Antibodies VRC01 and 10E8 Neutralize HIV-1 with High Breadth and Potency Even with Ig-Framework Regions Substantially Reverted to Germline

Ivelin S. Georgiev, Rebecca S. Rudicell, Kevin O. Saunders, Wei Shi, Tatsiana Kirys, Krisha McKee, Sijy O'Dell, Gwo-Yu Chuang, Zhi-Yong Yang, Gilad Ofek, Mark Connors, John R. Mascola, Gary J. Nabel and Peter D. Kwong

J Immunol 2014; 192:1100-1106; Prepublished online 3 January 2014;
doi: 10.4049/jimmunol.1302515
http://www.jimmunol.org/content/192/3/1100

References
This article cites 35 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/192/3/1100.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Antibodies VRC01 and 10E8 Neutralize HIV-1 with High Breadth and Potency Even with Ig-Framework Regions Substantially Reverted to Germline

Ivelin S. Georgiev,1 Rebecca S. Rudicell,1 Kevin O. Saunders,1 Wei Shi,1 Tatsiana Kirys, Krisha McKee, Sijy O’Dell, Gwo-Yu Chuang, Zhi-Yong Yang, Gilad Ofek, Mark Connors, John R. Mascola, Gary J. Nabel, and Peter D. Kwong

Abs capable of effectively neutralizing HIV-1 generally exhibit very high levels of somatic hypermutation, both in their CDR and framework-variable regions. In many cases, full reversion of the Ab-framework mutations back to germline results in substantial to complete loss of HIV-1-neutralizing activity. However, it has been unclear whether all or most of the observed framework mutations would be necessary or whether a small subset of these mutations might be sufficient for broad and potent neutralization. To address this issue and to explore the dependence of neutralization activity on the level of somatic hypermutation in the Ab framework, we applied a computationally guided framework-reversion procedure to two broadly neutralizing anti–HIV-1 Abs, VRC01 and 10E8, which target two different HIV-1 sites of vulnerability. Ab variants in which up to 78% (38 of 49 for VRC01) and 89% (31 of 35 for 10E8) of framework mutations were reverted to germline retained breadth and potency within 3-fold of the mature Abs when evaluated on a panel of 21 diverse viral strains. Further, a VRC01 variant with an ∼50% framework-reverted L chain showed a 2-fold improvement in potency over the mature Ab. Our results indicate that only a small number of Ab-framework mutations may be sufficient for high breadth and potency of HIV-1 neutralization by Abs VRC01 and 10E8. Partial framework revertants of HIV-1 broadly neutralizing Abs may present advantages over their highly mutated counterparts as Ab therapeutics and as targets for immunogen design. The Journal of Immunology, 2014, 192: 1100–1106.

Recent years have seen an explosion in the number of broadly neutralizing Abs (bNAbs) against HIV-1 (1–10). Many of these bNAbs have been shown to protect from or to provide control of infection (11–13) and, therefore, are of interest for passive-immunization approaches (14). An underlying characteristic of anti–HIV-1 Abs is the substantially increased levels of somatic hypermutation (15). Somatic hypermutation is part of the diversification of Abs that occurs during affinity maturation: this process occurs in activated B cells exposed to Ag within germinal centers where high-affinity Abs are selected over their low-affinity counterparts (16). Generally, chronic viral infections are associated with the generation of Abs with increased numbers of mutations compared with acute viral infections, suggesting that persistent Ag exposure plays a role in stimulating repeated rounds of somatic hypermutation and selection (17, 18). In the case of HIV-1, bNAbs mostly show higher mutation levels compared with weakly neutralizing Abs (18). Moreover, V-gene reversion to germline of several anti–HIV bNAbs results in lack of neutralization activity (18–20), indicating that somatic hypermutation is important for neutralizing breadth and potency (18).

Although somatic mutations occur preferentially within the CDR regions of Abs (21), large numbers of mutations in anti–HIV-1 bNAbs are also found within the Ab-framework regions (18, 19). Klein et al. (18) analyzed a set of anti–HIV-1 bNAbs targeting diverse epitopes on the HIV-1 envelope glycoprotein and found that full framework reversion to germline substantially reduces or completely abrogates neutralization activity for many of these Abs; function is partly restored in some Abs by allowing framework mature mutations in positions that are in direct contact with the Ag (18). The results of that study underline the importance of framework maturation for broad and potent neutralization by anti–HIV-1 Abs. However, it has been unclear whether most or all of the framework mutations would be necessary for retention of Ab function or whether some of these mutations could be reverted to germline with minimal effects on function.

To investigate this question, we selected two bNAbs that target different sites of vulnerability on the HIV-1 Env glycoprotein: the CD4 binding site (CD4bs) Ab VRC01 and the membrane-proximal external region (MPER) Ab 10E8. These Abs neutralize ∼90% and 98% of HIV-1 strains at an average potency of 0.33 and 0.22 µg/ml, respectively (4, 10). The variable regions of both of these Abs exhibit high degrees of amino acid mutation: VRC01 V-gene, 42% heavy/28% light and 10E8 V-gene, 22% heavy/17% light. The putative germline-reverted versions of these Abs are incapable of neutralizing HIV-1 viral strains (19; M. Louder and J. Mascola, unpublished observations). For VRC01, the mature CDRs, alone or in combination with the Ag-contacting framework residues, are not sufficient for potent neutralization: i.e., they were able to only weakly neutralize 0 and 3 of 10 tested strains, respectively (18). These results confirm the importance of framework mutations in VRC01.
However, we conjectured that not all mutations from germline would be necessary for retention of Ab-neutralization activity. To test this conjecture, we created a series of VRC01 and 10E8 variants with partial framework reversions to germline in both H and L chains and compared their neutralization activity to that of the mature Abs. This approach allowed us to explore the relationship between neutralization activity and the number of framework mutations in anti–HIV-1 bNAbs. Our results challenge the notion that extensive framework mutations are necessary for broad and potent neutralization by anti–HIV-1 bNAbs and suggest strategies for Ab redesign in the context of Ab-product optimization or immunogen design.

Materials and Methods

Design and analysis of partial framework revertants to germline

Different approaches for various degrees of Ab germline reversion, ranging from full V-gene reversion to selective point mutations, were described previously, with applications to a number of viruses (18, 19, 22–24). In our study, a CDR-grafting procedure typically used for humanization of non-human Abs (25) was used as the basis for our designs, in combination with structural modeling and mutation analysis. Specifically, the CDR regions (Kabat definition) of the mature Abs (VRC01 and 10E8) were grafted onto homologous human germline genes (in effect, this replaces the germline CDRs with their mature counterparts). Additionally, series of germline framework residues were selected for mutation to their mature counterparts (Fig. 1). Structural modeling, analysis, and visualization were used to determine which germline framework residues to mutate to mature. Specifically, mutations were selected based on a combination of several factors: whether the position is implicated in supporting CDR regions (25); the differences between germline and mature amino acid types (e.g., change in amino acid charge, hydrophobicity, or structural properties); and proximity to Ag or CDR regions, as determined by the Ag-bound crystal structure. The Ag-bound crystal structure of VRC01 was obtained from Protein Data Bank id: 3NGB (19). The Ag-bound crystal structure of 10E8 [Protein Data Bank id: 4G6F (10)] was not available at the time of the design and thus was therefore only used for retrospective analysis. Structural modeling, analysis, and visualization were performed using O/SREPAY (26, 27), MolProbity (28), and PyMOL (29). Throughout this article, the mAb-mH/mL nomenclature is used for designed partial framework revertants, where n is the number of framework mutations in the H chain and m is the number of framework mutations in the L chain: for example, VRC01-5fH/6fL refers to the VRC01 variant with grafted mature CDRs and 5 H chain and 6 L chain mature framework mutations. Designed H and L chains were paired in a sparse matrix that was deemed to sufficiently represent the diversity of the possible H-L chain combinations.

Protein expression and purification

Site-directed mutagenesis or gene synthesis was used to generate the CMV/R plasmids encoding the H or L chain framework revertants. Full-length IgG proteins were expressed by cotransfection of H and L chain plasmids into 293F cells, and Abs were purified with affinity chromatography using either Protein A Fast Flow Resin or HiTrap Protein A HP Columns (both from GE Healthcare).

Neutralization assays

Neutralization was measured using single-round-of-infection HIV-1 Env pseudoviruses and TZM-bl target cells, as described previously (30–32). Neutralization curves were fit by nonlinear regression using a five-parameter hill slope equation, as previously described (31). IC50 values were used for the computation of neutralization breadth and potency for the different Ab variants. Average neutralization potency for a given Ab was computed as the geometric mean of the IC50 values for a set of 21 diverse viral strains (7), where values > 50 μg/ml were set to 50. Neutralization breadth for a given Ab was computed as the percentage of strains with IC50 values < 50 μg/ml.

Results

Partial framework reversion of VRC01

A computational design procedure was used to create partial framework reversion variants of Ab VRC01 (see Materials and Methods and Fig. 1). The H chain of the mature VRC01 Ab contains 30 mutations within framework regions 1–4 (Fig. 2A). To test whether a subset of these mutations could be reverted back to germline without substantially affecting Ab activity, we created two partial framework revertants of the H chain, with five and eight mature framework mutations each (in the context of mature CDR regions), as well as the CDR-grafted version of the H chain (with mature CDR regions and no framework mutations) (Fig. 2). The selected mutations in the VRC01-5fH variant were in proximity to the Ag (distances of 3.6–7.7 Å) (Fig. 2A, 2B). The additional three mutations in the VRC01-8fH variant were deemed to represent substantial changes in amino acid type that were still in relative proximity to the Ag (distances of 6.7–8.8 Å). The remaining 22 residue positions of maturation were more distal to the Ag (mean distance of 16.3 ± 4.7 Å; range 8.3–24.9 Å) and represented more conserved amino acid changes and, thus, were not selected for study. Similarly, the mature VRC01 L chain contains 19 framework mutations, so we created partial framework revertants with mature CDR regions and 0, 6, and 10 mature framework mutations (Fig. 2). None of the framework mutations in the VRC01 L chain was in substantial proximity to the Ag (mean distance of 21.7 ± 6.9 Å; range 9.8–32.3 Å), so the reversion analysis was focused on framework interactions with the CDR regions and/or within the overall Ab structure. The VRC01-6fL L chain variant included mutations in proximity to the CDR regions (distances of 2.9–5.5 Å), as well as a two-residue FWR4 insertion that is not found in most other HIV-1

FIGURE 1. Procedure for partial reversion of Ab framework to germline. The CDR regions of the mature target Ab (yellow) were grafted onto the backbone of the putative germline precursor prior to somatic hypermutation (green) to obtain a chimeric CDR-grafted Ab. Additionally, based on sequence and structural considerations, residues were selected for mutation to their mature counterparts to obtain a partial framework reversion to germline of the target Ab with generally retained function.
bNAbs, including other Abs from the VRC01 class (9). Structural modeling identified an additional four mutations that could be of importance for the overall structure of VRC01, and the addition of these mutations formed the VRC01-10fL variant (Fig. 2).

HIV-1 neutralization by VRC01 partial framework revertants

A total of 10 H–L chain combinations using the 4 H chain and 4 L chain VRC01 variants was tested for neutralization activity. The mature VRC01 (VRC01-30fH/19fL, consisting of 30 H chain/19 L chain framework mutations) was among the best in terms of neutralization function (Fig. 3); however, the VRC01-30fH/10fL variant (with mature H chain and partially reverted L chain) consistently outperformed all other constructs when using neutralization potency-breadth curves as a metric (the percentage of neutralized viral strains at different IC50 potency cutoffs) (Fig. 4A). Six of the nine partial framework revertants maintained good neutralization activity. In fact, with the exception of VRC01-0fH/6fL (which did not express), VRC01-5fH/0fL, and VRC01-0fH/0fL, the revertants generally had an average potency within an ∼10-fold range from mature (Fig. 4B). The VRC01-0fH/0fL CDR-grafted construct showed complete lack of neutralization activity, in concordance with previous findings (18).

Partial framework reversion of Ab 10E8

The crystal structure of the 10E8–MPER complex was not available at the time of the designs and, thus, was used only for retrospective analysis. The H and L chains of mature 10E8 contain 19 and 16 mutations within framework regions 1–4, respectively (Fig. 5A). We created 3 H chain variants with mature CDR regions and 0, 2, and 10 framework mutations and 3 L chain variants with mature CDR regions and 0, 4, and 10 framework mutations (Fig. 5). The selected mutations in the 10E8-2fH and 10E8-10fH H chain variants and 10E8-4fL and 10E8-10fL L chain variants were deemed to represent more substantial changes in amino acid type and/or were found at positions that have been implicated in supporting CDR regions (Fig. 5C). Post hoc structural analysis revealed that the mutations in the 10E8-2fH H chain variant were in proximity to the Ag (distances of 3.6 and 6.9 Å), but the distances to the Ag or the CDR regions for the additional eight mutations in the 10E8-10fH H chain variant versus the remaining nine H chain mutations were not substantially different (18.1 ± 6.6 Å versus 20.6 ± 4.9 Å for Ag, 7.5 ± 3.4 Å versus 6.6 ± 2.1 Å for CDR regions) (Fig. 5A, 5B). None of the framework mutations in the 10E8-L chain was in
substantial proximity to the Ag (mean distance, 22.4 ± 6.8 Å; range, 14.2–33.0 Å; Fig. 5A). The 10E8-4fL L chain variant included mutations in proximity to the CDR regions (distances of 3.1–5.9 Å), but the distances to the CDR regions for the additional six mutations in the 10E8-10fL L chain variant versus the six remaining residue positions of maturation were not substantially different (10.3 ± 3.2 Å versus 8.3 ± 4.0 Å) (Fig. 5A, 5B).

HIV-1 neutralization by 10E8 partial framework revertants
A total of 10 H–L chain combinations using the 4 H chain and 4 L chain 10E8 variants were tested for neutralization activity. The mature 10E8 (10E8-19fH/16fL), along with the 10E8-19fH/10fL and 10E8-10fH/16fL variants, displayed the best neutralization potency and breadth (Figs. 3, 6A). Unlike VRC01, the 10E8 variant with no framework mutations (10E8-0fH/0fL) retained significant, but reduced, neutralization activity (Fig. 6B). As with VRC01, a significant correlation was found between the number of framework mutations and the neutralization potency for 10E8 variants (Fig. 6C; \( p = 0.0022 \)). Interestingly, however, the 10E8-0fH/4fL variant that contained no H chain and only four L chain framework mutations had improved neutralization potency compared with 10E8-0fH/0fL, with only an ∼3-fold decrease in potency relative to mature 10E8 (Figs. 6A, 6B).

Discussion
The high rate of mutation and the numerous other immune-evasion mechanisms of HIV-1 serve as a constant stimulus for HIV-1–reactive Abs to continuously evolve and mature as they target the ever-changing viral envelope. Perhaps as a consequence, broadly neutralizing anti–HIV-1 Abs typically have extreme numbers of variable domain (CDR and framework region) mutations. In the case of VRC01, the importance of framework mutations is underscored by the fact that, although VRC01-5fH/0fL showed virtually no neutralization activity, the addition of six framework mutations in the L chain resulted in VRC01-5fH/6fL being within 2-fold potency of the mature Ab. The precise role for each of these six mutations was not clear; however, three of these mutations (G66R, T69P, and F71Y) clustered closely to CDR L1 (Fig. 2B, 2C), suggesting that these mutations may help to support the CDR L1 loop in a conformation appropriate for interaction with Ag. We note that these mutations are generally not conserved in VRC01-class Abs from different donors, suggesting that Abs in this class may utilize diverse pathways to achieve broad and potent neutralization (1, 9).

In the case of Ab 10E8, a variant with no mature framework mutations showed reasonable, albeit ∼10-fold lower, neutralization activity. Thus, framework maturation in anti–HIV-1 Abs does not appear to be universally required for potent and broad neutralization activity. Although the overall number of framework mutations can affect neutralization activity, some mutations appeared to have a more substantial effect on Ab activity, so the specific selection of residues for germline reversion is also important. For example, the 10E8-2fH/4fL and 10E8-2fH/0fL variants had an ∼4-fold and ∼2-fold decrease in potency, respectively, compared with the analogous

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** HIV-1 neutralization by VRC01 and 10E8 Ab variants. For each Ab, shown are IC50 values (in μg/ml) for the wild-type mature Ab, a set of partial framework revertants, and a CDR-grafted variant in which the entire framework regions have been reverted to germline (columns; Ab names are based on the number of framework mutations in the H and L chains). Neutralization data were obtained for 21 diverse HIV-1 strains (rows) from clades A, B, and C. Values are colored according to potency, on a scale from green (least potent) to red (most potent), with white corresponding to a neutralization potency > 50 μg/ml.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** HIV-1 neutralization by framework revertants of Ab VRC01. (A) Neutralization breadth at different IC50 neutralization cutoff values. (B) Average (geometric mean) neutralization potency for the different combinations of H chain (HC) and L chain (LC) variants; gray indicates combination not tested. DNE indicates that the variant did not express. (C) Spearman correlation of neutralization potency versus number of framework mutations (heavy + light); colors same as in (A).
variants without the two H chain framework mutations (10E8-0fH/4fL and 10E8-0fH/0fL). Similarly, the VRC01-5fH/6fL variant had a ∼2-fold better potency than the substantially more mutated VRC01-8fH/19fL. In the case of 10E8-0fH/4fL, post hoc analysis of the 10E8-MPER peptide structure indicated that additional reversions of mutations that do not directly interact with the Ag or CDR regions may be possible (e.g., S2Y; Fig. 5). Further, the reversion analysis was focused solely on framework residues; however, it should also be possible to selectively revert CDR mutations, because not all mutations within the CDR regions are important for Ag recognition or overall Ab structure (33, 34). This process could allow for the generation of partially reverted Ab variants with even lower degrees of mutation and with similar, if not better, neutralization activity as their mature bNAb counterparts.

**FIGURE 5.** Framework revertants of Ab 10E8. (A) Sequence alignment (upper panel, H chain [HC]; lower panel, L chain [LC]) of a putative germline version, designed partial framework revertants, and mature 10E8. Residues that are identical to germline are shown as dots. CDR mutations are shown in yellow on gray background; framework mutations are colored according to the variant in which they are first introduced (orange, closest to germline; purple to blue, mature). For each residue position, the respective distance to Ag and CDRs is shown. (B) Mapping of framework mutations from germline backbone onto a 10E8 complex structure [colors same as in (A)]. (C) Selection criteria for framework mutations.

**FIGURE 6.** HIV-1 neutralization by framework revertants of Ab 10E8. (A) Neutralization breadth at different IC₅₀ neutralization cutoff values. (B) Average (geometric mean) neutralization potency for the different combinations of H and L chain variants; gray indicates combination not tested. (C) Spearman correlation of neutralization potency versus number of framework mutations (heavy + light); colors same as in (A).
Interestingly, we also found that reversion of selected mutations resulted in improved Ab variants compared with the mature Abs. In the case of VRC01, a combination of the mature H chain and an ~50% reverted L chain (VRC01-30fH/10fL) resulted in 2-fold improved potency over the mature Ab. For 10E8, two variants, 10E8-19H/10fL (mature H chain/~40% reverted L chain) and 10E8-10H/16fL (~50% reverted H chain/mature L chain), exhibited similar neutralization breadth and potency to the mature Ab. These results show the promise of partial framework reversion for Ab optimization.

Anti–HIV-1 bNAbs with lowered mutation levels may be of use both in vaccine design and as therapeutic agents. An effective prophylactic HIV-1 vaccine most likely will have to elicit bNAbs. Thus, the high mutation levels of most anti–HIV-1 bNAbs present a challenge for vaccine development and immunogen design, because the vaccine immunogen (or perhaps a series of different immunogens) would need to guide the Ab affinity–maturation process from germline activation through the appropriate intermediate stages of Ab development to the mature bNAb (35–37). However, bNAbs with lower levels of mutations, such as those presented in this article or from Ab ontogeny data from next-generation sequencing (1, 9), may be more easily inducible and can be suitable targets for immunogen design, alleviating the requirement for guiding the affinity-maturation process to extreme levels of hypermutation (alternatively, it may be that to achieve necessary CDR mutations, high overall levels of somatic hypermutation are required, resulting in a substantial number of “bystander” framework mutations). Abs with high mutation rates might also be problematic as clinical products because the likelihood of immunogenicity may be increased. Although, to our knowledge, no highly somatically mutated Abs have been evaluated for immunogenicity in humans, Abs that are closer to their germline ancestors (the genes that are commonly found in most individuals) may be more easily tolerated in the general population. Thus, partial germline reversion may be a useful addition to the armamentarium of technologies for neutralizing antibodies isolated from memory B cells in HIV-infected individuals. PLoS ONE 5: e8805.


7. For further reading on frameworks and their use, and WO2013070776 A1, titled “Neutralizing gp41 antibodies.”

8. Quadrilaterals


