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Identification of a novel broadly neutralizing antibody targeting HIV-1

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- Neutralization data for bNAbs was sourced from CATNAP.

Adapted figures from the co-publication ‘Restriction of HIV-1 Escape by a Highly Broad and Potent Neutralizing Antibody’ published in *Cell* 2020:

- Figure 8 ‘Identification of elite neutralizer IDC561 and characterization of his HIV-1 reactive B cell repertoire’
- Table 56 ‘HIV-1 Env reactive antibody clones isolated from IDC561’
- Table 3 ‘Half-maximal effective concentration and maximum optical density of isolated antibodies from IDC561 in ELISA’
- Table 4 ‘Neutralization activity of all tested antibodies from elite neutralizer IDC561’
- Figure 13 ‘Phylogenetic analysis of clone 4’
- Figure 19 ‘Comparison of serum neutralization and neutralization profile of bNAb 1-18’
- Figure 20 ‘Serum neutralization activity and neutralization activity against 42 pseudoviruses’
- Table 6 ‘Neutralizing activity compared between bNAb 1-18 and engineered variant of 1-18 without six amino acid insertion in CDRH1’

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For all who believe in science

Table of Contents

List of Abbreviations

Abbreviation	Meaning
1-18	Broadly neutralizing HIV-1 antibody 561_01_18
AAV	Adeno-associated viral vector
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ADCP	Antibody-dependent cellular phagocytosis
AID	Activation-induced deaminase
AIDS	acquired immune deficiency syndrome
APC	Antigen-presenting cell
ART	Antiretroviral therapy
ATP	Adenosine 5′-triphosphate
AZT	azidothymidine
bNAb	Broad neutralizing antibody
BSA	Bovine serum albumine
C..	Complement component
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CDC	U.S. Centers for Disease Control and Prevention
cDNA	complementary DNA
CDR	Complementary determining region
CDRH1/2/3	Heavy chain complementarity determining region 1/2/3
CoA	Coenzyme A
CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
DEAE	Diethylethanolamine
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	Ethylenediaminetetraacetic acid, Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
env	HIV-1 Envelope protein
ESCRT	Endosomal Sorting Complex Required for Transport
Fab	Fragment antigen binding
FBS	Fetal bovine serum
Fc	Fragment crystallizable region

Fw ..... Forward
FWR ..... Framework region
Gag ..... Group-specific antigen
GC ..... Guanin/cytosin
GFP ..... Green fluorescent protein
gp ..... Glycoprotein
HAART ..... Highly active retroviral therapy
HEPES ..... Hydroxyethylpiperazine ethane sulfonic acid
HF ..... High Fidelity
HIV-1 ..... Human immunodefiency virus 1
HLA ..... Human leukocyte antigen
IgBLAST ..... the international ImMunoGeneTics information system
IGEPAL ..... octylphenoxypolyethoxyethanol
IgG ..... Immunoglobulin G
L ..... Leader region
LB ..... Lysogeny broth
LDS ..... Lithium dodecyl sulfate
LUC ..... Luciferase
Mab ..... Monoclonal antibody
$MgCl_2$ ..... Magnesium chloride
MHC ..... Major histocompatibility complex
MOPS ..... (N-Morpholino)-propane sulphonic acid
MPER ..... Membrane proximal external region
mRNA ..... Messenger ribonucleic acid
nAbs ..... Neutralizing antibodies
NaCl ..... Natriumchlorid
NCT ..... ClinicalTrials.gov identifier
nef ..... Negative factor
NHP ..... Non-human primate
NP ..... Nonionic polyoxyethylene surfactant
NRTI ..... Nucleotide reverse transcriptase inhibitor
Opt5 ..... Optimized primer set 5
PBMC ..... peripheral blood mononuclear cell
PBS ..... Phosphate buffered saline
PCR ..... Polymerase chain reaction
PE ..... Phycoerythrin
PEI ..... Polyethylenimine

PEP ..... Postexposure prophylaxis
PIC ..... Pre-integration complex
pIgH/K/L .....
..... Protein expression vector for immunoglobulin heavy chain/kappa chain/lambda chain
pol ..... DNA polymerase
PreP ..... Preexposure prophylaxis
P-TEFb ..... Positive transcription elongation factor
RAG ..... Recombination-activating genes
rev ..... Regulator of expression of virion proteins
RNA ..... Ribonucleic acid
RT ..... Reverse Transcriptase
Rv ..... Reverse
RV144 ..... Thai HIV vaccine efficacy trial
SARS-CoV-2 ..... Severe acute respiratory syndrome coronavirus 2
SDS ..... Sodium dodecyl sulfate
SHIV ..... Simian/human-chimeric immunodeficiency virus
SLIC ..... Sequence ligase independent cloning
SOC ..... Super Optimal Broth with 20 MM Glucose
Taq ..... DNA polymerase from Thermophilus aquaticus
TasP ..... Treatment as prevention
tat ..... HIV-1 trans-activator
TC ..... Tissue culture
TdT ..... Terminale Desoxyribonukleotidyltransferase
Tris ..... Tris(hydroxymethyl)aminomethan
UN ..... United Nations
UNAIDS ..... Joint United Nations Programme on HIV/AIDS
V1/V2 ..... First/Second variable region of the HIV-1 gp120 envelope
V3 ..... Third variable region of the HIV-1 gp120 envelope
VDJ ..... Variability-diversity-joining
V_H ..... Immunoglobulin G heavy chain variable gene (also IGHV)
vif Viral infectivity factor
vpr ..... Lentivirus protein R
vpu ..... Viral protein U

Zusammenfassung

Obwohl seit bereits 40 Jahren an HIV-1 geforscht wird und seit mehr als 25 Jahren eine wirksame antiretrovirale Therapie (ART) existiert, stellt HIV-1 noch immer eines der großen Gesundheitsprobleme der heutigen Zeit in sogenannten Entwicklungs- als auch in Industrieländern dar. Mit einer aktuellen Neuansteckungsrate von 1,5 Millionen pro Jahr wurde das 2016 kalkulierte Ziel der UNAIDS um ein dreifaches verfehlt.[1] Daher benötigen wir neue Strategien, um die HIV-1 Pandemie zu bekämpfen.

Die Forschung rund um breit neutralisierende Antikörper (bNAbs) gegen HIV-1 war ein Wegbereiter für den schnellen klinischen Einsatz von einer Antikörpertherapie gegen SARS-CoV-2 und das Ebolavirus.[2,3] Dies zeigte, dass Antikörpertherapien effektiv gegen Infektionskrankheiten eingesetzt werden können. Grundsätzlich ist der Einsatz von bNAbs gegen HIV-1 sicher und zeigt antivirale Aktivität, führt aufgrund der hohen Mutationsrate von HIV-1 aber oft zu viralen Resistenzbildungen. Um dieses Problem zu überwinden, wurden Kombitherapien aus bNAbs eingesetzt, die die Zeit bis zur viralen Resistenzbildung im Vergleich zu Monotherapien verlängern können. Nichtsdestotrotz werden für eine dauerhafte Virussuppression neue potente bNAbs benötigt, um virale Fluchtmutationen zu verhindern.

Um dies zu erreichen, untersuchten wir das HIV-1 spezifische B Zell Repertoire von einem Individuum mit potenter Serumneutralization gegen HIV-1, ein Ansatz mit dem vormals bNAbs isoliert werden konnten. Hierbei handelte es sich um einen *Elite Neutralizer* IDC561, dessen Serumneutralization sich unter den besten 1% in einer Kohorte von 2.274 HIV-1 Infizierten befand. Aus HIV-1 reaktiven B Zellen von IDC561 amplifizierten wir Einzelzell-Sequenzen, welche uns eine weitere Klonierung und Produktion von Antikörpern erlaubte. Durch Testung der isolierten Antikörper auf ihre Bindungs- und Neutralisationseigenschaften konnten wir 1-18 isolieren, ein breit neutralisierender Antikörper, welcher eine außerordentliche Neutralizationsaktivität zeigte. Die Neutralizationsaktivität von 1-18 übertraf in vielen Bereichen die Neutralisationsaktivität der bisher klinisch eingesetzten bNAbs nicht nur im 12-Pseudoviren *global panel*, sondern auch in den 119-Pseudovirus *multiclade* and 100-Pseudovirus *clade C panel*. Dies macht den isolierten bNAb 1-18 zu einer vielversprechenden Option für eine HIV-1 Antikörpertherapie.

Summary

Despite 40 years of research and more than 25 years of effective antiretroviral therapy (ART), HIV-1 infection remains a tremendous public health problem in both developing and industrialized countries. With a recent incidence of 1.5 million the UNAIDS goals of 500,000 new infections calculated in 2016 were dramatically missed.[1] Therefore, new approaches to combat HIV-1 pandemic are needed.

HIV-1 research regarding 'Broadly neutralizing antibodies' has paved the way for developing effectively used antibody therapies against SARS-CoV-2 and Ebola virus, showing that antibody therapies can be effectively used against infectious diseases.[2,3] In general, bNAbs against HIV-1 were demonstrated to be safe and effective in infected individuals, but finally lead to viral escape because of the high mutation rate of HIV-1. Combi-therapies were implemented to address this problem and could prolong viral suppression compared to monotherapy. Still, for effective long-lasting viral suppression new potent bNAbs are needed to finally restrict viral escape.

To this end, we characterized the HIV-1 specific B cell repertoire of an individual with potent serum neutralization of HIV-1, a so-called Elite Neutralizer, as this approach was previously successful for the isolation of existing bNAbs. The serum neutralization of Elite Neutralizer IDC561 ranked among the top 1% of a cohort of 2,274 HIV-1 infected individuals. From IDC561's HIV-1 reactive B cell clones we amplified single cell sequences, allowing for antibody cloning and production. Testing isolated antibodies for their binding capacities and neutralizing activity lead to the isolation of 1-18, a broad and neutralizing antibody, which showed exceptional neutralizing activity. 1-18 exceeded neutralization activity in comparison to clinically advanced bNAbs not only in the 12-strain global panel but also in 119-pseudovirus multiclade and 100-pseudovirus clade C panels, which makes 1-18 a promising option for HIV-1 antibody therapy.

1 Introduction

1.1 HIV-1 as major health problem

In the early eighties an epidemic of clinical AIDS defining diseases especially in men having sex with men, intravenous drug users, and hemophilics was seen.[4–7] Sometime passed after it was discovered that a virus infecting $CD4^+$ T-lymphocytes, transmitted by sexual contacts, blood products, and needle sharing, caused the disease.[8] Mortality rates increased, and millions of people died without having suitable treatment options. The invention of highly active retroviral therapy in 1996 (HAART respective now ART) could turn HIV-1 infection from a death warrant to a chronic treatable disease. Thus, in most industrialized countries the HIV-1 pandemic became controllable. However, in some industrialized countries incidences are recently rising and AIDS-related deaths are still the fourth common cause of death in Africa.[9] Overall, until the present time the HIV-1 pandemic cost in total over 36 million lives.[10] Despite being subject of intensive research there is still no cure or vaccine against HIV-1 leaving the problem of the HIV-1 pandemic unsolved 40 years after its discovery.

1.2 The Human Immunodeficiency Virus 1

1.2.1 Epidemiology

According to the UNAIDS report in 2020, 37.7 million people worldwide were living with HIV-1, and 1.5 million got newly infected. 680,000 people died of AIDS related diseases.[1] Sixty percent of people living with HIV-1 worldwide are located in Sub-Saharan Africa, and in some regions of Sub-Saharan Africa, AIDS is even the leading cause of death. Approximately 20% of the population is HIV-1 positive in these regions.[9] In these regions AIDS not only represents a health issue but still is a massive social and economic problem.

In Germany, there were approximately 2,000 new infections diagnosed in 2020, and a total of 91,400 people were living with HIV-1.[11] This suggests that the HIV-1 pandemic in Germany is rather controlled. However, incidences in Eastern Europe have increased dramatically. Between 2010 and 2020, the number of new HIV-1 infections increased by 43%, and AIDS related deaths increased by 32%.[1]

Many global health programs have been established to combat the HIV-1 pandemic. In 2016, the UN set the goal that by 2020, 90% of people living with HIV should know their status, of which 90% should be on HIV-1 treatment, and of which 90% should be virally suppressed. However, in the evaluation of 2021, it was found that the set goals could not be reached. By 2020, 84% of people living with HIV-1 were aware of their HIV status, 73% in total of people

living with HIV-1 were on treatment and 66% were virally suppressed.[1] This suggests that progress in the effective treatment of HIV-1-infected individuals has been lagging.

In conclusion, even though great progress has been made, the HIV-1 pandemic is still a major global health problem requiring new strategies.

1.2.2 Structure

HIV-1 is the causative agent for AIDS. It is a Lentivirus from the family of Retroviruses with a cover by a lipoprotein membrane. In its conical capsid it contains single stranded, positive sensed RNA encoding for the nine virus genes: *Gag, pol, env, tat, rev, vif, vpr, vpu* and *nef* (Figure 1). *Gag* (group specific antigen) is encoding for the matrix, the capsid and the nucleocapsid. *Pol* (polymerase) is encoding for viral enzymes such as protease, reverse transcriptase and integrase. *Env* (envelope) is encoding for proteins of the envelope. On average, 14 HIV-1 envelope proteins spike the outer membrane.[12] As the envelope protein is the only protein on the surface of the virion, it is the only site where binding prevents HIV-1 entry and lead directly to neutralization. This characteristic makes it the main target for prevention strategies. Therefore, the structure will be described here more in detail.[13,14]

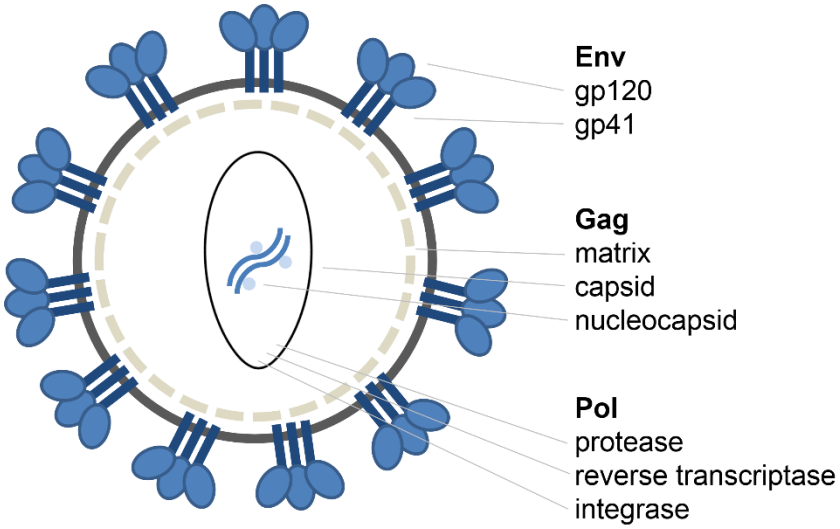


Figure 1. Structure of HIV-1

Each envelope protein is a transmembrane protein in the shape of a trimer consisting of three heterodimers. The stem is built of three non-covalently bound glycoprotein gp41, which is topped with three glycoproteins of gp120. This complex together is called gp160. The numbers indicate molecular weight. Antibodies can theoretically bind to the Env protein due to its outer exposure, but neutralization by antibodies is rare. One reason is, that the erroneous reverse transcriptase of HIV-1 produces a high variety of Env protein. When immune cells target the Env protein, selection pressure towards immune escape is build, making natural clearance

therefore difficult.[15,16] Another reason is that, although conserved epitopes exist, steric hindrance and a shield by mannose glycans make antibody binding difficult.[17,18]

The high variety of the Env protein and its structural conformation contribute to the inability of natural antibodies to effectively neutralize HIV-1, making spontaneous clearance of the infection challenging.

1.2.3 Replication cycle

Upon entering the bloodstream, HIV targets $CD4^+$ immune cells. After infection, they become the host and begin replicating new HIV-1 particles. Newly generated HIV-1 particles are then released to infect additional $CD4^+$ immune cells, perpetuating the infection cycle.

Viral entry:

After entering the blood stream, it comes to interactions between HIV and cells of the immune system. The viral envelope protein gp120 binds to the surface protein CD4 on predominately T cells, which play an important role in the defense of pathogens and act as an activator for further immune defense.[19]

Binding between the viral envelope protein gp120 and the CD4 receptor leads to conformation changes in gp120 (Figure 2). This enables the so-called V3 loop of the Env protein to bind the co-receptor of the T cell. This double binding is compulsory for infection.

After the gp120 trimer bound to the CD4 receptor and the coreceptor, it opens up, bringing the gp41 peptide closer to the cell membrane. The gp41 subunit is inserted in the host cell membrane (Figure 2). In a second step the envelope protein folds, which leads to **fusion** of the virus and cell membrane, and finally, the insertion of the HIV-1 capsid.[20]

HIV-1 can use two different cellular co-receptors: CCR5 or CXCR4.[21,22] The co-receptors play an important role in the infection process. A particular mutation in the CCR5-Gen leads to HIV-1 resistance, which is homozygous for approximately 1% of the world population. The only patients known to be or possibly be cured have been transplanted with stem cells enduring the homozygous CCR5Δ32 mutation.[23–25]

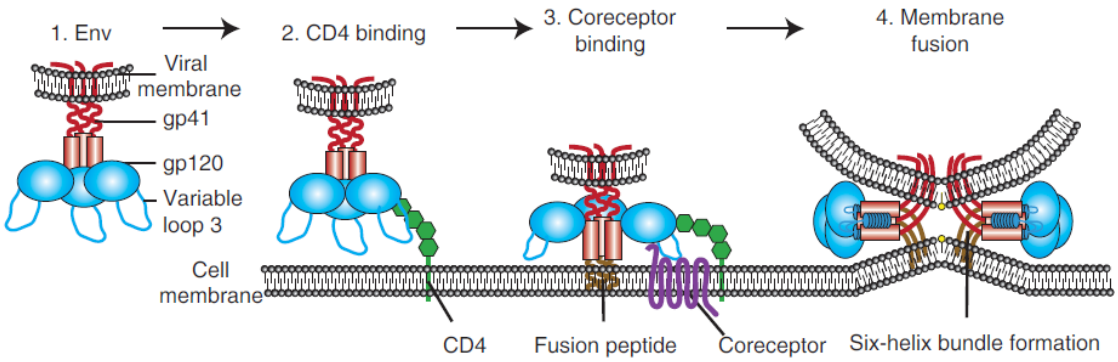


Figure 2. HIV-1 entry

The surface protein gp120 binds to the CD4 receptor of the host cell (1 and 2). This enables a binding between the V3 and the co-receptor CCR5 or CXCR4 of the host cell (3). The confirmation of gp120 changes and the fusion peptide gp41 is inserted in the host cell membrane. A second confirmation change takes place, the envelope protein folds in and the virus membrane und host cell membrane fuse (4). This figure is based on Wilen et al. (2012)[26] and used with friendly permission of Cold Spring Harbor Laboratory Press.

After HIV-1 entered the T cell, the capsid is transported towards the cell nucleus using microtubules. During this process inside the capsid the **reverse transcription** takes place. The enzyme reverse transcriptase rewrites the single stranded viral RNA in double stranded DNA. The enzyme is extremely erroneous and therefore produces DNA with multiple mutations. Some mutations cause replication to halt, while others lead to the emergence of various quasispecies within an individual. These quasispecies facilitate immune escape mechanisms, rendering immune defense ineffective or making drug resistance more likely.[27]

Shortly before entering the cell nucleus through nuclear pore or after, the capsid is **uncoated**. A part of the viral DNA bound to the viral protein integrase travels near the cell DNA. This complex is called pre-integration complex. Through the integrase the viral DNA gets attached to the cell DNA, cuts the cell DNA, and then **integrates** the viral DNA into the cell DNA. The present form is called provirus and can lay dormant for weeks, months or years.

After activation, the host cell's RNA Polymerase initiates the **transcription** and generates viral RNA and mRNA encoding for viral proteins. Normally, the multiprotein complex P-TEFb pauses the transcription and needs cellular signals for further transcription, but the viral protein *tat* bypasses this process, allowing for continuous transcription of the viral genome.[28]

The viral RNA and mRNA are transported by proteins bound to *rev* through the nuclear pore and exit the nucleus. Outside the nucleus some mRNA is translated by ribosomes into viral proteins, while other are packaged into new virions. The different components **assemble** close to the host cell membrane. The cell membrane buds, more viral proteins are recruited, and finally, the new virion is **released** by a protein called ESCRT.

After the release of the virion, it undergoes a process called **maturation**. The *gag* polymers are spliced by the viral protease, forming the nucleocapsid, the matrix, and the capsid.

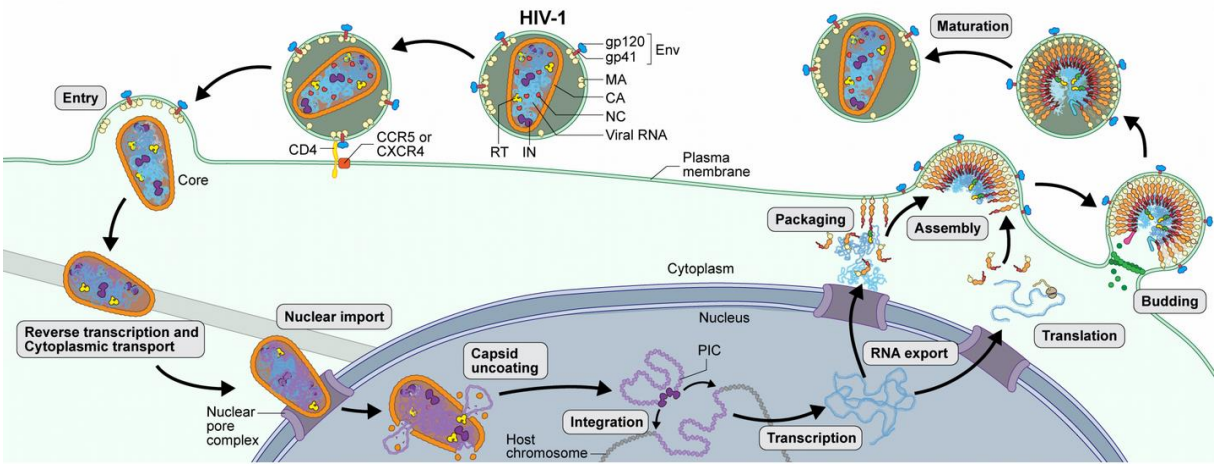


Figure 3. HIV-1 replication cycle

The HIV-1 virion binds to the CD4 receptor and subsequently to the co-receptor. The capsid enters the host cell. During the cytoplasmic transport on microtubules the reverse transcription takes place. The capsid is imported through a nuclear pore complex into the cell nucleus. The capsid shell breaks apart and the viral RNA bound to integrase (pre-integration complex PIC) travels to the host chromosome and is integrated. When the so-called provirus is activated, viral RNA is transcribed by the host RNA polymerase and then exported to the cytoplasma. Some mRNAs are translated into viral proteins, others are packing for building the viral RNA inside a new virion. *Gag* proteins assemble at the host cell membrane, causing budding of the virion. After the virion split up, the viral enzyme protease splices pre *gag* proteins into matrix, nucleocapsid and capsid proteins. This figure is used with courtesy of a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License, created by Janet Iwasa[29], https://creativecommons.org/licenses/by-sa/4.0/

A good strategy for HIV-1 prevention seems to be inhibiting the very first contact between gp120 and the CD4 receptor, but this approach could not be implemented so far. Another challenge is, that modern ART can pause the replication cycle by intervening in replication mechanisms after viral entry but cannot clear the infection because viral DNA lays dormant in immune cells. Therefore, new strategies for HIV-1 prevention and treatment are needed.

1.2.4 Clinical course

The HI-virus is mainly transmitted by blood or body fluids. The three most common routes are i) unprotected sexual activity with an infected individual, ii) blood transmission via needle sharing or more rarely contaminated blood products, and iii) mother-to-child transmission during pregnancy, birth or breastfeeding.[30]

The natural course of an HIV-1 infection has three stages and is closely linked with ongoing destruction of $CD4^+$ T cells.[31]

i) After infection, massive viral replication takes place, and the individual is very infectious. The innate and acquired immune system is activated and targets viral replication. Viral load decreases, and $CD4^+$ T cell counts recover partially. In this stage, it comes to an onset of

symptoms after approximately two weeks. The symptoms are nonspecific, such as fever, weight loss, exanthema, myalgia, and lymphadenopathy. It is also called 'Mononucleosis like syndrome' because the early stage is mistaken for mononucleosis so often.[32]

ii) It follows a stage with asymptomatic latency, which can last for years. High proliferation rates can initially compensate for the ongoing destruction of $CD4^+$ T cells, but as time passes the immune system gets exhausted. Viral load increases, and $CD4^+$ T cell count decreases. The two previous stages are referred to as CDC stage A.

iii) Typically, a $CD4^+$ T cell count below 200 per µl cannot be compensated for by increased reproduction. Symptoms due dysfunction of the immune system occur: opportunistic infections and HIV-1 associated malignant tumors. Initially, these are infections such as oral soor, herpes zoster and oral hair leukoplakia (CDC stage B), later so-called AIDS defining diseases appear (CDC stage C).[33]

Some individuals though have another more benignant clinical course. Approximately five percent of HIV-1 infected individuals stay without symptoms for over ten years without ART, the so-called 'Long term non progressors'. Less than one percent additionally sustain undetectable viral loads in most of this time and are called 'Elite controllers'.[34] Favorable factors for virological control without ART are certain HLA patterns (HLA B27, B58 and B27) or a heterozygous delta 32 mutation of the co-receptor CRX. It is discussed that HLA B expressing cells are more resistant to Nef mediated downregulation of MHC I receptors, a receptor that activates cytotoxic CD8 T cells and leads to cell destruction if the cell is infected. It is also described that Elite controllers have qualitatively better CD8 T cells responses.[34]

1.2.5 Treatment and prevention of HIV-1 infection

After the discovery of HIV as the causative of AIDS in the year 1983, the first drug, the nucleoside transcriptase inhibitor azidothymidine (AZT), was developed 1987, but it was not able to improve the clinical outcome due the fast development of viral resistance.[35,36] Also, the combination of two NRTIs was not yet successful.[37] The turning point was the invention of protease inhibitors and the use of multidrug regimes, which could finally limit viral escape and thus effectively suppress viral loads. Life expectancy under this novel regime increased rapidly.[38] Nowadays, the standard ART regime combines at least three drugs of two different drug classes. Drug classes inhibit the HIV-1 replication cycle at different steps as non-nucleoside and nucleoside transcriptase inhibitors, protease inhibitors, fusion or co-receptor binding inhibitors and integrase inhibitors. If taken regularly, ART can lower viral loads below

detection, extend life expectancy to almost normal, and hold up the progression to AIDS. If the viral load is completely suppressed, the individual is not even infectious anymore.

Nevertheless, ART has its negative sides. For many infected individuals, the daily intake of antiretroviral therapy is a psychosocial burden, being a daily reminder of the HIV-1 infection and a stigma, and going along with discrimination in society.[39] The life-long medication is not side-effect free but comes along with cardiovascular, renal, endocrinological, dermatological and neuropsychiatric side effects. ART is costly (standard ART regime costs approximately 36,000$ per year in the US)[40] and can be a reason why ART is not taken or not taken regularly especially in low-income countries.[41] Moreover, ART has many drug interactions and with an HIV population which lives longer and takes multiple medications for various health concerns, this can pose a challenge.[42]

All these reasons can negatively influence adherence, which is crucial for treatment success. Inadequate adherence to ART can lead to rising viral loads, disease progression, and the development of viral resistance. In some cases ART fails even taken continuously, especially in pretreated individuals the virus is likely to develop ART resistance.[43] World-wide, only two-third of HIV-1 infected individuals are virally suppressed, which means over 12 million individuals are not fully treated.[1]

To date, no effective vaccine is available against HIV. Existing prevention methods are pre-exposure prophylaxis (PreP), postexposure prophylaxis (PEP), the wide use of barrier methods and treatment as prevention (TasP), which all depend on adherence and the right use and are therefore as prevention methods limited. PreP can cause viral resistances if the individual was already infected.[44] Although efforts in awareness campaigns are large in Germany, the number of sexually transmitted diseases is recently steadily increasing and the HIV-1 incidence does not decline.[45]

In summary, present treatment and prevention methods are limited in their effectiveness and novel approaches are needed.

1.2.6 HIV-1 diversity

HIV-1 has four different groups: M, N, O and P. M is the major group and responsible for over 90 percent of the infections. Based on the genome, these branches can be subdivided in different subtypes or clades. It depends on the region which subtype is dominant, for example clade B is dominant in Europe, while clade C is dominant in Southern and Eastern Africa.[46] Apart from this genetic classification recombinant forms exist.[47] Different mechanisms are

described which contribute to the outstanding genetic variability of HIV-1. Already in one individual millions of variants of HIV-1 virions co-exist, the so-called ‘Quasispecies’.

In chronic HIV-1 infection on average 10 billion virions per day are produced. The error prone reverse transcriptase causes a mutation rate of 3.4×10^{-5}. The genome size is approximately 10^4 base pairs large. Therefore, a day of replicating HIV results in over 10^9 mutated virions.[48,49] These mutations can lead to an infected but defective provirus, non-infectious virions, or even more infectious virion.[50] Under selection pressure of the immune system or the presence of ART, virions become resistant and dominate the virus pool.[51] To lower the replication and therefore the chance for immune escape, immediate start with ART is of utmost importance.[52] Additionally, genetic recombination creates diversity. If a single cell becomes co-infected with two or more genetically diverse virions, two different RNA strands can be packaged in one virion.[53] If another cell gets infected, the reverse transcriptase can move back and forward between those two RNA templates, creating a novel recombinant transcript.[54] Even more resistant viruses evade if the patients are pretreated.[43]

Some regions are more susceptible to mutations than others. Early mutations occur mostly in the viral proteins Env, Vif, and Nef, leading to their high variability.[55] In other regions like Gag, mutations of the virus are associated with fitness cost, making them less likely to be selected for. These regions of the viral genome are called conserved. It is assumed that cytotoxic T lymphocytes responses of certain HLA B classes can select for Gag mutations which are associated with fitness cost, thereby temporarily controlling viral replication. In transmission partners without this genetic advantage, these mutations are reverted. After years, though, compensatory mutations develop, and disease progression continues.[51,56]

Viral diversity and resistance are the reasons why spontaneous clearance by the immune system remains elusive. It is the main problem of treatment and prevention strategies. New approaches to cope with viral diversity are therefore needed.

In conclusion, HIV-1 is a complex virus with different challenges that, to date, have not been resolved. These challenges are the reason why HIV-1 infection and AIDS are still not fully combated. The hidden surface structure and diversity, resulting by the error prone reverse transcription, makes an effective humoral immune response difficult. By inhibiting multiple viral enzymes with antiretroviral therapy, the virus replication can be kept under control but cannot clear the HIV reservoir; therefore, it cannot cure the HIV-1 infection. Moreover, treatment options have negative sides as side effects, the burden of daily medication and development of viral resistance. Despite intensive research of over four decades, effective vaccination

regimes do not exist. In conclusion, new treatment and prevention approaches are needed to end the HIV-1 pandemic.

1.3 Humoral immune response

1.3.1 B cell maturation

In the bone marrow, lymphoid stem cells differentiate to immature naïve B-lymphocytes whilst undergoing processes of gene recombination to generate a great diversity of B lymphocytes. The immature naïve lymphocytes are selected for self-tolerance. The now mature naïve lymphocytes leave the bone marrow over the blood stream and circulate between blood and secondary lymphoid tissue until they encounter a matching antigen.[57,58]

Activation of T helper cells

If a pathogen enters the blood stream, it is engulfed, processed, and presented on MHC II receptors by B cells, dendritic cells, monocytes or macrophages, the so-called antigen-presenting cells (APC). T helper cells with matching T cell receptor recognize the bound antigen on the surface of the APC. The APC then releases IL-1, which activates the T helper cell. The T helper cell with the matching T cell receptor proliferates.[57,59]

Activation of the B cell

The mature naïve B cell with the matching B cell receptor binds, phagocytizes, and processes the pathogen. The pathogen is displayed on an MHC II receptor. The pre-activated cognate T helper cell binds the displayed antigen on the B cell. It is the first signal for the activation of the B cell. The second signal is the ligation of the CD40 receptor on the T cell to the B cell.[57]

Proliferation and differentiation of the activated B cell

After their activation, B cells migrate into follicles of the secondary lymphoid organs and proliferate. They build the so-called germinal center in the middle of the follicle. Then, they undergo rounds of high affinity selection. The activated B cells whether differentiate to plasma cells and leave the lymphoid organs or differentiate to memory B cells, where they wait to be reactivated when the same pathogen attacks.[57]

1.3.2 B cell receptor/Antibody diversity

Antibodies (i.e. soluble B cell receptors) are formed of two identical light chains (25 kDa) and two heavy chains (75 kDa) in an epsilon shape (IgG). Each chain has a variable and a constant region. The variable region is responsible for the antigen binding. Therefore, it needs to bind pathogens with best possible affinity without being auto reactive. The constant region of the

heavy chain is responsible for the functions of the antibody. There exist five different types of constant regions. Moreover, there are two kinds of light chains: kappa κ and lambda λ. The normal Kappa κ/lambda λ ratio is approximately 2 to 1 and can range from 0.26 to 1.65.[60]

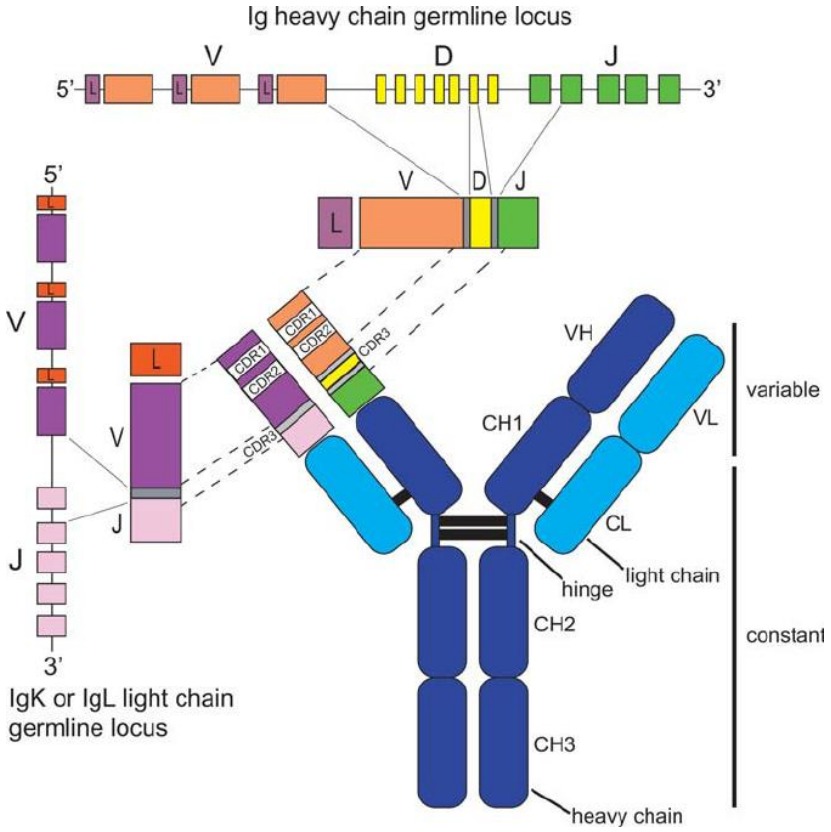


Figure 4. Structure of an IgG antibody

The antibody has an epsilon shape consisting of a light chain (light blue) and heavy chain (dark blue). The antigen-binding part (variable region) has the most variable structure, a result of the VDJ-recombination. The CDR3 region contains parts of the V-, D-, and J-gene and is therefore the most variable within the variable region. The leader region (L) is removed after translation. This figure is based on Boyd et al. (2014)[61] and used with kind permission of American Society for Microbiology – Journals.

Countless different antibodies (i.e., soluble B cell receptors) are created. Different processes contribute to the diversity: i) somatic recombination, ii) junctional diversification, iii) somatic hypermutation, and iv) affinity maturation.[62]

Somatic recombination and junctional diversification take place early in the development of the B cell in the bone marrow. These processes are antigen independent and randomly. During somatic recombination, a selection from gene segments is joined together in order to form a functional gene. This process is also called V(D)J recombination, referring to the segments which are joined together. For the heavy chain, there are 56 V, 23 D, and 6 J genes encoded. For the light chain, there are kappa and lambda as different entities. Kappa has 41 V and 5 J genes, lambda has 33 V and 5 J genes.[63] One of each gene is coincidental chosen and combined randomly, creating a vast diversity of heavy and light chains. Combined with the

combination of heavy and light chains, over 1.9 billion possible different antibodies are created.[57,62]

Junctional diversification describes the process of improper joining of gene segments. In this inaccurate process, enzymes like RAG and TdT fill up the gaps between the different gene segments. Exonucleases can lead to deletions at the junction. Junctional diversification adds up to over 10^{12} possible combinations.[62]

The modified recombined DNA is then transcribed and translated into proteins, followed by the formation of the antibody. On structural level, the variable region forms three complementary regions (CDR), which are located at the tip of the antibody and contribute strongly to the antigen-binding. The CDRs are flanked by framework regions (FWR), which contribute to the stability of the antibody and work as a frame. The CDR 1 and 2 are encoded in the V-segment, while the CDR 3 is encoded by a part of all three gene segments (subsequently two for light chains) and is therefore the most variable region.[57]

After the B cell is activated by antigens and T cells, somatic hypermutation and affinity maturation in the secondary lymphoid organs take place. Somatic hypermutation is created by the enzyme activation-induced deaminase (AID). This enzyme introduces deletions, insertions and substitutions into the variable region.[64] Mostly, these mutations are located in CDRs, which mainly contribute to antigen binding. However, it was found that mutations introduced in FWRs also influence antigen binding.[65] A region which is less mutated is the leader region. Located in front of the variable gene, it is responsible for heavy and light chain transportation. After translation, it is cleaved and discarded. Because of its tendency to be less mutated and the lack of encoding for the variable region, it is well suitable for primer strategies for antibody amplification.

After the first round of somatic hypermutation, affinity maturation begins. B cells with displayed B cell receptors are selected for affinity towards the antigen. The B cells with reduced affinity go into apoptosis, while the B cells with increased affinity undergo another round of somatic hypermutation. After several rounds of mutation and selection, the lymphocytes develop into plasma- or memory B cells.[57]

In summary, the diversification process combining somatic recombination, junctional diversification, and somatic hypermutation create an almost infinite number of possible distinct antibodies.

At last, the class of antibody is changed to become more adapted to the type of pathogen. This process is called isotype switching. It does not change the antigen binding site but only the constant region of the antibody.

1.3.3 Antibody mediated effector functions

The immune system combats infections through two primary mechanisms: the innate and adaptive immune response. The innate immune response serves as the first line of defense, comprising physical and chemical barriers, an inflammatory response, the attack by natural killer cells and phagocytes, and the activation of complement proteins. The adaptive immune response serves as a second line of defense. It is more specific but takes hours to days to develop. Cytotoxic T cells attack and destroy virus-infected cells, while activated T helper cells initiate the B cell maturation as described in Chapter 1.3.1. Mature B cells produce specialized antibodies to counteract pathogens through direct neutralization and Fc-mediated effector function.

Antibodies neutralize pathogens by binding to them, thus blocking their entry into host cells. The variable region of the antibody attaches to epitopes on the virus surface, preventing the virus binding to the host cell's receptor and infecting the host cell.[66] Additionally, the Fc region of the antibody primes the immune system against the pathogen through binding to the Fc receptors of immune cells and triggering Fc-mediated effector functions, such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent complement deposition (ADCD).[67]

Antibody-dependent cellular cytotoxicity is driven by the activation of natural killer cells through the engagement with their Fc receptor FcγRIIIa. The natural killer cells release the cytokines perforin and granzyme B, which causes the target cell to lyse and die.[67] Monocytes, macrophages and neutrophils engage in antibody-dependent cellular phagocytosis by binding through the Fc receptors FcγRIa and FcγRIIa and engulfing immune complex-opsonized cells.[68] Furthermore, the antigen:antibody complex activates the complement cascade, promoting antibody-dependent complement deposition. The binding of an antibody to an antigen triggers a conformational change, making the Fc region accessible to C1q, the initial protein of the complement cascade. This activation leads to classical pathway of complement activation, resulting in the formation of a membrane attack complex that lyses the pathogen.[57]

1.3.4 Antibody response against HIV-1

Soon after the infection with HIV-1, antibodies against the viral proteins Env, Pol and Gag. Screening on such antibodies can be used for diagnostical reasons, however, the elicited antibodies do not prevent infection. By standard ELISA an HIV-1 specific antibody response is detected on average after 22 days after infection.[69]

Tomaras et al. examined the HIV-1 specific antibody response more closely.[70] The earliest antibody response is found after eight days in shape of antibodies bound to the virion (immunocomplexes). Five days later, soluble gp41 antibodies are detected and after 27 days gp120 antibodies. These antibodies are mostly V3 and CD4 binding site antibodies. They are yet non-neutralizing and have little effect on plasma viraemia.[70]

Different mechanisms have been discussed why the early elicited HIV-1 antibodies cannot neutralize the virus. Some antibodies target mostly variable regions, which mutate quickly, while other antibodies target structures that are not present on the virion (e.g., precursor glycoproteins).[71] Still, non-neutralizing antibodies are discussed to contribute to the protective effect of the vaccine trial RV144 through their Fc mediated effector functions e.g. antibody-dependent cellular cytotoxicity.[72]

After some months, strain specific neutralizing antibodies develop, targeting the more variable regions on HIV-1. Because of the rapid turn-over of the virus and its penchant for mutations, escape mutants occur quickly, and the virus becomes resistant. Over several years of chronic infection and co-evolution, 10–30% of infected individuals develop some level of HIV-1 neutralizing cross clade serum activity.[73,74]

1.4 Broadly neutralizing antibodies targeting HIV-1

1.4.1 Discovery of bNAbs

In 1991, ten years after the first observed clinical case of AIDS, neutralizing antibodies (nAbs) from HIV-1 infected individuals could be isolated. These nAbs were produced using phage libraries and hybridomas. However, these first generation antibodies were lacking breadth and potency, or were auto reactive.[75–77] The techniques were cost and labor intensive and suppression of viremia could not be reached. The idea of human antibodies for therapy, prevention, or cure, was therefore abandoned. Around 2009, two milestones fueled the generation of broad neutralizing antibodies. A standardized neutralization panel allowed a high through put screening of sera from infected individuals and new single cell methods enabled an efficient generation of HIV-1 specific antibodies from memory B cells.[78–81] Then, the

generation of a stable HIV-1 Env trimer enabled the selection for antibody binding epitopes in the natural quaternary structure.[82] Since then, strategies for bNAb generation have been optimized and subsequently, hundreds of antibodies targeting the HIV-1 Env protein with improved breadth and neutralizing potency could be identified.[83–89]

1.4.2 Characteristics of bNAbs

Approximately 10–30% of HIV-1 infected individuals develop neutralizing heterologous activity 2–4 years after infection.[90] In 1% of HIV-1 infected individuals serum neutralizing is exceptional broad and potent.[91] They are called 'elite neutralizer'. An elite serum neutralization activity is closely linked to the development of bNAbs. Factors which are discussed to favor the development of bNAbs are: high viral loads, untreated infection, ethnicity, and potentially sex.[92–94] However, also in long-term non progressors with undetectable viral loads, neutralizing activity could be detected.[95]

BNAbs share specific characteristics. An elevated level of somatic hypermutations not only in the CDRs but also in the FWRs, an uncommonly long or short CDR3, the presence of insertions and deletions, and sometimes a higher level of polyreactivity can be found.[90] On structural base, protruding antibody loops are favored.[96] Some bNAbs classes spawn more potent and broader bNAbs than another (see chapter 1.4.4). However, little is known how bNAbs can be induced.

1.4.3 HIV-1 reactive B cell repertoire of elite neutralizer

Antigen-specific memory B cells normally account about 0.5% of the B cell repertoire.[83] How the HIV-1 specific B cell repertoire of some individuals becomes broad and potent and in others not, is still subject of ongoing research. However, eliciting antibodies targeting bNAb epitopes is seen to be the key for the development of a successful vaccine.

When it was investigated which factors can contribute to development of bNAbs, it was found that the viral strain of infection seems to play an important role. In non-human primates infected with SHIV carrying Env of the founder virus by elite neutralizers, co-evolutions between bNAb and virus could be mimicked.[97] Preexisting germline determinants again do not seem to play an important role.[98] However, preexisting long CDR3s and high somatic hypermutation rates in the B cell repertoire are correlated with neutralization breadth.[99]

1.4.4 Classes of broadly neutralizing antibodies targeting HIV-1

Many different bNAb epitopes have been discovered after the development of new antibody cloning techniques. They define the classes in which bNAbs are divided. Status 2022, there are six different known epitopes on the HIV-1 envelope protein plus multispecific engineered bNAbs. Namely the epitopes are the CD4 binding site (CD4bs), the V1/V2 loop, the V3 loop, the gp120/gp41 interface, the silent face, and the membrane proximal external region (MPER).[100]

The CD4 binding site describes the site where the virus attaches to the CD4 receptor of the T cell. This site is crucial for viral entry. Therefore, the sequence encoding for the CD4 binding site is relatively conserved compared to other regions of the Env protein.[84] Thus, the CD4 binding site is a favorable target.

CD4 binding site antibodies mimic the CD4 receptor and are V_H gene 1-2 or V_H gene 1-46 derived. They have the highest levels of somatic hypermutations. The CD4bs is recessed, therefore referred sometimes as ‘canyon’, and surrounded by glycans, which can shield the CD4 binding site against antibodies. For entering the CD4bs, corresponding bNAbs developed different structural features. The most potent CD4bs antibodies are VRC01-like bNAbs, which are V_H gene 1-2 derived and use a short five-residue light chain to bind the CD4bs surrounding glycans. Steric clashes are avoided by the short structure. N6 and N49P7 are the most potent in this class with near pan-strain-neutralizing activity (98–100%) and a potency of IC_{50} 0,1 µg/ml. N6 and N49P7 bind in special angle, which allows them to enter the canyon effectively. They have extensive mutations in the CDRH2 which buries a large surface area of gp120.[84] These explicit structural requirements could be the reason, why CD4 binding site bNAbs develop after a longer period of time than other classes of bNAbs.

V_H gene 1-46 derived antibodies as CH235.12 and 8ANC131 lack the five-residue light chain. CH235.12 derived in an individual where bNAbs maturation could be followed. It was seen that the maturation mode was driven by somatic hypermutation, resulting in more specific contacts with the CD4bs. V_H gene 1-46 derived antibodies are not that breadth and potent as the V_H1-2 derived antibodies.[100] Nevertheless, CD4bs bNAbs have the best combinations of breadth and potency compared to other classes of bNAbs.

V2 apex antibodies as CAP256VRC26.25, PGDM1400 and PG9 target the apex of the HIV-1 envelope protein. Typical for those bNAbs is the long CDRH3 region (≥ 24 residues), with which they penetrate the glycan shield. Insertions or deletions are not typical for V2 apex

bNAbs.[81,100,101] They belong to the most potent bNAbs (IC_{50} < 0.004 ug/ml) but have limited breadth (< 84%).[100]

V3 loop antibodies as 10-1074 and PGT121 target the V3 loop by approaching the N332 glycan on the V3 loop from different angles. Their neutralizing effect results from blocking the binding to the CD4 receptor.[102] The potency of the V3 loop antibodies falls within the middle range (IC_{50} of ~ 0.4 µg/ml) with relatively low breadth (~ 65%). They have insertions and deletions in the V-region and a long CDRH3. An exception is the bNAb BG18, which does not express insertions or deletions and therefore is a more favorable target for immunogen design.[100]

Most bNAbs targeting the MPER epitope are poly- or autoreactive, which are unfavorable features for therapy or prevention approaches. Some exceptions exist; for example, 10E8 and the newer MPER bNAbs as VRC42, PGZL1, LN01, DH511.2_K3 lack poly- or autoreactivity. The potency of the MPER bNAbs is limited, with 10E8 being the most potent, having an IC_{50} of ~ 0.299 µg/ml.[100,103,104]

BNAbs targeting the gp120/41 region include 8ANC195, PGT151, and 35O22. Some bNAbs are additionally reactive to the fusion peptide as VRC34. The fusion peptide plays a crucial role in HIV-1 entry as it inserts into the host cell membrane and opens up the way for transporting the virion into the host cell.[100] Potency and breadth of bNAbs targeting gp120/41 fall within in the middle range.

The so called ‘silent face’ is the most recent discovered epitope on the Env protein. VRC-PG505 and SF12 are targeting this epitope. SF12 has a coverage about 63% and potency about 0.221 µg/ml.[89,100]

Another approach includes the design of multispecific bNAbs, which target different sites at the same time. An example is the CrossMab 10E8.4/iMab, which is a combination of the MPER antibody 10E8 and ibalizumab. Ibalizumab is a monoclonal antibody targeting the CD4 receptor on T cells. This combination of blocking virus and CD4 receptor can extend the potency to an IC_{50} of 0.002 µg/mL and breadth larger than 97% in the 118-virus panel. Engineered antibodies like 10E84/iMab, though, have additional problems in comparison to native antibodies, such as aggregation instability due to their development process.[105]

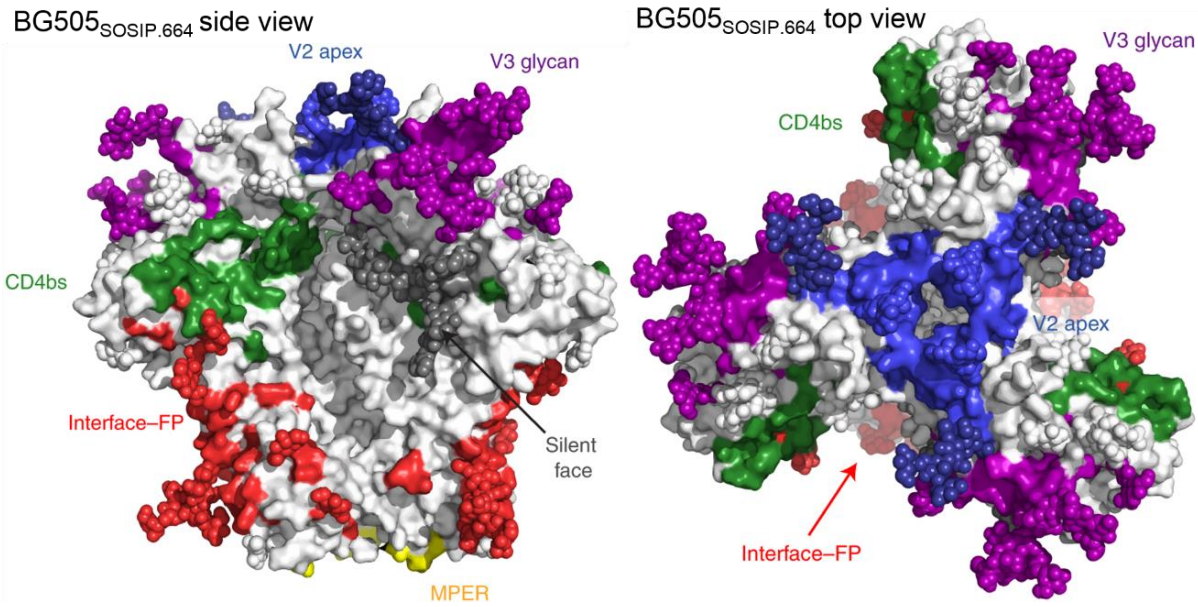


Figure 5. BNAb epitopes on the HIV-1 Env trimer

Colors indicate different epitopes. Blue: V1/V2 loop, purple: V3 loop, green: CD4bs, dark gray: silent face, red: gp120/gp41 interface, and yellow: MPER. As HIV-1 Env trimer a gp140 trimer from the HIV isolate BG505 is used. This modified graphic is based on Sok et al. (2018)[100], used with kind permission of Springer Nature.

1.4.5 bNAbs in therapy

Antibody therapies have revolutionized clinical therapies, especially in the field of hemato-oncologic diseases, and enabled the use of personalized medicine. Also, in the field of infectious diseases, antibody therapies have a great utility, as shown against the Ebola virus or against SARS-CoV2. Regarding HIV-1, the idea of using neutralizing antibodies had been abandoned for many years due to the lack of breadth and potency of the antibodies that were discovered. The discovery of broader and more potent second generation bNAbs could renew the hopes in using antibodies for HIV-1 therapy.

When the second generation bNAbs were evaluated in *in vivo* models, promising results could be obtained. At first, bNAbs were tested in humanized mice which are mice with depleted murine immune system and newly installed human immune system. Single bNAb studies showed that bNAb infusion in infected humanized mice lead to viral decline but ultimately rebounded. Combination bNAb studies, though, showed that the application of two bNAbs lead to sustained viral suppression up to 60 days.[106] This proof-of-principle advanced to *in vivo* studies in non-human primates (NHP), infected with chimeric human and simian SHIV. Here, as well, it could be shown that combination therapies lead to prolonged viral decline. For example, a single injection of a combination with PGT121, 3BNC117 and b12 lead to a decline up to 3.1 log of the plasma virus and had a median viral suppression of 56 days.[107] Finally, bNAbs had been tested in HIV+/- individuals and advanced from phase I to phase III studies,

showing preferable safety, pharmacokinetic and tolerability features.[108–111] While in HIV-1 infected individuals treated with the combination of PGDM1400, PGT121, and VRC07.523LS viral rebound occurred after a median of 20 days, viral suppression under 3BNC117 and 10-1074 sustained longer, in individuals with sensitive virus up to 20 weeks and more.[112] The authors assume that PGDM1400 and PGT121, as V1/V2 loop antibody and V3 loop antibody, respectively, are more susceptible to escape mutations than CD4bs antibodies.[113,114] Therefore, recent bNAb combination therapies are effective but limited by the development of escape mutations.

Effects from bNAbs

Therapeutic effects of bNAbs are mediated through Fab fragment and Fc effector mechanisms. By binding of the HIV Env of the Fab fragment, the virus cannot attach to the host membrane and viral entry is blocked. The Fc part of the antibody enhances defense functions of the immune system as described in Chapter 1.3.5. Studies with site-directed knock out mutations in the Fc region of CD4bs and V1/V2 bNAbs suggest that Fc-receptor mediated functions contribute 25–45% to the neutralization activity *in vivo*, however, depletion of NK cells, which play a major role in Fc effector functions, did not decrease the protective effect of PGT121 in macaques.[115,116] This data suggests that Fc effector functions might differ between bNAb classes. Whether Fc engineered antibodies can enhance the immune stimulation needs to be investigated further.

BNAbs can be modified to increase their half-life. One common mutation is the ‘LS’ variant, where two amino acids in the Fc region are exchanged and therefore reduce antibody degradation. With this modification, the half-life of bNAb VRC01 could be extended to 10 weeks.[109] Another approach is to design bispecific or trispecific bNAbs, which cover different epitopes on Env at the same time. Those approaches held promising results in *in vitro* testing and humanized mice. Recently, a trial, testing a trispecific antibody with fragments of VRC01, PGDM1400, and 10E8, is recruiting (NCT03705169), and the results are expected.

1.4.6 bNAbs in prevention

There have been over 100 vaccine trials, but the RV144 trial in Thailand was the only study which offered partwise success (reduction of the infection rate by 32.5%). However, an imitation study failed. The study was repeated in South Africa and had to be disrupted due ineffectiveness.[117,118] To date, no effective vaccine against HIV-1 infection exist.

One novel approach for prevention is the passive administration of bNAbs. BNAbs have shown to be protective against infection in monkeys if administered passively.[119,120] In 2016 to 2021,

an Antibody-mediated prevention study (AMP study) with the bNAb VRC01, including over 4,600 participants, was conducted to evaluate if HIV acquisition can be prevented by intravenous application of the bNAb VRC01. It could be shown, that the repeatedly administration of an intravenous infusion with VRC01 was safe and could prevent acquisition of susceptible HIV-1 strains in 75% of cases. However, in total, the HIV-1 acquisition in comparison to the placebo was not significantly reduced. A possible explanation could be the rate low rate of susceptible strains to VRC01 in the cohort (around 30%). These results show that passive administration of bNAbs is a possible key concept for prevention strategies, but novel and broader bNAbs are needed for combination therapies to address a wider range of viral strains.[121]

Other, more durable approaches include self-induction of bNAbs, for example, through vaccination. One approach is injecting modified immunogens or native-like glycosylated Env trimers. A study showed that by sequential injection of an epitope-modified immunogen in knock-in mice, expressing precursor germline PGT121 B cells could create neutralizing antibodies.[122] Clinical trials are ongoing, and results still need to be awaited.

Another strategy used well-established adeno associated viral vectors (AAV) to introduce gene-mediated antibody production. While in humanized mice and non-human primate models (NHP) promising results were shown, results in clinical trials were inconsistent. A trial with a AAV to induce PG19 could indeed show that the application was safe and well tolerated, but no serum concentration of PG19 was found. A clinical trial assessing AAV delivery of VRC07 could show that the production of VRC07 could be induced. The concentration was rather low at ~ 1 µg/ml, while in most clinical studies an antibody serum concentration at ~ 50 µg/ml is needed for antiviral effects. Moreover, three of eight participants developed antidrug antibodies, which inhibit antibody production.[111,123]

Although first results from clinical studies using bNAbs are promising, viral escape remains a significant challenge. Therefore, it is critical to identify bNAbs with high potency and breath, targeting new epitopes to prevent viral escape.

1.5 Aim of this thesis

In this thesis, the overall goal was to find novel broad and neutralizing antibodies targeting HIV-1 by investigating the immunological answer of an elite neutralizer. To this end, I performed a single B cell analysis of Elite neutralizer IDC561, an HIV-1 infected subject with exceptional neutralizing activity. After production of monoclonal HIV-1 specific antibodies of Elite neutralizer IDC561, I characterized isolated HIV-1 reactive antibodies *in vitro* for their binding capacity and neutralization activity. Identified promising HIV-1 reactive antibodies were

subjected to in-detail sequence analysis and tested for correlation with IDC561s serum neutralization activity.

2 Materials and Methods

2.1 Vote of the Ethics Committee

This project has a positive vote of the faculty of medicines Ethics Committee. Human samples were obtained under the protocols 13-364 and 16-054, approved by the Institutional Review Board of the University of Cologne.[88]

2.2 Material

Item	Manufacturer/ Composition	Source
Consumables		
96 well PCR plate, semi skirted	PEQlab/VWR	#81-35899
Alum plate cover foil	PEQlab/VWR	#82-0626-A
Amicon 30 kDa spin membranes	Millipore	UFC803024
Suspension Culture Dish 60mm Non-treated Polystren	Corning	430589
TPP Tissue Culture Dishes 150 mm, TC-treated	SRM	TPP93150
High binding ELISA plates, 96 wells	Corning	9018
Corning® 96 Well Clear Flat Bottom	Sigma Aldrich	CLS3628
Corning® 96 Well Black Flat Bottom	Sigma Aldrich	CLS3915
TC Flask T75,Cell+,Vented Cap	Sarstedt	833911302
Chemicals, reagents, recombinant proteins		
RNaseOUT	Promega	#10777-019
dNTPs, 25 mM each	Thermo Fisher	#R1122
RNasin	Promega	#N2515
RT Buffer (5x)	Invitrogen, Superscript IV Kit	#18090050
DTT 100 mM	Promega	#P1171
Reverse Transcriptase	Invitrogen, Superscript IV Kit	#18090050
Nuclease-free water, not DEPC treated	Thermo, Ambion	#AM9937
Random Hexamer Primer	Thermo Fisher	#S0142
NP-40	Thermo Fisher	#85124
10X PCR Buffer (-mg)	Thermo Fisher	#10966018
10X Green PCR Buffer (-mg)	Thermo Fisher	#11966034
50 mM magnesium chlorid	Thermo Fisher	#10966018
KB Extender	Thermo Fisher	#10966018
Platinum Taq DNA Polymerase	Thermo Fisher	#10966018
Agarose	AppliChem	#A8963, 0500
SYBR Safe	Invitrogen	#S33102
1 kb plus ladder	Thermo Fisher	#SM1331
5xPCR Buffer	NEB	NEB#B9027S
5x Q5 High GC Enhancer	NEB	NEB#M0493L
Q5 Hot Start High Fidelity DNA Polymerase	NEB	NEB#M0493L
Human antibody expression vector pIgH	Tiller et al., 2008	N/A
Human antibody expression vector pIgK	Tiller et al., 2008	N/A
Human antibody expression vector pIgL	Tiller et al., 2008	N/A
AgeI-HF	NEB	NEB#R3552L, 20000 U/ml
SalI-HF	NEB	NEB#R3138L, 20000 U/ml
BsiWI-HF	NEB	NEB#R3553L, 20000 U/ml
XhoI	NEB	NEB#R0146S, 20000 U/ml
10x CutSmart	NEB	NEB#B6004S
10x NEBuffer 2.1	NEB	NEB#M0203S
T4 DNA Polymerase	NEB	NEB#M0203S
NaCl	Carl Roth	7647-14-5
Branched polyethylenimine (PEI) 25 kDa	Sigma-Aldrich	9002-98-6
Protein G Sepharose 4 Fast Flow	GE Life Sciences	#17061805
0.1 M glycine (pH= 3.0)	Thermo Fisher	A13816.0E

1 M Tris (pH=8.0)	Thermo Fisher	AM9855G
$BG505_{SOSIP.664}$	J.P. Moore, Weill Cornell Medical College; Sanders et al., 2013	N/A
$YU2_{gp140}$ (fold-on trimer)	R. Wyatt, The Scripps Research Institute; Yang et al., 2000	N/A
Tris base	Carl Roth	77-86-1
acetic acetate	Carl Roth	64-19-7
EDTA	Carl Roth	6381-92-6
SOC medium	Thermo Fisher	15544034
Free-Style 293 Expression Medium	Thermo Fisher	#12338018
Penicillin/streptomycin	Thermo Fisher	15140122
Phosphate-buffered saline (PBS) pH 7.4	Thermo Fisher	10010056
MOPS	Carl Roth	1132-61-2
SDS	Carl Roth	151-21-3
InstantBlue™ Ultrafast Protein Stain	Sigma-Aldrich	ISB1L-1L
NaCl	Carl Roth	7647-14-5
Trypton	Carl Roth	91079-40-2
Yeast extract	Carl Roth	8013-01-2
Ampicillin	Thermo Fisher	11593027
Bovine Serum Albumine (BSA)	Sigma Aldrich	9048-46-8
Tween-20	Carl Roth	9005-64-5
ABTS Solution	Thermo Fisher	#002024
NuPAGE® LDS Sample Buffer (4X)	ThermoFisher	NP0007
DEAE-Dextran	Sigma Aldrich	D9885-10G
DMEM (w/o Glut/SP)	Gibco	11960-044
Gentamicin solution (50mg/ml)	Sigma Aldrich	G1397-10ML
Fetal bovine serum (FBS), inactivated	Sigma Aldrich	F4135
Sodium Pyruvate (100mM)	Gibco	11360-070
L-Glutamine (200mM)	Thermo-Fisher	25030024
HEPES (1M)	Biochrom	L1613
Trypsin-EDTA 0,25%	Gibco	25200056
FuGENE® 6 Transfection Reagent	Promega	E2691
L-Glutamine (200mM)	Thermo-Fisher	25030024
Antibiotic-Antimycotic (100 x)	Thermo Fisher	15240062
$MgCl_2$	Sigma Aldrich	M8266-100G
ATP	Sigma Aldrich	A2383- 10G
CoA	Sigma Aldrich	C4282-100MG
D-Luciferin, Sodium Salt	ZellBio/Goldbio	LUCNA-1G
IGEPAL (NP40)	Sigma Aldrich	I8896-50ML

Buffer, media and solutions	
1x TAE	40 mM Tris base, 20 mM acetate, 2mM EDTA ph8
20x MOPS SDS Running Buffer	50mM MOPS, 50mM Tris Base, 0.1% SDS, 1mM EDTA, pH 7.7
Lysogeny broth (LB) with Ampicillin (100 µg/ml)	50g NaCl, 50g Trypton, 50g yeast exstract in 5l VE-H20
Blocking buffer	3 % BSA in PBS
Washing buffer	0.05% Tween-20 in PBS
Growth medium 293E cells	FreeStyle293 Expression Medium DMEM, 0,2% Pen/Strep
Growth medium TZM.bl cells	10% heat-inactivated FBS, 2mM L-Glutamine, 1mM Sodium Pyruvate, 50µg gentamicin/ml and 25mM Hepes
Growth medium HEK293T cells	DMEM, 10% heat-inactivated FBS, 2mM L-Glutamine, 1mM Sodium Pyruvate, 1x Antibiotic/Antimycotic
Lyse/LUC-Mix	200mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 500µM CoA, 300µM ATP, 300µg/ml D-Luciferin (GoldBio)
Lysis Solution	Lysis Solution: 1% IGEPAL (NP40), 50 mM Tris-HCl, 30 mM NaCL, pH 7,7

Antibodies		
Monoclonal anti-HIV-1 Env 561_01_18	This thesis	N/A
Monoclonal anti-HIV-1 Env 561_01_55	This thesis	N/A

Monoclonal anti-HIV-1 Env 561_02_12	This thesis	N/A
Monoclonal anti-HIV-1 Env 1-18Δins	This thesis	N/A
Monoclonal anti-HIV-1 Env Patient IDC561 antibodies	This thesis	N/A
Monoclonal anti-HIV-1 Env 3BNC117	NIH Aids Reagent Program	Cat#12474; RRID: AB_2491033
Horseradish-peroxidase-conjugated anti-human IgG	Jackson ImmunoResearch	#109-035-098

Commercial kits		
GeneJET Gel Extraction and DNA Cleanup Micro Kit	ThermoFisher	# K0832
PureLink™ HiPure Plasmid Midiprep Kit	ThermoFisher	K210004
NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels	ThermoFisher	NP0322BOX
PageRuler™ Prestained Protein Ladder	ThermoFisher	26616

Cell lines, Bacteria and Viruses		
DH5α chemically competent cells (E.coli)	Thermo Fisher	EC0112
293-6E cells	NRC	NRC file 11565
HEK293T cells	ATCC	Cat#CRL-11268
TZM.bl cells	NIH AIDS Reagent Program; Platt et al.1998	Cat#8129
Global Panel: 12 HIV-1 Env-pseudotyped viruses	NIH Aids Reagent Program	Cat#12670
Cross Clade Panel: 119 HIV-1 pseudotyped viruses	M.S. Seaman, BIDMC; Seaman et al., 2010	N/A
Clade C Panel: 100 HIV-1 pseudotyped viruses	Hraber et al., 2017	N/A

2.3 Methods

2.3.1 Preparatory work

A cohort of 2,274 HIV-1 infected patients from Germany, Nepal and Tanzania was screened for their neutralizing activity against HIV-1 using a TZM.bl neutralization assay, testing isolated IgGs of these patients against a standardized panel of 12 HIV-1 strains.[88] This 'Global panel' expresses different envelope proteins and is therefore representative for most global HIV-1 strains. Around 13.1% of the screened patients showed broadly neutralizing activity, which qualified them as 'elite neutralizers'. One of these elite neutralizer is IDC561, ranking in the top 1% of the elite neutralizers (rank 22). IDC561 is a 48-year-old caucasian male, who was diagnosed with HIV-1 infection 21 years earlier, but controlled viremia for nearly 20 years.[88,124] After leukapheresis, peripheral blood mononuclear cells (PBMCs) were isolated from a blood sample through density-gradient centrifugation, followed by the separation of B cells. To isolate HIV-1 Env reactive B cells only, B cells were stained and sorted based on $CD19^{+}IgG^{+}DAPI^{-}$ criteria and a HIV-1 Env bait, either $BG505_{SOSIP.664}$-GFP^{+} or biotinylated $YU2_{gp140}{}^{+}$ labeled with Streptavidin-PE. Sorts were performed in 96-well plates for high throughput. For more detailed information about the preparatory work see corresponding paper.[88]

2.3.2 Single cell cDNA synthesis

For mRNA denaturation sorted B cells were incubated with a Random Hexamer Primer Mix consisting of 0.75 µl Random Hexamer Primer, 0.5 µl NP-40 and 0.15 µl RNaseOUT (40 U/µl) for 1 min at 65°C. Then, the plate was placed on ice for at least 2 min inducing primer binding. A Reverse Transcriptase Mix containing 3µl 5x RT Buffer, 0.5 µl dNTPs mix (25mM), 1µl DTT (100 mM), 2.05 µl RNase-free H_2O, 0.1 µl RNasin (40 U/µl), 0.1 µl RNaseOUT (40 U/µl) and 0.25 µl Reverse Transcriptase (Superscript IV, 200 U/µl) was added and incubated at room temperature for 10 to 15 minutes. Subsequently, the 96 well plates were incubated at 42°C, 25°C and 50°C each for 10 minutes, following 94°C for 5 minutes. Samples were processed right away or stored at −20°C for further use.

2.3.3 Single cell cloning nested/semi-nested PCR

Antibody sequences were amplified in two rounds. Products of the first round were used for SLIC-reaction (Sequence and Ligation Independent Cloning), second round products were sequenced by Sanger Sequencing and used for further sequence analysis. Heavy chains and light chains were amplified in separate reactions.

First round PCR amplification

For the 1st PCR 2954 µl of RNase free water, 412 µl of 10X PCR Buffer, 123.6 µl of 50 mM $MgCl_2$, 33 µl dNTPs (25 mM each), 16.5 µl of 5' Fw primers and 3' Rv primer each, 247 µl KB Extender and 16.5 µl Platinum Taq DNA Polymerase was mixed. With a multichannel pipette 38 µl of the 1st PCR mix was transferred into a 96 well PCR plate and 4 µl of cDNA was added. First-round PCR was run at 94°C for 2 minutes, following 50 cycles of 94°C for 30 seconds, 55°C for γ Opt5 primers, respectively 57°C for κ tiller primers or 60°C for λ tiller primers, and finally 72°C for 55 seconds.

Second round PCR amplification

For the 2nd PCR 1477 µl of RNase free water, 206 µl of 10X Green PCR Buffer, 61.8 µl of 50 mM $MgCl_2$, 16.5 µl dNTPs (25 mM each), 8.24 µl of 5' Fw primers and 3' Rv primer each, 123.5 µl KB Extender and Platinum Taq Green Hot Start DNA Polymerase were mixed. With a multichannel pipette 19 µl of the 2nd PCR mix was transferred into a 96 well PCR plate and 4 µl of 1st PCR amplimer was added. 2nd round PCR was run at 94°C for 2 minutes, followed by 50 cycles at 94°C for 30 seconds, 55°C for γ Opt5 primers, respectively 57°C for κ tiller primers or 60°C for λ tiller primers, and finally 72°C for 55 seconds.

2.3.4 Agarose gel electrophoresis

For checking the 2nd PCR product, agarose gel electrophoresis was performed. A 2% agarose gel was prepared with 5 µl SYBR Safe per 100ml gel. Eight microliters of the 2nd PCR product was loaded into the solidified gel, and 6 µl of GeneRuler 1 kb plus ladder was applied to the sides of each row to serve as a standard. Gel electrophoresis was run in 1x TAE Buffer at 182 V for approximately 20 minutes.

2.3.5 Sanger Sequencing

PCR products showing the correct band size on gel (~ 500 bp for heavy chains and ~ 450 bp for light chains) were sequenced by Sanger sequencing using the reverse primers of the 2nd PCR.

2.3.6 Antibody sequence analysis

The 2nd PCR product was sent for Sanger sequencing. First, antibody sequences were manually analyzed. This was conducted by (i) a quality check for stop codons, clean sequence output, a minimal length of 240 nts, (ii) annotation in IgBLAST[125], (iii) manual entry of V-, D-, J-gene, CDR3 amino acid sequence, percentage of identity to germline sequence, quality and special features like insertions and deletions and the bait, and (iv) assignment to clones

according to the retrieved information of V-, D-, J-gene, CDR3 sequence and length, and special features. At least one related light chain from each clone was duplicated according to protocol. Matching heavy and light chain sequence proceeded to third PCR. Later, manually performed antibody sequence analysis on plate 1–7 was completed by automatically sequence analysis from plate 8–14. Plate 1–7 was automatically rechecked as well. Automated sequence input was conducted as described in Schommers et al.[88]:

> *'Sequences with a mean Phred scoreR28 and a minimal length of 240 nucleotides were annotated with IgBLAST [125] and trimmed from framework region (FWR) 1 of the variable region to the end of the J gene. Base calls with a Phred score < 16 were masked and sequences with > 15 masked nucleotides, frameshifts, or stop codons were excluded from further analyses. To analyze the sequences for potential clonalities, all productive heavy chain sequences were grouped by identical V genes and the pairwise Levenshtein distance of their CDRH3s was determined. Individual sequences were grouped into clones when they shared the same V gene and had a minimal CDRH3 identity of 75%. After 10 rounds with a randomized input of sequences, the result that yielded the lowest number of unassigned (non-clonal) sequences was selected for further analyses. All clones were re-validated manually by the investigators in order to identify shared mutations. Sequences that were initially assigned to different clones but shared the same V-, D-, J-genes and amino acid and/or silent nucleotide mutations were subsequently grouped into subclones. Nucleotide sequence identity to germline was calculated using IgBLAST. The maximum-likelihood phylogenetic tree was calculated with PhyML with 1,000 bootstrap replicates (substitution model: general time reversible [GTR]; Geneious R10). [88,126] The best-scoring tree was rooted to IGHV1-46*01. Sequences were aligned using ClustalW (Geneious R10; cost matrix: BLOSUM; gap open cost: 10; gap extend cost: 0.1).'*

2.3.7 Antibody cloning

Third round PCR

After identifying clonal antibody sequences the respective 1st round PCR product was amplified. For the amplification PCR of one product, 24.1 µl RNase free H_2O, 10 µl 5x PCR Buffer, 0.4 µl 25 mM dNTP-Mix, 0.5 µl 50 µM forward primer, 0.5 µl 50 µM reverse primer, 10 µl Q5 High GC Enhancer and 0.5 µl Q5 Hot Start High Fidelity DNA Polymerase were mixed. Specific primers were individually chosen for the respective nucleotide sequence of the V- and J-region.[80] For the subsequent sequence ligase independent cloning, the primers contained the respective expression vector overhangs. After filling the 96 well plate with the respective mastermix, 4 µl of the template DNA was added and incubated at 98°C for 30 seconds,

following 35 rounds at 98°C for 10 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and finally were incubated for 2 minutes at 72°C. The PCR product was checked on a 2% agarose gel. PCR plates were sealed and stored at −80°C until further use.

Antibody expression vector preparation

Before cloning the 3rd round PCR product in the respective IgG1, kappa, or lambda expression vectors, human antibody expression vectors were cut and prepared.

For human IgG1 heavy chain expression vector:
10 µg of IgG1 vector DNA was digested with 5 µl AgeI-HF and 5 µl SalI-HF with 5µl 10x CutSmart in a final volume of RNase free H_2O of 50 µl at 37°C for 2 hours. Restriction enzymes were heat inactivated at 65°C for 20 minutes.

For human IgK light chain expression vector:
10 µg of IgG1 vector DNA was digested with 5 µl AgeI-HF and 5 µl BsiWI-HF with 5µl 10x CutSmart in a final volume of RNase free H_2O of 50 µl at 37°C for 2 hours.

For human IgL heavy chain expression vector:
10 µg of IgG1 vector DNA was digested with 5 µl AgeI-HF and 5 µl XhoI with 5µl 10x CutSmart in a final volume of RNase free H_2O of 50 µl at 37°C for 2 hours. Restriction enzymes were heat inactivated at 65°C for 20 minutes.

Plasmid purification

Cut plasmids were purified using the 'GeneJET Gel Extraction and DNA Cleanup Micro Kit' with the 'General clean up from enzymatic reactions protocol'. The DNA Purification Micro Column has a silica membrane that can bind DNA, while fragments and contaminants pass down the column. As preparatory step 40 ml Wash Buffer was diluted with 200 ml 96% Ethanol. Then, antibody expression vector reaction mixture, respectively, was adjusted to 200 µl with RNase free H_2O. 100 µl of Binding Buffer was added and mixed. 300 µl of 96% ethanol was added and mixed. The mixture was transferred into the DNA Purification Micro Column stuck into a collection tube. The column was centrifuged for 60 seconds at 14,000 G. The flow-through in the collection tube was discarded. Mixture was washed with 700 µl Wash Buffer, again centrifuged and the flow-through discarded. Washing step was repeated. The empty column was then centrifuged for an additional minute at 14,000 G to remove residual Wash buffer. Column was transferred into a 1.5 ml clean microcentrifuge tube. The cleaned vectors in the column were eluted. Therefore, 15 µl RNase free H_2O was placed in the center of the membrane of the column and centrifuged for one minute at 14,000 G. Column was discarded.

The purified vectors in the microcentrifuge tube were adjusted to a concentration of 80 ng/µl using RNase free H_2O and stored at −20°C for further use.

Third PCR product preparation

Third round PCR products were thawed on ice. The PCR product was transferred into microcentrifuge tubes. The PCR product was cleaned up using the 'GeneJET Gel Extraction and DNA Cleanup Micro Kit' with the 'PCR cleanup, dimers removal protocol'.

As preparatory step 44 ml Prewash Buffer was diluted with 11 ml 96% Ethanol respectively 40 ml Wash Buffer and 200 ml 96% Ethanol. Then, the PCR product was adjusted to 200 µl with RNase free H_2O. 100 µl of Binding Buffer was added and mixed. 300 µl of Binding Buffer was added and mixed. The mixture was transferred into the DNA Purification Micro Column stuck into a collection tube. The column was centrifuged for 60 seconds at 14,000 G. The flow-through in the collection tube was discarded. 200 µl of Prewash Buffer was added to the PCR product and again centrifuged. The flow-through was discarded. Same step was repeated twice with 700 µl Wash Buffer, respectively. The empty column was then centrifuged for an additional minute at 14,000 G to remove residual Wash buffer. Column was transferred into a 1.5 ml clean microcentrifuge tube. The cleaned PCR product in the column was eluted by placing 11 µl RNase free H_2O in the center of the membrane of the column and centrifuging for one minute at 14,000 G. The column was discarded. The cleaned PCR products were transferred into a 96 well plate and diluted with RNase free H_2O to reach a concentration of approximately 20 ng/µl. Plates with cleaned PCR products were sealed and stored at −20°C until further use.

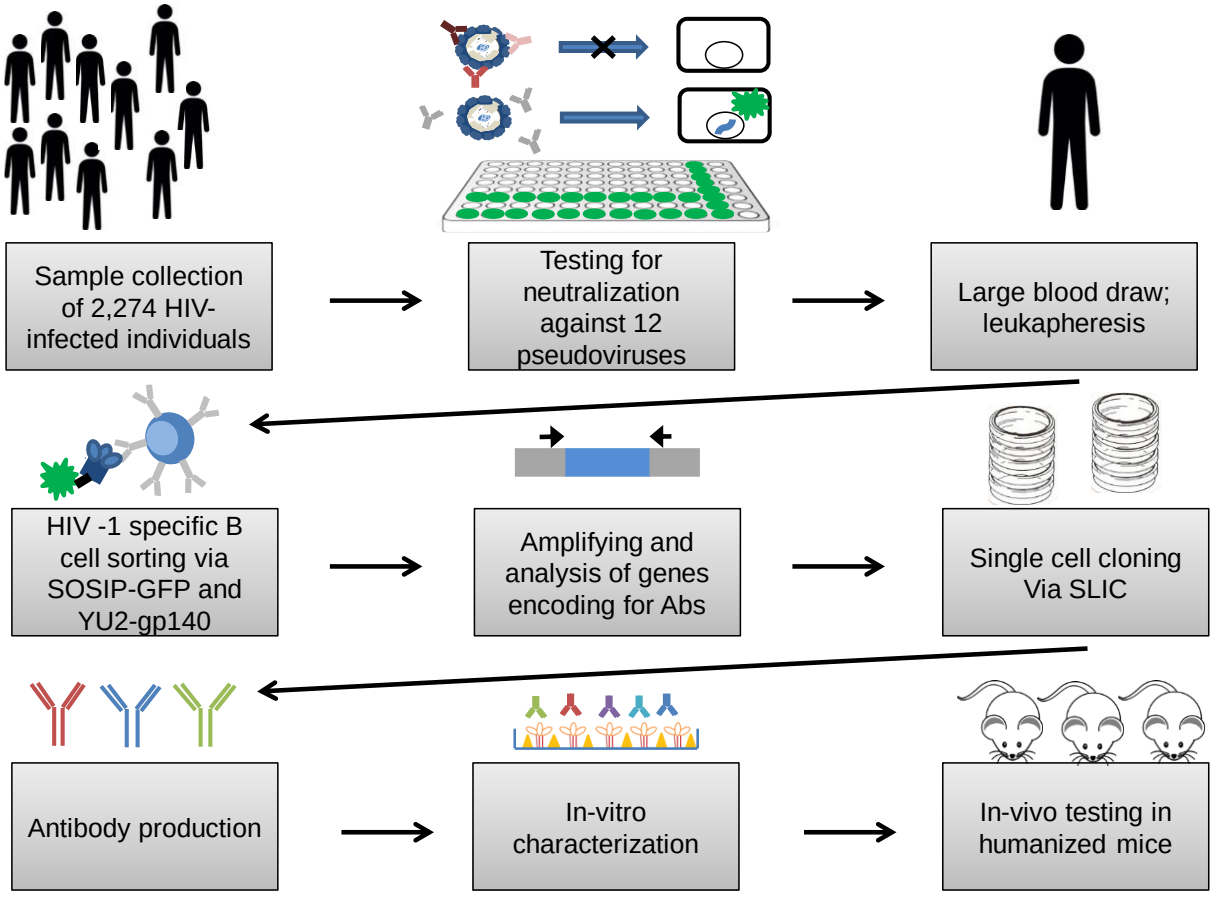


Figure 6. A pipeline for identification of new broadly neutralizing antibodies

After screening for elite neutralizers with exceptional cross-clade neutralization activity and neutralization breath, we will analyze the HIV-1 specific B cell response of those. This will reveal new broadly neutralizing antibodies targeting new epitopes.

Table 1. Primers used for cloning heavy, kappa, and lambda variable genes

Restriction sites are underlined.

		Name	Sequence	Reference
1. PCR HC	FW Opt5 Mix 1st	IGHV_Opt5_1_fw	CACCTGTGGTTCTTCCTCCTCC	Kreer et al., 2019
		IGHV_Opt5_2_fw	CACCTGTGGTTCTTCCTCCTGC	Kreer et al., 2019
		IGHV_Opt5_3_fw	ATGGAGTTTGGGCTGAGCTGG	Kreer et al., 2019
		IGHV_Opt5_4_fw	ATGGAGTTGGGGCTGAGCTG	Kreer et al., 2019
		IGHV_Opt5_5_fw	TGGAGTTTTGGCTGAGCTGGG	Kreer et al., 2019
		IGHV_Opt5_6_fw	ACTTTGCTCCACGCTCCTGC	Kreer et al., 2019
		IGHV_Opt5_7_fw	ATGGACTGGACCTGGAGCATC	Kreer et al., 2019
		IGHV_Opt5_8_fw	ATGGACTGGACCTGGAGGTTCC	Kreer et al., 2019
		IGHV_Opt5_9_fw	ATGGACTGCACCTGGAGGATC	Kreer et al., 2019
		IGHV_Opt5_10_fw	ATGGACTGGACCTGGAGGGTCTTC	Kreer et al., 2019
		IGHV_Opt5_11_fw	TCTGTCTCCTTCCTCATCTTCCTGC	Kreer et al., 2019
		IGHV_Opt5_12_fw	GGACTGGATTTGGAGGGTCCTCTTC	Kreer et al., 2019
		IGHV_Opt5_13_fw	GCTCCGCTGGGTTTTCCTTG	Kreer et al., 2019
		IGHV_Opt5_14_fw	TGGGGTCAACCGCCATCC	Kreer et al., 2019
		IGHV_Opt5_15_fw	GGCCTCTCCACTTAAACCCAGG	Kreer et al., 2019
		IGHV_Opt5_16_fw	TGGACACACTTTGCTACACACTCC	Kreer et al., 2019
	RV	3' Ozawa_cg_rt	AGGTGTGCACGCCGCTGGTC	Ozawa et al., 2006

1.PCR LCK	FW 5'LVk Mix	5′ L Vκ 1/2	ATGAGGSTCCCYGCTCAGCTGCTGG	Tiller et al., 2008
		5′ L Vκ 3	CTCTTCCTCCTGCTACTCTGGCTCCCAG	Tiller et al., 2008
		5′ L Vκ 4	ATTTCTCTGTTGCTCTGGATCTCTG	Tiller et al., 2008
	RV	3'Ck543	GTTTCTCGTAGTCTGCTTTGCTCA	Tiller et al., 2008

1.PCR LCL	FW 5'LVl Mix	5′ L Vλ 1	GGTCCTGGGCCCAGTCTGTGCTG	Tiller et al., 2008
		5′ L Vλ 2	GGTCCTGGGCCCAGTCTGCCCTG	Tiller et al., 2008
		5′ L Vλ 3	GCTCTGTGACCTCCTATGAGCTG	Tiller et al., 2008
		5′ L Vλ 4/5	GGTCTCTCTCSCAGCYTGTGCTG	Tiller et al., 2008
		5′ L Vλ 6	GTTCTTGGGCCAATTTTATGCTG	Tiller et al., 2008
		5′ L Vλ 7	GGTCCAATTCYCAGGCTGTGGTG	Tiller et al., 2008
		5′ L Vλ 8	GAGTGGATTCTCAGACTGTGGTG	Tiller et al., 2008
	RV	3′ Cλ	CACCAGTGTGGCCTTGTTGGCTTG	Tiller et al., 2008

2.PCR HC	FW Opt5 Mix 2nd	IGHV_Opt5_1_fw	CACCTGTGGTTCTTCCTCCTCC---	Kreer et al., 2019
		IGHV_Opt5_2_fw	CACCTGTGGTTCTTCCTCCTGC---	Kreer et al., 2019
		IGHV_Opt5_3_fw	ATGGAGTTTGGGCTGAGCTGG----	Kreer et al., 2019
		IGHV_Opt5_4_fw	ATGGAGTTGGGGCTGAGCTG-----	Kreer et al., 2019
		IGHV_Opt5_5_fw	TGGAGTTTTGGCTGAGCTGGG----	Kreer et al., 2019
		IGHV_Opt5_6_fw	ACTTTGCTCCACGCTCCTGC-----	Kreer et al., 2019
		IGHV_Opt5_7_fw	ATGGACTGGACCTGGAGCATC----	Kreer et al., 2019
		IGHV_Opt5_8_fw	ATGGACTGGACCTGGAGGTTCC---	Kreer et al., 2019
		IGHV_Opt5_9_fw	ATGGACTGCACCTGGAGGATC----	Kreer et al., 2019
		IGHV_Opt5_10_fw	ATGGACTGGACCTGGAGGGTCTTC-	Kreer et al., 2019
		IGHV_Opt5_11_fw	TCTGTCTCCTTCCTCATCTTCCTGC	Kreer et al., 2019
		IGHV_Opt5_12_fw	GGACTGGATTTGGAGGGTCCTCTTC	Kreer et al., 2019
		IGHV_Opt5_13_fw	GCTCCGCTGGGTTTTCCTTG-----	Kreer et al., 2019
		IGHV_Opt5_14_fw	TGGGGTCAACCGCCATCC-------	Kreer et al., 2019
		IGHV_Opt5_15_fw	GGCCTCTCCACTTAAACCCAGG---	Kreer et al., 2019
		IGHV_Opt5_16_fw	TGGACACACTTTGCTACACACTCC-	Kreer et al., 2019
	RV	3′ IgG (internal)	GTTCGGGGAAGTAGTCCTTGAC	Tiller et al., 2008
	Seq. Primer	3′ IgG (internal)	GTTCGGGGAAGTAGTCCTTGAC	Tiller et al., 2008

2.PCR LCK	FW	5′ Pan Vκ	ATGACCCAGWCTCCABYCWCCCTG	Tiller et al., 2008
	RV	3′ Cκ 494	GTGCTGTCCTTGCTGTCCTGCT	Tiller et al., 2008
	Seq. Primer	3′ Cκ 494	GTGCTGTCCTTGCTGTCCTGCT	Tiller et al., 2008

2.PCR LCL	FW 5'LV1 Mix	5′ L Vλ 1	GGTCCTGGGCCCAGTCTGTGCTG	Tiller et al., 2008
		5′ L Vλ 2	GGTCCTGGGCCCAGTCTGCCCTG	Tiller et al., 2008
		5′ L Vλ 3	GCTCTGTGACCTCCTATGAGCTG	Tiller et al., 2008
		5′ L Vλ 4/5	GGTCTCTCTCSCAGCYTGTGCTG	Tiller et al., 2008
		5′ L Vλ 6	GTTCTTGGGCCAATTTTATGCTG	Tiller et al., 2008
		5′ L Vλ 7	GGTCCAATTCYCAGGCTGTGGTG	Tiller et al., 2008
		5′ L Vλ 8	GAGTGGATTCTCAGACTGTGGTG	Tiller et al., 2008
	RV	3′ XhoI Cλ	CTCCTCACTCGAGGGYGGGAACAGAGTG	Tiller et al., 2008
	Seq.Primer	3′ XhoI Cλ	CTCCTCACTCGAGGGYGGGAACAGAGTG	Tiller et al., 2008

3.PCR HC	FW for pIgH Expression Vector	5´ AgeI SLIC VH 1	CTAGTAGCAACTGCAACCGGTGTACATTCCCAG GTG CAG CTG GTG CAG	Tiller et al., 2008
		5´ AgeI SLIC VH 1/5	CTAGTAGCAACTGCAACCGGTGTACATTCCGAG GTG CAG CTG GTG CAG	Tiller et al., 2008
		5´ AgeI SLIC VH 1-18	CTAGTAGCAACTGCAACCGGTGTACATTCCCAG GTT CAG CTG GTG CAG	Tiller et al., 2008
		5´ AgeI SLIC VH 1-24	CTAGTAGCAACTGCAACCGGTGTACATTCCCAG GTC CAG CTG GTA CAG	Tiller et al., 2008
		5´ AgeI SLIC VH 3	CTAGTAGCAACTGCAACCGGTGTACATTCTGAG GTG CAG CTG GTG GAG	Tiller et al., 2008
		5´ AgeI SLIC VH 3-11	CTAGTAGCAACTGCAACCGGTGTACATTCTCAG GTG CAG CTG GTG GAG	Tiller et al., 2008
		5´ AgeI SLIC VH 3-23	CTAGTAGCAACTGCAACCGGTGTACATTCTGAG GTG CAG CTG TTG GAG	Tiller et al., 2008
		5´ AgeI SLIC VH 3-33	CTAGTAGCAACTGCAACCGGTGTACATTCTCAG GTG CAG CTG GTG GAG	Tiller et al., 2008
		5´ AgeI SLIC VH 3-9	CTAGTAGCAACTGCAACCGGTGTACATTCTGAA GTG CAG CTG GTG GAG	Tiller et al., 2008
		5´ AgeI SLIC VH 4	CTAGTAGCAACTGCAACCGGTGTACATTCCCAG GTG CAG CTG CAG GAG	Tiller et al., 2008
		5´ AgeI SLIC VH 4-34	CTAGTAGCAACTGCAACCGGTGTACATTCCCAG GTG CAG CTA CAG CAG TG	Tiller et al., 2008
		5´ AgeI SLIC VH 4-39	CTAGTAGCAACTGCAACCGGTGTACATTCCCAG CTG CAG CTG CAG GAG	Tiller et al., 2008
		5´ AgeI SLIC VH 6-1	CTAGTAGCAACTGCAACCGGTGTACATTCCCAC TGC CAA CTG GTA CAA	Tiller et al., 2008
	RV for pIgH Expression Vector	3´ SalI SLIC JH 1/2/4/5	CCGATGGGCCCTTGGTCGACGC TGA GGA GAC GGT GAC CAG	Tiller et al., 2008
		3´ SalI SLIC JH 3	CCGATGGGCCCTTGGTCGACGC TGA AGA GAC GGT GAC CAT TG	Tiller et al., 2008
		3´ SalI SLIC JH 6	CCGATGGGCCCTTGGTCGACGC TGA GGA GAC GGT GAC CGT G	Tiller et al., 2008

3.PCR LCK	FW for pIgK Expression Vector	5′ AgeI SLIC Vκ 1-5	CTAGTAGCAACTGCAACCGGTGTACATTCTGAC ATC CAG ATG ACC CAG TC	Tiller et al., 2008
		5′ AgeI SLIC Vκ 1-9	CTAGTAGCAACTGCAACCGGTGTACATTCAGAC ATC CAG TTG ACC CAG TCT	Tiller et al., 2008
		5′ AgeI SLIC Vκ 1D-43	CTAGTAGCAACTGCAACCGGTGTACATTGTGCC ATC CGG ATG ACC CAG TC	Tiller et al., 2008
		5′ AgeI SLIC Vκ 2-24	CTAGTAGCAACTGCAACCGGTGTACATGGGGAT ATT GTG ATG ACC CAG AC	Tiller et al., 2008
		5′ AgeI SLIC Vκ 2-28	CTAGTAGCAACTGCAACCGGTGTACATGGGGAT ATT GTG ATG ACT CAG TC	Tiller et al., 2008
		5′ AgeI SLIC Vκ 2-30	CTAGTAGCAACTGCAACCGGTGTACATGGGGAT GTT GTG ATG ACT CAG TC	Tiller et al., 2008
		5′ AgeI SLIC Vκ 3-11	CTAGTAGCAACTGCAACCGGTGTACATTCAGAA ATT GTG TTG ACA CAG TC	Tiller et al., 2008
		5′ AgeI SLIC Vκ 3-15	CTAGTAGCAACTGCAACCGGTGTACATTCAGAA ATA GTG ATG ACG CAG TC	Tiller et al., 2008
		5′ AgeI SLIC Vκ 3-20	CTAGTAGCAACTGCAACCGGTGTACATTCAGAA ATT GTG TTG ACG CAG TCT	Tiller et al., 2008
		5′ AgeI SLIC Vκ 4-1	CTAGTAGCAACTGCAACCGGTGTACATTCGGAC ATC GTG ATG ACC CAG TC	Tiller et al., 2008
	RV for pIgK Expression Vector	3′ BsiWI SLIC Jκ 1/4	GAAGACAGATGGTGCAGCCACCGTACGTTT GAT YTC CAC CTT GGT C	Tiller et al., 2008
		3′ BsiWI SLIC Jκ 2	GAAGACAGATGGTGCAGCCACCGTACGTTT GAT CTC CAG CTT GGT C	Tiller et al., 2008
		3′ BsiWI SLIC Jκ 3	GAAGACAGATGGTGCAGCCACCGTACGTTT GAT ATC CAC TTT GGT C	Tiller et al., 2008
		3′ BsiWI SLIC Jκ 5	GAAGACAGATGGTGCAGCCACCGTACGTTT AAT CTC CAG TCG TGT C	Tiller et al., 2008

3.PCR LCL	FW for pIgL Expression Vector	5´AgeI SLIC VL 1	CTAGTAGCAACTGCAACCGGTTCCTGGGCCCAG TCT GTG CTG ACK CAG	Tiller et al., 2008
		5´AgeI SLIC VL 2	CTAGTAGCAACTGCAACCGGTTCCTGGGCCCAG TCT GCC CTG ACT CAG	Tiller et al., 2008
		5´AgeI SLIC VL 3	CTAGTAGCAACTGCAACCGGTTCTGTGACCTCC TAT GAG CTG ACW CAG	Tiller et al., 2008
		5´AgeI SLIC VL 4/5	CTAGTAGCAACTGCAACCGGTTCTCTCTCSCAG CYT GTG CTG ACT CA	Tiller et al., 2008
		5´AgeI SLIC VL 6	CTAGTAGCAACTGCAACCGGTTCTTGGGCCAAT TTT ATG CTG ACT CAG	Tiller et al., 2008
		5´AgeI SLIC VL 7/8	CTAGTAGCAACTGCAACCGGTTCCAATTCYCAG RCT GTG GTG ACY CAG	Tiller et al., 2008
	RV for pIgL Expression Vector	3´ XhoI SLIC CL	GAAGCTCCTCACTCGAGGGYGGGAACAGAGTG	Tiller et al., 2008

Colony PCR	HC	pIgH_K_L seq primer 1	ATCCACTTTGCCTTTCTCTC	Kreer et al., 2019
		3′ IgG (internal)	GTTCGGGGAAGTAGTCCTTGAC	Tiller et al., 2008
	LCK	pIgH_K_L seq primer 1	ATCCACTTTGCCTTTCTCTC	Kreer et al., 2019
		3′ Cκ 494	GTGCTGTCCTTGCTGTCCTGCT	Tiller et al., 2008
	LCL	pIgH_K_L seq primer 1	ATCCACTTTGCCTTTCTCTC	Kreer et al., 2019
		3′ Cλ	CACCAGTGTGGCCTTGTTGGCTTG	Tiller et al., 2008

Sequence ligase independent cloning (SLIC) reaction

Third round PCR products with adaptor sequences were cloned in the respective IgG1, kappa, or lambda expression vectors by ‘sequence ligase indepent cloning’. SLIC reaction was adapted from Boehmer *et al.*[127] The PCR product encodes for the variable region of the antibody and the expression vectors contains the respective constant regions, resulting in fully IgG1, kappa, or lambda antibody DNA.

All preparations were conducted on ice. First, a mastermix was created. For 1 µl of purified 3rd round IgG1, kappa, or lambda PCR product, respectively 6.8 µl RNase free H_2O, 1 µl of the cut IgG1, kappa, or lambda plasmid at 80 ng/µl, 1 µl 10 x NEBuffer 2.1 and 0.2 µl T4 DNA Polymerase was mixed. In a 96 well plate (subsequently called SLIC plate) 9 µl of the mastermix was transferred in each well with a multichannel pipette. 1 µl of purified 3rd round IgG1, kappa or lambda PCR product was added to respective mastermix. The SLIC plate was placed in a thermocycler at 24°C for exactly 2.5 minutes, causing the T4 DNA Polymerase to digest the overhangs in the direction 5’ to 3’. Reaction was stopped by placing the SLIC plate on ice for 10 minutes. DH5α chemically competent cells (*E.coli* cells which were made

previously competent to uptake of foreign DNA by chemical compounds) were removed from −80°C and thawed on ice. A new 96 well plate with 40 µl of competent cells in each well was prepared. 4 µl of SLIC reaction was added. Plate was incubated on ice for 20 minutes, attaching the plasmid from SLIC reaction to the membrane of chemically competent *E.coli*. The plate was heat-shocked at 42°C in the thermocycler for 45 seconds, so the plasmid was taken in. Following, the plate was incubated on ice for 2 minutes to reclose the bacterial membrane again. For recovery of *E.coli*, 50 µl of SOC medium was added to each well and placed on a shaker at 210 rpm at 37°C for 30 minutes. Plates were spun down at 300 G for 15 seconds to avoid spillover to other wells.

Pre-prepared LB-plates (Ø 6cm) with 100 µl/ml Ampicillin were set out to reach room temperature. Each well with transformed *E.coli* was streaked out on one LB-plate and was incubated at 37°C for 16 hours.

Colony PCR on transformed *E.Coli* and validation of antibody sequences

Sequences in transformed *E.coli* colonies were checked by performing a Colony PCR. For one reaction 19.75 µl RNase free H_2O, 2.5 µl 10x Green Buffer without magnesium, 0.75 µl 50 mM MgCl2, 0.2 µl 25 mM dNTP mix, 0.1 µl forward primer, 0.1 µl reverse primer, and 1.5 µl KB Extender were mixed. At least three *E.coli* colonies per antibody sequence were picked for colony PCR. The PCR was run at 94°C for 5 minutes, followed by 30 cycles of 30 seconds at 94°C, 42 seconds at 48°C and 60 seconds at 72°C, and finished with 10 minutes at 72°C. Colony PCR product was checked for successful transformation on a 2% agarose gel. Positive sequences were sent for Sanger sequencing and were compared with respective 2nd round PCR sequence.

Masterplate preparation

For every sequence in transformed *E.coli*, one colony was picked, and colonies were combined on a masterplate (15 cm dish with LB and 100 µl/ml Ampicillin). Masterplate was incubated at 37°C for 16 hours.

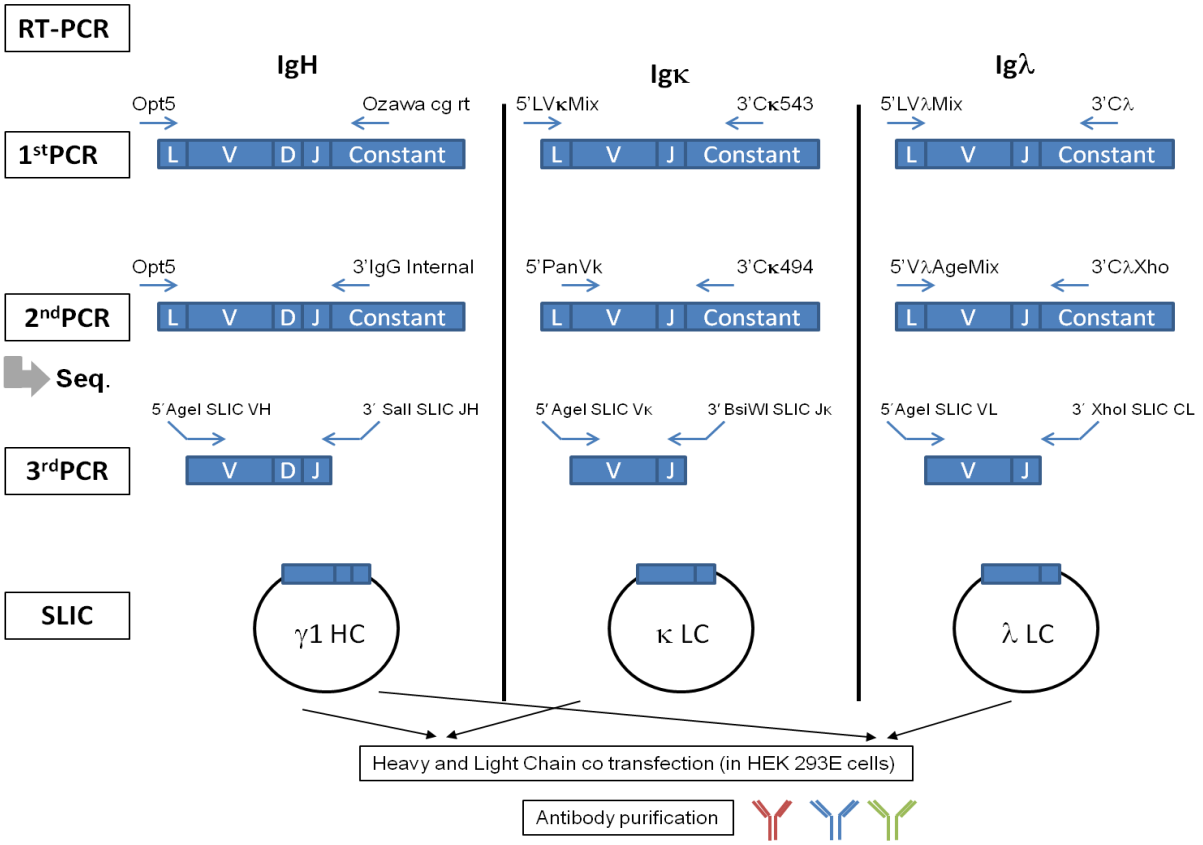


Figure 7. Strategy to clone and express human monoclonal HIV-1 antibodies

RT-PCR amplifed IgH and IgL genes with random hexamer primers. 1st PCRs are performed with primer mixes specific for the leader and reverse primers binding in the constant region. 2nd PCR primers are semi-nested for IgH, respectively nested for Igκ and Igλ. 2nd PCR products will be sent for sequencing. Analysis of B cell sequences encoding for IgH and IgL will reveal clonal sequences of interest. These sequences are cloned using the SLIC method. After cloning into corresponding expression vectors IgH and IgL plasmids are co-transfected into HEK 293-6E cells and harvested after 7 days.

2.3.8 Antibody production

Plasmid DNA preparation

To produce antibody plasmids *E.coli* bacteria with successfully transformed antibody plasmids were cultured from masterplate in 100 ml LB medium with Ampicillin (100 µg/ml) in a shaking incubator at 37°C. Plasmid DNA was isolated and purified using 'PureLink HiPure Plasmid Midiprep Kit'. As preparatory step, RNase A was added to the Resuspension Buffer to a concentration of 100 µg/ml and stored at 4°C. Columns were equilibrated with 10 ml Equilibration Buffer.

Preparation of the cell lysate: *E.coli* cells were harvested by spinning the tubes at 4,000 G for 10 minutes, forming a pellet at the bottom of the tube. The supernatant was poured off. The cell pellet was resuspended by adding 4 ml of Resuspension Buffer and mixing until homogenous. Cells were lysed by adding 4 ml Lysis Buffer and inverting the capped tube. 4 ml

of Precipitation Buffer was added and mixed immediately. Then, the lysate was clarified by centrifuging at 12,000 G for 10 minutes at room temperature.

Binding and washing of DNA: The supernatant was loaded onto the equilibrated column and drained by gravity, resulting in binding of the DNA. The columns contain a positive charged resin, which binds the negatively charged phosphate molecules of the backbone of the DNA. Then, the column was washed twice with 10 ml Wash Buffer and the flow-through discarded.

Elution and precipitation of DNA: A 15 ml centrifuge tube was placed under the column as elution tube. Then, 5 ml of Elution Buffer was added to the column. The high salt concentration causes the DNA to elute from the resin in the column. The column was discarded. The eluate was desalted and concentrated by mixing with alcohol. First, 3.5 ml isopropanol was added and mixed. The tube was centrifuged at 12,000 G for 30 minutes at 4°C, and the supernatant was discarded. The resulting pellet was resuspended with 3 ml 70% ethanol. The tube was again centrifuged at 12,000 G for 5 minutes at 4°C, following discarding of the supernatant. Pellets were air-dryed for 10 minutes. The DNA pellets were resuspended in 200 µg TE Buffer. The purified plasmid DNA was stored at −20°C until further use.

Transfection of HEK293-6E cells

For transfecting a culture of 100 ml HEK293-6E cells, 4.5 ml of 150 mM NaCl was mixed with 50 µg of the respective heavy and light chain plasmid and 0.34 ml polyethylenimine. Mix was added to HEK293-6E cells, kept in FreeStyle 293 Expression Medium with 0.2% penicillin/streptomycin at 37°C and in 6% CO2.

Antibody isolation

HEK293-6E cell cultures were centrifuged after 5−7 days at 4000 rpm for 20 minutes at 4°C. Supernatants were harvested and filtered through a 0.22 µm CA filter. Protein G Sepharose was washed by adding 1xPBS. Subsequently, the supernatants were enriched with 400 µl washed Protein G Sepharose per 100 ml HEK293-6E cell supernatant and incubated overnight rotating at 4°C. After equilibrating the chromatography columns twice with 1xPBS, protein G beads were washed twice with 1xPBS. Antibodies were eluted with 3.6 ml of 0.1 M Glycine (pH = 3) into 400 µl of 1 M Tris-HCl (pH = 8). Buffer was exchanged with 1x PBS until a Glycin dilution under 1:200 was reached, using Amicon Ultra 30 kDa spin columns. Concentrated antibody in 1x PBS was filled up in tubes and diluted to a final concentration of 0.5 mg/ml and stored at 4°C for further experiments.

SDS PAGE

SDS PAGE was used to check if antibody production worked, before the product proceeded to antibody isolation. In order to do so, 1 µl of an IgG antibody as a positive control was diluted with 19 µl PBS. The positive control and 20 µl of HEK293-6E cells culture supernatant was respectively mixed with 5 µl 1x NuPAGE™ LDS Sample Buffer and 2 µl 10x NuPAGE™ Reducing Agent. For reduction samples were heated at 70°C for 10 minutes. 20x NuPAGE™ MOPS SDS Running Buffer was diluted to 1x. Pre-prepared gel was rinsed with distillated water and placed into the gel chamber. XCell SureLock Mini-Cell™ was filled with 1x running buffer. 2 µl of prestained PageRuler™ Protein Ladder and 25 µl of each sample was filled into the wells. Gel was run at 200 V for 50 minutes. Then, the gel was developed in 10 ml InstantBlue™ Ultrafast Protein Stain. Supernatant was checked for a protein band at 50 kDa for heavy chain and 25 kDa for light chain. If showing bands at the appropriate height, supernatants progressed to antibody isolation.

2.3.9 HIV-1 Env ELISA

ELISA plates were coated overnight with HIV-1 Env glycoprotein solution at 2 µg/ml in PBS ($YU2gp_{140}$, $BG505_{SOSIP.664}$) at 4°C. Blocking Buffer incubated for 90 minutes at room temperature. Produced HIV-1 Env antibodies were applied as first antibody in a 1:5 dilution row in PBS, and then incubated again for 90 minutes at room temperature. As secondary antibody, Horseradish-peroxidase-conjugated anti-human IgG was applied in a 1:1,000 dilution in blocking buffer. After 60 minutes of incubation, ELISA plates were revealed with 100 µl ABTS solution per well, and absorbance was measured with a microplate reader (Tecan) at 415 nm. Between each step, plates were washed three times with washing buffer, and, before the last step, five times. Each HIV-1 Env antibody was applied in duplicates for each HIV-1 glycoprotein. Each plate contained antibody 3BNC117 as a standard and PBS as a negative control.

2.3.10 Neutralization assays

To characterize produced HIV-1 Env antibodies of elite neutralizer IDC561, neutralization assays were applied. First, the 'global panel' was used, containing 12 HIV-1 strains of different clades and being calculated to be representative for most global HIV-1 strains.[128] Same panel has been used to screen the serum IgG neutralization activity of HIV-1 infected individuals (see preparatory work). Then, antibodies with promising neutralization profile advanced to further characterization in a cross clade 119-pseudovirus panel and a clade C 100-pseudovirus panel (clade C is predominate in Sub-Saharan Africa and accounts for roughly half of the current HIV-1 infections).[129,130] Protocols were adapted from Sarzotti-Kelsoe *et al.*[131]

Pseudovirus production

Seeding: At day 0, 5 x 10^6 HEK293T cells were seeded in 12 ml of growth medium (DMEM, 10% FBS, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 1x antibiotic/antimycotic). HEK293T cells were incubated at 37°C at 5% CO_2 for 24h.

Transfection: At day 1, HEK293T cells were transfected by lipotransfection. 12 µg virus DNA plasmids (4 µg of Env clone DNA and 8 µg of backbone vector 'pSG3ΔEnv') were filled up to 100 µl with DMEM (without FCS and antibiotics). FuGENE (transfection agent) was mixed in a separate tube with 652 µl DMEM (without FCS and antibiotics). DNA mixture was transferred to FuGEne solution, mixed and incubated for 30 minutes at room temperature, in order to form complexes. Transfection complexes were added to 293T cells seeded at day 0. Cells incubated 6 h at 37°C at 5% CO_2 to allow the plasmid to enter the cells. After 6 h the medium, containing FuGene, was exchanged for fresh growth medium.

Harvesting: At day 2, the virus was collected. Therefore, the medium, containing produced virus, was collected in 50 ml tubes and centrifuged at 500 G for 5 minutes at 20°C to remove cells. Supernatant with virus was decanted into new tubes. FBS in the media was filled up to the end concentration of 20%. Supernatant was filtered through a 0.45 µm filter. Finally, produced virus was stored in aliquots and frozen at –80°C.

TZM.bl assay

After infection of the TZM.bl cells with HIV-1 pseudovirus, the Tat-regulated firefly luciferase (Luc) gene in the plasmid becomes activated and measurable luminescence is produced. When antibodies neutralize the HIV-1 pseudovirus, less cells get infected and leads to a measurable reduction of luminescence.

Produced HIV-1 Env antibodies were diluted from the top to the bottom in a 96-well plate in a 1:3 dilution series. 50 µl of virus dilution was added to all wells, including a virus control without pseudovirus and a cell control without antibodies nor virus. Plates were incubated at 37°C with 5% CO_2 for 1 hour. Then, 100 µl of a mixture of TZM.bl cells at a concentration of 10^4 cells/ml and DEAE-dextran at a final concentration of 10 µg/ml, were added to each well. Plates were incubated at 37°C and 5% CO_2.

Assay development

150 µl medium was removed, and 100 µl luciferin/lysis mix was added to each well. An incubation for 2 minutes at room temperature followed. The lysate was mixed, and 150 µl of

the lysate transferred to a black microtiter assay plate. Luminescence was measured in the luminometer for 1 second for each well. The cell control was used to subtract background relative luminescence. Inhibitory concentrations at 50% and 80% were determined in comparison to virus control. Murine leukemia virus (MuLV)-pseudotyped virus was used to determine unspecific activity. Every antibody was tested as duplicate. For assay development of the global panel, luciferin/lysis-buffer was used. For assay development of the 119-pseudovirus panel and the 100-pseudovirus clade C panel, Bright-Glo reagent was used.

The global panel was performed by Philipp Schommers (Cologne, Germany), and the 119-pseudovirus and 100-pseudovirus clade C panel by Michael Seaman (Boston, USA).[88]

2.3.11 Antibody variant cloning

To determine the effect of the 6 amino acid insertion [28]PYTDDD[33] in the CDRH1 on binding and neutralization, the heavy chain of antibody 1-18 was redesigned without insertion. In order to do so, following sequence was synthesized at 'Eurofins Genomics' as a gene fragment. The gene fragment was produced in a similar way as in the 3rd PCR protocol with the primers mentioned below. Specific primers were designed matching the original sequence obtained in 2nd PCR. The produced DNA proceeded to SLIC reaction and transformation.

561_1_18 original

CTAGTAGCAACTGCAACCGGTGCTCACTCCCAGGGTCGTTTGTTTCAGTCTGGGGCTGAGGTGAAGAGGCCTGGG
GCCTCAGTGAGGATTTCTTGTCGGGCAGACGACGACCCCTACACCGACGACGACACCTTCACCAAATACTGGACA
CACTGGATTCGACAGGCCCCTGGACAGCGACCTGAGTGGCTGGGGGTCATCAGCCCTCACTTTGCTCGGCCCATT
TACTCATATAAGTTCCGAGACAGACTCACGCTGACCAGGGACTCGTCCCTGACAGCCGTCTATTTGGAACTTAAA
GGTCTGCAACCTGACGACTCGGGCATTTACTTCTGTGCGCGAGATCCGTTCGGGGACAGGGCCCCCCACTATAAC
TATCACATGGACGTCTGGGGCGGAGGGACCGCGGTCATCGTCTCCTCTGCGTCGACCAAGGGCCCATCG

561_1_18 without six amino acid insertion

CTAGTAGCAACTGCAACCGGTGTACATTCCCAGGGTCGTTTGTTTCAGTCTGGGGCTGAGGTGAAGAGGCCTGGG
GCCTCAGTGAGGATTTCTTGTCGGGCAGACGACGACACCTTCACCAAATACTGGACACACTGGATTCGACAGGCC
CCTGGACAGCGACCTGAGTGGCTGGGGGTCATCAGCCCTCACTTTGCTCGGCCCATTTACTCATATAAGTTCCGA
GACAGACTCACGCTGACCAGGGACTCGTCCCTGACAGCCGTCTATTTGGAACTTAAAGGTCTGCAACCTGACGAC
TCGGGCATTTACTTCTGTGCGCGAGATCCGTTCGGGGACAGGGCCCCCCACTATAACTATCACATGGACGTCTGG
GGCGGAGGGACCGCGGTCATCGTCTCCTCTGCGTCGACCAAGGGCCCATCGG

Forward primer:

5´ AgeI SLIC VH 1-46 Clone 4

CTAGTAGCAACTGCAACCGGTGCTCACTCCCAG GGT CGT TTG TTT CAG

Reverse primer:

3´ SalI SLIC JH 6 Clone 4 AVIVSS

CCGATGGGCCCTTGGTCGACGC AGA GGA GAC GAT GAC CGC G

light blue: six amino acid insertion

red: beginning of the FWR1, ending of the FWR4

underlined: overlap with expression vector (part of the leader, part of the constant)

2.3.12 Statistical Analysis

Median V_H germline identity and CDRH3 length between clonal and non-clonal HIV-1 Env-reactive B cells were compared using the Mann-Whitney U-test. It was calculated in Prism (GraphPad), using the function 'analyze -> column analyses -> t tests and nonparametric tests -> unpaired, no assumption of gaussian distribution'. For the correlation of the neutralization activity in the global panel of the serum IgG from IDC561 with that of 1-18, spearman's rank correlation coefficient was calculated, using Prism (Graphpad). To do so, the function 'analyze -> correlation -> compute r between two selected data sets -> nonparametric Spearman correlation-> P value: Two-tailed. Confidence interval 95% -> P value style GP:1234)' was used. To determine if the removal of the six amino acid insertion in the CDRH3 of 1-18 changed binding and neutralization activity, Wilcoxon matched-pairs signed-ranks test was calculated in Prism (Graphpad). The function was 'analyze- > column analysis -> t-test (and nonparametric test) -> experimental design: pairs -> Use nonparametric test -> Wilcoxon matched-pairs signed rank test'.

3 Results

3.1 The HIV-1 Env reactive B cell Repertoire of Elite Neutralizer IDC561

A cohort of 2,274 HIV-1 infected individuals was screened for serum neutralization by testing isolated IgGs of each individual against a panel of 12 viral strains. Of those individuals, a minority showed elite neutralization characteristics. Elite neutralizer IDC561 was ranked among the top 1% of the cohort after ranking them, based on their IgG neutralizing activity. To identify corresponding antibodies that accounted for the potent neutralization activity of IDC561, we further analyzed HIV-1 Env reactive B cells.

To this end, we sorted $BG505_{SOSIP.664}$- and $YU2_{gp140}$-reactive B cells, and subsequently amplified and sequenced the immunoglobulin G variable heavy chain gene segment (V_H).

Based on obtained IgH sequences, we identified B cell clones and picked single sequences per clone for light chain production, allowing for cloning of respective antibody heavy and light chains, following antibody production and subsequent neutralization testing (Overview figure 8).

Sorting and clonality

In total, 1,168 B cells of individual IDC561 were sorted, with 531 and 637 B cells targeting $YU2_{gp140}$ and $BG505_{SOSIP.664}$, respectively. The frequency of $YU2_{gp140}$-reactive B cells was higher compared to B cells targeting $BG505_{SOSIP.664}$ (0.72% versus 0.08%). After obtaining HIV-1 Env reactive B cells, they were amplified using the optimized primer sets 'Opt5/oPR'.[132] Our novel primer sets bound in the leader segment of the antibody sequence variable region, enabling full-length amplification without primer-induced mutations. After a sequence quality check, 812 IgG heavy chain high quality sequences remained and were subjected to further characterization.

Of the 812 IgG heavy chain sequences, 445 (54.8%) originated from the $BG505_{SOSIP.664}$- and 367 (45.2%) from the $YU2_{gp140}$-single cell sort (Figure 8B). When analyzing for B cell clonality, defined as two or more members carrying the same V_H-, D_H-, J_H-gene with at least 75% similarity in the CDRH3 region, we found 560 heavy chain sequences to be clonally related (68.6%), belonging to 83 B cell clones. Of those, 18 clones shared V_H sequences of B cells identified in both $BG505_{SOSIP.664}$ and YU_{gp140} single cell sorts, while 14 clones and 51 clones were obtained from the $BG505_{SOSIP.664}$ and YU_{gp140} single cell sort only, respectively. Further, we analyzed clonal expansion in $BG505_{SOSIP.664}$ and $YU2_{gp140}$ single cell sorts. The mean number of V_H sequences belonging to a B cell clone was 9 (range 1 to 106) and 4 (range 1 to 20) for single cell sorts with $BG505_{SOSIP.664}$ and $YU2_{gp140}$, respectively (Figure 8B).

In conclusion, we were able to isolate HIV-1 Env reactive B cells from individual IDC561 by single cell sorting with $BG505_{SOSIP.664}$ and $YU2_{gp140}$. This allowed us to identify a large number of unique B cell clones, of which single prototypic antibodies were used for further downstream analyses.

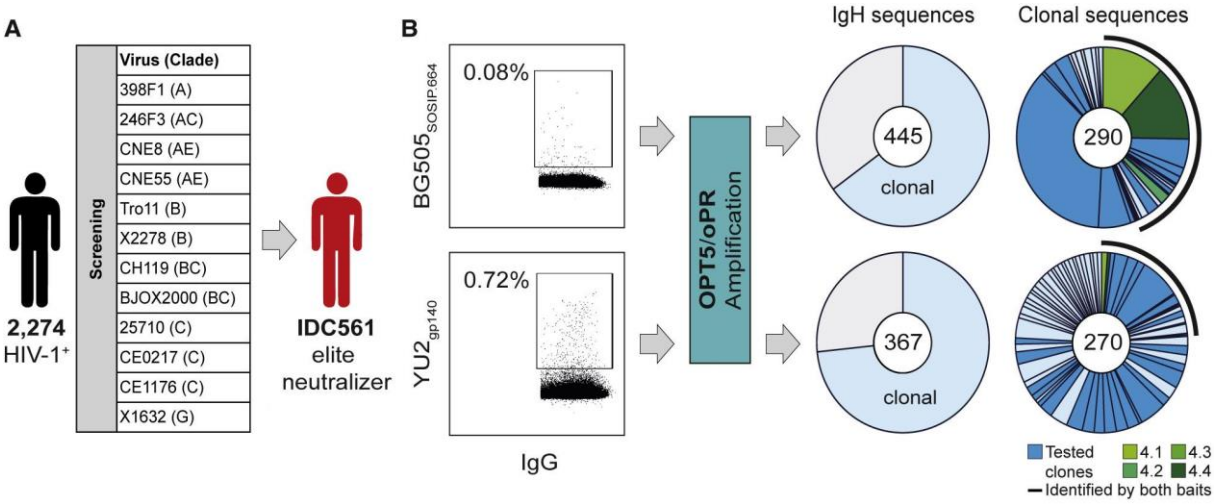


Figure 8. Identification of elite neutralizer IDC561 and characterization of his HIV-1 Env reactive B cell repertoire

(A) Screening of HIV-1 infected individuals and identification of individual IDC561 as an elite neutralizer. As screening method, the global panel with depicted virus strains was used. The virus clade is indicated in brackets. (B) Identification of HIV-1 Env reactive B cells and sequence analysis. On the left: B cells of IDC561 were sorted with baits $BG505_{SOSIP.664}$-GFP (at the top) and $YU2_{gp140}$ (at the bottom). Sequences were amplified using the novel Opt5/oPR primer sets. In the middle: Pie charts show distribution of clonal (light blue) and non-clonal sequences (light grey), respectively, for $BG505_{SOSIP.664}$-GFP (top) and $YU2_{gp140}$ (bottom). The number in the middle indicates the total number of HIV-1 Env reactive B cells IgH sequences. On the right: Clonal families in the HIV-1 reactive B cells repertoire. Each slice presents one clonal family and is proportional in size to the number of clonal members. The number in the middle indicates the number of clonal sequences for $BG505_{SOSIP.664}$-GFP (at the top) and $YU2_{gp140}$ (at the bottom). The clonal families in which antibodies were produced are colored green or dark blue. The black line at the outside marks clonal families which had been identified with both baits. (Adapted from Schommers *et al*.)[88]

Sequence analysis

We further compared sequential characteristics of clonal HIV-1 Env reactive B cells and non-clonal HIV-1 Env reactive B cells. To this end, the sequences were analyzed regarding their V_H gene segment, germline identity of the V-gene, and their CDHR3 length.

For HIV-1 Env reactive B cells clones, the most frequent V_H gene segments were IGHV1-69 (25.3 %), IGHV3-23 (12.1 %), and IGHV1-46 (8.43%). For non-clonal HIV-1 Env reactive B cells most frequent V_H-gene segments also were IGHV1-69 (10.7 %) and IGHV 3-23 (9.9%); third most frequent V_H- gene was IGHV 3-30 (9.5%), as shown in Figure 9.

The V_H-gene germline identity was displayed at the top of Figure 10. The median percentage of germline identity for clonal HIV-1 Env reactive B cells was 86.5% (range from 68.6% to 98.3%). Compared to clonal B cells, the median percentage of germline identity of non-clonal B cells was significantly higher (non-clonal B-cells: 93.2%, range from 64.9% to 100%; $p < 0.0001$).

Furthermore, we compared the median length of the CDRH3 of clonal and non-clonal HIV-1 Env reactive B cells. The median length of the CDRH3 in HIV-1 Env reactive B cell clones was 18 amino acids (range from 9 to 32 amino acids), while the median length of the CDRH3 of non-clonal HIV-1 Env reactive B cells was 16 amino acids (range from 6 to 30 amino acids; p = 0.0005, Figure 10, at the bottom).

In conclusion, clonal HIV-1 Env reactive B cells had longer CDHR3 lengths and higher levels of somatic mutations. The V_H-gene distribution of clonal and non-clonal HIV-1 Env reactive B cells was comparable.

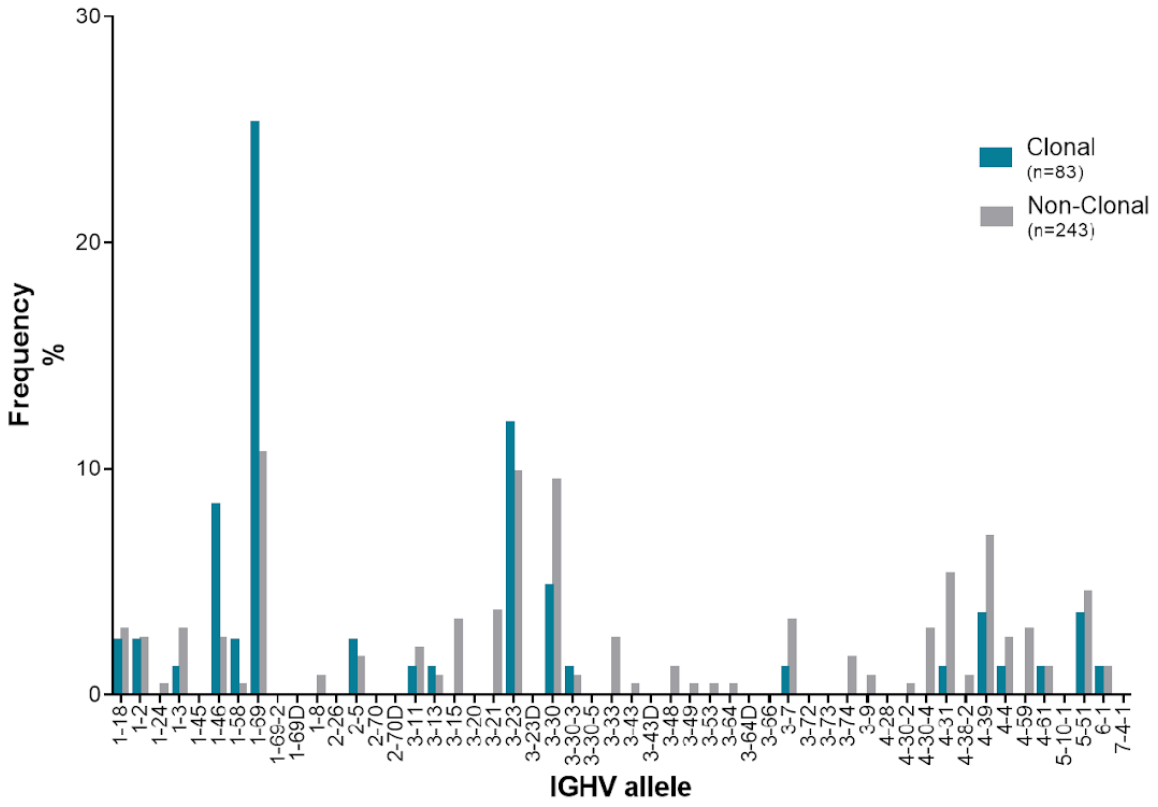


Figure 9. Comparison of V_H- gene of clonal and non-clonal HIV Env-reactive B cells of IDC561

Gene distribution was determined by annotation in the tool IgBlast using the IMGT database, created by Ye *et al*.[125] Blue columns indicate V_H-gene distribution for clonal HIV-1 Env reactive B cells. For each clone one sequence was considered, irrespective of the clone size. Grey columns indicate non-clonal HIV-1 Env reactive B cell sequences. 'N' describes the number of sequences. The frequency of V_H-genes is related to the total number of non-clonal HIV-1 Env-reactive B cells.

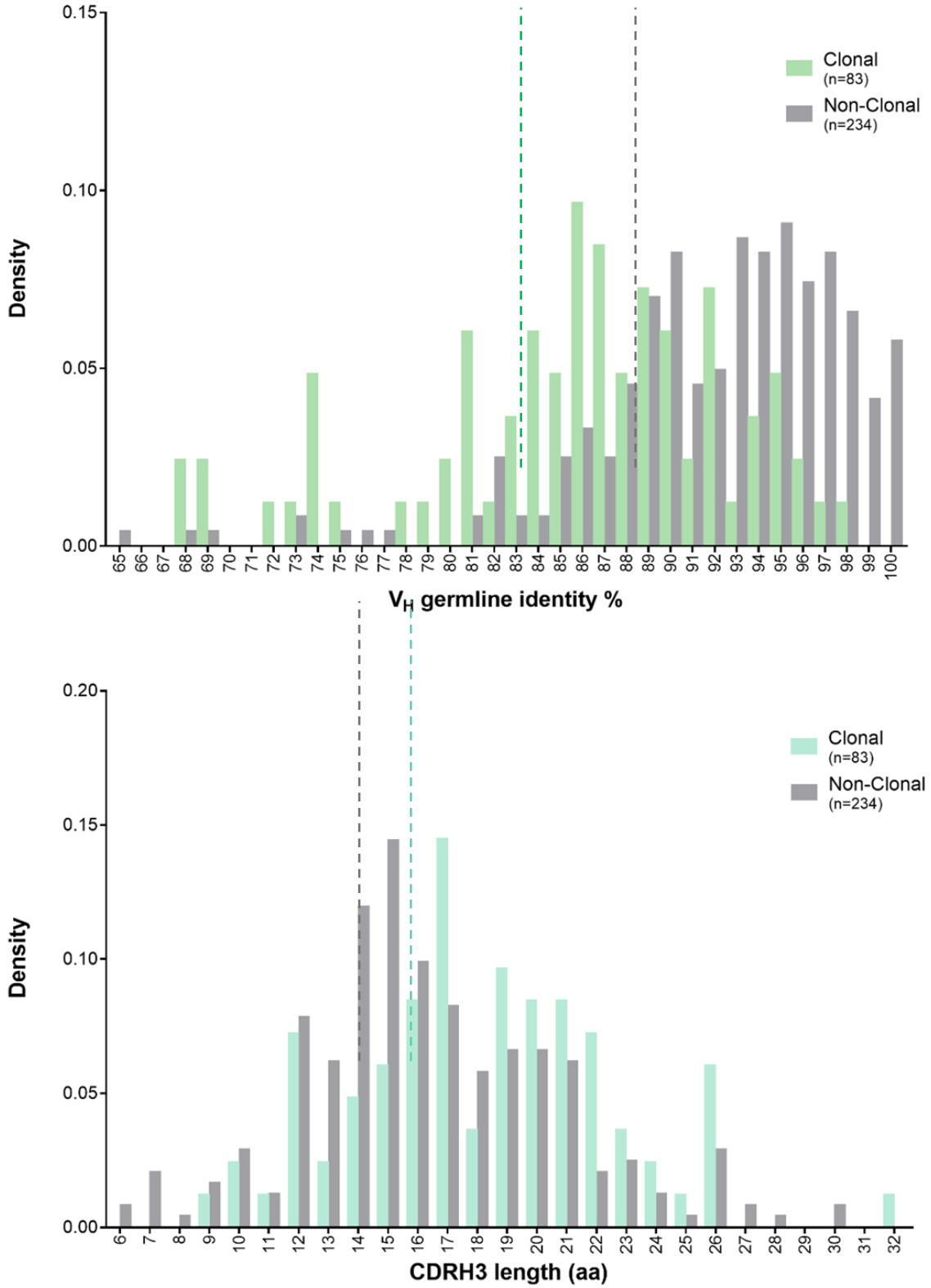


Figure 10. Comparison of V_H germline identity and CDRH3 length of clonal and non-clonal HIV Env-reactive B cells of IDC561

At the top is the V_H germline identity, and at the bottom, the CDRH3 length is shown. Colored columns indicate the V_H germline identity and CDRH3 length for clonal HIV-1 Env reactive B cells. Each clone was considered once, irrespective of the clone size. Grey columns indicate non-clonal HIV-1 Env reactive B cells. Dashed lines show medians.

Antibody cloning

In order to produce prototypic monoclonal antibodies of the identified B cell clones, we further amplified light chain sequences of these B cells, and subsequently cloned variable gene segments of heavy and light chains.

At least one member of 33 HIV-1 Env reactive B cell clones was produced. The produced antibodies represented the majority of the antibody sequences from the HIV-1 reactive B cells (73.6%). As depicted in Table 2, the production of antibodies included members of the most expanded B cell clones, clone 3 (106 members), clone 5 (42 members), and clone 4 (37 members). Further, we included antibodies of B cell clones with special features like large insertions or deletions.

In total, 56 produced monoclonal antibodies were selected for further binding and neutralization analyses (Chapters 3.2 and 3.3). We found that the clones 4, 53, 46, and 5 originated from the same lineage, even though they were found to have some different features, e.g. different CDRH3 lengths. Therefore, the B cell clones were counted as one clone comprising the subclones 4.1, 4.2, 4.3, and 4.4. New names are indicated in brackets in the following figures and tables for a better overview.

Overall, from the majority of the clonally expanded HIV-1 reactive B cells, antibodies could be produced. The produced monoclonal antibodies represented most HIV-1 Env reactive B cell clones of IDC561. Some clones were vastly expanded, some contained uncommon features, indicating a long process of B cell maturation.

Table 2. HIV-1 Env reactive antibody clones isolated from IDC561

The same color indicates the contribution to the same clone. In green, clone 4 and its subclones are shown. For the heavy chains, V_H-, D_H-, J_H- gene, CDRH3 sequence, and length are shown. For the light chains, assignment to kappa/lambda subtype, V_L- and D_L-gene, CDRL3 sequence and length are shown. Special features are described in 'comments.'

Clone	Antibody	Single cell sort		Heavy chain							Light chain						
		Clone members	Identifying Bait	V_H-gene	D_H-gene	J_H-gene	CDRH3 Sequence	CDRH3 Length	V_H-gene identity (%)	comments	κ/λ	V_L-gene	J_L-gene	CDRL3 Sequence	CDRL3 Length	V_L-gene identity (%)	comments
1	561_01_35	2	BG505	1-2*02	6-19*01	4*02	ARDRPSVGSGWYPGSD	16	91.9		λ	1-47*01	3*02	ASWDDRLSVGV	11	95.2	
2	561_01_04	18	BG505	1-69*01	6-19*01	2*01	AREGGSPVAAIYWFFDV	17	85.8		λ	1-40*01	1*01	QSYDGNNFV	9	93.7	
3	561_01_05	106	BG505	4-4*07	2-8*01	2*01	VRDRPGILIGAVPRFDF	17	88.4		κ	2-29*02	2*01	MQGIYLPHT	9	94.2	
4 (4.1)	561_01_18	37	BOTH	1-46*01	3-10*01	6*03	ARDPFGDRAPHYNYHMDV	18	67.2	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	79.1	
4 (4.1)	561_01_21			1-46*01	5-24*01	6*03	ARDPFGDMYPHYNYHMDV	18	67.8	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	78.4	
4 (4.1)	561_01_33			1-46*01	3-10*01	6*03	ARDPFGDMYPHYNYHMDV	18	68.2	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	78	
4 (4.1)	561_01_54			1-46*01	3-10*01	6*03	ARDPFGDMYPHYNSHMDV	18	68.5	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	78.4	
4 (4.1)	561_01_55			1-46*01	3-10*01	6*03	ARDPFGDMYPHYNYHMDV	18	67.5	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	77.7	
4 (4.1)	561_02_10			1-46*01	3-10*01	6*03	ARDPFGNMYPHYNYHMDV	18	68.2	6 AA Insertion in CDRH1	κ	3-20*01	4*01	QRYGGTPIT	9	78.4	
4 (4.1)	561_02_22			1-46*01	3-10*01	6*03	ARDPFGDMYPHYNYHMDV	18	68.8	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	77.7	
4 (4.1)	561_02_27			1-46*01	3-10*01	6*03	ARDPFGDMYPHYNYHMDV	18	68.5	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	78.7	
4 (4.1)	561_02_47			1-46*01	3-10*01	6*03	ARDPFGDMYPHYNYHMDV	18	67.8	6 AA Insertion in CDRH1	κ	3-20*01	3*01	QRYGGTPIT	9	76.7	
4 (4.1)	561_03_59			1-46*01	3-10*01	6*03	ARDPFGDRAPHYNYHMDV	18	66.9	6 AA Insertion in CDRH1	κ	3-20*01	4*01	QRYGATPIT	9	76.3	
4 (4.1)	561_05_18			1-46*01	5-24*01	6*03	ARDPFGDMYPHYNYHMDV	18	67.5	6 AA Insertion in CDRH1	κ	3-20*01	4*01	QRYGGTPIT	9	79.1	
4 (4.1)	561_08_10			1-46*01	3-10*01	6*03	ARDPFGDRAPHYNYHMDV	18	67.8	6 AA Insertion in CDRH1	κ	3-20*01	4*01	QRYGGTPIT	9	78.4	
4 (4.1)	561_09_23			1-46*01	3-10*01	6*03	ARDPFGERAPHYNYHMDV	18	70.7	6 AA Insertion in CDRH1	κ	3-20*01	4*01	QRFGATPIT	9	76.3	
4 (4.1)	561_10_07			1-46*01	3-10*01	6*03	ARDPFGDRAPHYNYHMDV	18	65.9	6 AA Insertion in CDRH1	κ	3-20*01	4*01	QRYGGTPIT	9	76.3	
53 (4.2)	561_08_52	2	BG505	1-46*01	6-13*01	6*03	ARDPLGEKSPAYSHHMDV	18	68.6	2 AA deletion in FR1	κ	3-20*01	4*01	QTYGAITPIT	10	75.7	2 AA insertion in FR3
53 (4.2)	561_09_89			1-46*01	1-26*01	6*03	ARDPLGEKSPAYSHHMDV	18	67.2	2 AA deletion in FR1	κ	3-20*01	4*01	QTYGAITPIT	10	76.1	2 AA insertion in FR3
46 (4.3)	561_09_71	7	BOTH	1-46*01	3-16*01	6*04	ARDSFGETFRHSGDQPYQMDV	21	73.3	2 AA deletion in FR1	κ	3-20*01	4*01	QSYGSITPLI	10	79.9	
5 (4.4)	561_01_23	42	BOTH	1-46*01	3-16*01	6*04	ARDSFGETFRGHDQPYQMDV	20	74.3	2 AA deletion in FR1	κ	3-20*01	4*01	QSYGSITPLV	10	82	
5 (4.4)	561_01_29			1-46*01	3-16*01	6*04	ARDSFGETFRGHDQPYQMDL	20	75.3	2 AA deletion in FR1	κ	3-20*01	4*01	QSYGSITPLV	10	81.3	
5 (4.4)	561_02_12			1-46*01	3-16*01	6*04	ARDPFGETFRGHDQPYRMDV	20	73	2 AA deletion in FR1	κ	3-20*01	4*01	QSYGSITPLV	10	81.3	
5 (4.4)	561_02_21			1-46*01	3-16*01	6*04	ARDPFGETFRGREQPYQMDV	20	74.7	2 AA deletion in FR1	κ	3-20*01	4*01	QSYGPITPLV	10	79.9	
5 (4.4)	561_03_07			1-46*01	3-16*01	6*04	ARDPFGETFRGHDQPYRMDV	20	72.3	2 AA deletion in FR1	κ	3-20*01	4*01	QSYGSITPLV	10	81.7	
5 (4.4)	561_03_78			1-46*01	3-16*01	6*04	ARDPFGETFRGHDQPYQMDV	20	73.6	2 AA deletion in FR1	κ	3-20*01	4*01	QSYGSITPLV	10	82.4	
6	561_01_30	2	BG505	3-30*18	4-17*01	6*03	VKDGEGVDYGDYTAYYYYMDV	21	95.4		λ	1-47*01	1*01	AAWDDSLSGRYV	12	98.6	
7	561_01_50	17	BOTH	3-13*01	3-3*01	6*03	TRGEAPYDFWGGHLDYHYFYMDI	23	72.6		κ	2-30*02	2*01	MQASHWPHA	9	81	
8	561_01_02	12	BOTH	5-51*01	5-24*01	3*02	ARPLCQGDVTCINAFDI	17	86.4		κ	4-1*01	2*01	EQFYDTPYT	9	92.9	
8	561_02_17			5-51*01	5-24*01	3*02	ARPFCQGDVTCINAFDI	17	88.5		κ	4-1*01	2*01	EQFYDTPYT	9	91.9	
9	561_02_32	7	BG505	4-34*01	3-10*01	6*03	VRYYPLHMDV	10	86.3		κ	2-24*01	2*01	MQASQFPHT	9	86.1	2 AA deletion in FR1
10	561_08_24	8	BG505	1-69*01	1-26*01	6*02	AVGSNVNGFFHMDH	14	67.2	6 AA deletion in FR3	λ	3-10*01	2*01	YLADRNINMGI	11	70.8	
11	561_02_73	9	BOTH	1-69*01	2-21*02	6*03	VRGGDVVGGSVDLTETDYYYQMDV	24	91		κ	3-15*01	2*03	QQYNNWPPYS	10	92.9	
13	561_05_16	5	YU2	1-18*01	2-8*02	4*02	ARDLNYPFCTETTCYETLAY	20	89.2		κ	1-39*01	2*01	QQSYSTPYT	9	93.2	
14	561_05_75	5	BOTH	1-2*02	3-3*01	6*03	ARAKTATMFDIIRNPKSGNNYMDA	24	85.4		κ	3-20*01	4*01	QQYATSVT	8	88	
17	561_05_54	4	YU2	1-69*01	3-9*01	4*02	EGRYKYGSSSTSGPFDS	17	83.7		κ	1-33*01	3*01	QQYDNIPPRAT	11	94	
18	561_05_11	8	YU2	1-69*01	3-9*01	4*02	VGRYKYRSASTSGPFDS	17	84.1		κ	1-33*01	3*01	QQCDNIPPRAT	11	69.3	
20	561_02_33	21	BOTH	1-69*01	3-9*01	4*02	VARYKYQHGNSGPFDS	16	87.5		κ	1-33*01	3*01	QQYDNVPPGAT	11	89.2	
21	561_05_02	5	YU2	1-69*01	3-3*01	3*02	ARNTISINGDDAFDI	15	89.8		λ	2-8*01	2*01	GSLVGNWDVI	10	86	6 AA deletion in CDR1
22	561_05_44	13	YU2	1-69*11	5-18*01	6*03	ARSSLVTVMDYEAEELEESHYYYMDV	26	91.5		κ	4-1*01	4*01	HQYYNTPLT	9	94.2	
23	561_06_44	3	YU2	1-69*12	3-10*01	6*03	ARSSLVTVMDYEAEELEESHYYYMDV	26	91.2		κ	4-1*01	4*01	QQYFSIPLT	9	92.2	
24	561_05_83	6	YU2	3-23*01	4-17*01	4*02	ARGPLLRFFDL	11	87.8		λ	2-23*02	2*01	SSYARGTSFL	10	90.9	
25	561_06_09	6	YU2	3-23*04	3-10*01	4*02	TKDQGLLRAHDY	12	91.9		λ	2-14*01	2*01	SSYTSSRTSS	10	94.9	
26	561_05_65	6	YU2	3-23*04	2/OR15-2a*01	4*02	ASILTAQISTFITDVGFLH	19	84.2		κ	1-9*01	2*01	HQLNSPRYT	9	92.3	
27	561_05_09	5	YU2	3-30*18	6-13*01	4*02	AKDKAPRARKQGFDF	15	84.8		κ	1D-12*01	2*01	QQANSFPFT	9	87.8	
28	561_06_69	6	YU2	3-30-3*02	5-18*01	4*02	ARGGFTYGLIFDY	13	84.7		κ	2-29*03	2*01	LQGVDLPPYT	10	90.2	
30	561_05_23	8	YU2	4-34*01	6-6*01	5*02	AGTKRVANRPKGGWFDT	17	88		κ	1-39*01	2*01	QQSYSTPYT	9	92.1	
32	561_05_10	7	YU2	4-34*01	3-16*01	5*02	ARLRAKNGYVDWFDP	15	87.6		λ	1-47*01	1*01	AAWDDSFDYV	10	89.4	
34	561_07_60	2	YU2	4-34*01	1-7*01	4*02	AGRLAGTRNFNS	12	89.7		κ	1-5*03	3*01	QQYNSIERLFT	11	89	
36	561_07_83	3	YU2	4-34*01	6-6*01	4*02	ARGPKGPRPFGGPADQ	16	89.8		κ	3-15*01	5*01	QQYYYWPSIT	10	93.9	
37	561_07_14	4	YU2	1-69*01	3-16*01	6*03	ARGSVDTVMEFGAGRWEENYFYYADV	26	88.1		κ	4-1*01	4*01	QQYFTIPLT	9	93.8	
40	561_07_07	6	YU2	3-23*01	6-25*01	4*02	ASVLAAQISTFITDVGFLH	19	85.3		κ	1-9*01	2*01	HQLNSPRYT	9	91.6	
41	561_05_78	6	BOTH	1-69*12	3-16*01	6*03	ATDGGAGAISITAADNPFDYDYSDYSYYLMDV	32	94.2		κ	3-20*01	3*01	QQYGSSPVT	9	94	
43	561_07_10	2	BOTH	3-7*03	3-9*01	4*02	VRYFDSRASEARLFDF	16	86.1		λ	3-10*01	1*01	YSTDKKGLHYV	11	85.8	
45	561_05_25	3	YU2	3-11*01	2-21*01	3*02	ARDRRQVLFKTGAGDAFSM	19	86.8		λ	3-21*02	3*02	QVWDYSRIHCV	11	91	
54	561_09_01	10	BOTH	1-69*06	6-6*01	4*02	ARSAAPRFRLDASGLGNLPH	20	70	9 AA insertion in CDRH2	κ	3-11*01	4*01	QQSSAWPLT	9	85.9	

AA: Amino acids

3.2 HIV-1 Env reactive antibodies isolated from individual IDC561 bind to HIV-1 Env glycoprotein

Monoclonal antibodies were tested for their binding capacities against the bait proteins $BG505_{SOSIP.664}$ and $YU2_{gp140}$, using an ELISA (Enzyme-linked Immunosorbent Assay). To verify whether binding curves on different ELISA plates were comparable, each plate included bNAb 3BNC117 as a reference. After validated comparability, the binding capacities of produced monoclonal antibodies were determined. To this end, the maximal optical density (OD_{415nm}) and half-maximal effective concentration (EC_{50}) in ELISA was measured.

First, the binding curves of the reference 3BNC117 were compared. As shown in Figure 11, the binding curves of the reference showed a similar binding pattern. Mean maximal OD_{415nm} of 3BNC117 was 1.53 (standard deviation ± 0.12) for $YU2_{gp140}$, and 1.49 (standard deviation ± 0.08) for $BG505_{SOSIP.664}$. Therefore, subsequently determined ODs of produced antibodies were obtained at comparable conditions.

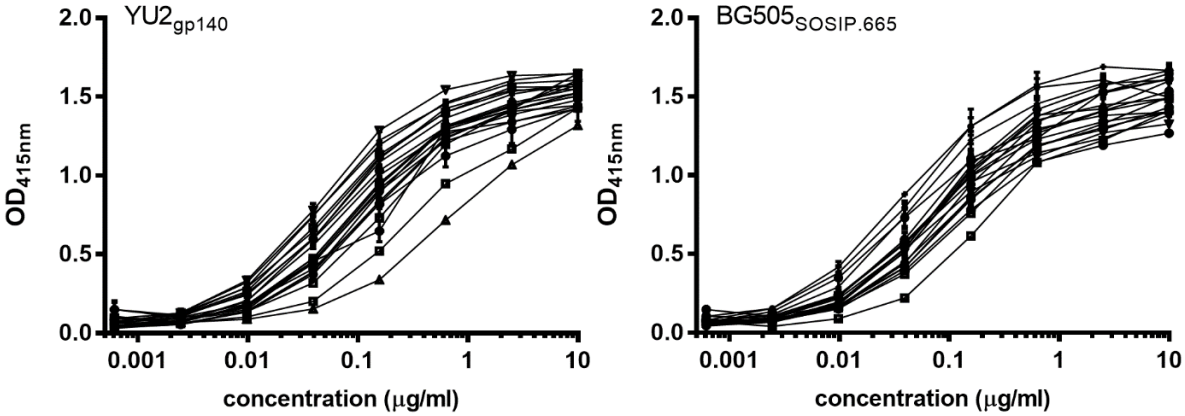


Figure 11. Binding of the reference antibody 3BNC117 to $YU2_{gp140}$ and $BG505_{SOSIP.664}$ of all plates

Binding curves of 3BNC117 to glycoprotein $YU2_{gp140}$ and $BG505_{SOSIP.664}$ are on the left and right side, respectively. Each line depicts the mean binding curve of duplicates of the reference antibody 3BNC117 per plate.

The binding of HIV-1 Env was defined as an OD larger than 0.1, which was detected in 70% of the B cell clones for both $BG505_{SOSIP.664}$ and $YU2_{gp140}$ (Figure 12 and Table 3). Strong binding, defined as an OD larger than 1.0, was detected in 35 antibodies for $BG505_{SOSIP.664}$ and in 39 antibodies for $YU2_{gp140}$. Ten and seven antibodies did not bind to $BG505_{SOSIP.664}$ and $YU2_{gp140}$, respectively. Antibodies that did not bind to $YU2_{gp140}$ also did not bind to $BG505_{SOSIP.664}$ either. Interestingly, non-binders to both baits included some B cell clones which were vastly expanded (clone 2: 18 members, clone 3: 106 members) and were found in different single cell sorts.

In conclusion, using HIV-1 Env-reactive single B cell sorting, we identified a large amount of HIV-1 Env binding antibody clones of elite neutralizer IDC561. Notably, twelve percent of the antibodies tested did not bind to the HIV-1 Env proteins utilized in ELISA, despite that their B cell clones were identified by bait-specific sorts. It is possible, that obtained B cells bound to a structure in FACS analysis that was not present in the HIV-1 Env glycoproteins used in ELISA. For $BG505_{SOSIP.664}$, the binding could be attributed to the green fluorescent protein (GFP), whereas for $YU2_{gp140}$, it might be the Avitag biotinylation signal or biotin itself. Alternatively, the HIV-1 Env non-reactive B cell clones could have been falsely detected as positive in the FACS analysis due to their extensive expansion.

Table 3. Half-maximal effective concentration and maximum optical density of isolated antibodies from IDC561 in ELISA

Values for optical density were measured at a wavelength of 415 nm. Isolated antibodies were tested up to a concentration of 10 µg/ml. Mean effective concentration (EC_{50}) indicates the concentration of the antibody where half of the maximal binding is seen. Antibodies marked with an 'x' are non-binders.

		$YU2_{gp140}$			$BG505_{SOSIP.664}$		
Clone	Antibody	Maximum OD_{415nm}	EC_{50} (µg/ml)		Maximum OD_{415nm}	EC_{50} (µg/ml)	
1	561_01_35	0.07	N/A	x	0.10	N/A	x
2	561_01_04	0.06	N/A	x	0.08	N/A	x
3	561_01_05	0.08	N/A	x	0.08	N/A	x
4 (4.1)	561_01_18	0.75	0.20		1.58	0.04	
4 (4.1)	561_01_21	0.51	0.20		1.40	0.09	
4 (4.1)	561_01_33	0.70	0.15		1.39	0.08	
4 (4.1)	561_01_54	0.62	0.27		1.40	0.11	
4 (4.1)	561_01_55	0.75	0.34		1.37	0.08	
4 (4.1)	561_02_10	0.33	0.63		1.25	0.16	
4 (4.1)	561_02_22	0.85	0.18		1.40	0.13	
4 (4.1)	561_02_27	0.72	0.36		1.22	0.07	
4 (4.1)	561_02_47	0.69	0.52		1.21	0.09	
4 (4.1)	561_03_59	0.93	0.23		1.58	0.14	
4 (4.1)	561_05_18	0.66	0.18		1.48	0.12	
4 (4.1)	561_08_10	0.99	0.18		1.59	0.12	
4 (4.1)	561_09_23	0.80	0.25		0.56	0.30	
4 (4.1)	561_10_07	0.79	0.21		0.70	0.21	
53 (4.2)	561_08_52	1.38	0.10		1.67	0.10	
54 (4.2)	561_09_89	1.42	0.07		1.20	0.12	
46 (4.3)	561_09_71	1.55	0.05		1.22	0.13	
5 (4.4)	561_01_23	1.58	0.06		1.52	0.06	
5 (4.4)	561_01_29	1.38	0.07		1.54	0.07	
5 (4.4)	561_02_12	1.47	0.06		1.64	0.06	
5 (4.4)	561_02_21	1.48	0.07		1.58	0.07	
5 (4.4)	561_03_07	1.49	0.07		1.65	0.08	
5 (4.4)	561_03_78	1.43	0.06		1.73	0.08	
6	561_01_30	0.07	N/A	x	0.08	N/A	x
7	561_01_50	1.63	0.31		1.67	0.08	
8	561_01_02	1.58	2.20		1.56	0.13	
8	561_02_17	1.51	0.99		1.37	0.04	
9	561_02_32	0.04	N/A	x	0.07	N/A	x
10	561_08_24	1.52	0.91		1.33	0.77	
11	561_02_73	1.44	0.10		1.57	0.21	
13	561_05_16	1.75	0.06		1.68	0.08	
14	561_05_75	1.54	0.05		0.07	N/A	x
17	561_05_54	1.50	0.04		1.18	3.93	
18	561_05_11	1.47	0.05		0.68	9.43	
20	561_02_33	1.42	0.04		1.10	5.13	
21	561_05_02	0.04	N/A	x	0.03	N/A	x
22	561_05_44	1.22	0.15		1.49	0.89	
23	561_06_44	0.99	0.47		1.30	1.76	
24	561_05_83	0.95	21.4		0.91	9.50	
25	561_06_09	1.19	0.43		1.53	0.94	
26	561_05_65	1.52	0.04		1.46	5.36	
27	561_05_09	1.01	0.25		0.04	N/A	x
28	561_06_69	1.23	0.06		0.56	N/A	
30	561_05_23	0.06	2.53	x	0.10	4.44	x
32	561_05_10	1.60	0.15		1.37	0.04	
34	561_07_60	0.92	1.35		0.35	0.13	
36	561_07_83	1.37	0.15		0.08	4.41	x
37	561_07_14	1.12	0.38		1.35	0.83	
40	561_07_07	1.31	0.12		1.16	4.03	
41	561_05_78	1.56	0.06		1.66	0.09	
43	561_07_10	1.54	0.22		1.73	0.13	
45	561_05_25	1.59	0.10		1.58	0.10	
54	561_09_01	1.04	0.89		0.41	0.73	
Control	3BNC117	1.54	0.11		1.50	0.08	

	EC_{50} (µg/ml)	OD_{415nm} (10 µg/ml)*
	> 5.00	< 0.25
	0.50 - 5.00	0.25 - 0.75
	0.25 - 0.50	0.75 - 1.00
	< 0.25	> 1.00

OD, optical density; EC_{50}, half-maximal effective concentration.

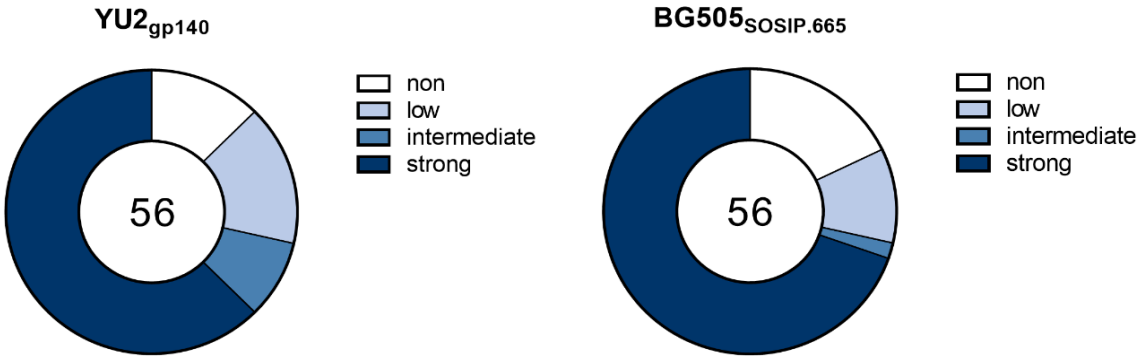


Figure 12. ELISA binding capacities of antibodies identified from elite neutralizer IDC561

Each slice is proportional to the number of antibodies. The number in the middle indicates the total number of tested antibodies. In white, antibodies with a maximum optical density at 415 nm lower than 0.25 are depicted. In light blue, antibodies with an OD from 0.25 to 0.75; in azure blue, antibodies with an OD from 0.75 to 1; and in dark blue, antibodies with an OD larger than 1 are depicted.

3.3 Neutralizing activity of antibodies identified from IDC561

IDC561-derived monoclonal antibodies were further evaluated for their neutralization potential. To this end, the antibodies were tested against the same 12-strain global panel used for screening the serum neutralization activity.

Antibodies of most clones showed no or only weak neutralizing activity. Weak neutralizing antibodies at maximum neutralized two virus strains with an IC_{50} between 0.35 and 23.4 µl/ml., suggesting that their role is limited for IDC561s serum neutralizing activity.

In contrast, all 23 members of the B cell clones 4.1–4.4 showed potent neutralizing activity against 92–100 % of the viruses in the 12-virus global (GeoMean IC_{50} 0.032–0.198 µl/ml) (Table 4). Among the four B cell clones, subclone 4.1 demonstrated the best neutralization capacity. Members of subclone 4.1 reached a coverage of 100% in the 12-virus panel with high potencies (1-18: GeoMean IC_{50} 0.035 µl/ml, 2-22: GeoMean IC_{50} 0.032 µl/ml). Antibody members of subclones 4.2, 4.3, and 4.4 were similarly potent (GeoMean IC_{50} of 0.033 to 0.115 µl/ml), but less broad. Members of these three clones neutralized all viruses of the 12- virus panel except clade G pseudovirus X1632. Clade A pseudovirus 398F1 was neutralized less potently by subclones 4.3 and 4.4 than by subclones 4.1 and 4.2 (IC_{50} of 0.87 compared to IC_{50} of 12.95 µl/ml).

In conclusion, we here identified broad and potent monoclonal antibodies that originate from four distinct B cell subclones with comparable features.

Table 4. Neutralization activity of all tested antibodies from elite neutralizer IDC561

Isolated antibodies were tested up to a maximum concentration of 25 µg/ml against a 12-virus panel, which was calculated to be representative of globally circulating viruses. Clades are written below virus strain names. Antibody 02-17 was tested up to a maximum concentration of 5 µg/ml, 05-54 was tested up to a maximum concentration of 10 µg/ml, and antibody 07-14 up to a maximum concentration of 17.5 µg/ml. Values indicate maximal inhibitory concentration (IC_{50}) for each antibody against the respective virus. The Geometric Mean was calculated from the IC_{50} values of the neutralized viruses. Coverage is defined by the percentage of viruses neutralized out of the twelve in the panel. (adapted from Schommers *et al*., 2020)

		IC_{50} (µg/ml)														
		398F1	CNE8	CNE55	246F3	X2278	Tro11	CH119	BJOX2000	25710	CE1176	CE0217	X1632	GeoMean*	Coverage %	Neutralization
Clone	Antibody	A	AE	AE	AC	B	B	BC	BC	C	C	C	G			
1	561_01_35	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
2	561_01_04	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
3	561_01_05	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
4 (4.1)	561_01_18	0,07	0,02	0,02	0,03	0,02	0,02	0,05	0,04	0,03	0,23	0,04	0,03	0,035	100	elite
4 (4.1)	561_01_21	0,33	0,05	0,02	0,04	0,03	0,03	0,90	0,05	0,05	0,54	0,11	0,07	0,086	100	elite
4 (4.1)	561_01_33	0,21	0,04	0,03	0,06	0,03	0,03	0,17	0,04	0,06	0,64	0,11	0,04	0,071	100	elite
4 (4.1)	561_01_54	4,48	0,08	0,07	0,09	0,07	0,06	0,59	0,13	0,08	1,86	0,25	0,14	0,198	100	elite
4 (4.1)	561_01_55	0,27	0,04	0,05	0,07	0,03	0,04	0,14	0,06	0,05	0,50	0,07	0,07	0,075	100	elite
4 (4.1)	561_02_10	2,36	0,06	0,08	0,12	0,03	0,04	0,33	0,10	0,11	2,95	0,27	0,17	0,172	100	elite
4 (4.1)	561_02_22	0,09	<0.01	0,03	0,01	0,02	0,02	0,06	0,02	0,04	0,38	0,03	0,03	0,032	100	elite
4 (4.1)	561_02_27	0,22	0,02	0,05	0,03	0,02	0,04	0,20	0,05	0,06	0,92	0,07	0,03	0,063	100	elite
4 (4.1)	561_02_47	0,46	0,02	0,04	0,03	0,02	0,03	0,15	0,04	0,05	0,98	0,08	0,05	0,065	100	elite
4 (4.1)	561_03_59	0,27	0,05	0,04	0,06	0,03	0,05	0,18	0,12	0,07	0,99	0,07	0,08	0,090	100	elite
4 (4.1)	561_05_18	0,29	0,03	0,04	0,04	0,02	0,05	0,22	0,05	0,06	0,87	0,06	0,05	0,073	100	elite
4 (4.1)	561_08_10	0,15	0,10	0,05	0,07	0,03	0,04	0,14	0,08	0,07	0,40	0,11	0,05	0,082	100	elite
4 (4.1)	561_09_23	3,97	0,05	0,03	0,07	0,03	0,03	0,23	0,09	0,06	0,39	0,10	2,51	0,135	100	elite
4 (4.1)	561_10_07	0,20	0,04	0,04	0,08	0,03	0,04	0,17	0,05	0,06	0,30	0,12	0,05	0,071	100	elite
53 (4.2)	561_08_52	0,29	0,04	0,04	0,10	0,03	0,05	0,05	0,04	0,13	0,45	0,05	>25	0,074	92	elite
54 (4.2)	561_09_89	0,35	<0.01	0,02	0,04	0,02	0,01	0,04	<0.01	0,04	0,27	<0.01	>25	0,033	92	elite
46 (4.3)	561_09_71	12,95	2,95	<0.01	2,63	0,02	0,01	<0.01	0,02	0,02	0,07	<0.01	>25	0,084	92	elite
5 (4.4)	561_01_23	0,87	0,04	0,02	0,07	<0.01	0,02	<0.01	0,01	0,03	0,09	0,01	>25	0,034	92	elite
5 (4.4)	561_01_29	10,14	0,51	0,04	0,77	0,03	0,02	0,04	0,03	0,06	0,30	0,01	>25	0,110	92	elite
5 (4.4)	561_02_12	3,22	0,08	0,03	0,22	0,03	0,04	0,05	0,03	0,08	0,36	0,04	>25	0,090	92	elite
5 (4.4)	561_02_21	7,27	0,11	0,03	0,45	0,03	0,04	0,08	0,02	0,18	0,34	0,04	>25	0,115	92	elite
5 (4.4)	561_03_07	9,10	0,14	0,04	0,31	0,04	0,04	0,10	0,03	0,08	0,46	<0.01	>25	0,111	92	elite
5 (4.4)	561_03_78	4,32	0,13	0,04	0,22	0,02	0,03	0,05	0,02	0,04	0,29	0,02	>25	0,075	92	elite
6	561_01_30	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
7	561_01_50	>25	>25	>25	>25	>25	3,24	>25	>25	>25	>25	>25	>25	3,24	8	weak
8	561_01_02	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
8**	561_02_17	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	2,17	>5	2,17	8	weak
9	561_02_32	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
10	561_08_24	>25	>25	>25	>25	>25	0,35	>25	>25	>25	>25	>25	>25	0,350	8	weak
11	561_02_73	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	n/a	0	-
13	561_05_16	>25	3,10	>25	>25	>25	>25	>25	>25	23,40	>25	>25	>25	8,52	17	weak
14	561_05_75	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
17	561_05_54	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	n/a	0	-
18	561_05_11	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
20	561_02_33	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
21	561_05_02	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
22	561_05_44	>25	>25	>25	>25	>25	>25	>25	>25	8,29	>25	>25	>25	8,29	8	weak
23	561_06_44	>25	>25	>25	>25	>25	>25	>25	>25	10,83	>25	>25	>25	10,83	8	weak
24	561_05_83	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
25	561_06_09	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	n/a	0	-
26	561_05_65	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
27	561_05_09	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
28	561_06_69	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
30	561_05_23	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
32	561_05_10	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
34	561_07_60	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
36	561_07_83	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
37	561_07_14	>17.5	>17.5	>17.5	>17.5	>17.5	>17.5	>17.5	>17.5	>17.5	>17.5	>17.5	>17.5	n/a	0	-
40	561_07_07	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
41	561_05_78	>25	>25	>25	>25	>25	>25	>25	>25	13,54	>25	>25	>25	13,54	8	weak
43	561_07_10	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-
45	561_05_25	>25	6,78	>25	>25	>25	>25	>25	>25	6,11	>25	>25	>25	6,44	17	weak
54	561_09_01	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	>25	n/a	0	-

IC_{50} (µg/ml)	Coverage %
<0.1	100-90
0.1-0.5	90-80
0.5-1	80-70
1-2	70-60
2-5	60-50
5-10	20-10
10-25	10-0
>25	0,00

3.4 Sequence features of V_H 1-46 derived bNAbs

For further characterization of the B cell clones of the here identified bNAbs, we compared them using a phylogenetic analysis and compared their sequential features. All four potently and broadly neutralizing B cell clones derived from the same variable gene for the heavy and light chain (IGHV 1-46 and IGLV 3-20, respectively). Furthermore, we analyzed respective antibody sequences for a phylogenetical relationship (Figure 13). Based on the phylogenetic

tree the B cell clones were subsumed as subclones of clone 4. Clone 4 was renamed to subclone 4.1, 53 to subclone 4.2, 46 to subclone 4.3, and 5 to subclone 4.4.

B cell clone 4 divides into a subclone 4.1, which has a six amino acid insertion in the CDHR1 and was the broadest and most potent subclone tested in the 12-strain global panel, and into the subclones 4.2, 4.3, and 4.4, which lack the six amino acid insertion but have a two amino acid deletion in FWR1 (Figure 13 and Figure 14). Subclones 4.1 and 4.2 had CDRH3 lengths of 18 amino acids. Subclones 4.3 and 4.4 had different CDHR3 lengths of 21 and 20 aa, respectively.

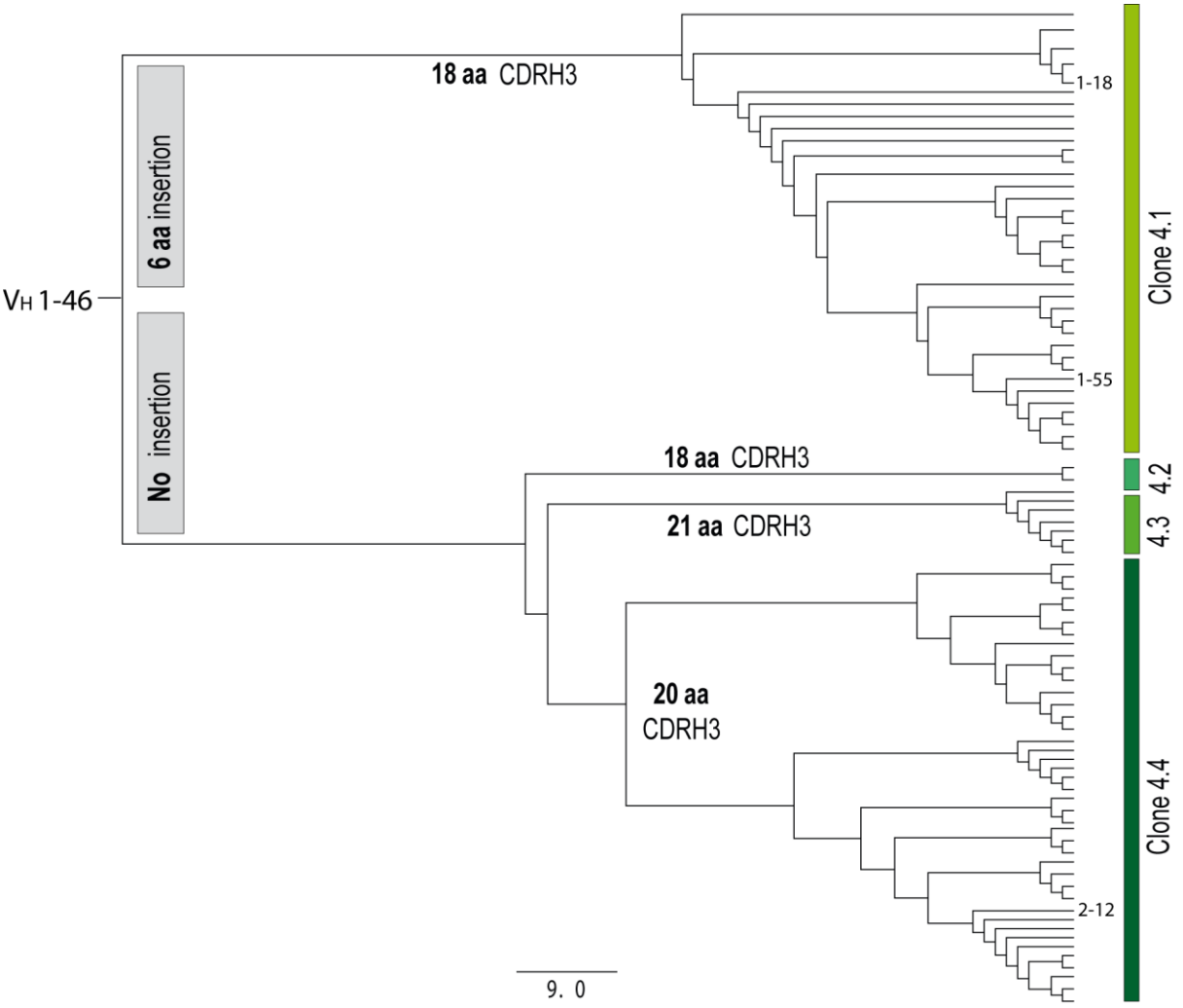


Figure 13. Phylogenetic analysis of clone 4

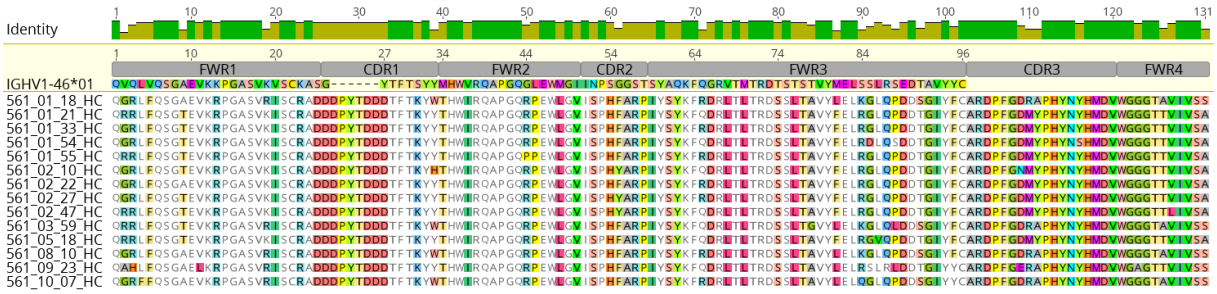
Sequences are rooted to germline sequence of V_H 1-46 (on the left site). Clone 4 is separated into distinct phylogenetic groups, either having the feature of the six amino acid insertion in the CDRH1 or not. The phylogenetic group without insertion splits up into different lengths of the CDR3. Sequences of 1-18, 1-55, 2-22, and 2-12 are marked, because the antibodies were selected for further neutralization analysis. (adapted from Schommers *et al.*, 2020)

In the following, we analyzed the sequences of subclones 4.1–4.4 were analyzed in more detail. The heavy chains of members from clone 4.1 all featured the distinctive motif

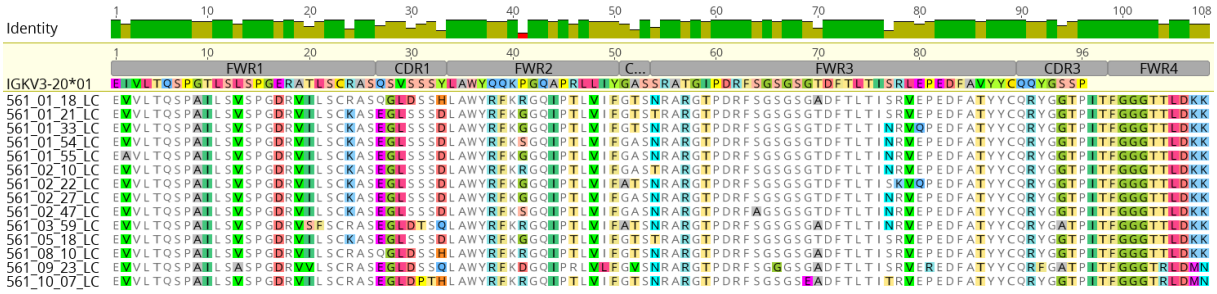
‘^{25}DDDPYTDDD^{33}’ in the CDRH1, which included an insertion of a six amino acids that extended the CDRH1 from 8 to 14 amino acids. Two-thirds of the motif consisted of aspartic acid, which charged the motif negatively. The CDR3 of the light chain had 9 residues (Figure 14, Clone 4.1). In addition to the above-mentioned features, subclones 4.2, 4.3, and 4.4 shared the same CDRL length of 10 amino acids. An outstanding feature of clone 4.2 is a two-residue insertion in the FWR3, which is not shared by another subclone (Figure 14, Clone 4.2–4.4).

All four subclones are highly mutated, with clone members displaying 65.9 to 75.3% identity to the germline for the variable gene. Subclone 4.1, with a mean V_H-gene germline identity of 68.1%, was the most mutated subclone within clone 4 and the second most mutated clone among all HIV-1 Env reactive B cell clones identified in IDC561.

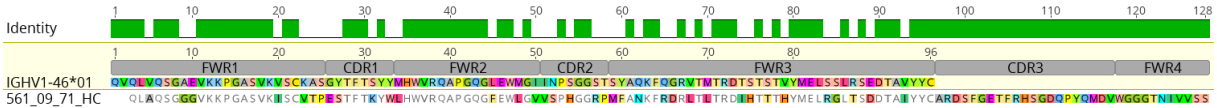
Clone 4.1 Heavy Chain Sequences



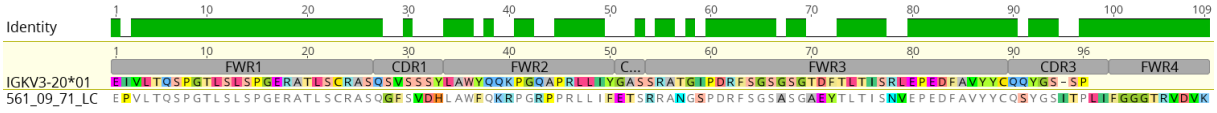
Clone 4.1 Light Chain Sequences



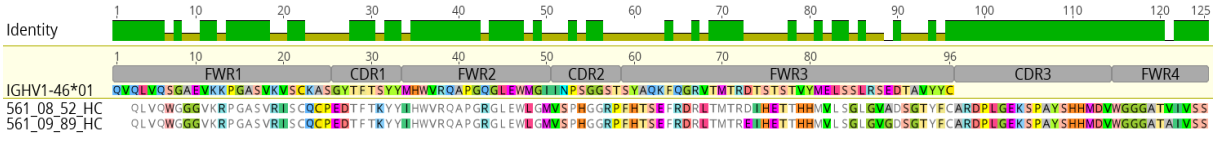
Clone 4.2 Heavy Chain Sequence



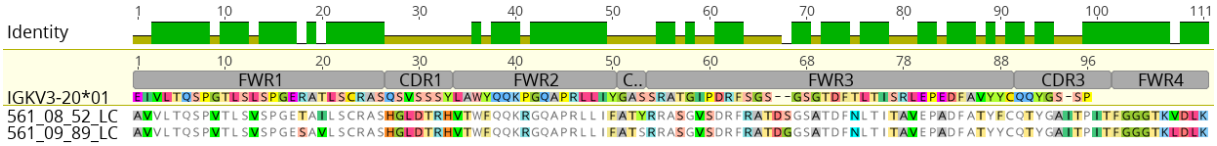
Clone 4.2 Light Chain Sequence



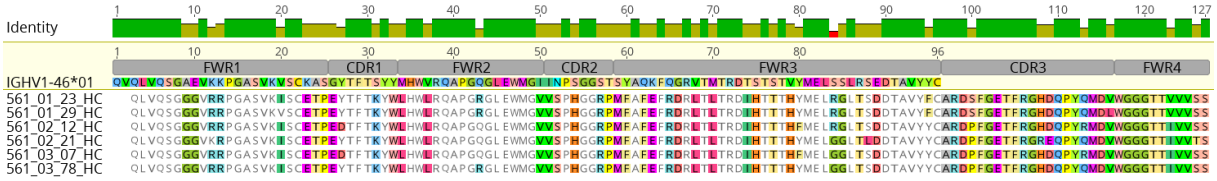
Clone 4.3 Heavy Chain Sequences



Clone 4.3 Light Chain Sequences



Clone 4.4 Heavy Chain Sequences



Clone 4.4 Light Chain Sequences

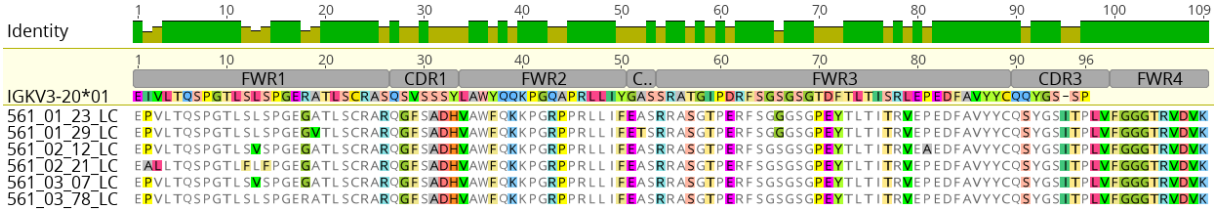


Figure 14. Heavy and light chain sequences of cloned and tested members of clone 4

At the top of the sequences, the germline variable gene (IGHV1-46*01 for heavy chains, respectively IGKV3-20*01 for light chains) is displayed. It is set as the reference sequence. A specific color is designated for each amino acid. In grey, the different regions of the antibody sequence are marked. Below clone 4 sequences are aligned to the reference sequence, and each amino acid divergent to the germline sequence is marked in color. Above the sequences, the identity of aligned sequences compared to germline is depicted. The higher the bar, the higher is the similarity between germline and aligned sequences. Alignments are calculated and created with Geneious version 10.2, created by Biomatters. Available from https://www.geneious.com.

3.5 Binding capacities of V_H 1-46 derived bNAbs

To assess the impact of sequence variations among subclones 4.1–4.4 on their binding capacities, we compared the binding capacities of representative members from each subclone in ELISA using HIV-1 Env glycoprotein $YU2_{gp140}$ and $BG505_{SOSIP.664}$. The binding capacity of subclone 4.1 was approximately half that of the subclones 4.2–4.4 for the HIV-1 Env glycoprotein $YU2_{gp140}$. Subclones 4.2, 4.3, and 4.4 had a similar binding capacities as the reference bNAb 3BNC117. However, the binding capacity to $BG505_{SOSIP.664}$ was similar for all subclones and comparable to the reference antibody.

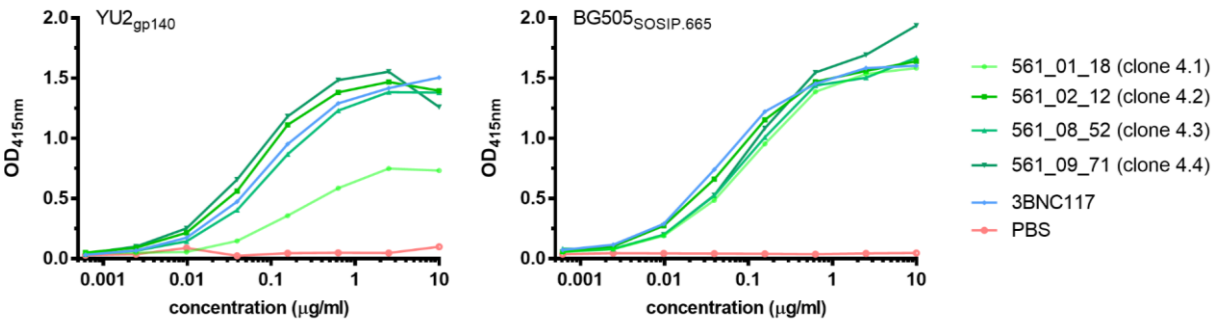


Figure 15. ELISA binding curves for antibodies from clone 4 isolated from elite neutralizer IDC561

A representative antibody was chosen for each subclone 4.1, 4.2, 4.3, and 4.4. On the left side, antibody binding curves of isolated antibodies to glycoprotein $YU2_{gp140}$ are depicted. On the right side, antibody binding curves of isolated antibodies against glycoprotein $BG505_{SOSIP.664}$ are depicted. In blue, the binding of reference antibody 3BNC117; in red, the negative control with PBS is shown.

In conclusion, isolated broadly neutralizing antibodies of the V_H 1-46-derived B cell clones are phylogenetically related and can be assigned to subbranches of one B cell clone (clone 4). Antibody sequences of B cell clone 4 are highly mutated and have unique features. The different binding mode of subclone 4.1 could be a result of the six amino acid insertion in the CDRH1.

To further characterize the properties of the isolated V_H 1-46-derived broadly neutralizing antibodies, selected antibodies were tested against extended pseudovirus panels (Chapter 3.6). This was followed by correlating the neutralization activity of the isolated bNAbs with IDC561's serum neutralization activity (Chapter 3.7). Finally, sequence characteristics were examined (Chapter 3.8).

3.6 1-18 is an exceptionally broad and potent antibody

In chapter 3.3, subclones 4.1–4.4 could be identified as B cell clones with extraordinary neutralization activity in the 12-strain global panel. To characterize the neutralization activity in more detail, the neutralization activity of representative antibodies 1-18, 1-55, 2-22 (members of clone 4.1), and additionally 2-12 (member of subclone 4.4) was tested in a 119-pseudovirus multiclade panel and 100-strain clade C panel. The panels allowed for in depth comparison with known bNAbs, which already advanced to clinical testing (CD4bs bNAbs: 3BNC117, N6, N49P7, VRC01; V2 loop bNAb: PGDM100; V3-N332 glycan bNAbs: PGT121, 10-1074).

1-18 and 1-55 were selected as representative members of the two early diving sub-branches in subclone 4.1. 1-18 was the third most mutated antibody from clone 4 (IGHV: 68% germline identity on nucleotide level, 51% germline identity on amino acid level). 1-55 was slightly less mutated than 1-18 (Figure 16). Additionally, 2-22 was selected as the most potent antibody tested against the 12-virus strain global panel and also belonged to clone 4.1. 2-12 was selected as a representative member of subclone 4.4, which was expanded similar to subclone 4.1.

	Heavy chain					Light chain			
	IGHV allele	Germline identity		special feature	CDRH3 length	IGKV allele	Germline identity		CDRL3 length
		nt	aa				nt	aa	
1-18	1-46*01	68%	51%	6 aa ins in CDRH1	18 aa	3-20*01	79%	71%	9 aa
1-55	1-46*01	68%	53%	6 aa ins in CDRH1	18 aa	3-20*01	78%	71%	9 aa
2-12	1-46*01	74%	61%	2 aa del in FR1	20 aa	3-20*01	81%	71%	10 aa

Figure 16. Characteristics of antibodies 1-18, 1-55, and 2-12.

Sequence information was obtained from the tool IgBLAST (Ye *et al*. 2013). Nt= nucleotides. Aa= amino acids.

When selected antibodies were tested against the 119-virus multiclade panel, 1-18 was the most potent and broadest antibody from clone 4 (1-18: Geometric Mean IC_{50} 0.048 µg/ml, coverage 96.6%). The neutralization activity of 2-12 (subclone 4.4) against the 119-virus panel was less broad (Geometric Mean IC_{50} 0.078 µg/ml, coverage 93.3%).

1-18s neutralization activity was more potent and broader than that of most known bNAbs in the 119-strain multiclade panel, except for N6, which had comparable characteristics (N6: Geometric Mean IC_{50} 0.086 µg/ml, coverage 97.5%). For a limited number of viruses, the potency of PDGM1400 was higher. However, the potency of 1-18 was thoroughly better than for the V3 loop antibodies PGT121 and 10-1074. When comparing the neutralization characteristics of 1-18 and N6 more closely, it was revealed that three viruses could not be neutralized by 1-18 but N6, and two viruses could not be neutralized by N6, but 1-18. Only one virus could not be neutralized by both bNAbs.

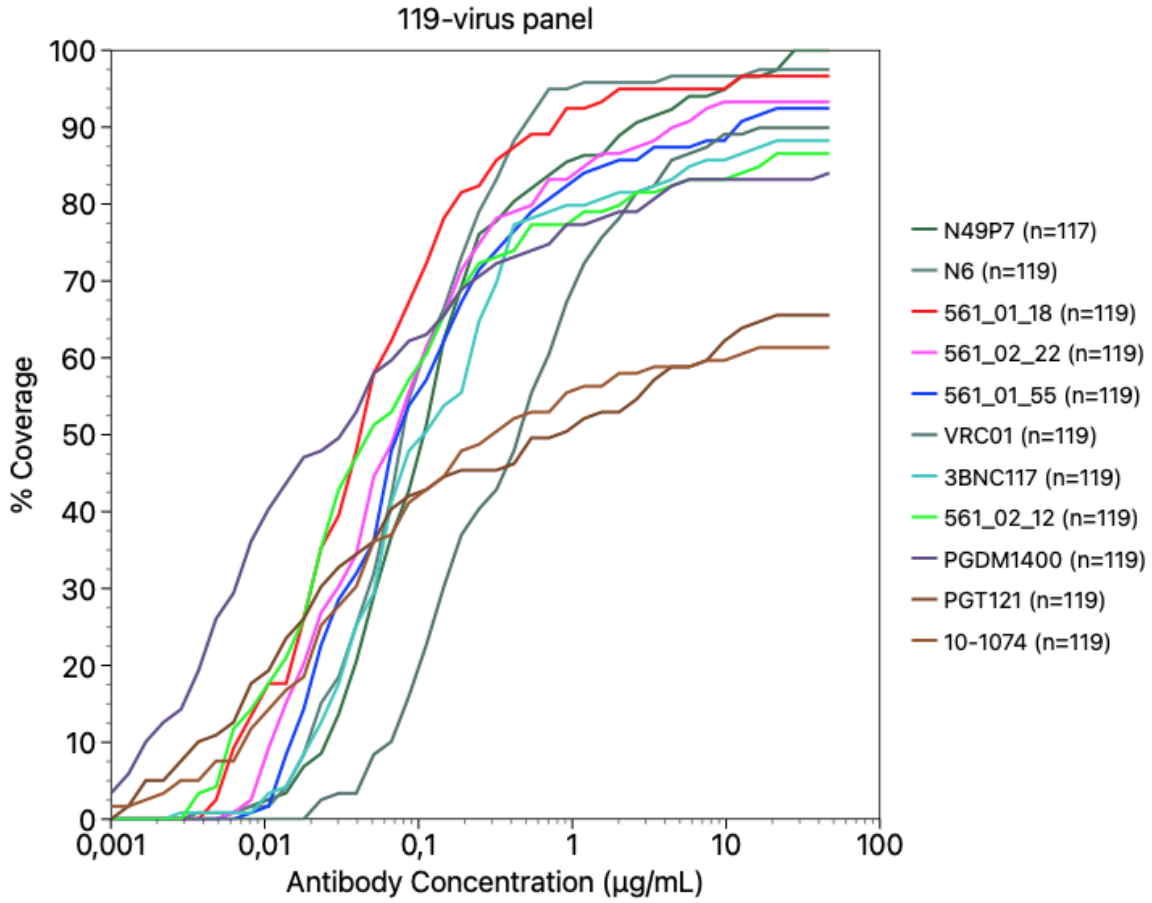


Figure 17. Neutralizing activity of 1-18, 1-55, 2-22 (clone 4.1), and 2-12 (clone 4.4) compared to bNAbs N6, N49P7, 3BNC117, VRC01, PGDM100, PGT121, 10-1074 on the 119-pseudovirus multiclade panel

Compared bNAbs are all in clinical testing. Neutralization data except 1-18, 1-55, 2-22, 2-12, and N6 were derived from CATNAP (Yoon *et al*., 2015). Curves indicate the percentage of pseudoviruses that are neutralized to 50% of the respective antibody concentration. On the y-axis, the percentage of neutralized pseudoviruses at the respective IC50 values is displayed. On the x-axis, the IC50 value for each antibody is shown. N49P7 was tested against 117 viruses from the 119-pseudovirus multiclade panel. 1-55 was tested in a previous version and therefore contained mutations at the start and end of the V genes (V_H: 2 aa mut., V_k: 4 aa mut.)

For further characterization, 1-18 and 2-12 were tested against a 100-strain clade C panel, which viruses are seen as globally predominantly in HIV-1 infections. They were then compared with known bNAbs. Here too, 1-18 demonstrated a highly active potent neutralization activity (GeoMean IC_{50} 0.074 µg/ml, coverage 90%). When compared to other bNAbs, 1-18s neutralization activity again exceeded the neutralization activity of other bNAbs apart from N6 (GeoMean IC_{50} 0.062 µg/ml, coverage 97%).

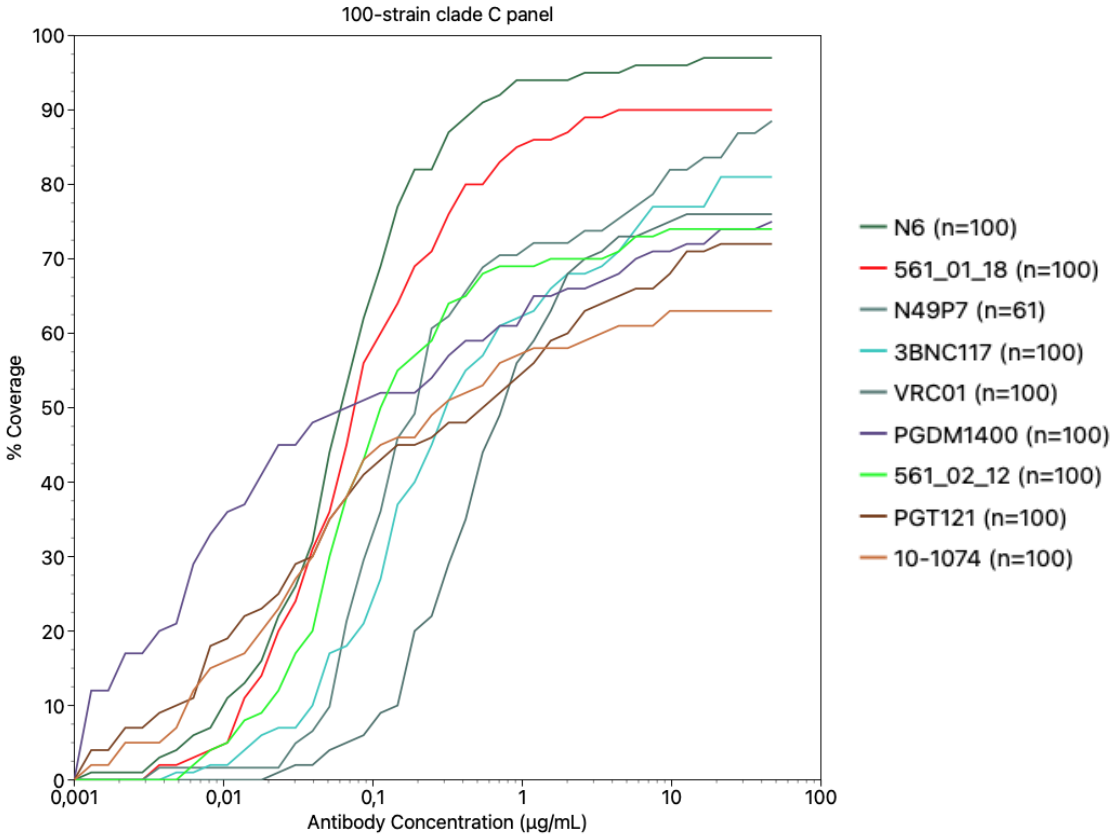


Figure 18. Neutralizing activity of 1-18 (clone 4.1) and 2-12 (clone 4.4) compared to bNAbs N6, N49P7, VRC01, 3BNC117, PGDM100, PGT121, 10-1074 in the 100-strain clade C panel

Compared bNAbs are all in clinical testing. Neutralization data of compared bNAbs were derived from CATNAP (Yoon *et al,.* 2015). Curves indicate the percentage of pseudoviruses that are neutralized to 50% of the respective antibody concentration. On the y-axis, the percentage of neutralized pseudoviruses at the respective IC50 values is shown. On the x-axis, the IC50 value for each antibody is shown. N49P7 was tested against 61 viruses from a 100-strain clade C panel.

In conclusion, 1-18 was the best bNAb among tested members of clone 4. It exceeded the neutralization activity of almost all CD4bs bNAbs described to date and rivals N6, which currently is best of its class. In the multiclade panel, as well as in the clade C panel, 1-18 had different neutralization characteristics than N6.

3.7 Clone 4 accounts for serum IgG neutralization activity

As described in Chapter 3.3, members of B cell clone 4 showed the broadest and most potent neutralization activity among antibodies isolated from IDC561. To determine whether clone 4 accounts for IDC561's elite serum neutralization, the clone 4 neutralization data was correlated with the serum IgG neutralization data against the 12-virus strain global panel by calculating spearman's rank correlation coefficient. Additionally, the neutralization data of 1-18 (as broadest and most potent clone 4 member) was compared to the serum neutralization activity in a larger virus panel.

The calculation of spearman's rank correlation coefficient revealed a strong and significant correlation between clone 4 members and IDC561 serum neutralization activities in the global pseudovirus panel ($r > 0.5$, $p < 0.05$, 11 from 23 antibodies) (Table 5). The significant correlation was a strong indicator that clone 4 accounts for the neutralizing activity of the serum IgG of IDC561. The comparison of 1-18 and serum neutralization activity in the global pseudovirus panel and additional 30-strain YU2 panel revealed an even stronger correlation ($r = 0.93$, $p < 0.0001$) (Figure 19 and 20).

In conclusion, antibodies of the B cell clone 4, especially bNAb 1-18, are the main contributors to the elite neutralization serum activity of IDC561.

Table 5. Correlation of neutralization activity of clone 4 with serum neutralization of IDC561

R stands for spearman's rank coefficient. Values above 0.5 indicate a strong correlation. Spearman's rank correlation also calculated the value for significance p. Values below 0.05 are statistically significant. IC50 values under 0.01 µg/ml were calculated with 0.01 µg/ml because lower IC50 values could not be determined by assay. Viruses were tested up to a concentration of 25 µg/ml. For IC50, values over 25 µg/ml could not be determined by assay and were therefore calculated as 25 µg/ml.

clone	correlated antibody	r	95% confidence interval	P (two-tailed)	Significant? (p < 0.05)
4.1	IgG IDC0561 vs. 561_01_21	0.7692	0.3327 to 0.9343	0.0049	Yes
4.1	IgG IDC0561 vs. 561_02_10	0.7413	0.2735 to 0.9255	0.0078	Yes
4.1	IgG IDC0561 vs. 561_09_23	0.6795	0.1543 to 0.9053	0.0179	Yes
4.1	IgG IDC0561 vs. 561_10_07	0.6737	0.1438 to 0.9033	0.0193	Yes
4.1	IgG IDC0561 vs. 561_01_54	0.6573	0.115 to 0.8978	0.0238	Yes
4.1	IgG IDC0561 vs. 561_01_55	0.6503	0.1029 to 0.8954	0.0257	Yes
4.1	IgG IDC0561 vs. 561_01_18	0.6351	0.07712 to 0.8901	0.0298	Yes
4.2	IgG IDC0561 vs. 561_08_52	0.6333	0.2133 to 0.9157	0.0119	Yes
4.4	IgG IDC0561 vs. 561_02_12	0.6182	0.1778 to 0.9096	0.0155	Yes
4.1	IgG IDC0561 vs. 561_01_33	0.6165	0.04661 to 0.8836	0.0362	Yes
4.4	IgG IDC0561 vs. 561_02_21	0.5364	0.05618 to 0.8857	0.0347	Yes
4.2	IgG IDC0561 vs. 561_09_89	0.7143	-0.1849 to 0.8205	0.1439	No
4.4	IgG IDC0561 vs. 561_03_07	0.7091	-0.07053 to 0.855	0.075	No
4.3	IgG IDC0561 vs. 561_09_71	0.619	-0.3914 to 0.7314	0.4259	No
4.1	IgG IDC0561 vs. 561_08_10	0.5804	-0.0954 to 0.706	0.0521	No
4.1	IgG IDC0561 vs. 561_05_18	0.5779	-0.01328 to 0.8697	0.0525	No
4.1	IgG IDC0561 vs. 561_02_47	0.5734	-0.02002 to 0.8681	0.0556	No
4.1	IgG IDC0561 vs. 561_03_59	0.5734	-0.02002 to 0.8681	0.0556	No
4.1	IgG IDC0561 vs. 561_02_22	0.5545	-0.2217 to 0.8075	0.1767	No
4.4	IgG IDC0561 vs. 561_03_78	0.4364	-0.05069 to 0.8603	0.0667	No
4.1	IgG IDC0561 vs. 561_02_27	0.3818	-0.2641 to 0.7913	0.22	No
4.4	IgG IDC0561 vs. 561_01_29	0.3545	-0.1542 to 0.8305	0.1215	No
4.4	IgG IDC0561 vs. 561_01_23	0.3347	-0.2241 to 0.8066	0.1768	No

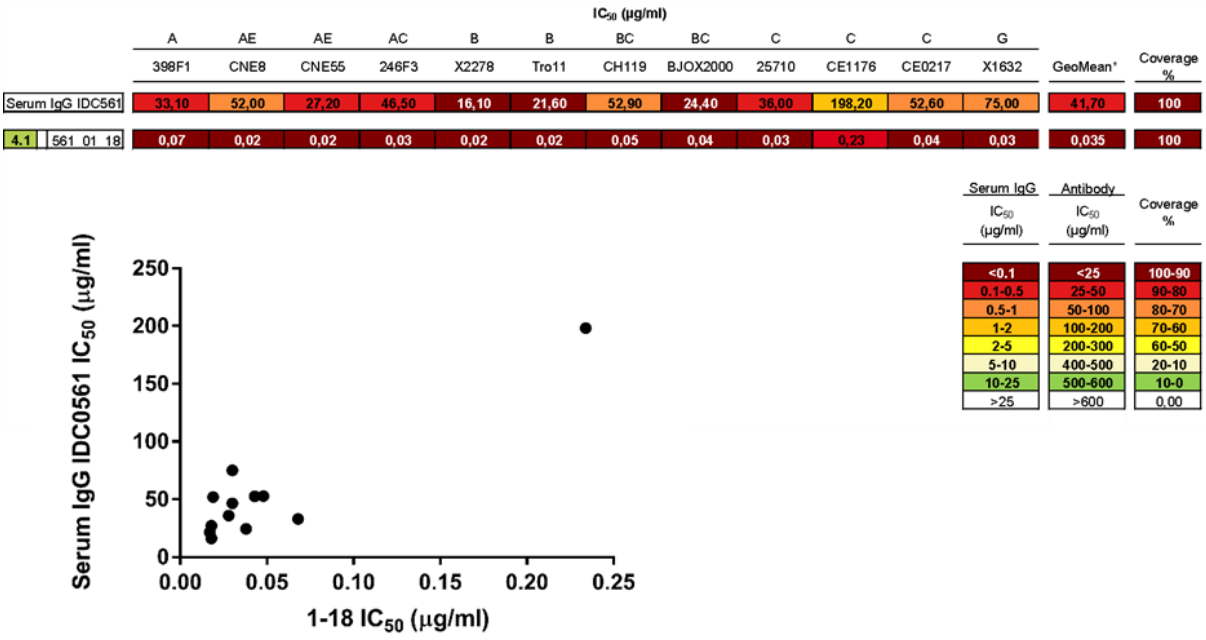


Figure 19. Comparison of serum neutralization and neutralization profile of bNAb 1-18

Above: Testing of neutralization characteristics of serum IgG of elite neutralizer IDC561 and bNAb 1-18 in the global panel. Respectively, IC_{50} values are shown for the 12 virus strains. The respective clade is written above virus strain names. GeoMean indicates the geometric mean of IC_{50} values of neutralized virus strains. Encoding colors for bNAb 1-18 are the same as for Table 4. Below: Correlation of serum IgG neutralization of IDC561 vs. neutralization characteristics of bNAb 1-18. The respective IC50 values for each strain were correlated. (p = 0.0298) (adapted from Schommers *et al.*, 2020)

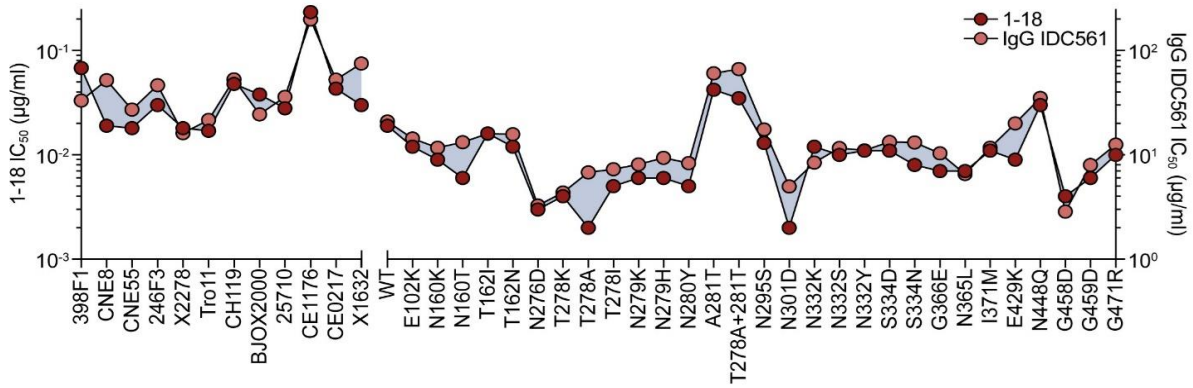


Figure 20. Serum neutralization activity and neutralization activity against 42 pseudoviruses

On the left side, IC_{50} values between serum and bNAb 1-18 are compared for the 12-virus strain global panel; on the right side, IC_{50} values are compared for 30 YU2 mutant pseudoviruses. The x-axis depicts the respective tested virus. ($r = 0.93$, $p < 0.0001$) (adapted from Schommers *et al*., 2020)

3.8 The unique CDRH1 of bNAb 1-18 is crucial for its neutralizing activity

In Chapter 3.3 it was found that antibody 1-18 is highly potent and broadly neutralizing. In order to evaluate the influence of sequence characteristics on the neutralization activity of bNAb 1-18 as described in Chapter 3.4, we engineered a variant lacking the six amino acid insertion (1-18ΔINS). Following, we assessed its binding capacity in ELISA and its neutralization activity against the 12 virus-strain global panel. Subsequently, we evaluated whether the six amino acid insertion removal significantly changed the neutralization activity by calculating the Wilcoxon matched-pairs signed-ranks test.

Removal of the six amino acid insertion did not affect the binding capacity to $BG505_{SOSIP.664}$ compared to the original antibody (Figure 21) ($p = 0.38$). However, to $YU2_{gp140}$, the binding capacity of the variant lacking the six amino acid insertion was slightly higher than that of the original antibody ($p = 0.0391$).

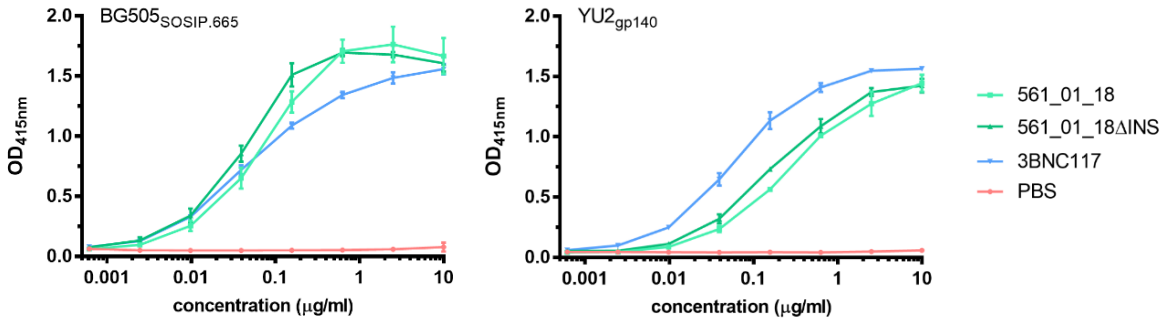


Figure 21. Binding capacity of 1-18 and 1-18ΔINS

On the left and right side, respective ELISA binding curves to $BG505_{SOSIP.664}$ and $YU2_{gp140}$ are shown. PBS is displayed as a negative control and the bNAb 3BNC117 as a positive control. All antibodies were tested in duplicates. Symbols show means, and error bars indicate standard deviation.

In terms of neutralization activity, removing the six amino acid insertion significantly reduced the neutralization activity (1-18Δins: GeoMean IC_{50} 0.114 µg/mL; 1-18: GeoMean IC_{50} 0.035 µg/mL, p = 0.012). To determine whether the six amino acid insertion was the only contributor to 1-18's superior neutralizing activity compared to other subclones, 1-18ΔINS was compared with member 08-52 of subclone 4.2. This subclone was phylogenetic related, lacked the insertion in the CDRH1, and had the same CDR3 length. 1-18ΔINS could still neutralize pseudovirus X1632, while bNAb 08-52 could not (Table 6). Likely, the six amino acid insertion was not the only feature that led to broad and potent neutralizing activity of 1-18.

In conclusion, removing the six amino acid insertion in 1-18 slightly increased the binding capacity to $YU2_{gp140}$ and decreased its neutralization activity. We propose that the six amino acid insertion in the CDRH1 is a crucial element for the exceptionally broad and potent neutralization activity of bNAb 1-18. However, 1-18ΔINS still exhibit elite neutralization characteristics, indicating that other sequence features also contribute to its neutralization activity.

Table 6. Neutralizing activity compared between bNAb 1-18 and engineered variant of 1-18 without six amino acid insertion in CDRH1

The motif ^{25}DDDPYTDDD^{33} was changed into ^{25}DD------^{27}. For comparison, the related subclone 4.2 (no six amino insertion and same CDRH3 length) is also depicted. The neutralizing activity was tested in the 12 virus-strain global panel. Geometric Mean was calculated from the IC_{50} values of neutralized viruses. Coverage determines how many out of twelve viruses were neutralized. (adapted from Schommers *et al.*)[88]

		IC_{50} (µg/ml)													
Clone	Antibody	398F1	CNE8	CNE55	246F3	X2278	Tro11	CH119	BJOX2000	25710	CE1176	CE0217	X1632	GeoMean*	Coverage %
4.1	561_01_18	0,07	0,02	0,02	0,03	0,02	0,02	0,05	0,04	0,03	0,23	0,04	0,03	0,035	100
(4.1)	561_01_18_ΔINS	0,97	0,04	0,11	0,13	0,03	0,03	0,03	<0,01	0,10	1,64	0,85	0,30	0,114	100
4.2	561_08_52	0,29	0,04	0,04	0,10	0,03	0,05	0,05	0,04	0,13	0,45	0,05	>25	0,074	92

IC_{50} (µg/ml)	<0.1	0.1-0.5	0.5-1	1-2	2-5	5-10	10-25	>25
Coverage (%)	100-90	90-80	80-70	70-60	60-50	20-10	10-0	0,00

4 Discussion

4.1 bNAbs as a new treatment and prevention approach

Monoclonal antibodies have been successfully implemented as standard treatment options in cancer therapy. Antibody therapy have also been developed against infectious diseases such as RSV-, Clostridium difficile- and Ebola infection.[133] Just recently, existing research pipelines for bNAb identification against HIV-1 and Ebola virus have been successfully adapted to rapidly identify and develop monoclonal antibodies as a treatment option against SARS-CoV-2.[134,135]

However, to date, no antibody therapy exists for HIV-1 infected patients, even though such therapies could offer several advantages over regular antiretroviral therapies (ART). HIV-1

reactive bNAbs could have better half-lives than ART, potentially have lesser long-term side effects, and have been shown to enhance the autologous immune reaction against HIV-1.[136] The implementation of HIV-1 neutralizing antibodies for therapy would require specific characteristics such as safety, a favorable pharmacokinetic profile, and both broad and highly potent neutralizing activity to target the diversity of HIV-1 and limit viral escape effectively.[88] While existing bNAbs were found to be safe and have favorable pharmacokinetic profiles,[108,109,111,137] the limitation of viral escape is the major hurdle for clinical practice. Monotherapy with single bNAbs in HIV-1 infected individuals leads to rapid viral escape after a few weeks.[137–140] For example, in individuals who underwent ART treatment interruption and were treated with the CD4bs bNAb VRC01, viral rebound occurred after 4 to 6 weeks, despite a sustained serum antibody concentration of 50 µg/ml.[140] The more potent CD4bs antibody 3BNC117 led after four infusions to a median viral suppression of 10 weeks.[138] Consistent with these results, Caskey *et al*. showed that a single infusion of V3 glycan bNab 10-1074 sustained viral suppression for 3 to 4 weeks, before viral resistances developed.[137]

Combination therapies with bNAbs targeting different epitopes have been tested to address this problem. Using the combination of 3BNC117 and 10-1074, the ART free time could be significantly extended from 3 to 10 weeks up to 20 to 42 weeks.[112,141] Also, in a group of ART-naïve HIV-1 infected individuals, combination therapy resulted in a prolonged viral suppression of 41.7 weeks.[141] However, some individuals did not respond to the bNAb combitherapy because of preexisting dual bNAb-resistance at the baseline. In bNAb sensitive individuals the ultimate viral rebound might be related to decreasing bNAb serum levels. Gaebler *et al*. determined a level above 10 µg ml^{-1} bNAb serum concentration necessary to maintain viral suppression.[112] Therefore, reliable determination of preexisting viral resistances by confidential prescreenings, choosing corresponding bNAb combinations, and maintaining high serum levels remain to be the challenges unsolved for long-term viral suppression.

Moreover, bNabs are being evaluated as a preventive measure. In a large-scale study with participants at high risk for acquiring HIV-1 infections, repeated application of the CD4bs bNAb VRC01 were shown to safely and effectively reduce the risk of acquiring VRC01-sensitive strains.[121] This study demonstrated that bNAbs are generally safe and suitable for prevention strategies. However, VRC01 did not prevent HIV-1 acquisition more effectively than a placebo, indicating that bNAb monotherapy is insufficient to offer protection against the remarkable diversity of HIV-1.[108,137,139] Consequently, broader and more potent combinations of antibodies for prevention need to be tested.

Therefore, new effective bNAbs with novel neutralization profiles for combination therapies and effective restriction of viral escape are needed.

4.2 Single-cell methods are effective for bNAb identification

Several approaches and protocols have been developed for the identification of B cell repertoires and generation of monoclonal antibodies.[83] These methods include the phage display technology, bait-protein specific single cell sorting and antibody cloning, and supernatant screening of single B cell cultures or immortalized B cells.[83,101,127,142–144]

The phage display technology was developed in 1985 by Smith *et al*. This technique involves the display of antibody repertoires in phage libraries, allowing the generation of large amounts of distinct antibodies relatively quickly.[142,145] However, the disadvantage of this method is that combined heavy and light chains of antibodies are randomly paired and do not display the natural antibody repertoire.[142]

When improved single-cell techniques were developed, second-generation bNAbs with much greater breadth and potency could be isolated.[12] The single-cell cloning technique and the screening of B cell supernatants from single-cell cultures or immortalized cells were both proven effective in isolating bNAbs.[89,101,124,144] However, unbiased single B cell cultivation has its disadvantages. Antigen-specific B cells normally comprise less than 0.5% of the memory B cell compartment.[83,124] Therefore, it is necessary to cultivate and screen thousands of B cells to identify antigen-specific neutralizing antibodies. This process is time- and cost-consuming, because cultivation of B cells takes several weeks and every supernatant has to be tested for neutralizing activity.[146] Another problem is that B cells are cultured in pairs, which bears the risk of incorrect light and heavy chain pairing. For example, for the isolation of antibody SF12, which targets the silent face, 44,000 memory B cells had been cultivated. Correct pairing of the heavy and light chain had to be reconfirmed by single B cell Env bait sorting data.[89] Therefore, antigen-specific single B cell sorting can be preferable over B cell culture to isolate novel bNAbs.

In our study, we confirmed that HIV-1 specific single B cell sorting can effectively isolate a naturally aroused broadly and potent neutralizing B cell clone. Newly established high-throughput pipelines with optimized PCR coverage has made single B cell cloning from single cell sorting more efficient.[83] Thus, we are now able to rapidly isolate antigen-specific neutralizing antibodies against a wide range of viruses like HIV-1 and, most recently, SARS-CoV-2.[147,148]

While single-cell sorting is still highly laborious and allows to analyze only several hundred single B cells, the novel LIBRA-sequencing technique allows for the analysis of several hundred thousand of antigen-binding B cells. LIBRA-seq combines antigen-specific and parallel paired heavy and light chain sequencing (LIBRA-seq) by using barcoded antigens. By using LIBRA-seq members of the VRC01 lineage could identified.[149] Whether this technique enables a less cost- and work-intensive approach to identify novel bNAbs, remains to be determined.

4.3 Identification of bNAbs by B cell analysis

To detect broadly neutralizing antibodies from IDC561, we first isolated HIV-1 Env specific B cells via FACS, amplified the V_H gene segments of the isolated B cells, and used this information to produce patient-derived monoclonal antibodies. For amplification, we used newly developed advanced primer sets.[132] The primer binding sites of the novel primer sets are located in the leader sequences of respective heavy and light chain sequences, which enables unraveling full-length sequences from the start of the FWR1 region. Thus, this primer set was superior to other primer sets in the amplification of highly mutated sequences given that the leader is mostly unchanged even for V_H genes. The novel primer sets could also be modified for the use in antibody cloning. In the previously utilized cloning technique with the 'Tiller' primer set, primers for attaching overhangs for subsequent cloning had to be individually chosen for each heavy and light chain, depending on the respective V- and J-gene. Primer mixes with multiple primers could not be used due to the length of the primers, resulting in labor-intensive primer picking for each antibody sequence. In the modified new primer set, the needed overhangs for cloning were combined with our optimized primer set, shifting the side of attachment to the leader sequence, and therefore circumventing the need to pick individual V- or J-gene specific primers for cloning PCR.[83]

In comparison to previously published primer sets for bNAb isolation, primers in previous studies align more downstream. The primers from the 'Tiler primer set' extend in the first round of PCR by three amino acids into the FWR1, and in the cloning PCR, they extend by even six amino acids.[143] This extension results in mutations introduced at the primer-binding sites, rendering these primers additionally less effective in amplifying highly mutated variable regions. For example, the here described bNAb 1-18 is also highly mutated at the beginning of the FWR1. Due to the use of established primer sets, three amino acids would have been reverted to the germline sequence, which was avoided by our optimized primers. In comparison, when established primer sets were used to generate known bNAbs such as VRC01, 3BNC117, and 10-1074,[85,87,150] the resulting antibody sequences did not contain

mutations at the beginning of the FWR1. The reason might be that this region only reflects the sequence of the used primer sets. Thus, for some bNAbs it remains uncertain whether an optimized primer set would have had beneficial effects on the binding and neutralizing capacities of some bNAbs.

For a long time, mutations in the FWRs were thought to be secondary to antibody affinity, because CDRs are the regions that are mainly important for antigen binding. The main task of the FWRs is to frame the CDRs and stabilize the variable domain. Often, antibodies with mutations in the FWRs are selected against because they can destabilize the overall structure of the variable domain. Therefore, somatic mutations are preferentially found in CDRs, where they alter the antibody binding site without affecting the structure.[151] However, when the impact of mutations in the FWRs for bNAbs was investigated, it was found that the reversion of mutations lowered binding and neutralization capacities. Even if only non-antigen-contacting mutations in FWRs were reverted to germline in VRC01, there was a significant reduction in neutralization activity.[65] In the case of 1-18, this suggests that although the mutated residues may not contact $BG505_{SOSIP.664}$ directly as revealed in structural analysis, these mutations could still be crucial for the neutralization activity of 1-18.

In this thesis, we further compared clonal HIV-1 reactive B cell sequences with non-clonal HIV-1 Env reactive B cell sequences, revealing a higher rate of somatic mutations and more extended CDRH3 regions in clonally expanded B cells. B cell clones with high levels of somatic mutations and extended CDRH3 region might have stronger binding capacities to HIV-1 and are therefore stimulated to expand.[84]

In line with this, known HIV-1 reactive bNAbs often carry rare sequential features such as high levels of somatic hypermutation, large insertions or deletions, and expanded CDRH3s.[102] However, when Griffith and McCoy compared bNAbs by their somatic hypermutation and CDRH3 length, higher levels of somatic mutations and longer CDRH3s in bNAbs were not necessarily correlated with a better neutralization activity.[84] On the other hand, Kreer *et al*. showed that the more improbable the generation of a bNAb is, calculated by the probability of long CDRH3s and somatic hypermutations, the better is its neutralization activity.[152] In our sequence analysis, the most mutated B cell clone (mean V_H-gene identity of 67.2%) had no neutralizing activity, and just recently, a bNAb with a relatively low level of somatic mutations and comparable neutralization activity to VRC01 had been found (V_H-gene germline identity 85 – 88%).[153] However, the isolated bNAb 1-18, having with an extraordinary neutralization activity, carried the feature of being highly mutated (mean V_H-gene identity of 67.5%). Therefore, it is not possible yet to predict neutralization activity by analyzing sequence

features, but CDRH3 length and somatic hypermutation are features which can lead to advanced neutralization activity.

In conclusion, optimized primer sites are advantageous over established primer sets. Moreover, it is not possible yet to predict the neutralizing activity of a monoclonal bNAb by sequence analysis, comparing features such as level of somatic hypermutation, length of CDRH3, or V_H-gene. Therefore, it is crucial to thoroughly analyze the HIV-1 reactive B cell repertoire to find the corresponding antibodies.

4.4 1-18 as novel CD4bs bNAb for clinical use

4.4.1 The CD4bs as preferable epitope

BNAbs target distinct epitopes on the Env trimer of HIV-1. These epitopes include the CD4bs, the V1/V2 loop, the V3 glycan patch, the gp120gp41 interface, and the membrane-proximal external region (MPER). When different bNAbs are compared regarding their neutralization profile, some distinct patterns were found in previous studies. HIV-1 reactive, V1/V2 targeting bNAbs have great potency but limited breadth, MPER antibodies have the highest breadth but low potency, and only bNAbs that target the CD4bs have a favorable combination of expanded breadth and high potency. Moreover, the CD4bs is of particular interest because it is relatively conserved to maintain interaction with the CD4 receptor, and therefore has a lower ease of escape.[96] When a triple combitherapy of the V3 glycan antibody PGT121, V1/V2 loop antibody PGDM1400, and CD4bs antibody VRC07-523LS was tested in viremic HIV-1 infected individuals, a rapid viral escape from PGDM1400 and PGT121 occurred, while rebound viruses were still susceptible to VRC07-523LS.[113] Therefore, the CD4bs is a preferable target for bNAbs.

VRC01-class CD4bs bNAbs, which are V_H1-2 derived, have high levels of somatic mutations and a short CDRL3 of 5 amino acids. They are among the most clinically advanced bNAbs.[154] Non-VRC01 class bNAbs include 8ANC195 and CH235.12, which are also highly mutated, are V_H1-46 derived, and have a normal CDRL3 length but are less potent and broad (GeoMean 0.648 µg/ml, coverage: 90%, and GeoMean 1.381 ug/ml, coverage: 77%).[104] Features of our isolated bNAb 1-18 like the V_H1-46 gene, somatic hypermutation, and the combination of high breadth and potency suggested the CD4bs as the targeted epitope. The neutralization fingerprint, which can predict epitope binding sites, supported this hypothesis by identifying a CD4bs activity in IDC561s serum. The CD4bs as targeted epitope of 1-18 was later revealed in a cryo-EM structure.[88] Therefore, 1-18 is the broadest and most potent non-VRC01-class

bNAb having features of V_H1-46 gene usage, high level of somatic mutations, and normal CDRL3 length.

Compared to VRC01-class bNAbs, of which some are already in advanced clinical testing, 1-18 exceeds the neutralization activity of all VRC01-class bNAbs and rivals N6s, that has a comparable neutralization activity (1-18: Geometric Mean IC_{50} 0.048 µg/ml, coverage 96.6%; N6: Geometric Mean IC_{50} 0.086 µg/ml, coverage 97.5%). When tested against primary virus isolates, which are more relevant for future clinical use than pseudoviruses, 1-18 could even exceed the neutralization activity of N6 (1-18: Geometric Mean IC_{50} 0.56 µg/ml, coverage 96%; N6: Geometric Mean IC_{50} 1.32 µg/ml, coverage 94.1%).[88]

4.4.2 Sequence features and neutralization of 1-18

The reduced neutralization capacity of 1-18Δins and 2-12 (a clone member without six amino acid insertion in the CDRH1) in comparison to 1-18, suggested that the insertion is crucial for neutralization. The insertion is part of a mainly negatively charged pattern by the dominance of aspartic acid (^{25}DDDPYTDDD^{33}). Aspartic acid is able to form salt bridges with positively charged proteins, which are the strongest non-covalent interactions between proteins. Gp120 is positively charged, which makes strong interactions between the Asp-enriched motif and the gp120 protomer imaginable. In a cryo-EM structure, it was finally confirmed that four Asp residues form coordinated interactions around the highly conserved Env residue $K207_{gp120}$.[88] Adjacent to the residue lies a positively charged patch on the V3 loop, which might have driven the selection of the other Asp residues.

Even when the relevance of the six amino acid insertion was confirmed, the neutralization activity was partly preserved in 1-18Δins and subclones without insertion. Compared to the subclones 4.2–4.4, 1-18Δins (modified member of subclone 4.1) still neutralized the clade G pseudovirus X1632. Structural analysis revealed that the amino acid F54HC of 1-18 mimics the CD4 receptor and targets the Phe43 pocket on HIV-1 Env, which could be a possible explanation for the better neutralization activity of 1-18Δins.[88] Among the subclones 4.2–4.4, the neutralization activity of subclone 4.2 was superior to that of subclones 4.3 and 4.4. Structural analysis later revealed that ^{28}GLD^{30} was a contact side in the light chain of 1-18, a pattern that was also present in subclone 4.2. This characteristic might have contributed to its better neutralization compared to subclones 4.3 and 4.4. However, which exact residues are responsible for the retained neutralization activity in subclones 4.2–4.4 remain unclear.

Compared to the other CD4bs bNAbs, 1-18 combines favorable features of known CD4bs bNAbs with additional favorable features. 1-18 holds an aromatic residue that mimics Phe43

of CD4 and targets the Phe43gp120 pocket.[88] This feature is also known for N6. Additionally, 1-18 contacts the adjacent gp120 protomer, as seen in 3BNC117. However, the buried surface area of 1-18 is larger than that of VRC01 and 3BNC117 via its six amino acid insertion in CDRH1, which can explain 1-18's exceptional breadth and potency.[88]

4.4.3 1-18 for clinical use

Essential features for the use of bNAbs in clinic are broadly and potent neutralizing activity, a favorable pharmacokinetic profile, the lack of autoreactivity, and most importantly, restriction of viral escape. In the following, I will evaluate antibody 1-18 for these criteria.

The neutralizing activity of 1-18 is superior in the 119-strain multiclade panel compared to the most known CD4bs, including VRC01 and 3BNC117. In terms of pharmacokinetics, the decline of 1-18 in humanized mice was slower than that of VRC01-class antibodies, and more comparable to 10-1074, which is the bNAb with the longest half-life in clinical testing. [155,156] Additionally, no autoreactivity of 1-18 was detected when tested against Hep-2 cells.[88]

Moreover, the superior neutralizing activity of 1-18 was also confirmed *in vivo*. In further experiments of our study, treatment of HIV-1 infected humanized mice with 1-18 resulted in a viral suppression over a period of 8 weeks with an average drop of viral load by 1.7 log10. In contrast, monotherapy with 3BNC117 or VRC01 resulted in viral rebound in most animals within the first two weeks and a far less viral load reduction (average drop 0.5 log10).[88] When 1-18 was applied to VRC01- or 3BNC117-pretreated humanized mice with viral rebound, viral suppression could be again obtained by 1-18, indicating that 1-18 could be an important combination partner for VRC01-class antibodies, which treatment and preventing success is limited due viral escape.[88,112,121]

All in all, we isolated a broadly neutralizing CD4bs bNAb, which showed to be a promising candidate for future treatment options. 1-18 combines needed requirements for the use in the clinic with i) a novel neutralizing profile, ii) the lack of autoreactivity, iii) a long half-life, and iv) the ability of suppression of viral escape.

4.5 Outlook

Neutralization activity and viral escape characteristics of bNAbs in *in vitro* studies and humanized mice were suitable to translate into treatment results of SHIV in non-human primates *in vivo* and into clinical studies.[119,157,158] The same viral escape which was observed in animal models using monotherapy was seen in clinical studies.[108,137,140] Therefore,

combination therapies of bNAbs were applied to overcome viral escape, resulting in prolonged viral suppression.[106,113]

Thus, for further evaluation of bNAb 1-18 future clinical studies are needed. The immune system of humanized mice, which was the animal model for testing 1-18 so far, does not yet reflect the human immune system in the same manner.[159] However, the neutralization activity in humanized mice depends also on the Fc receptor activity. Viral suppression in HIV-1 infected humanized mice was enhanced respective diminished using either an activating or inhibitory FcγR variant of the bNAbs. To finally evaluate the effect of 1-18 on HIV-1 infection studies in humans are needed. Current studies that test bNAb combinations are based on bNAbs targeting different epitopes.[112,113,141] Synergistic effects from overlapping epitopes are not expected, but as 1-18 showed another escape mechanism as VRC01, combinations of CD4bs bNAbs with 1-18 could be beneficial. Of note, the calculated breadth in a combination with 3BNC117 or N6 *in vitro* was 99%.[88]

In terms of prevention, our study did not yet evaluate the potential for protection against HIV-1. In the AMP trial it was seen that protection with VRC01 is limited due to preexisting viral resistance against administered bNAb. Because of its broad neutralization profile, 1-18 could be more effective for protection against the acquirement of HIV-1 infection.

For sufficient protection and restriction of viral escape a sufficient serum antibody concentration in treated individuals is essential. A successful treatment requires a minimal maintained concentration of 10 µg/ml.[112] Therefore, bNAbs have been engineered to extend their half-life. By exchanging two amino acids in the Fc domain, the affinity to the neonatal Fc receptor increases, and antibody degradation is reduced.[160] The half-life of bNAbs could be extended up to five times, for example, for VRC01 from 15 to 71 days.[109] For future applications, similar modifications for 1-18 are conceivable.

Finally, 1-18 is a highly promising antibody for future clinical use.

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6 Appendix

6.1 List of Figures

6.2 List of Tables

7 Publication of results

Results have been previously published in *Cell*:

Restriction of HIV-1 Escape by a Highly Broad and Potent Neutralizing Antibody

Schommers, P.*; Gruell, H.*; Abernathy, M. E.*; **Tran, M. K.**; Dingens, A. S.; Gristick, H. B.; Barnes, C. O.; Schoofs, T.; Schlotz, M.; Vanshylla, K.; Kreer, C.; Weiland, D.; Holtick, U.; Scheid, C.; Valter, M. M.; van Gils, M. J.; Sanders, R. W.; Vehreschild, J. J.; Cornely, O. A.; Lehmann, C.; Fatkenheuer, G.; Seaman, M. S.; Bloom, J. D.; Bjorkman, P. J.; Klein, F.

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