Title

Implications of the 375W mutation for HIV-1 tropism and vaccine development

Authors

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Abstract

HIV-1 vaccines need to induce broadly neutralizing antibodies (bnAb) against conserved epitopes in the envelope glycoprotein (Env) to protect against diverse HIV-1 clades. To achieve this, we need to understand how different amino acids affect the Env trimer structure to find a common strategy to readily produce Env vaccines of different subtypes. Previously, using a saturation mutagenesis strategy we identified single Env substitutions that open the CD4bs without modifying the trimer apex. One of these substitutions was a tryptophan residue introduced at position 375. Here, we introduced 375W into a large panel of 27 T/F, acute stage, chronic infection, and AIDS M-tropic, and non-M-tropic primary isolates from clades A, B, C, D and G, and circulating recombinant forms (CRFs) (CRF02_AG, and CRF01_AE), and a complex (cpx) (CRF13_cpx). To understand the effect of 375W mutation on Env trimer structure and tropism, we evaluated soluble (sCD4) and monoclonal antibody (mAb) neutralization (b6, 17b, b12, VCR01, 3BNC117, PGT128, 10-1074, PGT145, PG9 and PG16), as well as macrophage infection using wt and mutant Env+ pseudovirions. Broadly neutralizing Abs (bnAbs) such VCR01, and 3BNC117 neutralized almost all the primary isolates tested while the other bnAbs neutralized many but not all of our panel. B12 and VCR01 showed some tendencies to neutralize 375W macrophage-tropic (mac-tropic) and intermediate mac-tropic mutants more efficiently compared with non-mac-tropic mutants. In general, 375W did not impair neutralization of bnAbs. Envs that infected macrophages more efficiently than non-mac-tropic variants but did not reach the levels of highly macrophage-tropic brain reference Envs were classified as intermediate mac-tropic variants. Surprisingly, we observed intermediate mac-tropic primary isolates from clade C, D and G wt Envs that were not derived from the central nervous system (CNS). 375W substitution increased the affinity for sCD4 in all Envs of our panel and macrophage infection in Envs tested including a CRF01_AE X4 variant with the exception of one intermediate and mac-tropic variants. Increased sCD4 sensitivity and enhanced macrophage infection provide strong evidence that 375W confers exposure of the CD4bs across Envs from different clades/CRF/cpx and disease stages. Enhanced exposure of the CD4bs by 375W had little or no effect on exposure and sensitivity of CD4bs epitopes targeted by bnAbs. 375W is an ideal substitution for inclusion into HIV vaccines constructed from different subtype Envs, with aim to elicit neutralizing antibodies that target the CD4bs while maintaining exposure of other Env neutralization sites.

Introduction

HIV uses the CD4 receptor and a coreceptor to infect cells. The main coreceptors are CCR5 and CXCR4. Primary isolates that infect via CCR5 (R5 viruses) were classified as non-syncytium-inducing or macrophage tropic (mac-tropic) because these envelope glycoproteins (Envs) were unable to induce syncytia in T-cell lines or peripheral blood mononuclear cells (PBMCs) and infected primary macrophages. More recently, highly mac-tropic R5 viruses were detected in samples from brain and CSF tissue, but so far only in subtype B and C primary isolates [1-4] except for a semen sample from a patient infected by group M virus which did not correspond to any described subtype/CRF [3]. Moreover, these Envs are able to infect target cells that express low levels of CD4 receptors such as human macrophages, monocytes, microglial cells and hematopoietic progenitor cells [5]. On the other hand, variants that use the CXCR4 coreceptor (X4) are classified as T-tropic or syncytium-inducing due to their capacity to induce syncytia in T-cell lines and primary T-cells. Frequently, such viruses can use both CCR5 and CXCR4 coreceptors to infect cells and are termed dual tropic. Subsequently, non-macrophage tropic (non-mac-tropic) Envs that use CCR5 coreceptor were identified mainly in immune tissue, semen, and blood, and also in CSF [1, 2, 4].

All attempts to develop a potent HIV-1 vaccine have been unsuccessful. The immunogens investigated so far have not induced potent broadly neutralizing antibodies (bnAbs) that are effective against each of the different HIV-1 subtypes. An ideal immunogen candidate should recognize epitope structures common or similar across the huge range of Envelope (Env) variability of different HIV-1 clades. To achieve this, it is important that we improve our knowledge of how different amino acids and sites within Env impact the overall Env trimer structure and expose conserved epitopes such as the CD4 binding site (CD4bs) in the same way for all subtypes. It is therefore important to define sites and structures within the CD4bs that will need to be preserved in vaccines for the induction of neutralizing antibodies (nAbs). Thus, the CD4bs is a major target for cross-reacting bnAbs e.g. 3BCN177 and VRC01 and the development of vaccines that aim to elicit neutralizing antibodies. Moreover, ideal immunogens will not produce “off target” non-neutralizing Abs (non-nAbs) against occluded highly immunogenic epitopes including CD4-induced (CD4i), V3 loop, or trimer base of SOSIP immunogens [6]. The glycan shield is used by HIV-1 to mask neutralizing epitopes enabling escape from immune system pressure. Removing a glycan on an immunogen surface can expose an epitope that will induce Abs and/or create novel immunodominant areas such as in BG505 [7]. However, such glycan holes are not always present on different primary Envs. Moreover, amino acid changes in Env may modify the trimer structure, shifting the orientation of proximal glycans altering epitope exposure [8]. An HIV-1 vaccine designed to induce potent antibodies therefore needs to minimize the induction of highly immunogenic strain-specific epitopes e.g glycans, CD4i, the V3 loop, while exposing the CD4bs.

Previously, we used EMPIRIC, a saturation mutagenesis assay to identify Env substitutions that opened, or exposed the CD4bs without modifying the trimer apex [9]. The 375W substitution/mutant was one that was selected from a HIV-1 mutant library following cultivation in healthy donor peripheral blood mononuclear cells (PBMCs). In fact, position 375 was able to support a range of different amino acids that conferred replication as efficiently as (L, M, C, N, and Q) or better (H, T, Y, F and W) than wt (S).

A tryptophan at position 375 in gp120 of YU2 strain that fills the Phe 43 cavity [10] has been implicated in slight increases of sCD4 affinity, 2G12 glycan monoclonal antibody (mAb) neutralization sensitivity, resistance to b12 mAb (against CD4bs) and other non-neutralizing CD4bs Abs (b6 and F105) [10, 11] as well as inducing a conformational change closer to a CD4-bound state [10-12] and refolding the bridging sheet [13]. This 375W mutation can also be found in most HIV-2 and simian immunodeficiency (SIV) isolates such as SIVmac isolates including SIVmac239, SIV from sooty mangabey monkeys (SIVsmm) and some chimpanzees such as CPZ.TZ.06.TAN5, but it is not found in HIV-1 primary isolates [14]. SIV can infect cells that express low levels of CD4 in the surface, and a tryptophan at position 375 could help explain why it is less dependent of the CD4 receptor [15]. In SIV, the change of the tryptophan for a serine (typically found in HIV-1) reduces CD4 interaction and infection. Other amino acid changes in layer 1 of gp120 can decrease CD4 affinity but this effect is counteracted by 375W mutation [15]. SIV viruses that artificially carry HIV-1 Envs are called Simian-human immunodeficiency viruses (SHIVs). HIV-1 strains cannot infect via rhesus CD4 (rhCD4). However, the introduction of basic (H) or hydrophobic (Y, F and W) amino acids at position 375 into clade A, B, C and D SHIVs not only increases infection in rhCD4 cells but also enables replication with titers close to HIV-1 in human cells without altering the sensitivity to bnAb [16].

The effect of other mutations at position 375 has been studied in the context of different subtypes. 375N mutation in clade B HXB2 confers resistant to sCD4 and mAbs (39.13g, 1.5e, G13 and 448) that overlap the CD4bs [17]. A substitution of serine for threonine, at position 375 confers resistance to BMS-626529 attachment, an inhibitor that binds HIV-1 Env to block CD4 interaction in subtype B [18]. In a minority of CRF02_AG strains, histidine and methionine substitutions at 375 were genetically selected to induce drug resistance to this drug. CRF01_AE usually has histidine in position 375. When histidine is changed by a serine, viruses are less infectious [19] and a reduction of the antibody dependent cellular-mediated cytotoxicity (ADCC) was also observed [20]. Moreover, a tryptophan in position 375 in subtype B (YU2) and CRF01_AE reduced the gp120-gp41 association.

It is difficult to identify mutations that induce the same phenotype and/or conformational changes in the trimer for all clades including circulating recombinant forms (CRF) and complex (cpx) Envs. In this study, we tested the effect of 375W on exposure of CD4bs and a range of epitopes targeted by bnAbs. The CD4bs and such epitopes are the main targets for HIV-1 vaccines aimed at eliciting bnAbs. We tested a wide panel of diverse HIV-1 Envs in functional and neutralization assays and included transmitted/Founder (T/F), acute stage and late state mac-tropic and non-mac-tropic Envs of different subtypes such as clade A, B, C, D, and G, CRFs (CRF02-AG, CRF01-AE), and cpx variant (CRF13_cpx). In all Envs tested, the 375W substitution increased the exposure of the CD4bs as measured by sCD4 sensitivity as well as the capacity to infect macrophages, with the exception of highly mac-tropic Envs that already carried an exposed CD4bs. In summary, we describe for the first time, a substitution that increased sCD4 sensitivity and macrophage tropism in R5 Envs of all clades tested and done X4 Env as well as in primary Envs from viruses of different tropism phenotypes. Using mAbs against other Env sites including CD4i (b17), V3 loop/glycan (10-1047, and PGT128) and trimer association domain (TAD) V2q (PG9, PG16 and PGT145) we show that 375W did not confer significant alterations to other broadly neutralizing epitopes on Env. Thus, 375W confers strong exposure of the CD4bs without major effects on other broadly neutralizing epitopes on other sites of gp120. Finally, we identified primary, intermediate mac-tropic Envs in clade C, D and G that were not derived from central nervous systems (CNS). The role of such Envs in HIV pathogenesis is discussed.

Results

375W mutation increases sCD4 sensitivity.

To evaluate whether the 375W substitution influences soluble CD4 (sCD4) inhibition and therefore binding, we introduced 375W mutations by direct mutagenesis to a panel of Envs from different subtypes at different infection stages or time points (see Materials and Methods, Table 1). T/F (3T, 6T, 15T, and 19T, and Z1792M), acute stage (Z153M, Z185F, Z221M, and clones_251, _269, _278 and _258), chronic infection (0503M02138), and AIDS (92UG975.10) M-tropic (B33, B59, JR-FL, B100), and non-M-tropic (LN40, LN8, JR-CSF and LN58) primary isolates. 3T, 6T, 15T, 19T, B33, B59, JR-FL, B100, LN40, LN8, JR-CSF and LN58 Envs are subtype B and Z153M, Z221M, Z185F, and Z1792M Envs are clade C. We also tested other primary isolates from clade A (BG505), clade D (NKU3006, A03349M1, A08483M1, 57128), clade G (92UG975), circulating recombinant forms (CRFs) such as CRF02_AG (Clone_251 and Clone_278), CRF01_AE (0503M02138, and Clone_269), and a complex (cpx) variant (CRF13_cpx Clone_258). BG505 Env that represented a mother and child transmission, was obtained 6 weeks after birth and the infant was infected at delivery or via breastfeeding [21] and used in vaccine development.

We first tested neutralization of all Envs starting at a maximum 50ug/ml of sCD4 and subsequent 2-fold dilutions. However, we could not calculate the IC50s of non-M-tropic LN8, LN40, LN58, JR-CSF, 6T, 19T, Z153M, Z185F, Z221M, Z1792M, BG505, clone_269, 0503M02138, NKU3006, A03349M1 and clone_278 Envs since there was less than 50% inhibition at the maximum amount of sCD4. We know that these envelopes can confer infection of cells, so they must bind to the CD4 receptor even though they presumably have very low CD4 affinities. We decided to increase the sCD4 concentration up to 400ug/ml to calculate IC50s of these Envs. In figure 1, we represent the IC50 along with 95% confidence intervals. We found, independently of clade or disease stage, that IC50 values are lower for pseudovirions carrying Envs with the 375W substitution showing that this mutation increases sensitivity to sCD4 neutralization. These data confirm our previous results that the presence of a tryptophan in position 375 opens the CD4bs facilitating the interaction with the CD4 receptor [9]. Xiang et al. found a slight increase in sCD4 sensitivity for YU2 a subtype B isolate [10]. There is at least 1 log difference between WT and mutant IC50s. B_B33, B_LN40, B_B59, B_LN58, B_JR-CSF, AE_0503M02138, and G_92UG975 mutants were exceptions and showed less than 1 log more sensitive to sCD4. In this study, we found significant changes in the IC50 values (p <0.001) when we compared all WT with all 375W mutant Envs (Figure 2). In summary, a tryptophan at position 375W increases sCD4 binding in all clades, CRFs and cpx tested independently of disease state and compartment origin.

375W mutation changes tropism.

We next evaluated whether the presence of a tryptophan in 375 position influenced the biological property of tropism i.e. mac-tropism. M-tropic viruses are easier to neutralize due to a more accessible CD4 binging site (CD4bs) compared with non-M-tropic primary isolates [5]. We differentiated monocyte-derived macrophages (MDMs) from at least 3 healthy doors and infected with WT and mutant pseudoviruses in duplicate wells. Each experiment was repeated at least 3 times. Macrophage titers were normalized to TZM-bl titers (M&M). Clade B and C (figure 3A) were tested together and the other subtypes and CRF (figure 3B) were tested with all the mac- and non-mac-tropic reference Envs as positive and negative controls, respectively. We know that Central Nervous System (CNS)-derived Envs such as B33, B59, B100 and JR-FL are macrophage-tropic viruses that have been used extensively in our lab as positive controls for mac-tropism [1, 3]. LN40, LN8, LN58 and JR-CSF are non-mac tropic viruses and were obtained from the same patients as B33, B59, B100 and JR-FL, respectively. Envs that were not obtained from brain samples and has >1% macrophage titer but not reach the macrophage titer of our brain mac-tropic reference Envs, we considered intermediate mac-tropic Envs

We observed that mac-tropic B33, B59 and JR-FL 375W mutants reduce macrophage infectivity (Figure 3A). It is possible that these primary isolates evolved to infect cells with low levels of CD4 receptor and the introduction of this mutation could alter an optimally adapted trimer structure, this decreasing infectivity. However, the same mutation in the B100 mac-tropic Env did not affect macrophage infectivity. Surprisingly, G_92UG975 Env that we define as intermediate mac-tropic phenotype like clade C (Z185F, and Z221M) and D (D_A03349M1, D_A08483M1 and D_57128) intermediate Envs, showed the same reduction pattern as mac-tropic viruses. We found that the 375W mutation increases macrophage infectivity in all non mac-tropic and

intermediate variants except G_92UG975 as mentioned above. Our non mac-tropic reference (LN40, LN8, LN58 and JR-CSF) 375W mutants infected macrophages much more efficiently than their WT counterparts. LN40, LN8 and LN58 acquire intermediate mac-tropic phenotype when 375W substitution is present together with the other Clade B and C_Z153M. The presence of a tryptophan at position 375 in Z185F, Z221M and Z1792M Envs changed the phenotype to mac-tropic Envs while 3T and 19T were in borderline for mac-tropism.

We test subtype A, D and G, CRF and cpx variants with mac- and non mac-tropic references separately (Figure 3B). AE_0503M02138 was classified as X4 virus [22] and did does not infect macrophages well. However, it infects better than non-mac-tropic refence variants. When 375W substitution was introduced, an infectivity increase is observed changing to intermediate phenotype almost in the border of mac-tropism. Clade D (A03349M1, A08483M1 and 57128) and G (92UG975) Envs showed intermediate mac-tropic profile indicating the presence of such intermediate phenotypes outside of CNS (CSF and brain tissue) for non B and C subtypes

The 375W mutation also increased macrophage infection for CRF and cpx variants as well as all subtypes, except for G_92UG975. Thus, A_BG505, AE_Clone_269, D_57128, and cpx_Clone_258 variants become mac-tropic when a tryptophan in position 375 is present. Primary isolates such as AE_0503M02138, AG_Clone_251, D_NKU3006 D_A03349M1, D_A08483M1 exhibited intermediate phenotype but are borderline mac-tropic titers. We compared WT and mutant Envs for macrophage titers adjusted to TZM-bl titers (see Materials and methods) and found that they are significantly different ($p < 0.001$) (Figure 4). In summary, we found intermediate mac-tropic primary isolates outside the CNS from Clade C, D and G. 373W induces an enhanced interaction with CD4 (Figure 1 and 2) and increases the macrophage infection of primary isolates from different subtypes and at different disease stages.

Comparison of sCD4 neutralization IC50s and macrophage titers

We wanted to determine if there is a correlation between mac-tropism and sCD4 neutralization. We compared macrophage titers with sCD4 IC50s (Figure 5). We used the Spearman correlation test to calculate the P value because the data are not normally distributed. We found that there is a correlation between mac-tropism and CD4 binding as shown by the P value < 0.001.

This relationship does not explain why B_B100 primary Env and its 375W mutant have almost the same macrophage titers but the affinity for sCD4 of the mutants (measured by sCD4 inhibition) is 19 times higher. In addition, several mac-tropic (B_B33, B_B59, and B_JR-FL) and intermediate mac-tropic (G_92UG975) Envs lose macrophage infectivity when 375W is introduced. Thus, B_B33, B_B59, B_JR-FL and G_92UG975 mutants show around 4, 3, 15 and 10 more sensitivity to sCD4 compared with the wt. These observations are consistent with additional factors influencing mac-tropism.

The effects of 375W on mAb epitopes

B6 and 17b neutralization

T-cell-line adapted (TCLA) or laboratory HIV-1 strains that have been passaged in the lab without the presence of human immunity evolve Envs that are more accessible to antibody binding, gp120 shedding and trimer opening to expose the CD4bs [23]. On the contrary, primary isolates are subject to the immune pressure forcing a closed trimer. The monoclonal antibody (mAb) b6 interacts with an epitope overlapping the CD4bs and binds clade B lab strains such as HXBc2 [24]. b6 mAb binds to a very open CD4bs. We tested mAb b6 against subtype B and C primary isolates and it does not neutralize any these Env (data not shown) consistent with a CD4bs that is closed compared with the lab strains.

Following gp120 binding to CD4 there is a conformational change that exposes the coreceptor binding site and CD4-induced epitopes (CD4i). mAb 17b binds CD4i [25] including residues K121, R419, I420, K421, Q422 I423, and Y435 [26]. We tested the clade B and C Envs described here and 17b in the absence of sCD4 does not neutralize any (data not shown). These results demonstrate that 375W mutation does not induce a conformational change that exposes CD4i.

Neutralization by CD4bs mAbs

We were interested in understanding how the 375W mutation affects the exposure of CD4bs epitopes targeted by highly efficient bnAbs. To do so, we investigated b12, VRC01 and 3BNC117 mAbs in neutralization assays (Figure 6). VRC01 and 3BNC117 are mAbs that neutralize around 90% of the primary isolates from different clades.

<u>b12 mAb</u>

b12 mAbs recognizes the CD4bs region but it is not a potent bNAbs. Here, by using mAb12, only clade B, C and D Envs were neutralized. Of note, clade A (BG505), B (B100, LN40, LN8, 6T), C (Z153M, Z221M, Z1792M), and G (92UG975), CRF_AE (clone_269 and 0503M02138), CRF_AE (clone 251 and 278) and cxp (clone 258) Envs were all resistant to b12 mAb. Clade B LN40 is naturally resistant to b12 mAb due to the presence an asparagine at N386 together with an arginine at R373 which together block an Env pocket targeted by W100 on b12 [27].

There was not a significant difference between wt and mutant 375W Env sensitivity to b12 neutralization ($p = 0.22$). Although, we did notice a tendency for higher IC50s when the 375W substitution was present in non-mac-tropic viruses (Fig. 6 A). Other researchers have reported that the 375W mutation reduced b12 binding in gp120 monomers (ELISAs) and in neutralization assays [10, 11]. Interestingly, in mac-tropic (B_B33, B_59, and B_JR-FL) and intermediate mac-tropic Envs (D_A03349M1, D_A08483M1, and D_57128 except for C_Z185F), the b12 interaction is slightly increased or not change while it is always decreased in non-mac-tropic Envs (Figure 6A). Non-mac-tropic primary isolates have a more closed CD4bs and the introduction of 375W substitution opens CD4bs then as consequence of this structural change, the epitope is altered hampering b12 binding. The 375W conformational change resulting in a more open mac-tropic and intermediate mac-tropic trimer improves or does not alter the b12 interaction. This could show that 375W residue induces slightly different conformational changes in the trimer depending on if the previous Env was more closed or more open. In summary, we have observed some differences between mac-tropic and intermediate vs non-mac-tropic CD4bs structure via b12 mAb neutralization.

<u>The potent neutralizing VCR01 and 3BNC117 mAbs</u>

VRC01 mAb potently neutralized pseudovirions carrying most wt Envs tested (Fig. 6B) but did not neutralize B_LN58, C_Z185F, C_Z1792M, AG_Clone_278 and D_57128. We observed that the presence of a tryptophan in position 375 slightly decreased VRC01 sensitivity for some Envs shown by slight increases in the IC50 values but did not ever abrogate VRC01 neutralization (Fig. 6B). Significant differences between wt and mutant VRC01 neutralization titers were observed ($p=0.02$). Interestingly, in mac-tropic (B_B59, B_JR-FL and B_B100), and intermediate mac-tropic Envs (D_A03349M1, D_A08483M1, and G_92UG975), there is a neutralization shift of the 375W mutants towards sensitivity compared to WT except for B33 and Z221 Envs that did not change or increased neutralization sensitivity, respectively. These results are consistent with a slight change of trimer structure in the CD4bs region for these mac-tropic and intermediate mac-tropic Envs since the 375W substitution did not affect non-mac-tropic mutants in the same way.

3BNC117 is a very potent and broad mAb since it neutralized nearly all Envs tested here at lower IC50 compared to the IC50s reach by VRC01 mAb (Figure 6B and C). All Envs were neutralized with 3BNC117, except 15T and Z185F and Z1792M and Clone_278 Envs. Z185F, Z1792M, and Clone_278 Envs are also resistant to VRC01 bnAbs consistent with changes in the residues that affect both these bnAbs (Figure 6B and C). There were not significant differences between wt and mutant 3BNC117 neutralization titers ($p=0.41$). However, we did find a tendency of slightly increased resistance to 3BNC117 mAb in 375W mutants (Figure 6C).

In summary, we have corroborated some differences between mac-tropic and intermediate vs non-mac-tropic CD4bs structure by CD4 mAb neutralization when position 375 has a tryptophan residue. However, the 375W mutation does not abrogate the binding of potent CD4 mAbs including VRC01 and 3BNC117 as well as b12, leaving their epitopes accessible. Immunogens carrying 375W could therefore be used as a vaccine to induce CD4bs bNAbs.

Neutralization by V3 loop/glycan mAb

We previously reported that 375W confers a trimer conformation that opens the CD4bs without exposing the occluded GPGR V3 epitope that can potently induce non-neutralizing Abs such as 447-52D [9]. In this study, we evaluated the effect of the 375W mutation on neutralization sensitivity to mAbs 10-1047, and PGT128 that recognize conserved V3 loop/V3 glycan epitopes in our panel of 27 diverse HIV-1s.

N332 glycan has been reported as a target for 10-1074, while N332 together with N156, N301 and N157 for the PGT121 mab family. PGT128 mainly interacts with N332, N156 and N301 [28, 29]. 10-1074 has some minimal interactions with N156 and N301. N156 and N301 are present in all Envs investigated with the exception of AE_0503M02138 that does not have N301. However, B_B100, B_15T, C_Z153M, A_BG505, AE_0503M02138, D_NKU3006, and cpx_Clone_258 Envs do not have an asparagine at position 332 and were not neutralized by PGT128, nor by 10-1074 mAbs (Figure 7A). 10-1074 and PGT128 mAbs also bind the V3 peptide motif GDIR [28, 29]. All Envs tested here have this motif except for B_6T, C_Z221M, C_Z1792M and D_NKU3006 that have GNIR. Nevertheless, these latter Envs are neutralized by both mAbs with the exception of D_NKU3006 that does not have N332 glycan either.

When a tryptophan at position 375 is present, we found that mac-tropic (B_B33, B_B59, and B_JR-FL) and G_92UG975 (the higher intermediate mac-tropic) increase binding for 10-1074 mAb (Figure 7B). It could indicate 375W substitution in the mac-tropic and intermediate variants with more open CD4bs rearrange the apex of the trimer facilitating the interaction of 10-1074 mAb. However, this IC50 reduction was also observed in three non-mac-tropic Envs including B_LN58, B_19T and C_Z1792M indicating that there is not a clear neutralization pattern associated to mac-tropism. In PGT128 mAb, 375W mutants are less or more sensitive compared with the wt depending on the Env tested. IC50 values does not show significant different between wt and mutants in 10-1074 and PGT128 mAbs with p=0.49 and p=0.07, respectively.

Overall, these mAbs interact better with clade B and C compared with other subtypes, CRF and cpx in our panel. Our results indicate the 375W mutants retain sensitivity to broadly neutralizing V3 loop/glycan 10-1047 and PGT128 mAbs. Thus, 375W Env mutants that carry an exposed CD4bs (as we previously reported), maintain exposure of the vaccine-desirable, glycan dependent, V3 loop epitopes targeted by highly potent bnAbs.

Neutralization by V1V2 loop/glycan mAb

We next tested our primary Env panel against PGT145, PG9 and PG16 (Figure 8), less potent bnAbs that recognize N-linked glycosylation sites such as N160 and N156 and sometimes N173 as compensatory residue of V2 loops in the trimer apex [30]. PGT145 does not directly interact with N156 but the loss of that glycan abrogates its interaction with the trimer apex. N156 is present in all Envs tested. However, none of our Envs has an asparagine at position 173. N160 glycan is not present in B_LN40, B_15T, C_Z185F, D_NKU3006, and G_ 92UG975 Envs. None of these primary isolates were neutralized by PGT145, PG9 or PG16 mAb.

PGT145 targets a quaternary epitope simultaneously interacting with the 3 gp120 monomers in pre-fusion conformation [30]. 3BNC117 mAb binding in CD4bs was reported to increase the space between N160s from each of the 3 monomers and enhance the accessibility of PGT145 epitope [30]. IC50s would not be calculated in non-subtype B and C panel. K121A and R166A substitutions have also been associated with a reduction in PGT145 neutralization [30]. In our panel, all Envs tested have R166 except for B_3T, B_15T, C_Z185F, AG_clone_251 and _278. For K121, C_Z153M has T and AE_0503M02138 has I at this position. All these primary Envs are resistant to PGT145 mAb. Data obtained from the Envs that are sensitive to PGT145 mAb indicates that there are no changes or slightly increase in resistance to this mAb on 375W mutants (Figure 8A). However, the differences between wts and 375W mutants are not statistically significant (p=0.44).

PG9 and PG16 can also bind gp120 monomers while PGT145 bind quaternary Env structures [31]. PG16 preferentially bind to trimers while PG9 bind two of the three gp120 protomers in the trimer [32]. This difference could explain that PG9 and PG16 not always neutralize the same Env of our panel. A tryptophan at position 375 does not modify the affinity or increases resistance to these less potent bnAbs (Figure 8B and C). Differences

between WT and 375W mutant are significant with p= 0.0078 and p= 0.03 for PG9 and PG16mAb, respectively. It is possible that 375W substitution alters the apex structure of the trimer. In summary, 375W did not abrogate neutralization by these less potent bnAbs. However, there is a loss of affinity for PG9 and PG16 mAbs when 375W is introduced.

Discussion

We previously reported that the 375W mutation opens the HIV trimer and facilitates the binding to the CD4 receptor without exposing the V3 loop [9]. HIV trimers carrying 375W could therefore be good candidates to develop Abs against the CD4bs. In this study, 375W substitution increases macrophage infection in our Env panel. However, 375W mutation only reduced macrophage infectivity in the context of mac-tropic primary isolates. These results correlate with a previous data that show that 375W reduce infectivity in YU2 clade B mac-tropic [10, 11, 33]. An increase of sCD4 binding has been observed not only in Clade B primary isolates tested as previously described for YU2 too [10] but also in clade A, B, C and D SHIVs [16]. These findings support our results that 375W residue increases CD4 interaction in all Env tested.

The ideal HIV immunogens will likely present the CD4bs in an appropriate conformation to induce bnAbs that approach the CD4bs at an optimal angle [34]. Such immunogens will be presented as Env trimers and will not induce “off-target” non-nAbs against CD4i and V3 loop epitopes. Trimer based immunogens have been developed in the form of BG505 SOSIP.664. However, this immunogen induced the production of non-nAbs against the trimer base [7, 35]. Furthermore, CD4 binding induces conformational changes in BG505 SOSIP.664 that lead to the exposure of non-neutralizing epitopes (CD4i and V3 loop) and this seems to occur during immunization [35]. Env neutralization phenotypes are classified as tier 1A and B, 2 and 3 depending on the numbers of epitopes exposed and neutralization sensitivity. In tier 1, Envs that has open trimer were classified as tier 1A while tier 1B has more intermediate conformation. These Envs expose higher number of epitopes and are easier to neutralize. In contrast, tier 2 and 3 with closer trimer conformations expose less epitopes and are more resistant to neutralization [36]. Further studies showed that the derivative, DS-SOSIP.4mut, modified to stabilize the SOSIP trimer in a more prefusion-closed conformation, reduced CD4 binding and a V3-sensitive tier-1 response while increasing tier-2 antigenicity [6]. However, this construct did not induce bnAbs

In single molecule fluorescence resonance energy transfer (smFRET) experiments, different conformational states of Env were observed e.g. state 1 (a closed conformation), state 2 (asymmetric intermediate conformations with one protomer open) and state 3 (asymmetric open conformation with three protomer open that are bound to three CD4 molecules). The binding of a single CD4 molecule into one protomers of the trimer induced a state 3 conformation, while the other protomers (without CD4 bound) transitioned to state 2 [37]. The BG505 SOSIP.664 trimers predominantly stay in state 2 in smFRET [38] while primary isolate trimers exhibit more state 1 conformations [38, 39], indicating that they do not have the same structure as closed conformation in primary isolate Envs. SOSIP immunogens have been modified to prefusion-closed structures using introduced disulfide bonds. This approach may thus modulate the trimer structure, the presentation of epitopes and the capacity to enhance Ab breath in responses against the trimer. The introduction of 2 disulfide bonds in BG505 (non-mac-tropic Env) and JR-FL (mac-tropic Env) Envs stabilized the so-called state 2 conformation identified using smFRET [38] while such Envs are usually predominantly in a state 1 conformation. These novel approaches may eventually result in the identification of trimer that are immunogenic for bnAbs. We envisage that 375W will play an important role in the development of such immunogens.

We propose that HIV-1 375W Envs will good candidates for vaccines aiming to elicit bnAbs targeting the CD4bs. Since VRC01 and 3BNC117 neutralize 375W mutants as efficiently as wt, it may suggest that 375W immunogens would have the potential to generate bnAbs that have the appropriate angle of approach to the CD4bs. Our data show that such Envs retain V3/glycan epitopes (e.g targeted by PGT128 and 10-1074 mabs) exposed on mutant primary viruses of different clades, CRF and cpx. Such Envs may therefore be immunogens for bnAbs targeting such glycans. In addition, in the same way the glycans are removed to induce Abs against CD4bs [40], 375W immunogens could be used as a first boost to expose CD4bs followed by consecutive boosts with diverse, prefusion Env trimers, in attempts to mimick chronic infection. The advantage of the 375W mutation for vaccines, is that it exposes CD4bs and introduces the same conformational change in all clades, CRFs, and cpx as well as in Envs from different stages of infection tested. Recently, an HIV-1 mRNA vaccine was tested in rhesus macaques and heterologous tier-2 SHIV responses were observed [41]. This new vaccine platform has the advantage that can present different immunogens in their natural conformation at the same time and is highly appropriate for the inclusion of W375 trimer immunogens.

We found that bnAbs such as VRC01, and 3BNC117 interact with almost all wt Envs tested while 10-1074 and PGT128 are more effective neutralizing non-clade B and C variants. For VRC01 some significant IC50

differences were observed (p=0.02) between wt and mutant Envs. Thus, there is a tendency that the presence of a tryptophan in position 375 slightly decreased VRC01 sensitivity in non-mac-tropic variants, however, this did not ever abrogate VRC01 interaction. Interestingly, almost all mac-tropic, and intermediate mac-tropic Envs were neutralized with increased sensitivity for VRC01 mAb when a tryptophan is at position 375. 375W substitution also altered the b12 mAb interaction. In mac-tropic and intermediate mac-tropic variants, the 375W residue induced a slightly better exposure of b12 epitope compared with non-mac-tropic variants. It is possible that the structural change induced by 375W substitution alter the approach angle of these mAbs [42] causing different effects depending on each Env's tropism. No significant differences in the accessibility to 3BNC117, 10-1074 and PGT128 epitopes between variants with different tropism were observed when 375W substitution was present. These results show that small trimer structural changes can be detected between mac-tropic, intermediate and non-mac-tropic Envs using certain mAbs in neutralization assays.

We and others have reported on highly mac-tropic Envs derived from brain tissue, particularly in subjects with neuro-AIDS [3]. Based on our results here, we were able to define intermediate mac-tropic viruses from clade C, D and G, that have the capacity to infect macrophages at low levels compared with highly mac-tropic Envs. In children infected with clade C with the viral population that has a minor compartmentalization, variants were detected that could infect Affinofile cells via low CD4 at modest levels suggesting precursors of mac-tropic viruses [2]. Subtype differences in the capacity to induce HIV-associated cognitive impairments in Uganda and Ethiopia suggest different biological characteristics between clades [43]. For example, in Clade D infected patients, it is more common to develop HIV-1 dementia compared with that clade A infected individuals [44, 45]. However, infected individuals with this subtype have been reported to progress to AIDS more frequently compared with clade A [43, 46]. Here, we found that 3 of 4 clade D Env have an intermediate mac-tropic phenotype. HIV-related neurocognitive impairments have been observed in patients infected with clade G in Nigeria [47]. Surprisingly, our clade G Env also show intermediate mac-tropic phenotype. In South Africa, it has been found that some subtype C viruses induce cognitive impairments independent of tat polymorphism previously associated to neurocognitive impairments [48]. The clade C Envs supplied by Dr. Derdeyn and Dr. Hunter for this study come from Zambia in South Africa and these also show intermediate phenotype [49, 50]. Intermediate mac-tropic variants circulating in blood could invade the brain and quickly adapted to the environment increasing mac-tropism and the likelihood to develop HIV-1 dementia. An alternative explanation is that these viruses could be an adaptation of mac-tropic viruses released from brain to blood where immune pressure is higher.

It is possible that disease stage influences the presence of different phenotypes. All T/F primary isolates including A_BG505, C_Z1792M and clade B (3T, 6T, 15T, and 19T) are non-mac-tropic. It has been described that mac-tropic viruses are not favorably transmitted in ectocervical tissue [51]. Interestingly, some of viruses obtains in the acute phase show intermediate mac-topic phenotype from Clade C (Z185F, and Z221M). In this stage, the HIV-1 virus is in the higher peak of replication and a lot of variants arise in the viral population then it will be easy to find intermediate variants that could invade the brain establishing the infection on CNS.

Surprisingly, a tryptophan at position 375 slightly reduced macrophage infection in clade B brain Envs, although B100 was not affected. We could deduce these highly mac-tropic Envs carry trimers that are conformationally highly adapted to the brain environment and carry a succession of Env substitutions that confer this phenotype. Thus, the 375W substitution may have a slight negative impact on such optimally mac-tropic Envs. Of note, G_92UG975 mutant showed the same reduction in macrophage infection. It could indicate that this Env that has the higher value of intermediate mac-tropic Envs is in fact mac-tropic. However, we decide to maintain the classification as intermediate phenotype because has lower infection that our brain mac-tropic references. One single mutation can transform a non-mac-tropic virus to mac-tropic [52-54]. In this study, we observed the same effect in some subtype A, C, D, CRF01_AE and CRF13_cpx non-mac-tropic as well as intermediate variants when 375W substitution is introduced. Moreover, an X4 CRF01_AE (0503M02138) 373W mutant also had heightened, although intermediate mac-tropism. If an enhanced CD4bs exposure evolves in the blood or peripheral tissues, such Envs could be easier to neutralize by nAbs and this pressure likely reduces the presence of such variants there. Nevertheless, here we describe intermediate subtype mac-tropic viruses outside the CNS from different clades. The envelope structure and exposure of the CD4bs of such variants is unknown but could explain why Clade D infected individuals are more likely to develop dementia. More research is needed to understand the role of mac-tropism in peripheral tissues and to study mac-tropism in non-clade B Envs.

Figure Legends

Figure 1: sCD4 neutralization. Geometric mean IC50s and 95% confident intervals were calculated and plotted in GraphPad. Two different neutralization assays were performed, and each experiment was made in duplicate. The maximum concentration of sCD4 used to calculate IC_{50} was 400ug/ml. IC50s and confidence intervals for Wild type (WT) and 375W mutants are presented in blue (●) or orange (●) circles and error bars, respectively.

Figure 2: Compare the means of WT and 375W sCD4 neutralization IC50s from 2 experiments. 375W mutation decreases IC50 values compared with the WT Envs. The black line represents the median. WT and mutants were presented as light (●) and dark (●) gray circles. Analysis Wilcoxon test (paired nonparametric t-test) showed significant differences between WT and mutants IC50s (p value <0.001).

Figure 3: Percentage of macrophage titers vs TZM-bl titer. Each infection was repeated three times using differentiate MDM from at least 3 different healthy human blood donors. Titers in macrophages were normalized to TZM-bl titers. The data of subtype B and C (A), and other clades (B) are represented. Blue and orange bars represent WT and 375W mutant Envs, respectively. Black and blue line indicated higher titer of non-mac-tropic and the lower titer to consider that an Env is mac-tropic. In yellow, the region of intermediate mac-tropic Envs. SEM is represented on the top of each bar in black.

Figure 4: Comparison of the means of WT and 375W % macrophage titers/TZM-bl. The black lines represent the median. WT and mutant viruses were presented as blue (●) and orange (●) circles, respectively. Analysis using Wilcoxon test (paired nonparametric t-test) showed significant differences between WT and mutants in % macrophage titers/TZM-bl (p < 0.001).

Figure 5: Correlation of % macrophage titers/TZM-bl vs sCD4 IC50 means. WT and 375W mutant were represented in blue and orange, respectively. The analysis of Spearman test (nonparametric correlation model) showed significant correlation between tropism and sCD4 neutralization (p < 0.001).

Figure 6: IC50 values of CD4bs mAb neutralization. Geometric mean IC50s and 95% confident intervals were calculated and plotted in GraphPad for b12 (A), VRC01 (B), and 3BNC117 (C) mAbs. Two different neutralization assays were performed, and each experiment was made in duplicate. Confidence intervals for Wild type (WT) and 375W mutants are presented in blue or orange error bars, respectively. WT IC50s are represented by blue squares (●) and 375W mutant IC50s by orange circles (●).

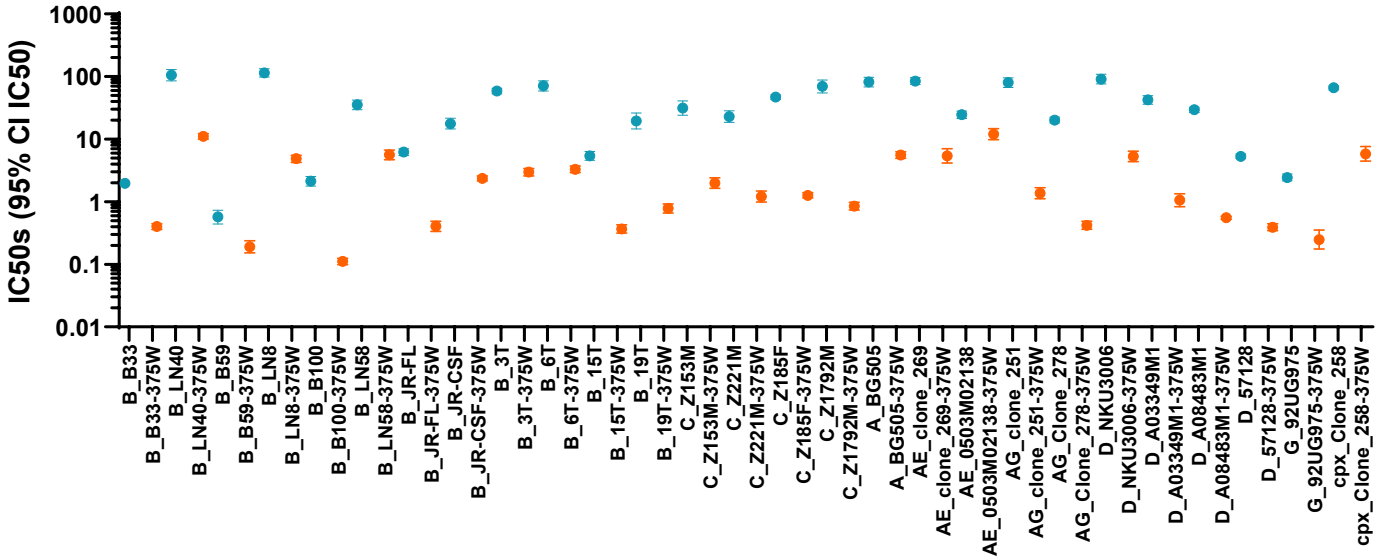
Figure 7: IC50 values of V3 loop/glycan mAb neutralization. Geometric mean IC50s and 95% confident intervals were calculated and plotted in GraphPad for PGT128 (A), and 10-1074 (B) mAbs. Two different neutralization assays were performed, and each experiment was made in duplicate. Confidence intervals for Wild type (WT) and 375W mutants are presented in blue or orange error bars, respectively. WT IC50s are represented by blue squares (●) and 375W mutant IC50s by orange circles (●).

Figure 8: IC50 values of V1V2 loop/glycan mAb neutralization. Geometric mean IC50s and 95% confident intervals were calculated and plotted in GraphPad for PGT145 (A), PG9 (B), and PG16 (C) mAbs. Two different neutralization assays were performed, and each experiment was made in duplicate. Confidence intervals for Wild type (WT) and 375W mutants are presented in blue or orange error bars, respectively. WT IC50s are represented by blue squares (●) and 375W mutant IC50s by orange circles (●).

Table 1: HIV-1 *env* clones used to prepare Env+ pseudovirions.

Table 2: mAbs used in this study.

Figure 1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0.01
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B_JR-CSF
B_JR-CSF-375W
B_3T
B_3T-375W
B_6T
B_6T-375W
B_15T
B_15T-375W
B_19T
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C_Z185F
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C_Z1792M
C_Z1792M-375W
A_BG505
A_BG505-375W
AE_clone_269
AE_clone_269-375W
AE_0503M02138
AE_0503M02138-375W
AG_clone_251
AG_clone_251-375W
AG_Clone_278
AG_Clone_278-375W
D_NKU3006
D_NKU3006-375W
D_A03349M1
D_A03349M1-375W
D_A08483M1
D_A08483M1-375W
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G_92UG975-375W
cpx_Clone_258
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Figure 2

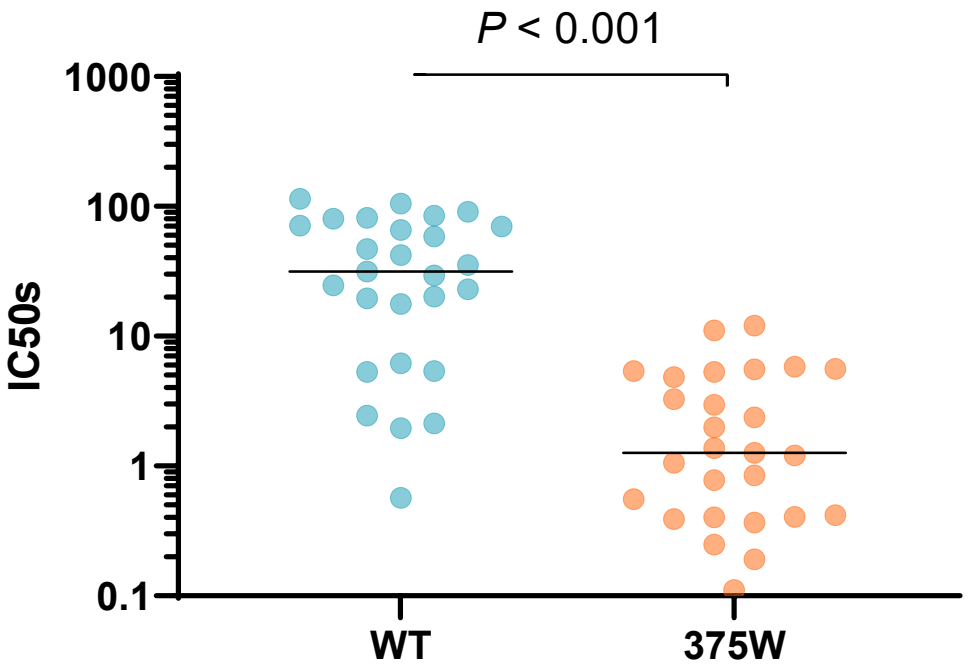
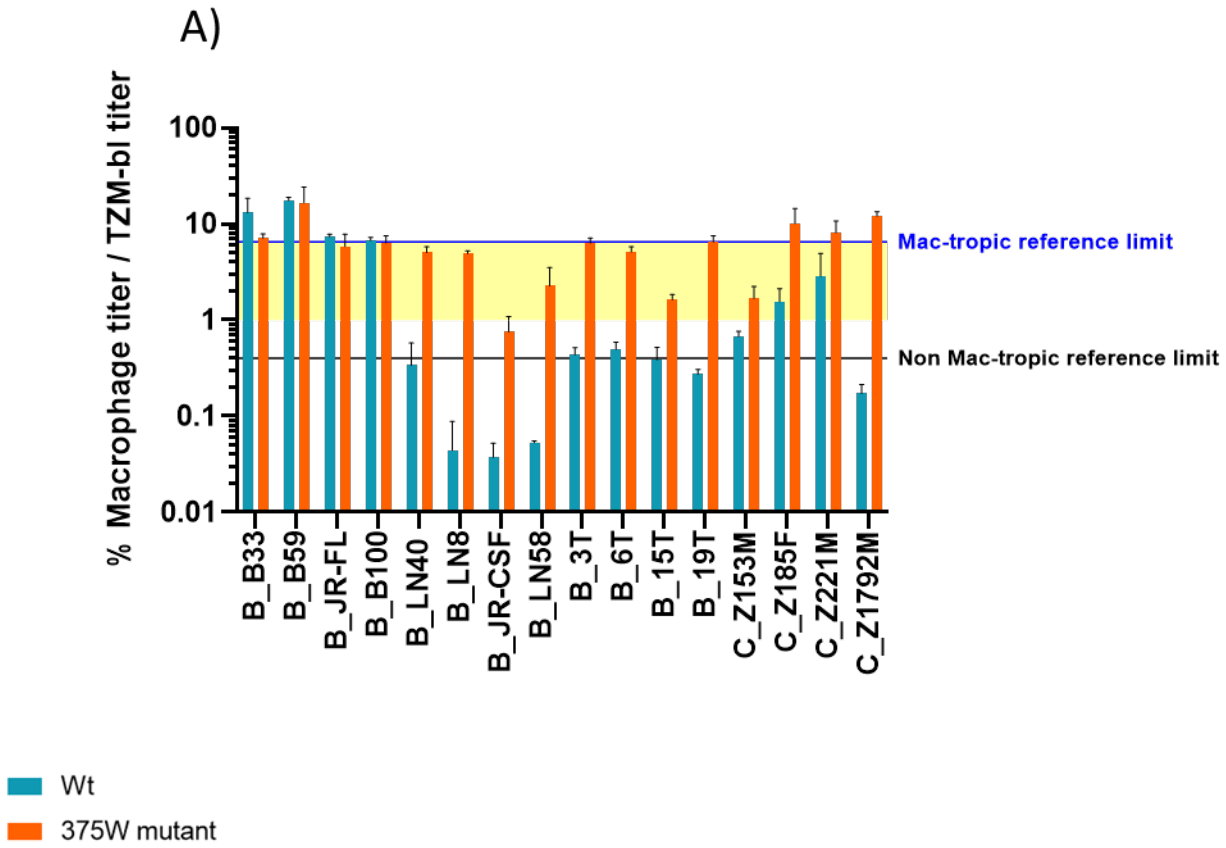


Figure 3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10
1
0.1
0.01
Mac-tropic reference limit
Non Mac-tropic reference limit
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B_JR-FL
B_B100
B_LN40
B_LN8
B_JR-CSF
B_LN58
B_3T
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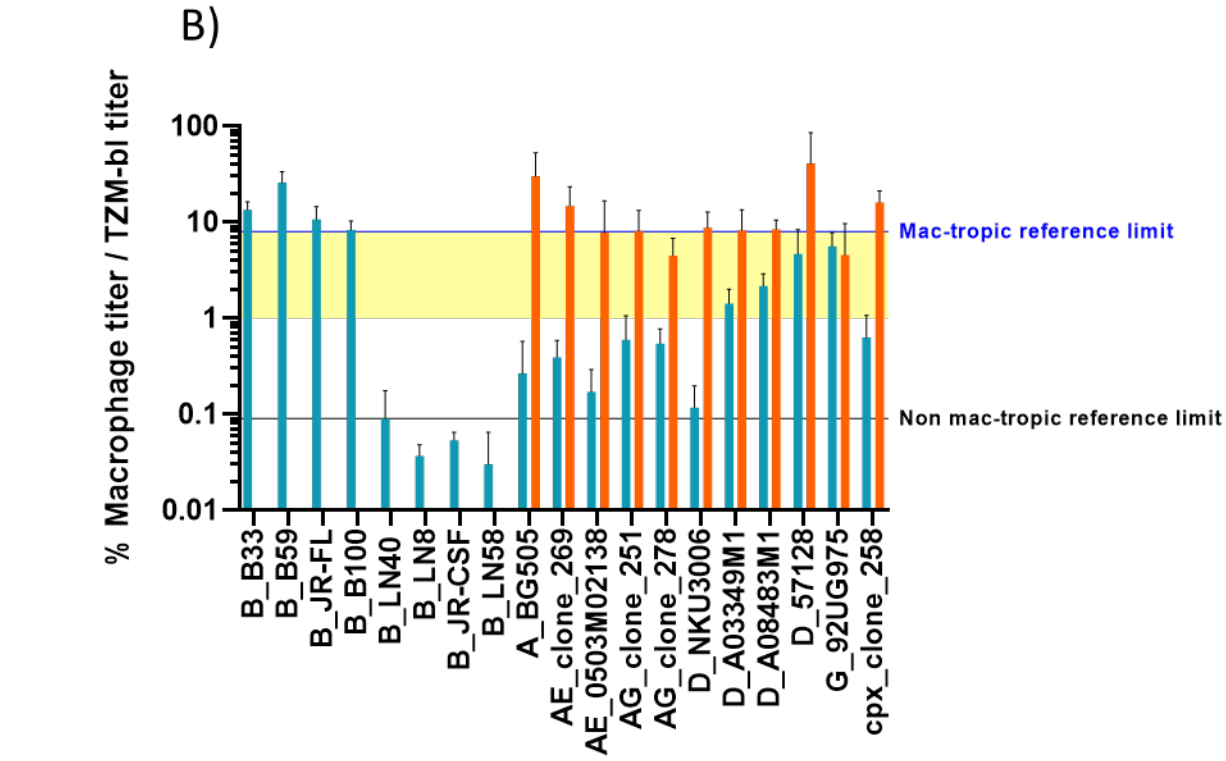
B_19T
C_Z153M
C_Z185F
C_Z221M
C_Z1792M
Wt
375W mutant


B)
% Macrophage titer / TZM-bl titer
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0.1
0.01
Mac-tropic reference limit
Non mac-tropic reference limit
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B_B59
B_JR-FL
B_B100
B_LN40
B_LN8
B_JR-CSF
B_LN58
A_BG505
AE_clone_269
AE_0503M02138
AG_clone_251
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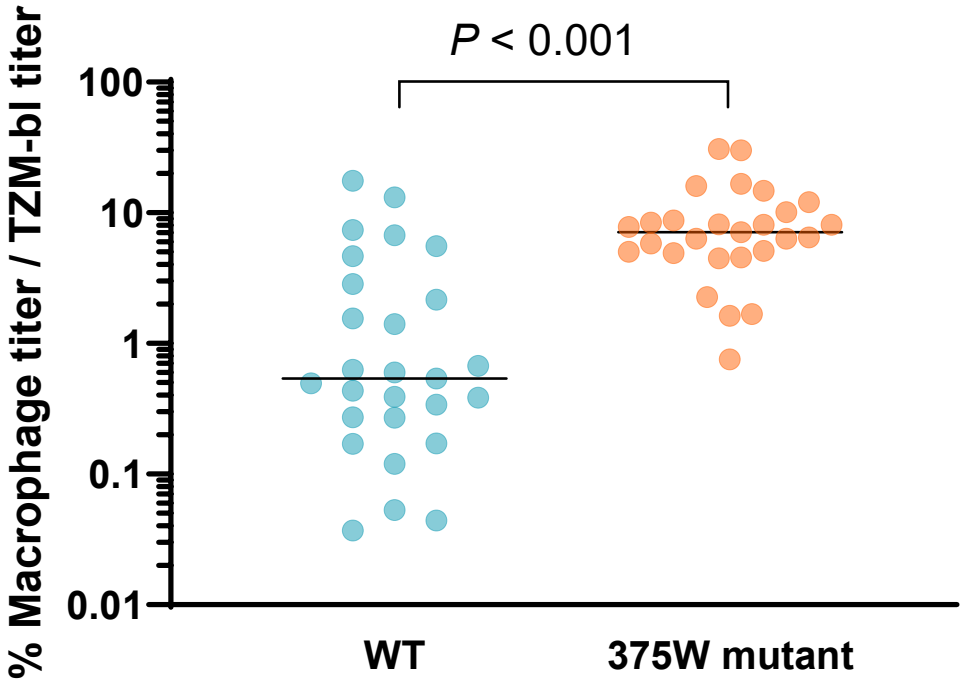
G_92UG975
cpx_clone_258

Figure 4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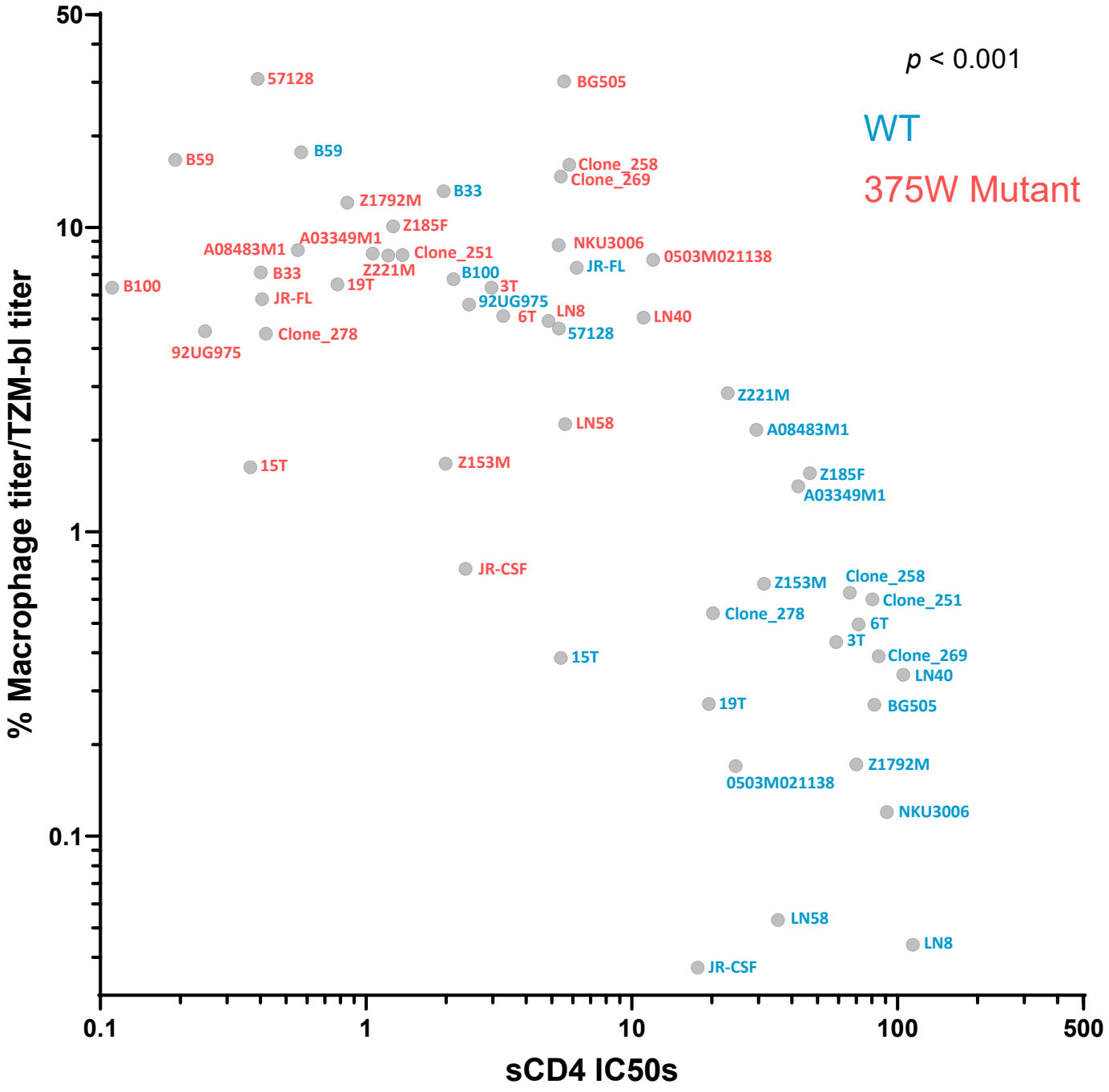
0.01
WT
375W mutant

Figure 5


p < 0.001
WT
375W Mutant
% Macrophage titer/TZM-bl titer
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0.1
0.1
1
10
100
500
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BG505
B59
B59
Clone_258
Clone_269
Z1792M
B33
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Z221M
B100
JR-FL
B100
19T
3T
JR-FL
92UG975
LN8
6T
LN40
57128
92UG975
Clone_278
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LN58
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15T
Z153M
Z185F
A03349M1
JR-CSF
Z153M
Clone_258
Clone_251
Clone_278
6T
3T
15T
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JR-CSF

Figure 6

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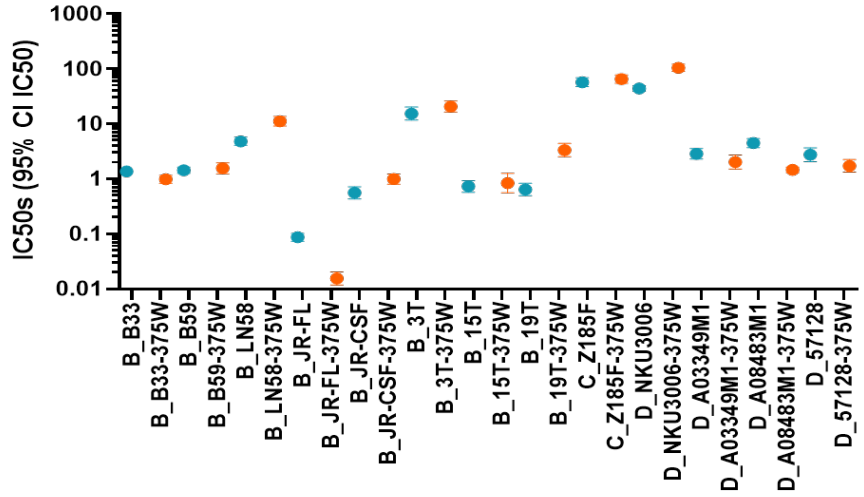
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0.1
0.01
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B_3T-375W
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B_19T
B_19T-375W
C_Z185F
C_Z185F-375W
D_NKU3006
D_NKU3006-375W
D_A03349M1
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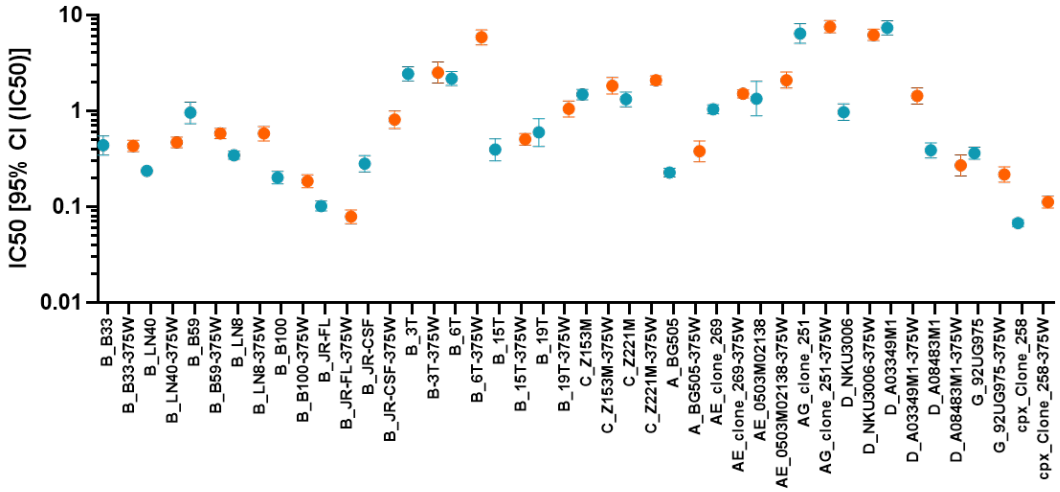
D_57128-375W

B)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0.01
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B_LN40
B_LN40-375W
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C_Z153M-375W
C_Z221M
C_Z221M-375W
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AE_clone_269-375W
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D_NKU3006-375W
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D_A08483M1
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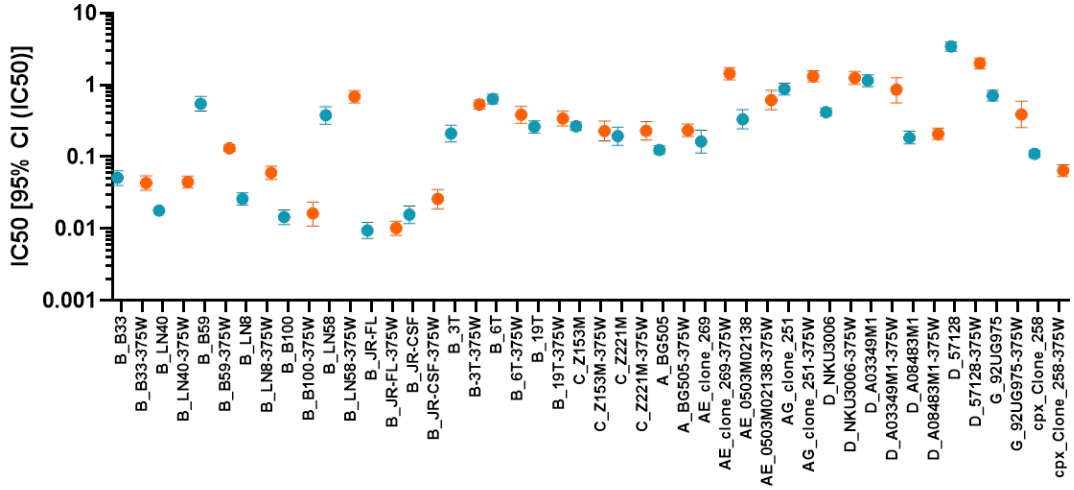
C)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C_Z153M-375W
C_Z221M
C_Z221M-375W
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A_BG505-375W
AE_clone_269
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AE_0503M02138
AE_0503M02138-375W
AG_clone_251
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D_NKU3006-375W
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Figure 7

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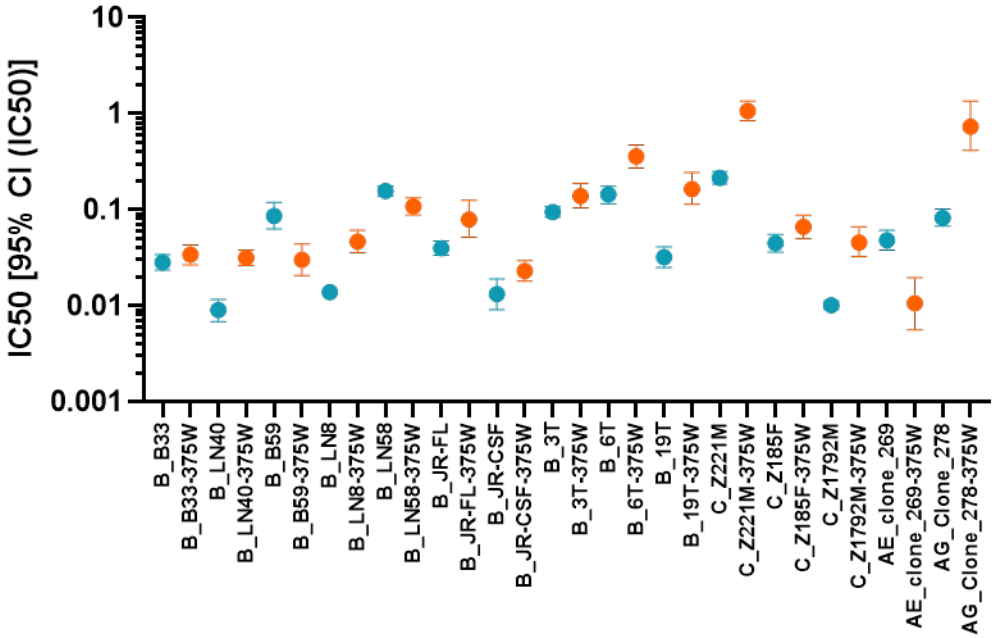
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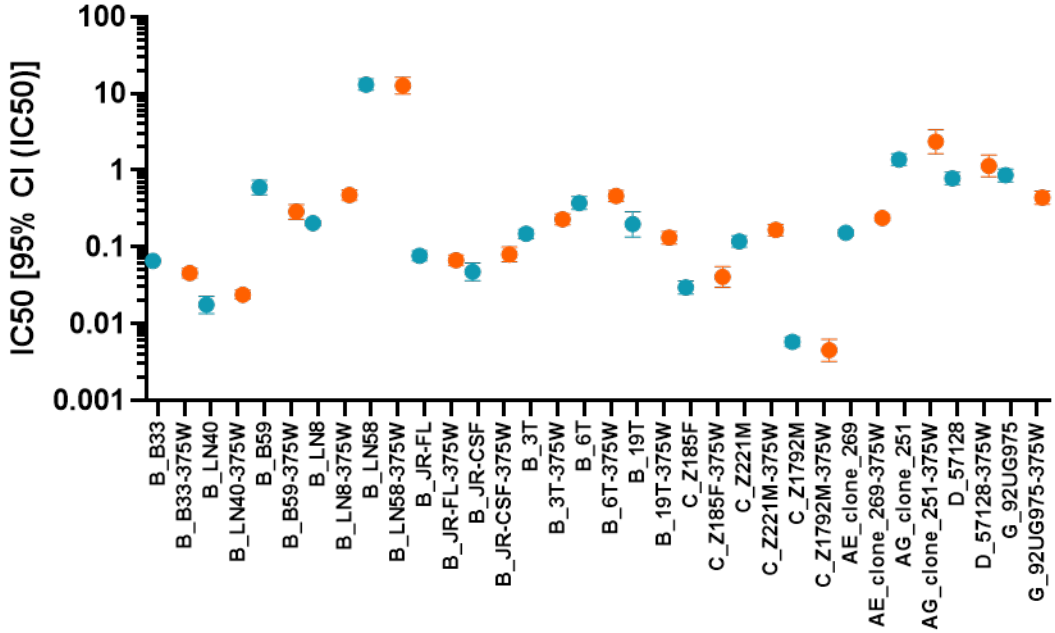
B)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C_Z221M-375W
C_Z1792M
C_Z1792M-375W
AE_clone_269
AE_clone_269-375W
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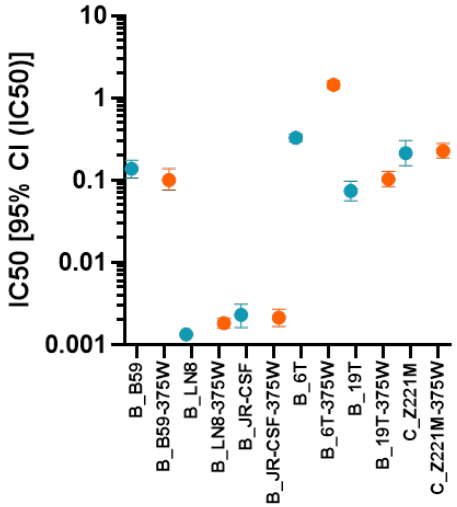
G_92UG975
G_92UG975-375W

Figure 8

A)


IC50 [95% CI (IC50)]
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0.1
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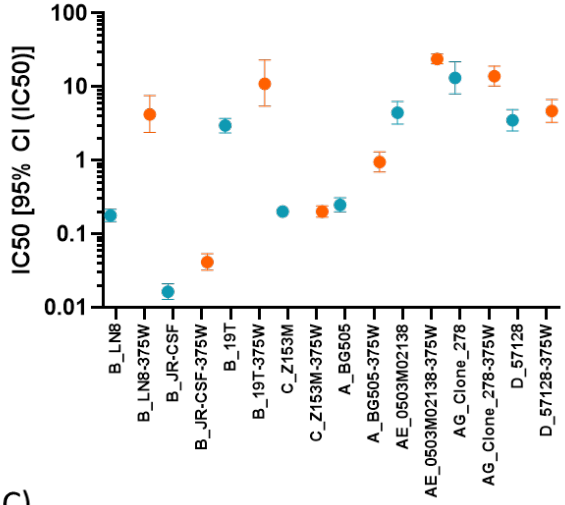
C_Z221M-375W

B)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B_19T
B_19T-375W
C_Z153M
C_Z153M-375W
A_BG505
A_BG505-375W
AE_0503M02138
AE_0503M02138-375W
AG_Clone_278
AG_Clone_278-375W
D_57128
D_57128-375W

C)

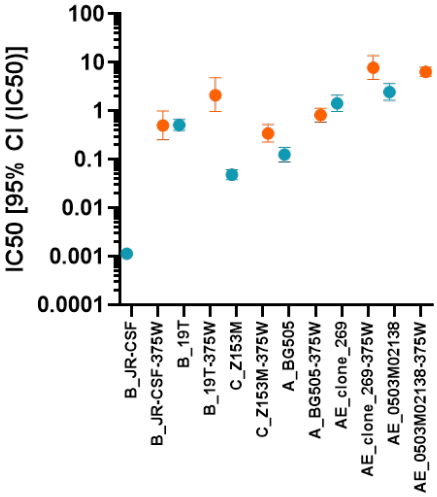
IC50 [95% CI (IC50)]
100
10
1
0.1
0.01
0.001
0.0001
B_JR-CSF
B_JR-CSF-375W
B_19T
B_19T-375W
C_Z153M
C_Z153M-375W
A_BG505
A_BG505-375W
AE_clone_269
AE_clone_269-375W
AE_0503M02138
AE_0503M02138-375W

Table 1

Env	Clade	Correceptor	Full name	Origin	References
Chronic infection Env					
Mac-tropic Env					
JR-FL	B	R5		Brain tissue	[27, 33, 55]
NA420 B33	B	R5		Proviral DNA from brain tissue	[1, 27, 33, 56-58]
NA20 B59	B	R5		Proviral DNA from brain tissue	[1, 33, 58]
E21 B100-1	B	R5		Proviral DNA from brain tissue	[3]
Intermediate mac-tropic Env					
92UG975	G **	R5	pCRII_92UG975.10	Cultured PBMC DNA	[59]
Non-mac-tropic Env					
JR-CSF	B	R5		CSF	[27, 33, 55]
NA420 LN40	B	R5	NA420 LN40/B33	Proviral DNA from Lymph Node tissue	[1, 9, 27, 56-58]
NA20 LN8	B	R5		Proviral DNA from Lymph Node tissue	[1, 9, 58]
E21 LN58-3	B	R5		Proviral DNA from Lymph Node tissue	[3]
0503M02138	AE	X4	96TH_0503M02138.EC1	Patient PBMCs cocultured with healthy donor PBMCs	[60]
Transmitted/founder (T/F) and Mother-to-child transmission (MTC) Env					
3T	B	R5	p1054.TC4.1499	Genomic DNA Plasma	[61]
16T	B	R5	p63358.p3.4013	Genomic DNA Plasma	[61]
15T	B	R5	p700010040.C9.4520	Genomic DNA Plasma	[61]
19T	B	R5	pPRB958_06.TB1.4305	Genomic DNA Plasma	[61]
Z1792M	C	R5	Z1792MPL18DEC07.3G7Env	Plasma ARN	[50]
BG505	A	R5	BG505-W6M-ENVC2	PBMCs DNA; MTC	[21]
Acute stage Env					
Z185F	C	R5	Z185FPB24AUG02ENV3.1	Blood	[49, 50, 62]
Z153M	C	R5	Z153MPL13MAR02ENV5.1	Plasma ARN	[49, 50]
Z221M	C	R5	Z221MPL7MAR03ENV2.1	Plasma ARN	[49, 50]
Clone_269	AE*	R5	CRF01_AE clone 269	Cultured PBMCs from patient	Unpublished
Clone_251	AG	R5	CRF02_AG clone 251	Cultured PBMCs from female patient	
Clone_278	AG	R5	CRF02_AG clone 278	Cultured PBMCs from male patient	
Clone_258	cpx***	R5	CRF13_cpx clone 258	PBMCs from male patient	
Non-T/F unknown stage Envs					
NKU3006	D	R5	00KE_NKU3006.EC1	Patient PBMCs cocultured healthy donor PBMCs	[60]
A03349M1	D	R5	99UG_A03349M1		
A08483M1	D	R5	99UG_A08483M1.VRC09A		
57128	D	R5	98UG_57128.VRC15		

* CRF01_AE clone 269 may be a potential CRF02-AG/CRF01-AE recombinant.
** pCRII_92UG975.10 Env has 39nt clade A in gp41.
*** CRF13_cpx carry genomic regions identified as subtypes A, G, J, and CRF01-AE [63].

Table 2

Reagent	Epitope	References
b6	CD4bs	[24]
b12		[64]
VRC01		[65]
3BNC117		[66]
PG9	Trimer association domain (TAD) V2q	[67]
PG16		[67]
PGT145		[68]
PGT128	V3 loop/glycan	[68]
10-1074		[66]
17b	CD4 induced (CD4i)	[25]

Materials and Methods

Cells and reagents.

293T/17 cells (ATCC CRL-11268) [57] were used for transfection of DNA plasmid constructs to prepare HIV-1 Env+ pseudovirions. HeLa TZM-bl cells from the HIV Reagent Program were used in to titrate pseudovirions and for neutralization assays [1]. HeLa TZM-bl carry β-galactosidase and luciferase reporter genes under the control of an HIV long terminal repeat. They express high levels of CD4, and CCR5, and are susceptible to infection by both R5 and X4 HIV-1 isolates [56, 69].

Soluble CD4 (sCD4) was from Progenics. MAbs b12, 2G12, PG9, and PGT145 were from HIV Reagent Program and Polymun Scientific. 17b, 447-52D, VRC01, PG16, PGT128, 3BNC117 and 10-1074 mAbs were from the HIV Reagent Program. b6 was provided by Dr. Dennis Burton, Scripps, La Jolla, CA.

Env primary isolates.

Expression plasmids carrying Envelopes (Envs) derived from molecular clones of transmitted/founder (T/F), acute stage, late disease stage macrophage-tropic and non-macrophage-tropic envelopes were provided by the HIV Reagent Program, Dr. Jonathan Ball [3], University of Nottingham, Nottingham, UK, Dr. Cynthia Derdeyn and Dr. Eric Hunter, Emory University, Atlanta, GA [62] or isolated in our laboratory [1]. These Env+ plasmids were used for pseudovirus production (Table 1).

Envelope genes from transmitted/founder (T/F) (3T, 6T, 15T, and 19T, and Z1792M), acute stage (Z153M, Z221M, and Z185F), chronic infection M-tropic (B33, B59, JR-FL, B100), non-M-tropic (LN40, LN8, JR-CSF and LN58) primary isolates were previously inserted in pSVIIIenv.

Direct mutagenesis.

375W substitutions in all the primary isolates were introduced by site-directed mutagenesis in plasmids carrying Envs using a QuikChange site-directed mutagenesis kit (Stratagene Inc.) and mutagenic primers to introduce each mutation [56]. 375W mutants of all the primary isolate Envs were obtained by direct mutagenesis in their original plasmids. The gp160 insert was sequenced to confirm the presence of the 375W mutation and the absence of any undesired mutation formed during the direct mutagenesis PCR.

Env pseudovirions production and titration.

Calcium phosphate was used to cotransfect plasmids carrying wild type (WT) or 375W mutant envs with pNL4.3 ΔEnv that does not express Env into 293T cells. This latter plasmid has a 2 amino acid insertion changing the reading frame in Env. Env+ pseudovirions from the cell supernatants were harvested 48 h after transfection, clarified (1,000 x*G* for 10 min), aliquoted, and stored at -180°C.

WT and mutant pseudoviruses were titrated in HeLa TZM-bl as described previously [1, 56, 57]. Infections were performed in duplicate. Briefly, serially diluted (10-fold dilutions) cell-free virus supernatant was incubated with HeLa TZM-bl. Infected cells were fixed 2 days post-infection in phosphate-buffered saline (PBS)-0.5% glutaraldehyde at 4°C and stained with X-Gal (0.5 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside/ml [Fisher Bioreagents Inc.] in PBS containing 3 mM potassium ferrocyanide, 3 mM potassium ferricyanide, 1 mM magnesium chloride for 3 h. Focus-forming units (FFU) were estimated by counting individual or small groups of blue-stained, infected cells and the numbers of FFUs/ml were then calculated.

Inhibition and Neutralization assays.

Neutralization assays with carried out using the panel of mAbs in table 2 and inhibition assays with soluble CD4 (sCD4) were performed by reduction in FFU following infection of HeLa TZM-BL cells with 200 FFU of wt or mutant Env+ pseudoviruses as described previously [57]. Two individual neutralization or inhibition experiments were performed in duplicate wells. Positive controls included untreated Env+ pseudovirions and the negative controls, non-infected cells. IC50s were then calculated (see IC50 determinations and statistics).

Infection of healthy donor macrophages

Human monocytes-derived macrophages (MDM) were obtained from healthy human blood donors after preparing blood monocyte monolayers and culturing for 2 days in medium containing macrophage colony-stimulating factor (R&D Systems) as described previously [56, 70]. Versene was used to wash macrophages, three times while incubating at 37°C for 10 min to loosen cell attachments the day prior to infection. Macrophages were then gently scraped off and reseeded into 48-well tissue culture trays.

1.25 x10^5 MDMs in 48-well tissue culture trays were treated with DEAE dextran [56]. Serial 10-fold virus supernatant dilutions were added, spinoculated and incubated for 2 h 15min at 37°C. Virus supernatant was rinsed twice with DMEM. After one week post infection, macrophages were washed once in PBS, then fixed in cold Methanol/Acetone and stained with X-Gal. The numbers of focus-forming units (FFUs) /ml were estimated [56]. Three individual infections were performed in duplicate wells. Error bars in figures were calculated from replicate wells for individual experiments.

Previously, we reported that there is a range of macrophage infectivity in Envs derived from brain samples [3]. Considering these data together with the reference viruses introduced in each assay as controls, we establish that the percentage of macrophage titer (titer normalized respect to TZM-bl titer) < 1% represent non mac-tropic viruses while >1% viruses show a good infection in macrophages. The lower value of the mac-tropic references after repeat the experiment 3 times represent the limit considered to define a mac-tropic Env. Envs that show % macrophage titer >1% and lower than the lowest brain mac-tropic reference macrophage titer were considered intermediate mac-tropic Envs.

IC_{50} determinations and statistics

GraphPad prism software (vX9.3.3) was used to analyze the data. Normalized data was evaluated in Excel and transformed in GraphPad. Sy.x and AICc were used to establish the goodness of fit and to decide the more appropriate model to calculate geometric mean IC50 and 95% CI of IC50. The nonlinear regression method used was a dose-response inhibition model.

Before applying a parametric or non-parametric test, we assessed if our samples are normally distributed using statistical tests or graphical methods. We applied two normality tests: 1)The Shapiro–Wilk test to calculate if normality was used because it is recommended and a more appropriate method for small sample sizes of less than 50 [71, 72] but you need to have unique values, and 2) Anderson-Darling test (the method of Royston) that keeps in mind the skewness and kurtosis to calculate how far the distribution is from asymmetry and shape Gaussian. Q-Q, frequency (histograms), violin, and box plots were used to corroborate the results of the two normality tests.

If our data was Gaussian distribution a paired t test was used to determine significant differences between the means of two groups of data, if not we applied two-tailed non-parametric Wilcoxon signed-rank test. We used an F test to decide if both normal distributed populations have the same standard deviation (SD), and then applied unpaired t test or t test Welch's correction. If the data is not normally distributed, two-tailed, nonparametric Mann-Whitney test (Wilcoxon rank sum test) is used. To test correlations, we used Spearman nonparametric correlation or Pearson correlation if the data follow a Gaussian distribution. P value asterisks as were as New England Journal of Medicine (NEJM) guidelines [<0.033 (*), <0.002 (**), <0.001 (***)].

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