitivity of the infectious clones with MVC resistant Env and PC to MVC was determined by the WST-8 assay as described in Materials and methods (C). Data are representative of results from 2 independent experiments. A summary table is also provided.

3.2 Sensitivity of MVC resistant clone without four mutations to anti-Env NMAbs and autologous plasma IgGs

By comparing sensitivities between PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (4m-), which was reverted the 4 mutations to PC, to anti-V3 (KD-247 and 447-52D), anti-CD4bs (b12 and 0.5∂) and anti-CD4i (4E9C) NMAbs, we examined whether the four mutations were responsible for sensitivity to the NMAbs or not. Removing all four mutations resulted in neutralization resistant to 4E9C and 0.5∂ NMAbs and autologous plasma IgGs (KP-5-IgG-1, -2, -5 and -7). The sensitivity were as same as the sensitivity of PC clone (Fig. 8A, 8B and Table 2). MVC-R (4m-) also showed less sensitive to KD-247 and 447-52D NMAbs compared to the sensitivity of MVC resistant virus containing all 4 mutations, MVC-R (T297I/M434I/V200I/K305R) (Fig. 8A). These results indicate that the four MVC resistant mutations highly influenced the increased sensitivity of MVC resistant infectious virus to different NMAb and also autologous plasma IgGs. The sensitivity of MVC resistant to anti-V3 NMAbs, KD-247 and 447-52D, might also influenced by other amino acid substitution therefore, MVC-R (4m-) clone was sensitive to both antibodies than PC clone.



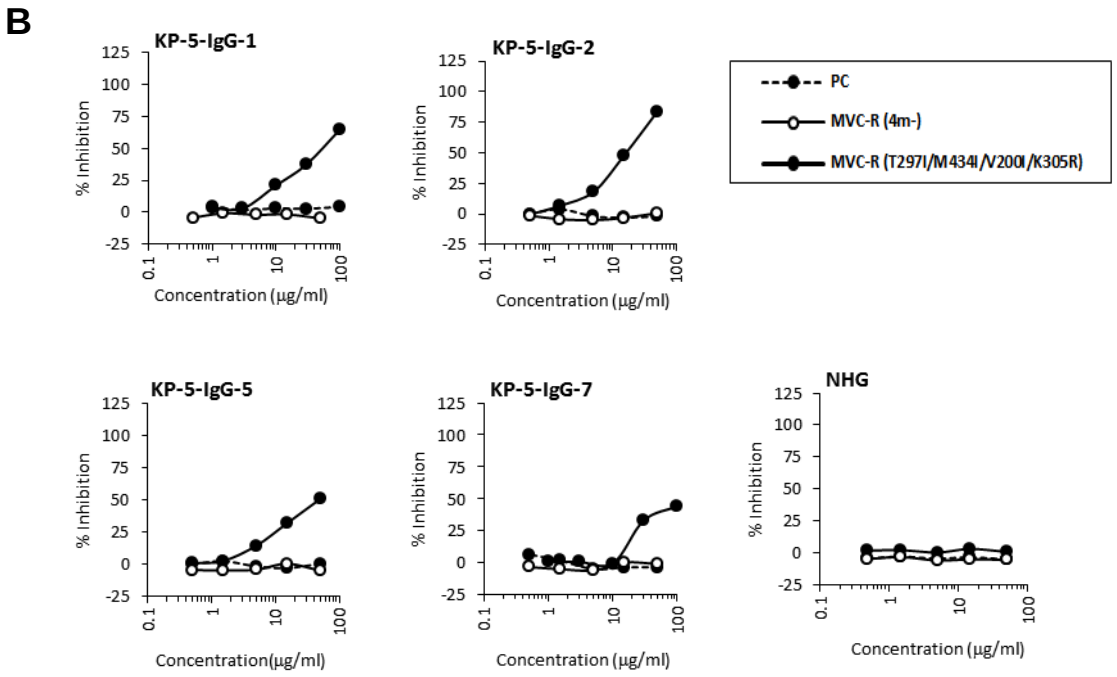


Figure 8. Sensitivity of PC, MVC (4m-) and MVC-R (T297I/M434I/V200I/K305R) infectious clones to NMAbs (A), autologous plasma IgGs (B). Sensitivity of the infectious clones with MVC resistant Env and PC to KD-247, 447-52D, b12, 0.5δ, 4E9C, autologous plasma IgGs (KP-5-IgG-1, -2, -5 and -7) and normal plasma IgG (NHG) was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

3.3 Sensitivity of infectious clones to NMAbs and autologous plasma IgGs

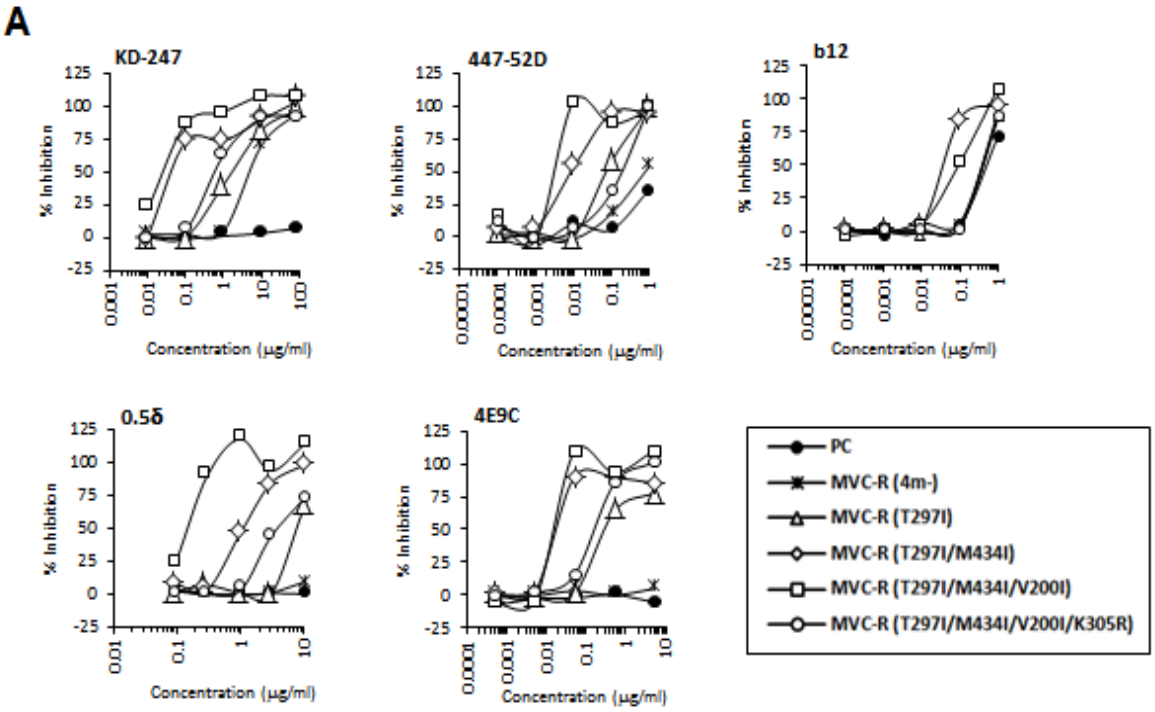
In order to determine the role of each MVC resistant mutation on sensitivity to NMAbs and autologous plasma IgGs, we used mutated-MVC resistant infectious clones mimicking the 4 mutations acquired during the *in vitro* selection process, MVC-R (4m-), MVC-R (T297I), MVC-R (T297I/M434I), MVC-R (T297I/M434I/V200I), MVC-R (T297I/M434I/V200I/K305R) and PC for the neutralization sensitivity assay (Fig. 7B).

The PC clone was highly resistant to KD-247, 447-52D, 0.5∂ and 4E9C NMAbs, but sensitive to b12. The MVC-R (4m-) clone showed sensitivity to KD-247, while the sensitivity to 447-52D, 0.5∂ and 4E9C NMAbs was comparable to the PC. Sequential accumulation of three Env mutations, MVC-R (T297I),

MVC-R (T297I/M434I) and MVC-R (T297I/M434I/V200I) resulted in a gradual increase in sensitivity to the NMAbs tested. However, the MVC-R (T297I/M434I/V200I/K305R) clone decreased sensitivity to all NMAbs compared to MVC-R (T297I/M434I) and MVC-R (T297I/M434I/V200I) (Fig. 9A and Table 2).

We also determined sensitivity of the infectious clones to autologous plasma IgGs. The MVC-R (T297I), MVC-R (T297I/M434I) and MVC-R (T297I/M434I/V200I) were highly sensitive to all autologous plasma IgGs compared to the virus harboring all four mutations, while the MVC-R (4m-) and PC were completely resistant (Fig. 9B and Table 2).

These results suggest that three MVC-resistant mutations, T297I, M434I and V200I, but not K305R, were responsible for sensitivity to NMAbs and autologous plasma IgGs singly or in combination.



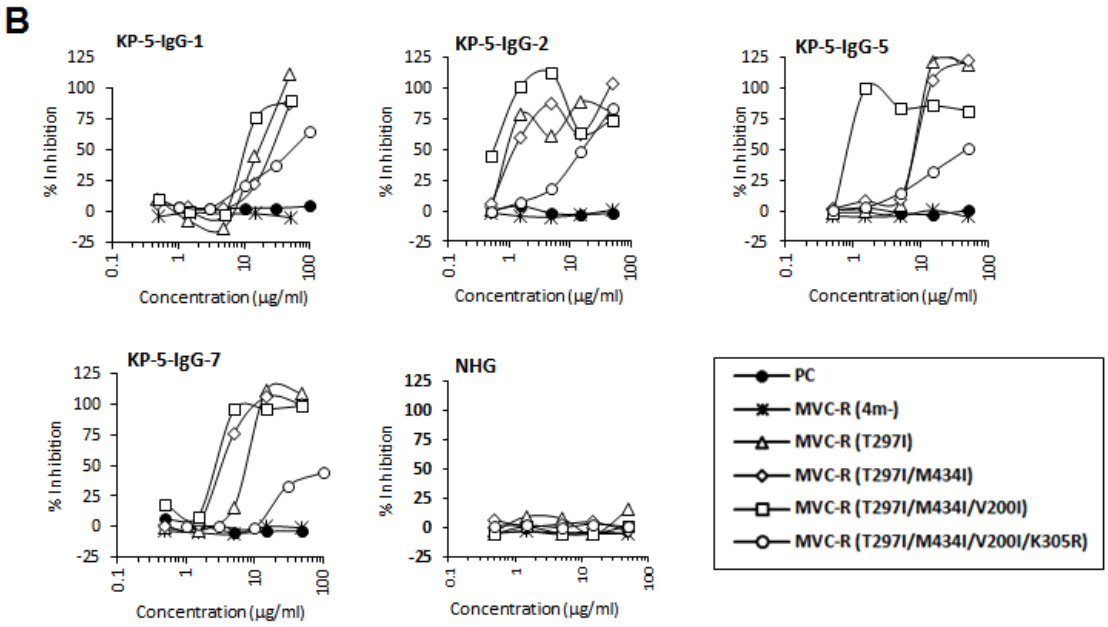


Figure 9. Sensitivity of infectious clones, PC and MVC-R (4m-) carrying the combination of MVC resistant mutations that arise during *in vitro* selection, to NMAbs (A) and autologous plasma IgGs (B). Sensitivity of the infectious clones with MVC resistant Env and PC to KD-247, 447-52D, b12, 0.5δ, 4E9C, autologous plasma IgGs (KP-5-IgG-1, -2, -5 and -7) and NHG was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

3.4 Sensitivity of clones with single mutation to NMAbs and autologous plasma IgGs

We accessed which mutation induced the greatest sensitivity to NMAbs and autologous plasma IgGs using infectious clones with a single mutation. As shown in Figure 10A and Table 2, the infectious clone with the M434I, MVC-R (M434I), was the most sensitive to all NMAbs compared to other single and 4-mutated MVC resistant clones. Sensitivity of the T297I single mutant clone to NMAbs was comparable to MVC-R (T297I/M434I/V200I/K305R). The V200I and the K305R mutant clones showed decreased neutralization sensitivity to all NMAbs with the exception of b12, compared to the MVC-R (T297I/M434I/V200I/K305R) clone (Fig. 10A and Table 2). The clones with M434I or T297I were equivalent or more sensitive to all autologous plasma IgGs compared to the MVC resistant virus with four mutations (Fig. 10B and Table 2). The V200I single mutated clone was more sensitive to two of four plasma

IgGs - KP-5-IgG-1 and -5, compared to the MVC resistant clone with all four mutations, while the K305R clone was completely resistant to autologous plasma IgGs tested (Fig. 10B).

These results indicate that the T297I and M434I mutations were responsible for increased sensitivity to NMAbs and autologous plasma IgGs particularly for the M434I clone to the NMAbs. The data also indicate that V200I was partially responsible for the increased sensitivity to autologous plasma IgGs, but not to the NMAbs tested, while the K305R single mutation within the MVC resistant Env backbone did not increase the sensitivity to either NMAbs or autologous plasma IgGs.

To investigate the role of the M434I mutation in virus sensitivity to NMAbs and autologous plasma IgGs, we constructed a clone reverted Ilu to Met at amino acid position 434, MVC-R (T297I/V200I/K305R), and tested the sensitivity to NMAbs and autologous plasma IgGs (Fig. 11 and Table 2). Unexpectedly, the 434 reverted clone showed decreased sensitivity to two of five NMAbs - KD-247 and 4E9C, and two of three plasma IgGs However, the neutralization sensitivity of the reverted clone was similar to the MVC resistant clone with all four mutations. It is possible that the mutation at position 434 was responsible for sensitivity to NMAbs and autologous plasma IgGs; however, other MVC resistant mutations, especially T297I and V200I, may compensate the loss of neutralization sensitivity by the reverted mutation.

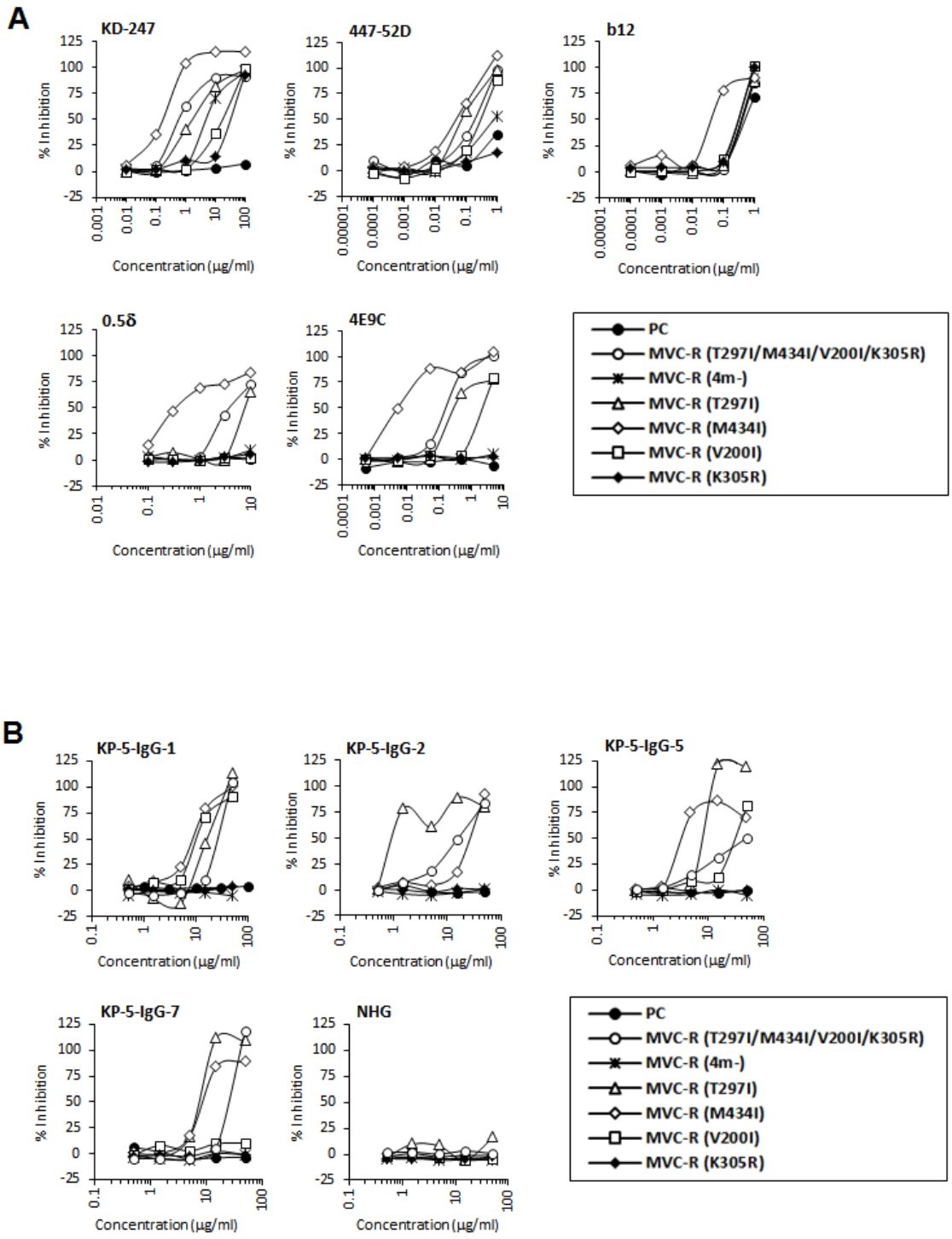


Figure 10. Sensitivity of PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (4m-) carrying V200I, T297T, K305R or M434I single mutation clones to NMAbs (A) and autologous plasma IgGs (B). Sensitivity of the infectious clones with MVC resistant Env and PC to KD-247, 447-52D, b12, 0.5δ, 4E9C, autologous plasma IgGs (KP-5-IgG-1, -2, -5 and -7) and NHG was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

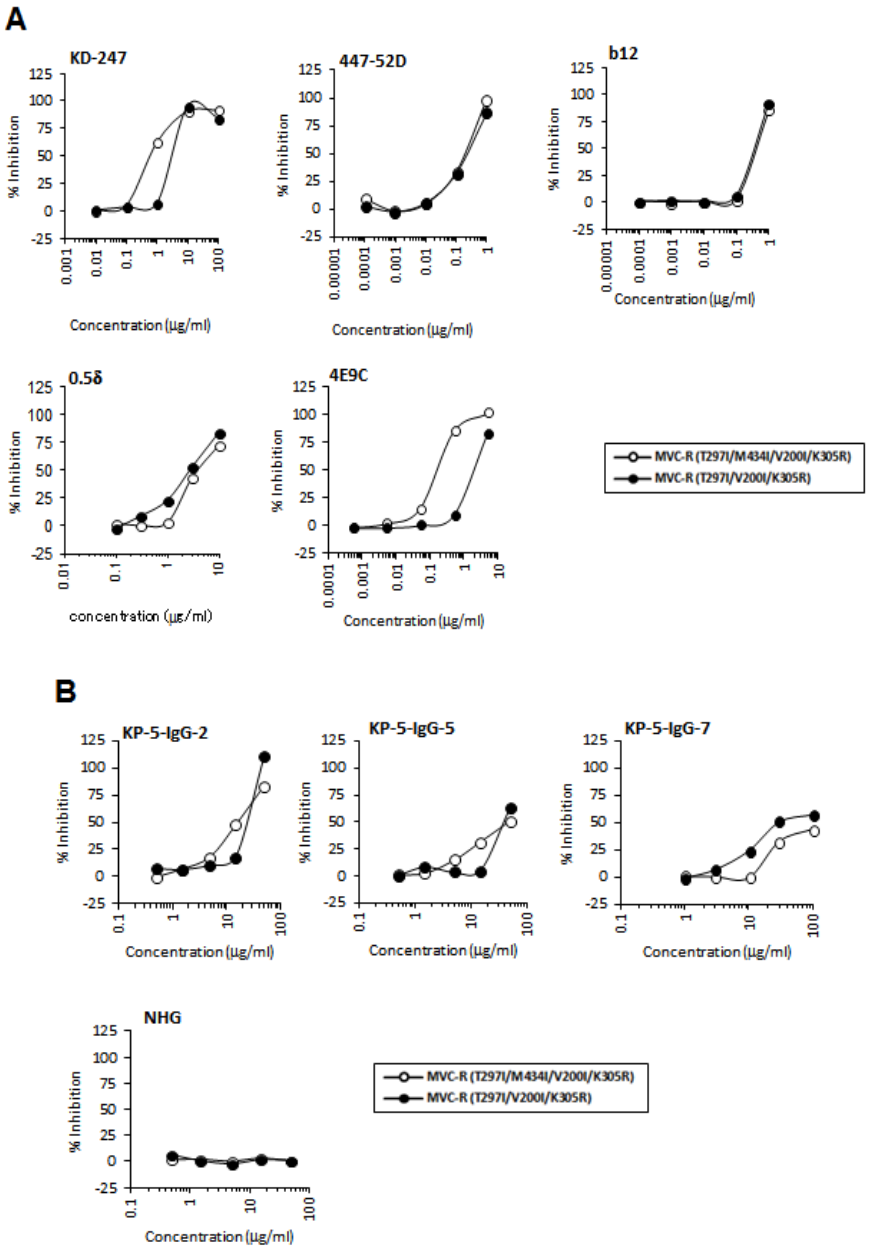


Figure 11. Sensitivity of MVC-R (T297I/M434I/V200I/K305R) and MVC-R (T297I/V200I/K305R) clones to NMAbs (A) and autologous plasma IgGs (B). Sensitivity of the infectious clones with MVC resistant Env and PC to KD-247, 447-52D, b12, 0.5δ, 4E9C, autologous plasma IgGs (KP-5-IgG-2, -5 and -7) and NHG was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

Table 2. Sensitivity of the infectious clones with MVC resistant mutations to NMAbs and autologous plasma IgGs

Infectious virus	IC_{50}[a] (FC[b])									
	KD-247	447-52D	b12	0.5δ	4E9C	KP-5-IgG-1	KP-5-IgG-2	KP-5-IgG-5	KP-5-IgG-7	NHG[c]
PC	>100(-)	>1(-)	0.47(-)	>10(-)	>5.0(-)	>50(-)	>50(-)	>50(-)	>50(-)	>50(1.0)
MVC-R (4m-)	**4.9(>20)**	0.8(>1)	0.35(1.3)	>10(1.0)	>5.0(1.0)	>50(1.0)	>50(1.0)	>50(1.0)	>50(1.0)	>50(1.0)
MVC-R (T297I)	**1.7(>59)**	**0.074(>14)**	0.35(1.3)	7.5(>1.3)	**0.30(>17)**	**16(>3.1)**	**1.0(>50)**	**7.7(>6.5)**	**7.4(>6.8)**	>50(1.0)
MVC-R (T297I/M434I)	**0.048(>2000)**	**0.0086(>116)**	**0.037(13)**	**1.1(>10)**	**0.018(>278)**	25(>2.0)	**1.2(>42)**	**7.9(>6.3)**	**3.3(>15)**	>50(1.0)
MVC-R (T297I/M434T/V200I)	**0.028(>3570)**	**0.0031(>322)**	**0.093(5.0)**	**0.15(>67)**	**0.015(>333)**	**10(>5.0)**	**0.56(>90)**	**0.88(>57)**	**2.7(>19)**	>50(1.0)
MVC-R (T297I/M434I/V200I/K305R)	**0.60(>166)**	**0.18(6)**	0.37(1.3)	3.9(2.5)	**0.16(>31)**	25(>2.0)	**16(>3.1)**	47(>1.0)	24(>2.0)	>50(1.0)
MVC-R (M434I)	**0.16(>625)**	**0.046(22)**	**0.041(11)**	**0.35(>29)**	**0.0051(>980)**	**8.6(>5.8)**	25(>2.0)	**3.3(>15)**	**8.7(>5.7)**	>50(1.0)
MVC-R (V200I)	**25(>4.0)**	**0.27(4)**	0.27(1.7)	>10(1.0)	2.0(>2.5)	**10(>5.0)**	26(>1.9)	29(>1.7)	>50(1.0)	>50(1.0)
MVC-R (K305R)	**29(>3.4)**	>1(1)	0.28(1.7)	>10(1.0)	>5.0(1.0)	>50(1.0)	>50(1.0)	>50(1.0)	>50(1.0)	>50(1.0)
MVC-R (T297I/V200I/K305R)	**3.0(>33)**	**0.22(>4.6)**	0.32(1.5)	**2.7(3.7)**	1.8(>2.8)	ND	23(>2.2)	38(>1.3)	26(>1.9)	>5(1.0)

[a] IC_{50}, μg/ml.

[b] FC, the fold change of IC_{50}. The IC_{50} fold change was calculated as the IC_{50} value of PC per IC_{50} value of each infectious clone.

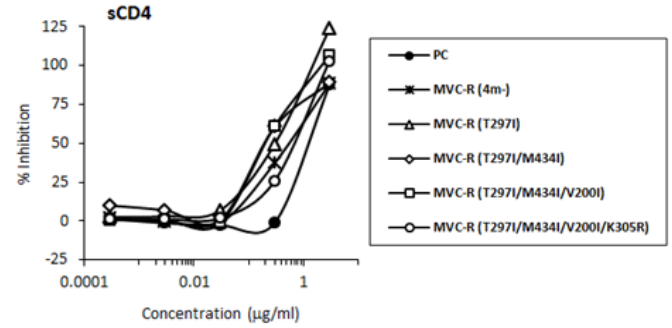
[c] NHG, normal human plasma IgG

Gray shows increased sensitivity (FC>3) to NMAbs or autologous plasma IgGs.

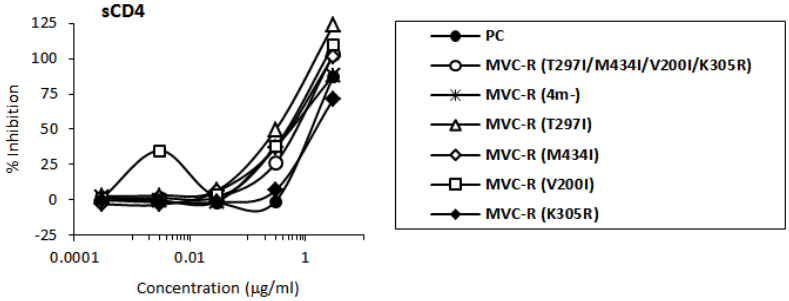
3.5 Sensitivity of MVC resistant infectious clones to sCD4

Sensitivity of MVC resistant virus to anti-CD4bs NMAbs suggested that MVC resistant mutations affected the CD4 binding area that induced uncovering epitopes for b12 and 0.5δ NMAbs. We examined the sensitivity of MVC resistant clones to soluble CD4 (sCD4) to determine the effect on MVC resistant mutations on the changes of CD4 binding site. The result showed that MVC-R (4m-) clone was sensitive to sCD4 than PC clone (Fig. 12). This MVC resistant virus might sensitive to sCD4 before obtained those four mutations in gp 120 region. Interestingly, MVC resistant clone carrying some of MVC resistant mutations increased sensitivity to sCD4 than MVC-R (4m-) clone. Except for MVC-R (T297I/M434I/V200I/K305R) and MVC-R (K305R), all MVC resistant clones was more than 2-folds sensitive to sCD4 than PC (Fig. 12). The MVC resistant clones carrying K305R expressed relatively high IC_{50} than other MVC resistant clones, while IC_{50} of MVC-R (T297I/M434I/V200I/K305R) and MVC-R (K305R) were 0.62 and 1.4 respectively (Fig. 12C). It indicates that K305R mutation confers neutralizing resistance to sCD4. This finding proofed that four MVC resistant mutations has impact on the CD4 binding site consequently, the sensitivity to anti-CD4bs was changed. Three MVC resistant mutations; T297I, M434I and V200I, might play a role on expose the epitope for anti-CD4bs antibody.

A



B



C

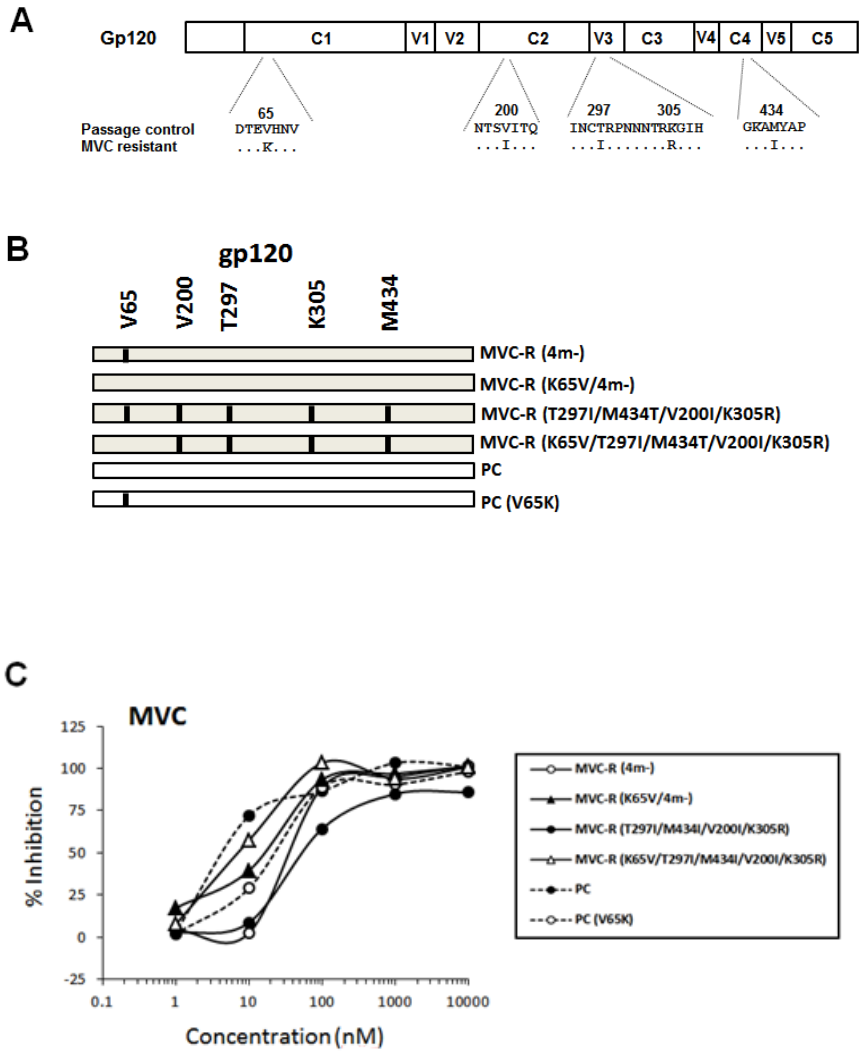
Infectious clone	IC_{50} (μg/ml)
PC	1.1
MVC-R (4m-)	0.53
MVC-R (T297I)	0.31
MVC-R (T297I/M434I)	0.20
MVC-R (T297I/M434I/V200I)	0.20
MVC-R (T297I/M434I/V200I/K305R)	0.62
MVC-R (M434I)	0.48
MVC-R (V200I)	0.44
MVC-R (K305R)	1.4

Figure 12. Sensitivity of infectious clones, PC and MVC-R (4m-) carrying the combination of MVC resistant mutations that arise during *in vitro* selection (A) and MVC-R (4m-) carries each single mutation (B) to sCD4. The IC_{50} value is shown in table C. Data are representative of results from 2 independent experiments.

3.6 The effect of V65K mutation to sensitivity of MVC sensitivity

From this resent study, we found that the MVC-R (4m-) infectious clone, which was prior to accumulation of four MVC resistant mutations was sensitive to anti-V3 MAbs, KD-247 and 447-52D, compared to PC virus (Fig. 8A and Table 2). It is possible that there are other mutations which appeared prior to four MVC resistant mutations that also influence to the sensitivity of both NMAbs. We detected V65K mutation in one of ten clones of the baseline KP-5 virus. This mutation appeared in C1 region of the *env* of MVC resistant（Fig. 13A）since passage 3 during the *in vitro* selection. However, V65K mutation disappeared from the population of PC virus from passage 7 (Yoshimura et al., 2014). Based on this observation, we continue the study on the impact MVC resistant mutation which acquired prior the four MVC resistant mutations on the neutralization sensitivity to NMAbs especially for anti-V3 antibodies. We introduced V65K mutation into *env* of PC meanwhile, substituted valine to lysine at position 65 of *env* of MVC-R (4m-) and MVC-R (T297I/M434I/V200I/K305R) clone, designated PC (V65K), MVC-R (K65V/4m-) and MVC-R (K65V/T297I/M434I/V200I/K305R), respectively (Fig. 13B). The neutralization sensitivity of these mutated clones to MVC, NMAbs and autologous plasma IgGs were determined.

PC (V65K) infectious clone decreased sensitivity to MVC compared to PC clone as the IC_{50} were 22 and 4.8 nM, respectively. However, MPI of both clones were comparable (98 and 101%, respectively) (Fig. 13C). MVC-R (K65V/4m-) and MVC-R (K65V/T297I/M434I/V200I/K305R) infectious clones reduced IC_{50} more then 2-fold compared to their parental clone MVC-R (4m-) and MVC-R (T297I/M434I/V200I/K305R), respectively. Introducing K65V into MVC-R (T297I/M434I/V200I/K305R) could increase MPI compared to the parental clone as showed 101 and 86%, respectively (Fig. 13C). These results suggest that the V65K mutation might be selected from quasispecies of baseline KP-5 virus at early passages under relative low concentrations of MVC by the *in vitro* selection. This mutation in the context of KP5 MVC resistant *env* confers resistance to MVC.



Infectious virus	MPI (%)	IC_{50} (nM)
MVC-R (4m-)	101	35
MVC-R (K65V/4m-)	101	16
MVC-R (T297I/M434I/V200I/K305R)	86	55
MVC-R (K65V/T297I/M434I/V200I/K305R)	101	7.1
PC	101	4.8
PC (V65K)	98	22

Figure 13. Impact of V65K mutation on the sensitivity of PC and MVC resistant infectious clones to MVC. Locations of V65K and others 4 MVC resistant mutations was demonstrated (A). Site-direct mutagenesis was used to generated PC (V65K) and MVC-R (4m-/K65V) and MVC-R (K65V/T297I/M434I/V200I/K305R) (B). Sensitivity of infectious viruses to MVC was presented in the sensitivity graph and table (C). Sensitivity of MVC resistant infectious clones is indicated in solid line. Sensitivity of PC infectious clones is indicated in dash line. Data are representative of results from 2 independent experiments.

3.7 The effect of V65K mutation to sensitivity of NMAbs and autologous plasma IgGs

The sensitivity of 65V mutated-MVC resistant infectious clones to NMAbs and autologous plasma IgGs was determined. MVC-R (K65V/4m-) clone reduced sensitivity to KD-247 and increased sensitivity to 447-52D, 4E9C NMAbs and autologous plasma IgGs (KP-5-IgG-5 and -7) compared to the parental clone, MVC-R (4m-) (Fig. 14 and Table 3). MVC-R (K65V/297I/M434I/V200I/K305R) infectious clone showed more than 2-folds increased sensitivity to all tested NMAbs and also increased sensitivity to autologous plasma IgGs compared to MVC-R (T297I/M434I/V200I/K305R) clone (Fig. 14 and Table 3). Taken together, the amino acid at position 65 in C1 region of HIV-1 KP-5 did not only affect on the sensitivity to MVC but also influence on the sensitivity to NMAbs and autologous plasma IgGs too. V65K mutation in *env* of MVC resistant clone confers neutralization resistance to all antibodies tested; however V65K without the combination of four MVC resistant mutations increases the sensitivity to KD-247 NMAb.

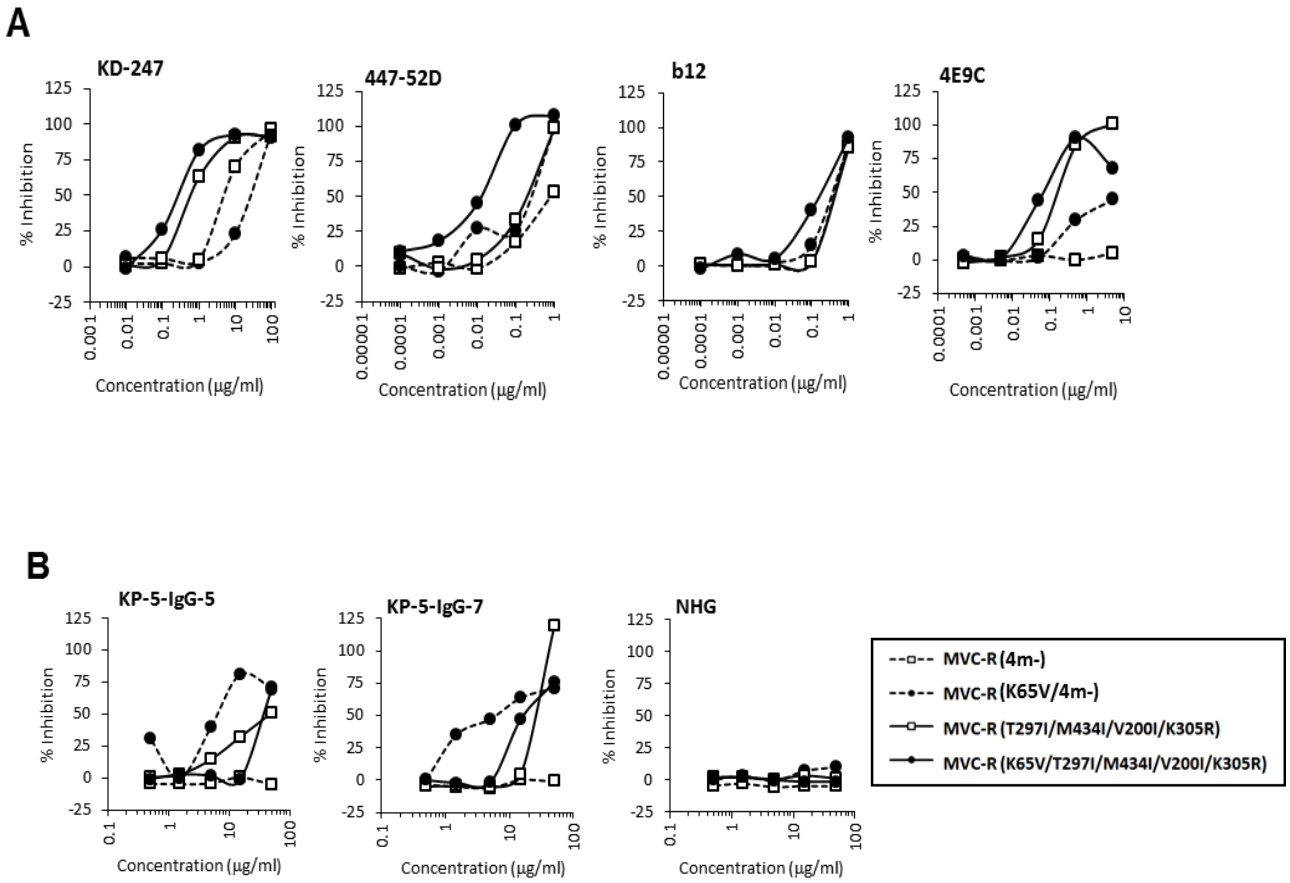


Figure 14. Sensitivity of MVC resistant and mutated MVC resistant infectious viruses to NMAbs (A) and autologous plasma IgGs (B). Valine was substituted at position 65 of MVC-R (4m-) and MVC-R(T297I/M434I/V200I/K305R) to obtain MVC-R(K65V/4m-) and MVC-R(K65V/T297I/M434I/V200I/K305R) infectious clones respectively. Sensitivity of the infectious clones to KD-247, 447-52D, b12, 4E9C, autologous plasma IgGs (KP-5-IgG-5 and -7) and NHG was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

V65K was also introduced into the *env* of PC. PC infectious clone was resistant to KD-247, 447-52D, 4E9C and all autologous plasma IgGs tested. Interestingly, PC (V65K) infectious clone became sensitive to KD-247, 447-52D, b12 and especially autologous plasma IgGs (Fig. 15B and Table 3).

These results suggested that the V65K mutation in a difference *env* background confer difference neutralization susceptibility to neutralizing antibodies.

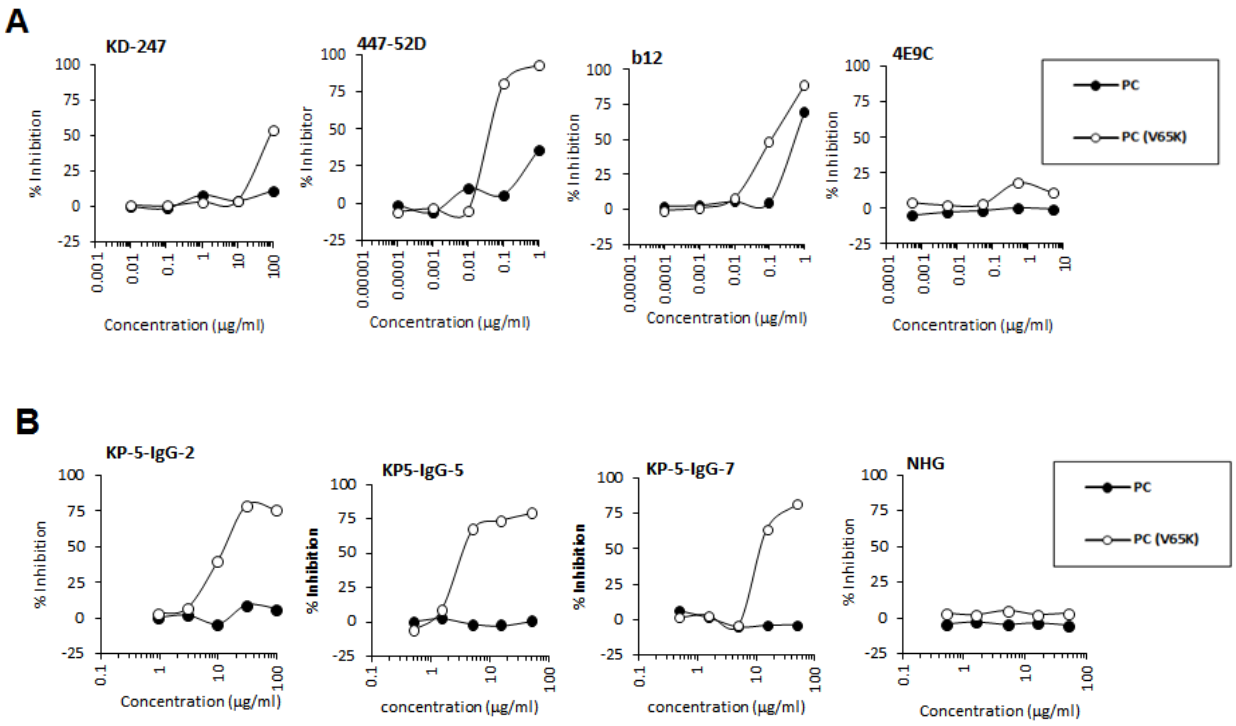


Figure 15. **Sensitivity of PC and PC (V65K) infectious clone to NMAbs (A) and autologous plasma IgG (B).** Sensitivity of the infectious clones to KD-247, 447-52D, b12, 4E9C, autologous plasma IgGs (KP-5-IgG-2, -5 and -7) and NHG was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

Table 3. Sensitivity of mutated and wild type MVC resistant and PC infectious clone to MVC, NMAbs and autologous plasma IgGs

Infectious clone	IC_{50} (μg/ml)							
	KD-247	447-52D	b12	4E9C	KP-5-IgG-2	KP-5-IgG-5	KP-5-IgG-7	NHG[a]
MVC-R (4m-)	4.9	0.80	0.35	>5	ND	>50	>50	>50
MVC-R (K65V/4m-)	24	0.22	0.3	>5	ND	6.6	6.0	>50
MVC-R (T297I/M434I/V200I/K305R)	0.60	0.18	0.37	0.16	ND	47	24	>50
MVC-R (K65V/T297I/M434I/V200I/K305R)	0.27	0.012	0.15	0.07	ND	36	17	>50
PC	>100	>1	0.47	>5	>100	>100	>100	>100
PC (V65K)	93	0.044	0.1	>5	14	3.5	11.97	>100

[a] NHG, normal human plasma IgG

ND, data not available

3.8 The effect of M434I and V65K mutations on the sensitivity of HIV-1 JRFL and YU2 strains to MVC and NMAbs.

We constructed chimeric clones with JRFL or YU2 *Env* to determine the effect of M434I and V65K mutations on the differences Env background. The JRFL and YU2 infectious clones carrying either M434I or V65K mutation showed similar neutralization profile to infectious clones carrying wild type envelope (Fig. 16). This result indicates that the effect of these single mutations on the sensitivity to MVC and NMAbs may be restricted by backgroud env sequence.

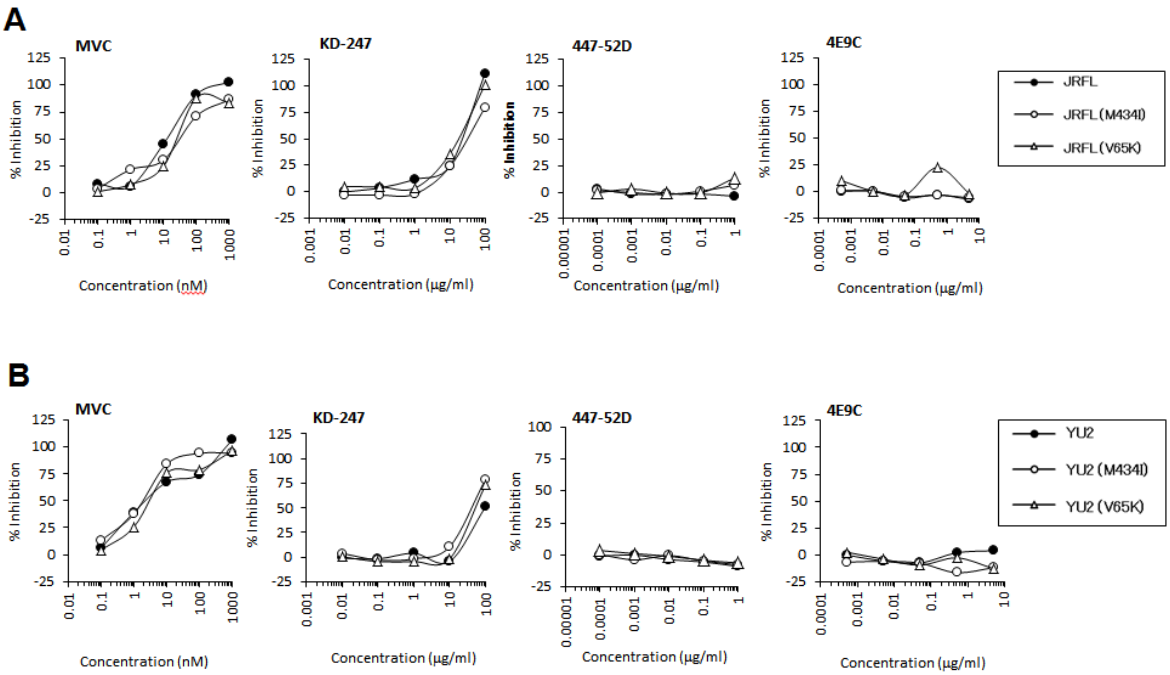


Figure 16. Sensitivity of chimeric infectious clones with JRFL (A) and YU2 (B) Env to MVC and NMAbs. Either M434I or V65K mutation was introduced into the envelopes of JRFL (JRFL (M434I) and JRFL (V65K), respectively) and YU2 (YU2 (M434I) and YU2 (V65K), respectively). Sensitivitiy of the infectious clones to MVC, KD-247, b12 and 4E9C was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

3.9 Sensitivity of MVC resistant infectious clones to heterologous sera from HIV-1 infected patients

To determine whether the infectious clones with MVC resistant mutations were sensitive to heterologous sera, we tested the sensitivity of MVC-R (T297I/M434I/V200I/K305R), MVC-R (M434I) and PC to sera obtained from ART naïve patients infected with HIV-1 subtype B and non-subtype B (A, C, G, AG and AE) (Table 4). The PC clone was resistant to all heterologous subtype B and 17 of 19 non-B sera. Two non-subtype B heterologous sera that neutralized the PC clone had low reciprocal dilutions that reduced virus infectivity by 50% (143 and 147). However, the MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) were sensitive to 57% (17 of 30) and 60% (18 of 30) of heterologous sera, respectively. The percentage of sera from subtype B subjects that neutralized the PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) were 0% (0 of 11), 55% (6 of 11) and 82% (9 of 11), respectively, while sera from

non-B infected subjects were 11% (2 of 19), 58% (11 of 19) and 47% (9 of 19), respectively. Interestingly, 3 of 11 sera from subtype B infected subjects and 6 of 19 samples from subjects infected with other HIV subtypes showed discordant neutralization results between the two mutant viruses, but the neutralization titer was always <500. Moreover, 67% (4 of 6) discordant non-B sera neutralized the MVC-R (T297I/M434I/V200I/K305R), while all discordant subtype B sera neutralized MVC (M434I), but not MVC-R (T297I/M434I/V200I/K305R) clones (Table 4).

Next, we compared the reciprocal dilutions of the sera among MVC-R (T297I/M434I/V200I/K305R), MVC-R (M434I) and PC clones. As shown in Fig. 17, both mutant clones were more sensitive to neutralization than the PC clone ($p < 0.01$). Additionally, the mean reciprocal dilution of sera to the MVC-R (M434I) clone was significantly higher ($p<0.05$) than the MVC-R (T297I/M434I/V200I/K305R) clone.

These findings indicate that MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) clones, especially the latter, are highly sensitive to heterologous sera obtained from ART naïve patients.

Table 4. **Neutralization of PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) by sera from drug naïve HIV-1 infected individuals**

Subtype	Sample ID	PC	MVC-R(T297I/M434I/V200I/K305R)	MVC-R(M434I)
B	ACC-B2	<100	**606**	**6211**
	ACC-B3	<100	**469**	**1389**
	ACC-B4*	<100	<100	**481**
	ACC-B5	<100	**1333**	**422**
	ACC-B6	<100	<100	<100
	ACC-B7*	<100	<100	**125**
	ACC-B8*	<100	<100	**334**
	ACC-B9	<100	**347**	**3846**
	ACC-B10	<100	**305**	**312**
	ACC-B11	<100	<100	<100
	ACC-B12	<100	**351**	**3333**
AG	502	147	**434**	**8603**
	983	<100	<100	<100
C	792*	<100	**134**	<100
	941	<100	**325**	**3077**
	947*	<100	<100	**289**
	23	<100	**288**	**1494**
G	696	<100	**264**	**136**
	511	<100	<100	<100
A	710*	<100	**390**	<100
AE	531	<100	<100	<100
	82	<100	**100**	**915**
	571	<100	**283**	**422**
	111	<100	**349**	**532**
	105*	<100	**165**	<100
	AE1	<100	<100	<100
	AE2*	143	<100	**195**
	AE3*	<100	**153**	<100
	AE4	<100	<100	<100
	AE5	<100	<100	<100

The values listed are the reciprocal serum dilutions that reduce virus infection by 50% stratified by serum and virus. The values highlighted in bold indicate reciprocal dilution ≧100. Asterisk (*) shows sera that were discordant between the MVC-R clones.

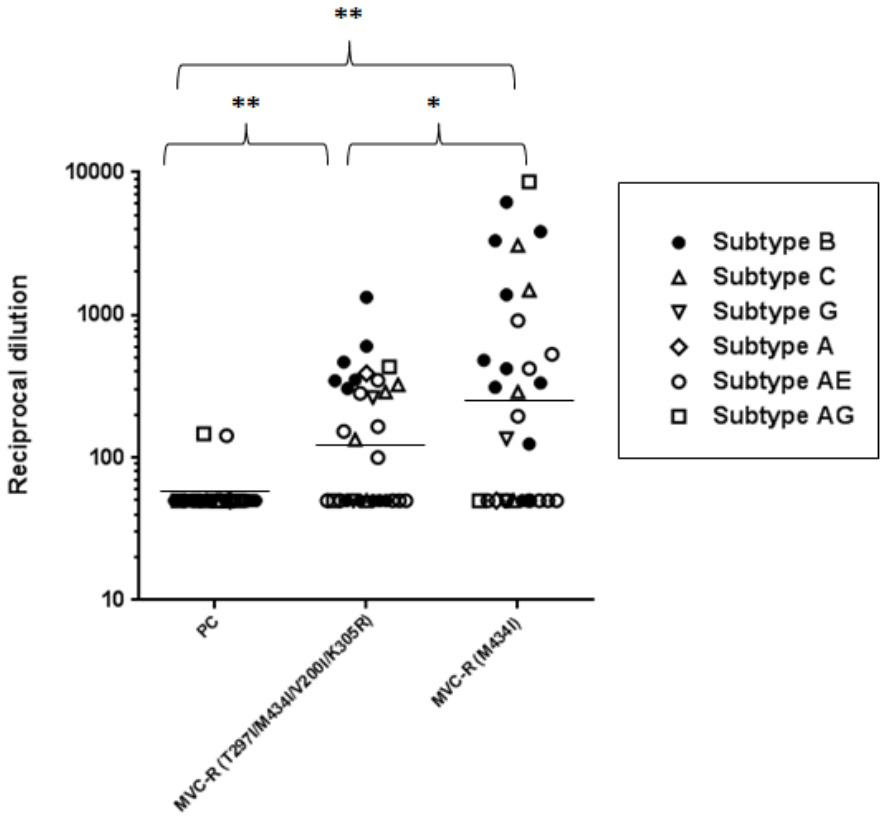


Fig. 17. **Neutralization of PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) by sera from ART naïve HIV-1 infected individuals.** Reciprocal dilution of the sera from ART naïve HIV-1 infected individuals against PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) were plotted using GraphPad Prism software. When the reciprocal dilution was less than 100, we used as 50 for calculating statistical significant. Asterisks correspond to statistically significant differences between groups (*$p<0.05$, **$p<0.01$). The reciprocal geometrical mean serum neutralizing titers are shown as horizontal bars.

Using heterologous ACC-B1 and ACC-B2 sera from patients treated with combination ART containing MVC, we measured the susceptibility of our clones to the subject's sera prior to and during treatment with MVC. ACC-B1 patient received ART prior started collecting sera while ACC-B2 patient was ART naïve before and at the time that ACC-B2-1 serum was collected. ACC-B1-1 serum, which was collected prior to initiation of the MVC containing regimen could neutralized all three infectious clones efficiently ,however sera collected 2 and 8 weeks after started treatment with MVC (ACC-B1-2 and ACC-B1-3) reduced the efficiency to neutralized three clones (Fig. 18 and Table 5). Likewise, sera collected after MVC treatment from ACC-B2 patient (ACC-B2-2 and ACC-B2-3) could neutralize all three clones better than the sera

collected before treatment with MVC (ACC-B2-1) (Fig. 18 and Table 5). Two MVC resistant clones were more than 2-fold sensitive to all sera than the PC infectious clone (Table 5).

Taken together, these results indicate that we can measure increasing neutralizing antibodies in infected patients' sera after initiating MVC using these infectious clones.

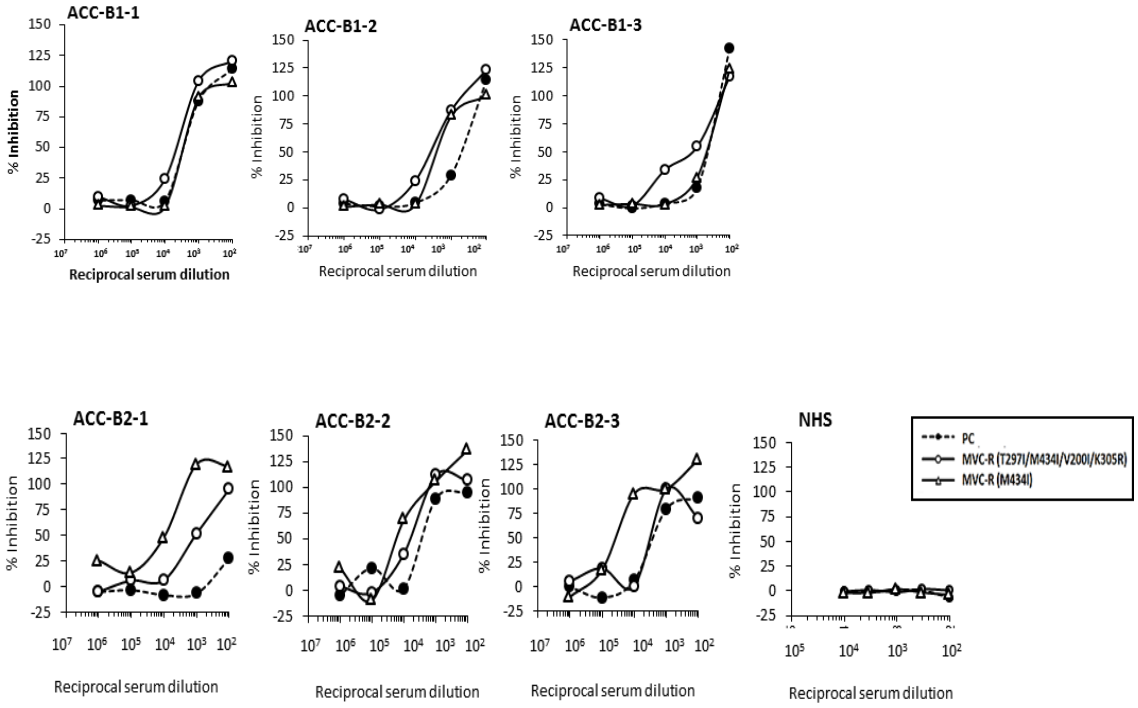


Figure 18. Sensitivity of PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) infectious viruses to heterologous sera from HIV-1 subtype B (ACC-B1 and ACC-B2) infected patients and normal human plasma (NHP). Sera were obtained from the patient 1 week before (ACC-B1-1 and ACC-B2-1), 2 (ACC-B1-1), 5 (ACC-B2-2) and 8 weeks (ACC-B1-3 and ACC-B2-3) after starting treatment with MVC. Sensitivity of the infectious clones with MVC resistant Env and PC to the sera was determined by the WST-8 assay as described in Materials and methods. Data are representative of results from 2 independent experiments.

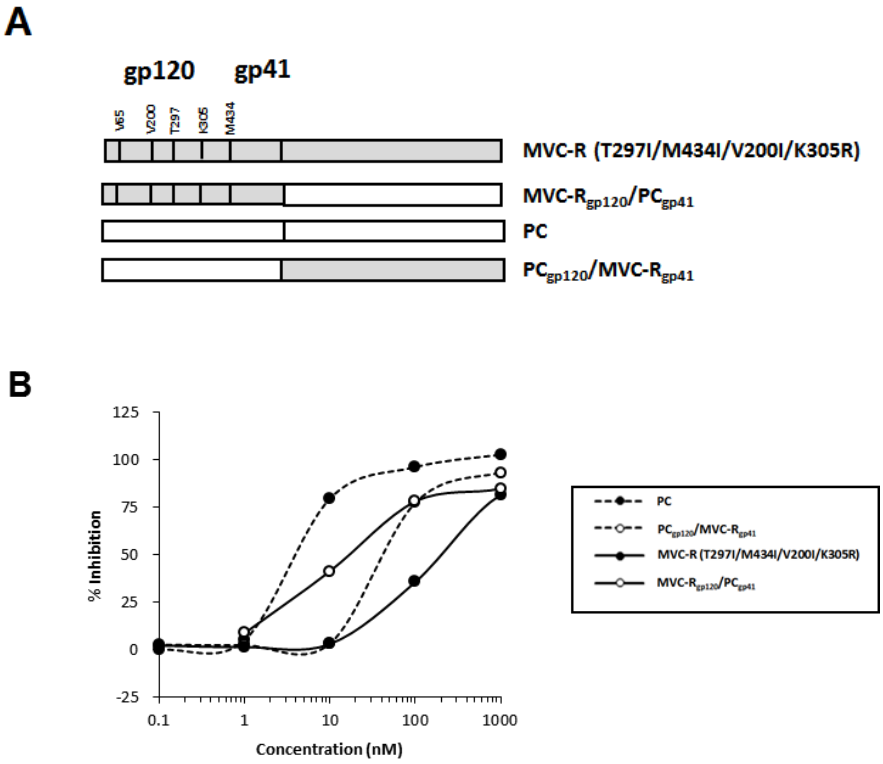
Table 5. Sensitivity of PC, MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) to sera from HIV-1 subtype B infected patients

Infectious clone	ACC-B1-1	ACC-B1-2	ACC-B1-3	ACC-B2-1	ACC-B2-2	ACC-B2-3
PC	2907	571	546	<100	2778	2500
MVC-R(T297I/M434I/V200I/K305R)	4762	3846	1639	1087	6667	3125
MVC-R (M434I)	2857	2500	578	9090	17241	41667

Number indicates reciprocal dilution of sera.

3.10 Sensitivity of gp120/gp41 chimeric infectious clones to MVC

The role of gp41 region of MVC resistant virus was also examined. The gp120/gp41 chimeric infectious clones were constructed as shown in figure 19A. The sensitivity of PC, MVC-R (T297I/M434I/V200I/K305R) and gp120/gp41 chimeric clones to MVC was determined. The gp41 region of MVC resistant virus apparently affected on the sensitivity to MVC as the PC carrying gp41 of MVC resistant virus (PC_{gp120}/$MVC\text{-}R_{gp41}$) increased IC_{50} (25 nM) compared to PC (4.8nM) (Fig. 19B). Furthermore, the MVC resistant carrying gp41 of PC virus reduced the IC_{50} to 6.2 compared to the MVC-R (T297I/M434I/V200I/K305R) (55 nM) (Fig. 19B). However, the MPI of chimeric clones did not significantly difference from PC, ranging 101 to 97. It indicates that amino acid substitution in gp41 region of MVC resistant virus also has impact on decreasing sensitivity to MVC by reduces $IC_{50,}$ but not increases MPI.



Infectious clone	MPI (%)	IC_{50} (nM)
PC	101	4.8
PC_{gp120}/MVC-R_{gp41}	98	25
MVC-R (T297I/M434I/V200I/K305R)	86	55
MVC-R_{gp120}/PC_{gp41}	97	6.2

Figure 19. Impact of gp41 region of MVC resistant virus on the sensitivity to MVC. The overlapping PCR was used to generated gp120/gp41 chimeric infectious clones (A). Sensitivity of PC, MVC-R (T297I/M434I/V200I/K305R) and gp120/gp41 chimeric infectious clones to MVC was presented in the sensitivity graph and table (B). Data are representative of results from 2 independent experiments.

3.11 Sensitivity of gp120/gp41 chimeric infectious clones to NMAbs

Inserting gp41 region of MVC-R (T297I/M434I/V200I/K305R) into PC *Env* (PC_{gp120}/MVC-R_{gp41} infectious clone) increased sensitivity to KD-247 even though the maximum of KD-247 concentration used in this experiment was not enough for PC_{gp120}/MVC-R_{gp41} clone to inhibit 50% of viral replication (Fig.20). Meanwhile, replacing the gp41 region of MVC-R (T297I/M434I/V200I/K305R) with gp41 region of PC

virus (MVC-R_{gp120}/PC_{gp41} infectious clone) resulted in more than two-fold reducing the sensitivity to KD-247, b12 and 4E9C NMAbs compared to the MVC-R (T297I/M434I/V200I/K305R) clone (Fig. 20 and Table 6). Taken together, gp41 region of MVC resistance virus also plays a role in increasing sensitivity to KD-247, b12 and 4E9C NMAbs. The MVC-induced mutation(s) in gp41 also influence on the neutralization sensitivity of this MVC resistance virus to NMAbs too.

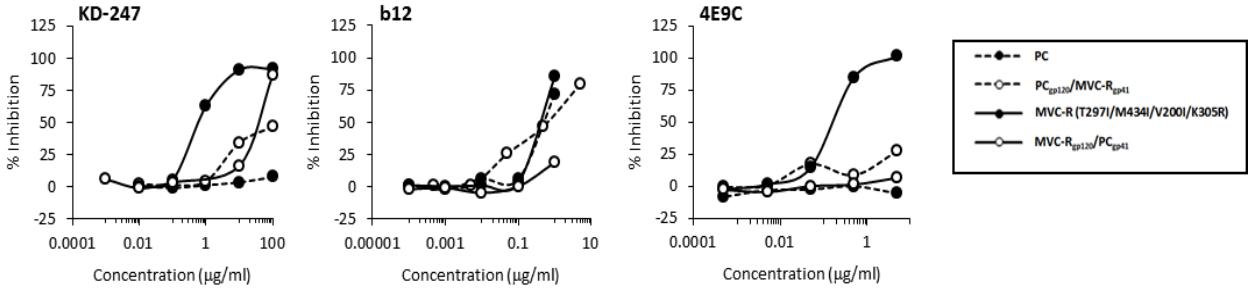


Figure. 20 Sensitivity of PC, MVC-R (T297I/M434I/V200I/K305R) and gp120/gp41 chimeric (MVC-Rgp120/PCgp41 and PCgp120/MVC-Rgp41) infectious clones to NMAbs (KD-247, b12 and 4E9C). Data are representative of results from 2 independent experiments.

Table 6. **Sensitivity of PC, MVC-R (T297I/M434I/V200I/K305R) and gp120/gp41 chimeric (MVC-Rgp120/PCgp41 and PCgp120/MVC-Rgp41) infectious clones to NMAbs**

Infectious clone	KD-247	b12	4E9C
PC	>100	0.47	>5.0
PC_{gp120}/MVC-R_{gp41}	>100	0.63	>5.0
MVC-R (T297I/M434I/V200I/K305R)	0.60	0.37	0.16
MVC-R_{gp120}/PC_{gp41}	30	>1.0	>5.0

Number indicates IC_{50} (µg/ml)

4. DISCUSSION

The MVC resistant virus escapes from drug pressure by acquiring mutations in the Env region. However, those mutations affect the structure of envelope, a target of neutralizing antibody (Pugach et al., 2008; Tilton et al., 2010; Yoshimura et al., 2014). In our previous study, the MVC resistant virus generated by *in vitro* selection occurred gradually following the acquisition of four MVC resistant mutations; T297I, M434I, V200I and K305R. In parallel with MVC resistance, the sensitivity to NMAbs and autologous plasma IgGs continuously increased during the accumulation of the four mutations. Three MVC resistant mutations; V200I, T297I and M434I, reside around the base of V3 and the K305R mutation is located at the V3 stem. All four mutations appear on the trimer apex site (Fig. 6) (Lyumkis et al., 2013; Yoshimura et al., 2014).

We have demonstrated that our MVC resistant virus became sensitive to NMAbs and autologous plasmas after acquisition of T297I mutation. The appearance of this mutation removes the N-linked glycan at position 295. The elimination of N295 in HIV-1 strain SF162 could increase the neutralization susceptibility of this mutant strain to anti-CD4 binding site, anti-V3, anti-CD4i and anti-gp41 NMAbs compared to the wild type SF162 (McCaffrey et al., 2004). The disappearance of glycan at position 295 in the MVC resistant virus might also allow improved the accessibility of NMAbs except for b12 (Table 2).

MVC resistant mutations apparently affected to the CD4 binding region as they changes the sensitivity of MVC resistant clones to sCD4 (Fig. 12). All four MVC resistant mutations are not in CD4 contact area however, those mutations somehow involves in alteration of CD4 binding site. The changing of CD4 binding site might be a reason of the increasing sensitivity of MVC resistant virus to anti-CD4bs.

The binding of HIV-1 Env to cellular CD4 induces Env conformation rearrangement of V1/V2 and V3 (Wilen, Tilton, and Doms, 2012) and leads to formation of bridging sheet. The Env CD4-bound state exposes CD4 binding and CD4i epitope that accessible for anti-CD4bs and CD4i antibodies (Kong et al., 2010; Myszka et al., 2000; Roitburd-Berman et al., 2013). KP-5 MVC resistant virus is highly sensitive to anti-CD4bs, anti-CD4i and anti-V3 NMAbs. We assume that this MVC resistant virus may have a CD4 bound-like Env structural.

Anti-V3, KD-247 and 447-52D, antibodies bind to the GPGR motif on the tip of the V3 loop (Hatada et al., 2010; Killikelly et al., 2013; Shibata et al., 2007). Our results indicate that the MVC resistant mutations outside the epitope of those anti-V3 antibodies changed the conformation of Env to expose the epitope(s) of KD-247 and 447-52D NMAbs. The Env conformation is also contribute to neutralization activity of both anti-V3 antibodies.

The K305R mutation located at the V3 stem conferred high resistance to MVC confirming that the mutation at the V3 loop is critical for MVC resistance (Berro et al., 2012; Soulie et al., 2008; Yuan et al., 2013). The emergence of the K305R mutation in addition to the other three mutations (T297I, M434I and V200I) conferred resistance to all NMAb and autologous plasmas (Table 2). This mutation, which locates near the tip of V3 loop, may affect the conformation of the trimer more than those around the base of the loop (di Marzo Veronese et al., 1993; Suphaphiphat, Essex, and Lee, 2007; Xiang et al., 2010; Yuan et al., 2013).

We demonstrate that the M434I mutation in the Env of MVC resistant virus may play a major role in increasing the sensitivity to NMAbs, autologous plasma IgGs and heterologous sera. Resistant mutations of entry inhibitors have frequently been reported in the C4 region of gp120 and increased the neutralization sensitivity to NMAbs (Berro et al., 2009; Vermeire et al., 2009). M434I, for instance, was observed in escape variants from the attachment inhibitor BMS-378806 and its derivatives (Lin et al., 2003; Zhou et al., 2014). Furthermore, some C4 mutations in the Env region also occur in neutralizing antibody sensitive variants (O'Rourke et al., 2012; Ringe et al., 2011; Vermeire et al., 2009). These C4 mutations, including M434I, are located in close proximity to the CD4 binding area. M434, a hydrophobic residue in the gp120 β21 strand, has a role in shielding and stabilizing hydrogen bonds between the β2 and β21 strands (Da, Quan, and Wu, 2011). M434 mutated to I434 might have an effect on stabilizing the CD4-bound conformation of the envelope (Da, Quan, and Wu, 2011; Pan, Ma, and Nussinov, 2005; Xiang et al., 2002; Zhou et al., 2011). Thus, it is possible that particular C4 mutations, such as M434I, impact exposure of the CD4 binding site epitope for neutralizing antibodies (O'Rourke et al., 2012; Ringe et al., 2011; Vermeire et al., 2009;

Yoshimura et al., 2014). In the present study, the M434I mutation showed enhanced neutralization sensitivity to anti-CD4bs, b12 and 0.5δ NMAbs and sCD4.

The M434I clone was also sensitive to sera obtained from HIV-1 subtype B infected patients (82%) compared to those with non subtype B infection (47%) (Table 4). The overall frequency of M434I is about 8% of the HIV-1 Los Alamos sequence database and is quite rare in subtype B (4%), but more common in recombinant subtypes (e.g. 23% of A1, 15% of CRF_02AG and 38% of CRF_A1C) (Zhou et al., 2011). It is possible that I434 has a greater impact on sensitivity to neutralization for subtype B compared to other HIV-1 subtypes so it is more prevalent in the latter group.

The MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I) were sensitive to over 50% of heterologous sera from treatment naïve patients whereas PC clone was sensitive to 6% of those sera. Comparing the reciprocal serum titers between the MVC-R (T297I/M434I/V200I/K305R) and MVC-R (M434I), the latter titer was significantly higher (Fig.17). This implies that the neutralization susceptibility of each MVC resistant clone to patient' sera is difference. The use of clones with different neutralizing antibody sensitivities may be useful in allowing the assessment of the evolution of sensitivity following MVC containing ART regimen initiation in subtype B infected patients.

Our preliminary data using heterologous sera from a patient treated with MVC containing ART (Fig.18 and Table 5) suggest that this treatment regimen might affect the induction of neutralization activity *in vivo* as the sera ACC-B2-2 and ACC-B2-3 which were collected after treatment with MVC could neutralized three KP-5 Env infectious clone better than sera obtain prior MVC treatment. This finding should be interpreted with caution due to the limited sample size and could be observed only one of two patients. The effect of ARV drug in sera on the neutralization susceptibility assay was also needed to be concerned, although all serum was diluted to minimized the inhibitory effect of ARV drug present in sera. Further studies are needed to investigate the effect of treatment with or without MVC on enhancing production of anti-HIV-1 neutralizing antibody using the clones described.

Following reversion of the four MVC resistant mutations, the clone MVC-R (4m-) was still sensitive to anti-V3 NMAbs (447-52D and KD-247). Amino acid substitutions other than these four mutations might be

involved in changing the neutralization sensitivity of the virus to anti-V3 antibodies. V65K mutation appeared in the viral population and selected by the low dose of MVC at the beginning of *in vitro* selection. This mutation may necessary for gaining MVC resistance phenotype as the presence of V65K mutation in PC and MVC resistant Env confers increasing of IC_{50} (Fig 12). The presence of V65K mutation with four MVC resistant mutations decreases sensitivity to NMAbs and autologous plasma IgGs, however it markedly increases sensitivity of PC virus to those antibodies. This indicates that a mutation in C1 region also influences to the neutralization susceptibility too. In 2014, Roederer et al. found that C1 mutations at position 45 and 47 of SIV Env are associated with monoclonal antibodies resistance and both mutations might mask the majority of epitopes on the viral Env (Roederer et al., 2014).

Introducing M434I or V65K mutation into JRFL and YU2 Env reveals that the effect of the M434I mutation on the sensitivity to NMAb and autologous plasma IgGs may be restricted by the backgroud *env* sequence (Fig. 16). Otherwise, different backbone may need different position in C4 region for increasing sensitivity to NMAbs. Moreover, introducing the M434I mutation into the Env of the PC virus impaired viral replication (data not shown).

Beside the mutations in gp120 region, the gp41 of MVC resistant virus also influences on sensitivity to MVC and NMAbs. Our result suggests that the gp41 region of MVC resistant virus decreases the sensitivity of MVC resistant virus to MVC and increases sensitivity of MVC resistant virus to NMAbs. However, the gp120 region is the major factor conferring sensitivity to NMAbs as PC_{gp120}/MVC-R_{gp41} clone shows not significantly increase the sensitivity to NMAbs compared to PC clone (Table 6). The CCR-5 inhibitor resistance variants harboring mutations in gp41 was also observed (Anastassopoulou et al., 2011; Anastassopoulou et al., 2009; Anastassopoulou et al., 2012; Vermeire et al., 2009). Mutations in gp41 region could enhance neutralization by anti-gp120 antibodies (Back et al., 1993; Blish, Nguyen, and Overbaugh, 2008; Kalia et al., 2005; Ringe and Bhattacharya, 2012). In 1993, Beck *et al.* suggested that gp41 mutation indirectly affect the neutralization epitopes of the CD4-binding site of gp120 or epitopes overlapping with this region (Back et al., 1993). In 2011, Lovelace *et al*. also reported that mutations in gp41 region enhance the neutralization sensitivity to anti-gp41, anti-gp120 and plasma (Lovelace et al., 2011). These suggest that

specific changes in envelope impact antibody binding and neutralization. The MVC resistant mutation in gp41 region and the mechanism of the mutation in gp41 effect the sensitivity of anti-gp120 antibodies is needed to be clarified.

5. CONCLUSION

A MVC resistant HIV-1 generated using *in vitro* selection exhibited high sensitivity to several NMAbs and autologous plasma IgGs. The resistant variant contained four mutations, T297I, M434I, V200I and K305R, which accumulated sequentially in the HIV-1 envelope. In this study, we examined the mutation most responsible for conferring enhanced neutralization sensitivity of the MVC resistant variant to several NMAbs and autologous plasma IgGs. We found that MVC resistant mutations play a role on changing the sensitivity to various NMAbs. Except for K305R, other mutations are most likely effect CD4 binding site. The changes of CD4 binding area may expose many epitopes of NMAbs. We propose that M434I mutation in C4 region has a major role of remarkable increase the sensitivity to NMAbs, autologous plasma IgGs and heterologous sera. Env changes due to MVC pressure especially, M434I mutation in the β21 strand in the C4 domain of gp120 induced resulted in alteration in the conformation of Env exposing discontinuous neutralizing epitopes in V3, CD4i and CD4bs forming the basis for enhanced virus neutralization with both autologous and heterologous peripheral blood antibodies. Other factors that also involve on the changing of neutralization sensitivity were also determined. We found that the gp41 region and pre-exist mutation in the Env of MVC resistant virus before the virus exposed to MVC also engage in changing the neutralization sensitivity. However, the ability of those mutations are limited only a particular Env background.

Our MVC resistant infectious clones exclusively express epitopes for several neutralizing antibodies. It may be a new candidate as an antigen for HIV vaccine for induces the anti-HIV antibody in human.

7. REFERENCES

Anastassopoulou, C. G., Ketas, T. J., Depetris, R. S., Thomas, A. M., Klasse, P. J., and Moore, J. P. (2011). Resistance of a human immunodeficiency virus type 1 isolate to a small molecule CCR5 inhibitor can involve sequence changes in both gp120 and gp41. *Virology* **413**(1)**,** 47 - 59.

Anastassopoulou, C. G., Ketas, T. J., Klasse, P. J., and Moore, J. P. (2009). Resistance to CCR5 inhibitors caused by sequence changes in the fusion peptide of HIV-1 gp41. *Proc Natl Acad Sci USA* **106,** 5318 - 5323.

Anastassopoulou, C. G., Ketas, T. J., Sanders, R. W., Klasse, P. J., and Moore, J. P. (2012). Effects of sequence changes in the HIV-1 gp41 fusion peptide on CCR5 inhibitor resistance. *Virology* **428**(2)**,** 86 - 97.

Arts, E. J., and Hazuda, D. J. (2012). HIV-1 Antiretroviral Drug Therapy. *Cold Spring Harbor Perspectives in Medicine* **2**(4)**,** a007161.

Asin-Milan, O., Chamberland, A., Wei, Y., Haidara, A., Sylla, M., and Tremblay, C. L. (2013). Mutations in variable domains of the HIV-1 envelope gene can have a significant impact on maraviroc and vicriviroc resistance. *AIDS Research and Therapy* **10:15**.

Back, N. K., Smit, L., Schutten, M., Nara, P. L., Tersmette, M., and Goudsmit, J. (1993). Mutations in human immunodeficiency virus type 1 gp41 affect sensitivity to neutralization by gp120 antibodies. *Journal of Virology* **67**(11)**,** 6897-6902.

Berro, R., Klasse, P. J., Moore, J. P., and Sanders, R. W. (2012). V3 determinants of HIV-1 escape from the CCR5 inhibitors Maraviroc and Vicriviroc. *Virology* **427**(2)**,** 158-165.

Berro, R., Sanders, R. W., Lu, M., Klasse, P. J., and Moore, J. P. (2009). Two HIV-1 variants resistant to small molecule CCR5 inhibitors differ in how they use CCR5 for entry. *PLoS Pathog* **5**(8)**,** e1000548.

Blish, C. A., Nguyen, M.-A., and Overbaugh, J. (2008). Enhancing Exposure of HIV-1 Neutralization Epitopes through Mutations in gp41. *PLoS Med* **5**(1)**,** e9.

Chan, D. C., Fass, D., Berger, J. M., and Kim, P. S. (1997). Core Structure of gp41 from the HIV Envelope Glycoprotein. *Cell* **89**(2)**,** 263-273.

Cormier, E. G., and Dragic, T. (2002). The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor. *J Virol* **76**(17)**,** 8953 - 8957.

Da, L.-T., Quan, J.-M., and Wu, Y.-D. (2011). Understanding the binding mode and function of BMS-488043 against HIV-1 viral entry. *Proteins: Structure, Function, and Bioinformatics* **79**(6)**,** 1810-1819.

Decker, J. M., Bibollet-Ruche, F., Wei, X., Wang, S., Levy, D. N., Wang, W., Delaporte, E., Peeters, M., Derdeyn, C. A., Allen, S., Hunter, E., Saag, M. S., Hoxie, J. A., Hahn, B. H., Kwong, P. D., Robinson, J. E., and Shaw, G. M. (2005). Antigenic conservation and

immunogenicity of the HIV coreceptor binding site. *The Journal of Experimental Medicine* **201**(9)**,** 1407-1419.

Delobel, P., Raymond, S., Mavigner, M., Cazabat, M., Alvarez, M., Marchou, B., Massip, P., and Izopet, J. (2010). Shift in phenotypic susceptibility suggests a competition mechanism in a case of acquired resistance to maraviroc. *AIDS* **24**(9)**,** 1382-1384 10.1097/QAD.0b013e328338b7a6.

di Marzo Veronese, F., Reitz, M. S., Gupta, G., Robert-Guroff, M., Boyer-Thompson, C., Louie, A., Gallo, R. C., and Lusso, P. (1993). Loss of a neutralizing epitope by a spontaneous point mutation in the V3 loop of HIV-1 isolated from an infected laboratory worker. *Journal of Biological Chemistry* **268**(34)**,** 25894-901.

Didigu, C. A., and Doms, R. W. (2012). Novel Approaches to Inhibit HIV Entry. *Viruses* **4**(2)**,** 309-324.

Dragic, T., Trkola, A., Thompson, D. A. D., Cormier, E. G., Kajumo, F. A., Maxwell, E., Lin, S. W., Ying, W., Smith, S. O., Sakmar, T. P., and Moore, J. P. (2000). A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5. *Proceedings of the National Academy of Sciences* **97**(10)**,** 5639-5644.

Eda, Y., Murakami, T., Ami, Y., Nakasone, T., Takizawa, M., Someya, K., Kaizu, M., Izumi, Y., Yoshino, N., Matsushita, S., Higuchi, H., Matsui, H., Shinohara, K., Takeuchi, H., Koyanagi, Y., Yamamoto, N., and Honda, M. (2006). Anti-V3 Humanized Antibody KD-247 Effectively Suppresses Ex Vivo Generation of Human Immunodeficiency Virus Type 1 and Affords Sterile Protection of Monkeys against a Heterologous Simian/Human Immunodeficiency Virus Infection. *Journal of Virology* **80**(11)**,** 5563-5570.

Fätkenheuer, G., Nelson, M., Lazzarin, A., Konourina, I., Hoepelman, A. I. M., Lampiris, H., Hirschel, B., Tebas, P., Raffi, F., Trottier, B., Bellos, N., Saag, M., Cooper, D. A., Westby, M., Tawadrous, M., Sullivan, J. F., Ridgway, C., Dunne, M. W., Felstead, S., Mayer, H., and van der Ryst, E. (2008). Subgroup Analyses of Maraviroc in Previously Treated R5 HIV-1 Infection. *New England Journal of Medicine* **359**(14)**,** 1442-1455.

FDA, U. S. (2014). Antiretroviral drugs used in the treatment of HIV infection.

Gray, E. S., Madiga, M. C., Hermanus, T., Moore, P. L., Wibmer, C. K., Tumba, N. L., Werner, L., Mlisana, K., Sibeko, S., Williamson, C., Abdool Karim, S. S., Morris, L., and and the, C. S. T. (2011). The Neutralization Breadth of HIV-1 Develops Incrementally over Four Years and Is Associated with CD4(+) T Cell Decline and High Viral Load during Acute Infection. *Journal of Virology* **85**(10)**,** 4828-4840.

Harada, S., Yoshimura, K., Yamaguchi, A., Boonchawalit, S., Yusa, K., and Matsushita, S. (2013). Impact of antiretroviral pressure on selection of primary human immunodeficiency virus type 1 envelope sequences in vitro. *Journal of General Virology* **94**(Pt 5)**,** 933-943.

Hatada, M., Yoshimura, K., Harada, S., Kawanami, Y., Shibata, J., and Matsushita, S. (2010). Human immunodeficiency virus type 1 evasion of a neutralizing anti-V3 antibody involves

acquisition of a potential glycosylation site in V2. *Journal of General Virology* **91**(5)**,** 1335-1345.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**(6510)**,** 123-126.

Huang, C. C., Tang, M., Zhang, M. Y., Majeed, S., Montabana, E., Stanfield, R. L., Dimitrov, D. S., Korber, B., Sodroski, J., and Wilson, I. A. (2005). Structure of a V3-containing HIV-1 gp120 core. *Science* **310**(5750)**,** 1025 - 1028.

Kalia, V., Sarkar, S., Gupta, P., and Montelaro, R. C. (2005). Antibody Neutralization Escape Mediated by Point Mutations in the Intracytoplasmic Tail of Human Immunodeficiency Virus Type 1 gp41. *Journal of Virology* **79**(4)**,** 2097-2107.

Kessler, J. A., McKenna, P. M., Emini, E. A., Chan, C. P., Patel, M. D., Gupta, S. K., Mark, G. E., Barbas, C. F., Burton, D. R., and Conley, A. J. (1997). Recombinant human monoclonal antibody IgG1b12 neutralizes diverse human immunodeficiency virus type 1 primary isolates. *AIDS Res Hum Retroviruses* **13**(7)**,** 575-82.

Killikelly, A., Zhang, H.-T., Spurrier, B., Williams, C., Gorny, M. K., Zolla-Pazner, S., and Kong, X.-P. (2013). Thermodynamic Signatures of the Antigen Binding Site of mAb 447-52D Targeting the Third Variable Region of HIV-1 gp120. *Biochemistry* **52**(36)**,** 6249-6257.

Kimura, T., Yoshimura, K., Nishihara, K., Maeda, Y., Matsumi, S., Koito, A., and Matsushita, S. (2002). Reconstitution of Spontaneous Neutralizing Antibody Response against Autologous Human Immunodeficiency Virus during Highly Active Antiretroviral Therapy. *Journal of Infectious Diseases* **185**(1)**,** 53-60.

Kondru, R., Zhang, J., Ji, C., Mirzadegan, T., Rotstein, D., Sankuratri, S., and Dioszegi, M. (2008). Molecular interactions of CCR5 with major classes of small-molecule anti-HIV CCR5 antagonists. *Mol Pharmacol* **73,** 789 - 800.

Kong, L., Huang, C.-c., Coales, S. J., Molnar, K. S., Skinner, J., Hamuro, Y., and Kwong, P. D. (2010). Local Conformational Stability of HIV-1 gp120 in Unliganded and CD4-Bound States as Defined by Amide Hydrogen/Deuterium Exchange. *Journal of Virology* **84**(19)**,** 10311-10321.

Kuhmann, S. E., Pugach, P., Kunstman, K. J., Taylor, J., Stanfield, R. L., Snyder, A., Strizki, J. M., Riley, J., Baroudy, B. M., and Wilson, I. A. (2004). Genetic and phenotypic analyses of human immunodeficiency virus type 1 escape from a small-molecule CCR5 inhibitor. *J Virol* **78,** 2790 - 2807.

Kuritzkes, D. R. (2009). HIV-1 Entry Inhbitors: An Overview. *Current opinion in HIV and AIDS* **4**(2)**,** 82-87.

Liao, H.-X., Lynch, R., Zhou, T., Gao, F., Alam, S. M., Boyd, S. D., Fire, A. Z., Roskin, K. M., Schramm, C. A., Zhang, Z., Zhu, J., Shapiro, L., Mullikin, J. C., Gnanakaran, S., Hraber, P., Wiehe, K., Kelsoe, G., Yang, G., Xia, S.-M., Montefiori, D. C., Parks, R., Lloyd, K. E., Scearce, R. M., Soderberg, K. A., Cohen, M., Kamanga, G., Louder, M. K., Tran, L. M.,

Chen, Y., Cai, F., Chen, S., Moquin, S., Du, X., Joyce, M. G., Srivatsan, S., Zhang, B., Zheng, A., Shaw, G. M., Hahn, B. H., Kepler, T. B., Korber, B. T. M., Kwong, P. D., Mascola, J. R., and Haynes, B. F. (2013). Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature* **496**(7446)**,** 469-476.

Lin, P.-F., Blair, W., Wang, T., Spicer, T., Guo, Q., Zhou, N., Gong, Y.-F., Wang, H. G. H., Rose, R., Yamanaka, G., Robinson, B., Li, C.-B., Fridell, R., Deminie, C., Demers, G., Yang, Z., Zadjura, L., Meanwell, N., and Colonno, R. (2003). A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *Proceedings of the National Academy of Sciences* **100**(19)**,** 11013-11018.

Lovelace, E., Xu, H., Blish, C. A., Strong, R., and Overbaugh, J. (2011). The role of amino acid changes in the human immunodeficiency virus type 1 transmembrane domain in antibody binding and neutralization. *Virology* **421**(2)**,** 235-244.

Lyumkis, D., Julien, J.-P., de Val, N., Cupo, A., Potter, C. S., Klasse, P.-J., Burton, D. R., Sanders, R. W., Moore, J. P., Carragher, B., Wilson, I. A., and Ward, A. B. (2013). Cryo-EM Structure of a Fully Glycosylated Soluble Cleaved HIV-1 Envelope Trimer. *Science* **342**(6165)**,** 1484-1490.

Maeda, Y., Yusa, K., and Harada, S. (2008). Altered sensitivity of an R5X4 HIV-1 strain 89.6 to coreceptor inhibitors by a single amino acid substitution in the V3 region of gp120. *Antiviral Research* **77**(2)**,** 128-135.

Marozsan, A. J., Kuhmann, S. E., Morgan, T., Herrera, C., Rivera-Troche, E., Xu, S., Baroudy, B. M., Strizki, J., and Moore, J. P. (2005). Generation and properties of a human immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor, SCH-417690 (SCH-D). *Virology* **338**(1)**,** 182 - 199.

Mascola, J. R., and Haynes, B. F. (2013). HIV-1 neutralizing antibodies: understanding nature 寰冭 pathways. *Immunological reviews* **254**(1)**,** 225-244.

McCaffrey, R. A., Saunders, C., Hensel, M., and Stamatatos, L. (2004). N-Linked Glycosylation of the V3 Loop and the Immunologically Silent Face of gp120 Protects Human Immunodeficiency Virus Type 1 SF162 from Neutralization by Anti-gp120 and Anti-gp41 Antibodies. *Journal of Virology* **78**(7)**,** 3279-3295.

Mikell, I., Sather, D. N., Kalams, S. A., Altfeld, M., Alter, G., and Stamatatos, L. (2011). Characteristics of the Earliest Cross-Neutralizing Antibody Response to HIV-1. *PLoS Pathogens* **7**(1)**,** e1001251.

Myszka, D. G., Sweet, R. W., Hensley, P., Brigham-Burke, M., Kwong, P. D., Hendrickson, W. A., Wyatt, R., Sodroski, J., and Doyle, M. L. (2000). Energetics of the HIV gp120-CD4 binding reaction. *Proceedings of the National Academy of Sciences of the United States of America* **97**(16)**,** 9026-9031.

O'Rourke, S. M., Schweighardt, B., Phung, P., Mesa, K. A., Vollrath, A. L., Tatsuno, G. P., To, B., Sinangil, F., Limoli, K., Wrin, T., and Berman, P. W. (2012). Sequences in Glycoprotein

gp41, the CD4 Binding Site, and the V2 Domain Regulate Sensitivity and Resistance of HIV-1 to Broadly Neutralizing Antibodies. *Journal of Virology* **86**(22)**,** 12105-12114.

Ogert, R. A., Ba, L., Hou, Y., Buontempo, C., Qiu, P., Duca, J., Murgolo, N., Buontempo, P., Ralston, R., and Howe, J. A. (2009). Structure-function analysis of human immunodeficiency virus type 1 gp120 amino acid mutations associated with resistance to the CCR5 coreceptor antagonist vicriviroc. *J Virol* **83**(23)**,** 12151 - 12163.

Ogert, R. A., Hou, Y., Ba, L., Wojcik, L., Qiu, P., Murgolo, N., Duca, J., Dunkle, L. M., Ralston, R., and Howe, J. A. (2010). Clinical resistance to vicriviroc through adaptive V3 loop mutations in HIV-1 subtype D gp120 that alter interactions with the N-terminus and ECL2 of CCR5. *Virology* **400**(1)**,** 145-155.

Pan, Y., Ma, B., and Nussinov, R. (2005). CD4 Binding Partially Locks the Bridging Sheet in gp120 but Leaves the β2/3 Strands Flexible. *Journal of Molecular Biology* **350**(3)**,** 514-527.

Pantophlet, R., and Burton, D. R. (2006). GP120: Target for Neutralizing HIV-1 Antibodies. *Annual Review of Immunology* **24**(1)**,** 739-769.

Poignard, P., Saphire, E. O., Parren, P. W. H. I., and Burton, D. R. (2001). GP120: Biologic Aspects of Structural Features. *Annual Review of Immunology* **19**(1)**,** 253-274.

Pugach, P., Ketas, T. J., Michael, E., and Moore, J. P. (2008). Neutralizing antibody and anti-retroviral drug sensitivities of HIV-1 isolates resistant to small molecule CCR5 inhibitors. *Virology* **377**(2)**,** 401-407.

Pugach, P., Marozsan, A. J., Ketas, T. J., Landes, E. L., Moore, J. P., and Kuhmann, S. E. (2007). HIV-1 clones resistant to a small molecule CCR5 inhibitor use the inhibitor-bound form of CCR5 for entry. *Virology* **361**(1)**,** 212 - 228.

Ramirez Valdez, K. P., Kuwata, T., Maruta, Y., Tanaka, K., Alam, M., Yoshimura, K., and Matsushita, S. (2014). Complementary and synergistic activities of anti-V3, CD4bs and CD4i antibodies derived from a single individual can cover a wide range of HIV-1 strains. *Virology* **475**(0)**,** 187-203.

Ratcliff, A. N., Shi, W., and Arts, E. J. (2013). HIV-1 Resistance to Maraviroc Conferred by a CD4 Binding Site Mutation in the Envelope Glycoprotein gp120. *Journal of Virology* **87**(2)**,** 923-934.

Ringe, R., and Bhattacharya, J. (2012). Association of Enhanced HIV-1 Neutralization by a Single Y681H Substitution in gp41 with Increased gp120-CD4 Interaction and Macrophage Infectivity. *PLoS ONE* **7**(5)**,** e37157.

Ringe, R., Sharma, D., Zolla-Pazner, S., Phogat, S., Risbud, A., Thakar, M., Paranjape, R., and Bhattacharya, J. (2011). A single amino acid substitution in the C4 region in gp120 confers enhanced neutralization of HIV-1 by modulating CD4 binding sites and V3 loop. *Virology* **418**(2)**,** 123-132.

Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P. D., Hendrickson, W. A., and Sodroski, J. (1998a). A Conserved HIV gp120 Glycoprotein Structure Involved in Chemokine Receptor Binding. *Science* **280**(5371)**,** 1949-1953.

Roche, M., Jakobsen, M. R., Sterjovski, J., Ellett, A., Posta, F., Lee, B., Jubb, B., Westby, M., Lewin, S. R., Ramsland, P. A., Churchill, M. J., and Gorry, P. R. (2011). HIV-1 Escape from the CCR5 Antagonist Maraviroc Associated with an Altered and Less-Efficient Mechanism of gp120-CCR5 Engagement That Attenuates Macrophage Tropism. *Journal of Virology* **85**(9)**,** 4330-4342.

Roche, M., Salimi, H., Duncan, R., Wilkinson, B., Chikere, K., Moore, M., Webb, N., Zappi, H., Sterjovski, J., Flynn, J., Ellett, A., Gray, L., Lee, B., Jubb, B., Westby, M., Ramsland, P., Lewin, S., Payne, R., Churchill, M., and Gorry, P. (2013). A common mechanism of clinical HIV-1 resistance to the CCR5 antagonist maraviroc despite divergent resistance levels and lack of common gp120 resistance mutations. *Retrovirology* **10**(1)**,** 43.

Roederer, M., Keele, B. F., Schmidt, S. D., Mason, R. D., Welles, H. C., Fischer, W., Labranche, C., Foulds, K. E., Louder, M. K., Yang, Z.-Y., Todd, J.-P. M., Buzby, A. P., Mach, L. V., Shen, L., Seaton, K. E., Ward, B. M., Bailer, R. T., Gottardo, R., Gu, W., Ferrari, G., Alam, S. M., Denny, T. N., Montefiori, D. C., Tomaras, G. D., Korber, B. T., Nason, M. C., Seder, R. A., Koup, R. A., Letvin, N. L., Rao, S. S., Nabel, G. J., and Mascola, J. R. (2014). Immunological and virological mechanisms of vaccine-mediated protection against SIV and HIV. *Nature* **505**(7484)**,** 502-508.

Roitburd-Berman, A., Dela, G., Kaplan, G., Lewis, G. K., and Gershoni, J. M. (2013). Allosteric induction of the CD4-bound conformation of HIV-1 Gp120. *Retrovirology* **10,** 147-147.

Shibata, J., Yoshimura, K., Honda, A., Koito, A., Murakami, T., and Matsushita, S. (2007). Impact of V2 Mutations on Escape from a Potent Neutralizing Anti-V3 Monoclonal Antibody during In Vitro Selection of a Primary Human Immunodeficiency Virus Type 1 Isolate. *Journal of Virology* **81**(8)**,** 3757-3768.

Soriano, V., Geretti, A. M., Perno, C.-F., F テ、tkenheuer, G., Pillay, D., Reynes, J., Tambussi, G., Calvez, V., Alcam テュ, J., and Rockstroh, J. (2008). Optimal use of maraviroc in clinical practice. *AIDS* **22**(17)**,** 2231-2240.

Soulie, C., Malet, I., Lambert-Niclot, S., Tubiana, R., Thevenin, M., Simon, A., Murphy, R., Katlama, C., Calvez, V., and Marcelin, A.-G. (2008). Primary genotypic resistance of HIV-1 to CCR5 antagonists in CCR5 antagonist treatment-naive patients. *AIDS* **22**(16)**,** 2212-2214 10.1097/QAD.0b013e328313bf9c.

Suphaphiphat, P., Essex, M., and Lee, T.-H. (2007). Mutations in the V3 stem versus the V3 crown and C4 region have different effects on the binding and fusion steps of human immunodeficiency virus type 1 gp120 interaction with the CCR5 coreceptor. *Virology* **360**(1)**,** 182-190.

Swenson, L. C., Chui, C. K. S., Brumme, C. J., Chan, D., Woods, C. K., Mo, T., Dong, W., Chapman, D., Lewis, M., Demarest, J. F., James, I., Portsmouth, S., Goodrich, J., Heera, J., Valdez, H., and Harrigan, P. R. (2013). Genotypic Analysis of the V3 Region of HIV from Virologic Nonresponders to Maraviroc-Containing Regimens Reveals Distinct Patterns of Failure. *Antimicrobial Agents and Chemotherapy* **57**(12)**,** 6122-6130.

Tan, Q., Zhu, Y., Li, J., Chen, Z., Han, G. W., Kufareva, I., Li, T., Ma, L., Fenalti, G., Li, J., Zhang, W., Xie, X., Yang, H., Jiang, H., Cherezov, V., Liu, H., Stevens, R. C., Zhao, Q., and Wu, B. (2013). Structure of the CCR5 Chemokine Receptor 寰滴 IV Entry Inhibitor Maraviroc Complex. *Science*.

Tilton, J. C., Wilen, C. B., Didigu, C. A., Sinha, R., Harrison, J. E., Agrawal-Gamse, C., Henning, E. A., Bushman, F. D., Martin, J. N., Deeks, S. G., and Doms, R. W. (2010). A maraviroc-resistant HIV-1 with narrow cross-resistance to other CCR5 antagonists depends on both N-terminal and extracellular loop domains of drug-bound CCR5. *J Virol* **84,** 10863 - 10876.

Tomaras, G. D., Yates, N. L., Liu, P., Qin, L., Fouda, G. G., Chavez, L. L., Decamp, A. C., Parks, R. J., Ashley, V. C., Lucas, J. T., Cohen, M., Eron, J., Hicks, C. B., Liao, H.-X., Self, S. G., Landucci, G., Forthal, D. N., Weinhold, K. J., Keele, B. F., Hahn, B. H., Greenberg, M. L., Morris, L., Karim, S. S. A., Blattner, W. A., Montefiori, D. C., Shaw, G. M., Perelson, A. S., and Haynes, B. F. (2008). Initial B-Cell Responses to Transmitted Human Immunodeficiency Virus Type 1: Virion-Binding Immunoglobulin M (IgM) and IgG Antibodies Followed by Plasma Anti-gp41 Antibodies with Ineffective Control of Initial Viremia. *Journal of Virology* **82**(24)**,** 12449-12463.

Tsamis, F., Gavrilov, S., Kajumo, F., Seibert, C., Kuhmann, S., Ketas, T., Trkola, A., Palani, A., Clader, J. W., and Tagat, J. R. (2003). Analysis of the mechanism by which the small-molecule CCR5 antagonists SCH-351125 and SCH-350581 inhibit human immunodeficiency virus type 1 entry. *J Virol* **77**(9)**,** 5201 - 5208.

Tsibris, A. M., Sagar, M., Gulick, R. M., Su, Z., Hughes, M., Greaves, W., Subramanian, M., Flexner, C., Giguel, F., and Leopold, K. E. (2008). In vivo emergence of vicriviroc resistance in a human immunodeficiency virus type 1 subtype C-infected subject. *J Virol* **82**(16)**,** 8210 - 8214.

UNAIDS (2015). Report to UNAIDS-HIV/AIDS Trends in Japan, March 2014.

Vermeire, K., Van Laethem, K., Janssens, W., Bell, T. W., and Schols, D. (2009). Human Immunodeficiency Virus Type 1 Escape from Cyclotriazadisulfonamide-Induced CD4-Targeted Entry Inhibition Is Associated with Increased Neutralizing Antibody Susceptibility. *Journal of Virology* **83**(18)**,** 9577-9583.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn, B. H., Kwong, P. D., and Shaw, G. M. (2003). Antibody neutralization and escape by HIV-1. *Nature* **422**(6929)**,** 307-312.

Westby, M., Lewis, M., Whitcomb, J., Youle, M., Pozniak, A. L., James, I. T., Jenkins, T. M., Perros, M., and van der Ryst, E. (2006). Emergence of CXCR4-using human immunodeficiency virus type 1 (HIV-1) variants in a minority of HIV-1-infected patients following treatment with the CCR5 antagonist maraviroc is from a pretreatment CXCR4-using virus reservoir. *J Virol* **80**(10)**,** 4909 - 4920.

Westby, M., Smith-Burchnell, C., Mori, J., Lewis, M., Mosley, M., Stockdale, M., Dorr, P., Ciaramella, G., and Perros, M. (2007). Reduced Maximal Inhibition in Phenotypic Susceptibility Assays Indicates that Viral Strains Resistant to the CCR5 Antagonist Maraviroc Utilize Inhibitor-Bound Receptor for Entry. *Journal of Virology* **81**(5)**,** 2359-2371.

WHO (2014). Global Health Observatory (GHO).

Wilen, C. B., Tilton, J. C., and Doms, R. W. (2012). HIV: Cell Binding and Entry. *Cold Spring Harbor Perspectives in Medicine* **2**(8).

Xiang, S.-H., Finzi, A., Pacheco, B., Alexander, K., Yuan, W., Rizzuto, C., Huang, C.-C., Kwong, P. D., and Sodroski, J. (2010). A V3 Loop-Dependent gp120 Element Disrupted by CD4 Binding Stabilizes the Human Immunodeficiency Virus Envelope Glycoprotein Trimer. *Journal of Virology* **84**(7)**,** 3147-3161.

Xiang, S.-H., Kwong, P. D., Gupta, R., Rizzuto, C. D., Casper, D. J., Wyatt, R., Wang, L., Hendrickson, W. A., Doyle, M. L., and Sodroski, J. (2002). Mutagenic Stabilization and/or Disruption of a CD4-Bound State Reveals Distinct Conformations of the Human Immunodeficiency Virus Type 1 gp120 Envelope Glycoprotein. *Journal of Virology* **76**(19)**,** 9888-9899.

Yoshimura, K., Harada, S., Boonchawalit, S., Kawanami, Y., and Matsushita, S. (2014). Impact of maraviroc-resistant and low-CCR5-adapted mutations induced by in vitro passage on sensitivity to anti-envelope neutralizing antibodies. *Journal of General Virology* **95**(Pt 8)**,** 1816-1826.

Yoshimura, K., Harada, S., Shibata, J., Hatada, M., Yamada, Y., Ochiai, C., Tamamura, H., and Matsushita, S. (2010). Enhanced Exposure of Human Immunodeficiency Virus Type 1 Primary Isolate Neutralization Epitopes through Binding of CD4 Mimetic Compounds. *Journal of Virology* **84**(15)**,** 7558-7568.

Yoshimura, K., Shibata, J., Kimura, T., Honda, A., Maeda, Y., Koito, A., Murakami, T., Mitsuya, H., and Matsushita, S. (2006). Resistance profile of a neutralizing anti-HIV monoclonal antibody, KD-247, that shows favourable synergism with anti-CCR5 inhibitors. *AIDS* **20**(16)**,** 2065-2073 10.1097/01.aids.0000247587.31320.fe.

Yuan, Y., Maeda, Y., Terasawa, H., Monde, K., Harada, S., and Yusa, K. (2011). A combination of polymorphic mutations in V3 loop of HIV-1 gp120 can confer noncompetitive resistance to maraviroc. *Virology* **413,** 293 - 299.

Yuan, Y., Yokoyama, M., Maeda, Y., Terasawa, H., Harada, S., Sato, H., and Yusa4, K. (2013). Structure and Dynamics of the gp120 V3 Loop That Confers Noncompetitive Resistance in R5 HIV-1JR-FL to Maraviroc. *PLOS ONE* **8(6)**.

Yusa, K., Maeda, Y., Fujioka, A., Monde, K., and Harada, S. (2005). Isolation of TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 Loop Library. *Journal of Biological Chemistry* **280**(34)**,** 30083-30090.

Zhou, N., Nowicka-Sans, B., McAuliffe, B., Ray, N., Eggers, B., Fang, H., Fan, L., Healy, M., Langley, D. R., Hwang, C., Lataillade, M., Hanna, G. J., and Krystal, M. (2014). Genotypic correlates of susceptibility to HIV-1 attachment inhibitor BMS-626529, the active agent of the prodrug BMS-663068. *Journal of Antimicrobial Chemotherapy* **69**(3)**,** 573-581.

Zhou, N., Nowicka-Sans, B., Zhang, S., Fan, L., Fang, J., Fang, H., Gong, Y.-F., Eggers, B., Langley, D. R., Wang, T., Kadow, J., Grasela, D., Hanna, G. J., Alexander, L., Colonno, R., Krystal, M., and Lin, P.-F. (2011). In Vivo Patterns of Resistance to the HIV Attachment Inhibitor BMS-488043. *Antimicrobial Agents and Chemotherapy* **55**(2)**,** 729-737.

Zolla-Pazner, S., Gorny, M. K., Nyambi, P. N., VanCott, T. C., and Nadas, A. (1999). Immunotyping of Human Immunodeficiency Virus Type 1 (HIV): an Approach to Immunologic Classification of HIV. *Journal of Virology* **73**(5)**,** 4042-4051.

Zwick, M. B., Labrijn, A. F., Wang, M., Spenlehauer, C., Saphire, E. O., Binley, J. M., Moore, J. P., Stiegler, G., Katinger, H., Burton, D. R., and Parren, P. W. H. I. (2001). Broadly Neutralizing Antibodies Targeted to the Membrane-Proximal External Region of Human Immunodeficiency Virus Type 1 Glycoprotein gp41. *Journal of Virology* **75**(22)**,** 10892-10905.