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Capture of SHIV particles by recombinant dimeric IgA specific to the immunodominant region of gp41

MASTER'S THESIS

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1 Introduction

1.1 The origins of HIV-1 & HIV-2 and the rising of an epidemic

More than 30 years after its discovery in humans, over 30 million people are currently globally living with HIV, and since 2000 about 25 million people have died of acquired immunodeficiency syndrome (AIDS)-related illnesses (www.unaids.org).

AIDS of humans is caused by two lentiviruses, human immunodeficiency virus type 1 (HIV-1) and 2. HIV-1 is divided up into groups M, N, O and P, whereas HIV-2 is composed of groups A to H. HIV-1 group M, termed main group, is responsible for the AIDS epidemic. Phylogenetic analysis showed the pandemic HIV-1 to have first emerged in colonial West Africa, around the area of Kinshasa (Leopoldville). The global spread of HIV-1 involved several diversifications, giving rise to the nowadays-existing nine subtypes (A-D, F-H, J, K). Moreover, also circulating recombinant forms have been described that arise when multiple subtypes infected the same population (Taylor et al. 2008).

According to Sharp and Hahn (2011), both HIVs are "the result of multiple cross-species transmissions of simian immunodeficiency viruses (SIVs) naturally infecting African primates". They further describe extensively the origin of the simian relatives of HIV-1 and HIV-2 as well as of the AIDS pandemic.

Old world monkeys are the natural hosts of more than 40 SIVs. Certain SIVs, such as SIV_{cpz} and SIV_{smm}, crossed the human species barrier during human-ape encounters in west central Africa, most likely in the context of bushmeat hunting. Phylogenetic analysis revealed the close genetic relationship of HIV-1 groups M and N to SIV_{cpz}. Common chimpanzees (*Pan troglodytes*) are the natural SIV_{cpz} reservoir, indicating a chimpanzee origin of HIV-1. Chimpanzees in turn are likely to have acquired SIV from other monkey species in the context of predation.

Group P is likely to have originated from SIV_{gor}, a SIV lineage found in wild-living gorillas. The source of HIV-1 group O still remains unknown.

Cross-species jump of SIV_{smm} gave rise to HIV-2 in humans and SIV_{mac} in macaques whereas SIV_{smm} is the corresponding SIV lineage in the sooty mangabeys. Fig. 1 gives an overview about the origins of human AIDS viruses.

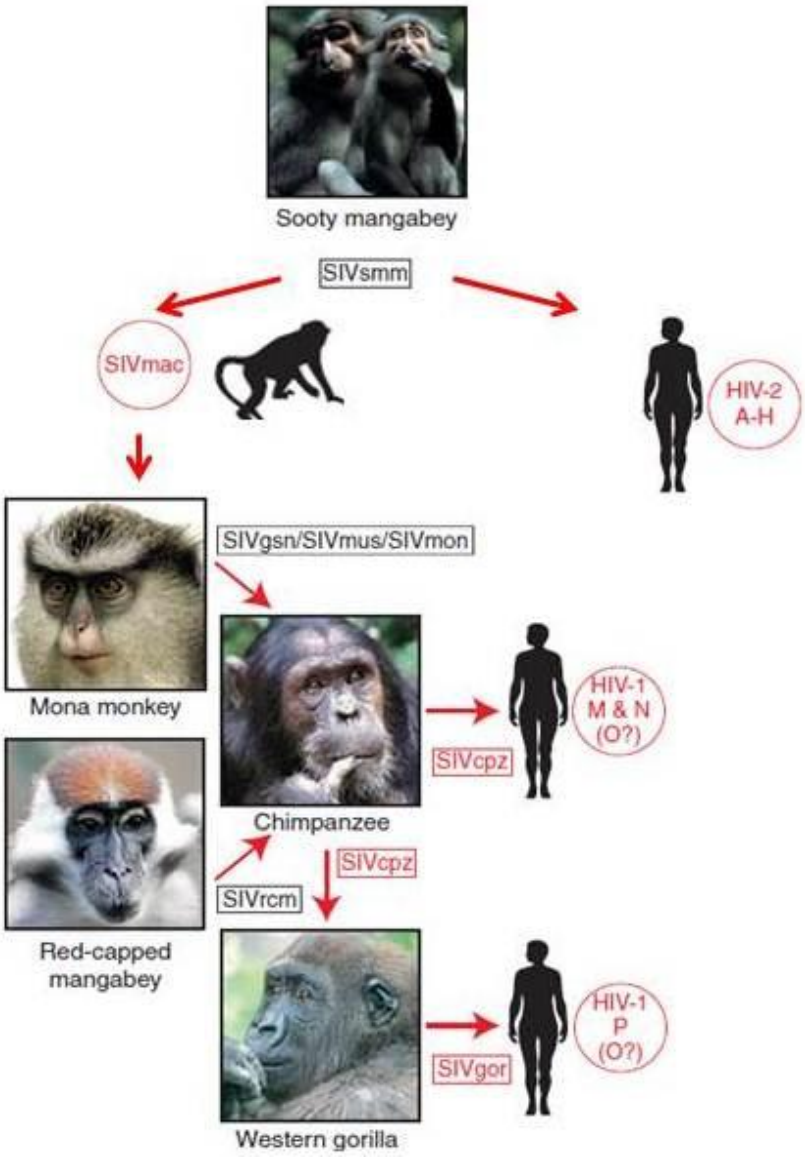


Fig. 1. Origin of human AIDS viruses

Several SIVs, such as SIV_{smm} and SIV_{cpz} have crossed the species barrier. These cross-species transmission events, highlighted in red, lead to the creation of HIV-1 and HIV-2. The four subgroups of HIV-1 have originated from independent transmission events. For further details, please consult the section above (modified after Sharp and Hahn 2011).

1.2 The search for a suitable animal model in HIV vaccine research

The lack of a suitable animal model recapitulating all features of human HIV infection still poses the biggest limitation on HIV vaccine development. As far as primates are concerned, research "has focused on the use of SIV or recombinant simian-human immunodeficiency virus (SHIV)". Hatziioannou and Evans (2012) described the feature of the distinct models and pointed out the challenges in finding a suitable AIDS animal model:

More than 40 different SIVs exist, each one endemically infecting their unique African primate species. However, these natural SIV hosts are not considered for HIV research because they do not develop disease as a result of infection.

In contrast, Asian monkeys, if infected with certain SIV strains, develop disease that closely resembles HIV-1 infection in humans such as high viral loads, $CD4^+$ T-cell depletion and opportunistic infections. Three Asian macaque species are commonly used as AIDS animal models: the rhesus macaque (*Macaca mulatta*), the pig-tailed macaque (*Macaca nemestrina*) and the cynomolgus macaque (*Macaca fascicularis*). The two most common SIV challenge strains were originally isolated from rhesus monkeys (RMs) of Indian origin and are well adapted in these animals for efficient replication: SIV_{mac251} and SIV_{mac239}. SIV_{mac251} is widely used as an uncloned challenge virus (Daniel et al. 1985), SIV_{mac239} is the first consistently pathogenic molecular SIV clone generated (Kestler et al. 1990).

All the SIV strains used in pathogenicity models were isolated after experimental or accidental macaque infections with SIV_{smm}. Consequently, these so generated SIV strains differ from HIV-1, with regard to their accessory proteins (Fig. 2). For instance, besides differences in co-receptor usage (Riddick et al. 2010), SIV is not sensitive to HIV-1 inhibitory drugs such as HIV-1 protease, reverse transcriptase or integrase inhibitors. Consequently, research focused on the development of recombinant viruses by substituting the gene of interest of an infectious SIV clone with its HIV counterpart (chimeric virions, designated SHIV). These recombinants have been instrumental in the evaluation of anti-HIV vaccines and the understanding of HIV gene evolution.

The insertion of HIV-1 *env* inserted into a SIV_{mac239} backbone, gave rise to SHIV (Fig. 2) and allowed for testing of drugs and vaccines targeted against the envelope glycoprotein (Env) of HIV-1.

The RT-SHIV, reverse transcriptase-SHIV, was created by replacing the SIV polymerase gene with the corresponding HIV-1 gene. Unlike parental SIVs, RT-SHIVs are sensitive to non-nucleoside reverse transcriptase inhibitors (Uberla et al. 1995).

HIV-1 replication in RM cells is blocked by cell endogenous TRIM5 and APOBEC3 proteins (Shibata et al. 1995). This problem was overcome by engineering resistance to TRIM5 and APOBEC3 proteins and creating a simian tropic human immunodeficiency virus (stHIV). Depending on the species, two distinct versions of stHIV exist:

Efficient replication in RM lymphocytes was achieved by substituting the *vif* gene and the sequence encoding for the capsid of HIV-1 with their SIV_{mac} counterparts (Hatziioannou et al. 2006, Kamada et al. 2006).

In case of pigtail macaques, replacement of the HIV-1 *vif* gene was sufficient (Hatziioannou et al. 2009) because the TRIMCyp protein of pigtail macaques does not inhibit HIV-1 replication (Liao et al. 2007).

Fig. 2 gives an overview of the genomic organization of HIV-1 and its modified versions.

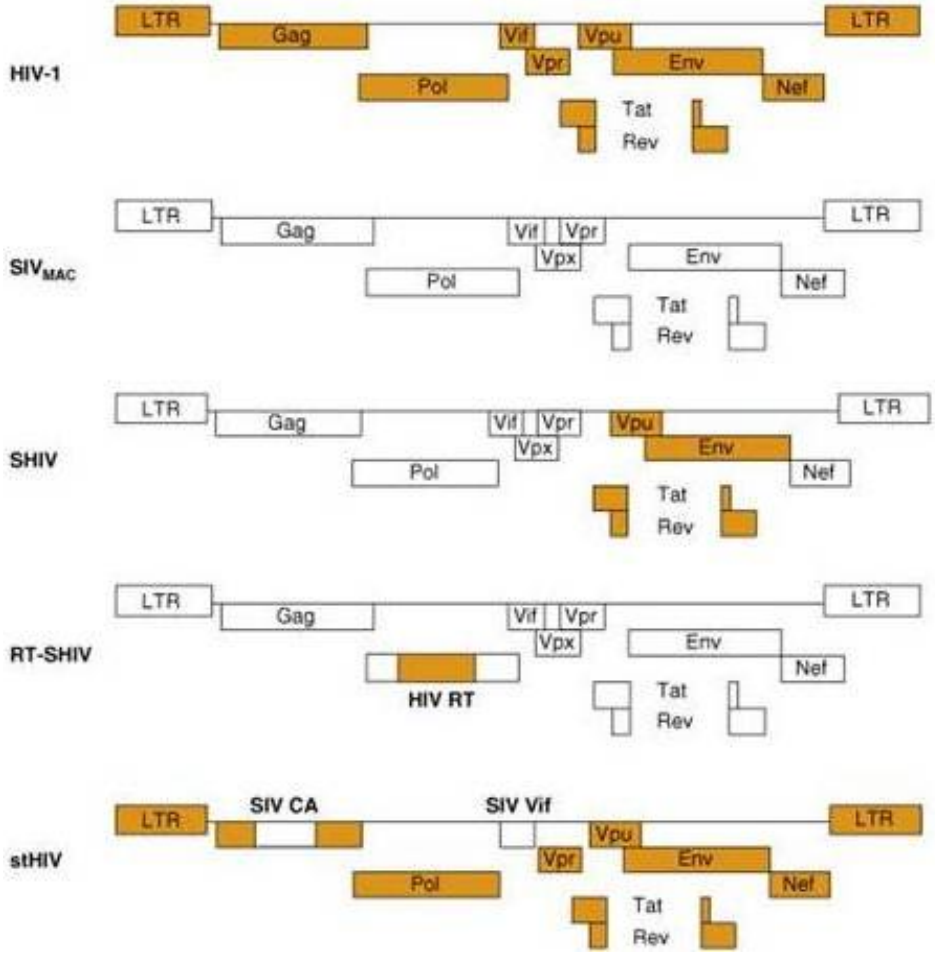


Fig. 2. Genomic organization of HIV-1 and its recombinant forms

The recombinant viruses mostly differ with regard to their accessory proteins and envelope proteins (Ambrose et al. 2007). For further details please refer to the text.

A number of SHIVs encoding Env of different clades have been generated so far, reflecting the diversity of HIV-1.

The more difficult-to-neutralize tier 2 viruses exhibit neutralization sensitivity profiles of typical primary HIV isolates, neutralization of the latter would better reflect the actual situation. The development of vaccines eliciting neutralizing antibody (nAb) responses against tier 2 viruses at sufficiently high titers however imposes a major challenge on HIV vaccine research (Siddappa et al. 2010). In this context, Siddappa et al. (2010) suggested a stepwise approach: Once protection against easy-to-neutralize tier 1 SHIVs was achieved, optimization of immunogens to induce protection against tier 2 viruses should be pursued. Following this concept, tier 1 and tier 2 are needed for vaccine design. Based on the R5 clade C SHIV of Song et al. (2006), SHIV-1157ipd3N4, its tier 1 counterpart was cloned: The HIV-1 clade C *env* of the "early" recently transmitted tier 1 SHIV-1157ip, derived from a recently infected Zambian infant's primary R5 HIV-C, was inserted into the backbone of tier 2 SHIV-1157ipd3N4 encoding a ''late'' form of the same Env, which had evolved in a SHIV-infected RM with AIDS. The so created chimera was termed SHIV-1157ipEL, EL standing for early late. The final SHIV-1157ipEL-p (i for infant origin, p for passage) chimera was derived by rapid passage through four RM for adaptation and has a tier 1 neutralization phenotype.

The HIV epidemic is dominated worldwide by HIV-1 clade C (Hemelaar 2012). Moreover, most primary HIV-1 isolates use CCR5 as their co-receptor (Dragic et al. 1996) and are therefore termed R5-tropic isolates. For better reflection of the genetic diversity of the epidemic, Song et al. (2006) created a pathogenic R5 SHIV encoding an HIV clade C Env. The Env came from a 6-month-old Zambian infant born to an HIV positive mother. The final construct was found to be a tier 2 virus and termed SHIV-1157ipd3N4: "i" refers to the infant origin, "p" to a serial passage history in RM (monkey adaptation) and "d" indicates virus reisolation from an infected animal with AIDS (defined by persistent depletion of $CD4^+$ cells to <200cells/µl). Viral replication was further accelerated by introduction of one additional NF-κB site in the 3'LTR ("N4").

To test the neutralization sensitivity of various HIV isolates, heterologous clade C tier 2 SHIV was cloned by Siddappa et al. (2009). The pediatric HIV-C *env* gene isolated from a 2-month-old Zambian infant, who died within 1 year of birth, was inserted into the backbone of SHIV1157-ipd3N4. The so generated molecular clone, SHIV-2873Ni, was serially passaged through five RMs, to obtain the adapted biological isolate SHIV-2873Nip.

HIV-1 clade A accounts for the majority of circulating strains in Kenya (Dowling et al. 2002). To be biologically relevant, the Ruprecht lab constructed tier 2 SHIV-KNH1144, a chimera encoding Env of the primary isolate HIV-A KNH1144 from Kenya. Infectious KNH1144 *env* was inserted into the proviral backbone of SHIV-1157ipd3N4 by swapping the *env* genes (Zhou et al. submitted).

Clade B tier 2 SHIV-SF162p3 is another recombinant virus of the SHIV family. Luciw et al. (1995) replaced the *tat*, *ref* and *env* genes of SIV_{mac239} with the corresponding genetic region of the non-pathogenic, macrophage-tropic, and CCR5-using primary $HIV\text{-}1_{SF162}$ strain to derive $SHIV_{SF162}$. Upon rapid serial intravenous passage of the virus, a variant designated tier 2 SHIV-SF162p3 was derived that maintained CCR5 co-receptor usage but exhibited enhanced pathogenicity (Harouse et al. 1999).

1.3 Immunoglobulin A: Structure and Variation

Immunoglobulin A (IgA) is the major antibody class present in the mucosal secretions of most mammals. It plays a pivotal role in protecting the mucosal epithelial barrier from invading pathogens. Variations in gene number, allotypes and molecular forms have been described (Woof and Kerr 2004). IgA molecules are expressed in many mammalian species of which most encode only one IgA heavy constant region (Cα) gene (Vaerman et al. 1969): Common species like mouse or dog express only one subtype of IgA. In contrast, the genome of hominoid primates (Chimpanzees, Gorillas, Gibbons and humans) encodes for two distinct Cα, arisen through gene duplication. This gives rise to the two existing subclasses IgA1 and IgA2. A notable exception is the rabbit with 13 Cα genes (Zhou and Ruprecht 2014).

Two allotypes of the human IgA2 subtype exist: IgA2 (m1) and IgA2 (m2). IgA2 (m2) (and IgA1) have disulfide bridges connecting heavy chain (HC) and light chain (LC) whereas the other allotype, IgA2 (m1) has not (Woof and Russell 2011).

Despite considerable sequence similarity, the two human IgA1 and IgA2 differ in their sterical arrangement and glycosylation pattern. Dimeric IgA1 (dIgA1) has a longer hinge region, resulting in a 16 ± 3 nm distance between two Fab fragments (Bonner et al. 2008) compared to 10 ± 2nm in dIgA2 (Bonner et al. 2009).The elongated 19 amino acid long hinge region of dIgA1 which is responsible for the T-like shape, makes IgA1 molecules more susceptible to bacterial proteases (Senior and Woof 2005). IgA1 molecules bear O-linked oligosaccharides on their surface (Mattu et al. 1998; Royle et al. 2003) whereas the IgA2 subclass lacks glycosylation (Tomana et al. 1976). All these structural differences between IgA1 and IgA2 antibodies are likely to result in different biological activities (Zhou and Ruprecht 2014).

In humans, serum IgA is mainly composed of monomeric forms comprising ~ 90 % IgA1 and 10 % IgA2 (Woof and Russell 2011). The ration between IgA1 and IgA2 varies among the human secretory effector sites, reaching over 90 % in nasal mucosa (Brandtzaeg and Johansen 2005). The various forms of IgA expressed in humans are depicted in Fig. 3 and 4.

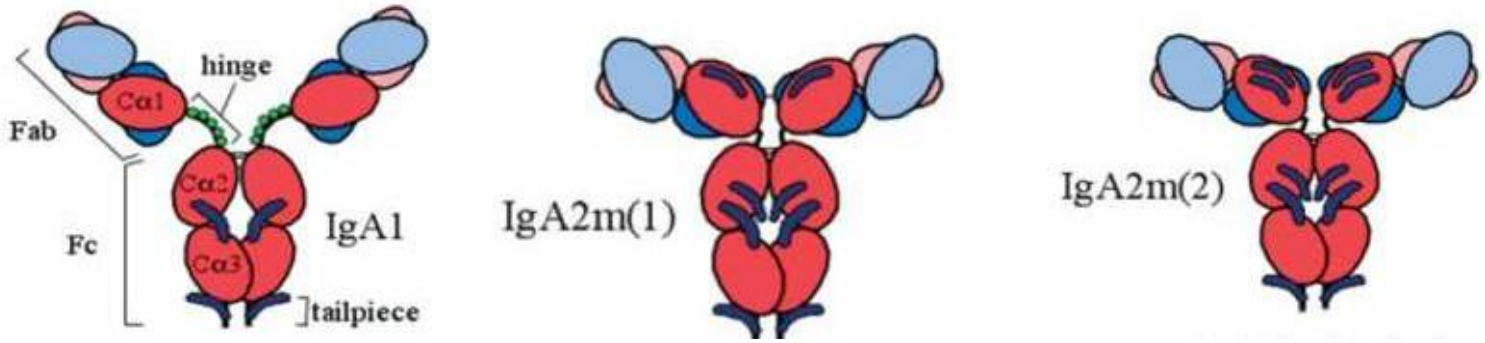


Fig. 3. Isotypes and Allotypes of IgA

The various isotypes and allotypes of IgA are shown: IgA1, IgA2m(1) and IgA2m(2). Each molecule is composed of two HC and two LC. Each HC is made up of four subdomains: the variable region of the HC (VH, pink) and the constant regions Cα1, Cα2 and Cα3 (red). The LCs are composed of only two subdomains, the variable region of the LC (VL, pale blue) and its constant region (CL, mid-blue). In IgA1 and IgA2m(2), disulfide bridges occur between the HC and LC. In the other allotype of IgA2, known as IgA2m(1), there are generally no HC – LC disulfide bonds but instead there is a disulfide bridge between the LC. *O*-linked sugars (on the IgA1 hinge) are depicted as green circles, while *N*-linked oligosaccharides are shown in dark blue (modified after Woof and Kerr 2006).

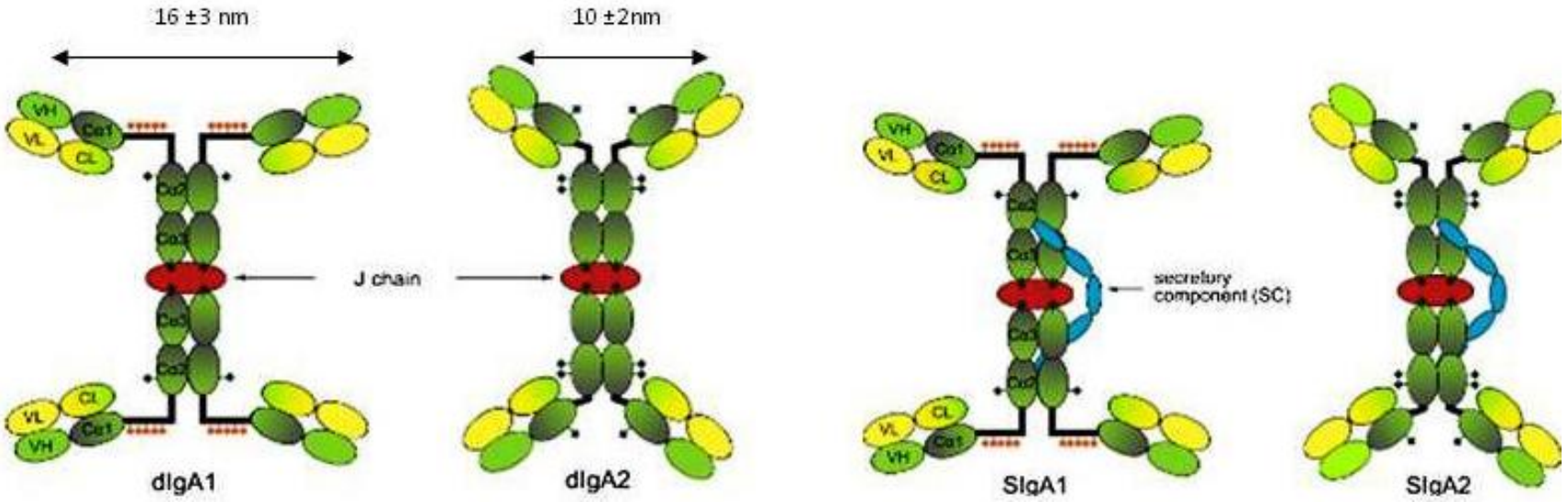


Fig. 4. Dimeric and secretory forms of human IgA.

Two monomeric molecules (for structure refer to Fig. 3) are covalently associated via a Joining chain (red) to form the dIgA1 and dIgA2. The secretory forms contain additionally a secretory component (blue). For simplification, only the secretory form of dIgA2(m2) is shown. O-linked oligosaccharides in the IgA1 hinge region are shown in orange. N-linked oligosaccharides are marked at the approximate locations in both IgA1 and IgA2 molecules via black dots (modified after Zhou and Ruprecht 2014).

1.3.1 The Formation of secretory IgA

After secretion of dIgA by plasma cells located in the lamina propria below the epithelium, the antibody dimer binds to the extracellular portion of the poly immunoglobulin receptor (pIgR) via the Joining chain (JC) (Braathen et al. 2007). After endocytosis of the receptor antibody complex, it gets transported through a series of endosomal compartments to the luminal side of the epithelium. Upon arrival at the apical surface, the extracellular portion of pIgR gets cleaved from its intracellular tail and becomes the secretory component (SC). Secreted dIgA in association with the SC is referred to as secretory IgA (Rojas and Apodaca 2002).

1.3.2 IgA: Standing guard at the mucosa

The major entry routes for the human immunodeficiency virus are mucosal surfaces of the genital or rectal tract (Pope and Haase 2003). DIgA reinforces various innate defense mechanisms to protect the mucosal surfaces against viral invasion: Immune exclusion, intracellular neutralization and immune excretion. All these mechanism were discovered using a transwell system. Such a system simulates a tight polarized epithelial monolayer and is a common *in vitro* model to study the mechanisms involved in the transmucosal passage of the human immunodeficiency virus (Bomsel et al. 1997).

Immune exclusion of HIV by IgA was shown by Devito et al. (2000): Plasma and mucosal IgA from HIV-1 exposed uninfected individuals inhibits HIV-1 transcytosis. The data suggest that IgA is transcytosed from the basolateral to the apical side of the epithelium where it encounters cell free virus and cross-links the virus particles to form large IgA-virion complexes; the virus gets excluded from the mucosal surface (Fig. 5). Thus, the process was termed immune exclusion.

Huang et al. (2005) demonstrated that anti-Env (gp160) monoclonal IgAs were also able to neutralize transcytosing viral particles by inhibiting their intracellular replication (Fig. 5). They used transwell systems consisting of polarized epithelial cells stably expressing pIgR on their basolateral surface. After transfection with proviral HIV DNA for 4 h, IgA monoclonal antibody (IgA mAb) (α gp120) was added basolaterally for 18 h. Virus in the apical supernatants and cell lysates was tested for infectivity and showed a significant reduction in viral titers. Moreover, IgA added at the basolateral side reduced the quantity of p24 in all three compartments (apical supernatant, basolateral medium, cell lysate). Taken together, IgA must be in the normal path of basolateral to apical transcytosis to mediate intracellular virus reduction by inhibited replication of the virus. In line with that, Bomsel et al. (1998)

demonstrated an intracellular co-localization of HIV virions with dimeric IgA during transcytosis. Virus and IgA are co-localized in the apical recycling endosome. The resulting IgA-HIV immune complexes are specifically recycled to the mucosal surface and most likely released at the apical pole as a consequence of SC cleavage at the epithelial apical membrane. This mechanism was called intracellular neutralization because it is a "protective function whereby antiviral IgA interferes with virus production via an intraepithelial cell action" (Wright et al. 2008).

Besides capture of transcytosing HIV virions on their way to the basolateral side (Bomsel et al. 1998), IgA also mediates the immune excretion of HIV: transportation of HIV from the basolateral surface across polarized epithelial cells to the apical surface and subsequent excretion (Wright et al. 2008). Wright and colleagues (2008) applied a cell-culture transwell systems consisting of pIgR expressing polarized epithelial cells as well. MAbs directed against Env (gp41 or gp160) were tested. IgA mAbs-HIV immune complexes were added to the basolateral side of the transwell system for 8 h. Apical excretion of HIV virus particles was observed in a concentration and time dependent manner and occurred at IgA concentrations found in human mucosal fluids. It correlated directly with the ability of the IgA mAbs to bind to virions as well as to the pIgR. Confocal laser scanning microscopy confirmed the constant association between the HIV particles and the IgA molecules throughout the whole excretion process. Virtually no HIV was observed in the absence of immune complexes.

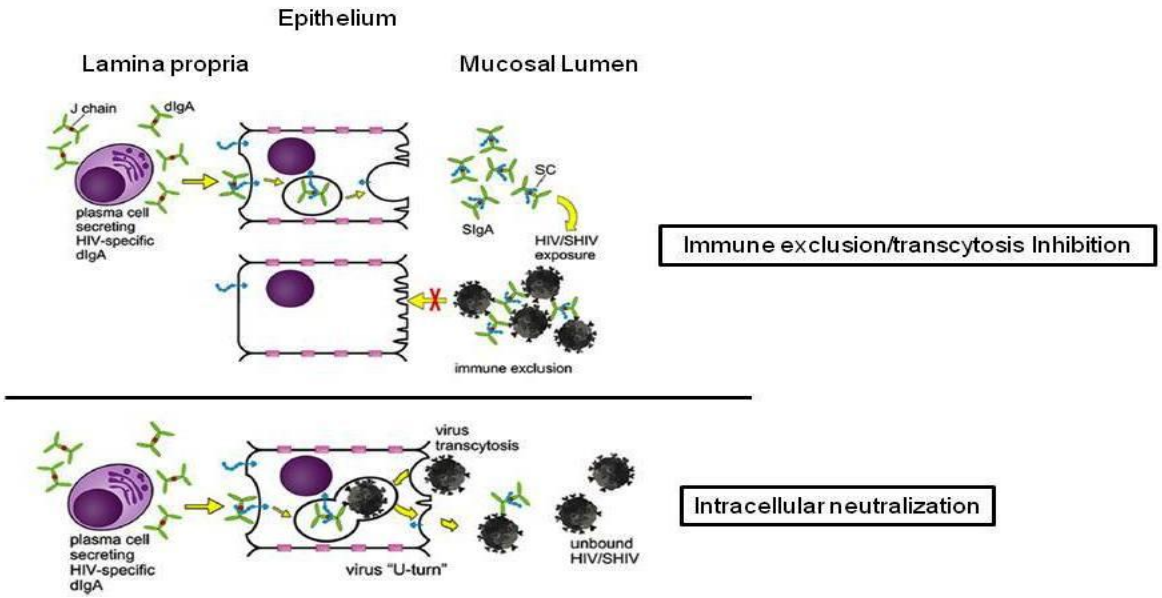


Fig. 5. Protective Mechanisms of dimeric IgA

The figure above shows immune exclusion, inhibition of transcytosis and intracellular neutralization. Mature plasma cells in the lamina propria produce HIV/SHIV-specific dIgA. DIgA interacts with the pIgR (blue) on the basolateral surface of epithelial cells and gets exported across the epithelial cells, a process mediated by pIgR. The latter undergoes proteolytic cleavage at the luminal side, which results in the generation of SC that is retained by dIgA molecules, releasing secretory IgA into the lumen. Secretory IgA binds to SHIV and prevents viral invasion of epithelial cells by forming large SHIV/dIgA complexes. These SHIV/dIgA complexes are now "excluded" from the basolateral side and thus cannot undergo transcytosis. The figure below describes intracellular virus neutralization. After the dIgA-pIgR complex is formed intracellularly, HIV/SHIV particles that have invaded the epithelial cells are bound and then excreted as virion-dIgA-pIgR complexes. This returns the secretory IgA-virion complex into the mucosal lumen (modified after Zhou and Ruprecht 2014).

1.3.3 IgA: Two sides of the medal

Literature is conflicting with regard to anti-HIV IgA responses. Despite its protective mechanisms mentioned above, IgA can promote HIV-1 infection or even lead to an increased risk of infection:

Enhancement of HIV replication *in vitro* by unfractionated, serum-derived polyconal IgA has been reported: Kozlowski and co-workers (1995) observed modest increases of viral replication of laboratory-adapted HIV_{IIIB} by IgA from seropositive individuals at various stages of HIV disease. Similar, low-level enhancement of viral replication by total serum IgA from seropositive individuals was observed in primary monocytes with the monocyte-tropic strain HIV-1_{Bal} (Janoff et al. 1995). The RV144 trial phase III clinical trial, a vaccine efficacy study, linked plasma anti-HIV Env IgA to an increased risk of infection (Haynes et al. 2012)

However, literature also supports a beneficial role of IgA: Kaul and co-workers (1999) correlated HIV-1 specific IgA in the genital tract of a cohort of HIV-1 resistant Kenyan sex workers with resistance to infection. The active immunization study of Bomsel et al. (2011) linked vaginal IgAs to protection from SHIV infection.

According to Zhou and Ruprecht (2014) "the jury is still out whether anti-HIV IgA responses will overall benefit or harm the host". To further elucidate anti-HIV IgA immune responses future studies will have to clearly define which forms of anti-HIV IgA are involved in the systemic circulation and in the various mucosal compartments.

1.3.4 IgA Isolation and Purification

In their review, Zhou and Ruprecht (2014) criticize the existing literature about IgA responses against HIV in that that most of the current publications do not distinguish between IgA subtypes and the various IgA forms (monomeric, dimeric, polymeric). This highlights the importance of distinguishing between the various IgA specific purification methods. Depending on the IgA isotype, isolation techniques are either based on plant sugars, bacterial molecules or mAbs.

Jacalin is a lectin derived from jackfruit seeds and was shown to precipitate human IgA (Kondoh et al. 1986). Gregory and co-workers (1987) used Jacalin agarose chromatography in a proof-of-concept study to separate human IgA1 from IgA2 in secretions. Jacalin-agarose binds specifically to the D-galactose moiety of IgA1. Despite preferential binding to IgA1, the lectin also binds IgA2, although with a lower apparent affinity than for IgA1 (Aucouturier et al. 1988).

Other purification techniques make use of bacterial proteins that help various bacteria to subvert IgA-mediated immunity. For streptococcus, literature describes the synthetic peptide "Sap", derived from the IgA (Fc)-binding region of a streptococcal M protein. Johnsson et al. (1999) describe the use of this synthetic 50-residue peptide for IgA subtype separation in various human secretions. The peptide was specific for all IgA1 and IgA2 forms. Sandin and colleagues (2002) used the same peptide for IgA depletion of human serum and finally named it Sap (Streptococcal IgA-Binding Peptide). Concerning Staphylococcus, Langley and co-workers (2005) showed the capability of staphylococcal superantigen-like proteins (SSL) to bind the various forms of IgA1 and 2 (monomeric and secretory forms).

Commercially available mAbs against the JC or the SC allows for selective enrichment of dimeric and secretory forms of IgA1 and IgA2. When specificity for only one subtype is desired, mAbs against the respective constant regions are recommended (Zhou and Ruprecht 2014).

1.4 Rationale/Background behind the Thesis

1.4.1 Passive immunization of rhesus monkyes with dimeric IgA

The passive immunization study of Watkins et al. (2013) provided direct proof that neutralizing mucosal dIgA can prevent SHIV acquisition in RMs. Different recombinant isotypes of the human neutralizing monoclonal antibody (nmAb) HGN194 (IgG1, dIgA1 and dIgA2), all directed against the conserved crown of the V3 loop in the HIV Env, were evaluated for their antiviral activity. They were applied intrarectally (i. r.) to three groups of six RMs each, 30 min before i. r. challenge with R5-tropic clade C SHIV-1157ipEL-p. A pharamcokinetic control study showed similar concentration levels in rectal fluid over time for all three antibody isotypes tested.

Despite similar neutralization patterns *in vitro*, HGN194 dIgA1 yielded the best protection against i. r. SHIV challenge in RMs. Protection could therefore not be correlated to neutralization patterns of the antibodies tested. Whereas only 17 % of RMs in the "dIgA2 group" stayed aviremic throughout, over 80 % of RMs that received dIgA1 before the i. r. challenge, remained virus-free.

Better protection correlated significantly with better capture of viral particles. HGN194 dIgA1 captured significantly more virus than its dIgA2 counterpart and was the only isotype blocking transcytosis of cell-free challenge virus across an epithelial barrier *in vitro*. This was attributed to the longer hinge region and its more open conformation allowing to accomodate twice as many virions as dIgA2.

1.4.2 Non-functional envelope proteins

Functional surface HIV-1 Env spikes consist of compact trimers of non-covalently associated gp120 and transmembrane gp41. In infected cells, Env is synthetized as a non-functional polyprotein precursor, gp160. During Env trafficking to the cell surface cellular furins cleave gp160 into external gp120 and non-covalently attached gp41. Trimerization of the gp120/gp41 heterodimer is required to render the Env fully functional and infectious (Freed and Martin 2001). However, variable gp160 processing and the heterodimer's conformational instability can result in the expression of non-functional Env species on the surface of the virion. Several virion capture assay (VCA) studies of the early 2000s proved their existence and gave a hint about their nature. Related to that, non-neutralizing monoclonal antibodies (non-nmAb) have been shown repeatedly to capture infectious virus particles in a highly specific fashion (Poignard et al. 2003, Burrer et al. 2005, Moore et al. 2006).

Burrer et al. (2005) isolated polyclonal IgG from HIV infected patients with various neutralization profiles and tested them for their ability to capture different primary HIV isolates. Virus capture of the various HIV isolates was observed for all polyclonal IgG samples. However, virus capture by polyclonal sample antibodies or the three control mAbs included (2F5, b12, 2G12) did not correlate with their neutralization profile.
Moreover, the capturing pattern was compared between gp120-depleted virus and normal virus particles. Gp120 shedding resulted in a decrease of the capture by the anti-gp120 mAb 2G12, while no significant difference was found for the anti-gp41 mAb 2F5. On contrary, the capture of the viral particles was significantly increased after shedding of gp120 for all the purified IgG samples. Peptide competition assays showed that the principal immunodominant region of gp41 was involved in virus capture.
Taken together, no correlation between virus capture and neutralization was found, neutralization is not predictive for virus capture. The immunodominant region of gp41 plays a role in virus capture by neutralizing antibodies (nAbs) and non-neutralizing antibodies (non-nAbs) directed against HIV Env.

The discrepancy between virus capture and neutralization is possibly explained by the fact that non-nAbs bind only to defective Env spikes:

Poignard et al. (2003) describe the existence of heterogeneous Env molecules on primary human immunodeficiency virus Type 1 particles: They compared the binding behaviour of two mAbs, b6 and b12, both directed against epitopes that overlap the CD4 binding site of gp120. However, b6 is non-neutralizing whereas b12 is neutralizing. It was shown that non-neutralizing b6 competes with neutralizing b12 for virus capture, but does not affect the neutralization activity of the b12. An explanation for virus capture by non-nAbs could therefore be that it occurs via an as yet unidentified alternative form of Env that is recognized by both neutralizing monoclonal antibodies (nmAbs) and non-neutralizing monoclonal antibodies (non-nmAbs). Their data suggest that only nAbs can bind to the functional Env leading to virus neutralization. However, both nAbs and non-nAbs can bind the non-functional Env leading to capture of the virus particle. According to them, HIV-1 primary virions can express a functional and a better accessible non-functional form of gp120. The non-functional form needs to be present at only a low density on the virion surface and permits capture but does not lead to neutralization.

Moore et al. (2006) showed evidence that these non-functional Env proteins could be non-trimeric arrangements of gp120/gp41 heterodimers and gp120-depleted gp41 stumps. Three different forms of HIV-1 Virus-like particles (VLPs) were used in their experiments: Wildtype (WT)-VLPs (unmodified gp160, wild type), SOS-VLPs (gp160 with an intermolecular disulfide bond between gp120 and gp41) and UNC-VLPs (gp160 cannot be cleaved due to an Env cleavage site mutation). A subtype B primary isolate Env, JR-FL, served as the Env prototype. Various mAbs directed against Env were tested for their ability to capture and neutralize VLPs.

Similarly to the findings of Burrer et al. (2005), the ability of each mAb to capture had no correlation with its neutralization activity. Despite being neutralizing, 2F5 did not capture efficiently. However, several non- or weakly neutralizing mAbs, e.g. b6 or 447-52D efficiently captured both WT-VLPs and SOS-VLPs. The anti gp41 antibodies 7B2 and 2.2B were able to capture WT-VLPs, suggesting that "gp120 shedding exposes gp41 stumps". As expected, these two antibodies failed in capturing SOS-VLPs because the possibility of gp120 shedding was eliminated in this mutant. According to this observed pattern of capture the following forms of Env have been suggested: WT-Env, non-functional gp120/gp41 monomers and gp41 stumps (Fig. 6 and Fig. 7). The non-functional monomers very likely originated due to variable gp160 processing. The non-covalent nature of the gp120/gp41 heterodimer renders it unstable which can lead to gp120 shedding leaving gp41 stumps expressed on the virion surface behind.

The suggested model for the Env surface heterogeneity of HIV (Fig. 7) is in line with the recent results of Stieh et al. (2015). They analyzed the potential distribution of Env forms across HIV-1_{Bal} with a panel of nAbs and non-nAbs. Virus capture of physical particles (p24) and of infectious particles (TZM-bl assay) was assessed. Capture of p24 did not correlate with capture of infectious virions which confirmed that other non-functional Env forms must be present on the virion surface besides the trimeric Env spike. NAbs VRC01, 2G12 and IF7 trapped over 90 % of infectious virions, but less than 35 % of viral particles. A small portion of infectious particles was captured by several non-nAbs targeted against gp41 cluster I. According to them, accessible gp41 cluster I which is part of the immunodominant region (Zolla-Pazner 2004) "most likely represents Env stumps which consist only of gp41 without any associated gp120", so called "gp41 stumps".

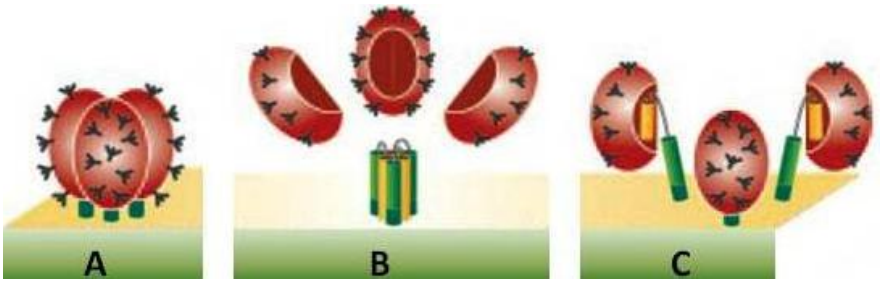


Fig. 6. Non-functional forms of Env on the HIV-1 membrane

Gp120 is shown in red with the outer neutralizing face in light red shading and the inner non-neutralizing face in darker red shading. Carbohydrate moieties are depicted as blue “tree”-like structures. gp41 is comprised of N-terminal (yellow) and C-terminal (green) transmembrane domains, separated by a disulfide-constrained loop. The membrane-proximal gp41 region exposed on the trimer is depicted in dark green. (A) Functional Env trimer, (B) gp120 shedding exposing gp41 stumps; (C) gp120/gp41 monomers (modified after Moore et al. 2006)

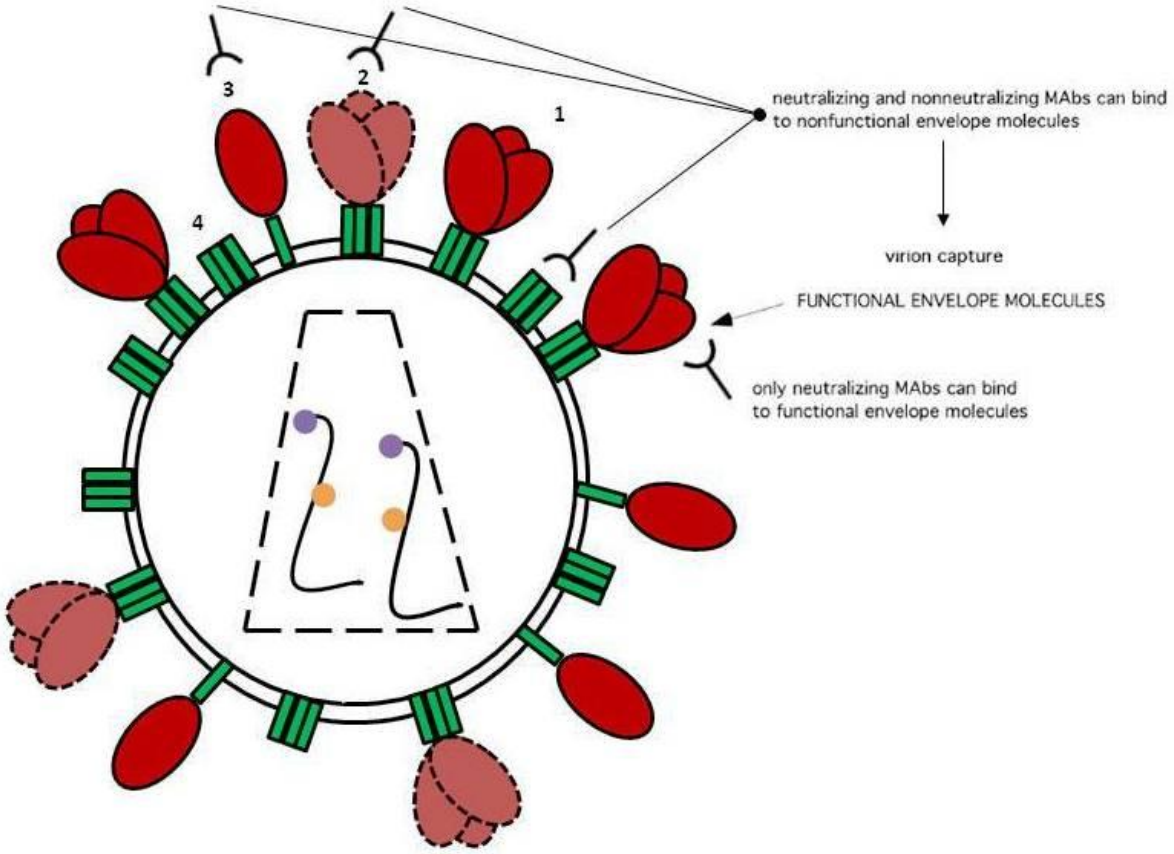


Fig. 7. Model for proposed heterogeneity of envelope molecules at the surfaces of primary HIV-1 particles

For better emphasis of the Env molecules only a simplified HIV virion with its capsid is shown. Functional Env spikes (1) consist of compact trimers of non-covalently associated gp120 (surface subunit, dark red ovoid shapes with solid black line) and gp41 (transmembrane subunit, green). Virion capture studies of the early 2000s suggest a heterogeneous Env surface of HIV. Besides functional gp160 trimer proteins, also non-functional Env species are found on the virion surface. Based on Burrer et al. (2005), Poignard et al. (2003) and Moore et al. (2006) these are:

Non-functional gp120 (2, light red ovoid shapes with broken black line), gp160 monomers (3), gp41 stumps (4). Red: gp120, green gp41, broken black line: capsid, purple: integrase, orange: reverse transcriptase. RNA molecules are depicted as wavy lines.

1.4.3 F240 IgG: A human monoclonal antibody reactive with the immunodominant region of HIV-1 glycoprotein 41

Cavacini et al. (1998) describe the isolation, production and functional characterization of a human mAb, F240IgG1, derived from an HIV-1-infected individual. ELISA revealed the isotype of F240 to be IgG1(κ).

Immunoblot analysis suggested antigen specificity of F240 for gp41 or its precursor gp160. Pepscan analysis mapped F240 specificity to the immunodominant region of the gp41 ectodomain. This epitope has been implicated in eliciting non-protective antibodies that enhance infection in the presence of complement. Consistent with this, F240 failed to neutralize laboratory isolates and enhanced viral infection in a complement-dependent manner.

1.5 Hypothesis & significant aims

As shown in the passive immunization study of Watkins et al. (2013) neutralization is not necessarily required for protection against mucosal SHIV challenge: The tested antibody versions of HGN194 showed the same epitope specificity and their neutralization profiles were similar. However, HGN194 dIgA1 was vastly superior in capturing virions than its dIgA2 counterpart.

Besides WT Env also non-functional Env species such as gp41 stumps are found on the virion surface (Poignard et al. 2003, Moore et al. 2006, Stieh et al. 2015). Besides Stieh et al. (2015), Burrer et al. (2005) showed that the immunodominant region of gp41 (encompassing the KLIC motive) was involved in virion capture. No direct correlation between virion capture and neutralization profiles was found for the tested antibodies.

The published F240 IgG1 directed against the immunodominant region of gp41 will be re-engineered into its dimeric versions, F240 dIgA1 and F240 dIgA2. Based on the findings of Watkins et al. (2013) whereas dimeric IgA1 was vastly superior in capturing virions, the underlying hypothesis of the thesis is as follows:

Re-engineered non-neutralizing F240 dimeric Immunoglobulin A antibodies, directed against the immunodominant region of gp41, successfully capture SHIV particles.

2 Material and Methods

2.1 List of buffers

Coating buffer pH 9.6

Dissolve one tablet of Carbonate/Bicarbonate (Sigma-Aldrich) in 200 ml of ultrapure water for 200 ml of the Carbonate/Bicarbonate Coating buffer pH 9.6.

PBST

For 1000 ml of PBST mix 100 ml of 10x PBS (Gibco, Life Technologies) with 900 ml of ultrapure water and add 500 µl of Tween20 (Sigma-Aldrich).

PBSTB

For 1000 ml of PBSTB mix 100 ml of 10x PBS with 900 ml of ultrapure water. Add 20 g of BSA (Sigma-Aldrich) and 500 µl of Tween20 (Sigma-Aldrich).

MPBST

For 200 ml of MPBST, mix 20 ml of 10x PBS with 180 ml of ultrapure water, add 8 g of with 4 % non-fat dry milk (Bio-Rad Laboratories) and 100 µl Tween20 (Sigma-Aldrich).

1x Tris-glycine-SDS (TGS) Buffer

For 1000 ml of TGS solution, mix 100 ml of 10x TGS concentrate (Bio-Rad Laboratories) with 900 ml of ultrapure water.

1x Tris-acetate-EDTA (TAE) Buffer

For 1000 ml of TAE solution, mix 20 ml of 50x TAE concentrate (Thermo Scientific) with 880 ml of ultrapure water.

Binding Buffer

For 200 ml of a 50 mM sodium phosphate buffer, mix 93.2 ml of 0.1M Na_2HPO_4 with 6.8 ml of 0.1 M NaH_2PO_4 and add 17.425 g of K_2SO_4. Add 40 ml of ultrapure water until K_2SO_4 is fully dissolved. Adjust the solution to pH 8.0 using Tris-base (Sigma-Aldrich). When pH 8.0 is reached, fill up with ultrapure water to 200 ml.

Elution Buffer (sodium phosphate)

For 200 ml of a 50 mM sodium phosphate buffer, mix 93.2 ml of 0.1M Na_2HPO_4 with 6.8 ml of 0.1 M NaH_2PO_4 and fill up with ultrapure water to 200 ml. Adjust the solution to pH 8.0 using Tris-Base (Sigma-Aldrich).

Elution Buffer (0.1 M α D-galactose)

For 1000 ml of 0.1 M α D-galactose solution, dissolve 9 g α D-galactose (Sigma-Aldrich) in 1000 ml of 1x PBS.

Elution Buffer (0.1 M glycine, pH 3.0)

For 1000 ml of 0.1 M glycin solution, dissolve 7.507 g L-glycine (Sigma-Aldrich) in 1000 ml of ultrapure water.

Elution Buffer (1 M Tris, pH 9.0)

For 500 ml of 1 M Tris solution, dissolve 60.57 g TRIS (Sigma-Aldrich) in 500 ml of ultrapure water.

2.2 SDS-PAGE

For supernatants, flow-through fractions, washes, eluates, 20 µl sample were mixed with 20 µl of 2x Laemmli Sample Buffer (Bio-Rad Laboratories). For samples of known concentration, the amount loaded is given in the respective gel legend. Ultrapure water was added to yield a final volume of 20 µl, 2x Laemmli buffer was added up to give 40 µl. After heating the mix on a heat block (Denville Scientific Inc.) for 2 min at 96 °C it was loaded on a Mini-PROTEAN® TGX Stain-Free™ Precast Gel (Bio-Rad Laboratories). Gel was run in 1x TGS buffer (Bio-Rad Laboratories) for 25 min at 200 Volt. For staining, gel was rinsed in distilled water 3 times and visualized by 1 h incubation in Imperial™Protein Stain (Thermo Scientific) and then decolorized under frequent distilled water changes..

2.3 Western Blot

0.1 µg of sample or control were loaded onto the native gel. For SDS-PAGE please refer to the section " SDS-PAGE". After SDS-PAGE, proteins were transferred onto a ready-to-use nitrocellulose membrane (0.2 µm pore size) provided with the iBlot®Gel Transfer Stacks (Gibco Life Technologies) using the iBlot® Gel Transfer Device (Gibco Life Technologies) according to the manufacturer's instructions. After blotting, blocking was performed with PBSTB over night at 4 °C. Depending on what antibody chain was detected, two distinct staining protocols were carried out the next day after blocking. For the JC, polyclonal rabbit-anti-human JC antiserum (Invivogen, 1:1000 in PBSTB) was added for 1 h. After extensive

wash in PBS the membrane was incubated for another hour with HRP-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, 1:2000 in PBSTB), followed by a washing step in PBST and water. Membrane was developed using Opti-4CN™ Substrate Kit (Bio-Rad Laboratories).

To detect alpha and kappa chains, HRP-conjugated goat anti-human alpha chain or HRP-conjugated goat anti-human kappa chain (Southern Biotech, 1:8000 in PBSTB) were added for 1 h. After a washing step in PBST and water, Opti-4CN™ Substrate Kit was used for membrane development.

2.4 Cell Lines, Production Media and Cultivation Conditions

2.4.1 Expi293 cells

Expi293 cells and Expi293 Expression Medium were purchased from Gibco Life Technologies. Cells were maintained in culture volumes varying from 30 ml to 120 ml at a cell density of 0.3 x 10^6 cells/ml at 37 °C under 8 % CO_2.

2.4.1.1 Cell Transfection (ExpiFectamine™ 293 Transfection Kit)

Expi293 cells were used for all transfections in this study. Cells were transfected with the ExpiFectamine™ 293 Transfection Kit (Gibco Life Technologies) according to the manufacturer's instructions. However, we used Expi293 expression medium instead of Opti-Mem® medium as a dilution medium. Briefly, Expi293 cells were seeded at 3 x 10^6 cells/ml the day before transfection and incubated for 24 h. The next day, cells were diluted to the recommended concentration by the manufacturer, 2.94 x 10^6 cells/ml. 1 µg of total DNA per 1 ml of culture (composed of plasmids encoding for respective HC, LC and JC) and ExpiFectamine™293 Reagent were diluted separately and incubated for exactly 5 min before being mixed and incubated for another 20 min. The DNA-reagent complex was finally added to the Expi293 cells. After 16 h, transfection enhancers I and II, both provided with the kit, were added to the transfected cells. After 6 days supernatant was harvested by centrifugation at 10.000 x g for 20 min. Clear supernatant was sterile filtrated using a Corning® 250 ml Vacuum Filter/Storage Bottle System (Corning Inc.). Filtered supernatant was stored at 4 °C until further usage.

2.5 Cloning of F240 dIgA1 and dIgA2

2.5.1 Linearization of pcDNA3.4

Linearized vector pcDNA3.4 was kindly provided by Dr. Anton Sholukh.

2.5.2 PCR of inserts for Gibson Assembly

All primers were designed in the lab and synthesized by Invitrogen. PCRs were carried out on a C1000 touch thermal cycler (Bio-Rad Laboratories) in a 50 µl reaction volume, composed of 25 µl Q5® High-Fidelity 2X Master Mix (New England Biolabs), 1 µl of respective plasmid (1 ng/µl), 1 µl of respective forward and reverse primers and 22 µl of dH_2O. Primers were used at a working concentration of 20 µM. For primer sequences consult Tab. 6 in the results section.

2.5.3 Extraction of amplified Plasmid Fragments for Gibson Assembly

Amplified inserts were separated on a 0.8 % Agarose gel and recovered using the NucleoSpin®Gel and PCR Clean-Up Kit (Macherey-Nagel GmbH & Co. KG) according to the manufacturer's instructions.

2.5.4 Gibson Assembly

Gibson assembly was carried out using the Gibson Assembly Kit (New England Biolabs) according to the manufacturer's instructions and recommendations. Assembly was carried out for 15 min at exactly 52 °C.

2.5.5 Transformation

Transformations were performed using NEB10-beta *E.coli* cells (New England Biolabs) according to the manufacturer’s High Efficiency Transformation protocol: Briefly, thawed cells were mixed with 5 µl of the Gibson assembly reaction. After being kept on ice for 30 min, cells were transformed by heat shock treatment for 30 sec at exactly 42 °C, followed by 5 min incubation on ice. 950 µl of super-optimal broth with catabolite repression medium was added and the mixture was incubated for 60 min at 37 °C in a bacterial shaker at 225 rpm. 10 µl of the bacterial suspension were distributed evenly on Luria-Bertani agar plates containing 100 µg/ml ampicillin and were incubated overnight at 37 °C.

2.5.6 Plasmid Mini Prep

On the next day after transformation, plates were harvested, single colonies picked and inoculated in 5 ml of ampicillin containing (100 µg/ml) Luria-Bertani medium. After overnight inoculation at 37 °C at 225 rpm, suspension colonies were harvested and plasmid mini prep was carried out using the Nucleo®SpinPlasmid QuickPure Kit according to the manufacturer's instructions (Macherey-Nagel GmbH & Co. KG).

2.5.7 Quality Control I of plasmids: Sequencing

Plasmids obtained by mini prep were sent for sequencing. Plasmids were sequenced in two reactions, one with the forward sequencing primer, the other with the reverse sequencing primer. For each reaction 800 ng of plasmid were mixed with 5 µl of primer (5 µM), dH_2O was added up to a final volume of 15 µl. Sequence analysis was done using the software SnapGene (SnapGene software (from GSL Biotech; available at snapgene.com)).

Tab. 1. Used sequencing primers

Sequencing Primer Name	Sequence
CMV-Seq-Fw	CGCAAATGGGCGGTAGGCGTG
pcDNA3.4-Seq-Rev	CAACATAGTTAAGAATACCAGTC

2.5.8 Plasmid Mega Prep

After confirmation of plasmid sequences, plasmid mega prep was carried out for subsequent large scale production of the antibodies. Briefly, NEB10-beta *E. coli* cells (New England Biolabs) were transformed with 1 µl of plasmid (1 µl) according to the manufacturer's High Efficiency Transformation protocol. Finally, 10 µl of the transformed bacterial suspension were distributed evenly on Luria-Bertani agar plates containing 100 µg/ml ampicillin and were incubated overnight at 37 °C.

Single colonies from each transformation were selected the next morning and inoculated in 5 ml of LBmedium containing 100 µg/ml ampicillin. The inoculated single colonies were incubated till the early afternoon in a bacterial shaker at 225 rpm at 37 °C. The 5 ml bacterial suspension culture was then inoculated into 250 ml of LB medium containing ampicillin (100 µg/ml). The big culture was incubated on a bacterial shaker overnight at 225 rpm at 37 °C.

The next day, plasmids were isolated from the 250 ml overnight cultures using the QIAGEN®Plasmid Mega Prep Plus Kit following the manufacturer's protocol, DNA was eluted in 2 ml of Elution Buffer (Quiagen).

2.5.9 Measurement of DNA concentration

Purified DNA was measured with the Nanodrop1000 Spectrophotometer. Using the 'Nucleic Acid' application module (DNA-50), 1.5 µl of each sample was pipetted on the fiber optic cable of the instrument's measurement pedestal. Absorbance at the wavelength of 260 nm determined the sample concentration in ng/ml.

2.5.10 Quality Control II of plasmids: Restriction digest

For final plasmid verification, restriction digestion was performed using restriction enzymes that gave a unique fragment pattern which allowed unequivocal identification. All verification digests used the same protocol. Each plasmid was single, double and triple digested.

Briefly, 200 ng (single digest) or 500 ng (double and triple digest) of plasmid DNA were digested with 0.5 µl of the required FastDigest Enzyme (Tab. 2) and 4 µl of Smart Cut 10x Buffer. Nuclease-free water was added to a final volume of 40 µl. The samples were digested for 2.5 h at 37 °C and then combined with 6x DNA loading dye and loaded onto a 1 % agarose gel containing 600 ng/ml ethidium bromide. The gel was run in 1x TAE buffer for 40 min at 100 V. Gel was photographed with the Molecular Imager® Gel Doc™ XR System (Bio-Rad Laboratories) and compared to the simulated agarose gel of SnapGene. Using the marker DNA ladder mix (Thermo Scientific), the samples were identified according to their digested fragments. All enzymes were purchased from New England Biolabs.

Tab. 2. Used enzymes and expected fragment sizes

Enzymes	Single Digest	Double Digest	Triple Digest
NdeI	✓	✓	✓
XbaI		✓	
AgeI			✓
AvrII			✓
Plasmid			
pcDNA3.4 F240-hA1	7483bp (linearized)	7059bp (XbaI - NdeI) 424bp (NdeI - XbaI)	3863bp(AvrII - NdeI) 1690bp(AgeI - AvrII) 1452bp (AgeI - AgeI) 478bp (NdeI - AgeI)
pcDNA3.4 F240-hA2	7439bp (linearized)	7015bp (XbaI - NdeI) 424bp (NdeI - XbaI)	3863bp (AvrII - NdeI) 3089bp (AgeI - AvrII) 478bp (NdeI - AgeI)
pcDNA3.4 F240-hk	6708bp (linearized)	6284bp (XbaI - NdeI) 424bp (NdeI - XbaI)	3863bp (AvrII - NdeI) 2367bp (AgeI - AvrII) 478bp (NdeI - AgeI)
pcDNA3.4 F240-hJ	6468bp (linearized)	6044bp (XbaI - NdeI) 424bp (NdeI - XbaI)	3863bp (AvrII - NdeI) 1690bp (AgeI - AvrII) 915bp (NdeI - AgeI)

2.5.11 Pilot IgA Monomer Expression Study

For successful production of dimeric F240 IgA1 (F240 dIgA1), it was necessary to ensure correct production and assembly of monomers as well as to find the optimal ratio of plasmids coding antibody chains for monomer expression. Based on the study of Schlatter et al. (2005), the following molar ratios of HC to LC plasmid were tested in a small scale transfection pilot study using the ExpiFectamine^{TM} 293 Transfection Kit: 10:1, 5:1, 5:2, 3:2, 1:1, 2:3, 1:2, 2:5, 1:5, 1:10. After 6 days, an SDS-PAGE and an end-point titration of all harvested supernatants were carried out. Based on the findings of this pilot study, an optimal HC:LC ratio was chosen for production of F240 dimeric IgA1.

2.5.11.1 Cell Transfection

Transfection was carried out in a 12-well cell culture plate (Corning Inc.), 2 ml transfection reaction volume per well. Expi293 cells were transfected with 2 µg of total DNA (composed of plasmids pcDNA3.4-F240-hA1 and pcDNA3.4-F240-hK, encoding for alpha-1 HC and kappa LC respectively). The positive control of the Expifectamine Transfection kit (full length rabbit IgG) was included to confirm successful cell transfection. For the negative control, no plasmids were added to the transfection mix. After 6 days supernatant was harvested by centrifugation at 5.000 rpm for 4 min. Clear supernatant was transferred into sterile 1.5 ml tubes (Eppendorf).

2.5.11.2 End-Point Titration

Nunc MaxiSorp® flat-bottom 96 well plates (Affymetrix eBioscience) were coated overnight at 4 °C with gp160 of HIV1084i at 1 µg/ml. Antigen-coated plates were washed with water and nonspecific sites were blocked with 200 µl PBSTB per well for 2 h at room temperature (RT). Serial 1:5 dilutions (in PBSTB) of the supernatants from the different transfection reactions were prepared and 100 µl were added to the respective wells, and incubated for 1 h at RT. The supernatant of the cell culture treated with transfection reagent only (no plasmids) served as a negative control. Plates were rinsed three times with water and incubated with 100 µl of peroxidase-conjugated goat anti-human alpha chain antibody (Southern Biotech, 1:8000 in PBSTB) for 1 h at RT. The wells were rinsed six times with water and incubated with 100 µl of 3,39,5,59-tetramethyl benzidine (TMB, Gibco Life Technologies). The reaction was stopped by adding 100 µl of 1N sulfuric acid per well and the optical density (OD) determined at 450 nm using a multiwell plate reader (Berthold Technologies).

For analysis, the average and Standard Error of the Mean of the respective dilution of the negative control was calculated. The average value plus three times the SEM was calculated and is further referred to as “normalization value“ (100 %). Supernatants from the distinct transfection reactions are referred to as "samples". Sample values of the respective dilution were normalized upon the normalization value, giving "sample normalization values". A sample was "positive" when its sample normalization value exceeded 100 %. If a sample was positive at a higher dilution in comparison to another sample, it contained more monomeric F240 IgA1.

2.5.12 Pilot Dimer Expression Study

Schlatter et al. (2005) determined the optimal HC:LC gene ratio for transient production of a certain recombinant mAb in Chinese hamster ovary cells to be 3:2, in a bi-cistronic expression system. On the basis of these findings, a pilot study for expression of F240 dIgA1 was carried out, using two different molar ratios of HC plasmid to LC plasmid, 3:2 and 2:3. JC plasmid was added on top with a 1:1 ratio [(HC:LC):JC]. The harvested supernatants were analyzed by SDS-PAGE and Western Blot. Produced F240 dIgA1 was purified by Thiophilic adsorption chromatography (TAC) followed by size-exclusion chromatography (SEC), and was tested for correct isotype and epitope specificity by ELISA. This pilot dimer expression study helped to determine the optimal conditions for further purification of larger batches of F240 dIgA1.

2.5.12.1 Cell Transfection

For pilot dimer expression study, 30 ml cultures of Expi293 cells were transfected with 30 µg of total DNA (composed of plasmids encoding for the respective HC, LC and JC, Tab. 4).

2.5.12.2 Thiophilic Adsorption Chromatography

TAC was used in the first place to purify F240 dIgA1 because it is a low cost method for protein purification. Expi293 supernatant was harvested on day six after transfection by centrifugation at 10.000 x g for 20 min. After sterile filtration through a Corning® 250 ml Vacuum Filter/Storage Bottle System (Corning Inc.), K_2SO_4 was added to a final concentration of 0.5 M. The mixture was stirred gently until the added salt had dissolved completely and clear supernatant was again sterile filtrated. IgA was purified according to the following procedure: 10 ml of thiophilic resin (G-Biosciences) was packed in a suitable plastic

column and equilibrated with 50 mM sodium phosphate buffer, containing 0.5 M K_2SO_4, pH 8.0 (Binding buffer). The pretreated sample was loaded onto the column, followed by washing of the column with Binding buffer to remove unbound protein. The adsorbed protein was then eluted with 50 mM sodium phosphate buffer, pH 8.0 (Elution buffer). All experiments were carried out at RT (20 °C). The eluate was collected in a 50 ml Falcon tube and concentrated using Amicon® Ultra 15 ml Centrifugal Filters with a molecular weight cut off of 50 kDa (Merck Millipore). Briefly, after an equilibration step with 1x PBS, three consecutive concentration steps were performed, each at 3.800 x g for 20 min. The concentrated fraction was further referred to as putative F240 dIgA1 and was stored at 4 °C until required.

2.5.12.3 Analytical Size-exclusion Chromatography

Concentrated putative F240 dIgA1 was further purified with analytical SEC by FPLC (ÄKTA Purifier) utilizing a Superdex 200 10/300 GL column (GE Healthcare). UV absorption at 280 nm was recorded with a high precision on-line monitor (UPC-900). Sample and buffers were mixed with a dynamic single chamber mixer (M-925) and loaded onto the column by a P-900 pump (all from GE healthcare). Degased 1x PBS was used as mobile phase and elution buffer with a flow rate of 0.5 ml/min. 200 µl of sample were loaded onto the column for each run, 100 fractions were collected automatically. 100 µg of HGN194 dIgA1 was run as a positive control to estimate size distribution of the collected fractions. Purified protein was stored at 4 °C.

2.6 Functional Characterization of F240 dIgA1

2.6.1 Epitope Specificity of pure F240 dIgA1 & Presence of Joining chain

Nunc MaxiSorp® flat-bottom 96 well plate (Affymetrix eBioscience) was coated overnight at 4 °C with four distinct antigens (gp160 SHIV-1157, gp120 1157 ipK195, HIV-1 gp41 MN and KLIC Peptide Mix) diluted to 1 µg/ml in coating buffer.

The next day, wells were blocked for 2 h at RT with 200 µl PBSTB each. Plate was washed three times with 300 µl water per well in an automated plate washing system (further referred to as washing step). Subsequently, samples, diluted on PBSTB to 250 ng/ml, were added for 1 h at RT, 100 µl each. After another washing step, 100 µl of polyclonal rabbit anti-human JC antiserum (Invivogen, 1:1000 in PBSTB) were added for 1 h at RT.

After washing away unbound antibody, HRP-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, 1:2000 in PBSTB) was added for 1 h at RT. Finally, wells were rinsed six times with water and incubated with 100 µl of TMB solution. The reaction was stopped by adding 100 µl of 1N Sulfuric acid per well and the optical density (OD) determined at 450 nm using a multiwell plate reader (Berthold Technologies). F240 IgG1 served as the antibody control.

Tab. 3. Controls of ELISA for epitope specificity
Six controls were included to exclude any cross-reactions of the antibodies involved. A green tick indicates that reagent was included, empty boxes indicate that particular reagent was omitted in the respective control.

	Antigen	F240	Polyclonal anti-human J-chain rabbit serum	Donkey anti-rabbit HRP
Control I	✓		✓	✓
Control II	✓	**F240 IgG1**		✓
Control III	✓	**Pure F240 dIgA1**		✓
Control IV	✓			✓
Control V				✓
Control VI			✓	✓

2.7 Large Scale Production: Dimeric F240 IgA1 and IgA2

2.7.1 Cell Transfection

Depending on the readiness of the cells, the available volume of Expi293 Expression medium as well as availability of free spots in the incubator on the day of transfection, transfections of either 30 ml, 60 ml or 120 ml Expi293 cell cultures were done. Moreover, the Expi293 cells required about two weeks to be adapted from the small 30 ml culture volume to the big 120 ml culture volume. Accordingly, Expi293 cells were transfected with either, 30 µg, 60 µg or 120 µg of total DNA (composed of plasmids encoding for HC, LC and JC).

Tab. 4. Plasmids for recombinant antibody production

F240 dIgA1	**F240 dIgA2**
pcDNA3.4 F240-hA1	pcDNA3.4 F240-hA2m(1)
pcDNA3.4 F240-hK pcDNA3.4-hJ	

The table above shows the plasmids used for producing F240 dIgA1 and F240 dIgA2. For details concerning cloning of the respective plasmid, please consult the section “cloning strategies” in the "Results" section.

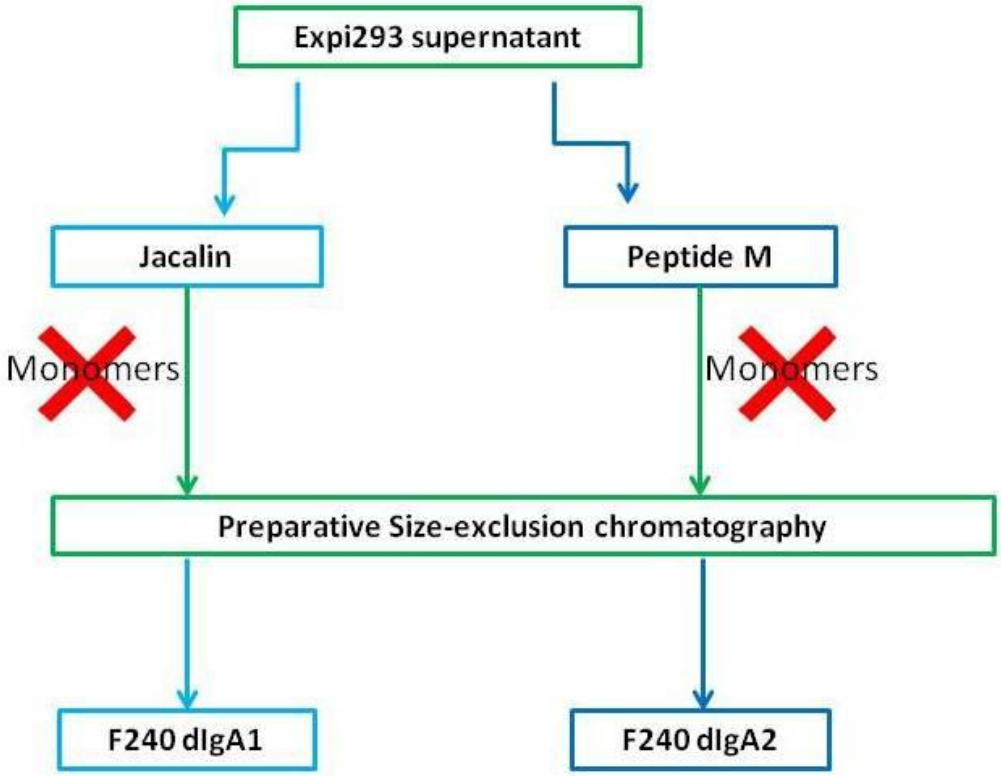


Fig. 8. Purification strategies for F240 dIgA1 and dIgA2

The purification strategy employed in this study exploited the molecular characteristics of the distinct subtypes of human immunoglobulin A. Common purification strategies are shown in green. Light blue shows the strategy employed for F240 dIgA1, dark blue refers to F240 dIgA2. Based on the glycosylation differences between IgA1 and IgA2 (refer to Introduction), Jacalin columns were used in this study to capture IgA1 and peptide M columns were used to capture IgA2 from Expi293 supernatants. The final separation of dimeric IgAs from monomeric IgAs and other remaining impurities was performed by SEC exploiting significant molecular mass difference between dIgA and other proteins. The final preparations of F240 dIgA1 and dIgA2 were analyzed by SDS-PAGE for purity.

2.7.2 Jacalin Affinity Chromatography

Ten ml of Jacalin Agarose (InvivoGen) were packed in a suitable plastic column (Bio-Rad Laboratories) and equilibrated with 10 column volumes of PBS (washing buffer). After equilibration, the supernatant was loaded onto the column with a flow rate of 0.45 ml/min. Unbound protein was washed away with 6 column volumes of washing buffer at a flow rate of 1 ml/min. Bound dIgA1 was eluted with elution buffer (0.1M α D-galactose). After each run, the column was washed with 9 column volumes of washing buffer. After a concentration step, eluate was stored at 4 °C until further usage.

2.7.3 Peptide M Affinity Chromatography

Ten ml of Peptide M Agarose (InvivoGen) were packed in a suitable plastic column (Bio-Rad Laboratories) and equilibrated with 10 column volumes of PBS (washing buffer). After equilibration, the supernatant was loaded onto the column with a flow rate of 0.45 ml/min. Unbound protein was washed away in the next step by washing the column with 6 column volumes of washing buffer at a flow rate of 1 ml/min. Bound dIgA2 was eluted with elution buffer (0.1 M glycine, pH 3) and immediately adjusted to pH 7.5 with Neutralization buffer. After each run, the column was washed with 9 column volumes of washing buffer. After a concentration step, eluate was stored at 4 °C until further usage.

2.7.4 Buffer Exchange and Protein Concentration

The eluates of the respective affinity chromatography were pooled and concentrated using Amicon® Ultra 15 ml Centrifugal Filters with a MWCO of 50 kDa (Merck Millipore). Briefly, after an equilibration step with PBS, three consecutive concentration steps were performed, each at 3.800 x g for 20 min. The concentrated eluate was stored at 4 °C until SEC.

2.7.5 Preparative Size-exclusion Chromatography

Preparative SEC was performed on an FPLC system (ÄKTA Purifier, GE Healthcare) composed of a high precision on-line monitor for measurement at 280 nm (UPC-900), a dynamic single chamber mixer (M-925) and a P-920 pump (all from GE Healthcare). The column used for separation was a HiLoad^{TM}16/60 SuperdexTM200 prep grade column (GE Healthcare) and the mobile phase was degased PBS. Proteins were loaded and eluted with a flow rate of 1 ml/min. Two ml sample aliquots were loaded onto the column for each run and 96 fractions were automatically collected. Chromatograms were recorded with the chromatography software UNICORN (Version 5.1, Amersham Biosciences, GE Healthcare), data were exported as MS Excel files and resulting chromatograms were plotted using

GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

2.7.6 Quantification of F240 dIgA1 and dIgA2

Concentration of IgA1/2 in the various intermediate purification products (Jacalin eluate, Peptide M eluate) as well as in the final preparation were measured with the Nanodrop1000 Spectrophotometer using the 'Protein' application mode (Protein 280), 1.5 µl of each sample was pipetted on the fiber optic cable of the instrument's measurement pedestal. Absorbance at the wavelength of 280 nm determined the sample concentration in ng/ml.

2.7.7 Analysis of Epitope Specificity of F240 dIgA1 and dIgA2

Antigen ELISA

Nunc MaxiSorp® flat-bottom 96 well plate (Affymetrix e Biosciece) was coated overnight at 4 °C with four distinct antigens: gp160 SHIV-1157, gp120 1157 ipK195, HIV-1-gp41 MN and KLIC Peptide Mix (peptides representing epitope of the parental F240 IgG1), diluted to 1 µg/ml in coating buffer. The next day, wells were blocked with 200 µl PBSTB for 2 h at RT. F240 dIgA1 and 2, HGN194 dIgA1 and 2 were added at 1 µg/ml in PBST. HGN194 dIgA1 and dIgA2 served as the antibody controls. For the assay controls please refer to the corresponding results section. After a washing step with water, HRP-conjugated goat anti-human alpha chain antibody (Jackson ImmunoResearch, diluted 1:2000 in PBSTB), was added to the wells for 1 h at RT. After three washing steps with water, wells were incubated with 100 µl of TMB solution. The reaction was stopped by adding 100 µl of 1 N sulfuric acid per well and the optical density (OD) determined at 450 nm using a multiwell plate reader (Berthold Technologies).

Peptide ELISA

Peptides used in the assay (Tab. 5) represented the epitope of parental F240 IgG1 antibody. Nunc MaxiSorp® flat-bottom 96 well plates (Affymetrix e Biosciece) were coated overnight at 4 °C with selected peptides of an HIV-1 Consensus Subtype C Env Peptide Library, 1 µg/ml in coating buffer each. Next day, wells were blocked with 200 µl PBSTB for 2 h at RT. After three washing steps with water, F240 dIgA1, F240 dIgA2, F240 IgG1 and Fm-6 IgG1 were added at 1 µg/ml. Parental F240 IgG1 and Fm-6 IgG1 served as antibody controls. Plates were incubated for 1 h at RT with HRP-conjugated goat anti-human alpha chain antibody

(Jackson ImmunoResearch) for F240 dIgA1 and 2 and with anti-human HRP (Jackson ImmunoResearch) for F240 IgG1 and Fm-6, each diluted 1:4000 in PBSTB. Plates were developed by incubation with 100 µl/well of TMB solution. Reaction was stopped by adding 100 µl of 1 N sulfuric acid per well and the optical density (OD) determined at 450 nm using a multiwell plate reader (Berthold Technologies).

Tab. 5. Peptide Sequences of Peptides 9329 – 9336

9329	ERYLKDQQLLGIWGC
9330	KDQQLLGIWGCSG**KL**
9331	LLGIWGCSG**KLIC**TT
9332	WGCSG**KLIC**TTAVPW
9333	G**KLIC**TTAVPWNSSW
9334	**C**TTAVPWNSSWSNKS
9335	VPWNSSWSNKSQEDI
9336	SSWSNKSQEDIWDNM

The table above gives the peptide sequences that were used in the peptide ELISA to exclude a shift in epitope specificity. The KLIC motive is highlighted in black.

2.8 Virion Capture Assay

96-well plates (NuncMaxisorp, Thermo Scientific) were coated with 100 µl of α-chain-specific goat anti-human serum IgA (diluted to 1 µg/ml in MPBST, Jackson ImmunoResearch) at 4 °C. Following blocking for 2 h with 200 µl MPBST, Abs were added at 5 µg/ml (100 µl) for 1 h at RT. After washing 5 times with PBS (200 µl/well) 100 µl of respective virus, serially diluted in 1x PBS to the desired input amount, was added overnight at 37 °C. Two assay controls were included for each assay, a virus only control (no antibodies, virus only) as well as an alpha control (omitting F240 dIgA1 and dIgA2). As a negative control purified human secretory IgA (Bio-Rad Laboratories), derived from human colostrum, was used.

The next day, wells were washed 5 times with 1x PBS (200 µl/well) before adding 125 µl disruption buffer mixture (25 µl disruption buffer per 100 µl TritonX-100) per well to release p27.

After incubation for 1 h, the amount of captured virus was assessed by p27 quantification of the virus lysate, using the p27 ELISA kit (ABL) according to the manufacturer's instructions, but using directly the 125 µl virus lysate as the sample input.

2.9 TZM-bl Neutralization Assay

96-well round bottom cell culture plates (Corning Inc.) were used for the assay. 100 µl of serially diluted (1:2 in TZM-bl medium) antibodies were added in duplicate to the dedicated wells followed by 50 µl of SHIV-1157ipEL-p.

As SHIV-1157ipEL-p will be used as the challenge virus in the subsequent passive immunization studies, it was therefore chosen as the test virus for the neutralization assay.

Virus was thawed and diluted in TZM-bl media to give a final dilution that produces a tissue culture infectious dose ($TCID_{50}$) of approximately 150,000 relative luciferase unit equivalents (RLUs) (1:125). Plate was then incubated for 1 h at 37 °C. During the incubation step, TZM-bl cell suspension was prepared to give a final cell density of 0.1×10^6 cells/ml per well. DEAE-Dextran (Sigma-Aldrich) was added to the cell suspension to give a final concentration of 15 µg/ml in the well. 100 µl of the TZM-bl cells were added to all wells and the plate was incubated for another 48 h at 37 °C. After two days, 150 µl of TZM-bl media was removed from all wells and 100 µl of Britelite Reagent added to each well (Promega) and incubated for

2 min at RT. Cell suspension was mixed, the remaining 150 µl transferred to a 96-well black plate (Bio-Rad Laboratories). Plate was read on a luminometer (Berthold Technologies) within 10 min. Percent neutralization was determined by calculating the difference in average RLU between virus control (cells + virus) and test wells (cells + serum sample + virus), dividing this result by the difference in average RLU between virus control (cell + virus, column) and cell control wells (cells only), and multiplying by 100. The antibody was non-neutralizing if it failed to score at least 50 % neutralization at any dilution.

2.9.1 TZM-bl media

Mix 500 ml DMEM (Gibco Life Technologies) with 50 ml of 10 % Fetal Bovine Serum, add 12.5 ml HEPES (1M) and 2.5 ml of gentamicin (10mg/ml) (all Sigma-Aldirch).

2.10 Gp160 Sequence Alignment

Gp160 sequences of the VCA-tested viruses were compared to each other using Megalign program of the LASERGENE bioinformatics software package (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed applying the Clustal V method of alignment with the preset default parameters (Gap Penalty=10, Gap Length Penalty=10).

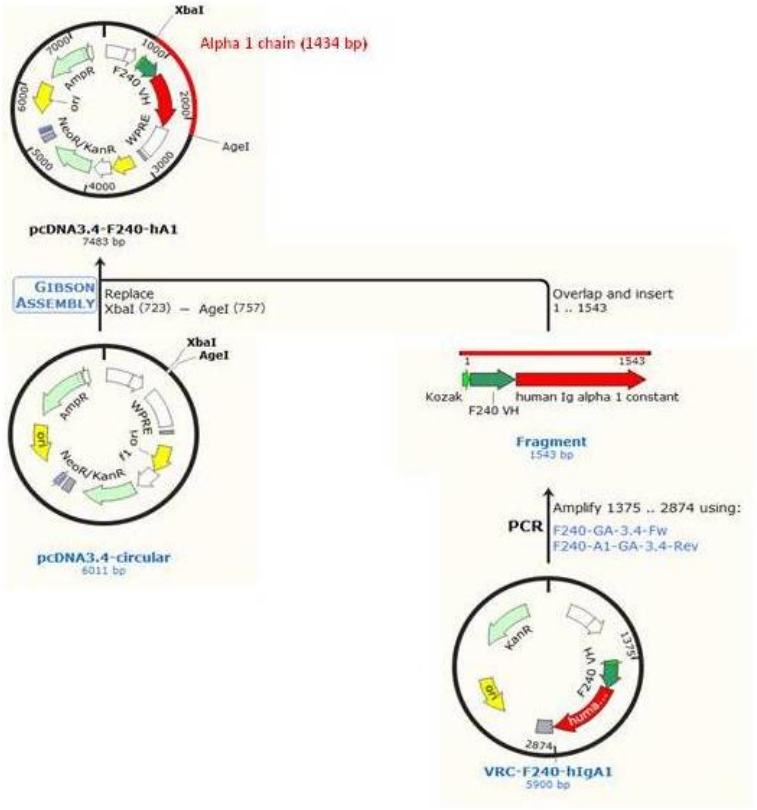
2.11 Plotting Graphs

All graphs in this thesis were plotted using GraphPad Prism (version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

3 Results

In this study we aimed to capture SHIV particles with dimeric versions of the already published F240 mAb. To achieve this goal we converted gp41-specific non-neutralizing mAb F240 of IgG1 isotype into IgA1 and IgA2. Toward this end, we cloned variable genes of F240 IgG1 into the corresponding IgA1 and IgA2 backbones. For this purpose primers had to be designed and were synthetized by GeneWiz. Corresponding vector regions and synthetized constructs were amplified by PCR and gel-purified. Gibson assembly was used to finally assemble PCR fragments into the respective plasmids encoding for HC, LC and JC. After cloning, plasmid integrity had to be tested by restriction digest before starting with preparative plasmid production. Once large-scale production of plasmids was finished, we expressed, purified and characterized F240 dIgA1 and F240 dIgA2 for epitope specificity, neutralization pattern and virion capture.

3.1 Cloning strategies



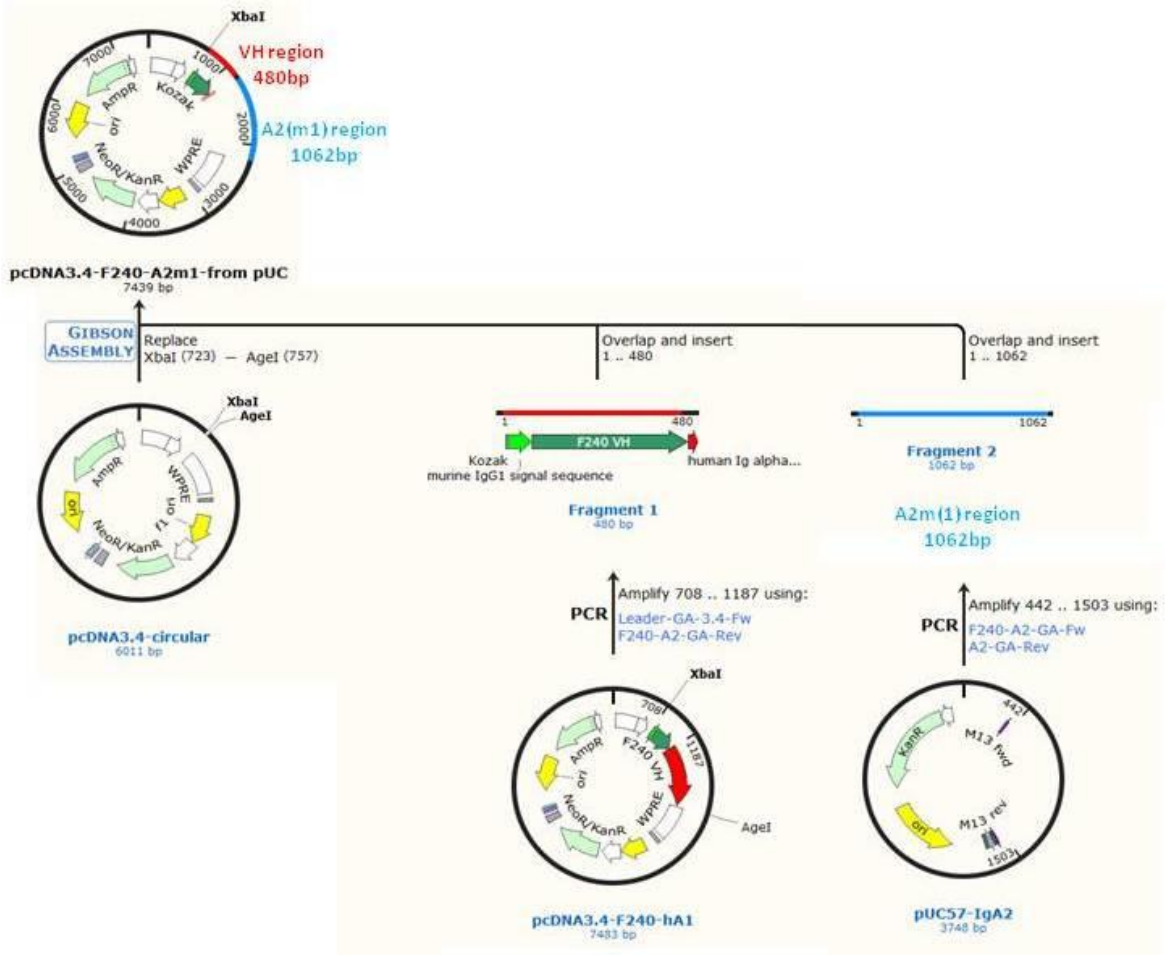
3.1.1 Cloning strategy for pcDNA3.4-F240-hA1

Sequences for full length human Ig alpha-1 chain (variable and constant region) were derived from GenBank (allele IGHA1*01, accession number constant region: >J00220; accession number variable region: >AF059199 (accessed 01/16/2015 at 07:00 p.m.). The full length human alpha-1 chain was provided inserted into the vector VRC-F240-hIgA1 and consisted of:

The Kozak sequence for translation initiation, a murine IgG1 signal sequence (light green), the HC variable region of F240 (“F240 VH”, dark green) and the HC constant region (“human Ig alpha 1 constant”, red).

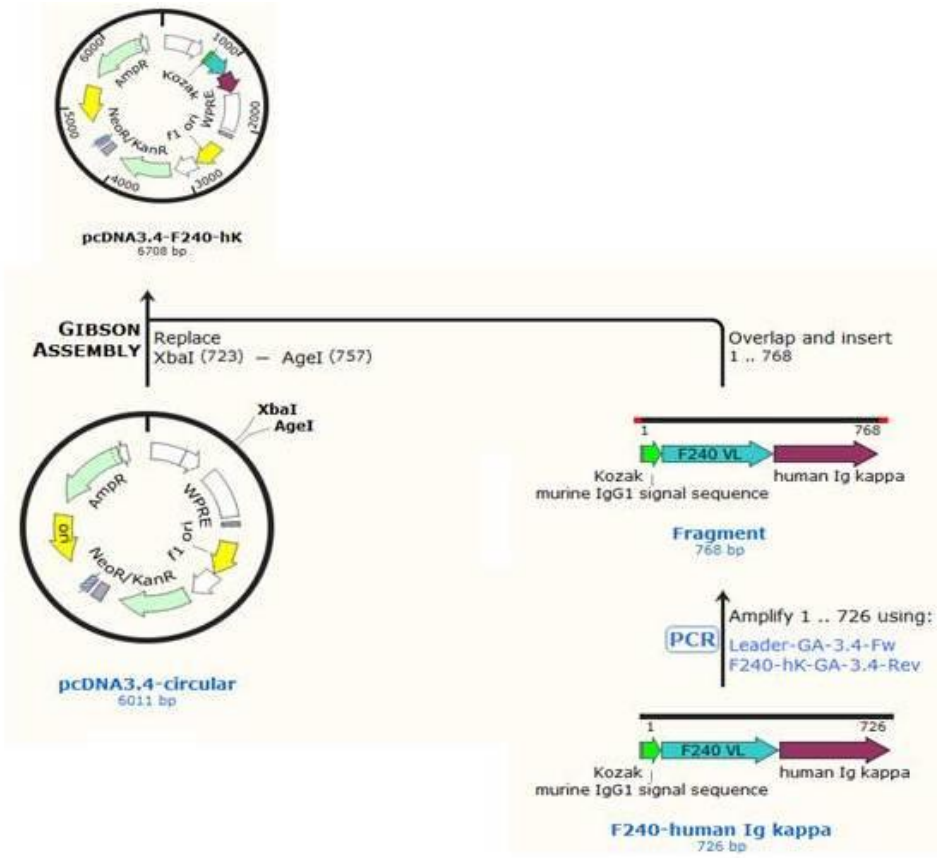
Using primers „F240-GA-3.4-Fw” and “F240-A1-GA-3.4-Rev”, the insert was amplified by PCR, introducing overlaps to the 5' end and 3' ends of the target vector pcDNA3.4, which are necessary for Gibson Assembly. Amplified and gel-recovered PCR fragment was finally Gibson-assembled to obtain the target construct "pcDNA3.4-F240-hA1".

3.1.2 Cloning strategy for pcDNA3.4-F240-A2m(1)



Sequence for constant heavy region of the human Ig A2m(1) was retrieved from GenBank (allele IGHA2*01, accession number >J00221 (accessed 01/16/2015 at 07:00 p.m.), synthesized by Genewiz and provided inserted into the vector pUC57 (pUC54-IgA2). Using the primers „F240-A2-GA-Fw" and "A2-GA-Rev", constant heavy region of IgA2 was amplified, introducing overlaps to the "F240 VH" fragment as well as to the 3' end of the target vector pcDNA3.4. As the variable region of the HC will be the same as in F240 dIgA1, it was amplified out of the already existing vector pcDNA3.4-F240-hA1 using the primers "Leader-GA-3.4-Fw" and "F240-A2-GA-Rev", introducing homologous regions to the 5' end of the target vector pcDNA3.4 and the 5' region of the A2m(1) region. Amplified and gel-recovered PCR fragments were Gibson-assembled to the final construct "pcDNA3.4-F240-A2m1-from pUC".

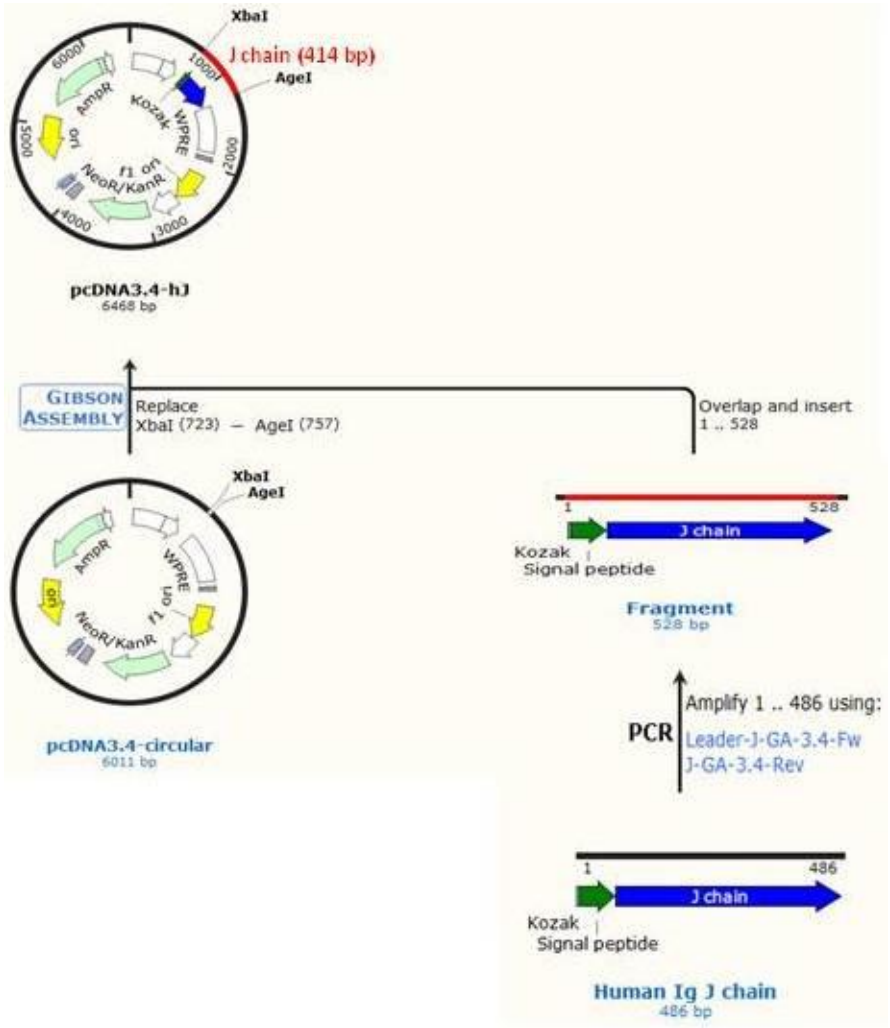
3.1.3 Cloning strategy for pcDNA3.4-F240-hK



Sequences for human Ig kappa chain (variable and constant region) were derived from GenBank, accession numbers: >AF059200, >AJ294735 (accessed 01/16/2015 at 07:00 p.m.). The full length human kappa chain was synthesized by Genewiz and provided inserted into the vector pUC57 and consisted of: The Kozak sequence for translation initiation, a murine IgG1 signal sequence (light green), the LC variable region of F240 (“F240 VH”, blue) and the LC constant region (“human Ig kappa”, purple).

Using the primers “Leader-GA-3.4-Fw” and „F240-hK-GA-3.4-Rev”, the insert was amplified by PCR, introducing overlaps to the 5' and 3' end of the target vector pcDNA3.4 which are necessary for the Gibson Assembly. Amplified and gel-recovered PCR fragments were Gibson-assembled to finally obtain the target construct "pcDNA3.4-F240-hK".

3.1.4 Cloning strategy for pcDNA3.4-hJ



Human JC precursor sequence (accession number >NM_144646.3, accessed 01/16/2015 at 07:00 p.m) was derived from GenBank. The sequence was synthesized by Genewiz, adding the Kozak sequence for translation initiation and the corresponding signal peptide. The whole construct (“Human Ig J-chain”) was provided inserted into the vector pUC57.

Using the primers „Leader-J-GA-3.4-Fw” and “J-GA-3.4-Rev”, the insert (“human Ig J-chain”) was amplified by PCR, introducing the overlaps to the 5' and 3' end of the target vector pcDNA3.4 which are necessary for the Gibson Assembly. Amplified and gel-recovered PCR fragment was Gibson-assembled to finally obtain the target construct "pcDNA3.4-hJ".

Tab. 6. List of Primers used for Insert Amplification

Plasmid cloned	Primer Name	Sequence
pcDNA 3.4-F240-hA1	F240-GA-3.4-Fw	ATCCAGCCTCCGGACTCTAGAACCACCATGGGATG GTCAT
	F240-A1-GA-3.4-Rev	ATCAAACTCATTACTAACCGGTTCAGTAGCAGGTGC CGTC
pcDNA 3.4-F240-A2m(1)	Leader-GA-3.4-Fw	ATCCAGCCTCCGGACTCTAGAACCACCATGGGATG GTCAT
	F240-A2-GA-Rev	CTTGGGGCTGGTCGGGGATGCTGAGGAGACGGTGA CCCGG
	F240-A2-GA-Fw	CCGGGTCACCGTCTCCTCAGCATCCCCG
	A2-GA-Rev	GATATCAAACTCATTACTAATCAGTAGCAGGTGCCG TCC
pcDNA 3.4-F240-hK	Leader-GA-3.4-Fw	ATCCAGCCTCCGGACTCTAGAACCACCATGGGATG GTCAT
	F240-hK-GA-3.4-Rev	ATCAAACTCATTACTAACCGGCTAACACTCTCCCCT GTTG
pcDNA3.4-hJ	Leader-J-GA-3.4-Fw	ATCCAGCCTCCGGACTCTAGAACCACCATGGGATG GTCAT
	J-GA-3.4-Rev	ATCAAACTCATTACTAACCGGTTGATCAGGATAGCA GGCA

Homology/Overlap to 5' end of pcDNA3.4, Homology/Overlap to 3' end of pcDNA3.4, Homology/Overlap to hA2m(1), Homology/Overlap to F240 VH

3.2 Plasmid Mapping

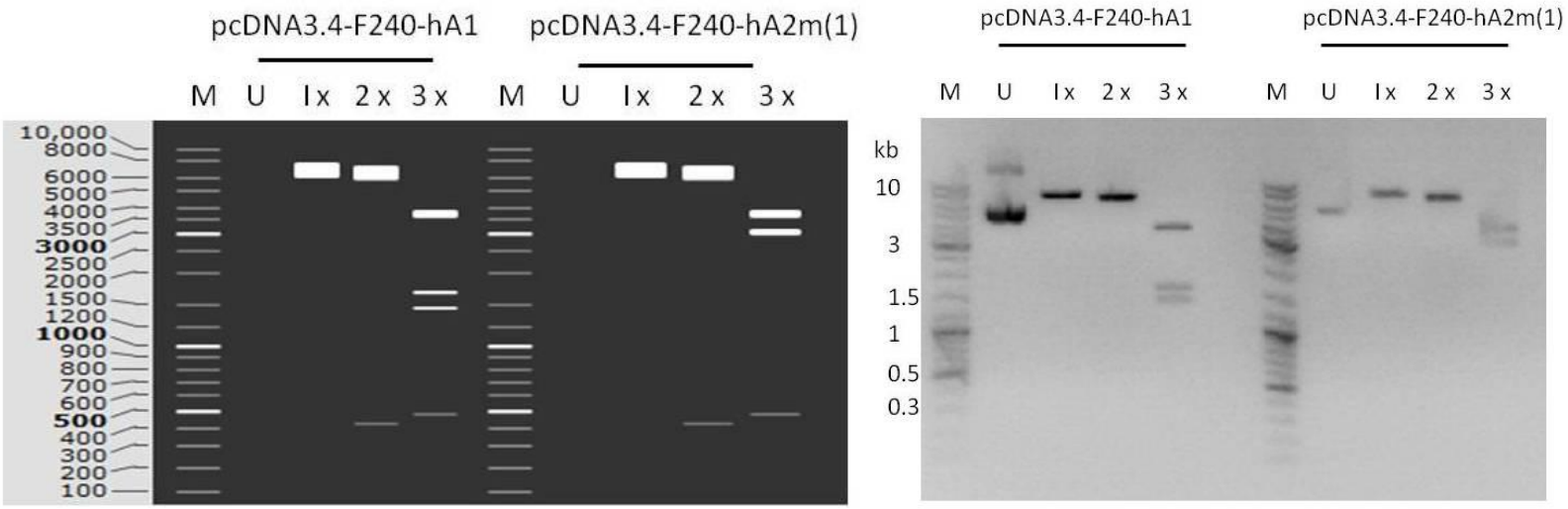


Fig. 9. Mapping of pcDNA3.4 F240-hA1 and F240-hA2m(1)

First, the plasmids pcDNA3.4-F240-hA1 and pcDNA3.4-F240-hA2m(1) were virtually digested in SnapGene. The real banding pattern was then compared to the virtual one of SnapGene: Plasmids were single (Ix, NdeI), double (2x, NdeI/XbaI) and triple (3x, NdeI/AgeI/AvrII) digested and analyzed on a 1 % agarose gel. The lane dedicated “U” (Undigested) was left empty because running the undigested plasmid was not possible within the programme. In the real gel, the lane "U" shows the undigested supercoiled plasmid. According to the simulation, both plasmids should display the band pattern of Tab. 2. Both plasmids displayed the expected simulated band pattern. The 424 bp, 478 bp band were too small to be visualized. DNA ladder mix (Thermo Scientific) was used as the marker in the simulation as well as in the real gel (15 µl).

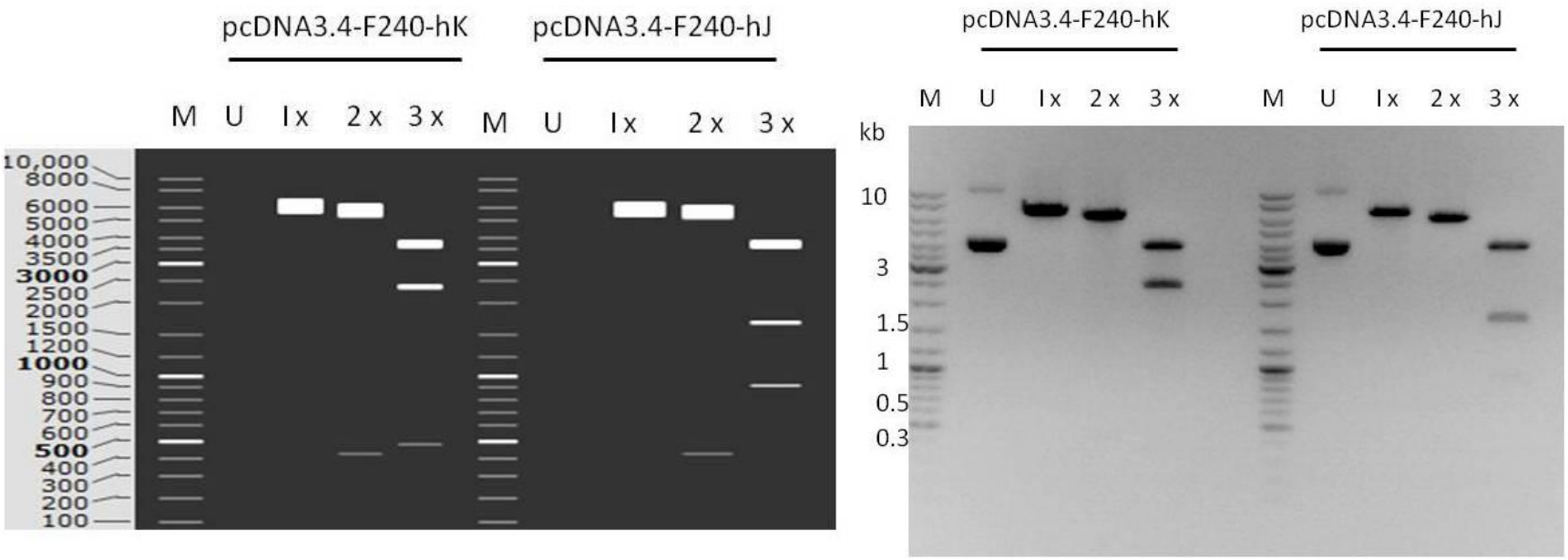


Fig. 10. Mapping of pcDNA3.4 F240-hk and pcDNA3.4 F240-hJ

First, the plasmids pcDNA3.4-F240-hK and pcDNA3.4-F240-hJ were virtually digested in SnapGene. The real banding pattern was then compared to the virtual one of SnapGene: Plasmids were single (Ix,NdeI), double (2x, NdeI/XbaI) and triple (3x, NdeI/AgeI/AvrII) digested and run on a 1 % agarose gel. The lanes dedicated “U” (Undigested) were left empty because running the undigested plasmid was not possible within the programme. In the real gel, the lane "U" shows the undigested supercoiled plasmid. According to the simulation, both plasmids should display the band pattern of Tab. 2. Both plasmids displayed the expected simulated band pattern. The 424 bp and 478 bp band were too small to be visualized. DNA ladder mix (Thermo Scientific) was used as the marker in the simulation as well as in the real gel (15 µl).

3.3 Pilot IgA Monomer Expression Study

3.3.1 SDS-PAGE of harvested supernatants

To check the expression of monomeric F240 IgA1, the harvested supernatants of the “pilot monomer expression study” were analyzed on SDS-PAGE. The chosen molar HC:LC ratio had an influence on the monomer expression. According to the SDS-PAGE, ratios 5:2 and 3:2 gave the best monomer expression (Fig. 11). The positive control, HGN194dIgA1, showed a band corresponding to the molecular weight of 350 kDa, as expected. Supernatant collected from the untransfected control cell culture didn’t show any band. A band with molecular weight around of 130 kDa was observed for the cell culture transfected with plasmids coding rabbit IgG (+Transfection). Possible difference in glycosylation could explain why monomeric F240 IgA1 showed a higher molecular weight than the full length rabbit monomeric IgG. All ratios tested lead to F240 IgA1 monomer expression at around 150 kDa and HC:LC ratios of 5:2 and 3:2 gave the best expression yield. Bands observed between ~55 kDa and ~100 kDa were, according to the negative control of the Expifectamine cell transfection (“-Transfection”), caused by the addition of transcriptional enhancers I and II to the transfected cells.

3.3.2 End Point Titration

Because SDS-PAGE analysis could not provide enough information to conclude on what molar HC:LC ratio results in a better yield of monomeric IgA1, we performed end-point titration ELISA to determine the highest yield of monomeric IgA1. Tab. 7 summarizes the percentage values obtained after the normalization that reflect relative content of monomeric IgA1. Supernatants “5:2” and “3:2” had the highest percentage values at each dilution, in both cases constantly decreasing with higher dilutions. From that we have concluded that supernatants "5:2" and "3:2" contained the highest amount of monomeric F240 IgA1. Thus, the molar HC:LC ratios “5:2” and “3:2” gave the best monomer expression.

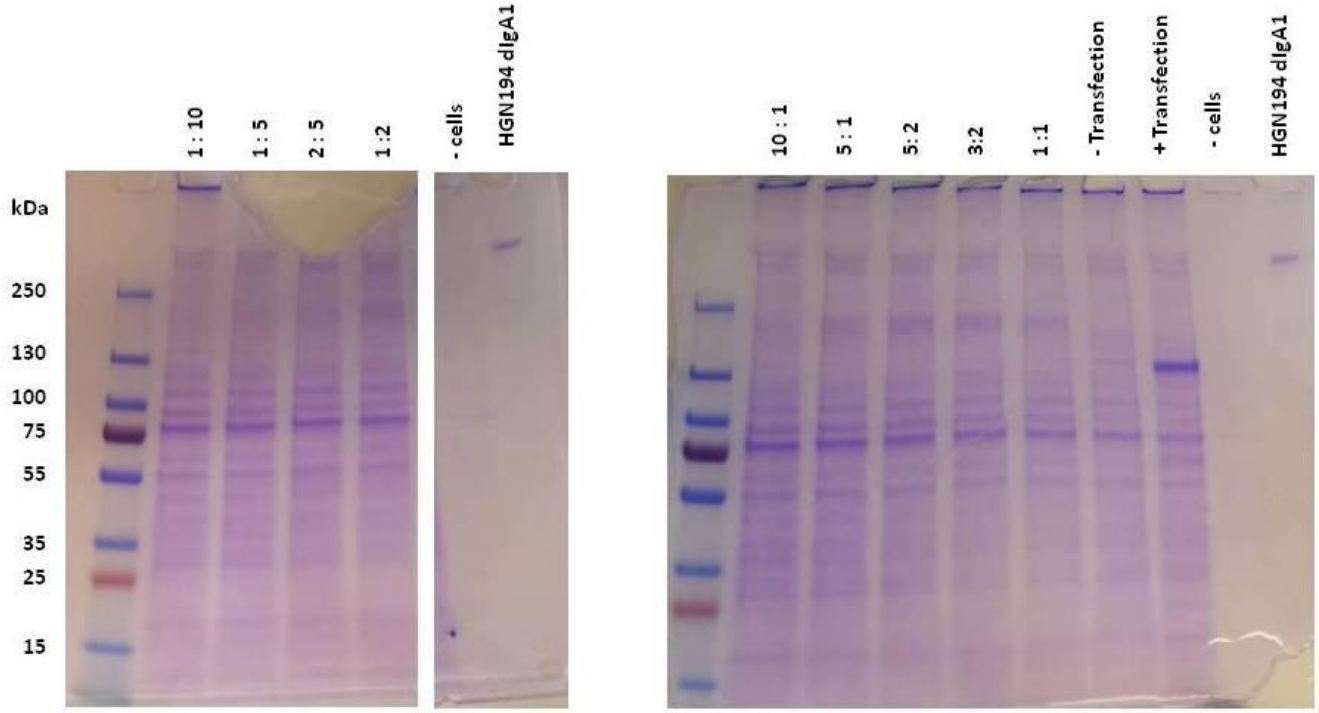


Fig. 11. SDS PAGE of the harvested supernatants

Expi293 cells were transfected with different molar ratios of HC plasmid to LC plasmid using the Expifectamine™ Transfection kit. Two negative and two positive controls were used: Supernatant from the Expi293 maintenance flask (20 µl) served as the negative control ("-cells") for the gel, HGN194 dIgA1 (0.5 µg) as a positive control for the gel. The positive control of the Expifectamine Transfection kit (full length rabbit IgG, "+Transfection", 20 µl) was included to confirm successful cell transfection. For the negative control of cell transfection ("-Transfection", 20 µl), no plasmids were added to the transfection mix. Different HC to LC plasmid ratios led to variable monomer expression.

Tab. 7. Sample Normalization Values after End-Point Titration of Harvested Supernatants

HC:LC	10 : 1	5 : 2	3 : 2	2 : 3	2 : 5	1 : 10
Dilution						
1 : 5	7981	9944	9006	8475	3484	2516
1 : 25	3207	3940	3753	3530	2889	1932
1 : 125	1916	2800	2563	2363	1458	721
1 : 625	663	1458	1321	1055	841	341
1 : 3125	198	496	457	335	**306**	**138**
1 : 15625	**111**	205	166	**166**	81	74
1 : 78125	81	**129**	**114**	90	56	49

An End Point Titration was carried out to compare the monomer expression in the different transfection reactions. Briefly, 96 well plates were coated with gp160 HIV1084i at 1 µg/ml and supernatants were added at serial 1:5 dilutions. The negative control of the cell transfection (no plasmids) was used as a negative control and also added at 1:5 dilution steps to the dedicated wells. Plates were developed with a peroxidase-conjugated goat anti-alpha chain antibody (Southern Biotech). For each negative control dilution, the average plus three times the standard error of the mean was calculated and used as the „normalization value“. This normalization value corresponded to 100 %, all sample values of the respective dilution were normalized upon that value.

A sample was positive when its normalization value exceeded 100 % ("positive sample", green values). If a sample was positive at a higher dilution in comparison to another sample, it contained more monomeric F240 IgA1.

3.4 Pilot Dimer Expression Study

3.4.1 SDS-PAGE and Western Blot analysis of harvested supernatants

Before aiming at large scale production of F240 dIgA1, correct assembly of dimeric antibody molecules containing JC had to be assured. A pilot dimer expression study was carried out: Expi293 cells were transfected with two different molar HC:LC ratios, 3:2 and 2:3. JC plasmid was added on top with a 1:1 ratio [(HC:LC):JC]. Six days after transfection, supernatants were harvested and analyzed on SDS-PAGE and Western Blot (Fig. 12).

SDS-PAGE confirmed successful dimer expression (Fig. 12). The transfection protocol worked, the positive control of transfection, whole length rabbit IgG (provided as plasmids together with the Expifectamine Kit), showed the expected band at ~130 kDa. Supernatant from the parental non-transfected Expi293 cell culture, the negative control ("Supernatant"), showed no bands. Both (HC:LC):JC ratios led to expression of F240 dIgA1 which appeared as distinct bands around 350 kDa.

Western Blot gave final confirmation of antibody expression, both alpha and kappa chains were detected. Controls showed the expected bands: HGN194 dIgA1 at ~350 kDa on the membrane developed with anti-alpha secondary antibody. As the positive control contains lambda LC, it was not visible on the membrane developed with an anti-kappa secondary antibody. HGN194 IgG1 used as a negative control showed no bands in either of the two membranes. Besides bands at ~350 kDa corresponding to F240 dIgA1, bands around 100 kDa and between 130 kDa and 220 kDa were observed. These bands were considered as byproducts of the F240 dIgA1 production, very likely partially assembled monomers. Subsequent chromatography steps were carried out to purify the dimers and remove these impurities. As on the gel, there were no obvious differences observed in the amount of dIgA1 expressed in cultures transfected with different HC:LC ratios. Thus, we pooled collected supernatants for TAC.

The obtained concentrated eluate will be further referred to as “putative F240 dIgA1” and was further purified by SEC. Finally, fractions containing F240 dIgA1 were pooled to give “pure F240dIgA1". Pure F240 dIgA1 was checked for presence of human JC as well as for correct epitope specificity within the same assay.

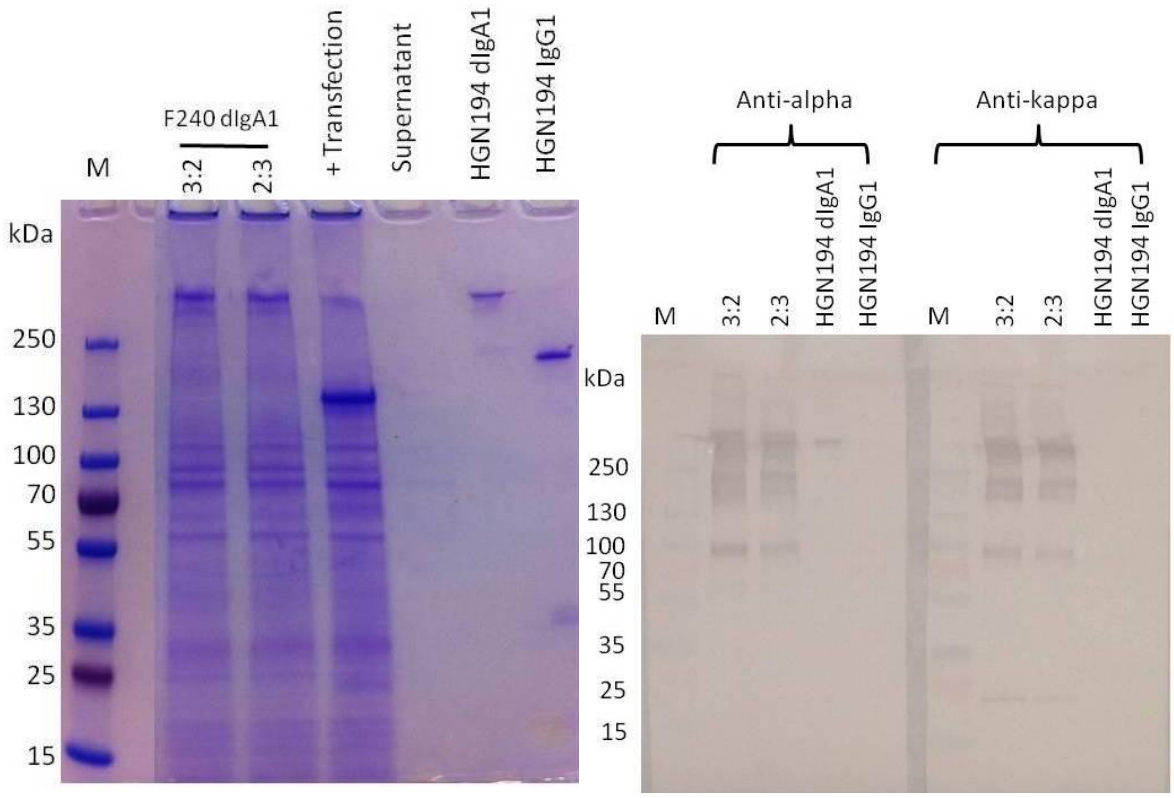


Fig. 12. Quality control of harvested supernatants (SDS-PAGE and Western Blot)

Expi293 cells were transfected with two different HC to LC plasmid molar ratios, 3:2 and 2:3. JC plasmid was added on top with a 1:1 ratio [(HC:LC):JC]. SDS-PAGE and Western Blot were performed to check supernatants for expression of F240 dIgA1. For Western Blot, HGN194 dIgA1and HGN194 IgG (0.1 µg each) served as the controls. For SDS-PAGE, 4 µg of HGN194 dIgA1 was run as a positive control, 4 µg of HGN194 IgG as an additional control. Both assays confirmed successful expression of dimeric F240 IgA1. M: Page Ruler Plus Prestained Protein Ladder (Thermo Scientific).

3.4.2 Purification of F240 dIgA1

3.4.2.1 Thiophilic Adsorption Chromatography

Sixty ml of F240 Expi293 supernatant was purified by TAC. Proteins were eluted as a single peak. The obtained eluate was concentrated down to 1 ml. Concentrated eluate is referred to as “putative F240 dIgA1”.

3.4.2.2 Size-exclusion Chromatography

Putative F20 dIgA1 was further purified by SEC (Fig. 14). Six runs, 200 µl each, were performed. Based on the size distribution of HGN194 dIgA1, fractions 24 - 51 of the putative F240 dIgA1 were analyzed on an SDS-Page (Fig. 15). The 315 kDa band representing pure dimers was observed from fraction 29 till fraction 33. Based on gel and chromatogram, fractions 27 till 34 of all runs were finally pooled and will be subsequently referred to as "pure F240 dIgA1". Fig. 17 gives a summary of the whole purification process and shows the progress of purification from the crude cell supernatant till the pure F240 dIgA1. 0.92 mg of F240 dIgA1 was finally isolated out of 60 ml of supernatant (Tab. 8).

3.4.2.3 Analysis of Epitope Specificity

Pure F240 dIgA1 was analyzed for presence of human JC and epitope specificity. ELISA revealed presence of JC in the F240 dIgA1. F240 dIgA1 and IgG1 successfully recognized gp160, gp41 and KLIC Peptide Mix, but did not react with gp120 that doesn’t contain epitope of F240 mAb (Fig. 16).

3.4.3 Choosing a HC:LC:JC ratio for large scale expression of F240 dIgA1 and dIgA2

We expressed dimeric F240 in a two-step approach. Fig. 13 depicts the proposed model of dimer production and assembly. It was assumed that efficient expression of monomeric IgA is critical for subsequent dIgA assembly. According to the pilot monomer expression study the best IgA1 monomer expression was achieved for HC:LC ratios equal 5:2 and 3:2. Because we didn’t see any difference between HC:LC ratios of 5:2 and 3:2, we choose HC:LC ratio equal 2:1 for the large scale production of F240 dIgA1. The plasmid encoding JC was added on top giving a final (HC:LC):JC molar ratio of (2:1):1. For large scale production of F240 dIgA2, the same HC:LC:JC ratio of (2:1):1 was chosen.

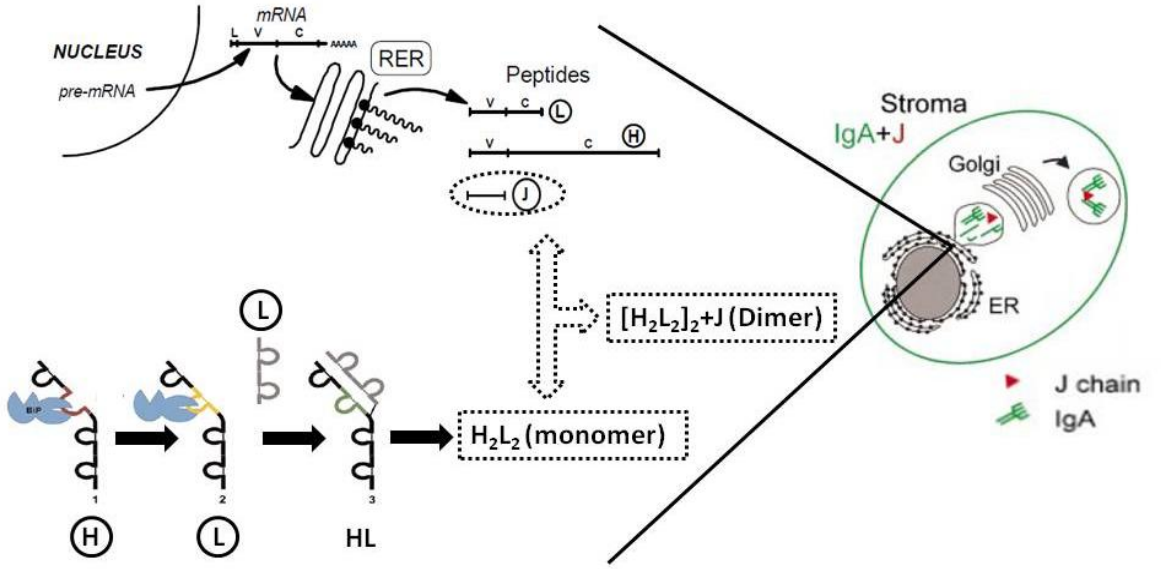


Fig. 13. Model for the production and assembly of immunoglobulin molecules

The genes for heavy (H encircled) and light (L encircled) chains are composed of a variable region (V) and a constant region (C) and have both an N-terminal leader sequence (L). After transcription and export of the corresponding m-RNAs into the cytoplasm, translation at the rough endoplasmic reticulum follows. In parallel to translation, subunit folding, separately for HC and LC, already begins. Both nascent chains are co-translationally translocated into the rough endoplasmic reticulum. Newly translated HC are bound to the chaperone BiP (blue), an ER resident chaperone molecule, via their unfolded CH1 domain (red, 1). After formation of an intramolecular disulfide bond in the CH1 domain (yellow, 2), the CH1 domain folds to its native structure (green) upon interaction with the complementary constant domain of the LC (gray). BiP prevents HC aggregation and presents the HC for proper interaction with LC. (Only one of the two HC and two LC in the final assembled antibody are depicted). The mature antibody structure (H_2L_2) is finally assembled during intracisternal transit through the endoplasmic reticulum and gets secreted after trafficking through the Golgi apparatus. For the expression of F240 dIgA1, it was assumed that correct monomer assembly is necessary for the later Joining of two monomers by the JC (for further details please consult the section "Pilot Monomer Expression Study" in the Materials and Methods section). Modified after Hebert and Gierasch (2009), Course Notes of “Medical Immunology 544 (Fall 2011)”, University of California, Irvine, College of Medicine; Johansen et al. (2000).

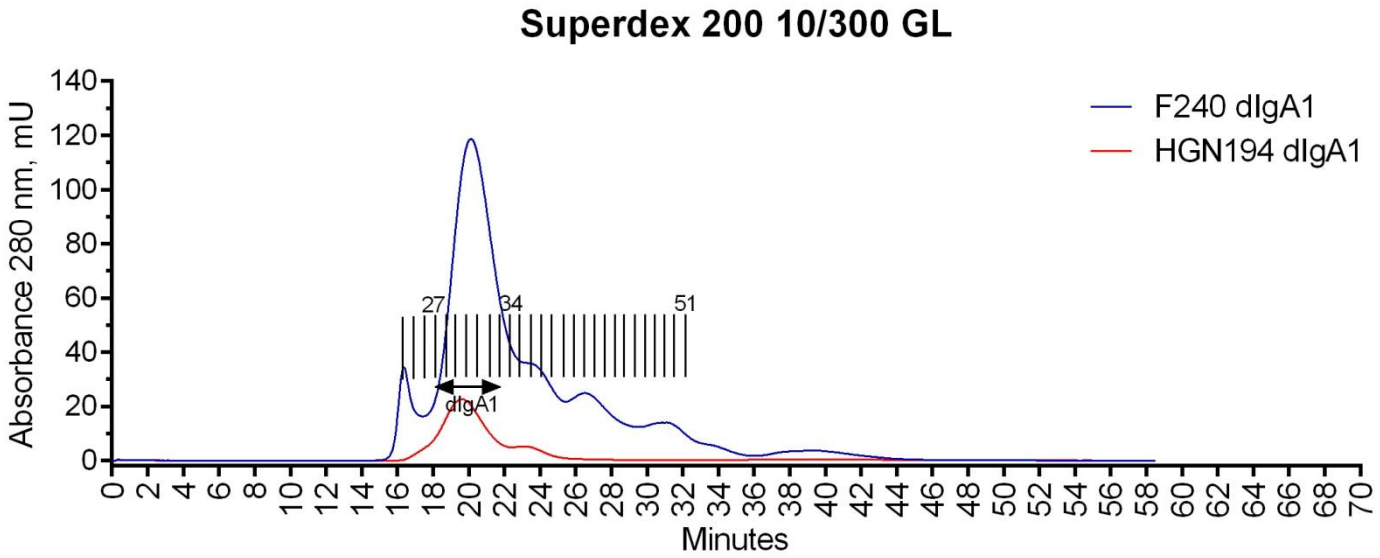


Fig. 14. Size-exclusion Chromatography of putative F240 dIgA1

Total IgA1 obtained by TAC was concentrated and further purified by analytical SEC on a Superdex 200 10/300 GL column (GE Healthcare). 100 µg of HGN194 dIgA1 was run as a positive control to estimate size distribution of the collected fractions. 200 µl TAC eluate aliquots were loaded onto the column and remaining monomeric impurities removed. Besides the main peak around fraction 33, monomeric (around fraction 38) and other low molecular weight impurities were detected in the putative F240 dIgA1. A subsequent SDS-PAGE was run to determine the size of the impurities. Chromatography fractions are indicated by vertical black lines.

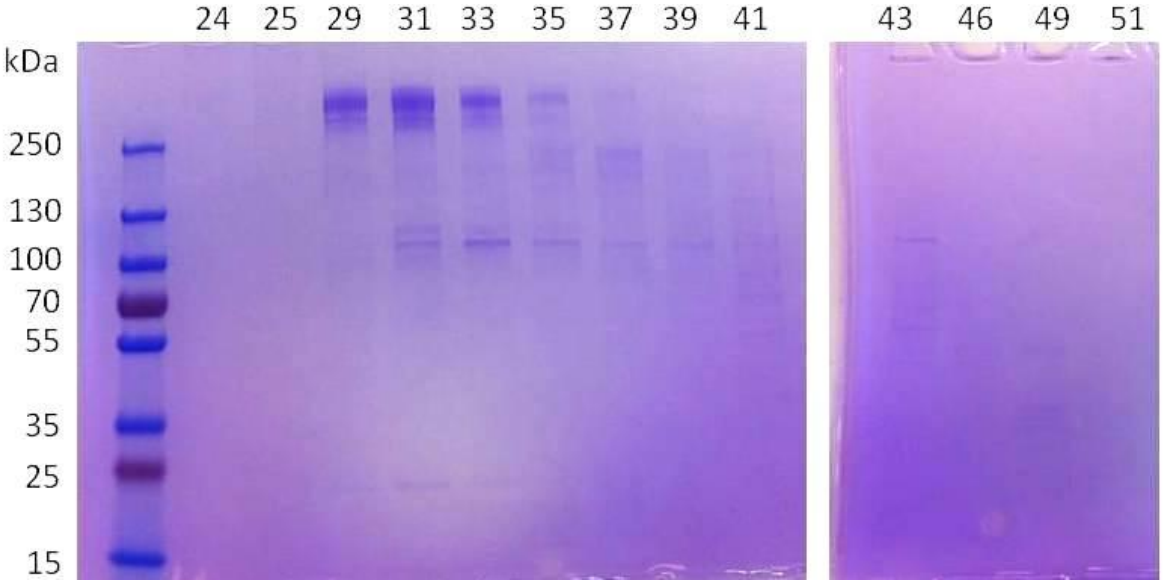


Fig. 15. SDS-PAGE of putative F240 dIgA1. Fractions 24 - 51.

Based on the size distribution of HGN194 dIgA1, fractions 24 till 51 of putative F240 dIgA1 were run on an SDS-PAGE. Due to space constraints on the gel, only 13 representative fractions could be analyzed. Fractions 29 till 33 showed clear bands of F240 dIgA1, unspecific protein bands around 100 kDa were not removed from the dimers by SEC. Finally, based on the gel and on the chromatogram, fractions 27 till 34 (indicated with numbers in the chromatogram) of all runs were pooled and stored at 4 °C till further usage in downstream assays.

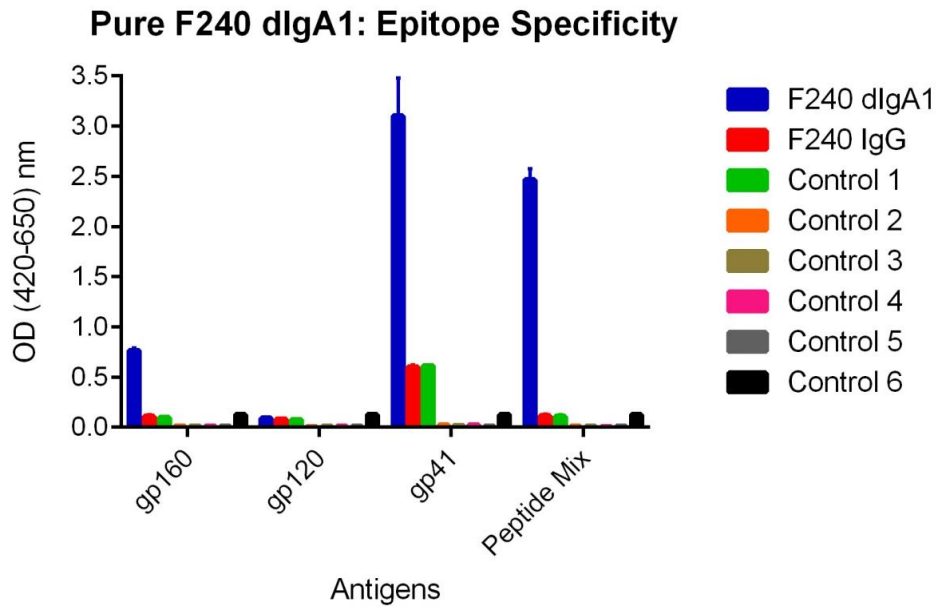


Fig. 16. Analysis of Epitope Specificity of F240 dIgA1

96-well plates were coated overnight at 4 °C with four distinct antigens (gp160 SHIV-1157, gp120 1157 ipK195, HIV-1-gp41 MN and KLIC Peptide Mix) diluted to 1 µg/ml in coating buffer. After blocking for 2 h in PBSTB at RT, samples (F240 IgG1 and F240 dIgA1) were added at 250 ng/µl. Polyclonal rabbit anti-human JC antiserum (InvivoGen), followed by HRP-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) were added. Six controls were applied to exclude any cross-reactions of the antibodies involved. Control I showed that there is some cross-reactivity of the polyclonal rabbit anti-human JC antiserum with the respective antigen tested. For further details, please refer to Tab. 3 in the Materials & Methods Section. F240 dIgA1 and IgG1 both bound to gp160, gp41 and KLIC Peptide Mix, but did not react with gp120 that doesn't contain epitope of F240 mAb.

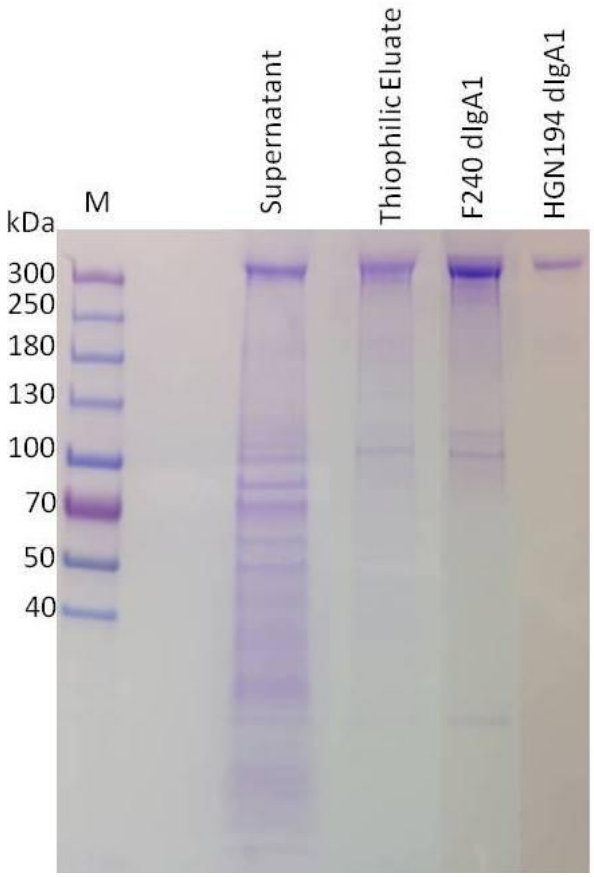


Fig. 17. Summary of F240 dIgA1 Purification Process (small scale)

In the first step, F240 dIgA1 was purified from the supernatant (“Supernatant”) by TAC. The obtained eluate ("thiophilic eluate", 4 µg loaded) was subjected to SEC. SEC was however not able to remove the unspecific protein bands around 100 kDa. The final preparation (“F240 dIgA1”, 4 µg) is composed of the pooled SEC fractions 27-34 and was concentrated as well as sterile filtrated through a 0.22 µm sterile filter. It contains dimeric F240 A1. The concentration measured by Nanodrop was 0.92 mg/ml. 1 ml of pure F240 dIgA1 was obtained. 1 µg of HGN194dIgA1 was run as a positive control. M: Spectra™ Multicolor High Range Protein Ladder (Thermo Scientific, 15 µl).

Tab. 8. Amount of IgA1 obtained along the purification

Purification Step	Protein concentration/amount	Amount (mg)
Harvest	0.060 l Supernatant	-
TAC	1 ml (3.94 mg/ml)	3.94 mg
SEC & Final Concentrating	1 ml (0.92 mg/ml)	0.92 mg

The table shows the amount of IgA1 obtained at each purification step. 60 ml of supernatants were collected ("Harvest") and gave a total of 3.94 mg of IgA1 after concentrating the pooled eluates of the TAC. SEC removed remaining impurities, pure dimeric SEC fractions (compare Fig. 15) were pooled to finally get pure F240 dIgA1. The final prep was concentrated to reduce the volume ("SEC & Final concentrating").

3.5 Large Scale Production: F240 dIgA1

On day six after transfection 1770 ml of F240 dIgA1 containing supernatant were harvested (Tab. 9). After pooling and sterile filtration, supernatant was checked for expression of F240 dIgA1 and presence of JC (Fig. 18). Corresponding controls showed the expected behaviour. SDS-PAGE confirmed dimer expression as did Western Blot the presence of the JC in the dimers.
Quality controlled supernatants were ready for Jacalin affinity chromatography.

3.5.1 Quality Control of harvested F240 dIgA1 supernatants

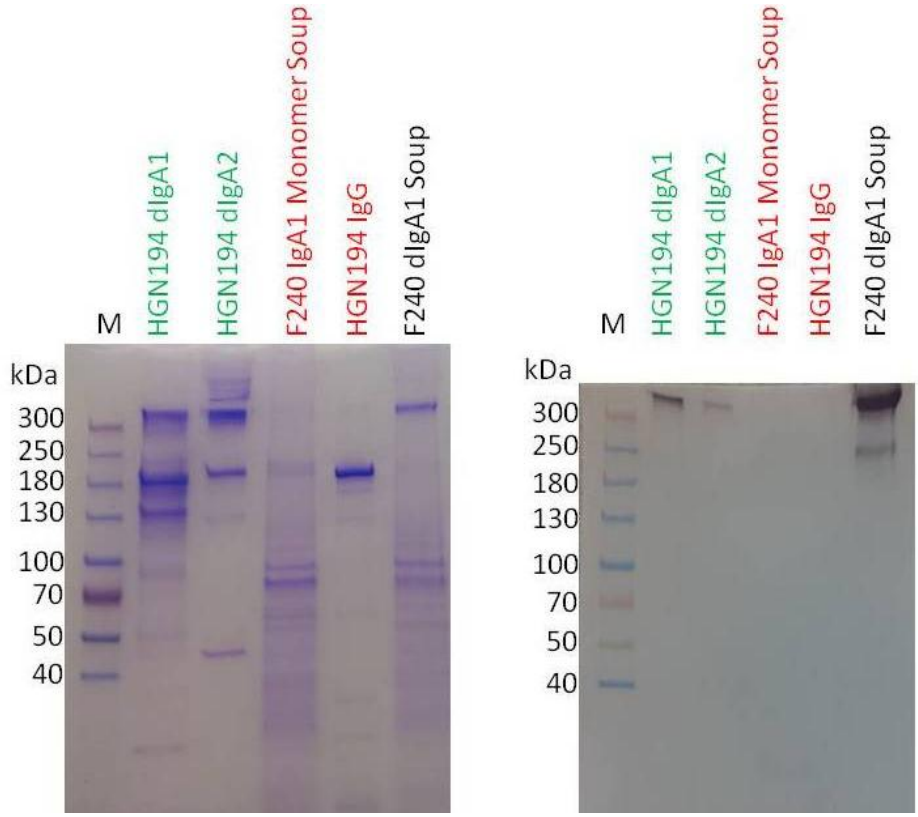


Fig. 18. Analysis of F240 dIgA1 for Joining chain

Presence of the JC was a prerequisite for all further purification steps. An SDS-PAGE and the corresponding Western Blot are shown. Negative controls are marked in red, positive controls are marked in green. As expected**,** HGN194 dIgA1 and dIgA2 (4 µg for SDS-PAGE, 0.1 µg for Western Blot) contained the JC. The monomer supernatant (no JC plasmid added at transfection) as well as HGN194 IgG (1.6 µg for SDS-PAGE, 0.1 µg for Western Blot) contained no JC.

3.5.2 Jacalin Affinity Chromatography

After having passed the quality control, dIgA1 had to be purified out of the pooled sterile filtered supernatants of the large scale transfections. As mentioned previously, 1770 ml sterile filtered Expi293 supernatant was available for purification. 4 runs, each with 443 ml supernatant, were performed. IgA was eluted as a single peak. A subsequent SDS-PAGE was run to confirm that dimers were eluted from the column (Fig. 19).

Despite minor dimeric protein losses in flow through and wash, jacalin chromatography successfully captured most of IgA1. Unspecific proteins between ~40 kDa and ~100 kDa were removed and enriched in the flow thorough together with protein smears. All eluates were pooled, concentrated and the amount of eluted IgA1, determined by Nanodrop, was 28.9 mg (Tab. 9).

3.5.3 Preparative Size-exclusion Chromatography

The remaining monomeric impurities around 180 kDa had to be removed by preparative SEC.

Two main peaks were observed, the first peak starting from fraction 31 and ending with fraction 35. The second peak started from fraction 37 and ended with fraction 41. Based on the chromatogram, we selected fractions 28-43 to be analyzed in an SDS-PAGE. SDS-PAGE revealed that the first peak contained pure dimers and that the second peak can be attributed to monomers. Thus, SEC successfully separated the dimers from the monomers (Fig. 20). Based on the SDS-PAGE and on the chromatogram, fractions 32-36 were pooled and considered "pure F240 dIgA1" (Fig. 21).

Fig. 22 gives a summary of the whole purification process and shows the progress of purification from the crude cell supernatant till the pure F240 dIgA1. 13.6 mg of F240 dIgA1 was isolated out of 1770 ml of supernatant. This finally gives an F240 dIgA1 yield of 7 mg/l.

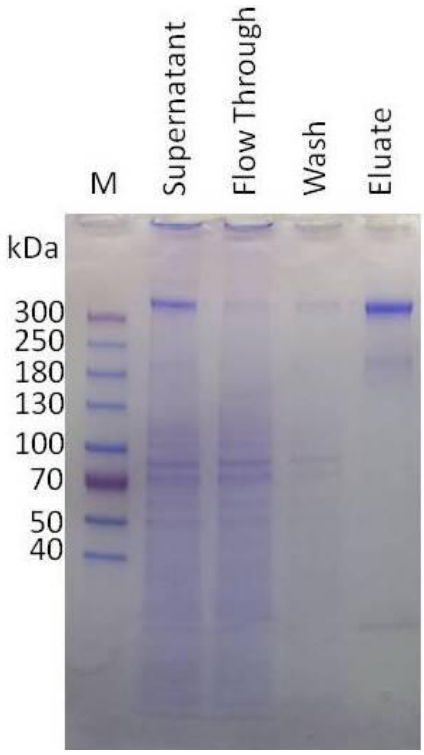


Fig. 19. SDS-Page of Jacalin Chromatography Fractions

The supernatant and the obtained chromatography fractions, Flow Through (unbound IgA1 and unspecific proteins), Wash (IgA1 molecules washed away from the column) and Eluate (eluted IgA1 specific molecules), are shown. Dimers were successfully enriched by jacalin chromatography and showed some monomeric impurities which needed to be removed by SEC. M: Spectra Color High Range Protein marker (15 µl, Thermo Fisher).

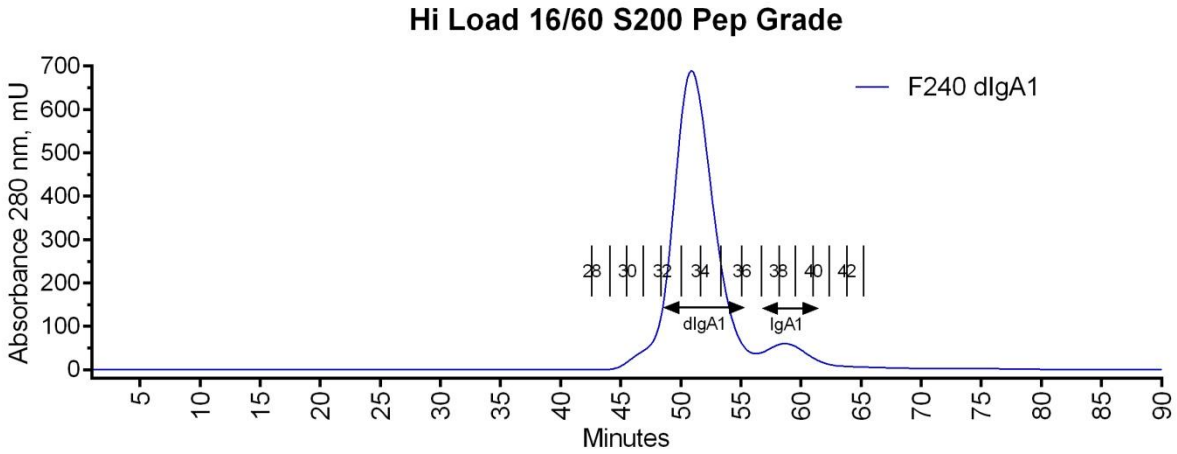


Fig. 20. Size-exclusion chromatogram of concentrated Jacalin eluate

Total IgA1 obtained by jacalin chromatography was concentrated and further purified by preparative SEC on a HiLoad^{TM}16/60 Superdex^{TM}200 pep grade column. 2 ml aliquots were loaded onto the column and remaining monomeric impurities removed. Besides the main peak around fraction 33 (dIgA1), a monomer peak was detected around fraction 38 (IgA1). Chromatography fractions are indicated by vertical black lines. A subsequent SDS-PAGE was run to decide which fractions are to be pooled (Fig. 21).

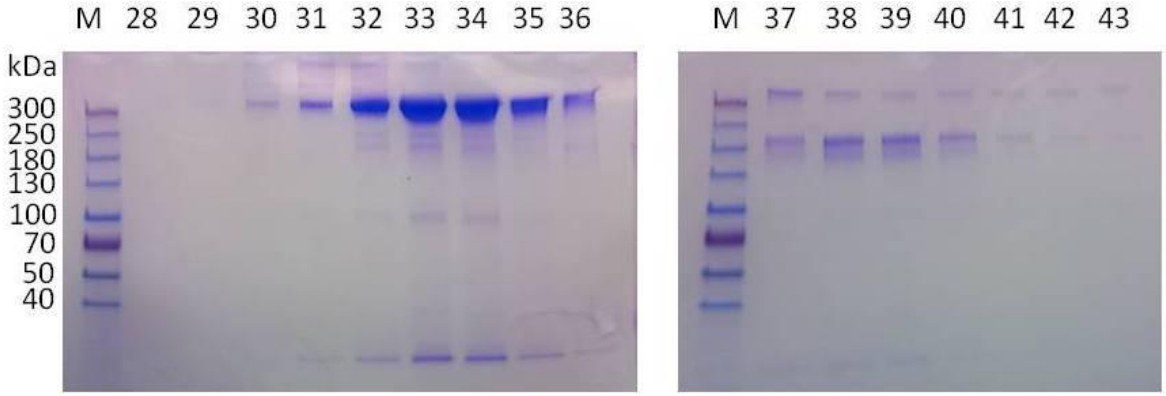


Fig. 21. SDS-PAGE of preparative size-exclusion chromatography fractions

SEC fractions 28 till 43 were loaded onto the gel. According to the gel pattern observed, fractions 32 till 36 (all containing the pure dimer, compare to the main peak in the chromatogram) were pooled for subsequent concentration and sterile filtration. It will further be referred to as "pure F240 dIgA1". Fractions 38-41 were monomers and excluded from further analysis. M: Spectra Color High Range Protein marker (15 µl, Thermo Scientific).

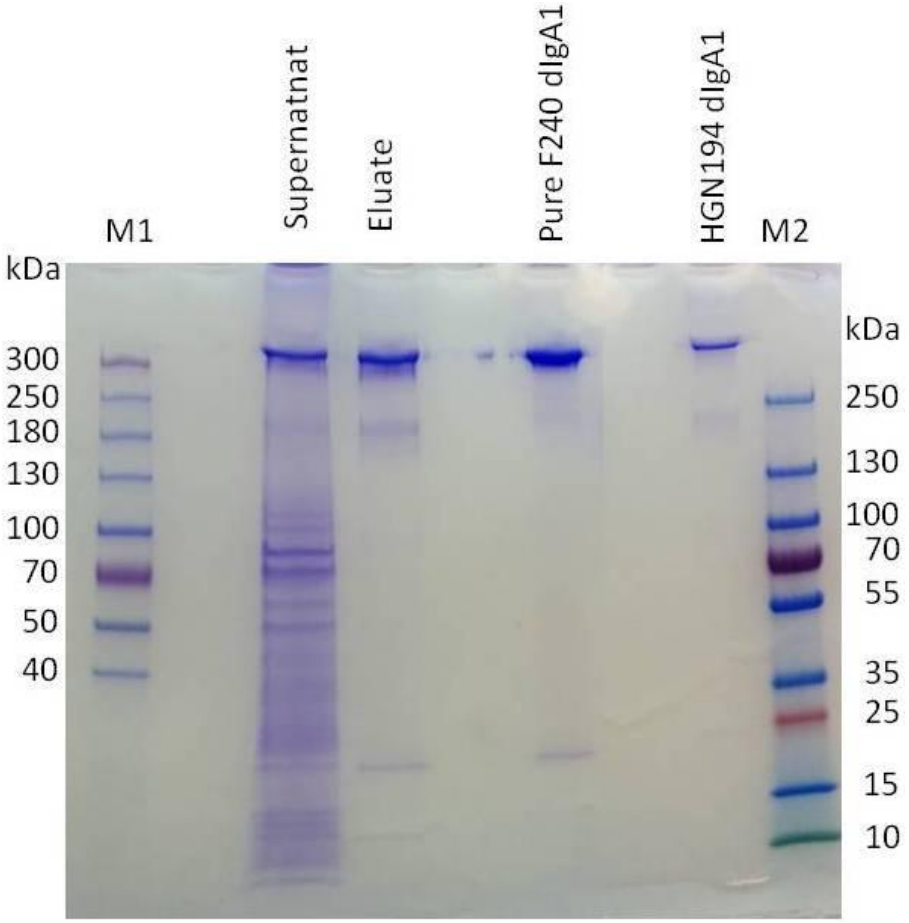


Fig. 22. Summary of F240 dIgA1 Purification Process (large scale)

A summary of the large scale purification process for F240 dIgA1 is shown. F240 dIgA1 was purified from the supernatant ("Supernatant") by jacalin affinity chromatography. The eluate ("Eluate") contained monomeric fractions around 180 kDa, which were successfully removed by SEC. The lower molecular weight bands below 40 kDa were caused by heat pre-treatment of the samples before loading them onto the gel. The final preparation ("pure F240 dIgA1", 4 µg) is composed of the pooled SEC fractions 32-36 and was concentrated as well as sterile filtrated through a 0.22 µm sterile filter. It contains only F240 dIgA1. The concentration of dIgA1 measured using Nanodrop at 280 nm was 1.51 mg/ml yielding 13.6 mg of pure F240 dIgA1, approximately. The positive control, HGN194dIgA1 (1.6 µg) is also indicated. M1: Spectra Color High Range Protein marker (15 µl); M2: Page Ruler Plus prestained protein ladder (both 15 µl, both Thermo Scientific).

Tab. 9. Yield of IgA1 along the purification

Purification Step	Volume of purification product (concentration)	Amount (mg)
Harvest	1.77 l of Supernatant	-
Jacalin	10.5 ml (2.75 mg/ml)	28.90 mg
SEC	37.5 ml (0.42 mg/ml)	15.75 mg
Final dIgA1 prep	9 ml (1.51 mg/ml)	13.60 mg

The table shows the amount of IgA1 obtained at each purification step. 1770 ml of supernatants were collected ("Harvest") and gave a total of 28.9 mg of IgA1 protein after concentrating the pooled eluates of the Jacalin chromatography ("Jacalin"). SEC removed any remaining impurities, pure dimeric SEC fractions (compare Fig. 21) were pooled to finally get pure F240 dIgA1 ("SEC"). Accepting some protein loss during the procedure, final preparation was concentrated to reduce the volume for later application in RM ("Final dIgA1 prep").

3.6 Large Scale Production: F240 dIgA2

About of 2380 ml of supernatant was finally harvested on day six after transfection. Pooled, sterile filtrated supernatant had to be checked for F240 dIgA2 expression as well as for presence of JC in the dimer. SDS-PAGE confirmed dimeric IgA2 expression and the presence of the JC was determined by Western Blot analysis (Fig. 23). In contrast to F240 dIgA1, F240 dIgA2 appeared as multiple bands around 300 kDa, showed however similar unspecific protein bands between ~40 kDa and ~100 kDa as the F240 dIgA1 supernatant.
Quality checked supernatants were aliquoted for peptide M affinity chromatography. Five runs, each with 476 ml supernatant were performed.

3.6.1 Quality Control of harvested F240 dIgA2 supernatants

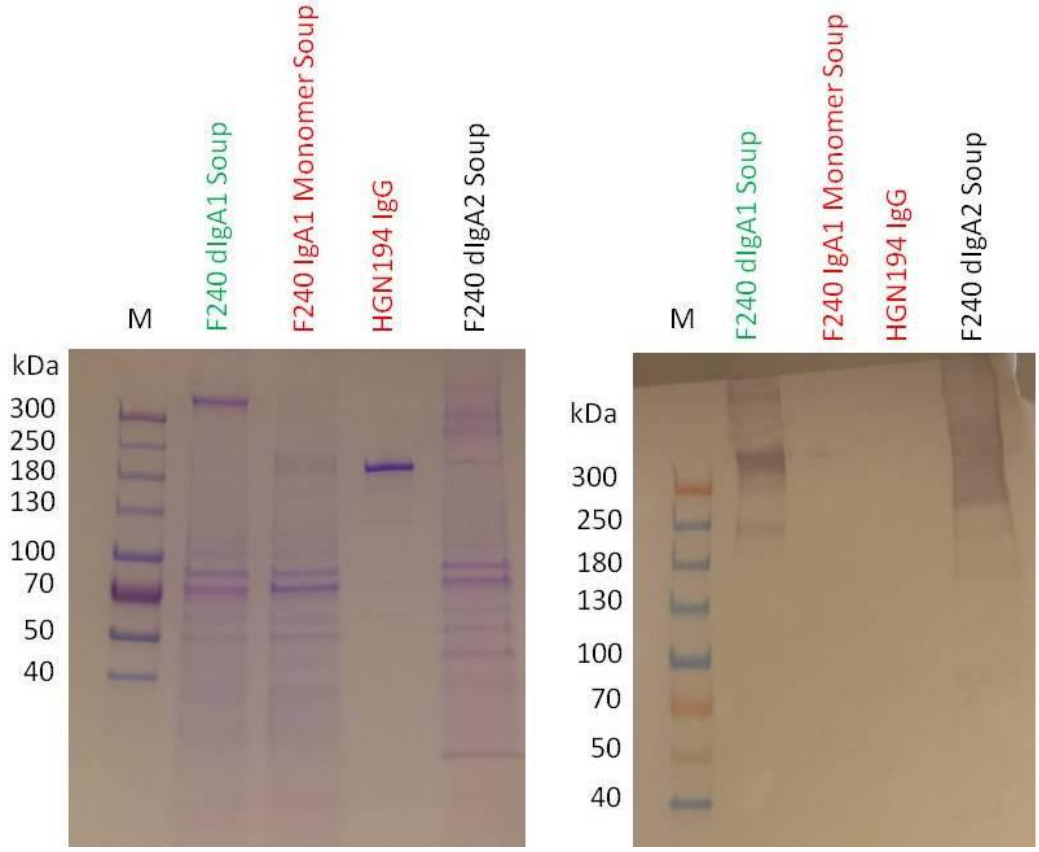


Fig. 23. Quality control of harvested F240 dIgA2 supernatants

Presence of the JC was a prerequisite for all further purification steps. An SDS-PAGE and the corresponding Western Blot are shown. Negative controls are marked in red, positive controls are marked in green. As expected, F240 dIgA1 had the JC (Fig. 18 for more details). The monomer supernatant (no JC plasmid added at the transfection) as well as the HGN194 IgG (1.6 µg for SDS-PAGE, 0.1 µg for Western Blot) had no JC present.

3.6.2 Peptide M Affinity Chromatography

We choose 2-step purification approach composed of Peptide M Affinity Chromatography followed by SEC to purify F240 dIgA2 from Expi293 cell supernatant (Fig. 8).

Despite some IgA2 losses along the way in the flow-through and wash fractions, the majority of IgA2 was successfully captured by Peptide M chromatography and eluted as a single peak. All eluates were pooled, concentrated and the amount of IgA2 in the 2380 ml of supernatant, determined by Nanodrop, was 84.75 mg (Fig. 24, Tab. 10).

The great majority of impurities, unspecific protein bands between ~ 40 kDa and ~100 kDa as well as protein smear, were removed during Peptide M chromatography. Besides dIgA2 band with molecular weight around 300 kDa, we observed protein bands corresponding to 40 kDa, ~50 kDa, ~130 kDa and 180 kDa.

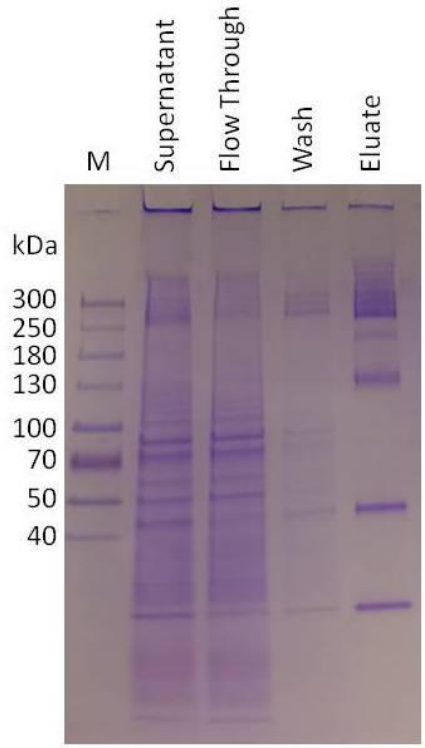


Fig. 24. SDS-PAGE of Peptide M Affinity Chromatography

The cell supernatant and collected fractions of the peptide M affinity chromatography, flow through (unbound IgA2 and unspecific proteins), wash (IgA2 molecules washed away from the column) and eluate (eluted IgA2 specific molecules) are depicted. IgA2 was successfully enriched in the eluate. M: Spectra Color High Range Protein marker (10 µl, Thermo scientific).

3.6.3 Preparative Size-exclusion Chromatography

The remaining impurities had to be removed by preparative SEC.

Six SEC runs were performed, each with 2 ml of F240 dIgA2. 3 main peaks were obtained in each run (Fig. 25). The fractions composing these three main peaks, 28-42, were analyzed on SDS-PAGE to determine which fractions are to be pooled. Based on the gel (Fig. 26), fractions of peak 2 (31-36) were pooled and resulted in about of 43 mg of "pure F240 dIgA2". Peak 1 (28-30) contained some higher molecular weight aggregates that could not be separated on this gel because they were stuck in the gel pouch. The third peak (37-42) was attributed to IgA2 monomers.

Fig. 27 gives a summary of the whole purification process. Starting from the crude cell supernatant, peptide M removed the major impurities. SEC removed remaining impurities around 130 kDa and 180 kDa. Finally, after concentrating, 42 mg of "pure F240 dIgA2" were obtained.

42 mg of F240 dIgA2 were isolated out of 2380 ml of supernatant. This finally gives an F240 dIgA2 yield of 17.6 mg/l.

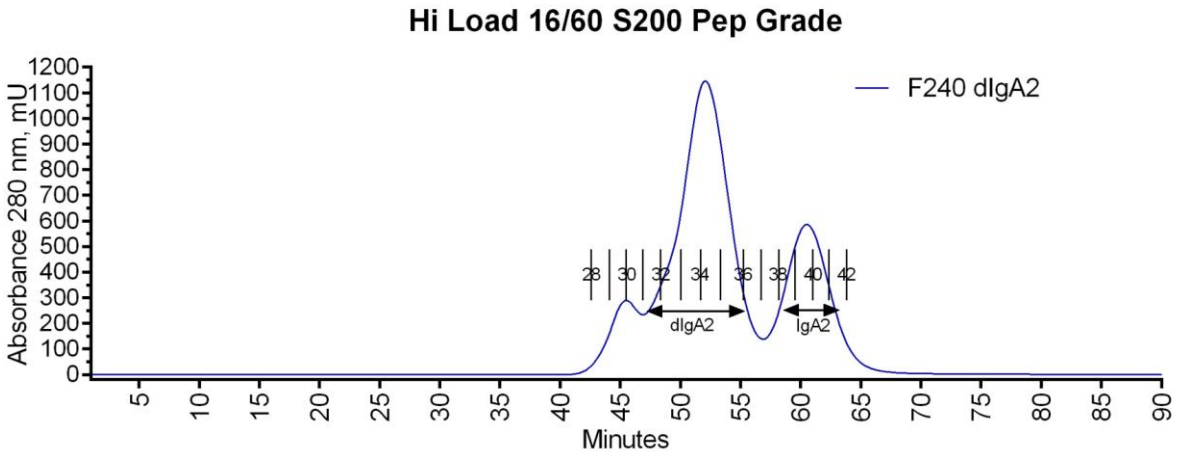


Fig. 25. Size-exclusion Chromatogram of concentrated peptide M eluate

IgA2 obtained by peptide M affinity chromatography was concentrated and further purified by preparative SEC on HiLoad^{TM}16/60 Superdex^{TM}200 pep grade column. 2 ml aliquots of eluate from Peptide M resin were loaded onto the column. Besides the main peak around fraction 34 (dIgA2), a monomer peak was detected around fraction 40 (IgA2) and a higher molecular weight peak around fraction 30. Chromatography fractions are indicated by vertical black lines. A subsequent SDS-PAGE was run to decide which fractions are to be pooled (Fig. 26).

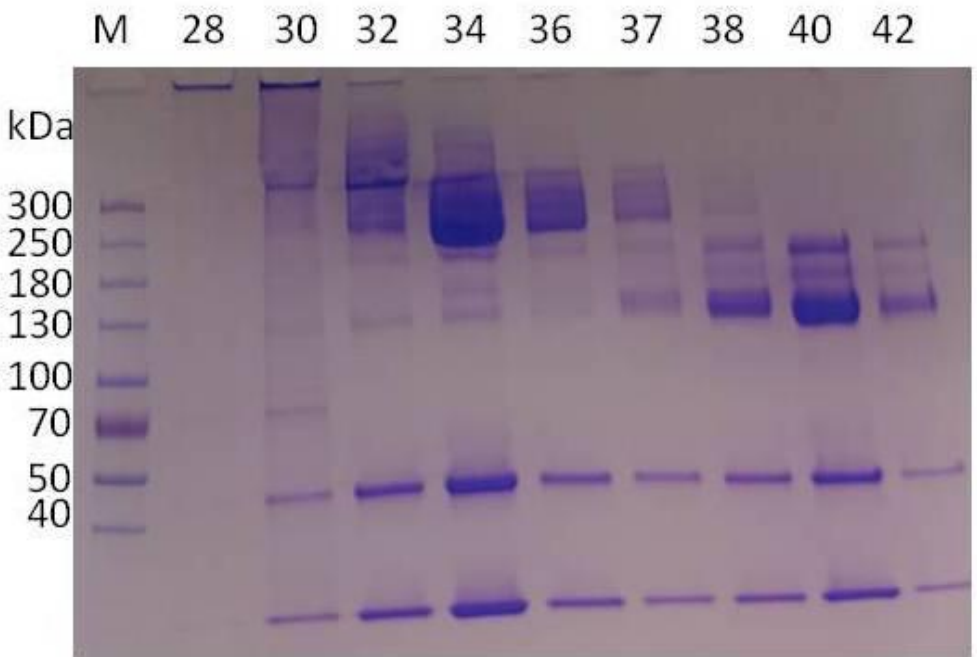


Fig. 26. SDS-PAGE of size-exclusion chromatography fractions

Based on the SEC chromatogram, fractions of the three peaks observed, 28-42 were loaded onto the gel. According to the gel pattern observed, fractions 31 till 36 (all containing the pure dimer, compare to the main peak in the chromatogram) were pooled for subsequent concentration and sterile filtration. Fractions 37-42 were considered the "monomer fraction" and not considered for further analysis. M: Spectra Color High Range Protein marker (10 µl, Thermo Scientific).

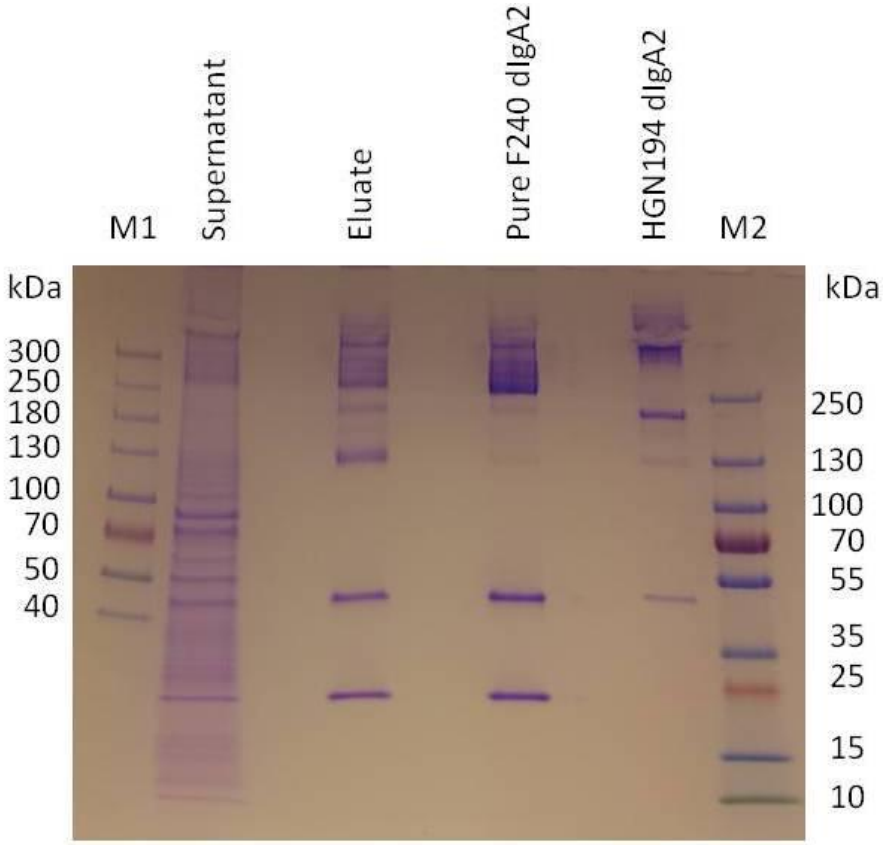


Fig. 27. SDS-PAGE of F240 dIgA2 at the different steps of purification

The pure dimeric F240 IgA2 was purified from the supernatant in a two-step approach. First, peptide M affinity chromatography was done to enrich only IgA2 molecules (“Eluate”). In the next step, the eluate was subjected to SEC to get rid of the monomer fraction. Finally, based on the SDS-PAGE of the obtained SEC fractions, only the fractions containing the pure dimer were pooled (“pure F240 dIgA2”). The lower molecular weight bands at 40 kDa and around 25 kDa were caused by heat pre-treatment of the samples before loading them onto the gel. HGN194 dIgA2 (4 µg) served as a reference for the molecular weight of dIgA2. M1: Spectra Color High Range Protein marker, M2: Page Ruler Plus Prestained protein ladder (both 10 µl, both Thermo Scientific).

Tab. 10. Yield of IgA2 along the purification

Purification Step	Volume of purification product (concentration)	Amount (mg)
Harvest	2.38 l of Expi293 cell supernatant	-
Peptide M	12 ml (7.74 mg/ml)	84.75 mg
SEC	50 ml (0.86 mg/ml)	43 mg
Final Concentrating	18.5 ml (2.33 mg/ml)	~ 42 mg

The table shows the amount of IgA2 obtained at each purification step. 2380 ml of supernatants were harvested in total ("Harvest") and gave a total of 84.75 mg of IgA2 protein after concentrating the pooled eluate of the peptide M chromatography ("Peptide M"). SEC removed any remaining impurities, pure dimeric SEC fractions (compare Fig. 26) were pooled to finally get pure F240 dIgA2 ("SEC"). Accepting minor protein loss during the procedure, final preparation was concentrated to reduce the volume for later application in animal experiments ("Final concentrating").

3.7 Characterisation of F240 dIgA1 and F240 dIgA2

3.7.1 Analysis of Epitope Specificity

There is evidence that switching of antibody isotype can cause alteration of epitope specificity (Tudor et al. 2012). After expression and purification of F240 dIgA1 and dIgA2, we analyzed epitope specificity of both mAbs in comparison to the parental F240 IgG. Toward this end, we performed ELISA using different HIV Env containing F240 epitope (Fig. 28). We also used mixture of overlapping peptides representing the exact F240 epitope. Both dIgA1 and dIgA2 versions of F240 successfully recognized gp160 and gp41 and did not react with gp120 that does not contain F240 epitope. Both mAbs also reacted with peptide mix representing F240 epitope.

Despite binding the same antigen, there is still the possibility of a slight epitope shift. To further address this issue we performed peptide ELISA using individual peptides as antigen. The dimeric mAbs recognized the same peptides as did their parental antibody F240 IgG1. Anti-SARS antibody Fm-6 used as a negative control did not bind to used peptides (Fig. 29).

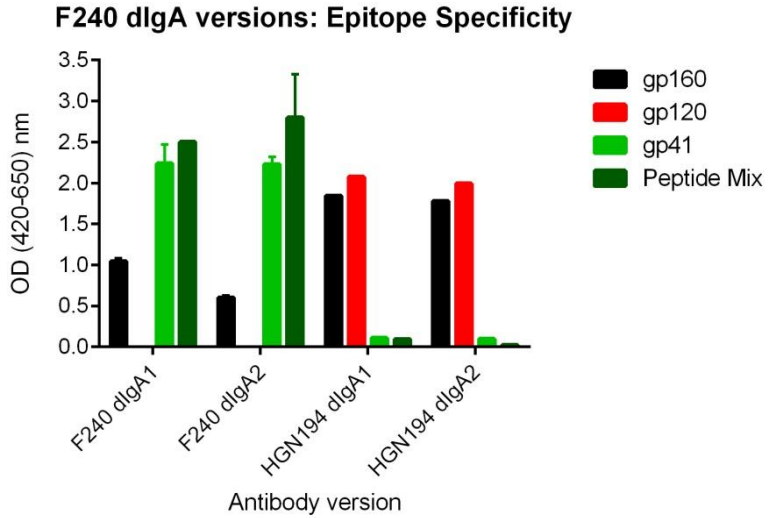


Fig. 28. Antigen ELISA of F240 dIgA1 and dIgA2

The final preparations of F240 dIgA1 and dIgA2 were tested for correct epitope specificity. Nunc MaxiSorp® flat-bottom 96 well plates (Affymetrix e Biosciece) were coated overnight at 4 °C with four distinct antigens: gp160 SHIV-1157, gp120 1157 ipK195, HIV-1-gp41 MN and KLIC Peptide Mix (peptides representing epitope of the parental F240 IgG1) at 1µg/ml. The next day, wells were washed and developed with an HRP-conjugated goat anti-human alpha chain antibody (Jackson ImmunoResearch). As expected two F240 dIgA versions bound to gp160, gp41 and peptide mix, but not to gp120. The HGN194 mAbs bound gp160 and gp120, but neither gp41 nor peptide mix.

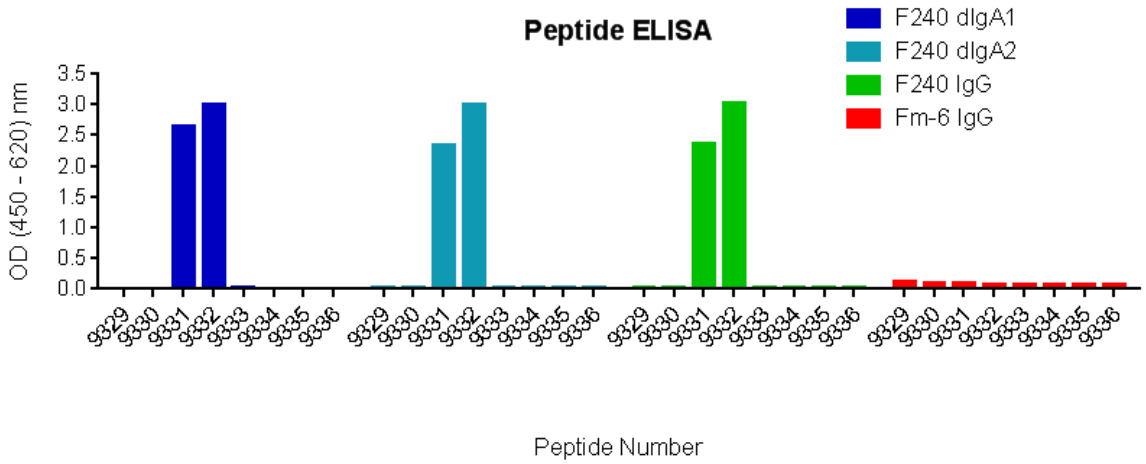


Fig. 29. Peptide ELISA of F240 dIgA1 and dIgA2

The final preparations of F240 dIgA1 and dIgA2 were analyzed for epitope specificity. Peptides of an HIV-1 Consensus Subtype C Env Peptide Library were used to represent the epitope of parental F240 IgG1. Anti-SARS Fm-6 served as negative control. F240 dIgA1 and dIgA2 showed the same pattern of peptide recognition as F240 IgG1, binding to peptides 9331 and 9332. For peptide sequences please consult Tab. 5 in “Materials and Methods”.

3.7.2 Neutralization of SHIV-1157ipEL-p by F240 dIgA1 and dIgA2

Next, we studied ability of F240 dIgA mAbs to neutralize immunodeficiency virus. The parental F240 IgG1 is known to be non-neutralizing. To test whether dIgA version preserved inability to neutralize virus we performed neutralization assay using TZM-bl cells and tier 1 SHIV-1157ipEL-p. This virus was chosen because of two reasons. First, this is easy-to-neutralize (tier 1) virus. Thus, if slight epitope alteration causes acquiring neutralization properties it would be easy to detect using tier 1 virus. Another reason was that the downstream animal experiment also will use this virus.

As expected, re-engineered F240 dIgA mAbs did not neutralize SHIV-1157ipEL-p as the parental F240 IgG (Fig. 30). The potent broadly nmAb VRC01 (anti-CD4 binding site) used as a positive control fully neutralized tested virus while Fm-6 used as a negative control also showed no neutralization.

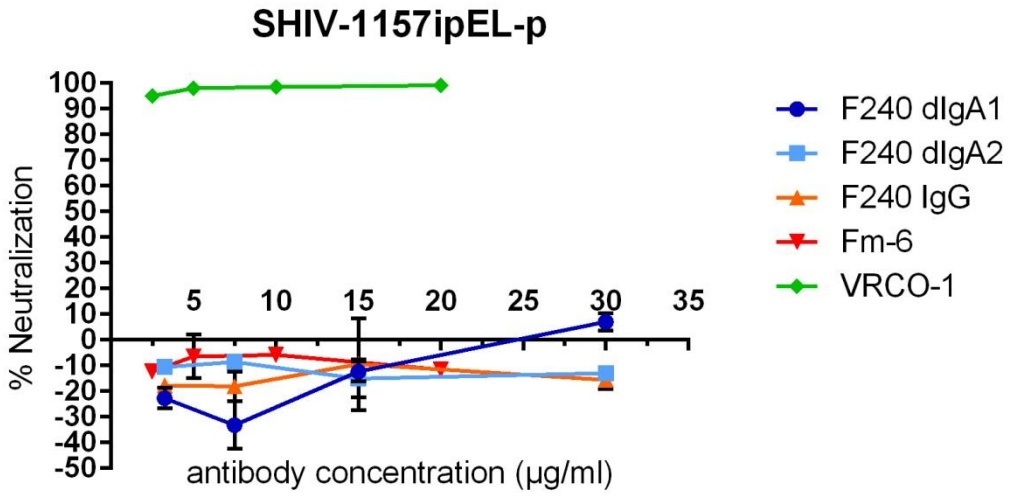


Fig. 30. TZM-bl neutralization assay for SHIV-1157ipEL-p

The neutralization pattern of dimeric F240 antibodies were compared to their parental form, F240 IgG1. Sample antibodies were tested starting from 30 µg/ml.

VRC01 is a very potent broadly nmAb and served as the positive control, it was used at a lower starting concentration, 20 µg/ml. VRC0-1 fully neutralized the test virus, Fm-6 did not. All F240 mAbs showed the same neutralization profile, they were all non-neutralizing. The X axis represents the antibody concentration in µg/ml. The Y axis shows the percentage of neutralization. Error bars represent the standard deviations measured in one experiment carried out in duplicate.

3.8 Virion Capture by F240 dIgA1 and dIgA2

Non-nmAb have been shown repeatedly to capture infectious virus particles in a highly specific manner (Poignard et al. 2003, Burrer et al. 2005, Moore et al. 2006). Recently, Stieh et al. (2015) added to that findings in that they found out that within their test virus, HIV_{BAL}, a small viral subpopulation was predominantly captured by non-nmAbs targeted against gp41 stumps. In this study, we tested non-neutralizing F240 dIgA1 and dIgA2 for capture of SHIV particles. Both antibodies recognize the immunodominant region of gp41, a region assumed to be highly conserved among the different viruses and exposed on gp41 stumps. Therefore, capture of any HIV or SHIV should be possible.

Purified human secretory IgA derived from human colostrum (AbD Serotec) was used as a negative control. Plates were coated with goat anti-human IgA antibodies (Jackson ImmunoResearch), blocked with MPBST and incubated with F240 dIgA1 and d IgA2 and the respective control antibodies. Viruses were standardized by p27 content and added in serial dilution. After overnight incubation plates were washed and captured virus was disrupted by 0.5 % Triton X-100 mixed with disruption buffer from p27 kit (ABL). The concentration of released p27 was measured by p27 quantification kit (ABL).

First, we tested tier 1 clade C SHIV-1157ipEL-p because it will be used as the challenge virus in downstream animal experiments involving F240 dIgA1 and dIgA2. Moreover, clade C is the most common subtype accounting for almost half of all infections (Stephens 2012). HGN194 dIgA1 and dIgA2, both directed against the V3 loop, served as positive controls because their behaviour towards tier 1 SHIV-1157ipEL-p was known. In the first place, we used only dIgA1 versions of F240 mAb and HGN194 (Fig. 31A). Human secretory IgA did not capture virus. Tier 1 SHIV-1157ipEL-p was trapped by F240 dIgA1. HGN194 dIgA1 also successfully captured tested virus. Of note, HGN194 dIgA1 captured tested virus to a better extent than F240 dIgA1 at the same virus input. As soon as we had dIgA2 version of F240 mAb we repeated the VCA experiment including both dIgA versions of F240 mAb and both dIgA versions of HGN194 mAb (Fig. 31B). Human secretory IgA gave no capture. Dimeric IgA1 versions of the tested antibodies showed better capture at the same virus input than dimeric IgA2 versions. HGN194 Abs captured SHIV-1157ipEL-p better than F240 dIgAs of the same isotype. For both VCAs, the virus input ranged from 17 ng to 25.5 ng.

F240 dIA1 and dIgA2 successfully captured tier 1 SHIV-1157ipEL-p. Assuming that gp41 stumps are conserved and accessible across viruses with different tier classifications, capture of tier 2 clade C SHIV-1157ipd3N4 should be possible. Therefore, in the next step, we decided to test tier 2 clade C SHIV-1157ipd3N4. HGN194 dIgA1 and dIgA2 have never been checked for capture of tier 2 SHIV1157-ipd3N4 before. Thus, besides F240 dIgA1 and dIgA2, also the dimeric HGN194 (anti-V3 loop) versions were analyzed for their capturing ability. In contrast to gp41 stumps, it is widely accepted that the V3 loop has a great sequence variability and that it is conformationally hidden in tier 2 viruses (Watkins et al. 2011). For the first assay, we only tested dIgA1 versions of F240 and HGN194. Human secretory IgA as well as HGN194 dIgA1 did not capture virus. F240 dIgA1 in turn trapped SHIV-1157ipd3N4 successfully (Fig. 32A). Once F240 dIgA2 was produced and purified, we repeated the VCA experiment including both dIgA versions of F240 mAb and both dIgA versions of HGN194 mAb. While tier 1 SHIV-1157ipEL-p was captured by both F240 dIgA1 and dIgA2 and HGN194 dIgA1 and dIgA2, tier 2 SHIV-1157ipd3N4 was trapped only by F240 dIgA versions, but not by HGN194 dIgA mAbs. Human Secretory IgA captured no virus. (Fig. 32B). For both VCAs, the virus input ranged from 28.1 ng to 78 ng.

Our dimeric F240 mAbs successfully trapped SHIV clade C: tier 1 SHIV-1157ipEL-p and tier 2 SHIV-1157ipd3N4. To exclude that capture was restricted to these two representative viruses of clade C, capture of heterologous clade C SHIV-2873Nip was tested. Fig. 33A shows the obtained results. Human secretory IgA did not capture any virus. SHIV-2873Nip got trapped by both, F240 dIgA1 and dIgA2. Of note, F240 dIgA1 captured to a better extent than did F240 dIgA2.

To find out whether virion capture is possible across other clades besides clade C, SHIVs representing different clades were used in the next set of experiments. Following clade C, HIV-1 clades A and B are the second and third most common clades in the current epidemic (Stephens 2012). We started with clade A which accounts for the majority of circulating strains in Kenya (Dowling et al. 2002). SHIV-KNH1144 (SHIV-A) belongs to clade A and was used as the test virus. Human secretory IgA did not capture any SHIV-A. SHIV-A was trapped by our dimeric F240 antibodies. Again, dIgA1 proved to be superior over its dIgA2 counterpart (Fig. 33B).

Followed by clade A, clade B, represented by SHIV-SF162p3, was tested. Human secretory IgA did not capture any virus. SHIV-SF162p3 was captured by F240 dIgA1 and dIgA2. Isotype A1 gave a better capture than did A2 (Fig. 33C).

Cross-clade capture was finished by checking the capture of SHIV-E. SHIV-E gave similar results in comparison to the already tested viruses: Re-engineered F240 successfully captured virus. The previously made observation of F240 dIgA1 being superior over F240 dIgA2 in trapping SHIV particles was confirmed (Fig. 33D).
Fig. 34 gives a summary of all viruses tested in this study.

3.9 Gp160 Sequence Alignment

Our dimeric F240 antibodies trapped all viruses tested, across different clades. This gave us a first hint that the epitope's amino acid sequence is conserved. For final confirmation, gp160 sequences of the VCA-tested viruses were compared to each other using Megalign program of the LASERGENE bioinformatics software package (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed applying the Clustal V method of alignment with the preset default parameters (Gap Penalty=10, Gap Length Penalty=10). The result of the multiple alignment is given in Fig. 35. Alignment revealed a high degree of sequence similarity of gp41's immunodominant region between the distinct clades. Together with the VCA data obtained, this clearly proves that the immunodominant region of gp41, including the KLIC motive, is highly conserved amongst the distinct clades ad representative viruses tested.

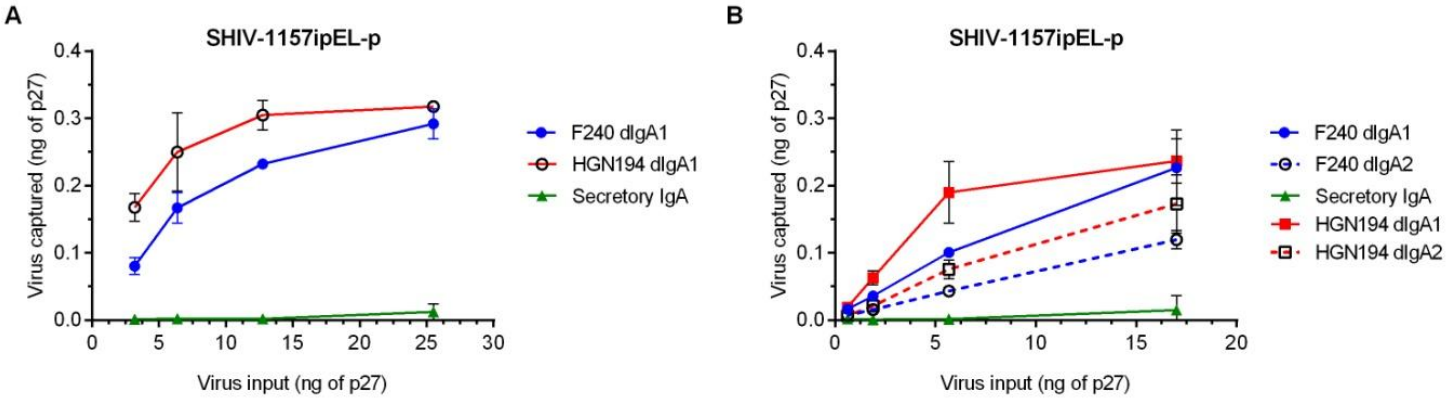


Fig. 31. Virion capture of SHIV-1157ipEL-p

F240 dIgA1 and dIgA2 were tested for capture of SHIV-1157ipEL-p. Human secretory IgA served as a negative control, HGN194 dIgA1 and dIgA2 served as positive controls. Plate was coated with goat anti-human IgA antibodies (Jackson ImmunoResearch), blocked with MPBST and incubated with F240 and HGN194 dIgAs. Viruses were standardized by p27 content and added in serial dilution. The next day, plates were washed, captured virus disrupted and released p27 measured by p27 quantification kit (ABL). The X axis represents the virus input per experimental well in ng of p27. The Y axis shows the amount of virus (ng of p27) captured per experimental well. The error bars represent the standard deviations measured in one experiment carried out in triplicate. Both, F240 and HGN194 antibodies successfully captured SHIV particles.

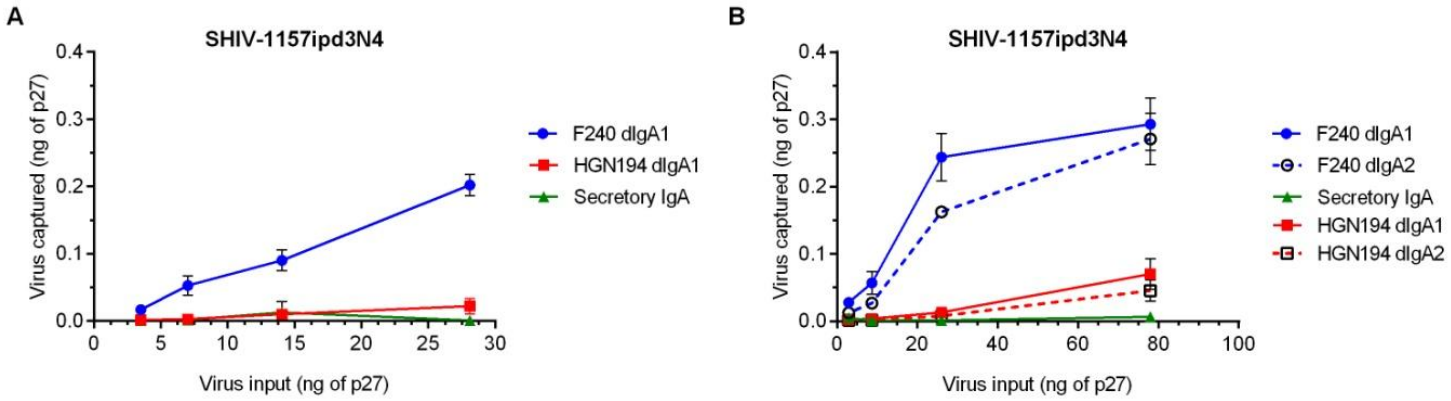


Fig. 32. Virion capture of SHIV-1157ipd3N4

F240 dIgA1 and dIgA2 were tested for capture of SHIV-1157ipd3N4. Human secretory IgA served as a negative control, HGN194 dIgA1 and dIgA2 served as positive controls. Plate was coated with goat anti-human IgA antibodies (Jackson ImmunoResearch), blocked with MPBST and incubated with F240 and HGN194 dIgAs. Viruses were standardized by p27 content and added in serial dilution. The next day, plates were washed, captured virus disrupted and released p27 measured by p27 quantification kit (ABL). The X axis represents the virus input per experimental well in ng of p27. The Y axis shows the amount of virus (ng of p27) captured per experimental well. The error bars represent the standard deviations measured in one experiment carried out in triplicate. Only F240 antibodies successfully captured SHIV particles.

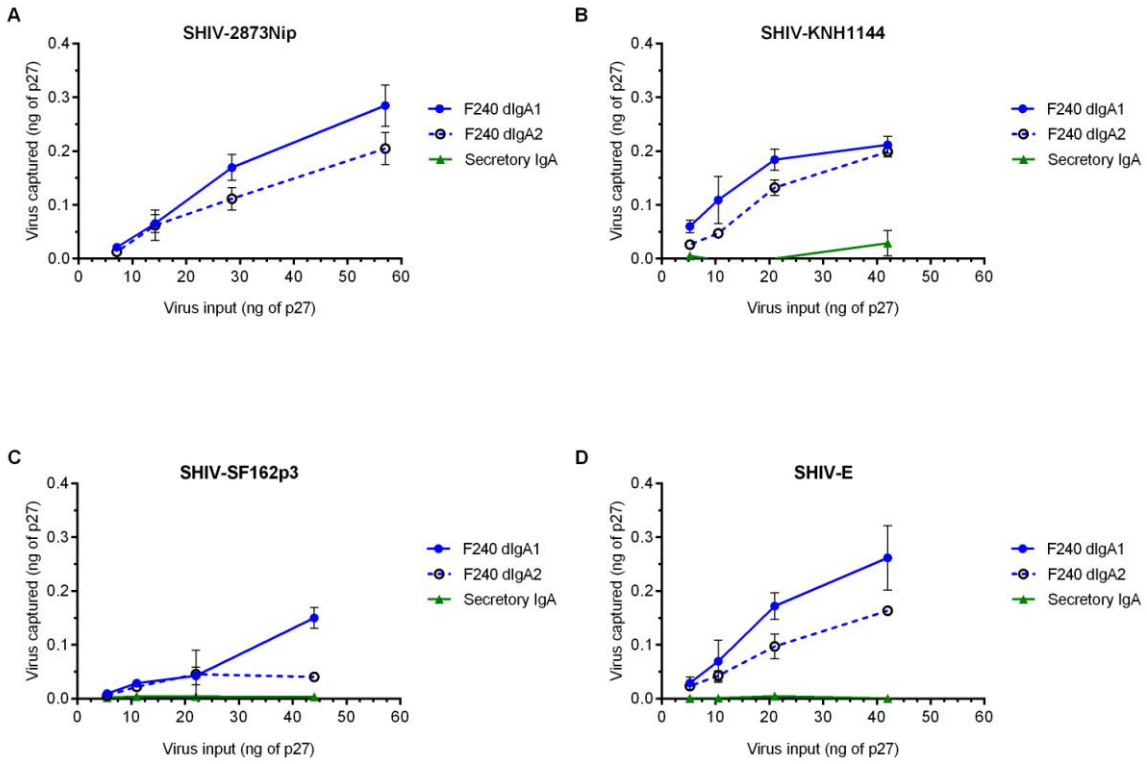


Fig. 33. Cross-clade virion capture of SHIV clade A, B, C and E

VCAs of SHIV-2873Nip (heterologous Clade C, Fig. A), SHIV-KHN1144 (Clade A, Fig. B), SHIV-SF162p3 (Clade B, Fig. C), and SHIV-E (Clade E, Fig. D) comparing F240 dIgA1 vs. F240 dIgA2. Human secretory IgA was used as a negative control. Plate was coated with goat anti-human IgA antibodies, blocked with MPBST and incubated with F240 dIgAs. Viruses were standardized by p27 content and added in serial dilution. After overnight incubation plates were washed, captured virus disrupted and the concentration of released p27 was measured by p27 quantification kit (ABL). The X axis represents the virus input per experimental well in ng of p27. The Y axis shows the amount of virus (ng of p27) captured per experimental well. The error bars represent the standard deviations measured in one experiment carried out in triplicate. Both F240 antibody isotypes captured viruses across distinct clades, F240 dIgA1 being superior over its dIgA2 counterpart.

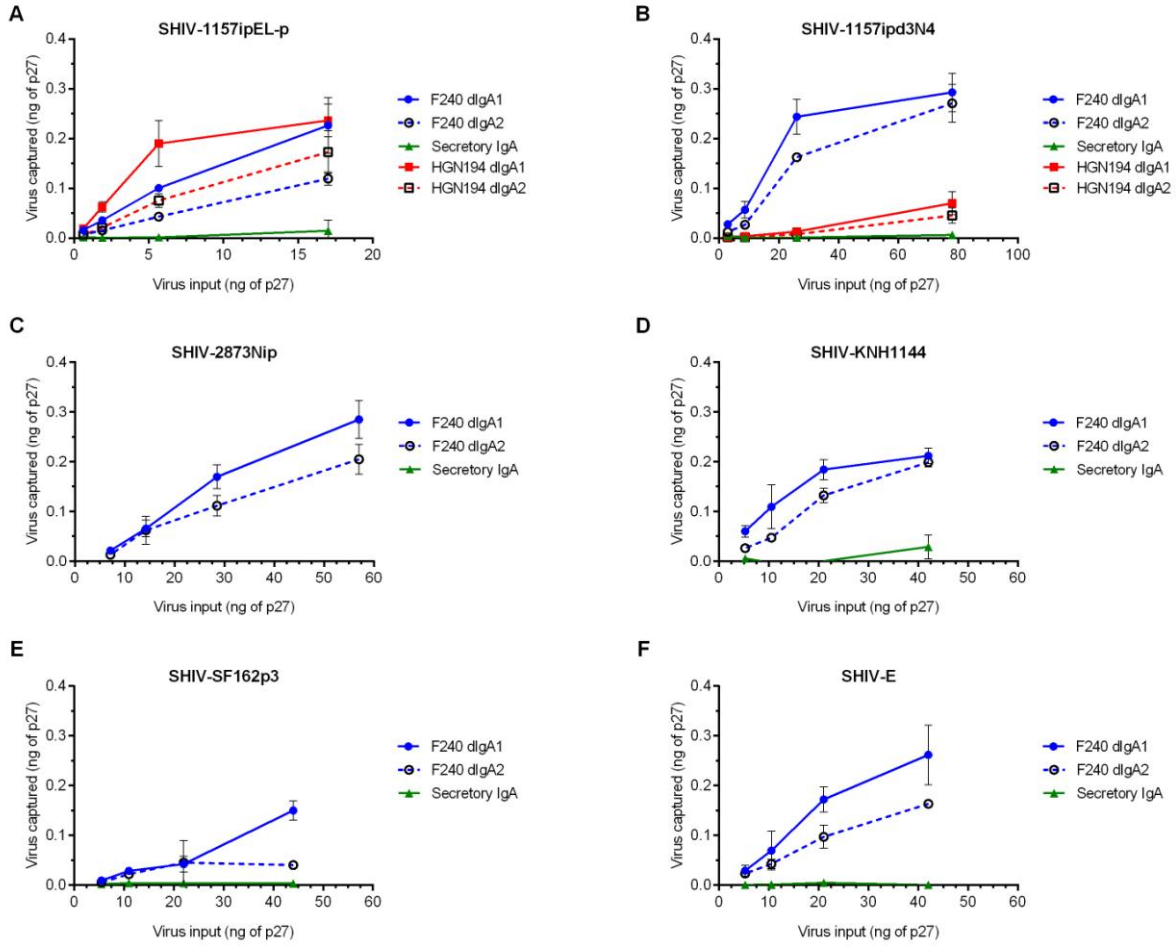


Fig. 34 Summary of all virion capture assays

For assay design, please refer to Fig. 33 and to the text. The figure shows all SHIVs tested in this study, each panel representing a different virus. Secretory IgA was used as a negative control in all assays. We chose tier 1 clade C SHIV-1157ipEL-p as the first test virus because it will be used as the challenge virus in the planned passive immunization study and included HGN194 dIgA1 and dIgA2 as additional positive controls (A). We further aimed at capturing its tier 2 version, clade C SHIV-1157ipd3N4 (B). For this virus, the capturing behaviour of HGN194 was unknown, so we included it in the assay besides F240 dIgA1 and dIgA2. We finished testing of SHIV clade C by trapping heterologous clade C SHIV-2873Nip (C). To check whether virion capture is possible across other clades besides clade C, cross-clade capture involving SHIVs of clades A, B and E (Panels D, E, F respectively) was carried out. All viruses in this study were successfully trapped by F240 dIgA1 and dIgA2. The negative control, secretory IgA, did not capture any virus in any of the experiments.

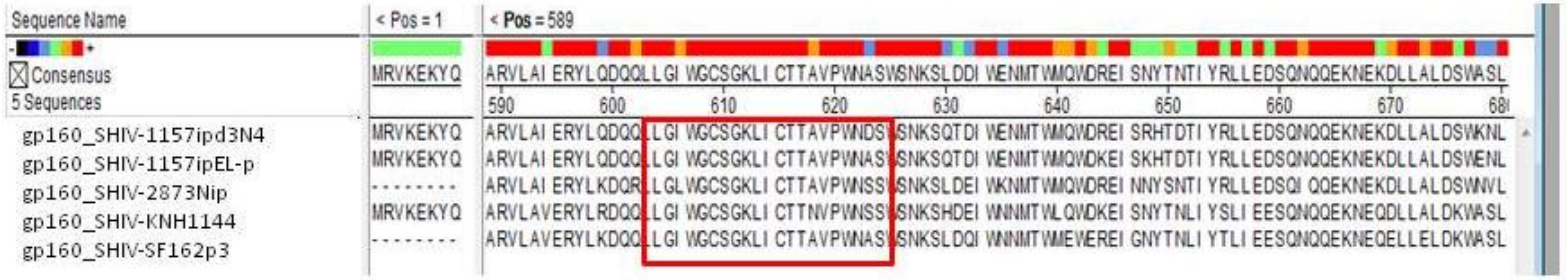


Fig. 35. Multiple Sequence Alignment of gp160 sequences

Gp160 sequences of all VCA-tested viruses were aligned to each other: SHIV-1157ipEL-p, SHIV-1157ipd3N4, SHIV-2873Nip, SHIV-KHN1144 and SHIV-SF162p3. SHIV-E gp160 could not be included because it is unpublished and there are no sequence data available yet. Multiple Alignment using the Clustal V method was performed. Red shows amino acids with a high degree of conservation. The KLIC region is highlighted by a red frame. The multiple alignment clearly proves that the immunodominant region of gp41, including the KLIC motive, is highly conserved amongst the distinct viruses/clades.

3.10 Conclusion

Our re-engineered non-neutralizing F240 dIgA1 and dIgA2 successfully trapped SHIV particles. VCA was predictive of a high degree of conservation of gp41's immunodominant region. Besides capture of clade C SHIVs, also cross-clade capture was achieved, SHIV clade A, B and E were bound by F240 dIgA1 and dIgA2. In line with that, multiple alignment of gp160 amino acid sequences showed a high degree of conservation of gp41’s immunodominant region across the distinct clades.

4 Discussion

This Master thesis is a proof-of-concept study that dimeric IgA antibodies directed against the disulfide loop-bonded immunodominant epitope of gp41 successfully capture SHIV particles.

4.1 Expression and Purification of F240 dIgA1 and dIgA2

For expression of dimeric IgAs we performed a two-step approach. It was assumed that correct and efficient monomer expression is important for the later correct dimer assembly via the JC. The efficiency of assembly of an antibody molecule during expression is strongly dependent of the ratio between HC and LC coding DNA. That is especially important for transiently transfected cell cultures. Thus, we decided to study different molar HC to LC ratios.

Once the optimal ratio for monomer expression was found, a pilot dimer expression study was performed to check correct production of dimers in the presence of JC in the Expi293 expression system. Taking into account the results of both pilot studies, a final decision for HC:LC:JC was made to express dimeric F240 in large scale (section "Choosing a HC:LC:JC ratio for large scale expression of F240 dIgA1 and dIgA2").

Two dimeric antibodies, F240 dIgA1 and dIgA2, were successfully cloned, expressed and purified from crude supernatant of Expi293 cells using distinct purification strategies. The supernatant contained many unspecific cellular proteins, which appeared as distinct protein bands between 130 kDa and 50 kDa. Additional protein smears could have been caused by released proteins in the course of cell death as the transiently transfected Expi293 cells were cultivated over six days.

Each expressed protein has their own inherent characteristics as it was the case with the two versions of F240. Besides different yields (A1: 7 mg/l; A2: 17.6 mg/l) F240 dIgA1 and F240 dIgA2 gave a different banding pattern on the gel. In contrast to F240 dIgA1, F240 dIgA2 appeared as multiple bands on the SDS-PAGE. We expressed the m(1) allotypic version of IgA2 in which HC and LC are not linked via disulfide bridges. Multiple high molecular weight bands were observed for F240 dIgA2 on the SDS-PAGE and likely arose through dissociating HC and LC. Also glycosylation differences must be taken into account,

not all produced antibody molecules necessarily share the same glycosylation pattern, this adds up to the appearance of multiple bands. The two additional bands observed at ~50 kDa and ~25 kDa could correspond to afore mentioned dissociated HC and LC.

The physiological assembly process of HC, LC and JC into dimeric antibodies is understudied in literature. This made it necessary to develop a new two-step approach with the underlying assumption: In course of dimeric antibody expression and assembly monomers are pre-assembled and then joined together by the JC.

First, we determined the optimal molar HC:LC plasmid ratio in a pilot monomer expression study where the best monomer expression was achieved at molar HC:LC ratios of 5:2 and 3:2. These results are in line with those of Schlatter et al. (2005). Schlatter and co-workers transiently expressed recombinant IgG and obtained the best antibody titer at HC:LC gene ratio of 3:2. Each antibody has their own optimal gene ratio for expression and it depends on the expression system and whether it is a transient or stable one.

In the second step, we expressed dimers using conditions that resulted in the best monomer production and adding the JC plasmid on top ("Pilot Dimer Expression Study"). TAC was performed for dimer purification because it is a cheap and easy way of immunoglobulin purification. Thiophilic resin preferably binds disulfide bridge containing proteins. However, TAC and subsequent SEC were not able to remove a 100 kDa protein from the dIgA preparation. Most likely this protein band was composed of disulfide-bonded cellular proteins that were additionally enriched by TAC along with the dimers. For large scale production of F240 dIgA1, we switched to Jacalin affinity chromatography. This approach made it possible to get almost rid of the 100 kDa protein band and to obtain pure F240 dIgA1. The underlying glycosylation differences between IgA1 and IgA2 made another change in chromatography techniques inevitable, changing to peptide M for F240 dIgA2 enrichment.

Minor dimeric F240 losses in flow-through and wash fractions of Jacalin and Peptide M affinity chromatography were accepted. It was easier to produce more supernatants and directly purify more antibody within the same time rather than reloading the same supernatant over and over again and improve the performance of the respective column.

The molar ratio [(2:1):1] [(HC:LC):JC] gave good dimer expression but produced additional monomers which had to be removed by SEC after the respective affinity chromatography. In course of this study sufficient antibody amounts had to be produced within a restricted time. Therefore, additional by-products and their later removal in a two-step approach were preferred over improving the expression conditions. Assuming that the JC is the driving protein behind monomer assembly into dimers, future expression approaches could aim at reducing monomer protein content by testing various molar JC plasmid amounts.

4.2 Isotype and Epitope Specificity

Constant heavy chain (CH1) domain of antibody can affect antibody epitope specificity. Tudor et al. (2012) re-engineered anti–HIV-1 human broadly nAb 2F5 IgG1 into IgA2 version and compared epitope specificities. Epitope mapping surprisingly showed that 2F5 IgA2 additionally recognized an N-terminal region of gp41 that was not bound by the parental antibody 2F5 IgG1. Possible explanations may include that a "given constant (C) region allosterically imposes constraints on variable (V) region structure and paratope" (Casadevall and Janda 2012). Therefore, in course of our studies, we had to ensure unaltered epitope specificity for F240 dIgA1 and dIgA2. Confirmation of binding to gp41 was not enough. A peptide ELISA had to be carried out to exclude any shifts in the recognition sequence between the dimeric F240 versions and F240 IgG1, their parental mAb.

4.3 The concept of non-neutralizing Antibodies

In our study we wanted to create a non-neutralizing dimeric F240 antibody. Neutralization assay clearly showed that F240 dIgA1 and dIgA2 are non-neutralizing.

The role of non-nAbs in prevention of HIV acquisition has been understudied in existing literature. One of the reasons may be that that research is still focussing on the induction of HIV-1 nAbs with broad reactivity. However, existing HIV-1 vaccine candidates have been unable to induce these broadly reactive nAbs (Mascola and Haynes 2013). Virus neutralization has long been considered the main protection mechanism, but one needs to admit that the term "neutralization" refers to an *in vitro* experiment whereby prevention of infection of cultured cells is measured (Dimmock 1984). And an *in vitro* measurement may or may not relate to the actual protection afforded by prior vaccination or infection.

Schmaljohn (2013) points out the major differences between nmAbs and non-nmAbs. Typically located on top of a protein's solvent face, neutralizing epitopes are generally more variable among natural virus isolates because they are less restricted in the generation of viable mutations than epitopes responsible for viral life cycle and molecular interactions.
Protective non-nmAbs however tend to be more reactive because their cognate epitopes are more highly conserved. Schmaljohn (2013) speaks of cryptic epitopes, epitopes that "map to virion Env proteins, yet appear to be masked and functionally absent from the surface of mature virions".
Hope (2011) introduces the term of "broadly binding antibodies" in this context, binding yet non-nAbs: Broadly binding antibodies can act in various ways such as trapping viral particles at the mucosal epithelial surfaces or mediate antibody-directed cell cytotoxicity.

NmAbs are not the only immune response that might confer protection. For Excler et al. (2014) "the induction of a high frequency and high titers of HIV specific functional non-nAb may represent an attractive hypothesis testing strategy in future HIV vaccine efficacy trials". Suggested mechanisms of virus inhibition by non-nAbs include for instance mucus retardation, aggregation, viral capture and transcytosis inhibition.
Neutralization escape variants of viruses can arise rapidly (resistant to nmAbs), circumstances under which non-nmAbs may be the best "arrow in the quiver" (Schmaljohn 2013).

Various animal data from viral infections and vaccine studies suggest that non-nAb without neutralizing activity *in vitro* may play a pivotal role in protecting against viral infection *in vivo*.
Hidajat et al. (2009) vaccinated monkeys with an adenovirus-SIV priming/Env boost approach and challenged them i. r. with SIV_{mac251}. Strong non-neutralizing serum antibody activities, such as antibody-dependent cellular cytotoxicity (ADCC) were induced in the vaccinated group and correlated with reduced acute viremia. Anti-Env IgA in the broncheoalveolar lavage and rectal secretions inhibited transcytosis.
Similar findings are reported by Xiao et al. (2010), using an adenovirus-HIV-SIV priming/Env boost approach. Both acute and chronic viremia were reduced by non-neutralizing anti-Env antibody activities: Strong ADCC in serum as well as transcytosis inhibition in rectal secretions.

4.4 Virion Capture

We first assessed the ability of F240 dIgA1 and dIgA2 to capture SHIV clade C of the tier 1 and tier 2 phenotypes. SHIV-1157ipd3N4 is a ‘‘late’’, tier 2 virus derived from a monkey after it developed AIDS (Song et al. 2006), whereas SHIV-1157ipEL-p (Siddappa et al. 2010) is a chimera of the ‘‘early’’ SHIV-1157ip Env (derived from a recently infected Zambian infant’s primary R5 HIV-C) and the ‘‘late’’, engineered backbone of SHIV-1157ipd3N4.
Besides secretory IgA, HGN194 dIgA1 and dIgA2 were included as additional antibody controls because their capturing behaviour towards early tier 1 SHIV-1157ipEL-p was known. The capturing behaviour of HGN194 dIgA1 and dIgA2 towards late tier 2 SHIV-1157ipd3N4 was unknown though. Therefore, we included them in the VCA involving late tier 2 SHIV-1157ipd3N4. HGN194 is a human mAb isolated from an individual infected with HIV-1 circulating recombinant form (CRF) and it targets a conformational epitope in the third hypervariable loop (V3) of HIV-1 gp120. The tier 1 SHIV-1157ipEL-p was captured by both F240 dIgA1 and dIgA2 and HGN194 dIgA1 and dIgA2, while tier 2 SHIV-1157ipd3N4 was trapped only by the F240 versions but not by dimeric HGN194 versions.
The extreme sequence variability of the V3 loop together with its conformation hiddenness in tier 2 viruses impedes recognition by specific Abs. For example, HGN194IgG was unable to neutralize tier 2 SHIV-1157ipd3N4 but efficiently neutralized tier 1 SHIV-1157ipEL-p (Watkins et al. 2011). The immunodominant region of gp41 is accessible for antibodies as within functional gp160 spikes as in the gp41 stumps on both tier 1 and tier 2 viruses. This explains superior virion capture by F240 dIgAs compared to dIgA versions of V3 loop-secific HGN194 mAbs for tier 2 SHIV-1157ipd3N4.

Besides capture of SHIV-1157ipEL-p and SHIV-1157ipd3N4, capture of heterologous clade C SHIV-2873Nip was achieved. This was a hint that the immunodominant region is conserved among the different viruses. To check that assumption, we also analyzed virion capture of SHIVs of different clades by F240 dIgA1 and dIgA2. All clades tested (A, B, C, E) were successfully trapped by our dimeric F240 antibodies, but not equally though. Clade B SHIV (SHIV-SF162p3) was captured at lesser extent compared with other viruses. This may likely be due to the fact that gp41 stumps are not equally accessible across different clades.

Further, we had to rule out that the observed (cross-clade) virus capture was just caused by the dimeric antibody backbone. Therefore, we included human secretory IgA as a negative

control in all VCAs. Human secretory IgA did not show capture in any of the assays. This excluded the possibility of a "dimeric antibody backbone effect" and confirmed that the capture observed was due to binding of F240 to its target epitope, the immunodominant region of the gp41 stumps.

As for SHIV-1157ipEL-p and SHIV-1157ipd3N4, dimeric IgA1 showed superior virion capture for all viruses tested. Such efficient virion capture depends on the dIgA1 molecular hinge region architecture where the distance between antigen-binding domains is about 16 nm compared to 10 nm in the dIgA2 molecule (Watkins et al. 2013). Also, affinity may play a role. An antibodies isotype can influence its affinity. Tudor et al. (2012) for example found that anti-HIV-1 gp41 2F5 antibody as an IgA2 version bound to gp41 with greater affinity than did the parental IgG1 version. Together with its beneficial molecular architecture, F240 dIgA1 may also have a greater affinity to gp41 and thus captured better than did F240 dIgA2. This aspect can addressed in future experiments using surface plasmon resonance to assess antibody affinity.

Taken together, the VCA experiments are in line with observations made by Watkins et al. (2013). There, higher degree of macaque protection against mucosal SHIV challenge by dIgA1 was linked to better virion capture by dIgA1. Better virion capture was due to the wide-open molecular architecture of dIgA1 compared with dIgA2 and IgG versions of the same antibody.

4.5 The role of mucosal IgA: recent and planned experiments

The role of mucosal anti-HIV IgA mAbs in HIV research has been neglected so far in the literature.

Watkins et al. (2013) were the first and only ones up to date to show cause and effect between dimeric IgA administration and prevention of mucosal SHIV acquisition. Protective effects of mucosal IgAs have been confirmed in the vaccine efficacy study of Bomsel et al. (2011). Virosomes displaying HIV-1 gp41 antigens protected the majority of RMs against mucosal SHIV challenge. Correlates of protection were identified as gp41-specific induced vaginal IgAs which showed HIV-1 transcytosis-blocking properties. Transcytosis inhibition was retained only upon IgG depletion of the mucosal secretions. This proves that vaccine-induced mucosal IgA was responsible for transcytosis inhibition of HIV *in vitro*.

Both studies identified either virion capture or inhibition of transcytosis or both as correlates of protection.

With their results Bomsel et al. (2011) call into question the paradigm "whereby circulating antiviral antibodies are required for protection against HIV-1 infection".

Watkins et al. (2013) showed that neutralizing mucosal dimeric IgA (dIgA) can prevent SHIV acquisition in RMs. Despite similar neutralization patterns *in vitro*, dIgA1 version of anti-V3 loop HGN194 yielded better protection against i. r. SHIV challenge than its dIgA2 counterpart. Protection could therefore not be correlated to different neutralization profiles. Virion capture and transcytosis inhibition were identified as correlates of protection. Our F240 dIgA1 and dIgA2 is a promising approach for *in vivo* studies. Both antibodies are non-neutralizing and capture viruses of different clades. Gp160 sequence alignment confirmed that their target epitope, the disulfide loop-bonded immunodominant epitope of gp41, is conserved among distinct clades.

Taking the concept of non-nAbs into account, follow-up experiments involving F240 dIA1 and dIgA2 are planned: A passive immunization study in RM with non-neutralizing F240 dIgA1 and dIgA2 will be carried out. Each group of RMs (six monkeys each) will get a single-dose i. r. challenge of antibody (1.25 mg) 30 min before a high-dose i. r. challenge with tier 1 SHIV-1157ipEL-p. Considering the already obtained *in vitro* data this study will show whether non-neutralizing dimeric IgA antibodies confer resistance against SHIV acquisition and thus break the still existing dogma that only nAbs are worth (re)searching for.

5 Zusammenfassung

Schleimhäute des Genital- und Rectaltraktes sind die Haupteintrittsrouten für das humane immunodefizienz virus (Pope und Haase 2003). Dennoch ist die Rolle von dimeren IgA Antikörpern, welche primär in den Schleimhäuten zu finden sind, nach wie vor unterbehandelt. Die jüngste passive Immunisierungsstudie von Watkins et al. (2013) zeigte erstmals einen Zusammenhang auf zwischen dimeren IgA Antikörpern und dem Schutz vor mocosaler Infektion durch simian human immunodeficiency virus (SHIV). Die beiden verwendeten Antikörper, dimeres IgA1 sowie dimeres IgA2 hatten sehr ähnliche Neutralisationsprofile. "Correlates of protection" waren "virion capture" und "transcytosis inhibition". Offensichtlich ist Neutralisierung nicht zwingend notwendig um Schutz vor Infektion zu gewährleisten. Dies steht dem Ansatz der bis dato gängigen HIV Vakzinforschung entgegen, wonach nur neutralisierende Antikörper Schutz bieten und man versuchen sollte, selbige zu induzieren.

Ziel dieser Masterarbeit war es, den nicht neutralisierenden humanen monoklonalen Antikörper F240 IgG1, reaktiv gegen die immunodominante Region der gp41 Ektodomäne, in seine dimeren Formen, F240 dIgA1 sowie F240 dIgA2m(1) zu konvertieren. Beide Antikörper wurden über transiente Transfektion von Expi293 Zellen hergestellt und mittels Jacalin Agarose bzw. Peptid M Agarose Chromatographie aufgereinigt. Der letzte Schritt bestand aus einer Size-exclusion Chromatographie auf einer Superdex 200 Säule.

Weiters sollten die beiden Antikörper charakterisiert werden und wurden auf Epitop Spezifität sowie Neutralisierungsprofil getestet. Beide dimeren Antikörper erkannten gp160, gp41 sowie überlappende Peptide, die das Epitope des Ausgangsantikörpers, F240 IgG1, repräsentieren. Das Neutralisierungsprofil war mit dem von F240 IgG1 ident, auch die dimeren Antikörper waren nicht neutralisierend.

Zu guter Letzt wurden F240 dIgA1 and dIgA2 auf "virion capture" gestestet. Tier 1 SHIV-1157ipEL-p und Tier 2 SHIV-1157ipd3N4 wurden erfolgreich von F240 dIgA1 und dIgA2 gebunden. "Cross-clade virion capture" unter Verwendung von Viren mehrerer Clades (A, B, C und E) wurde ebenfalls erfolgreich durchgeführt und zeigte die Konserviertheit besagter immunodominanter Region über die verschieden Clades hinaus auf.

F240 dIgA1 und dIgA2 werden in einer baldigen passiven Immunisierungsstudie an Rhesus Makaken getestet werden. In Anbetracht bereits generierter *in vitro* Daten wird diese Studie zeigen, ob nicht neutralisierende dimere IgA Antikörper Schutz vor einer mucosalen SHIV Infektion bieten und somit das existierende Dogma der HIV Vakzinforschung über neutralisierende Antikörper in Frage stellen.

6 English Summary

Despite the fact that mucosal surfaces of the genital or rectal tract serve as the major entry routes for HIV (Pope and Haase 2003), the role of dimeric IgA antibodies in the host's viral defense is understudied. The recent passive immunization study of Watkins et al. (2013) was the first to show a correlation between dimeric IgA antibodies and protection against mucosal SHIV challenge. Two dimeric antibodies, dIgA1 and dIgA2, were used that both exhibited similar neutralization profiles. Correlates of protection were identified as virion capture and transcytosis inhibition. Apparently, neutralization was not a prerequisite for protection. This challenges the widely assumed dogma of HIV vaccine development that only broadly neutralizing antibodies can confer protection and are worth being induced. So far, HIV vaccine development is still focused on the induction of broadly neutralizing antibodies that are difficult to induce, however.

In this master thesis, the non-neutralizing human mAb F240 IgG1, reactive against the immunodominant region of the gp41 ectodomain (Cavacini et al. 1998) was re-engineered into its dimeric versions, F240 dIgA1 and dIgA2m(1). Dimeric IgA1 and dimeric IgA2 were produced via transient transfection of Expi293 cells and purified using Jacalin agarose and peptide M agarose chromatography, respectively. Final purification was performed by size-exclusion chromatography on Superdex 200.

The aim was to produce and characterize F240 dIgA1 and F240 dIgA2(m1). Antibodies were tested for epitope specificity and neutralization profile. Both dimeric mAbs recognized gp160, gp41 and peptides representing the epitope of the parental F240 IgG1. The neutralization profile of F240 dIgA1 and dIgA2 as found similar to the parental F240 IgG1, both dIgA version were non-neutralizing.

Finally, dimeric antibodies were tested for their ability to capture virus in virion capture assays. F240 dIgA1 and dIgA2 successfully capture simian-human immunodeficiency viruses (SHIVs) containing HIV-1 Env of clades A, B, C and E.

The produced, characterized and functionally tested antibodies will be tested in an upcoming passive immunization study in rhesus monkeys. Considering the already obtained *in vitro* data this study will show whether non-neutralizing dimeric IgA antibodies confer resistance against mucosal SHIV acquisition and thus break the still existing dogma that only neutralizing antibodies are worth (re)searching for.

7 References

Ambrose Z, KewalRamani VN, Bieniasz PD, Hatziioannou T. 2007. HIV/AIDS: in search of an animal model. Trends in Biotechnology, 25(8):333-337.

Aucouturier P, Duarte F, Mihaesco E, Pineau N, Preud'homme JL. 1988. Jacalin, the human IgA1 and IgD precipitating lectin, also binds IgA2 of both allotypes. Journal of immunological methods, 113(2):185–191.

Bomsel M. 1997. Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. Nature medicine, 3(1):42-47.

Bomsel M, Heyman M, Hocini H, Lagaye S, Belec L, Dupont C, Desgranges C. 1998. Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM. Immunity, 9(2):277-287.

Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, Roger MG, Mouz N, Amacker M, Chalifour A, Diomede L, Devillier G, Cong Z, Wei Q, Gao H, Qin C, Yang GB, Zurbriggen R, Lopalco L, Fleury S. 2011. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. Immunity, 34(2):269-280.

Bonner A, Furtado PB, Almogren A, Kerr MA, Perkins SJ. 2008. Implications of the near-planar solution structure of human myeloma dimeric IgA1 for mucosal immunity and IgA nephropathy. Journal of immunology, 180(2):1008–1018.

Bonner A, Almogren A, Furtado PB, Kerr MA, Perkins SJ. 2009. The nonplanar secretory IgA2 and near planar secretory IgA1 solution structures rationalize their different mucosal immune responses. Journal of biological chemistry, 284(8):5077–5087.

Brandtzaeg P, Johansen FE. 2005. Mucosal B cells: phenotypic characteristics, transcriptional regulation, and homing properties. Immunological reviews, 206: 32-63.

Braathen R, Hohman VS, Brandtzaeg P, Johansen FE. 2007. Secretory antibody formation: conserved binding interactions between J-chain and polymeric Ig receptor from humans and amphibians. Journal of immunology, 178(3):1589–1597.

Burrer R, Haessig-Einius S, Aubertin AM, Moog C. 2005. Neutralizing as well as non-neutralizing polyclonal immunoglobulin (Ig)G from infected patients capture HIV-1 via antibodies directed against the principal immunodominant domain of gp41. Virology, 333(1):102-113.

Casadevall A, Janda A. 2012. Immunoglobulin isotype influences affinity and specificity. Proceedings of the National Academy of Sciences of the United States of America, 109(31):12272-12273.

Cavacini LA, Emes CL, Wisnewski AV, Power J, Lewis G, Montefiori D, Posner MR. 1998. Functional and molecular characterization of human monoclonal antibody reactive with the immunodominant region of HIV type 1 glycoprotein 41. AIDS Research and Human Retroviruses, 14(14):1271-1280.

Daniel MD, Letvin NL, King NW, Kannagi M, Sehgal PK, Hunt RD, Kanki PJ, Essex M, Desrosiers RC. 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. Science, 228(4704):1201-1204.

Devito C, Hinkula J, Kaul R, Lopalco L, Bwayo JJ, Plummer F, Clerici M, Broliden K. 2000. Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate. AIDS, 14(13):1917-1920.

Dimmock NJ. 1984. Mechanisms of neutralization of animal viruses. The Journal of general virology, 65 (Pt 6):1015-1022.

Dowling WE, Kim B, Mason CJ, Wasunna KM, Alam U, Elson L, Birx DL, Robb ML, McCutchan FE, Carr JK. 2002. Forty-one near full-length HIV-1 sequences from Kenya reveal an epidemic of subtype A and A-containing recombinants. AIDS, 16(13):1809-1820.

Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, Cayanan C, Maddon PJ, Koup RA, Moore JP, Paxton WA. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature, 381(6584):667-673.

Excler JL, Ake J, Robb ML, Kim JH, Plotkin SA. 2014. Nonneutralizing functional antibodies: a new "old" paradigm for HIV vaccines. Clinical and Vaccine Immunology, 21(8):1023-1036.

Freed EO, Martin MA. 2001. HIVs and their replication. In: Knipe DM, Howley PM, Editors. Fields Virology. Fifth edition. Philadelphia: Lippincott Williams & Wilkins, 2107-2185.

Gregory RL, Rundegren J, Arnold RR. 1987. Separation of human IgA1 and IgA2 using jacalin-agarose chromatography. Journal of immunological methods, 99(1):101–106.

Hatziioannou T, Evans DT. 2012. Animal models for HIV/AIDS research. Nature Reviews Microbiology, 10(12): 852–867.

Harouse JM, Gettie A, Tan RC, Blanchard J, Cheng-Mayer C. 1999. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. Science, 284(5415):816-819.

Hatziioannou T, Princiotta M, Piatak M Jr, Yuan F, Zhang F, Lifson JD, Bieniasz PD. 2006. Generation of simian-tropic HIV-1 by restriction factor evasion. Science, 314(5796):95.

Hatziioannou T, Ambrose Z, Chung NP, Piatak M Jr, Yuan F, Trubey CM, Coalter V, Kiser R, Schneider D, Smedley J, Pung R, Gathuka M, Estes JD, Veazey RS, KewalRamani VN, Lifson JD, Bieniasz PD. 2009. A macaque model of HIV-1 infection. Proceedings of the National Academy of Sciences of the United States of America, 106(11):4425-4429.

Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, Evans DT, Montefiori DC, Karnasuta C, Sutthent R, Liao HX, DeVico AL, Lewis GK, Williams C, Pinter A, Fong Y, Janes H, DeCamp A, Huang Y, Rao M, Billings E, Karasavvas N, Robb ML, Ngauy V, de Souza MS, Paris R, Ferrari G, Bailer RT, Soderberg KA, Andrews C, Berman PW, Frahm N, De Rosa SC, Alpert MD, Yates NL, Shen X, Koup RA, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Michael NL, Kim JH. 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. New England Journal of Medicine, 366(14):1275–1286.

Hemelaar J. 2012. The origin and diversity of the HIV-1 pandemic. Trends in molecular medicine, 18(3):182-192.

Hebert DN, Gierasch LM. 2009. The molecular dating game: an antibody heavy chain hangs loose with a chaperone while waiting for its life partner. Molecular cell, 34(6):635-636.

Hidajat R, Xiao P, Zhou Q, Venzon D, Summers LE, Kalyanaraman VS, Montefiori DC, Robert-Guroff M. 2009. Correlation of vaccine elicited systemic and mucosal non neutralizing antibody activities with reduced acute viremia following intrarectal simian immunodeficiency virus SIVmac251 challenge of rhesus macaques. Journal of Virology, 83(2):791–801.

Hope TJ. 2011. Moving ahead an HIV vaccine: to neutralize or not, a key HIV vaccine question. Nature Medicine, 17(10):1195–1197.

Huang YT, Wright A, Gao X, Kulick L, Yan H, Lamm ME. 2005. Intraepithelial cell neutralization of HIV-1 replication by IgA. Journal of immunology, 174(8):4828-4835.

Janoff EN, Wahl SM, Thomas K, Smith PD. 1995. Modulation of human immunodeficiency virus type 1 infection of human monocytes by IgA. Journal of infectious diseases, 172:855–858.

Johnsson E, Areschoug T, Mestecky J, Lindahl G. 1999. An IgA-binding peptide derived from a streptococcal surface protein. Journal of Biological Chemistry, 274(21):14521–14524.

Johansen FE, Braathen R, Brandtzaeg P. 2000. Role of J-chain in secretory immunoglobulin formation. Scandinavian Journal of Immunology, 52(3):240-248.

Kamada K, Igarashi T, Martin MA, Khamsri B, Hatcho K, Yamashita T, Fujita M, Uchiyama T, Adachi A. (2006). Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. Proceedings of the National Academy of Sciences of the United States of America 103(45):16959–16964.

Kaul R, Trabattoni D, Bwayo JJ, Arienti D, Zagliani A, Mwangi FM, Kariuki C, Ngugi EN, MacDonald KS, Ball TB, Clerici M, Plummer FA. 1999. HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers. AIDS, 13(1):23-29.

Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner A, Regier D, Sehgal P, Daniel M, King N, et al. 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science, 248(4959):1109-1112.

Kondoh H, Kobayashi K, Hagiwara K, Kajii T. 1986. Jacalin, a jackfruit lectin, precipitates IgA1 but not IgA2 subclass on gel diffusion reaction. Journal of immunological methods, 88(2):171-173.

Kozlowski PA, Black KP, Shen L, Jackson S. 1995. High prevalence of serum IgA HIV-1 infection-enhancing antibodies in HIV-infected persons. Masking by IgG. Journal of immunology, 154(11):6163–6173.

Langley R, Wines B, Willoughby N, Basu I, Proft T, Fraser JD. 2005. The staphylococcal superantigen-like protein 7 binds IgA and complement C5 and inhibits IgA-Fc alpha RI binding and serum killing of bacteria. Journal of immunology, 174(5):2926–2933.

Liao CH, Kuang YQ, Liu HL, Zheng YT, Su B. 2007. A novel fusion gene, TRIM5-CyclophilinAin the pig-tailed macaque determines its susceptibility to HIV-1 infection. Aids 21 (Suppl 8):S19–S26.

Luciw PA, Pratt-Lowe E, Shaw KE, Levy JA, Cheng-Mayer C. 1995. Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV). Proceedings of the National Academy of Sciences of the United States of America, 92(16):7490-7494.

Mascola JR, Haynes BF. 2013. HIV-1 neutralizing antibodies: understanding nature's pathways. Immunological Reviews., 254(1):225-44.

Mattu TS, Pleass RJ, Willis AC, Kilian M, Wormald MR, Lellouch AC, Rudd PM, Woof JM, Dwek RA. 1998. The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of N-glycosylation on Fcalpha receptor interactions. Journal of Biological Chemistry, 273(4): 2260–2272.

Moore PL, Crooks ET, Porter L, Zhu P, Cayanan CS, Grise H, Corcoran P, Zwick MB, Franti M, Morris L, Roux KH, Burton DR, Binley JM. 2006. Nature of nonfunctional envelope proteins on the surface of human immunodeficiency virus type 1. Journal of Virology, 80(5):2515-2528.

Poignard P, Moulard M, Golez E, Vivona V, Franti M, Venturini S, Wang M, Parren PW, Burton DR. 2003. Heterogeneity of envelope molecules expressed on primary human immunodeficiency virus type 1 particles as probed by the binding of neutralizing and nonneutralizing antibodies. Journal of Virology, 77(1):353-365.

Pope M, Haase AT. 2003. Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. Nature Medicine, 9(7):847-852.

Riddick NE, Hermann EA, Loftin LM, Elliott ST, Wey WC, Cervasi B, Taaffe J, Engram JC, Li B, Else JG, Li Y, Hahn BH, Derdeyn CA, Sodora DL, Apetrei C, Paiardini M, Silvestri G, Collman RG. 2010. A novel CCR5 mutation common in sooty mangabeys reveals SIVsmm

infection of CCR5-null natural hosts and efficient alternative coreceptor use in vivo. PLoS Pathogens, 6(8):e1001064.

Rojas R, Apodaca G. 2002. Immunoglobulin transport across polarized epithelial cells. Nature Reviews Molecular Cell Biology, 3(12):944-55.

Royle L, Roos A, Harvey DJ, Wormald MR, van Gijlswijk-Janssen D, el RM R, Wilson IA, Daha MR, Dwek RA, Rudd PM. 2003. Secretory IgA N- and O-glycans provide a link between the innate and adaptive immune systems. Journal of Biological Chemistry, 278(22): 20140–20153.

Sandin C, Linse S, Areschoug T, Woof JM, Reinholdt J, Lindahl G: Isolation and detection of human IgA using a streptococcal IgA-binding peptide. 2002. Journal of Immunology, 169(3):1357–1364.

Schlatter S, Stansfield SH, Dinnis DM, Racher AJ, Birch JR, James DC. 2005. On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. Biotechnology progress, 21(1):122-133.

Schmaljohn AL. 2013. Protective antiviral antibodies that lack neutralizing activity: precedents and evolution of concepts. Current HIV Research, 11(5):345-353.

Senior BW, Woof JM. 2005. The influences of hinge length and composition on the susceptibility of human IgA to cleavage by diverse bacterial IgA1 proteases. Journal of Immunology, 174(12):7792-7799.

Sharp PM, Hahn BH. 2011. Origins of HIV and the AIDS pandemic. Cold Spring Harbor Perspectives in Medicine, 1(1):a006841.

Shibata R, Sakai H, Kawamura M, Tokunaga K, Adachi A. 1995. Early replication block of human immunodeficiency virus type 1 in monkey cells. Journal of General Virology, 76 (Pt 11):2723– 2730.

Siddappa NB, Song R, Kramer VG, Chenine AL, Velu V, Ong H, Rasmussen RA, Grisson RD, Wood C, Zhang H, Kankasa C, Amara RR, Else JG, Novembre FJ, Montefiori DC, Ruprecht RM. 2009. Neutralization-sensitive R5-tropic simian-human immunodeficiency virus

SHIV-2873Nip, which carries env isolated from an infant with a recent HIV clade C infection. Journal of Virology, 83(3):1422-1432.

Siddappa NB, Watkins JD, Wassermann KJ, Song R, Wang W, Kramer VG, Lakhashe S, Santosuosso M, Poznansky MC, Novembre FJ, Villinger F, Else JG, Montefiori DC, Rasmussen RA, Ruprecht RM. 2010. R5 clade C SHIV strains with tier 1 or 2 neutralization sensitivity: tools to dissect env evolution and to develop AIDS vaccines in primate models. PLoS One, 5(7):e11689.

Song RJ, Chenine AL, Rasmussen RA, Ruprecht CR, Mirshahidi S, Grisson RD, Xu W, Whitney JB, Goins LM, Ong H, Li PL, Shai-Kobiler E, Wang T, McCann CM, Zhang H, Wood C, Kankasa C, Secor WE, McClure HM, Strobert E, Else JG, Ruprecht RM. 2006. Molecularly cloned SHIV-1157ipd3N4: a highly replication- competent, mucosally transmissible R5 simian-human immunodeficiency virus encoding HIV clade C Env. Journal of Virology, 80(17):8729-8738.

Stephens HA. 2012. Immunogenetic surveillance of HIV/AIDS. Infection, Genetics and Evolution, 12(7):1481-1491.

Stieh DJ, King DF, Klein K, Aldon Y, McKay PF, Shattock RJ. 2015. Discrete partitioning of HIV-1 Env forms revealed by viral capture. Retrovirology, 12:81.

Taylor BS, Sobieszczyk ME, McCutchan FE, Hammer SM. 2008. The challenge of HIV-1 subtype diversity. New England Journal of Medicine, 358(15):1590-1602.

Tomana M, Niedermeier W, Mestecky J, Skvaril F. 1976. The differences in carbohydrate composition between the subclasses of IgA immunoglobulins. Immunochemistry, 13: 325–328.

Tudor D, Yu H, Maupetit J, Drillet AS, Bouceba T, Schwartz-Cornil I, Lopalco L, Tuffery P, Bomsel M. 2012. Isotype modulates epitope specificity, affinity, and antiviral activities of anti-HIV-1 human broadly neutralizing 2F5 antibody. Proceedings of the National Academy of Sciences of the United States of America, 109(31):12680-12685.

Uberla K, Stahl-Hennig C, Böttiger D, Mätz-Rensing K, Kaup FJ, Li J, Haseltine WA, Fleckenstein B, Hunsmann G, Oberg B, et al. 1995. Animal model for the therapy of acquired

immunodeficiency syndrome with reverse transcriptase inhibitors. Proceedings of the National Academy of Sciences of the United States of America, 92(18):8210-8214.

University of California, Irvine, College of Medicine. http://jeeves.mmg.uci.edu/immunology/CoreNotes/CoreNotes.htm (accessed 01/17/15).

Vaerman JP, Heremans JF, Van Kerckhoven G. 1969. Communications. Identification of IgA in several mammalian species. Journal of Immunology, 103(6):1421-1423.

Watkins JD, Siddappa NB, Lakhashe SK, Humbert M, Sholukh A, Hemashettar G, Wong YL, Yoon JK, Wang W, Novembre FJ, Villinger F, Ibegbu C, Patel K, Corti D, Agatic G, Vanzetta F, Bianchi S, Heeney JL, Sallusto F, Lanzavecchia A, Ruprecht RM. 2011. An anti-HIV-1 V3 loop antibody fully protects cross-clade and elicits T-cell immunity in macaques mucosally challenged with an R5 clade C SHIV. PLoS One, 6(3):e18207.

Watkins JD, Sholukh AM, Mukhtar MM, Siddappa NB, Lakhashe SK, Kim M, Reinherz EL, Gupta S, Forthal DN, Sattentau QJ, Villinger F, Corti D, Ruprecht RM; CAVD Project Group. 2013. Anti-HIV IgA isotypes: differential virion capture and inhibition of transcytosis are linked to prevention of mucosal R5 SHIV transmission. AIDS, 27(9):F13-20.

Woof JM, Kerr MA. 2004. IgA function-variations on a theme. Immunology, 113(2):175-177.

Woof JM, Kerr MA. 2006. The function of immunoglobulin A in immunity. Journal of Pathology, 208(2):270-282.

Woof JM, Russell MW. 2011. Structure and function relationships in IgA. Mucosal Immunology, 4(6):590-597.

Wright A, Lamm ME, Huang YT. 2008. Excretion of human immunodeficiency virus type 1 through polarized epithelium by immunoglobulin A. Journal of Virology, 82(23):11526-11535.

Xiao P, Zhao J, Patterson LJ, Brocca-Cofano E, Venzon D, Kozlowski PA, Hidajat R, Demberg T, Robert-Guroff M. 2010. Multiple vaccine-elicited nonneutralizing antienvelope antibody activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. Journal of Virology, 84(14):7161–7173.

Zhou M, Ruprecht RM. 2014. Are anti-HIV IgAs good guys or bad guys? Retrovirology, 11:109.

Zolla-Pazner S. 2004. Identifying epitopes of HIV-1 that induce protective antibodies. Nature reviews. Immunology, 4(3):199-210.

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10 Index of Abbreviations

AIDS	Acquired immunodeficiency syndrome
Cα	IgA heavy constant region
dIgA	Dimeric Immunoglobulin A
HC	Heavy chain
HIV	Human Immunodeficiency Virus
Env	Envelope glycoprotein
Ig	Immunoglobulin
JC	Joining chain
LC	Light chain
RM	Rhesus Macaque
RT	Room temperature
SC	Secretory component
SEC	Size-exclusion chromatography
SHIV	Simian-human immunodeficiency virus
SIV	Simian immunodeficiency virus
TAC	Thiophilic adsorption chromatography
TCID50	Tissue culture infectious dose
nAbs	Neutralizing antibodies
non-nAbs	Non-neutralizing antibodies
mAbs	Monoclonal antibodies
nmAbs	Neutralizing monoclonal antibodies
non-nmAbs	Non-neutralizing monoclonal antibodies
pIgR	Poly immunoglobulin receptor
stHIV	Simian tropic human immunodeficiency virus
TMB	3,39,5,59-tetramethyl benzidine

VCA	Virion Capture Assay
VH	Variable region of the heavy chain
VL	Variable region of the light chain
VLPs	Virus-like particles
WT	Wildtype

11 Index of Companies

ABL, Rockville, MD, USA

Affymetrix e Biosciece, San Diego, CA, USA

Amersham Biosciences (bought up by GE Healthcare)

Berthold Technologies, Bad Wildbad, Germany

Bio-Rad Laboratories, Inc., Hercules, California, USA

(AbD Serotec, list here all companies that are under the brand of Bio-Rad)

Corning Inc., Corning, NY, USA

Denville Scientific Inc., South Plainfield, NJ, USA

Eppendorf, Hamburg, Germany

G-Biosciences, St. Louis, MO, USA

GE Healthcare Life Sciences, Chalfont St Giles, Buckinghamshire, UK

Genewiz, South Plainfield, NJ, USA

Gibco Life Technologies, Carlsbad, California, USA

GraphPad Software, Inc., La Jolla, CA, USA

InvivoGen, San Diego, CA, USA

Jackson ImmunoResearch Laboratories, West Grove, PA, USA

Macherey-Nagel GmbH & Co. KG, Düren, Germany

Merck Millipore, Billerica, MA, USA

New England Biolabs, Ipswich, MA, USA

Promega, Fitchburg, WI, USA

Qiagen, Valencia, CA, USA

Sigma-Aldrich, St. Louis, MO, USA

Southern Biotech, Birmingham, AL, USA

Thermo Scientific, Waltham, MA, USA