

UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Institut für Immunologie

Prof. Dr. med. Bernhard Fleischer

Generation of bispecific antibodies against the human immunodeficiency virus (HIV) envelope protein

Dissertation

Zur Erlangung des Grades eines Doktors der Medizin/Zahnmedizin
Der Medizinischen Fakultät der Universität Hamburg

vorgelegt von:

Malte L. Warncke
aus Göttingen

Hamburg 2012

Angenommen von der Medizinischen Fakultät am: 24.06.2013

Veröffentlicht mit Genehmigung der medizinischen Fakultät der Universität Hamburg

Prüfungsausschuss, der/die Vorsitzende:

Prof. Dr. Hans-Willi Mittrücker

Prüfungsausschuss, 2 Gutachter/in:

Prof. H.-J. Stellbrink

Die wissenschaftliche Tätigkeit, die zu dieser Arbeit geführt hat, wurde im Labor für molekulare Immunologie, geleitet von Michel C. Nussenzweig, an der Rockefeller University in New York City, U.S.A., von Dezember 2010 bis September 2011 geleistet.

The presented work was done at the laboratory of Molecular Immunology headed by Michel C. Nussenzweig at the Rockefeller University in New York City, U.S.A. from December 2010 till September 2011.

Table of Contents

Abbreviations	5
Aim	8
Introduction	9
HIV vaccine design	9
Strategies of HIV-1 to evade the humoral response	10
The antibody function depends on binding	11
Generation of bispecific antibodies against the HIV-1 envelope protein gp160	11
Material and Methods	12
Material	12
Bacteria.....	12
Cell lines	12
Chemicals, buffers and solutions.....	12
Commercial kits	13
Purification	13
Restriction enzymes	14
PCR and expression vector cloning.....	14
ELISA.....	15
SDS Page	16
Western-Blot.....	16
Equipment and lab material	16
Media and supplement for bacterial and tissue culture	17
Software.....	17
Methods	19
scFv PCR and expression-vector cloning.....	19
Transformation of DH10 β competent cells	20
Screening PCR.....	20
Preparation of plasmid DNA	21
Production and purification of bispecific antibodies	21
Tissue culture	21
Transfection	22
Antibody purification	23
Antibody concentration and buffer exchange	24
SDS – PAGE (Sodiumdodecylsulfate – Polyacrylamide Gel Electrophoresis).....	24
Fab preparation	24
ELISAs.....	25
SPR (surface plasmon resonance).....	26
Neutralization Assays	26

Results	28
Generation of anti-HIV-1 gp120/41 bispecific antibodies	29
The scFv-Fc construct preserves binding and neutralizing properties of the parental IgG	32
HIV-1 gp120/gp41-bispecific antibodies present simultaneously binding to gp120 and gp41	33
HIV-1 gp120/gp41-BiAbs recognize gp120 and gp41 simultaneously	36
Heteroligation enhances HIV-1 neutralization	37
Gp120/41-BiAbs require heteroligation for HIV-1 neutralization	40
Discussion	42
Abstract	45
Zusammenfassung	46
References	47
Publications	54
Eidesstattliche Versicherung	55

Abbreviations

10-188	anti-gp120 antibody that targets a linear epitope in the variable loop V3.
1-863	anti-gp120 antibody that binds to an epitope in the CD4bs.
4-42	anti-gp120 antibody that targets epitope in the CD4i.
5-25	anti-gp41 antibody that recognizes gp41 ^{ID} .
aa	a mino a cid(s)
Ab	a ntibody
AIDS	A cquired I mmune D eficiency S yndrome
B cell	B ursa dependent c ell
BCR	B Cell Antigen R eceptor
BiAb	B ispecific A ntibody
bNAbs	b roadly N eutralizing A ntibody
°C	degree C elsius
CD	C luster of D ifferentiation
CD4bs	CD4 binding site
CD4i	CD4 induced co-receptor binding site
CDR	C omplementary D etermining R egion
cDNA	c omplementary D esoxyribo N ucleid A cid
D	D iversity
Da	D alton
DMEM	D ulbecco's M odified E agle M edium
dNTP	d esoxyribo N ucleosid T ri P hosphate
E. coli	E scherichia c oli
EDTA	E thylene D iamine T etraacetic A cid
ELISA	E nzyme- L inked I mmuno S orbent A ssay
Env	mature e nvelope protein, comprising a homotrimer of non-covalently associated gp120-gp41 heterodimers.
Fab	F ragment antigen b inding
Fc	F ragment c rystalline
FBS	F etal B ovine S erum
g	g ram
G	G lycine
Glu	G lutamine

gp120	glycoprotein120 , surface protein (molecular weight: 120kDa).
gp120 ^{V3}	linear epitope in the variable loop V3 of gp120 .
gp140	glycoprotein140 , external domains of the HIV-1 envelope glycoprotein (gp120 and the gp41 ectodomain) containing all known neutralization epitopes.
gp160	glycoprotein160 (encoded by <i>env</i> gene, cleaved into gp120 and gp41 by host cell's own protease Furin).
gp41	glycoprotein41 , transmembrane protein (non-covalently-bound to gp120).
gp41 ^{ID}	immunodominant linear epitope of gp41 .
HEK 293T	Human Embryonic Kidney 293T
His	Histidine
HIV	Human Immunodeficiency Virus
IC ₅₀	Inhibitory Concentration (50%)
IgBLAST	Immunoglobulin Basic Local Alignment Search Tool
Ig(s)	Immunoglobulin(s)
IgA,D,E,G,M	Immunoglobulin A, D, E, G, M
IgH	Immunoglobulin Heavy chain
Ig γ	Immunoglobulin gamma heavy chain gene
Ig κ	Immunoglobulin kappa light chain gene
Ig λ	Immunoglobulin lamdba light chain gene
Ig μ	Immunoglobulin mu heavy chain gene
Iv	Intravenous
J	Joining
LB	Luria Bertani
LPS	LipoPolySaccharide
mM	milliMolar
M	Molar
mAb	monoclonal Antibody
mGO53	antibody that is neither specific to gp160 nor polyreactive.
MNR	Molar Neutralization Ratio
μ g	microgramm
μ l	microliter
ml	milliliter

min	minute(s)
mRNA	messenger RiboNucleic Acid
nm	nanometer
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pM	picoMolar
rpm	rounds per minute
RT	Room Temperature
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
RU	Response Units
S	Serin
scFv	single-chain variable Fragment
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
sec	second(s)
SIV	Simian Immunodeficiency Virus
SPR	Surface Plasmon Resonance
Taq	Thermus aquaticus
TCR	T Cell Receptor
Temp	Temperature
U	Units
UV	UltraViolet
V	Variable
WHO	Wolrd Health Organization
Yrs	Years

Aim

One of the evading strategies of human immunodeficiency virus (HIV) recently proposed is that the low density of its envelope protein gp140 expressed at the viral surface impedes bivalent binding of IgG antibodies to their target. Indeed, as measured by cryo-electron microscopy tomography, mature HIV virus expresses its gp140 surface protein at a very low density of only 10-15 viral spikes, with 90% of them being distributed randomly on the surface of virions (145 nm diameter) (Klein and Bjorkman, 2010). Thus, the average spike-to-spike distance would be too far apart for a bivalent antibody to bridge. Regarding this, a recent study showed that anti-gp140 neutralizing antibody, 2F5, acquires a dramatic enhancement of its neutralizing efficiency by binding bivalently to its natural epitope and to an artificial engineered one inserted in another region of gp140 (Wang and Yang, 2010). These results suggest that artificially engineered anti-HIV antibodies that bind bivalently to HIV gp140 may present an increased binding affinity and neutralizing efficiency.

Hence, we conducted this study to investigate if bispecific antibodies to HIV gp140 exhibit enhanced HIV-neutralizing activity. Our group has established an efficient method to clone and express human monoclonal antibodies from single B cells in different compartments (Wardemann et al., 2003). Tiller *et al* described in details the technical aspects of this methodological approach that is currently used in our laboratory (Tiller et al., 2008). We recently used this method to generate a broad spectrum of antibodies to HIV gp140 from HIV-infected individuals (Scheid et al., 2009). Considering the studies described above, we initiated a project that aims to generate and characterize HIV-bispecific antibodies bearing two antigen-binding sites, each of them specific for different epitopes of gp140 envelope protein.

Introduction

HIV vaccine design

Human immunodeficiency virus-1 (HIV-1), the agent causing acquired immune deficiency syndrome (AIDS), was discovered roughly 30 years ago, but a first vaccine candidate offering evidence of protection against infection was described only recently (Rerks-Ngarm et al., 2009). The extent of the level of protection and its mechanisms still require further description (Dolin, 2009). Hence, a vaccine that offers protection against infection by HIV-1 by stimulating the humoral and/or cellular components of the immune system has yet to be discovered.

Until now, a definitive case of a potent antibody response clearing or controlling the infection in a HIV-positive patient has not been described. However, a small percentage of infected individuals show a natural ability to suppress viral replication and progression of the disease. On the one hand, this rare phenomenon was explained by a robust cellular immune response primarily (Koup et al., 1994, McMichael and Rowland-Jones, 2001), but other cases have been reported where individuals developed broadly neutralizing serologic activity after 2-3 years of infection. (Deeks et al., 2006, Dhillon et al., 2007, Li et al., 2007, Binley et al., 2008, Doria-Rose et al., 2009, Gray et al., 2009, Sather et al., 2009, Simek et al., 2009). These broadly neutralizing antibodies (bNAbs) to gp-160, the surface spike of the HIV-1 virion, can be split into two categories: They are either specific to gp120 (b12, 2G12, PG9/PG16, HJ16 and VRC01) (Burton et al., 1991, Wu et al., 2010, Trkola et al., 1996, Walker et al., 2011) (Scheid et al., 2011) or gp41 (2F5, 4E10 and Z13) (Muster et al., 1993, Zwick et al., 2001). Further studies showed that passive

immunization with these antibodies protects monkeys from SHIV infection (Mascola and Montefiori, 2010, Zolla-Pazner, 2004, Mascola, 2007). Hence, it was proposed that a potent vaccine should elicit these antibodies in order to protect humans from the infection with HIV-1.

Strategies of HIV-1 to evade the humoral response

HIV efficiently escapes neutralizing antibodies. Two major explanations are: first, the envelope spike which comprises the glycoproteins gp120 and gp41 mutates rapidly (Wei et al., 2003, Moore and Sodroski, 1996, Albert et al., 1990) and second: other structural features, including steric occlusion (Labrijn et al., 2003), conformational masking (Kwong et al., 2002), a shield of host-derived carbohydrates (Wei et al., 2003), the protection of conserved regions at interfaces by oligomerization or in narrow pockets and the presence of highly flexible loops that shield conserved epitopes on the envelope spike (Kwong et al., 1998) (Wyatt et al., 1997). Furthermore, it was recently proposed that adequate germline genes with the capability to mature into neutralizing anti-HIV antibodies may be lacking (Xiao et al., 2009). However, this strategic repertoire is not unique to HIV. Recently an additional structural feature of HIV has been described that sets the virus apart in particular. In comparison to other viruses, the density of the HIV envelope spikes on the virus's surface is very low (Zhu et al., 2006, Klein et al., 2009, Klein and Bjorkman, 2010). This could impede bivalent binding of IgGs to the virus. Hence, the avidity of the anti-HIV antibodies would be reduced.

The antibody function depends on binding

Several studies have shown that the neutralizing activity of an antibody (Ab) is heavily influenced by its binding abilities. Higher neutralizing potency was often observed when an antibody had higher affinity or avidity for a critical epitope (Klein and Bjorkman, 2010) (Icenogle et al., 1983, Barbas et al., 1994, Schofield et al., 1997, Wu et al., 2005). Therefore, the neutralization of an antibody that binds bivalent to HIV should be higher than an Ab that binds only by virtue of one arm.

Generation of bispecific antibodies against the HIV-1 envelope protein gp160

In order to test our hypothesis, we engineered anti-HIV gp120/gp41 bispecific antibodies (BiAbs) comprising one arm targeting a number of different neutralizing gp120 epitopes and another arm directed against a non-neutralizing gp41 epitope. Three different constructs were produced and tested. All BiAbs showed simultaneous binding to gp120 and gp41. Neutralization essays showed enhanced neutralization of the anti-HIV gp120/41 BiAbs compared to the anti-gp120 immunoglobulin G (IgG) antibodies they were derived from. Viral neutralization is increased due to bivalent binding caused by heteroligation on gp160 antigens.

Material and Methods

Material

Bacteria

E. coli DH10B
Clontech
Palo Alto, U.S.A.

Cell lines

HEK 293T (ATCC, CRL-11268)
Invitrogen
Carlsbad, CA, U.S.A.

Chemicals, buffers and solutions

All chemicals used during this project were purchased from Sigma (St. Louis, MO, U.S.A.) at the highest level of purity possible.

Cresol Red ($C_{21}H_{17}O_5SNa$)
EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$)
Glycerol ($C_3H_8O_3$)
Glycine ($C_2H_5NO_2$)
Glacial acetic acid ($C_2H_4O_2$)
Potassium Chloride (KCl)
Potassium Phosphate (KH_2PO_4)
Polyethylenimine, branched (C_2H_5N)
Sodium Chloride (NaCl)
Sodium Phosphate ($NaH_2PO_4 \cdot 7H_2O$)
Sucrose ($C_{12}H_{22}O_{11}$)
Trizma® base ($C_4H_{11}NO_3$)
Tween®20 ($C_{58}H_{114}O_{26}$)

1x PBS pH=7.4	137mM NaCl 2.7mM KCl 4.3mM $Na_2HPO_4 \cdot 7H_2O$ 1.4mM KH_2PO_4
5x Loading buffer	60% sucrose 1 mM cresol red
50x TAE	2M Tris base 1M Acetate 0,5M EDTA (pH=8)

ELISA blocking buffer	2% BSA 1 μ M EDTA 0,05% Tween-PBS
HRP chromogenic substrate	ABTS solution, Invitrogen Carlsbad, CA, U.S.A.
0.45 mg/ml PEI solution	0.5M EDTA solution, pH=8.0 0.1M Glycine, pH=3.0 1M Tris-Base, pH=8
HisPur™ washing buffer	50mM sodium phosphate 300mM sodium chloride 10mM imidazole, pH=7.4
HisPur™ elution buffer	50mM sodium phosphate 300mM sodium chloride 150mM imidazole, pH=7.4
HBS-EP ⁺ running buffer	GE Healthcare Life Sciences Piscataway, NJ, U.S.A

Commercial kits

PureLink™ HiPure Plasmid Maxi Preplit	Invitrogen Carlsbad, CA, U.S.A.
NucleoSpin® Plasmid QuickPure Nucleic Acid & Protein Purification	Macherey-Nagel Dueren, Germany
Quickchange Site-Directed Mutagenesis kit	Stratagene La Jolla, CA, U.S.A.
Pierce® Silver Stain Kit	Thermo Scientific Waltham, MA, U.S.A.
Color Silver Stain Kit	Thermo Scientific Waltham, MA, U.S.A.
Pierce® Fab Preparation Kit	Thermo Scientific Waltham, MA, U.S.A.

Purification

Protein G Sepharose™ 4 Fast Flow	GE Healthcare Life Sciences Piscataway, NJ, U.S.A.
----------------------------------	---

HisPur™ Cobalt Resin

Thermo Scientific
Waltham, MA, U.S.A.

Restriction enzymes

AgeI

New England Biolabs,
Ipswich, MA, U.S.A.

BstXI

New England Biolabs,
Ipswich, MA, U.S.A.

PCR and expression vector cloning

HotStart Taq™ polymerase

Promega
Madison, WI, U.S.A.

Pfu Turbo® DNA polymerase

Adgilent
Santa Clara, CA, U.S.A.

10x Taq buffer

Qiagen,
Valencia, CA, U.S.A.

Desoxyribonukleosidtriphosphate

Invitrogen
Carlsbad, CA, U.S.A.

1kb Plus DNA marker

Promega
Madison, WI, U.S.A.

Nuclease-free water

Qiagen
Valencia, CA, U.S.A.

Forward primers 5'-3' sequence

5'AgeI/VH1 (Fig. 2)

New England Biolabs,
Ipswich, MA, U.S.A.

5'AgeI/VH1-18 (Fig. 2)

New England Biolabs,
Ipswich, MA, U.S.A.

5'AgeI/VH3 (Fig. 2)

New England Biolabs,
Ipswich, MA, U.S.A.

5'AgeI/VH4-39 (Fig. 2)

New England Biolabs,
Ipswich, MA, U.S.A.

5'(G₄S)₃-Vκ1-5 (Fig. 2)

New England Biolabs,
Ipswich, MA, U.S.A.

5'(G ₄ S) ₃ _V κ 2-30 (Fig. 2)	New England Biolabs, Ipswich, MA, U.S.A.
5'(G ₄ S) ₃ _V κ 3-15 (Fig. 2)	New England Biolabs, Ipswich, MA, U.S.A.
5'(G ₄ S) ₃ _V λ 1 (Fig. 2)	New England Biolabs, Ipswich, MA, U.S.A.

Reverse primers

3'JH4/5_(G ₄ S) ₃ (Fig. 2)	New England Biolabs, Ipswich, MA, U.S.A.
3'JH6_(G ₄ S) ₃ (Fig. 2)	New England Biolabs, Ipswich, MA, U.S.A.
3'J κ 1/4 BstXI (Fig. 2)	New England Biolabs, Ipswich, MA, U.S.A.
3'J κ 3 BstXI (Fig. 2)	New England Biolabs, Ipswich, MA, U.S.A.
expression vectors (for Ig γ 1, Ig κ , Ig λ) (Tiller et al., 2008)	Invitrogen Carlsbad, CA, U.S.A.

.

ELISA

Goat HRP-conjugated anti-human IgG antibodies	Jackson ImmunoResearch, Philadelphia, PA, U.S.A.
Anti-HIV gp140 IgG	The Lab of Molecular Immunology The Rockefeller University, NY, U.S.A.
gp41 overlapping peptides	Proteomics Resource Center, The Rockefeller University, NY, U.S.A.
gp120 ^{V3}	Proteomics Resource Center, The Rockefeller University, NY, U.S.A.
YU-2 gp120	The Lab of Molecular Immunology The Rockefeller University, NY, U.S.A.
YU-2 gp140	The Lab of Molecular Immunology The Rockefeller University, NY, U.S.A.
YU-2 gp120(D369R)	The Lab of Molecular Immunology The Rockefeller University, NY, U.S.A.

YU-2 gp120(I420R)	The Lab of Molecular Immunology The Rockefeller University, NY, U.S.A.
-------------------	---

gp41	Acris, Herford, Germany
------	----------------------------

SDS Page

3-8% separating gel	Invitrogen Carlsbad, CA, U.S.A.
---------------------	------------------------------------

Western-Blot

Anti-FLAG antibody	Sigma, St Louis, MO, U.S.A.
--------------------	--------------------------------

Anti-HIS antibody	BD Bioscience, Franklin Lakes, NJ, U.S.A.
-------------------	--

Anti-human IgG	BD Pharmingen Franklin Lakes, NJ, U.S.A.
----------------	---

Equipment and lab material

Everything used during this project was standard equipment and material found in every scientific laboratory. More advanced equipment and material that was necessary to complete this project is listed below.

Biacore T100	Biacore, Inc. GE Healthcare Life Sciences Piscataway, NJ, U.S.A
--------------	---

NanoDrop® ND-1000 Spectrophotometer	Thermo Scientific Waltham, MA, U.S.A.
-------------------------------------	--

Bio-Spin® chromatography columns	Bio-Rad Hercules, CA, U.S.A.
----------------------------------	---------------------------------

Tissue culture plates (150mm x 25mm)	Falcon, Becton Dickinson Labware Franklin Lakes, NJ, U.S.A.
--------------------------------------	--

Vivacell 70 Polyethersulfone – concentrator Centrifugal Filter Units	Sartorius AG Goettingen, Germany
---	-------------------------------------

Media and supplement for bacterial and tissue culture

Ampicillin 100X	Gibco, Invitrogen Carlsbad, CA, U.S.A.
Antibiotic-Antimycotic 100X	Gibco, Invitrogen Carlsbad, CA, U.S.A.
DMEM	Gibco, Invitrogen Carlsbad, CA, U.S.A.
Luria Bertani (LB) powder	Invitrogen Carlsbad, CA, U.S.A.
Nutridoma-SP	Roche Molecular Biochemicals Indianapolis, IN, U.S.A.
Sodium Pyruvate	Gibco, Invitrogen Carlsbad, CA, U.S.A.
Trypsin-EDTA	Gibco, Invitrogen Carlsbad, CA, U.S.A.
Ultra-low IgG FBS	Gibco, Invitrogen Carlsbad, CA, U.S.A.

Software

EditSeq™	DNASTAR Madison, WI, U.S.A.
MacVector	MacVector Inc. Cary, NC, U.S.A.
EndNote® X3	Thomson Scientific Philadelphia, PA, U.S.A.
ImageJ 1.42q software	NIH, Bethesda, MD, U.S.A.
Illustrator® CS4	Adobe San Jose, CA, U.S.A.
Photoshop® CS4	Adobe San Jose, CA, U.S.A.
Microsoft Office® 2008	Microsoft, Redmond, WA, U.S.A.

Scrubber 2

Center for Biomolecular Interaction
Analysis
University of Utah, U.S.A.

Methods

scFv PCR and expression-vector cloning

Single-chain variable fragments (scFvs) were generated by classical overlapping PCR using V_L and V_H DNA fragments cloned into human Ig-expressing vectors (Tiller et al., 2008) as templates, and encoding for the anti-gp140 monoclonal antibodies (mAbs) (Scheid et al., 2009) (Mouquet et al., 2011) or mGO53 control mAb (Wardemann et al., 2003). V_L and V_H DNA fragments were amplified using specific set of primers (Fig. 2) introducing a $(G_3S)_4$ linker sequence at the 3' and 5' ends of V_L and V_H DNA fragments, respectively (Fig. 2). PCR reactions were performed using 0.65 U of HotStart TaqTM polymerase (Promega) and comprised one cycle of 94°C for 10 min, 35 cycles of 94°C for 45 s, 59°C for 45 s and 72°C for 1 min, and a final elongation step of 72°C for 5 min.

PCR products were purified using NucleoSpin® Extract II kit (Macherey-Nagel) and assembled by overlapping PCR using specific 5'AgeI/ V_H and 3' BstXI/ J_k primers (Fig.2), and 0.32 U of Pfu Turbo® DNA Polymerase (Adgilent). PCR conditions comprised one cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 59°C for 45 s and 72°C for 1 min 30, and a final elongation step of 72°C for 10 min. Purified scFv fragments were then cloned into a modified human IgG1-expressing vector as described below, using AgeI and BstXI restriction sites. The classical vector (Tiller et al., 2008) was modified by PCR and cloning to introduce a FLAG or a Hexa-Histidine (HIS) tag at the c-terminus of the IgH constant domain 3 (CH3). The γ I-expressing vector was further modified by directed-site mutagenesis (QuickChange Site-Directed Mutagenesis kit, Stratagene) to remove BstXI site in the vector backbone and to introduce a C243A substitution. A “knob into hole” double mutation (a T366Y

substitution on the FLAG-tagged arm and a Y407T substitution on the HIS-tagged arm) was also introduced in the γ I-expression vector to increase the production of heterodimers as previously described (Ridgway et al., 1996) (Fig. 1A). Vectors containing scFv DNA fragment were isolated from transformed-DH10 β bacteria using plasmid DNA purification kits (NucleoSpin®Plasmid, Macherey-Nagel; or PureLink™ plasmid maxiprep kit, Invitrogen), sequenced and compared to the original PCR-product sequences (MacVector).

Transformation of DH10 β competent cells

3 μ l of the final plasmid were added to 5 μ l of DH10 β competent cells. After an incubation period of 15 min on ice, a heat shock in a 42°C warm water bath was performed for 45 sec. This procedure was followed by another incubation on ice for 5 min. The bacteria were grown in 100 ml LB medium for 1 h. Finally, the cells were transferred via material exhaust method onto selective agar plates containing 100 μ g/ml ampicillin. The plates were incubated for 17 h at 37°C.

Screening PCR

Bacterial colonies were screened by PCR to reveal the ones containing the insert. The parameters of this PCR were like the ones described before, but with a different set of primers and an altered number of cycles.

The 5' primer (Ab sense) (Tiller et al., 2008) used for all inserts was complementary to a sequence in the vector upstream of the insert site. Depending on the type of chain in question, either the 3' IgG-Internal (for Hc) (Tiller et al., 2008), the 3' C κ 494 (for κ C) (Tiller et al., 2008) or the 3' XhoI (for λ C) (Tiller et al., 2008) were added to the

mix. The PCR product was then analyzed by gel electrophoresis to check if the products' sizes turned out as expected. The correct colonies were picked and inoculated over night. A mini prep was performed to extract enough DNA for sequencing purposes. Sequencing was performed by Genewiz to confirm that the DNA not been altered during the process.

Preparation of plasmid DNA

Based on the results obtained from the sequencing, appropriate colonies were chosen for the generation of plasmid DNA. Plasmid DNA was prepared with the PureLink™ HiPure Plasmid Maxi Preokit (Qiagen). All steps were carried out according to the manufacturer's guidelines.

To summarize briefly, single colonies were grown overnight in 250 ml of LB medium containing 0,25 ml Ampicillin (1000X). After alkaline lysis and precipitation of the bacteria, the supernatant was loaded onto the supplied HiPure Maxi Column and washed once. The DNA was eluded from the column and precipitated again. After resuspension in TE Buffer the concentration of plasmid DNA was determined via NanoDrop®. Finally, the plasmid DNA was stored at -20°C till further use. Genewiz Inc. performed the sequencing of the product.

Production and purification of bispecific antibodies

Tissue culture

For the expression of the antibody molecules, the human embryonic kidney 293T (HEK 293T, ATCC, CRL-11268) cell line was utilized. Cells were cultured at 37°C

and 5% CO₂ in 20 ml complete Dulbeccos modified eagle medium (DMEM) containing 10% heat-inactivated ultra-low IgG fetal bovine serum (FBS), 1% sodium pyruvate and 1% antibiotic-antimycotic mix. The cells were split and redistributed every 2-3 days indicated by a plate coverage of 80%. First, 90% of the medium was removed from the plates and 5 ml of PBS were added. The PBS was removed and 3 ml of Trypsin-EDTA were added to detach the cells from the plate. With 7 ml of DMEM the cells were washed from the plates and resuspended in the appropriate amount of medium.

Depending on the cell's condition the splitting ratios varied from 1:3 to 1:5.

Transfection

At a confluency of 80% the HEK 293T cells were co-transfected by polythylenimine(PEI)-precipitation as previously described (Mouquet et al., 2011). The first step was to remove all medium and replace it with complete medium lacking any supplements. After 1 hour of incubation at RT, the medium was again replaced but the final medium was supplemented with 1% Nutridoma.

The transfection mix contained 15 ml 150 mM NaCl, equal amounts of ScFv γ 1_His- and ScFv γ 1_FLAG-expressing vectors (15 μ g of each plasmid DNA per plate) and 2 ml of 0.45/ml PEI. After 10 sec of vortexing, the mix was incubated for 10 min at RT. Each individual mix was distributed carefully in 1,7 ml portions over 10 plates. The supernatant was collected after 4 days of incubation. To remove cell remnants a centrifugation step at 1500 rpm for 10 min was carried out. The supernatants were stored at 4°C until the purification of the recombinant IgG molecules was performed.

Antibody purification

The BiAbs were affinity purified using Protein G sepharose beads (GE Healthcare) followed by HisPur™ cobalt-agarose (Pierce) and finally dialyzed in PBS. In detail, 50 µl of washed Protein G beads per 50 ml of supernatant were added to the harvested fluid and incubated overnight under gentle rotation at 4°C. The following day, the samples were centrifuged at 2000 rpm for 20 min at 4°C without deceleration. The beads were carefully resuspended in PBS and transferred onto chromatography spin columns (Bio-Rad). After 2 washes with PBS, the antibody molecules were eluted with 900 µl 0,1 M glycine (pH=3) into at least 4 Eppendorf tubes each containing 100 µl of 1 M Tris (pH=8). The protein concentration of each fraction was measured using the NanoDrop® photometer.

The second round of purification utilized the HIS-Tag to extract the hetero-dimeric BiAb. The fractions of the Protein G purifications were pooled and filled up with phosphate buffer (“mother solution”) to a final volume of 10ml. In order to reach the required imidazole concentration, 667 µl of the 150 mM HisPUR elution buffer were added. Each sample was incubated with 0,5 ml of washed HisPUR cobalt beads for 1 h under gentle rotation at RT. Afterwards, the samples were centrifuged at 2000 rpm for 20 min at 4°C without deceleration. The beads were carefully resuspended in Wash/Equi Buffer and transferred onto chromatography spin columns (Bio-Rad). After 2 washes with Wash/Equi Buffer, the antibody molecules were eluted with 500 µl HisPUR elution buffer into at least 4 Eppendorf tubes. The protein concentration of each fraction was determined by photometer.

Antibody concentration and buffer exchange

For neutralization testing, the produced antibodies have to be present at an adequate concentration in PBS. Hence, the buffer exchange and the concentration of the protein were performed simultaneously using a purification kit (Nucleospin®Plasmid, Machery-Nagel) according to the manufacturer's instruction.

SDS – PAGE (Sodiumdodecylsulfate – Polyacrylamide Gel Electrophoresis)

The size and purity of the recombinant env-proteins and antibodies were confirmed by SDS-PAGE. 500 ng of the antibodies were loaded on the 3-8% separating gel (Invitrogen). The gel was run for 50 min at 190 V. The protein purity was analyzed with the Color Silver Stain kit (Thermo Scientific) or Coomassie Blue G-250 staining. Relative quantification of stained protein bands was performed using ImageJ 1.42q software (NIH). To monitor the production of the heterodimer, the proteins were transferred onto nitrocellulose membranes followed by western blot analysis with anti-FLAG (Sigma), anti-HIS (BD Bioscience) or anti-human IgG (BD Pharmingen).

Fab preparation

Fabs were produced using papain digestion with the Fab Preparation Kit (Pierce®) according to the manufacturer's instructions. IgGs are split by immobilized papain and afterwards purified by Protein G Sepharose™. Finally, the purity of the products was evaluated on a G250 Coomassie blue-stained 4-12% NuPAGE (Invitrogen) (SDS-PAGE).

ELISAs

High-binding 96-well ELISA plates (Costar) were coated overnight with 100 ng/well of purified antigens YU-2 gp140, YU-2 gp120, YU-2 gp120(D368R), YU-2 gp120(I420R) (Mouquet et al., 2011) and gp41 (Acris, Herford) in PBS. Plates were washed and blocked for 2 h with 2% BSA, 1 μ M EDTA, 0.05% Tween-PBS (Blocking buffer) and then, incubated 2 h with IgG mAbs or BiAbs diluted at 26.7 nM and several consecutive 1:4 dilutions in PBS. After washings, the plates were revealed by incubation for 1 h with goat HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch) (at 0.8 μ g/ml in blocking buffer) and by adding 100 μ l of HRP chromogenic substrate (ABTS solution, Invitrogen). Optical densities were measured at 405 nm (OD_{405nm}) using an ELISA microplate reader (Molecular Devices). Background values given by incubation of PBS alone in coated wells were subtracted. To assay the antibody binding to the selected gp41 overlapping peptides (sequences shown in Fig. 6) and to gp120^{V3} peptide (RKSINIPGGRALYTTGEII) (Proteomics Resource Center, The Rockefeller University) (Scheid et al., 2009), the Abs were tested using a peptide ELISA method that was described previously (Mouquet et al., 2011). High-binding ELISA plates (Costar) were coated for competition assays with 100 ng/well of purified gp140 and gp41. Plates were blocked 2 h (blocking buffer) and incubated for 2 h with anti-HIV gp140 IgG and BiAbs at 26.7 nM in 1:2 serially diluted gp41⁵⁸⁹⁻⁶⁰⁸ peptide (gp41^{ID})-containing PBS (with a concentration ranging from 5.71 nM to 2.92 μ M). Plates were developed as described above. All ELISA experiments were performed at least twice.

SPR (surface plasmon resonance)

All surface plasmon resonance (SPR) experiments were performed by Dr. Hugo Mouquet with a Biacore T100 (Biacore, Inc) in HBS-EP⁺ running buffer (Biacore, Inc) at 25°C as described previously (Mouquet et al., 2010). On the CM5 chips (Biacore, Inc), the gp140, gp120 and gp41 proteins were immobilized at a concentration of 12.5 µg/ml by amine coupling at pH 4.5 resulting in an immobilization level of 100 RUs. For binding analysis IgG mAbs and BiAbs were injected through flow cells at 500 nM and 1000 nM, respectively, at flow rates of 35 µl/min with 3 min association and 5 min dissociation. The sensor surface was regenerated between the experiments with a 30 s injection of 10 mM glycine-HCl pH 2.5 at a flow rate of 50 µl/min. After subtracting the background (binding to control flow cells and signal of the HBS-EP⁺ running buffer), binding curves were plotted using Scrubber 2 software (Center for Biomolecular Interaction Analysis, University of Utah). To analyze the simultaneous binding of the anti-gp120/41 BiAb molecules to gp120 binding (binding 1) and to gp41 (binding 2), the anti-gp120/41 BiAbs and BiAb controls were injected through flow cells (at 700 nM) in HBS-EP⁺ running buffer at flow rates of 30 µl/min with 3 min association and 2 min dissociation. Purified gp41 (final concentration of 30 µg/ml) was injected in HBS-EP⁺ running buffer at a flow rate of 20 µl/min with 2 min contact time. The sensor surface was regenerated as described above.

Neutralization Assays

The essays were performed by M.S. Seaman at the Beth Israel Deacones Medical Center Boston as previously described (Montefiori, 2005). Briefly, neutralization is detected as reduction in firefly luciferase (Luc) reporter gene expression in TZM-bl

cells after a single infection. The HIV tat protein induces this effect. The cell line expresses CD4, CXCR4, CCR5. Furthermore, it contains a tat-responsive Luc reporter gene. The activity of the luciferase is quantified by luminescence and is directly proportional to the number of infected cells present in the sample. MuLV (murine leukemia virus) is used as a negative control. Env-pseudotyped viruses are prepared by transfection in 293T cells using a two-plasmid system. One plasmid expresses gp160 and the second is an appropriate env-defective HIV-1 backbone plasmid.

For the competition essays, purified anti-gp120/41 BiAbs were incubated with gp41^{ID} peptide at a final concentration of 0.53 μ M prior to neutralization testing. Decreases of neutralization activity were calculated by dividing the IC₅₀ values of the BiAbs in the presence of gp41^{ID} peptide by the results of the controls (BiAbs in the same concentration in 0.5% DMSO PBS without peptide).

Results

Dr. Hugo Mouquet and Malte L. Warncke performed all of the following experiments together, except for the entire surface plasmon resonance experiments that were carried out by Dr. Mouquet exclusively. He also wrote all SPR protocols present in this work (Mouquet et al., 2012).

Neutralization essays were performed by M.S. Seaman at the Beth Israel Deacones Medical Center Boston, U.S.A..

All sequencing was done by Genewiz, Inc., New Jersey, U.S.A..

Generation of anti-HIV-1 gp120/41 bispecific antibodies

We artificially engineered antibodies that bind simultaneously to both gp120 and gp41 subunits of HIV-1 gp160. They are scFv-Fc IgG-like molecules, also known as immunoadhesins, which contain two different binding sites specific either to gp120 or gp41 antigens (Fig. 1).

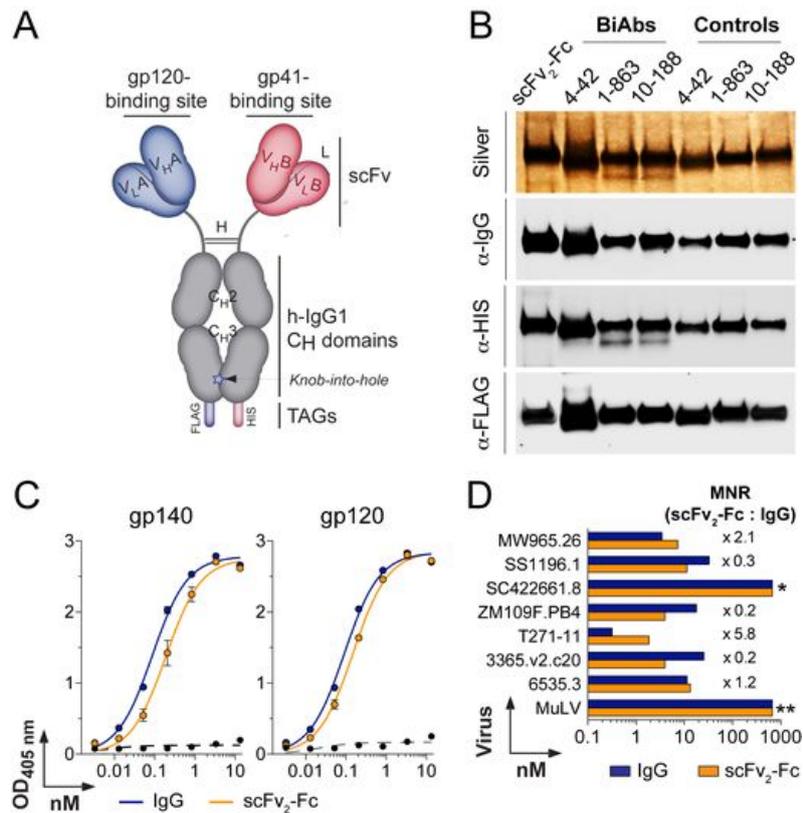


Figure 1. (A) Anti-Gp120/41 bispecific antibody (BiAb) made as scFv₂-Fc IgG-like molecule. One binding site targets gp120 while the other is directed against gp41. scFv, light-chain variable fragment; V_H, heavy-chain variable domain; V_L, light-chain variable domain; H, hinge; L, (G₄S)₃ linker; CH, heavy-chain constant domain; h-IgG1, human IgG1. (B) Western blot and silver staining of gp120/gp41 BiAbs and BiAb controls (anti-gp120/mGO53 heterodimers). (C) ELISA: Binding of 10-188 IgG antibody and scFv₂-Fc control to gp120 and gp140 antigens. (D) TZM-bl assay showing the neutralizing activity of 10-188 IgG and ScFv₂-Fc control. The x-axis shows the antibody concentration (nM) necessary to achieve IC₅₀ for each virus indicated on the y-axis. Displayed are the molar neutralization ratio (MNR) values comparing the IC₅₀ values. Error bars indicate the SEM. *, not neutralized; **, negative control.

scFv fragments were generated by overlapping PCR employing variable heavy- and light-chain domain (V_H and V_L) genes encoding for human anti-gp120, anti-gp41 or mGO53 (control antibody) (Table 1) (Wardemann et al., 2003, Mouquet et al., 2011, Scheid et al., 2009). mGO53 was used as a control because it neither binds to gp160

nor is it polyreactive (Table 1).

Table 1. Repertoire and reactivity of gp160-specific and control antibodies

mAb	Heavy chain							Light chain									
	VH	DH	JH	(-)	CDR3 (aa)	(+)	Length	VHmut	κ/λ	V κ/λ	J κ/λ	CDR3	Length	V κ/λ mut	Epitopes	Neut.	Poly.
1-863	3-30	3-3/3-9	4	6	GIQEDYDFWREYRELDY	2	17	24	κ	2-30/2-24	1	MQGTHWPRT	9	8	CD4bs	X	/
4-42	1-18	6-19/6-13/1-26	4	3	ENYSDBGWYEVGHFDL	1	15	41	κ	1-39	3	QQTYASVT	9	23	CD4i	X	/
10-188	3-49	3-16	6	5	YGDIYWAPPEDHDYYGMDV	0	19	36	λ	1-44	3	AAWDDSLNGPV	11	28	V3	X	/
5-25	1-69	1	4	2	RYKYLPGDQHMPWDY	3	15	35	κ	1-33	3	QQYDNLPRVT	11	13	gp41 ^{ID}	/	X
mGO53	4-39	2-15	4	1	FGYCSGGSCLPFDY	0	14	/	κ	3-15	1	QQYNNWPRGT	10	/	/	/	/

(-) and (+) indicate the numbers of negatively and positively charged aminoacids in the IgH complementary determining region (CDR3), respectively. VHmut and V κ/λ mut indicate the total number of mutations in the VH and VL Ig genes. CD4bs, CD4 binding site; CD4i, coreceptor CD4 induced site; V3, gp120 variable loop 3; gp41^{ID}, gp41 immunodominant epitope; Neut., neutralization activity; Poly., polyreactivity.

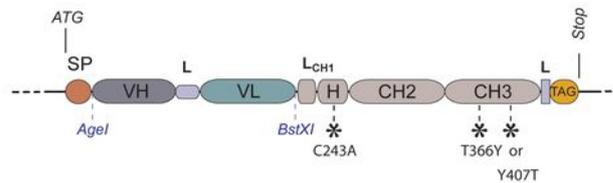
For the introduction of a flexible (Gly₃Ser)₄ linker between V_H and V_L domains specific primers were used (Fig. 2A).

A**Forward primers 5' - 3' sequence**

5' AgeI VH1 CTGCAACCGGTACATTCCCAGGTGCAGTGGTGCAG
 5' AgeI VH 1-18 CTGCAACCGGTACATTCCCAGGTTCAGCTGGTGCAG
 5' AgeI VH3 CTGCAACCGGTGACATTCTGAGGTGCAGTGGTGGAG
 5' AgeI VH4-39 CTGCAACCGGTACATTCCCAGGTGCAGCTGCAGGAG
 5'(G₄S)₃_Vk1-5 GGTGGAGCGGTTACGGCCGAGGTGGTTCTGGCCGGTGGCCGATCGGACATCCAGATGACCCAGTCTCCA
 5'(G₄S)₃_Vk2-30 GGTGGAGCGGTTACGGCCGAGGTGGTTCTGGCCGGTGGCCGATCGGATGTTGTGATGACTCAGTCTCCA
 5'(G₄S)₃_Vk3-15 GGTGGAGCGGTTACGGCCGAGGTGGTTCTGGCCGGTGGCCGATCGGAAATAGTATGACGACGACTCTCCA
 5'(G₄S)₃_Vλ.1 GGTGGAGCGGTTACGGCCGAGGTGGTTCTGGCCGGTGGCCGATCGGACTGTGTGACTGACGACCC

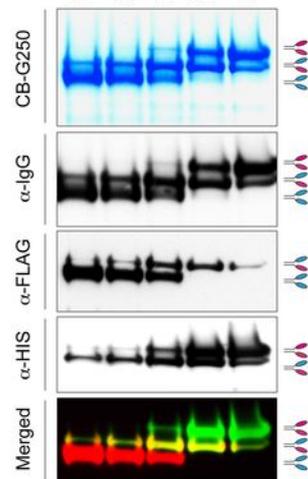
Reverse primers

3'JH4/5_(G₄S)₃ CGATCCGCCACCGCCAGAACCACCTCCGCCTGAACCGCCTCCACCCGCTGAGGAGACGGTGACCCAGGGTT
 3'JH6_(G₄S)₃ CGATCCGCCACCGCCAGAACCACCTCCGCCTGAACCGCCTCCACCCGCTGAGGAGACGGTGACCCAGGGTT
 3'Jκ1/4 BstXI GCCACCCAAGCTGCTGGATTGATYCCACCTTGGTCCCTTG
 3'Jκ3 BstXI GCCACCCAAGCTGCTGGATTGATATCCACTTGGTCCAGG

B**C**

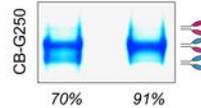
FLAG-vec : HIS-vec ratio

1:4 1:2 1:1 2:1 4:1

**D**

Eluats

Protein G HisPur



HIS-armed homodimer
 HIS/FLAG-armed heterodimer
 FLAG-armed homodimer

Figure 2. (A) Nucleotide sequences of the primers used to generate scFv DNA fragments by PCR. **(B)** Cloning strategy of the scFv DNA fragments into the modified human IgG1-expressing vector. VH, heavy-chain variable domain; VL, light-chain variable domain; H, hinge; L, linker; CH, heavy-chain constant domain. *, The aminoacid substitutions introduced in the vector by site-directed mutagenesis. **(C)** SDS-Page, Coomassie blue-stained (CB-G250) showing the production of the three possible antibody combinations according to the FLAG- and HIS-tagged vector ratio used for 293T-cell co-transfection. Western blot showing FLAG- or HIS-tagged molecules. **(D)** SDS-Page, Coomassie blue-stained (CB-G250) showing the production of HIS- and FLAG-tagged heterodimer after Protein G and HisPurTM cobalt affinity chromatographies. HIS- and FLAG-tags are visualized by Western blot analysis.

To monitor the proper heterodimer formation we introduced a unique HIS- or FLAG tag at the C-terminal of each scFv-Fc arm (Fig. 1A, Figs. 2B and 2C).

The scFv-Fc construct preserves binding and neutralizing properties of the parental IgG

To ensure that the binding and neutralizing properties of the original IgG are preserved when expressed as scFv-Fcs, we generated homodimeric scFvs (scFv₂-Fc) using 10-188, an anti-V3 antibody (Mouquet et al., 2011). ELISA experiments showed that purified 10-188 scFvFc₂ recognized artificial YU-2 gp140 trimers and YU-2 gp120 trimers with the same binding activity as 10-188 IgG antibody (Figs. 1B and 1C). In addition, the in vitro TZM-bl cell assay showed that the native neutralization profile of the 10-188 IgG antibody was similar to the 10-188 scFv₂-Fc (Fig. 1D, Table 2)

Table 2. In vitro TZM-bl neutralization assay of the anti-HIV gp120/gp41 BiAbs

Tier Clade	1								2	
	A	B				C		CRF02-AG	A	
Virus	DJ263.8	SF162.LS	BaL.26	SS1196.1	6536.3	MW965.26	ZM109F.PB4	T271-11	3365.v2.c20	MuLV
IgG										
1-863	1.90	2.20	7.90	38.4	>50	> 50	21.3	> 60	> 60	>50
4-42	15.2	0.70	7.90	8.70	>50	0.05	10.38	5.64	> 63	>50
10-188	0.59	0.04	0.52	4.90	1.71	0.01	3.98	0.32	25.5	>100
mGO53	>125	>125	>125	>125	>125	>125	> 42	> 42	> 42	>125
BiAb control										
1-863/mGO53	18.6	4.42	82	>184	>184	>184	174	> 220	> 220	>184
4-42/mGO53	>146	1.79	56	17.3	>146	0.84	> 38	> 38	> 38	>146
10-188/mGO53	2.17	0.23	3.92	11.3	6.92	0.12	68	2.22	31.1	>203
5-25/mGO53	>70	>70	>70	>70	>70	>70	> 31	> 31	> 31	>70
gp120/41 BiAb										
1-863/5-25	0.08	4.76	16.5	0.30	0.11	>164	178	> 239	> 239	>164
4-42/5-25	0.10	2.36	>51	0.03	>51	0.02	137	113	> 167	>51
10-188/5-25	0.05	0.18	4.34	0.03	0.08	0.05	100	2.92	70	>108
IgG Mix										
1-863+5-25	2.42	0.83	9.13	56	51	>60	ND	ND	ND	>60
4-42+5-25	9.05	0.80	33.7	9.27	>61	0.07	ND	ND	ND	>61
10-188+5-25	0.42	<0.03	0.79	2.54	2.78	<0.03	ND	ND	ND	>56

Numbers indicate antibody IgG concentrations in µg/ml to reach the IC₅₀ in the TZM-bl neutralization assay. For direct comparison, the BiAb concentrations to reach the IC₅₀ are expressed in µg/ml of IgG. > indicates that the IC₅₀ for a given virus was not reached at the concentration tested. MuLV, Murine leukemia virus, is the negative control.

In summary, the original binding and neutralizing properties of the anti-HIV antibody are preserved by the scFv₂-Fc.

HIV-1 gp120/gp41-bispecific antibodies present simultaneously binding to gp120 and gp41

For the production of the BiAbs, we chose three different human anti-gp120 antibodies with a modest neutralizing activity and one gp41 antibody with no neutralizing activity. The anti-gp120 antibodies recognize different epitopes: 10-188 binds selectively to a linear epitope in the variable loop V3 (gp120^{V3}) (Mouquet et al., 2011), while the other two target the CD4 binding site (CD4bs) and CD4 induced site (CD4i) (1-863 and 4-42, respectively) (Scheid et al., 2009) (Table 1).

The anti-gp120 arms were combined with the anti-gp41 antibody 5-25 that targets the immunodominant linear epitope of gp41 (gp41^{ID}) (Pietzsch et al., 2010). As a control we paired the anti-gp120 antibodies with the non-specific mGO53 (Wardemann et al., 2003). Our production comprised three gp120/gp41 BiAbs (10-188/5-25, 1-863/5-25 and 4-42/5-25) and their corresponding gp120/mGO53 controls (10-188/mGO53, 1-863/mGO53 and 4-42/mGO53). We reached a purity of roughly 90% using successive affinity chromatography (Figs. 2C and 2D).

As an additional control we produced anti-gp41 5-25/mGO53 heterodimer. To monitor the correct expression of the heterodimeric BiAbs anti-HIS and anti-FLAG western blot analysis was used (Fig. 1B).

The next step was to analyze the binding properties of the anti-gp120/gp41 and controls to gp140, gp120 or gp41. For that matter, we used surface plasmon resonance (SPR) and ELISA assays. The results show that all anti-gp120/gp41 BiAbs and controls recognized gp140 trimers, like the original anti-HIV IgG (Figs. 3A and 4A).

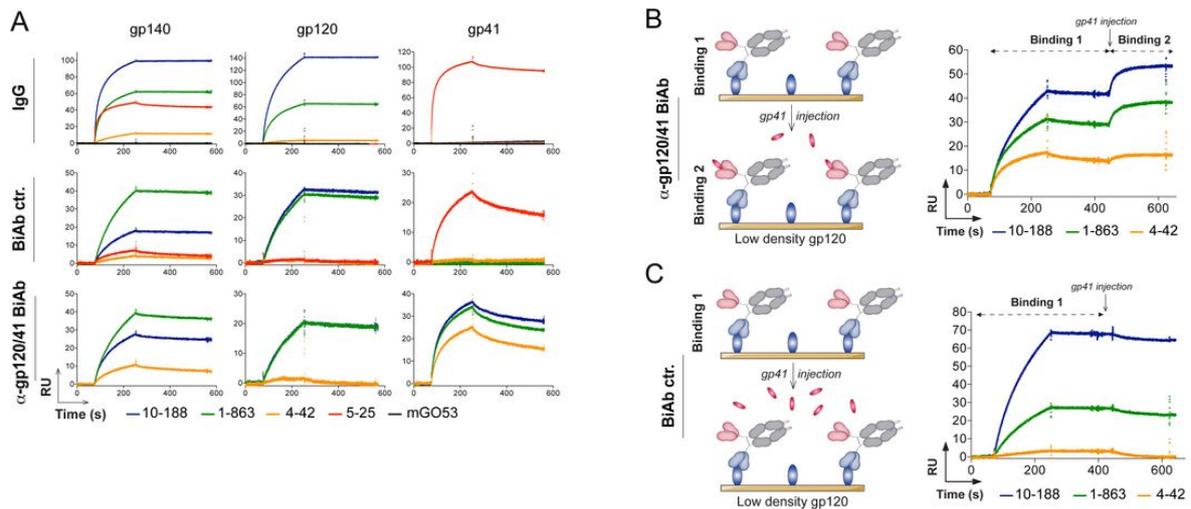


Figure 3. (A) Surface plasmon resonance (SPR) analysis of the interaction of the anti-gp120/gp41 BiAbs, IgG and BiAb controls with the gp140, gp120 and gp41 ligands immobilized at low density on the sensor chips (100 response units). Graphs show SPR sensorgrams over time for the binding of the selected antibodies. **(B)** SPR analysis of the anti-gp120/gp41 monovalently bound to gp120 immobilized on the low density chip (100 RU) (binding 1) with in solution-injected gp41 (binding 2), as illustrated by the schematic diagram. **(C)** SPR analyses show no interaction of the BiAb controls monovalently bound to gp120 immobilized on the low density chip (100 RU) (binding 1) with in solution-injected gp41, as depicted in the diagram on the left-hand side. RU, response units.

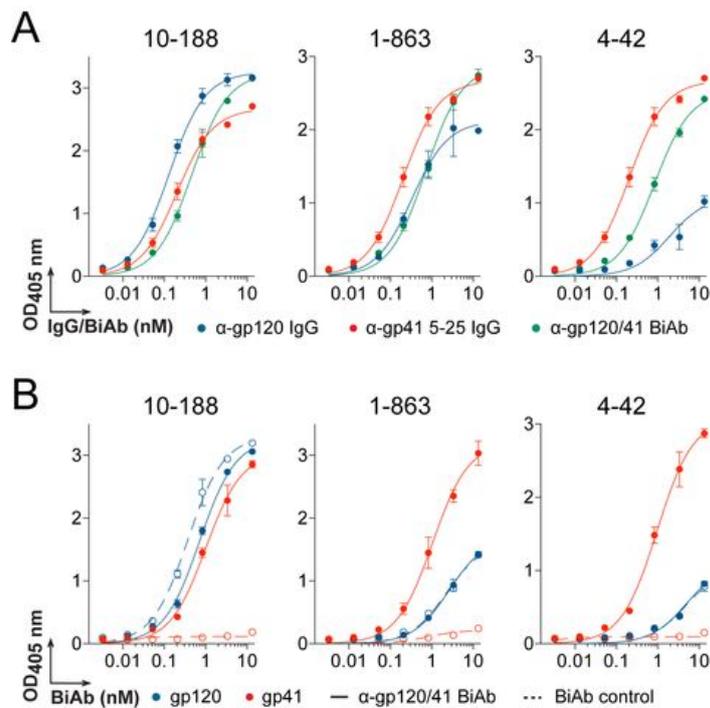


Figure 4. (A) ELISA: Binding analysis of anti-gp120/gp41 BiAbs and parental IgG to gp140. **(B)** Binding of anti-gp120/gp41 BiAbs and controls to gp120 and gp41 antigen measured by ELISA. X-axis shows the antibody concentration (nM) required to obtain the ELISA values ($OD_{405\text{ nm}}$) indicated on the y-axis. Error bars indicate the SEM.

Furthermore, anti-gp120/41 BiAbs bound to both gp120 and gp41 antigens while the

gp120/mGO53 and gp41/mGO53 BiAb controls only recognized either gp120 or gp41 (Figs. 3A and 4B). We performed ELISAs using gp120^{V3} and gp41^{ID} peptides, as well as two mutant proteins: gp120(D368R) which impedes the binding to the CD4bs (Li et al., 2007), and gp120(I420R) which blocks the binding to CD4i (Thali et al., 1991) to further prove that the anti-gp120/gp41 BiAbs and BiAb controls bind to gp140 with the original profile like the parental anti-gp120 and anti-gp41 IgG antibodies. BiAbs based on the 10-188 antibody bound to gp120^{V3} peptide and to both of the modified gp120 proteins, whereas the BiAbs derived from 1-863 and 4-42 presented a decrease in binding potency to gp120(D368R) and gp120(I420R) (Fig. 5).

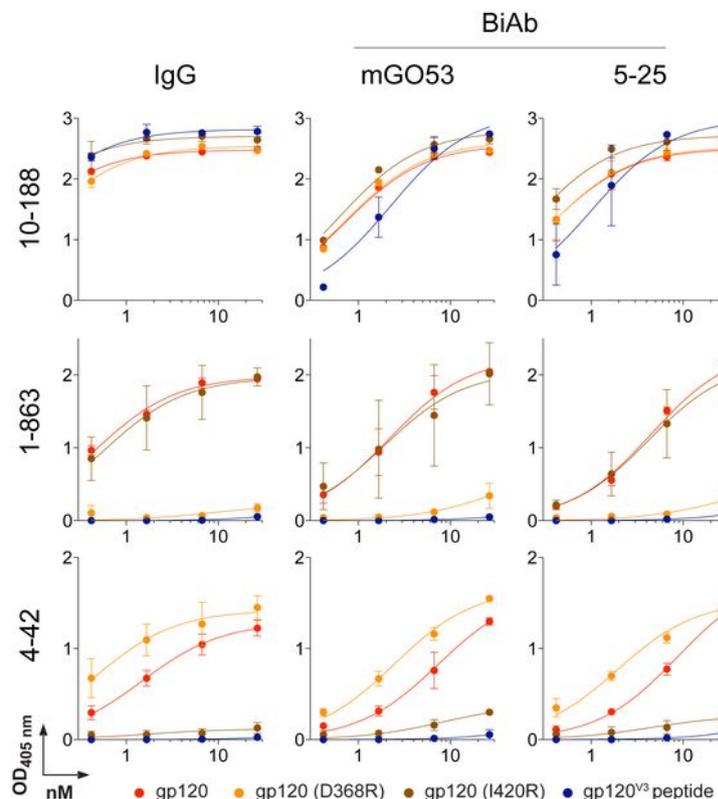


Figure 5. ELISA graphs show reactivity of anti-gp120/gp41 BiAbs, IgG and BiAb controls to gp120, gp120(D368R), gp120(I420R) and gp120^{V3} peptide. CD4bs- and CD4i-specific antibody reagents show no reactivity against gp120(D368R) and gp120 (I420R) mutant proteins. Only V3-specific antibodies (10-188 and derived BiAbs) reacted with gp120^{V3} peptide. Error bars indicate the SEM.

Further ELISA experiments showed that only anti-gp120/41 BiAbs and anti-gp41 5-25/mGO53 heterodimer bound to the gp41^{ID} protein (Fig. 6).

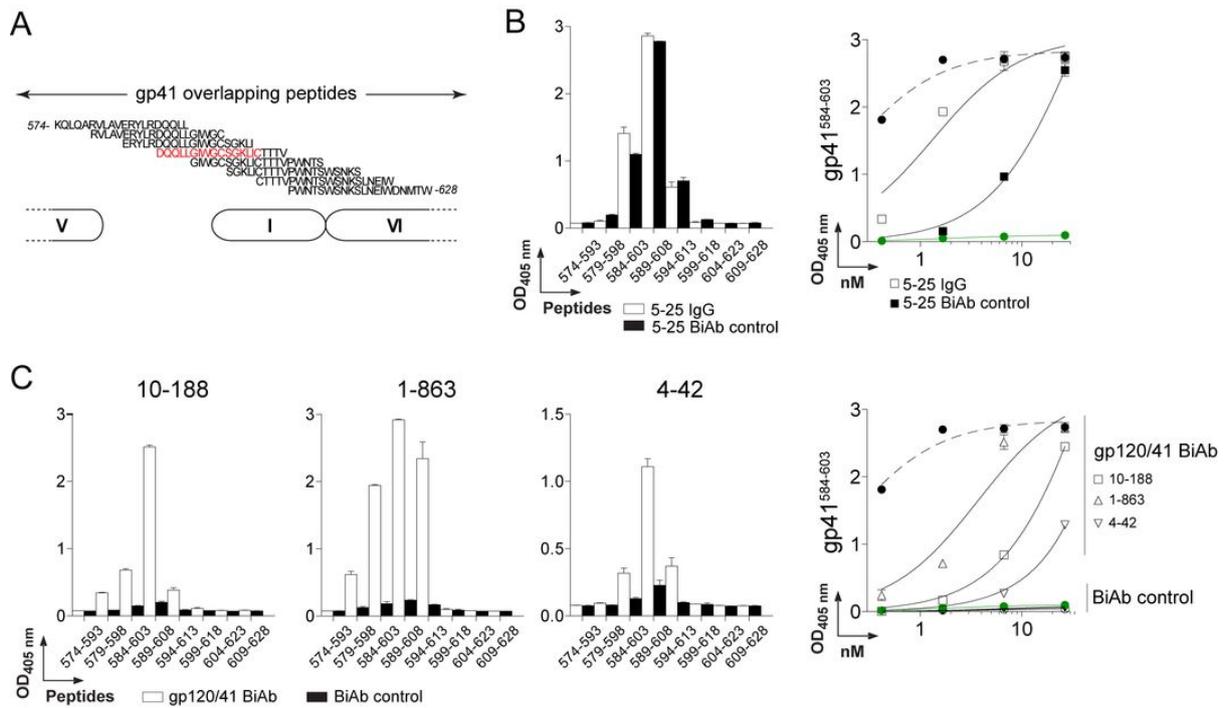


Figure 6. (A) Amino acid sequences of the gp41 overlapping peptides used in the ELISA assays presented in B and C. The immunodominant epitope (gp41^{ID}) marked red. Antigenic clusters shown on the bottom. **(B)** Bar graph shows the ELISA reactivity of 5-25 IgG and BiAb control (5-25/mGO53) against the selected gp41 peptides. ELISA graph shows the binding of 5-25 IgG and BiAb control against gp41⁵⁸⁴⁻⁶⁰³ that contains the gp41^{ID} epitope. The x-axis shows the antibody concentration (nM) required to obtain the ELISA values (OD_{405nm}) indicated on the y-axis. Green and black dotted lines show the negative (mGO53) (Wardemann et al., 2003) and positive (10-1255)(Mouquet et al., 2011) control antibodies. **(C)** Same as (B) except for anti-gp120/gp41 BiAbs and BiAb controls (anti-gp120/mGO53 heterodimers). 1-863/5-25 BiAb also bound strongly to peptides 579-598 and 589-608 that contains partial gp41^{ID} epitope suggesting that the artificial pairing of the 1-863 anti gp120 scFv-Fc with the anti-gp41 antibody arm may have slightly modified its specificity. Error bars indicate the SEM.

In conclusion, these results show that the binding and neutralizing specificities of the original IgG against gp120 and gp41 is conserved by the anti-gp120/gp41 BiAbs can bind both the gp120 and gp41 subunits of the anti-HIV surface protein.

HIV-1 gp120/gp41-BiAbs recognize gp120 and gp41 simultaneously

Surface plasmon resonance (SPR) “sandwich” experiments were performed to investigate if the anti-gp120/41 BiAbs can bind to gp120 and gp41 at the same time. Soluble gp41 was injected in solution over anti-gp120/41 or control BiAbs bound to

gp120 immobilized on SPR chips (Figs. 3B-C). While the anti-gp120 BiAb controls did not bind to gp41, their respective anti-gp120/gp41 BiAbs captured the soluble gp41 (Figs. 3B-C). Hence, the anti-gp120/gp41 BiAbs can capture their specific epitopes simultaneously without the disturbance of the binding of one arm with the other one.

Heteroligation enhances HIV-1 neutralization

TZM-bl cell infection assays were used to assess the *in vitro* neutralizing activity of our anti-gp120/41 BiAbs. Different pseudovirus variants were tested (Table 2). The first question we addressed was if neutralization potency and/or breadth were increased comparing the BiAbs to the regular anti-gp120 antibodies. Our results are expressed as molar neutralization ratio (MNR). This presentation method was chosen to account for any differences in the number of binding sites per antibody. To start off, in most cases the anti-gp120/41 BiAbs had far more neutralizing potency to the selected HIV variants than their respective control molecule (Fig. 7 and Table 2 and 3).

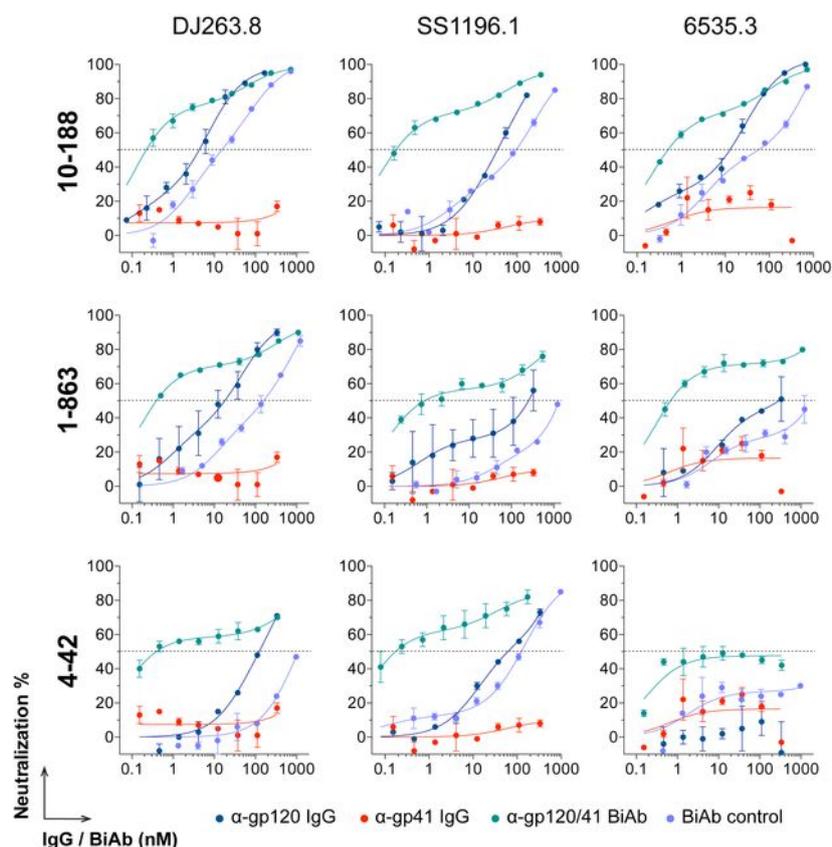


Figure 7. Neutralization curves of selected viruses for anti-gp120/gp41 BiAbs and controls. X-Axis shows antibody concentrations (nM) required to achieve 50% neutralization (IC_{50}) indicated by the dashed line. Error bars indicate the SD.

Table 3. Molar neutralizing IC_{50} ratios of anti-HIV gp120/gp41 BiAbs

Tier	1								2	
	A	B			C		CRF02-AG	A		
Clade	DJ263.8	SF162.LS	BaL.26	SS1196.1	6535.3	MW965.26	ZM109F.PB4	T271-11	3365.v2.c20	
Virus	DJ263.8	SF162.LS	BaL.26	SS1196.1	6535.3	MW965.26	ZM109F.PB4	T271-11	3365.v2.c20	
Anti-gp120 BiAb control vs anti-gp120/41 BiAbs										
1-863	232	0.9	4.9	>613	>1673	X	1.0	X	X	
4-42	>1460	0.8	X	577	X	42.0	\	\	X	
10-188	43.4	1.3	0.9	377	87	2.4	0.7	0.8	0.4	Fold
Anti-gp120 IgG vs anti-gp120/41 BiAbs										
1-863	23.8	0.5	0.5	128	>455	X	0.1	X	X	>200
4-42	152	0.3	/	290	X	2.5	0.1	0.1	X	5-50
10-188	11.8	0.2	0.1	163	21.4	0.2	0.04	0.1	0.4	2-5

Numbers indicate the molar neutralization ratio (MNR) values that compare the neutralizing activity of anti-gp120 BiAb with BiAb controls or with parental IgG as tested in the TZM-bl neutralization assay (see Table S2). In some cases, no neutralization of the tested viruses was observed for the 2 compared antibody molecules (X), for the anti-gp120/41 BiAb (/) or the BiAb control (/).

On top of that, our anti-gp120/41 BiAbs exhibited a higher neutralizing activity compared to their parental anti-gp120 IgG antibodies for many viruses tested

(Figs. 8A-D, Table 2 and 3).

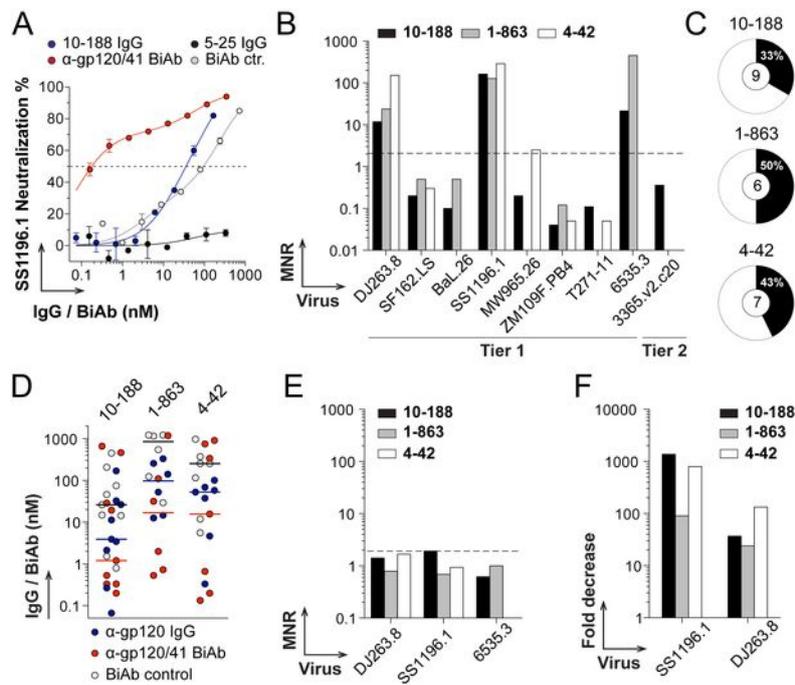


Figure 8. (A) Neutralization curves of SS1196.1 pseudovirus for 10-188 BiAb and controls. The x-axis shows the antibody concentration (nM) required to achieve 50% neutralization (IC_{50}) indicated by dashed line. (B) Bar graph shows the molar neutralization ratio (MNR) values comparing the IC_{50} concentrations of the anti-gp120/gp41 BiAbs and original anti-gp120 IgGs for each virus tested. (C) Pie charts show the frequency of virus variants showing enhanced neutralization with the anti-gp120/gp41 BiAbs compared to parental anti-gp120 IgG antibodies. (D) Dot graph shows IC_{50} concentrations of anti-gp120/gp41 BiAbs compared to controls for the neutralization of the selected HIV viruses. Median IC_{50} values are indicated for each group by horizontal lines. (E) Bar graph shows the MNR values comparing the IC_{50} concentrations of the parental anti-gp120 IgGs mixed with 5-25 anti-gp41 IgG and anti-gp120 IgG mAbs alone for selected viruses. (F) Bar graph shows the fold decreases for the neutralization activity of the anti-gp120/gp41 BiAbs in presence of gp41^{ID} peptide against viruses tested.

For example, the BiAb based on the 1-863 anti-gp120 antibody showed a 24-, 128- and over 455- fold increase in neutralization potency for 6535.5, SS1196.1 and DJ263.8 in comparison to it's original form (Figs. 8B, Fig. 7 and Table 2). In general, the BiAbs showed a 33%-50% increase in neutralization potency across all tested viral strains when compared to the IgGs they were derived from (Fig. 8C). Furthermore, the anti-gp120/41 BiAbs showed lower median IC_{50} concentrations for the neutralization of the viral strains than the IgG and BiAb control antibodies (Fig. 8D). Furthermore, the tested viruses (6535.3, DJ263.8 and SS119.1) showed an increase in sensitivity to neutralization by the BiAb compared to anti-gp120 IgG

controls, irrespective of the targeted antigen of the anti-gp120 arm (Figs. 8B and 7, Tables 2 and 3).

Gp120/41-BiAbs require heterologation for HIV-1 neutralization

To test if the enhanced neutralization was not just due to a synergetic effect by different BiAb molecules binding independently to gp120 and gp41, we prepared equimolar mixtures of anti-gp120 and anti-gp41 IgGs and Fab fragments. In coherence with our previous the results, neither the Fab nor the IgG showed an increase in neutralization activity for the tested HIV strains when compared to their parental anti-gp120 IgG (Fig. 8E, Fig. 9 and Tables 2).

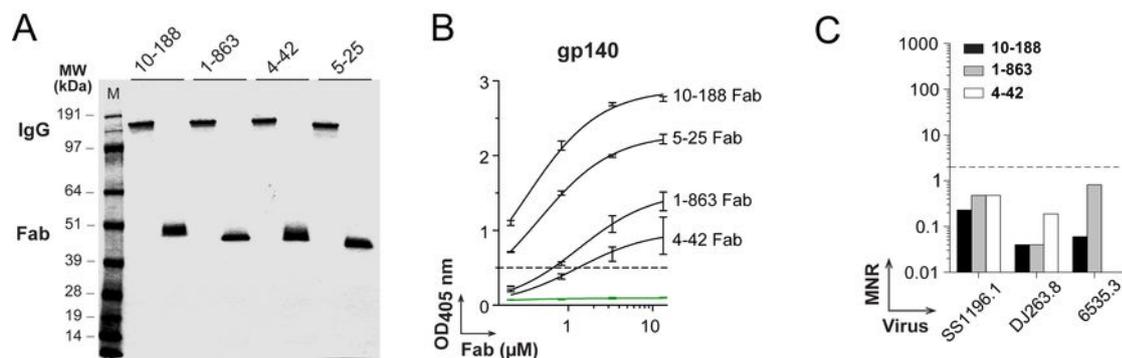


Figure 9. (A) SDS-Page, Coomassie blue-stained shows Fabs and IgGs of the parental anti-gp120 (10-188, 1-863 and 4-42) and anti-gp41 (5-25) antibodies used to generate gp120/gp41 BiAbs. MW; molecular weight. **(B)** Graph shows the ELISA reactivity of the Fab fragments presented in (A) against gp140. Green line shows the negative control antibody mGO53. **(C)** Bar graph shows the molecular neutralization ratio (MNR) values comparing the IC_{50} concentrations of the parental anti-gp120 Fab fragments mixed with 5-25 anti-gp41 Fab and anti-gp120 IgG antibodies for the selected viruses. Error bars indicate the SEM.

To verify that the increase in neutralization potency is dependent on the binding of the anti-gp41 arm of the BiAbs, competition neutralization assays were performed. Soluble gp41^{ID} was added to the mix to block the gp41-specific arm of the antibody. In the presence of the gp41^{ID} peptide (specific epitope of 5-25 anti-gp41 antibody),

the neutralizing activity of the anti-gp120/gp41 antibodies was abolished (Fig. 8F and 10).

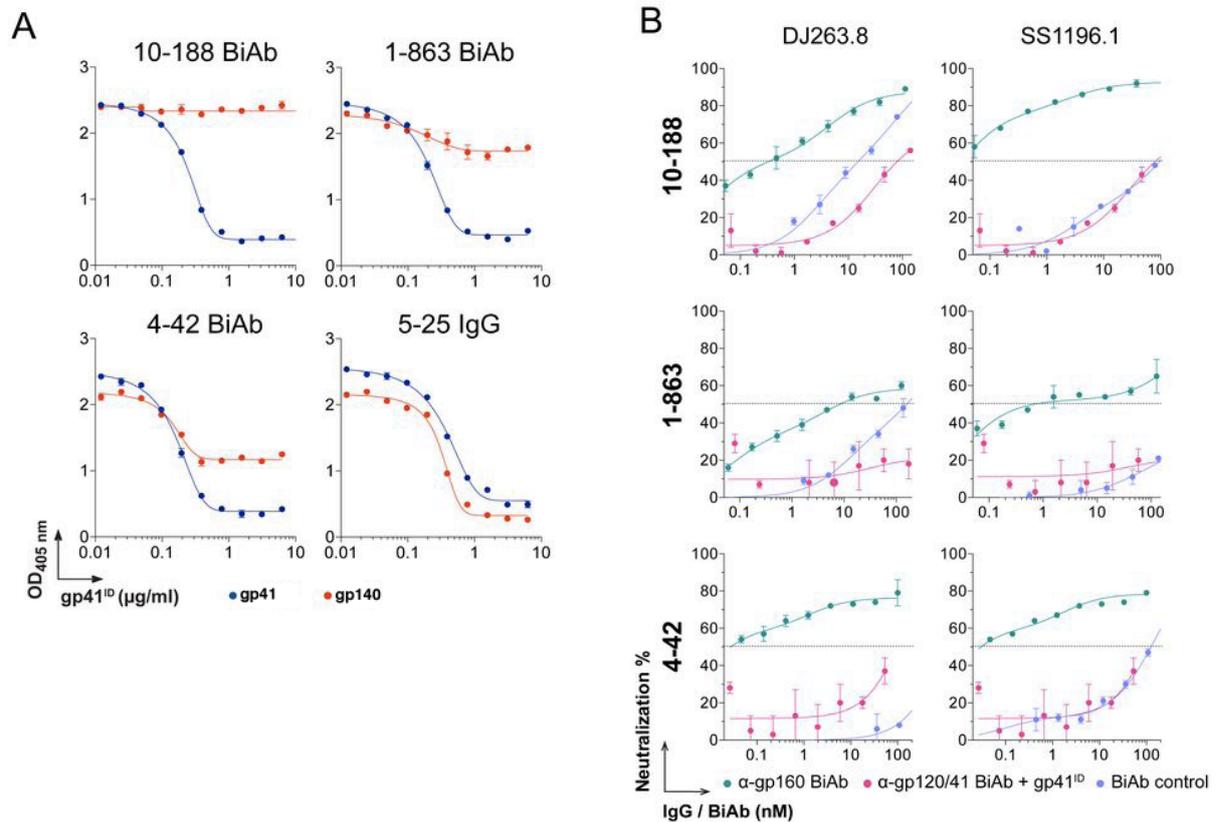


Figure 10. (A) Graphs show solution competition assay for binding to gp140(red)- and gp41(blue)-coated ELISA plates of anti-gp120/gp41 BiAbs and anti-gp41 IgG antibody control using gp41^{ID} peptide as competitor. Error bars indicate the SEM. **(B)** Graph shows neutralization curves of the selected viruses for anti-gp120/gp41 BiAbs in presence or absence of gp41^{ID} peptide. Neutralization curves of BiAb controls are presented for comparison purposes. X-axis shows antibody concentration (nM) required to achieve 50% neutralization (IC₅₀) indicated by the dashed line. Error bars indicate the SD.

Neutralization profiles of the BiAbs during these competition assays resembled the ones of their corresponding anti-gp120 BiAb control (Fig. 10B).

We conclude that simultaneous binding to gp120 and gp41 is required by the BiAbs to achieve an increase in neutralizing potency against sensitive viral strains.

Discussion

To this day, only few studies have investigated the relationship of an antibody's binding profile and its neutralization potency. One study in particular demonstrated that simultaneous antibody binding to influenza and respiratory syncytial virus provided an increase in apparent affinity and a higher neutralization activity. In contrast to HIV, these viruses present antigens at a very high density on their surface (Klein et al., 2009, Klein and Bjorkman, 2010) (Roost et al., 1995, Wu et al., 2005, Barbas et al., 1994). The number of randomly distributed gp160 trimers expressed on a mature HIV-1 virion is as low as 10-15. This has been shown by cryoelectron microscopy (Zhu et al., 2006, Klein and Bjorkman, 2010). Hence, the distance between spikes is likely to be too far to allow for an antibody to capture two spikes simultaneously (Zhu et al., 2006, Klein et al., 2009, Klein and Bjorkman, 2010). There are a several options to address this issue. In theory, bivalent binding could be achieved by modifying the architecture of the antibody, the number of binding sites (valency) or the specificity of the antibody to allow for intra-molecular binding of the envelope spike.

Previous studies showed that the polymerization (IgA and IgM) of the broadly neutralizing antibodies 2F5 and 2G12 lead to an increase in neutralization potency and breadth in comparison to the respective IgG molecule (West et al., 2009) (Wolbank et al., 2003). Furthermore, molecules have been artificially engineered which are able to cross-link spikes on the virion's surface. These molecules consist of a CD4i-directed Fab or IgG fused to CD4 hence, producing a compound that binds simultaneously to the CD4 binding site and the CD4 induced site on gp120. For selected pseudoviruses these constructs exhibited an increase in neutralization potency compared to their parental IgG antibodies (Dey et al., 2003, West et al.,

2010). Finally, enhanced neutralization potency was observed in a study where the 2F5 antibody bivalently recognized its original epitope expressed on gp41 and a second 2F5-epitope artificially grafted into the gp120-variable loop 4 (Wang and Yang, 2010). Apart from the previous methods, another way to achieve heterotypic bivalent binding involves targeting the surface spike and the lipids of the virion's envelope at the same time. The antibody's interaction with the surface lipids is essential for neutralization, although the exact mechanism is yet to be described (Alam et al., 2007, Alam et al., 2009, Scherer et al., 2010, Veiga et al., 2009). There are two possible explanations of this effect. First, the lipid binding may just increase the local concentration of the antibody and hence increase the number of antibodies binding to the envelope leading to a stronger neutralizing effect (Ofek et al., 2010, Julien et al., 2010, Scherer et al., 2010). Alternatively, bivalent heterotypic binding may cause the observed effect. For instance, the 21c antibody that recognizes both CD4 and the CD4 induced site shows that heteroligation due to a single arm increases neutralizing potency (Diskin et al., 2010). Furthermore, it has been described previously that the majority of anti-gp160 antibody repertoire display polyreactivity (Mouquet et al., 2010). These antibodies allow for simultaneous binding to a high-affinity antigen on a surface spike and a second low-affinity antigen on another molecular structure on the virion. These antibodies target a specific ligand with one arm and a different antigen with the second arm, which creates an increase in avidity (Mouquet et al., 2010).

To study the effect of heteroligation on neutralization potency, we artificially engineered bispecific antibodies with one neutralizing anti-gp120 arm paired with a non-neutralizing anti-gp41 arm. Surface Plasmon resonance and ELISA experiments prove that these molecules preserved the binding characteristics of their respective parental IgG and were able to bind gp120 and gp41 at the same time. The addition

of the anti-gp41 arm, but not the pairing with a non-specific arm (control construct), lead to an increase in neutralizing potency against some but not all HIV-1 strains tested compared to their original IgG antibodies. The neutralizing activity varied through viruses tested. This might be due to the fact that the architecture and the expression density of gp160 trimer differ between viral strains.

In conclusion, our artificially engineered anti-gp120/gp41 BiAbs showed an increase in neutralizing potency, although the exact molecular mechanism was not explored. Bivalency could either be achieved by inter- or intraspikes binding of the antibodies. The gain in avidity is most likely the reason for the increase in neutralizing potency of the anti-HIV BiAbs engineered in this study.

Abstract

The serologic response to HIV-1 in human beings is thought to be crucial for the development of a potent vaccine. Antibodies targeting the HIV-envelope protein, gp160, have been isolated from a small group of people that show very efficient neutralizing activity in vitro. Further studies showed that these antibodies protected monkeys against SHIV infection when transferred passively. Although these potent antibodies exist in infected humans, they are scarce. They are generated rarely, because the HIV-1 virus employs multiple strategies to evade the immune response including a glycan shield that protects the Env protein, mutation of viral surface protein gp160 and the expression of the surface epitope presenting spikes at a very low density. The latter is thought to interfere with the bivalent binding of the antibody. Hence, the apparent affinity or avidity of the antibody is decreased which leads to lower neutralization potency.

To counteract the problem of monovalent binding we engineered bispecific anti-HIV-1 antibodies (BiAbs) that can bind bivalently. These BiAbs comprise one scFv arm that binds to gp120 and a second arm to the gp41 subunit of gp160. Both arms persevered the binding characteristics of their original anti-HIV IgG while binding simultaneously to gp120 and gp41 as a heterodimer. These BiAbs show enhanced neutralizing activity against HIV-1 in vitro compared to the parental IgGs. In conclusion, heterologation might be crucial for antibody recognition and the neutralization of HIV.

Zusammenfassung

Von der durch HIV-1 im Menschen ausgelösten serologischen Reaktion wird angenommen, dass sie wichtige Aufschlüsse für die Entwicklung eines wirksamen Impfstoffs gegen das HI-Virus bietet. Aus einem kleinen Patientenkollektiv konnten Antikörper isoliert werden, die gegen das Hüllprotein gp160 gerichtet sind und starke Neutralisierungsaktivität *in vitro* zeigen. Darüber hinaus, schützt passive Gabe dieser Antikörper Affen vor Infektion mit SHIV. Diese Antikörper existieren in infizierten Individuen, sind aber rar. Sie werden selten gebildet, weil das HI-Virus vielseitige Mechanismen aufweist, welche es vor der immunologischen Antwort schützen. Zu diesen Strategien gehören: ein Glykanschild, welches Antigen präsentierende Hüllproteine verdeckt, die hohe Mutationsrate des Hüllproteinvorläufers gp160 und die sehr geringe Expressionsdichte der Oberflächenproteine. Letzteres könnte die bivalente Bindung des Antikörpers verhindern. Dadurch wird die Affinität des Antikörpers zu dem Virus negativ beeinflusst und dessen Neutralisierung behindert.

Um das Problem der monovalenten Bindung zu lösen, haben wir künstlich bispezifische anti-HIV-1 Antikörper (BiAbs) hergestellt, die in der Lage sind eine bivalente Bindung am Virion herzustellen. Diese BiAbs bestehen aus einem scFv-Arm, der gp120 bindet und einem zweiten Arm, der die gp41 Untereinheit von gp160 bindet. Beide Arme bewahren die Bindungscharakteristiken ihrer ursprünglichen anti-HIV IgGs. Als Heterodimer binden sie gp120 und gp41 gleichzeitig. Im Vergleich zu ihren ursprünglichen IgGs zeigten die BiAbs erhöhte Neutralisierung des HI-Virus *in vitro*. Aus diesen Ergebnissen schließen wir, dass „Heteroligation“ eines Antikörpers wahrscheinlich eine zentrale Rolle bei der Erkennung und Neutralisierung des HI-Virus spielt.

References

- ALAM, S. M., MCADAMS, M., BOREN, D., RAK, M., SCEARCE, R. M., GAO, F., CAMACHO, Z. T., GEWIRTH, D., KELSOE, G., CHEN, P. & HAYNES, B. F. 2007. The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. *J Immunol*, 178, 4424-35.
- ALAM, S. M., MORELLI, M., DENNISON, S. M., LIAO, H. X., ZHANG, R., XIA, S. M., RITS-VOLLOCH, S., SUN, L., HARRISON, S. C., HAYNES, B. F. & CHEN, B. 2009. Role of HIV membrane in neutralization by two broadly neutralizing antibodies. *Proc Natl Acad Sci U S A*, 106, 20234-9.
- ALBERT, J., ABRAHAMSSON, B., NAGY, K., AURELIUS, E., GAINES, H., NYSTROM, G. & FENYO, E. M. 1990. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. *AIDS*, 4, 107-12.
- BARBAS, C. F., 3RD, HU, D., DUNLOP, N., SAWYER, L., CABABA, D., HENDRY, R. M., NARA, P. L. & BURTON, D. R. 1994. In vitro evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. *Proc Natl Acad Sci U S A*, 91, 3809-13.
- BINLEY, J. M., LYBARGER, E. A., CROOKS, E. T., SEAMAN, M. S., GRAY, E., DAVIS, K. L., DECKER, J. M., WYCUFF, D., HARRIS, L., HAWKINS, N., WOOD, B., NATHE, C., RICHMAN, D., TOMARAS, G. D., BIBOLLET-RUCHE, F., ROBINSON, J. E., MORRIS, L., SHAW, G. M., MONTEFIORI, D. C. & MASCOLA, J. R. 2008. Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C. *J Virol*, 82, 11651-68.
- BURTON, D. R., BARBAS, C. F., 3RD, PERSSON, M. A., KOENIG, S., CHANOCK, R. M. & LERNER, R. A. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci U S A*, 88, 10134-7.
- DEEKS, S. G., SCHWEIGHARDT, B., WRIN, T., GALOVICH, J., HOH, R., SINCLAIR, E., HUNT, P., MCCUNE, J. M., MARTIN, J. N., PETROPOULOS, C. J. & HECHT, F. M. 2006. Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. *J Virol*, 80, 6155-64.
- DEY, B., DEL CASTILLO, C. S. & BERGER, E. A. 2003. Neutralization of human immunodeficiency virus type 1 by sCD4-17b, a single-chain chimeric protein, based on sequential interaction of gp120 with CD4 and coreceptor. *J Virol*, 77, 2859-65.

- DHILLON, A. K., DONNERS, H., PANTOPHLET, R., JOHNSON, W. E., DECKER, J. M., SHAW, G. M., LEE, F. H., RICHMAN, D. D., DOMS, R. W., VANHAM, G. & BURTON, D. R. 2007. Dissecting the neutralizing antibody specificities of broadly neutralizing sera from human immunodeficiency virus type 1-infected donors. *J Virol*, 81, 6548-62.
- DISKIN, R., MARCOVECCHIO, P. M. & BJORKMAN, P. J. 2010. Structure of a clade C HIV-1 gp120 bound to CD4 and CD4-induced antibody reveals anti-CD4 polyreactivity. *Nat Struct Mol Biol*, 17, 608-13.
- DOLIN, R. 2009. HIV vaccine trial results--an opening for further research. *N Engl J Med*, 361, 2279-80.
- DORIA-ROSE, N. A., KLEIN, R. M., MANION, M. M., O'DELL, S., PHOGAT, A., CHAKRABARTI, B., HALLAHAN, C. W., MIGUELES, S. A., WRAMMERT, J., AHMED, R., NASON, M., WYATT, R. T., MASCOLA, J. R. & CONNORS, M. 2009. Frequency and phenotype of human immunodeficiency virus envelope-specific B cells from patients with broadly cross-neutralizing antibodies. *J Virol*, 83, 188-99.
- GRAY, E. S., TAYLOR, N., WYCUFF, D., MOORE, P. L., TOMARAS, G. D., WIBMER, C. K., PUREN, A., DECAMP, A., GILBERT, P. B., WOOD, B., MONTEFIORI, D. C., BINLEY, J. M., SHAW, G. M., HAYNES, B. F., MASCOLA, J. R. & MORRIS, L. 2009. Antibody specificities associated with neutralization breadth in plasma from human immunodeficiency virus type 1 subtype C-infected blood donors. *J Virol*, 83, 8925-37.
- ICENOGLA, J., SHIWEN, H., DUKE, G., GILBERT, S., RUECKERT, R. & ANDEREGG, J. 1983. Neutralization of poliovirus by a monoclonal antibody: kinetics and stoichiometry. *Virology*, 127, 412-25.
- JULIEN, J. P., HUARTE, N., MAESO, R., TANEVA, S. G., CUNNINGHAM, A., NIEVA, J. L. & PAI, E. F. 2010. Ablation of the complementarity-determining region H3 apex of the anti-HIV-1 broadly neutralizing antibody 2F5 abrogates neutralizing capacity without affecting core epitope binding. *J Virol*, 84, 4136-47.
- KLEIN, J. S. & BJORKMAN, P. J. 2010. Few and far between: how HIV may be evading antibody avidity. *PLoS Pathog*, 6, e1000908.
- KLEIN, J. S., GNANAPRAGASAM, P. N., GALIMIDI, R. P., FOGLESONG, C. P., WEST, A. P., JR. & BJORKMAN, P. J. 2009. Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10. *Proc Natl Acad Sci U S A*, 106, 7385-90.
- KOUP, R. A., SAFRIT, J. T., CAO, Y., ANDREWS, C. A., MCLEOD, G., BORKOWSKY, W., FARTHING, C. & HO, D. D. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol*, 68, 4650-5.

- KWONG, P. D., DOYLE, M. L., CASPER, D. J., CICALA, C., LEAVITT, S. A., MAJEED, S., STEENBEKE, T. D., VENTURI, M., CHAIKEN, I., FUNG, M., KATINGER, H., PARREN, P. W., ROBINSON, J., VAN RYK, D., WANG, L., BURTON, D. R., FREIRE, E., WYATT, R., SODROSKI, J., HENDRICKSON, W. A. & ARTHOS, J. 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature*, 420, 678-82.
- KWONG, P. D., WYATT, R., ROBINSON, J., SWEET, R. W., SODROSKI, J. & HENDRICKSON, W. A. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature*, 393, 648-59.
- LABRIJN, A. F., POIGNARD, P., RAJA, A., ZWICK, M. B., DELGADO, K., FRANTI, M., BINLEY, J., VIVONA, V., GRUNDNER, C., HUANG, C. C., VENTURI, M., PETROPOULOS, C. J., WRIN, T., DIMITROV, D. S., ROBINSON, J., KWONG, P. D., WYATT, R. T., SODROSKI, J. & BURTON, D. R. 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J Virol*, 77, 10557-65.
- LI, Y., MIGUELES, S. A., WELCHER, B., SVEHLA, K., PHOGAT, A., LOUDER, M. K., WU, X., SHAW, G. M., CONNORS, M., WYATT, R. T. & MASCOLA, J. R. 2007. Broad HIV-1 neutralization mediated by CD4-binding site antibodies. *Nat Med*, 13, 1032-4.
- MASCOLA, J. R. 2007. HIV/AIDS: allied responses. *Nature*, 449, 29-30.
- MASCOLA, J. R. & MONTEFIORI, D. C. 2010. The role of antibodies in HIV vaccines. *Annu Rev Immunol*, 28, 413-44.
- MCMICHAEL, A. J. & ROWLAND-JONES, S. L. 2001. Cellular immune responses to HIV. *Nature*, 410, 980-7.
- MONTEFIORI, D. C. 2005. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. *Curr Protoc Immunol*, Chapter 12, Unit 12 11.
- MOORE, J. P. & SODROSKI, J. 1996. Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. *J Virol*, 70, 1863-72.
- MOUQUET, H., KLEIN, F., SCHEID, J. F., WARNCKE, M., PIETZSCH, J., OLIVEIRA, T. Y. K., VELINZON, K., SEAMAN, M. S. & NUSSENZWEIG, M. C. 2011. Memory B Cell Antibodies to HIV-1 gp140 Cloned from Individuals Infected with Clade A and B Viruses. *PLoS One*, 6, e24078.

- MOUQUET, H., SCHEID, J. F., ZOLLER, M. J., KROGSGAARD, M., OTT, R. G., SHUKAIR, S., ARTYOMOV, M. N., PIETZSCH, J., CONNORS, M., PEREYRA, F., WALKER, B. D., HO, D. D., WILSON, P. C., SEAMAN, M. S., EISEN, H. N., CHAKRABORTY, A. K., HOPE, T. J., RAVETCH, J. V., WARDEMANN, H. & NUSSENZWEIG, M. C. 2010. Polyreactivity increases the apparent affinity of anti-HIV antibodies by heteroligation. *Nature*, 467, 591-5.
- MOUQUET, H., WARNCKE, M., SCHEID, J. F., SEAMAN, M. S. & NUSSENZWEIG, M. C. 2012. Enhanced HIV-1 neutralization by antibody heteroligation. *Proc Natl Acad Sci U S A*, 109, 875-80.
- MUSTER, T., STEINDL, F., PURTSCHER, M., TRKOLA, A., KLIMA, A., HIMMLER, G., RUKER, F. & KATINGER, H. 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *J Virol*, 67, 6642-7.
- OFEK, G., MCKEE, K., YANG, Y., YANG, Z. Y., SKINNER, J., GUENAGA, F. J., WYATT, R., ZWICK, M. B., NABEL, G. J., MASCOLA, J. R. & KWONG, P. D. 2010. Relationship between antibody 2F5 neutralization of HIV-1 and hydrophobicity of its heavy chain third complementarity-determining region. *J Virol*, 84, 2955-62.
- PIETZSCH, J., SCHEID, J. F., MOUQUET, H., SEAMAN, M. S., BRODER, C. C. & NUSSENZWEIG, M. C. 2010. Anti-gp41 antibodies cloned from HIV-infected patients with broadly neutralizing serologic activity. *J Virol*, 84, 5032-42.
- RERKS-NGARM, S., PITISUTTITHUM, P., NITAYAPHAN, S., KAEWKUNGWAL, J., CHIU, J., PARIS, R., PREMSRI, N., NAMWAT, C., DE SOUZA, M., ADAMS, E., BENENSON, M., GURUNATHAN, S., TARTAGLIA, J., MCNEIL, J. G., FRANCIS, D. P., STABLEIN, D., BIRX, D. L., CHUNSUTTIWAT, S., KHAMBOONRUANG, C., THONGCHAROEN, P., ROBB, M. L., MICHAEL, N. L., KUNASOL, P. & KIM, J. H. 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med*, 361, 2209-20.
- RIDGWAY, J. B., PRESTA, L. G. & CARTER, P. 1996. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. *Protein Eng*, 9, 617-21.
- ROOST, H. P., BACHMANN, M. F., HAAG, A., KALINKE, U., PLISKA, V., HENGARTNER, H. & ZINKERNAGEL, R. M. 1995. Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proc Natl Acad Sci U S A*, 92, 1257-61.
- SATHER, D. N., ARMANN, J., CHING, L. K., MAVRANTONI, A., SELLHORN, G., CALDWELL, Z., YU, X., WOOD, B., SELF, S., KALAMS, S. & STAMATATOS, L. 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. *J Virol*, 83, 757-69.

- SCHEID, J. F., MOUQUET, H., FELDHahn, N., SEAMAN, M. S., VELINZON, K., PIETZSCH, J., OTT, R. G., ANTHONY, R. M., ZEBROSKI, H., HURLEY, A., PHOGAT, A., CHAKRABARTI, B., LI, Y., CONNORS, M., PEREYRA, F., WALKER, B. D., WARDEMANN, H., HO, D., WYATT, R. T., MASCOLA, J. R., RAVETCH, J. V. & NUSSENZWEIG, M. C. 2009. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. *Nature*, 458, 636-40.
- SCHEID, J. F., MOUQUET, H., UEBERHEIDE, B., DISKIN, R., KLEIN, F., OLIVEIRA, T. Y., PIETZSCH, J., FENYO, D., ABADIR, A., VELINZON, K., HURLEY, A., MYUNG, S., BOULAD, F., POIGNARD, P., BURTON, D. R., PEREYRA, F., HO, D. D., WALKER, B. D., SEAMAN, M. S., BJORKMAN, P. J., CHAIT, B. T. & NUSSENZWEIG, M. C. 2011. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science*, 333, 1633-7.
- SCHERER, E. M., LEAMAN, D. P., ZWICK, M. B., MCMICHAEL, A. J. & BURTON, D. R. 2010. Aromatic residues at the edge of the antibody combining site facilitate viral glycoprotein recognition through membrane interactions. *Proc Natl Acad Sci U S A*, 107, 1529-34.
- SCHOFIELD, D. J., STEPHENSON, J. R. & DIMMOCK, N. J. 1997. Variations in the neutralizing and haemagglutination-inhibiting activities of five influenza A virus-specific IgGs and their antibody fragments. *J Gen Virol*, 78 (Pt 10), 2431-9.
- SIMEK, M. D., RIDA, W., PRIDDY, F. H., PUNG, P., CARROW, E., LAUFER, D. S., LEHRMAN, J. K., BOAZ, M., TARRAGONA-FIOL, T., MIRO, G., BIRUNGI, J., POZNIAK, A., MCPHEE, D. A., MANIGART, O., KARITA, E., INWOLEY, A., JAOKO, W., DEHOVITZ, J., BEKKER, L. G., PITISUTTITHUM, P., PARIS, R., WALKER, L. M., POIGNARD, P., WRIN, T., FAST, P. E., BURTON, D. R. & KOFF, W. C. 2009. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. *J Virol*, 83, 7337-48.
- THALI, M., OLSHEVSKY, U., FURMAN, C., GABUZDA, D., POSNER, M. & SODROSKI, J. 1991. Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive neutralizing human monoclonal antibody. *J Virol*, 65, 6188-93.
- TILLER, T., MEFFRE, E., YURASOV, S., TSUIJI, M., NUSSENZWEIG, M. C. & WARDEMANN, H. 2008. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods*, 329, 112-24.
- TRKOLA, A., PURTSCHER, M., MUSTER, T., BALLAUN, C., BUCHACHER, A., SULLIVAN, N., SRINIVASAN, K., SODROSKI, J., MOORE, J. P. & KATINGER, H. 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *J Virol*, 70, 1100-8.

- VEIGA, A. S., PATTENDEN, L. K., FLETCHER, J. M., CASTANHO, M. A. & AGUILAR, M. I. 2009. Interactions of HIV-1 antibodies 2F5 and 4E10 with a gp41 epitope prebound to host and viral membrane model systems. *Chembiochem*, 10, 1032-44.
- WALKER, L. M., SOK, D., NISHIMURA, Y., DONAU, O., SADJADPOUR, R., GAUTAM, R., SHINGAI, M., PEJCHAL, R., RAMOS, A., SIMEK, M. D., GENG, Y., WILSON, I. A., POIGNARD, P., MARTIN, M. A. & BURTON, D. R. 2011. Rapid development of glycan-specific, broad, and potent anti-HIV-1 gp120 neutralizing antibodies in an R5 SIV/HIV chimeric virus infected macaque. *Proc Natl Acad Sci U S A*, 108, 20125-9.
- WANG, P. & YANG, X. 2010. Neutralization efficiency is greatly enhanced by bivalent binding of an antibody to epitopes in the V4 region and the membrane-proximal external region within one trimer of human immunodeficiency virus type 1 glycoproteins. *J Virol*, 84, 7114-23.
- WARDEMANN, H., YURASOV, S., SCHAEFER, A., YOUNG, J. W., MEFFRE, E. & NUSSENZWEIG, M. C. 2003. Predominant autoantibody production by early human B cell precursors. *Science*, 301, 1374-7.
- WEI, X., DECKER, J. M., WANG, S., HUI, H., KAPPES, J. C., WU, X., SALAZAR-GONZALEZ, J. F., SALAZAR, M. G., KILBY, J. M., SAAG, M. S., KOMAROVA, N. L., NOWAK, M. A., HAHN, B. H., KWONG, P. D. & SHAW, G. M. 2003. Antibody neutralization and escape by HIV-1. *Nature*, 422, 307-12.
- WEST, A. P., JR., GALIMIDI, R. P., FOGLESONG, C. P., GNANAPRAGASAM, P. N., HUEY-TUBMAN, K. E., KLEIN, J. S., SUZUKI, M. D., TIANGCO, N. E., VIELMETTER, J. & BJORKMAN, P. J. 2009. Design and expression of a dimeric form of human immunodeficiency virus type 1 antibody 2G12 with increased neutralization potency. *J Virol*, 83, 98-104.
- WEST, A. P., JR., GALIMIDI, R. P., FOGLESONG, C. P., GNANAPRAGASAM, P. N., KLEIN, J. S. & BJORKMAN, P. J. 2010. Evaluation of CD4-CD4i antibody architectures yields potent, broadly cross-reactive anti-human immunodeficiency virus reagents. *J Virol*, 84, 261-9.
- WOLBANK, S., KUNERT, R., STIEGLER, G. & KATINGER, H. 2003. Characterization of human class-switched polymeric (immunoglobulin M [IgM] and IgA) anti-human immunodeficiency virus type 1 antibodies 2F5 and 2G12. *J Virol*, 77, 4095-103.
- WU, H., PFARR, D. S., TANG, Y., AN, L. L., PATEL, N. K., WATKINS, J. D., HUSE, W. D., KIENER, P. A. & YOUNG, J. F. 2005. Ultra-potent antibodies against respiratory syncytial virus: effects of binding kinetics and binding valence on viral neutralization. *J Mol Biol*, 350, 126-44.

- WU, X., YANG, Z. Y., LI, Y., HOGERKORP, C. M., SCHIEF, W. R., SEAMAN, M. S., ZHOU, T., SCHMIDT, S. D., WU, L., XU, L., LONGO, N. S., MCKEE, K., O'DELL, S., LOUDER, M. K., WYCUFF, D. L., FENG, Y., NASON, M., DORIA-ROSE, N., CONNORS, M., KWONG, P. D., ROEDERER, M., WYATT, R. T., NABEL, G. J. & MASCOLA, J. R. 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science*, 329, 856-61.
- WYATT, R., DESJARDIN, E., OLSHEVSKY, U., NIXON, C., BINLEY, J., OLSHEVSKY, V. & SODROSKI, J. 1997. Analysis of the interaction of the human immunodeficiency virus type 1 gp120 envelope glycoprotein with the gp41 transmembrane glycoprotein. *J Virol*, 71, 9722-31.
- XIAO, X., CHEN, W., FENG, Y., ZHU, Z., PRABAKARAN, P., WANG, Y., ZHANG, M. Y., LONGO, N. S. & DIMITROV, D. S. 2009. Germline-like predecessors of broadly neutralizing antibodies lack measurable binding to HIV-1 envelope glycoproteins: implications for evasion of immune responses and design of vaccine immunogens. *Biochem Biophys Res Commun*, 390, 404-9.
- ZHU, P., LIU, J., BESS, J., JR., CHERTOVA, E., LIFSON, J. D., GRISE, H., OFEK, G. A., TAYLOR, K. A. & ROUX, K. H. 2006. Distribution and three-dimensional structure of AIDS virus envelope spikes. *Nature*, 441, 847-52.
- ZOLLA-PAZNER, S. 2004. Identifying epitopes of HIV-1 that induce protective antibodies. *Nat Rev Immunol*, 4, 199-210.
- ZWICK, M. B., LABRIJN, A. F., WANG, M., SPENLEHAUER, C., SAPHIRE, E. O., BINLEY, J. M., MOORE, J. P., STIEGLER, G., KATINGER, H., BURTON, D. R. & PARREN, P. W. 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J Virol*, 75, 10892-905.

Publications

MOUQUET, H., WARNCKE, M., SCHEID, J. F., SEAMAN, M. S. & NUSSENZWEIG, M. C. 2012.

Enhanced HIV-1 neutralization by antibody heterologation.
Proc Natl Acad Sci U S A, 109, 875-80.

MOUQUET, H., KLEIN, F., SCHEID, J. F., WARNCKE, M., PIETZSCH, J., OLIVEIRA, T. Y. K., VELINZON, K., SEAMAN, M. S. & NUSSENZWEIG, M. C. 2011.

Memory B Cell Antibodies to HIV-1 gp140 Cloned from Individuals Infected with Clade A and B Viruses.
PLoS One, 6, e24078.

SCHEID, J. F., MOUQUET, H., UEBERHEIDE, B., DISKIN, R., KLEIN, F., OLIVEIRA, T. Y., PIETZSCH, J., FENYO, D., ABADIR, A., VELINZON, K., HURLEY, A., MYUNG, S., BOULAD, F., POIGNARD, P., BURTON, D. R., PEREYRA, F., HO, D. D., WALKER, B. D., SEAMAN, M. S., BJORKMAN, P. J., CHAIT, B. T. & NUSSENZWEIG, M. C. 2011.

Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding.

Science, 333, 1633-7.

Acknowledged for protein expression.

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Unterschrift:

Malte Warncke

Hamburg,