Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Molecular Immunology 62 (2014) 219–226

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Characterization of a monoclonal antibody to a novel glycan-dependent epitope in the V1/V2 domain of the HIV-1 envelope protein, gp120

Rachel C. Doran*,[1], Javier F. Morales[1], Briana To, Trevor J. Morin, Richard Theolis Jr., Sara M. O'Rourke, Bin Yu, Kathryn A. Mesa, Phillip W. Berman

Department of Biomolecular Engineering, Baskin School of Engineering, University of California, Santa Cruz, 1156 High Street, MS-SOE2, Santa Cruz, CA 95064, United States

ARTICLE INFO

Article history:
Received 15 April 2014
Received in revised form 13 June 2014
Accepted 20 June 2014
Available online 11 July 2014

Keywords:
Gp120
Glycosylation
Epitope
V1/V2 domain
HIV
Monoclonal antibody

ABSTRACT

Recent studies have described several broadly neutralizing monoclonal antibodies (bN-mAbs) that recognize glycan-dependent epitopes (GDEs) in the HIV-1 envelope protein, gp120. These were recovered from HIV-1 infected subjects, and several (e.g., PG9, PG16, CH01, CH03) target glycans in the first and second variable (V1/V2) domain of gp120. The V1/V2 domain is thought to play an important role in conformational masking, and antibodies to the V1/V2 domain were recently identified as the only immune response that correlated with protection in the RV144 HIV-1 vaccine trial. While the importance of antibodies to polymeric glycans is well established for vaccines targeting bacterial diseases, the importance of antibodies to glycans in vaccines targeting HIV has only recently been recognized. Antibodies to GDEs may be particularly significant in HIV vaccines based on gp120, where 50% of the molecular mass of the envelope protein is contributed by N-linked carbohydrate. However, few studies have reported antibodies to GDEs in humans or animals immunized with candidate HIV-1 vaccines. In this report, we describe the isolation of a mouse mAb, 4B6, after immunization with the extracellular domain of the HIV-1 envelope protein, gp140. Epitope mapping using glycopeptide fragments and in vitro mutagenesis showed that binding of this antibody depends on N-linked glycosylation at asparagine N130 (HXB2 numbering) in the gp120 V1/V2 domain. Our results demonstrate that, in addition to natural HIV-1 infection, immunization with recombinant proteins can elicit antibodies to the GDEs in the V1/V2 domain of gp120. Although little is known regarding conditions that favor antibody responses to GDEs, our studies demonstrate that these antibodies can arise from a short-term immunization regimen. Our results suggest that antibodies to GDEs are more common than previously suspected, and that further analysis of antibody responses to the HIV-1 envelope protein will lead to the discovery of additional antibodies to GDEs.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Recombinant forms of the HIV-1 envelope (Env) protein have long been studied as HIV vaccine immunogens (Lasky et al., 1986; Berman et al., 1990). The Env protein is synthesized as a 160 kDa precursor, gp160, which then undergoes maturational cleavage to yield gp41, a membrane-bound protein that mediates virus fusion, and gp120, a peripheral membrane protein that is responsible for CD4 and chemokine receptor binding and virus tropism. In virus particles, the envelope proteins gp120 and gp41 are associated by non-covalent interactions and form a trimeric spike structure. Both gp120 and gp41 are highly glycosylated, with approximately 50% of their molecular mass attributed to N-linked glycosylation. Since both gp120 and gp41 possess epitopes recognized by neutralizing antibodies, multiple vaccine development efforts have investigated the immunogenicity of these proteins. However, after more than 30 years of effort, none of the candidate vaccines described to date have been effective in eliciting broadly neutralizing antibodies (bNAbs). For many years, the inability to elicit bNAbs was attributed to the inability to accurately replicate the trimeric structure of the Env protein found on the surface of viruses or virus-infected cells. However, the recent discovery of bNAbs to glycan-dependent epitopes (GDEs) on monomeric HIV-1 (Walker et al., 2009, 2011; McLellan et al., 2011; Pejchal et al., 2011; Kong et al 2013) has

* Corresponding author. Tel.: +1 831 459 3720; fax: +1 831 459 1970.
E-mail address: racdoran@soe.ucsc.edu (R.C. Doran).

[1] Indicates equal contributions to the design and execution of the experiments in this study.

http://dx.doi.org/10.1016/j.molimm.2014.06.025
0161-5890/© 2014 Elsevier Ltd. All rights reserved.

raised the possibility that the inability to elicit bNAbs was due to: (1) the inability to accurately replicate the specific glycan structure of envelope proteins on the surface of viruses and virus-infected cells and (2) our inability to direct antibody responses to GDEs. Indeed, little is known about immunization regimens or adjuvant formulations that favor the formation of antibodies to GDEs.

Of particular interest is the GDE landscape within the first and second variable (V1/V2) domain of gp120. Although the V1/V2 domain is known as a "variable" region (Leonard et al., 1990), numerous glycosylation sites within the V1/V2 domain exhibit a high degree of conservation (Zolla-Pazner and Cardozo, 2010; Go et al., 2011). Previously, it was thought that glycans on gp120 were poorly immunogenic. This characteristic, in addition to the unusually large number of glycosylation sites on gp120, was thought to be a major mechanism, glycan shielding, responsible for immune escape (Wei et al., 2003; Wyatt et al., 1995; Bunnik et al., 2008; Rong et al., 2007; van Gils et al., 2010, 2011). However, the recent discovery of bN-mAbs to GDEs suggests that these epitopes are more immunogenic than previously imagined and that a vaccine targeting GDEs might help to overcome the problem of virus variation. Thus, we have begun to investigate the magnitude, specificity, and frequency of antibodies to GDEs resulting from immunization with recombinant Env proteins.

At this early stage of investigation, all antibodies to GDEs of the HIV-1 envelope protein are informative; however, antibodies to the V1/V2 domain of gp120 are of particular interest. First, the V1/V2 domain contains the GDEs recognized by several bN-mAbs (e.g., PG9, PG16, CH01-4, PGT145) (Walker et al., 2009, 2011; Pejchal et al., 2011; Sanders et al., 2002; Trkola et al., 1996; Mouquet et al., 2012; Bonsignori et al., 2011). Second, non-neutralizing antibodies to the V1/V2 domain represent the only antibody response found to correlate with protection in the RV144 HIV vaccine trial, which included immunization with the ALVAC-HIV canarypox vector vaccine (vCP1521) and the AIDSVAX B/E recombinant gp120 subunit vaccine (Berman, 1998; Berman et al., 1999; Rerks-Ngarm et al., 2009; Haynes et al., 2012). The lack of correlation between neutralizing antibodies and protection in the RV144 trial caused investigators to consider numerous ways by which non-neutralizing antibodies against the V1/V2 domain may confer protection against infection (Liu et al., 2013). Such methods may involve viral inactivation through antibody-dependent cell-mediated virus inhibition, virion aggregation, or inhibition of virion mobility and transport across mucosal surfaces (Liu et al., 2013). Therefore, GDEs in the V1/V2 domain recognized by both neutralizing and non-neutralizing antibodies represent intriguing targets for candidate vaccines to prevent HIV-1 infection.

2. Materials and methods

2.1. Production of HIV envelope proteins gp120, gp140, and gp120 fragments

Recombinant gp140 was prepared as described previously (Berman et al., 1989, 1990) from the 108060_Q655R clinical isolate (O'Rourke et al., 2009). From this sequence, the extracellular domain of gp160 (gp140), full length gp120, and nine overlapping fragments of 108060_Q655R were created and expressed as previously described (Nakamura, 2012). All proteins contained a flag epitope from herpes simplex virus glycoprotein D (gD) fused to the amino terminus as described previously (Lasky et al., 1986; Berman et al., 1988). All proteins were purified by immunoaffinity chromatography using an immunosorbent prepared with 34.1, a mAb to the gD flag epitope. Point mutations N130H or T132A were introduced into a V1/V2 fragment of gp120 from the MN strain of HIV-1 by site-directed mutagenesis, using a QuikChange Lightning kit (Agilent, Santa Clara, CA). The resulting construct was verified by confirmatory sequencing. Plasmids for protein expression were transfected into FreeStyle™ 293-F cells (Invitrogen, Carlsbad, CA).

2.2. Deglycosylation studies

Digestion with the enzyme peptide-*N*-glycosidase F (PNGase F) was used to remove N-linked glycans on HIV gp120 proteins or fragments of gp120. The enzyme with its respective buffers was obtained from New England Biolabs (Ipswich, Mass) and used per manufacturer's instructions. Briefly, 200 μg of recombinant gp120 or V1/V2 fragment was denatured in 10× denaturation buffer. Samples were boiled at 100 °C for 10 min, then mixed with 10× reaction buffer and 5000 units of PNGase F. Digests were carried out at 37 °C for 12 h. To confirm digest completion, the digest product was analyzed by polyacrylamide gel electrophoresis (PAGE) using precast polyacrylamide gels (4–12% Bis-Tris) in MOPS running buffer (NuPAGE®, Invitrogen). Proteins were transferred to nitrocellulose paper (Novex, Life Technologies, Carlsbad, CA). The 4B6 mAb was used as the primary antibody, and the goat anti-mouse IgG/M conjugated to HRP (American Qualex Antibodies, San Clemente, CA) was used as the secondary antibody. Glycan-independent control mAbs included 34.1 (anti-gD) and 1088 (anti-V1/V2).

2.3. Immunizations

BALB/c mice from Charles River (Hollister, CA) were initially immunized with 5 μg of 108060_Q655R gp140 incorporated in Freund's Complete Adjuvant, and then boosted multiple times over a four week period with 5 μg of immunogen incorporated in Freund's Incomplete Adjuvant.

2.4. Monoclonal antibody production

Splenocytes were harvested from immunized mice, and fused to the mouse sp2/0 cell line to create immortalized hybridoma cell lines using standard procedures (Kohler and Milstein, 1975). To identify antibodies that bound the gp120 region, hybridomas were selected using HAT media, and hybridoma subclones were screened for secreted antibody reactivity against gD-tagged 108060_Q655R-rgp120. Hybridoma supernatants that tested positive for reactivity against 108060_Q655R-rgp120 in an enzyme-linked immunosorbent assay (ELISA) were subcloned, and the secreted mAbs were further characterized by ELISA. These assays were carried out in flat bottomed 96 well microtiter plates (Nunc Maxisorp®, Affymetrix, Santa Clara, CA) and coated with 2 μg/mL of protein in PBS, overnight at 4 °C. After 12 h, plates were blocked with PBS containing 1% BSA and 0.02% sodium azide for 1 h at room temperature. After 1 h, PBS solution was removed, and plates were washed four times. For initial screening, 60 μL of hybridoma supernatant obtained directly from the 96 well culture plate was added to the gp120-coated plates. Plates were incubated with gentle agitation for 1 h at room temperature, washed four times in PBS, and incubated for 1 h at room temperature with goat-anti-mouse, or goat-anti-human, HRP-conjugated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Plates were then washed four times in TBST. Plates were developed with OPD solution and stopped with 50 μL of 3 M H_2SO_4. Absorbance was read at 492 nm. The isotype of mAb 4B6 was determined using the Pierce Rapid ELISA Mouse Antibody Isotyping kit (Thermo Fisher Scientific Inc., Rockford, IL).

2.5. Immunoassays

ELISAs were used to detect antibody binding to gp120s from clade B, C, and CRF01_AE isolates and to overlapping fragments

of 108060_Q655R-rgp120. The gp120s and V1/V2 fragments used in these studies were similar to those described previously (Nakamura, 2012; Smith, 2010). Epitopes were further mapped using a library of overlapping peptides 15 amino acids in length derived from the MN sequence (NIH AIDS Reagent Program, Germantown, MD; catalog number 6541). Peptide ELISAs were performed with an initial coating of peptide at 5 μg/mL, and subsequent steps followed the ELISA described above. Antibody binding to peptide was tested in duplicate. The PG9 mAb used in competition assays was purchased from Polymun Scientific (Klosterneuburg, Austria).

2.6. Neutralization assays

A TZM-bl neutralization assay (Montefiori, 2005) was used to evaluate the neutralization potential of the mAbs described in this paper. The viruses used in this assay were from the clade C isolate MW965, and from clade B isolates MN, 108060_Q655R, QHO692, PV04, and JR-FL. Plasmids for the construction of all pseudoviruses with the exception of 108060_Q655R were kindly provided by Dr. David Montefiori (Duke University, Durham, NC). The positive control used for virus neutralization assays consisted of a mixture of the monoclonal antibodies b12, 2G12, and 2F5 (Tri-mAb).

2.7. Sequence alignments and amino acid numbering

The sequences of gp120s compared in this study were aligned using MAFFT (Katoh and Toh, 2008). The numbering of the amino acids described in this paper is provided using the HXB2 standard reference sequence (Los Alamos National Laboratories, HIV Sequence Compendium, Los Alamos, NM, hiv.lanl.gov/content/sequence/HIV/compendium.html).

3. Results

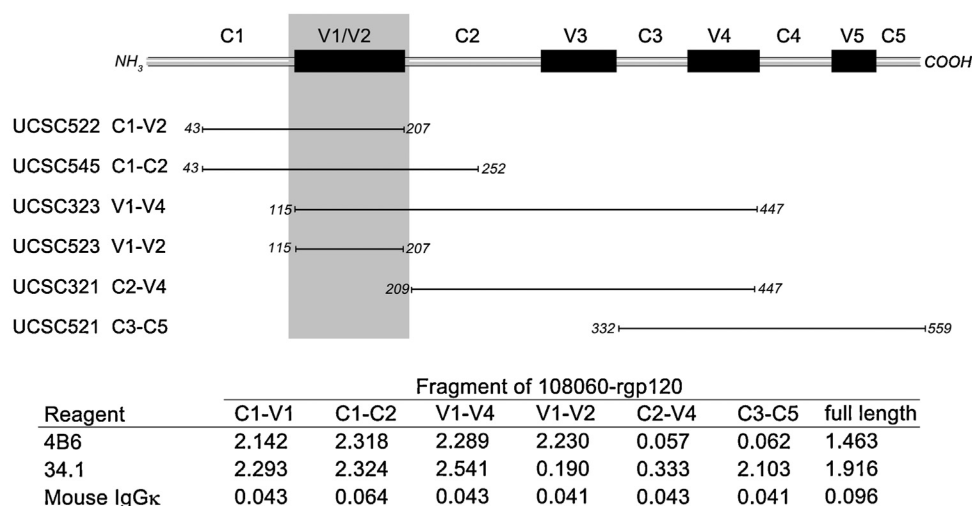
3.1. Immunization with 108060_Q655R _rgp140 and initial mAb screening

In previous studies, we characterized an envelope protein from the clade B clinical isolate 108060_Q655R (O'Rourke et al., 2009). This isolate contains a point mutation in gp41 that appeared to increase neutralization sensitivity to broadly neutralizing, monoclonal and polyclonal antibodies by destabilizing the pre-hairpin fusion intermediate. We postulated that immunization with this envelope protein might provide access to epitopes in gp120 and gp41 (e.g., the membrane proximal external region, or MPER) that are normally concealed until the formation of the 6-helix coiled-coil structure is triggered by the engagement of the CD4 and chemokine receptors (O'Rourke et al., 2009). Although immunization with gp140 prepared from the 108060_Q655R envelope protein did not result in antibodies with exceptional neutralizing activity, mouse hybridomas were isolated as reagents to investigate the antibody response against the unique structural features of this molecule.

To map the epitopes recognized by mAbs secreted by these hybridomas, a series of glycopeptide fragments was prepared. These were designed to contain overlapping sequences of the 108060_Q655R-rgp140, similar to those previously described (Nakamura, 2012) (Fig. 1, Supplementary Fig. S1). Briefly, fragments of the 108060_Q655R-rgp140 sequence were designed to contain consecutive constant and variable domains of HIV gp120. The smallest fragment contained only the V1/V2 domain (Supplementary Fig. S1F), while the largest fragment included sequences from the V1/V2 domain through the C4 domain (Supplementary Fig. S1B). Constructs were appended with an N-terminal gD flag epitope for purification purposes and were expressed by transient transfection in FreeStyle^{TM} 293-F cells. In order to maintain as much tertiary structure as possible, the constructs were designed to preserve the disulfide bridges found in the full-length gp140. As described previously (Nakamura et al., 1993, 2012), fragments expressed by this approach are typically glycosylated, and maintain the disulfide structures required for recognition by a variety of conformation-dependent mAbs.

3.2. Isolation and characterization of mAb 4B6

Analysis of mAb binding to a panel of six different 108060_Q655R fragments (Fig. 1, Supplementary Fig. S1 panels A–F) led to the discovery of the hybridoma clone, 4B6. The mAb from this clone was found to bind to all 108060_Q655R fragments that contained the V1/V2 domain (UCSC522, UCSC545, UCSC323, and UCSC523), including the short fragment (UCSC523) that included only the V1/V2 domain (Fig. 1, Supplementary Fig. S1). In contrast, 4B6 did not bind to the two fragments (UCSC321 and UCSC521) that lacked the V1/V2 domain (Fig. 1). These data suggested that 4B6 recognizes an epitope located within the V1/V2



	Fragment of 108060-rgp120						
Reagent	C1-V1	C1-C2	V1-V4	V1-V2	C2-V4	C3-C5	full length
4B6	2.142	2.318	2.289	2.230	0.057	0.062	1.463
34.1	2.293	2.324	2.541	0.190	0.333	2.103	1.916
Mouse IgGκ	0.043	0.064	0.043	0.041	0.043	0.041	0.096

Fig. 1. Mapping of the 4B6 epitope using gD-tagged fragments of 108060-rgp120. Binding of 4B6 to various fragments of the 108060 envelope protein was assayed by ELISA. All fragments were bound by the positive control antibody to the gD tag (34.1). A mouse IgGκ mAb served as a negative control. Values represent absorbance (492 nm) from single point ELISA using undiluted 4B6 cell culture supernatant. Shading indicates minimal length of epitope bound by the 4B6 antibody.

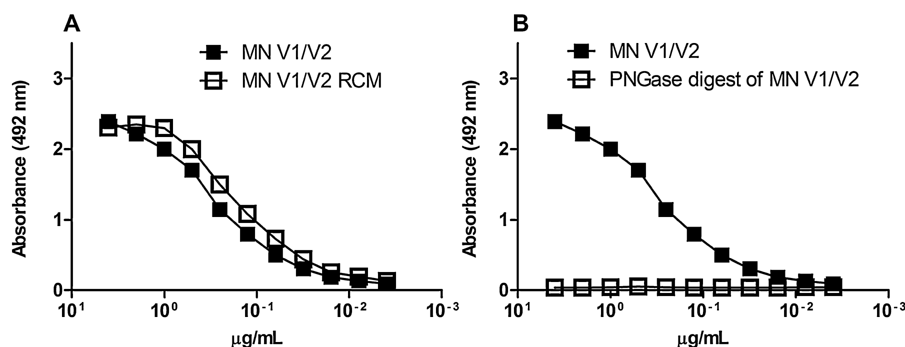


Fig. 2. Effect of secondary structure and glycosylation on mAb 4B6 binding. A fragment corresponding to the V1/V2 domain of MN-rgp120 expressed in 293 HEK cells was mock treated, treated by reduction and carboxymethylation (RCM), or treated with PNGase to remove N-linked carbohydrate as described in Materials and Methods. The resulting proteins were coated onto microtiter plates and 4B6 binding was measured by ELISA. (A) The binding of 4B6 to RCM V1/V2 domain (open squares) and native V1/V2 domain (closed squares). (B) The binding of 4B6 to PNGase-treated V1/V2 domain (open squares) or mock digested V1/V2 domain (closed squares).

domain of 108060_Q655R_rgp120. Isotype analysis of 4B6 revealed that it belonged to the IgG2a subclass (data not shown).

In order to further map the 4B6 epitope, we attempted to measure antibody binding to a series of overlapping of synthetic peptides, 15 amino acids in length, from the V1/V2 domain of the MN strain of HIV-1 (NIH AIDS Reagent Program). These synthetic peptides contained neither the glycosylation nor the disulfide structures present in the secreted fragments used for the initial screening. In contrast to rabbit antibodies to MN-rgp120 that bound to multiple peptides, 4B6 was unable to bind to any of the synthetic peptides in this panel (Supplementary Table S1). This result suggested that 4B6 might recognize a conformation-dependent epitope. To further investigate this possibility, we measured binding to the native and the reduced and carboxymethylated (RCM) forms of the V1/V2 domain of MN-rgp120 (Supplementary Fig. S1, panel G). Surprisingly, we found that 4B6 bound to both the native and the RCM MN V1/V2 fragment (Fig. 2A). This result demonstrated that 4B6 recognized a conformation-independent epitope in the V1/V2 domain of both MN and 108060_Q655R gp120. The observation that 4B6 bound to the RCM form of MN V1/V2 but not to the synthetic peptides indicated that a factor other than conformation was required for 4B6 binding. We hypothesized that 4B6 binding may be dependent on the glycosylation present in the V1/V2 fragment expressed in mammalian cells, but not in the synthetic peptides.

3.3. Sequence comparison and in vitro mutagenesis to localize the 4B6 epitope

To further localize the epitope recognized by 4B6, we evaluated its ability to bind to a panel of seventeen purified gp120s with diverse sequences expressed in mammalian cells (Fig. 3). These included eleven clade B gp120s including MN, IIIB, SC422, TRO.11, JRFL, WITO (Seaman et al., 2010), and five clinical isolates 108060_Q655R, UCSC101, UCSC109, UCSC127, and UCSC195. Additionally, we measured binding to five clade C gp120s described previously (CN97001, CN98005, IN98026, TZ97005, and ZA97010) (Smith, 2010) and to the CRF01_AE A244-rgp120 (Berman et al., 1999). Of the gp120s tested, only eight of the clade B sequences were bound by the 4B6 antibody (Fig. 3).

To localize the epitope recognized by 4B6, the sequences of the 4B6 binding and non-binding envelope proteins were aligned, and the amino acid sequences were compared to identify polymorphisms that segregated with 4B6 binding (Fig. 3). The sequence alignments implicated several predicted N-linked glycosylation sites (PNGSs) that were present in the gp120s that bound 4B6, and not present in gp120s unable to bind 4B6. The most promising site from this comparison was asparagine at position 130 (N130). Of note, while the clade B sequence JR-FL contains the N130 residue, it lacks the required the serine (S) or threonine (T) of the canonical N-X-S/T motif necessary for N-linked glycosylation. Therefore, mAbs dependent on glycosylation at N130 would not be expected to bind to JR-FL.

In vitro mutagenesis studies were then carried out to investigate the dependency of 4B6 binding on position 130. To prevent glycosylation at this position, we independently substituted for either the N or the T of the canonical N-X-T/S N-linked glycosylation motif. First, we replaced N at position 130 with histidine (H), because examination of multiple HIV sequence data sets revealed that histidine is the second most common amino acid at position 130 after N. The results of this study are shown in Fig. 4A. We found that replacement of N with H at position 130 completely abolished the binding of 4B6 to the V1/V2 fragment of MN-rgp120. To confirm this finding, we replaced threonine (T) at position 132 with alanine (A) in an independent construct. This substitution also disrupted the N130 PNGS and abolished the binding of 4B6 to the V1/V2 fragment of MN-rgp120 (Fig. 4B). In contrast, neither of these mutations had any effect on the binding of a positive control anti-V1/V2 mAb (1088), whose binding has been established to be independent of glycosylation (Nakamura, 2012). Together, these studies indicate that 4B6 binding is dependent on the PNGS at position 130.

3.4. PNGase treatment destroys the epitope recognized by the 4B6 mAb

To verify that 4B6 recognizes a glycan-dependent epitope, we treated the MN V1/V2 fragment with peptide-*N*-glycosidase F (PNGase F). This enzyme cleaves N-linked glycans at the first *N*-acetylglucosamine (GlcNac), effectively removing any carbohydrates on a glycoprotein. In ELISA assays (Fig. 2B), we observed that PNGase treatment destroyed the binding of the 4B6 mAb to the MN V1/V2 domain fragment. To confirm that the PNGase treatment ran to completion, and to verify the identity of the species bound by 4B6, immunoblot studies were carried out. For these experiments, the mock- and enzyme-treated proteins were probed with either 4B6 (Fig. 5A) or an anti-gD control mAb, 34.1 (Fig. 5B). We observed that the native and mock-treated V1/V2 fragment exhibited a molecular mass that was higher than expected, approximately 50 kDa. This anomalous migration was attributed to the high carbohydrate content of the V1/V2 domain which contains 9 PNGSs in 131 amino acids, including the gD tag. As expected, treatment with PNGase F resulted in a molecular mass consistent with the calculated (17 kDa) value. These results further suggested that 4B6 recognizes a GDE in the V1/V2 domain.

Clade/Envelope		Binding to 4B6	120 130	140 150
B	HXB2		SLKPCVKLTPLCVSLK	CTD-LKNDTNTNSS-------SGRMIMEKG---EIKN
B	108060	+	SLKPCVKLTPLCVTLN	CTDK---LRNDAF-----G--VNNT--MEG---EMKN
B	MN	+	SLKPCVKLTPLCVTLN	CTD-LRNTTNTNNSTDNNNSKSEGTI-KGG---EMKN
B	UCSC101	+	SLKPCVKITPLCVTLN	CTD-LEEGTSSNN--------SSYQRGEEG---EIKN
B	UCSC109	+	SLKPCVKLTPLCVTLN	CTDLYTNTTSKSN-----G-TTNDTNNMSH---DMKN
B	UCSC127	+	SLKPCVKLTPLCVTLN	CTDAEVTRKTNTT-------SGDWEKVKKG---EIKN
B	UCSC195	+	SLKPCVKLTPLCVTLN	CTD-LKNATNITN--------SEGGMREGG---EIKN
B	SC422	+	---PCVKLTPLCVTLN	CTDELRNGTYANV-----------TVTEKG---EIKN
B	TRO.11	+	SLKPCVKLTPLCVTLN	CTD---NITNTNTNSSKNSSTHSYNNSLEG---EMKN
B	JRFL	-	---PCVKLTPLCVTLN	CKD-V-NATNTTND-------SEGTM-ERG---EIKN
B	WITO	-	---PCVKLTPLCVTLH	CTNV---TISSTN-----GSTANVT--MRE---EMKN
E	A244	-	SLKPCVKLTPPCVTLH	CTNAN----LTKANLTNVNNRTNVSNIIGNITDEVRN
B	IIIB	-	SLKPCVKLTPLCVSLK	CTD-LKNDTNTNSS-------SGRMIMEKG---EIKN
C	CN97001	-	SLKPCVKLTPLCVTLE	CRNVS----------SNSNGAHNET--YHESMKEMKN
C	CN98005	-	SLKPCVKLTPLCVTLE	CRNVS----------SNG----TET--YNESVKEVKN
C	IN98026	-	GLKPCVKLTPLCVTLE	CK--------------DANYTHNET--YNEIKKEMKN
C	TZ97005	-	SLKPCVKLTPLCVTLK	CGNVTISNDTTYNNVSNVNAAYNSD--MRE---ELKN
C	ZA97010	-	SLKPCVKLTPLCVTLR	CTN---ANRTEVK--INITGNYNVS--MNE---EIKN

			160 170 180 190	200
B	HXB2		CSFNISTSIRGKVQKEYAFFYKLDIIPIDNDT--------------TSYKLTS	CNTSVITQACPK
B	108060	+	CSFNTTTSLRDKIQKEYALFYKLDVVQIKNNN------NS----NYTSYRLIN	CNTSVITQACPK
B	MN	+	CSFNITTSIGDKMQKEYALLYKLDIEPIDNDS--------------TSYRLIS	CNTSVITQACPK
B	UCSC101	+	CSFNITTRLREKVQKEYALFYKLDIIAMDNKTNA------------TRYRLIS	CNTSTITQACPK
B	UCSC109	+	CSFNVTTALRDRVTKEYALFYKLDVEPIDNNN------HS-----YANYRLIN	CNTSTITQACPK
B	UCSC127	+	CSFDAINT-KNKVQKQYALFDTLNVVSIDDDNNSNNNSNNNNNTNYSDFRLTK	CDTSVIRQACPK
B	UCSC195	+	CSFNITTSLRDRVQKEYALFYKLDVEPIDDDKNSTDNNST----NYTNYRLIS	CNTSVITQACPK
B	SC422	+	CSFNITTAIRDKVQKTYALFYRLDVVPIDNNH---GNSSS----NYSNYRLIN	CNTSVITQACPK
B	TRO.11	+	CSFNITAGIRDKVKKEYALFYKLDVVPIEEDK------DT----NKTTYRLRS	CNTSVITQACPK
B	JRFL	+	CSFNITTSIRDEVQKEYALFYKLDVVPIDNNN--------------TSYRLIS	CDTSVITQACPK
B	WITO	-	CSFNTTTVIRDKIQKEYALFYKLDIVPIEGKN------TN------TGYRLIN	CNTSVITQACPK
E	A244	-	CSFNMTTELRDKKQKVHALFYKLDIVPIEDNN------------DSSEYRLIN	CNTSVIKQACPK
B	IIIB	-	CSFNISTSIRGKVQKEYAFFYKLDIIPIDNDT--------------TSYKLTS	CNTSVITQACPK
C	CN97001	-	CSFNATTVVRDRKQTVYALFYRLDIVPLTKKN----SSEN----SSEYRLIN	CNTSAITQACPK
C	CN98005	-	CSFNATTVLRDRKKTVHALFYRLDIVPLNDEN----SGKN----SSEYYRLIN	CNTSAITQACPK
C	IN98026	-	CSFNTTTELRDRKQKVYALFYRLDIVSLNENN--EKNSSN----SSE-YRLIN	CNTSAITQACPK
C	TZ97005	-	CSFNMTTEVRDKKQNVYALFYKLDIVPIDGNK----SISS----NFSEYRLIN	CNTSAITQACPK
C	ZA97010	-	CSFNATTEIRDKKQKVYALFYRSDLVPLKEDS----SGEN----NSSKYILIN	CNTSTITQACPK

Fig. 3. Amino acid sequence alignment of V1/V2 domains from gp120s used for 4B6 binding studies. The binding of 4B6 to gp120s from 17 different isolates was measured by ELISA. The gp120s able to bind 4B6 are indicated by plus signs (+) and those unable to bind are indicated by minus (−) signs. The sequences of all of the gp120s were aligned using MAFFT (Katoh and Toh, 2008). The location of the N130 PNGSs that matched the pattern of 4B6 binding is indicated by white on black lettering. The location of other predicted glycosylation sites that failed to match the pattern of 4B6 binding is indicated by black on blue lettering. Numbering is provided with reference to the standard HXB2 sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Location of N130 in the V1/V2 domain structure

Recent structural studies have shown that the V1/V2 domain forms a 4-stranded β-sheet structure with the different strands identified as A, B, C, and D (McLellan et al., 2011; Julien et al., 2013) (Fig. 6). We observed that N130 is located near the middle of the A strand and located between cysteines C126 and C131 that form disulfide bonds with cysteines in the D and B strands, respectively. Comparison of gp120 sequences showed that the N130 glycan is conserved among clade B viruses (Go et al., 2011). Examination of the 3-D structure of the V1/V2 domain showed that this N130 is located in close spatial proximity to the N160 and N156 glycosylation sites that are critical for the binding of the broadly neutralizing PG9 mAb (Fig. 6) (Walker et al., 2009; McLellan et al., 2011).

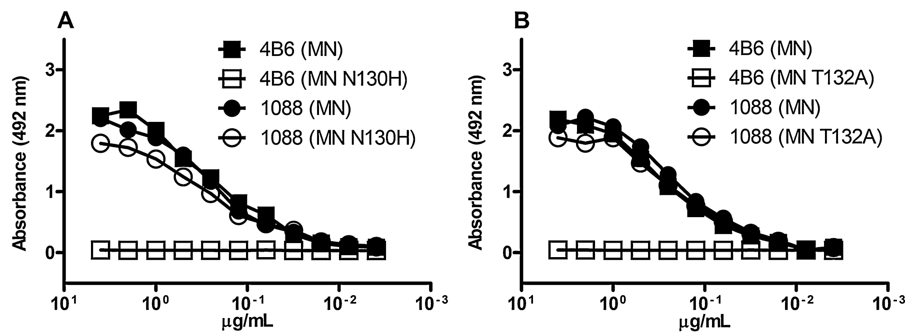


Fig. 4. 4B6 binding to MN V1/V2 fragments mutagenized to delete the N130 glycosylation site. The N130 glycosylation site was disrupted by two independent mutations targeting the canonical N-X-T/S sequon required for N-linked glycosylation. These included replacement of the codons specifying asparagine (N) at position 130 with the codon for histidine (H), or replacement of the codon specifying the threonine (T) at position 132 with the codon for alanine (A). (A) Comparison of the binding of the glycan-dependent 4B6 mAb (squares) or the non-glycan-dependent 1088 mAb (circles) to the native V1/V2 fragment (shaded symbols) or to the V1/V2 fragment with the N130H mutation (open symbols). (B) Comparison of the binding of the glycan-dependent 4B6 mAb (squares) or the non-glycan-dependent 1088 mAb (circles) to the native V1/V2 fragment (shaded symbols) or to the V1/V2 fragment with the T132A mutation (open symbols).

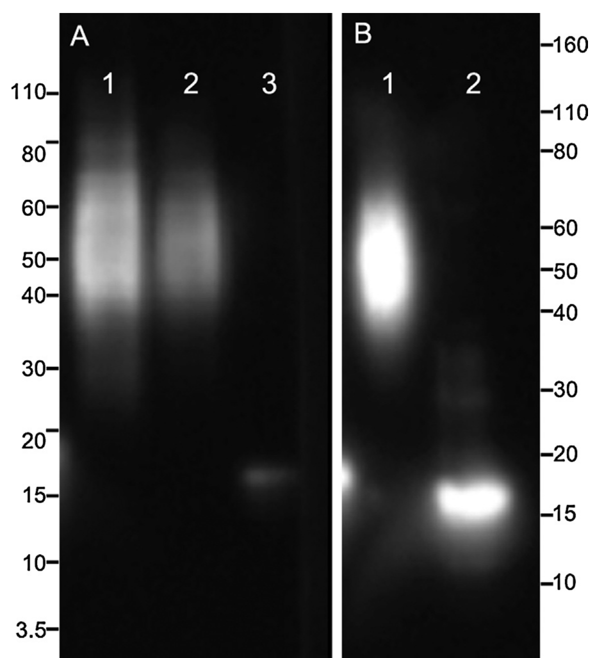


Fig. 5. Analysis of 4B6 binding to PNGase-treated MN V1/V2 fragments by immunoblot. A fragment corresponding to the V1/V2 domain of MN-rgp120, expressed in 293 cells, was either treated with PNGase or treated with PNGase digest buffer alone (mock treated), and subjected to polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose paper (Novex, Life Technologies, Carlsbad, CA) and allowed to react with either the 4B6 mAb (panel A) or with 34.1, a mAb to the gD flag epitope (panel B). When probed with 4B6, untreated and mock-treated V1/V2 fragment migrates as a diffuse band of approximately 50 kDa (panel A, lanes 1 and 2); when treated with PNGase, the V1/V2 fragments runs as a sharp band of approximately 17 kDa (panel A, lane 3). When probed with the 34.1 mAb to HSV gD, untreated V1/V2 fragment migrates as a diffuse band of approximately 50 kDa (panel B, lane 1); when treated with PNGase, the V1/V2 fragment runs as a sharp band of approximately 17 kDa (panel B, lane 2). The mobility of molecular weight standards is shown in the left and right margins.

However, when we measured PG9 binding to envelope proteins lacking the N130 glycosylation site, the binding of PG9 appeared to be unaffected (data not shown).

3.6. Neutralization assays

The ability of 4B6 to neutralize various clade B or C viruses was determined in a TZM-bl neutralization assay (Montefiori, 2005). We found that 4B6 was not able to neutralize any of the clade B isolates MN, 108060_Q655R, QHO692, PV04, and JR-FL, nor the clade C isolate MW965 (Supplementary Table S2). Examination of the sequences of these viruses showed that all but JR-FL contained the glycosylation site at N130.

4. Discussion

This study documents the isolation of a mouse mAb (4B6) to a novel GDE in the V1/V2 domain of the HIV envelope protein, gp120. Four new findings derive from these studies. First, we show that antibodies to GDEs in the V1/V2 domain of gp120 can result from immunization with recombinant gp120, whereas previously such antibodies have been isolated only from HIV-infected humans or chimpanzees (Walker et al., 2009, 2011; Bonsignori et al., 2011; Trkola et al., 1995; Vijh-Warrier et al., 1996; Wu et al., 1995). Second, we show that antibodies to GDEs can result from a relatively short immunization schedule and that continuous exposure to gp120 over a long period of time, as occurs during chronic HIV infection, is not required to elicit antibodies to GDEs. Third, we show that the 4B6 epitope is common among clade B viruses. Finally, we show that while 4B6 binds to GDEs in the V1/V2 domain, this binding is not sufficient for virus neutralization, and that additional factors determine whether an antibody possesses virus neutralizing activity.

Historically, N-linked glycans have been considered poorly immunogenic, and it has been surprising to discover that a large percentage of the broadly neutralizing antibodies in sera from HIV-infected individuals are directed to GDEs on gp120 (Walker et al.,

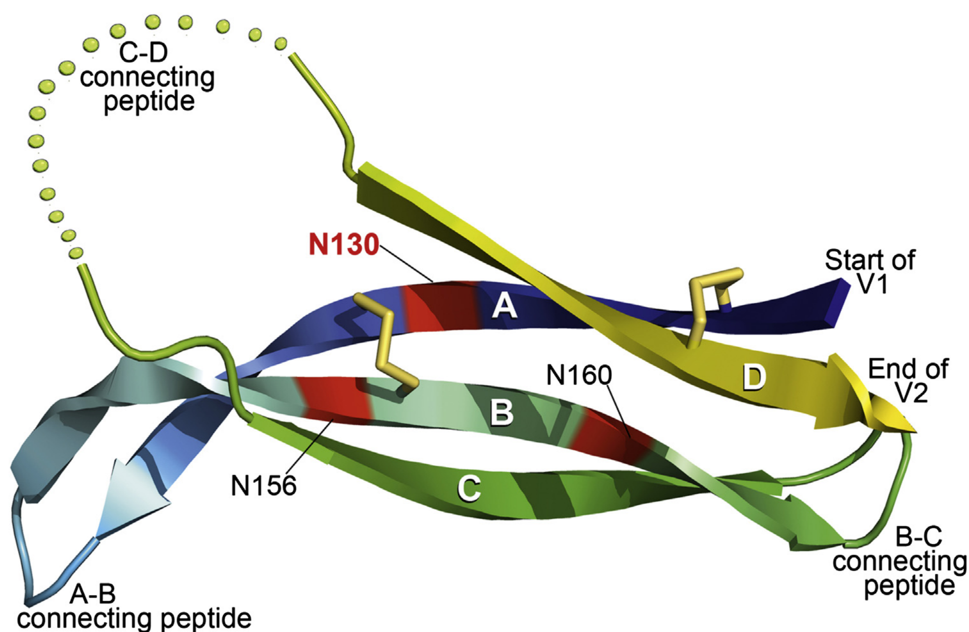


Fig. 6. Diagram of the V1/V2 domain. The 4-stranded β-sheet structure of the V1/V2 domain reported by McLellan et al. (2011) was modified to show the approximate location of glycosylation sites (red segments) recognized by the 4B6 mAb (N130) and the broadly neutralizing PG9 mAb (N156 and N160) (Walker et al., 2009). Letters (A–D) indicate each strand of the β-sheet structure. The two disulfide bonds at positions C126 and C131 flanking the N130 glycosylation site are also indicated (yellow). Other features including the B–C connecting peptide required for PG9 binding are also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2009, 2011; Pejchal et al., 2011). While it has been well documented that N-linked glycans found on the HIV envelope protein function as a "glycan shield" to protect large regions of the gp120 structure from antibody binding by steric hindrance, we now know that this shield is imperfect, and that glycans can themselves become targets for neutralizing antibodies (Moore et al., 2012). Recent studies of mAbs from HIV-infected humans have identified at least two different clonal lineages of bNAbs that recognize GDEs in the V1/V2 domain. These include the PG9 lineage that recognizes glycans at positions 156 and 160, and the PGT121/122 lineage that recognizes glycans at position 137 (Kwong and Mascola, 2012; Julien, 2013). Thus far, antibodies from humans recognizing the N130 GDE have not been described. However, recent pepscan analysis of clinical specimens from VAX003 and RV144 vaccine trials revealed that the portion of the V1/V2 domain containing N130 PNGS is highly immunogenic (Gottardo, 2013). The observation that the N130 site is contained within a region that is particularly visible to the humoral immune response supports the hypothesis that antibodies to the N130 GDE may additionally be found in human sera.

Studies showing that GDEs are major targets of bNAbs have prompted considerable interest in developing vaccines able to elicit antibodies to this class of epitope. However, numerous uncertainties remain concerning the best approach to elicit antibodies to GDEs in gp120. We know little regarding the optimal formulation or immunization regimen to elicit antibodies to GDEs. In humans, bNAbs to GDEs are seldom found until 2–3 years post-infection, following years of continuous exposure to viral antigens (Walker et al., 2009, 2011; Bonsignori et al., 2011). Based on these kinetics, it has been postulated that antibodies to GDEs might only occur as the result of continuous exposure to highly glycosylated viral antigens, or as a consequence of breakdown in immune tolerance of the glycan structures resulting from HIV infection. Indeed, several groups are pursuing the possibility of "guided" immunization strategy with viral proteins recovered from the sequential virus isolates thought to have guided the evolution of antibodies to GDEs with broadly neutralizing activity (Kwong and Mascola, 2012; Bonsignori et al., 2012; Klein et al., 2013). It is postulated that this approach might be effective in driving the evolution of antibodies with the atypical structures found in most bNAbs, such as long CDR H3 domains (>20 amino acids) resulting from unusual VDJ splicing, or highly mutated antibody genes (>100 nucleotide changes) resulting from unusually high levels of somatic mutation (Kwong and Mascola, 2012). Moreover, bNAbs to GDEs often appear to bind simultaneously to two different glycan moieties (e.g., N156 and N160 in the case of PG9, and N302 and N332 in the case of PGT128) (Kong et al., 2012). Our isolation of 4B6 demonstrates that antibodies to GDEs can arise in healthy, uninfected animals as a result of a short intensive immunization regimen with recombinant envelope protein. Further work to sequence and solve the structure of this antibody would provide insight as to whether its inability to neutralize HIV results from a lack of structural features (e.g., long CDR H3 domain, etc.) common to bNAbs.

Finally, these studies highlight the possibility that antibodies to GDEs are more common than previously appreciated. This insight has implications for our understanding of the protective immune responses to the highly glycosylated proteins of other pathogens such as cytomegalovirus (Britt and Vugler, 1989), SARs-CoV, influenza, and West Nile virus (Vigerust and Shepherd, 2007). Until recently, vaccines were developed to epitopes that were primarily amino acids or carbohydrates, but not a combination of both. It now appears that another important class of epitope exists, exemplified by 4B6 and by bNAbs to HIV that depend on both amino acid and N-linked glycan contacts. Further studies evaluating the frequency and specificity of these antibodies, as well as studies aimed to elucidate the best method to elicit such antibodies, may present important medical insights and applications.

Funding

This work was supported by grants from the National Institutes of Health, National Institute of Drug Abuse (R01 DA 26801-01A1) and National Institute of Allergy and Infectious Diseases (R01 AI 089378 01). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional funding was provided by the Henry M. Jackson Foundation (#683948).

Acknowledgments

We thank Dr. Aaron Vollrath for his assistance with sequence analysis, and Ann Durbin for expert technical assistance in the production of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2014.06.025.

References

Berman, P.W., 1998. Development of bivalent rgp120 vaccines to prevent HIV type 1 infection. AIDS Res. Hum. Retroviruses 14 (Suppl 3), S277–S289.
Berman, P.W., Nunes, W.M., Haffar, O.K., 1988. Expression of membrane-associated and secreted variants of gp160 of human immunodeficiency virus type 1 in vitro and in continuous cell lines. J. Virol. 62 (9), 3135–3142.
Berman, P.W., et al., 1989. Expression and immunogenicity of the extracellular domain of the human immunodeficiency virus type 1 envelope glycoprotein: gp160. J. Virol. 63 (8), 3489–3498.
Berman, P.W., et al., 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature 345 (6276), 622–625.
Berman, P.W., et al., 1999. Development of bivalent (B/E) vaccines able to neutralize CCR5-dependent viruses from the United States and Thailand. Virology 265 (1), 1–9.
Bonsignori, M., et al., 2011. Analysis of a clonal lineage of HIV-1 envelope V2/V3 conformational epitope-specific broadly neutralizing antibodies and their inferred unmutated common ancestors. J. Virol. 85 (19), 9998–10009.
Bonsignori, M., et al., 2012. HIV-1 antibodies from infection and vaccination: insights for guiding vaccine design. Trends Microbiol. 20 (11), 532–539.
Britt, W.J., Vugler, L.G., 1989. Processing of the gp 55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. J. Virol. 63 (1), 403–410.
Bunnik, E.M., et al., 2008. Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. J. Virol. 82 (16), 7932–7941.
Go, E.P., et al., 2011. Characterization of glycosylation profiles of HIV-1 transmitted/founder envelopes by mass spectrometry. J. Virol. 85 (16), 8270–8284.
Gottardo, R., et al., 2013. Plasma IgG to linear epitopes in the V2 and V3 regions of HIV-1 gp120 correlate with a reduced risk of infection in the RV144 vaccine efficacy trial. PLoS One 8 (9), e75665.
Haynes, B.F., et al., 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N. Engl. J. Med. 366 (14), 1275–1286.
Julien, J.P., et al., 2013. Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science 342 (6165), 1477–1483.
Katoh, K., Toh, H., 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform. 9 (4), 286–298.
Klein, F., et al., 2013. Antibodies in HIV-1 vaccine development and therapy. Science 341 (6151), 1199–1204.
Kohler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256 (5517), 495–497.
Kong, L., et al., 2012. Toward a carbohydrate-based HIV vaccine. In: Klyosov, A. (Ed.), Glycobiology and Drug Design. American Chemical Society, Washington, D.C., pp. 187–215.
Kong, et al., 2013. Supersite of immune vulnerability on the glycosylated face of HIV-1 envelope glycoprotein gp120. Nat Struct Mol Biol 20 (7), 796–803.
Kwong, P.D., Mascola, J.R., 2012. Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. Immunity 37 (3), 412–425.
Lasky, L.A., et al., 1986. Neutralization of the AIDS retrovirus by antibodies to a recombinant envelope glycoprotein. Science 233 (4760), 209–212.
Leonard, C.K., et al., 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. 265 (18), 10373–10382.
Liu, P., et al., 2013. Infectious virion capture by HIV-1 gp120-specific IgG from RV144 vaccinees. J. Virol. 87 (14), 7828–7836.

McLellan, J.S., et al., 2011. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. Nature 480 (7377), 336–343.
Montefiori, D.C., 2005. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr. Protoc. Immunol. 64, 12.11.1–12.11.17.
Moore, P.L., et al., 2012. Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. Nat. Med. 18, 1688–1692.
Mouquet, H., et al., 2012. Complex-type *N*-glycan recognition by potent broadly neutralizing HIV antibodies. Proc. Nat. Acad. Sci. U.S.A. 109 (47), E3268–E3277.
Nakamura, G.R., et al., 2012. Monoclonal antibodies to the V2 domain of MN-rgp120: fine mapping of epitopes and inhibition of alpha4beta7 binding. PLoS One 7 (6), e39045.
Nakamura, G.R., et al., 1993. Strain specificity and binding affinity requirements of neutralizing monoclonal antibodies to the C4 domain of gp120 from human immunodeficiency virus type 1. J. Virol. 67 (10), 6179–6191.
O'Rourke, S.M., et al., 2009. Novel ring structure in the gp41 trimer of human immunodeficiency virus type 1 that modulates sensitivity and resistance to broadly neutralizing antibodies. J. Virol. 83 (15), 7728–7738.
Pejchal, R., et al., 2011. A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. Science 334 (6059), 1097–1103.
Rerks-Ngarm, S., et al., 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N. Engl. J. Med. 361 (23), 2209–2220.
Rong, R., et al., 2007. Role of V1V2 and other human immunodeficiency virus type 1 envelope domains in resistance to autologous neutralization during clade C infection. J. Virol. 81 (3), 1350–1359.
Sanders, R.W., et al., 2002. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J. Virol. 76 (14), 7293–7305.
Seaman, M.S., et al., 2010. Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. J. Virol. 84 (3), 1439–1452.
Smith, D.H., et al., 2010. Comparative immunogenicity of HIV-1 clade C envelope proteins for prime/boost studies. PLoS One 5 (8), e12076.
Trkola, A., et al., 1995. Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. J. Virol. 69 (11), 6609–6617.
Trkola, A., et al., 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. 70 (2), 1100–1108.
van Gils, M.J., et al., 2010. Rapid escape from preserved cross-reactive neutralizing humoral immunity without loss of viral fitness in HIV-1-infected progressors and long-term nonprogressors. J. Virol. 84 (7), 3576–3585.
van Gils, M.J., et al., 2011. Longer V1V2 region with increased number of potential N-linked glycosylation sites in the HIV-1 envelope glycoprotein protects against HIV-specific neutralizing antibodies. J. Virol. 85 (14), 6986–6995.
Vigerust, D.J., Shepherd, V.L., 2007. Virus glycosylation: role in virulence and immune interactions. Trends Microbiol. 15 (5), 211–218.
Vijh-Warrier, S., et al., 1996. Synergistic neutralization of human immunodeficiency virus type 1 by a chimpanzee monoclonal antibody against the V2 domain of gp120 in combination with monoclonal antibodies against the V3 loop and the CD4-binding site. J. Virol. 70 (7), 4466–4473.
Walker, L.M., et al., 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 326 (5950), 285–289.
Walker, L.M., et al., 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature 477 (7365), 466–470.
Wei, X., et al., 2003. Antibody neutralization and escape by HIV-1. Nature 422 (6929), 307–312.
Wu, Z., et al., 1995. Characterization of neutralization epitopes in the V2 region of human immunodeficiency virus type 1 gp120: role of glycosylation in the correct folding of the V1/V2 domain. J. Virol. 69 (4), 2271–2278.
Wyatt, R., et al., 1995. Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. J. Virol. 69 (9), 5723–5733.
Zolla-Pazner, S., Cardozo, T., 2010. Structure-function relationships of HIV-1 envelope sequence-variable regions refocus vaccine design. Nat. Rev. Immunol. 10 (7), 527–535.