Characterization of human anti-HIV-1 Env-specific monoclonal antibodies *in vitro* and *in vivo*

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Summary

The development of an effective vaccine preventing HIV-1 infection remains of paramount importance (Fauci, 2008). Passive transfer studies using potent broadly neutralizing monoclonal antibodies, identified in a small number of HIV-1 infected individuals, can provide sterilizing immunity against SHIV infection in macaques and appear to delay HIV-1 rebound in humans (Hessell et al., 2009a; Hessell et al., 2009b; Hessell et al., 2010; Mascola et al., 2000; Mehandru et al., 2007; Shibata et al., 1999; Trkola et al., 2005). Thus antibodies, which are key components of the adaptive immune response elicited by most vaccines, are also thought to be essential for protection against HIV-1 infection (Karlsson Hedestam et al., 2008; Plotkin, 2008).

Winning the enormous challenge of developing a protective vaccine preventing HIV-1 infection requires further understanding of the virus's interaction with the immune system. The characterization of conserved epitopes on the viral envelope protein (Env) that are immunogenic in humans and targeted by neutralizing antibodies is an important step in vaccine design. Here we report on the fine features of the anti-HIV-1 Env response in individuals that mount a broadly neutralizing serologic response against HIV-1 (aim one and two). In an effort to further our understanding of antibody-mediated protection *in vivo* we developed a mouse model that enables us to study HIV-1 entry. We show that an antibody's ability to inhibit viral entry comprises its bioavailability, its direct neutralizing activity and its effector functions (aim three).

Zusammenfassung

Die Entwicklung eines effektiven HIV-Impfstoffes, ist für die Eindämmung der weltweiten AIDS Pandemie von entscheidender Bedeutung (Fauci, 2008). Ein schützender Impfstoff sollte breit-neutralisierende Antikörper induzieren, da diese in Transfer Experimenten sterilisierende Immunität vermitteln (Karlsson Hedestam et al., 2008; Plotkin, 2008; Hessell et al., 2009a; Hessell et al., 2009b; Hessell et al., 2010; Mascola et al., 2000; Mehandru et al., 2007; Shibata et al., 1999; Trkola et al., 2005).

Die Charakterisierung von konservierten Epitopen auf dem viralen Hüllprotein (Env), die immunogen im Menschen sind und von neutralisierenden Antikörpern gebunden werden, ist von grosser Bedeutung für die Impfstoffentwicklung. Anti-gp41 Antikörper, kloniert von Individuen mit breit-neutralisierender Serum Aktivität gegen HIV-1, erkennen diverse antigene Determinanten auf Env_{gp41} und reduzieren virale Infektion *in vitro* nur bei sehr hohen Konzentrationen (Pietzsch et al., 2010b). Bindungsstudien mit Env_{gp120} Mutanten zeigen, dass eine Gruppe von Antikörpern (anti-core) an ein zuvor unbeschriebenes konformationelles Epitop bindet. Das core Epitop umfasst Aminosäuren D474/ M475/ R476 auf Env_{gp120}, ist stark konserviert und notwenidg für optimale Infektion (Pietzsch et al., 2010a). Wenig ist über die *in vivo* Wirkungsweise von neutralisierenden Antikörpern bekannt. Mit Hilfe eines neuen Mausmodells, das die HIV Eintrittsfaktoren exprimiert, zeigen wir, dass neutralisierende Antikörper abhängig von ihrer Bioverfügbarkeit, ihrer neutralisierenden Aktivität und ihrer Effektor Funktionen eine Infektion inhibieren (Pietzsch et al., 2012).

List of Publications

I Pietzsch J, Scheid JF, Mouquet H, Seaman MS, Broder CC, Nussenzweig MC. **Anti-gp41 antibodies cloned from HIV-infected patients with broadly neutralizing serologic activity.** *J Virol.* 2010 May;84(10):5032-42.

JP designed the study; analyzed data; expressed monoclonal antibodies; performed IgG adsorption, competition ELISAs, MPER peptide ELISAs, deglycosylation; and drafted the manuscript.

II Pietzsch J, Scheid JF, Mouquet H, Klein F, Seaman MS, Jankovic M, Corti D, Lanzavecchia A, Nussenzweig MC. **Human anti-HIV neutralizing antibodies frequently target a conserved epitope essential for viral fitness.** *J Exp Med.* 2010 Aug 30;207(9):1995-2002.

JP designed the study; analyzed data; expressed recombinant proteins; performed site-directed mutagenesis, performed ELISAs and FACS-based binding assay; developed the fusion; and drafted the manuscript.

III Pietzsch J, Gruell H, Bournazos S, Donovan BM, Klein F, Diskin R, Seaman MS, Bjorkman PJ, Ravetch JV, Ploss A, Nussenzweig MC. **A mouse model for HIV-1 entry.** *PNAS.* Published online before print 2012 Sep 10, doi: 10.1073/pnas.1213409109

JP designed the study; developed the *in vivo* neutralization system; analyzed data; cloned the hCCR5-2A-hCD4 construct; performed flow cytometry binding assays; produced HIV-1 pseudovirus and performed *in vivo* infection; performed histological detection of hCCR5 and hCD4 in liver sections; perfomed serum antibody ELISAs; performed bioluminescence imaging; and drafted the manuscript.

Additional publications not included in this thesis

Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, Ott RG, Anthony RM, Zebroski H, Hurley A, Phogat A, Chakrabarti B, Li Y, Connors M, Pereyra F, Walker BD, Wardemann H, Ho D, Wyatt RT, Mascola JR, Ravetch JV, Nussenzweig MC. **Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals**. *Nature*. 2009 Apr 2;458(7238):636-40.

Mouquet H, Scheid JF, Zoller MJ, Krogsgaard M, Ott RG, Shukair S, Artyomov M, Pietzsch J, Connors M, Pereyra F, Walker BD, Ho DD, Wilson PC, Seaman MS, Eisen HN, Chakraborty AK, Hope T, Ravetch JV, Wardemann H, Nussenzweig MC. **Polyreactivity increases the apparent affinity of anti-HIV antibodies by heteroligation.** *Nature*. 2010 Sep 30;467(7315):591-5.

Forbi JC, Pietzsch J, Olaleye VO, Forbi TD, Pennap GR, Esona MD, Adoga MP, Agwale SM. **Urban-rural estimation of hepatitis C virus infection sero-prevalence in north Central Nigeria.** *East Afr J Public Health.* 2010 Dec;7(4):367-8.

Scheid JF, Mouquet H, Ueberheide B, Diskin R, Klein F, Olivera TYK, Pietzsch J, Fenyo D, Abadir A, Velinzon K, Hurley A, Myung S, Boulad F, Poignard P, Burton D, Pereyra F, Ho DD, Walker BD, Seaman MS, Bjorkman P, Chait BT, Nussenzweig MC. **Convergence of broad and potent anti-HIV antibodies that mimic CD4 binding.** *Science.* 2011 Sep 16;333(6049):1633-7.

Mouquet H, Klein F, Scheid JF, Warncke M, Pietzsch J, Oliveira TYK, Velinzon K, Seaman MS, Nussenzweig MC. **Memory B cell antibodies to HIV-1 gp140 cloned from individuals infected with clade A and B viruses.** *PLoS ONE.* 2011 Sep; 6(9):e24078.

Klein F, Gaebler C, Mouquet H, Sather DN, Lehmann C, Scheid JF, Kraft Z, Liu Y, Pietzsch J, Hurley A, Poignard P, Feizi T, Morris L, Walker BD, Faetkenheuer G, Seaman MS, Stamatatos L, Nussenzweig MC. **Broad neutralization by a combination of antibodies recognizing the CD4 binding site and a new conformational epitope on the HIV-1 envelope protein.** *J Exp Med.* 2012 Jul 30;209(8):1469-79.

Abbreviations

Ab	Antibody
AdV	Adenovirus
AIDS	Aquired immune deficiency syndrome
CD4bs	CD4 binding site
CD4is	CD4 induced site
CDC	Centers for Disease Control and Prevention
CHR	Carboxy-terminal heptad repeat region
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope glycoprotein
FP	Fusion peptide
GFP	Green fluorescent protein
HIV-1	Human immunodeficiency virus type 1
i.v.	Intravenously
IC_{50}	Inhibitory concentration 50
ID	Immunodominant region
IgG	Immunoglobulin G
M-tropic	Macrophage tropic HIV strains
mAbs	Monoclonal antibodies
MPER	Membrane-proximal external region
NHR	Amino-terminal heptad repeat region
PBS	Phosphate buffered saline
PR	Polar region
RNA	Ribonucleic acid

Introduction

Historical introduction

In the early 1980s five cases of opportunistic infections, such as pneumonia and candidiasis, were described among previously healthy young homosexual men in California (Gottlieb, 2006). The disease, which was associated with rapid depletion of $CD4^+$ T lymphocytes, was defined as the "Aquired Immune Deficiency Syndrome" (AIDS) by the Centers for Disease Control and Prevention (CDC)(Gottlieb et al., 1981; Hymes et al., 1981; Masur et al., 1981; Siegal et al., 1981). In 1983 Françoise Barré-Sinoussi together with Luc Montagnier isolated the causative agent of AIDS from infected patients (Barresinoussi et al., 1983; Gallo et al., 1983; Klatzmann et al., 1984a), which was later termed the human immunodeficiency virus type 1 (HIV-1) (Coffin et al., 1986; Ratner et al., 1985).

HIV-1 components and life cycle

HIV-1 is a (+) single stranded RNA virus, which belongs to the Lentivirus genus of the *Retroviridae* family. *Retroviridae,* which are enveloped by a host-derived lipid membrane, encode three major structural genes: gag, pol, and env. The Gag-Pol precursor is processed by the viral protease into: protease, reverse transcriptase, integrase, matrix, capsid and nucleocapsid proteins. The Env precursor gp160 is cleaved into a gp120 exterior subunit and a gp41 transmembrane subunit by a host furin protease (Moulard and Decroly, 2000; Wyatt and Sodroski, 1998). In addition, three regulatory proteins and four accessory proteins are encoded in HIV-1 (reviewed in (Peterlin and Trono, 2003)).

Most HIV-1 infections occur by sexual exposure through the genital tract or rectal mucosa. Cell-free virus enters target cells upon binding of its trimeric envelope spike (gp160) to human CD4 (Klatzmann et al., 1984b) and subsequently to a co-receptor. Several chemokine co-receptors including human CXCR4 (Feng et al., 1996) and human CCR5 (Deng et al., 1996; Dragic et al., 1996; Trkola et al., 1996a) have been identified. CCR5 is of particular interest since it is used by M-tropic HIV isolates for transmission and establishing an infection (Spijkerman et al., 1995; van't Wout et al., 1994). Furthermore, a homozygous deletion in the CCR5 allele confers resistance against HIV-1

acquisition (Huang et al., 1996; Liu et al., 1996) and can also lead to long-term control of HIV after stem cell transplantation (Hutter et al., 2009). Upon fusion, the viral RNA is released in the cytosplasm and reverse transcribed into double-stranded complementary DNA, which is integrated into the host chromosome by the viral integrase. Viral transcripts are expressed and transported to the plasma membrane for viral assembly and release from the infected cell (reviewed in (Ganser-Pornillos et al., 2008)).

Antibody response against the envelope glycoprotein of HIV-1

Neutralizing antibodies contribute to protective effects upon successful vaccination, and thus it is believed that they will be essential for the development of an effective HIV-1 vaccine (Karlsson Hedestam et al., 2008; Plotkin, 2008; Stamatatos et al., 2009).

Soon after HIV's discovery it became evident, that the envelope glycoprotein (Env) is the only virally encoded surface protein (Wyatt et al., 1998). The functional envelope spike consists of a trimer, which is formed by heterodimers of gp41 that are non-covalently linked to gp120 (reviewed in (Burton et al., 2005)). Several regions of gp120 and gp41 are crucial for virus function and subsequently immune protection. The highly conserved CD4 binding site (CD4bs) of gp120 forms a binding pocket for initial viral attachment to CD4. This attachment leads to conformational changes in the bridging sheet of gp120 (also known as the CD4 induced site, CD4is) allowing for the subsequent interaction with chemokine receptors. On gp41 the membrane-proximal external region (MPER), the immunodominant region, the heptad repeat regions and the fusion peptides are of particular interest for viral fusion with its target cell (reviewed in (Zolla-Pazner, 2004)).

Early findings by Robin Weiss and Marjorie Robert-Guroff et al. showed that sera of HIV-infected patients show neutralizing activity *in vitro* (Robert-Guroff et al., 1985; Weiss et al., 1985). Historically, the only known epitopes, which are targeted by broadly neutralizing monoclonal antibodies, were the MPER, the CD4bs and mannose residues on gp120 (Trkola et al., 1995). Human IgG b12 was the first broadly reactive neutralizing human monoclonal antibody, which was generated using phage display technique and targets the CD4bs (Burton et al., 1991; Burton et al., 1994; Scanlan et al., 2002). 2F5 and

4E10 are human monoclonal antibodies that were generated by electrofusion and target the MPER region of gp41 (Buchacher et al., 1994; Zwick et al., 2001). 2G12 is a monoclonal antibody that was also generated by electrofusion and targets a cluster of carbohydrate moieties on gp120 (Buchacher et al., 1994; Trkola et al., 1996b). Even though these antibodies broadly neutralize HIV-1 *in vitro* (Figure 1 left), they are only single examples and have not been re-isolated from other individuals, nor have they been induced by immunization. Importantly, the reasons why some individuals develop broadly neutralizing antibodies are unclear, but it is most likely related to the duration of infection suggesting the necessity of persistent viral stimulation for their generation (Gray et al., 2011; Sather et al., 2009).

Basic understanding of the humoral immune response targeting the HIV envelope trimer further advanced over the last few years. The development of single Env-specific B cell sorting, high-throughput micro neutralization assays and deep sequencing enabled the cloning of novel broadly and potently neutralizing antibodies (Figure 1 right). For instance, PG9 and PG16 were cloned after direct functional screening of antibody-containing tissue culture supernatants from activated memory B cells from an HIV-infected African donor (Walker et al., 2009). They target a glycosensitive epitope on the trimeric envelope protein, which spans variable loop V1/V2 (Walker et al., 2009). PGT128 was also cloned after direct functional screening of antibody-containing tissue culture supernatants. It targets high-mannose glycans in proximity to the gp120 V3 loop (Walker et al., 2011). HJ16 was isolated from memory B cells using EBV immortalization (Corti et al., 2010). It recognizes a novel epitope in proximity to the CD4bs (Corti et al., 2010). VRC01 was isolated using an antigen-specific memory B cell sorting technique (Scheid et al., 2009a; Scheid et al., 2009b) utilizing a resurfaced glycoprotein specific for the CD4bs (Wu et al., 2010). Sorting was followed by single-cell polymerase chain reaction on cDNA to amplify immunoglobulin G genes for recombinant protein expression. VRC01 binding mimics CD4 as antagonist, inducing a conformational change allowing for exposure of the co-receptor binding region (Wu et al., 2010; Wu et al., 2011; Zhou et al., 2010). Developing a new primer set to complement for a high degree of somatic mutations increased recovery of

immunoglobulin G genes and led to the discovery of 3BNC117 and NIH45-46 (Diskin et al., 2011; Scheid et al., 2011). Both 3BNC117 and NIH45-46 showed similar binding patterns to VRC01 and resemble CD4 binding in that they cause a conformational change leading to the exposure of the CD4is (Scheid et al., 2011).

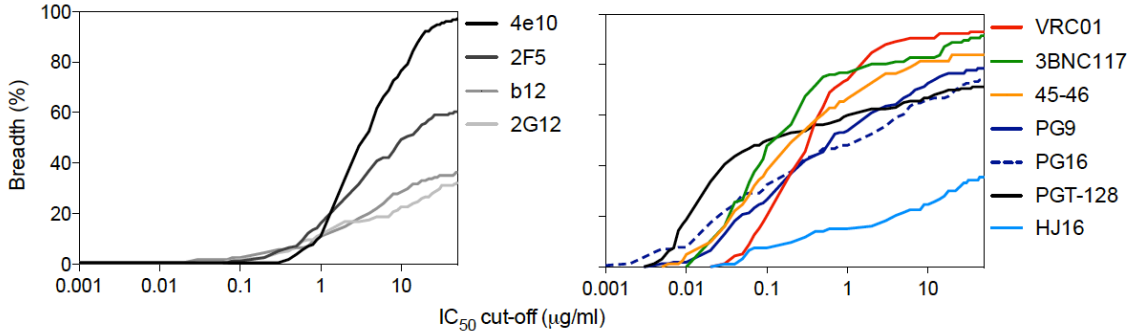

Figure 1: Neutralizing breadth and potency of anti HIV-1 Env monoclonal antibodies. Cumulative frequency distribution of IC_{50} values (µg/ml) of broadly neutralizing antibodies tested against a virus panel. The y-axis shows the cumulative frequency (breadth) of the IC_{50} values up to the concentration shown on the x-axis (potency). (left) shows the traditional broadly neutralizing monoclonal antibodies 4e10, 2F5, b12 and 2G12. (right) shows the most recently cloned broadly neutralizing antibodies VRC01, 3BNC117, 45-46, PG9, PG16, PGT-128 and HJ16. To generate the curves, the neutralizing raw values have been derived from the individual papers (Corti et al., 2010; Diskin et al., 2011; Scheid et al., 2011; Walker et al., 2011; Walker et al., 2009; Wu et al., 2010; Wu et al., 2011; Zhou et al., 2010).

HIV-1 continues to represent a major global health problem, with the development of an effectively protecting vaccine being of paramount importance. Solving this massive challenge demands further understanding of the biology of the virus, and its interaction with the immune system. A better understanding of epitopes targeted by monoclonal antibodies elicited in HIV-infected individuals, as well as obtaining information on how certain antibodies mediate protective effects *in vivo* may contribute to the development of an antibody-based HIV-1 vaccine.

Aims

The overall aim of this thesis was to characterize the HIV-1 Env-specific B cell response in HIV-1 infected individuals. The specific aims of the individual papers were:

I To characterize the monoclonal gp41-specific B cell response from HIV-infected patients with broadly neutralizing serologic activity.

II To define a new epitope which is frequently targeted by human anti-HIV neutralizing antibodies and to prove the hypothesis that it is relevant for viral fitness.

III To develop a mouse model to study HIV-1 entry and blocking by human neutralizing antibodies *in vivo*.

Results and Discussion

The materials and methods used in this thesis are described in detail in the attached papers.

I Anti-gp41 antibodies cloned from HIV-infected patients with broadly neutralizing serologic activity (Pietzsch et al., 2010b)

The HIV envelope glycoprotein gp41 is highly immunogenic (Barin et al., 1985) with an early onset of free plasma antibodies specific for gp41 being characteristic for an acute HIV-1 infection (Tomaras et al., 2008). However, little is known about the monoclonal anti-gp41 memory B cell response in individuals with high titers of broadly neutralizing serologic activity. In order to characterize the gp41-specific B cell response from HIV-infected patients with variable viral loads and high titers of broadly neutralizing antibodies, we single cell sorted their memory B cells with gp140 and cloned their antibodies (Scheid et al., 2009a; Scheid et al., 2009b).

To determine whether anti-gp41 antibodies contribute to the broadly neutralizing serologic activity in those patients, we adsorbed purified serum IgG on recombinant gp41. Testing the gp41-depleted ($gp41^{neg}$) and gp41-binding ($gp41^{pos}$) fractions in *in vitro* neutralization assays and comparing their activity to unfractionated IgG, revealed that anti-gp41 antibodies did not contribute to the neutralizing activity of the patient sera studied. These results are consistent with previous reports showing that broadly neutralizing serologic activity is usually not mediated by anti-gp41 antibodies (Opalka et al., 2004; Tomaras et al., 2008), with the exception of rare antibodies targeting the membrane-proximal external region (MPER) (Gray et al., 2007).

Next, we wanted to characterize the epitopes targeted by the monoclonal anti-gp41 antibodies derived from these patients. The gp41 ectodomain consists of the following regions: the N-terminal fusion peptide (FP), the polar region (PR), the amino-

terminal and carboxy-terminal heptad repeat regions (NHR and CHR, respectively), the immunodominant region (ID), and the MPER (Figure 2a) (Earl et al., 1997).

We performed competition experiments with well-characterized anti-gp41 antibodies that target clusters I to VI on the gp41 ectodomain (Figure 2b) (Earl et al., 1997; Scheid et al., 2009a).

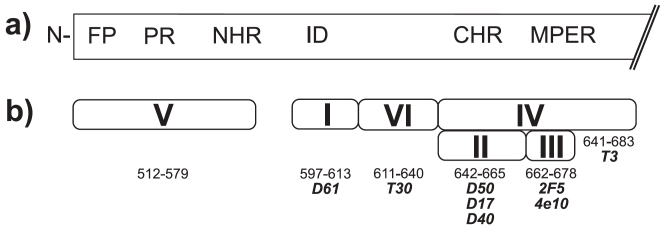

Figure 2: HIV-1 gp41. (a) Diagrammatic scheme of the gp41 ectodomain with the fusion peptide (FP), the polar region (PR), the amino-terminal and carboxy-terminal heptad repeat regions (NHR and CHR, respectively), the immunodominant region (ID), and the MPER (Earl et al., 1997). **(b)** The clusters I to VI are indicated. The amino acid residues (LAI sequence) are shown below the different clusters. Antibody standards used in competition ELISAs are shown in italics.

We found that anti-cluster I, -cluster II, -cluster IV, and -cluster VI antibodies accounted for all of the anti-gp41 binding by monoclonal antibodies isolated from patients with broadly neutralizing serologic activity (Figure 3). Notably, the cluster distribution of anti-gp41 antibodies varied between patients. When all patients were combined, the largest number of unique B cell clones was directed to cluster IV (53%), followed by cluster II (49%) and cluster I (32%).

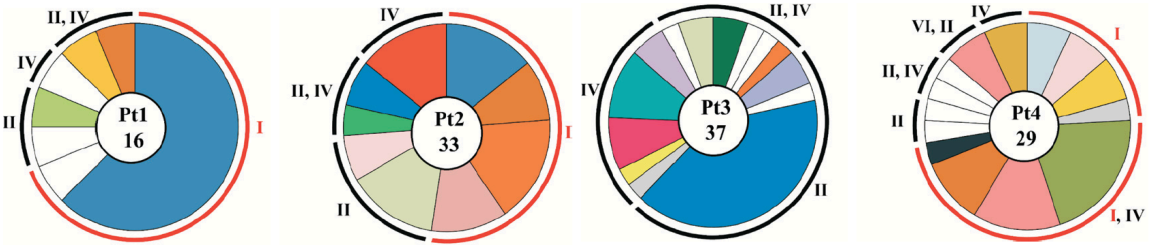

Figure 3: Clonal expansion and cluster distribution of anti-gp41 antibodies. Pie charts show the distribution of anti-gp41 antibodies to the different clusters (I, II, IV, and VI). Patients (Pt1 to Pt4) are indicated. The number in the center indicates the number of antibodies; slices are proportional to the size of the unique clones (Scheid et al., 2009a). The expanded cluster I antibodies are highlighted in red.

Previous reports showed that none of the cloned anti-gp41 antibodies showed neutralizing activity at concentrations up to 50 μg/ml (IC_{50}) (Scheid et al., 2009a). To investigate whether any of the anti-gp41 antibodies showed neutralizing activity at higher concentrations, we performed *in vitro* neutralization screenings with antibody concentrations of up to 2,380 μg/ml against two difficult to neutralize tier 2 viruses (TRO.11 and RHPA4259.7). We found that only some cluster I monoclonal antibodies showed neutralizing activity and only at very high concentrations ranging from 433 to 1,712 μg/ml (IC_{50}).

We conclude that anti-gp41 antibodies cloned from patients with broadly neutralizing serologic activity and variable viral loads did not account for the patients' neutralizing serologic activity. In the patients studied, we found that monoclonal antibodies target cluster I, II, IV, and VI; but none recognized the fusion peptide, the polar region, the N-terminal heptad repeat or the MPER peptide. Investigating the neutralizing activity of the monoclonal antibodies revealed that only a fraction of anti-gp41 cluster I antibodies reduced viral infection *in vitro* and only at very high concentrations.

II Human anti-HIV neutralizing antibodies frequently target a conserved epitope essential for viral fitness (Pietzsch et al., 2010a)

To characterize the human antibody response to the HIV-1 envelope glycoprotein, we cloned 502 anti-HIV-1 gp140 antibodies from the memory B cell compartment of six individuals with broadly neutralizing antibodies and variable viral loads (Scheid et al., 2009a). We found that in these patients, the memory B cell response to gp140 is composed of high affinity antibodies binding to the gp120 variable loops, the CD4 binding site (CD4bs), the CD4 induced site (CD4is), several epitopes on gp41 (Pietzsch et al., 2010b; Scheid et al., 2009a; Scheid et al., 2009b). In addition to these antibodies, we found a group of antibodies, termed "core", that bound gp120, $gp120_{core}$ (a mutant that lacks variable loops V1-V3; (Kwong et al., 1998)), $gp120_{D368R}$ (a mutant that interferes with the binding of CD4bs antibodies and soluble CD4; (Li et al., 2007; Olshevsky et al., 1990; Pantophlet et al., 2003; Thali et al., 1991)), and $gp120_{I420R}$ (a mutant that interferes with the binding of CD4is antibodies; (Thali et al., 1993)) (Scheid et al., 2009a). Furthermore, anti-CD4bs and some anti-CD4is antibodies inhibit the binding of anti-core antibodies, suggesting that anti-core antibodies recognize an epitope in vicinity of the CD4bs and CD4is (Scheid et al., 2009a).

In order to fine map the epitope recognized by anti-core antibodies, we assayed all cloned anti-core antibodies for binding to a set of HIV_{YU-2} gp120 alanine mutants by ELISA. Control antibodies included the anti-CD4bs antibody b12 (Burton et al., 1991; Burton et al., 1994; Scanlan et al., 2002) and the anti-variable-loop antibody 1-79 (Scheid et al., 2009a). Mutations that caused a reduction of antibody binding to 60% or less when compared with binding to wild type gp120 protein were considered significant. Alanine-mutated residues were initially spread across gp120 and later refined based on initial binding results. Assaying 72 alanine mutations, we found three residues (D474, M475, and R476) that inhibited the binding of anti-core antibodies, but had no effect on b12 binding, when mutated to alanine (Figure 4). These residues cover a stretch from a direct CD4 contact residue (D474) up to the α5 helix at the outer-domain/inner-domain junction of gp120 (M475, R476).

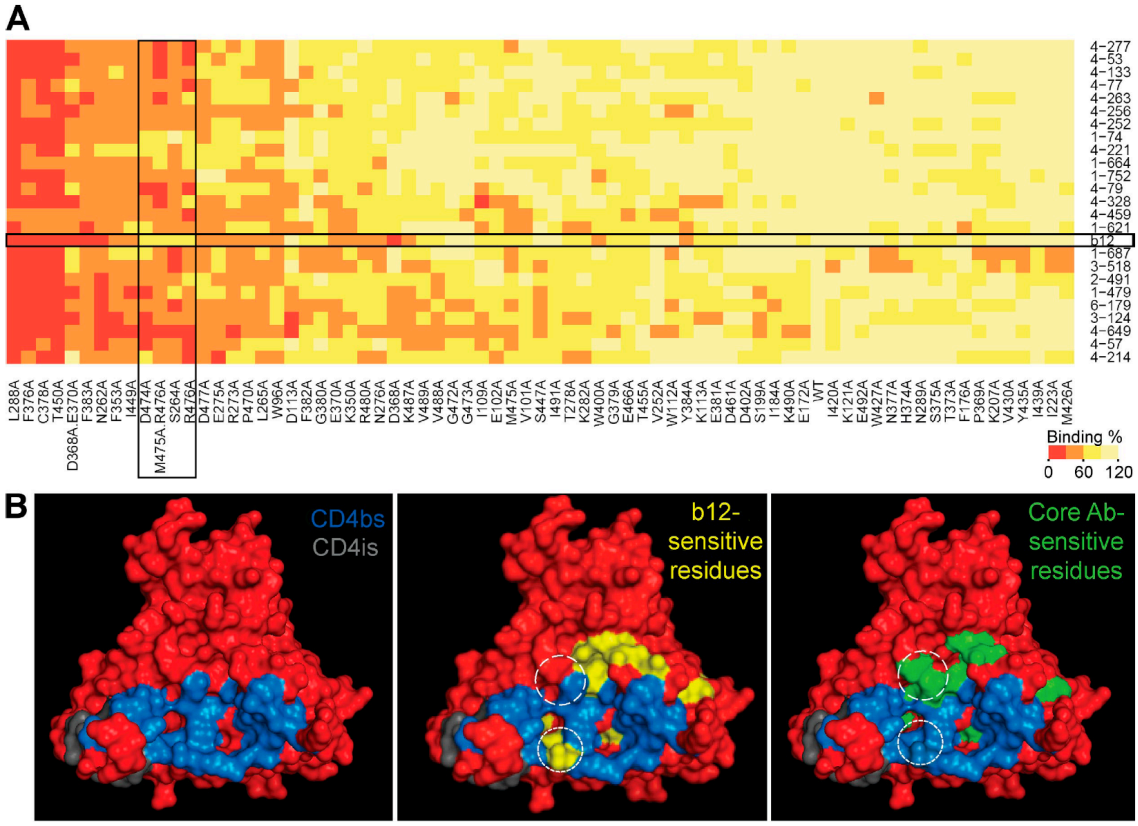

Figure 4: Mapping of the HIV-1 gp120 core epitope. (A) Heat map summarizes the binding of the different anti-core antibodies and b12 to gp120 alanine mutants. Red and orange fields indicate <60% binding, whereas yellow shows no difference compared with the wild type gp120 control. **(B)** Surface diagram of gp120 (PDB ID: 3DNO; (Liu et al., 2008)) showing the CD4bs in blue, the CD4is in gray, the b12 binding sites in yellow, and the core-sensitive residues in green. Residues that distinguished between the anti-CD4bs epitope and anti-core epitope are highlighted with circles.

To confirm the initial mapping studies, which were performed on HIV_{YU-2} gp120, we produced recombinant trimeric HIV_{BaL} gp140 and corresponding core epitope mutant HIV_{BaL} $gp140_{D474A/M475A/R476A}$ and performed ELISA assays and surface plasmon resonance studies (SPR) with two randomly selected anti-core antibodies (4-79 and HGS2 (Corti et al., 2010)), and controls (b12 (Burton et al., 1991) and a variable loop-specific antibody 2-1092 (Scheid et al., 2009a)). We found that the control antibodies retained their high-affinity binding to HIV_{BaL} $gp140_{D474A/M475A/R476A}$, whereas binding of the two anti-core antibodies tested (4-79 and HSG2) was greatly reduced when measured by ELISA and undetectable when measured by SPR (Figure 5).

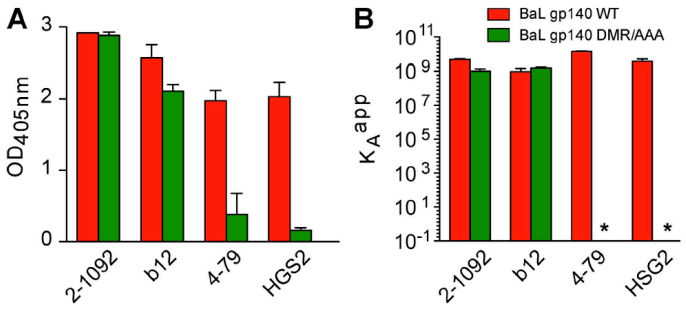

Figure 5: Binding to soluble HIV-1 gp140 wild type and core mutant. (A) Graphs show optical density at 405 nm (OD_{405nm}) for the selected IgG antibodies as measured by capture ELISA with purified HIV_{BaL} gp140 wild type and HIV_{BaL} $gp140_{D474A/M475A/R476A}$. Error bars represent the SD from at least two independent experiments. **(B)** Graphs show apparent K_A (K_A^{app}, M^{-1}) for the selected IgG antibodies as measured by surface plasmon resonance (SPR) on chips derivatized with HIV_{BaL} gp140 wild type and HIV_{BaL} $gp140_{D474A/M475AR/476A}$. Error bars represent the SEM from at least two independent experiments. * indicates that no binding to HIV_{BaL} $gp140_{D474A/M475AR/476A}$ was detected.

Given the fact that most anti-core antibodies cloned from different patients recognize a common epitope, we asked whether D474, M475, and R476 might be conserved across different HIV-1 strains. We analyzed 1,963 sequences covering all clades (QuickAlign; http://www.hiv.lanl.gov) and found that 56% contained all three of these residues and 35% showed strong homology.

To investigate the impact of D474/M475/R476 in viral fusion we developed an assay to measure fusion between BOSC.23 cells co-expressing cell-surface HIV_{BaL} gp160 trimer lacking the cytoplasmic tail (gp160Δc) and GFP, and TZM.bl cells co-expressing mCherry and HIV-1 entry factors (Seaman et al., 2010; Wei et al., 2002). Fusion between TZM.bl and BOSC.23 cells expressing the gp160Δc trimer was readily detected by flow cytometry. In contrast, there was little fusion of cells expressing the mutant trimer HIV_{BaL} $gp160\Delta c_{D474A/M475A/R476A}$ ($p=0.0078$; unpaired one-tailed t test; Figure 6a). Fusion events were confirmed by image-based flow cytometry (ImageStream) (Figure 6b).

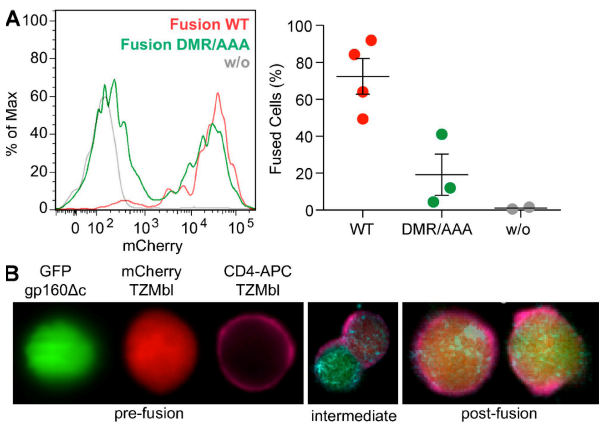

Figure 6: Fusion (A) (left) Histogram plots summarize fusion between BOSC.23 cells expressing GFP (in grey) or HIV_{BaL} gp160Δc (in red) or HIV_{BaL} $gp160\Delta c_{D474A/M475A/R476A}$ (in green) with mCherry expressing TZM.bl cells. (right) Fusion events (%) with mean and SEM are shown. (B) Shows image-based analysis of fusion events. (left) Single staining compensation controls for BOSC.23 cells expressing HIV_{BaL} gp160Δc and GFP, mCherry expressing TZM.bl cells and CD4-APC stained TZM.bl cells. (middle) Shows an intermediate fusion event. (right) Shows the colocalization of the GFP-mCherry-APC signal after fusion.

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We conclude that anti-core antibodies recognize a previously unknown conformational epitope (D474/M475/R476) in vicinity of the CD4bs that includes the α5 helix at the outer-domain-inner-domain junction of gp120. The core epitope is highly conserved across different HIV-1 isolates and is indispensible for optimal viral fusion, and hence viral fitness. Thus, the core epitope, a frequent target of anti-HIV-1 neutralizing antibodies cloned from patients with broadly neutralizing serologic activity should be included as a target for vaccine design.

III A mouse model for HIV-1 entry (Pietzsch et al., 2012)

Rapid progress in the development of an effective vaccine against HIV-1 infection has been hindered in part by the lack of a widely available small animal model. Current animal models, which include rhesus macaques and immunodeficient humanized mice, are neither readily available nor amenable to genetic modifications (Boberg et al., 2008; Denton and Garcia, 2009).

HIV-1's entry into target cells is mediated by binding of the trimeric envelope spike (gp160) to human CD4 (hCD4) (Klatzmann et al., 1984b) and a coreceptor. Several chemokine receptors including CXCR4 (Feng et al., 1996) and CCR5 (Deng et al., 1996; Dragic et al., 1996; Trkola et al., 1996a) have been identified and serve as coreceptors for HIV infection. CCR5 is of particular interest since it is used by M-tropic HIV isolates for transmission and establishing an infection (Spijkerman et al., 1995; van't Wout et al., 1994).

Here, we describe a hCCR5- and hCD4-expressing, luciferase reporter mouse that can be used to measure HIV-1 pseudovirus entry and to study antibody-mediated protection against initial infection *in vivo*.

Co-expresion of hCCR5 and hCD4 was achieved by linking the two proteins on a single polyprotein transcript separated by a ribosomal skip 2A peptide sequence (Szymczak et al., 2004). To express high levels of the HIV-1 entry factors *in vivo*, we generated a recombinant human adenovirus (AdV) encoding hCCR5-2A-hCD4 and administered this vector (AdV-hCCR5-2A-hCD4) intravenously (i.v.), which resulted in strong hepatic expression of hCCR5 and hCD4 as evidenced by fluorescence microscopy (Figure 7) (Berkner, 1988; He et al., 1998).

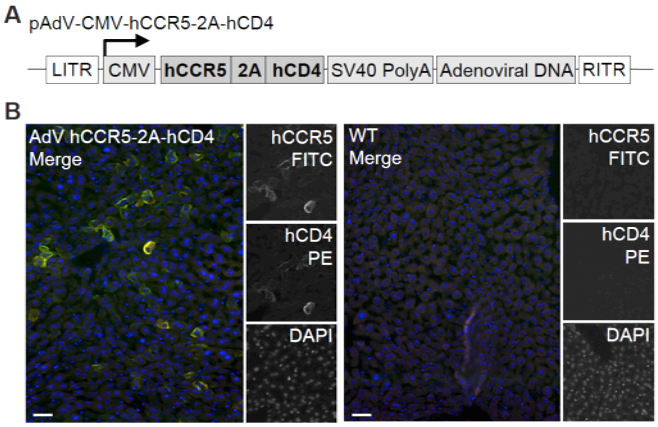

Figure 7: Adenoviral delivery of AdV-hCCR5-2A-hCD4. (A) Schematic diagram of the adenovirus construct used to deliver hCCR5-2A-hCD4. hCCR5-2A-hCD4 are under control of the CMV promoter. (B) hCCR5 and hCD4 were detected in fixed frozen liver sections by immunofluorescence microscopy one day after delivery of 10^{11} adenoviral particles of AdV-hCCR5-2A-hCD4 through the lateral tail vein. Scale bar depicts 30µm.

To determine whether hCCR5 and hCD4 expressing mice can be used to measure HIV-1 entry *in vivo*, we transduced mice that carry an inducible loxP-STOP-loxP luciferase reporter (Gt(ROSA)26Sor$^{tm1(Luc)Kaelin}$; hereafter termed ROSA^{flox}STOP-Luc) (Dorner et al., 2011; Safran et al., 2003) with AdV-hCCR5-2A-hCD4. One day later, mice were challenged i.v. with gp160$_{YU-2}$ expressing pseudovirus (HIV$_{YU-2}$) encoding Cre recombinase. Productive viral entry by hCCR5/ hCD4 positive cells would result in the expression of Cre recombinase capable of excising the transcriptional stop element and consequently inducing luciferase expression. Bioluminescence activity, imaged in an optical luminometer (IVIS), increased longitudinally and peaked between day 4 and 5 after i.v. pseudovirus injection, compared to control mice (Figure 8). We conclude that HIV pseudovirus entry can be measured quantitatively in living mice expressing hCCR5 and hCD4.

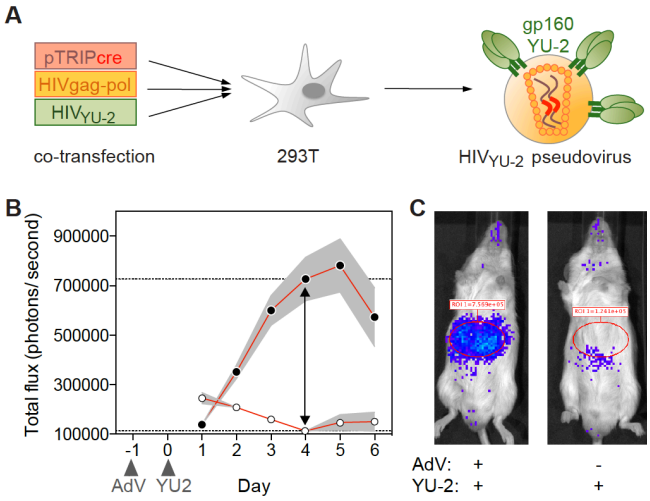

Figure 8: HIV$_{YU-2}$ pseudovirus entry *in vivo*. (A) Diagram summarizes method for producing HIV$_{YU-2}$ pseudovirus. (B-C) ROSA^{flox}STOP-Luc mice were injected with AdV-hCCR5-2A-hCD4 at day -1, followed by HIV$_{YU-2}$ injection at day 0. Bioluminescence was acquired longitudinally in (B) and at day 4 in (C). (B) Graph shows the mean total photon flux (± SEM) for n=6 infected mice (black circles) versus controls that did not receive the AdV-hCCR5-2A-hCD4 virus, n=3 mice (empty circles). (C) Representative images of mice from (B).

Next, we wanted to investigate whether antibodies are able to block HIV-1 entry in this mouse model. We selected 6 potent broadly neutralizing anti-HIV antibodies to examine their effects on HIV entry *in vivo*: NIH45-46^{G54W} (Diskin et al., 2011), 3BNC117 (Scheid et al., 2011), 3BNC60 (Scheid et al., 2011), VRC01 (Zhou et al., 2010), b12 (Burton et al., 1994) and PG16 (Walker et al., 2009). The selected antibodies show various degrees of *in vitro* neutralizing activity (IC_{50}) in the TZM-bl cell assay against HIV_{YU-2} ranging from 0.01 to 2.30 μg/ml (Figure 9A). Antibodies were administered individually at doses ranging from 1μg to 200μg subcutaneously one day before challenge with HIV_{YU-2} and luciferase expression was measured 4 days later. We determined the serum antibody levels at the time point of pseudovirus injection by ELISA and found that they varied among the selected monoclonal antibodies (Figure 9B). In contrast to the HIV neutralizing antibodies that blocked entry with broad degrees of efficacy, an isotype control antibody (mGO53) (Wardemann et al., 2003) had no significant effect on entry compared to the PBS control (Figure 9C). Fifty percent inhibition of entry was achieved after injection of 100-200μg of VRC01 or PG16 or b12, while the same level of inhibition was obtained with as little as 4-6μg of 3BNC117 or 3BNC60 or NIH45-46^{G54W} (Figure 9D). We conclude that an antibody's ability to block HIV-1 entry *in vivo* can be measured directly in HIV-LUC_{AdV} mice.

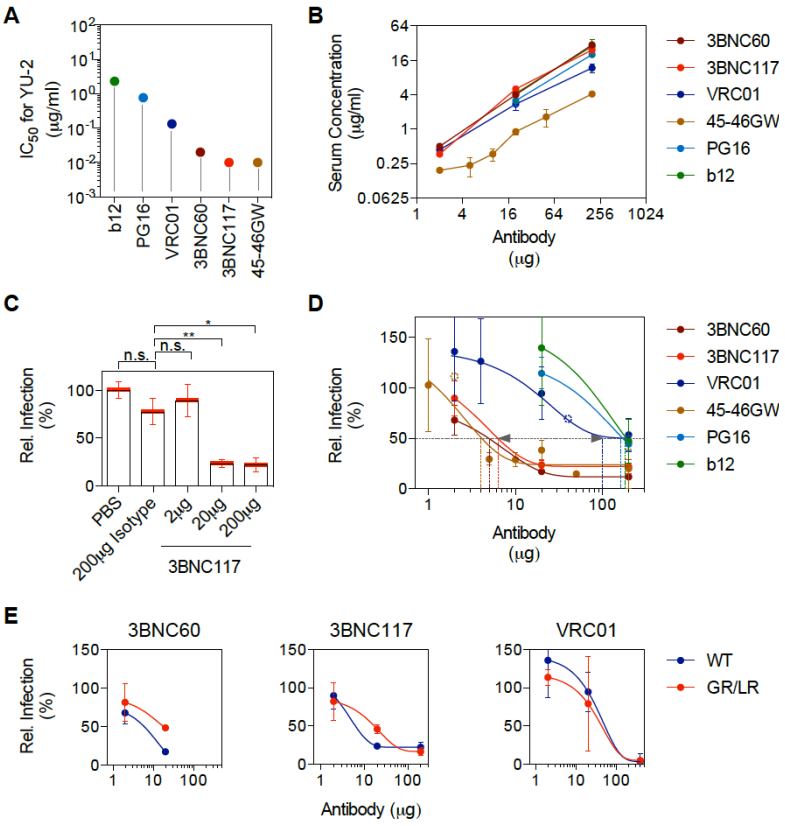

Figure 9: *In vitro* and *in vivo* neutralization data. (A) Broadly neutralizing monoclonal antibodies were tested in the TZM-bl assay for neutralizing activity against HIV_{YU-2}. Plot shows the IC_{50} (µg/ml) against HIV_{YU-2} for the individual monoclonal antibodies. (B) The serum antibody concentration at the time point of pseudovirus injection was determined by ELISA. The injected antibody amount (µg) is plotted against the determined serum concentration of the monoclonal antibody (µg/ml). (C-D) Relative infection (% compared to PBS control) plotted against the amount of antibody injected (µg) in bar graph (C; six to seventeen mice per group, mean ± SEM; *p<0.05; **p<0.01) or dose response curves (D; three to nine mice per group, except empty circles represent a single mouse, mean ± SEM). (E) Comparison of the *in vivo* neutralizing activity of the WT form and GR/LR mutant of 3BNC60, 3BNC117 and VRC01 (three to nine mice per group, mean ± SEM).

To examine the role of antibody effector functions in protection against HIV entry in this system, we introduced mutations (G236R/L328R) into the antibody Fc domain that eliminated binding to mFcγRs and complement. Introducing these mutations did not alter antibody binding to the HIV envelope protein or neutralizing activity *in vitro*. Dose response experiments were performed to compare the *in vivo* activity of $3BNC60^{GR/LR}$, $3BNC117^{GR/LR}$ and $VRC01^{GR/LR}$ to wild type controls. Mutant $3BNC60^{GR/LR}$ and $3BNC117^{GR/LR}$ showed decreased activity at doses of 20 µg of injected antibody when compared to the wild type form ($p=0.0027$ and $p=0.0044$ respectively, Figure 9E). Though statistically significant, the differences were small and not found at higher or lower doses of 3BNC60 or 3BNC117 or at any tested dose of VRC01. We conclude that antibody Fc effector functions appear to make a contribution to the inhibition of HIV-1 entry, however additional studies will be necessary to fully evaluate this component.

Discussion and Future Directions

Broadly protective antibodies against HIV-1 Env are present in the human B-cell repertoire of some infected individuals. However, inducing these types of antibodies by active immunization has not been possible so far. A major future challenge is to understand how to build immunogens capable of stimulating B cells to make these antibodies. Equally important, immunogens inducing non-neutralizing antibodies should be excluded as far as possible while retaining immunogenicity. Knowing the epitopes that are targeted by human anti HIV-1 Env antibodies could support the design of such immunogens.

We dissected the memory B cell response against gp41 in six patients with broadly neutralizing serologic activity and found that anti-gp41 antibodies target six different antigenic clusters on the gp41 ectodomain without significant immunodominance. None of the cloned antibodies showed neutralizing activity at physiologic concentrations. Thus we conclude that a gp41 immunogen would most likely not induce any neutralizing antibodies and should be excluded from further pre-clinical and clinical trials (aim one, (Pietzsch et al., 2010b)). In support of this idea, the recent RV144 vaccine trial excludes gp41 components from the vaccine regimen, focusing only on gp120 antigens (Rerks-Ngarm et al., 2009).

Analyzing the memory B cell response against gp120 in six patients with broadly neutralizing serologic activity revealed that 32% of all HIV-neutralizing antibodies recognize a single conserved immunodominant epitope (‘core’), which is essential for viral infectivity. We conclude that the ‘core’ epitope should be considered as one target for vaccine design (aim two, (Pietzsch et al., 2010a)). A recent study characterizing the neutralizing phenotype of circulating viruses highlights, that anti-core antibodies but not broadly neutralizing CD4bs antibodies are able to neutralize a viral clone isolated 22 years post infection (Sather et al., 2012).

Little is known about the protective activity of broadly neutralizing antibodies *in vivo*, mainly due to the lack of an easily accessible small animal model for HIV-1 entry. We show that a hCCR5- and hCD4-expressing, luciferase reporter mouse can be used to

measure HIV-1 pseudovirus entry and antibody-mediated protection against initial infection *in vivo*. We conclude that an antibody's ability to block viral entry *in vivo* is a function of its bioavailability, its direct neutralizing activity and its effector functions. Our results suggesting an involvement of Fc effector functions in protection are in agreement with the macaque SHIV model (Hessell et al., 2007; Hessell et al., 2009a), but extend those findings by revealing the importance of the antibody Fc region on pre-entry protection against HIV-1 *in vivo* (aim three, (Pietzsch et al., 2012)). This HIV-LUC_{AdV} mouse model may prove useful for pre-clinical testing of candidate vaccines. Alternatively, a luciferase reporter mouse expressing hCD4 and hCCR5 ubiquitously might allow for a more physiologic route of HIV-1 entry (Gruell and Pietzsch manuscript in preparation).

Besides neutralizing antibodies, which directly interfere with viral entry, also non-neutralizing antibodies might mediate protection (Hope, 2011; McElrath and Haynes, 2010). The recent RV144 Thai vaccine trial suggested an inverse correlation between IgG plasma concentrations of anti-HIV binding antibodies and infection risk (Haynes et al., 2012; Rerks-Ngarm et al., 2009). However, the mechanisms that mediate the protective effects in RV144 are poorly understood, since the vaccine assessed did not elicit broadly neutralizing antibodies. Non-neutralizing binding antibodies that mediate effector functions such as antibody-dependent cellular cytotoxicity, complement activation and phagocytosis might be responsible for the protective effects and should be investigated in future pre-clinical *in vivo* studies.

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Appendix – Original Publications

I Pietzsch J, Scheid JF, Mouquet H, Seaman MS, Broder CC, Nussenzweig MC. **Anti-gp41 antibodies cloned from HIV-infected patients with broadly neutralizing serologic activity.** *J Virol.* 2010 May;84(10):5032-42.

II Pietzsch J, Scheid JF, Mouquet H, Klein F, Seaman MS, Jankovic M, Corti D, Lanzavecchia A, Nussenzweig MC. **Human anti-HIV neutralizing antibodies frequently target a conserved epitope essential for viral fitness.** *J Exp Med.* 2010 Aug 30;207(9):1995-2002.

III Pietzsch J, Gruell H, Bournazos S, Donovan BM, Klein F, Diskin R, Seaman MS, Bjorkman PJ, Ravetch JV, Ploss A, Nussenzweig MC. **A mouse model for HIV-1 entry.** *PNAS.* Published online before print 2012 Sep 10, doi: 10.1073/pnas.1213409109

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Anti-gp41 Antibodies Cloned from HIV-Infected Patients with Broadly Neutralizing Serologic Activity

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Anti-gp41 Antibodies Cloned from HIV-Infected Patients with Broadly Neutralizing Serologic Activity▽

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Most HIV-infected individuals develop antibodies to the gp120 and gp41 components of the viral spike; however, only a fraction of these individuals mount a broadly neutralizing serum response against HIV. We have cloned anti-HIV antibodies from the memory B-cell compartment of six individuals with variable viral loads and high titers of broadly neutralizing antibodies. Here, we report on the features of the anti-gp41 response in these patients. Competition experiments with previously characterized antibodies targeting defined epitopes on the gp41 ectodomain showed antibodies directed against the "immunodominant region" (cluster I), the carboxy-terminal heptad repeat (cluster II), and the membrane-proximal external region (cluster IV). On the other hand, antibodies directed against the amino-terminal part of the molecule, including the fusion peptide, polar region, and the N-terminal heptad repeat, were not detected. When all patients' data were combined, unique B-cell clones targeting cluster I, II, and IV accounted for 32%, 49%, and 53% of all anti-gp41-reactive B cells, respectively; therefore, no single region was truly immunodominant. Finally, although we found no new neutralizing epitopes or HIV-1-neutralizing activity by any of the gp41 antibodies at concentrations of up to 50 μg/ml, high concentrations of 7 out of 15 anti-cluster I antibodies neutralized tier 2 viruses.

The trimeric envelope spike of the human immunodeficiency virus (HIV) consists of three heterodimers of the transmembrane glycoprotein (gp41) and the surface glycoprotein (gp120) (59). Whereas gp120 carries the CD4 and chemokine receptor binding sites, gp41 is crucial for fusion between the viral particle and the cell membrane (Fig. 1a). The glycine-rich fusion peptide, located at the amino-terminal region of gp41, is normally covered by gp120 but is transiently exposed for interaction with the target cell membrane when gp120 binds to its receptors (14). The fusion peptide is followed by a serine/threonine-rich polar region and heptad repeats, which form leucine zippers that mediate assembly of the coiled-coiled form of gp41 in response to gp120 engagement (8, 22, 24, 52). Finally, the membrane-proximal external region (MPER) also plays a role in virus-host membrane fusion (38); however, the mechanism by which it enhances fusion is not known.

Some regions of gp41 are accessible to antibodies on the native gp140 trimer; however, others are exposed to the immune system only after gp120 shedding (40). In addition, otherwise cryptic gp41 epitopes are uncovered during viral fusion with the cell membrane (13). Consistent with gp41 exposure to the immune system, serologic studies of infected individuals indicate that there is a strong humoral response to gp41 during HIV infection (35) which precedes the response against gp120 (26).

Antibodies to gp41 have been isolated from phage display libraries, as have Epstein-Barr virus (EBV) immortalized B cells from infected individuals (4, 53). Some of these anti-gp41 antibodies can neutralize HIV infection *in vitro* and interfere with the virus *in vivo* (4, 6, 50). However, there has yet to be a systematic study of the anti-gp41 memory B-cell response of individuals with high titers of broadly neutralizing anti-HIV antibodies.

In order to document the nature of the anti-gp41 antibody response in HIV-infected individuals with high titers of broadly neutralizing antibodies, we studied 131 such antibodies, accounting for 47 unique B-cell clones, which we obtained from the memory B-cell compartments of six patients with low-to-moderate HIV viral titers (43). Each unique clone was composed of up to 15 clonal members that were either identical or related by somatic mutation. The largest number of unique B-cell clones, 53%, was directed to a conformational epitope which neighbors the MPER (cluster IV), 49% were directed to the carboxy-terminal heptad repeat (cluster II), and 32% were directed to the previously identified "immunodominant region" (cluster I), of which 60% recognize a linear peptide (amino acids 579 to 604). Furthermore, B cells producing antibodies to this region comprise large expanded clones. In total, 57 out of the 131 anti-gp41 and 502 anti-gp140 antibodies cloned were directed to cluster I, some of which show tier 2 virus-neutralizing activity at high antibody concentrations.

MATERIALS AND METHODS

Plasma samples. The HIV-1-infected patients were part of the Elite Controller Study of the Partners Aids Research Center (patients 2, 3, and 5) and clinical

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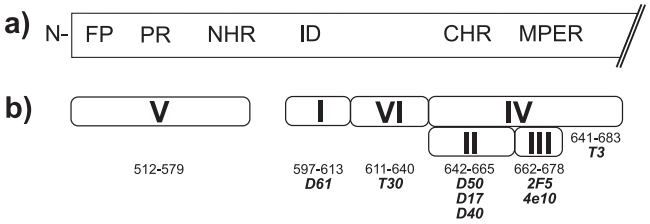

FIG. 1. HIV-1 gp41. (a) Diagrammatic representation of the gp41 ectodomain with the fusion peptide (FP), the polar region (PR), the amino-terminal and carboxy-terminal heptad repeat regions (NHR and CHR, respectively), the immunodominant region (ID), and the MPER (12). (b) The clusters I to VI are indicated. The amino acid residues (LAI sequence) are shown below the different clusters. Antibodies used in competition ELISAs are shown in italics.

protocols at the Aaron Diamond Research Center (patient 1) and the National Institute of Allergy and Infectious Diseases (patients 4 and 6). The uninfected volunteer (healthy control [HC]) was recruited at the Rockefeller University. All work with human samples was performed in accordance with approved Institutional Review Board protocols (43).

Monoclonal antibodies. Cloning of anti-human HIV-1 gp140 antibodies was performed as described previously (43, 46). All IgGs were expressed by cotransfection in HEK-293 cells (43, 46).

IgG adsorption and elution of gp41-positive IgG fractions. Biotinylated HIV-1 gp41 (Prospec) contained the full-length extracellular domain of strain IIIB (amino acids [aa] 513 to 674). To coat streptavidin-magnetic beads (Dynal M-280 Streptavidin; Invitrogen) with HIV-1 gp41, 10 mg of beads was incubated with 100 μg of protein at room temperature for 45 min on a shaking platform. IgG was purified, dialyzed against Dulbecco's phosphate-buffered saline (DPBS; Gibco) and incubated with the coated and washed beads for 1 h at a ratio of 10 mg of coated beads per 10 mg of IgG. Following magnetic bead removal, the IgG was adsorbed five more times to ensure maximal adsorption. Beads containing the gp41-positive IgG fraction were washed with DPBS (Gibco) three times before specific antibody elution with 0.1 M glycine buffer, pH 3, into 1 M Tris-HCl buffer, pH 8.

Competition ELISA. Specificity of HIV-1 gp41 binding antibodies was determined by competition enzyme-linked immunosorbent assays (ELISAs). Cloned anti-gp41 antibodies were biotinylated (EZ-Link Micro Sulfo-NHS-Biotinylation Kit; Pierce), and their specificities were initially determined by competition with previously characterized anti-gp41 cluster antibodies (Fig. 1b): D61 to cluster I amino acids 597 to 613 (12); D40, D17, and D50 to cluster II amino acids 642 to 665 (12); 4E10 and 2F5 to cluster III amino acids 662 to 678 (60); T3 to cluster IV amino acids 641 to 68 (12); and T30 to cluster VI amino acids 611 to 640 (12). The biotinylated antibodies 4E10 and 2F5 (cluster II), 2-378 and 2-55 (cluster I; the first number in designations of this type indicate the patient), 1-763 (cluster II and IV), and 3-255 (cluster IV) were then used in direct competition experiments to determine the properties of the remaining anti-gp41 antibodies.

ELISAs were performed with high-binding capacity ELISA plates (Costar) coated with 50 μl of gp41 (ectodomain aa 541 to 682; strain HxB2) (Acris, Herford) at 5 μg/ml in phosphate-buffered saline (PBS) overnight at room temperature. This protein preparation contains three major immunospecific bands migrating between 20 and 30 kDa, minor bands between 20 and 30 kDa, bands at 14 and 7 kDa, and an aggregation smear at 35 kDa and greater, as indicated by the manufacturer (Acris, Herford, Germany) (see Fig. 6a). Plates were washed three times with 200 μl of ultrapure water per well and incubated with 100 μl of blocking buffer (2 mM EDTA and 0.05% Tween-20 in PBS) for 30 min at room temperature and washed again. Biotinylated antibodies (0.8 μg/ml) were mixed with 4-fold serial dilutions of the competing antibodies starting at 10 μg/ml before they were applied to the coated ELISA plate. After 2 h of incubation at room temperature, plates were washed as described above. Bound biotinylated antibodies were detected with streptavidin-horseradish peroxidase (HRP) conjugate (AbD). The complex was detected with an HRP substrate kit (Bio-Rad) and measured at 405 nm to calculate the half-maximal (50%) inhibitory concentration (IC_{50}) of the unbiotinylated antibodies.

MPER peptide ELISA. Peptide ELISAs were performed as previously described (32). Briefly, ELISA plates (Costar) were coated with 50 μl of the peptide recognized by 2F5 (SQNQQEKNEQELLALDKWAS; underlining refers to the minimal epitope) or 4E10 (LWNWFDITKWLWYIKIFIMI) at 5 μg/ml in PBS and incubated overnight at room temperature. After plates were washed three times with PBS–0.1% Tween 20 (PBST), wells were blocked with PBS–1% Tween 20–5% sucrose–3% milk powder for 1 h at room temperature. Serial dilutions of human IgG (starting at 100 μg/ml in PBST–1% bovine serum albumin [BSA]) were added, and samples were incubated for 1 h at room temperature and visualized with peroxidase-conjugated affinity-purified goat anti-human IgG (Jackson) using an HRP substrate kit (Bio-Rad). Controls were HC IgG, polyclonal HIV-1 immune globulin (HIV IG), 2F5, and 4E10 (Polymun Scientific, Austria), each of which was included in every experiment.

Deglycosylation. To remove N-linked glycans, 50 μg of gp41 was treated with peptide *N*-glycosidase F (PNGase F; New England Biolabs) in 50 mM sodium phosphate at 37°C under nondenaturing conditions for 12 h. Deglycosylation was confirmed by band shift on an SDS-polyacrylamide (PA) gel with Coomassie blue staining and by lectin precipitation (agarose-bound *Lens culinaris* agglutinin; Vector Laboratories, Burlingame, CA), followed by elution with 0.5 M methyl α-D-mannopyranoside (Sigma) of the glycosylated, but not deglycosylated, gp41.

Neutralization assays. Neutralization assays were performed as described previously (27, 29). Briefly, neutralization was detected as a reduction in firefly luciferase reporter gene expression after infection of TZM-bl cells with HIV-1 envelope glycoprotein (Env) pseudovirus variants. Murine leukemia virus was used as a negative control to rule out nonspecific toxicity by the antibodies.

RESULTS

Serologic neutralizing activity. We cloned antibodies to gp140 from the memory B-cell compartment of six patients with variable viral loads and high titers of broadly neutralizing antibodies by staining cells with artificially trimerized gp140 (43). Although we did not find any monoclonal antibodies with broad neutralizing activity among the 502 cloned IgG antibodies to gp140, combinations of antibodies showed a breadth of neutralizing activity at high concentrations (43, 54). To determine whether antibodies to gp41 might contribute to the neutralizing activity in the serum of these patients, we adsorbed purified serum IgG on gp41. The depleted and gp41 binding fractions were tested for gp41 binding by ELISA, as well as for neutralizing activity on a previously described panel of tier 1 and tier 2 viruses (23). Anti-gp41 binding antibodies were efficiently depleted and recovered after adsorption on gp41 (Fig. 2a).

Unfractionated IgG neutralized HIV at concentrations ranging from 2 μg/ml up to 1,138 μg/ml (Table 1). For two of three patients studied, removal of the anti-gp41 binding antibodies resulted in a 2- to 3-fold increase in neutralizing potency; however, patient 3 showed a smaller increase and, for some viruses, a decrease in neutralizing activity after the removal of gp41 binding antibodies (Fig. 3). Consistent with the observation that anti-gp41 antibody depletion did little to alter the overall neutralizing activity, the purified anti-gp41 antibodies were significantly less active than unfractionated IgG in neutralization assays. We conclude that the anti-gp41 antibodies did not significantly contribute to the neutralizing activity of the patient sera studied. These findings are consistent with the observation that broad neutralizing activity in serum is not usually due to anti-gp41 antibodies (35, 48), with the exception of rare anti-MPER antibodies (18). However, since there are several distinct conformational states of gp41 during viral entry, including the prefusion, prehairpin intermediate, and postfusion conformations, we cannot rule out the possibility that not all anti-gp41 antibodies have been adsorbed and depleted by the soluble form of the protein used here.

Anti-gp41 binding epitopes. To characterize the epitopes recognized by the cloned anti-gp41 memory antibodies in the six patients with variable viral loads and high titers of broadly

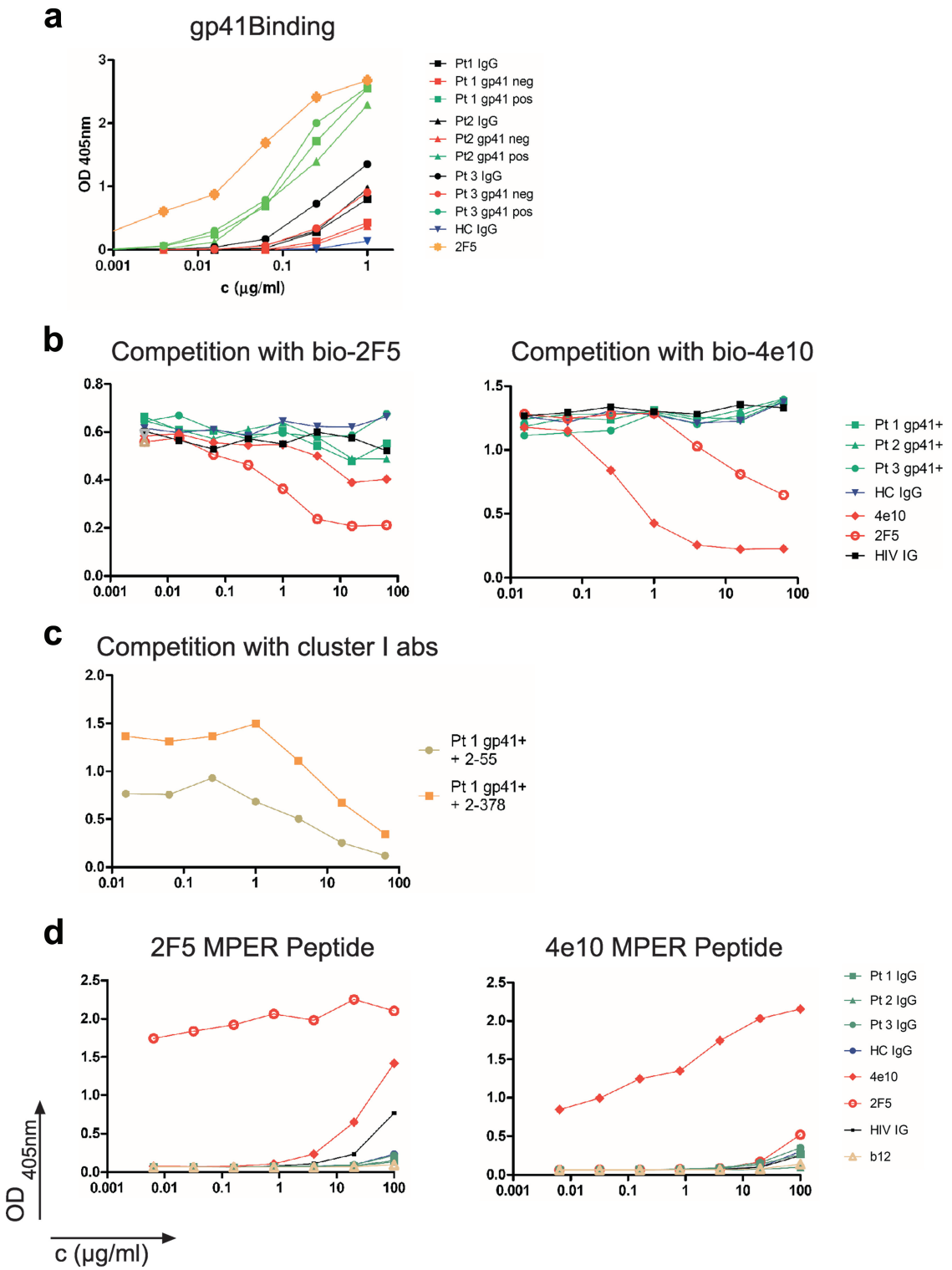

FIG. 2. gp41 ELISA. (a) The result of the IgG gp41 adsorption. The affinity-purified gp41 binding IgG fractions (pos) and the anti-gp41-depleted (neg) IgG fractions are shown for each of three different patients (Pt1 to Pt3), according to the legend on the figure. HC, healthy control; OD, optical density. (b) Graphs show lack of competition between the affinity-purified anti-gp41 serum IgG fractions and biotinylated 2F5 or 4E10. Controls are unbiotinylated 2F5 and 4E10 as well as HC IgG and HIV IG. (c) Competition between affinity-purified anti-gp41 serum IgG and anti-cluster I antibodies (2-55 and 2-378). (d) Results of the ELISAs with the 2F5 and 4E10 MPER-specific peptides. Fractions are colored as indicated on the figure.

TABLE 1. TZMbl neutralization data of gp41-adsorbed IgG

Patient and sample[a]	Neutralizing activity (IC_{50} [μg/ml])[b]							
	Tier 1 virus (clade)					Tier 2 virus (clade)		
	MW965.26 (C)	BaL.26 (B)	SS1196.1 (B)	SF162.LS (B)	DJ263.8 (A)	6535.3 (B)	RHPA4259.7 (B)	TRO.11 (B)
Patient 1								
IgG	**14.1**	**49.0**	**157.2**	**9.6**	**128.8**	**114.8**	**308.8**	**198.6**
$gp41^{neg}$	**7.1**	**22.3**	**108.5**	**4.2**	**117.3**	**67.1**	**148.2**	**64.8**
$gp41^{pos}$	**11.5**	>55	>55	>55	>55	NT	NT	>55
Patient 2								
IgG	**2.0**	**20.3**	**92.9**	**6.0**	**132.5**	**203.6**	**388.6**	**1.138**
$gp41^{neg}$	**1.2**	**10.2**	**69.4**	**2.5**	**67.8**	**273.5**	**252.1**	>1.005
$gp41^{pos}$	**16.2**	>35	>35	**>35***	>35	NT	NT	>35
Patient 3								
IgG	**10.1**	**27.2**	**140.1**	**24.0**	**25.4**	**489.9**	**57.4**	**106.5**
$gp41^{neg}$	**9.7**	**21.4**	**105.1**	**22.4**	**27.4**	**963.5**	**77.3**	**92.7**
$gp41^{pos}$	**23.9**	>50	>50	>50	>50	NT	NT	>43

[a] IgG refers to the neutralizing activity in patients' total IgG fraction, whereas $gp41^{neg}$ refers to the activity in the gp41-depleted fraction, and $gp41^{pos}$ refers to the activity in the eluate of anti-gp41 antibodies after gp41 binding.

[b] Values in boldface represent the concentrations at which the tested serum samples showed 50% neutralization. *, values that almost reached the IC_{50} at the highest concentration tested; NT, not tested.

neutralizing serum antibodies, we performed competition ELISAs with well-characterized anti-gp41 antibodies (12, 43). We examined all of the previously reported unique anti-gp41 antibodies cloned from memory B cells (43). The following anti-gp41 antibodies were used as standards (Fig. 1b) (11, 12, 57): D61, which recognizes an amino-terminal determinant, corresponding to the immunodominant region, and spans amino acids 597 to 613 (cluster I; LAI strain) (12); D40, D17, and D50, which bind to a region (cluster II) that includes amino acids 642 to 665 located at the amino terminus of the MPER; 4E10 and 2F5, which bind to separate but adjacent peptides in the MPER that span amino acids 662 to 678 (cluster III); T3, which binds a conformational epitope neighboring the MPER and spans amino acids 641 to 683 (cluster IV); and T30, which binds the carboxy terminus of cluster I, amino acids 611 to 640 (cluster VI).

Despite some degradation in the gp41 protein preparation (see Fig. 6a), anti-cluster I, -cluster II, -cluster IV, and -cluster VI antibodies accounted for all of the anti-gp41 binding by the monoclonal antibodies isolated from patients with high titers of broadly neutralizing antibodies (Fig. 4 and 5 and Table 2). However, the distribution of unique, nonclonal antibodies varied between patients. For example, cluster I antibodies were most abundant in patient 2, and cluster II antibodies were most abundant in patient 3. When combined, the largest number of unique B-cell clones was directed to cluster IV (53%), followed by cluster II antibodies (49%) and cluster I antibodies (32%) (the sum shown in Table 2, 134%, results from antibodies that bind to two clusters). Although none of the antibodies tested interfered with the binding of the MPER-specific cluster III antibodies 4E10 and 2F5, antibodies to clusters II and IV, which flank this region, were common; therefore, this region of the molecule is accessible to the immune system *in vivo*. In-

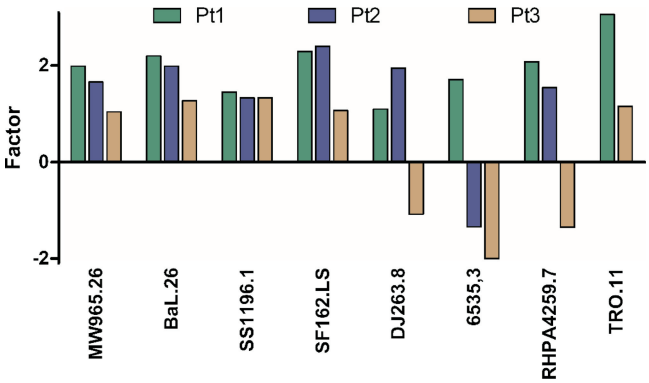

FIG. 3. Neutralizing activity. Shown is the increase or decrease in the neutralizing activity of IgG against different pseudoviruses for patients 1 to 3 after depletion of the gp41 binding antibodies by gp41 coupled to magnetic beads.

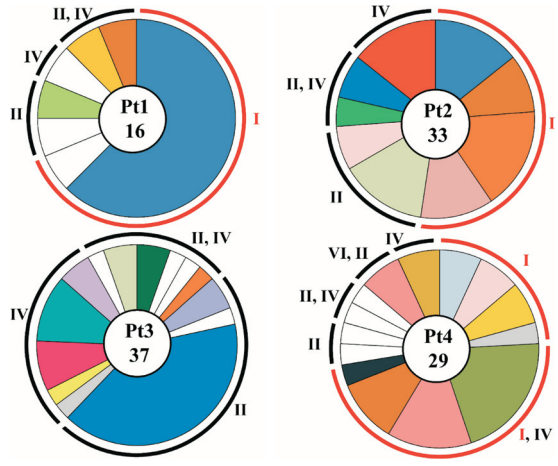

FIG. 4. Clonal expansion and cluster distribution of anti-gp41 antibodies. Pie charts show the distribution of anti-gp41 antibodies to the different clusters (I, II, IV, and VI). Patients (Pt1 to Pt4) are indicated. The number in the center indicates the number of antibodies; slices are proportional to the size of the unique clones (43). The expanded cluster I antibodies are highlighted in red.

Asigned clusters	Competing antibodies	Biotinylated antibodies												
		4e10	2F5	2-378	2-55	1-763	3-255	4-147	2-474	3-334	3-140	2-512	3-18	4-380
		III	III	I	I	II, IV	IV	II, VI	II, IV	IV	II, IV	IV	II, IV	II, IV
II	D40	36	17	100	nd	25	25	100	5	100	9	100	7	7
IV	T3	100	48	87	nd	13	8	100	3	9	5	9	7	5
VI	T30	50	100	100	nd	51	100	11	100	58	100	21	64	100
I	D61	100	100	4	nd	100	100	100	100	100	100	100	100	100
II	D17	15	7	29	nd	7	100	3	5	69	6	100	6	6
II	D50	16	6	24	nd	7	17	100	3	100	6	24	4	4
Self inhibition		7	8	4	4	5	6	4	5	6	6	11	8	11
IV	1-11	100	100	100	100	28	9							
I	1-27 ID	100	41	5	3	100	11							
II, IV	1-96	100	28	100	100	9	10							
II	1-167	100	88	100	100	6	16							
II	1-491	100	72	100	100	11	22							
II, IV	1-763	100	46	100	100	5	5							
I	2-55	100	100	12	4	100	100							
II	2-150	100	47	65	31	8	100							
II	2-275	100	44	100	100	8	100							
I	2-378 ID	100	100	4	4	100	100							
II, IV	2-474	100	76	64	100	100	23							
IV	2-512	100	100	100	100	100	48							
II, IV	2-1290	100	100	38	48	10	12							
II, IV	3-18	100	69	100	50	20	34							
IV	3-64	100	36	100	100	17	5							
II	3-125	100	100	100	100	9	28							
II, IV	3-140	100	52	100	100	19	18							
IV	3-160	71	40	100	100	24	6							
II, IV	3-204	100	20	100	69	10	13							
IV	3-233	100	66	100	100	100	12							
IV	3-255	100	100	100	100	100	6							
II, IV	3-296	100	39	100	100	12	7							
IV	3-334	47	54	100	100	42	24							
II	3-384	60	29	100	100	9	31							
II, IV	3-461	100	40	100	100	7	15							
II	3-474	53	53	100	100	6	100							
II, IV	3-650	100	21	100	100	14	13							
I	4-20 ID	nd	nd	5	4	100	nd							
IV	4-63	100	100	100	100	100	28							
I, IV	4-95 ID	100	75	5	3	100	10							
I, IV	4-104	100	100	6	4	100	14							
VI, II	4-147	100	100	100	100	100	100							
I, IV	4-153 ID	100	100	4	2	100	15							
I	4-157	100	57	6	3	100	47							
I	4-251	100	100	5	3	100	27							
II	4-283	100	100	51	100	10	29							
II	4-342	100	100	100	100	5	40							
I, IV	4-357	100	97	9	4	100	12							
II, IV	4-380	100	100	100	100	100	24							
I	4-431 ID	nd	nd	5	2	88	nd							
II, IV	4-613	100	100	100	100	10	7							
I	5-25	100	100	5	3	100	21							
IV	6-91	100	100	29	100	100	15							
II	6-129	100	100	100	100	5	24							
III	4e10	7	21											
III	2F5	43	8											

IC_{50} (μg/ml)
≤ 15
≤ 50
≤ 100

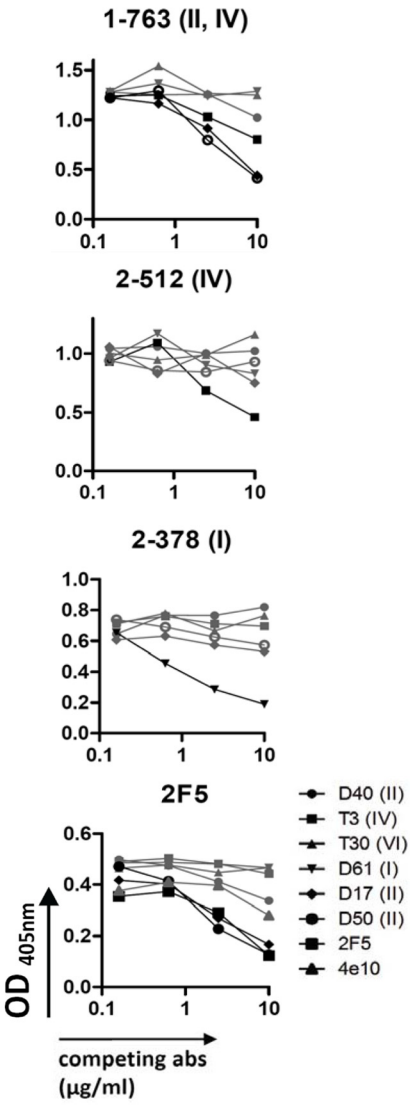

FIG. 5. gp41 hot-spot inhibition map with IC_{50}s (μg/ml). ID, the competing antibody reacts with the immunodominant peptide; ND, not done. Representative ELISA inhibition graphs are also shown (b). Black lines indicate inhibition. OD, optical density; abs, antibodies.

deed, each of the patients showed several antibodies that inhibited binding by both anti-cluster II and -cluster IV antibodies but not by cluster III antibodies, despite the proximity of these epitopes. The most likely explanation for the overlap between cluster II and cluster IV antibodies is the fact that cluster II antibodies recognize an epitope that is contained within the larger, conformationally dependent cluster IV epitope (12).

Each of the unique antibodies tested was a member of a clone of antibodies ranging in size from 1 to 15 members. Together, 47 unique B-cell clones were expanded to account

TABLE 2. Number and percent distribution of anti-gp41 antibodies

Patient no.	No. of unique B-cell clones (%)	No. of clonal members (%)	No. of clones (%) by cluster(s)					
			I	II	IV	II, IV	I, IV	VI, II
1	7		2	2	1	2	0	0
		16	11	2	1	2	0	0
2	9		4	2	1	2	0	0
		42	22	9	6	5	0	0
3	14		0	3	5	6	0	0
		37	0	18	11	8	0	0
4	14		4	2	1	2	4	1
		29	7	2	2	2	14	2
5	1		1	0	0	0	0	0
		3	3	0	0	0	0	0
6	2		0	1	1	0	0	0
		4	0	3	1	0	0	0
Total B-cell clones (single cluster)[a]	47 (100)		11 (23)	10 (21)	9 (19)	12 (26)	4 (9)	1 (2)
Total B-cell clones (two clusters)[a]	(134)		15 (32)	23 (49)	25 (53)			
Total clonal members (single cluster)		131 (100)	43 (33)	34 (26)	21 (16)	17 (13)	14 (11)	2 (2)
Total clonal members (two clusters)		(124)	57 (44)	53 (40)	52 (40)			

[a] Totals reported refer to the sum of the individual clones or clonal members binding their cluster(s). Totals for a single cluster indicate the binding of the antibodies as mapped; totals for two clusters distribute the antibodies that bound to two clusters evenly to their single clusters.

for a total of 131 independent antibodies. Within cluster I, antibody clones against linear (amino acids 579 to 604 [53]) and nonlinear epitopes were highly expanded in all of the patients except patient 3, who showed no antibodies to this region. Anti-cluster I antibodies comprised 15 different clonal families, with a total of 57 members. When the detected antibodies were combined, 32% of all unique anti-gp41 B-cell clones and 44% of all the anti-gp41 antibodies we obtained targeted this region. In contrast, antibodies to clusters II and IV comprised 49% and 53% of all unique anti-gp41 B-cell clones, respectively, and 40% each of all the anti-gp41 antibodies (the sum is greater than 100% because some antibodies appear to bind to two epitopes) (Table 2). Thus, antibodies to cluster I of gp41 are highly represented but not immunodominant in the memory B-cell compartment of the patients studied (53).

To confirm the absence of MPER-directed 2F5- and 4E10-like antibodies in the patients' sera, we performed competition ELISAs between affinity-purified anti-gp41 patient IgG and biotinylated 4E10 and 2F5. Controls included HIV IG, which did not inhibit 4E10 or 2F5 binding, as well as unbiotinylated 4E10 and 2F5 and IgG from noninfected individuals. Consistent with the results obtained with the monoclonal antibodies, the patients' affinity-purified anti-gp41 IgG did not block the binding of 4E10 or 2F5 to gp41 (Fig. 2b). However, as expected, the same affinity-purified fraction (shown for patient 1) blocked the binding of cluster I antibodies (2-55 and 2-378) (Fig. 2c). To increase the sensitivity of the assay, we performed ELISAs with anti-2F5 and -4E10 peptides (Fig. 2d). HIV IG bound to the 2F5 peptide at very high concentrations (>25 μg/ml), indicating that only rare or low-affinity antibodies to this peptide exist in HIV IG, and we found no binding to either peptide by our patients' IgG. We conclude that none of the patients studied produced significant titers of antibodies against the 2F5- and 4E10-like MPER epitopes.

To determine whether binding of any of the anti-gp41 antibodies was carbohydrate dependent, we deglycosylated gp41 with PNGase F to remove N-linked glycans and repeated the ELISAs. Deglycosylation was confirmed by a shift in mobility on denaturing polyacrylamide gels (Fig. 6a) and by lectin precipitation (Fig. 6b). Glycosylated gp41 bound to *Lens culinaris* lectin-Sepharose, whereas deglycosylated gp41 did not (Fig. 6b). We found one monoclonal anti-gp41 antibody (4-157) whose binding was carbohydrate dependent (Fig. 6c). This antibody was mapped to cluster I by competition experiments, but did not bind to the immunodominant peptide (amino acids 579 to 604).

Monoclonal neutralizing activity. None of the anti-gp41 antibodies showed neutralizing activity at concentrations up to 50 μg/ml (43). To determine whether any of the 47 anti-gp41 antibodies showed neutralizing activity at higher concentrations, we performed *in vitro* neutralization assays with antibody concentrations of up to 2,380 μg/ml on two tier 2, clade B, Env-pseudotyped viruses from primary isolates (TRO.11 and RHPA4259.7). We found that 7 out of 15 cluster I antibodies were able to neutralize TRO.11 at high concentrations ranging from 433 to 1,712 μg/ml (IC_{50}). Among all other antibodies, only one antibody against cluster IV showed neutralization at 1,276 μg/ml (Table 3). We conclude that, among anti-gp41 antibodies, those directed to cluster I show neutralizing activity but only at very high concentrations.

DISCUSSION

gp41 is highly immunogenic and elicits antibodies in almost all HIV-infected individuals, with titers that can exceed $1{:}10^6$ (2, 53). These titers are 25- to 625-fold higher than anti-gp120 titers (35). In addition, gp41 differs from gp120 in that all regions of the protein appear to be targeted by the human antibody response (35).

Serologic examination of 23 randomly selected HIV-1-positive individuals showed that the fusion peptide and the polar region induce low-to-medium serum antibody titers (35). In contrast, high serum antibody titers (up to $1{:}7 \times 10^5$) were documented against the N- and C-terminal heptad repeats (35), the membrane-proximal region (35), and the cluster I

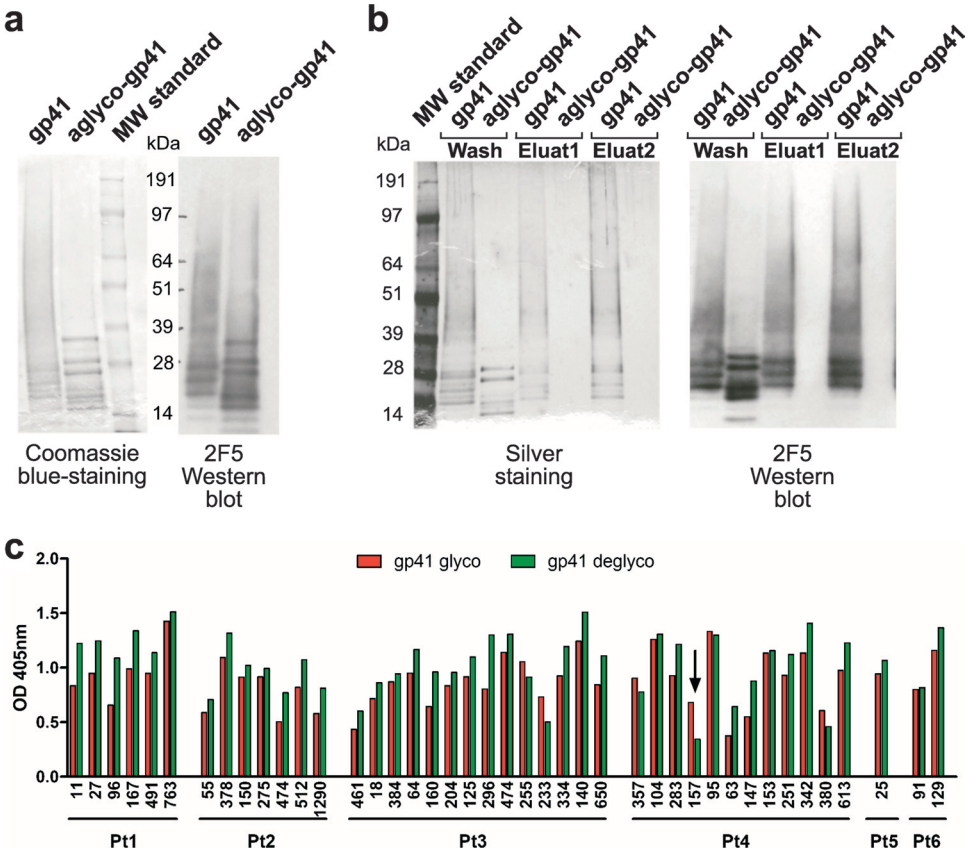

FIG. 6. Rare glyco-dependent binding by anti-gp41 antibodies. (a) Coomassie blue-stained polyacrylamide gel and Western blot analysis with MAb 2F5 of control and deglycosylated gp41. As specified by the manufacturer (Acris), the protein shows three major immunospecific bands between 20 and 30 kDa, minor bands between 20 and 30 kDa, bands at 14 and 7 kDa, and an aggregation smear at 35 kDa and above. The aggregation smear condenses in major specific bands after deglycosylation. (b) Deglycosylation was further confirmed by *Lens culinaris* lectin precipitation, followed by elution with 0.5 M methyl α-D-mannopyranoside of glycosylated gp41 but not of the deglycosylated form. (c) Graph summarizes differences in binding of anti-gp41 antibodies to intact (red bars) and deglycosylated (green bars) gp41 as measured by ELISA (OD at 405 nm). A significant decrease in binding is indicated by a black arrow.

region (53). The latter was identified as immunodominant by the observation that 53 out of 53 HIV-positive patients' sera tested showed reactivity to a synthetic 12-mer peptide from this region (LGLWGCSGKLIC) (15, 16). Consistent with this peptide's immunogenicity, injection of the peptide coupled to keyhole limpet hemocyanin into rabbits resulted in serum antibody titers of $1{:}1 \times 10^6$ (15). Although antibodies to the antigens that are membrane proximal are also present in most patients' sera, these do not appear to resemble the broadly neutralizing 2F5- and 4E10-like antibodies (18, 56). Instead, the anti-MPER antibodies that were commonly found among patients appear to be directed to a peptide partially overlapping the 2F5 and 4E10 epitope (amino acids 665 to 683) (35). Similarly, gp41-immunized rabbits produced antibodies to cluster I but failed to produce anti-MPER antibodies (58).

In contrast to the serologic studies described above, which were performed on immunized rabbits and randomly selected HIV-1-infected individuals, our patients were selected for broad neutralizing antibody responses, relatively low viral titers, and moderate $CD4^+$ T-cell counts (43). In addition, the B cells from which our antibodies were cloned were selected for their binding to soluble trimeric gp140 (54), which is recognized by most anti-gp41 antibodies, including those binding to the MPER, the C-terminal heptad repeat, cluster I, and the fusion peptide polar region (antibody 5F3) (6). However, antibody D3 mapped to cluster V, which includes the fusion peptide, the polar region, and the N-terminal heptad repeat, does not recognize the synthetic trimer (data not shown); therefore, antibodies with this type of reactivity to gp41 would not be detected in our analysis.

We found that 47 unique clones of anti-gp41 antibodies were variably expanded, with each containing 1 to 15 members, for a total of 131 independently cloned antibodies. All of these antibodies could be assigned to clusters I, II, IV, and VI, but none recognized the fusion peptide, the polar region, or the N-terminal heptad repeat or the MPER peptide. This finding is consistent with both the absence of antibodies to the MPER peptide in the patient serum and the inability of a control anti-cluster V antibody to bind to the artificial gp140 trimer. Unique antibodies reacting with cluster I, the immunodominant region, were not predominant in the collection; they represented only 32% of all the unique B-cell clones. Instead, a conformational epitope neighboring the MPER (cluster IV) was the dominant immunogen. However, anti-cluster I clones

TABLE 3. TZMbl neutralization data

Patient no.	Antibody no.	Cluster[a]	Neutralizing activity (IC_{50} [μg/ml])[b]	
			TRO.11	RHPA4259.7
1	11	IV	>500	>500
	27	I-ID	**433**	>500
	96	II, IV	>500	>500
	167	II	>500	>500
	491	II	>500	>500
	696	I-ID	**651**	>500
	763	II, IV	>500	>500
2	55	I	>1,030	>1,030
	149	I-ID	**>1,030***	>1,030
	150	II	>1,500	>1,500
	275	II	>1,500	>1,500
	378	I-ID	>1,560	>1,560
	474	II, IV	>1,500	>1,500
	512	IV	**1,276**	>1,500
	1007	I-ID	>1,000	>1,000
	1290	II, IV	>1,500	>1,500
3	18	II, IV	>1,500	>1,500
	64	IV	>1,500	>1,500
	125	II	>1,500	>1,500
	140	II, IV	>1,500	>1,500
	160	IV	>1,500	>1,500
	204	II, IV	>1,500	>1,500
	233	IV	>1,500	>1,500
	255	IV	>1,500	>1,500
	296	II, IV	>1,500	>1,500
	334	IV	>1,500	>1,500
	384	II	>1,500	>1,500
	461	II, IV	>1,500	>1,500
	474	II	>1,500	>1,500
	650	II, IV	>1,500	>1,500
4	20	I-ID	**508**	>1,100
	63	IV	>1,500	>1,500
	95	I-ID, IV	>670	>670
	104	I, IV	**1,712**	>2,380
	147	VI, II	>1,500	>1,500
	153	I-ID, IV	>1,500	>1,500
	157	I	>1,500	>1,500
	251	I	**841**	**1,234**
	283	II	>1,360	>1,360
	342	II	>1,500	>1,500
	357	I, IV	>665	>665
	380	II, IV	>1,500	>1,500
	431	I-ID	>690	>690
	613	II, IV	>1,500	>1,500
5	25	I	**964**	>1,050
6	91	IV	>1,500	>1,500
	129	II	>1,500	>1,500

[a] ID, binds to the immunodominant peptide.
[b] Both TRO.11 and RHPA4259.7 are tier 2, clade B viruses. Values in boldface represent the concentration at which the tested monoclonal antibodies showed 50% neutralization. *, values that almost reached the IC_{50} at the highest antibody concentration tested.

were disproportionately expanded, accounting for 44% of all anti-gp41 antibodies when all of the distinct clonal family members were considered. Among the 15 unique clones of anti-cluster I antibodies, 9 recognized the linear peptide, and 6 were against conformational determinants in this region. Similarly, 5 out of 10 antibodies produced from seven seropositive patients by EBV immortalization were directed to cluster I, and of these, 4 were directed to the linear peptide (53). In contrast, phage display antibody libraries that were constructed from patients and that were selected on recombinant gp41 showed a much lower frequency of cluster I binders (5 out of 25) (4). However, antibody cloning by phage display is biased by multiple rounds of selection and amplification; in addition, the process may not reflect the native antibody repertoire because heavy and light chains are paired randomly, potentially creating novel combining sites. Thus, the frequency of specific antibodies estimated by this method may not be an accurate reflection of the antibodies found in a patient's B-cell repertoire. We conclude that in patients with high titers of broadly neutralizing HIV antibodies and variable viral loads, anti-cluster I antibodies account for 32% of all unique B-cell clones; these are preferentially expanded to account for 44% of all anti-gp41 antibodies, a significant fraction of which are directed to the linear peptide comprised of amino acids 579 to 604. The gp41 antibodies found in the patients studied might result from B-cell stimulation by the intact virion or by nonfunctional membrane-associated gp41 on the surface of infected cells or even damaged virions (7, 19, 31).

Our studies reveal several previously unappreciated epitopes on gp41. For example, four antibodies cloned from the memory B cells were inhibited by monoclonal antibodies directed to both cluster I and IV but not by antibodies to cluster VI, which is between the two. Inhibition by anti-cluster I and –cluster IV but not anti-cluster VI suggests that the former two are closely opposed to each other in the trimer, either in *cis* or in *trans* between two different gp41 molecules in the trimer.

In addition to conformational epitopes within cluster I, we found a novel carbohydrate-dependent epitope in the same region of gp41. This region contains three well-conserved, predicted glycosylation sites (21). Like 2G12, which recognizes a carbohydrate-dependent epitope on gp120 (42), gp41 binding by antibody 4-157 is sensitive to deglycosylation, which thereby establishes that the cluster I region of gp41 is indeed glycosylated (21).

Among the 345 amino acid residues in gp41, one-third show up to 90% conservation among HIV-1 group M isolates (20). These include highly conserved residues critical for the formation of the coiled-coil gp41 pocket (28), for gp120-gp41 interactions (55), and for envelope biosynthesis (45). These critical residues are found primarily in the heptad repeats and the cluster I region. Indeed, mouse anti-cluster I antibodies exhibit extensive cross-reactivity with primary envelope proteins from divergent HIV-1 clades, indicating the presence of a highly conserved secondary antigenic structure in the cluster I loop region (12). Given the importance of these regions in the function of the viral spike and their relatively high level of conservation, these might be important targets for neutralizing antibodies if they were accessible either on the virion or as membrane-associated gp41 postfusion on the surface of infected cells. Consistent with this idea, the IgG fraction of serum from rabbits immunized with gp41 neutralized 52% of 21 HIV primary isolates in a peripheral blood mononuclear cell (PBMC)-based assay, with IC_{50}s lower than 50 μg/ml (58). Finally, several groups of investigators have recently shown that a small fraction of HIV-infected patients develops anti-

gp41 neutralizing antibody responses, mainly due to anti-MPER antibodies (3, 39, 47).

Despite the serologic data noted above, there are only a few neutralizing monoclonal anti-gp41 antibodies. D5 is a phage-derived antibody obtained from a naïve single-chain variable-fragment (scFv) library that inhibits the assembly of fusion intermediates *in vitro* by binding to the N-terminal heptad repeat (27). Antibody m44, another phage-derived antibody, recognizes a conformational epitope implicated in the binding of the C-terminal heptad repeat (57). Less is known about how this antibody inhibits infection, but the mechanism may be related to the one used by D5 since both target the heptad repeats that form six-helix coiled-coiled fusion intermediates *in vitro*. Clone 3 is specific for the immunodominant region and shows neutralizing activity against a diverse group of laboratory-adapted HIV-1 strains (10, 51). This antibody was produced by EBV-transformed PBMCs from an asymptomatic HIV-1-positive donor (10). Other antibodies against this region show complement-mediated infection-enhancing activity *in vitro* (36, 37).

Finally, there are three broadly neutralizing antibodies directed to the membrane-proximal external region: 2F5 (33, 49), 4E10, and Z13 (60). Such antibodies are rare (3) and were detected only in the sera of 3 out of 156 chronically HIV-1-infected individuals (17). The poor immunogenicity of this region may be attributed to its lack of exposure on the surface of the native virus (9, 34, 41, 44; reviewed in reference 30). The neutralizing activity of these antibodies appears to be dependent on binding to both the MPER and to the viral membrane (25). It has been proposed that antibody binding to the viral membrane serves to concentrate the antibody in the region of the MPER, thereby favoring its interaction with this poorly exposed antigenic epitope (1). Consistent with the idea that lipid binding may be important in the function of the anti-MPER antibodies, liposomes containing MPER peptide induced multispecific antibodies that simultaneously recognize the lipid and the MPER antigen; they also neutralized HIV SF162 in a PBMC-based neutralization assay (25).

We found that none of the gp41 antibodies cloned from the memory B cells of the six individuals studied were able to neutralize HIV at concentrations of up to 50 μg/ml (43). Surprisingly, 7 out of 15 antibodies to cluster I neutralize one of two tested tier 2 viruses at high concentrations. Although it is unlikely that an individual anti-cluster I antibody would have a biological effect, large numbers of gp41 antibodies might enhance neutralizing activity due to additive or even synergistic neutralizing effects (43). Antibody binding to the cluster I hinge region between the heptad repeats might interfere with the formation of the prehairpin fusion intermediate by steric hindrance (27). Why such high concentrations of cluster I antibodies are necessary for neutralization remains unclear (5); one possibility is that this region is poorly accessible to antibodies binding the intact virion.

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Human anti–HIV-neutralizing antibodies frequently target a conserved epitope essential for viral fitness

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The identification and characterization of conserved epitopes on the HIV-1 viral spike that are immunogenic in humans and targeted by neutralizing antibodies is an important step in vaccine design. Antibody cloning experiments revealed that 32% of all HIV-neutralizing antibodies expressed by the memory B cells in patients with high titers of broadly neutralizing antibodies recognize one or more "core" epitopes that were not defined. Here, we show that anti-core antibodies recognize a single conserved epitope on the gp120 subunit. Amino acids D474, M475, R476, which are essential for anti-core antibody binding, form an immunodominant triad at the outer domain/inner domain junction of gp120. The mutation of these residues to alanine impairs viral fusion and fitness. Thus, the core epitope, a frequent target of anti–HIV-neutralizing antibodies, including the broadly neutralizing antibody HJ16, is conserved and indispensible for viral infectivity. We conclude that the core epitope should be considered as a target for vaccine design.

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Abbreviations used: CD4bs, CD4 binding site; CD4is, CD4 induced site; EBV, Epstein-Barr virus; SPR, surface plasmon resonance; VL, variable loop.

A fraction of patients infected with HIV-1 develop broadly neutralizing antibodies against the virus (McMichael et al., 2010). In vitro studies indicate that these antibodies can reduce infectivity by interfering with virus–target–cell interactions or by blocking viral fusion (Dimmock, 1993; Robbins et al., 1995; Shibata et al., 1999; Zolla-Pazner, 2004). In addition, passive administration of mABs with broadly neutralizing activity to macaques or humans can provide sterilizing immunity or delay HIV-1 rebound (Emini et al., 1992; Gauduin et al., 1995; Mascola et al., 2000; Trkola et al., 2005). Therefore, it is generally believed that reproducing this type of serologic activity by immunization would be important for the development of an effective HIV vaccine (Stamatatos et al., 2009).

Although several different broadly neutralizing mABs that target HIV-1 envelope epitopes have been described (Zolla-Pazner, 2004; Burton et al., 2005), there have been few comprehensive efforts to clone and characterize the antibodies from patients with broadly neutralizing serologic responses. In an effort to understand the human antibody response to HIV-1, we cloned 502 anti-HIV-1 gp140 antibodies from the memory B cell compartment of six individuals with variable viral loads and high titers of broadly neutralizing antibodies (Scheid et al., 2009). We found that the memory B cell response to gp140 is composed of high affinity antibodies binding to the gp120 variable loops (VLs), the CD4 binding site (CD4bs), the induced coreceptor-binding site (CD4is), several different epitopes on gp41 (Pietzsch et al., 2010), and a group of potentially heterogeneous antibodies to one or more epitopes near the CD4bs, termed "core" (Scheid et al., 2009). The core antigen was not characterized molecularly; however, antibodies to this region accounted for 18% of all anti-gp140

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antibodies and 32% of all antibodies with neutralizing activity (Table S1; Scheid et al., 2009). Anti-core was the largest single group of neutralizers in the six patients studied. In addition, antibodies with characteristics similar to anti-core antibodies were also reported in a collection of mABs obtained from EBV-transformed B cells ("partial CD4 binding site" antibodies; Corti et al., 2010).

Anti-core antibodies bind to gp120, $gp120_{core}$ (a mutant that lacks V1-V3; Kwong et al., 1998), $gp120_{D368R}$ (which interferes with binding by CD4 and anti-CD4bs antibodies; Olshevsky et al., 1990; Thali et al., 1991; Pantophlet et al., 2003; Li et al., 2007), and $gp120_{I420R}$ (a mutant that interferes with the binding of anti-CD4–induced site [CD4is] antibodies; Thali et al., 1993). Anti-core antibodies do not bind to a stabilized $gp120_{core}$ protein that retains CD4 and b12 binding sites, but is mutated to reduce the flexibility of gp120 to improve presentation of conserved but discontinuous epitopes (Zhou et al., 2007; Scheid et al., 2009). Furthermore, anti-CD4bs and some anti-CD4is antibodies inhibit the binding of anti-core antibodies, suggesting that anti-core antibodies recognize an epitope that is closer to the CD4bs than to the CD4is (Scheid et al., 2009).

Here, we report on the characteristics of this new epitope. The data show that anti-core antibodies target a conformational epitope on gp120 found within the α5-helix of the molecule, which is highly conserved across different HIV-1 clades. This high degree of conservation correlates to viral fitness, as mutating the epitope results in loss of infectivity.

RESULTS

Fine mapping of anti-core antibodies cloned by single cell sorting

To map the epitope or epitopes recognized by anti-core antibodies, we assayed all anti-core antibodies for binding to 72 different alanine mutants of HIV-1 gp120 by ELISA. Controls included the anti-CD4bs antibody b12 (Burton et al., 1994; Saphire et al., 2001) and an anti–variable-loop antibody (1–79; Scheid et al., 2009). Mutations that reduced antibody binding to 60% or less compared with the WT protein were considered significant. The mutated residues were primarily spread across gp120 to cover a broad range of candidate binding sites, and then refined based on initial binding results. In particular, we included residues from the variable-loop 2 (VL2), the silent face, the CD4bs, the CD4is, the Phe 43-cavity (Kwong et al., 1998), in addition to residues that lie proximal or distal to these sites (Fig. 1 A and Fig. S1).

We found nine mutations that altered the binding of nearly all anti-core antibodies and b12, despite being physically distant from the CD4bs recognized by b12. Based on their position and physical chemical characteristics, these residues (L288, I449, T450, L265A, S264, C378, N262, F383, and F376) appear to be required to maintain the structural integrity of the molecule.

As previously demonstrated, alanine substitutions in the CD4bs (E370A, D368A, and D368A/E370A), or in close proximity to the CD4bs (N276A, R480A, and Y384A) reduce binding of the anti-CD4bs antibody b12 (Pantophlet et al., 2003) and CD4, respectively (Olshevsky et al., 1990; Fig. 1, A and B). These mutations had little or no effect on anti-core antibody binding (Fig. 1, A and B). Thus, the binding characteristics of the anti-CD4bs and anti-core antibodies are distinct. However, as expected from the antibody blocking experiments (Corti et al., 2010; Scheid et al., 2009), anti-core and anti-CD4bs antibody binding overlapped to a significant extent, with varying levels of sensitivity for F376A, P470A, W96A, E275A, and D477A (Fig. 1, A and B).

Among the 72 mutants, we found three mutations (D474A, M475A, and R476A) that inhibited the binding of anti-core antibodies, but had no significant effect on b12 (Fig. 1 C). These adjacent residues are in close proximity to the CD4bs and cover a stretch from a direct CD4 contact residue (D474) up to the α5 helix at the outer-domain/inner-domain junction of gp120 (M475, R476; Fig. 1 E). Among the 24 anti-core antibodies, only 2 were insensitive to these 3 mutations. Of the remaining 22, 16 were sensitive to both D474A and R476A (Fig. 1 D); in addition, 8 of these also showed altered binding to M475A (Fig. 1 D).

To determine whether anti-core antibodies recognize a linear peptide epitope containing D474, M475, and R476 we performed ELISAs with the DTNGTEIFRPGGG DMRDNWR peptide. Controls included a V3-loop peptide (NNNTRKSINIGPGRALYTT) that was recognized by an anti-V3-loop antibody (2–59; Scheid et al., 2009). We found that none of the 24 anti-core antibodies recognized this peptide (Fig. S2). Thus, anti-core antibodies recognize a conformational epitope containing amino acids D474, M475, and R476 in gp120 (Fig. 1, A–E).

Antibodies from EBV-transformed B cells

mABs with properties similar to anti-core that were able to completely or partially block the binding of sCD4 to Env-coated ELISA plates, henceforth defined as CD4bs- or partial CD4bs–specific mAbs were recently obtained from EBV immortalized memory B cells (Corti et al., 2010). To determine whether these antibodies (HGF12, HGI46, HGS2, HGW26, HGZ1, and HJ16) show binding characteristics similar to anti-core antibodies, we assayed all anti–partial CD4bs antibodies for binding to core epitope alanine mutants by ELISA (Fig. S3). Two of the antibodies (HGF12 and HGI46) could not be mapped; two others (HGW26 and HGZ1) were sensitive to D368A/E370A mutation and are likely to be conventional anti-CD4bs antibodies. HGS2 and HJ16 were sensitive to D474A, R476A, and M475A/R476A, but not to D368A; therefore, they target the same epitope as anti-core antibodies. Among these antibodies, HJ16 is of special interest because it is a broad and potent HIV neutralizer with very peculiar features, such a preferential neutralization of isolates not neutralized by b12 and an almost exclusive capacity to neutralize tier 2 isolates (Corti et al., 2010).

Trimeric BaL gp140

To confirm the mapping studies on trimeric gp140 of a different HIV strain, we produced BaL gp140 trimer and

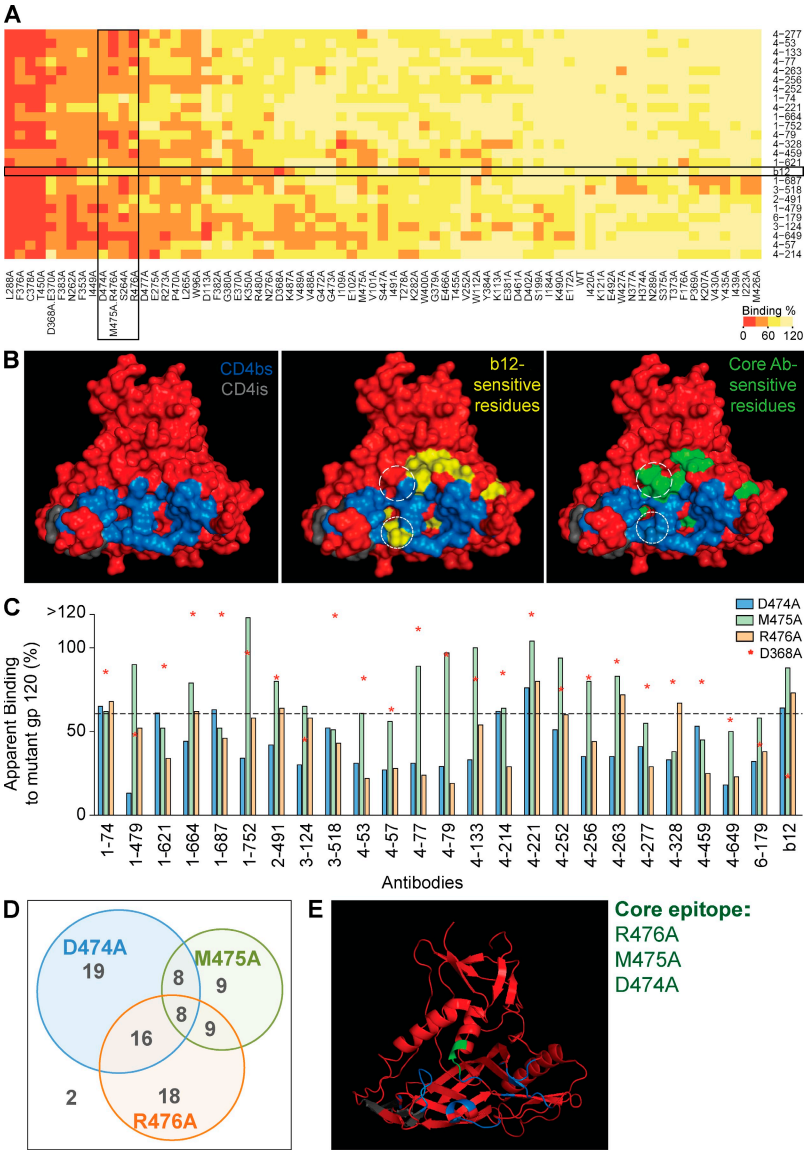

Figure 1. Mapping of the HIV-1 gp120 core epitope. (A) Heat map summarizes the binding of the different anti-core antibodies and b12 to gp120 alanine mutants. Red and orange fields indicate <60% binding, whereas yellow shows no difference compared with the WT gp120 control. The mapping of the anti-core epitope has been confirmed by three independent experiments. (B) Surface diagram of gp120 (PDB ID: 3DNO; Liu et al., 2008) showing the CD4bs (blue), the CD4is (gray), the b12 binding sites (yellow), and the core epitope (green). Residues that distinguished between the anti-CD4bs epitope and anti-core epitope are highlighted with circles. (C) Diagram shows the apparent binding of anti-core antibodies and b12 to mutant gp120$_{D474AM475AR476A}$ relative to gp120 WT in percent. The red star indicates binding to D368A. (D) Venn diagram summarizes the sensitivity for anti-core antibodies binding to D474A, M475A, and R476A. (E) Ribbon diagram of gp120 (PDB ID: 3DNO; Liu et al., 2008) shows the CD4bs (blue), the CD4is (gray), and the core epitope (green).

Binding to cell surface BaL gp160Δc trimer

The gp120 monomer and the artificial gp140 trimer lack yet to be defined features found on cell surface expressed gp160 viral spikes. To examine anti-core antibody binding to membrane-anchored molecules, we measured binding to a native gp160 trimer lacking the cytoplasmic domain of the spike (gp160Δc) expressed on the surface of BOSC.23 cells. Anti-core antibodies (4–79 and HGS2) bind the gp160Δc trimer, but not the mutant trimer (BaL gp160Δc$_{D474A/M475A/R476A}$). In contrast, b12 and the anti-VL antibodies bound to both gp160Δc and the gp160Δc mutant membrane anchored proteins. We conclude that the core epitope is accessible on the functional and fusion competent (see below) form of the HIV-1 spike and that it differs from the epitope recognized by the b12 anti-CD4bs antibody (Fig. 2 C).

corresponding core epitope mutant (BaL gp140$_{D474A/M475A/R476A}$) and repeated the ELISAs with two randomly selected anti-core antibodies (4–79 and HGS2), b12, and anti-VL controls. All of the antibodies tested showed similar binding characteristics on gp120 and the gp140 trimers (Fig. 2 A and Fig. S4).

To obtain a more precise measurement of the affinities of the antibodies to WT and mutant trimeric gp140 we performed surface plasmon resonance (SPR) experiments. The two anti-core antibodies, 4–79 and HGS2, bound to WT gp140 with high affinity K_As of 3.8×10^9 and 1.4×10^{10}, respectively, but binding to the mutant gp140$_{D474A/M475A/R476A}$ could not be detected. In contrast, control antibodies targeting the VL (2–1092) and the CD4bs (b12) retained their high-affinity binding to gp140$_{D474A/M475A/R476A}$, with K_As ranging from 9.6×10^8 to 4.8×10^9 (Fig. 2 B, Fig. S5, and Table S2).

Core epitope conservation

Given that most anti-core antibodies from different patients recognize a common epitope, we explored the possibility that D474, M475, and R476 might be conserved across different HIV-1 strains. Among 1,963 sequences analyzed from all clades (QuickAlign; http://www.hiv.lanl.gov), 56% contained all three of these residues and 35% of the remaining showed strong homology. Thus, the core epitope shows a moderate degree of precise conservation based on the primary amino acid sequence, but is highly conserved if homology is considered (Table S3).

Fusion and infectivity

To elucidate the possible roles of D474, M475, and R476 in fusion we developed an assay to measure fusion between BOSC.23 cells coexpressing BaL gp160Δc trimer and GFP,

with TZM.bl cells expressing red fluorescent protein (mCherry). Fusion between TZM.bl and BOSC.23 cells expressing the gp160Δc trimer, but not empty control GFP+ BOSC.23 cells, was readily detected by flow cytometry (Fig. 3 A). In contrast, there was little if any fusion of cells expressing the mutant spike (BaL gp160$\Delta c_{D474A/M475A/R476A}$).

To confirm that the events detected by flow cytometry represent fusion as opposed to binding, we repeated the experiments and analyzed them by image-based flow cytometry (ImageStream100; Amnis). Fusion was the predominant event when BOSC.23 cells expressed the gp160Δc trimer (Fig. 3 B). We conclude that D474, M475, and R476 are required for optimal fusion.

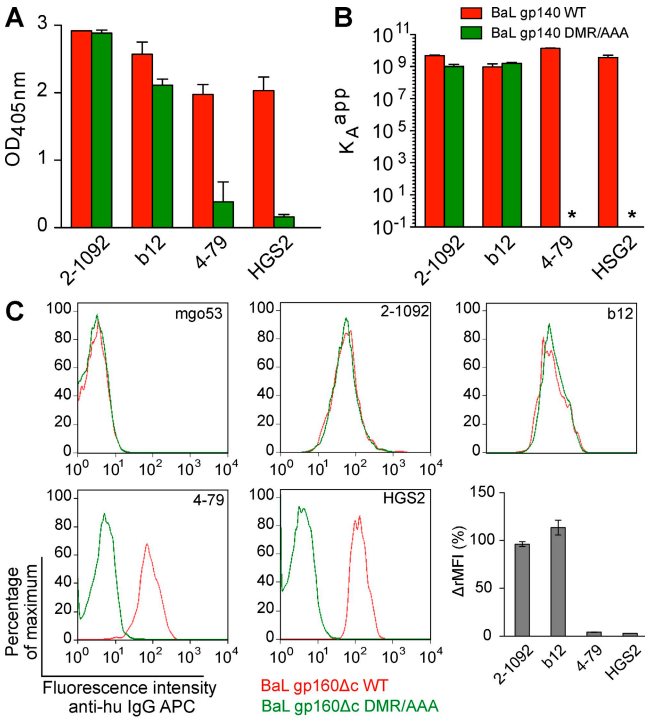

Figure 2. Binding to soluble and cell surface trimeric gp160Δc. (A) Graphs show optical density at 405 nm (OD_{405nm}) for the selected IgG antibodies as measured by capture ELISA with purified BaL gp140 WT and BaL $gp140_{D474A/M475A/R476A}$. See also Fig. S4. Error bars represent the SD from at least two independent experiments. (B) Graphs show apparent K_A (K_A^{app}, M^{-1}) for the selected IgG antibodies as measured by surface plasmon resonance (SPR) on chips derivatized with BaL gp140 WT and $BaL_{D474A/M475AR/476A}$. See also Fig. S5 and Table S2. Error bars represent the SEM from at least two independent experiments. * indicates that no binding to BaL $gp140_{D474A/M475A/R476A}$ was detected. (C) (left) Histogram plots show the binding of the selected antibodies to BaL gp160Δc and BaL $gp160\Delta c_{D474A/M475A/R476A}$ expressed on GFP-positive BOSC.23 cells. Controls include mgo53 (Wardemann et al., 2003), mAbs 2–1092 (anti-VL) and b12 (anti-CD4bs). BOSC.23 cells gated on GFP^{high} expression. The number of binding events as percentage of the maximum was plotted against APC fluorescence intensity. (right) Graphs show apparent differences in the relative median fluorescence intensity (ΔrMFI) for the selected IgG antibodies between BaL gp160Δc and BaL $gp160\Delta c_{D474A/M475A/R476A}$. Error bars represent the SD from at least two independent experiments.

To investigate the role of D474, M475, and R476 in viral infectivity, we assayed for the infection of TZM.bl cells with HIV-1 YU-2 and BaL pseudo-virus mutants (Fig. 3 C). YU-2 $gp160_{D474A/M475A/R476A}$ pseudo viruses showed a decrease in infectivity of four orders of magnitude at constant p24 levels (Table S4). The BaL pseudo virus was more sensitive to the mutation of the core epitope. R476A and M475A/R476A mutants decreased infectivity by two and three orders of magnitude, respectively, and we could not detect infection with the D474A mutant.

Collectively, the data from cell fusion and pseudo-virus infection experiments indicate that residues D474, M475, and R476 are important for optimal viral infectivity.

DISCUSSION

Although strong evidence suggests that any antibody that can bind to the HIV spike will neutralize the virus, only a small number of epitopes that induced broadly neutralizing antibodies upon HIV infection have been defined. These include the α1→2 mannose residues recognized by 2G12 (Trkola et al., 1996; Scanlan et al., 2002), the central region of the CD4bs bound by b12 (Barbas et al., 1992), and the V1/V2

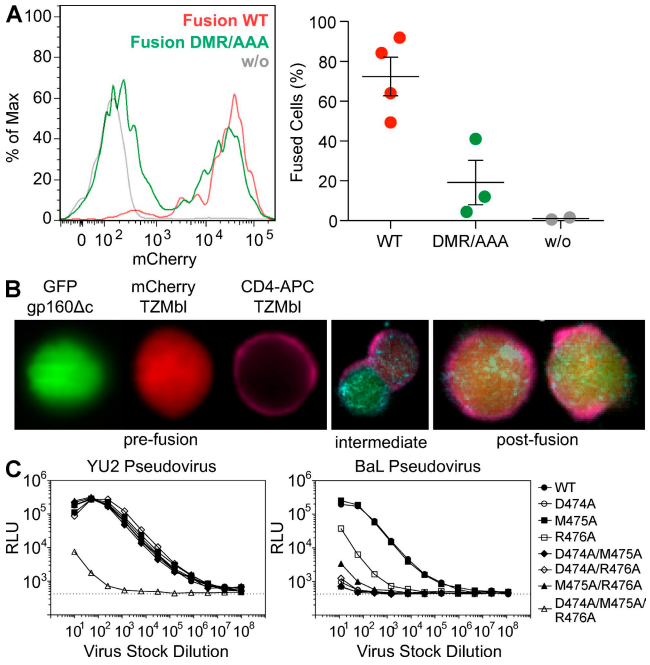

Figure 3. Fusion and infectivity. (A) (left) Histogram plots summarize fusion between BOSC.23 cells expressing GFP alone (w/o) or BaL gp160Δc or BaL $gp160\Delta c_{D474A/M475A/R476A}$ with mCherry expressing TZM.bl cells. (right) Fusion events (%) with mean and standard deviation for at least two independent experiments. (B) Image-based analysis of fusion. (left) Single staining compensation controls for BOSC.23 cells expressing BaL gp160Δc and GFP, mCherry expressing TZM.bl cells and CD4-APC stained TZM.bl cells. (middle) An intermediate fusion event. (right) The colocalization of the GFP-mCherry-APC signal after fusion. Experiment has been performed twice. (C) Graphs show the titration curves for TZM.bl cell infection by YU2 and BaL pseudoviruses, which has been performed twice. RLU relative light unit.

and V3 loops recognized by PG9 and PG16 (Walker et al., 2009; Walker and Burton, 2010). Unfortunately, vaccination approaches using recombinant HIV envelope proteins and derivatives specifically engineered to elicit broadly neutralizing antibodies have all been disappointing to date. Thus, the identification of additional immunogenic targets of neutralizing antibodies on the HIV viral spike remains an important issue in HIV vaccine research (Mascola and Montefiori, 2010). Optimal vaccine epitopes should be immunogenic in humans, conserved among HIV isolates and required for optimal viral infectivity. The core epitope fits all of these criteria.

Anti-core antibodies represent 18% of all anti-gp140 antibodies cloned from the memory B cell compartment of patients with high titers of broadly neutralizing antibodies (Scheid et al., 2009), but most importantly, they represent the most frequent neutralizers, accounting for 32% of all HIV-1 neutralizing antibodies (Scheid et al., 2009). In contrast, 18% of the neutralizing antibodies cloned in the same study showed binding properties similar to the anti-CD4bs antibody b12, 27% were anti-VL directed, and 23% recognized the CD4is (Scheid et al., 2009).

Neutralizing antibodies targeting a region other than the "central region of the CD4bs" were found in the sera of patients with broadly neutralizing serologic activity (Li et al., 2007). Anti-core plus anti-CD4bs containing serum and IgG fraction eluted from $gp120_{core}$ (which lacks VL 1–3, and therefore the CD4is) was more potent in viral neutralization than the anti–CD4bs-antibody–enriched fraction alone (Li et al., 2007). Thus, the serologic data are in agreement with the antibody-cloning experiments; both indicate that anti-core antibodies contribute significantly to the overall neutralizing activity in human serum.

Most antibodies that bind to HIV neutralizing epitopes are restricted in their neutralizing activity, including antibodies to the CD4bs. For example, among the antibodies that are like b12 and sensitive to the D368A mutation, most show neutralizing activity against tier 1 viruses, but much more limited activity against tier 2 viruses (Wyatt and Sodroski, 1998; Mascola, 2003; Pantophlet and Burton, 2006; Karlsson Hedestam et al., 2008; Scheid et al., 2009; Stamatatos et al., 2009). Similarly, most anti-core antibodies are not broad but are instead restricted to neutralize one or another strain of HIV-1. However, like the traditional anti-CD4bs antibodies, an occasional anti-core antibody, HJ16 exhibits a breadth of neutralizing activity comparable to, and generally complementary to b12, being able to neutralize approximately one third of all HIV-1 isolates tested, irrespective of clade (Corti et al., 2010). HJ16 also showed selective neutralization of tier 2, and not tier 1, isolates, making it particularly interesting as a template for vaccine design (Corti et al., 2010). Moreover, HJ16 was obtained from a clade C–infected patient, whereas the antibodies we cloned were obtained from clade B infected patients; therefore, the core epitope is immunogenic in humans even across different HIV isolates and clades.

Anti-core antibodies recognize a common epitope in close proximity to the CD4bs. We would therefore like to suggest that this group of antibodies should be referred to anti-CD4bs/DMR. Although this group of antibodies recognizes the same epitope, they are heterogeneous with respect to their neutralizing breadth and potency (Scheid et al., 2009; Corti et al., 2010). Similarly, traditional anti-CD4bs antibodies sensitive to the D368R mutation also show a wide range of neutralizing activity (Chen et al., 2009; Scheid et al., 2009; Corti et al., 2010). The structural basis for the difference is not entirely clear for either of the two classes of antibodies despite extensive structural information on the more traditional CD4bs antibodies. For example, there were only subtle differences between the structures of CD4bs antibodies F105 and b12 bound to a fragment of gp120, despite enormous differences in neutralizing activity (Chen et al., 2009). Further understanding of the molecular basis of differences in the neutralizing activity of these antibodies may require determining the structure of their cocrystals with native gp160.

Anti-CD4bs/DMR antibodies recognize a conformational epitope that includes the α5 helix at the outer-domain–inner-domain junction of gp120. The core epitope is highly conserved across different HIV-1 isolates and is essential for optimal infectivity, and therefore for viral fitness. Importantly, anti-CD4bs/DMR antibodies resemble b3 and b6 antibodies that were isolated as Fab fragments from a phage display library and lack neutralizing activity (Burton et al., 1991; Barbas et al., 1992; Pantophlet et al., 2003). Based on the comparative analysis of the structure of b12, which is broadly neutralizing, and b6, which is not, it has been suggested that a stabilized core protein, which does not allow b6 (Pantophlet et al., 2003; Zhou et al., 2007) or anti-core antibody (Scheid et al., 2009) binding, should be used as a vaccine immunogen (Dey et al., 2007, 2009; Mörner et al., 2009). However, antibodies to the core epitope can account for a significant fraction of the neutralizing activity in sera (Li et al., 2007), and of the antibodies produced by memory B cells (Scheid et al., 2009; Corti et al., 2010), including potent and broadly neutralizing antibodies (Corti et al., 2010). Therefore, we would like to suggest that an optimal HIV vaccine immunogen may require inclusion of the core epitope.

MATERIAL AND METHODS

mABs. Anti-human HIV-1 gp140 antibodies were described in (Barbas et al., 1992; Wardemann et al., 2003; Tiller et al., 2008; Scheid et al., 2009; Corti et al., 2010). All IgGs were expressed by cotransfection in HEK-293T cells (American Type Culture Collection). All work with human samples was performed in accordance with approved Institutional Review Board protocols (Scheid et al., 2009).

Mutagenesis and expression of recombinant gp120. Amino acid numbering refers to the HXB2CG reference sequence (Korber et al., 1998). Alanine mutations were introduced into pYU2gp120 (obtained from J. Sodroski, Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, MA) using the QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutagenesis primers were designed using Stratagene's web-based QuickChange primer design program. All site-directed mutations generated in this study were verified by DNA sequencing.

Recombinant proteins were produced in HEK-293T cells (American Type Culture Collection) grown in Dulbecco's modified Eagle's medium

(Invitrogen) supplemented with 1% (vol/vol) antibiotic-antimycotic (Invitrogen), 1% (vol/vol) L-glutamine (Invitrogen), and 1% (vol/vol) nutridoma-sp (Roche) that were transiently transfected with WT or mutant pYU2gp120 plasmids (50 µg). 3 d after transfection, culture supernatants were harvested and stored at 4°C and supplemented with complete protease inhibitor cocktail tablets (Roche) until use in ELISA.

ELISAs. To determine concentrations of the expressed gp120 proteins, ELISA plates (high-binding capacity; Corning) were coated with affinity-purified sheep anti–HIV-1gp120 antibody (Aalto Bio Reagents) recognizing a linear peptide at the carboxy-terminus (497–511; APTKAKRRVVQREKR) in PBS overnight at 5 µg/ml. The gp120 mutants were captured and detected with an anti-VL antibody (1–79; Scheid et al., 2009). The protein concentration of the gp120 mutants was determined using a gp120 standard and adjusted to 20 µg/ml.

To determine the relative binding of anti-core antibodies to the different gp120 mutants (MUT) compared with gp120WT, ELISA plates were coated as described in the previous paragraph. After washing with ultra pure water and blocking with 2 mM EDTA and 0.05% Tween-20 in PBS for 30 min at room temperature, mutant proteins were captured at 20 µg/ml for 2 h. Anti-core antibodies were incubated with the washed plates at serial dilutions in PBS-10% sheep serum (Equitech-Bio). Bound antibodies were detected with peroxidase conjugated affinity purified goat anti-Human IgG (Jackson ImmunoResearch Laboratories) using an HRP substrate kit (Bio-Rad Laboratories). The ELISA graphs were used to determine the linear range of antibody binding. The optical density (OD_{405nm}) for binding of a given antibody to gp120MUT divided by the OD_{405nm} of the same antibody to gp120WT was considered the percentage of apparent binding.

Peptide ELISA. Peptide ELISAs were performed as previously described (Mouquet et al., 2006). In brief, ELISA plates (Corning) were coated with 50 µl of a peptide containing the core epitope (underlined; DTNGTEIFRPGGGDMRDNWR) at 5 µg/ml, in PBS over night at room temperature. After washing three times with PBS-0.1% Tween 20 (PBST), wells were blocked with 1% PBS, 5% Tween20, and 3% sucrose milk powder for 1 h at room temperature. Serial dilutions of anti-core antibodies (starting at 4 µg/ml in PBST-1% BSA) were added, incubated for 1 h at room temperature, and visualized with peroxidase-conjugated affinity purified goat anti–human IgG (Jackson ImmunoResearch Laboratories) using an HRP substrate kit (Bio-Rad Laboratories). Controls included a V3-loop peptide (NNNTRKSINIGPGRALYTT) that was recognized by an anti-V3-loop antibody (2–59).

Mutagenesis and expression of recombinant gp140. Codon-optimized human BaL gp160 plasmid (pSF219; obtained from A.L. DeVico, University of Maryland, Baltimore, MD) was subcloned into pCDNA3.1. Mutations at the proteolytic cleavage site were introduced (arginines 508 and 511 to serines), as well as the trimerization domain from the C terminus of bacteriophage T4 fibritin inserted, thus leading to pBaLgp140Δ683(-/FT) (Yang et al., 2002). Alanine mutations (D474A/M475A/R476A) were introduced as described and confirmed by sequencing. Recombinant proteins were produced in HEK-293T cells as described and purified by lectin and nickel-chelate affinity chromatography (Freeze, 2001; Petty, 1996).

Surface plasmon resonance. All experiments were performed with a Biacore T100 (Biacore, Inc.) in HBS-EP+ running buffer (Biacore, Inc.) at 25°C. Samples were analyzed in kinetic experiments performed at least in duplicate. BaL gp140 WT and DMR/AAA mutant proteins at 62.5 µg/ml were immobilized on CM5 chips (Biacore, Inc.) by amine coupling at pH 4.5, resulting in an immobilization level of 5,000 RUs. For kinetic measurements on the gp140 WT- and gp140 DMR/AAA-derivatized chips, IgGs were injected through flow cells at 333 nM and 4 successive 1:2 dilutions in HBS-EP+ running buffer (Biacore, Inc.) at flow rates of 40 µl/min with 3-min association and 5-min dissociation. The sensor surface was regenerated between each experiment with a 30-s injection of 10 mM glycine-HCl, pH 2.5, at a flow rate of 50 µl/min. Off rate (s^{-1}), on rate ($k_{a1}[M^{-1}s^{-1}$, $k_{a1}[RU^{-1}s^{-1}]$), and binding constants ($K_{A1}[M]$, $K_{A2}[RU^{-1}]$) were calculated after subtraction of backgrounds (binding to a control flow cell and signal of the HBS-EP+ running buffer) using Biacore T100 Evaluation software using the kinetic analysis and the bivalent model. K_{A1} value was used as an estimation of apparent K_A (K_A^{app}) because K_{A1} represents the majority of the binding.

Expression of the native gp160Δc trimer and FACS-based binding assay. Codon-optimized human BaL gp160 plasmid (pSF219; obtained from A.L. DeVico, University of Maryland, Baltimore, MD), whose intracellular tail was deleted (gp160Δc), was subcloned into pMX-IRES-GFP (Robbiani et al., 2008). Alanine mutations (D474A/M475A/R476A) were introduced as described in the previous paragraph and confirmed by sequencing. Human embryonic kidney BOSC.23 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (vol/vol) fetal calf serum, 1% (vol/vol) antibiotic-antimycotic (Invitrogen), and 1% (vol/vol) L-glutamine (Invitrogen). FuGENE 6 (Roche) was used for transient transfection according to the manufacturer's instructions. 24 h later, cells were harvested with enzyme-free cell dissociation buffer (Invitrogen) and stained with dialyzed human mABs (2–1092, 4–79, HGS2, mgo53, and b12) at 0.4 µg/ml for 30 min at 4°C. Primary antibodies were visualized with APC-conjugated mouse anti–human IgG antibody (BD) according to the manufacturer's instructions. Flow cytometry was perfomed on FACSCalibur (BD). The median fluorescence intensity (MFI) was calculated using FlowJo software. The differences in relative MFI (ΔrMFI) between gp160Δc and MUT BaL gp160Δc were calculated according the following formula: $rMFI = 100 \times (1 - [\{MFI - MFI_{MUT}\}/MFI])$.

Fusion assay. BaL gp160Δc and gp160Δc D474A/M475A/R476A were expressed on BOSC.23 cells, harvested as described, and mixed with mCherry positive TZM.bl cells in excess. The cell suspension was centrifuged at 657× g and 37°C for 1 h and incubated for one additional hour at 37°C and 5% CO_2, then treated with 0.05% Trypsin-EDTA. Cells were assayed on ImageStream100 (Amnis) and BD LSR II (BD Biosciences) and analyzed with FlowJo software. Fused cells (%) = $n(GFP^+mCherry^+)/ [n(GFP^+mCherry^+) + n(GFP^+mCherry^-)]$.

HIV-1 pseudovirus infectivity assay. Alanine mutations were introduced in pYU-2Env and pBaL-Env as described above. Clones were cotransfected with pHIVΔEnv in HEK-293 T cells. The virus stocks were titrated on TZM.bl cells to measure the median tissue culture infective dose ($TCID_{50}$; Li et al., 2005). p24 ELISAs (ZeptoMetrix) were performed according to manufacturer's instructions.

Online supplemental material. The supplemental material includes results of the mapping ELISA (Figs. S1, 3, and 4), the peptide ELISA (Fig. S2), SPR sensograms (Fig. S5), TZM.bl neutralization assay (Table S1), SPR affinities (Table S2), conservation of the core epitope (Table S3), and the pseudo virus infectivity assay (Table S4). Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101176/DC1.

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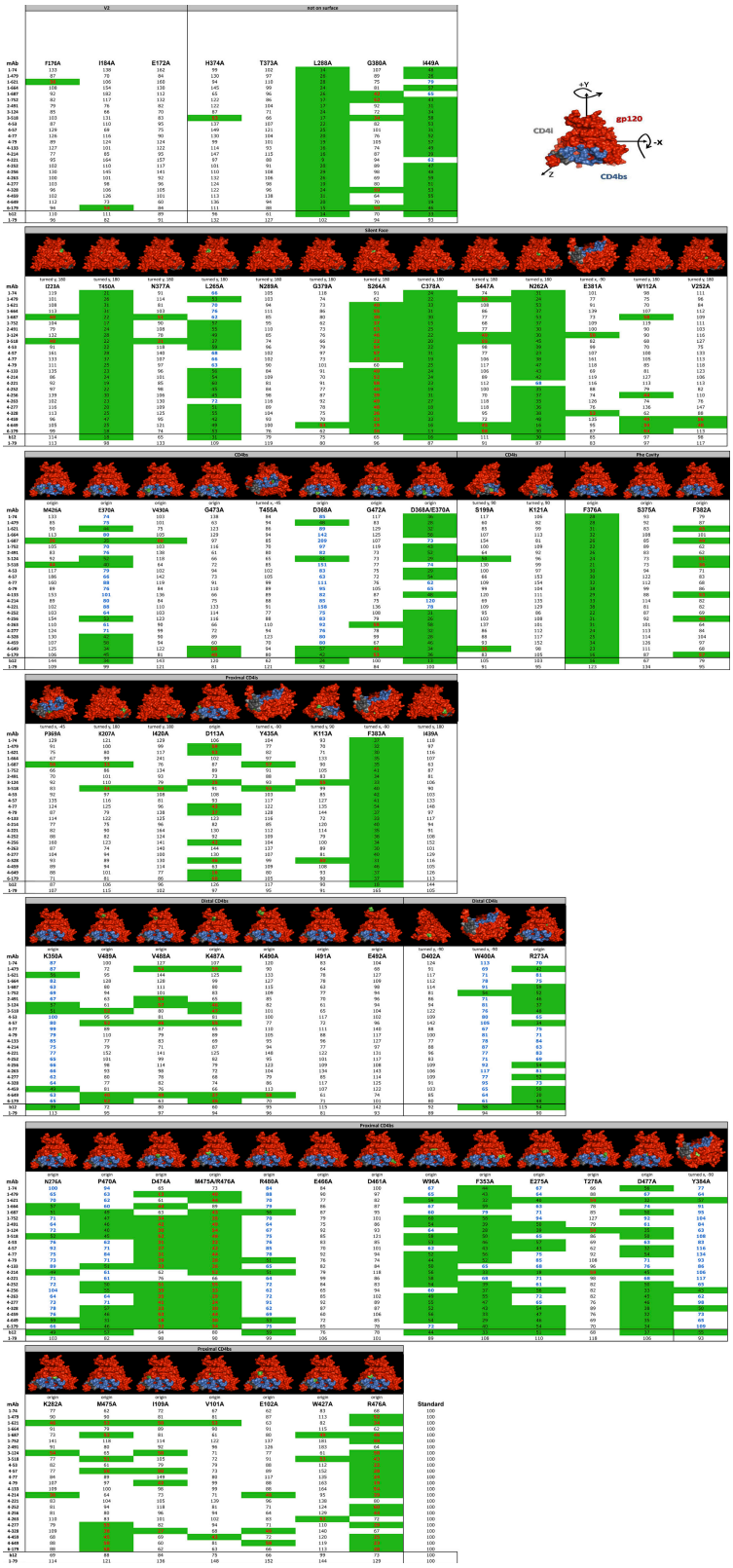


Figure S1. Numbers indicate the apparent binding (%) of the anti-core antibodies to the different gp120 mutant proteins. Green fields indicate a significant decrease (<60%) in binding. Red (knock-down) and blue (no knock-down) values highlight differences in binding properties compared with anti-CD4bs antibody b12. To confirm anti-core epitope, ELISA assays have been performed in three independent experiments.

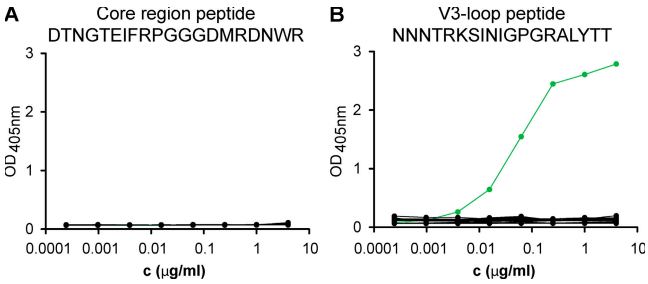
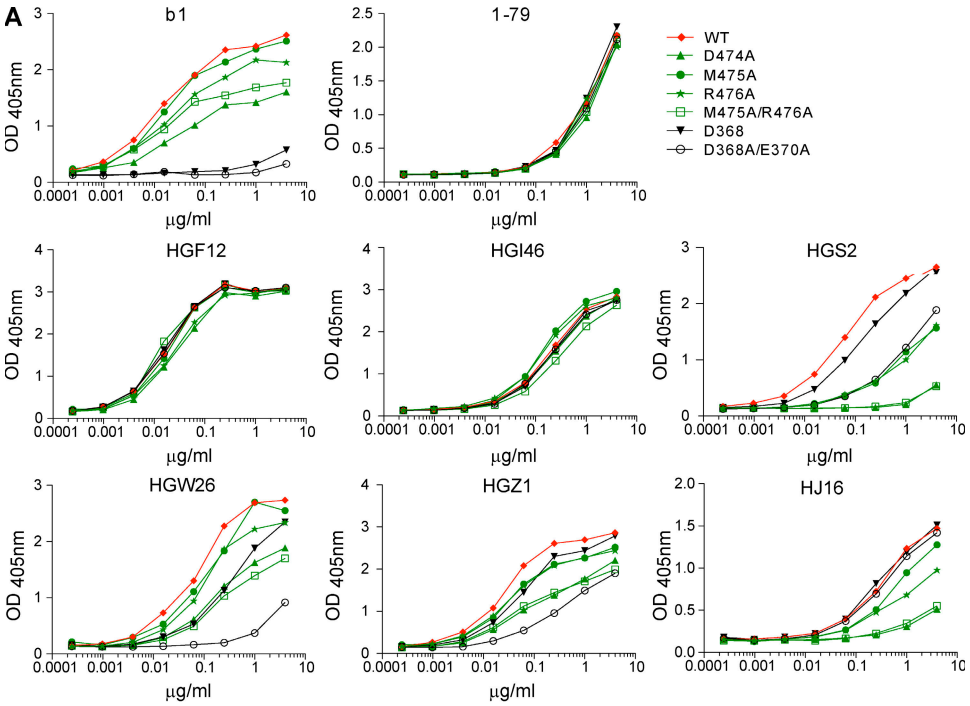


Figure S2. Peptide ELISA. (a) The ELISA graphs show that none of the anti-core antibodies (black lines) recognizes a peptide (DTNGTEIFRPGGGDMRD-NWR) containing the core epitope. (b) Control ELISA shows that the anti-V3 loop antibody (green line) 2–59 (Scheid et al. *Nature* .458:636–640) recognizes the V3-loop peptide (NNNTRKSINIGPGRALYTT). Results are representative for two independent experiments. c, concentration. OD, optical density.



B

	WT	D474A	M475A	R476A	M475A/ R476A	D368A	D368A/ E370A
b12	100	53	99	82	75	10	7
1-79	100	83	99	106	89	107	94
HGF12	100	80	93	82	120	107	101
HGI46	100	91	120	114	78	96	94
HGS2	100	7	28	29	8	78	31
HGW26	100	53	81	82	45	50	9
HGZ1	100	50	79	77	54	70	26
HJ16	100	25	77	55	28	96	93

Figure S3. Fine mapping of "partial CD4bs" antibodies. (a) Shows ELISA binding curves for the different partial CD4bs antibodies (HGF12, HGI46, HGZ1, HGS2, HGW26, HJ16; Corti et al. PLoS ONE. 5:1) to gp120 YU2 WT (in red) and different alanine mutants (black and green lines). Controls included the CD4bs antibody b12 and a variable loop antibody 1–79 (Scheid et al. *Nature*. 458:636–640). Alanine mutants are within the "core" epitope (green lines) and the CD4bs (black lines). (b) Apparent binding (%) of the tested antibodies to the different gp120 mutant proteins was calculated at the linear range of the curves. Red fields indicate a significant decrease (<50%) in binding. Shown are representative results of two independent experiments.

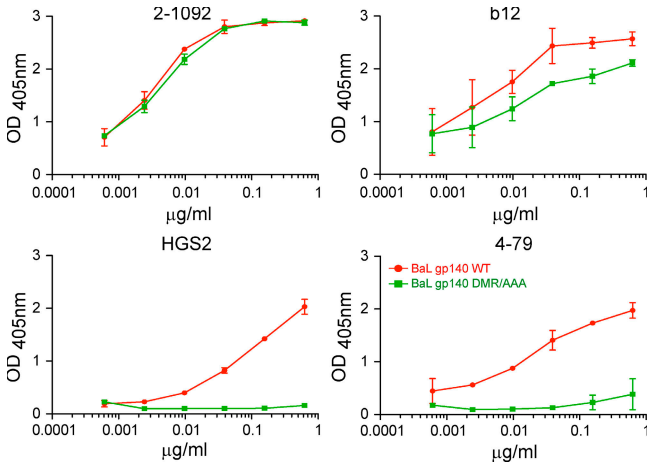


Figure S4. Core epitope mapping on soluble BaL gp140. ELISA curves for different antibodies binding BaL gp140 WT and BaL gp140 D474A/M475A/R476A are shown. Controls include an anti-VL antibody (2–1092; Scheid et al. *Nature* .458:636–640) and b12. Anti-core antibodies 4–79 (Scheid et al.) and HGS2 (Corti et al. *PLoS ONE*. 5:1) are sensitive to the mutant protein. Data shown are mean values calculated from two independent experiments (Standard error bars are indicated).

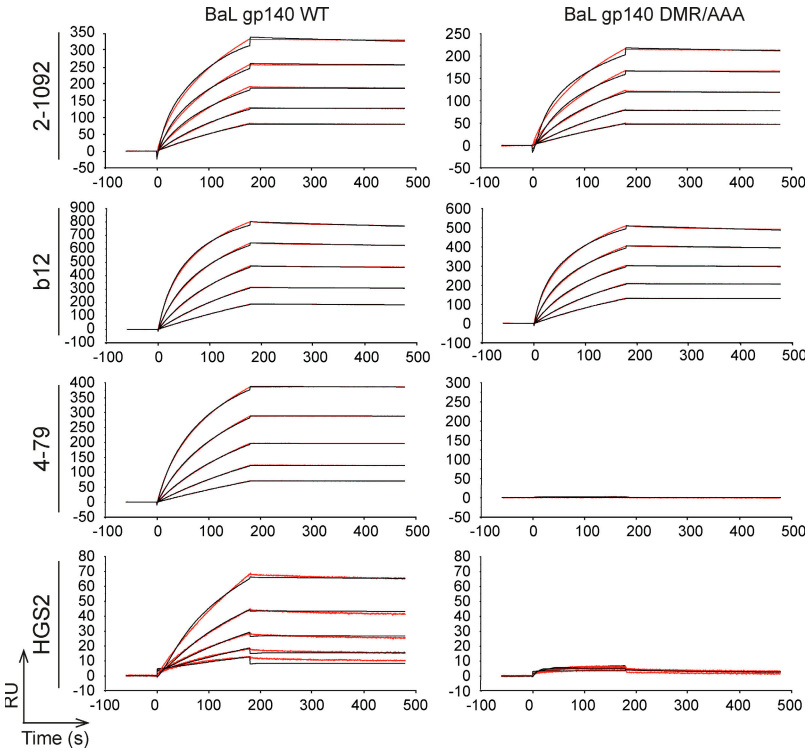


Figure S5. SPR. SPR analyses of the interaction of the selected analytes with the BaL gp140 WT and BaL gp140 DMR/AAA-immobilized chips. Graphs show SPR sensograms over time for the binding of the analytes (IgG antibodies). RU; response units. Experiment has been performed in duplicates.

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Table S1. TZMbl neutralization data (IC_{50}, μg/ml)

Strain	Tier/ Clade	1-74	1-479	1-621	1-664	1-687	1-752	2-491	3-124	3-518	4-53	4-57	4-77	
		anti-core antibodies												
MW965.23	1/C	>50	**<0.02**	**9.8**	>50	>50	>25	**<0.01**	>25	>25	>25	**0.1**	>25	
DJ263.8	1/A	**0.16**	**0.7**	>50	**2.9**	>50	**1.7**	**0.3**	>25	>25	**0.5**	**1.6**	**0.76**	
SF162.LS	1/B	>50	**1.1**	>50	**1.1**	>50	**2.9**	**1.6**	**0.2**	>25	**0.4**	**3.5**	**7.7**	
SS1196.1	1/B	13	30.4	>50	>50	>50	>25	**21.4**	>25	>25	**>25X**	**75.4**	>25	
BaL.26	1/B	**2.75**	**12.2**	>50	**19.2**	>50	>25	**8.2**	>25	>25	**1.4**	**35.7**	**>25X**	
6535.3	2/B	**42.3**	**24.2**	>50	**19.6**	>50	>25	**4.6**	ND	>25	**>25X**	**14.5**	**>25X**	
RHPA4259.7	2/B	>50	>50	>50	>50	>50	>25	>25	ND	>25	>25	>100	>25	
TRO.11	2/B	>50	>50	>50	>50	>50	>25	ND	ND	>25	>25	>100	>25	
CAAN5342.A2	2/B	ND	ND	ND	ND	ND	ND	>25	ND	ND	ND	ND	ND	
SC422661.8	2/B	>50	>50	>50	>50	>50	>25	**79.1**	ND	>25	>25	>100	**>25X**	
THRO4156.18	2/B	ND	ND	ND	ND	ND	ND	**>25X**	ND	ND	ND	ND	ND	
PVO.4	2/B	>50	>50	>50	>50	>50	>25	ND	ND	>25	>25	>100	>25	
Strain	Tier/ Clade	4-79	4-133	4-214	4-221	4-252	4-256	4-263	4-277	4-328	4-459	4-649	6-179	b12
		anti-core antibodies												
MW965.23	1/C	>25	**1.1**	>25	>100	**0.06**	**4.5**	>25	>25	**>25X**	>30	**0.1**	**0.1**	**0.2**
DJ263.8	1/A	**0.85**	**0.7**	**0.6**	**0.6**	**0.8**	**2.4**	>25	**0.7**	**3.8**	>30	**6.3**	**2.1**	>25
SF162.LS	1/B	**12.7**	**1.9**	**1**	**0.2**	**1.7**	**8.3**	**3.2**	**1.3**	>25	**1.3**	**6.8**	**1.9**	**0.01**
SS1196.1	1/B	>25	>50	>25	>100	>30	>25	**>25X**	>25	>25	>30	>50	>50	**0.3**
BaL.26	1/B	**>25X**	**6.9**	**4.7**	**2**	**7.5**	**>25X**	**>25X**	**4.6**	>25	>30	**41.9**	**44.1**	**0.2**
6535.3	2/B	**>25X**	**22.7**	>25	>100	>30	**>25X**	23.5	>25X	>25	**18.5**	**42.9**	**27.2**	**1.4**
RHPA4259.7	2/B	>25	>50	>25	>100	>30	>25	>25	>25	>25	>30	>100	>50	**0.1**
TRO.11	2/B	>25	>50	>25	>100	>30	>25	>25	>25	>25	>30	>100	>50	>50
CAAN5342.A2	2/B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SC422661.8	2/B	**>25X**	**>50X**	>25	**>100X**	>30	>25	>25	>25	>25	>30	>100	>50	**0.2**
THRO4156.18	2/B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PVO.4	2/B	>25	>50	>25	>100	>30	>25	>25	>25	>25	>30	>100	>50	>50

Values represent IC_{50}s in μg/ml for anti-core antibodies and b12 in a TZMbl-based neutralization assay. Bolded values show inhibition at the concentrations tested. X indicates that this given antibody almost reached an IC_{50} at the highest concentration tested. (Scheid et al. *Nature*. 458:636-640)

Table S2. Affinity of IgG antibodies to BaL gp140 WT and BaL gp140 DMR/AAA ligands measured by surface plasmon resonance

IgG antibody	Ligand	k_{a1}	k_{d1}	K_{A1}	k_{a2}	k_{d2}	K_{A2}	fit
		1/M/s	1/s	1/M	1/RU/s	1/s	1/RU	
4-79	gp140 WT	3.2E+05	2.4E-05	1.4±0.09E+10	2.3E+00	2.2E+02	1 ± 0.04E-02	bivalent analyte
	gp140 DMR/AAA	/	/	/	/	/	/	bivalent analyte
HGS2	gp140 WT	1.6E+06	4.1E+04	3.8 ± 1.4E+09	1.1E-03	2.6E-02	1.1 ± 0.3E-02	bivalent analyte
	gp140 DMR/AAA	/	/	/	/	/	/	bivalent analyte
2-1092	gp140 WT	1.0E+06	2.1E-04	4.8 ± 0.5E+09	2.4E+00	1.3E+02	1.8 ± 0.1E-02	bivalent analyte
	gp140 DMR/AAA	2.7E+05	2.8E+04	1 ± 0.4E+09	9.4E-03	2.1E-01	4.1 ± 0.5E-02	bivalent analyte
b12	gp140 WT	2.6E+05	3.8E-04	9.6 ± 5.5E+08	6.8E-02	1.0E+01	1.2 ± 0.6E-02	bivalent analyte
	gp140 DMR/AAA	4.4E+05	2.8E-04	1.6 ± 0.3E+09	7.0E-03	4.3E-01	1.7 ± 0.1E-02	bivalent analyte

Data shown are mean values calculated from at least two independent experiments. SEM (± SEM) are indicated for K_{A1} and K_{A2} values. M, mol/l; s, second; /, no binding detected. See sensograms shown in Fig. S5.

Table S3. Conservation of the core (DMR) epitope.

DMR variant	No.	Percentage
Summary of all variants regardless of subtype (2229 sequences)		
---	1,229	55.1
N-K	519	23.3
N--	156	7
--K	127	5.7
Summary for subtype A1 (84 sequences)		
---	60	71.4
N--	12	14.3
N-K	5	6
Summary for subtype B (697 sequences)		
---	450	64.6
N-K	117	16.8
N--	79	11.3
Summary for subtype C (585 sequences)		
---	261	44.6
N-K	245	41.9
--K	40	6.8
Summary for subtype D (84 sequences)		
---	70	83.3
N-K	6	7.1
Summary for subtype 01_AE (111 sequences)		
NIK	72	64.9
N-K	17	15.3
Summary for subtype 02_AG (72 sequences)		
---	66	91.7

Shown is the analysis for the conservation of the DMR epitope, nt 7644 to 7652 (HXB2 numbering), amino acids 474 to 476 (amino acid position relative to protein start in HXB2 CDS_env). Analysis was performed by using QuickAlign software. DMR region was aligned to current (date 7/26/10) HIV Web alignment database (2,229 sequences; www.hiv.lanl.gov). Alignments are shown for the total number of sequences and for different subtypes. Only variants that are present in >5% of analyzed sequences are listed.

Table S4. Pseudo-viral infectivity

Isolate	Mutant	$TCID_{50}$	p24 ELISA
			ng/ml
YU2	WT	3,024,892	73
	D474A	4,826,599	99
	M475A	8,488,980	67
	R476A	6,391,838	54
	D474A/M475A	3,727,468	57
	D474A/R476A	6,841,400	132
	M475A/R476A	3,497,846	70
	D474A/M475A/R476A	475	101
BaL	WT	283,738	328
	D474A	0	232
	M475A	208,823	264
	R476A	2,089	352
	D474A/M475A	0	271
	D474A/R476A	0	312
	M475A/R476A	160	260
	D474A/M475A/R476A	0	335

Values represent $TCID_{50}$ (50% tissue culture infectious dose) and p24 levels (nanogram/milliliter) for different Env pseudovirus clones. The experiment was independently performed twice.

A mouse model for HIV-1 entry

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Passive transfer of neutralizing antibodies against HIV-1 can prevent infection in macaques and seems to delay HIV-1 rebound in humans. Anti-HIV antibodies are therefore of great interest for vaccine design. However, the basis for their in vivo activity has been difficult to evaluate systematically because of a paucity of small animal models for HIV infection. Here we report a genetically humanized mouse model that incorporates a luciferase reporter for rapid quantitation of HIV entry. An antibody's ability to block viral entry in this in vivo model is a function of its bioavailability, direct neutralizing activity, and effector functions.

HIV-1 (HIV), the causative agent of AIDS, represents a formidable global challenge, with the development of an effective vaccine being of paramount importance (1–4). Rapid progress in this area has been hindered in part by lack of a widely available small animal model for HIV entry. Currently available animal models include nonhuman primates and immunodeficient humanized mice, neither of which is readily available or amenable to genetic modifications (5, 6).

Some viral pathogens exhibit a narrow host range, one of those being HIV. HIV's entry into target cells is mediated by binding of its trimeric envelope spike (gp160) to human CD4 (hCD4) (7) and subsequently to a coreceptor such as human CXCR4 (8) or human CCR5 (hCCR5) (9–11). hCCR5 is of particular interest because it seems to be the primary coreceptor used for transmission (12, 13), as evidenced by the finding that homozygous deletion in the CCR5 allele confers resistance against HIV-1 acquisition (14, 15) and can also lead to long-term control of HIV after stem cell transplantation (16). Finally, HeLa cells and other HIV-resistant cells, including mouse cells, support viral entry when they are engineered to express hCD4/hCCR5/hCXCR4 (17–19).

Here, we describe a hCCR5- and hCD4-expressing luciferase reporter mouse that can be used to measure HIV pseudovirus entry and antibody-mediated protection against initial infection in vivo.

Results

hCCR5-2A-hCD4 Construct. To overcome HIV's host-restriction at the level of viral entry, we coexpressed hCCR5 and hCD4 on a single poly-protein transcript separated by a ribosomal skip 2A peptide sequence (hCCR5-2A-hCD4) (Fig. 1*A*) (20). Coexpression of hCCR5 and hCD4 was verified in transfected HEK293T cells ($293T_{hCCR5\text{-}2A\text{-}hCD4}$) by flow cytometry (Fig. 1*B*). The ability of these proteins to support viral entry was confirmed by infection of $293T_{hCCR5\text{-}2A\text{-}hCD4}$ with an $HIV_{YU\text{-}2}$ pseudotyped lentivirus encoding GFP (Fig. 1*C*). We conclude that the hCCR5-2A-hCD4 mRNA supports cell surface expression of the two proteins that restrict HIV entry into mammalian cells.

Adenoviral Delivery of hCCR5-2A-hCD4. Recombinant adenoviruses provide an established tool for efficient gene delivery and expression (21, 22). To express high levels of the factors that restrict HIV entry in vivo, we used a recombinant human adenovirus (AdV) serotype 5 encoding hCCR5-2A-hCD4 under control of the CMV promoter (AdV-hCCR5-2A-hCD4) (21, 22) (Fig. 2*A*). AdV serotype 5 targets the liver after i.v. injection (23–26). We confirmed that i.v. administration of this vector resulted in hepatic expression of both hCD4 and hCCR5, as measured by fluorescence microscopy (Fig. 2*B*). On the basis of immune fluorescence, we estimate that 30–40% of the cells in the liver express hCD4 and hCCR5.

In Vivo Infection with $HIV_{YU\text{-}2}$-Pseudotyped Lentivirus. To determine whether hCCR5- and hCD4-expressing mice can be used to measure HIV entry in vivo, we transduced mice that carry an inducible loxP-STOP-loxP luciferase reporter [$Gt(ROSA)26Sor^{tm1(Luc)Kaelin}$] (27, 28) with AdV-hCCR5-2A-hCD4 (HIV-LUC_{AdV} mice). One day later, HIV-LUC_{AdV} mice were challenged i.v. with $gp160_{YU\text{-}2}$-pseudotyped lentivirus ($HIV_{YU\text{-}2}$) encoding Cre recombinase (Fig. 3*A*). Productive viral uptake by hCCR5-2A-hCD4 expressing cells would result in the expression of Cre recombinase capable of excising the transcriptional stop element and consequently inducing luciferase expression. Bioluminescence activity, imaged in an optical luminometer (IVIS; Caliper Life Sciences), increased longitudinally and peaked between day 4 and 5 after i.v. pseudovirus injection into HIV-LUC_{AdV} mice (Fig. 3 *B* and *C*). Despite a limited dynamic range and variation by as much as one order of magnitude between mice, there was a highly significant difference between experimental and control groups ($P < 0.0001$; Fig. 3*D*). Taken together, the data indicate that HIV pseudovirus entry can be measured quantitatively in living mice.

Neutralizing Human Anti-HIV Monoclonal Antibodies Mediate Protection in HIV-LUC_{AdV} Mice. Antibodies are key components of most protective vaccines (29, 30) and thus are thought to be essential for protection against HIV infection. In support of this idea, passive administration of potent broadly neutralizing monoclonal antibodies can provide sterilizing immunity against simian/HIV (SHIV) infection in macaques (31–35), and they seem to delay HIV rebound in humans (36, 37). In addition, plasma concentration of anti-HIV IgG antibodies specific for the V1V2 loop region was inversely correlated with infection risk in the recent RV144 vaccine trial (38, 39). However, the mechanisms that mediate the protective effects in RV144 are poorly understood, as exemplified by the finding that the vaccine assessed in the RV144 trial did not elicit broadly neutralizing antibodies (38–40).

We selected six potent, broadly neutralizing anti-HIV antibodies to examine their effects on HIV entry in vivo. NIH45-

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The authors declare no conflict of interest.

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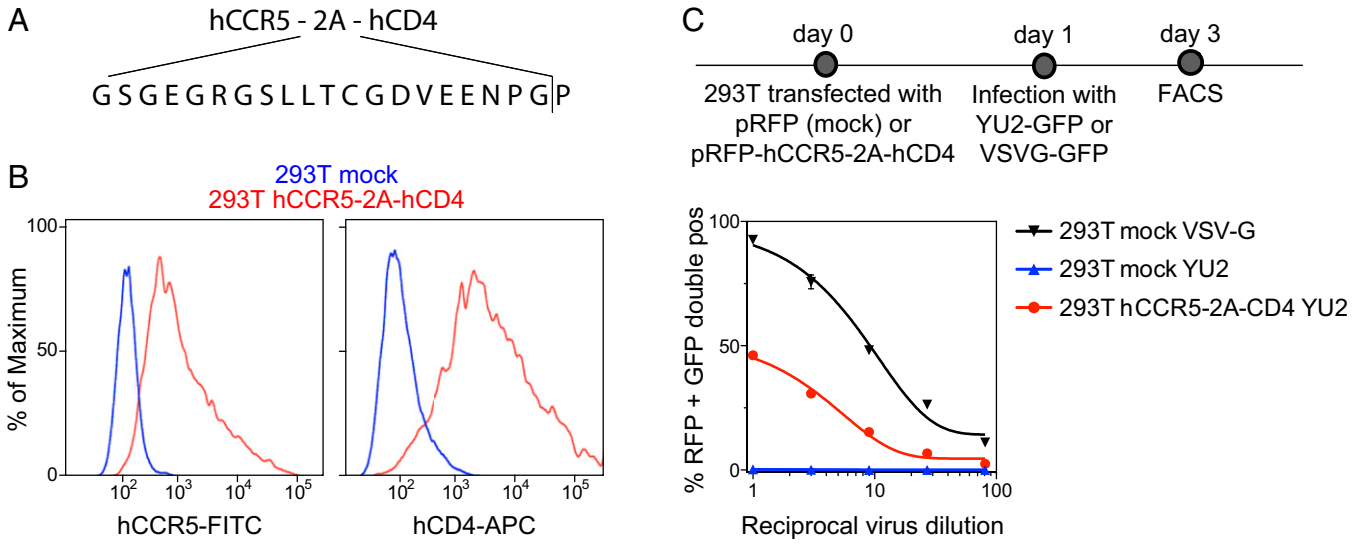

Fig. 1. Construction of hCCR5-2A-hCD4. (*A*) Schematic diagram of the hCCR5-2A-hCD4 construct showing the sequence of the ribosomal skip 2A peptide sequence. (*B*) Representative histogram plots showing the surface expression of hCCR5 and hCD4 on 293T cells transfected with hCCR5-2A-hCD4 [allophycocyanin (APC); fluorescein isothiocyanate (FITC)]. (*C*) Functional expression of hCCR5 and hCD4. 293T cells transfected with hCCR5-2A-hCD4-IRES-RFP were infected with YU2-GFP. Graph shows the percentage of RFP-positive cells that are also GFP positive. Mock-transfected 293T cells served as negative control and infection with VSV-G-GFP as positive control.

46^{G54W} (41), 3BNC117 (42), 3BNC60 (42), VRC01 (43), and b12 (44) all target the CD4 binding site, whereas PG16 (45) targets the V1/V2 loop region. These antibodies have varying levels of neutralizing activity (IC_{50}) in the TZM-bl cell assay against HIV_{YU-2} in vitro, ranging from 0.01 to 2.30 μg/mL (Fig. 4*A*). Antibodies were administered individually at doses ranging from 1 μg to 200 μg s.c. 1 d before challenge with HIV_{YU-2}, and luciferase expression was measured 4 d later. Serum antibody levels determined at the time of pseudovirus injection varied among the selected monoclonal antibodies. For example, high levels were achieved after 3BNC117 injection, whereas antibody concentrations for NIH45-46^{G54W} were fourfold lower (Fig. 4*B*). The different serum antibody levels are in keeping with the half-life of these antibodies, with 3BNC117 having $t_{1/2}$ = 48.6 h and NIH45-46^{G54W} having $t_{1/2}$ = 24.1 h after i.v. injection (Fig. S1).

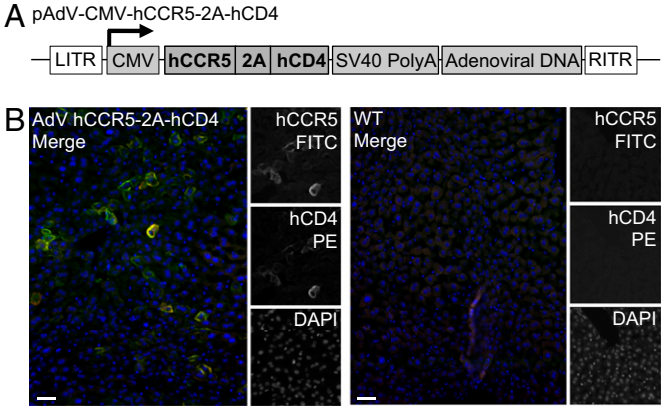

Fig. 2. Adenoviral delivery of AdV-hCCR5-2A-hCD4. (*A*) Schematic diagram of the AdV construct used to deliver hCCR5-2A-hCD4. hCCR5-2A-hCD4 is under control of the CMV promoter. (*B*) hCCR5 and hCD4 were detected in fixed frozen liver sections by immunofluorescence microscopy 1 d after delivery of 10^{11} adenoviral particles of AdV-hCCR5-2A-hCD4 through the lateral tail vein [fluorescein isothiocyanate (FITC); phycoerythrin (PE)]. Representative images of two experiments are shown. (Scale bar, 30 μm.)

In contrast to the HIV neutralizing antibodies that blocked entry with varying degrees of efficacy, an isotype control antibody (mGO53) (46) had no significant effect on entry compared with the PBS control (Fig. 4 *C* and *D*). Fifty percent inhibition of entry was achieved after injection of 100–200 μg of VRC01 or PG16 or b12, whereas the same level of inhibition was obtained with as little as 4–6 μg of 3BNC117 or 3BNC60 or NIH45-46^{G54W}. For 3BNC117 and 3BNC60 this equals a serum concentration of ∼1 μg/mL, which is 100 times greater than the in vitro IC_{50} (Fig. 4*B*). We conclude that the ability of antibodies to inhibit HIV entry in vivo can be measured directly in HIV-LUC_{AdV} mice.

Antibody Fc Involvement in Entry Inhibition. Neutralizing antibodies can protect against HIV infection in vitro in the absence of innate effector cells or complement (47–49). However, the mechanisms by which they mediate protection against HIV in vivo are poorly understood. Complement seems to be essential in antibody-mediated postexposure protection in hu-SCID mice (50), whereas Fc receptors but not complement are important in antibody-mediated preexposure protection in macaques (51), but neither study resolves the question of whether antibodies protect from entry by direct viral clearance or by mediating clearance of infected cells or a combination of both.

To examine the role of antibody effector functions in protection against HIV entry we introduced mutations (G236R/L328R) into the antibody Fc domain that eliminated binding to mFcγRs and complement (Fig. S2) but did not alter antibody binding to the HIV envelope protein or neutralizing activity in vitro (Fig. S3 *A*–*C* and Tables S1 and S2) (52). Dose–response experiments were performed to compare the in vivo activity of $3BNC60^{GR/LR}$, $3BNC117^{GR/LR}$, and $VRC01^{GR/LR}$ to WT controls. GR/LR mutant and WT forms of the individual antibodies were present at similar serum concentrations at the time of pseudovirus injection (Fig. S3*D*). Mutant $3BNC60^{GR/LR}$ and $3BNC117^{GR/LR}$ showed decreased activity at doses of 20 μg of injected antibody compared with the WT form (P = 0.0027 and P = 0.0044, respectively; Fig. 4*E*). Although statistically significant, these differences were small and not found at higher or lower doses of 3BNC60 or 3BNC117 or at any tested dose of

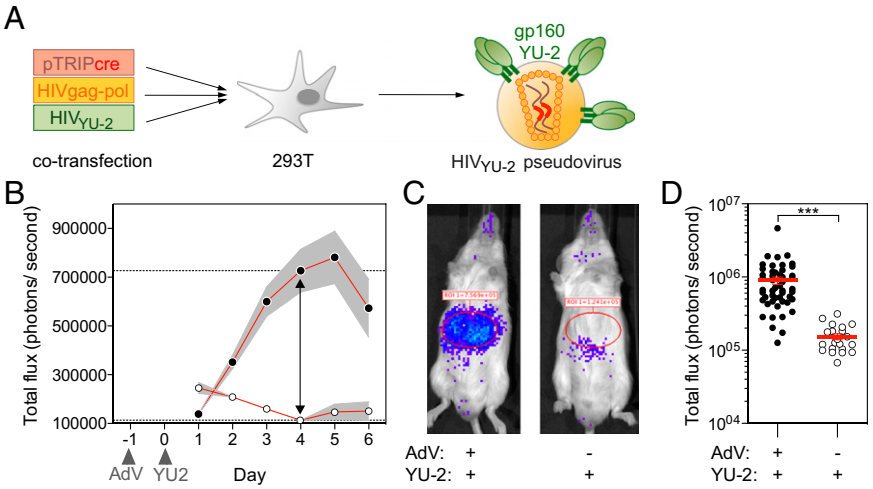

Fig. 3. HIV pseudovirus entry and protection in vivo. (*A*) Diagram summarizes method for producing HIV_{YU-2} pseudovirus. (*B–D*) $ROSA^{flox}$STOP-Luc mice were injected with AdV-hCCR5-2A-hCD4 at day −1, followed by HIV_{YU-2} injection at day 0. Bioluminescence was acquired longitudinally in *B* and at day 4 in *C* and *D*. (*B*) Graph shows the mean total photon flux (±SEM) for $n = 6$ infected mice (filled circles) vs. controls that did not receive the AdV-hCCR5-2A-hCD4 virus, $n = 3$ mice (open circles). (*C*) Representative images of mice from *B*. (*D*) Dot plot shows total photon flux of mice receiving AdV-hCCR5-2A-hCD4 and HIV_{YU-2} (filled circles) or HIV_{YU-2} only (open circles). Each dot represents an individual mouse, error bars represent SEM. ***$P < 0.0001$.

VRC01. Thus, although Fc effector function seems to make a contribution to the inhibition of HIV entry, additional studies will be necessary to fully evaluate this component.

Nonneutralizing Antibodies in Entry Inhibition. Although plasma concentration of anti-HIV IgG specific for the V1V2 loop region was inversely correlated with infection risk in the recent RV144 vaccine trial, broadly neutralizing antibodies were not found (38, 39). Whether nonneutralizing monoclonal antibodies can mediate protection in vivo remains controversial (53). For example, b6, a phage-derived monoclonal anti-HIV antibody with limited in vitro activity showed little or no protection against vaginal SHIV challenge in macaques (54). However, the macaque experiments were performed on a relatively small number of subjects compared with the RV144 trial and may not have been sufficiently powered to detect effects of the magnitude found in the trial (38, 54). To investigate whether nonneutralizing monoclonal antibodies can reduce viral entry, we selected two antibodies that bind to the HIV_{YU-2} trimer expressed as soluble protein (55) but that do not reach an IC_{50} against HIV_{YU-2} at concentrations of up to 50 μg/mL in vitro: 1-79 targets the V3 loop (55), and 1-74 recognizes an epitope in proximity to the CD4 binding site (55). Both of these antibodies were tested for their ability to block entry in vivo by injecting mice with 200 μg as described above. Neither reduced entry significantly compared with the isotype control ($P = 0.1848$ and $P = 0.0830$, respectively; Fig. 4*F*). When testing 1-79 and 1-74 for binding to the HIV_{YU-2} trimer expressed on the surface of 293T cells, we found that their binding is relatively weak compared with the neutralizing antibodies examined above (Fig. 4*G*). Thus, antibodies that bind weakly to the HIV trimer and do not neutralize the virus in the TZM-bl assay seem to have little or no effect reducing HIV entry in vivo. Additional studies with antibodies such as those derived from the RV144 trial will be necessary to fully evaluate the contribution of nonneutralizing antibodies in entry inhibition.

Discussion

Basic understanding of the humoral immune response to HIV has advanced significantly over the last several years. Antibody cloning experiments have revealed that humans can develop potent and broadly active serologic activity to the HIV trimer by producing combinations of antibodies to many different epitopes (55, 56), or by producing unique monoclonal antibodies to specific target sites, such as the CD4 binding site (42, 57). Moreover, broadly neutralizing human monoclonal antibodies can prevent chimeric SHIV infection in nonhuman primate models (31–35), and antibodies seem to be the only correlate of protection in the recent RV144 vaccine trial (38, 39).

However, progress toward an effective antibody-based vaccine has been elusive. One of the significant challenges in this area of research has been the development of adequate animal models to test vaccines or to study the mechanisms of action of broadly neutralizing antibodies. In vitro assays for antibody neutralization such as the TZM-bl assay, which is a reproducible and reliable assay for neutralizing antibody activity, do not take into account Fc-mediated antibody effector function or bioavailability (18). Moreover, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI) assays, which are commonly used to try to measure effector function in vitro, are far more difficult to reproduce and standardize than the TZM-bl assay and are limited in that they include only a fraction of the effector cells that participate in antibody-mediated effector activity in vivo (49).

Although they cannot be infected with HIV, rhesus macaques are the current gold standard for testing vaccines because they can be challenged with simian immunodeficiency virus (SIV) (58), or with chimeric SHIV (59). However, there are a number of important issues with these models, including the fact that SIV and SHIV do not produce the same disease in rhesus macaques as HIV in humans, nor are they identical to HIV in terms of the requirements for effective vaccination (60). In addition, there is the problem of limited availability of macaques, enormous expense, inability to control for genetic heterogeneity, and finally ethical considerations.

Humanized mice (hu-mice) containing human cell- and, in some cases, tissue-transplants represent an alternative to macaques (reviewed in ref. 61). This model may be particularly useful for pharmacologic studies of anti-HIV drugs (62) and passive immunization (63), but it is limited in some important ways. Adaptive immune responses, and in particular antibody-mediated immune responses to HIV, are not optimal in these mice, and therefore they are not amenable to studies that require

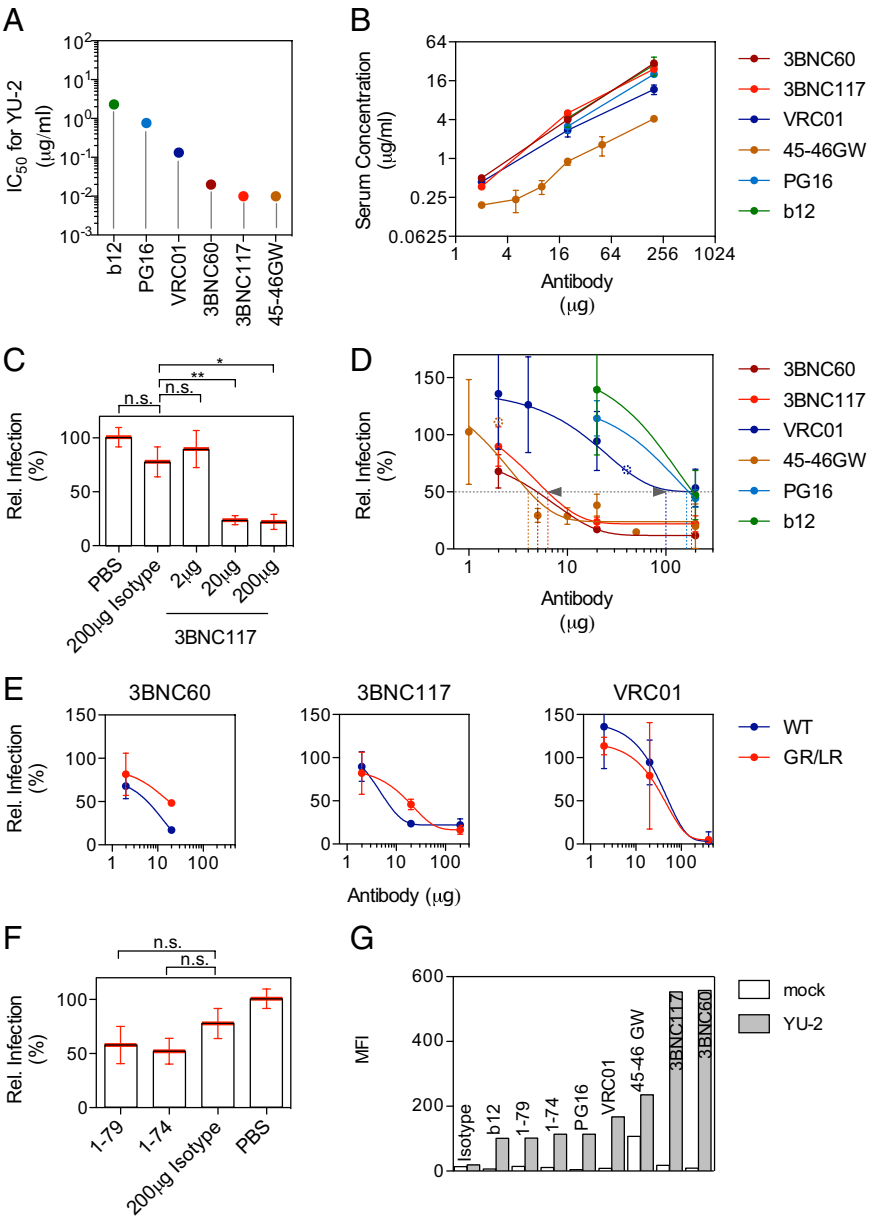

Fig. 4. In vitro and in vivo neutralization data. (*A*) Broadly neutralizing mAbs were tested in the TZM-bl assay for neutralization of HIV_{YU-2}. Plot shows the IC_{50} in μg/mL against HIV_{YU-2} for the individual monoclonal antibodies. (*B*) Serum antibody concentration after 1 d, the time point of pseudovirus injection, as determined by ELISA. The injected antibody amount (μg) is plotted against the determined serum concentration of the monoclonal antibody (μg/mL). (*C* and *D*) Relative infection (% compared with PBS control) plotted against the amount of mAb injected (μg) in bar graph (*C*; six to 17 mice per group, mean ± SEM; $*P < 0.05$, $**P < 0.01$) or dose–response curves (*D*; three to nine mice per group, except open circles represent a single mouse, mean ± SEM). (*E*) Comparison of the in vivo neutralizing activity of the WT form and GR/LR mutant of 3BNC60, 3BNC117, and VRC01 (three to nine mice per group, mean ± SEM). (*F*) In vivo neutralizing activity of nonneutralizing antibodies 1-79 and 1-74 (14 to 20 mice per group, mean ± SEM). (*G*) Binding of antibodies to the envelope of HIV_{YU-2} expressed on the surface of 293T cells. Graph shows the mean fluorescent intensity (MFI) for the different monoclonal antibodies binding to HIV_{YU-2} expressed on 293T cells. Mock-transfected cells served as negative control.

vaccination (64–67). Equally important, genetic experiments are not possible in chimeric mice containing mouse and human cells.

In contrast to hu-mice, the HIV-LUC_{AdV} mouse model is fully immunocompetent, providing a platform for combined immunization and challenge studies using the i.v. route of infection. This route of exposure, which occurs primarily among i.v. drug users, represents a growing global health challenge (68). Additional advantages of the HIV-LUC_{AdV} mice include the use of replication-deficient pseudovirus (18) as opposed to infectious virus and the possibility of genetic modifications. However, HIV-LUC_{AdV} mice express hCCR5/hCD4 in hepatocytes, a nonphysiological target of HIV, owing to preferential hepatic expression of the Coxsackie and adenovirus receptor (CAR) in mice (69, 70). Although infection of other cell types might be achieved, it would require transgenic expression of human CAR (hCAR); for example, under control of the ubiquitin promoter using the available hCAR transgenic mouse (71) or targeting hCAR expression to specific cell types using tissue-specific promoters (72). Alternatively, if expression is high enough, transgenic mice expressing hCCR5/hCD4 under a T cell-specific

promoter (19) could be used after breeding to Gt(ROSA)26Sor$^{tm1(Luc)Kaelin}$ reporter mice. Still another alternative that could be used in combination with humanized mice would be infectious molecular clones that stably express luciferase (Env-IMC-LucR) (73).

Previous experiments in hu-mice and macaques showed that broadly neutralizing antibodies can interfere with HIV infection in vivo (50, 51). However, these experiments could not distinguish whether protection from infection was due to the effects of antibodies on infected cells or to blocking HIV entry directly, or a combination thereof. The availability of HIV-LUC$_{AdV}$ mice allowed for quantitative assessment of potent broadly neutralizing antibodies in vivo and revealed that the antibodies block viral entry directly because this model does not support HIV envelope expression on the surface of infected cells. This means that ADCC would not influence viral control in this system; however, other effector mechanisms, such as Fc-dependent viral uptake by Kupffer cells or other phagocytes, could play a role in viral clearance (74).

A large number of antibodies were compared directly using pseudotyped virus expressing the envelope of HIV$_{YU-2}$, a difficult-to-neutralize tier 2 virus. The HIV-LUC$_{AdV}$ mouse model made it possible to perform dose–response experiments with a significant number of mice (220 in total), something that would be very difficult to achieve in macaques, for which the number of well-characterized SHIV envelopes and experimental animals are very limited. We find that neutralizing activity is related to both the in vitro activity in TZM-bl assays and to bioavailability in vivo. For example, VRC01 did not perform as well as 3BNC60 after s.c. injection of equivalent doses, likely owing to the lower bioavailability of VRC01 and the weaker in vitro neutralizing potency. Conversely, NIH45-46^{G54W}, which had equal potency in vitro but lower bioavailability than 3BNC117, was similar in its ability to the latter in blocking infection in HIV-LUC$_{AdV}$ mice. The low bioavailability of NIH45-46^{G54W} is probably related to its high levels of polyreactivity, as evidenced by its binding to mock-transfected HEK293T cells (Fig. 4*G*). Finally, our results suggest an involvement of Fc effector functions in entry protection, and therefore our experiments are in agreement with the macaque SHIV model (51) but extend those findings by revealing the importance of antibody dose on the effect of the antibody Fc region on protection against HIV in vivo. Additional insights might also be gained by evaluation of antibodies targeting glycan residues (75) or antibodies targeting the membrane-proximal external region (74).

In conclusion, HIV-LUC$_{AdV}$ mice resemble the TZM-bl assay system in that they serve as an assay for HIV entry into heterologous cells, with the important additional dimension of doing so in vivo where the effects of antibody effector functions and bioavailability can be measured.

Materials and Methods

Animals. Gt(ROSA)26Sor$^{tm1(Luc)Kaelin}$ (ROSA^{flox}STOP-Luc) mice (27) were purchased from The Jackson Laboratory. ROSA^{flox}STOP-Luc mice contain the firefly luciferase reporter under control of the ROSA26 promoter. Cre-mediated excision of the transcriptional stop cassette results in luciferase expression. Mice were bred and maintained at the Comparative Bioscience Center at The Rockefeller University according to guidelines established by its Institutional Animal Committee.

AdV Constructs. hCD4 and hCCR5 were PCR-amplified from pT4B (76) and pc.CCR-5 (9, 77) respectively.

hCD4 forward: GTCGACGCCACCATGAACCGGGGAGTCCCTTT
hCD4 reverse: GCGGCCGCCATTCATTCATTCAAATGGGGCTACATGTCTT
hCCR5 forward: GTCGACGCCACCATGGATTATCAAGTGTCAAG
hCCR5 reverse: GCGGCCGCCATTCATTCATTCACAAGCCCACAGATATTT

hCCR5 and hCD4 were linked by the ribosomal skip T2A peptide sequence (GSGEGRGSLLTCGDVEENPGP) (20) and inserted into pMX-IRES-mCherry (78) using EcoRI and BamHI sites.

The adenoviral construct expressing hCCR5-2A-hCD4 was created using the AdEasy Adenoviral Vector System (Agilent Technologies) according to the manufacturer's instructions. All plasmid constructs were verified by DNA sequencing.

A detailed description of flow cytometry, production of recombinant AdVs, HIV pseudovirus production and in vivo infection, histological detection of hCCR5 and hCD4, serum antibody ELISA, bioluminescence imaging, neutralization assays, recombinant protein expression and purification, site-directed mutagenesis, gp140 ELISA, surface plasmon resonance, C1q binding and C3 fixation assays, mAb binding to cell surface gp160, immune complex binding assay, and statistical analysis are provided in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Flow Cytometry. hCCR5 and hCD4 surface expression on 293T cells was confirmed by flow cytometry using an LSRFortessa cytometer (BD Biosciences). Cells were stained with fluorescein isothiocyanate (FITC) mouse anti-human CD195 (CCR5) (BD Pharmingen) and allophycocyanin (APC) mouse anti-human CD4 (BD Pharmingen) according to manufacturer's instructions. Data were analyzed using FlowJo software (Treestar).

Production of Recombinant Adenoviruses. Adenoviral stocks were generated as previously described (1). Briefly, AdV-hCCR5-2A-hCD4 (AdV serotype 5) was transfected into HEK293 cells (ATCC) using the calcium-phosphate method. At full cytophatic effects, transfected cultures were harvested and freeze–thawed. Supernatants were serially passaged two more times. For virus purification, cell pellets were resuspended in 0.01 M sodium phosphate buffer (pH 7.2) and lysed in 5% sodium deoxycholate, followed by DNase I digestion. Lysates were centrifuged and the supernatant was layered onto a 1.2- to 1.46-g/mL^{-1} CsCl gradient, then spun at 95,133 × *g* on a Beckman Optima 100K-Ultra centrifuge using an SW28 spinning-bucket rotor (Beckman-Coulter). Adenovirus bands were isolated and further purified on a second CsCl gradient using an SW41.Ti spinning-bucket rotor. Resulting purified adenoviral bands were isolated and twice dialyzed against 4% (wt/vol) sucrose. Adenovirus concentrations were determined at 10^{12} times the OD_{260} reading on a FLUOstar Omega plate reader (BMG Labtech). Adenovirus stocks were aliquoted and stored at −80 °C. For in vivo transduction, 10^{11} particles were injected through the lateral tail vein.

HIV Pseudovirus Production and in Vivo Infection. HIV_{YU-2} pseudovirus was generated by cotransfection of pTRIPcre [nls-cre cloned into pTRIP (2, 3) using XbaI/XhoI sites], HIV gag-pol (4), and $pSVIIIenv_{YU-2}$ (5) in HEK293T cells (ATCC) using X-tremeGENE 9 (Roche) according to the manufacturer's instructions. Plasmids were transfected at a 1.42:1:1.68 ratio (pTRIPcre:HIV gag-pol:$pSVIIIenv_{YU-2}$). Pooled supernatants were clarified by centrifugation at 300 × *g*, filtered through a 0.45-μm filter unit (Thermo Scientific) to remove cell debris and concentrated 20-fold using a stirred cell (Millipore) with a 300,000 nominal molecular weight limit (NMWL) cutoff membrane. Viral preparations were quantified using a p24 ELISA kit (PerkinElmer) according to manufacturer's instruction. For in vivo infection, 500 μL of concentrated pseudovirus (c_{p24} = 1.835 ± 0.3918 μg mL^{-1}, mean ± SEM) were injected through the lateral tail vein.

Histological Detection of hCCR5 and hCD4. Livers of mice injected with AdV-hCCR5-2A-hCD4 were harvested 24 h after infection and fixed in 4% (vol/vol) paraformaldehyde, 10% (wt/vol) sucrose for 1 h at 4 °C, followed by incubation in 30% (wt/vol) sucrose overnight at 4 °C. Tissue was frozen in optimal cutting temperature compound (Ted Pella, Inc.) at −80 °C. Tissue sections (8 μm) were cut on Superfrost Plus microscope slides (Fisher Scientific). Sections were immunostained with phycoerythrin (PE) mouse anti-human CD4 and FITC mouse anti-human CCR5 (both BD Pharmingen). Nuclei were detected using ProLong Gold + DAPI (Invitrogen). Images were captured on an Axioplan 2 imaging fluorescence microscope (Zeiss) using Metavue Software (Molecular Devices) and processed in ImageJ (National Institutes of Health).

Serum Antibody ELISA. ELISA plates (Corning) were coated with goat anti-human IgG (Jackson ImmunoResearch Laboratories) at 2.5 μg mL^{-1} overnight. Plates were blocked with 2 mM EDTA and 0.05% Tween-20 in PBS (blocking buffer) for 1 h at room temperature. Serial dilutions of mouse serum in PBS were incubated and detected with an HRP-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories) at a 1:1,000 dilution in blocking buffer. Samples were subsequently developed with ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (Invitrogen). Purified human IgG was included to generate the standard curve.

Bioluminescence Imaging. Mice were injected with 10^{11} adenoviral particles i.v. and mAbs s.c. 24 h before i.v. injection with HIV_{YU-2} pseudovirus. At day 4 after pseudovirus infection mice were anesthetized with 2% (vol/vol) isoflurane and injected i.p. with 4.5 mg D-Luciferin (Caliper Life Sciences). Bioluminescence was acquired using an IVIS Lumina II platform (Caliper Life Sciences).

Neutralization Assays. TZM-bl neutralization screens were performed as previously described (6).

Briefly, neutralization was detected as reduction in luciferase reporter gene expression after single round infection in Tzm.bl cells. Murine leukemia virus was used as a negative control.

Recombinant Protein Expression and Purification. Recombinant proteins were expressed in HEK293T cells (ATCC). Human monoclonal antibodies were purified using Protein G Sepharose 4 Fast Flow (GE Healthcare) as previously described (7). Antibodies were dialyzed against PBS and sterile filtered (0.22 μm). Endotoxin (LPS) contamination was quantified by the Limulus amebocyte lysate assay (Associates of Cape Cod) and levels were <0.005 EU mg^{-1}. His-tagged recombinant proteins were purified using His-Tag isolation and pull-down dynabeads (Invitrogen). Purity was assessed by SDS/PAGE and Coomassie staining and was estimated to be >90%.

Site-Directed Mutagenesis. G236R/L328R mutations of the hIgG1 constant region were introduced using the QuikChange site-directed mutagenesis Kit II (Agilent Technologies). For the introduction of the G236R mutation the following primer pairs were used: forward: 5′-GCACCTGAACTCCTGAGGGGACCGTCAGTCTTCCTC; reverse: 5′-GAGGAAGACTGACGGTCCCCTCAGGAGTTCAGGTGC. For the L328R mutation: forward: 5′-GGTCTCCAACAAAGCCCGCCCAGCCCCCATCGAG; reverse: 5′-CTCGATGGGGGCTGGGCGGGCTTTGTTGGAGACC. Mutated plasmid sequences were validated by direct sequencing (Genewiz).

gp140 ELISA. Recombinant gp140 (8) (50 ng per well) was immobilized onto high-binding 96-well microtiter plates (Nunc). After blocking with PBS + 2% (wt/vol) BSA + 0.05% Tween 20 for 2 h, plates were incubated for 1 h with serially diluted IgG antibodies in PBS, followed by HRP-conjugated goat anti-human IgG (1 h; 1:5,000; Sigma). Plates were developed using the 3,3′,5,5′-Tetramethylbenzidine (TMB) two-component peroxidase substrate kit (KPL).

Surface Plasmon Resonance. All experiments were performed with a Biacore T100 SPR system (Biacore, GE Healthcare) at 25 °C in HBS-EP^{+} buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% (vol/vol) surfactant P20]. For the determination of the affinity of mouse FcγRs for human IgG1

and Fc domain variants, soluble ectodomains of mouse FcγRI, FcγRII, FcγRIII, and FcγRIV diluted at 20 μg mL^{-1} in 10 mM sodium acetate (pH 4.5, or pH 5 for FcγRI) were immobilized on Series S CM5 chips by amine coupling, resulting in a density of 2,000 response units (RU). Recombinant IgG samples were injected through flow cells at seven different concentrations (ranging from 2,000 to 31.25 nM; 1:2 successive dilutions) at a flow rate of 30 μL min^{-1} for 120 s, followed by a 300-s dissociation step. After each assay cycle, the sensor surface was regenerated with a 30-s injection of 25 mM NaOH at a flow rate of 30 μL min^{-1}. For the measurement of anti-gp140 IgG affinity for gp140 or 2-CC core (9), IgG antibodies (diluted at 20 μg mL^{-1} in 10 mM sodium acetate, pH 4.5) were immobilized on Series S CM5 chips by amine coupling at a density of 1,000 RU. Recombinant gp140 trimer or 2-CC core were injected through flow cells at a flow rate of 20 μL min^{-1}, with the concentration ranging from 31.25 to 1,000 nM (1:2 successive dilutions). Association time was 120 s, followed by 300 s dissociation. At the end of each cycle, sensor surface was regenerated with 50 mM NaOH (50 μL min^{-1}; 30 s). Background binding to blank immobilized flow cells was subtracted, and affinity constants were calculated using BIAcore T100 Evaluation software using the 1:1 Langmuir binding model.

C1q Binding and C3 Fixation Assays. Mouse C1q binding to mAbs and mAb-mediated fixation of mouse serum C3 were measured by ELISA. Antibodies were serially diluted (100–0.1 μg mL^{-1}) in PBS and coated overnight (4 °C) onto high-binding 96-well microtiter plates (50 μL per well). After washing with PBST [PBS + 0.05% (vol/vol) Tween-20], plates were blocked with protein-free blocking buffer (Pierce). Normal mouse serum [3% (vol/vol)] was added and incubated for 60 min with gentle shaking. For the detection of C1q binding, biotinylated mouse monoclonal anti-C1q antibody (JL-1, Abcam) was added at 0.5 μg mL^{-1}, whereas for the C3 fixation assay, biotinylated chicken polyclonal anti-C3 antibody (ab14232, Abcam) was used at a final concentration of 1 μg mL^{-1}. Streptavidin HRP (Invitrogen) was used at a dilution of 1:2,500, and plates were developed and analyzed as described above.

mAb Binding to Cell-Surface gp160. HEK293T cells were transfected with pMX-$gp160^{YU.2}$Δc-IRES-GFP (8). Mock-transfected cells (carrying pMX-IRES-GFP) were used as controls. Cells were maintained in DMEM supplemented with 10% (vol/vol) FBS and 10 μg mL^{-1} puromycin. Cells were detached (5 mM EDTA in PBS), resuspended at 10^7/mL in PBS + 5% (vol/vol) FBS + 2 mM EDTA, and incubated with the various anti-gp140 mAbs (mGO53 was used as a negative control) at a final concentration of 20 μg mL^{-1} for 30 min at 4 °C. Cells were then incubated for 20 min at 4 °C with Alexa Fluor 647-conjugated goat anti-human IgG (1 μg mL^{-1}; Invitrogen) and analyzed on a BD FACSCalibur cytometer (BD Biosciences). Data were analyzed using FlowJo (Treestar) software.

Immune Complex (IC) Binding Assay. CHO cells expressing mouse FcγR were previously described (10). Cells were maintained in DMEM supplemented with 10% (vol/vol) FBS and 0.1 mM MEM nonessential amino acids with the corresponding selection agent. ICs were generated by incubating mGO53 hIgG1 WT and GR/LR mAb (1 mg mL^{-1}) with Alexa Fluor 647-conjugated goat $F(ab')_2$ anti-human $F(ab')_2$ (200 μg mL^{-1}; Jackson Immunoresearch) for 20 min at 37 °C. ICs were centrifuged for 1 min at 14,000 × *g* to remove large aggregates, and supernatant was serially diluted (100-0.1 μg mL^{-1} based on hIgG concentration) in PBS +0.5% (wt/vol) IgG-free BSA (PBS-B) and immediately used for the IC binding assay. FcγR-CHO cells were detached using 0.01% trypsin + 5 mM EDTA and resuspended at 5×10^6 cells mL^{-1} in PBS-B. Cells were incubated for 30 min on ice, washed twice with PBS-B, and IC binding was quantified using a BD FACSCalibur cytometer (BD Biosciences). As control to determine the specificity of the FcγR-IC interaction, cells were preincubated with function blocking anti-FcγRII/III (2.4G2) or anti-FcγRIV (9E9) antibodies (final concentration 10 μg mL^{-1}) 10 min before the addition of the ICs. Data were analyzed using FlowJo (Treestar) software.

Statistical Analysis. Statistical analysis was performed using Prism software (GraphPad). Statistics were calculated using an unpaired one-tailed *t* test. Asterisks ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$) indicate significant difference from the control group. Columns and error bars represent mean ± SEM.

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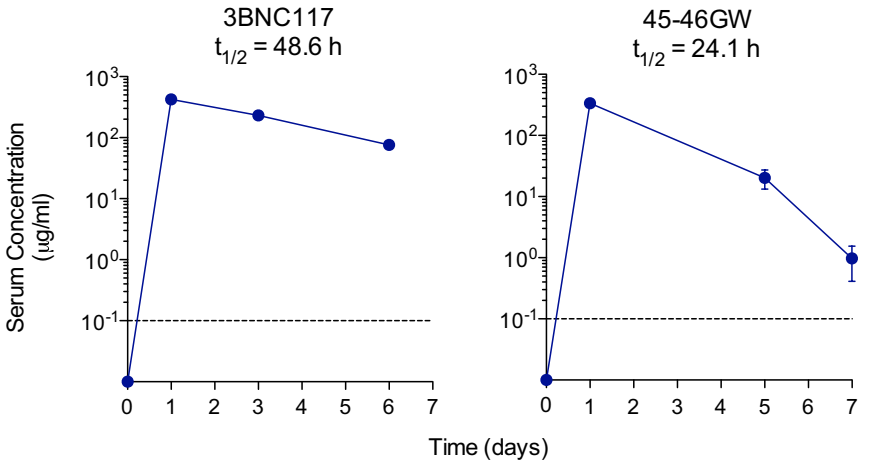

Fig. S1. Antibody half-life. Antibody serum concentration in NOD Rag1$^{-/-}$ IL2Rγ^{NULL} mice was determined by ELISA after i.v. injection of 0.5 mg antibody at day 0. Graphs show the actual serum concentration (mean ± SEM, three mice each) of 3BNC117 (*Left*) and 45-46^{G54W} (*Right*) plotted against the time after monoclonal antibody injection. Dotted line indicates limit of detection. The mean half-life ($t_{1/2}$) was calculated by using a one-phase exponential decay model between day 1 and day 5–7.

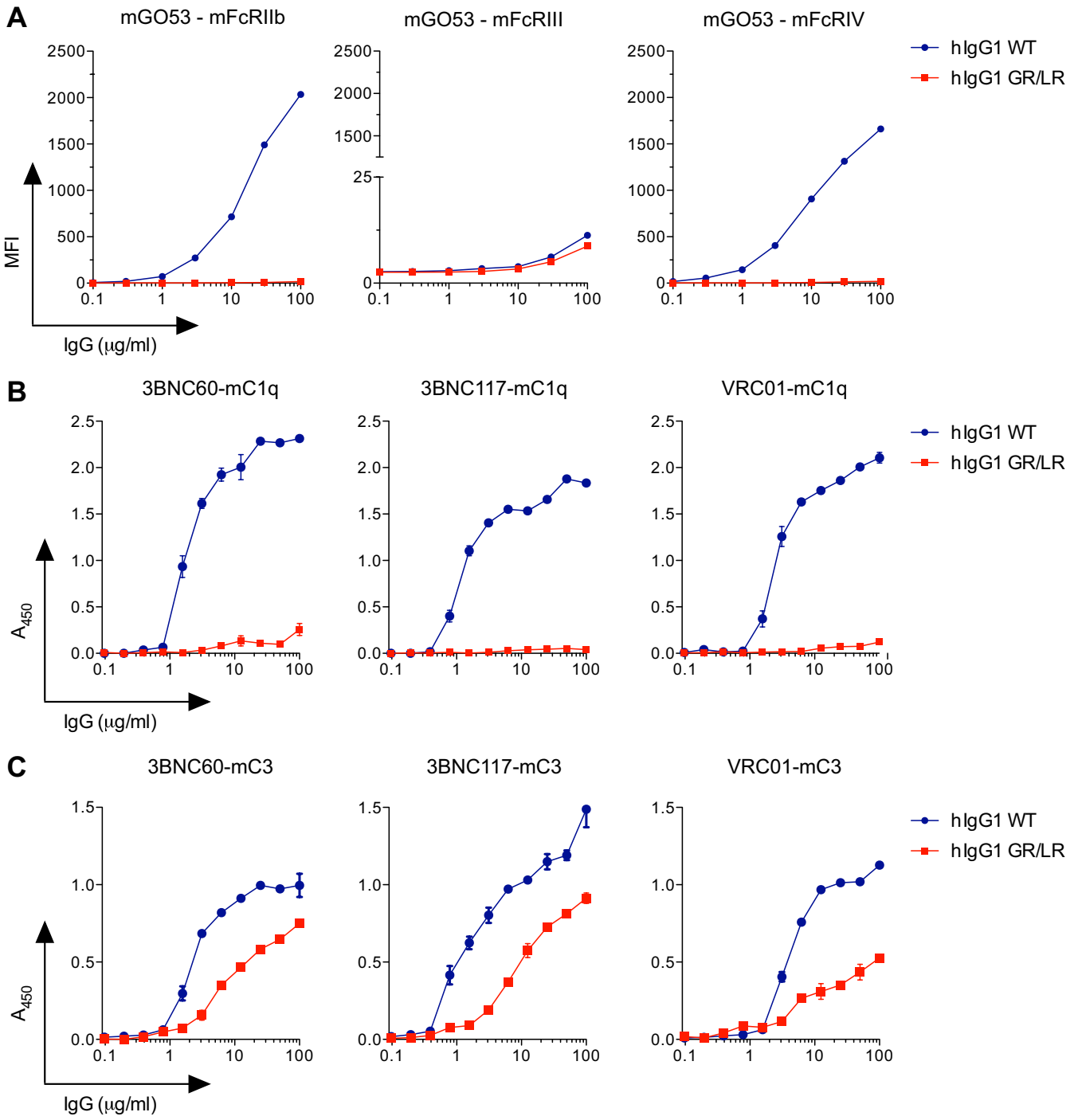

Fig. S2. Antibody Fc: hIgG1 WT vs. hIgG1 G236R/L328R. (*A*) Binding of hIgG1 (WT or G236R/L328R) after forming immune complexes with Alexa Fluor 647-conjugated goat F(ab')$_2$ anti-human F(ab')$_2$ to mouse FcγRIIb, mFcγRIII, and mFcγRIV stably expressed on CHO cells. Graph shows the mean fluorescent intensity (MFI) vs. the monoclonal antibody concentration. (*B* and *C*) Binding of 3BNC60, 3BNC117, and VRC01 hIgG1 WT and GR/LR mutant to mouse C1q (*B*) or mouse C3 (*C*) as determined by ELISA. Graph shows the absorption at 450 nm vs. the monoclonal antibody concentration.

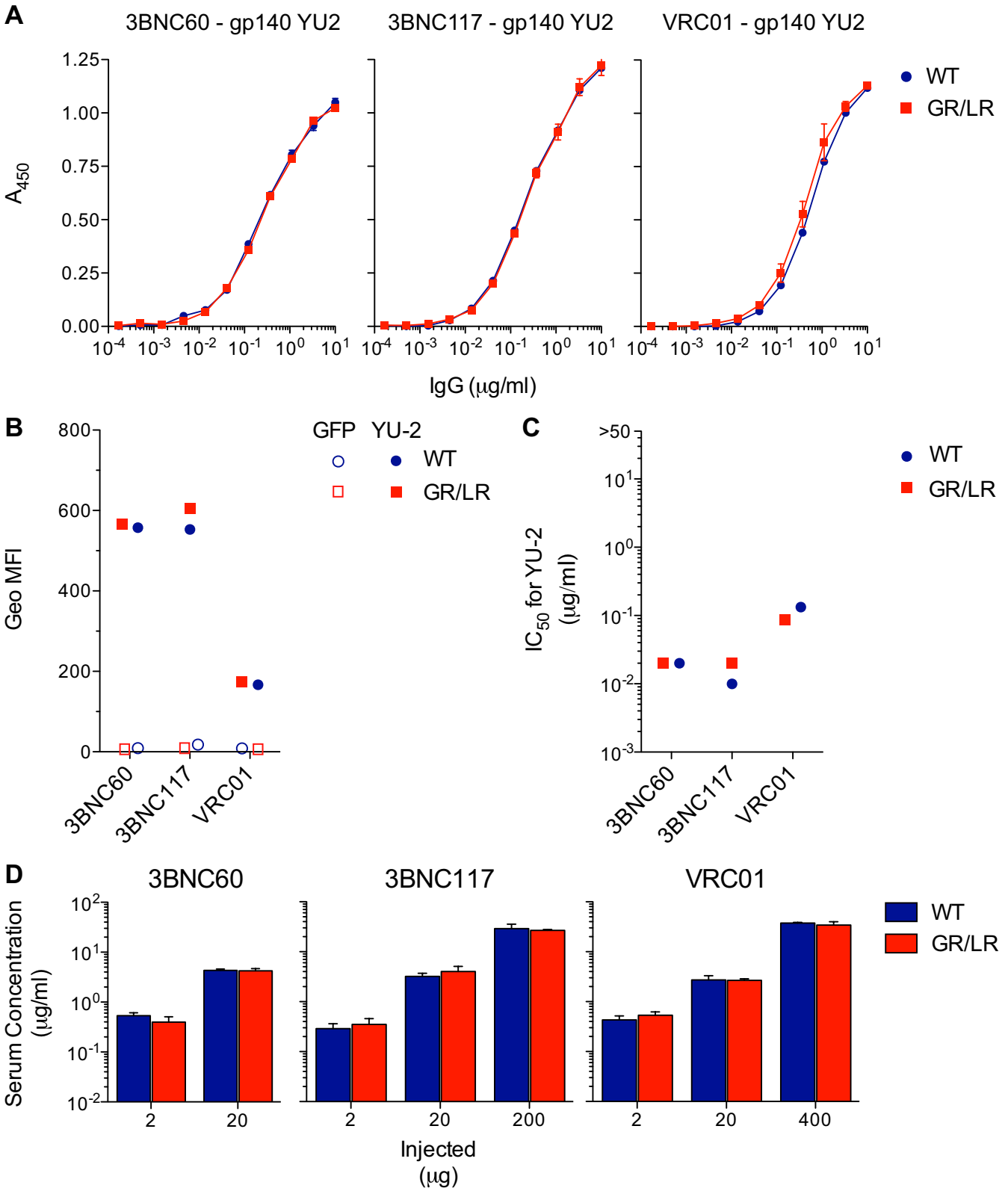

Fig. S3. Antibody functionality: hIgG1 WT vs. hIgG1 G236R/L328R. (*A*) ELISA of 3BNC60, 3BNC117, and VRC01 show comparable binding to gp140-YU2 for WT and GR/LR. Graph shows the absorption at 450 nm vs. the monoclonal antibody concentration. (*B*) 3BNC60, 3BNC117, and VRC01 were tested for binding to HIV_{YU-2} Env expressed on 293T cells. Binding was detected with anti-human IgG AF 647. 293T cells expressing GFP served as negative control. Representative graph shows the MFI for the individual antibodies. (*C*) In vitro neutralization of HIV_{YU-2} by WT and GR/LR mutants of 3BNC60, 3BNC117, and VRC01, as assessed by the TZM-bl neutralization assay. The IC_{50} in μg/mL against HIV_{YU-2} for the individual monoclonal antibodies is shown. (*D*) Serum antibody concentration after 1 d, the time point of pseudovirus injection, as determined by ELISA. Graph compares the actual monoclonal antibody concentration in serum (μg/mL) between WT and GR/LR mutant of 3BNC60, 3BNC117, and VRC01 (three to four mice per group, mean ± SEM).

Table S1. Comparison of the affinities of anti-gp140 antibodies in Fc domain variants

mAb	Fc variant	ka (1/Ms)	kd (1/s)	KD (M)
3BNC60	WT	1.4×10^{4}	1.73×10^{-4}	1.2×10^{-8}
	G236R/L328R	1.36×10^{4}	1.79×10^{-4}	1.31×10^{-8}
3BNC117	WT	1.5×10^{4}	1.29×10^{-4}	8.63×10^{-9}
	G236R/L328R	1.78×10^{4}	1.18×10^{-4}	7.9×10^{-9}
VRC01*	WT	2.5×10^{4}	6.25×10^{-5}	2.5×10^{-9}
	G236R/L328R	2.28×10^{4}	7.2×10^{-5}	3.17×10^{-9}

Affinities were determined using recombinant gp140 trimer or 2-CC core (*) and calculated by fitting the SPR sensograms to the 1:1 Langmuir binding model.

Table S2. Affinities of Fc domain variants for mouse FcγR

Variable	mFcγRI	mFcγRIIb	mFcγRIII	mFcγRIV
hIgG1 WT	4.2×10^{-7}	1.2×10^{-5}	$>10^{-4}$	9.4×10^{-7}
hIgG1 G236R/L328R	n.d.b.	n.d.b.	n.d.b.	n.d.b.

n.d.b., no detectable binding.