HIV-1, how llamas help us fight the AIDS pandemic

HIV-1, hoe lama's ons helpen de AIDS pandemie te bestrijden

(met een samenvatting in het Nederlands)

Proefschrift

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door

Nika Mindy Strokappe

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Promotoren: Prof. dr. ir. C.T. Verrips
Prof. dr. J.J.W. de Haard

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Hello?

I know you're out there...

I can hear you metabolizing oxygen and expelling carbon dioxide.

Jim Parsons (as Sheldon, Big bang theory, 2010)

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Chapter 1

Introduction

HIV-1 background

In 1981 the first cases of patients with symptoms of infections by all kinds of opportunistic pathogens, were described by Michael Gottlieb[1]. He found that the immune system in these patients was severely compromised, due to the disappearance of their helper T-Lymphocytes. This phenomenon was named Acquired ImmunoDeficiency Syndrome (AIDS). Two years later, the causative agent was discovered by Françoice Barré-Sinoussi *et al.*[2] and was given the name Lymphadenopathy-Associated Virus (LAV). Their discovery was published in May 1983 in Science. In that same Science issue, the team of Robbert Gallo published what they thought was causing AIDS and named it Human T Lymphotropic Virus type III (HTLV III)[3]. In 1986, a far less virulent and less infective variant of HIV was identified and named LAVII. When it was confirmed that LAV and HTLV III were identical, the virus was renamed Human Immunodeficiency Virus type-1 (HIV-1) and LAVII was renamed to HIV-2. HIV-1 is by far the most problematic of the two types and is spread around the world, whereas HIV-2 is mainly confined to West-Africa[4].

HIV-1 is thought to be originated from Simian Immunodeficiency Viruses (SIV) and more specifically from the ones found in chimpanzees. The jump from apes to humans is believed to have occurred, somewhere in West central Africa, in the beginning of the 20th century. This is thought to have happened on at least three independent occasions, giving rise to the 3 different groups of HIV-1, M (main), O (outlier) and N (non-M, non-O). Group M viruses are by far the most common and are divided into subgroups A to K and a number of circulating recombinant forms (CRFs). Subgroup, or clade, C and A strains are the most prevalent, especially in Africa[5]. From Africa, a clade B virus was most likely brought to Haiti around 1966. From there it travelled further around the world from 1969 onwards, making clade B the most prevalent outside of Africa[6]. The two oldest known HIV-1 sequences, ZR59 and DRC60, were obtained from patient material from Kinshasa from 1959 and 1960 respectively[7, 8]. The genetic diversity of the Env gene between both samples was 11.7%. According to our current grouping, ZR59 belongs to the clade D viruses and DRC60 to clade A. These data shows that even back in the 60's the genetic background of HIV-1 was rather diverse, also indicating that the last common ancestor occurred in the beginning of the 20th century[8].

To date an estimated 34 million people are living with an HIV-1 infection. The number of people newly infected with HIV-1 in 2010 is believed to be about 2.6 million. Due to more and better education about preventing HIV-1 infection, this number has decreased by over 20% since 1997, when about 3.2 million people were newly infected. The number of AIDS related deaths has also significantly decreased. After a peak around 2005 with an average of 2.2 million deaths annually, this number has dropped down to 1.8 million in 2010. Eastern Europe, central Asia and central and south America, are the only parts of the world in which the number of people dying from AIDS related causes is still increasing. Although the numbers of new infections and deaths are both declining, the number of people living with HIV-1, however is still steadily rising. The main reason for this phenomenon is that

treatments are improving and the access to treatments is rising, increasing the life expectancy of infected individuals. It is estimated that since 1995, around 2.5 million deaths have been averted in the low and middle income countries, due to the increased access of these patients to anti-retroviral therapies. Sub-Saharan Africa, where HIV originated from, is the region that is still most affected by HIV, with 68% of all people living with HIV and 70% of the new infections and 72% of all AIDS related deaths occurring there[9, 10]. An overview of the distribution of HIV and the different clades is shown in figure 1.

After more than 30 years of research, still no vaccine or cure has been found against HIV-1 infection. Treatment is possible, but is expensive and has serious side effects. The best options in the fight against HIV-1 would be either an effective vaccine or a preventative method that is acceptable for use by the people who need it.

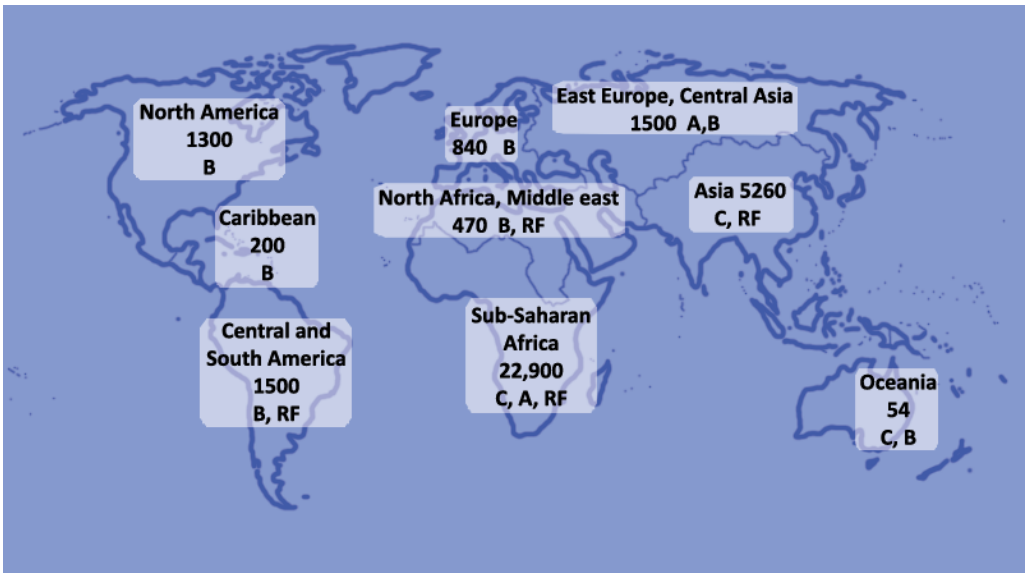
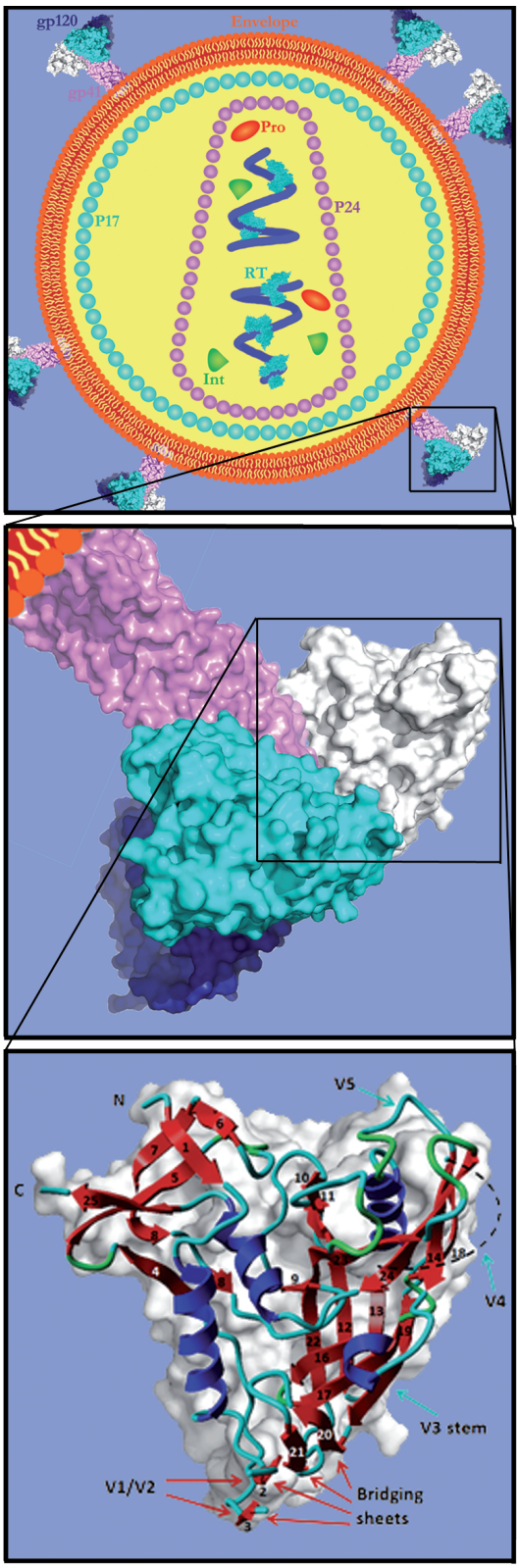


Figure 1: Global distribution of HIV-1. Number of HIV-1 infected people per WHO region[10], given in thousands, together with the most prevalent clades per region, RF stands for recombinant forms[11].

Virus structure

HIV-1 is a member of the family of retroviridae (retroviruses) and more specifically of the lentivirus genus. Retroviruses are enveloped viruses that carry their genetic information in the form of a single stranded RNA (ssRNA) molecule. Upon infection the ssRNA is transcribed into double stranded DNA (dsDNA) by reverse transcriptase after which the dsDNA is integrated into the host's genome. The group of the lentiviruses is characterized by a long latency period between infection and the onset of disease, which generally is between eight and twelve years in the case of HIV-1[12].

A schematic representation of an HIV virus particle is given in figure 2a. The HIV particle consists of two copies of ssRNA and the proteins reverse transcriptase, protease and integrase, in a capsid composed of p24 proteins. This capsid is surrounded by a matrix



composed of p17. The outer layer of the virion consists of a host cell derived lipid bilayer, known as the envelop. Within the envelop, trimeric protein dimers are residing, named the envelop spikes, or Env's. Functional Env is made up of three copies of gp41, which are inserted in the bilayer by their trans-membrane regions, with on top, three copies of gp120 (figure 2b). Figure 2c shows a representation of a single gp120 molecule in which its variable domains marked V1-V5 and its beta strands numbered 1-25. V1/V2 together with beta strand 20 and 21 form the so-called bridging sheets, which are very important during infection. Gp41 and gp120 are cleaved from a single precursor molecule, gp160, by the viral protein, protease. On average, there are only about 14 complete Env spikes on present on a virion[13]. Although some of the spikes are clustered, the distance between the functional spikes is usually more than 15 nm,

Figure 2: The structure of HIV-1. a) Schematic representation of an intact HIV-1 particle. b) Structure of a functional envelope spike. In white, light blue and dark blue are the three gp120 subunits (PDB ID: 3DNN)[20]. In lavender is a representation of the three gp41 subunits (PDB ID: 2X7R)[21]. As there is no crystal structure known of an intact trimer, this trimer is made up the crystal structure of liganded gp120, superimposed onto an electron microscopy picture of the entire unliganded trimer. c) A cartoon of a single liganded gp120 molecule, shown with the surface of gp120 in the background. There are five variable regions shown designated V1-V5. As some of the variable regions are very flexible, they are not (fully) shown V1, V2 and V3 and V5 are shortened and V4 is removed (V4 is marked with a dashed line). The beta strands are numbered 1-25. V1/V2 together with beta strand 20 and 21 form the bridging sheets.

which is the distance of the two binding domains of conventional antibodies[14]. Next to the functional Env, it is believed that there are four types of other "spikes" present on the envelop; a non-functional conformation of Env, uncleaved gp160 precursor proteins, monomeric gp41-gp120 dimers and gp41 stumps which have shedded the gp120 molecules[15]. Functional Env is the only target for the host's neutralizing antibodies. There are multiple mechanisms by which HIV-1 avoids the generation of these antibodies or, when they have been generated, escapes from these antibodies. The first evasive strategy is that the gp120 part of Env is heavily glycosylated. The glycans contribute up to 50% of gp120's total molecular weight of approximately 120kDa[16] and cover most of the potentially neutralizing epitopes. The glycan canopy is present in such a way that the appropriate receptors are capable of binding their epitope, but it sterically hinders the binding of any antibody which is slightly bigger or binds a slightly different epitope compared to the receptor[17]. The second strategy is that gp120 physically shields its vulnerable sites, by conformational masking[18], revealing them only after binding of the appropriate cellular receptors. Third, HIV-1 has a high mutation rate, generating an enormous diversity in circulating virions. Actually, only 1 in 1,000 to 10,000 viral particles seems to be able to infect a target cell, the others are defective[19]. The high amount of non-functional particles acts as a decoy for the host's antibodies. This low proportion of functional virions is not an evolutionary disadvantage once the patient has an established infection[17], as viral genetic code is integrated into the host's genome, ensuring a continues supply of new particles. The infection rate of new cells is slow at this stage, but over the years the virus slowly gains territory.

Method of infection

Transmission of HIV-1 primarily occurs by sexual contact[22]. Other modes of transmission are sharing needles, blood transfusions and the transference from mother to child.

The first step of infection by HIV-1 is the binding of viral Env to the cellular CD4 receptor, which is present on macrophages and on a subgroup of T-lymphocytes. There is some evidence, that DC-SIGN, a lectin present on the cell surface of dentritic cells and macrophages, first binds HIV-1 and subsequently transfers the virus to its target cells[23]. However, binding of DC-SIGN will aid infection, but it is not an absolute necessity for infection[24], whereas CD4 binding is. Upon CD4 binding, Env undergoes a large conformational change (figure 3, step 1). The gp120 monomer rotates relatively to the gp41 stem[25] and the V1/V2 loop and the β20-β21 loop (bridging sheets) rearrange, exposing the V3 loop and together form the co-receptor binding site (figure 2c)[26]. Dependent on the stage of infection, the co-receptor site will be specific for either CCR5 or CXCR4. In the initial stages of HIV-1 infection the virus is always specific for the CCR5 receptor, later CXCR4 specific viruses will start to emerge. Binding of the co-receptor (figure 3, step 2) will induce another conformation in which gp41 will fully extend and anchor itself in the target cell membrane (figure 3, step 3). Subsequently gp41 will fold back onto itself, forming a bundle of six helices and initiating fusion of the virion and target cell membranes (figure 3, step

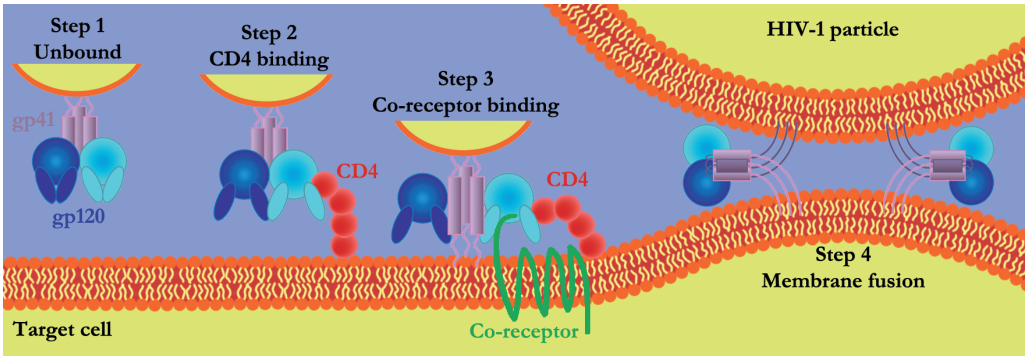


Figure 3: Binding and fusion of an HIV-1 particle with its target cell. Step 1) HIV-1 envelope spike in the unliganded conformation. Step 2) Binding of the envelope spike to the cellular CD4 receptor, the bridging sheets and V3 loop rearrange to reveal the co-receptor site. Step 3) binding of the co-receptor to the envelope spike, gp41 changes conformation and anchors itself into the target cell membrane. Step 4) gp41 further changes its conformation, pulling both membranes closer initiating membrane fusion.

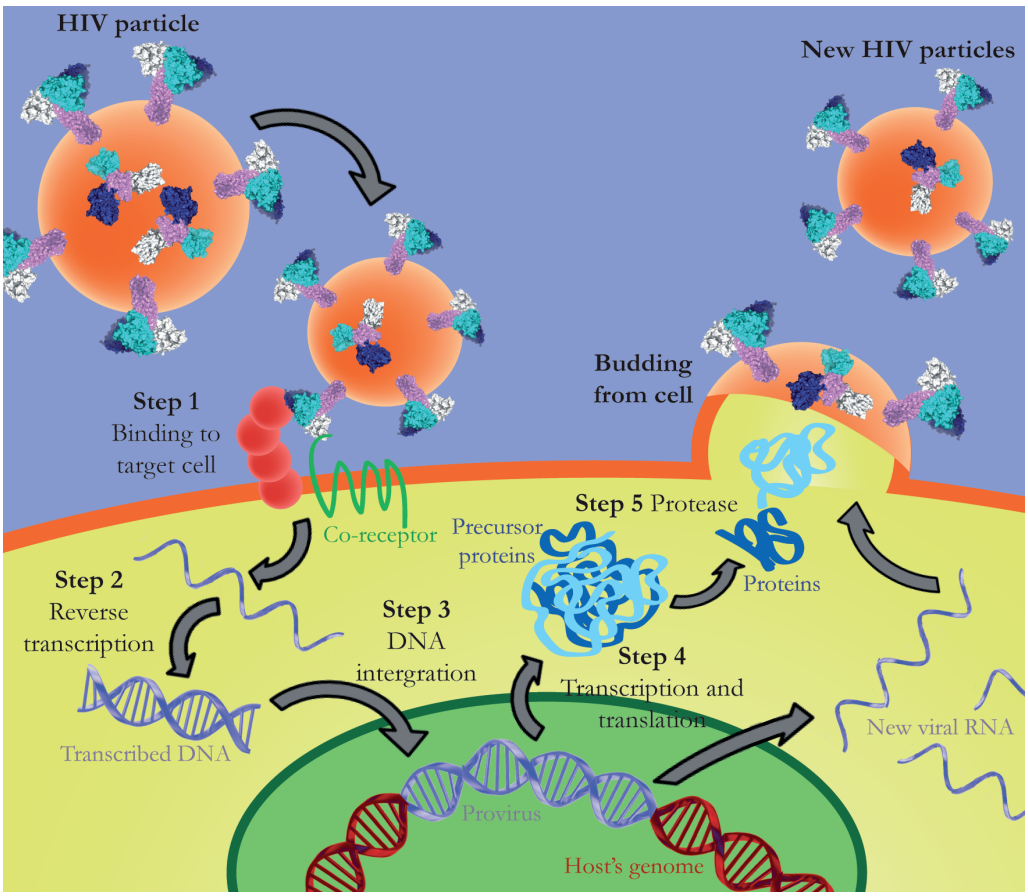


Figure 4: Replication cycle of HIV-1. Step 1) HIV-1 binding to the target cell membrane. Step 2) transcription of the viral genetic code into dsDNA by reverse transcriptase. Step 3) Intergration of the viral genome into the hosts genome by integrase. Step 4) Transcription and translation of the viral genome by the host's machinery. Step 5) Processing of the viral proteins by protease. Step 6) Assembling of new HIV-1 particles and budding of the newly assembled HIV-1 particles.

4). Drugs that target the virus during all these initial steps of infection are referred to as entry inhibitors (figure 4, step 1).

After membrane fusion, the matrix and capsid fall apart, releasing the HIV-1 ssRNA genome and the proteins from within the capsid. Reverse transcriptase will transcribe the viral ssRNA into double stranded cDNA (figure 4, step 2). This step of infection can be inhibited by two different types of inhibitors. The first are the Nucleoside (or nucleotide) analog Reverse Transciptase Inhibitors (NRTI's) which are analogs of the normal building blocks of DNA. They differ from normal nucleotides in such a way that they prevent the coupling of the next building block, stopping DNA transcription. The NRTI's were the first type of anti-retroviral drugs developed, but they have an adverse effect on normal DNA replication to some extend as well[27]. Furthermore there are the Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI's). They block transcription by binding to reverse transcriptase thereby preventing its function.

After the viral genetic information is processed into copy DNA, it is inserted into the host genome by the viral protein integrase. The resulting endogenous virus is referred to as provirus (figure 4, step 3). The provirus can be dormant for years, serving as a viral reservoir[28]. The class of Integrase inhibitors prevents this specific step in the viral replication cycle. After insertion, new virions can be produced, to that end the virus makes use of the host's RNA polymerase to transcribe copies of its DNA.

The ssRNA copies of the viral genome serve as a template for the translation of all the viral proteins. This translation is performed by the host's ribosomes and the products are proteins as well as polyproteins. The polyproteins get processed into the final viral proteins by the viral protease (figure 4, step 5). Protease is the last step in the replication cycle targeted by anti-retroviral drugs. These drugs are referred to as protease inhibitors. After all HIV-1 proteins have been produced, new HIV-1 particles are assembled at the host's cell membrane and bud of from the infected cell (figure 4, step 6).

Three to six weeks after infection with HIV-1, patients enter the acute phase of infection in which high viral titers are found throughout the body. At this stage, patients develop both a humoral as well as a cellular immune response against the virus which in turn contributes to the sharp drop in viral titers around the ninth week. The infection then enters a phase of "clinical latency" which is generally asymptomatic. During this phase, a patient's T-cell count gradually drops and the viral titers slowly rise. After an average of eight to twelve years, the T-cells are depleted below 500 cells/μl and the first symptoms start to emerge. From this moment, the viral titer in the blood starts to rise quickly. A patient is diagnosed with AIDS if the number of T-cells drops below 200 cells/μl. At this stage a patient is vulnerable to all kinds of opportunistic infections, which finally leads to death in most cases[12]. Of a small number of patients, the T-cell counts do not seem to decline after the acute phase and AIDS will not manifest. These individuals are referred to as long-term non-progressors and they are the source of many of the known broadly neutralizing monoclonal antibodies (mAbs)[29, 30].

Immunology

Innate immunity:

In the human body there are two distinct immune systems, the innate and the adaptive immune system. If a pathogen enters the body for the first time, it will first encounter the innate immune system. Innate immunity is characterized by its quick response (within minutes or hours) and it is active from the moment one is born. This system does not rely on memory, but on preprogrammed ways to detect and get rid of invaders.

The first barrier of the body is the almost impenetrable epithelial surface layer that covers the whole body. The cells of this barrier are secreting antimicrobial enzymes and peptides. If a pathogen manages to breach this barrier, it will encounter the macrophages and dendritic cells that are residing within this cell layer. Macrophages (Greek for large eaters) will chew up as much of the pathogens as they can handle and by doing so, get activated and launch an inflammatory response. Dendritic cells will "nibble" of the pathogens as well, but their main mode of action is to transport them to the lymph nodes where they present the pathogens to cells of the adaptive immune system. The cells of the innate immune system identify pathogens with a set of receptors that recognize molecules commonly found on, and characteristic for invaders, like lipopolysaccharide (LPS).

Next to cells, innate immunity also relies on a system of plasma proteins, called the complement system. Proteins of the complement system can either recognize pathogens by themselves or be directed to them by antibody-pathogen complexes. Once bound to the surface of a pathogen, these proteins, of which most possess enzymatic activity, will attract other proteins of the complement system and cleave them into two parts. The first part will remain bound to the pathogen, soon covering it largely (opsonizing). The second part will act on the nearby blood vessels, making them more permeable to antibodies, complement proteins and a whole battery of immune cells. The complement covered pathogens can be easily recognized by phagocytic cells, like the macrophages, which will "eat" them and display parts of them to the adaptive immune system.

When a macrophage gets activated by a pathogen, it will secrete all kinds of signaling molecules, mainly cytokines and chemokines. Most of these molecules act locally by increasing vascular permeabilization and by making the site of infection better accessible through the local destruction of tissue. Furthermore, they recruit other cells, like neutrophils, basophils and natural killer cells and they help with the activation of cells of the adaptive immune system. Some of these molecules also have systemic effects, like raising protein production and causing fever. During fever the body's temperature is raised to create an inhospitable environment for pathogens.

Usually a response from the innate immune system is enough to combat invaders. Even at this very moment there will be multiple sites in your body where this system is active, think of a small scratch, mosquito bite or a pimple. In case its response is not enough, the innate immune system will try to keep the pathogens under control until the adaptive immune system takes over[31].

Adaptive immunity:

Adaptive or acquired immunity is characterized by its specificity and slow response to first time invaders. It is being activated by the presence of a certain level of an antigen, which generally occurs after an invader escapes or overwhelms the innate immune system. Activation is aided by the signaling molecules released by the complement system and activated macrophages and dendritic cells. Upon activation it takes around four days before there is a measurable response and on average around 12 days until the response is of sufficient level to fend of the pathogen. The reason for this is that antigen specific B-Lymphocytes (B-cells) and T-Lymphocytes (T-cells) need to mature, proliferate and differentiate before they can effectively combat invaders.

Pathogen derived antigen is brought to the lymph nodes by antigen presenting cells, like dendritic cells. Here the antigen is presented to circulating naïve T cells. If a naïve T-cell is capable of binding the antigen, it will differentiate into either a cytotoxic or a helper T-cell.

Cytotoxic T-cells carry the CD8 receptor on their membranes and specifically kill cells that are infected by a pathogen. There is a specialized system by which cytotoxic T-cells cells are able to recognize infected cells. All cells in the body use a special receptor to present small peptides, derived from all proteins inside them, on their cell surface. If a cell becomes infected, it will therefore present peptides from the pathogen as well. If a cytotoxic T-cell recognizes these particular peptides, it will kill this cell by perforating its membrane and starting-up programmed cell death (apoptosis).

Helper T-cells have CD4 and CCR5 receptors on their cell membrane and "help" with the activation of macrophages and B-cells by producing a range of signaling molecules. These molecules function in helping macrophages to leave the bloodstream or agglutinate and in the activation, proliferation and differentiation of immune cells, they can even kill certain immune cells. Before a helper T-cell can "help" with activating other immune cells, it first needs to be activated itself. This occurs by interacting with antigen presenting cells. When T-cells get activated, they will start to express the CXCR4 receptor as well.

Another type of immune cells are the B-cells. Their main function is to secrete antibodies that aid the humoral immune response in many ways. The B-cells develop in the bone marrow from which they travel to the lymph nodes, where they meet with antigens and T-cells. The B-cells stay dormant until an antigen binds to their surface anchored immunoglobulin. Upon binding the antigen is taken in, digested into small peptides, which will be presented in the same fashion as all other cells present protein fragments. If a helper T-cell recognizes this displayed foreign peptide, it will in turn activate the B-cell. After activation, somatic hypermutation (maturation) of the immunoglobulin will start upon rapid proliferation of the parental B-cell. If the interaction between antigen and immunoglobulin is strong enough, the B-cell will be stimulated to keep proliferating. Their offspring will either become long lasting memory B-cells or antibody secreting plasma cells. B-cells expressing a low affinity immunoglobulin will not be stimulated enough and will die by apoptosis[31].

Once an adaptive immune response has developed, memory cells are made which re-

main in a hibernating state within the body until it is being re-infected with the particular pathogen. This results in long lasting immunity against that specific pathogen. For example, in 1918 there was pandemic outbreak of a uniquely virulent influenza virus strain, known as the Spanish flu. Recently (2007) memory B-cells able to recognize the Spanish flu have been isolated from individuals that survived the 1918 pandemic flu outbreak. This indicates that memory B-cells are able to stay in circulation for many decades, in this case even 89 years[32]. After re-infection these memory cells will launch a quick defense against the invader and may even conquer infection before symptoms start to emerge.

The effect of HIV-1 on the immune system

To get into the body, the HIV-1 particles have to cross the mucosal epithelia. An intact epithelial cell layer is difficult to penetrate, but the layer may be compromised by lesions or underlying disease, opening a door for pathogens like HIV. Within these cell layers, the virus meets with macrophages and dendritic (or Langerhans) cells. Normally these cells either "eat" the invaders or transport them to the lymph nodes to present them to various immune cells, thereby eliciting an immune response. In general this is a good thing, however macrophages and dendritic cells carry CD4 and CCR5 and can thus be infected by HIV-1[33]. Even if the dendritic cell does not get infected, but manages to bring the virion to the lymph node, it can have disastrous effects. The lymph nodes are home to the naïve and helper T-cells, which also carry CD4 and CCR5. Even though an immune response is being elicited, HIV-1 found a way to use the host's system, designed to defeat invaders, as a perfect environment to establish infection.

The body will develop a strong immune response against the virions and infected cells that express viral proteins, destroying them in the process. However, in the provirus form, the virus can stay undetectable for the immune system. From these viral reservoirs, a constant supply of new virus particles is launched. Most of the viral particles will be neutralized, but every once in a while, a mutant version is not recognized and will be able to infect new cells[17]. The immune system will quickly adapt, but generally stays one step behind. Very slowly, over time, more cells become infected until a threshold is reached and the body is no longer capable of controlling the infection. The viral load will quickly rise, infecting most CD4 carrying cells. If the CCR5 carrying macrophages, dendritic cells, helper T-cells and, when the virus finally switches to the CXCR4 co-receptor, also the activated T-cells are all infected, a large part of the immune system is incapacitated. Pathogens will no longer be recognized and B-cells and cytotoxic T-cells will not be activated anymore, thus the body will then have to do with little more than a crippled complement system.

Microbicides

Treatment of HIV-1 is not only expensive, but in general it has serious side effects like nausea, problems with vision, anorexia and insomnia[34]. NRTI's have the specific side effect to be toxic on the mitochondria, which are the main providers of energy in the body,

causing lactic acid production (also the underlying cause of muscle ache) and finally liver failure[27]. Sticking to a tight schedule of taking medication at set time points is also very invasive in the patient's life and in almost 10% of people who receive treatment it is the reason for not taking the medication[34]. Therefore the prevention of contraction of HIV-1 would be preferential over treatment.

Condom use gives the most reliable protection against sexually transmitted HIV-1 infection and numerous other sexually transmitted diseases (STD) as well. However, there are various reasons against condom use. First of all, there could be religious considerations against condom usage. Second, not all women around the world are in the position to enforce the use of a condom. Third, in view of procreation, condom usage is not desirable. Thus something better will have to be developed.

Microbicides are substances designed to destroy infectious agents including bacteria and viruses. The ideal microbicide should be inexpensive, save to use over prolonged periods of time, accepted by society and above all, efficacious[35].

The price of a microbicide should be low enough to be afforded by people in the poorest countries of the world, to ensure its wide availability. The product should have sufficient funding by governments or foundations as well, to make it freely available to those who still cannot afford it. This all should ensure the product is widely used, as the effect of the microbicide will be enhanced if more people use it, an effect known as herd immunity.

Safety indicates that the microbicides should not compromise the body's natural defense system in any way. Damage or irritation of the epithelial cell layer may increase the risk of infection as the subsequent activation of the immune system recruits the cells that are vulnerable for HIV-1 infection toward this site. Vaginally used microbicides will have the additional requirement that they should not raise the vaginal pH as that increases the risk of infection as well. In addition to the above, the microbicide should not be toxic or carcinogenic either, as it will not be a very useful product if it protects you form HIV, but gives you cancer.

Acceptance is one of the most important aspects as people actually need to use the product in order for it to be effective. Compliance can be enhanced by creating a product without adverse effects. It should be easy or even beneficial to use, for instance creating a microbicide that can be used as a lubricant will make its use preferential to many people. Another possibility is to make the use of the product unnoticeable to others.

Antibodies

Humans have a total of five different isotypes of immunoglobulins (antibodies), IgA, IgD, IgE, IgG and IgM (figure 5). They are all made up of two so called heavy and two light chains. The heavy chains consist of three constant domains (four for IgM and E), referred to as CH1-3, and a variable domain designated as V_H. The light chains consist of only one constant domain (CL1) and a variable domain, designated as V_L. The light chains are linked to the heavy chains by sulphur bridges formed between the CH1 and CL1 domains. All

these isotypes do not only have distinct functions, their distribution throughout the body also differs[31].

The main function of IgA is to neutralize pathogens by binding to it, although it can activate the complement system or cause opsonization of the pathogen as well. A small proportion of IgA resides in the serum, where it is in a monomeric form[31]. The majority of IgA is however, secreted toward the mucosal tissues and it is the major immunoglobulin found in breast milk[36]. Before transportation through the epithelial cell layer, IgA will dimerize via its 18 amino acid tail, called the J-chain. The J-chain is also necessary during its transport which is mediated by the Polymeric Immunoglobulin Receptor (pIgR)[37]. A healthy adult human secretes around five grams of IgA a day, mainly into the gastro intestinal tract[38].

IgD is expressed on the surface of mature naïve B-cells before it switches isotype. Its concentration in the serum is, with an average of 40 μg/ml, very low compared to the other antibodies. There is little known about the exact function of this soluble IgD[36].

The serum concentration of IgE is is even lower than that of IgD, reaching only around 40 ng/ml. Its sole purpose is to sensitize mast cells and this is the isotype related to all kinds of allergic reactions[31].

IgG is an isotype that is only found among mammals and marsupials[39]. In humans, IgG appears in four different subtypes, IgG_{1-4}. IgG_1 and $_3$ are very broad in their functions, as they are capable of neutralizing, opsonizing, sensitizing natural killer and mast cells and activation of the complement. IgG_2 and $_3$ mainly just neutralize. The IgG's are quite abundant in the serum, with their total concentration being around 14 mg/ml. They are transported across the placenta and their diffusion out of the bloodstream is very high[31].

IgM's main function is to activate the complement system, but it can neutralize and induce opsonization as well. IgM is like IgD expressed on the cell surface of mature naïve B-cells as well. Just like IgA it can polymerize via its J chain, but it is forming pentamers rather than dimers. As IgM is expressed in the beginning of the B-cell response, its affinity is usually low. The pentameric IgM compensates its low affinity by having a very high avidity due to its 10 antigen binding sites. Pentameric IgM is like IgA secreted by the pIgR toward the mucosal tissues and into breast milk[36, 40].

All B-cells start with IgM expressed on their surface, this is also the first antibody made in the immune response. As B-cells mature, IgD will be co-expressed with IgM, however, upon activation IgD will no longer be expressed. Based on the signals the B-cells receive from T-cells, they can switch their antibody isotype from IgM to either IgA, IgE or IgG. This process, referred to as isotype switching, involves recombination on DNA level. No matter which isotype is produced, it is always processed in two ways, one to be surface anchored, the other to be secreted[31].

Birds, reptiles and amphibians lack IgG, but have IgY, which is now thought to be the ancestor of both IgG and IgE type antibodies. IgY is also made up of a light and a heavy chain, the heavy chain has 1 variable and 4 constant domains, the light has only 1 variable

and 1 constant domain. The light chain is linked to the heavy chain by a disulfide bridge between both first constant domains. There is also a version where constant domains 3 and 4 are missing (IgY ΔFc)[41].

All antibodies are binding their antigens with their variable domains. Both V_H and V_L have three complementary determining regions (CDRs) which together determine the specificity of the antibody. As there is a high variety in pathogens and their antigens, the body should carry a high variety of antibodies as well. However, the human genome is not nearly large enough to code for all variations individually, instead a highly sophisticated method has evolved to create this large diversity with use of relatively limited genetic information[31].

The V_H domain is encoded by multiple gene segments. The first segment is designated as the V gene, as it encodes most of the V_H domain. Then there is a diversity (D) gene which codes for most of the highly diverse CDR3 region. Lastly there is a joining (J) gene, which joins the V_H domain to the first constant domain. In humans there are 40 different V genes, 25 D genes and 6 J genes to pick from. Somatic recombination links one of each gene segments together to form the V_H domain, which will then be linked to the constant

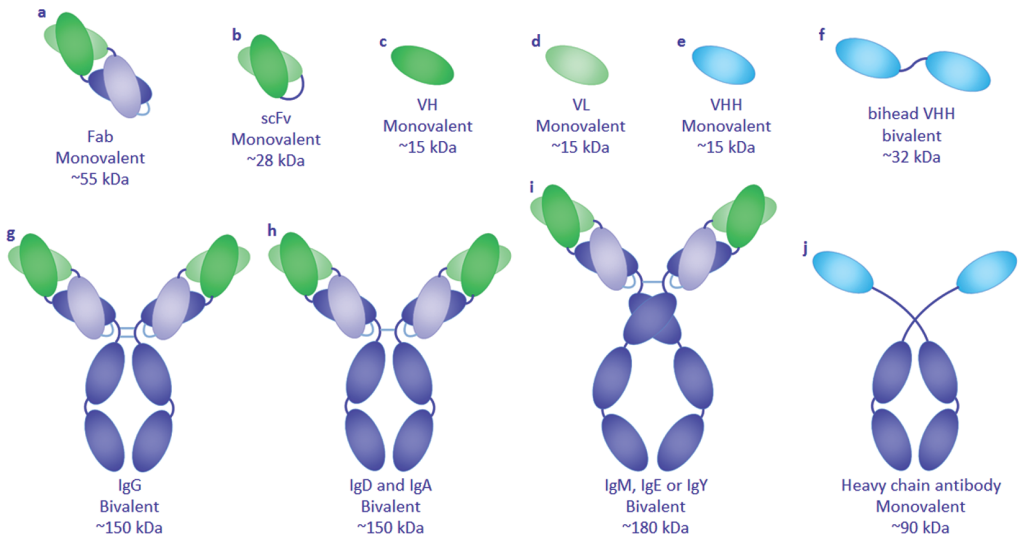


Figure 5: Different types of antibodies and antibody fragments. A schematic representation of different types of antibodies together with some of their commonly used fragments. Their name, respective molecular weight and whether they are mono or bivalent is indicated underneath their image. a) Fab fragment, consisting of VH, VL, CH1 and CL domains. b) Single chain Fv antibody fragment, consisting of VH and VL, physically linked together. c) VH, the variable domain of a heavy chain. d) VL, the variable domain of a light chain. e) VHH, the variable domain of a heavy chain only antibody. f) Bihead VHH, two VHH linked together by a linker of variable size. g) Representation of immunoglobulin G, consisting of a VH, VL, CL and 3 CH domains, the heavy chains are linked by two cysteine bridges. h) Representation of immunoglobulin D or A consisting of a VH, VL, CL and 3 CH domains, the heavy chains are linked by one cysteine bridge. i) Representation of immunoglobulin M, E[31] or Y[41] consisting of a VH, VL, CL and 4 CH domains[31]. j) Representation of a heavy chain only antibody (subtype of IgG) their hinge region may be either 12 or 35 amino acids in length[42].

domain. The reading frame of the V and J genes are fixed, but the D genes can be inserted in three different frames. Without taking mutations into account, this system thus generates 18,000 different combinations[31].

The V_L domain is created in a similar manner, however it lacks the D genes and is encoded on two loci in the genome (λ and κ). The λ locus encodes for 30 V and 4 J genes and further 4 different constant regions. The basic diversity here is thus 120, as the constant regions do not contribute to antigen specificity. The κ locus 40 V and 5 J genes, which brings the total light chain diversity to 320.

There are thus over 5.7 million different gene combinations. This is however still not enough to be able to recognize all antigens with high specificity and affinity. Therefore there are two more mechanisms that increase the repertoire. Firstly, for the V_H domains, the area between V - D and D - J is filled up with random nucleotides and some existing nucleotides may even be nibbled off[31]. Secondly, in about 5% of human antibodies it is found that two D genes are inserted between the V and J genes instead of one[43].

The increase in the antibody's affinity, after its associated B-cell has been activated, is accomplished by somatic hypermutation. This process creates point mutations throughout the gene fragments encoding the V_H and V_L domains, and is mainly concentrated around certain "hotspots". B-cells with disruptive mutations will not receive the necessary proliferation signals and will die. Those with the highest affinity antibodies will receive most proliferation signals from helper T-cells, and will thus become the most abundant. Approximately two weeks after infection the body is equipped with a battery of antibodies, which are sufficient in affinity and which will stay in circulation for some time. From the associated B-cells, memory cells are formed, which remain dormant until the body is re-infected with the pathogen. During re-infection, the dormant B-cells will respond immediately, clearing infection quickly. This process is also the basis of vaccines.

Llama antibodies

Members of the *Camelidae* family (camel, dromedary, llama, alpaca etc.) are carrying two special forms of IgG, not found in other mammals. These IgG's, designated IgG_2 and $_3$, are devoid of their light chain and are referred to as heavy chain only antibodies (HCAb) and are represented in figure 5j. The "Fab fragment" of these antibodies therefore consists of only a single antigen binding domain (figure 5a). This antibody fragment is referred to as the Variable domain of the Heavy chain of a Heavy chain only antibody, the V_{HH} or VHH (figure 5e). The conventional IgG present in these animals is IgG_1 which is a ~170 kDa molecule consisting of two ~50 kDa heavy chains and 2 ~30 kD light chains. IgG_2 is made up of two ~45 kDa heavy chains and IgG_3 is a ~43 kDa heavy chain dimer. All IgG antibodies of *Camelidae* are capable of binding to protein A. The heavy chain only antibody fraction coming off a protein A column can be as high as 75% in some members of the *Camelidae* family[44].

In both IgG_2 and $_3$ the CH1 domain is deleted, linking framework 4 of the variable fragment directly to the hinge region. Where IgG_3 has a normal hinge region of 12 amino acids, IgG_2 is carrying a much longer hinge of 35, which was probably derived from motifs normally present in conventional IgG_1 and $_3$[44]. There are two explanations for the lack of CH1. First, in the production of conventional antibodies, Binding Immunoglobulin Protein (BIP) binding to CH1 will prevent the antibody from being secreted until a light chain is attached to the heavy chain[45]. in HCAbs a light chain should not be attached to the heavy chain and thus BIP will prevent its secretion. As HCAbs should be secreted, deletion of CH1, containing the BIP binding motive will allow their secretion without a light chain. Second, the CH1 domain is the place where normally the light chain is linked to the heavy chain. It is rather hydrophobic, and that is probably why it was deleted, as leaving this domain intact will cause solubility issues. This is observed for V_H fragments derived from conventional antibodies, as they frequently precipitate. The variable fragments of both heavy and light chain also have a hydrophobic interaction to keep them together, this may cause problems with solubility as well if this surface is exposed. To increase the solubility of the VH without light chain L45, located in the in the VH-VL interface, is most often mutated to an arginine in VHH[44].

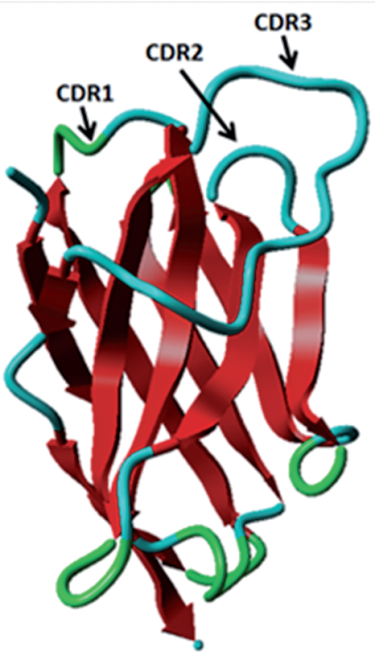


Figure 6: Schematic representation of a VHH. Ribbon representation of VHH D7 (PDB ID: 2XA3)[125] with beta strands in red and CDR 1, 2 and 3 indicated

Even though the llama VHH is lacking the antigen binding surface of the light chain, it is proven that it is capable of binding its antigen just as specific as mAbs and with comparable affinities[46, 47].

VHH - pro's and con's

There are numerous reasons to use VHH as treatments and prophylactics. First of all, they are relatively small proteins, which enhances their tissue penetration compared to full length conventional antibodies[48]. Their small size also makes them good candidates for targeting difficult to reach epitopes. Elaborating on this, heavy chain only antibodies, and thus the VHH as well, have a preference for binding into cavities[49]. VHH are able to have similar interaction areas as conventional antibodies, but with a much smaller footprint[47]. This makes them perfect for targeting enzymatic clefts and other difficult to reach epitopes.

Additionally, VHH have been shown to be rather thermo stable. In a comparison between mouse mAb's and VHH, 64% of VHH still gave a signal above half V_{max} after 2 hour incubation at 90°C whereas for the mAbs, incubation above 70°C completely abrogated

binding. The same study further revealed that two out of eight VHH were even able to specifically bind their antigen at 90°C[46]. Another study revealed that the high thermo stability of the VHH is most probably due to proper refolding after denaturation[50].

Further, VHH generally give high production yields, for instance in *Saccharomyces cerevisiae*[51]. However, they have been described to be produced in various other expression systems as well, among which is tobacco and potato [52-54]. This ease of production makes them relatively cheap to produce[46].

Another benefit of VHH is that they are relatively simple to isolate (compared to scFv's). As they lack the V_L domain there is no need for proper V_H/V_L pairing which keeps the cloning and library construction simple.

Recently it has been described that VHH can pass the blood brain barrier without the aid of other proteins, albeit with low transfer rates. These VHH were shown capable of translocating by themselves without needing to bind to specialized translocation receptors. They most likely do so because of their basic PI or their positive charge in combination with their small size[55, 56]. Further it was shown for one of these VHH that it was capable of binding its target within the cytoplasm of cells. Normally disulfide bridges do not form in the cytoplasm of cells, which makes normal antibodies not able to stay in their proper conformation[56].

There are a few disadvantages to these molecules as well. Because of their small size their half-life within the human body is limited, as they get rapidly cleared by the kidneys. Making them bigger, for instance by linking two VHH together increases their half-life. Rapid clearing is not always a disadvantage though, for whole body imaging it is perfect. In mice it has been shown that a two hour delay between injection of the dye conjugated VHH and imaging is optimal for visualizing a tumor. For conventional antibodies the optimum was shown to be around 24 hours[48]. Quick imaging would be rather patient friendly as with conventional antibodies he or she would have to come back after a day.

As the VHH does not have the Fc region, it does not have any effector function like antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In contrast to full length antibodies, there will be no activation of the complement system or opsonization. The VHH will therefore have to neutralize solely through its binding to the antigen. It is however possible to re-assemble a full length heavy chain antibody of VHH with various effector functions, but the production cost will rise significantly.

VHH against HIV-1

The ideal step to prevent HIV-1 infection would be to block it from binding its target cell. Blocking the following conformational changes, needed for membrane fusion, would also be an option. All subsequent steps happen inside the target cell, which is difficult to target with an antibody. VHH would thus be best suited as entry inhibitors. The site on the virus that makes the first contact with the target cell is the CD4bs, therefore this would

be a logical target for an antibody to block binding. Next to this, the CD4bs may be the only site that is largely accessible on the naïve trimer. A molecule that targets the CD4bs, should ideally do so by binding completely within the area that CD4 itself binds[17]. Another advantage of targeting the CD4bs is that this area is reasonably conserved. The reason for this being that the virus needs to be able to bind CD4 in order to infect and mutations in this area will most likely affect CD4 binding as well.

CD4 binds to gp120 with its first domain. This domain is a so called immunoglobulin domain, which basically means that it resembles the structure of domains found in immunoglobulins[57]. Normal antibodies have a binding site that consists of two immunoglobulin domains next to each other. This is why CD4bs mAbs usually do not bind fully within the CD4bs. There are mAbs that try to compensate for their bulkiness by either binding with only their heavy chain (b12[58]) or by binding with a CDR3 that is extremely long for conventional antibodies (PG9 and 16[59]) As the binding site of a VHH is made up of only one immunoglobulin domain it would be perfect to fit completely within the CD4bs.

Another advantage of a VHH against HIV-1 would be that they have been shown to inhibit syncytia formation. Hereby the assembled viral proteins on the membrane of the infected cells make them fuse with healthy cells, just like the virus would do, forming huge cells. This phenomenon is also observed for HIV infected cells. VHH may also be beneficial to block cell to cell transmission of the virus, as they may be able to reach these synapses better than the much larger conventional antibodies.

Multivalent VHH

One of the disadvantages of a VHH compared to a conventional antibody is that it has only one binding domain instead of two. Therefore, the VHH does not have the benefit of avidity (improved affinity due to having multiple binding sites). To increase the affinity of a VHH, they can be linked together, to get so called, bi- tri- and quadra-heads. These multivalent constructs can consist of identical VHH (homo specific) or different VHH (hetero specific). Some multivalent VHH have shown a remarkable increase in potency, up to 4000 times, as well as in breadth[60, 61].

When two VHH are coupled together, they are linked "head to toe" on DNA level. A linker is attached to the C terminus of the first VHH, subsequently followed by the second VHH. The linker length can be varied, in order to create the optimal distance between the VHH. The optimum differs per construct, based on the distance between the targeted epitopes. Theoretically there is no maximum size of the linker, however linkers above 35 amino acids in length start to give cloning as well as translational problems. To reach epitopes further apart, tri-headed VHH can be used, in which VHH1 and 3 will bind to the desired epitopes. Additionally, by using multivalent VHH is that it is possible to target two different epitopes on the same molecule, fixing it in a certain position.

A further benefit of multivalent VHH is that they have an extended half-live in the bloodstream, compared to monovalent VHH. The normal half-life of a VHH in humans

is thought to be around 2 hours, which is much too short for treatment purposes. Multimerization can extend the residence time of a VHH in the bloodstream, as the size of the molecule increases which makes it less prone to a-specific clearance by the kidneys. The half-life can further be improved by the linkage of an additional VHH targeting other "long-lived" blood proteins like immunoglobulins[62]. This stretches the time the construct is in the bloodstream, but lowers its tissue penetration[48].

In some cases the integrity of the C terminal VHH of a bihead is compromised, as the linker between the VHH is in close proximity to its binding area, especially close to CDR1. In case of hetero-specific constructs, linking the VHH in opposed directions may solve these problems.

Aim

As clade B viruses are by far the most common in the western society, most research is done on clade B viruses. Most of the drugs have been developed against and tested on viruses belonging to clade B as well. A fair number of these drugs do show efficacy toward non-B clades, but they could perform better if they were specifically raised against these other clades. Another disadvantage of the drugs designed for the western world countries is that they generally are very expensive. Thus, even though there are drugs that can target the clades that roam in the developing world, many of the patients cannot afford them.

There may be yet another problem with drugs, specially designed for patients in the high income countries. Some drugs may not function as they should if given to patients that are malnourished or infected with parasites or have other underlying diseases. Therefor there is also a need for drugs that are specifically designed for people in the poorest countries of the world.

The costs for the development and testing of a product are very high. It would be cost efficiently to develop a drug that is as broad as possible and limits the risk of viral escape. Further it would be beneficial if the product would be able to function alongside the current treatments and would not induce cross-resistance toward these other drugs.

The objective of this thesis is to identify VHH that are active against the viral clades that are most prominent in the low income countries, clades C, A and CRF02_AG. These VHH should be very broad and potent. Preferable these VHH would target different sites on the virion in order to be used in combination with each other. A mix of VHH could potentially complement each other's breadth and together they may neutralize close to 100% of all viral strains. A further benefit of a mix is that it has been shown that the targeting of different epitopes at once lowers the risk of viral escape.

Chapter 2

Llama antibody fragments recognizing various epitopes of the CD4bs neutralize a broad range of HIV-1 subtypes A, B and C.

Adapted from:

Llama antibody fragments recognizing various epitopes of the CD4bs neutralize a broad range of HIV-1 subtypes A, B and C.

Nika Strokappe[1a], Agnieszka Szynol[1a], Marlèn Aasa-Chapman[2], Andrea Gorlani[1], Anna Forsman Quigley[2], David Lutje Hulsik[3], Lei Chen[4], Robin Weiss[2], Hans de Haard[1] and Theo Verrips[1*]

[1] Biomolecular Imaging, Department Biology, Faculty of Science, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
[2] UCL/MRC Centre for Medical Molecular Virology, Division of Infection and Immunity, University College London, Gower Street, London, WC1E 6BT, United Kingdom
[3] Unit of Virus Host Cell Interactions (UVHCI), UMI 3265, Université Joseph Fourier-EMBL-CNRS, Grenoble, France
[4] Vaccine Research Centre, NIAID/NIH, Bethesda, MD 20892, USA
[a] N.M.S. and A.S. contributed equally to this work

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Abstract

Many of the neutralizing antibodies, isolated to date, display limited activities against the globally most prevalent HIV-1 subtypes A and C. Therefore, those subtypes are considered to be an important target for antibody-based therapy. Variable domains of llama heavy chain antibodies (VHH) have some superior properties compared with classical antibodies. Therefore we describe the application of trimeric forms of envelope proteins (Env), derived from HIV-1 subtype A and CRF07_B/C, for a prolonged immunization of two llamas. A panel of VHH, which interfere with CD4 binding to HIV-1 Env were selected with use of panning. The results of binding and competition assays to various Env, including a variant with a stabilized CD4-binding state ($gp120_{Ds2}$), cross-competition experiments, maturation analysis and neutralization assays, enabled us to classify the selected VHH into three groups. The VHH of group I were efficient mainly against viruses of subtype A, C and CRF07_B/C. The VHH of group II resemble the broadly neutralizing antibody (bnmAb) b12, neutralizing mainly subtype B and C viruses, however most had a broader neutralization profile. A representative of the third group, 2E7, had an even higher neutralization breadth, neutralizing 21 out of the 26 tested strains belonging to the A, CRF02_A/G, B, CRF07_BC and C subtypes. To evaluate the contribution of certain amino acids to the potency of the VHH a small set of the mutants were constructed. Surprisingly this yielded one mutant with a slightly improved neutralization potency against 92UG37.A9 (subtype A) and 96ZM651.02 (subtype C). These findings and the well-known stability of VHH indicate the potential application of these VHH as anti-HIV-1 microbicides.

Introduction

Neutralizing antibodies against the human immunodeficiency virus type 1 (HIV-1) are powerful tools not only for understanding the virus structure [20, 58, 63, 64] and the mechanism of cellular entry [15, 65], but also for passive immunization [66-68]. Many monoclonal antibodies specific for HIV-1 envelope proteins, gp120 and gp41, have been isolated both from immunised animals and infected individuals. However, only a few of these are broadly neutralizing. These rare antibodies, including b12, 2G12, 2F5, 4E10 and X5 [69, 70] have all been derived from HIV-1 subtype B infected patients and, besides 4E10, display limited activity against the globally most prevalent HIV-1 subtype C [71-74]. More recently other promising broadly neutralizing monoclonal antibodies (bnmAbs), notably PG16, PG9 [29, 59, 75], HJ16 [76], VRC01-03 [30] and 3BNC60 and 117[77] have been described. Many of these bnmAbs recognize the CD4bs and the sometimes relatively small differences in the interaction area, derived from X-ray data, resulted in quite different neutralization potencies [30, 77, 78]. Isolation and characterisation of novel bnmAbs, with specific attention to non-subtype B viruses, may aid the design and development of a vaccine capable of inducing a broadly protective antibody immune response. Additionally, such antibodies may be developed as specific entry inhibitors for inclusion in anti HIV-1 microbicides [79].

Llamas, and other *Camelidae*, possess conventional antibodies and heavy chain antibodies. The latter are devoid of light chains [44] and the Variable domain of the Heavy chain of the Heavy chain antibody (VHH) is therefore solely responsible for antigen recognition. The specificities and affinities of VHH are comparable to those of IgGs even though the size of a VHH is only approximately 15kDa., compared to the 150kDa. of IgG [46, 47]. On average, VHH have longer complementarity determining regions 3 (CDR3) [80-82], a feature that may facilitate binding into deeper cavities on the antigen surface [49]. Grooves and cavities play a crucial role in multiple biological activities as these often form the specific interaction site between two molecules [81]. Fitting into the CD4bs is thought to be important for potent neutralization of HIV-1 via binding to the envelope spike [59, 83]. Moreover the small size of VHH may be an important property to inhibit transmission of HIV in the small viral synapsis [84]. The high stability [46, 80, 85-87] and the often excellent expression yield of VHH in microbial fermentations [51, 88, 89] make VHH realistic candidates for the development of microbicides to protect against HIV infections.

We have shown that neutralizing VHH can be raised in llamas immunized with gp120 of HIV-1_{CN54} [90]. Although the selected VHH exhibited neutralizing effects against HIV-1 primary isolates of subtype B and to a lesser extent subtypes C, they did not neutralize HIV-1 subtypes A, CRF02_AG and D.

In the present study, we immunised two llamas with a mixture of two different antigens, $gp140_{CN54}$ (subtype CRF07_B/C) and $gp140_{UG37}$ (subtype A) to promote the development of broadly reactive VHH. Here we describe the selection of VHH from immune phage display libraries on trimeric Env, by competition with sCD4. This resulted in the isolation of a number of VHH that not only competed with broadly neutralizing anti-CD4 binding

site antibody (b12) for binding to HIV-1 Env proteins, but also revealed neutralizing activities against a panel of primary HIV-1 strains, including subtypes A, B and C. We classified the neutralizing VHH into three groups based on sequence analysis and alignment against germline V-, D- and J-genes, binding and competition experiments, and neutralization assays. These data demonstrate the diversity of epitopes recognized by these VHH and suggest various mechanisms of HIV-1 entrance inhibitions.

Materials and Method

Ethics statement

The prolonged Llama immunizations were approved and performed according to the guidelines of Utrecht University Animal Ethical Committee (approval ID: 2007. III.01.013).

Proteins

Soluble CD4 (sCD4) was purchased from R&D systems (cat 514-CD/CF), Concanavalin A (ConA; cat. C2010) and biotinylated ConA (cat. C2272) were purchased from Sigma-Aldrich Chemie BV, The Netherlands. Recombinant HIV-1 Env $gp140_{CN54}$ (0699), $gp120_{IIIB}$ (0607) and $gp120_{CN54}$ (6015) used for binding and competition assays, and the anti-CD4bs monoclonal antibody (mAb) b12 (3065) were obtained from the Centre for AIDS Reagents, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and were donated by Polymun, Immunodiagnostics, Immune Terchnology and Drs D P Burton and P Parren, respectively. Envelope proteins $gp140_{UG37}$ and $gp140_{CN54}$ used for immunization were kindly provided by Dr S. Jeffs, Wright-Fleming Institute, Division of Medicine, Imperial College London, London, UK. Envelope proteins $gp120_{YU2}$ and its modified variant $gp120_{Ds2}$ were kindly provided by Dr. P. Kwong, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, USA. The anti-llama IgG_3 mAb 8E1 was kindly provided by BAC BV, Naarden.

Viruses

HIV-1 IIIB (0101) and MN (0102*)* were obtained from the Centre for AIDS Reagents, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and was donated by Drs *R* Gallo & M Popovic and propagated in H9 and C8166 cells, respectively. Virus stocks of HIV-1 envelopes pseudotyped with the pSG3Δenv vector and replication-competent HIV-1 molecular clones were produced by transfection of 293T cells [91]. The subtype B (THRO4156.18, TRJO4551.58, 6535.3) and C (Du156.12, Du422.1, ZM197M.PB7, ZM214M.PL15, ZM233M.PB6, ZM109F.PB4, ZM135M.PL10a, CAP45.2.00.G3) HIV-1 Reference Panels of Env. Clones [91] were obtained through the AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH (USA). HIV-1 subtype

CRF02_AG (T257-71, T266-60, T278-50 and T33-7) gp160 clones, subtype CRF07_BC gp160 clones (CH038.12, CH064.20, CH091.9, CH110.2 and CH181.12), 96ZM651.02 and MS208.A1 p160 clones were kindly provided by Dr D. Montefiori (Duke University Medical Centre, Durham, USA) through the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA-VIMC) as part of the Collaboration for AIDS Vaccine Discovery (CAVD). Virus 92UG037.A9 is a gp120 clone of the primary isolate 92UG37 [92] cloned into the pHXB2Δenv vector [93].

Binding sCD4 and b12 antibodies to gp140 and 120 molecules

To determine the functionality of the different Env, their interactions with sCD4 and b12 were tested. MaxiSorp microtitre plates (cat 442404, Nunc GmbH & Co. KG, Germany) were directly coated with Env, serially diluted in PBS, and incubated at 4°C overnight. After treatment with 4% skimmed milk powder (Marvel) in PBS (4% MPBS) for 1h at room temperature (RT), 50 µL sCD4 [3 µg/mL] or 50 µL b12 [100 ng/mL] in 1% MBPS was added and incubated for an additional 1h at RT, shaking. Soluble CD4 was detected with L120 (mouse anti CD4, 1:10,000 in 1% MPBS; NIBSC) and b12 was detected with rabbit anti-human IgG (1:10,000 in 1% MPBS; DAKO). Finally, peroxidase-conjugated donkey anti-mouse or donkey anti-rabbit IgG (1:5,000 in 1% MPBS; Jackson Immunoresearch, West Grove, PA, USA) were added. Plates were washed three times with PBST (PBS supplemented with 0.05% Tween 20) between each step. The complexes were visualised by *o*-Phenylenediamine (OPD) according to method described by Verheesen *et al.* [94].

Immunisation of *Lama glama* with $gp140_{UG37}$ and $gp140_{CN54}$

Two *Lama glama* were injected intramuscularly with mixture of $gp140_{CN54}$, and $gp140_{UG37}$, 50 µg of each protein in commercially available Stimune adjuvant (CEDI Diagnostics, Lelystad, The Netherlands). First boosting was given on day 7, with the same immunogens doses as the first injection. The following booster injections were given on days 14, 21, 28, 35 and 113 with mixture containing 25 µg of each gp140. Ten millilitres blood samples were collected at days 0 (before injection), 21 and 113. To construct immune libraries, 150 ml blood samples were collected at day 43 and 122.

To assess the llamas' immune response, MaxiSorp microtitre plates were coated with 50 µL $gp140_{CN54}$, $gp140_{UG37}$ or $gp120_{IIIB}$ [5 µg/mL] as described above. After blocking with 200µL 4% MPBS serial dilutions of pre-immune and immune sera were incubated for 1 h. Detection of bound llama single chain antibodies was performed by incubation with the IgG3 specific mAb 8E1 [95] followed and peroxidase-conjugated donkey anti-mouse all in 50 µL. Complexes were detected as described above.

Phage library construction

To construct immune libraries, 150 ml blood samples were collected at 122 day, and peripheral blood lymphocytes (PBLs) were purified by Leucosep (cat 227290, Greiner Bio-

One BV, The Netherlands). Total RNA was extracted from PBLs as described by Chomczynski *et al.* [96] and random primed complementary DNA (cDNA) was synthesised using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, cat. 18080-051). After purification of the cDNA with QIAquick PCR Purification Kit (Qiagen, cat 28106), the cDNA was used as template for PCR using the combination of the leader and CH2 based primers [94] which resulted in an amplification of the conventional and heavy-chain IgG repertoire gene fragments. Due to the lack of C_H1 region in heavy-chain antibodies, the amplified gene fragments of conventional and heavy-chain antibodies were separated on agarose gel. Subsequently, a *Sfi*I restriction site was introduced upstream of FR1 in a nested PCR using the gel purified heavy chain amplicon as template. Since a *Bst*EII restriction site naturally occurs in 90% of the FR4 of llama heavy-chain antibodies genes [97] the repertoire of PCR-amplified genes was cut with *Bst*EII and *Sfi*I and the resulting 300-400 bp fragments were purified from agarose gel. Finally cDNA fragments were ligated into a phagemid vector for display on filamentous bacteriophage [98] and electroporated in *E. coli* TG1 (K12, _(*lac-pro*), *supE*, *thi*, *hsdD5/F'traD36, proA+B+*, lacIq, *lacZ*_M15).

The rescue with helper phage VCS-M13 and polyethylene glycol precipitation was performed as described previously [99]. Phage stock containing 5×10^{11} pfu/ml was prepared and used for subsequent biopanning.

Selection of clones competing with sCD4 for binding to gp140

To select phages that specifically bound to CD4 binding site of gp140 the modified competitive elution method [90, 100, 101] using sCD4 as selective eluant was applied. Wells of MaxiSorp microtitre plates were coated with 100 µL $gp140_{CN54}$ [2.5 or 0.5 µg/mL] in PBS overnight at 4°C. Blocking was performed with 2% MPBS. After washing the plate with PBS, 5×10^9 phages, which were preincubated in blocking buffer for 30 min at RT, were added to the wells and incubated for 2 hours at RT. Next, the coated wells were extensively washed with PBS. Subsequently, 100 µL sCD4 [30 µg/mL] or 100 µL triethylamine (TEA) 100mM were added and the plates incubated for 30 min at RT. The eluates were removed, the TEA eluted phage was neutralized with half volume of 1M Tris pH 7.5, and subsequently 10-fold serial dilutions in PBS were prepared. 10 µl of each dilution was used for infection of 190 µL log-phase *E. coli* TG1. After infection at 37°C for 30 min. without shaking, 5 µL of bacterial suspensions were spotted on LB agar plates supplemented with 100 µg/mL ampicillin and 2% glucose ($LB/Amp^{100}/Glu^{2\%}$) to determine the enrichment of the first round. Moreover, 75 µL of eluate was used for infection 0.5 mL log-phase *E. coli* TG1 to rescue phages [99], and were subsequently applied for second round of selection. The conditions of the following selection round were identical to the first one.

Screening ELISA

At the end of the second round, 100 µL serially diluted infected *E. coli* TG1 were plated on $LB/Amp^{100}/Glu^{2\%}$ agar plates and single colonies were picked and grown in 2YT broth

containing 100 μg/mL ampicillin and 2% glucose (2×YT/Amp^{100}/Glu$^{2\%}$) in 96-well microtitre plate format.

Expression of the VHH from single clones was performed in 96 deep-well plates (cat. AB-0932, Westburg B.V, The Netherlands) according to the modified method described by Marks *et al.* [99]. Briefly, 1 mL of 2×YT/Amp^{100}/Glu$^{0.1\%}$ broth was inoculated with 10 μL overnight culture and grown with shaking at 37°C until OD_{600}=1 was reached. Expression of the protein was induced by adding IPTG (final concentration of 1mM) and the cultures were grown for additional 4 hours with shaking at 37°C. After harvesting bacteria by centrifugation for 15 min at 4566 ´ g and freezing pellets overnight at - 20°C, bacteria were resuspended in 100 μL PBS and shaken for 2h at 4°C. Next, spheroplasts were pelleted by centrifugation for 15 min 4566 ´ g at 4°C and supernatants (i.e. periplasmic fractions) containing VHH were taken for screening assays.

Periplasmic fractions were screened for their ability to interfere with binding of monoclonal antibody (mAb) b12 to $gp140_{CN54}$ by direct competitive enzyme-linked immunosorbent assay (ELISA). This approach was chosen because of the weak interaction between $gp140_{CN54}$ and sCD4 in our ELISA setup that prevented screening of individual clones for competition with sCD4. For this purpose, wells of MaxiSorp microtitre plates were coated with 50 μL b12 [2 μg/mL] in PBS overnight at 4°C. Next, the b12-coated wells were blocked with 4% MPBS for 1 hour. In the meantime, mixtures of 5-fold diluted periplasmic fractions and 1 μg/mL $gp140_{CN54}$ (final concentration) in 1% MPBS were prepared and incubated for 1 hour at room temperature. Then 50 μL of the mixtures were transferred into blocked, b12-coated wells and incubated for an additional 1 hour. To detect bound, non-inhibited $gp140_{CN54}$ biotinylated concanavalin A (ConA) was used at concentration 2 μg/mL in 1% MPBS followed by addition of streptavidin-HRP conjugate. Complexes were detected as described above. Positive clones, which gave a low signal in the b12 competition assay, were selected and one-way sequencing was performed by application M13Rev primer [94] (ServiceXS, Leiden, The Netherlands). For further characterisation, VHH genes were recloned into an *E.coli* production vector and after expression the VHH were purified by means of immobilised metal affinity chromatography (IMAC) as it has been described by Verheesen *et al.* [102].

Characterisation of selected VHH

Sequence comparisons

To analyse the maturation and to classify the selected VHH, we grouped them according to the use of the germline V, D and J segments using a database with twenty three different V gene segments of *Lama glama*; seven D gene segments of *Vicuña pacos;* and five J gene segments of *Lama glama* [103] supplemented with two missing J gene segments of *Vicuña pacos* [104]. To analyse the sequences, WHAT IF's [105] implementation of combined DNA codon/amino acid alignments of all germ line genes against selected VHH sequences was performed. The D gene segments were translated in three readings frames and the best

fitting D-segment was used to analyse maturation of CDR3. The DNA/amino acid sequence format dictates that the triplet and the corresponding amino acid (so each codon is followed by its cognate amino acid e.g. cagQgtgVcagQ) remained associated in-phase in subsequent alignment procedures. The aligned DNA/ amino acid sequences allowed fitting short sequences, such as D gene segments, and differentiate between silent and functional mutations.

VHH binding to various Env

To test binding of purified VHH to various Env, $gp120_{IIIB}$ (clade B), $gp140_{CN54}$ (its gp120 is representing clade C), $gp140_{UG37}$ (clade A) $gp120_{YU2}$ (clade B) and its modified variant $gp120_{Ds2}$ were directly coated on Nunc MaxiSorp plates in 50 µL PBS, [5 µg/mL] by overnight incubation at 4°C. After blocking with 4% MPBS as described above, VHH diluted in 1% MPBS were allowed to interact with Env. Subsequently, bound VHH were detected with mouse anti-C-Myc (9E10) antibodies, which recognised C-terminal Myc tag incorporated in VHH, and the detection was followed by incubation with donkey anti-mouse – HRP conjugate. The signals were quantified by colorimetric assay described above. The experiments were performed in triplicates. Binding activities of 2E7 variants were tested in a similar manner, with exception of coating wells with 50 µL [4 µg/mL] Env and detection of VHH with rabbit anti-llama VHH serum [106] followed by goat anti-rabbit HRP conjugate.

Competition assays

To test possibility of epitope overlapping of selected VHH and b12 monoclonal antibodies we applied the method described by Kuroki [91, 107]. Microtitre plates were coated with 50 µL, [2 µg/mL] of b12. Equal volumes of 2 µg/mL gp120IIIB, gp140CN54 or gp140UG37 and serially diluted VHH were pre-incubated, and subsequently added to the mAb b12 coated and blocked wells. After 1 hour, bound HIV-1 Env proteins were detected by ConA as described above. The experiments were performed in triplicates. The absorbance at 490 nm (A490) of each tested sample was expressed as a percentage of positivity (PP), calculated by the following equation: PP = 100 * ((A490sample - A490min) / (A490max - A490min), where A490min is a A490 of sample without VHH and without Env; and A490max is A490 of samples without VHH for the particular Env and finally A490sample is the signal of sample with both the VHH and Env [108, 109]. To determine the descriptive measures such as mean (X) and standard deviation (S.D.), the results (expressed as PP) were processed with SPSS version 16.0 for Windows.

Cross-competition assays between selected VHH were performed in a similar manner. The only exceptions were: coating plates with 50 µL [3 µg/mL] of VHH and pre-incubation of equal volumes of 50 µg/mL VHH with 5 µg/mL $gp140_{CN54}$ or $gp140_{UG37}$. As a negative control an anti EGFR VHH was used [97]. The experiments were performed in duplicates. PP was calculated as described above with the difference that A_{490min} is a A_{490} of sample, where the same VHH were used in solution as the immobilized VHH; and A_{490max}

is A_{490} of samples, where anti EGFR VHH were applied as the VHH in solution for the particular immobilized VHH.

HIV neutralization assay

The HIV-1 neutralizing activities of the VHH were assessed in the TZM-bl cell based assay, as described previously [110] Briefly, 3-fold serial dilutions of purified VHH starting from 50 μg/mL were performed in duplicate in 10% (v/v) fetal calf serum (FCS) supplemented DMEM growth medium (Invitrogen, Paisley, UK). 200 $TCID_{50}$ of virus was then added to each well and the plates were incubated for 1 hour at 37°C. TZM-bl cells were subsequently added (1 x 10^4 cells/well) in growth medium supplemented with DEAE-dextran (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 15 μg/mL. Assay controls included replicate wells of TZM-bl cells alone (background control), and TZM-bl cells with virus assayed (virus control). No virus inactivation was observed with a negative control VHH. Following 48 hours incubation at 37°C, all 100 μL of the assay medium was removed and 100 μL of Bright-Glo luciferase reagent (Promega, Madison, WI, USA) was added to each well. The cells were allowed to lyse for at least 2 minutes, and the luminescence was then measured using a luminometer. The 50% inhibitory concentration (IC_{50}) titres were calculated as the VHH concentration that achieved a 50% reduction in relative luminescence units (RLU) compared to the virus control RLU, after subtraction of the background control RLU from both values. The calculations were performed using the XLFit4 software (ID Business Solutions, Guildford, UK).

Construction, expression and purification of VHH variants

Alanine scan was performed on residues N27, V29 (CDR1); D32 (FW2) and Y98, Y99, G100, R100b, Y100c, D101, Y102 (CDR3) of 2E7 by site-directed mutagenesis (SDM) with application of the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to instructions provided by manufacturer. In addition, the same method was applied for introduction of back-to-germline-mutations in CDR1: N27S, V29F, and in FW2: D32N. The introduced mutations were verified by plasmid sequencing (ServiceXS, Leiden, The Netherlands). Expression and purification of mutated 2E7 variants were performed as previously described by Verheesen *et al.* [102].

Results

Immune response and library construction

To obtain VHH specific for HIV-1 Env, two *Lama glama* were immunized with a cocktail of $gp140_{CN54}$ (subtype CRF07_B/C, with most of the gp120 representing subtype C) and $gp140_{UG37}$ (subtype A). The induction of a humoral immune response was followed by testing sera of the animals before and after immunization by ELISA. Immunizations resulted in the induction of a specific heavy chain antibody response towards both immunogens (figure 1). The titer of anti-$gp140_{UG37}$ antibodies in immune sera was slightly higher than

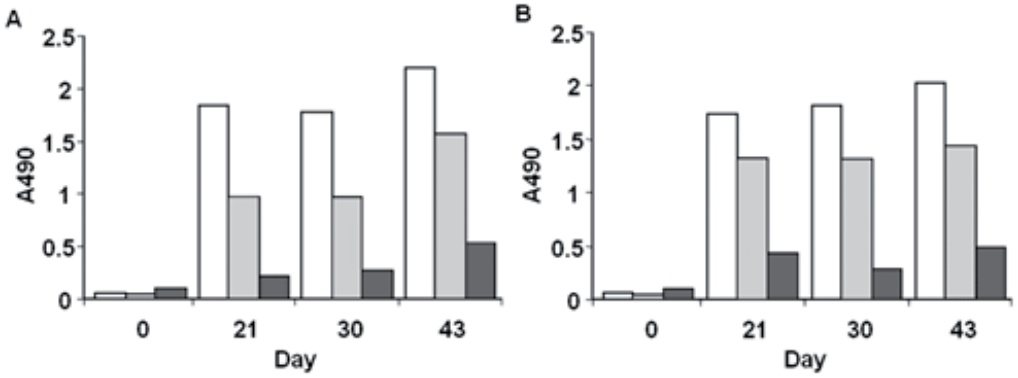

Figure 1: Heavy chain antibody response of llama 8 and 9. Heavy chain antibody response in llama 8 (A) and llama 9 (B) to $gp140_{UG37}$ (white bars), $gp140_{CN54}$ (light grey bars), $gp120_{IIIB}$ (dark grey bars) at indicated days following initial immunization. Sera from llamas were collected, diluted 1000 fold and tested by ELISA for the presence of specific IgG_3 heavy chain antibodies coated recombinant HIV-1 Env.

of anti-$gp140_{CN54}$. In addition, the immune sera were also reactive with $gp120_{IIIB}$ (subtype B). These data clearly demonstrate the successful induction of a humoral immune response towards the HIV-1 Env proteins.

Since the immune response was good, library construction was continued. The synthesis of the VHH repertoires resulted in two libraries: llama 8 and llama 9, of approximately 10^7 transformants each.

VHH selection by competitive elution with sCD4

The specific competitive elution method required binding of sCD4 to $gp140_{CN54}$. Therefore we tested how well Env was recognised by sCD4 in ELISA. As shown in supporting figure 1, sCD4 bound to $gp140_{UG37}$ in a dose dependant manner (0.37 – 10 μg/mL). However, binding of sCD4 to $gp140_{CN54}$ was only detectable at high concentrations (10 μg/mL) of Env. Therefore a high concentration of sCD4 was used as elution method during phage display, to ensure that all CD4 binding sites of the coated Env proteins were saturated, thus preventing rebinding of phages displaying VHH that recognize this site.

Two rounds of selection were performed. In the first round, 1.5 x 10^6 phages were non-specifically eluted by TEA from $gp140_{CN54}$ coated at 2.5 μg/mL and 100 fold lower outputs were obtained from the 0.5 μg/mL coating. Approximately 15 fold less phages were eluted with sCD4 from the 2.5 μg/mL coat and approximately the same number of phages were found for the 0.5 μg/mL coated wells as compared to TEA elution. For the second round, the rescued phages from 2.5 μg/mL $gp140_{CN54}$ eluted with sCD4 were used and the selection procedure was repeated. Surprisingly no difference was observed between number of phages eluted with sCD4 and with irrelevant protein BSA for llama 8 library. However, in case of the llama 9 library, ten times more phages were eluted from 2.5 μg/mL $gp140_{CN54}$ with sCD4 compared to BSA, thereby indicating a successful competitive elution with sCD4. Out of 280 individual clones screened from the selection, approximately 87% of

the clones were able to bind specifically to $gp140_{CN54}$.

In order to narrow down the investigation to clones that potentially were able to bind to CD4bs we performed a competition assay with b12. The setup of the sCD4 competition ELISA on $gp140_{CN54,}$ applied for selection of VHH with the specific competitive elution method, could not be used for screening purpose, because a too high concentration of sCD4 was required (10 μg/ml) to get a signal. Therefore we tested the ability of b12 to bind Env, since the b12 binding epitope overlaps partially with CD4bs [58]. In contrast to sCD4 binding, b12 binding to $gp140_{UG37}$ was observed at concentrations above 3.3 μg/mL (Supporting Fig. 1). For $gp140_{CN54}$, a decent signal was already observed at concentrations as low as 1 μg/mL. Therefore, this setup was used as the competition ELISA for screening purpose. Approximately 11% of the clones were able to compete with b12 (data not shown) and therefore selected for further characterisation.

Sequences analysis

The 30 competing clones were sequenced and based on deduced amino acids sequence 17 unique VHH were found, which were divided into seven families based on the DNA/amino acid alignment with the 23 V, the 7 D and the 7 J genes [103, 104] (figure 2). The maturation analysis revealed that four germline V gene segments were used to encode the VHH identified in the mAb b12 competition assay. Eleven VHH were derived from V_d gene, three VHH from V_g, two from the V_o gene and another one from the V_m gene. Probably two different D genes have been used in the group derived from V_d gene and on that basis two subpopulations of VHH could be distinguished in this group. Sequences of all selected VHH revealed that only J3 or J7 gene segments contributed in the formation of the VHH CDR3 loops. A notable feature of the selected VHH is a relatively long CDR3 loop in most of the selected VHH. This panel of VHH has an average length of 16.4 residues compared to an average of 12.7 for the human heavy chain CDR3 and 8.5 for mice [111]. Examination of amino acids sequences showed that besides strictly conserved disulfide bridge (C22-C92) typical of the immunoglobulin fold, an extra pair of cysteines is present in the sequences of most of the newly selected VHH. The VHH originated from the V_d gene have an extra cysteine at position 50, which forms an S-S bridge with a cysteine at various positions in CDR3. This feature has been previously observed by Vu *et al.* [82]. Noteworthy is the fact that VHH 1B5 and 1H9, which derive from V_g gene segment, contain a cysteine at position 52a in CDR2 and a second additional cysteine at position 71 in FW3. Interestingly, both cysteines were not present in the original germline sequence, and were never seen before in our hands, nor in the antibody database Fungen (fungen.wur.nl) or the protein data bank (www.wwpdb.org). The introduction of this new location of a cysteine bridge must be important for the function of these VHH. Evidence for such maturation step is provided by 1E1, which is closely related to 1B5, but lacks the additional cysteines and is less potent than 1B5, indicating that the S-S bridge is important for binding and neutralization. This observation was confirmed by replacement of both cysteines in 1B5 (data not shown).

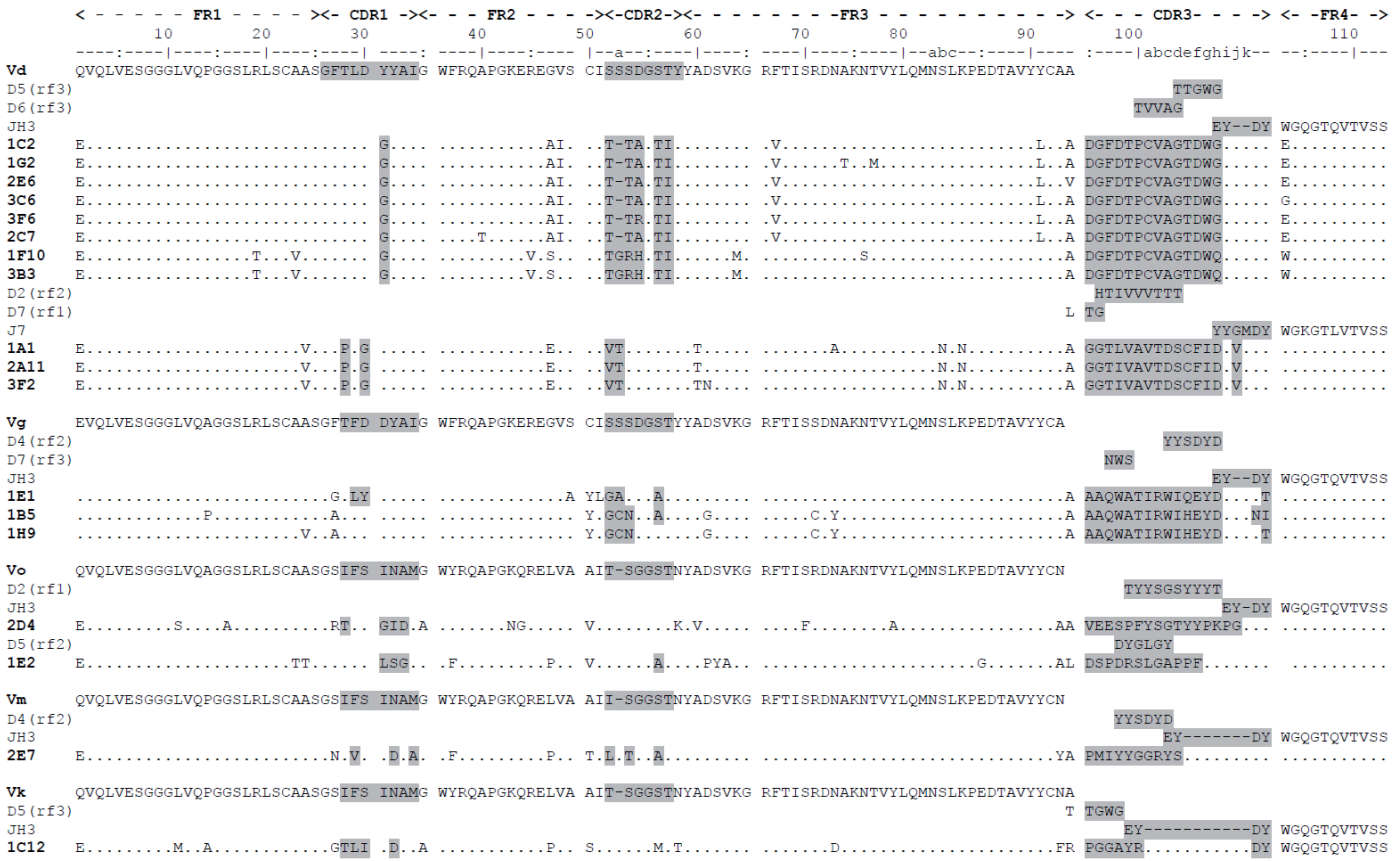

Figure 2: Alignment of the VHH sequences against the germline V, D and J segments. Amino acids sequences of HIV-1 Env specific VHH aligned against Lama glama V and J germ line genes and D germ line genes of Vicuña pacos. The reading frame of the most likely D germline genes are marked. Numbering of amino acid according to Kabat, however the CDRs of the VHH are redefined.

Binding of selected VHH to Env

Purified VHH were tested in ELISA to characterise their ability to recognise directly coated the HIV-1 Env proteins $gp140_{CN54,}$ $gp140_{UG37}$, $gp120_{IIIB}$, $gp120_{YU2}$ and its modified variant $gp120_{Ds2}$ (table 1, supporting figure 2). All selected VHH bound reasonable well to both $gp140_{CN54}$ and $gp140_{UG37,}$ with the exception of 2D4, which demonstrated limited binding to $gp140_{CN54}$. 1F10 and 1C2, as well as 1B5 and 1H9, which belong to two different families, were the best binders to $gp140_{CN54}$ and $gp140_{UG37}$ and reached half of maximal signal at concentrations below 0.63 μg/mL (or 42 nM). Only members of the 1B5 family (1B5, 1H9, 1E1) were able to bind to subtype B derived Env, although less efficiently than to $gp140_{CN54}$ and $gp140_{UG37}$. 1B5 and 1H9 reached half of maximum signal at a concentration between 82 and 330 nM of all subtype B Env proteins tested. In contrast, 1E1 lacking the second S-S bridge present in the other members of this family bound very weakly to $gp120_{IIIB}$ as well as to $gp120_{YU2}$, and binding to $gp120_{Ds2}$ was not detectable. As a comparison, previously selected VHH A12 [90] bound to $gp120_{IIIB}$ as good as 1B5 and 1H9, but was unable to bind to $gp120_{Ds2}$ (data not shown). Furthermore, A12 bound to $gp140_{UG37}$ very well but poorly to $gp140_{CN54}$.

	HIV-1 envelope protein				
VHH	**$gp140_{UG37}$**	**$gp140_{CN54}$**	**$gp120_{IIIB}$**	**$gp120_{YU2}$**	**$gp120_{Ds2}$**
1C2	+++	+++	-	-	-
1F10	+++	+++	-	-	+
1E1	++	+++	+	+	-
1B5	+++	+++	++	++	++
1H9	+++	+++	++	++	++
1E2	++	++	-	-	-
2E7	++	++	+	-	-
A12	+++	+	+++	N.D.	N.D.

Table 1: Summary of VHH binding to HIV-1 Env. The amount of VHH required to give half-maximal A_{490} was estimated from the respective binding curves. +++ half-maximal binding at < 0.63 μg/mL; ++, 0.63-10 μg/mL; +, > 10 μg/mL; -, no binding was observed even at the highest amount of VHH. N.D., not done.

Competition assay between b12 and VHH

To assess whether the selected VHH bind to HIV-1 Env in a way that they may interfere with CD4 binding, we performed a competition assay with b12 (table 2, supporting figure 3). We chose to work with the anti-CD4bs mAb instead of sCD4, because of the poor sCD4 binding to $gp140_{CN54}$ shown previously (supporting figure 1). Note that the epitope of b12 does overlap with the CD4bs but it is not exactly the same, so competition with b12 does not necessarily mean competition with CD4. The competition with b12 was assessed by using $gp120_{IIIB,}$ $gp140_{CN54}$ and $gp140_{UG37}$, as it is known that CD4 binding sites may differ between various HIV-1 subtypes [58, 112]. Our data show that all VHH inhibited binding of b12 to $gp140_{CN54}$ and 1C2, 1B5, 1H9 and A12 prevented the binding of b12 to $gp140_{CN54}$ at concentrations lower than 30 nM.

	HIV-1 envelope protein		
VHH	**gp140$_{UG37}$**	**gp140$_{CN54}$**	**gp120$_{IIIB}$**
1C2	+++	++	-
1F10	++	++	-
1E1	++	++	+
1B5	++	++	++
1H9	+++	++	++
1E2	++	++	-
2E7	++	++	+
A12	+++	++	++

Table 2: Summary of VHH competition with mAb b12 for binding to HIV-1 Env. The amount of VHH required to reduce the b12 signal by 50% of its maximum was estimated from the respective competition curves. +++ < 0.44 μg/mL; ++ 0.44-12 μg/mL; + > 12 μg/mL; -,no competition was observed even at the highest amount of VHH.

A

Immobilized VHH	Soluble VHH									Epitope group
	1C2	1F10	1E1	1B5	1H9	2D4	1E2	2E7	1C12	
1C2	0.0	0.3	82.2	103.4	106.0	117.6	20.8	72.5	79.1	I
1F10	-1.1	0.0	57.0	89.4	129.3	141.5	14.0	79.8	99.7	I
1E1	7.5	14.6	0.0	0.8	0.4	23.3	10.8	5.8	80.0	II
1B5	4.9	12.3	1.2	0.0	-1.2	35.8	12.3	-1.2	61.7	II
1H9	-2.4	6.3	-4.0	-9.5	0.0	28.6	0.8	0.0	59.5	II
2D4	86.1	101.5	46.6	33.5	39.5	0.0	71.7	36.2	101.9	
1E2	50.6	79.0	78.0	86.2	86.6	92.1	0.0	0.0	5.3	
2E7	41.9	74.8	78.9	84.0	96.6	95.6	3.0	0.0	3.6	III
1C12	72.1	113.3	120.5	128.1	122.0	121.5	-0.6	3.9	0.0	

B

Immobilized VHH	Soluble VHH									Epitope group
	1C2	1F10	1E1	1B5	1H9	2D4	1E2	2E7	1C12	
1C2	0.0	2.9	57.6	57.0	53.7	109.0	2.2	64.3	82.5	I
1F10	2.3	0.0	26.2	25.1	26.8	103.7	7.6	24.8	84.5	I
1E1	0.3	2.9	0.0	0.6	-1.6	82.0	-0.6	-2.9	91.0	II
1B5	-6.2	21.0	2.5	0.0	13.4	48.3	162.2	-6.7	26.9	II
1H9	16.7	-8.3	16.7	-33.3	0.0	95.8	-45.8	-75.0	166.7	II
2D4	75.0	53.4	61.6	51.9	42.5	0.0	55.8	38.0	142.5	
1E2	26.1	71.2	76.7	67.0	72.6	92.9	0.0	1.1	7.9	
2E7	15.1	34.7	37.3	52.1	42.4	85.9	1.8	0.0	14.2	III
1C12	28.3	91.7	115.9	120.6	121.9	139.4	7.3	12.1	0.0	

Figure 3: Competition between different VHH for binding toward recombinant Env. Competition matrix of VHH to gp140$_{UG37}$ (A) and gp140$_{CN54}$ (B). The values shown represent the mean binding of Env in presence of the competitor VHH. The effect of competition is expressed in percent of positivity and marked mutual cross-competition (<30%) are shown by light grey cells and weak mutual cross-competition (>75%) by dark grey cells.

Cross-competition assay

To further characterise the selected VHH, we tested whether they compete with each other or if they can bind to Env simultaneously. For this purpose we applied the competition assay described by Kuroki [107]. Since CD4bs and b12 epitopes differ between various Env [58, 112], the experiments were performed with both $gp140_{CN54}$ and $gp140_{UG37}$ (figure 3). Marked mutual cross-competition together with similarity between CDRs was taken as evidence of overlapping epitopes. To define strength of competition we followed the rules described by Tzartos [113]. Although slightly more marked cross-competition reactions were observed for $gp140_{CN54}$ than for $gp140_{UG37}$, the general pattern of cross-competition was similar for both Env. 2E7 binding to its epitope was hampered by 1E2 and 1C12, and vice versa. Noteworthy is the fact that all three VHH derive from various V genes and differ in CDR3 sequences.

Based on those data and sequence and maturation data the selected VHH were classified into three neutralizing groups (I-III) (figure 3). Group III is composed of 2E7, which originate from germline V_m gene. The other members of this group have been selected by family specific rescreening of library 9 (data not shown).

Neutralization assay

Functional characterisation of the VHH was assessed in the TZM-bl cell based neutralization assay developed by Derdeyn *et al.* [114], Wei *et al.* [115] and Li *et al.* [91]. The lowest VHH concentration required to achieve 50% reduction of infectivity (IC_{50}) was determined against a panel of 26 HIV-1 strains derived from clade A, B, C, CRF02_AG and CRF07_BC origin. The results presented in the table 3 show that the VHH from different groups have different neutralization profiles. In contrast to the previously described VHH A12, D7 and C8 [90], as well as mAb b12, which showed the most potent activity against HIV-1 subtype B, some of the newly selected VHH were active against subtype C and CRF07_B/C. Overall, VHH 1B5 and 2E7 were the most broadly neutralizing VHH demonstrating inhibitory activity against respectively 18 and 21 out of 26 viruses tested, predominantly tier 2 neutralization sensitivity class. VHH 1C2 and 1F10 were active against all subtype A, C and CRF07_BC viruses tested, but inactive against most of the subtype B and CRF02_AG strains.

Effect of CDR1 and CDR3 mutations on 2E7 activity

As shown above, 2E7 VHH revealed the broadest cross-subtype neutralization activities, yet its neutralization potency was lower than b12. Therefore we were interested in determining the influence of single amino acids substitutions in 2E7 on Env binding and neutralization potency. We were particularly interested in CDR1 and CDR3 regions since they have been shown to be mainly involved in interaction with antigen [116, 117]. Surprisingly, the back to germline mutation of the residue on position 29 (V29F) seems to enhance binding of 2E7to $gp140_{CN54}$. The alanine scan showed a lack of effect of D32A on binding to Env and improved binding of V29A mutant to $gp140_{CN54}$.

			IC50 in TZM-b1 cells (µg/mL)										
Virus	Subtype	Tier	1C2	1F10	1E1	1B5	1H9	2D4	1E2	2E7	A12	C8	b12
92UG37.A9	A	nd	16	17	•	•	•	•	•	16	•	•	•
MS208.A1	A	1	0.3	0.1	4.1	0.1	0.2	37	4.5	2.9	•	•	0.6
T257-71	CRF02_AG	2	•	•	•	•	•	•	•	45	•	•	•
T266-60	CRF02_AG	2	•	•	•	5.5	8.7	•	15	9.0	•	•	•
T278-50	CRF02_AG	2	•	•	•	10	11	•	•	•	•	•	25
T33-7	CRF02_AG	2	•	38	•	•	•	•	•	38	•	•	•
MN	B	1	6.7	17	•	•	•	•	39	17	0.2	4.5	0.1
IIIB	B	1	•	•	39	6.4	5.6	•	•	10	0.02	0.3	0.04
JRFL	B	nd	•	•	•	48	42	•	•	36	•	•	8.6
6535.3	B	2	•	•	•	30	21	•	•	41	0.1	28	2.5
THRO4156.18	B	2	•	•	•	21	24	•	•	•	6.2	18	0.5
TRJO4551.58	B	2	•	•	•	40	33	•	•	•	16	•	•
96ZM651.02	C	2	27	2.2	34	11	9	•	•	18	0.1	4.3	•
Du156.12	C	2	33	11	•	4.6	37	•	•	8.9	•	•	<1.9
Du422.1	C	2	37	24	•	•	•	•	•	22	•	•	<1.9
ZM197M.PB7	C	2	8.2	5.2	39	16	8.8	•	•	38	6.0	24	7.4
ZM214M.PL15	C	2	6.0	4.3	•	32	•	•	•	22	•	•	<1.9
ZM233M.PB6	C	2	0.2	0.2	•	48	•	•	17	6.4	7.0	38	•
ZM109F.PB4	C	2	1.7	1.7	•	•	•	•	•	•	0.8	38	•
ZM135M.PL10a	C	2	2.6	1.9	•	20	13	•	40	17	•	•	•
CAP45.2.00.G3	C	2	5.8	38	28	7.0	3.7	•	•	30	•	•	<1.9
CH038.12	CRF07_BC	2	2.0	0.8	•	•	•	•	•	13	•	•	<1.9
CH064.20	CRF07_BC	2	4.5	1.5	•	15	22	32	•	20	•	•	•
CH091.9	CRF07_BC	2	14	4.5	•	8.2	38	50	•	36	•	•	•
CH110.2	CRF07_BC	2	6.3	2.6	•	•	40	•	18	16	•	•	•
CH181.12	CRF07_BC	2	2.5	0.8	17	5.9	7	50	•	•	•	•	<1.9
VHH epitope group:			I	I	II	II	II			III			

Table 3: Neutralization of the different viruses by the VHH and mAb b12. Comparison of the neutralization potencies of the VHH and mAb b12 performed in a TZM-b1 neutralization assay against 26 viruses. IC_{50} values above 50 µg/mL are marked as • and IC_{50} values of A12, C8 and mAb b12 are cited from Forsman *et al.*[90]. The putative epitope groups of some of the VHH are given at the bottom. Not determined is marked as Nd.

An alanine scan of the CDR3 region of 2E7 (supporting table 1) revealed that the binding to both Env was unchanged for most mutants. The exceptions were Y98A, Y99A and Y100cA mutants, which bound worse to $gp140_{UG37}$ and $gp140_{CN54}$. Remarkably, mutation of R100bA completely reduced binding of mutant to $gp140_{CN54}$, but not $gp140_{UG37}$.

To verify the influence of the mutations on biological function of the VHH, the neutralization assays were performed against four viruses (table 4). The ZM233M.PB6 virus

			IC50 in TZM-b1 cells (μg/mL)					
Virus	**Subtype**	**Tier**	**2E7 WT**	**V29A**	**Y98A**	**R100bA**	**Y100cA**	**Y102A**
92UG37.A9	A	Nd	16	9	28	16	13	12
96ZM651.02	C	2	18	9	23	20	28	14
Du156.12	C	2	9	4	11	5	6	3
ZM233M.PB6	C	2	6	14	45	33	28	23

Table 4: Neutralization potencies of wild type VHH 2E7 and mutated variants in TZM-bl neutralization assay. Comparison of neutralization potencies of VHH 2E7 wild type and 5 mutants against a panel of 4 viruses. IC_{50} values are given in μg/mL. Not determined is marked as Nd.

was the most resistant and any mutation tested had an adverse effect on 2E7 potency. In contrast Du156.12 virus was the most sensitive to all mutants except Y98A mutant, which revealed decreased potency against all viruses. Interestingly alanine substitution at position 29 slightly enhanced the potency of 2E7 VHH against 96ZM651.02, 92UG37.A9 and Du156.12, making this residue an interesting start point for future *in vitro* maturation studies. This is in agreement with previously observed enhancement of binding affinities.

Discussion

HIV-1 subtype C viruses have become predominant epidemic strains in the world (http://www.unaids.org). The well described broadly neutralizing antibodies of human origins b12, 2G12, 4E10 and 2F5 overall show limited neutralization of subtype C viruses[71, 72, 118, 119]. More recent, potent antibodies PG9, PG16 and VRC01-03, have been selected from blood cells of HIV patients[29, 30, 59, 83]. Nonetheless selection of novel anti HIV-1 neutralizing antibodies is crucial for a better understanding of immune responses to non-subtype B viruses. Further, it is important for the development of better and cheaper microbicides, effective against the most prevalent subtypes, as the recently tested tenofovir gel only reached 39% protection[120].

In the current study, two llamas were immunized with a cocktail of recombinant Env proteins of HIV-1 subtype A ($gp140_{UG37}$) and CRF07_BC ($gp140_{CN54}$) to select VHH against non-subtype B HIV-1 using competitive elution. From the 280 clones tested, 30 clones competed with b12. Sequence analysis revealed 17 unique VHH that were clustered into 7 families based on V-, D- and J- gene usage during their maturation. Our previous data, described by Forsman *et al.* in 2008 [90] demonstrated that competition between sCD4 and VHH-phage complexes results in the release of a phage population enriched in sCD4 competitors. Free gp140 is thought to sample many conformations[121] and only the CD4-bound conformation promotes the virus entry process[122]. Thus it is reasonable to assume that during selection, VHH could recognize different conformations of Env, and subsequent elution with sCD4, could release not only CD4bs binders, but also VHH that interact with regions involved in transition to CD4-bound conformation and therefore could reveal

neutralization activities.

The lack of crystal structures of Env in complex with VHH prevents us to exactly localize VHH epitopes on Env However, four sets of experimental data, i.e. binding to Env (table 1), sequences (figure 2), cross-competition data (figure 3) and neutralization data (table 3), allowed us to categorize the selected VHH into epitope groups. Moreover, current knowledge of Env complexes with sCD4, b12 and F105[123] and the VHH interactions with mutated Env helped us to deduce the localisation of the VHH epitopes. The structural analysis studies have revealed that both sCD4 and b12 recognize non-linear epitopes on Env, ranging from amino acid 124 to 477, and that F105 binds to a different site than b12 [58, 124]. This information has been applied by the group of Peter Kwong to construct mutant Env, in which the bridging sheet was tethered to the inner domain [78]. We used both mutated ($gp120_{Ds2}$) and wild type $gp120_{YU2}$ Env for VHH characterisation. Similarly to most of the antibodies that target the CD4bs [58] the previously selected high affinity VHH A12 and D7 [90] most likely recognize the cavity under the bridging sheets as they bound to $gp120_{YU2}$, but not to $gp120_{DS2}$.

More recent studies show that VHH A12 and D7[90, 125] most likely bind to an epitope equal or closely related to the epitope recognized by F105 [126]. Since F105 is not a broad neutralizing antibody, this epitope will most likely not yield broad VHH either and thus there is a need for VHH that recognize other epitopes. From the VHH selected in this study, the VHH of group I (represented by 1F10 and 1C2) did not bind to subtype B Env, showed different competition and neutralization patterns than VHH of groups II and III and therefore they may bind to B20/B21, which is part of the bridging sheets and forms the loop between the cavity and the outer domain. The VHH belonging to group II (1B5, 1H9, 1E1) most likely recognize the outer domain contact site for CD4 of Env [58], since these VHH interacted with both $gp120_{Ds2}$ and $gp120_{YU2}$. However, in contrast to b12, 1B5 recognized a larger number of viruses including non B viruses and neutralized in total 18 out of 26 viruses.

Based on these considerations and the 3D structures of Env we propose that the three major groups of anti-HIV VHH bind to Env in the cavity below the bridging sheets (A12/D7), the bridging sheet themselves (1F10) and the outer domain (1B5). 2E7, representing group III, bound slightly to $gp120_{IIIB}$, but not to $gp120_{YU2}$ nor to $gp120_{Ds2}$ and had the broadest neutralization spectrum of HIV-1 subtypes (21 out of 26) of any CD4bs targeting antibodies derived from immunizations. The epitope recognized by 2E7 differs from the antigenic determinants of the 1B5 group as is shown by competition experiments and the various spectra of viruses neutralized by group II and III. Cross-competition between 2E7 and 1E2 or 1C12 probably resulted from alteration of the antigenic determinant conformation by the competing VHH or steric occlusion. To further map the exact location where the VHH bind to Env, competition assays can be performed with more mAbs of known specificity. Also their binding to two additional Yu-2 mutants, D368R which is not bound by sCD4 or CD4bs mAbs [127], or I420R which is no longer bound by CD4i specific

mAbs, can be tested[76]. The large breadth of 2E7 and 1B5 makes them interesting candidates for further studies. For that reason we carried out a restricted number of mutations on basis of the maturation analysis and this provided us with both an improved neutralizing 2E7 variant and insight, which amino acids of CDR1 and CDR3 take part in Env binding.

Although the VHH epitopes still remain to be precisely localized with biophysical methods, our data strongly suggest that the neutralizing VHH recognized various epitopes. *In vitro* maturation of 2E7 and 1B5 and construction of hetero-specific biheaded VHH[60], in particular biheads consisting of VHH derived from group I and II or II and III, might broaden virus cross-neutralization and enhance their potency. The effect of bivalency was recently described with a molecule that combined the binding domains of sCD4 and 17b with a 35 amino acid linker. This construct neutralized all 47 strains tested coming from clade A, B, C, D, F, CRF01_AE and CRF02_A/G, 90% with an IC_{50} below 4 μg/ml[128].

The evidence that cell-to-cell transfer may be a major factor in spreading the HIV-1[84, 129] and the fact that VHH are small enough to operate in the viral synapsis may increase the potential of anti-HIV-1 VHH as functional ingredient in microbicides just as their generally high stability[130]. As microbicides are generally tested on simian-human immunodeficiency virus (SHIV) a small scale assay was performed to evaluate that. 1B5 and 2E7 were active against type B SHIV, 1B5 and 1F10 against type C SHIV (to be published). Finally, the new VHH recognizing three different areas of the CD4bs may provide useful insight into vaccine development.

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Chapter 3

Broadly neutralising single-chain llama antibody fragments targeting novel gp120 and gp41 epitopes on the HIV-1 envelope spike.

Broadly neutralising single-chain llama antibody fragments targeting novel gp120 and gp41 epitopes on the HIV-1 envelope spike.

Anna Forsman Quigley*[1,2], Nika M Strokappe*[3], Laura E McCoy[1,2], Lucy Rutten[3], Shiang Tan[1,2], Marlen MI Aasa-Chapman[1,2], Michael S Seaman[4], Agnieszka Szynol, Ying Ying Liu[3], Hans de Haard[3], Robin A Weiss[1,2], Theo Verrips[3].

[1]Wohl Virion Centre and [2]Medical Research Council (MRC) Centre for Medical Molecular Virology, Division of Infection and Immunity, London, UK
[3]Biomolecular Imaging, Department of Biology, Institute of Biomembranes, Faculty of Science, Utrecht University, 3584 CH Utrecht, Netherlands
[4]Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215
*Both authors contributed equally to this work.

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Abstract

Even though HIV-1 medications are getting better by the minute, there is still an urgent need for effective preventative measures. Recent studies, such as the CAPRISA 004 trial[120], have shown that the topical administration of microbicides may be the basis of such a prevention method[198].

The major target for monoclonal antibodies (mAbs) on HIV-1 is its envelope glycoprotein (Env) spike, consisting of a trimeric gp41 stem, associated with three copies of gp120. A handful of broadly neutralizing mAbs that were derived from long term non-progressors, gave us insights into which epitopes on the envelope spike may be best to target. First, the CD4 binding site, which is the first contact point between virus and target cell. Second, the co-receptor binding site, with which Env interacts following CD4 binding. Targeting this site has one major disadvantage: it is shielded from antibody binding until CD4 is bound to gp120. Third, is gp41 which has been proven to be a difficult target for neutralizing mAbs as it is shielded by gp120 and close to the viral envelope. However, CD4 binding to gp120 results in substantial conformational changes in gp120 and probably also an initial conformation change in gp41, making some neutralizing epitopes more accessible to antibodies.

Here we use small antibody fragments, derived from the heavy chain only antibodies found in *Camelidae*, termed VHH. We assumed that these VHH, which are about ten times smaller than conventional mAbs, are small enough to target non-CD4 binding site related epitopes like the co-receptor binding site and gp41. We hypothesized that the VHH could recognize epitopes presented by the native envelope conformation, as CD4i and gp41

epitopes are well exposed for only a relatively short time during the viral entry process. By targeting these sites the VHH can bind the virus at any time point, making sure it is in close proximity to it when necessary, potentially lowering the concentration of VHH needed for neutralization.

Introduction

Over the years, broadly neutralizing mAbs against HIV-1 have been isolated from infected patients. Even though recently many more neutralizing mAbs have been described [29, 30, 76, 77, 131, 132], they are still more an exception then rule[70, 133, 134].

HIV is an enveloped virus and the only viral protein present on the virion surface is Env. The Env spike structure consists of three gp41 molecules that make up the stem and anchor the spike in the viral membrane. On top of the gp41 trimer are three copies of non-covalently bound gp120 subunits. Gp120 is the main target of broadly neutralizing mAbs, but some mAbs do target the less accessible gp41[135-137]. Gp120 is a heavily glycosylated protein[16] that undergoes major conformational changes on binding to CD4 and only then reveals its vulnerable co-receptor site. This shielding of conserved epitopes[18] narrows the time that they are presented to the body's immune system, greatly reducing the efficacy of neutralizing antibodies against these sites[63].

Members of the *Camelidae* family have, in addition to conventional antibodies, a subtype of IgG that lacks the light chain[44]. The antigen binding domain of this heavy chain only antibody is called a VHH and with an average molecular weight of only 15 kDa, it is the smallest known antigen recognizing domain that is derived from nature. VHH have a number of qualities that make these small molecules have big potentials. First, VHH are generally quite stable in a wide range of temperature and pH[46, 50, 130]. Second, they lack light chains, therefore when screening for a specific antibody function it is not necessary to "find" the proper light chain to pair it with. Third, VHH are easily expressed in a bacterial or yeast system, which makes them relatively cheap to produce[46, 51] and fourth they are hardly immunogenic in humans, as they have around 90% sequence similarity to human antibodies. Not having a light chain can be beneficial to a heavy chain only antibody as the narrow paratope may facilitate deeper penetration into molecules, such as into enzymatic clefts[49, 138]. The disadvantage of lacking the light chain is of course that the antigen binding surface is reduced. However, it is thought that VHH compensate for this by generally having longer Complementary Determining Regions (CDR) 1 and 3[42], which further facilitates the deeper penetration into clefts.

Previously, we have shown that neutralising VHH can be selected from llamas immunised with recombinant monomeric gp120 derived from the HIV-1 CRF07_BC isolate CN54 [90, 125, 139]. However, these VHH were relatively limited in their neutralisation breadths and potencies [90]. Therefore, we hypothesised it may be advantageous to immunize with recombinant trimeric gp140 as previous studies have shown that trimeric Env proteins may

be better at eliciting neutralizing antibodies against the HIV-1 spike[140].

In addition, a previous study has shown that trimeric Env immunogens were more able to induce cross-subtype-neutralising antibodies than monomeric gp120[141]. To further increase the likelihood of generating broader VHH, a mixture of two recombinant gp140 immunogens were administered to the llamas in this study. These recombinant Env were derived from HIV-1 primary isolates of subtype A (92UG037) and CRF07_BC (CN54) and were injected intramuscularly into two llamas (llama 8 and 9) for six times. Furthermore, we extended the immunization schedule with an additional boost, 77 days later, at day 122 to give the llamas' immune system more time to mature the antibodies. Recently we have described the subsequent isolation of broadly neutralising VHH using two distinct isolation strategies[142, 143].

In the first of the two studies[143], a CD4bs-targeted selection strategy was applied, similar to that used for the gp120-immunised llamas[90].This involved panning the VHH libraries on immobilised gp140 followed by a competitive elution with sCD4, to promote the enrichment of VHH targeting CD4bs-related epitopes on gp140. Subsequently the VHH were screened for the ability to compete with mAb b12 for binding to HIV-1 Env. This resulted in the isolation of six groups of VHH with greater cross-reactive neutralisation properties (up to 80% of strains tested) than the VHH obtained from the gp120-immunized animals. In the second study[142], a novel VHH isolation strategy was applied, in which the panning step was excluded. This eliminates the enrichment for VHH that bind the recombinant Env protein as it is coated on a plate, as excellent binding does not necessarily mean that it is neutralizing. Instead, the VHH library from llama 8 was subjected to a direct neutralisation screen, resulting in the isolation of VHH J3, which targets an epitope located almost exclusively within the CD4bs. The neutralisation breadth (96%) and potency of J3 are comparable to that of the few best mAbs, derived from natural infection[142].

Previous directed selection strategies have all been aimed at isolating VHH targeting CD4bs-related epitopes on HIV-1 Env[90, 139]. Whilst the CD4bs-related epitopes are important targets for vaccine design and for broadly neutralising antibodies in natural infection, isolating VHH to additional, non-CD4bs-related epitopes is also of interest. This as the use of multiple molecules attacking HIV simultaneously, is much more effective than using only one[144]. In this study, we focus on all regions of HIV-1 Env exposed upon binding to CD4, termed CD4-induced (CD4i) epitopes, but also the epitopes that are unaffected by CD4 binding.

Some human mAbs to CD4i sites, such as 17b[145] and X5[146], have been found to be relatively broadly neutralising as Fab fragments[145, 146]. This indicates that these type of antibodies target epitopes that are to some extend conserved among HIV-1 strains[17, 147]. Anti-CD4i antibodies have also been reported to be induced in human vaccine trials[148], as well as fol-

lowing immunisation in macaques[149, 150], where they correlated with virus control in a SHIV challenge[149]. This indicates that these antibodies are of importance in the fight against HIV. However, it is thought that full length IgGs of these CD4i mAbs are unable to bind the functional HIV-1 Env spike due to steric hindrance[63]. A further disadvantage is that CD4i antibodies have been found to neutralise HIV-1 primary isolates much better or even only in the presence of sCD4[63, 145-147, 151].

We hypothesised that the small size of VHH would make them suitable for targeting the conserved but possibly occluded CD4i epitopes. To this end, we combined a directed selection strategy with direct screening for neutralization. Therefore the VHH libraries from llama 8 and 9 were panned on immobilised gp140 bound to sCD4, followed by a competitive elution with anti-CD4i mAb 17b. This promotes the selection of VHH targeting epitopes similar to the CD4i epitopes recognised by 17b. Subsequently, a total elution using alkaline shock was performed to enrich for VHH recognising additional, novel epitopes exposed on CD4-bound gp140. VHH related to those derived from the 17b elution were also found via a completely different selection strategy, focussing on b12-like antibodies. Briefly, this involved panning on an immobilised Yu-2_{DS2} mutant gp120[78] and used b12[152] for the elution step. Thus, as related VHH were identified by both selection methods all VHH derived from these selections were included in the screening.

These selection experiments resulted in the isolation of a set of VHH with distinct, and in some cases very broad, neutralisation profiles. However, none of the VHH showed a marked increase in binding to gp140 in the presence of sCD4, compared to gp140 alone. This indicates that they do not recognise CD4i epitopes, in the sense that their epitopes are more accessible to the VHH upon sCD4 binding to Env. This is beneficial for VHH neutralisation, as the VHH can bind the trimer at all times and not only in the small timeframe after CD4 binding. On the other hand, none of the VHH competed with sCD4 for binding to Env, indicating that they do bind epitopes outside of the CD4bs.

Material and Methods

Monoclonal antibodies.

MAb b12[152] and 17b[145] were obtained through the Centralized Facility for AIDS Reagents (CFAR), National Institute for Biological Standards and Controls (NIBSC), (original source, D Burton & Dr J Robinson respectively).

Recombinant HIV-1 envelope proteins.

Recombinant trimeric $gp140_{UG37}$ (subtype A) for immunizations and ELISAs was kindly provided by S. Jeffs, Imperial College London, recombinant trimeric $gp140_{CN54}$ was provided by Polymun for the immunizations, for ELISA it was obtained via NIBSC (EVA699, Polymun Scientific). Recombinant $gp120_{IIIB}$ (EVA657) and CN54 (ARP699) and gp41 (ARP680) were obtained from the CFAR, NIBSC for ELISAs. Yu-2_{DS2} gp120 was kindly

provided by L. Chen (Dr. P Kwongs lab)

Viruses.

HIV-1 IIIB (ARP101) and YU2 were obtained from the Centralized Facility for AIDS Reagents (CFAR), NIBSC. IIIB was donated by R. Gallo and M. Popovic (University of Maryland School of Medicine, Baltimore, MD) and was propagated in H9 cells. HIV-1 CRF07_BC primary isolate 97CN54 was obtained from EMPRO and was propagated in peripheral blood mononuclear cells. All other replication competent virus stocks were prepared from HIV-1 molecular clones by transfection of 293T cells. The 92BR025.C1 (C111) molecular clone was obtained by amplifying gp120 from cells infected with the WHO panel peripheral blood mononuclear cell–grown isolate and inserting it into the pHXB2 backbone[90]. HIV-1 envelope pseudotyped viruses were produced in 293T cells by co-transfection with the pSG3Δenv plasmid[153]. The subtype B and C HIV-1 Reference Panels of Env Clones[91,119] were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH. HIV-1 subtype CRF07_BC gp160 clones, subtype CRF02_AG gp160 clones (263-8, T278-50, T266-60) and the 92UG037, 93MW965.26 and 96ZM651.02 gp160 clones were kindly provided by Dr D. Montefiori (Duke University Medical Center, Durham, NC) through the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA2 VIMC) as part of the Collaboration for AIDS Vaccine Discovery (CAVD). All additional pseudoviruses were produced at the CAVD Preclinical Neutralizing Antibody Core laboratory.

Cells.

TZM-bl cells (Derdeyn et al., 2000; Li et al., 2005; Wei et al., 2002) were obtained through the NIH AIDS Research and Reference Reagent Program from J. C. Kappes, X. Wu, and Tranzyme, Inc., and cultured in Dulbecco's modified Eagle medium (Invitrogen) containing 10% (v/v) fetal calf serum (FCS).

Immunization of Lama glama and construction of VHH phage library.

This is described in detail by Strokappe et al. and McCoy et al[142,143]

Panning on envelope proteins

CD4 bound gp140 protein: MaxiSorp plates were coated overnight at 4°C with 100 µl of serially diluted $gp140_{UG37}$ or $gp140_{CN54}$ in sterile PBS, starting at a concentration of 10 µg/ml. The next day, the plates were blocked with 4% Marvel in PBS (MPBS) for 1 hour. After washing the plates with PBS/0.05% Tween 20 (PBST) for 3 times, 100 µl of saturating amounts of sCD4 in 1% MPBS was added and allowed to bind for 1 hour at room temperature (RT). PEG precipitated phages, displaying VHH from library 8 and 9 were preincubated 1:10 in 2% MPBS, for 30 minutes before adding them to plate which was washed again for 3 times with PBST. Phages were allowed to bind to the antigen for

2 hours while shaking at RT, before the plate was washed for 40 times with PBST to remove unbound phages. The plate was washed an additional 3 times with PBS to remove all remaining tween. Bound phages were eluted with Triethylamine (TEA) and rescued by infecting log phase TG1 bacteria.

The outputs from the 10 μg/ml coated wells were used for the production of phages used as second round input. To purify these phages, PEG precipitation was performed. The method used in the second round of selection was similar to that of the first round, with a few exceptions, here the plates were coated with 2.5, 0.5 and 0 μg/ml $gp140_{UG37}$ and $gp140_{CN54}$, and 10 times less phages were used. Next to a total elution, competitive elution was done, using an excess of 17b. A total of 756 individual clones were picked for further screening.

Modified gp120 protein: For the panning on the modified gp120 a similar protocol to that of the sCD4 bound gp140 was used, with some exceptions. MaxiSorp plate was coated overnight at 4°C with 100 μl of 5, 1.67, 0.56 and 0 μg/ml $gp120_{DS2}$ {{54 Chen,L. 2009}} in sterile PBS. The step involving sCD4 binding was omitted, all other steps were identical.

The outputs from the 5 μg/ml coated wells were used to produce phages for the second round input. For the second round of panning 2, 0.4 and 0 μg/ml $gp120_{DS2}$ was used. Instead of using 17b, a 50 times molar excess of b12 was used. A total of 190 individual clones were picked for further screening.

Production and purification of VHH

The clones selected for initial screening were expressed in 96 well format. Each clone was expressed in 1 ml of 2×Yeast Tryptone medium supplemented with 100 μg/ml ampicillin and 0.1% glucose, followed by induction of VHH production with 0.1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). The periplasmic extract from each well was harvested by freeze thawing.

Larger quantities of VHH were necessary for those selected in the screen. Therefore the VHH gene was transferred into our production vector pCAD51. Expression from the pCAD51 vector incorporates a C terminal myc and 6-His tag to the VHH and removes the bacteriophage gene III product. VHH were purified by means of the attached 6His-tag using TALON Metal Affinity Resin (Clontech).

HIV neutralisation assay

The HIV-1 neutralising activity of the VHH were assessed in the TZM-bl cell based assay, as developed by [91, 114, 115]. The neutralization activity of the VHH was carried out in two separate laboratories: UCL and the CAVD Pre-Clinical Neutralizing Antibody Laboratory at Harvard Medical School. Each VHH-virus combination was tested in duplicate/triplicate on one or more occasions. No neutralisation was observed with negative control VHH or with a negative control pseudovirus bearing a murine leukaemia virus (MuLV) envelope. Briefly, 50 μl periplasmic fractions (for the screen) or 3-fold serial dilutions of purified

VHH starting from 50 μg/mL (for IC50 calculations) were added in 10% (v/v) fetal calf serum (FCS) supplemented DMEM growth medium (Invitrogen, Paisley, UK). Note that the periplasmic fractions were passed through a 0.2 μM PDVF membrane to remove bacterial contaminants that could adversely affect the cell-based assay. 200 TCID50 of virus was then added to each well and the plates were incubated for 1 hour at 37°C. TZM-bl cells were subsequently added (1 x 104 cells/well) in growth medium supplemented with DEAE-dextran (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 11 μg/mL. Assay controls included replicate wells of TZM-bl cells alone (background control), and TZM-bl cells with virus assayed (virus control). No virus inactivation was observed with a negative control VHH. Following 48 hours incubation at 37°C, 100 μL of the assay medium was removed and 100 μL of Bright-Glo luciferase reagent (Promega, Madison, WI, USA) was added to each well. The cells were lysed for 2 minutes, and the luminescence measured using a Glomax luminometer. The 50% inhibitory concentration (IC50) titres was calculated as the VHH concentration that achieved a 50% reduction in relative luminescence units (RLU) compared to the virus control RLU, after subtraction of the background control RLU from both values. VHH 50% inhibitory concentration (IC50) were calculated using the XLFit4 software (ID Business Solutions, Guildford, United Kingdom).

Sequence analysis and Bio-informatics

VHH sequences have been clustered using ClustalX analysis, and the result of this clustering was displayed as a phylogenetic tree (figure 2 a and b). The final tree was constructed by FigTree version 1.3.1 software. We also determined the most likely maturation patterns of these VHH using the knowledge of 23 V- and 7 J-genes as determined in *Lama glama* or *Vicugna pacos* (figure 2c) (unpublished).

Binding and competition ELISA's

Binding to Env: MaxiSorp plates were coated with 2.5 μg/ml $gp140_{UG37}$, $gp140_{CN54}$, $gp120_{IIIB}$ or gp41, overnight at 4°C. The next day, the plates were blocked with 4% MTBS for 1 hour, after which 3 times serially diluted VHH (starting from 5) in 1% MTBS were added. The VHH were detected with 1:2000 mouse anti Myc (9E10) via their C-terminal Myc tag, followed by 1:5000 AP conjugated anti mouse antibody.

Competition with sCD4: MaxiSorp plates were coated with 10 μg/ml sCD4 in 0.1 M NaHCO3, pH 8.5, overnight at 4°C. Plates were blocked with 4% Marvel in TBS (MTBS) and at the same time serially diluted VHH was allowed to bind to 0.5 μg/ml $gp140_{UG37}$. VHH-gp140 mix was added to the plates and gp140 was detected by 10 μg/ml D7324 (Aalto Bio Reagents) followed by 0.5 μg/ml AP conjugated rabbit anti sheep (catalogue number. ab6748-1, Abcam, Cambridge, UK).

Competition with mAbs: MaxiSorp plates were coated with 2.5 μg/ml UG37 gp140, overnight at 4°C. Plates were blocked and serially diluted VHH in 1% MTBS added. Subsequently 5 μg/ml b12 and 17b were added and detected by 0.5 μg/ml AP conjugated goat

anti human IgG (Harlan SeraLab).

Competition with VHH: MaxiSorp plates were coated with 5 μg/ml VHH in 0.1 M NaHCO3, pH 8.5, overnight at 4°C. Plates were blocked and at the same time a serial dilution of the competing VHH was allowed to bind to 0.5 μg/ml UG37 gp140. VHH-gp140 mix was added to the plates and gp140 was detected by human serum, followed by 0.5 μg/ml AP conjugated goat anti human IgG.

For all steps, the added proteins were allowed to bind for 1 hour, shaking, RT, before washing three times with 0.05% Tween20 supplemented TBS. All antibodies and sCD4 were added in 1% MTBS. Before adding the detection agent, the plate was washed an additional three times with TBS, to remove all Tween remnants. Then the amount of bound AP conjugated antibody was visualized by adding 100 μl per well of Lumi-Phos Plus substrate (Aureon Biosystems). Chemiluminescence was detected after 30 min. incubation at 37°C using a Glomax luminometer.

Results

Immunisation of llamas and construction of phagemid libraries

Two *Lama glama*, designated llama 8 and llama 9, were immunised with a mixture of recombinant $gp140_{UG37}$ and $gp140_{CN54}$, derived from HIV-1 primary isolates 92UG037 (subtype A) and 97CN54 (CRF07_BC). Following the relatively long immunisation, the anti-gp140 antibody response in serum was evaluated in ELISA. Since a satisfactory serum anti-gp140 antibody response was induced in both animals[143], phage libraries containing the cloned VHH repertories were constructed. This resulted in two libraries of around 10^7 transformants each. Further details are discussed by Strokappe *et al.* [142, 143] and McCoy *et al.*[142].

Selection of VHH binding to recombinant HIV-1 Env proteins

In order to enrich for VHH which bind epitopes exposed on CD4-bound HIV-1 Env, we employed a directed selection strategy where the phage libraries from llama 8 and 9 were panned on immobilised $gp140_{UG37}$ or $gp140_{CN54}$ which had been pre-incubated with saturating concentrations of sCD4. Prior to carrying out the panning, we confirmed that sCD4 and 17b bind $gp140_{UG37}$ and $gp140_{CN54}$ in ELISA (figure 1). Binding to $gp120_{IIIB}$ was also tested as monomeric Env is useful during further characterization of the VHH. As shown in figure 1a, sCD4 binds all three Env, however the binding to $gp140_{CN54}$ did not yield high signals. Given the superior binding, a coating concentration of 2.5 μg/ml was used for panning.

Figure 1b shows the subsequent binding of 17b to the sCD4 bound Env next to the binding to Env alone. For all three Env, 17b binds more strongly in the presence of sCD4, confirming that 17b is a good candidate for competitive elution of VHH targeting CD4i epitopes.

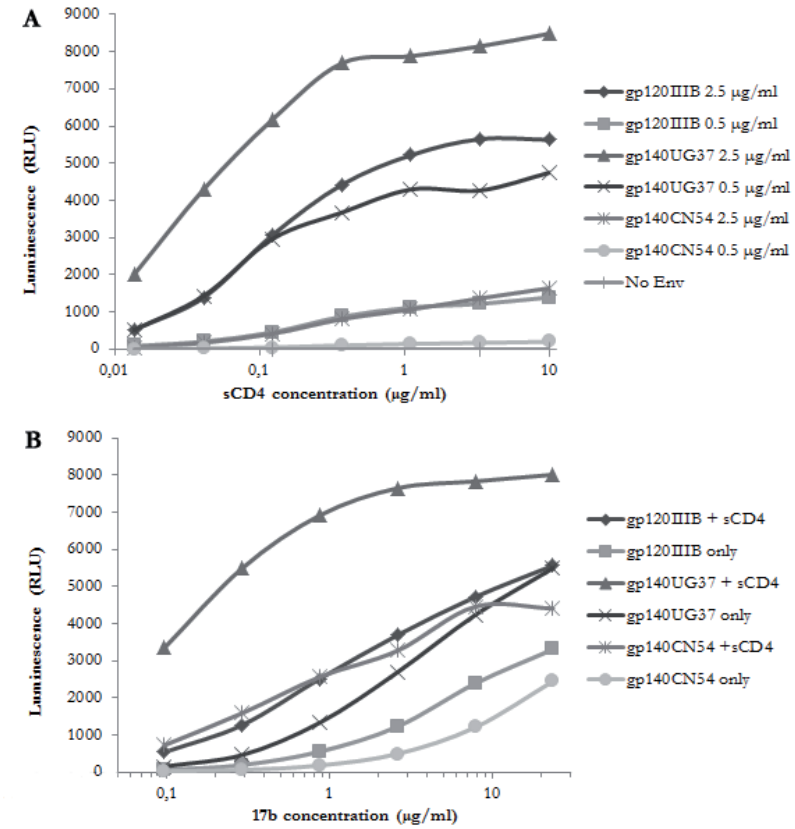


Figure 1: The binding of sCD4 and 17b to Env. A) The binding of sCD4 against two concentrations of $gp120_{IIIB}$, $gp140_{UG37}$ and $gp140_{CN54}$ in ELISA. B) The binding of mAb 17b to $gp120_{IIIB}$, $gp140_{UG37}$ and $gp140_{CN54}$ in the presence and absence of saturating concentrations of sCD4 in ELISA.

In the first round of panning, all phages binding to sCD4 bound gp140 were enriched. For the second round, following the enrichment of phages binding to gp140 in the CD4i conformation, a more specific elution was performed, using 17b. Here an excess of 17b prevents rebinding of the phages to the 17b epitope after their release. From the different outputs a total of 756 individual clones were picked for further screening.

Parallel to this, two rounds of panning were performed on a recombinant gp120 Yu-2 mutant [78]. This mutant $gp120_{DS2}$ has an additional disulphate bond, to mimic the CD4 bound conformation. The first round of panning yielded an enrichment of approximately 10^3 for library 8 and 10^2 for library 9 for the highest concentration compared to the non-coated well. The second round of panning lead to an enrichment $>10^6$ for the total elution of both library 8 and 9, b12 eluted about 10 times less (Data not shown). A total of 190 clones were picked for further screening.

Screening of the individual clones

The 756 individual clones picked from the panning on gp140-sCD4 complexes, were screened for their neutralization capacity. Briefly, periplasmic fractions containing monoclonal VHH, were tested against at least 2 different viruses. A clone capable of decreasing

viral infectivity more than 40% was marked as neutralizing. Overall library 9 produced a higher percentage of neutralizing VHH compared to library 8 (31.1% vs. 15.5%) and the total elution yielded more neutralizing VHH compared to 17b elution (30.2% vs. 16.2%). A total of 161 neutralizing clones were identified. Neutralizers were rescreened on additional, tier 2 and 3, virus isolates, albeit still in a non-purified format and the 60 most promising VHH were sequenced.

The 190 clones that derived from the panning on $gp120_{DS2}$ were screened by testing their periplasmatic fractions for their ability to compete with b12. The 22 clones that decreased b12 binding by at least 50% were sequenced.

Figure 2 a and b show the phylogenetic trees generated for the VHH obtained during these panning experiments, with the addition of the previously described VHH [142, 143]. In both trees, the sequences derived from the VHH that were panned on $gp120_{DS2}$ all grouped together, with the exception of 1H1, which is non-neutralizing. This clustering may be due to their different elution or screening method, or to panning on $gp120_{DS2}$. All other VHH sequences are distributed evenly around the trees, apart from a large group in the llama 9 tree (top left quadrant) that has only one VHH derived from panning on gp140-sCD4. This indicates that the epitope of these VHH may no longer be present on the gp140-sCD4 complex, for instance if their epitope overlaps with that of CD4. Furthermore, there are two small groups distinguishable in the llama 8 tree that are derived solely from the total elution on gp140-sCD4 complexes.

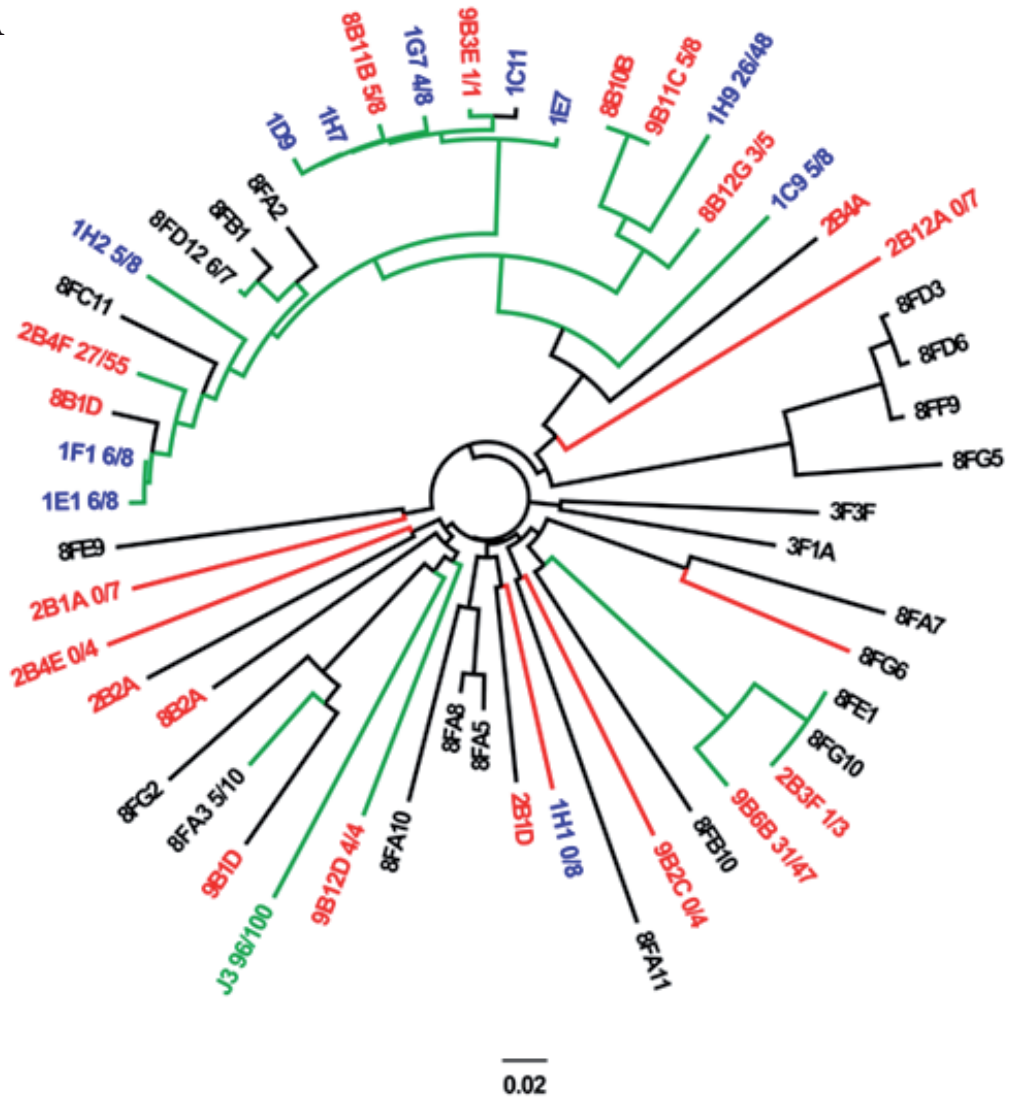
Representatives of the different families were tested for their neutralization capabilities on several virus isolates, in purified format (data not shown). Based on neutralization potency, targeted epitope and sequence analysis, a total of six VHH were selected for further characterization. A sequence alignment of these VHH together with the previously described 2E7 is shown in figure 2c. The VHH are aligned with the germline sequences of which they are most likely derived. Note that 11F1B has an insertion of 6 amino acids in CDR2, which was observed only once in the 1012 sequences analysed

The six selected VHH neutralise HIV-1 of several subtypes

The neutralisation activities of the selected VHH were evaluated against a panel of viruses expressing Env derived from subtype A, B, and C, as well as CRFs 01_AE, 02_AG, and 07_BC viruses, in the TZM-bl neutralisation assay[91, 114, 115]. No neutralisation was observed with a negative control VHH[98] or with a negative control pseudovirus bearing a murine leukaemia virus (MuLV) envelope (Table 1).

The related VHH 1H9 and 2B4F neutralised a similar set of viruses, although 1H9 was able to neutralise three viruses that 2B4F did not. They neutralised 26 out of 48 (54%) and 27 out of 55 (49%) viruses tested, respectively. Both VHH were most broadly reactive against viruses expressing Env of subtype B, C, and CRF07_BC, neutralising around 50% of the subtype B viruses, up to 73% of subtype C and 100% of the CRF07_BC viruses.

A



0.02

C

```
          -------------------------<  CDR1 >------------------<     CDR2    >
Vp+J4  QVQLVESGGGLVQAGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVAAI     TSGGSTN
11F1F  EVQLVESGGALVQAGRSLRLSCAASGNAFTIDAAAWYRQAPGKQREPVATI     LSGGTTN
2E7    EVQLVESGGGLVQPGGSLRLSCAASGNIVSIDAAGWFRQAPGKQREPVATI     LTGGATN

Vp+J4  QVQLVESGGGLVQAGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVAAI     TSGGSTN
5B10D  EVQLVESGGGLVQAGGSLTLSCEASGTIFSINGQGWFRQAPGKQREPVAFI     TSGGMTN

Vk+J5  QVQLVESGGGLVQPGGSLRLSCAASGSIFSINAMGWYRQAPGKQRELVAAI     TSGGSTN
11F1B  EVQLVESGGGLVQPGGSLRLSCAASGNILGENTMNWYRQAPGKERELVAGVTNLRSTYYPDGVK

Vc+J4  QVQLVESGGGLVQPGGSLRLSCAASGFTLDDYAIGWFRQAPGKEREGVSCI    SSSDGSTY
2B4F   EVQLVESGGGLVQAGGSLRLSCAASGFTSDDYAIGWFRRAPGKEREGVSYI    SIEDGSTY
1H9    EVQLVESGGGLVQAGGSLRLSCVCSGFSSDEYAIGWFRQAPGKEREGVSYI    SREDGSRH

Vs+J4  QVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVAAI    SWSGGSTY
9B6B   EVQLVESGGGLVQTGGSLRLSCTASGQTSIIDAVGWFRQAPGKEREFVSAI    SWSDNNIY
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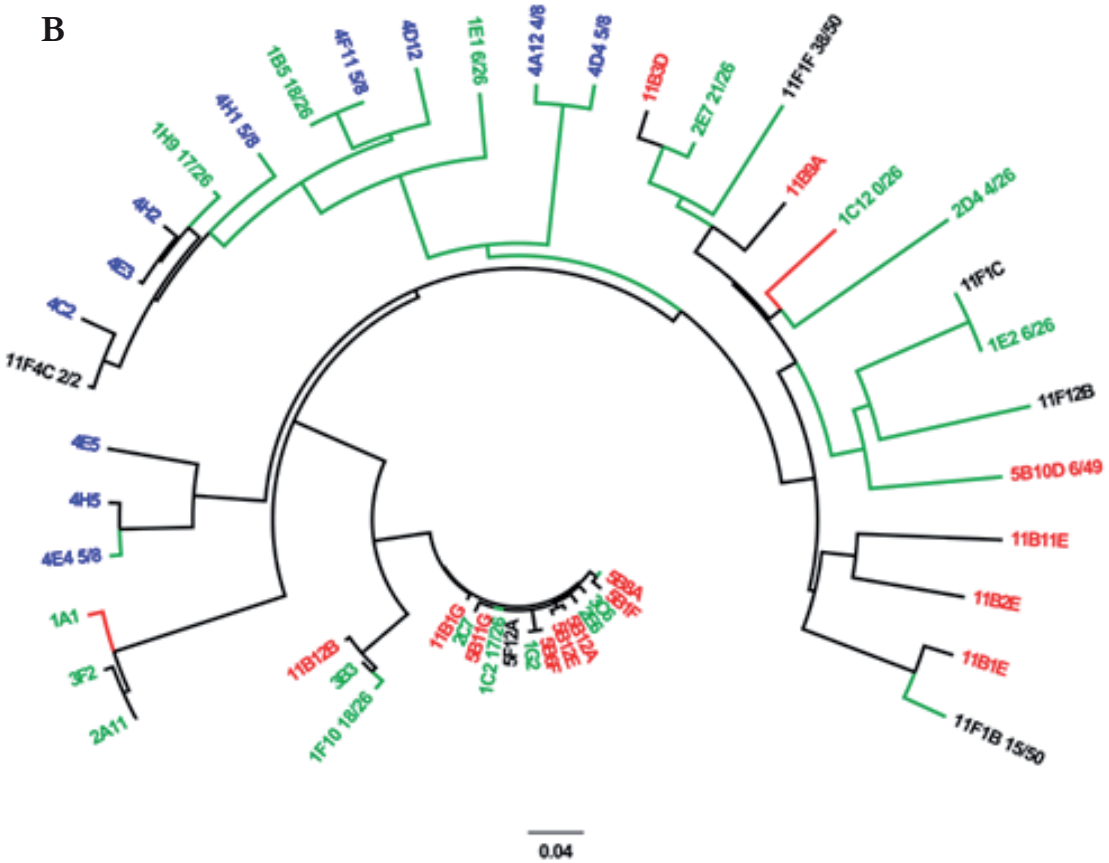

Chapter 3

Figure 3: Phylogenetic trees and sequence aligment of VHH derived from llama 8 and 9. Phylogenetic trees of all VHH identified from llama 8 (A, top left) and llama 9 (B, top right). The color of the VHH name indicates the origen of the VHH (Black: gp140-sCD4 TEA elution, Red: gp140-sCD4 17b elution, Blue: $gp120_{DS2}$ b12 elution, Green: previously described), the color of the lines indicates whether the VHH is neutralizing (Green: neutralizing, Red: non-neutralizing, Black: not tested in purified form). The numbers behind some of the the VHH names indicate how many viral strains the VHH is capable of neutralizing. C (bottom, both pages) shows an alignment of the VHH together with the germline they are most likely derived from. CDR 1, 2 and 3 are marked.

```
-------------------------------------<        CDR3        >-----------
YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA                    EYDYWGQGTQVTVSS
YADSVKGRFTISRDNVKNTVYLQMNSLKPEDTAVYYCYV      PMVYYSGR      YNDVWGQGTQVTVSS
YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCYA      PMIYYGGR      YSDYWGQGTQVTVSS

YADSVRGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA                    EYDYWGQGTQVTVSS
YADHVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA   EDSPPNYRCSGEW     CFDYWGQGTQVTVSS

YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCNA                   PQPEYWGQGTLVTVSS
YADSVKGRFTVSRDNAKNAVYLQMNSLKPEDTAVYYCNL       FEHRF       PPATYWGQGTQVTVSS

YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA                    EYDYWGQGTQVTVSS
SADSVKGRFTISSDYAKNTVYLQMNGLKPEDTAVYYCAA   AILGDSVRWLQEY     DYDYWGQGTQVTVSS
YADSVKGRFTISTDYARNTVYLQMDSLKPEDTGVYYCAA   ALLGDYVLWLPEY     PYNYWGQGTQVTVSS

YADSVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA                    EYDYWGQGTQVTVSS
YEDSVKGRFTISRDNAKDTVYLQMDSLKPEDTAVYYCAARNRPPDFLTNYFRVLYYREGAYDYWGQGTQVTVSS
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			IC50 µg/ml in TZM-bl cells					
Virus	Subtype	Tier	1H9	2B4F	5B10D	9B6B	11F1B	11F1F
MS208.A1	A	1	1,2	1,8	>50	32	17	8,3
3718.V3.C11	A	2	>50	>50	>50	24,7	>50	30,45
92UG037.1	A	2	>50	>50	>50	>50	>50	31
Q461.E2	A	2	>50	>50	>50	15,5	34,7	11,75
Q769.D22	A	2	>50	>50	>50	36	>50	41,41
Q259.D2.17	A	2	>50	>50	>50	>50	>50	49,55
242-14	CRF02_AG	1B	>50	>50	>50	32,5	>50	24,44
271-11	CRF02_AG	1B	>50	>50	>50	>50	>50	39,8
DJ263.8	CRF02_AG	1B	30,92	>50	>50	>50	>50	>50
263-8	CRF02_AG	2	2,79	5,59	>50	43,3	49,7	23,99
T266-60	CRF02_AG	2	•	17	•	•	•	•
T257-31	CRF02_AG	3	>50	>50	>50	>50	42,3	17,35
T278-50	CRF02_AG	3	•	>50	>50	>50	>50	>50
620345.C1	CRF01_AE	2	>50	>50	>50	>50	>50	>50
703357,2	CRF01_AE	2	>50	>50	>50	>50	>50	46,76
C1080.C3	CRF01_AE	2	>50	>50	>50	>50	>50	>50
R2184.C4	CRF01_AE	2	>50	>50	>50	>50	>50	>50
IIIb	B		•	0,6	>50	18	>50	14
SF162.LS	B	1a	2,47	10,75	>50	30,7	>50	44,35
Bal.26	B	1B	0,84	1,52	>50	21	>50	28,3
SS1196.1	B	1	12	9,5	•	•	>50	•
A/C10.0.29	B	2	16,17	37,62	>50	25,2	>50	>50
CAAN5342.A	B	2	>50	>50	>50	28,2	>50	28,95
QH0692.42	B	2	5,59	7,55	>50	>50	>50	>50
REJO4541.67	B	2	>50	>50	>50	>50	39,5	20,02
RHPA4259.7	B	2	47,69	>50	>50	>50	>50	>50
SC422661.8	B	2	9,73	10,42	>50	>50	>50	>50
TRO.11	B	2	>50	>50	>50	41	>50	39,11
WITO41600.3	B	2	>50	>50	>50	18,5	>50	19,04
THRO4156.18	B	2	>50	>50	>50	42,5	>50	35,64
PVO.4	B	3	27,44	40,51	>50	>50	>50	>50
TRJO4551.58	B	3	>50	>50	>50	35,3	>50	28,86
CH064.20	CRF07_BC	2	3,67	7,94	>50	5,4	44,3	11,58
CH091.9	CRF07_BC	2	•	12	•	•	•	•
CH110.2	CRF07_BC	2	22,19	36,94	>50	5	>50	15,68
CH111.8	CRF07_BC	2	6,12	7,47	>50	13,8	>50	21,05
CH181.12	CRF07_BC	2	0,72	1,07	>50	10,6	>50	27,67
CH115.12	CRF07_BC	3	•	11	•	•	•	•
CH038.12	CRF07_BC	3	•	23	•	•	•	•
92BR025.C1	C	?	•	2	•	•	•	•
TV1.21	C	1	>50	>50	>50	19,9	>50	27,75
93MW965.26	C	1	0,16	0,23	>50	2,1	12,4	2,28
ZM109F.PB4	C	1B	29,17	>50	>50	39,7	>50	45,82
ZM197M.PB7	C	2	3,23	4,62	>50	35,2	>50	32,52
96ZM651.02	C	2	3,4	2,3	•	•	•	•
CAP210.2.00.	C	2	38,53	>50	>50	•	42,3	20,26
CAP45.2.00.G	C	2	0,61	0,99	>50	>50	>50	19,77
Du156.12	C		21,16	28,49	21,1	6	13	5,25
Du172.17	C	2	>50	>50	45,3	•	31,7	9,81
Du422.1	C	2	>50	>50	48,5	6,2	40,2	10,09
ZM135M.PL1	C	2	3,69	5,51	21,3	6	14,3	5,55
ZM214M.PL1	C	2	4,34	6,1	>50	37	>50	>50
ZM233M.PB	C	2	0,65	2,64	17,2	1,7	6	2,17
ZM249M.PL1	C	2	>50	>50	>50	12,7	20,9	6,79
ZM53M.PB12	C	2	2,93	>50	46,9	12,4	35,8	15,64
MuLV	n/a	n/a	>50	>50	>50	>50	>50	>50
Total neutralization:			54%	49%	13%	65%	31%	75%

Left page: Table 1: Neutralization table. The IC_{50} values in μg/ml of 6 VHH against 55 HIV-1 strains and a negative control pseudovirus bearing a murine leukaemia virus (MuLV) envelope**.** Clade and if known the tier of the viruses are indicated. • indicates that the particular combination was not tested. The neutralization activity of the VHH was determined in duplicates or triplicates.

Against viruses of subtype A and CRF02_AG, both 2B4F and 1H9 were less reactive, neutralising only 3 out of 12 or 14 viruses tested, respectively. Both VHH were unable to neutralise any of the 4 CRF01_AE viruses included in this study (table 1).

9B6B displayed a slightly broader neutralisation profile compared to 1H9 and 2B4F, neutralising 30 of 46 viruses (65%). However, the IC50 values for 9B6B, against the tested viruses, were generally lower than those of 1H9 and 2B4F. Out of the 30 viruses neutralised, only 7 (23%) were neutralised with IC_{50} values below 10 μg/ml. Like 1H9 and 2B4F, 9B6B was most effective against viruses expressing Env of subtype B, C and CRF07_BC, neutralising 67% of the subtype B viruses, 92% of subtype C and all 4 CRF07_BC viruses. 9B6B was also not able to neutralise any of the CRF01_AE viruses. Unlike 1H9 and 2B4F, 9B6B also neutralised 67% of the subtype A viruses and 33% of the CRF02_AG viruses (table 1).

5B10D was the least broad and least potent out of the selected VHH, neutralising only 6 out of 48 viruses (13%), all belonging to subtype C, with IC_{50} values in the range of 17.2-48.5 μg/ml.

11F1B also showed a limited neutralisation breadth and potency , neutralising 15 out of 49 viruses (31%), with IC_{50} values in the range of 6.0-49.7 μg/ml. However, the viruses it neutralised were spread over all subtypes tested, with the exception of CRF01_AE viruses.

11F1F was found to be the broadest of the selected VHH, neutralising 36 out of the 48 viruses tested (75%), with IC_{50} values in the range of 2.2-49.6 μg/ml. Compared to 1H9 and 2B4F, it had limited potency, neutralising only 15% with an IC_{50} of less than 10 μg/ml. However, 11F1F neutralised all subtype A and CRF07_BC viruses, 87% of subtype C, 67% of CRF02_AG and 63% of subtype B viruses. It was also the only VHH that neutralised 1 of the 4 CRF01_AE viruses, albeit with an IC_{50} value of 46.8 μg/ml.

Binding of the selected VHH to recombinant HIV-1 Env proteins

The ability of the VHH to bind $gp140_{UG37}$ and $gp140_{CN54}$ was tested, as these Env trimers were used during both immunisation and panning. To delineate which epitopes the VHH bind on the trimeric Env, binding to $gp120_{IIIB}$ and gp41 was also evaluated.

As is shown in figure 3, all VHH were able to bind to $gp140_{UG37}$ and $gp140_{CN54}$ proteins, only 5B10D showed relatively weaker binding to $gp140_{UG37}$. 1H9 and 2B4F were the only two VHH able to bind to $gp120_{IIIB}$. Their binding is however less than that against the gp140's, not reaching their V_{MAX} even at 5 μg/ml. Binding to gp41 resulted in lower signal than was seen for the other Env proteins, however 11F1B and 11F1F were still detectable at a concentration of 20 ng/ml. 9B6B and 5B10D showed a very minimal binding toward gp41, with a measurable binding only at 444 ng/ml and above.

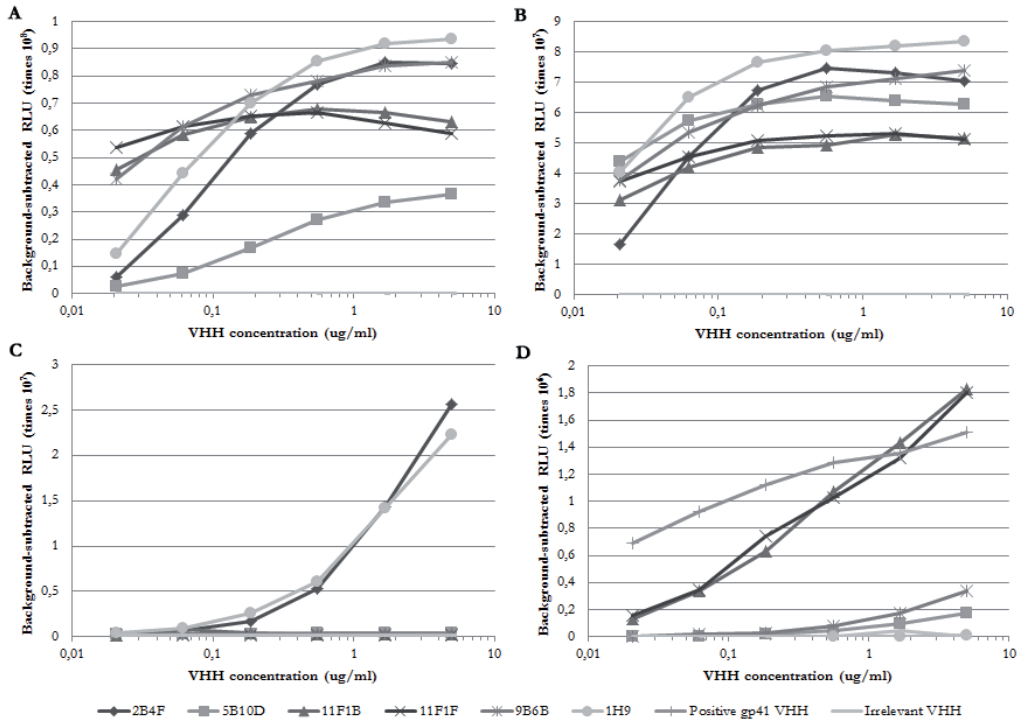

Figure 3: Binding of VHH against various Env. A and B, binding of the VHH against UG37 and CN54 gp140 respectively. C binding against IIIB gp120 and D gp41. 5A1 is a previously identified VHH against gp41, which was used as a positive controll for gp41 binding.

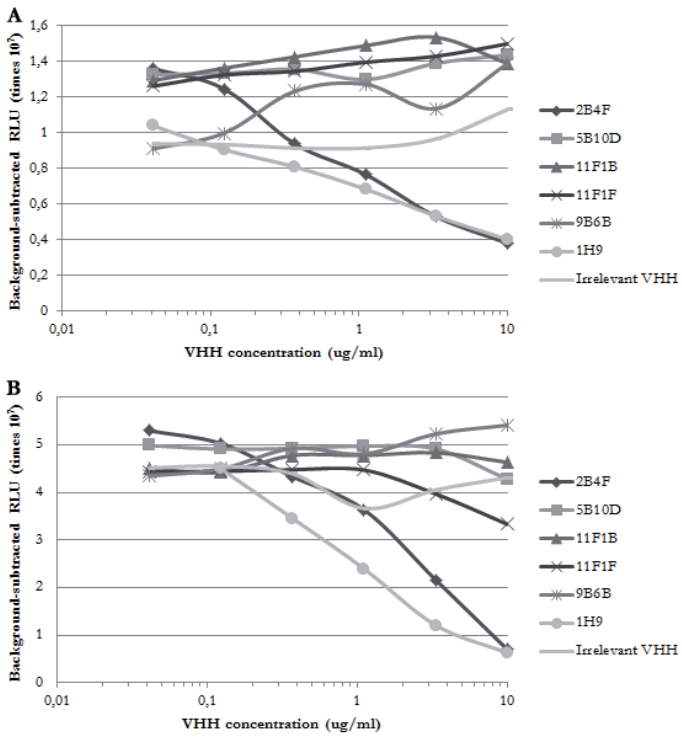

Figure 4: VHH inhibition of mAb b12 and 17b binding to UG37 in ELISA. The inhibition of b12 (A) and 17b (B) binding toward UG37 gp140 by the individual VHH.

VHH	Llama	Selection	Elution	Screen	Neutralisation (% viruses)	Binding				Binding enhanced by sCD4	Inhibition			VHH cross-competition profile
						gp140UG37	gp140CN54	gp120IIIB	gp41		sCD4	b12	17b	
81H9	8	gp120DS2	b12	b12 comp.	52	+	+	+	-	NT	-	+	+	A
2B4F	8	gp140UG37 + sCD4	17b	Neutr.	47.4	+	+	+	-	-	-	+	+	A
9B6B	8	gp140CN54 + sCD4	17b	Neutr.	66	+	+	-	+	-	-	-	-	B
5B10D	9	gp140UG37 + sCD4	17b	Neutr.	12.2	+	+	-	+/-	-	-	-	-	B
11F1B	9	gp140CN54 + sCD4	pH	Neutr.	29.4	+	+	-	+	-	-	-	-	B
11F1F	9	gp140CN54 + sCD4	pH	Neutr.	74.5	+	+	-	+	-	-	-	-	B

Table 2: VHH selection and characterisation overview. This table gives an overview of the properties of the different VHH. From which library they are derived, either from llama eight or nine. On which Env they were panned and whether sCD4 was present during panning, indicated with + sCD4. Their elution method, either a competitive elution with mAb b12 (b12), or 17b (17b), or pH shock (pH). How they were screened, either on their ability to compete with b12 (b12 comp.) or for their neutralisation ability (Neutr.). Their ability to bind various Env, their ability to compete with sCD4, b12 or 17b as indicated by +, binding or inhibition; -, lack of binding or inhibition; and whether their binding affinity increased by sCD4, - not increased, NT, not tested; and finally their cross-competition pattern for binding to $gp120_{UG37}$, either A or B.

VHH binding competition analysis

To gain a better insight into the epitopes targeted by these VHH, competition assays were conducted. First, all VHH were tested for their ability to compete with two known human neutralising antibodies b12[152, 154] and 17b[145]. Figure 4 shows that only 1H9 and 2B4F compete with both b12 and 17b, all other, 5B10D, 9B6B, 11F1B and 11F1F, do not compete with either antibody and thus bind to distinct epitopes (table 2).

Following the competition with known antibodies, the VHH were tested for their ability to compete with one another for Env binding. Figure 5 demonstrates that 9B6B, 11F1B and 11F1F compete with each other and also with 5B10D for binding to $gp140_{UG37}$. Competition with 5B10D is not as profound as it is for the others, but this could be explained by the lower affinity of 5B10D for $gp140_{UG37}$ (figure 3). In addition, these competition assays showed that 1H9 and 2B4F do not interfere with the binding of 9B6B, 11F1B or 11F1F to $gp140_{UG37}$.

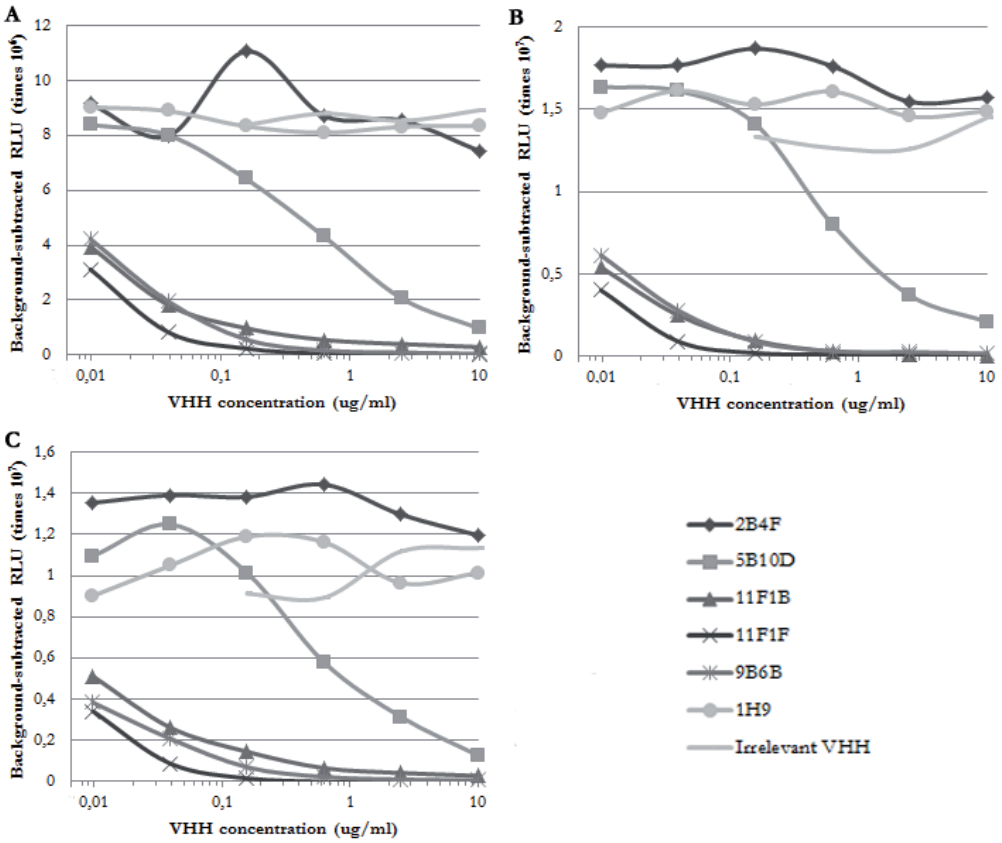

Figure 5: Cross competition between VHH. Cross-competition of 9B6B (A), 11F1B (B) and 11F1F (C) with the individual other VHH. Signals indicate the binding of UG37 gp140 toward the coated VHH after its preincubation with the competing VHH.

Discussion

In this study we focused on the identification of neutralizing VHH which target non-CD4bs related epitopes. Ideally, a neutralizing VHH should be able to bind to the naïve virus, i.e. non CD4-bound envelope spike and be highly cross-reactive against the multiple circulating strains of HIV-1. It was decided to select VHH by sequential rounds of panning on immobilized sCD4 bound gp140, to avoid enriching for VHH that bind to the CD4bs. The interaction of sCD4 with gp140 will however results in conformational changes that may either close off or reveal new epitopes on the Env trimer. To select against VHH that solely bind to these CD4i epitopes, which may be limited in terms of efficacy due to the short time frame in which such epitopes are available during virus entry, the neutralization screen was performed in the absence of sCD4.

From panning on three different Env with use of three different elution methods, nearly a thousand clones were isolated and subsequently screened for the ability to neutralize HIV-1. Six VHH, 2B4F, 1H9, 9B6B, 5B10D, 11F1F and 11F1B, were selected for

further characterization. They range in neutralisation breadth from 13% to 75% and vary in terms of potency as reflected by median IC50 values between 4 –35 ug/ml. In addition, these VHH bind distinct non-CD4bs epitopes as highlighted by their competition profiles (figure 4 and 5 and table 2).

Immunization with a cocktail of gp140 from two different subtypes has been hypothesized to give rise to antibodies that have a wider neutralization breadth[141]. This study together with our previous studies support this view, as VHH with up to 96% breadth were identified from these two llamas. However, without employing similar methods to identify VHH from llamas immunized with the individual components of this immunization regimen, this hypothesis cannot be proven.

In addition to the previous successful isolation of broad and potent anti-HIV-1 VHH that target the CD4bs[142, 143], in this study the same llama VHH libraries were used to identify broadly neutralizing VHH directed against other Env epitopes. This is in agreement with the hypothesis that recombinant trimeric gp140 is highly flexible and may "wobble" between conformational states revealing its conserved epitopes occasionally[121, 155].

Since the CD4bs related VHH have previously been extensively described, our aim was to identify neutralizing VHH targeting alternative sites on the Env trimer. To this end, phage display was used for panning on immobilized sCD4 bound gp140. It was hypothesized that this would enrich for VHH able to bind to the CD4 induced conformation and all other exposed epitopes, excluding only CD4bs-related epitopes. Indeed, this method identified numerous neutralizing VHH, targeting epitopes on gp120 as well as gp41, as is shown in figure 3.

Interestingly, the sequences of the VHH isolated via panning on sCD4-gp140 are similar to those identified during a different selection, aimed to elute b12- like VHH. This indicates that panning on $gp120_{DS2}$ with a subsequent b12 elution is capable of yielding VHH that bind non CD4bs-related epitopes and VHH that can compete with 17b. This is logical given that b12 and 17b epitopes overlap to a certain degree, thus a b12 elution is likely to yield some VHH which can compete with 17b as well. However, since the epitope of b12 overlaps to a greater extent with the binding site of CD4, it was expected that many of the eluted VHH would compete with sCD4 as well, nevertheless VHH from the 1H9/2B4F family do not. The DS2 mutant of $gp120_{Yu\text{-}2}$ [78] has a mutation designed to mimic the CD4 bound conformation of gp120 to some extent. Therefore it is possible that this mutation makes the 17b epitope more available. Furthermore, it may be that this mutation makes the mutant gp120 oscillates toward a CD4i conformation more frequently than wild type Env, thereby shifting the balance toward non-CD4bs epitopes.

Both selection methods identified VHH which phylogenetic analysis revealed form part

of a large family of VHH clones, two of these VHH were further characterized. These two VHH, 1H9 and 2B4F, showed a high degree of sequence similarity and similar neutralisation, binding, and competition profiles. In ELISA, both VHH bound to the immunogens, $gp140_{UG37}$ and $gp140_{CN54}$, and to $gp120_{IIIB}$, but not gp41, indicating that they recognise epitopes on gp120 rather than gp41. This conclusion is further supported by the (cross-) competition studies, which reveal that both 2B4F and 1H9 compete with b12 and 17b, but not with any of the other VHH described here. Additionally, both VHH do not bind CD4-induced epitopes in the sense that their binding is enhanced by CD4 binding to HIV-1 Env, in contrast to the enhanced binding seen with the human mAb 17b. Preliminary SPR studies revealed that 2B4F and 1H9 bind $gp140_{UG37}$ with affinities in the low nanomolar range (Webb et al. manuscript in preparation). Taken together, these findings indicate that VHH 1H9 and 2B4F recognise novel neutralising epitopes on HIV-1 Env that may overlap with the b12 and/or 17b epitopes, but not that of sCD4 and that these epitopes are independent of CD4 binding.

The remaining four VHH, one from llama 8 (9B6B) and three from llama 9 (5B10D, 11F1B and 11F1F), share much less sequence homology. Moreover, they exhibit different neutralisation properties in particular with regard to breadth. On the other hand, their binding to Env and competition profiles were alike (table 2). All of these VHH only bound recombinant Env that contained at least part of gp41 (figure 4). This suggests that gp41 forms a major component of the epitope recognised by these VHH, further studies are required to establish whether their epitope lays solely on gp41. However, 9B6B and to a greater extent, 5B10D, bound only weakly to gp41. Although it should be noted that 5B10D also showed a weaker binding toward $gp140_{UG37}$ than the other VHH.

9B6B, 5B10D, 11F1B and 11F1F all competed with each other for binding to UG37 gp140, indicating that they recognise related epitopes, or that their mutual cross-competition is the result of steric hindrance. Higher concentrations of 5B10D were needed to achieve inhibition of binding of 9B6B, 11F1B and 11F1F to $gp140_{UG37}$, in keeping with the observed lower affinity of 5B10D for $gp140_{UG37}$ (figure 3). These VHH did not compete with 1H9, 2B4F, b12, 17b, or sCD4, thus they do not bind to any of these epitopes, which is in accordance with their ability to bind gp41.

The high proportion of gp41 binding VHH identified in this study (four out of six), is to some extend due to the exclusion of VHH that were closely-related to VHH previously identified as they are all binding gp120. Further, as the immunization was carried out with soluble recombinant gp140, there was no viral envelope in close proximity to gp120, which could otherwise shield gp41. This may have led to epitopes on gp41 being better presented during both immunization and panning, than during natural infection. Additionally, the gp140 immunogens are cleaved just before the transmembrane domain of gp41, thus their N terminals are not fixed together in a membrane as is the case in the infectious Env spike. This may have resulted in a higher degree of flexibility in the gp41 part of the immunogen

trimer, causing it to be more exposed during llama immunization.

It has been found that CD4i targeting antibodies neutralize HIV-1 primary isolates only in the presence of sCD4[63,145-147]. This may as well be the reason for not finding all 17b like VHH, as our screening setup did not include pre-incubating the viruses with sCD4. Such a screen may eliminate VHH that exclusively recognize the CD4i conformation. To screen in such a way was decided, as VHH recognizing their epitope before binding of CD4 hold the advantage that they can bind the virus long before it meets its target cell. This omits the necessity of the VHH being in close proximity of the virus at the moment of CD4 binding. This may allow the concentration of the VHH in the body to be lower as the VHH has a larger window of time to bind its target.

17b elution is not likely to yield gp41 binders, however it is not impossible. 17b elution will enrich for VHH-phages binding to the 17b epitope, however low affinity VHH like 5B10D may elute by chance, because of their high K_{off} rate. Further, binding of 17b may cause conformational changes in gp41 causing VHH-phages to be forced off their epitope, especially if their binding is not very strong.

Overall, the VHH described here provide valuable insight into which epitopes can be targeted by antibodies elicited following immunization, albeit in llamas. This may still be informative for the design of a vaccine against HIV-1. In particular, further study to characterize the epitope bound by 11F1F may prove useful given that this VHH is broadly neutralizing. In addition, 1H9, 2B4F, 9B6B and 11F1F could potentially be used in a microbicide against HIV-1. Some success has been seen with a vaginal microbicide containing an anti-retroviral drug[120]. Microbicides containing mAb would be costly to produce and most likely require refrigeration to maintain their functionality. VHH may be advantageous in such a context as they are in general very stable[130]. Furthermore, VHH targeting epitopes on Env that are not readily accessible to full length mAbs, such as CD4i epitopes[63], could be effective in a microbicide context to prevent infection. The VHH described here are beneficial in addition to the recently described VHH J3, which neutralizes 96% of strains tested with a median IC50 of 0.9 μg/ml. Notably, of the four strains not neutralized by J3, at least two are neutralized by this new set of VHH: 1H9 and 2B4F neutralize CAP45.2.00.G3 very potently (IC50 of 0.6 and 1 μg/ml respectively) and 11F1F neutralizes both CAP45.2.00.G3 as well as Du172.17. 620345.C1 was not neutralized by 1H9, 2B4F or 11F1F and X2160_c25 was not tested. Therefore, the VHH found described in this study are highly suitable for use in combination with other VHH such as J3 for use in a microbicide as their combined breadth will increase. Furthermore, using two or more VHH in a cocktail has the additional advantage that the virus needs to escape all VHH to be able to infect and thus the more neutralizing VHH are present, the more difficult infection will be[144]. In addition, these VHH may be expressed as bi-specific bivalent VHH, linking them for example with the anti-CD4bs VHH J3, as previous studies have shown that bivalent VHH can exhibit

improved potency and/or breadth (manuscript in preparation)[60, 61].

In conclusion, the VHH found in this study are not be as good as our previously found J3, but since they target novel epitopes, they are still very valuable. The use of these VHH in combination with, for instance J3 or as a bi-specific VHH in a microbicide, may cover an even broader range of viral strain and may reduce the risk of the elicitation of escape mutants.

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Chapter 4

Potent and broad neutralization of HIV-1by a llama antibody elicited by immunization

Adapted from:

Potent and broad neutralization of HIV-1 by a llama antibody elicited by immunization

Laura E. McCoy[1,2], Anna Forsman Quigley[1,2], Nika M. Strokappe[4], Bianca Bulmer-Thomas[1,2], Michael S. Seaman[5], Daniella Mortier[6], Lucy Rutten[4], Nikita Chander[1,2], Carolyn J. Edwards[1,2], Robin Ketteler[3], David Davis[6], Theo Verrips[4], and Robin A. Weiss[1,2]

[1]Wohl Virion Centre and [2]Medical Research Council (MRC) Centre for Medical Molecular Virology, Division of Infection and Immunity; and
[3]MRC Laboratory for Molecular Cell Biology; University College London, London WC1E 6BT, England, UK
[4]Biomolecular Imaging, Department of Biology, Institute of Biomembranes, Faculty of Science, Utrecht University,3584 CH Utrecht, Netherlands
[5]Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215
[6]Department of Virology, Biomedical Primate Research Centre, 2280 GH Rijswijk, Netherlands

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Abstract

Llamas *(Lama glama)* naturally produce heavy chain only antibodies (HCAbs) in addition to conventional antibodies. The variable regions in these HCAbs (VHH) demonstrate comparable affinity and specificity for antigens to conventional immunoglobulins despite their much smaller size. To date, immunizations in humans and animal models have yielded only antibodies with limited ability to neutralize human immunodeficiency virus type 1 (HIV-1). In this study, a VHH phagemid library generated from a llama that was multiply-immunized with recombinant trimeric HIV-1 envelope proteins (Env) was screened directly for HIV-1 neutralization. One VHH, L8CJ3 (J3), neutralized 96 of 100 tested HIV-1 strains, encompassing subtypes A, B, C, D, BC, AE, AG, AC, ACD, CD and G. J3 also potently neutralized chimeric simian-human immunodeficiency virus (SHIV) strains with HIV subtypes B and C Env. The sequence of J3 is highly divergent from previous anti-HIV-1 VHH and its own germ line sequence. J3 achieves broad and potent neutralization of HIV-1 via interaction with the CD4-binding site of HIV-1 Env. This study may represent a new benchmark for immunogens to be included in B-cell based vaccines, and supports the development of VHH as anti-HIV-1 microbicides.

Introduction

More than thirty years ago, the first cases of acquired immune deficiency syndrome (AIDS) were reported, followed two years later by the identification of the causative agent of this disease, HIV. There were approximately 2.6 million new infections in 2009, of which 1.8 million occurred in sub-Saharan Africa where the majority of new infections continue to occur (www.unaids.org). The need for a safe and efficacious HIV preventive vaccine is as urgent as thirty years ago and remains the best long-term strategy to prevent the transmission of HIV/AIDS[156]. In addition to the lack of a prophylactic vaccine, at the moment, no strong correlate of protective immunity has been directly established. However, considering classical vaccinology and the principles underlying the partial immune control of HIV-1 infection, it has been postulated that an effective vaccine will need to produce coordinated B and T cell responses[157-159]. Notably, passive immunization with monoclonal antibodies derived from HIV-1 infected individuals has demonstrated the potential for neutralizing antibodies (nAbs) as a protective component of a vaccine-induced immunological memory response[64, 160-163]. Consequently, demonstrating, as we do here, that immunization can induce antibodies able to broadly neutralize HIV-1 is a major goal of HIV-1 vaccine development[70, 164].

Potent nAbs, which neutralize up to 90% of virus strains tested, have been recently isolated[29, 30, 77, 131, 165] from patients, substantially raising the benchmark for nAbs elicited by immunization. Notably, many of these nAbs neutralize HIV-1 via interaction with the CD4-binding site (CD4bs) of Env and a considerable number of such nAbs have now been isolated from multiple donors[76], validating the CD4bs as target for protective antibodies. Furthermore, the neutralization activity in many broadly neutralizing human sera is reported to be mediated by CD4bs targeting antibodies[127]. However, immunization in a variety of animal models has rarely produced any mAbs with potency or breadth comparable even to the long-established human nAbs such as b12[152]. Moreover, the potency and breadth of immunization elicited nAbs[166, 167], has not by any means approached the standard of the newly described human monoclonals obtained during HIV-1 infection[29, 30, 131], although recently robust Env-specific memory B cell responses have been seen in immunized non-human primates (NHP)[168]. Neutralization with plasma samples was predominantly restricted to clade B tier 1 viruses with one representative from clades A and C neutralized and detectable and less potent neutralization against some tier 2 viruses[168]. It should be noted that antigen-binding fragments (Fab) of CD4-induced epitope monoclonal Abs (mAbs), such as 17b[145], exhibit the ability to neutralize viruses that the corresponding full-length IgG cannot[146] and that it has been possible to induce 17b-like mAb following immunization[148]. However, the breadth of the 17b Fab was evaluated as neutralizing HIV-1 with comparable efficiency to b12 IgG[146], which in contrast was less effective in Fab form[63, 146] as are the majority of Fabs compared to their corresponding whole antibody molecules[169].

The natural occurrence of llama heavy-chain only antibodies[44], and the independent neutralization properties of their variable domains, i.e., the single-domain VHH, enabled

screening of the VHH without the need for random recombination of heavy and light chains. Preliminary immunization of llamas with monomeric gp120 previously led to the isolation of neutralizing VHH against HIV-1[90, 125, 139]. Given the limitations of the previous anti-HIV VHH, a new neutralization screening process was developed to isolate the extremely broad and potent VHH J3 described herein. The power of the screening process was increased by removing the biopanning stage, which favors VHH that bind to recombinant HIV-1 Env, but does not distinguish between virus neutralizing and non-neutralizing binding properties. Consequently, in this study, the direct neutralization screening of a phagemid VHH library, generated from a llama immunized with two recombinant HIV gp140 proteins, yielded a llama VHH that neutralizes 96% of HIV-1 isolates tested via interaction with a CD4bs epitope. This is a marked improvement on anti-HIV-1 mAbs or antibody fragments previously isolated following immunization, including but not limited to other anti-HIV-1 VHH, and demonstrates for the first time that a mammalian immune response to HIV-1 Env can elicit an antibody which as a VHH fragment is as broadly cross-reactive against a large panel of HIV-1 variants as the best full-length conventional human mAbs produced as a result of natural infection.

Materials and methods

Monoclonal antibodies.

MAb b12[152] and 2F5[135, 170] were obtained through the Centralized Facility for AIDS Reagents (CFAR), National Institute for Biological Standards and Controls (NIBSC), supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and were donated by D. Burton & H. Katinger respectively.

Recombinant HIV-1 Env proteins.

Recombinant trimeric gp140 from HIV-1 92UG037 (subtype A) for immunizations and ELISAs was kindly provided by S. Jeffs, Imperial College London. Recombinant trimeric gp140 derived from HIV-1 CN54 for immunizations was provided by Polymun Scientific. Recombinant D368R and wild type monomeric gp120 from HIV-1 YU2 (subtype B) for ELISAs was kindly provided by J. Mascola, NIH, Maryland, USA. Recombinant gp120 from HIV-1 IIIB (EVA657) and recombinant trimeric gp140 from HIV-1 CN54 (ARP699) for ELISAs were obtained from the CFAR, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and were donated by Immunodiagnostics and Polymun Scientific respectively.

Viruses.

HIV-1 IIIB (ARP101) and YU2 were obtained from the CFAR, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the

Bill and Melinda Gates GHRC-CAVD Project. IIIB was donated by R. Gallo and M. Popovic. HIV-1 CRF07_BC primary isolate CN54 was obtained from EMPRO. HIV-1 IIIB was propagated in H9 cells and CN54 was propagated in peripheral blood mononuclear cells. All other replication competent virus stocks were prepared from HIV-1 molecular clones by transfection of 293T cells. The 92BR025.C1 (C111) molecular clone was obtained by amplifying gp120 from cells infected with the WHO panel peripheral blood mononuclear cell-grown isolate and inserting it into the pHXB2 backbone[90]. HIV-1 Env pseudotyped viruses were produced in 293T cells by co-transfection with the pSG3Δ*env* plasmid[153]. The subtype B and C HIV-1 Reference Panels of Env Clones[91, 119] were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA. HIV-1 subtype CRF07_BC gp160 clones, subtype CRF02_AG gp160 clones (263-8, T278-50, T266-60) and the 92UG037, 93MW965.26 and 96ZM651.02 gp160 clones were kindly provided by D. Montefiori (Duke University Medical Center, Durham, NC) through the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA2 VIMC) as part of the Collaboration for AIDS Vaccine Discovery (CAVD). All additional pseudoviruses were produced at the CAVD Pre-clinical Neutralizing Antibody Core laboratory. Virus CVS-11 pseudotyped with rabies virus G protein (Wright et al., 2008), was kindly provided by E. Wright (University of Westminster, UK).

Cells.

TZM-bl cells[91, 114, 115] were obtained through the NIH AIDS Research and Reference Reagent Program from J. C. Kappes, X. Wu, and Tranzyme, Inc., and cultured in Dulbecco's modified Eagle medium (Invitrogen) containing 10% (v/v) fetal calf serum (FCS).

Immunization of Lama glama and construction of VHH phage library.

Llama 8 was immunized with recombinant gp140 derived from HIV-1 CN54 and 92UG037. Immunizations and VHH library construction were carried out as described previously[98]. In brief, the llamas received seven intramuscular injections as per table S1. Each injection consisted of a freshly prepared 4.5-ml water-in-oil emulsion prepared by vigorously mixing 2 volume units of antigen (50 or 100 μg) with 2.5 volume units of the adjuvant Stimune (CEDI Diagnostics). This study was carried out in strict accordance with the Dutch Experiments on Animals Act 1997. In accordance with article 18 of the Act the protocol was assessed and approved by the Animal Ethics Committee of the Utrecht University (Permit Number: DEC#2007.III.01.013). All efforts were made to minimize discomfort related to immunizations and blood sampling. The animal welfare officers of the Utrecht University checked the mandatory administration and supervised the proper conduct of procedures and the well-being of the llamas that were used. The anti-Env immune response in sera was verified in an ELISA against immobilized recombinant $gp120_{IIIB}$. Total RNA was isolated from peripheral blood lymphocytes collected post-immunization (on day 122), and cDNA was prepared. The VHH repertoire was amplified and cloned into the

pCAD50 phagemid vector. To obtain recombinant bacteriophages expressing the VHH as fusion proteins with the M13 bacteriophage gene III product, transformed TG1 *E. coli* cells were grown to logarithmic phase and then infected with helper phage M13KO7. The phage particles were precipitated with polyethylene glycol.

Isolation of anti-HIV-1 VHH through direct HIV-1 neutralization screening.

Bacteria expressing the cloned VHH repertoire were plated onto agar containing 100 µg/ml ampicillin and 2% syncytial stain (1 g methylene blue, 0.33 g basic fuschin in 200 ml methanol). Individual clones were picked using a Norgren CP7200 colony picker (Rapid-Pick, Hudson Robotics) into 384-well master plates. 2816 individual clones were expressed in TG1 *E. coli* cells in a 96-well plate format. Each clone was expressed in 1 ml of 2x TY medium containing 100 µg/ml ampicillin and 0.1% glucose, followed by induction of VHH production with 0.1 mM isopropyl-ß-D-thiogalactopyranosid. Bacterial pellets were frozen at -80 °C for a minimum of 1 h and then thawed and re-suspended in phospho-buffered saline (PBS). The periplasmic extract from each well was separated from bacterial debris by filtration through a 0.2 µM PDVF membrane and screened for the ability to neutralize HIV-1. To enable semi high-throughput screening and characterization of VHH, neutralization was measured using 200 50% tissue culture infective doses of virus in the TZM-bl cell-based assay developed by Derdeyn *et al.*[91,114,115], with Bright-Glo luciferase reagent (Promega) using a Glomax plate reader (Promega). DNA from the individual VHH which neutralized all viruses to less than 20% seen with control was purified, sequenced and re-cloned into the pCAD51 expression vector followed by transformation into TG1 cells for purification and further characterization. The HIV-1 neutralization potencies of llama 8 sera were also evaluated in TZM-bl cells as described above. Serum samples were heat-inactivated in order to destroy complement by incubation at 56°C for 1 hour prior to use in neutralization assays. Three-fold serial dilutions of llama sera were then tested, starting at a 1:5 dilution.

VHH purification and neutralization profiling.

Expression from the pCAD51 vector incorporates a 6-His- and a c-Myc-tag to the C-terminus of the VHH and removes the bacteriophage gene III product. The VHH were purified by means of the attached His-tag using TALON Metal Affinity Resin (Clontech). The neutralization activity of the VHH was assayed in duplicate/triplicate at either UCL or VIMC laboratories. No virus inactivation was observed with a negative control VHH or with a pseudovirus bearing a rabies virus G-protein Env or murine leukemia virus Env. VHH 50% IC50 titers were calculated using the XLFit4 software (UCL-ID Business Solutions) or the Labkey Neutralizing Antibody Tool[171].

SHIV.

Molecularly cloned SHIV 89.6p and HIV-1 89.6 were obtained from J. Sodroski, Dana-

Farber Cancer Institute, Boston, USA, through the NIH AIDS Research & Reference Reagent Program. SHIV1157ipEL-p and a molecular clone of SHIV1157ipd3N4 were kindly provided by R. Ruprecht, Dana-Farber Cancer Institute, Boston, USA, and SHIVSF162p3 was a generous gift of C. Cheng-Mayer, Aaron Diamond AIDS Research Center, New York, USA. SHIVSF162P4 was obtained from the Division of Acquired Immunodeficiency Syndrome (DAIDS), NIAID, Bethesda, USA.

Pseudotyped SHIV viruses were prepared by E.J. Verschoor and Z. Fagrouch of the Department of Virology, BPRC, essentially as described by[115]. In short, the full-length *env* genes were amplified from molecularly cloned viruses or from viral RNA, and the PCR products were cloned into the expression plasmid pcDNA3.1 (Life Technologies Europe B.V., The Netherlands). Individual clones were sequenced and selected for their suitability to produce pseudoviruses in a small-scale infection assay on TZM-bl indicator cells prior to performing neutralization assays[110]. For this purpose, small stocks of pseudotyped viruses were prepared by transfection of 293T cells with a mixture of the pcDNA-*env* plasmid, and the pSG3Δ*env* plasmid, that contains an Env-deficient molecular clone of HIV-1 SG3[153]. After incubation, cell-free virus stocks were produced by low-speed centrifugation, followed by filtration through a 45 μM filter, and used to infect TZM-bl cells. Viruses that induced luciferase activity were selected for the pseudovirus neutralization assay.

The neutralization activity of the VHH was assayed in duplicate at the BPRC laboratory in the TZM-bl cell-based assay developed by Derdeyn et al.[91, 114, 115] containing DEAE-Dextran (15 μg/ml), and assayed with Britelite Plus Reagent (Perkin Elmer) according to manufacturer's instructions using a Victor light plate reader (Perkin Elmer). VHH IC50 titers were calculated using the Luc5Samples02NotProtected.xls program (courtesy of D. Montefiori).

ELISAs.

Clear 96-well Maxisorp plates (Nalgene) were coated overnight with 2 μg/ml of HIV-1 $gp120_{IIIB}$ or $gp140_{UG37}$. Plates were blocked using 5% milk powder in tris-buffered saline (TBS). Serial dilutions in TBS supplemented with 0.05% Tween (TBS-T) containing 1% milk powder (TMT) of the VHH to be assayed and of a negative control VHH were then added to the plates in triplicate wells and the plates were incubated at room temperature for 1 h and subsequently washed four times with TBS-T. The wells were then incubated with 0.5 μg/ml of mouse anti-c-Myc-horse radish peroxidase conjugated antibody (cat. No. 11814150001, Roche Diagnostics) in TMT for 1 h at room temperature. After six washes with TBS-T, TMB ELISA substrate (Thermo scientific) was added and the plates were incubated at 37°C for 0.5 h. Absorbance at 450 nm was detected and background-subtracted data were plotted against VHH concentration.

Immune response ELISAs.

50 μl of 2 μg/ml of $Gp140_{CN54}$ and $Gp140_{UG37}$ were coated on MaxiSorp™ plates

(Nunc) overnight at 4°C. After blocking with 4% milk powder in PBS serial dilutions of pre-immune serum (day 0) and immune plasma (day 122) were incubated for 1 h in triplicate. The serum and plasma were diluted in 1% milk powder in PBS by serial dilution from 500-fold to 32000-fold with 2-fold dilution steps in between. Detection of bound llama single chain antibodies was performed by incubation with mAb 8E1 followed by an incubation with peroxidase-conjugated donkey anti-mouse Ig (1:5,000 in 1% milk powder in PBS). Anti-llama IgG3 mAb 8E1 was kindly provided by BAC BV, Naarden. 50 µl of O-phenylenediamine containing 0.03% H_2O_2 was added to the wells. After approximately 5 minutes the reaction was stopped with 1 M H_2SO_4. The absorbance of the reaction was measured at 490 nm.

Competition ELISAs.

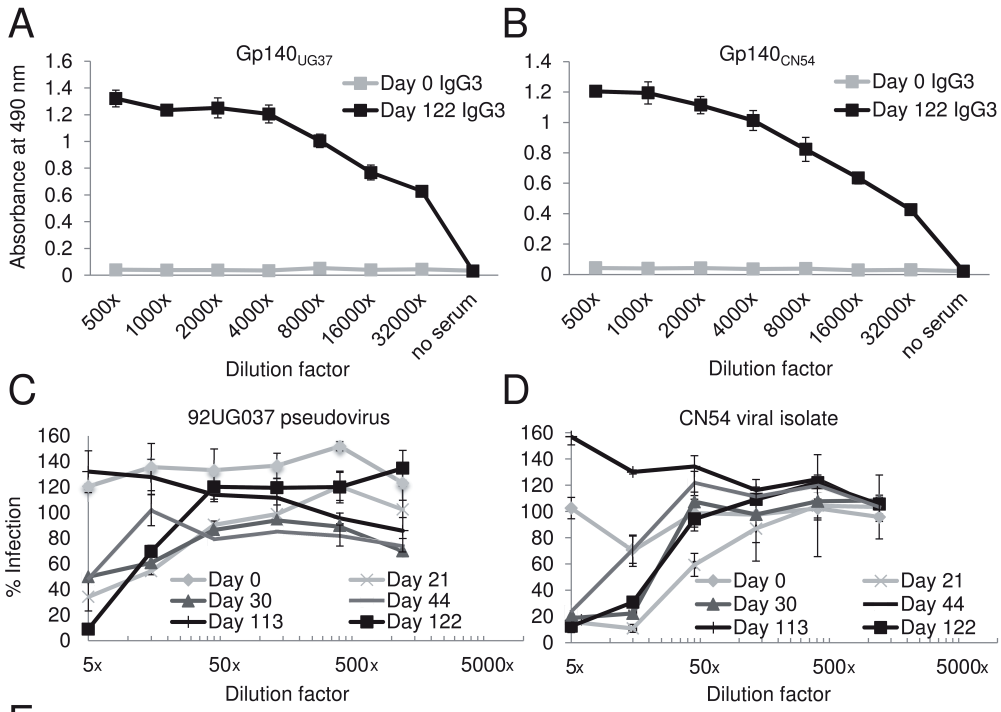
Clear 96-well Maxisorp plates (Nalgene) were coated overnight with 2 µg/ml of $gp140_{UG37}$ and washed three times with PBS with 0.05% tween (PBS-T). Plates were blocked using 4% milk powder in PBS, and then washed three times in PBS-T. Serial dilutions in PBS-T supplemented with 1% milk powder (PBS-M) of the VHH to be assayed and of a negative control VHH were then added to the plates in duplicate/triplicate wells as indicated in the figure legends and the plates were incubated at room temperature for 1 h. The wells were then incubated either 1 µg/ml b12 or 0.1 µg/ml sCD4 in PBS-M for 1 h at room temperature. After three washes with PBS-T, the wells were incubated with either 1 in 5000 goat anti-human peroxidase or 1 in 10000 L120 in PBS-M for the b12 and sCD4 wells respectively and incubated at room temperature for 1 h. After three washes, the sCD4 wells were incubated for an additional hour with 1 in 5000 and donkey anti-mouse peroxidase in PBS-M at room temperature. Binding was detected for both b12 (Fig. 5C) and sCD4 (figure 5A) competition assays by washing the wells six times with PBS-T and adding O-phenylenediamine supplemented with 0.03% hydrogen peroxide for 45 min at room temperature. Absorbance at 490 nm was detected and background-subtracted data were plotted against VHH concentration.

Alternatively, white 96-well Maxisorp plates (Nalgene) were coated overnight with 1 µg/ml of HIV-1 $gp140_{UG37}$ (figure 5B). Plates were blocked using 5% milk powder in TBS. Serial dilutions in TMT of the mAb/VHH to be assayed and of a negative control mAb/VHH were then added to the plates in triplicate wells and the plates were incubated at room temperature for 1 h and subsequently washed four times with 0.05% TBS-T. The wells were then incubated with 0.5 µg/ml of biotinylated VHH J3 in TMT for 1 h at room temperature. J3 was pre-biotinylated using a EZ-Link Micro Sulfo-NHS-Biotinylation Kit (Pierce), according to manufacturer's instructions. After four washes with TBS-T, conjugated streptavadin alkaline phosphatase was added at 0.125 µg/ml and the plates were incubated at room temperature for 1 h. After six washes with TBS-T, Lumi-phos substrate (Thermo scientific) was added and the plates were incubated at 37°C for 0.5 h. RLU were detected and background-subtracted data were plotted against VHH concentration.

Results

Llama immunization and phagemid library generation

A *Lama glama* (designated llama 8) was injected intramuscularly with a mixture of gp140 trimers derived from a subtype BC HIV-1 strain, CN54 ($gp140_{CN54}$), and a subtype A strain, 92UG037 ($gp140_{UG37}$). Llama 8 was immunized in parallel with another llama and the library generated from the other llama is described elsewhere[143]. Previously, llamas immunized with monomeric HIV-1 proteins had been shown to produce anti-HIV-1 VHH with limited breadth and neutralization potency[90, 125, 139]. In addition, the neutralization activity induced by human immunization trials (VAX04) with monomeric Env has been



E

Virus:	92UG037	CN54	IIIB	Bal.26	93MW956.26	96ZM965.01
Subtype:	A	BC	B	B	C	C
Day 122 ID50:	1:11.5	1:12.5	1:169	1:45	1:>10000	1:77

Figure 1: Llama 8 immune response evaluation. (A) Serial dilutions of llama Sera obtained on days 0 and 122 was incubated on ELISA plates pre-immobilized with Gp140UG37 or (B) Gp140CN54 recombinant ENV. Binding was assessed as described in the methods. All samples were assayed in triplicate. (C) Sera taken on days 0 and 122 were pre-incubated with the 92UG037 pseudovirus or (D) CN54 viral isolate and neutralization activity was assessed as described in the text. All samples were assayed in duplicate. Error bars represent standard deviation from the mean. (E) IC50 titers (μg/ml) for sera taken on day 122 against both homologous viruses and the indicated heterologous viruses. Experiments were repeated in duplicate and on two independent occasions, except for the heterologous virus neutralization assays which were carried out once in duplicate.

reported to be inferior to that induced by trimer immunization of NHP[168]. Hence, this subsequent immunization schedule included trimeric Env with the aim that the functional glycoprotein spike of infectious HIV-1 would be more accurately mimicked and presented to the llama immune system. The protein immunogens were administered with Stimune adjuvant seven times as described in the immunization schedule depicted in table. S1. The llama immune response was satisfactory, as indicated by an increase in the ability of post-immunization sera, relative to pre-immunization sera, to bind to the immunogen Env proteins $gp140_{UG37}$ $gp140_{CN54}$ in enzyme-linked immuno-sorbent assay (ELISA) (figure 1A and B). Retrospectively, sera from llama 8 were assessed for neutralization abilities against pseudovirus with 92UG037 or the CN54 primary isolate. Neutralization against both viruses was seen with sera taken 122 days after immunization compared to sera taken on day 0 (figure 1C and D). For the neutralization sensitive viruses IIIB and 93MW956 appreciably higher ID50 values were seen with the day 122 sera compared to the ID50 values of against the homologous viruses (figure 1E). In contrast, the ID50 value obtained for Bal26 (tier 1b neutralization sensitivity) was only slightly higher than those calculated for the homologous viruses, and the ID50 for 96ZM956 (tier 2) was similar to the ID50 values for the homologous viruses (figure 1E). Immune phagemid libraries were constructed using blood collected from llama 8 on day 122 and library construction followed. Briefly, RNA was extracted from purified peripheral blood lymphocytes[96] from post-immunization blood at day 122 and cDNA generated to enable the amplification of the conventional and heavy-chain IgG repertoire. The heavy-chain only antibody cDNAs were separated by gel electrophoresis and used as a template in a nested PCR which enabled the isolation of the VHH repertoire via the insertion of restriction sites. The resulting cDNA fragments were ligated into a phagemid vector for display on filamentous bacteriophage M13[98, 172, 98, 98] and electroporated in *E. coli* TG1 cells. Rescue with helper phage VCS-M13 and polyethylene glycol precipitation was performed as described previously[99] and a phage stock containing 5×10^{11} pfu/ml was generated. The library from llama 8 had a diversity of more than 10^7 and VHH inserts in more than 90% of the phagemids.

Direct neutralization screening of the phagemid library 8

Previously, VHH from immunized llamas were isolated from phagemid libraries via sequential rounds of biopanning on immobilized proteins to enrich the libraries for VHH which bind specifically to the protein target under investigation. For example, the previously described anti-HIV-1 VHH[90] were isolated from fractions of a phagemid library which had been previously enriched for the ability to bind to $gp120_{IIIB}$ and then compete with soluble CD4 (sCD4). However, it is well-established that some anti-HIV-1 mAbs that can bind efficiently to recombinant Env do not neutralize functional virus and are thus termed non-nAbs[164]. Indeed, one of the means by which HIV-1 evades a protective human immune response is by eliciting the production of non-nABs or strain-specific nAbs[173]. Therefore, it was hypothesized that enriching the phagemid library for VHH that bind most efficiently

could result in the preferential selection of strongly binding VHH over strongly neutralizing VHH and thus that a direct neutralization screen would be preferable to isolate broad and potently neutralizing VHH. This hypothesis was supported by the successful isolation of two broad and potent human mAbs, PG9 and PG16, via a high-throughput neutralization screen of human B cells[29]. Consequently, a new screening method was developed whereby the VHH were assessed directly for their ability to neutralize HIV-1 pseudovirus without any prior selection for the ability to bind to Env or compete with characterized anti-HIV-1 mAb or sCD4.

The direct neutralization screening method involved robotically isolating 2816 VHH clones plated out onto agar from the phagemid library into 384-well master plates. The VHH clones were expressed in 96-well plates and targeted to the periplasm of the *E. coli*. Subsequently, the VHH-containing periplasmic extract was harvested by freeze-thawing followed by filter sterilization and assessed for the ability to neutralize the subtype A MS208.A1 and subtype C 93MW956.1 HIV-1 pseudoviruses in parallel via the TZM-bl cell-based assay, whereby infection by HIV-1 elicits the expression of firefly luciferase and can be quantitatively measured by light emission. Neutralization was defined as reduction in relative light units (RLU) to less than 40% of non-HIV specific VHH[91, 114, 115] and stratified as strong if the RLU were reduced to less than 20% and intermediate if reduced to less than 40%. As both MS208.A1 and 93MW956.1 are classified as tier 1 pseudoviruses[174], and thus relatively easy to neutralize, the VHH identified as neutralizing to both a strong and intermediate level in the initial screen were re-expressed and assessed for their ability to neutralize four tier 2/3 viruses: subtype C ZM214M.PL15, subtype B THRO4156.18, subtype A 92UG037 and circulating recombinant form (CRF)02_AG T257-31. Individual sequences were obtained for 8 of the 2816 VHH clones screened that were able to neutralize one or more of these tier 2 pseudoviruses. The level of antigen-specificity in VHH that did not neutralize in this screen was not comprehensively investigated, although to date 48 unique sequences, have been obtained from llama 8 via a combination of neutralization screening and panning selection. Interestingly, periplasmic extract containing one VHH, designated J3, neutralized five of the six screening viruses. Therefore, J3 was subcloned into an expression vector, to allow improved protein production with a C-terminal His tag, to enable purification via affinity chromatography and quantification of both antibody yield and neutralization potency.

J3 neutralizes 96% of HIV-1 strains tested

Initially, VHH J3 was titrated from a starting concentration of 50 μg/ml against the pseudoviruses used in the direct neutralization screen. For the first two strains tested, MS208.A1 and 93MW956.1, 50% inhibitory concentration (IC50) values were calculated as 6.5 μg/ml and <0.023 μg/ml respectively (table S2). Furthermore, all four pseudoviruses in the second screening panel were also neutralized to less than 50% of total virus (table S2). Thus J3 neutralized representatives from subtypes A, B, C and CRF02_AG. Further

neutralization assays undertaken in two laboratories revealed that J3 also neutralizes pseudoviruses, replication-competent molecular clones and T-cell grown isolates from subtypes D, G and CRF AE, AC, ACD and BC (table S2). Notably, among both the B and C viruses tested are pseudoviruses derived from transmitter/founder isolates, which are all neutralized with IC50 values of less than 1 μg/ml and 2 μg/ml for subtypes B and C respectively.

When the neutralization data are stratified by subtype, J3 exhibits 100% neutralization of all viruses from subtypes A, B, D and CRFs AG, AC, ACD, BC and CD (Fig. 2, Table S2). The median IC50 values are 2.77, 0.74, 3.09, 0.6, 0.99, 4.42, 0.32 and 2.34 μg/ml for these subtypes respectively and interestingly all CRF07_BC strains were neutralized with IC50 values of less than 1 μg/ml. Furthermore, J3 neutralized 90, 88 and 86% of viruses from the other three remaining subtypes C, G and CRF01_AE respectively. In total, J3 neutralized 96% of 100 strains tested (figure 2, table S2), significantly more than any other anti-HIV mAbs elicited following immunization in an animal model. This includes previously characterized anti-HIV VHH which are either limited in terms of breadth[90, 125, 139] and/or potency[143]. The breadth of J3 neutralization compares favorably to the 78-93% neutralization seen with the recently isolated human mAbs which arose during HIV-1 infection[29, 30, 131, 165].

Neutralization data for a substantial subset of the viruses tested in this study have previously been reported for other human nAbs which target the CD4bs. Thus, the neutralization profile of J3 for this subset of 69 viruses was compared to that for VRC01/2, b12 and CD4-Ig[30] which were previously tested in the same laboratory (table S3). Within this subset, neutralization (defined as an IC50 value <50 μg/ml) was observed for 88.4, 85.5, 47.8, 92.8 and 94.2% of viruses for VRCO1, VRC02, b12, CD4-Ig and J3 respectively. VRCO1/2

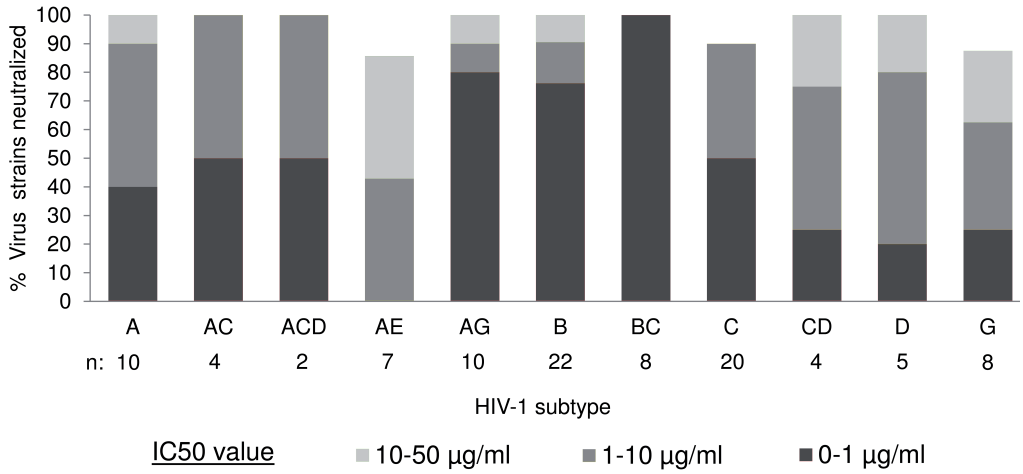


Figure 2: Breadth of J3 HIV-1 neutralization. Percentage of virus strains neutralized by J3 stratified by subtype as described in Materials and methods. To aid comprehension, the columns have been subdivided to show the percentage of strains from each subtype that were neutralized with IC50 values falling into the intervals indicated in the key. The neutralization activity of each VHH was assayed in duplicate or triplicate, and negative controls included mouse leukemia and rabies pseudoviruses. The majority of the panel of viruses were assayed once.

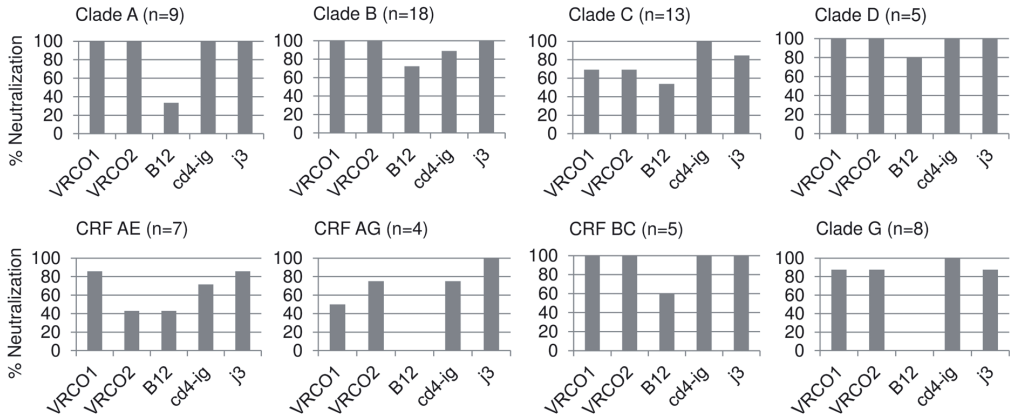


Figure 3: Comparison of breadth of neutralization achieved by VRCO1/2, b12, CD4-Ig and J3. Percent neutralization for the matched subset of viruses assayed against by J3, VRC01/2, b12, and CD4-Ig (using data published by Wu et al. [2010][30]) was stratified by clade.

and J3 neutralized all viruses from subtypes A, B, D and BC, whereas b12 neutralized 33.3, 72.2, 80 and 60% respectively for these subtypes (figure 3). In addition, J3 neutralized all the CRF02_AG viruses, whereas VRCO1 neutralized 50% and b12 failed to neutralize any AG viruses (figure 3). Viruses from subtypes C, CRF01_AE and D were neutralized in a comparable manner by J3 and VRC01/2, and in both cases more effectively than by b12 (figure 3). However, it is important to note that these comparisons are based on IC50 values in µg/ml and not molar values. Interestingly, there are two viruses (AE 620345.c1 and C Du172.17) out of the subset of 69 which both VRC01/2 and J3 failed to neutralize (Table S3). Another clade C virus, CAP45.2.00.G3, was effectively neutralized by VRCO1/2 but not by J3 and within the subset of 69 viruses there are six viruses which are resistant to VRCO1/2 but sensitive to J3. A total of four viruses in this study were resistant to neutralization by J3, and as described, two of these are also resistant to VRCO1/2 mediated neutralization, while the other two, X2160_c2 and 620345.c1 are susceptible. Unfortunately, viral sequence data for the latter has yet to be established, so sequence analysis of the other three resistant viruses was undertaken and it was found that no single amino acid mutation is present in all three resistant viruses that is not present in susceptible viruses. However, in all three cases there are multiple mutations relative to the sequences of closely related virus sequences. Therefore, it is hypothesized that multiple clusters of mutations contribute to the J3 resistance as has been described for naturally-occurring viral resistance to VRCO1/2 [175], albeit in a distinct way as indicated by the different susceptibilities of distinct viruses to neutralization by VRC01/2 or J3.

SHIV PSEUDOVIRUS	CLADE	IC50 µg/ml in TZM-bl cells J3 VHH
SHIVsf162p4	B	0.0685
SHIVsf162p3	B	0.2154
SHIV89.6p	B	0.4215
SHIV89.6	B	0.1528
SHIV1157IPD3N4	C	0.01976
SHIV1157IP EL-p	C	0.006205

Table 1: SHIV neutralization by J3. VHH J3 neutralization activity was assessed in the indicated SHIV pseudoviruses, as described in the text. The derivation of SHIV1157IPD3N4 and SHIV1157IP EL-p is described in detail by Humbert et al.176 The SHIV neutralization activity of J3 was assayed in duplicate on TZM-bl cells as described in the text, the subtype B SHIVs assays were undertaken once and the subtype C SHIVs were assayed in two independent experiments.

J3 neutralizes all SHIV strains tested

To further characterize J3, and to evaluate its potential as an anti-HIV microbicide, its ability to neutralize SHIV pseudoviruses was investigated. J3 was found to potently neutralize six SHIV pseudoviruses from subtypes B and C, with IC50 values all below 0.5 µg/ml. The clade C SHIVs were in fact potently neutralized with IC50 values of less than 0.02 µg/ml. The strains assayed included one derived from SHIV1157IP EL-p, a clade C SHIV strain that has been used in recent mucosal challenges in NHP[176] and SHIV1157IPD3N4 (table 1), a highly replication-competent, mucosally transmissible clade C R5 SHIV, which rapidly induces abnormalities in immune parameters and could therefore be used to assess post-acute viremia levels as read-out parameters of vaccine or microbicide efficacy[177]. Thus, the potent neutralization of these strains by J3 indicates this VHH is a suitable candidate for inclusion in microbicide formulations to be tested in NHP models.

J3 binds to immunogen HIV Env trimers and competes for the CD4bs

J3 binding specificities were examined in ELISA (see methods) where it bound to both the llama immunogen trimer proteins $gp140_{CN54}$ and $gp140_{UG37}$ (figure 4A and B). Sigmoidal concentration-dependent binding was observed in a similar fashion to that seen with the previously isolated, but less broadly neutralizing VHH, A12[90, 125, 139]. However, the binding curves are not identical for both VHH as their interactions with Env are distinct from one another, indicating that their interactions with Env are qualitatively different as reflected in the strikingly superior neutralization profile of J3. In addition, J3 binds to monomeric $gp120_{IIIB}$ derived from HIV-1 strain IIIB demonstrating that it achieves such a wide breadth of neutralization by interacting with HIV-1 Env in a monomer- rather than trimer-specific fashion (figure 4C). Thus, J3 binds to Env from subtypes A, B and CRF07_BC in a comparable way to the less broadly neutralizing VHH A12. However, the nature and consequences of the two distinct VHH binding to Env are clearly diverse as A12 binds to $gp140_{UG37}$

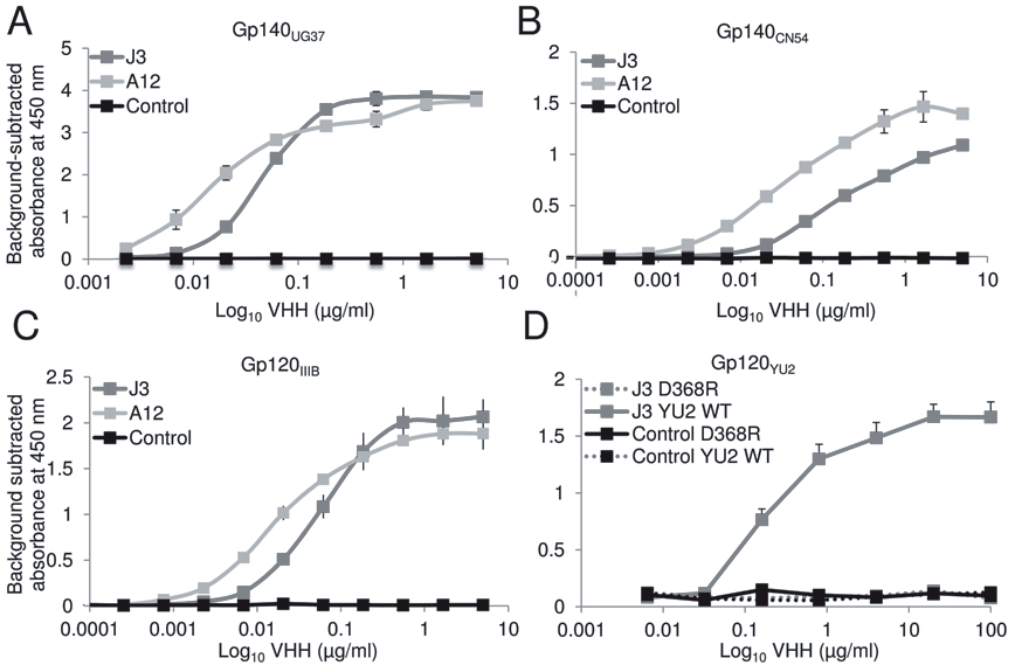


Figure 4: Binding to trimeric and monomeric Env in ELISA. (A) VHH binding to $gp140_{UG37}$, subtype A and (B) $gp140_{CN54}$, subtype CRF07_BC, (C) monomeric $gp120_{IIIB}$, subtype B and (D) wild-type monomeric $gp120_{YU2 \text{ or its}}$ D368R mutant, subtype B. All error bars represent standard deviation from the mean. All data are representative of at least two independent experiments.

but is unable to neutralize the 92UG037 HIV-1 pseudovirus. Therefore, the difference seen in neutralization breadth could not have been easily predicted by binding ability. This in turn confirms the rationale behind the direct neutralization screen undertaken, that VHH binding to recombinant Env is not functionally equivalent to HIV-1 neutralization as has already been established for human mAbs[178].

To assess whether J3 binds, as does A12, to a CD4bs-related epitope, the interaction of J3 with a mutant Env (D368R) derived from HIV-1 strain YU2 ($gp120_{YU2}$) was evaluated compared to wild type protein. The mutant Env contains a single residue mutation at position 368 of an aspartic acid to an arginine which abolishes CD4 binding[127]. The binding of J3 was evaluated by ELISA, which revealed that the D368R mutation in Env prevents J3 binding as compared to interaction seen with the wild type Env (figure 4D). Many CD4bs antibodies have previously been characterized from infected patients[76, 77, 179, 180]] and their epitopes characterized via structural and mutational methods, including the use of the D368R mutant Env in binding studies. Thus, from this preliminary epitope analysis it can be concluded that the J3 epitope is similarly affected by mutation of residue 368 as are VRCO1 and b12 but not HJ16 , which can bind to the D368R Env[30, 76]. However, the observation that distinct viruses are resistant to neutralization by J3 from those that are resistant to the highly-characterized broadly neutralizing VRC01 implies there is also variation in the

residues precisely targeted by VRC01 and J3 and structural studies are underway to define the key contacts of J3 within the CD4bs[126].

Competition ELISA studies further showed J3 pre-incubation with gp140 competes with sCD4 for binding to the HIV-1 Env (figure 5A), as does the previously described VHH A12, in contrast to the negative control (an anti-HIV-1 VHH which is not specific for the CD4bs). In addition, pre-incubating J3 with the HIV-1 Env competes with the anti-CD4bs human mAb b12[58], as does pre-incubation with the VHH A12, whereas the control VHH does not compete with b12 (figure 5B). Furthermore, pre-incubation of A12 with HIV-1 Env competes with biotinylated J3 for binding to gp140, although unlabelled J3 itself appears to bind with a higher affinity than A12 and thus competes more efficiently with biotinylated J3. This implies that J3 and A12 bind at least partially overlapping epitopes within the CD4bs but that J3 probably has a higher affinity for its epitope within the CD4bs than does A12. Whereas the control in this case, the gp41-specific mAb 2F5[135], does not compete with biotinylated J3 binding (figure 5C). Thus J3 competes with both sCD4 and a previously characterized human mAb, b12[152], indicating J3 targets the CD4bs effectively to achieve its neutralization breadth across a wide range of HIV-1 subtypes by

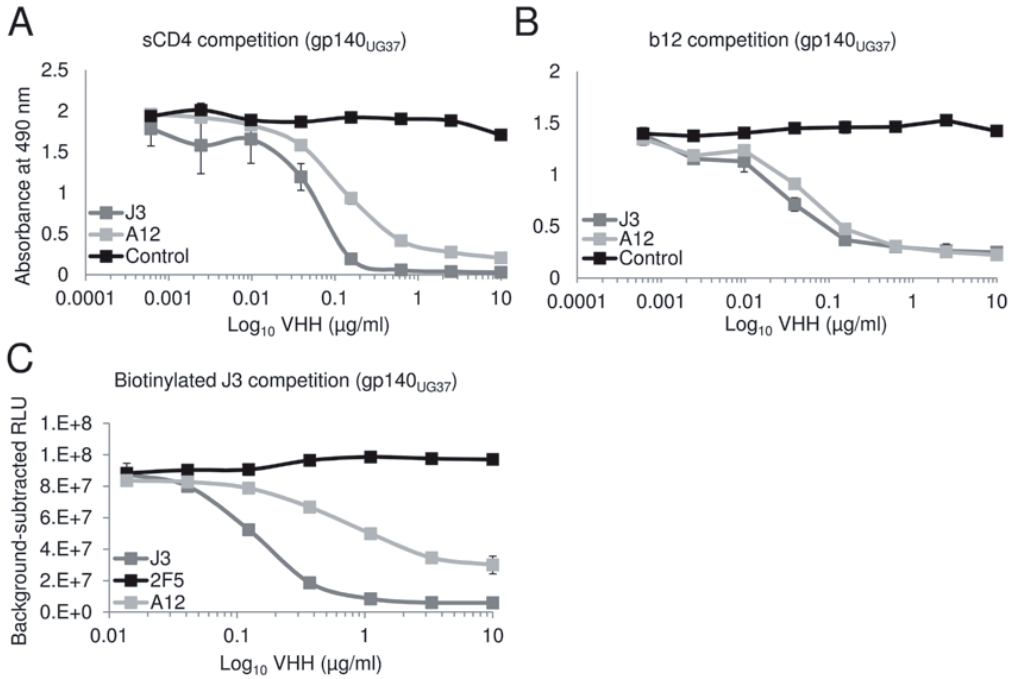


Figure 5: J3 competition for the CD4bs of HIV-1 Env in ELISA. (A) Dose-dependent competition of VHH A12, J3, and a negative control VHH with sCD4 for binding to $gp140_{UG37}$. (B) Dose-dependent competition of VHH A12, J3, and a negative control VHH with mAb b12 for binding to $gp140_{UG37}$. (C) Dose-dependent competition of VHH J3, A12 and a negative control mAb, 2F5, with biotinylated J3 for binding to $gp140_{UG37}$.

```
          ----------FW1----------__CDR1__-------FW2--------__CD32__---
Germline:QVQLVESGGGLVQAGGSLRLSCAASGRTFSSYAMGWFRQAPGKEREFVAAISWSGGSTYYAD
      J3:EVQLVESGGGLVQAGGFLRLSCELRGSIFNQYAMGWFRQAPGKEREFVAGM   GAVPHYGE

          ----------------FW3-----------------_____CDR3______----FW4----
Germline:SVKGRFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA           EYDYWGQGTQVTVSS
      J3:FVKGRFTISRDNAKSTVYLQMSSLKPEDTAIYFCARSKSTYISYNSNGYDYWGRGTQVTVSS
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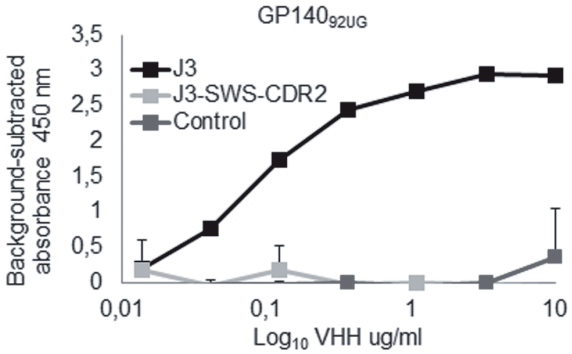

Figure 6: J3 amino acid and germ line sequences. (A) J3 amino acid sequence as determined by purified pCAD51 A12 DNA and J3 germ line determined based on literature data and DNA analysis of the area on which the V and J genes encoding VHHs are located on the lama glama and vicuña pacos genomes. Residues which deviate from germ line outside the CDRs are highlighted in yellow, and those inside CDR1 and 2 in red. Note, the portion of the VHH sequence arising from the D genes (mapping to CDR3) are too small to enable alignment via ClustalW or BLAST with D genes from lama glama or vicuña pacos, no significant association is found, hence the gap in the germ line depicted.(B) VHH binding to subtype A gp140UG37 was assessed by ELISA. Recombinant Env were immobilized and triplicate serial dilutions of Mutant, wild type J3 or a negative control VHH were added, and binding was detected as described in the Methods. All data are representative of at least two independent experiments.

interacting with invariant residues that are conserved in Env. The precise nature of these interactions will be defined by structural studies.

Affinity maturation of J3 resulted in a shortened CDR2

Analysis of the amino acid sequence (figure 6A) of J3 revealed it is part of a distinct phylogenetic family as compared to previously described anti-HIV-1 VHH[90, 125, 139] (figure S1). The most striking aspect of the sequence is a deletion in complementarity determining region (CDR)2, reducing its size from 8 to 5 amino acids. Insertions and substitutions in both CDR2 and 3 of human anti-CD4bs mAbs have been shown to be of critical importance for potency by altering the interaction with the inner domain and bridging sheet of Env[179]. The deletion in the CDR2 of J3 may be required to enable the precise binding of this extremely broad neutralizing anti-HIV-1 VHH to its epitope as a mutant J3 in which the corresponding three germ line residues have been re-inserted can no longer bind to Env (figure 6B). The role of CDR2 in the J3-Env interaction will be further clarified by structural studies. Notably, such a deletion can be considered rare

as a comparison of sequences of 1014 different VHH showed only one other CDR2 deletion of three amino acids (data not shown), which was also a CD4bs specific VHH, however unrelated to J3.

This deletion in CDR2 is the result of affinity maturation as it is not present in the germ line sequence (figure 6A) determined for J3 (determined using 23 V genes from *Lama glama* and 7 J genes from *Vicuña pacos* (data not shown)). The 23 unique V genes encoding VHH based on analysis of over 180 clones which can be separated in several families based on the presence of the following motifs KEREG, KQREL and KEREF. J3 is derived from the family typified by the KEREF motif. Within this family there are two very similar germ lines, designated Vt and Vs. Both these germ line sequences encode the same amino acid sequence. However, by aligning the J3 DNA sequence with that of both Vs and Vt germ lines using both Blast and ClustalX programs shows Vt to be the most closely aligned germ line to J3. However, it should be noted that in the absence of the sequencing of the full *Lama glama* genome it is possible that additional V-

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                          FRI-IMGT             CDR1-IMGT       FR2-IMGT
                           (1-26)               (27-38)         (39-55)
                  1        10        20        30         40        50
                  .........|.........|...... ...|........ .|.........|.....
AJ879486 Homosap
    IGHV3-23*04F: EVQLVESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVSA
               J3: EVQLVESGG.GLVQAGGFLRLSCELR GSIF....NQYA MAWFRQAPGKEREFVA.

                  CDR2-IMGT            FR3-IMGT
                  (56-65)              (66-104)
                      60         70        80        90        100
                  ....|..... ....|.........|.........|.........|....
AJ879486 Homosap
   IGHV3-23*04 F: ISGS..GGST YYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AK
               J3:  ..GM..GAVP HYGEFVK.GRFTISRDNAKSTVYLQMSSLKPEDTAIYFC
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                          FRI-IMGT             CDR1-IMGT      FR2-IMGT
                           (1-26)               (27-38)        (39-55)
                  1        10        20        30         40        50
                  .........|.........|...... ...|........ .|.........|.....
AJ879486 Homosap
   IGHV3-23*04 F: EVQLVESGG.GLVQPGGSLRLSCAAS GFTF....SSYA MSWVRQAPGKGLEWVSA
               A12: AVQLVESGG.GLVQAGGSLRLSCTAS GRIS....SSYD MGWFRQAPGKEREFVAA

                  CDR2-IMGT            FR3-IMGT
                  (56-65)              (66-104)
                      60         70        80        90        100
                  ....|..... ....|.........|.........|.........|....
AJ879486 Homosap
   IGHV3-23*04 F: ISGS..GGST YYADSVK.GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC AK
               A12: ISWS..GGTT DYADSVK.GRFAISKDNAKNAVSLQMNSLKPEDTAVYYC
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Figure 7: Phylogenetic relationship of heavy chain V genes from llamas and humans. J3 and A12 sequences were aligned with human germ line IgVH sequences through the VQUEST tool of IMGT (www.IMGT.org) (accession AJ879486). (B) J3 sequence was aligned with human germ line IgVH sequences through the VQUEST tool of IMGT (www.IMGT.org) (accession AJ879486). Residues which differ in the VHH sequence as compared to the human germ line are underlined.

genes exist which are more closely-related to J3 than is Vt. A family-specific approach[139] will be employed to isolate further VHH clones related to J3 and enable estimation of the frequency of J3-like clones in the library. However, there is some disparity between the low level of neutralization seen with the post-immune sera compared to the breadth and potency of J3. Given the previous isolation of multiple VHH from the same V gene germ line it is unlikely this disparity is due to a rare germ line precursor but rather sub-optimal stimulation and proliferation of the J3-secreting B cell clone. In addition, no J3-inhibitory activity was present in the post-immune sera which may have masked the J3 neutralization activity (data not shown). Based on the screening protocol used in this study the frequency of J3-like clones is less than 0.04% of the VHH repertoire and 0.012% of the total antibody repertoire. Therefore, future immunogens aiming to elicit a J3-like response will need substantial improvement to increase the amount of specific stimulation.

In addition to the shortened CDR2, there are twenty five separate sites where residues deviate from their germ line sequence counterparts, including residues located both in the CDRs and in framework regions (figure 6A). These residues have been altered during the maturation of the J3 heavy-chain only antibody and may play important roles in the function of the VHH. This hypermutation from the germ line confirms that J3 was indeed the result of affinity maturation in response to an antigen which the llama immune system encountered, in this case the two subtype HIV-1 Env immunogens used in immunization.

Analysis of nAbs isolated from HIV-1 infected patients has implied that a substantial degree of affinity maturation is necessary to produce effective neutralization[29, 77, 181] and the divergence of J3 from its germ line sequence is characteristic of such affinity maturation. Recently, a convergence of residues found in the heavy chains of patient-derived broadly neutralizing anti-HIV-1 mAbs has been reported[77]. To establish how similar llama heavy-chain only antibodies are to human heavy chains the nucleotide sequences of both J3 and A12 was compared to available human heavy chain sequences (figure 7). The human VH3-23*04 sequence shares 79.57% homology with the V gene segment of J3 and 84.38% homology with that of A12. Notably, this level of homology was not greatly different from the 85.60% homology that J3 shares with its precursor llama germ line V gene sequence indicated in figure 6. Furthermore, the human JH*02 sequence shares 83.33% homology with the J gene segment of J3 and 78.72% homology with A12 (figure 7).

Discussion

The isolation of J3 represents a significant improvement on previous nAbs derived from immunized animals as in single-domain VHH form it has a comparable breadth and potency to the best nAbs obtained from a limited number of natural human infections. In contrast, previous nAb clones characterized from immunized animals have only

exhibited limited breadth[90, 168]. A caveat to this is the observation that sera with 17b-like binding specificity can be induced following immunization of humans[148] and it should be noted that 17b, and other antibodies to CD4-induced epitopes, are less broadly neutralizing as full-length mAbs than in Fab form. However, given the previously reported decrease in neutralization ability seen with the Fab of b12[63], it appears the CD4bs of Env is not per se more easily targeted for neutralization by small antibody fragments as is the CD4-induced sites, presumably due to the required presence of the co-receptor interacting with Env in the latter case. As J3 neutralizes via interaction with the CD4bs it is unlikely that its breadth is a function solely of the smaller size of VHH. Therefore, whether the breadth and potency of J3 are conserved in a full-length heavy-chain only llama antibody will be investigated. Thus, this study reports for the first time a neutralizing antibody response following experimental immunization with a breadth equivalent to that seen in the best antibody responses in natural infection.

Llama 8 was multiply-immunized with trimeric gp140 Env derived from a subtype A and CRF07_ BC viruses in the presence of the adjuvant Stimune. The resulting phagemid VHH library was screened via a selection method based on neutralization function rather than pre-enriching a polyclonal mixture of VHH for the ability to bind to recombinant HIV-1 Env and/or compete with nAbs. However, to isolate a VHH specific for a particular binding site on Env, the original biopanning method remains advantageous, as it enables targeted selection of VHH via enriching for those VHH that compete with a known nAb to a specific region. As Env glycoproteins were used to elicit J3, and given patient-derived nAbs have been shown to provide protection from infection when passively infused into NHP[64, 161, 182, 183] these recombinant immunogens represent valid vaccine candidates for HIV. However, given the weak level of serum neutralization observed, they will require further modification to induce high titer J3-like broad cross-neutralizing serum responses.

J3 neutralizes 96% of all strains tested in this study which included 100 representatives from a wide range of subtypes and CRFs. In fact, J3 neutralizes 100% of subtype A, B, D and CRFs AC, ACD, AG, BC, and CD, and above 85% of the remaining subtypes tested C, G and CRF01_AE. In an analysis of viruses previously tested against VRCO1/2 and b12, J3 neutralized 94.2% of viruses compared to VRCO1 which neutralized 88.4% of the subset of 69 viruses. Sensitivity to the full-length human nAb and the llama VHH were similarly distributed across different subtypes in this subset. However, at the level of individual viruses, resistance to VRCO1 did not confer resistance to J3 and vice versa. No clear pattern of residues associated to J3 resistance was discernible from analysis of the available sequences for viruses which were not neutralized by J3. While the evaluation of J3 against additional viruses may provide further insight into key residues for J3-mediated neutralization it may be more thorough to evaluate the neutralization sensitivity of a panel of engineered viral mutants to define the functional J3 epitope in combination with escape mutant studies.

J3 binds to both the trimeric immunogens that llama 8 received, as well as monomeric $gp120_{IIIB}$ and $gp120_{YU2}$. J3 targets the binding site of Env for the HIV-1 cellular receptor CD4 as confirmed by the loss of binding seen with the CD4bs D368R $gp120_{YU2}$ mutant relative to wild type $gp120_{YU2}$. In addition J3 competes with sCD4 itself as well as with the well-characterized human mAb b12. Competition studies with A12, a previously isolated anti-CD4bs VHH, showed that binding assays, while demonstrating a level of difference between the two VHH, were not able to detect the disparity between these two VHH in terms of neutralization ability. Together with the level of neutralization breadth observed, these findings indicate that J3 recognizes part of the Env CD4bs and very few if any of the adjacent amino acids, although this remains to be defined in detail.

This study describes the isolation of an extremely broad and potent HIV-1 neutralizing VHH, J3, from a screen of almost 3000 clones. This method provides a relatively quick screening process compared, for example, to the 30000 human B cell clones screened to isolate the PG9 and PG16 nAbs[29]. Thus, this method could be used alongside testing the immune sera for neutralization to evaluate new HIV-1 immunogens in the established llama model, accelerated by the advantage of heavy-chain only antibodies that they do not require heavy and light chain random recombination. Furthermore, the independently functional nature of the VHH antibody fragments[184] coupled with their intrinsic stability[130] makes them suitable candidates for anti-HIV-1 microbicide development. Thus, broad and potent neutralizing VHH isolated in this way, such as J3, can both aid progress towards an HIV-1 vaccine and simultaneously provide a useful reagent for anti-HIV therapeutics and/or prophylaxis.

Recently, the precedent for an anti HIV-1 microbicide has been established by the successful phase III trial of an antiretroviral-containing gel[120]. Furthermore, immunological microbicides, such as mAbgel[185], are under investigation. A VHH with the breadth of J3 has potential as the active component of such a microbicide due to their intrinsic temperature and pH stability of VHH[130] and the ease and low cost with which they can be manufactured as compared to full-length human nAbs. In addition, J3 neutralizes a range of SHIV strains as well, making it an ideal candidate for SHIV mucosal challenge experiments either via passive immunization or in a microbicide formulation. The potential use of microbicides is not mutually exclusive with that of a vaccine, as the two could be complementary in a situation whereby an effective microbicide could limit the probability of infection and thus support a vaccine-induced immune response.

In conclusion, the isolation of such a potent and broad VHH fragment from an animal immunized with a relatively simple combination of recombinant protein immunogens argues for the potential of vaccination to elicit cross-reactive protective anti-HIV antibodies and further study of this VHH will provide insight into how to re-capitulate the elicitation of such antibodies, hopefully in conventional IgG format. VHH J3 neutralizes 96% of all strains tested to date, these include a great variety of HIV-1 subtypes. This highly cross-reactive anti-HIV-1 VHH differs from previous anti-HIV-1 VHH not only in its breadth

but in terms of the immunization procedure undertaken, the screening method by which it was isolated and its phylogentically distinct and matured sequence. The breadth of neutralization achieved via targeting the CD4bs demonstrated by J3 has only previously been seen as a result of natural infection. The definition of the precise CD4bs epitope targeted by J3 will be clarified by structural studies[126] and may be used to optimize future immunogens, which can be easily and efficiently evaluated using this redefined screening process for llama VHH libraries.

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Online supplementary data

Table S1 shows the llama immunization protocol with HIV-1 Env. Table S2 contains the individual IC50 (μg/ml) titers obtained for J3 against 100 viruses. Table S3 reiterates the IC50 titers (μg/ml) for VRC01/2, b12, and CD4-Ig as published[30] for a subset of 69 matched viruses against which J3 was also tested. Fig. S1 illustrates the phylogenetic relationship of the J3 and A12 VHH families.

Chapter 5

Bivalent VHH are superior to monovalent VHH in the combat against HIV-1

Bivalent VHH are superior to monovalent VHH in the combat against HIV-1

Nika M Strokappe[1,6], Laura E McCoy[2,3], Gillian Dekkers[1,2,3], Alexandre M J J[4] Bonvin[4], Michael S Seaman[5], Theo Verrips[1,6], Robin A Weiss[2,3], Lucy Rutten[1]

[1]Biomolecular Imaging, Department of Biology, Faculty of Science, Utrecht University, 3584 CH Utrecht, Netherlands
[2]Wohl Virion Centre and [3]Medical Research Council (MRC) Centre for Medical Molecular Virology, Division of Infection and Immunity
[4]Computational Structural Biology, Bijvoet Center for Biomolecular Research, Faculty of Science, Utrecht University, 3584 CH Utrecht, Netherlands
[5]Division of Viral Pathogenesis, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215
[6]QVQbv, Padualaan 8, 3584CH Utrecht, The Netherlands

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Abstract

A number of llama derived single domain antibodies (VHH), we have previously selected against the envelope spike of HIV-1, neutralize the virus with great breadth. The broadest of them all, VHH J3, targets the CD4 binding site (CD4bs) and neutralizes 96% of the HIV-1 strains tested. J3 is a promising microbicide against HIV-1, by itself or in combination with other HIV-1 neutralizing VHH that do not bind to the same epitope. Here we describe a novel structurally different VHH, 3E3, which neutralizes 80% and binds to a nearly identical epitope as J3 does. Based on the binding modes of other CD4bs targeting broadly neutralizing antibody-gp120 complexes, a 3D model of the gp120-3E3 complex was generated. Furthermore, we studied the cross-competition between 16 of our HIV-1 neutralizing VHH with the seven broadest VHH for antigen binding. The results of this indicated that our VHH recognize a total of four independent neutralizing epitopes on gp120. To increase the breadths and potencies of J3 and 3E3, they were fused to VHH that recognized epitopes other than the CD4bs. J3 or 3E3 linked to either 2E7 or 11F1F, showed increases in neutralization potencies compared to the molar mixes of the individual VHH. J3 linked to 2E7 yielded the largest improvement, i.e. a 1400 fold improvement in potency against the SVPC15 pseudovirus. In conclusion, because of their large breadths and high potencies, some of our bivalent VHH are very promising molecules for the use as microbicides to prevent HIV-1 infections.

Introduction

Even after 30 years of research, AIDS caused by HIV-1 still remains one of the largest health problems, especially in the developing countries. Nowadays, AIDS can be treated and in the developed countries it is regarded as a chronic disease. However, treating AIDS has two major disadvantages, first of all it is very expensive and second, it has many adverse effects for the patient. Annually an estimated 1.8 million people, of whom 1.2 million in Africa, die an AIDS related death, mainly because treatment is not available to them. Therefore, prophylactic drugs against HIV-1 infection or at least affordable therapeutics are urgently needed.

Llamas and other members of the *Camelidae* family have, besides conventional antibodies comprising heavy and light chains, antibodies that lack the light chain (IgG_2 and IgG_3), the so-called Heavy chain only Antibodies (HcAbs). The antigen binding fragment of these HcAbs consists of a single domain referred to as the Variable domain of the Heavy chain of a Heavy chain only antibody, or VHH. In a HcAb, the VHH domain is linked directly to CH2 domain by a special hinge region, and thus the HcAb lacks the CH1 domain[42, 44]. VHH domains can be produced separately and have been shown to bind diverse antigens with high affinities[46, 47, 60]. A major advantage of the use of VHH over the use of conventional antibodies or conventional Fab fragments, is that they are easier and cheaper to produce and more stable due to their much lower complexity[46, 51, 130]. Furthermore, VHH are much smaller, which allows them to bind epitopes that cannot be reached by conventional antibodies[47, 49]. The most conserved epitopes on the envelope proteins (Env) of HIV-1, the CD4bs and the co-receptor (CCR5) binding site, are difficult to reach by conventional antibodies. This is due to conformational shielding and the shielding by glycans surrounding these binding sites[17, 18]. Therefore, especially in the case of targeting HIV-1, the small size of a VHH is expected to be a great advantage.

Over the years we have identified a great number of potent and broadly neutralizing VHH against HIV-1[142, 143, 186]. One of these VHH, J3, is broader neutralizing than the broadest neutralizing conventional antibody isolated from long-term non-progressors. J3 neutralizes 96 out of the 100 HIV-1 strains that were tested. The other VHH neutralize up to 82% of the tested strains. Some are even able to neutralize the few strains that are not neutralized by J3. Previous data suggests that these VHH target multiple non-overlapping epitopes on Env. Furthermore, linking two VHH together into a mono- or bi-specific biheads may result in a great improvement in potency and breadth[20, 60].

The aim of this study was to create a molecule with an even better potency and breadth than J3. We describe a novel broadly neutralizing VHH, called 3E3, which binds to nearly the same epitope as J3 does. A competition assay was performed to find suitable partner VHH for our broadest and most potent VHH. Linkage of J3 and 3E3 to these partner VHH yielded molecules with enhanced potencies compared to the mixes of the individual VHH. Some linked VHH also showed increased neutralization breadths compared to either individual VHH. Overall, these bi-specific biheads are promising molecules for the use

as microbicides to prevent HIV-1 infections. An additional benefit of bi-specific VHH is that the probability that escape mutants will be generated will be much less than for mono-specific VHH[144].

Materials and methods

Proteins

Monoclonal antibodies b12 (EVA3065, by Dr D. P. Burton and Dr P. Parren)[152], 17b (ARP3071 by Dr J. Robinson) [145], 2F5 (EVA 3063, by Dr H. Katinger), 4E10 (ARP3239, by Polymun), and L120 (ARP359, by Becton Dickinson) the recombinant proteins gp120IIIB (EVA607, by ImmunoDiagnostics), gp140UG37 (ARP698, by Polymun), gp140CN54 (ARP699 by Polymun), gp41 (ARP680) and human soluble CD4 (EVA609, by ImmunoDiagnostics) were obtained through the Centralized Facility for AIDS Reagents (CFAR), the National Institute for Biological Standards and Controls (NIBSC).

Viruses

Replication competent virus stocks were prepared from HIV-1 molecular clones by transfection of 293T cells. HIV-1 envelope pseudotyped viruses were produced in 293T cells by co-transfection with the pSG3Δenv plasmid. The subtype B and C HIV-1 Reference Panels of Env Clones [91, 119] were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA. The 96ZM651.02 gp160 clone was kindly provided by Dr D. Montefiori (Duke University Medical Center, Durham, NC) through the Comprehensive Antibody Vaccine Immune Monitoring Consortium (CA2 VIMC) as part of the Collaboration for AIDS Vaccine Discovery (CAVD). All additional pseudoviruses were produced at the VIMC laboratory.

Cells

TZM-bl cells[91, 114, 115] were obtained through the NIH AIDS Research and Reference Reagent Program from J. C. Kappes, X. Wu, and Tranzyme, Inc., and cultured in Dulbecco's modified Eagle medium (Invitrogen) containing 10% (v/v) fetal calf serum (FCS).

VHH

2E7, 1F10, 1B5 and 1E2 have been described by Strokappe *et al.*[143]. 1H9, 2B4F, 11F1F, 11F1B, 5B10D and 9B6B have been described by Forsman Quigley *et al.*[186] 8FA3 is came from these selections and screenings, but was not further described. It was isolated from llama 8, panned on gp140CN54 and eluted with TEA. A12 and C8 have been described by Forsman *et al.*[90]. J3 has been described by McCoy *et al.* [142]. 2H10 has been described by Lutje Hulsik *et al.*[61].

Immunization of *Lama glama* and construction of the VHH phage library.

Llama 9 was immunized with recombinant gp140 derived from HIV-1 CN54 and

92UG037. Immunizations and VHH library construction were performed as described previously[142, 143].

Selection of 3E3 by phage display

Phage display selection of CD4bs VHH was performed as described previously[90]. In short, for the first round of panning MaxiSorp plates (NUNC) were coated overnight at 4°C with 100 µl of 5, 1.67, 0.56 and 0 µg/ml gp120Yu-2DS2[78] in sterile phosphate buffered saline (PBS). The next day, the plates were blocked with 4% skim milk powder (Marvel) in PBS (MPBS) for 1 hour. Polyethylene $glycol_{6000}$ (PEG) precipitated phages, displaying VHH from llama 9 were preincubated 1:10 in 2% MPBS for 30 minutes prior to adding them to a plate, which was washed three times with PBS supplemented with 0.05% Tween-20 (PBST). Phages were allowed to bind the antigen for 2 hours, while shaking. Unbound phages were removed by washing 40 times with PBST. The plate was washed an additional three times with PBS to remove all remaining Tween. Bound phages were eluted by pH shock with 1 M Triethylamine (TEA) and rescued by infecting log phase TG1 *E. coli*. The outputs from the 5 µg/ml coated wells were used to produce phages for the second round. For the second round of panning 2, 0.4 and 0 µg/ml gp120Yu-2DS2 was used. Next to a TEA elution, a specific elution with sCD4 was done. A 50 times molar excess sCD4 was added to the wells and incubated for 2 hours, while shaking. All eluted phages were rescued by infecting log phase TG1 *E. coli*. 190 individual clones were screened for their ability to compete with sCD4. Each clone was expressed in 1 ml of 2×Yeast Tryptone medium supplemented with 100 µg/ml ampicillin and 0.1% glucose. At an optical density at 600nm of approximately 1, VHH production was induced by 0.1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). The VHH from the periplasmic extracts were harvested by freeze thawing. The VHH were screened for their ability to compete with sCD4, therefore MaxiSorp plates were coated with 100ng per well gp120Yu-2DS2, overnight at 4°C. After blocking, 25 µl 2% MPBS and 25 µl periplasmic fraction was added to the plates. After washing, 2.5 ng sCD4 was added to each well, followed by 1:10,000 L120 (mAb against CD4, NIBSC)and 1:5,000 of 1 mg/ml peroxidase conjugated donkey anti-mouse IgG (DAMPO) (Jackson Immunoresearch). In all steps, the added proteins were allowed to bind for one hour, while shaking at room temperature, before washing three times with PBST. To the antibody and sCD4 solutions, MPBS was added to achieve a final concentration of 1% MPBS. Before adding the detection agent, the plate was washed an additional three times with PBS. Subsequently, the amount of peroxidase conjugated antibody was visualized by adding o-Phenylenediamine (OPD) supplemented with 0.03% H_2O_2. After 30 minutes, the reaction was stopped using 0.33 M H_2SO_4. The absorption was measured at 490 nm.

Isolation of anti–HIV-1 VHH through direct HIV-1 neutralization screening.

For the direct neutralization screening, bacteria expressing the cloned VHH repertoire were plated onto agar plates containing 100 µg/ml ampicillin and 2% syncytial stain (1 g

methylene blue and 0.33 g basic fuchsin in 200 ml methanol). Individual clones were picked using a Norgren CP7200 colony picker (RapidPick; Hudson Robotics) and transferred to 384-well master plates. 1,056 individual clones were expressed in TG1 *E. coli* cells in a 96-well plate format. Each clone was expressed in 1 ml of 2× TY medium containing 100 μg/ml ampicillin and 0.1% glucose, followed by induction of VHH production with 0.1 mM IPTG. Bacterial pellets were frozen at −80°C for a minimum of 1 h and then thawed and resuspended in PBS. The periplasmic extract from each well was separated from the bacterial debris by filtration through a 0.2-μM polyvinylidene fluoride membrane and screened for the ability to neutralize HIV-1. To enable semi–high-throughput screening and characterization of VHH, neutralization was measured using 200 50% tissue culture infective doses of virus in the TZM-bl cell-based assay developed by [91, 114, 115] with Bright-Glo luciferase reagent (Promega) using a Glomax plate reader (Promega).

Production and purification of VHH

Large quantities of VHH were needed for subsequent assays. Therefore the *VHH* genes were transferred into our production vector pCAD51. Expression from the pCAD51 vector results in the incorporation of a C-terminal myc and 6-His tag to the VHH and the removal of the bacteriophage gene III product. Overnight cultures were inoculated 1:100 into 2×Yeast Tryptone (Carl Roth) supplemented with 0.1% glucose and 100 μg/ml ampicillin. At an OD_{600} of approximately 1.2 VHH production was induced by 0.1 mM IPTG. Cells were incubated either four hours at 37°C or overnight at 24°C and spun down. The periplasmic extract from each well was harvested by freeze thawing. VHH were purified by means of the attached 6-His-tag using TALON Metal Affinity Resin (Clontech).

Cross-competition assay

VHH that were found to be neutralizing were included in a large competition screen among the VHH themselves to determine whether they had overlapping epitopes. To be able to detect only one of the two VHH that are present during this assay, part of them had to be biotinylated. Therefore NHS-LC-LC-biotin (Thermo scientific, Cat. No: 21343), was added to the VHH in a 10:1 molar. This mix was incubated at room temperature for 1 h. Unbound biotin was removed by dialysis. The biotinylated VHH were titrated for their binding to gp140UG37, gp120IIIB and gp140CN54.

For the competition assay, MaxiSorp plates were coated, overnight at 4°C, for gp140UG37 a concentration of 100 ng per well was coated for all VHH, for gp120IIIB 250 ng was used and for gp140CN54 100 ng per well was used, except for J3, in which case 250 ng was used. The next day, the plates were blocked with 4% MPBS, shaking at room temperature. After three washes with PBST, 50 μl of 100 μg/ml of the competing (non-biotinylated) VHH was added to the wells and allowed to bind for 1 hour. This amount of VHH should be sufficient to cover the antigen, but to make sure the competing VHH is actually binding, a binding assay in which the VHH was detected by 1:2,000 anti Myc-tag

(9E10 , in house production) and 1:5,000 DAMPO was performed in parallel. Competing VHH not showing binding were excluded from further analysis. 10µl of the biotinylated VHH was added to the competing VHH in all of the wells, in the amount determined based on the previously described titration. The added amounts being for gp140UG37: 1B5 (3.2 ng/well), 1F10 (6.5), 1H9 (12.5), 2E7 (1.5), 3E3 (6.5), 11F1F (1.3) and J3 (8.5), for gp120IIIB: 1B5 (150 ng/well), 1F10 (NT), 1H9 (45), 2E7 (NT), 3E3 (40), 11F1F (NT) and J3 (10), and for gp140CN54: 1B5 (32 ng/well), 1F10 (10), 1H9 (20), 2E7 (32), 3E3 (NT), 11F1F (30) and J3 (32). NT means that it was not tested, due to a lack of detectable signal. After an additional hour of incubation, the plate was washed three times with PBST followed by addition of 50µl 1:2,000 HRP conjugated Streptavidine (1:1,000 for 3E3 on UG37 and 1B5 on IIIB). After washing three times with PBST followed by washing with PBS, the amount of Strep-HRP present in the wells was visualized by adding 50 µl *o*-Phenylenediamine, supplemented with 0.03% H_2O_2. After 30 minutes, the reaction was stopped using 25 µl of 1 M H_2SO_4. The signals were measured for their absorption at 490 nm. These values were then converted to percentages, in which competition with itself was regarded as minimal binding (maximal competition), i.e. 0%, and the competition against an irrelevant VHH as the unhindered binding, i.e. 100%.

Binding to various Env proteins.

MaxiSorp plates were coated overnight at 4°C with 100 ng per well gp140UG37, gp120IIIB or gp41. The following day, the plates were blocked with 200 µl 4% MPBS, while shaking at room temperature. After three washes with PBST, 50 µl serially diluted VHH (mono or bivalent), starting at 1 µM, were added to the wells and allowed to bind for 1 h at room temperature (RT), while shaking. VHH (mono or bivalent) were detected via their C-terminal Myc tag, by 1:3,000 mouse anti Myc (9E10) and 1:5,000 DAMPO. All were incubated 1 h at RT while shaking, and washed three times with PBST. The presence of peroxidase in the wells was visualized as stated above.

Competition assay with mAbs and sCD4

MaxiSorp plates were coated overnight at 4°C with 100 ng per well gp140UG37 (for b12, 17b, sCD4 and partially 2F5 competition) or gp41 (4E10 and partially 2F5 competition). After blocking and washing, 50 µl of 1 µM VHH (mono or bivalent) were added to the wells and allowed to bind for 1 h at RT while shaking. 10 µl of competitor was added to the wells in a final concentration of 0.4 µg/ml for b12, 1 µg/ml for 17b, 0.07 µg/ml for 4E10, 0.07 µg/ml for 2F5 in the gp140UG37 coated wells and 0.6 µg/ml for 2F5 where gp41 was coated. After 1 hour incubation (RT, shaking), the plates were washed three times with PBST. Competitors were detected with 1:5,000 peroxidase conjugated goat anti human (Jackson Immunoresearch) for the human mAbs b12, 17b, 2F5 and 4E10 and 1:10,000 L120 followed by 1:5,000 DAMPO for sCD4. All were incubated 1 hour (RT, shaking) and

washed three times with PBST. The presence of peroxidase in the wells was visualized as stated above.

Construction of 3D model

A 3D model of VHH 3E3 interacting with gp120 based on published crystal structures of classical antibodies in complex with gp120, and using sequence data concerning 3E3 was made. In addition to the cysteine bridge present in all VHH, 3E3 has an additional cysteine bridge between the CDR3 Cys100a and the framework Cys50, which stabilizes the conformation of the CDR3 loop of 3E3. A model of 3E3 was generated with the program Modeller 9.9 using the VHH D7[125] as a template structure. This template structure does not have an additional cysteine bridge as present in 3E3, so this bridge was not automatically created in the model either. Using the molecular building program Coot 0.6.2 for Windows[187], the CDR3 loop was bent, to create this second cysteine bridge.

Prior to docking on gp120 using the program HADDOCK v2.1, the geometry of the model of 3E3 was refined with CNS (Crystallography and NMR System)[188]. The restraints used in the modeling with HADDOCK were based on published data of the HIV-1 neutralizing anti-CD4bs antibodies b12, b13, F105, VRC01, VRC03, NIH45-46 and VRC-

PDB code	Antibody	D368 binding	I371 binding	In cavity like Phe43$_{CD4}$	Large conformational change of the bridging sheet
3U7Y	NIH45-46	R71	G54,G55,A56	-	no
3HI1	F105	R100F(2.9), R94(3.6), H32(3.7)	A99(3.8), V100(4.3)	-	yes
3RJQ	A12	K95(3.7), R97(4.2)	-	-	yes
3IDX	B13	K52(3.6)	Y53a(4.0)	Y53a	yes
2NY7	b12	Y100a(2.7), N52(3.4)	Y53(3.6)	-	yes
3SE8	VRC03	R71(2.9)	W54(3.9)	W54	no
3SE9	VRC-PG04	R71(2.8)	A55(3.5), T53(3.7)	-	no
3NGB	VRC01	R71(2.9)	A56(3.8)	-	no
-	J3	H56	Y100	Y100	no
1G9N	CD4	R58	F43(3.2)	F43	reference point

Table 1: Interactions of various CD4bs antibodies gp120. The binding modes of various ligands to gp120. Indicated is which amino acid of the ligand binds to D368, an amino acid crucial for CD4 binding to gp120, whether or not there is an amino acid penetrating into the Phe43$_{CD4}$ pocket and whether the ligand induces a large conformational change of the bridging sheets compared to their conformation in the CD4 bound state.

PG04[58, 78, 126, 179, 180, 189, 190] (table 1). 3E3 gives a similar competition pattern as J3 does in the competition assay. The exact epitope of J3 is known from the co-crystal structure by Chen *et al.* [126]. This formed the basis for modeling 3E3 toward the CD4bs. In all antibody-gp120 complex crystal structures, in which the antibody is targeting toward the CD4bs, two interactions seen in CD4 binding to gp120, seem to be key in the binding of antibodies as well. Firstly, D368 always makes an interaction with a basic residue. Secondly, in the CD4-Phe43 binding cavity, when intact, either a phenylalanine or a tyrosine binds at an equivalent position. We reasoned that 3E3, based on our biochemical and neutralization data, should also bind in a similar fashion. The CDR3 of 3E3 contains an arginine, a tyrosine and a phenylalanine. In HADDOCK two restraints were given. The first was that there had to be an interaction between the R100d in CDR3 and D368 of gp120. The other was that either Y100 or F100f should be in the CD4-Phe43 binding pocket. The best model in terms of HADDOCK scores had the Y100 in the Phe43-binding pocket and the arginine make a salt bridge to D368 of gp120.

Construction of bi-specific biheads

To construct bi-specific biheads, the N- and C-terminal fragments were generated in separate PCR reactions. The specific primer used, defines the length of the linker that connects the two VHH. For the N terminal fragments, the following primers were used: Forward; M13rev (5'-GAGCGGAT AACAATTTCACACAGG-3') and reverse; R5SGBam (5'-AGTAGGATCCGCCACCTCCTGAGGAGACCGTGAC CTGGGTCCC-3') in case of a 15 a.a. linker. For the C-terminal fragments, the following primers were used: Forward; F10GSBam (5'- TCTTGGATCCGGCGGGGGAGGTAGTGGGGGTGGGGGCTCAGAGGTGCA GCTGGTGGAGTCTGGG-3') and M13for (5'-GCCAGGGTTTTCCCAGTCACGA-3') .

The PCR was performed using DreamTaq green (Fermentas, Cat. No: EP0712). As DNA template, 0.5 µl of the bacterial glycerol stock was used, and 2.5 µl of a 5 µM stock of each of the respective primers was used. The PCR reaction was as follows: 5 min at 95°C, then 34 cycles of 30 s at 94°C, 30 s at 55°C, 45 s at 72°C, followed by a 10 min extension period at 72°C. The PCR product was cleaned using the NucleoSpin® Extract II kit, the PCR clean-up protocol, (Machery-Nagel, Cat. No: 740609.250) and eluted in 26 µl.

N-terminal fragments were digested by adding 3 µl Buffer Green and 1 µl SfiI (Fermentas, Cat. No. ER1821), incubation at 50°C for 90 min. Subsequently, 1 **µl** BamHI (Fermentas, Cat. No. ER0051) was added to the mix followed by an additional 90 minutes incubation at 37°C. The C-terminal fragments were digested by adding 6 µl of buffer Tango, 1 µl Eco91I (Fermentas, Cat. No. ER0391) and 1 µl BamHI to 22 µl eluate. The mix was incubated for 3 h at 37°C. The digested fragments were separated on a 1% agarose gel, the 350 bp band that corresponds with the VHH was excised and purified using NucleoSpin® Extract II kit, gel extraction protocol. The N- and C-terminal fragments were ligated for 1 h at RT in a 1:1 ratio into SfiI/NcoI/Eco91I digested pCAD51 vector using

T4 DNA Ligase (Fermentas, Cat. No: EL0011). The construct was then transformed into chemically competent TG1 E. coli and subsequently plated onto Luria Broth agar plates supplemented with 2% glucose and 100 μg/ml ampicillin.

HIV neutralisation assay

The HIV-1 neutralising activities of the VHH were assessed in the TZM-bl cell based assay, as described previously[90]. Briefly, 3-fold serial dilutions of purified VHH were added in 10% (v/v) fetal calf serum (FCS) supplemented DMEM growth medium (Invitrogen, Paisley, UK). 200 TCID50 of virus was then added to each well and the plates were incubated for 1 hour at 37°C. TZM-bl cells were subsequently added (1 x 104 cells/well) in growth medium supplemented with DEAE-dextran (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 11 μg/mL. Assay controls included replicate wells of TZM-bl cells alone (background control), and TZM-bl cells with virus assayed (virus control). No virus inactivation was observed with a negative control VHH. Following 48 hours incubation at 37°C, the assay medium was replaced by Bright-Glo luciferase reagent (Promega, Madison, WI, USA). The cells were allowed to lyse for 2 minutes, and the luminescence was then measured using a luminometer. The 50% inhibitory concentration (IC50) titres were calculated as the VHH concentration that achieved a 50% reduction in relative luminescence units (RLU) compared to the virus control RLU, after subtraction of the background control RLU from both values. The calculations were performed using the XLFit4 software (ID Business Solutions, Guildford, UK).

Results

Selection of 3E3

The VHH 3E3 was identified in two different ways. First it was isolated from the phagemid library via two sequential rounds of biopanning on immobilized gp120Yu-2_{DS2}. In the first round, phages were eluted with TEA, in the second round with soluble CD4 (sCD4). Additionally a new screening method was used whereby the VHH were assessed directly for their ability to neutralize HIV-1 pseudovirus without any prior selection for the ability to bind to Env or compete with characterized anti–HIV-1 mAb or sCD4. In this way J3 has been found previously[142]. Individual sequences were obtained for the VHH clones that were able to neutralize one or more of the chosen tier 2 pseudoviruses. Periplasmic extract containing one VHH, designated 3E3, neutralized all of the six screening viruses. Therefore, 3E3 was selected for further characterization. 3E3 was shown to lack binding to a gp120-D368R mutant[127]. D368 is an amino acid, crucial to CD4 binding and antibodies that fail to bind to this mutant gp120 are said to be CD4bs binders. Therefore 3E3 most likely binds to the CD4bs.

Cross-competition assay

Sequence analysis of the HIV-1 neutralizing VHH that we obtained earlier, showed that

a large number of quite different VHH had been selected[90, 142, 143]. Based on this analysis we classified the VHH and subdivided them into different families and sub-families. However sequence differences do not necessarily mean that the VHH binding epitopes are different as well. In order to make an educated decision about what VHH may be best to link to J3 and 3E3, the broadest neutralizing VHH, a large competition assay was performed. 16 of the HIV-1 neutralizing VHH were studied for their cross-competition with the 7 best VHH for antigen binding. The 7 VHH were biotinylated in order to be able to detect them. Figure 1 shows the averages of the competition assays that were performed on three different Env antigens. For some VHH, the competition was not tested on all three antigens, because they showed insufficient binding towards the antigen. An irrelevant VHH that lacks binding to any of the antigens, even at concentrations as high as 100 μg/ml was chosen as a negative control for competition (data not shown).

	1B5	1H9	2B4F	4D4	1F10	2E7	11F1F	11F1B	1E2	93E3	J3	33E1	8FA3	A12	C8	Irr
1B5	0	2	-6	-9	49	78	71	64	100	87	88	61	41	75	85	100
1H9	7	0	4	2	23	77	88	93	91	116	101	58	43	56	72	100
1F10	68	59	56	79	0	89	88	73	81	89	84	62	69	90	82	100
2E7	105	141	116	127	54	0	7	7	5	126	112	101	115	107	113	100
11F1F	115	127	111	142	77	0	0	-2	10	149	95	104	134	92	104	100
3E3	131	86	115	111	84	80	48	75	90	0	6	14	10	10	23	100
J3	125	101	119	104	122	138	95	94	116	1	0	4	2	3	13	100

Figure 1: Cross-competition assay. Competition among the VHH is presented as a percentage. The signal observed for VHH competing with themselves was defined as 0% , signal during competition with an irrelevant VHH was defined as 100%. The VHH in the columns were the competing VHH, present in excess, the binding of the VHH represented in the rows was measured.

Competition is presented as a percentage. The signal observed for VHH competing with themselves was defined as 0%, since both the biotinylated VHH and the competing VHH then bind to identical epitopes, with equal affinities. The signal during competition of biotinylated VHH with an irrelevant VHH was defined as 100%.

Competition up to 30% (red background) indicates that the two VHH most likely have overlapping epitopes. Competition between 30% and 75% (orange background) means that even though there is not very strong competition, the two tested VHH are most likely not able to bind simultaneously. Competition between 76% and 125% (light green background) indicates that a molar excess of non- biotinylated VHH does not interfere with the binding of the biotinylated VHH. Finally, competition of 125% or more (dark green background) indicates that the competing VHH may even enhance the binding of the biotinylated VHH, like sCD4 enhances 17b binding.

The pattern of the levels of competition indicates that the VHH recognize four different epitopes on Env. VHH 1B5 and 1H9 compete with a similar set of VHH (2B4F, 4D4 and to some extend 1F10). Furthermore, they fully compete with each other. This suggests that their epitopes may be the same. The same holds true for 2E7 and 11F1F, which are

phylogenetically related. Note that the extraordinarily broadly neutralizing and potent J3 shares its epitope within the CD4bs with 3E3. 1F10 does not seem to compete with many of the other VHH, except with 1H9/2B4F and to some extent 1B5, indicating that it binds to a separate epitope that may have some overlap with the epitope of 1H9/2B4F and 1B5.

Overall, 2B4F (a family member of 1H9) and 4D4 bind epitopes overlapping with that of 1B5/1H9. 11F1B and 1E2 bind epitopes overlapping with the one of 2E7/11F1F. The epitopes of 33E1 (a less potent VHH related to J3), 8FA3, A12 and C8 overlap at least partially with that of J3/3E3.

Binding to Env

The binding of the monovalent and bivalent bi-specific VHH (bi-specific biheads) is shown in figure 2. The level of binding to the antigens is indicated by a minus sign, which indicates no binding, or one, two or three plus signs indicating good binding by an increasing degree. All VHH bound to gp140UG37. Seven VHH (2E7, 11F1F, 11F1B, 1E2, 5B10D, 9B6B and 2H10) did not bind gp120IIIB, but did bind gp41. One VHH, i.e. 1F10, bound neither gp120IIIB nor gp41, which may be due to both proteins being derived from clade B.

All bi-specific biheads bound to gp140UG37 as well as to gp120IIIB. All bi-specific biheads that contain a VHH that binds to gp41, bound gp41 as well, albeit with lower affinity when 2E7 or its family member 11F1F were N-terminally. Interestingly, for some bi-specific biheads the binding affinity is increased just by the linkage to a partner, like for J3 linked to 2E7 or 11F1F. J3 binds gp120IIIB with moderate affinity, whereas 2E7 or 11F1F do not, but by linkage of J3 to 2E7 or 11F1F the binding affinity to gp120IIIB is increased.

Competition with mAbs and sCD4

To obtain insight into where the different groups of VHH (1B5/1H9/2B4F, 1F10, 2E7/11F1F/1E2 and J3/3E3) bound the Env trimer, all VHH were entered into a competition assay with sCD4 and the neutralizing mAbs b12[152] and 17b[145] (figure 2). b12 binds mainly to the CD4bs and 17b to the CCR5 binding site. The VHH that were able to bind gp41 were entered into a competition assay with the neutralizing mAbs 2F5 and 4E10, which both bind gp41[135-137]. The level of competition is expressed in the same fashion as for the cross-competition, however, 0% was defined as no signal of the competitor (sCD4, b12, 17b, 2F5 or 4E10) and 100% as the signal where no VHH was added to the competitor.

Members of the 1B5/1H9 epitope group as well as 1F10 all competed with 17b, but not b12 or sCD4. In fact the binding of sCD4 seems to be enhanced to some extent. 2E7/11F1F members do not seem to compete with any of the five competitors tested, indicating that they are binding to a novel epitope. CD4bs binders 3E3, J3 and 33E1 all competed with sCD4 as well as b12 and most interestingly they enhanced the binding of 17b up to four times. 8FA3, A12 and C8 competed with all of the gp120 targeting competi-

	Binding			Competition				
	UG37 gp140	IIIB gp120	IIIB gp41	b12	17b	sCD4	2F5	4E10
1B5	++	+	-	122	4	127	nt	nt
1H9	+	+	-	115	5	135	nt	nt
2B4F	+	+	-	118	5	176	nt	nt
4D4	+	+	-	84	8	130	nt	nt
1F10	+++	-	-	101	21	129	nt	nt
2E7	+++	-	+++	87	79	112	74	85
11F1F	++	-	+++	98	127	155	124	75
11F1B	++	-	+++	95	131	148	121	73
1E2	+	-	+++	100	84	150	64	80
3E3	+	+	-	2	430	0	nt	nt
J3	+	+	-	8	295	0	nt	nt
33E1	+	+	-	18	323	0	nt	nt
8FA3	+	+	-	1	5	0	nt	nt
A12	+	+	-	1	4	0	nt	nt
C8	+	+	-	36	10	5	nt	nt
3H9	+	+	-	74	21	70	nt	nt
5B10D	+	-	+++	101	94	88	98	82
9B6B	++	-	+++	91	103	98	92	81
2H10	+	-	+	99	102	92	10	58
B5 (irr)	-	-	-	95	103	95	98	83
J3-15-2E7	+++	+++	+++	7	126	0	79	83
J3-15-11F1F	++	++	+++	5	203	0	80	84
3E3-15-2E7	++	+	+++	1	405	0	99	96
3E3-15-11F1F	+++	++	+++	2	427	0	75	84
11F1F-15-3E3	+	+	+	2	413	0	96	89
J3-15-1F10	+	+	-	6	43	0	nt	nt
3E3-15-1F10	++	+	-	2	194	0	nt	nt
J3-15-1H9	++	++	-	2	5	0	nt	nt
3E3-15-1H9	+	+	-	1	41	0	nt	nt
2E7-15-1H9	++	+	+	87	9	85	95	82
11F1F-15-1H9	++	+	++	91	5	105	83	86
H9 (irr)	-	-	-	81	96	80	103	86

Figure 2: Binding and competition of VHH. Binding assays were performed starting from 1 μM, – indicates no binding, + some binding, ++ good binding and +++ excellent binding, based on at which concentrations the binding of the molecule was still detectable above certain levels. Competition is shown in percentages, in which 0% is no signal and 100% is the signal of the competitor when no VHH was added to it. B5 is an irrelevant monovalent VHH and H9 is an irrelevant bi-specific bihead (albeit with a 25GS linker).

tors, i.e/ sCD4, b12 and 17b. 2H10 is the only VHH that competes with the gp41 targeting mAb 2F5 and it slightly competes with 4E10 binding as well. None of the VHH abrogated the binding of 4E10 to gp41. The monovalent irrelevant VHH B5 did not compete with nor enhance the binding of any competitors.

Bi-specific biheads made of CD4bs targeting VHH J3/3E3 linked to gp41 targeting 2E7/11F1F were all still able to enhance 17b binding, albeit less profound for the ones that included J3. When J3 was linked to the 17b competitor 1H9, 17b binding was completely abrogated, however with 3E3 linked to 1H9, 17b was still able to bind to some extent. When J3 was linked to 1F10, the level of 17b binding was decreased by 50%, however the combination of 3E3 and 1F10 still doubled the level of 17b binding. The irrelevant bi-specific bihead H9 did not alter the binding of any competitor.

Bi-specific bihead Neutralization

Bi-specific biheads were constructed between the broadest VHH in combination with the best VHH that did not compete with them. All of these bi-specific biheads were screened for their ability to outperform the molar mix of their monovalent components (data not shown). Four bi-specific biheads were selected for further characterization. Table 2 shows the neutralization properties of these bi-specific biheads in combination with their individual components and the molar mix of both components. The factor of increase in potency between the molar mix of the individual components and the bi-specific bihead is indicated by "fold change". For the viral strains 92UG037 and Bal.26 no large

Pseudovirus:	92UG037	96ZM965	bal26	SVPC15
J3-15GS-11F1F	12,8	1,3	2,09	0,11
J3 +11F1F	19	100,5	8,9	22,5
J3	22	112	6,88	49,9
11F1F	>1000	>1000	>1000	>1000
Fold change	1,5	77,3	4,3	204,5
3E3-15GS-11F1F	2,45	0,1	0,54	0,32
3E3+11F1F	2,45	80	3,2	72
3E3	6,8	1200	2,7	290
11F1F	>1000	>1000	>1000	170
Fold change	1,0	800,0	5,9	225,0
J3-15GS-2E7	10	nt	1,3	0,02
J3+2E7	19	nt	5,7	28
J3	17	nt	5,6	87
2E7	>1000	nt		360
Fold change	1,9	tbc	4,4	1400,0
3E3-15GS-2E7	160	40	5,02	250
3E3+2E7	80	130	11	>1000
3E3	270	140	10	>1000
2E7	170	>1000	>1000	>1000
Fold change	0,5	3,3	2,2	>4

Table 2: Improvement of neutralization potency. The neutralization potencies of four bi-specific biheads compared to the molar mix of their individual components, against four different pseudoviruses. The increase in potency is marked by "fold change". For the completeness of the figure, the potency of the individual components of the bi-specific biheads are given as well. The IC_{50} values are given in nM. Nt means not tested and tbc, to be calculated.

A	Pseudovirus:	Du172	96ZM965
	J3	>1500	36,0
	1F10	116,7	166,7
	J3-15GS-1F10	101,4	5,3

B	Pseudovirus:	CH038	96ZM965
	3E3	>1500	14,7
	1F10	20,0	136,7
	3E3-15GS-1F10	189,2	6,8

Table 3: Improvement of neutralization breadth. a) The neutralization potencies of J3-15GS-1F10 compared with the potencies of J3 and 1F10 alone against the pseudoviruses Du172 and 96ZM965. b) The neutralization potencies of 3E3-15GS-1F10 compared with the potencies of 3E3 and 1F10 alone against the pseudoviruses CH038 and 96ZM965. IC_{50} values are given in nM.

increases in potency of bi-specific bihead neutralization was observed, unlike for 96ZM and SVPC15. 96ZM neutralization by 3E3-15GS-11F1F (3E3 linked to 11F1F with a GGGGSGGGGSGGGGS linker) is increased 800 fold when the two VHH are physically linked and SVPC15 neutralization by J3-15GS-2E7 is increased even by 1400 fold.

Table 3 shows the neutralization of either J3 or 3E3 linked to 1F10, in both cases the bi-specific bihead is capable of neutralizing a viral strain that is not neutralized by either J3 or 3E3. J3 does not neutralize Du172, IC_{50} >1.5 μM, whereas 1F10 neutralizes with an IC_{50} of 117 nM. Linked together, J3-15GS-1F10 neutralizes Du172 with an IC_{50} of 101 nM, indicating that the potency of 1F10 is not altered by its linkage to J3. Furthermore, the potency of the bi-specific bihead compared to that of either individual VHH was improved when the bi-specific bihead was tested against a strain that was neutralized by both J3 and 1F10, i.e. 96ZM965. Here the IC_{50} of J3 and 1F10 were 36 nM and 167 nM respectively whereas the bi-specific bihead had an IC_{50} of 5.3 nM. The combination of 3E3 and 1F10, was able to neutralize CH038, which 3E3 alone did not. However the IC_{50} of the bi-specific bihead was ten times higher than that of 1F10 alone, 189 nM compared to 20 nM. When the bi-specific bihead was tested against 96ZM965, the IC_{50} dropped slightly compared to either monovalent VHH, to 6.8 nM.

Modeling of 3E3 on gp120

3E3 contains four cysteines, which most probably make two sulphur bridges, C22-C92 and C50-C100a. All cysteines in 3E3 are completely conserved among all family members (data not shown). For modeling, an ideal template model would be a crystal structure with the same set of sulphur bridges. Unfortunately, there is no known crystal structure of a VHH with a C50-C100a bond. Therefore, we choose to make a model of 3E3 using our VHH D7 (2XA3)[125] as a template structure, using the program Modeller 9.9. Using the molecular building program Coot 0.6.2 for Windows[187], the CDR3 loop was bent in such a way that a cysteine bridge was formed.

Modeling the binding of an anti-CD4bs VHH to gp120 is far from straightforward. Firstly, gp120 is a very flexible molecule and anti-CD4bs antibodies have been shown to bind to a large variety of conformations of gp120. Furthermore, anti-CD4bs antibodies display many different kinds of interactions and a have a different angle of approach than

CD4. As a basis for the modeling we searched for similarities between anti-CD4bs antibodies and CD4 binding modes. The CD4bs on gp120 has two hotspots for CD4 binding, a hydrophobic cavity, the so-called Phe43CD4 cavity, in which Phe43 of CD4 binds, and the acidic residue Asp368 to which Arg59 of CD4 binds[191]. Structures of Fabs in complex with gp120 have been solved for the HIV-1 neutralizing anti-CD4bs antibodies b12, b13, F105, VRC01, VRC03, NIH45-46 and VRC-PG04[58, 78, 126, 179, 180, 189, 190]. Recently the crystal structures of the anti-CD4bs VHH A12 and J3 have been solved in complex with gp120 (unpublished results)[126]. The conformations of the bridging sheet of gp120 proteins in all different complexes differ from each other. In CD4 the V1/V2 loops forming the bridging sheet fold back on the gp120 structure, whereas in some antibody-gp120 complexes these loops dangle outwards or are not even visible, because of their high flexibility. The structures with the largest conformational changes of the V1/V2 loops with respect to the CD4 bound conformation, are those of the antibodies F105, A12, b13 and b12 (see table 1). Distances are between brackets). The antibodies VRC01, VRC03, NIH45-46, VRC-PG04 and J3 all bind to a gp120 conformation resembling that of the conformation observed in the gp120-CD4- 17b complex structures, which in fact resembles that of recently solved unliganded gp120 structures[192]. The neutralization breadths of F105, b13, b12 and A12 are ~3%, ~3%, ~40 and ~42%, respectively. In comparison, the neutralization breadths of VRC01, VRC03, NIH45-46, VRC-PG04 and J3 are ~94%, ~56%, ~94%, ~74% and ~96%. There seems to be a good correlation between the conformation of the bridging sheet and the neutralization breadth of the antibody. The closer the conformation of the bridging sheets resembles that of the CD4 bound conformation, the broader the neutralization seems to be. 3E3 is very broadly neutralizing, so it most likely binds gp120 in a CD4-bound resembling conformation. The fact that 3E3, like CD4, enhances 17b binding supports this notion.

Most antibodies do not stick an aromatic residue in the $Phe43_{CD4}$ cavity. As a matter of fact, the cavity is not present in most complexes due to large conformational changes of the bridging sheets. Only VRC03 and J3 have an aromatic residue in the $Phe43_{CD4}$ cavity. In NIH45-46 a glycine in present at the approximate position of the main chain of W54 of VRC03. In NIH45-46(G54W), for which no crystal structure is available yet, W54 most likely sticks in the $Phe43_{CD4}$ cavity. NIH45-46(G54W) displays a higher potency than NIH45-46, supporting the importance of such an interaction. I371 (or V371), which is one of the residues that delineates the entrance of the $Phe43_{CD4}$ cavity in gp120, is involved in hydrophobic interactions in nearly all anti-CD4bs antibody-gp120 complex structures.

In all anti-CD4bs antibodies and VHH, except for b12, at least one positively charged residue (a His in J3) makes a salt bridge with $Asp368_{gp120}$ (table 1). It is therefore likely that 3E3 also binds to $Asp368_{gp120}$ with a positively charged amino acid. Indeed 3E3 does not bind to D368R mutant gp120 (data not shown).

Based on the observations of the gp120-antibody complex structure (table 1), for the modeling of 3E3 on gp120 we assumed that 3E3 would bind to both hotspots of the

CD4bs. The gp120 structure of the complex with CD4 and 17b (PDB ID 2NXY)[58] was chosen as the starting model for gp120. We assumed that a positively charged amino acid of 3E3 should contact D368 of gp120, and that an aromatic amino acid of 3E3 sticks into the $Phe43_{CD4}$ cavity of gp120. 3E3 has one well exposed positively charged amino acid, i.e. R100d, and two aromatic residues in the CDR3, i.e. Y100 and F100f. Therefore, in HADDOCK two modeling runs using different restraints were performed. In one run we restrained the an interaction between R100d of 3E3 and D368 of gp120, and between Y100 of 3E3 and the $Phe43_{CD4}$ cavity. In the other run we restrained the interaction between R100d of 3E3 and D368 of gp120, and between F100f and the $Phe43_{CD4}$ cavity.

The best model in terms of HADDOCK scores had the Y100 in the $Phe43_{CD4}$ binding cavity and the arginine binding to D368 of gp120. In this model, as expected, the CDR2, which is three amino acids shorter than its germ line counterpart, interacts with gp120,

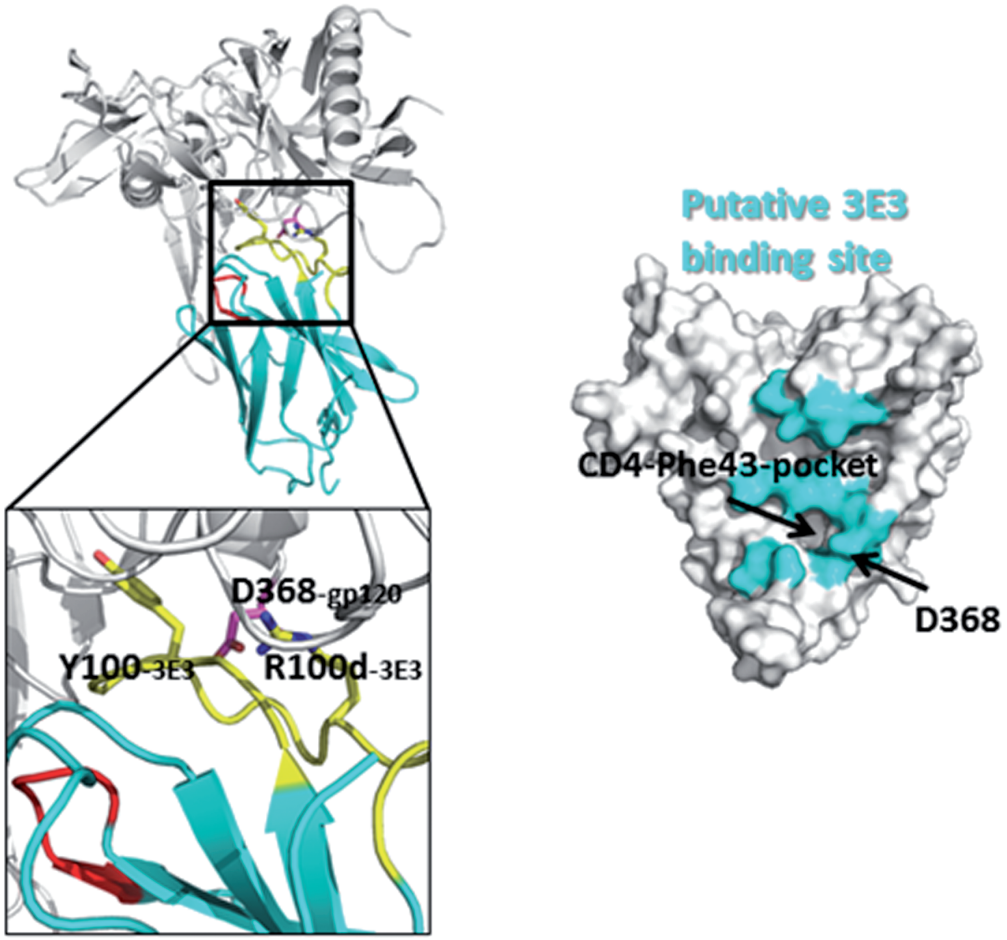

Figure 3: 3E3 model. On the left a model of the binding of VHH 3E3 toward gp120 (PBD ID 2NXY) [58] is shown. The zoom box highlights the three amino acids that are most crucial for 3E3 binding, R100d of 3E3 interacts with D368 of gp120 and Y100 of 3E3 is penetrating into the $Phe43_{CD4}$ cavity. On the right the model of the footprint of 3E3 on the gp120 molecule is shown in cyan.

and the epitope of 3E3 overlaps quite well with that of CD4 (figure 3). The model with the phenylalanine in the cavity had lower HADDOCK scores, the CDR2 hardly interacts with gp120 and the epitope overlaps much less accurately with that of CD4. 3E3 thus likely interacts with both hotspots in the CD4bs of gp120. In the best model, 3E3 binds to little residues outside the CD4bs. Only Phe27 of 3E3 interacts with Val275 and Asp276 of gp120, outside the CD4bs.

Discussion

Our previous studies yielded a large number of VHH that neutralize HIV-1[90, 142, 143, 186]. The two top VHH, J3 and 3E3 neutralize 96% and 80% of the viruses tested with a median IC_{50} of 0.9 and 0.7 μg/ml, respectively. These VHH are among the best HIV-1 neutralizing antibodies, but still not all viral strains could be neutralized by them and some strains were only neutralized by high concentrations of VHH. In the past the linkage of two VHH to form so called biheads often proved to increase the potency and/or breadth of the VHH[60, 61, 61]. Targeting two different epitopes is very beneficial as it lowers the chance of escape mutants as the virus would need to alter two epitopes to escape[144]. Furthermore it would likely increase the neutralization breadth, as it was shown by Doria-Rose *et al.* that for broadly neutralizing mAbs the potency and breadth of mixes targeting independent epitopes increase more than that of mixes targeting the same epitope[193]. Since we have a large number of VHH to choose from we decided to explore the possibility of using two different VHH in one bi-specific VHH. Therefore, we investigated whether our VHH were able to bind Env simultaneously.

Loss of signal in the competition assay may not only be caused by overlapping epitopes, but may also be caused by steric hindrance or more likely by conformational changes as the result of binding of one of the two VHH. The use of a large excess of competing VHH was chosen to minimize the effects that may result from large differences in affinity between the two tested VHH, because when a competing VHH releases from its epitope, the epitope becomes available for the labeled VHH to bind to it. However, when an excess of competitor is present, the chance that another competitor binds to the available epitope is higher, in contrast to the underrepresented labeled VHH. Steric hindrance and conformational changes brought upon binding of the competing VHH may give a false indication of competition. I.e. competition does not necessarily mean that epitopes of the two VHH are indeed overlapping, however, lack of competition does indicate that epitopes are not overlapping. The aim of this assay was to find compatible partners to generate bi-specific biheads, therefore, the cause of the observed competition is not important, but only the observation that VHH are able to bind simultaneously.

Binding of monovalent VHH to the Env proteins showed some interesting results. 2E7 and 1E2 were thought to bind the CD4bs, but they seem to target gp41 instead. 1F10 binds neither gp120IIIB nor gp41 (both from a clade B virus), whereas it does bind to gp140UG37 (trimeric, from a clade A virus). This may be explained by having an epitope

which includes both gp41 and gp120, or requiring a trimeric conformation of Env, i.e. having an epitope overlapping either two gp120 or gp41 molecules. Further it may be explained by the possibility that 1F10 does not recognize any clade B Env. The latter is in agreement with the observation that 1F10 does not neutralize clade B viruses[143]. Some of the bi-specific biheads show an increased affinity for their antigens compared to that of the monovalent VHH. For example, J3-15GS-2E7 binds gp120IIIB very well, even though 2E7 does not bind gp120IIIB and J3 binds to gp120IIIB only weakly. An explanation for this is that the conformational changes induced by J3 binding[126] to gp120IIIB may allow 2E7 to bind as well.

The lack of competition of 1B5, 1F10, 1E2, 1H9 and 2E7 with b12 and sCD4 was interesting as well, because these VHH were previously all selected for their competition with b12, whereas in the competition assay described here they did not seem to compete anymore. This may be due to the setup of this particular experiment, which differs from earlier setups. Previously, 1B5, 1F10, 1E2 and 2E7 were screened for b12 competition by coating b12 to the plate and subsequently detecting the binding of the VHH-gp120 complex. Here Env was coated and subsequently b12 was detected. Coating of Env may prevent the VHH from changing the Env's conformation upon binding, which would otherwise prevent b12 from binding. 1H9 was selected and screened on a mutant gp120 in which an additional S-S bridge was keeping the bridging sheets in a particular conformation, this may be why on non-mutated gp140UG37 1H9, 4D4 and 2B4F do not compete with b12. The fact that 3E3, J3 and 33E1 enhance the binding affinity of 17b by over 400%, indicates that their binding causes similar conformational changes as CD4 causes. Bi-specific biheads made up of a 17b competing as well as an enhancing VHH either do not compete very strongly with 17b or still enhance its binding affinity. This can be explained by the possibility that the VHH causing the enhancement, changes the conformation of gp120 in such a way that the other VHH, the ones competing with 17b, no longer sterically hinder 17b's binding. Furthermore, it may be that the conformational change lowers the affinity of the competing VHH to such extend that it is no longer able of competing with 17b. Of all VHH, 2H10 is the only VHH that had been shown to bind the MPER of gp41[61] and it was also the only one that competed with 2F5.

Based on the results of the cross-competition assay, four different epitopes were found and none of them seem to overlap with each other, except for 1F10, which may overlap partially with that of 1B5/1H9/2B4F. The competition experiment further identified an abundance of potential VHH pairs for making bi-specific biheads. The decision was made to continue with only those combinations that were either between the best VHH (percentage of neutralization) or VHH that could complement each other's neutralization breadths. When VHH J3 or 3E3 were linked to either 2E7 or 11F1F the neutralization potency of the molecules compared to the molar mix of the components showed an increase up to 1400 fold. Linkage of J3 or 3E3 to 1F10 showed neutralization of a viral strain not neutralized by either J3 or 3E3, which indicates that these bi-specific biheads may potentially have

a neutralization breath above the 96% of J3.

Since the competition assay showed a competition pattern for 3E3 similar to that of CD4bs binder J3, we decided to model the interaction of 3E3 with the CD4bs of gp120. A 3D model of a 3E3-gp120 complex was generated based on knowledge gained from 3D structures of other anti-CD4bs antibody-gp120 complexes and on CD4-gp120 complexes (table 1). There were indications that 3E3 may, similarly to CD4, also have two key interactions, i.e. with D368 and with the $Phe43_{CD4}$ cavity. During the modeling we restrained these two interactions. In the best model that was obtained R100d of 3E3 interacts with D368 of gp120 and Y100 binds in the $Phe43_{CD4}$ cavity of gp120 (figure 3). This model agrees with all experimental data of 3E3. Firstly, we expect 3E3 to bind within the CD4bs with no or very few amino acids binding outside the CD4bs, because of its large neutralization breadth. In the model only one amino acid of 3E3, namely Phe27, interacts with Val275 and Asp276 of gp120, outside the CD4bs. The side chain of Phe27 is probably flexible, so it may adjust its position when binding to viruses with other residues at positions 275 or 276, which is thus in agreement with the broad neutralization. Furthermore, the tip of the CDR2 interacts with the bridging sheet of gp120. If the CDR2 would be longer, there would be major steric hindrance, which would prevent binding of 3E3 to gp120. Binding of the CDR2 to gp120 is also expected, because 3E3 has an extraordinarily short CDR2 loop, which is three amino acids shorter than its germ line. Such short CDR2 loops are very rare. Only two of the 1012 VHH sequences present in our data base have a CDR2, which is three amino acids shorter than its germ line. Interestingly, J3 also has a deletion of three amino acids compared to its germ line, which has been shown to be essential for the functioning of J3. The model indicates that the short CDR2 is crucial for binding of 3E3 to gp120. Furthermore, the model shows that it is possible for 3E3 to bind to gp120 in the CD4 bound conformation. This agrees well with the observation that 3E3, like CD4, enhances 17b binding (figure 2).

Conclusion

This study describes the selection of a novel broadly neutralizing VHH, 3E3. Competition experiments combined with modeling revealed that 3E3 binds an epitope located almost exclusively within the CD4bs. The competition together with the binding assays gave some insights into the putative binding areas of our other VHH as well. Furthermore, this study shows that linkage between J3 and 3E3 and VHH that bind independent epitopes results in molecules with increased breadth and potency compared to the individual VHH. When applied in microbicides these molecules are potentially very potent. Such bi-heads, coupled to Fc are potentially also useful as therapeutics.

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Chapter 6

General conclusions

The aim of this thesis was to identify VHH that are both broad and potent HIV-1 neutralizers. These VHH should be active against the HIV-1 clades that are most prevalent in the developing countries, i.e. clade C, A and CRF02_AG. The VHH should target different epitopes on the virus so they can be used in combination with each other, to improve their combined breadth and potency[193] and to prevent the emergence of escape mutants[144].

In chapter 2 we described the selection and characterization of five families of VHH of which three are very broadly neutralizing. 2E7 was the broadest, neutralizing 81% of the tested viral strains from clades A, CRF02_AG, B, C and CRF07_BC. 1B5 was slightly less broad, neutralizing 69% of the strains spread over all tested clades. 1F10 neutralized 70%, but hardly any strains belonging to clades CRF02_AG and B. 1E2 was neutralizing only 23%, but the neutralized strains were spread over all tested clades. All of these VHH were eluted from gp140CN54 with sCD4 and screened for their ability to compete with b12.

In chapter 3, more broadly neutralizing VHH were identified, all targeting epitopes not related to the CD4bs. Two of these VHH, 1H9 and 2B4F, were thought to interact mainly with the co-receptor binding site as they competed with 17b and b12, but not sCD4. They were neutralizing around 50% of the viral strains, from CRF02_AG, B, CRF07_BC and C, but not A or CRF01_AE. The other VHH, 5B10D, 9B6B, 11F1B and 11F1F, targeted gp41. The broadest VHH was 11F1F, which is a family member of 2E7 and neutralized 76% of the strains tested, coming from all six different clades. 9B6B neutralized 65% from all clades except CRF01_AE.

The in chapters 2 and 3 identified VHH were the broadest known antibodies, derived from immunization, however it seemed like what they gained in breadth, they lost in potency. In chapter 4 we showed that not all VHH have to "choose" between potency and breadth as VHH J3 is capable of neutralizing 96 out of 100 viruses tested, with a median IC50 of 0.9 μg/ml. This VHH does not bind the D368R mutant of gp120[127], thus binds to the CD4bs. J3 most likely is very broad because it targets an epitope mainly within the CD4bs, which is not shielded by glycans or easily mutated, because the virus needs to be able to interact with CD4 in order to be infectious.

In chapter 5 we described another very broadly neutralizing VHH, 3E3, which neutralizes 80% of the tested viral strains. Competition experiments in combination with modeling revealed that 3E3 targets a similar epitope as J3 does, which in agreement with its exceptional neutralization breadth and potency. Furthermore, it was shown that our previously identified VHH (chapters 2-5, Forsman *et al.*[90] and Lutje Hulsik *et al.*[61]) target at least five different epitopes on Env, of which one (1F10) slightly interferes with the binding toward the epitope of 1H9/1B5. It was also shown that the physical linkage of two VHH, targeting different epitopes on Env, increases the breadth and potency of the molecule compared to either VHH separately.

The exact binding sites of many of the broadly neutralizing mAbs are known, because they have been crystalized in complex with a gp120 molecule. For our VHH A12 and J3 a co-crystal structure with gp120 has been solved in the lab of Peter Kwong[126]. This knowledge may help us to determine the binding areas of our other VHH, exemplified by the modeling of 3E3 described in chapter 5.

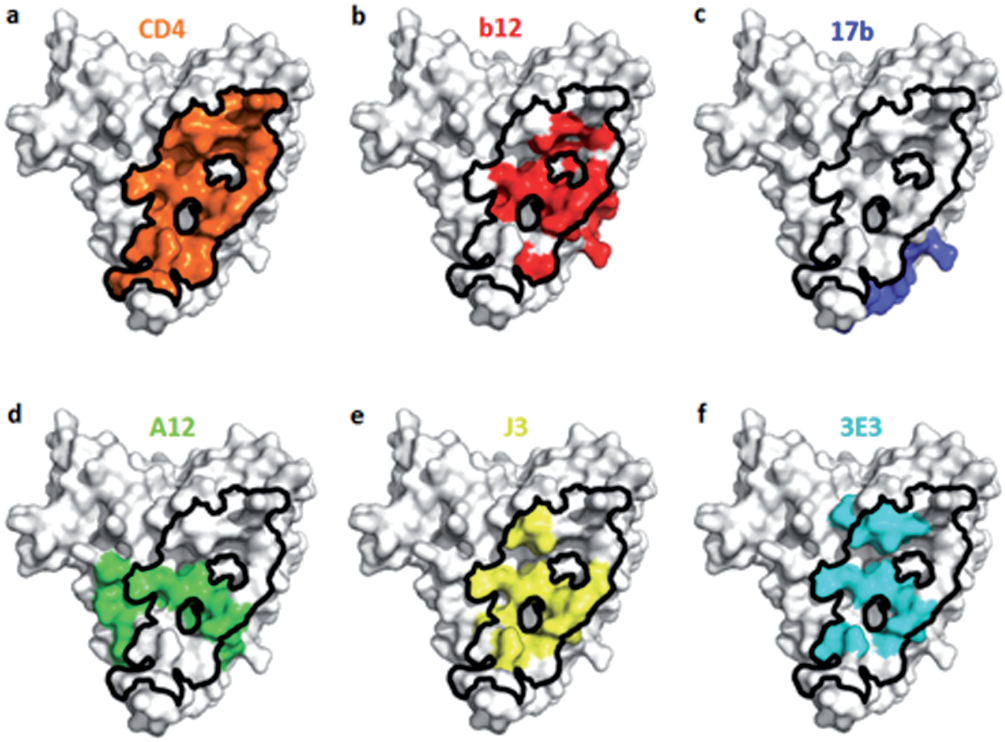

Figure 1: Footprints of ligands on gp120. The footprints of several gp120 ligands. The CD4bs is indicated with a thick black line. Note that binding of most antibodies changes the conformation of gp120 significantly so their actual binding area will be different, but for easier comparison all footprints are shown on the crystal structure of gp120 in complex with sCD4 and 17b (PDB ID: 2NXY)[58]. The footprint of sCD4 marked in orange (a)[58], b12 marked in red (PDB ID: 2NY7)[58] (b), 17b marked in blue (c)[58]. Much of the 17b binding site is not visible in this figure as it is underneath the molecule. The footprint of A12 is in green (d)[126], A12 binding pushes away the bridging sheets and binds underneath, which is not visible in this picture as the bridging sheets are in front of it, but most of the bottom part of the CD4bs outline is bound by A12 as well. The footprint of J3 is depicted in yellow (e)[126] and the putative footprint based on the model of 3E3 in cyan (f)[194].

Figure 1a shows the footprint of CD4 on the gp120 monomer, in b-f an outline of this footprint is marked by a thick black line. The footprints of two broadly neutralizing mAbs, b12 and 17b, are shown in figure 2b and c respectively. The footprints of three of our VHH are shown in figure 2d-f. These are the only VHH from which we are relatively sure where their exact binding epitope is on gp120.

For our other VHH it is less well know where their epitopes are on the envelope spike and thus an exact footprint cannot be given. For many of our VHH, binding and competition data provided insights into their epitopes, but for 1H9 we have some additional data. 1H9 was shown to compete with b12 and 17b. Because the epitopes of 17b and b12 have one amino acid in common, i.e. R419, therefore, 1H9 most likely binds around this amino acid. 1H9 does not compete with sCD4 and its binding is not altered by the pre-incubation of Env with sCD4[186]. This indicates that it is binding toward the co-receptor binding site of gp120, however not in the same sCD4 dependent manner as the co-receptors or 17b. This view is supported by preliminary alanine scan data which show that T388 and R419 are very important for the binding of 1H9 toward gp120. Furthermore, two escape mutants raised against 1B5 in which either P417 or R419 in gp120 were mutated, were tested for cross-resistance against 1H9. As expected they showed resistance to 1H9 as well[195]. The residues on gp120 which have been shown to be important for 1H9 binding are highlighted in purple in figure 2c.

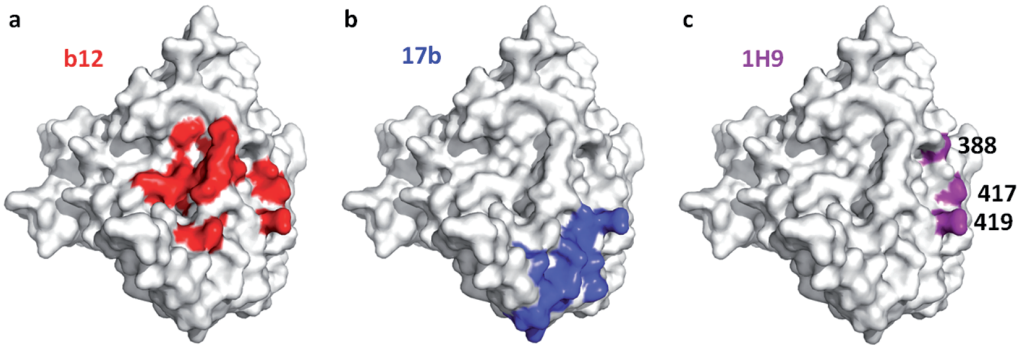

Figure 2: Putative 1H9 binding area. The gp120 monomer (PDB ID: 2NXY)[58] is tilted back and to the left compared to figure 1, both around 45°, in order to better visualize the b12, 17b and 1H9 binding areas. b12 (PDB ID: 2NX7)[58] is depicted in red (a), 17b in blue (b) and three amino acids crucial for 1H9 binding in purple.

Based on the information obtained from experiments performed in chapters 2 to 5 we have tried to get insight into the locations of the binding epitopes of the other VHH groups as well. Competition data as well as the preliminary data of escape mutant study mentioned above[195] indicate that 1B5 binds to an epitope similar to that of 1H9. The same holds true for 2B4F which is not only highly related to 1H9, but also shows similar binding and competition profiles to 1H9.

The binding of VHH 1F10 is somewhat hampered by the binding of 1B5/1H9/2B4F and the other way around. Furthermore, it was shown that it is competing well with 17b. 1F10 was shown to bind trimeric gp140UG37 and gp140CN54 well, but not gp120IIB or gp41[194], which may indicate that it targets an epitope that covers more than one gp120 molecule. Therefore, it is reasonable to suggest that 1F10 is binding approximately at the dark blue oval indicated in figure 3. Note that 1F10 is not neutralizing clade B viruses very well, which may also be an explanation for the absence of binding to gp120IIIB, and for

the very weak binding to gp120Yu-2_{DS2}[143]. Future testing of the binding of 1F10 to trimeric Env of a clade B or to gp120 of a clade A or C may provide further insight to the area targeted by 1F10.

1E2 and 2E7 have been shown to bind gp140UG37, gp140CN54[143], gp120CN54 (data not shown) and gp41[194]. They are not competing with b12, 17b, sCD4, 2F5 or 4E10 in the setup used in chapter 5. Therefore we propose that the epitope of 1E2 and 2E7 are located approximately at the red circle of figure 3. The same holds true for the 2E7 family member, 11F1F. VHH 5B10D, 9B6B and 11F1B all bind gp41 exclusively, and do not compete with any mAbs or with sCD4 tested in chapter 5 either. We propose their binding epitope to be around the brown circle indicated in figure 3. Our previously identified VHH 2H10 binds roughly the same epitope as 2F5[61], its epitope is highlighted by a light blue circle in figure 3.

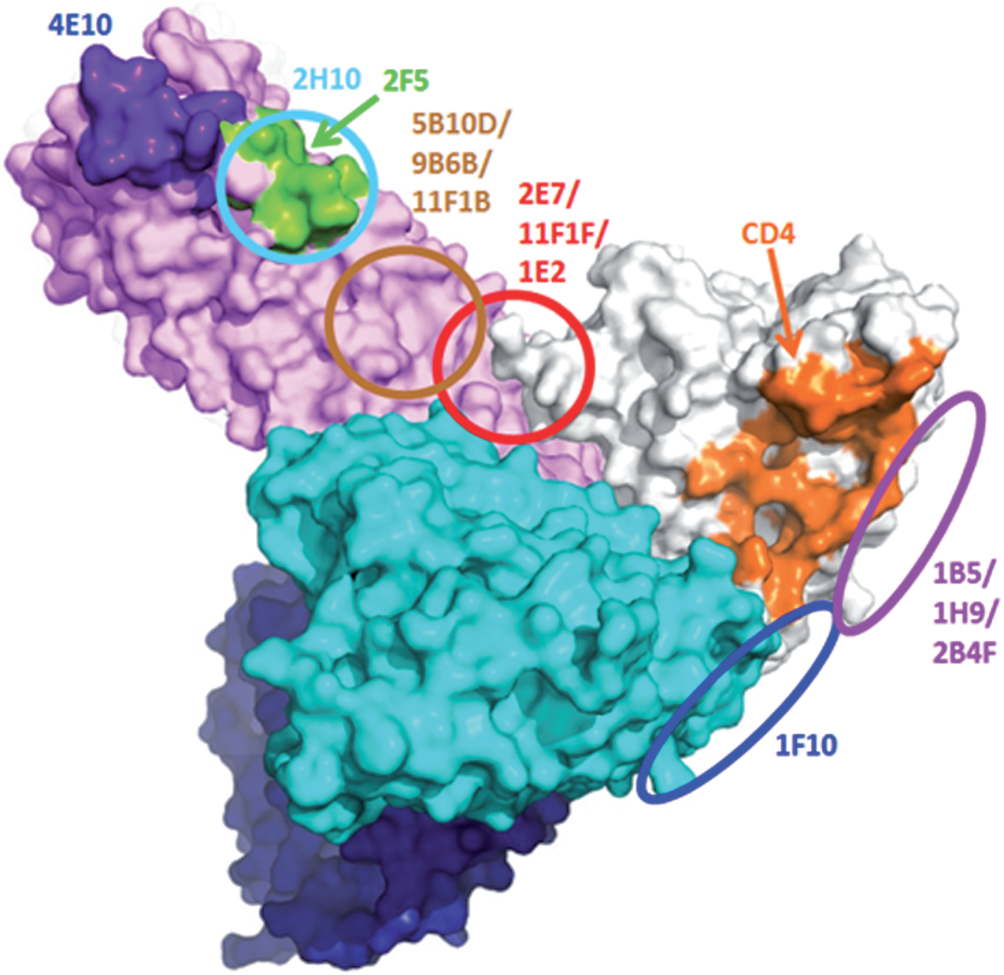

Figure 3: Putative binding sites of our VHH. Schematic representation of the envelope spike (PDB IDs: 3DNN for the gp120 trimer, 2X7R for the gp41 hexamer). The exact binding site of sCD4 is highlighted in orange, that of 4E10 in blue (top left) and 2F5 in green. The putative binding area of 1B5/1H9/2B4F is indicated with a purple oval, 1F10 with a blue oval, 2E7/11F1F/1E2 with a red circle, 5B10D/9B6B/11F1B with a brown circle and 2H10[61] with a cyan circle.

In the search for a successful treatment against HIV-1 there is a need for products that do not elicit cross-resistance with the currently used drugs[196]. The here described VHH have been proven to target different, mostly non-related epitopes. The risk of cross resistance among these VHH is therefore likely to be low. As these VHH are entry inhibitors, the chance of cross resistance toward other anti-HIV drug classes, targeting different HIV proteins, is negligible as well. The here described VHH could even be used in combination with these other classes of drugs (reverse transcriptase inhibitors, intergrase inhibitors and protease inhibitors). Since the more spots on HIV-1 are being targeted simultaneously, the less likely the virus is to escape all these drugs. This would reduce the risk of resistance even more.

At the moment, there is only one fusion inhibitor approved that targets Env, Fuzeon also known as Enfuvirtide or T20[197]. This peptide drug binds to the N terminal heptad repeat region of gp41, and may thus compete with VHH that also target this region, which might be 2E7/11F1F/1E2 or 5B10D/9B6B/11F1B. For 2H10 the combination with Fuzeon would not be recommended as Fuzeon is a gp41 derived peptide and contains the 2H10 epitope. Therefore 2H10 and Fuzeon would be neutralizing each other's function.

Overall our VHH are very good candidates for inclusion in microbicides or treatment cocktails. However, whatever drug/treatment/preventative method we will be able to offer, it will always have to be given in combination with education. HIV patients should feel encouraged to seek treatment and not feel ashamed. The position of HIV patients in society should improve, and the taboo should be broken. It is important to keep in mind that if people do not use our products for whatever reason, it will fail, regardless of how potent or broad it is.

In conclusion, a number of VHH described in this thesis are extremely broad and potent neutralizers of HIV-1. The biheads obtained by the linkage of these VHH to VHH targeting different epitopes on Env are even more potent and broad in their neutralization. Combinations of these mono- or bi-headed VHH are very good candidates for use as prophylactic or treatment of HIV-1. Further studies should prove whether they perform as well in *in vivo* models as they do in the *in vitro* studies.

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Samenvatting

Al meer dan 30 jaar is Humane Immunodeficiëntie Virus type 1 (HIV-1) één van de grootste gezondheidsproblemen van deze wereld. Het merendeel van alle met HIV geïnfecteerden (67%) leeft in de Afrikaanse landen ten zuiden van de Sahara. De meesten van hen heeft geen toegang tot de nodige medicatie en overlijd uiteindelijk aan een AIDS gerelateerde ziekte. Hier in de westerse wereld zijn er inmiddels redelijk goede behandelmethoden en is een HIV infectie te zien als een chronische ziekte. Helaas hebben de meeste medicijnen veel bijwerkingen en is het strikte regime van het innemen van de medicatie erg invasief in het leven van de patiënt. Kortom, het beste zou zijn om te kunnen voorkomen dat mensen besmet raken met HIV.

De meest voor de hand liggende methode om infectie te voorkomen is door middel van een vaccin. Hierbij word een onderdeel van de ziekteverwekker toegediend aan het lichaam zodat het immuunsysteem hier een reactie op kan voorbereiden. Zodra het lichaam dan in aanraking komt met de infectieuze variant van deze ziekteverwekker, kan het gelijk reageren en word men doorgaans niet ziek. Vaccins worden al veelvuldig gebruikt tegen bijvoorbeeld rode hond, mazelen en de bof. Helaas is het tot nu toe nog niet gelukt om een vaccin te maken dat effectief is tegen de vele verscheidene varianten van HIV-1.

Een andere mogelijkheid om HIV besmetting te voorkomen is het gebruik van een microbicide. Een microbicide is een stofje wat de infectiviteit van microben, zoals bacteriën en virussen, verminderd. Het doel is om hun infectiviteit dusdanig te verminderen, dat het immuunsysteem de rest kan uitschakelen.

In deze thesis word een speciaal type antilichaam beschreven afkomstig van kameelachtigen. Dit antilichaam mist een onderdeel ten opzichte van de "normale" antilichamen waardoor het gedeelte waarmee het aan zijn antigen bind een stuk kleiner is. Dit antigen bindende gedeelte word een VHH genoemd en kan onafhankelijk van de rest van het antilichaam gebruikt worden, waardoor ze nog kleiner worden. Deze VHHs zijn over het algemeen zeer stabiel en makkelijk, en dus ook goedkoop, te produceren.

Het vinden van goede VHHs tegen HIV kan het onderzoek naar HIV op twee manieren ondersteunen. Ten eerste zouden deze moleculen op zichzelf een goed ingrediënt van een microbicide kunnen vormen. Ten tweede kunnen de bindingsplaatsen van deze VHHs een aanwijzing geven welke stukken van HIV gevoelig zijn voor neutralisatie. Dit is belangrijk voor de ontwikkeling van een werkend vaccin.

Door deze hele thesis heen worden VHHs gebruikt die afkomstig zijn van twee lama's (8 en 9). Deze lama's zijn langdurig gevaccineerd met twee verschillende HIV envelop eiwitten afkomstig uit Afrika.

Hoofdstuk 2 beschrijft de eerste selecties die met de VHHs van lama 8 en 9 uitgevoerd zijn. Gedurende deze selectie zijn de VHHs als het ware "losgeweekt" van een HIV envelop eiwit door middel van CD4. CD4 is de receptor waarmee HIV aan zijn target cel bind. Het idee hierachter is dat je voornamelijk VHHs krijgt die aan de CD4 bindingsplaats van HIV binden. Theoretisch zouden deze VHHs dus de binding van HIV aan CD4 moeten kunnen voorkomen. Van de zes geïdentificeerde VHH families, zijn er vijf die HIV kunnen neutraliseren. De beste VHH, 2E7, neutraliseert 21 van de 26 geteste virussen, wat een verdubbeling is ten opzichte van de benchmark van die tijd (b12 met een neutralisatie van 41%). Helaas zijn deze VHHs niet erg potent en is er dus relatief veel VHH nodig om de

virussen te kunnen neutraliseren.

De selecties die in hoofdstuk 3 beschreven zijn richten zich op alle gedeelten van het HIV envelop eiwit behalve de CD4 bindingsplaats. De envelop eiwitten die in deze selecties gebruikt zijn hebben CD4 gebonden waardoor deze plek al bezet is en de VHHs hier niet kunnen binden. De focus ligt voornamelijk de co-receptor bindingsplaats van het envelop eiwit. Hierom is er gebruik gemaakt van een antilichaam dat de co-receptor bindingsplaats herkent (17b) om de VHHs los te weken. Daarnaast is er ook een hoge pH gebruikt om alle andere VHHs los te maken die deze CD4 gebonden eiwitten herkennen. Verder is er een ook nog een gemuteerde variant van het envelop eiwit gebruikt. Er zijn twee neutraliserende VHHs tegen de co-receptor zijde gevonden en vier die de gp41 stam van het envelop eiwit herkennen.

In hoofdstuk 4 word een andere methode van het screenen van VHHs beschreven, waarbij niet eerst een selectie op binding word uitgevoerd. Goede binding van een VHH staat namelijk niet gelijk aan goede neutralisatie, aangezien de plaats van binding net zo belangrijk is. Daarnaast kan het voorkomen dat een VHH een eiwit dat op een plaat gecoat is niet herkent, maar wel het intacte virus. Een directe screening op neutralisatie heeft dus als voordeel dat je geen neutraliserende VHHs misloopt vanwege hun slecte binding. Het nadeel is echter dat je ook zeer veel niet relevante VHHs moet screenen, die er normaalgesproken bij de selectie al tussen uit worden gehaald. Door de komst van betere "high troughput" assays werd het mogelijk om duizenden VHHs te screenen, en zo werd VHH J3 gevonden. J3 bind aan een zo goed als identieke plaats als dat CD4 bind, neutraliseert 96 van de 100 geteste virussen en is daarnaast ook zeer potent.

In hoofdstuk 5 word een nieuwe VHH, 3E3, beschreven die net als J3 aan de CD4 bindingsplaats van het HIV envelop eiwit bind. 3E3 is iets minder breed neutraliserend als J3, maar met 80% toch nog steeds zeer goed en daarnaast ook zeer potent. Omdat uit eerder onderzoek is gebleken dat de breedte en potentie van VHHs sterk verbeterd kan worden als ze aan elkaar gelinkt worden is er een competitie experiment uitgevoerd om te bepalen welke VHHs tegelijkertijd aan HIV kunnen binden. Het fysiek linken van een partner VHH aan zowel J3 als 3E3 heeft moleculen opgeleverd die beter waren dan de individuele VHHs. 2E7 gelinkt aan J3 is wel 1400 keer potenter dan de mix van 2E7 met J3 terwijl 1F10 gelinkt aan J3 in staat is een virus te neutraliseren dat J3 alleen niet neutraliseert.

In totaliteit lijken de VHHs die hier beschreven zijn op 4 onafhankelijke plaatsen op het envelop eiwit van HIV te binden. Deze gebieden kunnen belangrijk zijn voor de ontwikkeling van een vaccin tegen HIV, omdat het aangeeft waar kennelijk de zwakkere plekken van het virus zitten. Het linken van VHHs die aan de verschillende gebieden binden zorgt ervoor dat de breedte en of de potentie van de VHHs stijgt. De hier beschreven moleculen zouden ook zeer goede ingrediënten zijn in microbiciden tegen HIV. Op dit moment word J3 dan ook geproduceerd voor een Makaken studie.

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Bazinga, here we are... The last piece of my booklet that I'm writing and most probably the first part that you are reading. Like Herman Finkers already mentioned in his version of Macbeth "Hoewel het slot van het stuk aan het begin van het verhaal zat, is pas aan het einde de kop eraf"... I'll try my best not to forget anyone, but if I do: Sorry!

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Thank you all!!!

Sincerely,

Nika

Curriculum Vitae

Nika Strokappe was born on Thursday August 16th 1984 in Apeldoorn, the Netherlands. During high school she started working as a volunteer in a youth center in Beekbergen where she did some planning, bartending, cashiering and later on also event planning. Helping bands like Normaal as a local crew member got her interested in light techniques, which became a side job for a couple of years. After finishing high school at the Heemgaard in Apeldoorn she started a bachelor in Biology at Utrecht University in 2003. After a broadly oriented first year, her focus shifted toward molecular cell biology, immunology and virology. After finishing her bachelor in 2006 she started the master science teacher education. A mini-internship on a high school confirmed her interest in teaching biology. During this master a six month research internship was obligatory as well and thus she contacted Theo Verrips, as his group was working with the tiny antibodies named VHH. The internship was about identifying VHH against gp41, which is part of the HIV-1 envelope protein. This internship regained her interested in research and finally it was elongated to nine months after which she switched masters to bio molecular sciences, which she finished in 2008. She started her Ph.D. in 2008 as well in the group of Theo Verrips with quest of finding broadly neutralizing VHH against HIV as the main subject. Further she worked on identifying VHH against Rotavirus and bacterial toxins as well. After finishing the practical part of her Ph.D. she started a course in Dutch sign language. At the moment she is working as scientist at QVQ BV, a startup company working with VHH.

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