HIV-1 Envelope Glycoprotein Trimer Immunogenicity Elicited in the Presence of Human CD4 Alters the Neutralization Profile

Mattias N.E. Forsell,^{1,2} Krisha McKee,¹ Yu Feng,^{1,*} John R. Mascola,¹ and Richard T. Wyatt^{1,*}

Abstract

The HIV-1 envelope glycoproteins (Env) gp120 and gp41 are the sole virally derived components on the surface of the virus. These glycoproteins mediate receptor binding and entry and are targets for neutralizing antibodies. The most highly validated protein region on Env that is a target for broadly neutralizing antibodies is the conserved CD4 binding site. Mimetics of Env have been used in attempts to elicit antibodies to the CD4 binding site. Some trimers, such as the soluble foldon trimers used here, elicit 5–10% of the Env-directed B cell response to this conserved region. As these trimers, or other Env versions, advance into clinical development, there is both considerable interest and concern as to whether binding to the abundant CD4 present on the surface of T cells and macrophages may blunt potentially protective antibody responses to this site. Here, we utilized rabbits transgenic for human CD4 to evaluate the role of CD4:Env interaction *in vivo* relative to the elicitation of Env-directed antibodies following immunization. We analyzed responses to trimers both capable and incapable of recognizing human CD4 with high affinity. We demonstrated that the presence of human CD4 *in vivo* did not significantly affect the overall elicitation of Env binding or CD4bs-directed antibodies. However, the presence of CD4 did reduce the capacity of elicited serum antibodies to neutralize the clade C isolate, MW965. Reduction of HXBc2 neutralization was associated with the CD4 binding-incompetent trimers. These results highlight an important consideration regarding CD4 binding-competent trimeric Env immunogens as they enter the clinic for human vaccine trials.

Introduction

THE HUMAN IMMUNODEFICIENCY VIRUS type-1 (HIV-1) envelope glycoproteins (Env) gp120 and gp41 are the sole virally derived components exposed on the outside of an infectious virus particle. Env-based candidate immunogens are often used in both experimental and clinical approaches designed to determine if vaccine-induced protection against HIV is achieved. The recent clinical trial, RV144, demonstrated moderate efficacy, albeit of a relatively nondurable nature, using Env candidate immunogens to protect against the “real world” strains circulating in Thailand.¹ The protective effect induced by this vaccine candidate is associated with the induction of antibodies that target the major variable (V) regions of Env, V1, and V2.^{2,3} These results provide one potential explanation for the limited efficacy of this vaccine, as the majority of the residues located in these regions

demonstrate relatively high variability among the diverse array of HIV-1 strains. With the exception of the rare, infection-induced V1/V2-directed broadly neutralizing antibodies (bNAbs),⁴ most antibodies that are directed at variable regions of Env are susceptible to rapid genetic drift, or immune-mediated selection, with the rapid escape of ensuing HIV-1 strains. However, if a vaccine would induce antibodies that are aimed at more conserved regions of Env, such antibodies may have the potential to significantly increase the efficacy of protection.⁵ This protective efficacy is supported by the ability of passively infused potent CD4 binding site (CD4bs)-directed bNAbs to protect nonhuman primates (NHPs) from mucosal challenge by a relatively antibody-resistant simian–human immunodeficiency virus (SHIV) inoculum.⁶

Generally, the receptor binding regions of the HIV-1 spike proteins need to remain highly conserved for a virus to be

¹Vaccine Research Center, NIAID, National Institutes of Health, Bethesda, Maryland.

²Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden.

*Current affiliation: IAVI Neutralizing Antibody Center at TSRI and Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California.

infectious since the human receptors are monomorphic. Upon binding to CD4, gp120 Env undergoes a large conformational change that exposes or forms the coreceptor binding site (CoRbs). Subsequent Env binding to the coreceptor, typically CCR5 or CXCR4, induces additional conformational changes that allow fusion of the virus-to-target-cell membranes to mediate insertion of HIV genetic material into susceptible target cells. Although the CoRbs represents one of the most conserved regions on Env, CoRbs-directed antibodies cannot neutralize primary HIV-1 isolates,⁷ presumably due to steric or conformational occlusion of this highly conserved region.⁸ In contrast, several studies clearly demonstrate the capacity of monoclonal CD4bs-directed antibodies derived from several independently infected individuals to potently and broadly neutralize primary HIV-1 strains *in vitro*.^{9–11} In addition, and as mentioned, passive infusion of several of these CD4bs-directed bNAbs protects against experimental SHIV challenge of NHPs,^{6,12} and the broad neutralization exhibited by some sera derived from HIV-1-infected “slow progressors” maps to this site.^{11,13,14} Consistently, most Env-based vaccine candidates to date retain an intact CD4bs, as a fraction of antibodies that target this relatively well-conserved site possesses the potential to confer a high degree of protection against diverse HIV-1 isolates. However, it remains a puzzle why CD4bs-directed antibodies of the broadly neutralizing type are detected only following prolonged HIV-1 infection, but are not induced at detectable levels by current experimental vaccine regimens.¹⁵

A number of *in vitro* studies investigated whether Env binding impacts the functionality and activation of CD4⁺ cells and it has also been suggested that this interaction is detrimental for TLR signaling in human dendritic cells.¹⁶ In contrast, CD4 binding was not found to affect the ability of primary human dendritic cells to engulf, process, and present an Env-pp65 fusion immunogen to CD4⁺ T cells.¹⁷ Moreover, NHPs injected with CD4 binding-competent or -incompetent Env generated a similar anti-Env T cell response.¹⁸ These data indicate that while Env:CD4 binding can negatively affect CD4⁺ human cells *in vitro*, the overall effect *in vivo* may be limited (at least in the context of non-CpG-containing adjuvants¹⁸). Even though the anti-Env B cell responses elicited by CD4 binding-competent versus -incompetent Envs appear similar in the study by Douagi *et al.*, it is not possible to completely rule out whether the Env:CD4 interaction directly affected the ability of B cells to elicit anti-Env-directed antibodies. We hypothesized that this could occur either by occlusion of the CD4bs or by locking Env into a conformation different from that found on free infectious, prereceptor-engaged virions.^{19,20} As a consequence, B cells would be exposed to less Env immunogen presenting the CD4bs or “different” targets (e.g., CD4-induced epitopes) than would be optimal for the elicitation of CD4bs-directed neutralizing antibodies.

Using rabbits engineered previously to be transgenic for human CD4 (huCD4 rabbits),²¹ we demonstrate the requirement of *in vivo* CD4 binding for elicitation of CoRbs-directed antibodies (abs) after immunization with Env.²² These data suggest that indeed, some fraction of trimeric Env engages endogenous human CD4 to potentially occlude immune responses, especially B cells, to this conserved neutralization determinant. Here, we utilized these transgenic huCD4 rabbits to further evaluate the impact of human CD4

on the elicitation of anti-Env abs after immunization with CD4 binding-competent and -incompetent Env-based trimeric immunogens. The data presented here suggest that while the presence of the human CD4 *in vivo* did not significantly affect bulk elicitation of Env binding abs following repeated inoculation with Env trimers, nor elicitation of CD4bs-directed binding antibodies, its presence did promote the generation of CoRbs-directed neutralizing antibodies not detected in wild-type (wt) rabbits and subtly affected the capacity of the elicited IgG to neutralize the HIV-1 neutralization-sensitive strain, MW965.

Materials and Methods

Rabbits and Env trimer injections

The huCD4 transgenic strain of New Zealand white rabbits used in this study was generated previously by microinjection, as reported by Snyder *et al.*²¹ Here, huCD4 rabbits were bred from a founder pair and offspring were screened for the presence of huCD4 transgene by flow cytometry at Spring Valley Laboratories, Woodbine, MD. Limits of the pace and efficiency of the breeding process did limit the numbers of huCD4⁺ animals available within a reasonable time frame for the studies reported here. Wt and huCD4 transgenic female New Zealand white rabbits were injected intramuscularly in each hind leg with a total of 50 μ g recombinant Env in Abisco100 (ISCONOVA) according to the manufacturer's instructions and 50 μ g/ml of monophosphoryl A (Sigma). Injections were performed every 4 weeks and the animals were bled for serum 2 weeks after each injection. Detection of CoRbs-directed antibodies in serum from Env-injected animals was previously shown to correlate with the detection of human CD4 on rabbit CD4⁺ cells by flow cytometry.²² Consequently, we here assessed the elicitation of CoRbs-directed antibodies after Env injection to phenotypically verify the genotype of wt and huCD4 rabbits. Animals were housed at Spring Valley Laboratories. All procedures were preapproved by the appropriate NIH and company Ethical Committee on Animal Experiments.

Protein production and characterization

Protein production of soluble trimers was performed as previously described.²² Proteins were produced by transient transfection of 293Freestyle suspension cells. The highly glycosylated and His6-tag containing YU2gp140-F (Env)²³ or YU2gp140-F D368R (Env.368R)²² trimers or YU2-derived monomeric gp120 were captured and purified from the serum-free media by a three-step process. First, the trimers were captured by their high-mannose glycans using lentil-lectin affinity chromatography (GE Healthcare). After extensive washing with phosphate-buffered saline (PBS) to elute nonspecifically bound proteins, the trimers were eluted by mannose-containing buffer and captured in the second step via the C-terminal His-tag by nickel-chelation chromatography (GE Healthcare) then washed and eluted with a 300 mM imidazole-containing PBS buffer. Finally, the trimers were separated from lower molecular weight Env forms by the third step of size exclusion chromatography using a Superdex200 26/60 prep grade column and the ÄKTA Fast protein liquid chromatography system (GE Healthcare). The fractions containing the trimeric peak were concentrated

using Amicon spin columns. The design, production, and purification of the HXBc2 gp120-based core TriMut and TriMut368/370 proteins used for the serum adsorption experiments to determine the presence of CD4bs-directed antibodies were previously described.^{24,25}

ELISA binding and adsorption assays

The capacity of serum IgG or the control CD4bs-directed monoclonal antibodies (mAbs) b12 or VRC01 to bind different proteins was measured by enzyme-linked immunosorbent assay (ELISA). Briefly, high-protein binding MaxiSorp plates (Nunc) plates were adsorbed with 100 or 200 ng protein (trimers, monomers, or cores) in PBS per well and incubated overnight at 4°C. After washing, the plates were blocked with PBS containing 2% nonfat milk for 1 h at room temperature (RT). Serum was diluted in the PBS-milk blocking buffer (bb) and added to the ELISA plate wells. After 1 h incubation at RT, the wells were washed and incubated with horseradish peroxidase conjugated to antirabbit IgG. Following extensive washing, binding of serum IgG to the adsorbed Env immunogens was assessed by colorimetric change of the peroxidase enzyme immunoassay substrate (3,3',5,5'-tetramethylbenzidine; Bio-Rad) after the reaction was terminated by adding 1 M H₂SO₄. The optical density (OD) was read at 450 nm. The 50% effective reciprocal dilutions (ED₅₀) to Env, Env.368R, gp120, TriMut, and TriMut368/70 were calculated from titration curves derived from individual animals by using nonlinear regression curve fitting after logarithmic transformation of the reciprocal dilution factors of antiserum (GraphPad Prism 5.0). To determine differential binding at the CD4bs, the half maximal binding values of serum to the TriMut to TriMut368/70 (CD4bs defective) were similarly assessed from the individual titration curves. The percent CD4bs-directed antibodies of total gp120 binding antibodies was then calculated as $(ED_{50TriMut} - ED_{50TriMut368/70}) \cdot 100 / ED_{50gp120}$.

For selective adsorptions, serum samples or mAbs were coincubated with bovine serum albumin (BSA), TriMut, or TriMut368/70 (all 25 µg/ml) for 1 h prior to addition to wells coated with TriMut. The differential binding at the CD4bs of coated TriMut was then determined by calculating the difference of soluble TriMut or TriMut368/70 to adsorb serum abs or mAb at subsaturating conditions as $(ED_{50BSA} - ED_{50TriMutX}) \cdot 100 / ED_{50BSA}$, where the difference in binding capacity is accounted for by CD4bs-directed abs.

Isolation of serum IgG

Serum samples were diluted to the 50% Env binding ELISA titer and then pooled according to immunogen group. Pooled IgG from these samples was extracted and purified using recombinant protein A-conjugated Sepharose Fast Flow beads (Amersham). The bead-adsorbed IgG was separated from other serum components by centrifugation of the beads for 3 min at 5,000×g. The beads were washed twice with 1.2 ml PBS containing 0.5 M NaCl, once with PBS, and then all liquid was removed above the bead pellet. To elute captured IgG from the protein A beads, each pellet was resuspended in 135 µl of 0.1 M glycine (pH 2.0) for 1 min at RT and then centrifuged at 500×g for 3 min. The supernatant containing the IgG was then removed and the pH was adjusted to neutrality by adding 15 µl of 1 M Tris (pH 8.5). The

resulting purified IgG was quantified by Nanodrop analysis at OD 280 nm.

Neutralizing assays and serum adsorptions

Analyses of HIV-1 and HIV-2 neutralizing in serum samples were performed as previously described.^{22,26} Briefly, HIV pseudoviruses were prepared by cotransfecting 293T cells with an Env expression plasmid containing a full gp160 *env* gene and an *env*-deficient HIV-1 backbone vector (pSG3 Env) and viruses with different Envs were secreted into the media. To these pseudoviruses, a single dilution of sera or plasma was used and the percent neutralization was calculated compared to controls with no sera added to the pseudovirus stock. To determine the dilution of the sera that resulted in a 50% reduction in RLU against selected viruses, serial dilution of the sera was performed and the neutralization dose-response curves were fit by nonlinear regression using a four-parameter hill slope equation programmed into JMP statistical software (JMP 5.1, SAS Institute Inc., Cary, NC). The results are reported as the serum neutralization ID₅₀ or 1/IC₅₀, which is the reciprocal value of the serum dilution or the inverse IgG concentration resulting in a 50% reduction in viral entry. Statistical analysis was done using the Mann-Whitney nonparametric test (GraphPad Prism 5.0).

To examine the contribution of CD4bs-directed antibodies to neutralizing capacity we performed differential inhibition of neutralization. Each serum sample was preincubated with 12.5 µl of TriMut core protein (100 µg/ml), TriMut368/370 (100 µg/ml; CD4bs defective, or cell culture medium, respectively, for 1 h at 37°C. For each serum, three neutralization curves derived from the assays performed in parallel were analyzed to reveal whether virus neutralization was directed to the CD4bs by differential inhibition of neutralization using the isogenic probes. To compare the percentage of CD4bs-directed neutralization between animals grouped by regimen, differences of neutralization absorbed by TriMut and TriMut368/70 at 1:12 serum dilution were plotted. Statistical analysis of CD4bs-directed neutralization detection was done using the Mann-Whitney nonparametric test (GraphPad Prism 5.0).

Results

Elicitation of immunogen-specific IgG after immunization of rabbits with Env trimers

To evaluate the capacity of *in vivo* Env:CD4 interaction to alter the potential of Env-based immunogens to induce specific IgG after immunization, we combined the use of the huCD4 transgenic rabbits with Env immunogens that differ in their capacity to bind primate CD4. That is, we immunized both wt and huCD4 rabbits with both unmodified trimers (termed Env) and trimers containing a 368 D-to-R alteration in their CD4 binding region (termed Env.368R). This non-conservative substitution on the molecular surface of the CD4bs eliminates completely binding by CD4 and recognition by most antibodies directed against this conserved site *in vitro*^{22,27} and would therefore eliminate CD4 interaction *in vivo* as we demonstrated previously.¹⁸

Accordingly, we immunized wt or huCD4 rabbits four times with wt Env or Env.368R. To assess the overall response to Env, as well as the relative immunogenicity of the

altered CD4bs between Env and Env.368R, we measured the binding capacity of pooled serum IgG derived from each group of immunized animals to each respective antigen with ELISA after two (Fig. 1A) and four (Fig. 1B) injections. To derive a more precise comparison of the response to Env between wt and huCD4 rabbits, we compared the 50% binding titers (ED_{50} ; effective dilution) of individual animals after two (post 2) and four injections (post 4) (Fig. 1C). We found that the wt rabbits produced higher quantities of Env-specific serum IgG after two, but not after four injections. This confirmed that after repeated injections, the huCD4 transgenic rabbits were a valid immunogenicity animal model for assessment versus wt rabbits.

This analysis did not rule out the elicitation of antibodies with specificity for the altered CD4bs in an Env.368R trimer context in each rabbit type. We therefore determined the 50% serum binding to both Env and Env.368R in each individual wt and huCD4 rabbit and assessed the ratio of binding between the two. We found that binding to Env.368R was favored by serum from wt rabbits after two injections with Env.368R trimers, expressed as a ratio of the 50% binding

titers between Env/Env.368R, but not after injection with unmodified Env trimers ($p=0.02$). In contrast, we found no difference in the Env/Env.368R binding ratio in serum from huCD4 rabbits (Fig. 1D, left panel). Of note, the differential elicitation of Env.368R-specific antibodies after two injections of wt rabbits was not detected after four injections, indicating that elicitation of 368R-specific antibodies was transient in nature (Fig. 1D, right panel). This demonstrated that overall, both wt and huCD4 rabbits elicited similar levels of binding abs after four injections, regardless of the Env immunogen type.

Analysis of trimer-elicited CD4bs-directed IgG

To focus our analysis on abs directly targeting the gp120 CD4bs, we took advantage of two Env-derived protein “cores” that we developed recently.²⁴ These cores lack the major variable regions 1, 2, and 3 (V1/V2/V3) and focus binding evaluations on the conserved regions of gp120 that remain intact in the core, primarily the CD4bs. These cores also contain three mutations in the gp120 CoRbs, or bridging

FIG. 1. Env-binding antibodies in serum from injected animals. Serum IgG-binding enzyme-linked immunosorbent assay (ELISA) to Env or Env.368R from wild-type (wt) or huCD4 rabbits after two (A) and four (B) injections. (C) The effective reciprocal serum dilution yielding 50% maximal binding (ED_{50}) to Env gp140 14 days after two (post 2) and four (post 4) homologous injections in the wt and huCD4 transgenic rabbits. (D) The ratio of ED_{50} values from Env and Env.368R-immunized rabbits is shown after two and four injections of the wt and huCD4 rabbits. The ED_{50} values were determined by nonlinear regression analysis, and statistical analysis was done using the Mann-Whitney nonparametric test (* $p < 0.05$, GraphPad Prism 5.0).

sheet, that eliminate recognition of antibodies specific to this conserved site (I423M, N425K, and G431E), removing this type of binding specificity from the analysis (see below). The core with the three mutations alone is called TriMut and the isogenic core that also contains two CD4bs mutations, D368R and E370A, is called TriMut368/70. This matched pair of cores can be used for the detection of CD4bs-directed abs by differential binding.²⁴ CD4bs-directed abs in the serum will bind to the TriMut core protein and not the TriMut368/70 core protein.

Based upon this differential analysis, comparing serum binding to TriMut versus TriMut368/70, we detected CD4bs-directed abs in both wt and huCD4 rabbits following four immunizations with the unmodified Env trimers

(Fig. 2A). Although the shifts in the binding curves between the two cores are subtle, they are detected in each Env-immunized animal, regardless of wt or huCD4 genotype, indicating that there were no large differences in the elicitation of CD4bs-directed abs between the two rabbit types. As an additional validation of the sensitivity of the assay, we did not detect shifts in the binding curves between the two TriMut cores in any of the animals injected with Env.368R, likely due to the large side chain mismatch in the CD4bs (Fig. 2A). Note that gp120 will be recognized by V-region-directed abs elicited by the Env trimers as well as CoRbs-directed antibodies, and the differential binding between gp120 and the TriMut core detected in Fig. 2A and B reflects the selective loss of recognition by these specificities.

FIG. 2. Detection of CD4bs-binding abs by direct binding. **(A)** ELISA serum IgG binding from wt and huCD4 rabbits 14 days after four injections with Env or Env.368 to gp120 (black), TriMut core (red), or TriMut368/70 (black). Since TriMut core will be recognized by CD4bs-directed antibodies (abs), but not TriMut368/70 core, the difference in ED₅₀ values between the two targets determines the contribution of CD4bs-directed abs. We used the V-region and N- and C-terminal-deleted cores to increase sensitivity of CD4bs-directed ab detection. Binding to gp120 determines total binding to all elements of gp120. **(B)** The proportion CD4bs-directed abs in each serum sample was assessed by calculating the ED₅₀ differential between TriMut and TriMut368/70 relative to gp120 binding. **(C)** The capacity of the control CD4bs-directed monoclonal antibodies (mAbs) b12 and VRC01 to bind the respective coat proteins is shown. The ED₅₀ values in **B** were determined by nonlinear regression analysis, and statistical analysis of binding ratios was done using the Mann-Whitney nonparametric test (**p* < 0.05, GraphPad Prism 5.0).

By assessing the difference between the serum 50% TriMut and TriMut368/70 binding capacities relative to the 50% gp120 binding capacity (defined as ED_{50} for the 50% effective dilution, see Materials and Methods) from respective animals, we determined that approximately 15–20% of the gp120-specific antibodies were directed against the CD4bs when the Env trimers were used as immunogens (Fig. 2B). This is consistent with our previous estimates of the percentage of CD4bs-directed B cells elicited in NHPs after vaccination with Env.²⁸ As expected, this analysis also demonstrated a lack of CD4bs-directed antibodies in serum from animals injected with the control immunogen, Env.368 (wt, $p=0.0156$. huCD4, $p=0.0286$). The CD4bs-directed antibodies will bind to unmodified gp120 as well, and we validated the antigenicity of all the target proteins used here with the known CD4bs-directed mAbs, b12, and VRC01 (Fig. 2C). As anticipated, the b12 or VRC01 control mAbs demonstrated severely reduced or abrogated capacity to bind TriMut368/70, as compared to TriMut and gp120 recognition.

In addition, because the decreases in direct binding to the three target probes were relatively subtle, we sought an independent means to confirm these differentials. We therefore utilized a different ELISA format where serum samples were coincubated with BSA (negative control) or nonsaturating levels of TriMut or TriMut368/70 in solution prior to the addition of the serum to TriMut-coated wells. This assay detects binding to a fixed amount of TriMut target protein after adsorption of control CD4bs-directed mAbs or serum abs. We used three adsorption proteins: BSA (negative control), TriMut core, or the CD4bs-defective TriMut368/70 core. As expected, coincubation with soluble TriMut resulted in a marked reduction of binding by the control mAb, b12, to the homologous TriMut-coated protein (Fig. 3A, left panel). In contrast, coincubation with the isogenic CD4 binding-defective TriMut368/70 core, or the BSA negative control, did not result in a reduction of b12 binding to the TriMut coat protein, validating the assay. Consistent with our data in Fig. 2, we detected consistent shifts in binding curves to TriMut in

serum samples from animals injected with Env but not Env.368 (Fig. 3A, middle and right panels). By assessing the 50% serum binding titer (ED_{50}) after coincubation with TriMut or TriMut368/70, we determined that these shifts were detected in both the wt and transgenic rabbits to the same degree (Fig. 3B). And consistent with the direct binding data, no shifts were detected in sera from either rabbit type immunized with the Env.368 immunogen (Fig. 3A and B).

Since Env was capable of eliciting CD4bs-directed abs in both wt and huCD4 rabbits, this result suggests that after four inoculations, potential *in vivo* CD4 binding to Env did not significantly influence the stimulation of B cells and abs capable of recognizing the CD4bs.

Detection of CoRbs-directed antibodies as an indicator of Env:CD4 interaction *in vivo*

Binding of CD4 by Env is required for the elicitation of most, or all, CoRbs-directed antibodies *in vivo*.^{22,29} We and others have previously demonstrated that Env can directly bind to splenocytes from huCD4 rabbits but not to splenocytes from wt rabbits, and that this interaction is CD4 specific.^{21,22} To determine that the stimulation of CD4bs-directed antibodies in huCD4 rabbits was not due to the inability of Env to bind human CD4 in these transgenic animals, we assessed the ability of serum samples to cross-neutralize HIV-2 in the presence of sCD4. Since the CoRbs, but not other regions, is highly conserved between HIV-1 and HIV-2 Env, when using HIV-1 Env-based immunogens, this assay specifically detects the presence of elicited CoRbs-directed antibodies in serum. We previously demonstrated that the elicitation of CoRbs-directed abs after immunization with Env correlates with binding of Env to human CD4 in rabbits.^{22,26} After two and four inoculations, HIV-2 neutralization was detected only in serum (Fig. 4A) or the purified IgG fraction of pooled serum (Fig. 4B) from huCD4 rabbits injected with Env trimers. This analysis verified the ability of Env to engage human CD4 following immunization *in vivo* and to subsequently elicit CD4-induced Env-specific abs.

FIG. 3. Selective adsorption of CD4bs-binding abs. (A) Representative TriMut-binding curves of the control mAb, b12, and the experimental post 4 serum IgG from Env-injected and Env.368-injected animals (wt shown) to core gp120 after coincubation with BSA, TriMut, or TriMut368/70 cores as competitors. (B) Relative elicitation of CD4bs-directed abs was assessed by calculating the difference between the adsorption capacity of TriMut (homologous to coat proteins) and TriMut368/70 (lacking the capacity to bind CD4bs-directed antibodies). The ED_{50} values in B were determined by nonlinear regression analysis, and statistical analysis of binding ratios was done using the Mann–Whitney nonparametric test (* $p < 0.05$, GraphPad Prism 5.0).

FIG. 4. Elicitation of antibodies that target the coreceptor binding sites (CoRbs) of Env. (A) Serum samples after two and four injections or (B) purified IgG from wt and huCD4 rabbits after two and four injections with Env or Env.368R were assessed for their ability to neutralize HIV-2 in the presence of sCD4. The effective reciprocal serum dilution (ID₅₀) or concentration (IC₅₀) where 50% neutralization was reached is shown. Statistical analysis was done using the Mann–Whitney nonparametric test (* $p < 0.05$, GraphPad Prism 5.0).

Trimer-elicited neutralizing antibodies display differential CD4bs-related properties

After the relative quantification of Env binding antibodies in serum from injected animals, we sought to determine if the qualitative effects in the neutralizing capacities were elicited in the different experimental regimens. Therefore, we next assessed the potential of serum to confer *in vitro* neutralization of a panel of different HIV-1 isolates using the standardized TZM-bl assay. Consistent with rabbits being outbred, we found a relatively high variability of responses between the neutralization capacity in the sera derived from different animals, which was limited to relatively sensitive tier 1 isolates or the “tier 2-like” homologous YU2 virus (Fig. 5). While the median neutralization between groups varied, we found no statistically significant difference in the potency of serum to neutralize the homologous YU2 strain, which is resistant to abs directed to the V3 region, or the variable region 3-sensitive strain, SF162 (Fig. 5A). In contrast, a statistically significant difference ($p = 0.04$) was detected between the ability of serum from Env and Env.368R-injected wt rabbits to neutralize the HXBc2 virus (Fig. 5B). A similar trend was observed in the huCD4 rabbits as well.

These results are consistent with HXBc2 being neutralized by 368D-dependent antibodies that target the CD4bs as previously demonstrated.¹⁸ In this instance, and others, HXBc2 is a good sentinel virus for CD4bs-directed antibodies since it is not matched in sequence to any of the V

FIG. 5. *In vitro* neutralization of HIV-1. Serum samples from wt or huCD4 rabbits after four injections with Env or Env.368R were assessed for their potency at neutralizing the HIV-1 homologous tier-2 strain YU2 and the heterologous tier-1 strains SF162 (A), HXBc2 (B), and MN (C). In addition, the IgG fractions of pooled serum samples from wt rabbits injected two and four times with Env or Env.368 or huCD4 rabbits injected with Env were assessed for their capacity to neutralize MN (D). The reciprocal serum dilution or concentration where 50% neutralization was achieved is shown (ID₅₀ and IC₅₀, respectively). Statistical analysis was done using the Mann–Whitney nonparametric test (* $p < 0.05$, GraphPad Prism 5.0).

region neutralizing targets such as V1, V2, or V3 present on the ab-eliciting YU2 trimeric immunogens. Note that the aspartic acid at amino acid position 368 of Env, altered in the Env.368R trimer immunogen, lies roughly in the center of the CD4bs, altering the molecular surface of this conserved neutralization determinant, explaining why the HXBc2 neutralization capacity is so dramatically reduced in the response to this immunogen. We detected a similar trend, which did not achieve statistical significance (wt $p = 0.07$, huCD4 $p = 0.06$), for the capability of serum samples to neutralize the MN virus (Fig. 5C). This was not as pronounced an effect as that detected for HXBc2, perhaps because MN is sensitive to other neutralizing specificities. A similar 368 D-dependent trend was detected when assessing the MN-neutralization potential of purified IgG fractions of serum pools from wt rabbits injected with Env or Env.368R trimers or huCD4 rabbits injected with Env (Fig. 5D).

FIG. 6. Neutralization of MW965. Serum samples from wt or huCD4 rabbits after four injections with Env or Env.368R were assessed for their potency to neutralize MW956 (A). Serum samples from wt or huCD4 rabbits after four injections with Env were assessed for neutralization of MW965 after adsorption with the TriMut and TriMut368/370 cores. The resulting net reduction, indicative of CD4bs-directed neutralizing antibodies, is shown (B). The IgG fractions of pooled serum samples from wt rabbits injected two and four times with Env or Env.368 or huCD4 rabbits injected with Env were assessed for their capacity to neutralize MW965 (C). The reciprocal serum dilution or concentration where 50% neutralization was reached is shown (ID₅₀ and IC₅₀, respectively). Statistical analysis was done using the Mann–Whitney non-parametric test (* $p < 0.05$, GraphPad Prism 5.0).

For the tier 1 clade C HIV-1 strain, MW965, we did detect a significant difference in the neutralization capacity between serum samples derived from Env-injected wt rabbits and the huCD4 rabbits ($p = 0.03$, Fig. 6A). This difference was not detected for Env.368R-injected wt and huCD4 rabbits (Fig. 6A). These data suggest that *in vivo* binding of CD4 by Env may have some impact on the elicited neutralizing responses, since the effect appeared independent of the 368 D-to-R mutation present in the YU2 trimer Env368 immunogen. To further investigate potential reasons for this effect in the MW965 virus context, we assessed the presence of CD4bs-directed neutralizing abs in serum from Env-injected wt and huCD4 rabbits by using the TriMut and TriMut 368/70 cores described above. Since the bridging sheet mutations present in both of these cores eliminate CD4 binding, but do not effect recognition by CD4bs-directed abs,³⁰ they can be added directly to the neutralization assay to determine ab differences at the CD4bs. By this differential adsorption, the relative fraction of CD4bs-directed abs was elevated in Env-injected wt rabbits, compared to abs elicited in the huCD4 rabbits; however, this differential trend was not statistically significant ($p = 0.1$) (Fig. 6B).

These data indicated that at least part of the MW965 neutralization capacity in serum samples from Env-injected wt animals was due to 368D-dependent CD4bs-directed antibodies, but that elicitation of similar antibodies appeared suboptimal in Env-injected huCD4 animals. Purified IgG from animals from these groups demonstrated a similar trend, with IgG of pooled serum from Env-injected wt rabbits better neutralizing MW965 than IgG of pooled serum from Env-injected huCD4 rabbits (Fig. 6C). In contrast, IgG from wt rabbits had a similar potency of neutralizing MW regardless of immunization with Env or Env.368R, demonstrating that the CD4bs-directed neutralization also was partially 368D independent, or dependent on antibodies that target the unbound Env conformation (Fig. 6C). We next assessed the neutralization capacity of the serum to neutralize more resistant isolates and determined that neither regimen nor the genotype of rabbits resulted in the elicitation of abs capable of neutralizing the more resistant tier-2 HIV-1 strains RHPA.7, TRO.11, 286.36, and ZM215.8 (not shown).

In sum, we conclude that the overall potency and breadth of neutralization after Env or Env.368 immunization of wt or

huCD4 rabbits are relatively similar, with the more sensitive tier 1 HXBc2 and MW965 viruses revealing subtle differences in the Env-elicited neutralizing capacities directed at the conserved CD4bs.

Discussion

We here address the role of *in vivo* CD4 binding by Env-based immunogens for the elicitation of neutralizing antibodies against HIV-1. While it is well established that CD4bs-directed abs are elicited (generally at low levels) in HIV-1-infected patients and in NHPs after vaccination,²⁸ it has not been determined if these responses are influenced by Env binding to its primary receptor *in vivo*. We partially addressed this issue previously by comparing neutralizing antibody responses after immunization of NHP with Env or Env.368.¹⁸ While the overall potency of immune serum to neutralize different HIV-1 strains *in vitro* was similar between the groups, serum from Env.368-injected animals could not neutralize the strain HXBc2. This was not surprising, as this virus is highly sensitive to CD4bs-directed antibodies that recognize an aspartic acid in position 368 of Env, whereas the Env.368R instead contains a nonconservative arginine in this position. Consequently, this phenotype was likely due to antigen design rather than a direct lack of CD4 binding by the immunogen.

To further pursue this line of investigation we utilized rabbits that were transgenic for human CD4 in comparison to wt rabbits whose CD4 does not bind HIV Env with high affinity.^{21,22} This allowed for immunization with Env under conditions where Env:CD4 binding was solely dependent on the *in vivo* presence or absence of human CD4. After two injections of wt animals, we determined that Env.368R-injected animals had elicited IgG that favored Env.368R binding. Since we did not observe a strong elicitation of similar antibodies after four injections, 368R-directed antibodies had diminished over time, similar to what we previously described for the elicitation of gp41-directed antibodies in mice and nonhuman primates.^{31,32} After four injections, we found a similar potency of serum to bind both Env and Env.368R regardless of rabbit genotype after both heterologous and homologous immunization. These results showed that the wt and huCD4 rabbits used in this study were

equally capable of generating a quantitative anti-Env response after repeated immunizations. Considering a number of studies describing CD4bs-directed antibodies in HIV-1-infected individuals,^{10,11,13,14} and in nonhuman primates following immunization with the YU2 foldon trimeric model immunogens used in this study,²⁸ it was not surprising to also find CD4bs-directed antibodies in both wt and huCD4 rabbits after immunization with Env. Since the levels of CD4bs-directed antibodies were similar between both rabbit strains used here, our data additionally suggest that *in vivo* CD4 binding does not significantly influence quantitative elicitation of antibodies with such specificity.

To investigate if this was also true for qualitative elicitation of antibodies, we assessed the potency of serum to neutralize different strains of HIV-1 in a number of *in vitro* settings. We found that the majority of HIV-1 strains tested were equally neutralized by serum from both groups of Env-injected rabbits. Consistent with our previous study, the strain HXBc2 was not neutralized by serum from wt or huCD4 rabbits if Env.368R was used as the immunogen. However, we also found that serum samples from huCD4 rabbits had a reduced capacity to neutralize the clade C strain MW965, as compared to serum samples from wt rabbits after injection with Env. This reduction was therefore not due to the design of the model foldon Env trimers, but instead appeared to be dependent on the *in vivo* presence of the primate CD4 expressed on the surface of T cells in huCD4 rabbits. We did not find compelling evidence that this was directly linked to reduced induction of CD4bs-directed antibodies; thus the data do not provide full support for the CD4-dependent occlusion hypothesis. It was proposed previously that slight alterations in conformation may alter the immunogenicity of Env *in vivo*.³³ Therefore it is possible that the reduced potential of serum from huCD4 rabbits to neutralize the clade C MW965 isolate is partially due to the induced conformation change in Env upon *in vivo* CD4 binding.

We conclude that for the CD4 binding-competent Env trimer immunogens used in the current study, although *in vivo* CD4 binding does occur, this interaction does not appear to compromise the ability of Env-based immunogens to generate neutralizing antibodies in rabbits in a substantial manner. There may be exceptions to that general rule, which may occur by subtle alterations of CD4bs-directed neutralizing activity, such as that observed for the cross-clade, subtype C isolate, MW965. While this does not affect the overall, and somewhat limited, neutralization capacities elicited by the current “model immunogen” foldon trimers used in the current study, it may more profoundly affect the neutralizing capacity elicited by more well-ordered trimers recently described³⁴ through high-affinity interaction of the gp120 CD4bs with human or primate CD4. The data presented here define a foundation for assessing such alterations in preclinical immunogenicity testing in nonhuman primates or future clinical testing in human subjects.

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Author Disclosure Statement

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Address correspondence to:

Richard T. Wyatt

IAVI Neutralizing Antibody Center at TSRI

and Department of Immunology and Microbial Science

The Scripps Research Institute

10550 North Torrey Pines Road

La Jolla, California 92037

E-mail: wyatt@scripps.edu