Single N277A Substitution in C2 of Simian Immunodeficiency Virus Envelope Influences Vaccine-elicited CD4i Neutralizing and Anti-V2 Antibody Responses

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Abstract

An effective HIV vaccine remains elusive, and immunogens capable of eliciting protective host humoral immunity have not yet been identified. Although HIV/SIV infections result in the abundant production of CD4-induced (CD4i) antibodies (Abs), these Abs are not protective due to steric restrictions following gp120 binding to CD4 on target cells. Here we report that both DNA- and vaccinia-based vaccines encoding SIV_{mac239} gp160 readily elicited high levels of CD4i Abs in experimental animals. We identified a highly conserved N-linked glycosylation site N277 in the C2 region which strongly affected the immunogenicity of the CD4i Ab domain. Moreover, a single N277A substitution significantly enhanced the immunogenicity of the V2 domain yielding higher titers and frequency of anti-V2 Ab responses as determined by ELISA and yeast antigen display mapping, respectively. Importantly, immune sera elicited by the N277A-mutated gp160 exhibited elevated antibody-dependent cellular cytotoxicity (ADCC) activity. ADCC activity correlated positively with the anti-V2 Ab titer yet, inversely with CD4i Ab titer. Thus, we identified a determinant of the CD4i domain that might affect vaccine-elicited anti-V2 Ab and ADCC responses to SIV_{mac239}. Our findings may have implications for design of immunogens to direct B cell recognition in the development of an Ab-based HIV vaccine.

Keywords

HIV, CD4-induced, mutagenesis, V2, ADCC, vaccine

1. Introduction

The entry of human immunodeficiency virus (HIV) into target cells requires the binding of viral envelope glycoprotein to the primary receptor CD4 and a coreceptor (either CCR5 or CXCR4). CD4 binding induces conformational changes in gp120, leading to the exposure of the coreceptor-binding site [1]. Antibodies targeting the gp120 structures exposed after CD4 binding are defined as CD4-induced (CD4i) antibodies (Abs) [2, 3] Based on the structural analysis, immunogenic determinants on gp120 that can elicit CD4i Abs are considered as the CD4i domain, which is a

conserved and discontinuous structure located proximal to the bridging sheet [4, 5]. In the presence of soluble CD4 (sCD4) or its mimics, CD4i Abs may compete with gp120 for coreceptor binding and block viral entry.

High titers of CD4i Abs are elicited against a broad range of HIV type 1 (HIV-1) subtypes (A-H), and even HIV type two (HIV-2) strains [2, 3], but most CD4i Abs can neutralize HIV-1 only in the presence of sCD4 [6-8]. CD4i Abs are generated early during HIV-1 infection. In contrast to broadly neutralizing antibodies (bNAbs), the generation of CD4i Abs does not require complex and slow B cell maturation [9-12]. Similarly high titers of CD4i Abs were also detected in the sera of rhesus macaques experimentally infected with SIV [13-16], one of the most relevant animal models of HIV/AIDS. However, CD4i Abs are inefficient in neutralizing virus, as during natural infection gp120-CD4 binding results in a sterically hindering environment on the cell membrane [17].

While the induction of bNAbs is considered to be a reliable readout for the evaluation of effective vaccines, accumulating evidence from both non-primate animal experiments [18] and the RV144 clinical trial [19, 20] has suggested a potential role for non-neutralizing Ab functions, such as Ab-dependent cellular cytotoxicity (ADCC), in protection against SHIV/HIV. In particular, an IgG Ab response specific to the envelope V1V2 region was correlated with reduced risk of infection [19, 21-23]. Further analysis using the Systems Serology approach has indicated that the V1V2 response may coordinate IgG1/IgG3 responses and Ab functionality to drive protective humoral immunity in vaccinated uninfected individuals [20]. Here, we sought to characterize the immunodominant CD4i domain on gp120 and to identify mutations to direct antibody responses towards immunorecessive antigenic sites that may be relevant to protection.

2. Material and methods

2.1. Cell lines and virus.

TZM-bl, and 3T3.T4 cells were kindly provided by the NIH AIDS Research and Reference Reagent Program and maintained according to the standard protocols provided. The 293T-rhCCR5 cell line was generated in our lab as previously described [14]. 293T-rhCCR5 cells were maintained in DMEM

supplemented with 0.5 μg/ml puromycin. SIV_{mac39} pseudovirus was prepared by co-transfecting 293T cells with pSIV-Luc-vpr$^-$env$^-$ and the vector expressing SIV_{mac39} Env, as described previously [14]. The *env* sequence of SIV_{mac239} is identical to those used in previous structural studies and has a premature stop mutation at codon 736 [24, 25]. This mutation increases the rate of cell surface expression and viral packaging, but does not affect the entry properties of the virus [26, 27].

2.2. Animals and immunization schedule.

Our animal experimental protocols were approved by the Committee on the Use of Live Animals in Teaching and Research, Laboratory Animal Unit (LAU), The University of Hong Kong. Animals were handled in accordance with approved guidelines. Female BALB/c mice (5-8 weeks old) were bred under standard pathogen-free conditions in LAU. For DNA vaccination, each mouse was immunized with 50 μg of an endotoxin-free DNA construct expressed by plasmid pVAX carrying SIV_{mac239} wild type (WT) Env or different Env mutants (Fig. 3A) three times at a two-week intervals by intramuscular electroporation as described previously [28]. For the vaccinia-based vaccination, each mouse was immunized twice with 10^6 PFU of $MVTT_{SIVgpe}$, expressing SIV Gag-pol and Env, at a three-week intervals by intranasal and oral inoculation as previously decribed [29]. A higher dose of 10^8 PFU of $MVTT_{SIVgpe}$, however, was used to immunize Chinese macaques that were 3 to 6 years of age twice at a four-week interval by intranasal and oral inoculation [29]. For all animals, blood samples were taken for serum antibody analysis two weeks after the final immunization.

2.3. Neutralization assay.

The protocol for viral neutralization has been described previously [28, 30, 31]. sCD4 was incubated with SIV_{mac239} pseudotyped virus at a non-inhibitory concentration of 1 μg/ml before the addition of serially diluted sera.

2.4. gp120 binding assays.

Binding of gp120 to cell surface CD4 or CCR5 was assessed by flow cytometry using biotinylated

SIV_{mac239} gp120 (Immune Tech, NY), or SIV_{mac239} gp120 fused with a rabbit Fc tag (gp120-Fc), which does not contain the Fc receptor binding region. These gp120-Fc proteins were expressed by transfection of 293T cells, purified by Protein G-Agarose from culture supernatant and quantified by ELISA. 293T-rhCCR5 and 3T3.T4 cells were used for binding. In brief, 4 μg of gp120-biotin or gp120-Fc protein pre-incubated with or without 1/10 diluted immune serum or sCD4 (10 μg/ml) was incubated on a shaker with 10^5 cells at room temperature for an hour. After washing, bound gp120-biotin or gp120-Fc was measured by flow cytometry.

sCD4 was pre-coated on microplates (μg/well), and binding of recombinant gp120, in the presence or absence of 1/10 diluted immune serum was assessed by rabbit anti-SIVgp120/gp160 polyclonal Abs (MyBioSource, CA) staining, detected by anti-rabbit IgG (H+L)-HRP. The anti-CD4 MAb (clone RPA-T4) was used as a positive control that blocks the sCD4-gp120 binding.

Biotinylated 7D3 binding to the gp120-sCD4 complex was assessed by ELISA. Biotinylated 7D3 binding to gp120-pre-coated microplates was detected with Streptavidin-HRP. Before the addition of biotin-labeled 7D3, gp120 was preincubated with sCD4 (10 μg/ml) and immunized sera (msDNA) or control sera (msPBS).

2.5. Combination assay.

Combination of immune serum and MAb 7D3 against Env-pseudotyped SIV_{mac239} in TZM-b1 cells was determined using MacSynergy II software as previously described [31, 32]. Synergy volumes between −50 and 50 were defined as additive effects; between 50 to 100 indicates a slight synergistic effects; >100 a highly synergistic effect; and < −50 an antagonistic effect.

2.6. Antigen yeast display analysis.

Antibody reactive antigens were comprehensively analyzed using the yeast display technique, as previously described [23, 33].

2.7. ELISA for measuring SIV–specific antibody.

High-binding 96-well microplates (Corning, NY) were coated overnight with purified SIV_{mac239} pseudoviral particles (2 □g/well) or purified native gp120 (Immune Tech, NY, 0.05 □g/well) in a coating buffer (pH 9.6) containing 0.53% Na_2CO_3, 0.42% $NaHCO_3$ and 0.1% sodium azide. Plates were washed and blocked with PBS containing 5% nonfat milk and 0.5% BSA (blocking buffer). Serially diluted sera were added and incubated for 1 hour at 37°C. After three washes with PBS containing 0.05% Tween 20 (PBST), plates were incubated with HRP-conjugated goat anti-mouse antibodies (Bethyl, TX) for 1 hour at 37°C. The plates were then washed three times with PBST, developed with the substrate 3,3',5,5'- Tetramethylbenzidine (Sigma, MO), stopped with 2M H_2SO_4 and then analyzed at optical density deviation at 450 nm using the VICTOR3 Multilabel Plate Reader (PerkinElmer, MA). The endpoint antibody titer was defined as the highest serum dilution, yielding a value greater than twice of the background level.

For the detection of V2-specific Abs, the microplates were incubated with 100 µl streptavidin (5 µg/ml, Sigma, MO) in the same coating buffer. Plates were washed and blocked with the blocking buffer, followed by incubation with 100 µl SIV_{mac239} V2 peptide (3 µg/ml) containing an amino-terminal biotin tag (CIAQDNCTGLEQEQMISCKFNMTGLKRDKKKEYNETWYSADLVCEQGNNTGNESRCY).

2.8. ADCC assay.

The ADCC assay was performed as previously described with modifications [20, 34]. We used the LIVE/DEAD Cell-Mediated Cytotoxicity Kit (Invitrogen) to assess the ADCC activity of each immune serum following manufacturer's instructions. Briefly, $CEM.NKR_{CCR5}$ cells pulsed with recombinant SIV_{mac239} gp120 (6 µg/ml) was used as the target cells. These cells were then labeled with the target-cell marker 3,3'-dioctadecyloxacarbocyanine (DiO, Invitrogen), and incubated with sera from PBS control and immunized mice at a final dilution of 1:50 in a 96-well microplate for 30 min at room temperature. NK cells (effector cells) isolated from splenocytes of naive mice with Dynabeads® FlowComp™ Mouse CD49b (Invitrogen) were added at a 5:1 effector/target cell (E:T) ratio together with the cell membrane impermeant propidium iodide nucleic acid stain (PI, Invitrogen). Cultures

were incubated for 4 hours at 37°C in 5% CO_2 and then the percentage DiO^+ cells also stained with PI was analyzed using the FACSCalibur flow cytometer (BD Biosciences).

2.9. Statistical analysis.

Statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad software, Inc, La Jolla, CA). Our data is represented as mean with standard errors (SEM). Two-tailed *t* tests were used to analyze the statistical significance of data with normal distribution whereas two-tailed Mann-Whitney tests were used for data without normal distribution. *P* values less than 0.05 (*), 0.01 (**) or 0.001 (***) were considered statistically significant.

3. Results

3.1. Prevalence of CD4i Abs in SIV infected and vaccinated animals.

To analyze the generation of SIV-directed CD4i Abs, we infected rhesus macaques with SIV_{mac239} or immunized them with a recombinant vaccinia-based vector carrying the SIV_{mac239} envelope ($MVTT_{SIVgpe}$) [29]. As observed in HIV-1 infection, CD4i Abs were detected in the sera of both acutely infected and vaccinated rhesus macaques (Fig. 1). Using TZM-bl cells that express both membrane-bound human CD4 and CCR5, we found that sera of these animals neutralized SIV_{mac239} in the presence of 1 μg/ml sCD4 (Fig. 1A and B). Unexpectedly, vaccinating mice with a DNA vaccine pVAX-SIV_{mac239}Env (msDNA) or $MVTT_{SIVgpe}$ (msVac) also induced potent SIV-specific CD4i Abs (Fig. 1A and B). This finding contradicts a previous report indicating that human CD4 is essential for the induction of HIV-specific CD4i Abs [35], while our results suggest that induction of SIV-specific CD4i Abs is not absolutely dependent on human or rhesus CD4. To exclude the influence of human CD4 on neutralization in TZM-bl cells, we further assessed the capacity of serum samples to inhibit infection of 293T-rhCCR5 cells that expressed rhesus CCR5 but not CD4 on the cell surface (Fig. 1C). Again, only in the presence of 1 μg/ml human sCD4 did sera from either infected or vaccinated rhesus macaques exhibit potent SIV-specific neutralization. It should be noted that 1 μg/ml of sCD4 alone had no significant inhibitory effect on SIV_{mac239} infection of either TZM-bl or 293T-rhCCR5 cells (Fig.

1D and 1E). These results demonstrate the high prevalence of SIV-specific CD4i Abs in vaccine- and infection-elicited antibody responses in animals.

3.2. Vaccine-elicited CD4i Abs target the CCR5 binding site on SIV_{mac239} envelope.

Since the coreceptor-binding site of HIV-1 gp120 is highly conserved and elicits high levels of CD4i Abs [2, 3], we sought to determine whether the CCR5 binding site (CCR5bs) on SIV also elicits CD4i Abs. Sera from non-immunized mice (msPBS) and immunized mice (msDNA) did not affect the binding of soluble SIV gp120 to 3T3.T4 ($CD4^+CCR5^-$) and 293T-rhCCR5 ($CD4^-CCR5^+$) cells (Fig. 2A). Moreover, SIV gp120 did not bind to cell surface rhCCR5 unless there was sCD4 (Fig. 2B, red). Critically, the sCD4-induced binding of SIV gp120 to rhCCR5 was significantly inhibited by the sera of immunized mice (Fig. 2B, green) but not by the sera of non-immunized mice (Fig. 2B, blue). We also found that immune sera did not influence the binding of SIV gp120 to sCD4 (Fig. 2C). Therefore, immune sera appear to target primarily the CCR5bs.

Next, we evaluated the cross-reactivity of 7D3, a monoclonal antibody (MAb) that recognizes the CCR5bs of $SIV_{CP\text{-}Mac}$ gp120. 7D3 was previously reported to act as a CD4i MAb against SIV_{mac239} [36]. As a control, we included MAb KK8 (NIH AIDS Research and Reference Reagent Program), a non-neutralizing V1/V2-diretced MAb [37, 38]. Consistent with previous reports, we found that 7D3 neutralized SIV_{mac239} in the presence of sCD4, while Mab KK8 did not (Fig. 2D). To understand the competition of vaccine-induced CD4i-NAbs with 7D3, we measured their synergistic effect in a checkerboard experiment and the data was analyzed using MacSynergy II software [39]. No synergistic effect was found between 7D3 and vaccine-induced CD4i-NAbs (Fig. 2E) due to a low synergy volume/antagonism volume of 17.97/-140.06, suggesting that they probably shared the same CCR5bs. We then performed a competition assay to examine whether vaccine-induced Abs could compete with 7D3 for binding with the gp120-sCD4 complex. Sera from mice immunized with pVAX-SIV_{mac239}Env (msDNA) significantly reduced the binding of 7D3 to the SIV_{mac239} gp120-sCD4 complex (Fig. 2F). In contrast, sera of the PBS control group did not inhibit 7D3 binding. Taken

209 together, our results indicate that vaccine-elicited CD4i Abs primarily target the CCR5bs of SIV
210 gp120.
211
212 ### *3.3. Mutations affect three highly conserved functional domains of SIV_{mac239} Env.*
213 In order to identify determinants of SIV CD4i Abs, we focused on highly conserved regions across
214 SIV, HIV-1 and HIV-2 sequences that are relevant to CCR5bs or CD4i conformational changes. These
215 include HIV residues I420 and K432 (corresponding to I433 and K445 in SIV, Fig. 3A) within the
216 bridging sheet that are critical for CCR5 binding and for the binding of a monoclonal CD4i Ab [4], as
217 well as residues 216-220 (β4 sheet) and N262 in HIV gp120 (corresponding to N277 in SIV gp120 C2
218 region) that are buried in the closed state but exposed in the CD4-induced open state [25]. Site-directed
219 mutagenesis was used to substitute these residues with alanine at the corresponding sites of SIV_{mac239}
220 gp120 (Fig. 3A). Western blot analysis indicated that none of these substitutions affects the level of
221 protein expression in transfected 293T cells (Fig. 3B, top). Importantly, the mutant Envs could no
222 longer mediate entry in the single-round pseudovirus assay (Fig. 3C), confirming the dependency of
223 viral infectivity on these highly conserved sites.
224 We then introduced these mutations into SIV_{mac239} gp120 conjugated with the rabbit Fc fragment
225 (239gp120-Fc) to characterize the role of these amino acids in receptor and coreceptor binding. None of
226 these mutations affected the release of soluble 239gp120-Fc from transfected 293T cells, as
227 determined by Western blot analysis (Fig. 3B, bottom). Interestingly, these mutants exhibited different
228 effects on receptor and coreceptor binding. Mutations in the β4 sheet diminished the binding of
229 239gp120-Fc to both CD4 or CCR5 (Fig. 3D and 3E). These results were expected due to the structural
230 impairment of a cysteine loop. Dual I433A and K445A substitutions abrogated the binding ability of
231 239gp120-Fc to CCR5 but had no significant influence on the interaction with CD4, confirming that
232 these two residues were conserved to preserve CCR5 binding in both HIV and SIV [4]. The single
233 N277A substitution, however, significantly impacted 239gp120-Fc binding to both CD4 and CCR5,
234 suggesting a critical role for this amino acid in envelope conformation and function.

To further understand the influence of these aforementioned mutations on gp120 epitopes, we analyzed the ability of MAbs with well-defined epitopes to bind viral Env mutants expressed on the surface of 293T cells. The binding of KK8, which recognizes a conformational epitope spanning V1/V2 region, to these Env mutants did not differ from its binding to wild type (WT) Env (Fig. 3F). In contrast, the binding of the N277A mutant to 7D3 was significantly reduced (Fig. 3G). These results suggest that these three highly conserved regions in SIV Env play different roles in viral entry, interacting with CD4/CCR5 and binding to the CD4i Ab 7D3.

3.4. Single N277A mutation destroys the immunogenic domain of CD4i Abs.

To determine the influence of the β4, I433A/K445A and N277A mutants on the immunogenicity of SIV_{mac239} Env, individual DNA vaccines encoding the corresponding SIV Env mutants were generated and used to immunize mice. The levels of Abs binding SIV_{mac239} viral particle (VP) induced by β4 and I433A/K445A mutants did not differ significantly from that induced by WT Env (msWT) (Fig. 4A**)**. The N277A mutant, however, generated significantly lower levels of VP-specific Abs. In contrast the antibody response to SIV gp120 did not differ significantly between WT and the three Env mutant vaccines (Fig. 4B). In the absence of sCD4, no neutralizing activity was detected in immune sera induced by either WT or mutated Env (Fig. 4C). In the presence of sCD4, both β4 and I433A/K445A mutants elicited similar levels of CD4i Abs in mice as WT Env. In contrast, CD4i Ab induction was significantly lower in N277A-immunized mice (Fig. 4D). Furthermore, sera from mice immunized with N277A Env mutant competed significantly less potently with 7D3 binding to the gp120-sCD4 complex, than WT immune sera (Fig. 4E). These results indicated the essential role of N277 of SIVgp120 in inducing CD4i Abs.

3.5. Enhancement of vaccine-elicited V2 antibody response correlates with ADCC activity.

To further assess the different antigenic profiles of WT Env and the N277A mutant, a yeast antigen display library was used to map the binding sites of vaccine-elicited polyclonal antibodies. We used a

yeast library including displays of 10^6 overlapping peptide fragments from the complete SIV_{mac239} Env [23]. After incubating this library with sera of mice immunized with either vaccine, 190 msWT-reactive and 113 msN277A-reactive yeast clones were obtained by FACS sorting and individually sequenced (Fig. 5A). Two immunodominant regions in WT Env were identified at the N-terminal region of gp120 and the ectodomain of gp41 (Fig. 5B). The frequency of peptides recognized by msWT was much greater in gp41 (67% of area under the curve (AUC)) than in gp120 (33% of AUC). Moreover, this dominant immunogenic domain focuses on the HR1/HR2 region, in agreement with the previously reported immunodominance of this gp41 region in SIV_{mac239} Env [40, 41]. The frequency of peptides recognized by msN277A was greater in gp120 (66% of AUC) than in gp41 (34% of AUC). These results suggest a major shift of the immunodominant region in SIV Env caused by the single N277A substitution. Furthermore, the recognition frequencies of V1 and V2 domains by antibodies in msN277A (15% and 12%) were two and four times higher than that of msWT (7% and 3%), respectively. In addition, the N277A mutant elicited a significantly higher level of V2-binding antibodies than WT Env, as detected with ELISA using a synthetic V2 peptide as the coating antigen (Fig. 5C). Moreover, N277A immune sera competed more efficiently with KK8, the V1/V2-directed MAb for binding to the gp120-sCD4 complex than WT immune sera (Fig. 5D). Our findings indicate that the single N277A substitution not only influences viral entry (Fig. 3D) and the immunogenicity of the CD4i-domain (Fig. 4C), but also affects antibody responses to the SIV V2 region (Fig. 5C).

Because no neutralizing activity was detected in immune sera induced by either WT or mutated Env, an ADCC assay was performed to determine the Fc-mediated functionality of non-NAbs. FACS analysis confirmed the binding of SIV_{mac239} gp120 to $CEM.NKR_{CCR5}$ cells (Fig. 6A). gp120-pulsed $CEM.NKR_{CCR5}$ cells were then used as targets to investigate the ADCC activity of each immune serum [34]. The percentage of target cells killed in the presence of the serum of mice administered the N277A mutated Env (1:50) was about twice that of those administered WT Env (Fig. 6B). Moreover, the anti-V2 antibody titer positively correlated with the ADCC activity of the immune sera (Fig. 6C). In contrast, ADCC activity was inversely correlated with the CD4i NAb titer (Fig. 6D). To confirm that N277A-induced Abs specific to V2 mediate ADCC, we used the synthetic V2 peptide to block sera binding to the target cells, and observed decreased ADCC activity of N277A sera but not WT sera

(Fig. 6E). These results indicated that the N277A vaccination resulted in an enhanced anti-V2 antibody response, which was positively correlated with elevated ADCC activity.

4. Discussion

In this study, we show that the induction of SIV CD4i Abs is not restricted by the type of vaccine (DNA vs vaccinia-based), animal species (mouse vs macaque) or infection status (vaccine vs experimental SIV infection) (Figs. 1-2). Importantly, for the first time we demonstrate that a single N277A amino acid substitution in the conserved C2 region of SIV Env not only abolishes viral entry (Fig. 3D) and the immunogenicity of the CD4i domain (Fig. 4C) but also directs vaccine-elicited antibody response to the V2 region (Figs. 5B-C), which is correlated with enhanced ADCC activity (Fig. 6B-C). These findings provide new insights for B cell immune recognition and the design of immunogens for a protective HIV vaccine.

SIV Env gp120 residue N277 was previously reported to be essential for the formation of sugar cluster I by mediating interactions between glycan 277 and glycan 294 [42]. Consistent with our results (Fig. 3C), the loss of glycan 277 is reported to reduce SIV infectivity, suggesting its critical role in viral entry [43, 44]. Here we show that glycan 277 is also critical for gp120 conformation, influencing the immunogenicity of the CD4i domain (Fig. 4D). DNA immunization with the N277A mutant resulted in a reduced Ab response to the CD4i domain, but an increased Ab response to the V2 region. Similarly, loss of the corresponding glycan in HIV (N262) also produced non-infectious progeny [45, 46], confirming the highly conserved nature of this N-glycan across genetically divergent HIV and SIV strains (Fig. 3A). Interestingly, our preliminary data indicate that an HIV-1 DNA vaccine carrying the N262A mutant induced an enhanced anti-V2 antibody response (data not shown). Future studies remain necessary to carefully evaluate the role of the HIV-1 N262 glycan in HIV vaccine design.

Directing B cell recognition towards the CD4i Ab domain may represent a mechanism of immune evasion employed by HIV/SIV. It has been well documented that CD4i Abs might constrain virus to CD4-dependencey by preventing spontaneous exposure of the coreceptor binding site for

neutralization [3, 47, 48]. In this sense, CCR5 could be the original receptor of ancestral HIV/SIV. This theory is supported by the existence of non-pathogenic CD4-independent viruses [13, 15, 49]. To establish persistent infections, however, HIV/SIV has probably evolved an immune evasion mechanism, adapting to CD4-dependency to avoid CCR5 site directed neutralization. As such, these types of CD4i Abs offer limited neutralizing potential *in vivo* due to inefficient access to the hidden epitope [17].

New vaccine design, therefore, should overcome this immune evasion mechanism by directing B cell recognition from this non-protective but immunodominant CD4i domain to other protective antigenic sites. A possible novel vaccine strategy is to eliminate the CD4i domain from gp120. A monoclonal antibody 71B7 targeting the N277-glycan region (epitope 271-290) does not exhibit detectable coreceptor blocking neutralizing activity [36]. We now show that the N277-glycan is not part of the gp120 bridging sheet for the binding of CD4i Abs. However, it affects the induction of CD4i Abs. The induction of an enhanced anti-V2 antibody response suggests a shift in B cell responses after the introduction of a single N277A amino acid substitution in the CD4i Ab domain. Our results, therefore, provide new evidence that SIV B cell recognition domains can differ significantly from antibody targets.

The potential function of non-bNAbs in preventing HIV infection has been clearly demonstrated in the RV144 trial, in which the level of V2-binding antibodies was inversely correlated with the risk of HIV-1 infection [21]. Here we found that the immune serum elicited by the N277A mutant Env, albeit still non-neutralizing against $SIV_{mac239,}$ enhanced ADCC activity, and was positively correlated with the V2 antibody titer (Fig. 6B-C). Future studies should determine whether V2-directed ADCC antibodies can protect against a low-dose repeat SIV challenge in macaques, a better model for predicting protection of HIV/SIV immunogens [22]. In addition, since SIV and HIV Envs are genetically and structurally diverse (both in the inner domain and the variable loops), future experiments should carefully determine whether our findings could be useful for HIV vaccine design.

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Contributions: XT and ZC conceived and designed the experiments. XT, JG, LC, CS and LL performed the experiments. CS, LC and LZ shared materials and assays. XT, JG, LC, TZ, HW, LC, LZ and ZC analyzed the data. XT, JG and ZC wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. Vaccine- and infection-elicited CD4i Abs in rhesus macaques and mice. Neutralization of SIV_{mac239} infection of TZM-bl cells by immune sera of rhesus macaques (rh) or mice (ms) in the presence (A) and absence (B) of human sCD4. The sera of macaques vaccinated with vaccinia $MVTT_{SIVgpe}$ (rhVac, n=4) or experimentally infected with SIV_{mac239} (rhSIV, n=4) were compared with that received PBS only (rhPBS, n=4). The sera of mice vaccinated with vaccinia $MVTT_{SIVgpe}$ (msVac, n=4) or a DNA vaccine carrying SIV_{mac239} Env (msDNA, n=4) were also compared with sham vaccinated mouse sera (msPBS, n=4). The capacity of these sera to inhibit infection of 293T-rhCCR5 cells in the presence (C) and absence (data not shown) of human sCD4 was also assessed. SIV_{mac239} entry into TZM-bl (D) and 293T-rhCCR5 (E) cells was not inhibited in the presence of 1 μg/ml sCD4. Error bars represent SEM from 4 animals per group.

Figure 2. Vaccine-elicited CD4i Abs target the CCR5bs on SIV_{mac239} envelope. The capacity of mouse immune sera to block SIV_{mac239} gp120 binding to CD4 and CCR5 on 3T3.T4 cells was assessed by

flow cytometry (A) or on 293T-rhCCR5 cells (B) in the presence or absence of sCD4. The sera of DNA (msDNA) or sham (msPBS) vaccinated mice were assayed at a dilution of 1:30 using APC-conjugated streptavidin. (C) Binding of SIV_{mac239} gp120 to sCD4 was not affected by the addition of immune serum (msDNA) as analyzed by ELISA. (D) Neutralization of SIV_{mac239} by MAb 7D3 and KK8 in TZM-bl cells in the presence or absence of sCD4. (E) 7D3 and vaccine-induced CD4i Abs did not synergize to neutralize SIV_{mac239} infection of TZM-bl cells in the presence of sCD4. (F) Biotinylated 7D3 binding to the gp120-sCD4 complex was blocked by immunized sera (msDNA), but not control sera (msPBS), as determined by ELISA.

Figure 3. Mutations that affect three highly conserved functional domains of SIV_{mac239} Env. (A) Sequence alignments and crystal structure of three CD4i-related domains β4, N-glycan 277 and residues I433 and K445. The number corresponds to the location of core structural sequence of SIV_{mac32H} gp120 [50]. Mutated amino acids in SIV_{mac239} Env are highlighted in boxes. (B) Expression of SIV_{mac239} gp160 (top) and soluble fusion gp120-rabbit Fc (bottom) of corresponding mutant Envs in 293T cells assessed by Western blot. Untransfected 293T cells were used as negative control (NC). (C) Infectivity of pseudotyped SIV_{mac239} and its variants in TZM-bl cells 48 h after viral entry. (D-E) Binding of SIV_{mac239} gp120-rabbit Fc or its variant proteins to huCD4 on 3T3.T4 cells (D) or rhCCR5 on 293T-rhCCR5 cells (E) with or without sCD4 (10 μg/ml) was assessed by flow cytometry. (F-G) Interaction of SIV_{mac239} and mutant Envs expressed on the surface of 293T cells with specific murine MAbs KK8 (F) and 7D3 (G) was evaluated by flow cytometry staining with Alexa Fluor® 647 Goat Anti-Mouse IgG (H+L) Antibody. *P* values were calculated by Mann-Whitney tests.

Figure 4. A single N277A mutation affects the immunogenicity of SIV CD4i domain**.** (A) ELISA binding titer of various mouse immune sera (ms) to SIV_{mac239} viral particles. Four groups of mice (n=8) were immunized thrice via i.m./EP with equal amounts of DNA (50 μg) vaccines encoding WT and mutant Envs. Each symbol represents a mouse. *P* values were calculated by *t* tests. (B) ELISA binding titer of various immune sera to soluble SIV_{mac239} gp120. Each symbol represents a mouse. (C-D) Neutralization of SIV_{mac239} infection of TZM-bl cells in the absence of sCD4 (C) or of 293T-rhCCR5

cells in the presence of 1 μg/ml sCD4 (D). The mean ± SE of each group is shown. (E) Blocking of biotinylated 7D3 binding to the gp120-sCD4 complex by immunized sera (msN277A or msWT) at a dilution of 1:40, determined by ELISA. *P* values were calculated by *t* tests. Each solid symbol represents a mouse and each empty symbol represents a value determined by ELISA without serum.

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Figure 5. Shift of N277A vaccine-elicited antibody responses towards SIV V2 region. (A) Distribution of overlapping nucleotide sequences of positive yeast clones along the full-length SIV_{mac239} *env* sequence. Antibody reactive yeast clones were sorted by flow cytometry after binding to pooled polyclonal msWT (top) and msN277A (bottom) sera and subsequently sequenced individually. (B) Frequency and distribution of deduced peptide sequences of antibody reactive yeast clones along the complete SIV_{mac239} Env glycoprotein. The percentages represent the area under the curve (AUC) of total positive yeast clones analyzed in the experiments. (C) ELISA binding titer of various mouse immune sera (ms) specific to the V2 region of WT Env (anti-V2) (n=8). The experiment is representative of three independent experiments. *P* values were calculated by Mann-Whitney tests. (D) Blocking of biotinylated KK8 binding to the gp120-sCD4 complex by immunized sera (msN277A or msWT) at a dilution of 1:40 as determined by ELISA. *P* values were calculated by *t* tests. Each solid symbol represents a mouse and each empty symbol represents a value determined by ELISA without serum.

Figure 6. ADCC responses of immune sera elicited by WT and mutant Envs. (A) SIV_{mac239} gp120 coating of $CEM.NKR_{CCR5}$ cells was detected by anti-SIV gp120 MAb (KK8) followed by rabbit anti-mouse IgG conjugated with FITC. (B) The percentage of killed target cells by ADCC. (C) Linear regression analysis between anti-V2 antibody titer and ADCC induced by sera from immunized animals. *P* values were determined by *t* tests. (D) Linear regression analysis between CD4i NAb titer and ADCC induced by sera from immunized animals. (E) ADCC activities of sera of mice immunized

with N277A mutated or WT Env in the presence or absence of the V2 peptide (10 ug/ml). *P* values were determined by *t* tests. Each symbol represents a mouse.