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Characterisation of Neutralising and Functional Antibody Responses to Different HIV-1 Env Vaccines in Bovines

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To my grandmother, Nena
and her ducks with jumpers

A mi abuela, la Nena
y sus patos con chaleco

Abstract

Two main challenges have impeded the development of an effective HIV-1 envelope (Env) vaccine, with antibodies eliciting neutralisation of virions as well as Fc-effector functions, such as antibody-dependent cytotoxicity (ADCC), phagocytosis (ADP) or complement deposition (ADCD). On one hand, designing the right Env vaccine to elicit humoral or cellular protection has been challenging and, to date, SOSIP-Env trimers which are covalently constrained in the closed, pre-fusion conformation are the best vaccine candidate over uncleaved (Unc), open-structured trimers. On the other hand, eliciting heterologous neutralising antibodies in several animal models (including humans) has been difficult. Cows nevertheless produce unique antibodies with long CDRH3 regions, capable of accessing neutralising epitopes beneath the glycan shield, inaccessible for other animals. We tested how differences in clade and/or structure of HIV-1 Env vaccines affect the neutralising activity and Fc-effector functions of antibodies elicited, using recombinant trimers of clades A (KNH1,BG505), B (AD8, PSC89) and C (MW), which exposed either an open structure (Unc gp140) or a closed structure (SOSIP gp140).

KNH1/BG505 SOSIP gp140 vaccine elicited the best neutralising IgGs against heterologous tier-2 pseudoviruses with high potency and breadth. While AD8 Unc gp140 also induced neutralisation, it was against tier-1 pseudoviruses. Nevertheless, it was the only vaccine able to elicit IgGs that engaged CD32 (FcgRIIa), induced phagocytosis and complement-activation. The different antibody profile observed with both vaccines was explained by the Env immunogen structure, as KNH1/BG505 SOSIP gp140 induced mostly IgGs targeting the V1/V2 loop, whereas AD8 Unc gp140 induced antibodies targeting CD4-binding site and CD4-induced epitopes. In addition, analysis of IgG repertoires from animals of KNH1/BG505 SOSIP 100 and AD8 Unc 500 groups showed that KNH1/BG505 SOSIP gp140 induced higher rates of somatic hypermutation in germline genes compared to AD8 Unc gp140, with each animal

presenting a unique antibody profile, and with germline antibodies already presenting high affinity towards HIV-1 Env trimers, as high levels of affinity maturation were not required to obtain antibodies with high neutralising activity.

Overall, the results in this work show that open structured trimers elicit antibodies which highly activate antibody-effector functions, while SOSIP trimers focus antibody responses to concealed neutralising epitopes. The high neutralising responses observed in bovines against HIV-1 Env are due to antibodies which do not need high levels of somatic hypermutations and, in particular for KNH1/BG505 SOSIP, this antigen induced high levels of affinity maturation, probably favouring the improvement of both binding and neutralisation. Our study suggests that an effective vaccine regimen may include both uncleaved gp140 and SOSIP gp140, in order to target epitopes required for antibody-dependent effector functions as well as neutralisation, or a new trimeric structure with flexibility in the gp120-gp41 interface, exposing both epitopes involved in Fc-effector functions as well as neutralising ones.

Declaration

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This is to certify that:

I. This thesis comprises only my original work towards the degree of Doctor of Philosophy, except where indicated in the preface.
II. Due acknowledgements have been made in the text to all other materials used.
III. The thesis is fewer than 100,000 words in length, exclusive of tables, figures, bibliography, and appendices as approved by the Research Higher Degrees Committee.

_____

Natalia Andrea Salazar-Quiroz

Preface

This work was carried out in the HIV Molecular Virology Laboratory, part of the Department of Microbiology & Immunology of the University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, under the supervision of Prof. Damian Purcell, Dr. Georges Khoury and Dr. Behnaz Heydarchi.

My contribution to the experiments within each chapter is as follows:

- Chapter 3: 100%

- Chapter 4: 85%

- Chapter 5: 70%

In addition, other people have contributed with experimental data in this work. I acknowledge the important contributions from the following people: Jack Edwards (15%) on **Chapter 4**, and Dr. Shuo Li (10%) and Dr. Behnaz Heydarchi (20%) on **Chapter 5**.

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I would also like to give a big thanks to the collaborators of Purcell Lab: A. Prof. Marit van Gils and Prof. Rogier Sanders from the Academic Medical Center, Dr. Bruce Wines from the Burnet Institute, and Dr. Martin Corcoran and Prof. Gunilla Karlsson-Hedestam from the Karolinska Institutet. They provided important elements for chapters three, four and five of this work, respectively, and hence their involvement in this work was crucial for its success.

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List of publications

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1. Literature Review

1.1 HIV-1 Summary

The Human Immunodeficiency Virus type 1 (HIV-1) is a Lentivirus, part of the *Retroviridae* family, and the causative agent of the Acquired Immunodeficiency Syndrome (AIDS). Since studies linked HIV-1 to AIDS in 1983 (Barre-Sinoussi et al., 1983; Gallo et al., 1984), there is an estimation of 37.9 million people living with HIV, of which 1.7 million are newly infected (UNAIDS, 2018). Over the last decades, access to anti-retroviral therapies and use of prophylactic methods such as condoms and pre-exposure prophylaxis (PrEP) have helped to decrease the rates of virus transmission and deaths related to HIV infection (Martin et al., 2011; Mujugira et al., 2011; Baeten et al., 2012; Choopanya et al., 2013). Still, such prophylactic strategies have some application limits particularly due to socio-cultural context. Together with lack of a functional cure for HIV-1 infection and failure to achieve the goal set by UNAIDS 500,000 cases per year (UNAIDS 2018), it is evident that there is still an urgent need for a global preventative vaccine.

1.1.1 HIV-1 structure and life cycle

As a member of the *Retroviridae* family, HIV-1 is a (+) ssRNA virus with two identical plus-stranded RNA genomes within an infectious virion. After entering cells, viral genome must enter the nucleus and undergo reverse transcription to cDNA, as other members of the *Retroviridae* family, before integrating into the host DNA, entering a state of provirus. Provirus then uses the cellular RNA polymerase II to express the structural polyproteins Gag, Gag-Pol and Env, plus other non-structural proteins from a new 9kb genomic RNA (Gallo et al., 1988), allowing replication. New virions are formed internally by p55 Gag and p160 Gag-Pol precursor polyproteins. While cleavage within Gag by the viral protease domain produces matrix, capsid and nucleocapsid proteins (Gottlinger et al., 1989), cleavage within Pol results in the viral protease, integrase and reverse transcriptase (Jacks et al., 1988). Env, on the other

hand, is expressed from a 4kb spliced RNA and appears on the surface of budded virions to produce the viral spikes (Figure 1-1).

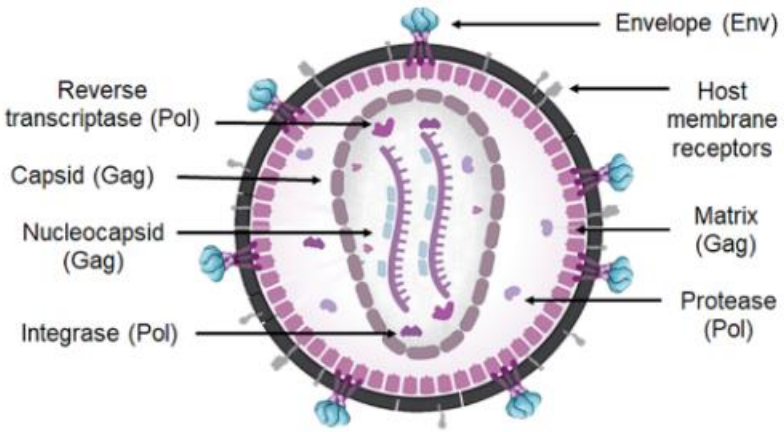


Figure 1-1: Structure of HIV-1. HIV-1 viral particles are composed of different structural proteins, synthesised from the genes *gag*, *pol* and *env* which produce the polyproteins Gag, Pol, and Env, respectively. Further cleavage of polyproteins results in maturation of virions, which present the proteins indicated in the figure. As reference, all structural proteins have indicated their polyprotein origin in parenthesis (Adapted from Thomas Splettstoesser (www.scistyle.com)).

In particular, Env is expressed through the endoplasmic reticulum (ER) and Golgi as a gp160 precursor that is later cleaved by the cellular protease furin, forming the transmembrane glycoprotein gp41, and the surface glycoprotein gp120, both called according to their molecular weight in kDa (Allan et al., 1985; Robey et al., 1985; Veronese et al., 1985). Before cleavage by furin or furin-related proteases, the glycoprotein precursor gp160 is arranged at the viral surface as a trimeric structure, usually called “envelope spike”, and it represents the only surface antigen for HIV-1, in both virions and infected cells. In consequence, an envelope spike is formed by three gp120 plus three gp41 glycoproteins (Figure 1-2).

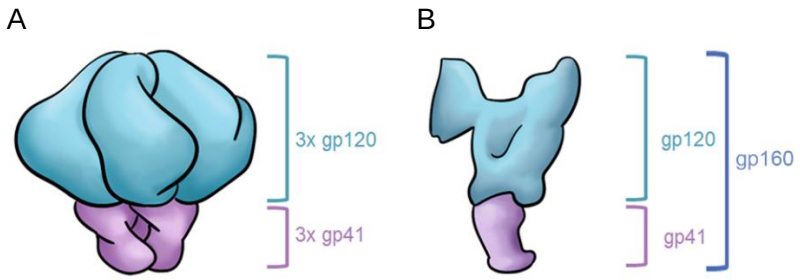


Figure 1-2: Structure of HIV-1 Envelope. A) Envelope spike represents the only viral surface antigen, formed by a heterotrimer of glycoproteins gp120 and gp41. B) Monomeric subunit of the envelope spike, called gp160 and formed by gp120 and gp41, which is product of the polyprotein Env.

As the only surface viral protein, Env mediates HIV-1 infection of CD4+ cells, mainly immune cells: T lymphocytes and macrophages (Koppensteiner et al., 2012). HIV-1 infection occurs in a sequence of events: interaction of the host cell's CD4 receptor with Env gp120, exposure of the coreceptor binding site within gp120 and binding to a co-receptor: CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4) (Feng et al., 1996; Kwong et al., 1998; Rizzuto et al., 1998; Chen et al., 2005). Binding of either CCR5 or CXCR4 determines the cell tropism of a viral isolate: CCR5 is predominantly used by HIV-1 to infect macrophages and primary CD4+ T cells, whereas CXCR4 is only used in T cells and laboratory T-cell lines. Viruses are then defined as R5 if they only use CCR5, X4 if they only used CXCR4, or R5X4 or "dual-tropic", if they can use both coreceptors to infect (Berger et al., 1998). Regardless of viral tropism, binding of either CCR5 or CXCR4 by gp120 allows the exposure and further insertion of a fusion peptide, located in the amino terminus of gp41, into the host cell membrane, inducing a conformational change that draws near both virus and cell and causes fusion of both lipid membranes, releasing the viral core

inside the infected cell cytoplasm (Chan et al., 1997; Weissenhorn et al., 1997; Chen et al., 2005; Buzon et al., 2010) (Figure 1-3).

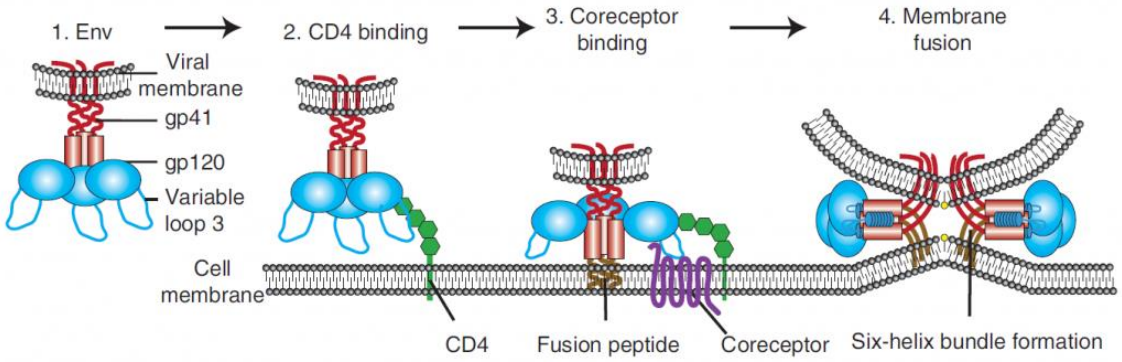


Figure 1-3: Interaction between HIV-1 Env and susceptible cells for viral entry. 1) HIV-1 Env is formed by glycoproteins gp120 and gp41 in an heterotrimeric arrangement; 2) gp120 interacts with cellular receptor CD4 in CD4+ T cells and macrophages, exposing co-receptor binding site (Co-RBS) epitope; 3) gp120 binds to CCR5 or CXCR4 co-receptors and fusion peptide is inserted into host plasma membrane; 4) conformational changes within gp41 produces a six-helix bundle which draws near viral and host membranes, inducing membrane fusion (Wilen et al., 2012).

In order to successfully infect a cell, HIV-1 genome must be imported to the nucleus and integrate into the cell genome. To achieve this, both RNA strands are first reverse transcribed into double-stranded DNA inside of the viral capsid, while the capsid interacts with dynein and microtubule-associated protein 1 (MAP1) for transportation of reverse transcription complexes (RTCs) during their maturation into pre-integration complexes (PICs), one per virion (McDonald et al., 2002; Fernandez et al., 2015). After synthesis of the first DNA strand, viral capsid disassembles in a process called uncoating (Cosnefroy et al., 2016) which is inhibited if reverse transcription is blocked (Iordanskiy et al., 2006; Hulme et al., 2011). Uncoating is crucial, as capsid-protected

DNA cannot enter the nucleus due to size constraints (Pante and Kann, 2002; Ganser-Pornillos et al., 2007; Lowe et al., 2010). Once the viral capsid is destabilised near the nuclear pore, interactions between the non-structural protein Vpr with protein vpr-interactin protein 1 (RIP-1) allows entry of one PIC into the nucleus (Heinzinger et al., 1994; Zhao et al., 1994; Popov et al., 1998), in a process independent of mitosis (Katz et al., 2003). Finally, viral integrase initiates integration of viral genome into active transcription sites within the host cell genome (Craigie and Bushman, 2012; Engelman and Singh, 2018). From here, infected cells become either productively infected (and thus, produce infectious virus) or latently infected (meaning that viral genome is present in the cell but not expressed) and their state will depend on their activation and further stimulation (Colin and Van Lint, 2009).

In productively infected cells, transcription is mediated by the host cell DNA polymerase from the viral 5' LTR promoter, which produces one full-length, 9 kb mRNA. Production of the full 9 kb transcript is inefficient unless viral protein Tat is present, which can be produced through inefficient transcription and regulated by a conserved RNA structure within genome called Tat IRES-modulator of tat mRNA (TIM-TAM) (Khoury et al., 2020). Tat binds to a Trans-activation region (TAR) located in the 5' region of the nascent transcript, recruiting host factors such as pTEFb, increasing processivity of RNA polymerase II and allowing extension of transcription along the viral genome. This full-length transcript can produce up to 40 different mRNAs of three general sizes through alternative splicing: 1) a series of fully spliced 2 kb mRNAs that encode regulatory proteins Tat, Rev and Nef; 2) a series of partially spliced 4 kb transcripts that encode regulatory proteins Vif, Vpu and Vpr, along with structural protein Env; and 3) an unspliced 9 kb mRNA which produces structural proteins Gag and Gag-Pol (Purcell and Martin, 1993).

During the formation of new viral particles, Env is produced as gp160 in the endoplasmic reticulum (ER) and glycosylated with N-linked carbohydrates (Montefiori

et al., 1988; Stansell and Desrosiers, 2010) and O-linked carbohydrates (Bernstein et al., 1994). Before exiting the ER, gp160 forms trimeric structures and is cleaved into gp120 and gp41 at the Golgi apparatus (Stein and Engleman, 1990) by furin or furin-like proteases (Hallenberger et al., 1992; Moulard and Decroly, 2000), with subunits associated through non-covalent interactions (Kowalski et al., 1987). Gag and Gag-Pol on the other hand are produced in the cytoplasm and form complexes that migrate to the plasma membrane within hours of production (Tritel and Resh, 2000), and with the co-translational addition of an N-terminal myristyl group which increases its affinity towards membranes (Gottlinger et al., 1989; Bryant and Ratner, 1990), Gag and Gag-Pol complexes accumulate in lipid rafts (Ganser-Pornillos et al., 2008). After the recruitment of two copies of newly synthesised RNA genome which form a diploid structure through an RNA kissing loop, interactions with zinc-finger motifs in the nucleocapsid domain of myristated p55 Gag assemble both genome and capsid precursors at the plasma membrane, and together with recruiting of Env through interaction with the matrix domain of p55 Gag, the curvature in the host cell plasma membrane allows budding of new virions, excised by the host cell ESCRT machinery (Sundquist and Krausslich, 2012). Once the particles are released, virions undergo a final maturation step mediated by the activation of viral protease, which self-cleaves and then cleaves proteins from precursor Pol, a step needed for viral infectivity (Briggs and Krausslich, 2011).

These new mature particles can now infect new cells, and infected cells can undergo cell death, become productively infected or latently infected (Killian and Levy, 2011). The path followed by infected immune cells will later determine disease progression, which can take years to manifest symptoms, along with activation of innate and adaptive immune response during the acute phase of infection.

1.1.2 Transmission and pathogenesis of HIV-1 infection

A person living with HIV can transmit the virus through one of three routes: 1) sexual intercourse, 2) bloodstream, translated into contact with infected needles or blood transfusion and 3) mother-to-child transmission. This is due to virus being found in sexual fluids, blood, and breastmilk (Zagury et al., 1984; Ho et al., 1985; Vogt et al., 1986; Albert et al., 1987; Borzy et al., 1988; Stiehm and Vink, 1991). Additionally, mothers can also infect their children during pregnancy (Maury et al., 1989; European Collaborative, 1992). Other groups of risks include haemophiliacs (Kitchen et al., 1984) and people in need of blood transfusions (Feorino et al., 1985). For sexual intercourse, transmission is dependent on viral load (Quinn et al., 2000), and high viral load along with genital lesions correlate with transmission of HIV-1 between serodiscordant couples (Gray et al., 2001). In consequence, choosing known seronegative sexual partners over untested partners and condom usage reduce relative risk of HIV-1 infection (Varghese et al., 2002).

CD4+ T cells are susceptible to HIV-1 at the site of infection. Virus can also enter Langerhans cells by endocytosis in the vaginal mucosae (Hladik et al., 2007). Alternatively, dendritic cells (DCs) can internalise HIV-1 in rectal mucosae (Gurney et al., 2005) or in blood (Patterson et al., 1991, Patterson, 1987 #449; Weissman et al., 1995) by DC-SIGN receptor (Arrighi et al., 2004; Gurney et al., 2005) and transport it into lymphoid organs where it can then infect CD4+ T cells (Geijtenbeek et al., 2000, {Weissman, 1995 #17). In bloodstream, virus can be found in PBMCs and cell-free, and plasma viremia is a reliable marker for disease progression (Coombs et al., 1989). At transmission, most circulating virus derives from a single clone, which establishes infection and thus is called transmitted/founder (T/F) virus (Pang et al., 1992; Zhu et al., 1993; Derdeyn et al., 2004). Antibodies against T/F virus are produced at the start of the infection, but they fail in neutralisation.

Since HIV-1 infects CD4+ immune cells, primary infection starts with rapid drop in the numbers of CD4+ T-lymphocytes, particularly at the gastro-intestinal tissue (Douek, 2007). Patients can exhibit influenza-like symptoms during the first days post-infection (Little et al., 1999) in concordance with high viral titres, which decline after weeks of infection to undetectable levels (Clark et al., 1991). After 3 to 4 weeks post infection, CD4+ T cell levels stabilise due to innate and adaptive immune responses controlling viral load and in consequence, replication of virus and cell death progress slowly. Despite initial immune control, HIV continues to replicate generating a high level of mutated virus, arising from a 10^{-4} reverse transcription error rate, and in following years (8 to 10), functional immune control of HIV is lost.

1.1.3 AIDS

It is estimated that HIV-1 appeared around 1930's from SIVcpz, a retrovirus from chimpanzees which could have changed tropism into humans, and it took 50 years to see consequences of infection in human population (Greene, 2007; Sharp and Hahn, 2011). In early 1980's, several cases of homosexual men with opportunistic infections and aggressive cases of Kaposi's sarcoma appeared around the world. In 1982, the Centre for Disease Control (CDC) named the disease Acquired Immunodeficiency Syndrome (AIDS), due to the observation of deteriorated immune system within affected patients. In following years, it was discovered that the impairment in their immune response was due to a reduction in the levels of circulating CD4+ T cells and in 1983, HIV-1 was profiled as the causative agent of AIDS (Greene, 2007).

Without therapy, patients die from an immunodeficiency that has many disease manifestations. In the final state, AIDS is defined as circulating CD4+ T cells under 200 cells per mm^3, where upon the immune function is so undermined that patients become vulnerable to several opportunistic infections from diseases, such as pneumocystis pneumonia and tuberculosis (Killian and Levy, 2011). After the advent of

combination antiretroviral therapy in 1995, HIV-1 is treated as a chronic infection and AIDS has been dramatically reduced, however lifelong HIV infections remain, and the main ongoing heath concern are co-morbid diseases associated with ongoing chronic inflammation (Yoshimura, 2017).

1.1.4 Prevention and treatment for HIV-1 infection

To date, no vaccine to prevent viral acquisition or a cure for infected patients exists; therefore, the major focus is on prevention of viral transmission. Condom usage, disposal of used syringes, blood supply screening and usage of antiretroviral drugs during pregnancy and delivery have helped to prevent transmission through all transmission routes (Eisinger and Fauci, 2018). Usage of antiretroviral drugs in non-infected individuals has shown to additionally prevent acquisition of HIV-1, a method called pre-exposure prophylaxis (PrEP). This strategy has shown positive outcomes in men who have sex with men (MSM), high-risk heterosexual individuals and drug users (Riddell et al., 2018). All these methods are effective but require high adherence (van Ameijden et al., 1992; Galel et al., 1995; Weller and Davis-Beaty, 2002; Desai et al., 2017)

For treatment of HIV-infected individuals, more than 30 different FDA-approved antiretroviral drugs are available which target different processes of HIV-1 replicative cycle. Depending on which process they block, antiretrovirals can be classified into six groups: nucleoside-analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitors, protease inhibitors (PIs), fusion inhibitors or coreceptor antagonists (Arts and Hazuda, 2012). These drugs have helped extend life expectancy for HIV-positive patients, closing the gap between infected and uninfected people worldwide to an average of 8 years of life term (Marcus et al., 2016). Furthermore, viral load in infected patients can be reduced

to levels at which they cannot infect their partners, a concept named “Undetectable equals untransmittable” or “U = U” (Eisinger et al., 2019).

Current preventive measurements and treatments are effective when used correctly. Nevertheless, their effectiveness depends of socio-cultural factors, which affects the delivery of prevention programs or antiretroviral drugs (Chillag et al., 2002; Poundstone et al., 2004). In some developing countries, access to drugs is not immediate, condom usage cannot be forced to partners and campaigns for HIV testing have not been effective enough to prevent new cases (UNAIDS, 2018). One way to overcome these socio-cultural issues and diminish the amount of new infections is the usage of a universal vaccine. Vaccine trials against HIV-1 now mostly aim to elicit the production of broadly neutralising antibodies (BnAbs), and in some the additional activation of T cell responses (Lelievre and Levy, 2016). To date, most of the vaccine trials have focused on inducing BnAbs through targeting HIV-1 Env, but most have lacked effectiveness for different reasons outlined below.

1.2 Antibody production and HIV-1

The plasma gamma globulin antibodies, or immunoglobulins (Igs), are proteins secreted by plasma cells that differentiate from antigen-activated B lymphocytes (Nutt et al., 2015). Antibodies with protective activity against HIV-1 can block binding of virus to target cells, as well as recruit other immune cells for elimination of free virus or infected cells. For both functions, binding to native Env spikes is required, but antibodies produced in the initial phase of infection usually have low affinity for HIV-1 Env. Moreover, the entire set of antibodies that an animal can produce, called “antibody repertoire”, target numerous sites with different affinities against a target, called “antigen”, and offer alternative isotypes engaging different cellular receptors.

Different species present different antibody repertoires against an antigen, making difficult to ensure that successful results observed in animal vaccine trials will be replicated in humans. Therefore, it is important to know how antibodies are produced, the events that lead to the production of BnAbs, and the challenges in different animal models to elicit them. This thesis investigates a unique animal model for HIV-1 Env antibody production in response to vaccination that results in the production of BnAbs, standing in stark contrast to many other animal hosts. The following section of this literature review describes the molecular events that determine antibody production, so that the unique aspects leading to BnAbs in bovines can be explored in the following experimental chapters.

1.2.1 Antibody structure, assembly, and generation of antibody repertoire

Antibodies (Abs) are formed by two heavy (H) and two light (L) chains, with disulphide bridges between each L and H chain, and both H chains together into a “Y” shape (Figure 1-4). H chain has one variable domain (VH) and three constant domains (CH1, CH2 and CH3), whereas L chain has one variable domain and one constant domain only (VL and CL, respectively) (Schroeder and Cavacini, 2010). Abs can also be divided into two “antigen binding fragments”, Fabs, formed by VH-VL and CH1-CL, and a crystallisable portion, Fc, formed by CH2-CH2 and CH3-CH3 (Edelman et al., 1969; Segal et al., 1974; F A Saul, 1978; Marquart et al., 1980; Novotny and Haber, 1985).

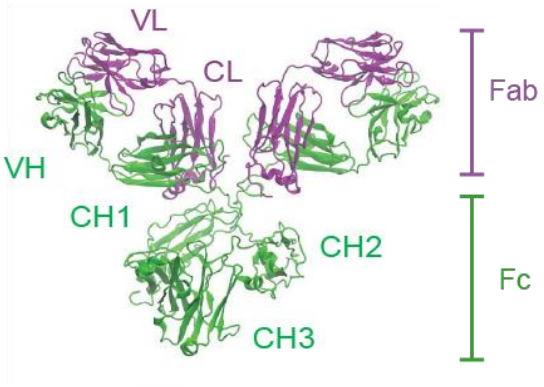


Figure 1-4: Immunoglobulin structure and subunits. Antibodies are formed by two heavy chains (green) and two light chains (purple), which are divided into β-sheet domains. Variable domains of both chains (VH-VL) and their first constant domain (CH1-CL) form the antigen binding fraction on an immunoglobulin, Fab, whereas the second and third constant domains from the heavy chain forms the crystallisable fraction, Fc. (Image adapted from Singer et al., 2014).

The Fab region (VH/VL and CH1/CL) of an Ab is responsible for binding to the antigen, whereas the Fc portion (CH2 and CH3) engages either Fc receptors (FcRs) located on the surface of immune cells, or proteins of the complement cascade. In each Fab region, the V domains determine the specificity of an Ab for a specific site within an antigen, called *epitope.* Antigens can thus present multiple epitopes which can then be detected by the antigen binding site of an antibody, called *paratope.* On the other hand, the constant region of Fc portion confers one of the following *classes* of an Ab: IgM, IgD, IgG, IgE and IgA (Schroeder and Cavacini, 2010). Each Ab class is elicited at different timepoints following antigen exposure and can affect the outcome of an immune response by engagement of FcRs or other immune complexes (ICs).

Abs are produced by B lymphocytes as part of the adaptive immunity. B cells develop in the bone marrow (where their name comes from) and circulate to other lymph

nodes, where they finalise differentiation. Initially, B cells do not secrete any Ab, but they express B cell receptors (BCRs) on their surface. BCRs are composed of a membrane bound Ig, that are embedded on the B cell surface. The BCRs can interacts with CD79a and CD79b, proteins to activate B cell signalling (Schroeder and Cavacini, 2010). Generally, secreted Abs are considered structurally similar to membrane bound Igs, however in some cases structural differences may affect antigen binding (Iype, 2019). Once a membrane bound BCR interacts with a pathogen, the B cell clonotypes are activated to differentiate into plasma cells that efficiently secrete Abs (Nutt et al., 2015). This secreted version of Ig is expressed from an unspliced version of the mRNA that does not utilise a weak 5' splice donor within an exon containing a promoter-proximal termination-polyadenylation site. Whereas mRNA expressing the BCR membrane-bound Ig co-transcriptionally uses the weak 5' splice donor to remove as intron the promotor-proximal poly(A) signal and instead include a new 3' exon that incorporates a transmembrane domain of the membrane bound version and uses an alternative poly(A) signal for mRNA termination (Bruce et al., 2003).

BCRs are formed by the rearrangement of a multifamily of genes located at different locus for H and L chain (Leder, 1982; Tonegawa, 1983). Each Ab locus has a collection of different *germline* genes: the H chain locus presents variable (V), diversity (D), joining (J) and constant (C) genes, whereas the L chain locus has V, J and C genes only (Tonegawa, 1983). Ab diversity is produced in different ways depending on specie (de los Rios et al., 2015), but in all cases, formation of a H chain occurs by one unique V, D, J germline gene rearrangement and a C gene, while Ab L chain consist of one unique V, J germline gene rearrangement and a C gene. In consequence, the rearrangement of different combinations of these genes, called V(D)J recombination, contributes to diversity. Each B cell precursor has a unique gene rearrangement for each H and L chain loci, therefore, each B cell clone presents a different BCR (Nutt et al., 2015) (Figure 1-5).

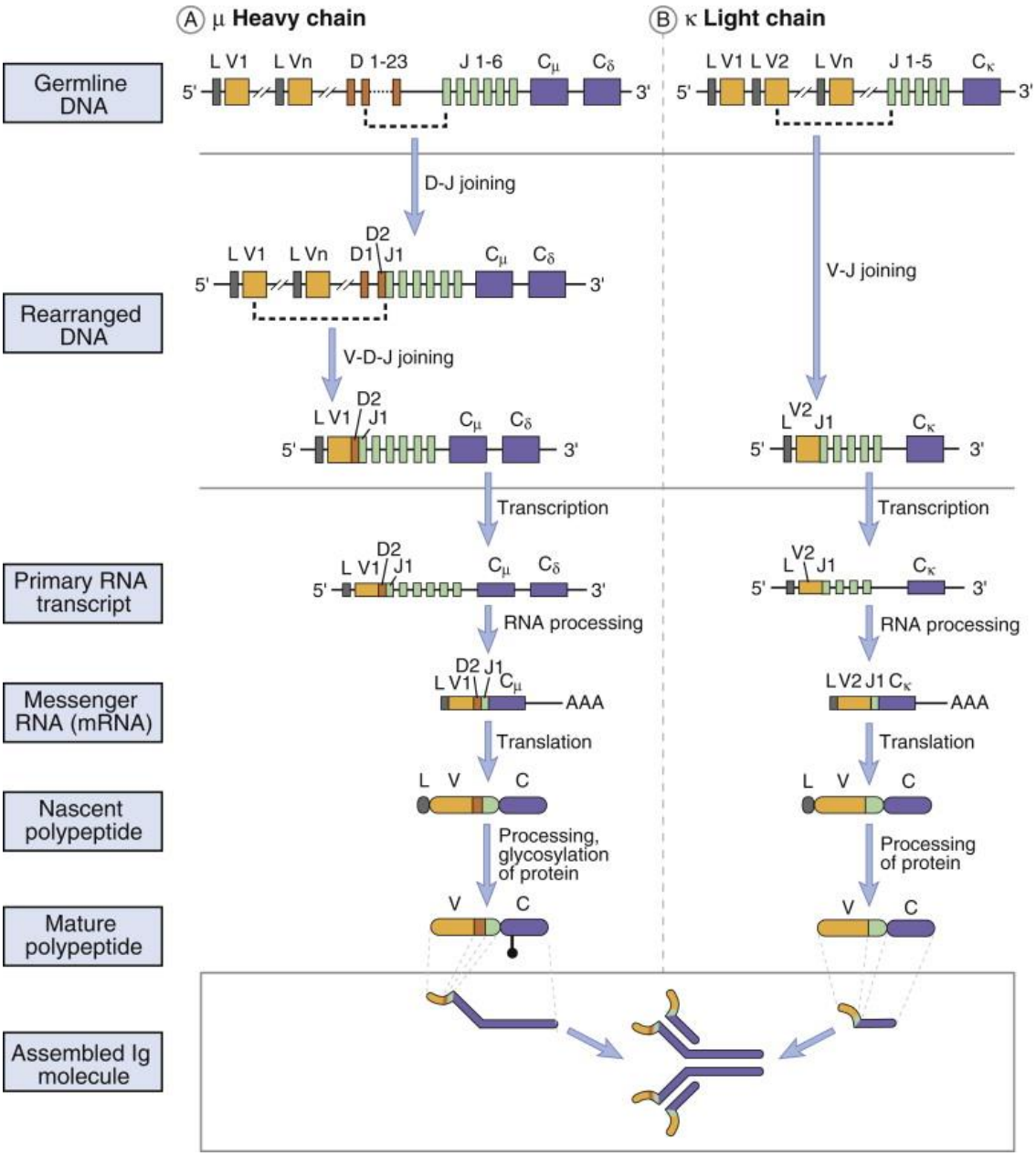


Figure 1-5: V(D)J recombination for generation of antibody repertoire. Germline genes for H and L chains are encoded in separated loci, which are later rearranged for the generation of a H and L chain specific for each cell. In heavy chains (A), V(D)J recombination starts joining one D gene to a J gene, and later joining one V gene to the rest of the chain. When the DNA is transcribed into RNA, non-coding sequences are spliced out in order to generate a unique gene with one V, D, J and constant (C) gene. In L chains (B) the process is similar, with the difference that no D gene is present and thus only joining between one V and one J gene occurs. (Source: Abbas, Litchman and Pillai, 2017).

Generation of diversity starts with V(D)J recombination. Insertion of nucleotides in gene junction points during the V(D)J recombination process also contributes in diversity (called “junctional diversity”). Junction points are defined by flanking sequences, consisting of a highly conserved heptamer motif and a less conserved nonamer motif, called recombination signal sequences (RSS) (Max et al., 1979; Sakano et al., 1979). Recognition of these sequences by the proteins RAG-1 and RAG-2 induces imprecise breaks between the coding gene and the RSS, which then are repaired by the nonhomologous end-joining system, leaving “P” nucleotides. Because joins in the coding end are imprecise, DNA repair by the enzyme terminal deoxynucleotidyl transferase (TdT) is needed, which adds non-germline templated nucleotides (N nucleotides) to the sequence. Overall, joins in different points of the coding ends during the V(D)J recombination and the addition of N nucleotides confer another level of diversity (Schroeder and Cavacini, 2010) (Figure 1-6).

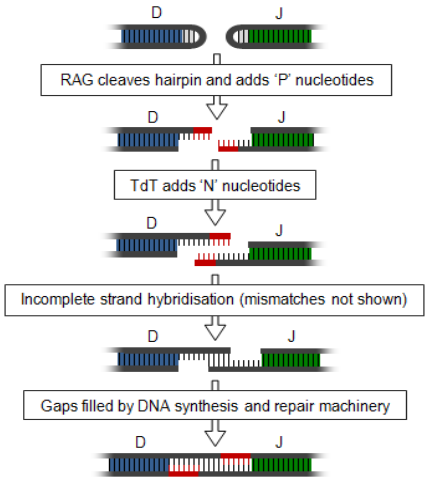


Figure 1-6: Generation of junctional diversity in during V(D)J recombination. Enzymes RAG-1 and RAG-2 cut DNA at the recombination signal sequences and insert “P” nucleotides shown in red. Then, TdT repairs the DNA strand by adding new “N” nucleotides (also shown in red), which are complementary. After hybridisation at the N nucleotide area, DNA is finally repaired at the points were no complementary nucleotides are present. (Source: Wilson, 2009).

A final process in which diversity arises in the Ab repertoire happens after B cells are activated. Upon activation, an activated plasma cell can re-enter into a lymph node, where along with T follicular helper (Tfh) cells, initiates a process in which point mutations accumulate in the V regions of both Ab H and L chains. These modifications are called somatic hypermutations (SHMs) (Bothwell et al., 1981; Tonegawa, 1983; Neuberger, 2008), and its purpose is to enable B cells to produce Abs with higher affinity to bacteria, viruses and other pathogens.

Before the events of somatic hypermutation, addition of N and P nucleotides plus V(D)J recombination establishes the antibody repertoire before encountering antigens. Moreover, both features confer to a BCR and, therefore, to an Ab, its unique specificity. Within the V region of both H and L chains, three loops are exposed at the paratope site, called *complementarity determining regions* (CDRs). While CDR1 and CDR2 are encoded by V genes and vary depending on the V germline gene chosen to produce a BCR and, after activation, somatic hypermutations (Janeway and Travers), CDR3 is encoded by the joining point between V-D and D-J genes for the heavy chain, and between V-J for the light chain. This determines that variability of CDR3 is much higher than CDR1 and CDR2, and that affinity of certain Ab towards their antigen might be driven by the CDR3 sequence and structure. In between CDRs, more conserved regions called *framework regions* (FR) flank each CDR loop and stabilise both VH and VL domains (Figure 1-7).

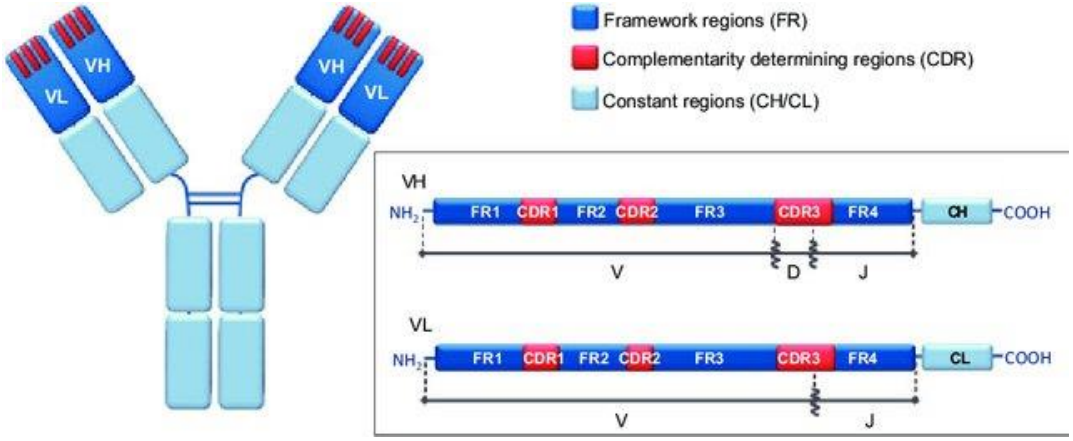


Figure 1-7: Generation of somatic diversity and CDRs. Ab V domains are formed by framework regions (FR, blue) and Complementarity determining regions (CDR, red). Formation of the V domain of both H and L chains occur by the recombination of V, D and J genes in the H chain (VH, upper chain), and of V and J genes in the L chain (VL, lower chain). This DNA rearrangement, called V(D)J recombination, confers somatic diversity to the Ig repertoire, which also forms the CDR3 region in both chains. Together with CDR1 and CDR2, encoded in the V gene, they form the contact site of an Ab for their cognate antigen (Source: Anisimova, 2014).

1.2.2 B cell development, activation, and differentiation

B cell lineage is defined during early stages of development (Melchers, 2015). Its precursors are found in bone marrow and express the markers CD45R/B220, CD24 and c-Kit, and lack CD19 (Li et al., 1996; Ogawa et al., 2000). Cells at this stage, called Pro-B or pre-Pro-B cells (Hardy and Hayakawa, 2001) have not undergone V(D)J recombination yet (Allman et al., 1999), meaning that they do not express a BCR. After rearrangement of their heavy chain, they are called Pre-B, and after further rearrangement of their light chain, they are classified as immature or transitional B cell, expressing a full BCR of IgM class (Hardy and Hayakawa, 2001).

Between immature and transitional state, the newly formed BCR is tested by the central B-cell tolerance system in order to eliminate autoreactive B cells. As an indication, studies in humans and mice show that between 50% and 75% of immature B cells in bone marrow are specific for self-antigens, whereas only between 20% and 40% of transitional B cells found in spleen and blood are autoreactive (Grandien et al., 1994; Wardemann et al., 2003). After exiting bone marrow, B cells are still under surveillance for self-reactivity by peripheral tolerance mechanisms, which drives them into anergy if self-reactive (Pillai et al., 2011) reducing the level of autoreactivity to 10%-30% (Goodnow et al., 1989). The small fraction of autoreactive B cells, which surpass the selection and become mature B cells, present a low level of autoreactivity (Hayakawa et al., 2003; Wardemann et al., 2003; Wen et al., 2005) and are a population available for the evolution of BnAbs against HIV-1 Env during infection and after vaccination (Mouquet et al., 2010; Liu et al., 2015). HIV presents many self-epitopes as a method of immune evasion, restricting the capacity for effective bNAb responses.

B cells that survive the central and peripheral self-antigen immune tolerance mechanisms complete their maturation by increasing the expression of CD21 and decreasing expression of the pre-B cell marker CD10 to background levels (Malaspina et al., 2006; Benitez et al., 2014). After this, mature naïve B cells migrate to peripheral lymphoid tissues where can be activated by direct contact through their BCR with their cognate antigen. Depending on their location in the lymph node follicle they can be activated by T-follicular helper cells. Low affinity antigen interactions promote an ability to recirculate between lymph node, spleen and Peyer's patches, and these B cells will mostly be activated in absence of T-helper cells. On the contrary, high affinity antigen interaction promotes residence in the follicular niche where these cells can be activated by T-helper cell stimulation (Allman and Pillai, 2008). T cell-dependent activation of B cells is important because it induces changes into the antibody isotype

of an Ig produced by plasma cells, a phenomenon called *class-switch recombination* (CSR), allowing the use of distal constant region exons for the production of IgG, IgA and IgE even before the formation of germinal centres where affinity maturation occurs (Roco et al., 2019).

T cell-dependent activation of B cells occurs in four phases, as these cells are initially activated by their antigen, then migrate to T-cell-rich zones in secondary lymphoid organs, interact with antigen-primed T cells and migrate to extrafollicular foci within the lymph node to proliferate and differentiate into plasma cells (MacLennan et al., 2003). At this point, class-switching has already occurred, and the antibodies secreted by plasma cells have no significant difference with the BCR expressed on their B-cell progenitor prior to activation. Activated B cells can later improve binding against their cognate antigen, by re-entering the lymph node and interacting with T follicular helper (Tfh) cells, forming a germinal centre where the process of affinity maturation initiates (Nutt and Tarlinton, 2011; Shlomchik and Weisel, 2012). During this process, the enzyme activation-induced cytidine deaminase (AID) is responsible for introducing SHMs as small mutations within H and L chain loci of activated B cells during clonal expansion (Bothwell et al., 1981; Tonegawa, 1983; Neuberger, 2008), and newly formed BCRs are tested for binding against their antigen in presence of Tfh, which will activate different stimulatory signals mediated by interactions such as antigen-BCR and CD40-CD40L (in Tfh–B cell, respectively), allowing survival of high-affinity B cells (Liu et al., 1989). Introduction of SHM is important to refine and improve binding of Igs to their target which, in consequence, improve the neutralising activity of an Ab response (Sok et al., 2013).

Along with producing long-lived plasma cells with SHM, GCs also produce long-lived memory B cells, which are dormant cells residing in secondary lymphoid organs or other tissues and can be activated upon re-encounter with their antigen. Memory B

cells are formed before the production of long-lived plasma cells, and their BCRs possess lower levels of SHM than their counterparts (Smith et al., 1997; Weisel and Shlomchik, 2017). Nevertheless, memory B cell activation is needed to induce heterologous neutralisation in viruses, which is not achieved by long-lived plasma cells alone, as seen in West Nile virus and Influenza A virus (Purtha et al., 2011; Leach et al., 2019). GC-independent memory B cells, generated in the primary response phase, can also protect from viral infections and their involvement has been observed against influenza A virus infection under the effects of rapamycin, which inhibits the formation of GCs (Keating et al., 2013), and Dengue virus, whose neutralising responses are related to IgM+ memory B cells, with low levels of SHM (Burton et al., 2018).

Overall, an effective Ab response must stimulate the production of IgG-secreting plasma cells with high levels of SHM, along with memory B cells that target conserved sites within an antigen. Generation of these responses against HIV-1 depends of the engagement of Env to a BCR, as cell division and levels of SHM are proportional to the affinity of that BCR to its antigen, implying that antigens with high affinity are needed to elicit a strong antibody response against a pathogen (Gitlin et al., 2014).

1.2.3 Antibody responses against HIV-1 in infected patients

During the acute phase of infection, Abs generated against HIV-1 Env are mostly non-neutralising and thus, fail to control infection (Tomaras et al., 2008). IgM-based Abs have been observed at this stage, which correlates with release of short-lived plasmablasts without CSR (Fink, 2012), along with IgG3 responses which decline rapidly (Yates et al., 2011). Due to the infection of CD4+ T cells, destruction of GCs has been observed in gastrointestinal tract at early stages of infection (Levesque et al., 2009), and in lymph nodes during AIDS (Xu et al., 2015). This interferes with antigen-specific activation and proliferation of B cells, together with other effects related to

diminished T cell function. This results in hypergammaglobulinemia (high secretion of IgG antibodies) and autoantibody production (Lane et al., 1983), and low levels of resting memory (RM) and activated memory (AM) B cells (Titanji et al., 2006; Kardava et al., 2014). Despite this, infected patients present high levels of tissue-like memory (TLM) B cells (Moir et al., 2008; Amu et al., 2014), which are regulated by IgG3 (Kardava et al., 2018) and can produce type-specific neutralising antibodies with low levels of SHM (Meffre et al., 2016).

Maintaining virus control with antibodies elicited against T/F viruses is challenging, mainly because HIV-1 Env exhibits high rate of mutations from the start of infection. Viral mutation rate is higher than selective evolution rate of the host immune system, causing a dramatic heterogeneity of Env structures arising, even in the same individual (Wei et al., 2003; Bonsignori et al., 2017). In addition, changes in the glycosylation pattern masks potential neutralising epitopes (Binley et al., 2010), and the heterogeneity of conformations present on viral particle surface increases the likelihood of eliciting non-neutralising antibodies (Kwong et al., 2002). Due to these difficulties, it is important to target epitopes on HIV Env-1 that are conserved even during the evolution of the virus during immune escape, because these represent functional motifs that are required for viral replication. BnAbs are a subgroup of neutralising antibodies which target these conserved sites within Env on viruses from different clades across the globe. BnAbs target one of five known conserved neutralising epitopes within Env, called “supersites”: (1) CD4 binding site (CD4bs), (2) the exposed V1V2 loop apex, (3) the V3 glycan domain, (4) the gp120-140 interface and (5) the membrane-proximal external region (MPER) of gp41 (Haynes and Mascola, 2017) (Figure 1-8). Only around 20% of infected patients induce BnAbs, and only after years of infection (Sather et al., 2009; Liu et al., 2015), showing that eliciting BnAbs is challenging because these epitopes are strongly anergic and often lead to self-reactive auto-antibodies.

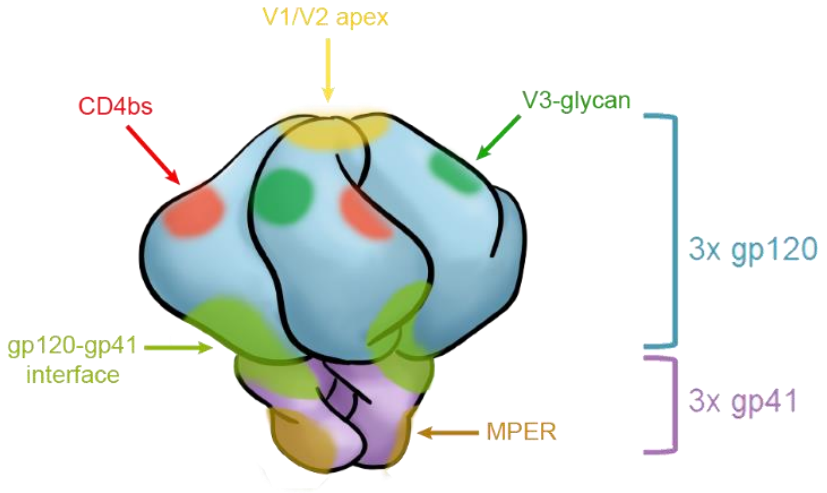


Figure 1-8: Supersites of vulnerability targeted by broadly neutralising antibodies (BnAbs) on HIV-1 Env. Neutralising antibodies isolated from patients after years of infection target conserved sites within gp120 or gp41, present in isolates from different clades and subtypes, and that are important for viral infection. Color codes: red: CD4bs; yellow: V1/V2 apex; green: V3-glycan domain; light green: gp120-gp41 interface; brown: MPER.

1.2.4 Events involved in the generation of BnAbs against HIV-1 Env

When present, BnAbs against HIV-1 often display rare properties like unusual high levels of SHM and long CDR3s. For example, human CD4bs BnAbs present H chains formed by either VH1-2 or VH1-46 genes and acquire SHM rates of around 30% (Wu et al., 2010). V1/V2 apex Abs do not require high levels of SHM (~15%), but their CDRH3 regions are very long, between 24 and 32 residues (Pejchal et al., 2010; McLellan et al., 2011). V3-glycan targeting BnAbs present moderate values of SHM (15-23% in VH; 6-14% in VL) and CDRH3s slightly longer than the human average size (between 18 and 24 residues), but both features result from high levels of non-templated nucleotide insertions and deletions during AID (Walker et al., 2011). The fact that SHM occurs in GCs evidences impairments in the production of BnAbs:

infected patients display damage in lymph node architecture (van Grevenynghe et al., 2008), supporting the reasons behind their low frequency during HIV infection. In addition, several of these BnAb sequences were isolated from elite controllers, patients who can control infection without antiretroviral therapy (ART) and present levels of resting memory B cells similar to uninfected patients (Rouers et al., 2017). In concordance, chronic HIV-1 infected patients can improve their antibody response against Env under treatment with ART drugs, increasing memory B cells frequency and hence improving their functional profile (Moir et al., 2010).

Polyreactivity and autoreactivity are also common features in cloned BnAbs (Liu et al., 2015). Around 75% of gp140 trimer binding Abs in chronic infected patients are reactive with common autoantigens like single and double strand DNA, LPS and cardiolipin (Liu et al., 2015). When unmutated common ancestors (UCAs) for polyreactive/autoreactive anti-Env Abs were generated, 50% of them fail to bind Env gp140 but kept their polyreactivity, suggesting that HIV-1 Env evolves to resemble selfantigens and evade through anergy (Mouquet et al., 2010). MPER antibodies 2F5 and 4E10 are known to be poly- and autoreactive, respectively (Haynes et al., 2005; Mouquet et al., 2010; Yang et al., 2013). They target human proteins kynureinase (KYNU) and splicing factor unit 3b subunit 3 (SF3B3), two proteins with epitopes similar to HIV-1 gp41 epitopes. HIV-1 Env vaccination of opossums, which naturally carry a mutation that abrogates the shared epitope of KYNU, induced high amounts of 2F5-like antibodies (Yang et al., 2013). Due to the anergic elimination of poly- and auto-reactive B cells by central and peripheral tolerance mechanisms, this result confirmed that MPER-directed BnAbs are not produced under normal circumstances (Verkoczy et al., 2011; Doyle-Cooper et al., 2013; Verkoczy et al., 2013).

Genetic fitness of the human antibody germline repertoire to produce antibodies against HIV-1 Env must be analysed, in addition to the requirement for high levels of

SHM that impairs production of neutralising Abs and the elimination of poly- and autoreactive B cell precursors, Unmutated repertoires display low affinity for HIV-1 Env (Xiao et al., 2009; Mouquet et al., 2010; Zhou et al., 2013), explaining the need of high levels of SHM in order to initiate binding. Transgenic mice expressing the Ig V locus with unmutated common ancestors (UCAs) predicted for VRC01 and PGT121 require immunisation with sequential mutated Env trimers that iteratively improve binding to levels observed in these two BnAbs. Induced polyclonal Abs have high rates of SHM resembling the reference monoclonal antibodies (mAbs) isolated in humans (Escolano et al., 2016; Jardine et al., 2016). In addition, high levels of glycosylation in gp120 and gp41 promote early Ab responses to exposed epitopes that are not covered by the glycan shield. These epitopes are not necessarily neutralising and change during infection (Binley et al., 2010). This implies that in order to access neutralising epitopes occluded in the glycan shield, antibodies with long CDRH3s are beneficial. The average CDRH3 length reported in the human antibody repertoire is around 15 residues, and it is shown that human B cells expressing long CDRH3s are naturally eliminated by the immune system before reaching full development (Shiokawa et al., 1999; Meffre et al., 2001). This means that the CDRH3 size of 30 residues observed in some BnAbs is rarely achieved.

1.2.5 Role of Fc-effector functions and antibody-mediated cellular immunity in HIV-1

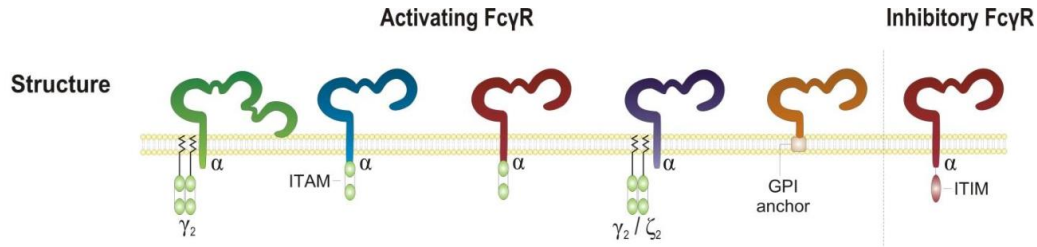
The results from the RV144 Thai trial of ALVAC/gp120 which showed protective efficacy (Rerks-Ngarm et al., 2009) refocused attention on functions elicited by non-neutralising Abs, which emerged as correlates of protection in that vaccine trial (Haynes et al., 2012; Tomaras and Plotkin, 2017). Foremost interest was then turned to immune responses mediated by components of the immune system engaged through an Ab Fc portion, known as Fc-effector functions (Chan et al., 2015). Unlike

BnAbs, Abs eliciting Fc-effector functions only need to bind to their cognate antigen forming immune complexes (ICs) of size and stoichiometry such that allows one of two things: 1) the crosslinking of two or more Fc receptors (FcRs) bound to two or more Ab Fc-regions, or 2) binding of one C1q molecule, the complement component initiating the classical pathway cascade, to five IgG Fc-regions or one IgM Fc-region (Pierson et al., 2007; Lux et al., 2013; Peschke et al., 2017; Robinett et al., 2018). For HIV-1, activation of Fc-effector functions have been associated with control of patient viremia (Alsahafi et al., 2015; Toyoda et al., 2015; Ackerman et al., 2016; Madhavi et al., 2017), reduced risk of virus transmission in humans (Mabuka et al., 2012; Milligan et al., 2015) and lower HIV infection in non-human primates (Gomez-Roman et al., 2005; Xiao et al., 2012; Fouts et al., 2015; Bradley et al., 2017). This highlights the importance of considering antibody-dependent effector functions in designing HIV vaccines

There are several different FcRs, depending on the immunoglobulin class the receptor binds to: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) bind IgGs, FcεRI binds IgE, and FcαRI (CD89) binds IgA (Hogarth, 2015). In their extracellular portion, almost all receptors present two globular domains composed of β-strands (Garman et al., 1998; Maxwell et al., 1999; Sondermann et al., 2000; Zhang et al., 2000; Radaev and Sun, 2002) with the exception of FcγRI that possess 3 globular domains (Lu et al., 2011), and interaction occurs by the engagement of the CH2 domain of an Ab, located at the start of the Fc region (Wines et al., 2000; Shields et al., 2001) with only one of the globular domains of an FcR (Radaev and Sun, 2002). Although extracellular region of an FcR is variable within receptors, their intracellular domain is highly conserved (Bournazos, 2019). All FcRs present either immunoreceptor tyrosine-based activation motifs (ITAMs) (Reth, 1989; Cambier, 1995) that autophosphorylate upon activation, or immunoreceptor tyrosine-based inactivation motifs (ITIMs) (Daeron et al., 2008) which are phosphorylated by recruitment into a complex with an ITAM-containing receptor

(Isakov, 1997; Getahun and Cambier, 2015). It is established that ITAM motifs will activate an immune response, whereas ITIMs will inhibit it (Getahun and Cambier, 2015).

Some FcRs have more than one member in their family, as for example CD32 includes FcγRIIa/b/c, and CD16, FcγRIIIa/b, which can elicit different functions. FcγRIIa and FcγRIIIa are activators of Fc-effector functions, whereas FcγRIIb acts as an immune checkpoint for autoimmune humoral responses (Baerenwaldt et al., 2011). The valency of ICs when bound to FcRs can also regulate the immune response, as the ratio of ICs to monomeric IgGs must be high in order to activate Fc-effector functions (Boruchov et al., 2005). In addition to subtypes of FcRs, different polymorphisms have been found in some receptors. FcγRIIa presents the alleles G27/R131 and W27/H131, where the last residue in discord affects interaction of its receptor to IgGs (Warmerdam et al., 1990). In FcγRIIIa, two allotypes have been characterised, V158 and F158, with V158 presenting higher affinity for IgGs than F158 (Ravetch and Perussia, 1989). Both alleles for each receptor are co-dominant but are expressed at different levels in discrete populations and are linked to several autoimmune diseases (Li et al., 2014). Additional regulation of Fc-receptor effector functions is mediated by differences in affinity of IgG subclasses to each FcR, establishing a binding hierarchy depending on the IC composition (Nimmerjahn and Ravetch, 2005; Bruhns et al., 2009; Wines et al., 2016). For instance, FcγRIIa binds with higher affinity to human IgG3 and with lower affinity to human IgG1, but both isotypes bind with equal affinity to FcγRIIIa (Dijstelbloem et al., 2001). Glycosylation of the Fc portion of antibodies also modulates engagement of their CH2 domain to an FcR (Niwa et al., 2004; Dekkers et al., 2017). A summary of the different FcγR receptors, alleles, expression levels in immune cells and affinities for different Igs can be found on figure 1-9.



Name	FcγRI	FcγRIIa	FcγRIIc	FcγRIIIa	FcγRIIIb	FcγRIIb
CD	CD64	CD32a	CD32c	CD16a	CD16b	CD32b
Gene	*FCGR1A*	*FCGR2A*	*FCGR2C*	*FCGR3A*	*FCGR3B*	*FCGR2B*
Affinity	High	Low to medium	Low to medium	Low to medium	Low to medium	Low to medium
Alleles	¤	H_{131} R_{131}	Q_{57} $stop_{57}$	F_{158} V_{158}	HNA1a/b/c	I_{232} T_{232}
IgG1	6×10^7	5×10^6 3×10^6	1×10^5 -	1×10^6 2×10^6	2×10^5	1×10^5 ND
IgG2	-	4×10^5 1×10^5	2×10^4 -	3×10^4 7×10^4	-	2×10^4 ND
IgG3	6×10^7	9×10^5 9×10^5	2×10^5 -	8×10^6 $\mathbf{1\times10^7}$	1×10^6	2×10^5 ND
IgG4	3×10^7	2×10^5 2×10^5	2×10^5 -	2×10^5 2×10^5	-	2×10^5 ND
Expression pattern:						
B cell	-	-	-	-	-	+
NK cell	-	-	+#	+	-	-*
Mono/Macro	+	+	+#	+	-	+/-
Neutrophil	(+)	+	+#	-	+	+/-
Dendritic cell	+	+	-	-	-	+
Basophil	-	+	-	-	+/-	+
Mast cell	(+)	+	-	-	-	-
Eosinophil	-	+	-	-	-	-
Platelet	-	+	-	-	-	-

Figure 1-9: Summary of Fc-gamma receptors (FcγR) and features. Structural scheme for all described FcγRs is shown. FcγRIIb presents an ITIM motif in the carboxi-terminal end (indicated in red), while all the other receptors present ITAM domains (indicated in green). Alleles found for each receptor are indicated below, as well as expression pattern in different immune cells (Source: Lassauniere, 2015).

Two immune-effector functions can be activated through FcR engagement: antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP). ADCC is initiated by activation of FcγRIIIa on NK cells, macrophages and neutrophils (Kramski et al., 2013; Smalls-Mantey et al., 2013; Worley et al., 2018), and leads to killing of infected cells. On the other hand, activation of the receptor FcγRIIa, present in monocytes, macrophages and neutrophils induces ADCP (Anania et al., 2019). Protection mediated by ADCC can enhance the anti-HIV activity of BnAbs. Mutations in the Fc portion of CD4bs BnAbs including b12, 3BNC117 and 10-1074 significantly decrease protection afforded to macaques during SHIV challenge (Hessell

et al., 2007; Bournazos and Ravetch, 2015). Interestingly, no changes were observed when the Fc portion of the V3-glycan BnAb PGT121 was mutated (Parsons et al., 2019). These results show that importance of Fc-effector functions in conferring protection depends on the epitope targeted by an Ab. Quantity of Env expressed on cell surface also affects ADCC activation (Smalls-Mantey et al., 2013) and is tightly regulated by endocytosis through a conserved signal in the gp41 cytoplasmic tail (Rowell et al., 1995; Boge et al., 1998). In consequence, internalisation of Env by infected cells protects them from ADCC (von Bredow et al., 2016).

BnAbs targeting the CD4bs, gp41 ectodomain and the V2 and V3 loops elicit ADCP (Perez et al., 2009; Ackerman et al., 2013; Chung et al., 2014; Tay et al., 2016; Musich et al., 2017) which clears HIV-1 infected cells (Gach et al., 2017) and free virus (Tay et al., 2016), and blocks viral replication in antigen-presenting cells (Holl et al., 2006). Non-neutralising antibodies targeting the V1/V2 loop can also mediate phagocytosis, as observed in the RV144 trial (Liao et al., 2013; Yates et al., 2014). However, the breadth of Envs targeted by non-neutralising antibodies is limited compared to BnAbs (Liu et al., 2013). Phagocytosis of free virus depends on the amount of virion-associated Env, or the strain of Env used as target, with phagocytosis reported for BaL-coated virions (Tay et al., 2016), but not for JRFL- or NL4-3-coated virions (Gach et al., 2017). This viral strain distinction however was not evident for phagocytosis of infected T cells (Gach et al., 2017).

Binding of IgGs to FcγRs is dependent on their isotypes. IgG3 presents the highest affinity towards both FcγRIIa and FcγRIIIa, followed by IgG1, IgG2 and IgG4 (Kimberly et al., 2002; Wines et al., 2016). Experiments with human antibodies have shown that IgG3 is the main driver of ADCP, followed by IgG1 and lastly IgA (Tay et al., 2016).

Table 1-1: Binding hierarchy of IgG molecules to Fc-gamma receptors. Low-affinity FcγRs (FcγRIIa and FcγRIIIa) and their allotypes are listed on the table. Affinity of IgGs towards each receptor is different, thus making a binding hierarchy between Ig molecules. (Sources: Kimberly et al., 2002; Wines et al., 2016).

FcγR	**Allotype**	**Binding Hierarchy**
FcγRIIa	H131	IgG3 > IgG1 ~ IgG2 >> IgG4
	R131	IgG3 > IgG1 >> IgG2 ~ IgG4
FcγRIIIa	V158	IgG3 > IgG1 > IgG2 >> IgG4
	F158	IgG3 > IgG1 > IgG2 >> IgG4

Soluble phase Fc-effector functions also impact on HIV-1 infection independent of FcR engagement. Complement-dependent cytotoxicity (CDC) depends only on soluble components, and unlike ADCC or ADCP, can be activated by both innate and adaptive immune responses. Abs activate the classical pathway when the C1 complement component is bound by ICs containing IgG1, IgG3 or IgM and initiates a proteolytic cascade reaction involving other complement proteins. This ultimately ends in formation of a membrane attack complex (MAC) that forms a pore in the plasma membrane, resulting in cell lysis (Ricklin et al., 2010). Fc glycosylation can modulate binding of C1q to ICs (Dekkers et al., 2017), as well as proximal cell surface expression of CD55, also called complement decay-accelerating factor (DAF), which degrades the C3bBb complement convertase, protecting cells from MAC formation (Fujita et al., 1987; Nicholson-Weller and Wang, 1994) (Figure 1-10).

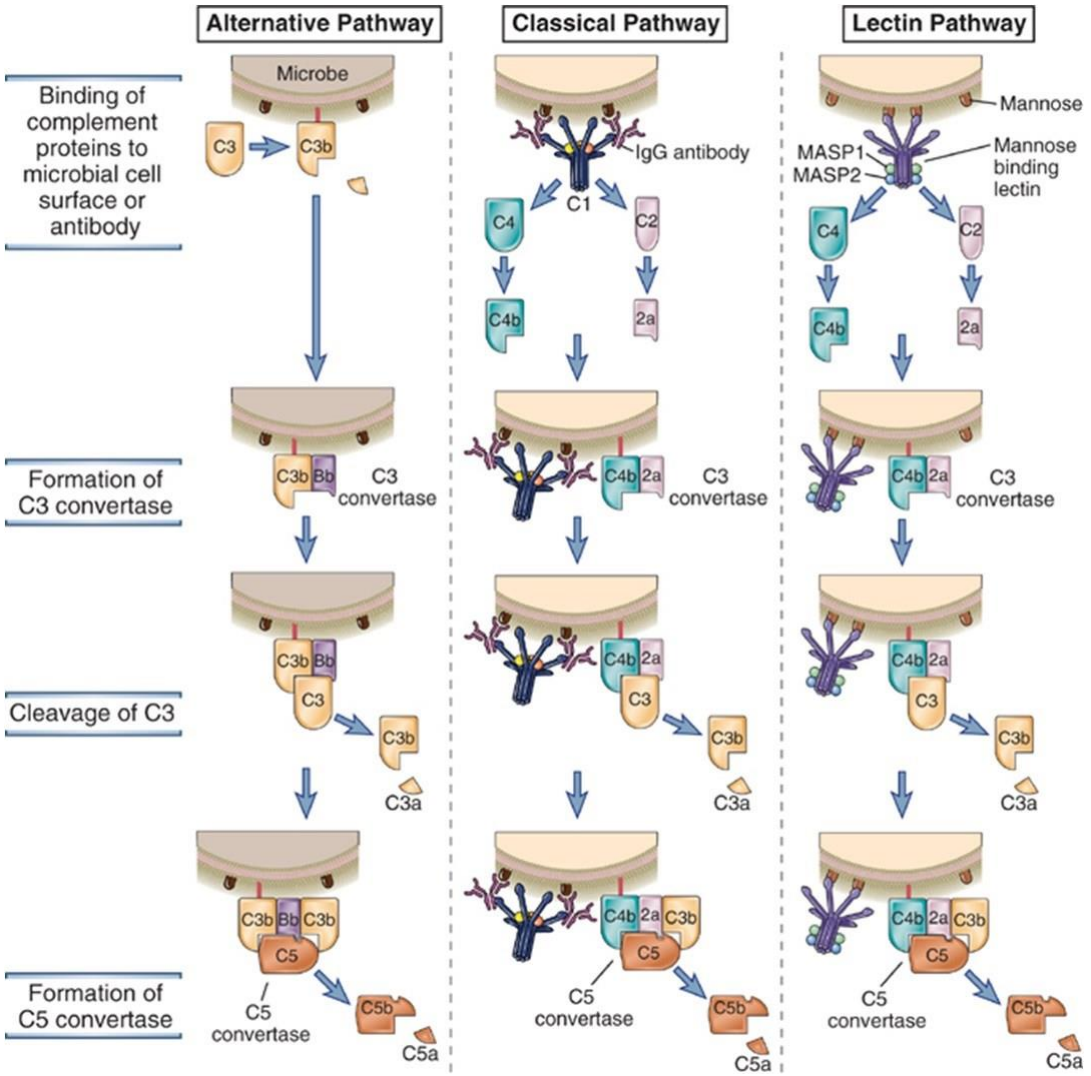


Figure 1-10: Complement activation through classical pathway. Complement can be activated by three different pathways. When activated through classical pathway, immune complexes recruit protein C1q which induces formation of enzyme C3 convertase. Once protein C3 is cleaved by C3 convertase, C3b is deposited on the surface of infected cell, inducing formation of another enzyme, C5 convertase. Activation of C5 convertase recruits other proteins of the complement cascade to form the membrane attach complex (MAC) and produce cell lysis (not shown in figure) (Source: Abbas, Litchmann and Pillai, 2017).

Complement activation against HIV-1 was one of the first Fc-effector functions studied (Perricone et al., 1987; Robinson et al., 1987; Senaldi et al., 1990; Spear et al., 1991; Spear et al., 1993) and it is mostly mediated by the classical pathway. Protective

effects of CDC during HIV-1 infection are mediated by direct virolysis and by opsonisation of virions with a coating of complement proteins (Sullivan et al., 1996; Sullivan et al., 1998; Aasa-Chapman et al., 2005). However, CDC effectiveness is unclear because enhanced infectivity has also been observed following complement adhesion on virion (Robinson et al., 1988; June et al., 1991; Montefiori et al., 1996; Huber et al., 2006). Apex-binding BnAbs PG9 and PG16 activate complement but the CD4bs BnAb VRC01 does not (Mujib et al., 2017). This was considered important in understanding the role of CDC activation by apex-binding Abs that were detected in the RV144 trial (Perez et al., 2017). In addition, non-neutralising antibodies can activate complement and when these are produced during the acute phase of infection, they correlate with lower viral loads (Huber et al., 2006). In non-human primate studies, however, protection against SHIV challenge was mediated mostly through Fc-receptor responses and not through complement activation (Hessell et al., 2007). CDC can also be activated by HIV-1 Env due to direct interactions between C1q and gp120 (Haurum et al., 1993; Susal et al., 1996) and gp41 (Ebenbichler et al., 1991; Thielens et al., 1993). Indeed, regions on gp41 that usually interact with gp120 can also interact with C1q, showing a structural homology between gp120 and C1q (Stoiber et al., 1996).

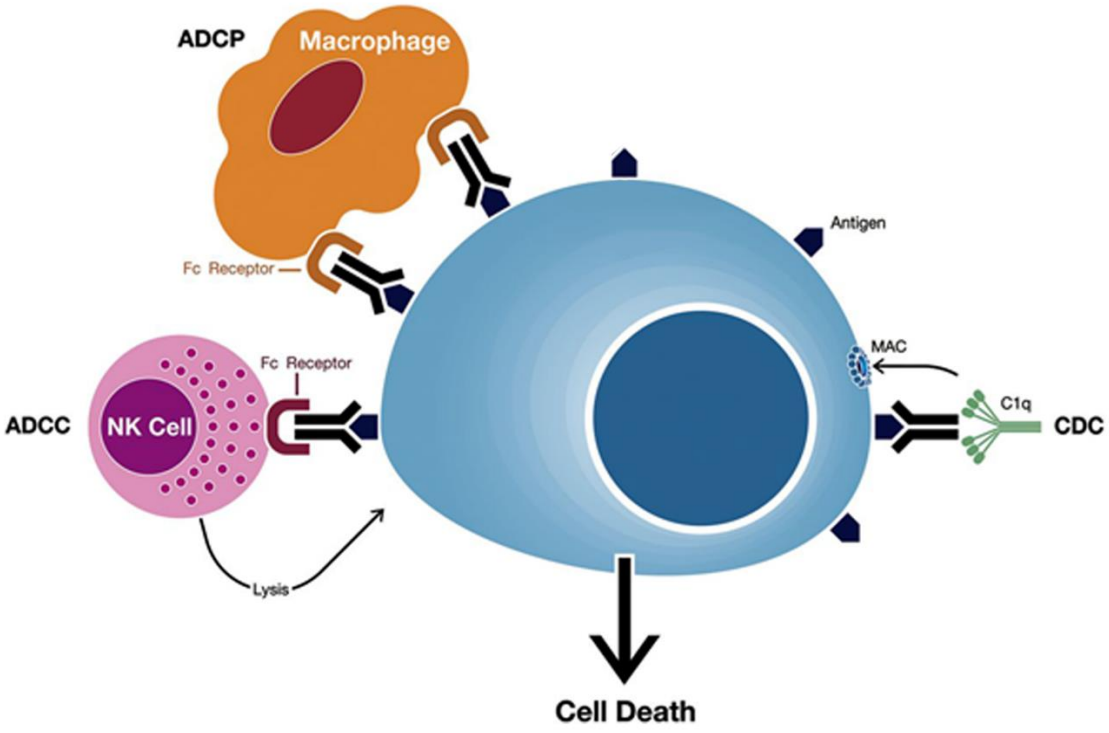


Figure 1-11: Summary of Fc-effector functions elicited by antibodies. When antibodies engage antigen at the surface of infected cells, the Fc portion can be recognised by Fc receptors on immune cells and activate, for example, ADCC in the case of NK cells, or ADCP in the case of macrophages. In parallel, antibodies can activate CDC through the classical pathway of complement activation. In all cases, activation of Fc-effector functions will derive into death of infected cell (van de Donk et al., 2016).

1.3 Antibody-based HIV vaccines

To date, vaccine trials have failed to induce BnAbs in humans. Indeed, the only successful HIV-1 vaccine trial, RV144, showed protection without inducing tier-2 neutralising antibodies (Rerks-Ngarm et al., 2009). Instead, it induced an immune response by activating CD4+ T cells and B cells, producing high levels of IgGs against epitopes in the V1/V2 loop of Env and eliciting antibody-dependent cellular cytotoxicity (ADCC) (Bonsignori et al., 2012), antibody-dependent cellular phagocytosis (ADCP)

(Chung et al., 2015) and antibody-dependent complement deposition (ADCD) (Perez et al., 2017), while each of these protective mechanisms were blocked by IgA (Tomaras et al., 2013). Because protection against HIV infection can be mediated by BnAbs or Abs eliciting Fc-effector functions, further investigation is required to know what factors in vaccines have impacted on the induction of BnAbs or Abs with effector-functions, and what modifications can produce a protective or therapeutic response through vaccination.

1.3.1 Vaccine trials in animal models

Animal trials for HIV-1 vaccines have been performed in mice, rabbits, guinea pigs and non-human primates. While the B cell repertoire in these animal models are different to humans, elicitation of BnAbs can reveal the antigenicity of different Env vaccines.

CDRH3s in mice are slightly shorter than humans, around 13 residues (Shi et al., 2014), due to restrictions based on CDRH3 length which are applied before maturation of B cells (Ivanov et al., 2005). Even with short CDRH3 sequences, mice vaccinated with uncleaved trimers can raise heterologous neutralising antibody against clade B viruses (Yang et al., 2001; Grundner et al., 2004). Vaccination with conformationally stabilised antigen, such as Con6 Env trimers (an early version of a consensus Env sequence incorporating many different clades), raised neutralising antibodies against clade B viruses but not against clade C viruses (Gao et al., 2005). When BG505 SOSIP gp140 was used as immunogen, it elicited only autologous neutralisation (de Taeye et al., 2015).

Guinea pigs have an antibody repertoire with slightly smaller CDRH3s than humans (between 6 and 16 residues) (Matsuzawa et al., 2018). When Con6 gp120 was used as the immunogen in guinea pigs, weak neutralising antibodies against primary isolates were isolated (Gao et al., 2005). Recently, uncleaved trimers induced better

neutralising responses such as ConS (a consensus sequence with shorter variable loops (Liao et al., 2006)) and ConT (Liao et al., 2013), both inducing antibodies with heterologous neutralisation but limited breadth. Better results were obtained, however, by vaccinating with Envs from T/F strain viruses, inducing mild potency antibodies with high breadth in guinea pigs (Liao et al., 2013).

Rabbits present CDRH3 of similar length range to humans (Wu et al., 1993; Lavinder et al., 2014). When Con6 gp120 (same sequence as in Gao et al., 2005) was used, it elicited antibodies that competed with the CD4bs BnAb VRC01 on BG505 SOSIP gp140 and in gp120 Envs (Qin et al., 2014). Although this response was not tested for neutralisation, trimeric vaccines have achieved heterologous neutralisation, using both uncleaved (Yang et al., 2001) and SOSIP (Klasse et al., 2018) Env antigens. Recently, ConM, an improved consensus sequence for producing a SOSIP-stabilised trimer, was tested in rabbits where it elicited autologous but not heterologous neutralisation (Sliepen et al., 2019). It is unclear if the poor neutralisation breadth was due to the vaccine itself or the pool of animals used for the study.

Of all the available animal models, rhesus macaques are the closest to humans. Their antibody repertoire includes CDR3 heavy and light chain lengths similar to humans, usually around 15 and 10 residues respectively (Vigdorovich et al., 2016; Fu et al., 2017). Both species present three distinctive loci for heavy (1 locus) and light (2 loci) chains (Sundling et al., 2013). Although humans present more genes in the heavy chain locus, macaques present more genes in the kappa light chain locus (Rhesus Macaque Genome et al., 2007), meaning that available genes for Ab production are different between these two species. Macaques vaccinated with gp140 trimers with a foldon motif (gp140-F trimers) (Yang et al., 2002; Morner et al., 2009) elicited Abs targeting similar binding sites to human Abs (Sundling et al., 2013), which confirm macaques are appropriate animal model to assess vaccine efficiency. Use of ConS as

a DNA vaccine with an Adenovirus-based boost expressing ConS Env at the cell surface resulted in neutralising antibodies against clades A, B and C (Santra et al., 2008). Recombinant AD8 uncleaved gp140 has also elicited neutralising Abs against a panel of pseudoviruses four weeks post vaccination, however these Abs were unable to confer protection against challenge (Center et al., 2009). Lastly, ConM SOSIP gp140 exhibited only autologous neutralisation (Sliepen et al., 2019).

1.3.2 Vaccine trials in humans

As mentioned before, no vaccine trial has been successful in eliciting neutralising antibodies in humans. Initial vaccines tested used recombinant gp120, based on results observed in nonhuman primates in early studies (Berman et al., 1990; Mooij et al., 1998; Stott et al., 1998). These vaccines did not achieve protection in clinical trials, as they achieved poor induction of neutralising Abs (Flynn et al., 2005; Gilbert et al., 2005; Pitisuttithum et al., 2006). By that time, the need to present native trimers in order to elicit BnAbs was unknown.

Another approach was to produce recombinant viral particles using an adenoviral backbone. Two trials using exclusively adenoviral particles with clade B Env on surface, STEP (Buchbinder et al., 2008) and Phambili (Gray et al., 2011), were tested in America and South Africa, respectively. No neutralising Abs were elicited by them. In addition, a correlation between Ad5 seroprevalence and increased chances of infection was found in the STEP trial (Gray et al., 2011). Due to the enhancement of infection showed in STEP, the Phambili study was cancelled.

The RV144 Thai trial was conducted by using a canarypox backbone that packaged DNA encoding polyproteins Gag, Pol and Env, and a clade B Env gp120 as boost (Rerks-Ngarm et al., 2009). Results from this trial were previously mentioned in section 1.2.5. Later, approaches from the RV144 trial and the STEP trial were combined in the

HVTN 505 vaccine trial,conducted in USA using a DNA vaccine encoding clade B Gag, Pol and Nef, and Envs from clades A, B and C, and a boost of a rAd5 vector expressing a clade B Gag-Pol fusion protein and Envs of clades A, B and C (Hammer et al., 2013). The study, however, was halted due to lack of efficacy and the risk of increasing chances of infection, plus cross-reactivity found with microbiota in rAd5 seropositive patients (Williams et al., 2015).

1.3.3 Env conformations and effects on eliciting protective antibodies

Antigen presentation is a crucial feature in vaccination studies. The results from previous vaccine trials exhibit that in order to elicit BnAbs, a trimeric Env conformation similar to the native viral spike is needed (VanCott et al., 1997; Forsell et al., 2009; Lelievre and Levy, 2016). Following this observation, several attempts have been made to generate trimeric Envs, mostly by truncating the endodomain and transmembrane region of gp41 in order to produce exclusively the outer part of the spike, called gp140 (Earl et al., 1994) (Figure 1-12 B). However, this structure can be disassociated easily so several modifications had been introduced for further stabilisation. One of the developed stabilised trimers, called uncleaved (Unc), lacks cleavage site between gp120 and gp41 (Earl et al., 1994). The most recent stabilised trimer is a SOSIP version in which gp120 subunits are connected to gp41 subunits by disulphide bonds, and an isoleucine-to-proline substitution at position 559 stabilises the gp41 ectodomain into its prefusion conformation (Binley et al., 2000; Sanders et al., 2002; Klasse et al., 2013) (Figure 1-12 A).

While Unc trimers exhibit higher flexibility compared to its cleaved counterpart and enhances epitope availability (Haim et al., 2013; Schiffner et al., 2013) and binding of polyclonal antibodies (Pancera and Wyatt, 2005; Li et al., 2012), CD4-induced (CD4i) epitopes such as the Co-Rbs and the cluster A epitope are more exposed in Unc

trimers and apex epitopes are abrogated such as the ones engaged by mAbs PG16 and PGT145 (Yasmeen et al., 2014); in addition, it alters epitopes in the V3 loop and CD4bs compared to native trimers (Castillo-Menendez, 2018) (Figure 1-12 C). SOSIP trimers on the other hand have a more homogeneous structure (Castillo-Menendez et al., 2018; Castillo-Menendez et al., 2019), and present similar structure to native trimers in single-molecule FRET experiments (Stadtmueller et al., 2018). Due to the compact conformation of SOSIP trimers, apex epitopes are better exposed (Yasmeen et al., 2014) whereas cluster A epitopes, a subgroup of CD4i epitopes, are not accessible (Sanders et al., 2002) (Figure 1-12 D).

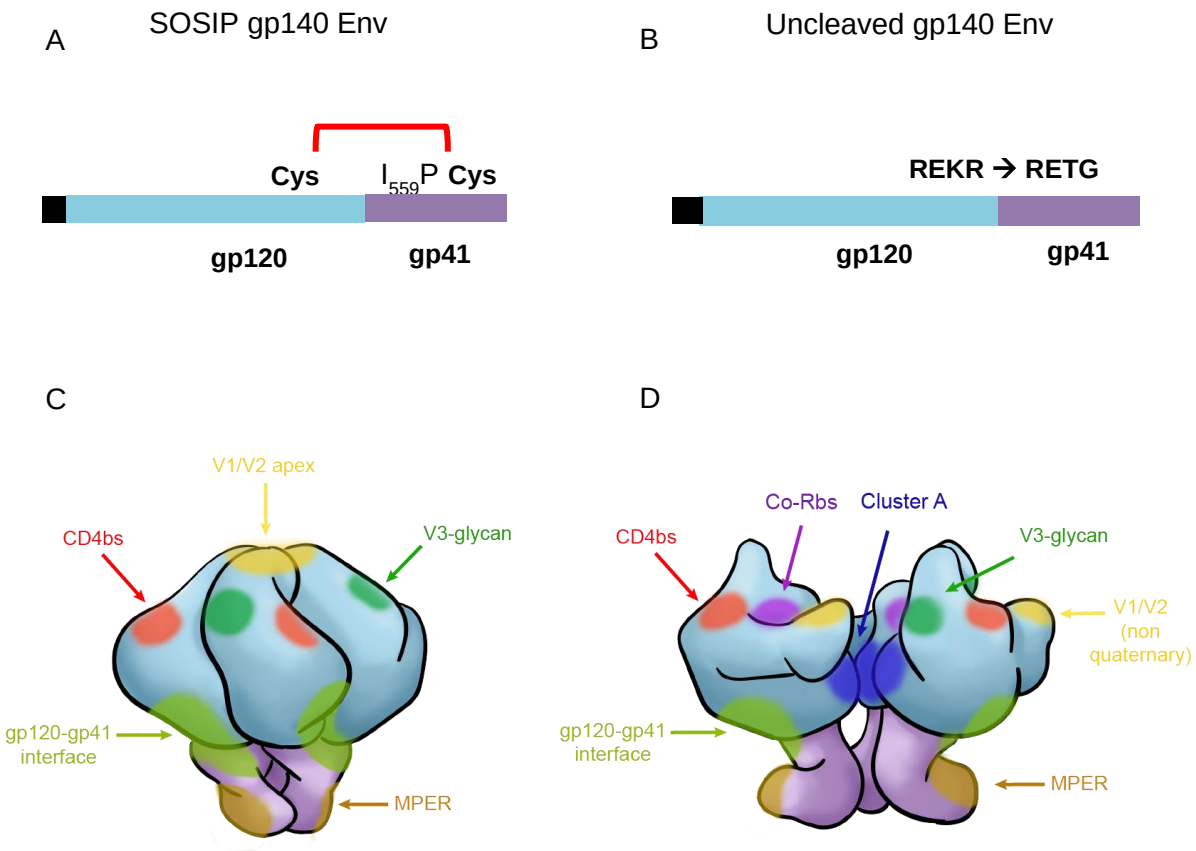


Figure 1-12: Sequence modifications and epitopes exposed by SOSIP (A, C) and uncleaved (B, D) gp140 Env trimers. While SOSIP trimers bind gp120 to gp41 through an artificial disulphide bond and an isoleucine-to-proline substitution (A), uncleaved trimers have a deletion in the cleavage site between used by furin-like proteases (B). The different modifications added to stabilise the trimeric structure by these two methods change the conformation Env present (C, and D), and hence, both trimers expose different epitopes, some of which are not neutralising but have importance into eliciting Fc-effector functions.

Conformation of Env affects the activation of Fc-effector functions, which implies that it is important to choose stabilised Envs to elicit protective antibodies through these means. Downregulation of surface CD4 receptor by Nef and Vpu reduces ADCC levels (Pham et al., 2014). While CD4 receptor is presented on the cell surface, it interacts with HIV-1 Env, exposing CD4-induced (CD4i) epitopes that are important for eliciting ADCC. Thus, downregulation of CD4 receptor also reduces the exposure of these epitopes, protecting infected cells from ADCC (Veillette et al., 2014; Veillette et al., 2014). Additionally, Vpu aids to protect infected cells by blocking tetherin, which impedes virion budding from the plasma membrane, reducing the accumulation of surface Env and resulting in protection of infected cells from ADCC responses (Alvarez et al., 2014; Arias et al., 2014).

The native Env trimer is metastable and three conformational states have been described (Munro et al., 2014) (Figure 1-13). The viral spike transits between a closed Env conformation, state 1, and a more open conformation, state 2. When Env binds to CD4 receptor, it stabilises into a thermodyamically favoured state 2 structure. This exposes CCR5 or CXCR4 binding sites that, when engaged, rearrange the Env into state 3 from which it cannot return (Herschhorn et al., 2016). Downregulation of CD4 stabilises Env in state 1 and Envs in this conformation are susceptible to ADCC only by CD4bs BnAbs, whereas Envs in state 2 and 3 are additionally susceptible to CD4-induced (CD4i) antibodies (Prevost et al., 2018; Richard et al., 2018). Recently, a new asymmetric Env conformation called state 2A has been described, which is present when soluble CD4 or CD4 mimetic compounds are bound plus Co-RBS and anti-cluster A Abs, simulating co-receptor engaging. This state requires opening of Env, and primary CD4+ T cells that present this conformation become susceptible to ADCC (Alsahafi et al., 2019).

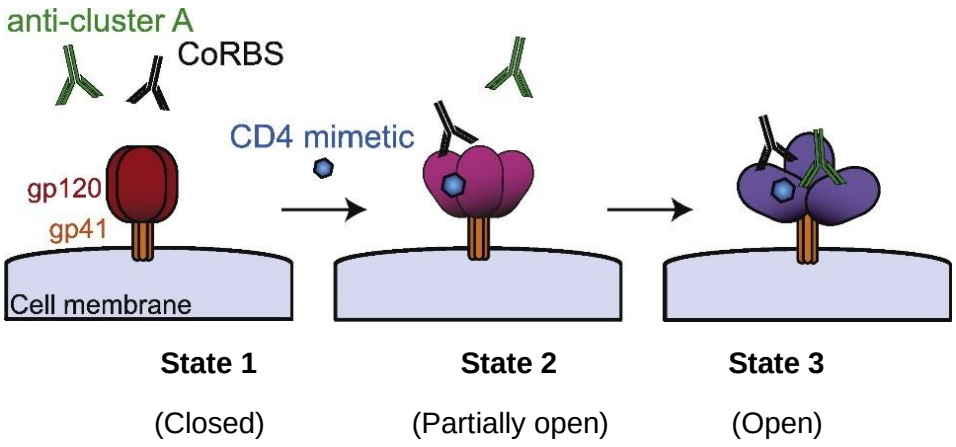


Figure 1-13: Conformations of HIV-1 Env during viral entry and susceptibility to Fc-effector functions. Before infection, Env in found in a closed conformation, called state 1. After engagement with CD4 receptor, or with CD4 mimetic compounds, Env changes into state 2, a partially open state where is susceptible to antibodies targeting the Co-receptor binding site (Co-RBS). Finally, binding of Co-RBS antibodies, CCR5 or CXCR4 changes Env into state 3, an open conformation where is vulnerable to anti-cluster A antibodies which activate strong ADCC responses (Richard et al., 2016).

1.4 Bovine antibodies and neutralising responses against HIV-1 Env

In recent years, our lab group and other researchers have successfully produced BnAbs against HIV-1 in cows. Bovines possess an antibody repertoire with long average CDRH3 lengths of between 25 and 28 residues (De Genst et al., 2006; Wang et al., 2013). This property of long CDRH3s has also been reported in camelids that have up to 24 residues with average size of between 13 and 17. In addition, cows are also able to produce up to 10% Abs with CDRH3 over 50 residues, called ultra-long CDRH3s (Heydarchi et al., 2016). As mentioned, the average CDRH3 length in human Abs is between 8 and 16 residues, while bovines can naturally produce Abs with longer CDRH3. Taking into account that most BnAbs present long CDRH3s, we hypothesised that vaccination of cows with native-like Envs could elicit strong

neutralising responses. Previous results obtained by our laboratory and other groups support this idea, and knowledge about how these ultra-long sequences are produced is important because it helps defining strategies to elicit potent neutralising Abs, if possible, with similar features to those in humans.

1.4.1 Immunoglobulin repertoire and antibody production in bovines

A complete analysis of H and L chain loci of the bovine Ig repertoire showed interesting features, including that half of V genes in the H chain locus (IGHV) are pseudogenes not used for producing Abs (Ma et al., 2016). For this reason, total number of genes used to construct BCRs in the bovine immune system is limited, and diversity in their repertoire is dependent mainly on SHM of the CDRH3 sequences (Stanfield et al., 2018). Ultra-long sequences are however not restricted to IgGs, as they have been found in other isotypes such as IgM (Walther et al., 2013).

The structure of ultra-long CDRH3s in bovines is unique, as it forms a stalk and a knob, which protrude from the tip of the variable domain (Wang et al., 2013). The stalk is structured by β-sheets sustained by the presence of cysteines in even numbers, forming disulphide bonds that maintain the stalk in a rigid position (Saini et al., 1999), along with conserved aromatic residues that are meant to stabilise the descending stalk sheet (Stanfield et al., 2016). The knob, on the other hand, is highly variable in sequence and structure and only conserves one β-strand, which works as a scaffold for contact loops sustained by disulphide bonds (Stanfield, Wilson and Smider, 2016). Interestingly, sequence analysis of ultra-long CDRH3s in different bovine Abs revealed that specific genes are required for their formation: IGHV1-7, IGHD8-2 and IGHJ2-4 (Deiss et al., 2019) (Figure 1-14). IGHV1-7 presents an insertion of 8 nucleotides before the RSS motif used for recombination, producing a frameshift that translates into the sequence “CTTVHQ”. This differs from the “CAR/T” sequence found at the

start of common CDRH3s found in all ultra-long CDRH3s with slight variations (Koti et al., 2010; Stanfield et al., 2018). Additionally, germline gene IGHD8-2 has the longest D gene in the whole repertoire (148 nucleotides) (Shojaei et al., 2003), and is prone to insertions and deletions by the enzyme AID during SHM (Muramatsu et al., 2000), due to the presence of the motif “A/G,G,T/C,A/T” in 19 places within this gene. These bovine genomic peculiarities explain the high variability found in structured knob (Haakenson et al., 2018). Finally, IGHJ2-4 is the most used gene in the bovine repertoire, encoding the descending β-strand of the stalk (Stanfield et al., 2018). To accompany this ultra-long sequence, it is apparent that L chain uses the germline gene V30, as it has been found in other characterised bovine IgGs with ultra-long CDRH3s (Saini et al., 2003; Sok et al., 2017). V30 belongs to the lambda L chain locus; indeed, adult bovines mostly use the lambda chain locus instead of kappa (Arun et al., 1996; Pasman et al., 2010).

Antibodies without ultra-long CDRH3 present SHM levels and structure similar to Abs from humans and other species. Unlike their counterparts, these Abs do not need to maintain the scaffold for ultra-long sequences and, instead, need to undergo high levels of SHM in order to improve binding towards their cognate antigen (Stanfield et al., 2018).

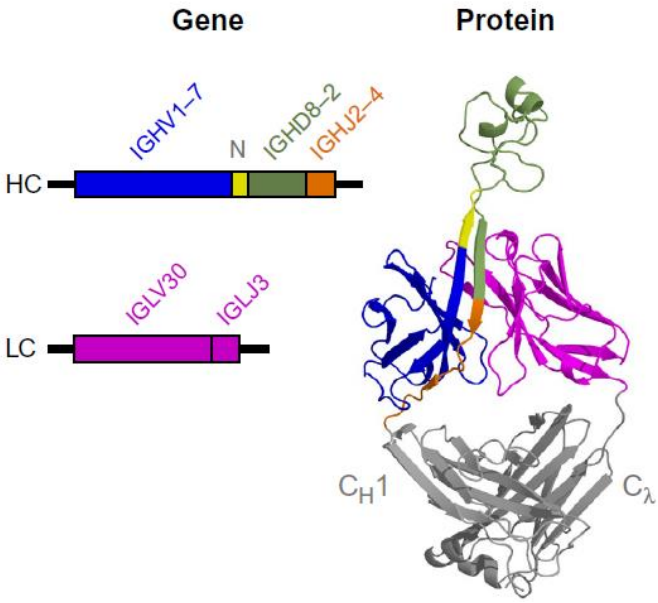


Figure 1-14: Architecture of antibody variable region with ultra-long CDRH3 in bovines. Ultra-long CDR3 in the heavy chain (HC) presents a structure of stalk and knob that protrudes out of the variable region of an antibody. Germline gene IGHV1-7 contributes with the ascending part of the stalk (blue), and IGHJ2-4 with the last part of the descending stalk (orange). Germline gene IGHD8-2 on the other hand contributes with the knob along with part of the descending stalk (green). Light chain (LC) does not have an ultra-long CDR3, and genes such as IGLV30 and IGLJ3 can contribute to stabilise the structure of stalk and knob at the heavy chain (Source: Stanfield et al., 2018).

1.4.2 Previous results from vaccination of cows with HIV-1 envelope glycoproteins

Due to the complications in developing BnAbs, particularly the degree of affinity maturation required to neutralise HIV-1 virus (Verkoczy et al., 2010; Verkoczy et al., 2011; Doyle-Cooper et al., 2013), Env vaccination in human and most laboratory animals has not yet been successful. In contrast, cows produced neutralising Abs against Env with just a relatively short and simple vaccination regimen. So far, only two groups have performed vaccination of bovines with recombinant Env trimers (Kramski

et al., 2012; Heydarchi et al., 2016; Sok et al., 2017). The different vaccination regimens and antigens are likely to have directed a different outcome from the immune response. In both studies, however, elicitation of cross-clade neutralisation was found.

Bovines produce large quantities of Igs during pregnancy, storing it in colostral milk (first milk received by calves). Unlike in humans, Abs cannot cross bovine placenta, thus the only method to transfer immune protection to newly born calves is by drinking their mother's milk (Balfour and Comline, 1962). This fact allows the production of large quantities of IgGs by vaccinating cows during pregnancy and collecting colostrum for harvesting after calving. Our laboratory showed the most efficient strategy for inducing bovine BnAbs was by initiating the vaccination before conception and boosting during pregnancy. Two different vaccine regimens were successful, either AD8 uncleaved gp140 alone, or a mixture of uncleaved Envs from clade A, B and C (UG8, AD8 and MW, respectively). Cows commencing vaccination late in pregnancy did not induce high titres of BnAbs in colostrum compared with the long duration group that developed broad neutralising activity against a panel of tier 1 and 2 viruses from clades A, B and C (Kramski et al., 2012). In addition, bovine Abs could induce activation of human monocytes (Kramski et al., 2012). Interestingly, bovine polyclonal Abs competed for binding of CD4bs mAbs b12 and VRC01 (Kramski et al., 2012; Heydarchi et al., 2016), implying presence of CD4bs Abs. In addition, we showed that cysteines and aromatic residues in the CDRH3 domain played a critical role in antigen binding of two bovine mAbs selected from one of the animals, indicating that binding against HIV-1 Env is also dependent on these residues in bovine Ab as observed in human BnAbs (Heydarchi et al., 2017).

One cow from this vaccination study, which presented the highest neutralising activity of the group, was selected for repeated ongoing vaccination to test for any improved neutralising breadth (Heydarchi et al., 2016). Long sequential vaccination with an

autologous immunogen did not improve neutralisation. More investigation is required to understand whether a low affinity maturation rate in the bovine immune system has resulted in such response. Unfortunately, the database available to measure affinity maturation rates in bovines is scarce and incomplete and needs further improvement.

Thereafter, another group used BG505 SOSIP gp140 to vaccinate another small group of cows (Sok et al., 2017). This HIV-1 Env was already tested in mice (de Taeye et al., 2015), but only showed high autologous neutralisation. The vaccination scheme induced cross-clade neutralisation at day 42, a feature not achieved in any other animal model. A CD4bs bovine mAb, called NC-Cow1, was isolated showing 72% neutralisation against cross-clade isolates (n = 117) with a high potency (IC50 value of 0.028 µg/ml). This Ab had an ultra-long CDRH3 of 60 residues with higher and broader neutralisation than other CD4bs antibodies, such as VRC01 (Sok and Burton, 2018).

1.5 Project Aims

Rapid elicitation of neutralising Abs in bovines serves as an advantageous method for testing HIV-1 Env vaccines. Additionally, conservation of the CH2 region of antibodies between species allows the study of Fc-effector function activation using bovine Abs. By analysing both neutralisation and activation of Fc-effector functions, new questions can be asked regarding type of Env vaccine and function elicited, since the level of protection conferred when both functions are present is unknown. Furthermore, investigation into the events required by bovines to produce neutralising antibodies against HIV are of interest, which can be commercially used in the future to produce high amounts of protective antibodies, or monoclonal antibodies with high and broad neutralising activity. To date, no comparison has been made between modifications to stabilise Env trimers and ability to elicit both neutralisation and Fc-effector functions. In

addition, because heterologous neutralisation has been induced with both Unc- and SOSIP-stabilised trimers in bovines, it is unclear in this model how these neutralising Abs are generated, and the levels of affinity maturation required for them to reach such levels of affinity for their antigen. It is possible that the UCA of those Igs already present high binding, regardless of the Env trimer presented to the immune system.

Taking together the points above, the aims of this thesis are the following:

1- Investigate the effects of stabilisation of different HIV-1 Env trimers in eliciting neutralising antibodies in bovines
2- Investigate the effects of stabilisation of different HIV-1 Env trimers in eliciting Fc-effector functions with human proteins, and compare these responses with aim 1
3- Analyse differences in the antibody profiles elicited with relevant vaccines, in terms of affinity maturation required and levels of SHM.

The results of this study will help understanding the way bovine Abs are produced against HIV-1, serving as a guide in the design of a vaccine regimen able to induce both neutralising activity and Fc-effector functions.

1.5.1 Vaccination trial design:

Based on the results from Kramski et al., 2012 and Heydarchi et al., 2016, a new vaccination study was planned. This time, the study was performed in 2 phases: the first one, same as in these two previous studies, included pregnancy in order to collect colostral milk with high quantity of anti-HIV-1 Env IgGs; the second one did not include pregnancy and instead, cows presenting high autologous binding titres after phase 1 were revaccinated with either the same immunogen, or with a different protein in terms of structure and clade. In phase 1, vaccination was initiated before conception, with following boosts being administered during pregnancy. The goal of this phase was to study differences in antibody responses and protective functionality exclusively due to

the vaccine and the amount received. Phase 2 of the study included only one final boost after calving and colostrum collection. Because at this timepoint antibody response against each vaccine is defined and sustained, as shown in Heydarchi et al., 2016, phase 2 focused in studying the effect of changing immunogen during vaccination on the antibody neutralising profile.

The immunogens used in the study were chosen based on structure, clade and glycosylation levels. For phase 1, same immunogens used in Sok et al., 2017 and Kramski et al., 2012 were included: clade A BG505 SOSIP gp140 and clade B AD8 Unc gp140, respectively. At the start of the vaccination scheme, clade A KNH1 SOSIP gp140 was used before using BG505 SOSIP gp140, meaning that sequence was slightly changed but clade was maintained. Two other Unc trimers were used: clade C MW Unc gp140, and clade B PSC89 Unc gp140. This last one was collected and sequenced from a pre-seroconverter isolate and presents low number of N residues in its sequence, indicating low levels of glycosylation compared to the other Envs used.

In terms of immunogen amounts, cows were separated into five groups: two received 100µg of immunogen (KNH1/BG505 SOSIP, or AD8 Unc gp140), whereas the other three groups received 500µg (AD8 Unc, MW Unc and PSC89 Unc gp140). By having AD8 Unc gp140 supplied at both 100µg and 500µg, the quantity of immunogen administered can be compared. In addition, antibody profiles elicited by the two groups AD8 Unc gp140 and KNH1/BG505 SOSIP gp140 at the same amount (100µg) can be contrasted in relation to structure or clade changes and, by having all uncleaved trimers at the same amount (500µg), the effects of glycosylation levels and clades in the antibody response can be compared.

Table 1-2: Summary of gp140 immunogens used in vaccination scheme. Four different HIV-1 Env trimers were chosen based on their clade, stabilising modification and glycosylation level. During this work, all gp140 trimers will be named by their Env name and stabilising modification.

Env name	Clade	Stabilising Modification	Features
AD8	B	Uncleaved	Previously tested in bovines (Kramski et al., 2012; Heydarchi et al., 2016)
KNH1/BG505	A	SOSIP	Previously tested in bovines (Sok et al., 2017)
PSC89	B	Uncleaved	T/F strain. Low glycosylation sites
MW	C	Uncleaved	Clade C Env. Included to compare to clade B AD8 Env.

Analysis of Ab response was performed in blood samples during the vaccination course, after each vaccination, and in colostral milk, after phase 1 of the scheme. Because the analysis of aim 2 required high amounts of IgGs, it was performed only on IgGs from colostral milk, which represents the end of phase 1 of vaccination course. For both aims 1 and 2, in order to test certain functions such as binding site and phagocytosis, new proteins were produced with an Avi tag, which can later be biotinylated, allowing the capture of such protein. In consequence, these Avi-tagged proteins are not from the same batch as vaccines, but they share the same nucleotide and amino acid sequence. Finally, aim 3 was performed on PBMCs collected at different timepoints during the vaccination course. As it will be mentioned later, this analysis was only performed in cows vaccinated with relevant vaccines, but after phase 1 and 2 of the vaccination course, allowing recapitulation of Ab response during vaccination, and the comparison between relevant vaccines.

2. Materials and Methods

2.1 Reagents

2.1.1 Chemicals

Unless stated otherwise, all chemical reagents were purchased from Sigma-Aldrich (Missouri, USA) after quality check for analytical grade purity assessment.

2.1.2 Oligonucleotides

Oligonucleotides without probes used in this thesis were synthesised by Sigma-Aldrich. Stocks were delivered at 100µM in 100 µl of water, and working solutions were prepared at 10µM by dilution in nuclease-free water.

Table 2-1: Oligonucleotides used in this thesis. ID number in Purcell Lab's database, sense (forward (F) or reverse (R)), source and target are indicated.

Oligo ID	Sense	Sequence (5' → 3')	Source	Notes
3503	F	GCACATTGTAATGTCAGTAAAGC	Sigma-Aldrich	Used for site-directed mutagenesis
3504	R	GCTTTACTGACATTACAATGTGC	Sigma-Aldrich	Used for site-directed mutagenesis
0687	F	GGCAGTCTAGCAGAAGAAG	Sigma-Aldrich	Targets HIV-1 Env
0241	F	GTCGTAACAACTCCGCCCCATTG	Sigma-Aldrich	CMV promoter
1378	R	CCAATACTACTTCTTGTGGG	Sigma-Aldrich	Targets HIV-1 Env
0945	R	CCATAGTGCTTCCTGCTGCT	Sigma-Aldrich	Targets intron sequence downstream of Env
3748	F	GGAGTTCAGACGTGTGCTCTTCCGATCTHHHHHACA HHHHHACAHHHHNHACACCAGGGGGAAGACTCTC	Sigma-Aldrich	Targets 5' end of 5' RACE PCR product

3749	R	CTAATACGACTCACTATAGGGC-(T)30VN	Sigma-Aldrich	Targets poly-A tail of 5’ RACE PCR product
3580	F	AATGATACGGCGACCACCGAGATCTACACCCTAT CCTACACTCTTTCCCTACACGACGCTCTTCC GATCTCTTGTTGCCGTTGAGCTCCTC	Sigma-Aldrich	Targets sequence in primer 3748. Adds Read1 adapter for Illumina sequencing
3572	R	AATGATACGGCGACCACCGAGATCTACACATATCG GAACACTCTTTCCCTACACGACGCTCTTCCGATCT GTTCGGGGAAGTAGTCCTTGAC	Sigma-Aldrich	Targets IgG C region. Adds Read2 adapter for Illumina sequencing
3573	R	AATGATACGGCGACCACCGAGATCTACAC ATATCGGAACACTCTTTCCCTACACGACG CTCTTCCGATCTGGGAATTCTCACAGGAGACG	Sigma-Aldrich	Targets IgM C region. Adds Read2 adapter for Illumina sequencing
4040	F	TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCACCCGAGAGTCTTCTCCC	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4041	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGG TACGGGAGAGACTTGCAGGAGTGGTGACTCTC	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4042	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGCTTTGTGCTGTCAGCCCCNCAGAGGAGG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4043	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGAC AGGGACCCTCCTCTTTGTGCTGTCAGCCCC	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing

4044	F	TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGGCCCCCAGAGGGGTCCTGTCCC	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4045	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGC TTTGTGCTGTCAGCCCCCAGAGGGGGCCTGTCC	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4046	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCTTTGTGCTGTCAGCCCCCACAGG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4047	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCTTTGTGCTGTCAGCCCCCACCGG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4048	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCTTTGTGCTCTCAGCCCCCACAGG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4049	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCTTTGTGCTCTCAGCCCCCACCGG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4050	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GTGCTGTCAGCCCCCAGAGGTCCTG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4051	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GTGCTGTCAGCCCCAGAGGTCCTGTCC	Sigma-Aldrich	Targets IGHV leader sequence.

				Adds Read1 adapter for Illumina Nextera sequencing
4052	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCTTTGTGCTGTCAGCCCCAGAGG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4053	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCTTTGTGCTGTCAGTCCCAGAGG	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4054	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGATGAACCCACTGTGGACCCTC	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4055	F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA GCTTTCGGGGCTGTGGTGGAGGC	Sigma-Aldrich	Targets IGHV leader sequence. Adds Read1 adapter for Illumina Nextera sequencing
4056	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGHHHHHACAHHHHHACAHHHHNHCTTT CGGGGCTGTGGTGGAGGC	Sigma-Aldrich	Targets IgG C region. Adds Read2 adapter for Illumina Nextera sequencing
4057	R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GHHHHHACAHHHHHACAHHHHNH CAGGACACCAGGGGGAAGACTCTC	Sigma-Aldrich	Targets IgG C region. Adds Read2 adapter for Illumina Nextera sequencing

Biotinylated oligonucleotides with Cy5 and BHQ probes were synthesised by Integrated DNA Technologies (Iowa, USA). Stocks were delivered lyophilised and resuspended in nuclease-free water in order to prepare 100µM, while working solutions were prepared at 15µM by dilution in nuclease-free water.

Table 2-2: Biotinylated oligonucleotides used for ADP-SHIP assay.

Probe ID	Sequence (5' → 3')	Source	Notes
FIP-Cy5	Cy5-TCAGTTCAGGACCCTCGGCT-Biotin	IDT	Biotinylated ssDNA probe. Presents Cy5 fluorophore
Qpc-BHQ	AGCCGAGGGTCCTGAACTGA-BHQ2	IDT	ssDNA probe. Presents BHQ2 quencher.

2.1.3 Antibodies

Table 2-3: Antibodies used in this thesis. All antibodies were IgG isotype, as a whole molecule or as F(ab')2 fragment.

Antibody	Host	Specificity	Source (cat no)	Notes
Anti-bovine IgG	Rabbit	Bovine Fc	Sigma-Aldrich (cat no SAB3700018)	Used as capture antibody in ELISA
Anti-bovine IgG-HRP	Rabbit	Whole molecule	Sigma-Aldrich (cat no A5295)	Used as detection antibody in ELISA. HRP-conjugated
Anti-bovine IgG-HRP	Sheep	Whole molecule	Biorad (cat no AAI23P)	Used as detection antibody in ELISA. HRP-conjugated
F(ab')2 anti-biotin	Goat	Whole molecule	Rockland (cat no 700-101-098)	Used as capture antibody in ELISA

Anti-human IgG-HRP	Goat	Human Fc	KPL (cat no 474-1002)	Used as detection antibody in ELISA. HRP-conjugated
VRC01	Human (recombinant)	HIV-1 Env (CD4bs)	NIH AIDS Reagent Program (cat no 12033)	Monoclonal antibody. Used for epitope characterisation
B12	Human (recombinant)	HIV-1 Env (CD4bs)	NIH AIDS Reagent Program (cat no 2640)	Monoclonal antibody. Used for epitope characterisation
PGT121	Human (recombinant)	HIV-1 Env (V3)	NIH AIDS Reagent Program (cat no 12343)	Monoclonal antibody. Used for epitope characterisation
PGT145	Human (recombinant)	HIV-1 Env (V1/V2)	NIH AIDS Reagent Program (cat no 12703)	Monoclonal antibody. Used for epitope characterisation
A32	Human (recombinant)	HIV-1 Env (cluster A)	NIH AIDS Reagent Program (cat no 11438)	Monoclonal antibody. Used for epitope characterisation
17B	Human (recombinant)	HIV-1 Env (Co-Rbs)	NIH AIDS Reagent Program (cat no 4091)	Monoclonal antibody. Used for epitope characterisation
Anti-human C3b-FITC	Mouse	C3/C3b/iC3b	Cedarlane (cat no CL7631F)	Used for complement activation assays

2.1.4 Plasmids

Plasmids used for this thesis were for protein expression or pseudoviral particles. As stated in table 8, most of these plasmids were available in Purcell Lab before initiating experiments; if not, its production is presented in section 2.3.2.

Table 2-4: Plasmids used in this thesis. Most plasmids were acquired or previously produced in-lab, except those referenced in other sections of Materials and Methods.

Plasmid name	Size (bp)	Protein expressed	Selection marker	Source and references	Notes
pN1- AD8-140Age6H	7730	AD8 Unc gp140 – 6H	Kanamycin	In-lab (CG) (Center et al., 2009; Heydarchi et al., in preparation)	Used for vaccine production and detection proteins
pN1-AD8140.SOSIP664.6H	7670	AD8 6R SOSIP .664 gp140	Kanamycin	In-lab (CG) (Heydarchi et al., in preparation)	Used for vaccine production
pN1-BG505-664-SEKS-PPI4	8582	BG505 Unc gp140	Ampicillin	Prof. Rogier Sanders (Sanders et al., 2015)	Used for vaccine production
pN1-MW-140	7706	MW Unc gp140	Kanamycin	In-lab (RC)	Used for vaccine production
pN1-PSC89.51-140AgeI	7730	PSC89 Unc gp140	Kanamycin	In-lab (RC)	Used for vaccine production
pN1-AD8-140-Avi	7748	AD8 Unc gp140-Avi	Kanamycin	Section 2.3.2	Expresses Avi tag on C-terminal. Can be biotinylated by BirA enzyme
pN1-AD8140SOSIPv4.1-Avi	7688	AD8 SOSIP v4.1-Avi	Kanamycin	In-lab (CG) (Heydarchi et al., in preparation)	Expresses Avi tag on C-terminal. Can be biotinylated by BirA enzyme
pN1-AD8120-Avi	7787	AD8 gp120-Avi	Kanamycin	In-lab (CG)	Expresses Avi tag on C-terminal. Can be biotinylated by BirA enzyme
pDR-NLΔEnv.nef-EGFP	12293	Gag + EGFP reporter	Ampicillin	NIH AIDS Reagent Program (Cat no 11100) (Zhang et al., 2004)	Backbone for pseudovirus production. Incorporates EGFP gene as production reporter

pDR-NLΔEnv.nef LucR-E-	13079	Gag/Pol + firefly luciferase reporter	Ampicillin	NIH AIDS Reagent Program (Cat no 3418) (Connor et al., 1995)	Backbone for pseudovirus production. Incorporates firefly luciferase gene as production reporter
pSVIII-MuLV	---	MuLV gp160	Ampicillin	NIH AIDS Reagent Program (discontinued) (Lodge et al., 1998)	Used for pseudovirus production
pSVIII-MN	7912	MN gp160	Ampicillin	NIH AIDS Reagent Program (discontinued)	Used for pseudovirus production
pCAGGS SF162 gp160	8054	SF162 gp160	Ampicillin	NIH AIDS Reagent Program (cat no: 10463) (Cheng-Mayer et al., 1997; Stamatatos et al., 1998)	Used for pseudovirus production
pSVIII-PSC89	7921	PSC89 gp160	Ampicillin	In-lab (CM)	Used for pseudovirus production
ZM109F.PB4	8257	ZM109F.PB4 gp160	Kanamycin	NIH AIDS Reagent Program (cat no: 11314) (Derdeyn et al., 2004)	Used for pseudovirus production
pBG505.W6M.ENV.C2.N332	8611	BG505 gp160	Ampicillin	Section 2.3.1	Used for pseudovirus production
pSVIII-JR-CSF	7921	JR-CSF gp160	Ampicillin	NIH AIDS Reagent Program (discontinued) (Zwick et al., 2001)	Used for pseudovirus production
pHIS-AD8-Env02	7017	AD8 gp160	Kanamycin	In-lab (RC) (Center et al., 2009)	Used for pseudovirus production

2.1.5 Recombinant HIV-1 Env for vaccination and assays

Table 9 shows all recombinant HIV-1 Env used in this thesis. Unc trimers were purified by lentil-lectin and size exclusion chromatography as previously described (Center et al., 2009; Sanders et al., 2013). On the other hand, SOSIP-modified trimers were purified by 2G12- or PGT145-coupled beads and size exclusion chromatography (de Taeye et al., 2015).

Table 2-5: HIV-1 Env proteins used for this thesis. Abbreviations: CG: Dr. Christopher Gonelli; MvG: Dr. Marit van Gils; RS: Prof. Rogier Sanders; JM: Dr. John Moore.

Env protein	Tag	MW (kDa)	Vaccine (Y/N)	Source	Notes
AD8 gp120	n/a	120	N	Produced by CG	No tag
AD8 gp120-Avi	Avi	120	N	Produced by CG	Contains Avi tagged, can be biotinylated
AD8 Unc gp140	n/a	450	Y	Produced by CSIRO	No tag
AD8 Unc gp140-Avi	Avi	450	N	Section 2.3.4	Contains Avi tagged, can be biotinylated
AD8 6R SOSIP.664 gp140	n/a	450	Y	Produced by CG	No tag
AD8 6R SOSIP.664 gp140-Avi	Avi	450	N	Produced by CG	Contains Avi tagged, can be biotinylated
BG505 Unc gp140	n/a	450	Y	Produced by CG	No tag
BG505 SOSIP.664 gp140	n/a	450	Y	Produced by RS	No tag
BG505 SOSIP,v5 gp140-Avi	Avi	450	N	Produced by MvG	Contains Avi tagged, can be biotinylated
KNH1 SOSIP gp140	n/a	450	Y	Produced by JM	No tag
MW Unc gp140	n/a	450	Y	Produced by CSIRO	No tag
PSC89 Unc gp140	n/a	450	Y	Produced by CSIRO	No tag

2.2 Mammalian cell culture

2.2.1 Cell lines

Cell lines used in this thesis were originally purchased from commercial suppliers, or acquired from the NIH AIDS Reagent Program (Maryland, USA). All cell lines were stocked in liquid nitrogen (-180°C) and grown in a tissue culture incubator at 37°C, 5% CO_2; in particular, Expi293F cells were grown in a shaker incubator at 37°C, 5% CO_2.

Table 2-6: Cell lines used in this thesis.

Cell line	Organism	Cell type (growth)	Growing media	Source (Cat no)
HEK293T	Human	Epithelial (adherent)	DMEM + 10% FBS	ATCC (CRL-3216)
Expi293F	Human	Epithelial (suspension)	Expi293 media	ThermoFisher (A14527)
U937	Human	Monocyte/lymphocyte (suspension)	RPMI + 10% FBS	ATCC (CRL-1593.2)
Cf2th CCR5+	Canine	Thymocyte/lymphocyte (adherent)	DMEM + 10% FBS 25mM HEPES 50mg/ml hygromycin, 500µg/ml G418, 1µg/ml of puromycin	NIH AIDS Reagent Program (4662)
TZMbl	Human	Epithelial (adherent)	DMEM + 10% FBS, 25mM HEPES	NIH AIDS Reagent Program (8129)
CEM.NKR CCR5+	Human	Lymphoblast (suspension)	RPMI + 10% FBS	NIH AIDS Reagent Program (150240)

2.2.2 Maintenance of cell lines

All cell lines were initially thawed in their growing media at 37°C, centrifuged at 500 x g for 5 minutes, washed from supernatant and resuspended in fresh growing media, before storing them in incubator. Adherent cells were kept in T75 flasks (Corning, cat

no CLS430641U) in a total volume of 10 ml of growing media, and passaged three times a week using trypsin-versene after washing flasks with PBS 1x, and diluting cells at 1/10 from total volume. Suspension cells were kept in T25 flasks (Corning, cat no CLS430639) in a total volume of 10ml, or in T75 flasks in a total volume of 25 ml; for both cases, cells were passaged twice a week by centrifugation of total content of flasks, replacement of supernatant by fresh growing media and dilution of cells at 1/5. Expi293F cells were kept in 30ml of Expi293 media in a polycarbonate Erlenmeyer shaker flask (Corning, cat no 431407), and passaged twice a week by centrifugation of total content of flasks, replacement of supernatant by fresh growing media and dilution of cells at 1/2.

Expansion of cell stocks was performed at early passage (no more than passage 10). New stocks were frozen initially at -80°C, and 24 hours later transferred to liquid nitrogen, in FBS + 10% DMSO and stored in cryotube vials (Nunc, cat no CLS430488). Thawed cells were expanded to no longer than passage 25.

2.3 Plasmid production

2.3.1 Production of pBG505.W6M.ENV.C2.N332

Plasmid pBG505.W6M.ENV.C2.N332 was produced by site-directed mutagenesis of plasmid pBG505.W6M.ENV.C2, which lacks an N-glycan site at position 332 of BG505 Env gp160. Mutation was introduced by using oligos ODP 3504 and 3505 and performing a whole plasmid PCR, using Phusion High-Fidelity DNA polymerase (New England BioLabs, cat no: M0530S) under the following conditions:

- Plasmid (100ng): 10µl
- Primers (10uM): 2.5 µl
- HF buffer 5x: 10 µl

- Phusion enzyme: 0.5 µl
- dNTPs: 1 µl
- DMSO: 1.5µl
- H_2O: 22 µl

, for a total of 50 µl. Band around 10000 bp was purified using Nucleospin(c) gel and PCR clean-up (Macherey Nagel), and product was eluted in 30µl. Then, 10 µl of product was treated with DpnI following manufacturer's indications (using CutSmart buffer) to degrade template, while 10 µl was untreated as negative control. DpnI was inactivated by heating sample for 20 mins at 70°C. Then, NEB© Stable competent E. coli cells (New England, BioLabs; catalog number C3040I) were transformed with 10ul of each tube, and colonies were picked, lysed for plasmid purification and sent for Sanger sequencing (AGRF, Victoria, Australia). From those, one colony which showed insertion of T332N mutation and no other mutations in BG505 gp160 ORF sequence was used for further experiments.

2.3.2 Production of plasmid pN1-AD8-140-Avi

Plasmid pN1-AD8-140-Avi was produced by modifying plasmid pN1-AD8140SOSIPv4.1-Avi, following a strategy of restriction enzyme digestion and further ligation. ORF expressing AD8 SOSIP gp140 in pN1-AD8140SOSIPv4.1-Avi was excised and ORF of AD8 Unc gp140 from plasmid pN1-AD8-140AgeI6H was inserted in its place.

In details, plasmids pN1-AD8140SOSIPv4.1-Avi and pN1-AD8-140AgeI6H were digested by enzymes NheI and PstI (New England BioLabs, Ipswich, MA, USA; catalog numbers R0131S and R0140S, respectively), at 37°C for 1 hr. Then, reaction from plasmid pN1-AD8140SOSIPv4.1-Avi (vector) was dephosphorylated for 30 minutes at 37°C using Antarctic Phosphatase (New England BioLabs, catalog number M0289S). Both digestion products were visualised in a 1.2% agarose gel, and bands

corresponding to vector (from pN1-AD8140SOSIPv4.1-Avi, 5291 bp) and insert (from pN1-AD8-140AgeI6H, 2457 bp) were cut and purified using Nucleospin Gel and PCR Clean (Macherey-Nagel, Duren, Germany; catalog number 740609.50). Finally, ligation was performed using T4 ligase enzyme (New England BioLabs; catalog number M0202S) following manufacturer's indications, at a vector-to-insert ratio of 1:3. Ligation product was used to transform NEB© Stable competent E. coli cells (New England, BioLabs; catalog number C3040I), colonies were picked, grown and lysed for plasmid purification, and pN1-AD8-140-Avi plasmid sequence was verified by Sanger sequencing (AGRF, Victoria, Australia).

2.4 Recombinant protein production

2.4.1 AD8 Unc gp140-avi production

AD8 Unc gp140-Avi protein was produced by transfecting Expi293 cells in suspension with plasmid pN1-AD8-140-Avi, using Expi293 transfection kit (Invitrogen) under manufacturer's indications. 72 hours post transfection, 100ml of supernatant was collected, clarified by centrifugation (3000 x g for 15 min) and loaded on a lentil lectin column for affinity chromatography. Coupling was performed loading supernatant twice, and elution by using buffer containing 20mM Tris, 500 mM NaCl and 300 mM methyl-D-glucoside at pH 7.4. After elution, sample was loaded on an Amicon Ultra-15 tube (Merck, MWCO 30 kDa) for buffer exchange into PBS 1x, by centrifugation at 3000 x g for 30 min twice. Finally, 1 ml of resuspended sample in PBS 1x was loaded in a HiTrap size exclusion chromatography column (GE Healthcare), 500µl fractions were collected in 1.5 microcentrifuge tubes and evaluated by SDS-PAGE and Coomassie staining. Fractions which showed trimeric structure were then pooled together and concentrated using an Amicon Ultra-15 tube (Merck, MWCO 30 kDa).

Protein concentration was measured by spectrophotometry and protein conformation by ELISA against mAbs VRC01, PGT121, A32, 17b and PGT145.

2.5 Vaccination Scheme and Sample Collection

Thirty-two Holstein Friesian cattle *(Bos Taurus)* used for the study were kept in an experimental open-gazing cattle farm, under the Scientific Premises License from the Department of Economic Development, Jobs, Transport and Resources (DEDJTR). Ethics and animal welfare were evaluated and supervised by Reef Pharmaceuticals' Animal Ethics Committee, and the study was approved by the Agricultural Research and Extension Animal Ethics Committee. All vaccinations were applied subcutaneously into the flank, mixing 1 ml of antigen in PBS and 1 ml of Seppic Montanide (ISA206) adjuvant.

For phase 1 of the study, animals were vaccinated over a period of 33 weeks and were divided into 5 groups according to the Env strain and amount of immunogen: 100µg KNH1/BG505 SOSIP, 100µg AD8 Unc, 500µg AD8 Unc, 500µg PSC89 Unc and 500µg MW Unc. The prime vaccine was injected at week 0 before conception, followed by 3 boosts with approximately 7 weeks intervals. Blood samples were collected before each boost (Weeks 0, 7 and 16) and 5 days after the last boost (at week 33). Colostral milks were also collected between weeks 46 to 52 for testing mucosal transmission of HIV-1 (Reef Pharmaceuticals) (Figure 2-1 A).

For phase 2 of the study, ten animals with high titre of IgGs against autologous Env binding in enzyme-linked immunosorbent assays (ELISAs), were selected for further revaccination after calving (at week 54). Cows vaccinated with KNH1/BG505 SOSIP gp140 were revaccinated with either 50µg or 100µg BG505 SOSIP gp140, while other animals received one of the following revaccination regimens: 50µg BG505 SOSIP

gp140, 50µg BG505 Unc gp140, 100µg AD8 Unc gp140 or 100µg AD8 6R SOSIP.664. (Figure 2-1 B).

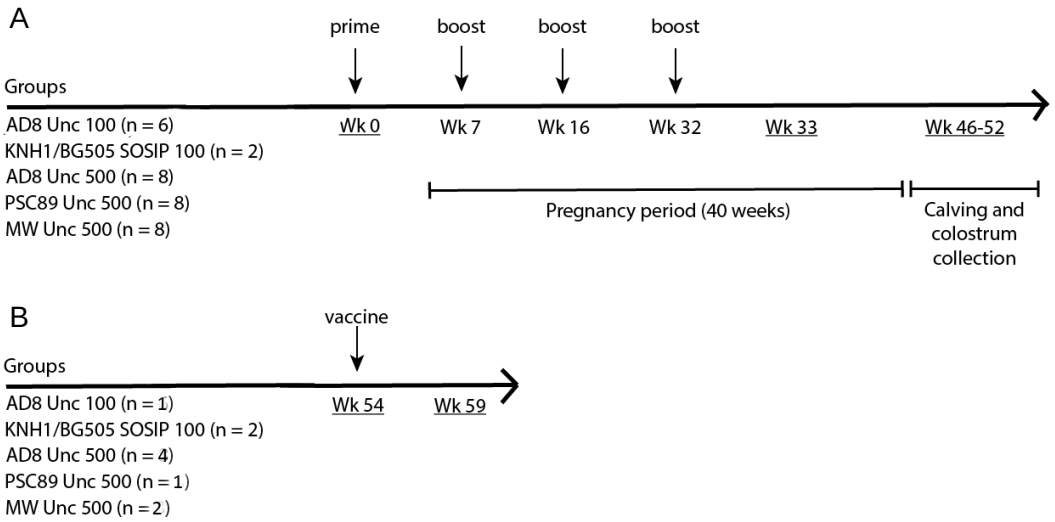


Figure 2-1: Vaccination scheme used for bovine vaccination. During phase 1 (A), animals divided into five groups were vaccinated at weeks 0, 7, 16 and 32. Artificial insemination and conception was performed before second immunisation, and in consequence, calving and colostrum collection occurred between weeks 46 and 52. Sera was collected at week 33, one week after last vaccination. For phase 2 (B), animals were revaccinated once with either same immunogen or a different Env gp140 trimer, at week 54 from first immunisation, and sera was collected at week 59.

Table 2-7: Vaccination groups and identity of cows in each group during phase 1. Thirty-two Holstein Friesian cows were divided into five groups and vaccinated with one of the following HIV-1 Env gp140 trimers: AD8 Unc, KNH1/BG505 SOSIP, PSC89 Unc or MW Unc. Table delivers information about samples collected (sera or colostral milk) and vaccinations received.

Cow ID#	gp140 Env received	Sera collected (Y/N)		Colostrum collected (Y/N)
	Wks 0, 7, 16 and 32	Wk 0	Wk 33	Wk 46 - 52
AD8 Unc 100				
623	4 x 100µg AD8 Unc	Y	Y	Y
2005		Y	Y	Y
3096		Y	Y	Y
6714		Y	Y	Y
9511		Y	Y	Y
9516		Y	Y	Y
		Total: 6/6		Total: 6/6
KNH1/BG505 SOSIP 100				
609	3 x 100µg KNH1 SOSIP + 1 x 100µg BG505 SOSIP	Y	Y	Y
617		Y	Y	Y
		Total: 2/2		Total: 2/2
AD8 Unc 500				
35	4 x 500µg AD8 Unc	Y	Y	Y
537		Y	Y	Y
647		Y	Y	Y
1134		Y	Y	Y
2150		Y	Y	Y
8203		Y	Y	Y
8434		Y	Y	Y
9533		Y	Y	Y
		Total: 8/8		Total: 8/8
PSC89 Unc 500				
26	4 x 500 µg PSC89 Unc	Y	Y	N (n.p.)
2223		Y	Y	Y
3333		Y	Y	Y
5682		Y	Y	Y
7333		Y	Y	N (mastits)
9506		Y	Y	Y
9545		Y	N (dead)	N (dead)
9552		Y	Y	Y
		Total: 7/8		Total: 5/8
MW Unc 500				
657	4 x 500µg MW Unc	Y	Y	Y
698		Y	N (dead)	N (dead)
2036		Y	Y	Y
2179		Y	Y	Y
5586		Y	Y	Y
5641		Y	Y	Y
9244		Y	Y	Y
9540		Y	Y	Y
		Total: 7/8		Total: 7/8

Table 2-8: Vaccination groups and identity of cows in each group during phase 2. Ten Holstein Friesian cows, representative of the initial thirty-two animals from phase 1, were revaccinated with one of the following HIV-1 Env gp140 trimers: AD8 Unc, AD8 SOSIP, BG505 Unc, or BG505 SOSIP. Table delivers information about samples collected (sera or colostral milk) and vaccinations received

Cow ID#	Group (amount vaccine received)	gp140 Env received after calving	Sera collected (Y/N)	
	Wks 0, 7, 16 and 32	Wk 54	Wk 54	Wk 59
2005	**AD8 Unc 100 (4 x 100µg)**	**100µg AD8 Unc gp140**	**Y**	**Y**
609	KNH1/BG505 SOSIP	100µg BG505 SOSIP gp140	Y	Y
617	(3 x 100µg + 1 x 100µg)	50µg BG505 SOSIP gp140	Y	Y
8434	AD8 Unc 500 (4 x 500µg)	100µg AD8 SOSIP gp140	Y	Y
9533		50µg BG505 Unc gp140	Y	Y
35		100µg BG505 SOSIP gp140	Y	Y
537		50µg BG505 Unc gp140	Y	Y
9506	PSC89 Unc 500 (4 x 500µg)	50µg BG505 SOSIP gp140	Y	Y
5586	MW Unc 500 (4 x 100µg)	100µg AD8 SOSIP gp140	Y	Y
9244		50µg BG505 SOSIP gp140	Y	Y
			Total: 10/10	

2.6 Sample preparation

Sera samples were collected from blood at weeks 0, 33, 54 and 59. Blood samples were collected in a red top Vacutainer© collection tube (BD, catalogue number 367820), incubated at room temperature (RT) for 2 hrs and centrifuged at 1,300*x g* for 12 minutes (mins) at RT. Supernatant was then collected from each tube, aliquoted in 1.5 ml Eppendorf™ tubes and stored at -80°C.

Purified IgGs from colostral milk were prepared as mentioned previously (Kramski et al., 2012; Heydarchi et al., 2016). In brief, colostrum was defatted and pasteurised by centrifugation at 10,000*x g* for 30 mins at 4°C, incubated at 63°C for 30 mins and by centrifuged at 10,000*x g* for 10 mins. Thereafter, casein was removed using 0.2M sodium acetate (pH 4.0) with incubation at 37°C for 2 hours (hrs) and centrifugation at 10,000*x g* for 30 mins. The final colostral whey was used for IgG purification using

protein G sepharose column (GE Healthcare) according to manufacturer's instructions. Quality of purification was verified by SDS-PAGE and Coomassie blue staining, and concentration of IgG in purified samples was measured by spectrophotometer using the formula:

$$C_{(\frac{mg}{ml})} = \frac{A_{280nm}}{\varepsilon_{percent}} \times 10$$

Where $\varepsilon_{percent}$ is the protein extinction coefficient and equals 13.7. Therefore, each concentration was calculated as:

$$C_{(\frac{mg}{ml})} = \frac{A_{280nm}}{1.37}$$

2.7 Quantification of Total IgG in sera

In order to evaluate Ab responses elicited by vaccination, bovine IgG in sera was measured by capture ELISA. Samples were loaded along with a standard curve of bovine polyclonal IgG, starting from 10 µg/ml, in half-log dilutions; standard curve was carried in every plate and it reached a curve with average optical density (O.D.) values of 1.45 at 10 µg/ml, and of 0.27 at 3.17 x 10^{-3} µg/ml. To start, 96 well plates (Corning clear flat bottom) were coated with a 3µg/ml rabbit anti-bovine IgG (Fc specific) (Sigma) in coating buffer (200mM Tris-HCl, 100mM NaCl, pH 8.8) and incubated overnight at 4°C. Then, plates were washed with PBST (PBS + 0.01% tween 20) and PBS, and blocked with 5% casein in PBS for 1 hr at RT. Serial dilution of serum samples in 5% casein were added and incubated for 3 hrs at RT. Afterward, 1/1000 dilution of Horse Radish Peroxidase (HRP)-conjugated rabbit anti-bovine (whole molecule) (Sigma, cat no: A5295) premixed with 1% normal rabbit serum (NRS) was added and incubated for 1 hr at RT. Finally, colour development was performed using TMB (Sigma, cat no: T5525) according to manufacturers' instruction and reaction was stopped using 1M H_2SO_4. Absorbance was measured at 450 nm against a reference of

690 nm. Values for standard IgG carried in each plate were used to calculate a standard curve, which was finally used to intrapolate IgG concentrations in serum samples.

2.8 Assessment of binding against autologous vaccine

2.8.1 Direct ELISA for assessment of HIV Env IgGs in sera and colostrum

IgG titres against autologous or heterologous Envs were detected in sera and purified IgGs from colostrum. Briefly, 96-well plate was coated with 1µg/ml recombinant Env gp140 proteins (BG505 SOSIP, AD8 Unc, PSC89 Unc or MW Unc) overnight at 4 °C and blocked with 5% casein as mentioned above. Sera or purified IgG samples were added and incubated for 3 hrs at RT. Afterward, 1/1000 dilution of HRP-conjµgated sheep-anti-bovine IgG (BioRad, cat no. AAI23P) was added to the wells and incubated for 1 hr at RT. Finally, colour development was performed using TMB reagent as mentioned above.

2.8.2 Competition ELISA for epitope characterisation

To investigate epitopes targeted by bovine IgGs on HIV-1 Env, a competition ELISA was performed using human BnAbs as previously described (Kramski et al., 2012; Heydarchi et al., 2016). For IgGs from cows vaccinated with AD8 Unc gp140, a direct ELISA was performed: plate was coated with 1µg/ml AD8 Unc gp140 overnight at 4°C and blocked with 5% skim milk in PBS. Then, purified IgGs from colostral milk (1mg/ml final concentration) was added and incubated for 2 hrs at RT. Afterwards, human BnAbs VRC01, b12, PGT121, A32 and 17b (NIH AIDS Reagent Program) were added in serial dilutions and incubated for 2 hrs at RT. Finally, the plate was incubated with 1/1000 dilution of HRP-conjugated goat anti-human IgG (γ) (KPL, cat no: 474-1002) for 1hr at RT. TMB was used for colour development.

For IgGs from cows vaccinated with BG505 SOSIP gp140, a capture competition ELISA was performed: plates were coated with sheep D7324 antibody overnight at 4°C and blocked with 5% skim milk in PBS, followed by the addition of 100 ng/well of BG505 SOSIP 664-D7 gp140. Then, purified IgGs from colostral milk (1mg/ml final concentration) was added and incubated for 2 hrs at RT. Afterwards, human BnAbs VRC01, PGT121 and PGT145 (NIH AIDS Reagent Program) were added in serial dilutions and incubated for 2 hrs at RT. Finally, the plate was incubated with 1/1000 dilution of HRP-conjugated goat anti-human IgG (γ) (KPL, cat no: 474-1002) in 5% skim milk plus 2% normal sheep serum (NSS), for 1hr at RT. As in the direct ELISA, TMB was used for colour development.

In both cases, signal from each human BnAb will show a reduction in binding of such BnAb if polyclonal antibodies previously blocked its epitope. Quantification of reduction was performed by calculating the area under the curve (AUC) after plotting values with GraphPad Prism v7. Values of AUC were averaged between all animals within each group.

2.8.3 Fcγ receptor binding assessment

Binding of bovine Abs to Fc-gamma (γ) receptors was studied with an Fcγ receptor dimmer ELISA (Wines et al., 2016). Experiments were performed by Jack Edwards under my supervision. Briefly, 96-well plates (Nunc high protein-binding capacity polystyrene 96-well ELISA plates) were coated with 1µg/ml recombinant gp140 trimers (AD8 Unc, MW Unc or BG505 SOSIP) overnight at 4°C, blocked with 1% BSA in PBS and incubated at saturating conditions (500µg/ml) with polyclonal IgGs purified from colostrum for 3 hr at RT. Then, recombinant biotinylated Fcγ dimers for low affinity receptors FcγRIIa (allotypes R131 and H131) and FcγRIIIa (allotypes F158 and V158) were added and incubated for 1 hr at 37°C. High sensitivity Streptavidin HRP-conjugated (Thermo Fisher, cat no: 21130) was added at 1/10,000 dilution in 1% BSA-

PBS for 1 hr at 37°C, plates were then washed 4x with 1% BSA-PBS followed by 4x PBS before developing the colorimetric reaction as mentioned before. Binding curves of receptor dimers were obtained by plotting the OD observed at 450 nm minus signal at 690 nm in *GraphPad Prism v7*.

2.9 Assessment of neutralising activity in sera and IgGs from colostrum

2.9.1 Pseudovirus production

HIV pseudoviruses were produced as mentioned before (Montefiori et al., 2004; Kramski et al., 2012). Backbone plasmids pNL4-3ΔEnvEGFP (Cat no: 11100, obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID) (Zhang et al., 2004) and pNL-4.3.Luc.R-E- (Cat no: 3418, obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID) (Connor et al., 1995) were used to produce pseudoviruses for TZM-bl and CF2th/CD4/CCR5/CXCR4 (Cf2th) canine cells neutralisation assays, respectively. Pseudoviruses were produced by co-transfecting one backbone plasmid and Env expressing plasmids in HEK 293T cells using Lipofectamine 2000 (Thermo Fisher) according to the manufacturer's instructions in an envelope-to-backbone ratio of 1:9 (except for MN, which was 1:5). Env expression plasmids encoded *env* genes of viruses MuLV (pSVIII-MuLV), AD8 (pCMV-AD8), SF162 (pCAGGS SF162 gp160), MN (pSVIII-MN), (Kramski et al., 2012; Heydarchi et al., 2016), PSC89 (pSVIII-PSC89), BG505 (pBG505.W6M.ENV.C2.N332) and ZM109F.PB4 (NIH AIDS reagent, Cat No. 11314). Supernatants of transfected cells were collected at 48 hrs post transfection and stored at -80°C. Viral titres were calculated by infecting either TZM-bl cells or Cf2th cells with pseudovirus in half-log dilutions, and luciferase activity was evaluated 48 hours post-infection as described before (Gonelli et al., 2019).

2.9.2 Neutralisation assays

TZM-bl neutralisation assay was performed for sera samples collected during vaccination course at week 0 (before vaccination), week 33 (after the 3rd boost and before calving), week 54 (after calving and before the final boost) and week 59 (after the final boost). Assay was performed as described before (Montefiori et al., 2004). Briefly, 50µl of each of the seven pseudoviruses produced in section 2.3.9.1 (200 $TCID_{50}$) were mixed with serial dilutions of serum samples in 100µl final volume in 96-well plates (Corning, flat bottom, non-pyrogenic), and incubated for 1 hr at 37°C. Thereafter, 10^4 TZM-bl cells (containing 125mM of DEAE-Dextran (Sigma) were added to each well and the plates were incubated for 72 hours at 37°C. Infectivity of the pseudoviruses in the presence of antibodies was investigated by measuring relative luminescence units (RLUs) using luminescence reporter reagent BriteLite Plus (PerkinElmer) and FLUOstar Omega (BMG Labtech) plate reader. Because TZM-bl cells express firefly luciferase under the control of HIV-1 Long Terminal Repeat (LTR), amount of luciferase expressed is directly proportional to viral infection and replication.

Cf2th neutralisation assay was performed for purified IgGs from colostrum (Kramski et al., 2012). Briefly, 10^4 Cf2th cells per well were seeded in growth media (DMEM, 10% FBS, 25mM HEPES, 50mg/ml hygromycin, 500µg/ml G418, 1µg/ml of puromycin) overnight at 37°C. 50ul pseudoviruses (200 $TCID_{50}$) were mixed with a dilution series of polyclonal IgGs and incubated for 1 hour at 37°C. Afterwards, the antibody-virus mixtures (in presence of 125mM of DEAE-Dextran) were added to pre-seeded CF2th cells. The plate was incubated for 72 hours at 37°C and luminescence was measured as mentioned above. Same as in TZM-bl cells, infectivity of pseudoviruses in the presence of antibodies was investigated by measuring relative luminescence units (RLUs) using luminescence reporter reagent BriteLite Plus (PerkinElmer) and FLUOstar Omega (BMG Labtech) plate reader. In this case, pseudoviruses were

produced with a firefly luciferase reporter and virus-antibody mixture was used to infect Cf2th cells. Infection and further viral replication is also directly proportional to the amount of luciferase expressed.

In both cases, infections were performed using 150 x 10^3 RLUs, equivalent to 200 $TCID_{50}$. Baseline luciferase signal was around 500 for Cf2th cells, and around 2000 for TZM-bl cells.

2.9.3 Analysis of results

For TZM-bl assays, neutralisation curves for each animal were plotted in triplicate, and dilution at which 50% of neutralisation was reached (ID_{50}) was calculated based on such curves. For Cf2th cells, neutralisation curves for each animal were plotted and the concentration at which 50% of neutralisation was reached (IC_{50}) was calculated based on such curves. Then, average values were calculated against each pseudovirus, where low IC_{50} values are coloured in red whereas high IC_{50} values are colourless.

To evaluate differences between neutralising activity at weeks 54 and 59, fold change between both timepoints was calculated as it follows:

$$Fold\ change = \frac{ID50_{week\ 59}}{ID50_{week\ 54}}$$

, where values above 1 represent an improvement in neutralisation against that pseudovirus, while values below 1 indicate a decrease in neutralisation.

2.10 Assessment of phagocytosis mediated by bovine IgGs

2.10.1 Biotinylation of Avi-tagged proteins

Proteins with Avi-tag were biotinylated as described before (Fairhead and Howarth, 2015), with minor modifications. Briefly, 1 mg Env protein was incubated with 5µg BirA enzyme in 1 ml of Tris pH 9.0 plus 0.05M bicine buffer, 10mM ATP, 10mM MgOAc and 50µM D-biotin, at 37°C for 3 hours. Afterward, Env proteins were buffer exchanged using Amicon Ultra 0.5 tube (Molecular weight cut-off (MWCO) of 2kDa) and stored in PBS at -80°C until usage.

2.10.2 ADP-SHIP assays

ADP-SHIP assays were performed as described previously (Ana-Sosa-Batiz et al., 2014) with minor modifications. Three µl (50% slurry) of FITC-labelled NeutrAvidin FluoSpheres 1µm (Invitrogen) was incubated with 1µg biotinylated recombinant HIV-1 Env (AD8 Unc gp140, AD8 gp120, AD8 SOSIP gp140 or BG505 SOSIP gp140) and 3µl of 150µM biotin- and Cy5- labelled fluorescent internalisation probe (FIP_{Cy5}) (Integrated DNA Technologies, Coralville, IA, USA) in 100ul of PBS, overnight at 4°C. After washing beads with 2% BSA-PBS, 10µl of a 1/10 dilution of coated beads was opsonised with half-log dilutions of bovine polyclonal IgGs for 2 hours at 37°C. After 2 rounds of washes with 2% BSA-PBS, 1 x 10^5 of U937 cells (ATCC CRL-1593.2) per reaction was added and incubated for 16 hours at 37°C. Finally, 1µg/ml of BHP-conjugated complementary quenching probe (QP_c, Integrated DNA Technologies, Coralville, IA, USA) was added to quench signal from surface-bound beads for 10 mins on ice before fixing cells and acquiring the signal in LSR Fortessa flow cytometer (BD Biosciences). Data of Cy5 signal in the FITC positive population was collected, and phagocytosis index was calculated as the product of the percentage of Cy5 positive

events (% Cy5+) and the mean fluorescence intensity (MFI) of total FITC population in the Cy5 channel (MFI Cy5 intensity – FITC+).

2.11 Assessment of complement activation by bovine IgGs

Complement deposition was measured as C3b activation, as described previously (Barouch et al., 2015; Ackerman et al., 2016). Briefly, 1 x 10^6 CEM NKr CCR5+ cells (Cat no: 4376, obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID) (Howell et al., 1985; Lyerly and Morse, 1999; Trkola et al., 1999), equivalent to 10 reactions, was pulsed with 6µg recombinant HIV-1 Env (AD8 Unc gp140, AD8, gp120, BG505 SOSIP gp140, PSC89 Unc gp140 or MW Unc gp140) for 1 hr at RT in 100ul of RPMI. In a total volume of 50 µl, 500µg/ml purified IgGs were added in veronal buffer 0.1% gelatin (Sigma), incubated for 15 mins at RT, and 1/10 dilutions of freshly isolated HIV negative human plasma in veronal buffer 0.1% gelatin was then added and incubated for 20 mins at RT to allow complement activation. Cells were washed twice before staining with anti-C3b human FITC antibody (Cedarlane, 1/50 final dilution) for 20 mins at RT, fixed with 2% paraformaldehyde, and analysed in a FACS Canto II flow cytometer (BD Biosciences). Measurement of complement activation was taken as a relative fluorescence (RF) value (%FITC+ events x MFI in FITC for single cells) with the cut-off set at 2 times above the RF of the non-immune control.

2.12 Preparation of IgM and IgG library for sequencing

2.12.1 RNA extraction and cDNA production

RNA was extracted from bovine PBMCs with the NucleoSpin© RNA plus kit (Macherey Nagel, catalogue no 740984.50), following the manufacturer's indications. DNA from extracted RNA was degraded by using DNAseI (Sigma-Aldrich, catalogue number AMPD1), following manufacturer's indications and incubating samples at RT for 15 min, followed by heat-inactivation of DNAseI at 70 degrees for 10 min. Samples were then stored at -80 degrees for further usage.

For cDNA production, 200 ng of RNA from each sample were incubated with 500 ng of OligoDTs and 200 ng of random hexamers, plus 200 units/ul of SuperScript III reverse transcriptase (ThermoFisher, cat no 18080085) in a two-step process. Indications of reagents used in each step can be found in table 13:

Table 2-9: Reagents and protocol used for cDNA production

Step 1: 65 degrees for 5 min, cool down for 1 min

Reagent	Amount or Volume
RNA template	200 ng
OligoDTs	500 ng
Random Hexamers	200 ng
H2O	For 13 ul volume

Step 2: 55 degrees for 1 hr

Reagent	Amount or Volume
FS buffer 4x	4 ul
RNAseOUT RNAse inhibitor	1 ul
SuperScript III enzyme	1 ul

After reaction, cDNA was purified using Nucleospin Gel and PCR Clean (Macherey-Nagel, Duren, Germany; catalog number 740609.50). Samples were eluted in 20ul of ultra-pure water and stored at -20°C before usage.

2.12.2 PCR for 5' Rapid Amplification of cDNA Ends (5'RACE) library

5' RACE PCR protocol was performed by Dr. Shuo Li as shown previously in the literature (Vazquez Bernat et al., 2019), in a two-step process. First step involved the amplification of cDNA by using 1µl of primers ODP 3748 and ODP 3749 at 10uM, and later the addition of read1 and read2 adapters for Illumina MiSeq sequencing, using the primers ODP 3580 (forward, read 1 adapter) and ODP 3572 and 3573 (reverse, read 2 adapter for IgG and IgM sequences, respectively). Reaction was performed using the KAPA HiFi Hotstart Ready PCR Kit (Roche, cat no: KK2601) according to the manufacturer's indications, using the following conditions:

- cDNA: 10ul
- Read1 U primer (ODP 3580) (10uM): 0.5 ul
- Read 2 primer (ODP 3572 or 3573) (10uM): 1 ul
- KAPA mix: 10.5 ul

PCR libraries were then sent to AGRF (Victoria, Australia) for addition of P5 and P7 adapters used in Illumina sequencing systems. For this, primers targeting Read1 and Read2 adapters were used.

2.12.3 PCR for Multiplex library

Multiplex PCR was performed as shown previously in the literature (Vazquez Bernat et al., 2019). First step involved the amplification of cDNA and addition of adapters for Illumina Nextera sequencing, by using 2µl of a 10mM mix of primers from ODP 4040 to 4055 (forward, Read1 adapter for leader sequences within IGHV), and 1ul of either

ODP 4056 or ODP 4057 at 10uM (reverse, read 2 adapter for IgG and IgM sequences, respectively). For more information about the design of primers ODP 4040 to 4055, please refer to section 2.13. Reaction was performed using Phusion High-Fidelity DNA polymerase (New England BioLabs, cat no: M0530S), according to the manufacturer's indications, under the following conditions:

- cDNA: 2 ul
- Read 1 primer mix: 2 ul
- Read 2 primer (ODP 4056 or 4507): 1 ul
- HF buffer 5x: 4 ul
- Phusion enzyme: 0.2 ul
- dNTPs: 0.4 ul
- H_2O: to make 20 ul

PCR products were visualised by 1% agarose gel electrophoresis, and bands between 450 and 550 bp were cut and purified using Nucleospin Gel and PCR Clean (Macherey-Nagel, Duren, Germany; catalog number 740609.50). Then, PCR libraries were then sent to AGRF (Victoria, Australia) for addition of P5 and P7 adapters used in Illumina sequencing systems. For this, primers targeting Read1 and Read2 adapters were used.

2.13 Next-Generation Sequencing

2.13.1 Sequencing of cDNA libraries and data retrieve

Both 5' RACE and Multiplex PCR library sequencing were performed by AGRF (Victoria, Australia) in Illumina© sequencers. Due to the length constrains of these

devices (300 bp), samples were sequenced from both extremes, delivering two files per sample in fastq format: R1 (read from 5' end) and R2 (read from 3' end).

Sequencing of 5' RACE libraries was performed in an Illumina© MiSeq system, using the following adapters:

- Read 1 adapter: 5′ ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'
- Read 2 adapter: 5′ GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3'

On the other hand, sequencing of Multiplex libraries was performed in an Illumina© MiSeq system, using the following adapters:

- Read 1 adapter: 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG '3
- Read 2 adapter: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG '3

For both sequencing experiments, files with R1 and R2 reads were retrieved from AGRF servers as compressed fastq files and stored in one unique folder containing both R1 and R2 reads.

2.13.2 Sequence analysis using IgDiscover

IgDiscover version 0.11 (Corcoran et al., 2016) was installed in a PC running Ubuntu version 20.4 LTS (Canonical Group Limited) under a Windows Subsystem for Linux compatibility layer (WSL, Microsoft, USA) and Python 3.7 as programming language. Program was executed following IgDiscover documentation sheet (available at http://docs.igdiscover.se/en/stable/), using a command structure as follows:

```
Igdiscover init –-db database –-reads input_R1.fastq.gz output
```

, where:

- `database`: folder with V, D and J genes to use in analysis
- `input_R1.fastq.gz`: Read 1 of input folder, in fastq format and compressed in gz

- `output`: Output folder which will be created when command is executed

This created an editable configuration file called “igdiscover.yaml”, which allows editing of settings in each program run. For all experiments, first run was performed with 2 iterations, while runs including novel alleles were performed with only 1 iteration. Merging was performed with PEAR (Pair-End rAad meRger, (Zhang et al., 2014)) and all other variables remained untouched for 5’ RACE library analysis. For Multiplex library analysis, a barcode length of 21 residues was included in settings.

Results of sequence analysis were evaluated using “assigned.tab” file. Rows in “assigned.tab” were filtered using LibreOffice version 6.2.4.2 (The Document Foundation, USA) and Microsoft Excel (part of Microsoft Office 360, Microsoft, USA). New alleles were collected from iterations 1 and 2 and pasted on V gene files accordingly.

2.13.3 Sequence analysis using AbMining ToolBox

AbMining ToolBox version 1 (D'Angelo et al., 2014) was installed in a PC running Ubuntu version 20.4 LTS (Canonical Group Limited) under a Windows Subsystem for Linux compatibility layer (WSL, Microsoft, USA) and Python version 2.7 as programming language. Program was used editing the script ”cdr3_pipeline.py”, which works with nucleotide annotation, to be as follows:

```
cdr3_pattern =
r'(TT[TC]|TA[CT])(TT[CT]|TA[TC]|CA[TC]|GT[AGCT]|TGG)(TG[TC])(([GA][AG
CT])|TC)[AGCT]([ACGT]{3}){5,72}TGGG[GCT][GCT]'
```

This extends the size of recognized CDR3 region to 75 residues. Modification was saved in a separate file as “cdr3UL_pipeline.py”. Then, cdr3UL_pipeline.py was used

for analysis of merged reads generated with PEAR under the IgDiscover pipeline. Results of sequence analysis by program were evaluated using “bins_output.dna.cdr3.csv” file. Counts and analysis were performed using LibreOffice version 6.2.4.2 (The Document Foundation) and Microsoft Excel (part of Microsoft Office 360, Microsoft, USA).

2.13.4 Production of Phylogenetic trees and Cluster analysis

Multiple alignment of sequences was performed using MUSCLE version 3.8.15 (Edgar, 2004), included in the Miniconda3 package (Anaconda Inc.), installed in a PC running Ubuntu version 20.4 LTS (Canonical Group Limited) under a Windows Subsystem for Linux compatibility layer (WSL, Microsoft, USA) and Python version 3.7 as programming language.

For primer design targeting leader sequences within IGHV genes, data of leader sequences obtained by IgDiscover in 5’ RACE PCR libraries was clustered with a maximum iteration of 2, and clusters with common sequences were reduced to 16 common sequences, which were later used in the design of primers ODP 4040 to 4055 for Multiplex PCR libraries.

For phylogenetic analysis of bovine monoclonal antibodies, sequences of mAbs isolated by Dr. Behnaz Heydarchi from cows 35, 8434 and 617, which presented ultra-long CDRH3, were analysed along with their putative germline gene through MUSCLE, with no maximum iteration. Maximum likelihood trees were generated using RAxML version 8 (Stamatakis, 2014), using 10000 bootstrap replicates, and then phylogenetic trees were visualised and edited in Dendroscope 3 (Huson and Scornavacca, 2012).

2.14 Statistical Analysis

All assays represent 3 separate experiments with each run performed in duplicate, unless stated otherwise. Statistical significance was evaluated using *GraphPad Prism version 7* (GraphPad Software Inc.). Differences between groups were evaluated by one-way or 2-way ANOVA tests when indicated in results section. P values < 0.05 were considered to denote significant differences. ns = non-significant, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3. Assessment of neutralising antibody responses in cows against different stabilised HIV-1 Env trimers

3.1 Background

Production of neutralising antibodies by using recombinant HIV-1 Env has been challenging. As evidence, BnAbs appear in about 20% of patients only after years of infection (Sather et al., 2009; Liu et al., 2015), and previous vaccination trials in humans have not been successful to produce BnAbs (Buchbinder et al., 2008; Pitisuttithum et al., 2006; Flynn et al., 2005; Gilbert et al., 2005; Gray et al., 2011; Rerks-Ngarm et al., 2009). Structure-based reverse vaccinology has shown that usage of trimers is more effective in eliciting neutralisation compared to monomeric gp120 (VanCott et al. 1997; Lelievre and Yves, 2016, Forsell et al., 2009), however, production of such trimeric structures requires stabilising the interactions between gp120 and the ectodomain of gp41, which falls apart very easily as they interact without any covalent link.

Stabilising modifications such as deletion of the cleavage site between gp120 and gp41 (Earl et al., 1994) or the introduction of an artificial disulphide bond connecting both subunits (Binley et al., 2000; Sanders et al., 2002) has resulted in the production of Unc- and SOSIP-modified trimers, respectively. Autologous neutralisation has been achieved with SOSIP trimers in mice and rabbits (de Taeye et al., 2015; Sliepen et al., 2019) and heterologous neutralisation was induced with both modifications in the same species (Grundner et al., 2004; Yang et al., 2001; Gao et al., 2005; Liao et al., 2013; Yang et al., 2001; Klasse et al., 2018; Center et al., 2009). None of the trials performed in macaques, however, has conferred protection, and neutralising titres in other animals were mild compared to the neutralising activity observed with BnAbs.

As mentioned in section 1.4, cows present advantages in the production of Abs with neutralising activity against HIV-1 isolates (Kramski et al., 2012; Heydarchi et al., 2016; Sok et al., 2017) due to their long CDRH3 antibody repertoire (De Genst et al., 2006; Wang et al., 2013), In cows, both Unc- and SOSIP-stabilised trimers were able

to induce BnAbs. To date, however, no comparison has been made regarding which of these vaccines is more effective in eliciting BnAbs. Both clade and structural differences of Unc- and SOSIP-stabilised trimers were compared in this chapter.

Vaccination was performed as indicated in section 2.5 and in figure 2-1. Phase 1 involved 5 groups of animals which were immunised with either KNH1/BG505 SOSIP gp140, AD8 Unc gp140, MW Unc gp140 or PSC89 Unc gp140. All groups received a prime before conception (week 0), and 3 boosts during pregnancy (week 7, 16 and 32). Blood samples were collected after each vaccination, and timepoints before (week 0) and after phase 1 (week 33) were evaluated. For phase 1, binding to autologous and heterologous Env vaccines, neutralising activity, and binding epitopes were evaluated. Phase 2 involved ten cows representing each one of the five initial groups and were vaccinated further with either the same vaccine or a different one. In this case, only binding to vaccine used on phase 1 and neutralising activity was tested to evaluate the efficiency of phase 2 vaccination.

As our previous results showed that sequential immunisation with the autologous immunogen could not improve anti-HIV neutralisation response, we aimed to investigate whether modifications in HIV-1 Env immunogen could result in an improved anti-HIV response. Our hypothesis was that either further stabilisation of trimeric structure or change of Env strain could boost antibody neutralisation response in the vaccinated animals.

3.2 Quantification of total IgG in bovine sera during phase 1 and 2

To evaluate if vaccination changed IgG concentration, sera samples before and after phase 1 and 2 of the scheme were collected for each animal and tested in a capture ELISA for total bovine IgG. The amount of bovine IgGs in each sera sample was compared to a standard curve of bovine IgGs, and results were compiled in figure 3-1 and table 3-1, where each dot represents an animal and error bars indicate the standard deviation of the whole group.

No significant differences were found in total IgG concentration during phase 1 (week 0 and 33) for groups AD8 Unc 100, AD8 Unc 500 and PSC89 Unc 500 (values of week 0 v/s week 33: 74.76 mg/ml vs 85.03 mg/ml for AD8 Unc 100, 106.54 mg/ml vs 84.04 mg/ml for AD8 Unc 500, and 70.38 mg/ml vs 66.35 mg/ml for PSC89 Unc 500). KNH1/BG505 SOSIP 100 and MW Unc 500 presented increased IgG concentration at week 33, with total values of 188.48 mg/ml vs 16.03 mg/ml at week 0, and of 101.95 mg/ml vs 47.29 mg/ml at week 0, respectively. This suggests that the immunogenicity of KNH1/BG505 SOSIP gp140 and MW Unc gp140 is higher than other vaccines.

IgG concentration decreased at the start of phase 2 (week 54) in all groups, probably due to calving, reaching similar values between groups: 14.74 mg/ml for AD8 Unc 100, 19.57 mg/ml for KNH1/BG505 SOSIP 100, 15.87 mg/ml for AD8 Unc 500, 12.96 mg/ml for PSC89 500, and 16.9 mg/ml for MW Unc 500. By the end of phase 2 (week 59), IgG concentration increased almost 10-fold in AD8 Unc 500 and MW Unc 500 groups (112.26 mg/ml for AD8 Unc 500 and 175.14 mg/ml for MW Unc 500), compared to the values obtained at week 54. In other groups, these values boosted nearly 3-fold (47.13 mg/ml for AD8 Unc 100, 54.42 mg/ml for KNH1/BG505 SOSIP 100 and 50.75 mg/ml for PSC89 500). Compared to week 0, three groups increased their total IgG concentration at the end of phase 2: KNH1/BG505 SOSIP 100, AD8 Unc 500 and MW Unc 500.

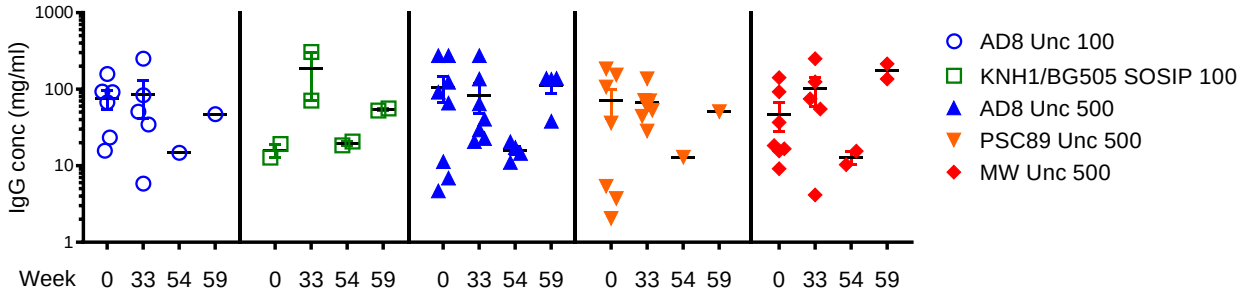


Figure 3-1: Total IgG concentration during phase 1 and phase 2 of vaccination scheme. Samples at weeks 0, 33 (phase 1), 54 and 59 (phase 2) were used for a capture ELISA against bovine IgGs. Each point represents one animal in one of the 5 groups: AD8 Unc 100 (blue open circles), KNH1/BG505 SOSIP 100 (green open squares), AD8 Unc 500 (blue closed triangles), PSC89 Unc 500 (orange closed triangles) or MW Unc 500 (red closed diamonds). Bars in each timepoint indicate average of the group ± SEM. Results from n = 3 replicates.

Table 3-1: Values of total IgG concentration per group, over the vaccination course. Averages plus/minus SEM were calculated for each group at the defined timepoint.

	AD8 Unc 100	KNH1/BG505 SOSIP 100	AD8 Unc 500	PSC89 Unc 500	MW Unc 500
Week	Average (mg/ml)	Average (mg/ml)	Average (mg/ml)	Average (mg/ml)	Average (mg/ml)
0	74.76 ± 21.34	16.03 ± 3.23	106.54 ± 9.6	70.38 ± 29.22	47.29 ± 19.09
33	85.03 ± 43.29	188.48 ± 117.31	84.04 ± 35.18	66.35 ± 13.07	101.95 ± 41.93
54	14.74 ± 0	19.57 ± 1.14	15.87 ± 2.04	12.96 ± 0	12.9 ± 2.6
59	47.13 ± 0	54.42 ±1.8	112.26 ± 24.7	50.75 ± 0	175.14 ± 39.23

3.3 Assessment of autologous binding in bovine sera

In order to evaluate whether this upsurge in total IgG titre was due to higher immunogenicity of KNH1/BG505 SOSIP gp140 and MW Unc gp140, HIV-specific IgG titre in sera was investigated, using a direct ELISA with Env vaccines, using each immunogen in each group. Serial dilutions of sera were performed in order to find endpoint titres of each animal. Based on data obtained in section 3.2, endpoint titres

were later transformed into endpoint concentration, which indicates the minimal concentration at which polyclonal mixture presents reactivity against its antigen and allows assessment of autologous binding.

After phase 1 (week 33), only cows vaccinated with AD8 Unc gp140 showed mean endpoint concentrations below 100 µg/ml: 52.85 µg/ml for AD8 Unc 100 and 44.6 µg/ml from AD8 Unc 500 group (Figure 3-2). The other three groups presented higher endpoint concentrations (461.32 µg/ml, 469.8 µg/ml and 305.09 µg/ml for KNH1/BG505 SOSIP 100, PSC89 Unc 500 and MW Unc 500, respectively). These results indicate that most of the antibodies generated by KNH1/BG505 SOSIP gp140 and MW Unc gp140 do not bind against the vaccine in a direct ELISA setting. PSC89 Unc 500 on the other hand presented the highest endpoint concentration and a total IgG concentration similar to week 0, meaning that PSC89 Unc gp140 was not highly immunogenic (Figure 3-2, section “week 33”).

After calving and right before phase 2 (week 54), all vaccinated groups presented similar endpoint concentrations between 128.96 µg/ml (MW Unc 500) and 195.69 µg/ml (KNH1/BG505 SOSIP 100). It is possible that at this point, transfer of IgGs into colostral milk and further decrease of antibodies in sera could have diminished differences in binding titres between groups. At week 59 (after phase 2), antibodies against autologous antigen decreased in all groups (average endpoint concentration values of 471.32 µg/ml , 289.43 µg/ml, 745.99 µg/ml, 570.71 µg/ml and 1286.93 µg/ml for AD8 Unc 100, KNH1/BG505 SOSIP 100, AD8 Unc 500, PSC89 Unc 500 and MW Unc 500 group, respectively). In contrast, only one animal from KNH1/BG505 SOSIP 100 and AD8 Unc 500 groups improved binding titres compared to week 54 (from 184.32 µg/ml to 16.65 µg/ml for KNH1/BG505 SOSIP 100 group and from 206.22 µg/ml to 120.93 µg/ml for AD8 Unc 500 group). Overall, groups vaccinated with AD8 Unc gp140 presented the highest affinity for their immunogen at week 33; these binding titres declined after calving and did not improve after phase 2 of vaccination.

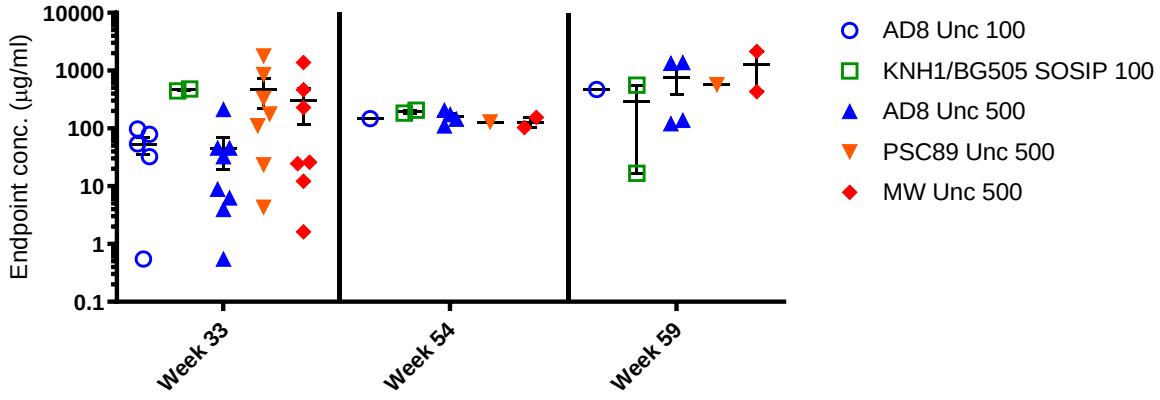


Figure 3-2: Autologous Env binding of bovine IgGs in sera of vaccinated cows. Binding was measured at weeks 33, 54 and 59 against the same gp140 vaccine through direct ELISA. Data points show IgG concentration of each animal in one of the 5 groups: AD8 Unc 100 (blue open circles), KNH1/BG505 SOSIP 100 (green open squares), AD8 Unc 500 (blue closed triangles), PSC89 Unc 500 (orange closed triangles) or MW Unc 500 (red closed diamonds). Bars in each timepoint indicate average plus/minus SEM of the group. Endpoint concentration indicates the minimal concentration at which the polyclonal antibodies presented reactivity against Env antigen. Results from n = 3 replicates.

Table 3-2: Autologous Env binding of IgGs in sera of vaccinated cows. Binding was assessed at weeks 33, 54 and 59. Averages plus/minus SEM were calculated for each group at the defined timepoint.

	AD8 Unc 100	KNH1/BG505 SOSIP 100	AD8 Unc 500	PSC89 Unc 500	MW Unc 500
Week	Average (µg/ml)	Average (µg/ml)	Average (µg/ml)	Average (µg/ml)	Average (µg/ml)
33	52.85 ± 38.36	461.32 ± 23.29	44.6 ± 70.52	469.8 ± 649.54	305.09 ± 501.83
54	147.43 ± 0	195.69 ± 16.07	158.71 ± 40.73	129.56 ± 0	128.96 ± 36.79
59	471.31 ± 0	289.43 ± 385.77	745.99 ± 711.94	570.71 ± 0	1286.92 ± 1211.74

3.4 Assessment of autologous and heterologous binding in bovine colostrum

After phase 1 of vaccination, delivery of calves stimulated secretion of the first batch of colostrum in pregnant cows. As mentioned before, colostrum contains high concentration of antibodies compared with sera and bovine milk, as bovine placenta impedes antibody transfer and calves are required to drink colostrum in order to acquire protection (Balfour and Comline, 1962). Therefore, vaccination of pregnant cows could induce large amount of anti- HIV-1 antibodies in colostrum (Kramski et al., 2012).

Direct ELISAs were performed against autologous antigen (vaccine immunogens) for sixteen animals demonstrating high binding titres in sera and representing all five groups from phase 1 (Figure 3-3). Bovines from groups vaccinated with AD8 Unc gp140 and MW Unc gp140 presented the lowest endpoint concentrations (0.304 µg/ml, 0.216 µg/ml and 0.308 µg/ml for AD8 Unc 100, AD8 Unc 500 and MW Unc 500, respectively) showing strong binding, whereas cows from groups KNH1/BG505 SOSIP gp140 and PSC89 Unc gp140 showed divergent binding affinity (4.055 µg/ml v/s 0.753 µg/ml for KNH1/BG505 SOSIP 100, and 2.343 µg/ml v/s 1.245 µg/ml for PSC89 Unc 500). The result of colostrum binding was in agreement with binding profile of sera samples.

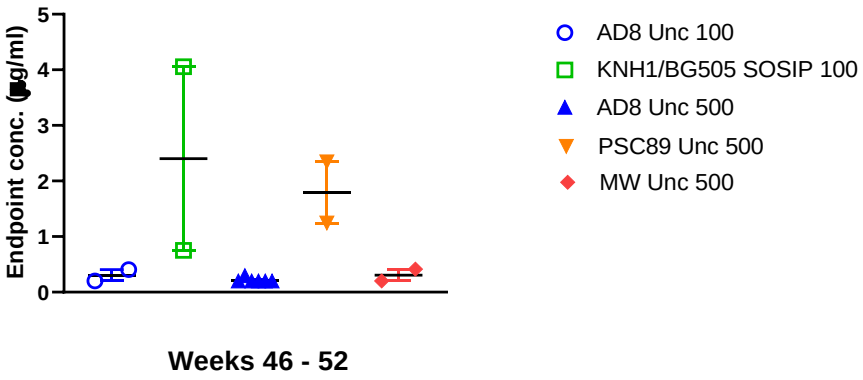


Figure 3-3: Autologous binding against vaccine immunogens by IgGs from colostrum. Binding curves were plotted by serial dilutions of IgGs, starting from 200 µg/ml, and a non-linear regression was calculated. A cut-off of two times the optical density (O.D.) of a non-immune sample was used to determine the endpoint concentration of samples. Groups are as follows: AD8 Unc 100 (blue open circles), KNH1/BG505 SOSIP 100 (green open squares), AD8 Unc 500 (blue closed triangles), PSC89 Unc 500 (orange closed triangles) or MW Unc 500 (red closed diamonds). Bars in each timepoint indicate average plus/minus SEM of the group.

Table 3-3: Average endpoint concentration of purified IgGs from colostrum samples. Binding was assessed with colostral milk collected between weeks 46 to 52. SEM values are indicated on the right of each value.

	AD8 Unc 100	KNH1/BG505 SOSIP 100	AD8 Unc 500	PSC89 Unc 500	MW Unc 500
Week	Average (µg/ml)	Average (µg/ml)	Average (µg/ml)	Average (µg/ml)	Average (µg/ml)
46 - 52	0.304 ± 0.104	2.404 ± 1.651	0.216 ± 0.016	1.793 ± 0.549	0.308 ± 0.108

Due to limitations in amount of available sera samples, further characterisation of Env binding for vaccinated cows was performed using purified IgGs from colostrum samples. As shown in figure 3-4, Env binding of bovine purified IgGs was assessed against AD8 Unc gp140, BG505 SOSIP.664 gp140, PSC89 Unc gp140 and MW Unc gp140. AD8 Unc gp140 groups reached the highest affinity to all tested Env proteins (average endpoint concentrations between 0.2 µg/ml and 0.657 µg/ml for AD8 Unc

100 group, figure 3-4 A, and between 0.2 µg/ml and 0.547 µg/ml for AD8 Unc 500 group, figure 3-4 B). MW Unc gp140 also induce IgGs with high affinity for AD8 Unc gp140 (endpoint concentrations between 0.2 µg/ml and 1.076 µg/ml) and BG505 SOSIP.664 gp140 (endpoint concentrations of 0.2 µg/ml) but not for PSC89 Unc gp140 (between 1.245 µg/ml and 2.343 µg/ml) (Figure 3-4 E). PSC89 Unc 500 group elicited Abs with high affinity against MW Unc gp140 (endpoint concentration of 0.2 µg/ml), but with lower affinity to AD8 Unc gp140, PSC89 Unc gp140 and BG505 SOSIP.664 gp140 (values between 0.762 µg/ml and 4.148 µg/ml against AD8 Unc gp140, and for BG505 SOSIP.664 gp140) (Figure 3-4 D). Interestingly, KNH1/BG505 SOSIP 100 group elicited Abs that bound better to both PSC89 Unc gp140 and MW Unc gp140 than to BG505 SOSIP.664 gp140 (compare average endpoint concentrations of 0.2 µg/ml for both PSC89 Unc gp140 and MW Unc gp140, against 2.404 µg/ml against BG505 SOSIP.664 gp140) (Figure 3-4 B). Overall, AD8 Unc gp140 generated Abs with the highest binding breadth, followed by MW Unc gp140. On the other hand, PSC89Unc gp140 and KNH1/BG505 SOSIP did not induce Abs with high potency and breadth in terms of binding.

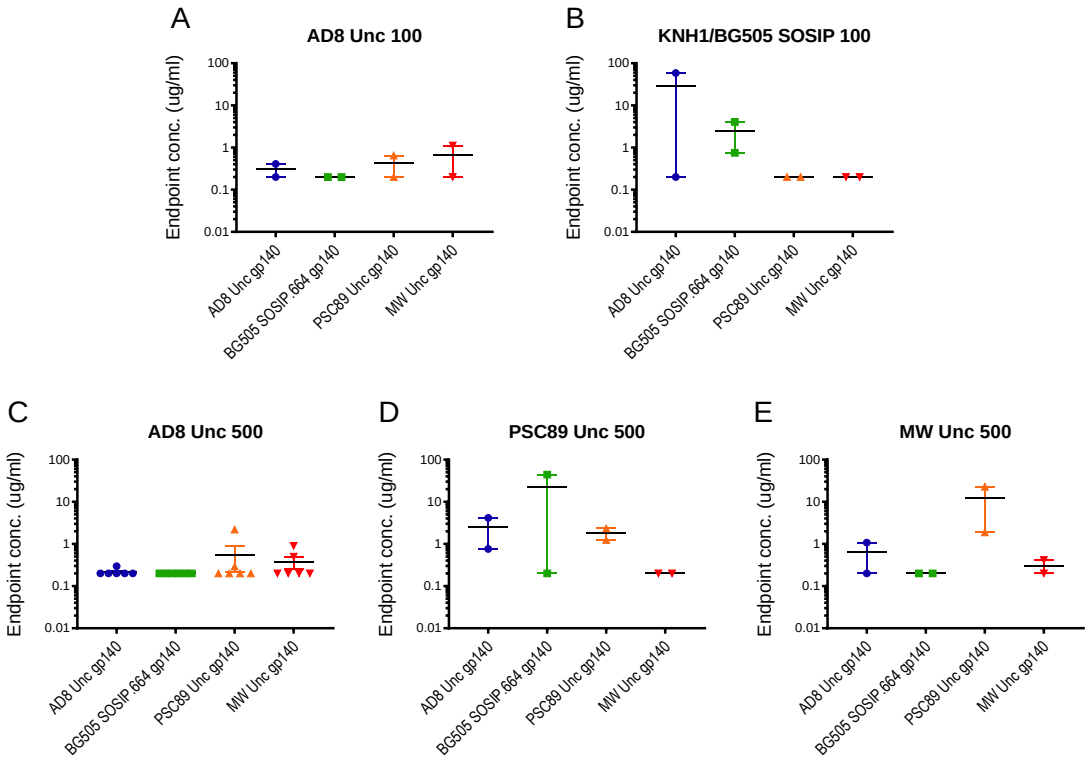


Figure 3-4: Heterologous binding against different vaccines by purified IgGs from colostrum. Measurements were performed in samples from 16 cows representing all five groups of vaccinated animals. Binding curves were plotted by serial dilutions of IgGs, starting from 200 µg/ml, and a non-linear regression was calculated. A cut-off of two times the optical density (O.D.) of a non-immune sample was used to determine the endpoint concentration of samples. Groups are as follows: A) AD8 Unc 100; B) KNH1/BG505 SOSIP 100, C) AD8 Unc 500, D) PSC89 Unc 500 or E) MW Unc 500. Antigens are noted at the bottom of each data group and indicated with different symbols: AD8 Unc gp140 as blue circles, BG505 SOSIP.664 gp140 as green squares, PSC89 Unc gp140 as orange triangles and MW Uncgp140 as red triangles. Bars in each timepoint indicate average plus/minus SEM of the group.

Table 3-4: Average values of endpoint concentration of purified IgGs from bovine colostrum. Samples represent weeks 46 – 52, and binding was assessed against different vaccines used in study, in order to measure heterologous binding. SEM values are indicated on the right of each value.

	AD8 Unc 100	KNH1/BG505 SOSIP 100	AD8 Unc 500	PSC89 Unc 500	MW Unc 500
gp140 antigen	Average (μg/ml)	Average (μg/ml)	Average (μg/ml)	Average (μg/ml)	Average (μg/ml)
AD8 Unc	0.3 ± 0.1	29.32 ± 29.12	0.22± 0.02	2.46 ± 1.7	0.64 ± 0.44
BG505 SOSIP.664	0.2 ± 0	2.4 ± 1.65	0.2 ± 0	22.21 ± 22.01	0.2 ± 0
PSC89 Unc	0.42 ± 0.22	0.2 ± 0	0.55 ± 0.33	1.8 ± 0.55	12.38 ± 10.5
MW Unc	0.66 ± 0.46	0.2 ± 0	0.37 ± 0.12	0.2 ± 0	0.31 ± 0.11

3.5 Neutralising activity of IgGs in sera and colostral milk during phase 1 and 2

After measuring autologous and heterologous binding and confirming the presence of Abs against HIV-1 Env vaccines, IgGs from sera and colostrum were evaluated for neutralising activity. As a negative control, sera from week 0 for each animal was carried during the experiments and a non-HIV-1 Env was used in order to measure off-target neutralisation (MuLV Env) (Figure 3-5).

Overall neutralising activity of antibodies improved after phase 1 of vaccination (week 33). At week 54, a decline was observed and in phase 2 of vaccination (week 59), neutralising activity was enhanced but did not reach to the activity recorded for week 33. Specifically, sera from groups MW Unc 500 and PSC89 Unc 500 presented mild neutralising activity at week 33 against tier 1 and tier 2 pseudoviruses including autologous neutralisation (ID_{50} against PSC89 of 35.82, and against MW of 11.56). For PSC89 Unc 500, however, neutralising activity was maintained only against tier 1A MN at week 54 and lost at week 59 (ID_{50} value at week 54 of 31.56) while for MW Unc 500, neutralising activity was only maintained against JR-CSF at both weeks 54 and 59 (ID_{50} values of 45.04 and 41.27, respectively). AD8 Unc gp140 induced antibodies with higher neutralising activity than other Unc gp140 Env vaccines, and both groups AD8

Unc 100 and AD8 Unc 500 presented higher ID_{50} values than PSC89 Unc 500 and MW Unc 500. At week 33, both AD8 Unc gp140 groups neutralised tier 1A pseudoviruses MN, SF162 and PSC89, and tier 2 BG505 and JR-CSF (ID_{50} values in same order of 86.57, 18.12, 69.62, 192.24 and 46.80 for AD8 Unc 100, and of 126.93, 73.23, 192.52, 27.21 and 41.16 for AD8 Unc 500), while at week 54 only neutralisation against MN and JR-CSF was carried out. At week 59, AD8 Unc 100 improved neutralisation against MN and JR-CSF but did not recover neutralising activity against SF162, PSC89 or BG505 (ID_{50} values for MN and JR-CSF of 304.98 and 30.75, respectively). On the other hand, AD8 Unc 500 recovered neutralising activity against SF162, PSC89 or BG505 at week 59, plus improved neutralisation against MN and BG505, while neutralisation against other pseudoviruses was less potent (improved ID_{50} values against MN and BG505 of 538.54 and 182.2, respectively). According to the results, increasing five times the amount of AD8 Unc gp140 improves the neutralising activity of sera antibodies, however neither group presented autologous neutralisation against AD8 pseudovirus.

KNH1/BG505 SOSIP 100 elicited broad neutralising antibodies against all tested pseudoviruses It is possible that the sensitivity of the two cell lines used in the assay (Cf2th for IgGs from colostrum and tested at week 33 (ID50 values between 38.16 against PSC89 and 69.71 against BG505) and it was the only group that could neutralise tier 2 pseudovirus AD8 at this timepoint (ID50 of 70.66). At week 54, neutralisation against BG505 and JR-CSF was carried over, whereas neutralisation against other pseudoviruses was lost. At week 59, neutralisation was recovered and even improved against MN, SF162, BG505, JRCSF and AD8 (ID50 values of 165.89, 136.16, 538.26, 68.65 and 133.77, respectively). Overall, KNH1/BG505 SOSIP gp140 showed to be the best immunogen to elicit neutralisation against the panel of pseudoviruses tested, in terms of potency and breadth.

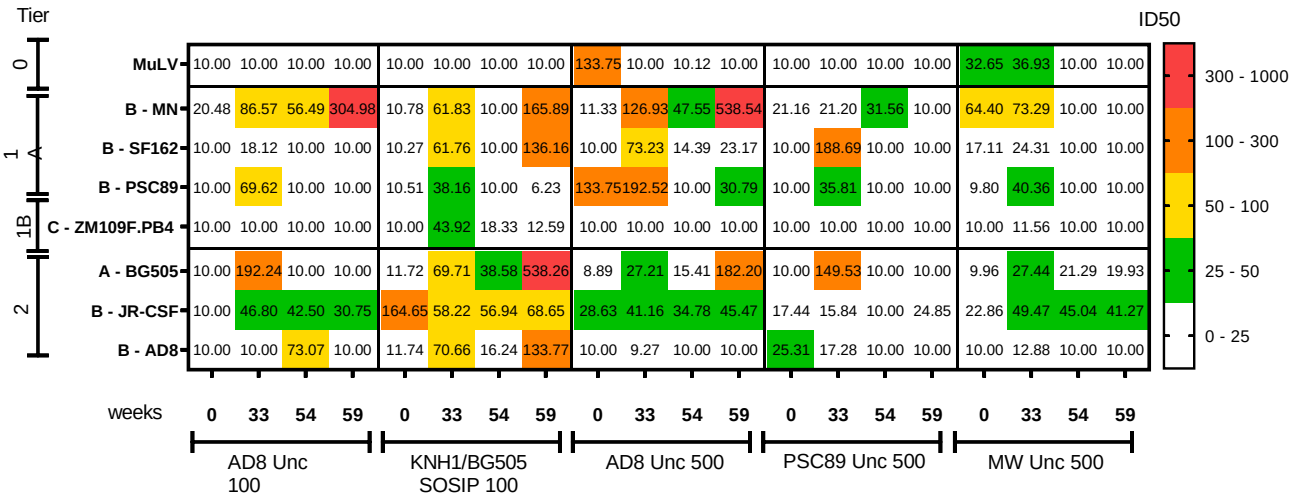


Figure 3-5: Neutralising activity measured in sera from vaccinated cows with different HIV-1 Envs. Neutralisation assays were performed with serial dilutions of sera from phase 1 (weeks 0 and 33) and phase 2 (weeks 54 and 59) of the vaccination course, against a panel of pseudoviruses from clades A, B and C and from tier 1a, 1b and 2. Luminescence levels were measured 48 hours post infection. As negative control, MuLV (Murine Leukemia Virus) Env was used. Neutralisation was calculated as percentage of inhibition from signal given by a positive control without antibodies, and ID50 values (dilution that reaches 50% of neutralisation) were calculated by plotting a non-linear curve over neutralisation curves. Heatmap shows average ID50 values from cows within a group against each pseudovirus. Scale shows no neutralisation from a value of ID50 below 25 (white values), to the highest neutralisation achieved at ID50 = 1000 (red values).

Similar experiments were performed using purified IgGs from colostrum. In this case, however, pseudoviruses were produced with a firefly luciferase reporter and virus-antibody mixture were used to infect Cf2th cells. Nevertheless, infection and further viral replication is also directly proportional to the amount of luciferase expressed (Figure 3-6).

Due to the high amount of total IgGs in sera, overall neutralisation was weaker in colostrum samples compared to sera samples. It is possible that the sensitivity of the two cell lines used in the assay (Cf2th for IgGs from colostrum and TZMbl cell for sera samples) differs as well, making measurement of neutralizing activity on Cf2th cells harder than on TZMbl cells. Such sensitivity differences can be related to the method by which each produce the luciferase reporter: while TZMbl cells have an integrated copy of an LTR-driven luciferase reporter, which responds to Tat produced in infected cells, Cf2th cells do not carry an LTR reporter, and instead express the luciferase reporter included into the pseudotyped lentivector genome. It could also be due to the depletion of certain components in colostral milk, which are still present in serum, that aid neutralising activity. Nevertheless, neutralisation observed with purified IgGs from colostrum agreed with neutralising activity of sera samples at week 33. Cows vaccinated with AD8 Unc gp140 (100 µg and 500 µg) induced neutralising antibodies against MN with similar values (IC_{50} of 82.5 µg/ml for AD8 Unc 100 and of 83.0 µg/ml for AD8 Unc 500). PSC89 Unc 500 group also presented neutralising antibodies against MN (IC_{50} of 308.5 µg/ml) and JR-CSF (IC_{50} of 398.5 µg/ml), while MW Unc 500 group did not show neutralisation against any pseudovirus. Again, KNH1/BG505 SOSIP 100 group showed the broadest neutralisation, with three of seven pseudoviruses being neutralised (tier 1 MN, and tier 2 BG505 and AD8) and was the only group that could neutralise AD8 pseudovirus (IC_{50} of 344.6 µg/ml). Together with the data obtained from sera after phase 1 and phase 2 of the vaccination course, our data suggest the combination of KNH1 SOSIP gp140 and BG505 SOSIP gp140 elicited antibodies with the highest and broadest neutralising activity in cows.

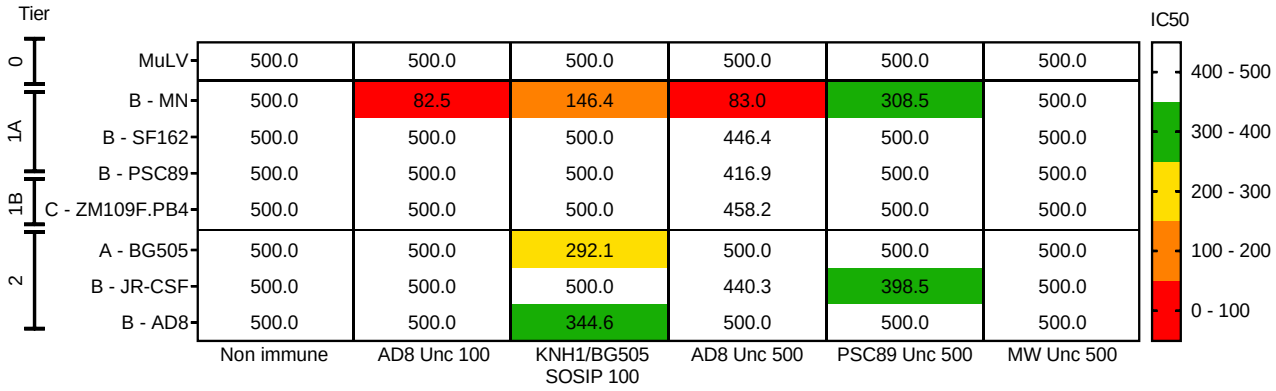


Figure 3-6: Neutralising activity of purified IgGs from colostral milk from vaccinated cows with different HIV-1 Envs. Assay was performed with serial dilutions against a panel of pseudoviruses from clades A, B and C and from tier 1a, 1b and 2. Luminescence levels were measured 48 hours post infection. As negative control, MuLV (Murine Leukemia Virus) Env was used on pseudoviruses. Neutralisation was calculated as percentage of inhibition from signal given by a positive control without antibodies, and IC50 values (concentration in µg/ml that reaches 50% of neutralisation) were calculated by plotting a non-linear curve over neutralisation curves. Heatmap shows average IC50 values from cows within a group against each pseudovirus. Scale shows no neutralisation from a value of IC50 = 500 (white values), to the highest neutralisation achieved at IC50 near zero (red values).

3.6 Evaluation of the efficiency of phase 2 vaccination

Phase 2 of the vaccination scheme started between two and eight weeks after calving and colostrum collection. Ten cows representing all five initial groups (cow 2005 from AD8 Unc 100 group, cows 609 and 617 from KNH1/BG505 SOSIP 100 group, cows 35, 537, 8434 and 9533 from AD8 Unc 500 group, cow 9506 from PCS89 Unc 500 group and cows 5586 and 9244 from MW Unc 500 group) that presented high autologous binding titres, were selected for phase 2 to evaluate whether extending vaccination regimen with either autologous Env or different immunogen (in terms of structure or clade) could improve the neutralising activity of bovine antibodies.

As a control, cows 609 and 617 from KNH1/BG505 SOSIP 100 group were revaccinated again with BG505 SOSIP gp140. In addition, cow 2005 which initially was vaccinated with 100ug of AD8 Unc gp140, was revaccinated with the same antigen as well. In other cows originally vaccinated with AD8 Unc gp140, the antigen was changed for AD8 SOSIP gp140 (cow 8434), BG505 Unc gp140 (cows 537 and 9533) and or BG505 SOSIP gp140 (cow 35), which allowed to test directly whether changing from the second-best immunogen to the best one improves neutralisation profile, or by any variant of these two immunogens (AD8 Unc gp140 and BG505 SOSIP gp140). Finally, Cows in PSC89 Unc 500 and MW Unc 500 groups were revaccinated with BG505 SOSIP gp140 (cow 9244) or AD8 SOSIP gp140 (cow 5586). In order to evaluate whether these changes could improve neutralisation potency and breadth following re-vaccination, we compared the ID50 values at weeks 54 and 59.

Further vaccination of AD8 Unc 100 group with autologous immunogen decreased by 90% autologous neutralisation (0.1-fold change for AD8) and 30% neutralisation against JRCSF (0.7-fold change), while showing only an improvement against MN (5.4-fold change). On the other hand, revaccination of KNH1/BG505 SOSIP 100 group with BG505 SOSIP gp140 improved drastically both autologous and heterologous neutralisation (fold changes of 16.6, 13.6, 11.3, 2 and 6.2 against MN, SF162, BG505, JR-CSF and AD8 pseudoviruses, respectively), while slightly decreased neutralising activity against PSC89 and ZM109F.PB4, with fold changes of 0.6 and 0.8, respectively (Figure 3-7).

Modifications in immunogen structure from AD8 Unc gp140 in phase1 to AD8 SOSIP gp140 in phase 2 as well as using different clade (BG505 Unc gp140) could improve heterologous neutralisation in AD8 Unc gp140 group. Revaccination with BG505 Unc gp140 in phase 2 for this group of animals showed the highest increment of ID50 values against 5 pseudoviruses: MN, SF162, PSC89, BG505 and JR-CSF (fold changes of 19.6, 1.3, 4.1, 33.6 and 3.1). On the contrary, change of both structure and

subtype (BG505 SOSIP gp140) only improved tier-1 neutralisation against MN (fold change of 4.9) and SF162 (fold change of 1.1), whereas neutralisation against JR-CSF was dropped (fold change of 0.3). Moreover, improvement in neutralisation in MW Unc 500 group was observed only following re-vaccination with AD8 SOSIP gp140 (fold changes of 3 and 5.4 against BG505 and JR-CSF, respectively) whereas a boost with BG505 SOSIP gp140 reduced neutralising activity against the same pseudoviruses (fold changes of 0.3 and 0.4 for BG505 and JR-CSF, respectively). Altogether, our results suggest that a drastic change in immunogen during boosts does not help improving neutralisation, which could be due to low engagement of plasma cells, previously activated with one vaccine and thus cannot undergo affinity maturation.

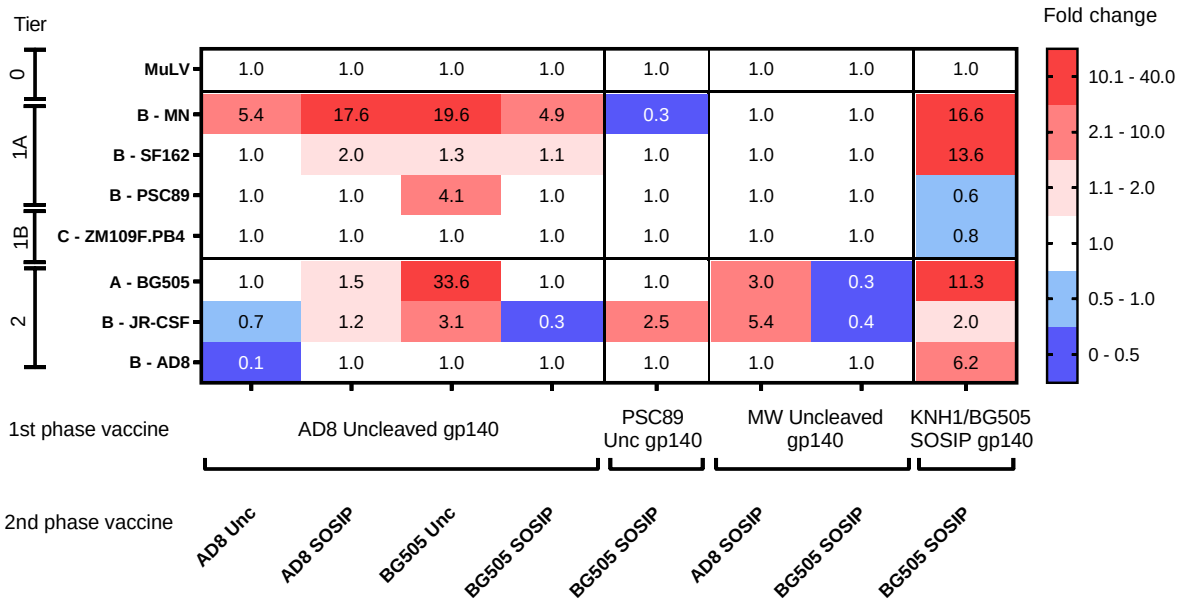


Figure 3-7: Effects of changes in immunogen on neutralising activity (phase 2). Difference between ID50s at weeks 59 and 54 were calculated in terms of fold-change and arranged in a heat map, where increased ID50 are indicated in red (above 1) and decreased ID50 is indicated in blue (below 1).

3.7 Neutralising potency and breath in vaccinated cows

To investigate how vaccination regimen impacted on anti-HIV neutralisation potency and breadth, samples collected from cows that underwent both phase 1 and 2 of vaccination scheme were analysed. As shown in figure 3-8, at week 0 samples showed very low neutralisation background while the highest breadth was achieved after phase 1 (week 33). 6 out of 8 groups showed high neutralising breadth, with KNH1/BG505 SOSIP gp140 – BG505 SOSIP gp140 group presenting the highest breadth value (n = 6, 85.7%). The lowest breadth was observed in group AD8 Unc that was revaccinated with AD8 Unc or BG505 Unc immunogens in phase 2. Due to calving and termination of pregnancy, neutralisation activity dropped significantly in all groups (except AD8 Unc gp140 – AD8 Unc gp140) at week 54. In phase 2 of vaccination (week 59), neutralisation potency increased compared to week 33 but the breadth could not be recovered, and the values could not reach to the breadth obtained at week 33. The highest potency was achieved for vaccination group of KNH1/BG505 SOSIP as well as AD8 Unc gp140 group that was revaccinated with AD8 SOSIP or BG505 SOSIP in phase 2 (ID50 values between 120 and 160).

In conclusion, results showed that maximum breadth was achieved at week 33, (phase 1 of the vaccination), whereas maximum potency was achieved at week 59, (phase 2). It is important to notice that by week 59, high potency was achieved but did not reach the same neutralising breadth observed at week 33. Additionally, among tested vaccines, AD8 Unc gp140 and KNH1 /BG505 SOSIP gp140 played critical roles in phase 1 to elicit antibodies with high breadth, while AD8 SOSIP gp140, BG505 Unc gp140 and BG505 SOSIP gp140 were involved in the induction of high potency during phase 2.

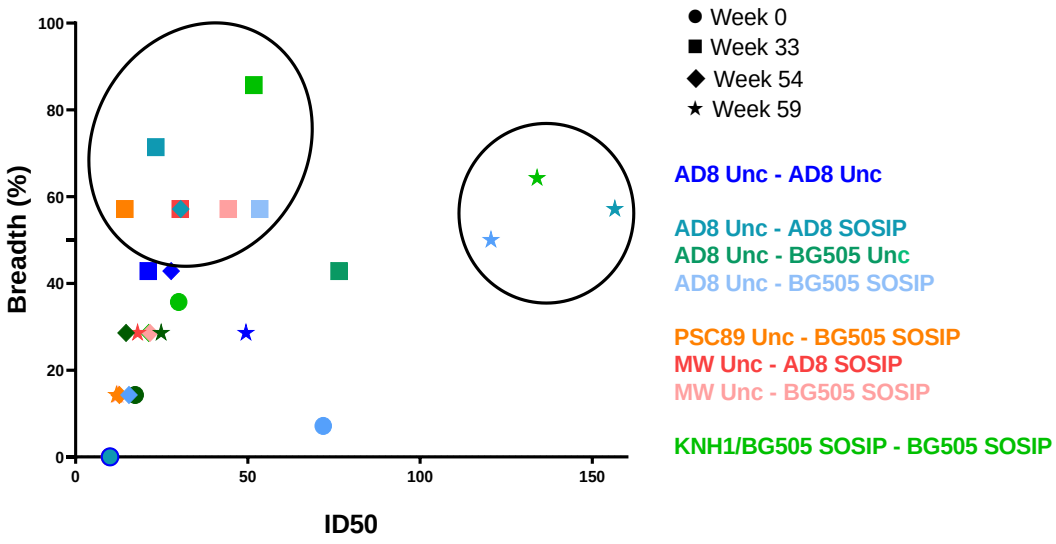


Figure 3-8: Analysis of neutralising breadth versus potency in cows vaccinated with different Env vaccines. Average ID50 and percentage of neutralised viruses (100% = 7 pseudoviruses) were calculated for each vaccinated groups that received different combination of vaccines in phase 1 and 2. Symbols indicate different timepoints (circles: week 0; squares: week 33; diamonds: week 54; stars: week 59) whereas colours indicate vaccine immunogens.

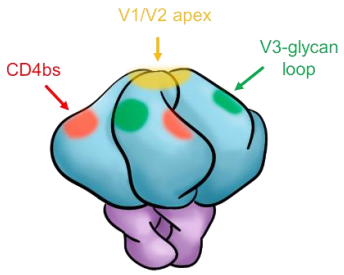
3.8 Bovine antibodies targeting neutralising epitopes on HIV-1 Env

In order to investigate differences in specificity in antibodies elicited against AD8 Unc gp140 and KNH1/BG505 SOSIP gp140, a competition ELISA was performed against human broadly neutralising antibodies. One possibility is that structural differences between these two HIV Env immunogens would change epitopes targeted by both antibody mixtures. Another possibility is that antibodies from both groups target the same epitopes, but KNH1/BG505 SOSIP gp140 elicited antibodies with higher or broader affinity.

First, an assessment of neutralising epitopes exposed within AD8 Unc gp140 and BG505 SOSIP gp140 was made. In order to do this, an ELISA with a list of different BnAbs targeting specific epitopes within HIV-1 Env was performed (table 3-5).

Table 3-5: List of broadly neutralising antibodies used for epitope characterisation. Antibodies targeting CD4 binding site (CD4bs), the V3-glycan loop and the V1/V2 apex were used to assess the presence of neutralising epitopes

Epitope	BnAb
CD4bs	VRC01
	B12
V3-glycan	PGT121
V1/V2 apex	PGT145



To choose human BnAbs for competition ELISA, a panel of BnAbs were tested in ELISA assay against BG505 SOSIP gp140 and AD8 Unc gp140. The result showed that both antigens bound CD4bs mAb VRC01 and V3-glycan loop mAb PGT121. Differences of binding were found with CD4bs mAb B12, which bound only to AD8 Unc gp140. In addition, V1/V2 apex mAb (PGT145) bound only to BG505 SOSIP gp140 (figure 3-9), but not to AD8 Unc gp140. This implies that CD4bs in both proteins could be spatially different, and that apex epitopes such as the V1/V2 apex were present in BG505 SOSIP gp140, but not in AD8 Unc gp140.

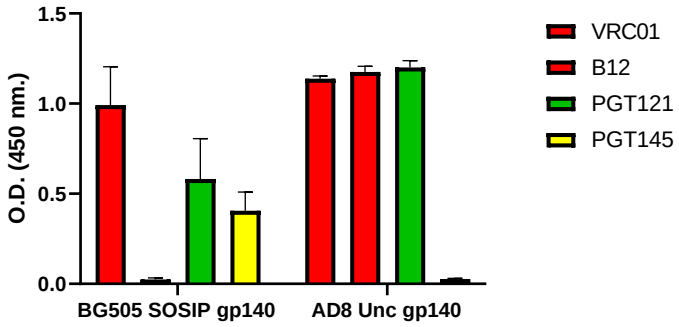


Figure 3-9: Binding of BnAbs to BG505 SOSIP gp140 and AD8 Unc gp140 vaccines. In order to assess the presence of neutralising epitopes, ELISAs against BG505 SOSIP gp140 and AD8 Unc gp140 were performed with BnAbs VRC01, B12, PGT121, and PGT145, which target the epitopes CD4 binding site (red), V3-glycan loop (green) and V1/V2 apex (yellow).

When competition ELISAs were performed, results showed that KNH1/BG505 SOSIP vaccination induced Abs competing with PGT145 antibody (reduction of AUC from 88.9 to 53.89) demonstrating that this group of animals induced Abs against V1/V2 apex epitope or its vicinity. No significant competition against VRC01 or PGT121 was achieved, indicating that probably the titre of bovine Abs targeting these two epitopes was not enough to generate competition against mentioned human BnAbs. (figure 3-10 A). On the other hand, AD8 Unc gp140 vaccine elicited IgGs that could compete against both VRC01 (AUC decrease from 52.09 to 39.6) and B12 (AUC decrease from 64.27 to 46.4), which targets CD4bs on Env (Figure 3-10 B). Overall, both vaccines induced bovine antibodies against well-known BnAb epitopes: KNH1/BG505 SOSIP gp140 elicited antibodies mainly against V1/V2 apex, whereas AD8 Unc gp140 elicited antibodies targeting CD4bs.

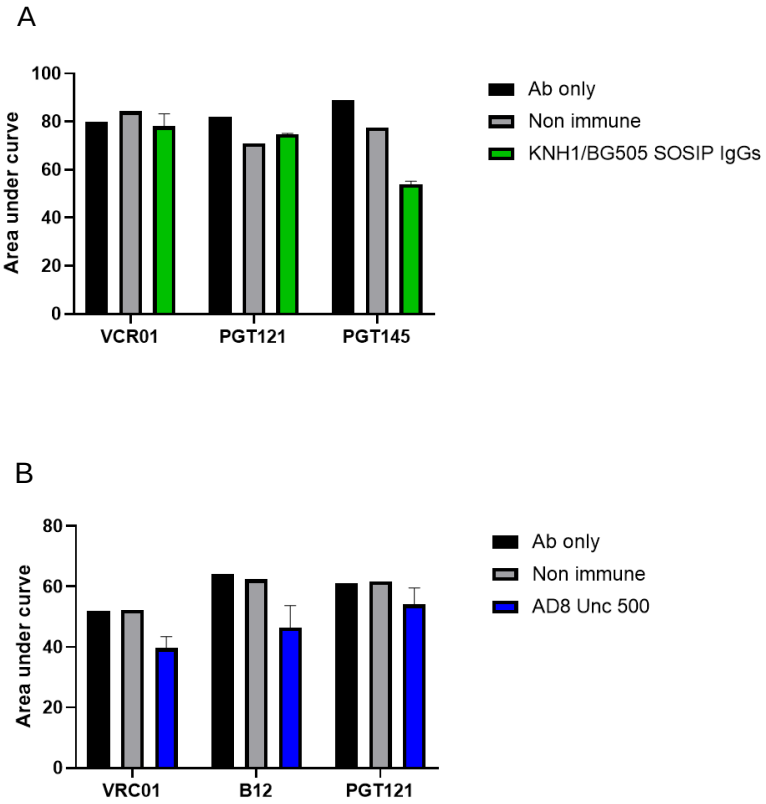


Figure 3-10: Epitope characterisation of polyclonal IgGs from KNH1/BG505 SOSIP 100 and AD8 Unc 500 groups. Competition ELISAs were performed in order to block binding of BnAbs VRC01, b12, PGT121 and PGT145 by previously binding polyclonal IgGs from vaccinated cows. Binding curves were plotted and area under each curve (AUC) was calculated using GraphPad Prism v7. Green bars show decrease of binding by IgGs from KNH/BG505 SOSIP 100 group, whereas blue bars show decrease by IgGs from AD8 Unc 500 group. In both cases, an average of AUC was calculated, with error bars showing SEM from the whole group (Results from n = 3 experiments).

3.9 Discussion

Here we have compared, for the first time, different HIV-1 Env vaccines in their capacity to elicit neutralising Abs in cows. The results of this chapter agree with data from previous vaccinations using HIV-1 Env trimers (Kramski et al., 2017; Heydarchi et

al., 2016; Sok et al., 2017). As mentioned previously, the average CDRH3 length in bovines fit around the same lengths observed in human BnAbs against Env (reviewed in Heydarchi, Salazar-Quiroz and Purcell, 2016), which are unusual for humans (between 8 and 16 residues). This may be one of the reasons behind the rapid elicitation of BnAbs (few weeks) observed in cows, while on the contrary, it takes years in humans to induce such Abs. Whether the neutralising responses observed in this vaccination study or in previous ones were only due to ultra-long CDRH3 Abs or also due to average-size CDRH3s is a feature needing to be addressed. Considering the potent anti-HIV-1 neutralising response that was observed only at week 33 (phase 1) post-vaccination in all vaccinated groups, it is possible that an effective immune response against HIV-1 Env requires lower levels of affinity maturation in cows compared with humans (Sok et al., 2017). Unfortunately, due to the different number of animals in each vaccination group, we were unable to perform statistical analysis to check how significantly different the neutralising profiles were.

As shown in SPR experiments with BnAbs VRC01, B12, PG16 and PGT145 (Yasmeen et al., 2014; Castillo-Menendez et al., 2018; 2019; Stadtmueller et al., 2018), SOSIP trimers resemble more native Env structure than Unc trimers. Nevertheless, bovines elicited Abs with heterologous neutralisation by using both modifications in Env trimeric structures in different studies (Kramski et al., 2012; Heydarchi et al., 2016; Sok et al., 2017). In this study, we also showed that both AD8 Unc gp140 and KNH1/BG505 SOSIP were able to induce heterologous BnAbs but the latter vaccine induced broader and more potent responses. These two vaccines induced antibodies targeting different epitopes: Abs from AD8 Unc groups targeted CD4bs, whereas Abs in KNH1/BG505 SOSIP 100 group targeted the V1/V2 apex.

As shown in previous studies, cleavage of gp160 reduces binding of antibodies to HIV-1 Env compared to Unc trimers (Pancera and Wyatt, 2005; Li et al., 2012). Unc trimers present more flexibility, which increases the availability of epitopes within Env (Haim,

Salas and Sodroski, 2013; Schiffner et al., 2013). However, uncleaved trimers bind to both neutralising and non-neutralising antibodies with similar affinity (Si et al., 2003; Pancera and Wyatt, 2005; Chakrabarti et al., 2011) and further investigation is required to estimate what portion of CD4bs Abs raised in AD8 Unc vaccination are neutralising or non-neutralising. It is possible that epitopes such as CD4bs are more available in uncleaved trimers than in cleaved trimers such as SOSIP-modified trimers, although the work from Sok et al., in 2017 showed that BG505 SOSIP gp140 induced a strong response against CD4bs. Therefore, differences observed in our study could be related to the initial use of KNH1 SOSIP gp140, which could have induced an antibody response against V1/V2 loop epitopes, unlike BG505 SOSIP gp140. The absence of competition for both epitopes, however, cannot discard presence of antibodies targeting such epitopes. For instance, previous vaccination of cows with BG505 SOSIP gp140 allowed the isolation of a CD4bs antibody, Nc-Cow1 (Sok et al., 2017). Additionally, a family of mAbs with high neutralising activity has been isolated from one of the animals from KNH1/BG505 SOSIP 100 group, they all compete with CD4bs antibodies and do not bind to Envs with mutations in the CD4bs epitope (Heydarchi et al., in preparation). Additionally, presence of V1/V2 apex binding antibodies in KNH1/BG505 SOSIP vaccination is in concordance with previous studies, showing that apex epitopes are present in SOSIP-modified trimers, (where antibodies like PG16, PG9 and PGT145 bound to BG505 SOSIP gp140), whereas absent in BG505 Unc gp140 (Yasmeen et al., 2014).

While SOSIP gp140 is the closest structure to native HIV-1 Env, it exposes the first heptad-repeat (HR1) in gp41 ectodomain with a different mode compared with native trimers (Castillo-Menendez et al., 2019). Uncleaved trimers, also presents V3-glycan loop and CD4bs epitopes slightly different to native trimers as indicated by cross-linking/mass spectrometry experiments (Castillo-Menendez, 2018). These structural

differences could affect the profile of Abs elicited by both vaccines which in addition to clade difference could vary the Abs elicited in our vaccinated animals.

Differences in clade can affect the Ab response against HIV-1 Env and present a bias in epitopes targeted. For instance. clade B Envs tend to generate CD4bs antibodies, whereas non-clade B Envs induce antibodies against the V2 loop (Rusert et al., 2016), which correlates with our results. In our study, AD8, PSC89 and MW presented the same modification, but they elicit different antibody responses. A sequence analysis of the proteins produced by plasmids pN1- AD8-140AgeI, pN1-PSC89.51-140AgeI and pN1-MW-140 (figure 3-11) showed differences above 50% in all variable regions (except V3), the constant region C5 and the heptad repeat 2 (HR2). Specifically, residues at positions 279 and 283 from the D-loop at C2, 461 and 467 from V5, and 471, 474 and 476 from C5 are different between sequences. These regions are contact residues for CD4 receptor and are important for binding of CD4bs antibodies VRC01 and B12 (Li et al., 2011). Our results could explain differences observed in the elicitation of neutralising antibodies targeting CD4bs, which could be due to the architecture of the CD4bs in each Env vaccine, with AD8 presenting the highest advantage.

Taking into account structural and clade differences present in the vaccines used for this study, changes in structure and clade improved the neutralising response of antibodies from cows initially vaccinated with AD8 Unc gp140. Revaccination (phase 2) with AD8 SOSIP gp140 or BG505 Unc gp140, but not with BG505 SOSIP gp140, showed an improvement in AD8 Unc groups. SHM is needed in order to improve neutralisation potency but it is also needed to generate breadth, since affinity for conserved epitopes is refined and selected (Sok et al., 2013). Failure in the improvement of both potency and breadth by priming with AD8 Unc gp140 (phase 1) and boosting with BG505 SOSIP gp140 (phase 2) could be due to simultaneous changes in immunogen structural and clade.

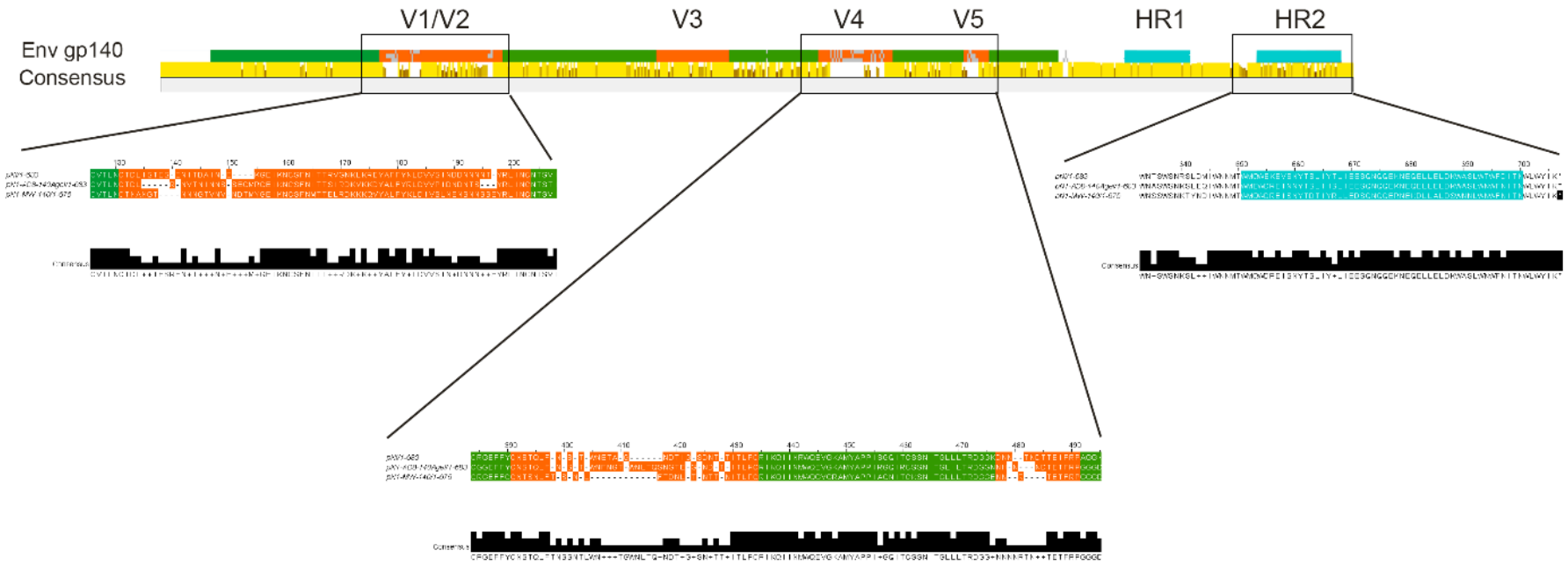


Figure 3-11: Sequence analysis of uncleaved gp140 Envs used in vaccination study. A sequence alignment was performed with the protein sequence from plasmids pN1-AD8-140AgeI, pN1-PSC89.51-140AgeI and pN1-MW-140. Variable regions are indicated on top of consensus sequence and within sequence in orange, constant regions are indicated in green, and heptad repeats of gp41 ectodomain are indicated in cyan. Boxes zoom in regions where differences below 50% consensus were found (also indicated in yellow in full sequence and in black in zoom boxes). Alignment was generated by MUSCLE (Edgar et al., 2014) and visualised through Jalview version 1.8.3.

When new immunogens are introduced in a vaccination study, affinity maturation in germinal centers will depend on binding between new BCRs formed in germinal centres by the first immunogen and Tfhs activated with the new immunogen. It is possible that for AD8 Unc gp140 / BG505 SOSIP gp140 vaccination, boosting with BG505 SOSIP gp140 might not have been sufficiently efficient to promote survival of B cells (Liu et al., 1989). In contrast, boosts with AD8 SOSIP gp140 or BG505 Unc gp140 could have activated Tfh cells with better binding for new BCRs, most likely due to their resemblance of clade and structure, respectively, with AD8 Unc gp140. As evidence, previous studies using sequential immunisation with different immunogens was performed using identical Env clade until last stages of vaccination, improving neutralising breadth to different extents with phylogenetically close immunogens (Malherbe et al., 2011; Escolano et al., 2016; Klasse et al., 2016; Mohan et al., 2018; Torrents de la Pena et al., 2018).

In summary, KNH1/BG505 SOSIP gp140 was the most efficient vaccine in inducing low Env binding Abs as well as broad and potent BnAbs. On the contrary, AD8 Unc gp140 induced strong Env binding Abs but with mild neutralising activity. PSC89 Unc 500 and MW Unc 500 groups presented low heterologous binding and low neutralisation, which might be due to differences in key residues for contact of CD4bs Abs such as VRC01 and B12. Finally, changes in structure or clade, but not both simultaneously, improved HIV-1 neutralisation potency but not breadth. In conclusion, it is apparent that usage of SOSIP-modified trimers played the critical role in increasing neutralisation profile of Abs elicited, whereas Unc trimers elicited both neutralising and non-neutralising Abs, decreasing its effectiveness into blocking viral entry and infection.

Assessment of Fc-effector functions mediated by anti-HIV bovine antibodies elicited in vaccinated cows

4.1 Background

An effective HIV-1 vaccine can confer protection not only through elicitation of neutralising antibodies, but also through activation of Fc-effector functions like ADCC, ADCP and CDC, which can eliminate viral particles and infected cells (Gach et al., 2018; Tay et al., 2017; Ackerman et al., 2012). Results from the RV144 vaccine trial, using a combination of a canarypox particle expressing Gag and Env and recombinant gp120 Env in a prime/boost strategy (Rerks-Ngarm et al., 2009), showed failure on inducing broadly neutralising antibodies while antibody-mediated Fc-effector functions could result in the protective immunity observed. Sixty percent of protection achieved during the first year of study (Robb et al., 2012) was mediated by CD4+ T cells and B cells producing high antibody titres against V1/V2 loop epitopes on HIV-1 Env (Karasavvas et al., 2012; Zolla-Pazner et al., 2014), which were then linked to mediate ADCC (Bonsignori et al., 2012), ADCP (Chung et al., 2015) and CDC (Perez et al, 2017). Ab dependent Fc-effector functions in the HIV-1 infected individuals have shown to control viremia (Ackerman et al., 2016; Madhavi et al., 2017; Mwimanzi et al., 2013; Toyoda et al., 2015, Alsahafi et al., 2015) and reduce risk of viral transmission in humans (Mabuka et al., 2012; Milligan et al., 2015). Studies in non-human primates have also demonstrated the importance of antibody-dependent Fc effector functions in mediating protection (Bradley et al., 2017; Fouts et al., 2015; Gomez-Roman et al., 2005; Xiao et al., 2012).

An important feature of Fc-effector functions is that Abs mediating such functions do not necessarily need to be neutralising. For instance, ADCC against HIV-1 can be elicited not only by BnAbs but also with non-neutralising Abs targeting the co-receptor binding site (Co-RBS) and the cluster A epitope (Bruel et al., 2017; Pollara et al., 2013; Richard et al., 2018; Ding et al., 2016; Richard et al., 2015, 2016, 2017). Unlike BnAbs that require blockage of receptor binding during infection or impeding conformational changes, activation of Fc-effector functions only requires binding to their cognate

antigen. To commence an antibody dependent effector function, it is essential the presence of immune complexes (ICs), formed by several antibodies accumulated in close proximity in contact with the antigen. Formation of ICs allows either the crosslinking of Fc receptors (FcRs) bound to 2 antibodies' Fc-regions, or binding of one C1q molecule, part of the complement cascade, to five IgGs' Fc-region or one IgM Fc-region (Pierson et al., 2007; Lux et al., 2013; Wang et al., 2016; Peschke et al., 2017; Robinett et al., 2018). For FcRs, ICs must promote clustering of receptors that will later cross-activate Src family kinases and finally Syk kinase in the intracellular portion, initiating signalling cascades (Kimura et al., 1996). For C1q, on the other hand, ICs need to interact with several globular domains in C1q, allowing the autoactivation of serine proteases C1r and C1s which will later activate the complement cascade (Thielens et al., 2017).

We previously showed that anti-HIV bovine Abs could mediate ADCC by human monocytes (Kramski et al., 2012b). Since the sequence of the Abs CH2 domain in cow and human is similar and both domains share important contact residues for binding to FcRs and C1q, we aimed to investigate whether anti-HIV bovine antibodies could mediate other antibody-dependent effector functions in human cells. We first assessed interaction of bovine IgGs with FcRs and based on those results, further functional assays were performed. As activation of antibody dependent functional assays initially require binding of bovine IgGs to HIV Env, we hypothesised that vaccine groups which elicited high titre of Env binding IgGs, but not necessarily high titre of neutralising IgGs, will induce higher antibody dependent effector functions.

Based on the facts that activation of these functions requires the formation of ICs along with results from chapter 3, the hypothesis in this chapter is that Fc-effector functions are dependent of the binding titres of a vaccine and its structure. As such, AD8 Unc gp140 that presented the highest and broadest binding titres is suspected to

elicit Fc-effector functions. Additionally, since V1/V2 apex binding IgGs were able to induce effective ADCC and CDC responses in the RV144 trial, we aimed to investigate whether KNH1/BG505 SOSIP 100 group which also produced Abs against such epitope was able to induce the mentioned Ab dependent effector functions. Due to the lack of sufficient sera samples, the analysis of this chapter was focused only on purified IgGs from colostrum samples collected after phase 1 of vaccination and no assessment was performed on the effect of vaccines used in phase 2 in inducing Ab dependent effector functions.

4.2 Binding of purified IgGs from bovine colostrum to recombinant human Fcγ-receptors

The first step into evaluating Fc-effector functions was to confirm binding of bovine IgGs to human FcRs. To do this, we used a novel FcγR assay established by Dr. Bruce Wines at the Burnet Institute, using recombinant dimeric FcγR ectodomains as a probe of the FcR function (Wines et al., 2016). In order to replicate the events involved into activation of immune cells, recombinant FcRs were produced in a dimeric form; this way, engagement of IgG's Fc portions will be favoured if there are two molecules bound in close proximity, as found in ICs.

As shown in figure 4-1, among the tested human FcγRs, bovine IgGs could only bind to FcγRIIa, allotype R131. From all the groups tested, AD8 Unc 500 showed the highest binding to this receptor (average O.D. of 1.021), followed by AD8 Unc 100 and MW Unc 500 (average O.D. of 0.436 and 0.433, respectively). However, no binding was observed for KNH1/BG505 SOSIP 100 and PSC89 Unc 500 groups (average O.D. values of 0.11 and 0.249, respectively). Our findings suggest that Fc-effector functions reported from anti-HIV bovine IgGs (Kramski et al., 2012) might have been

mediated by FcγRIIa, leading to phagocytosis instead of ADCC, as indicated in the article.

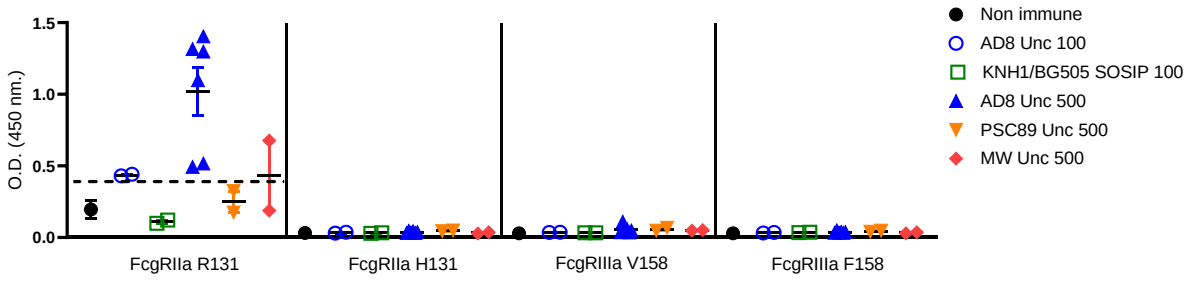


Figure 4-1: Assessment of binding of FcRs to IgGs bound to autologous Env vaccines. HIV-1 Env trimers used in the vaccination were bound to ELISA plates and 500 µg/ml of purified IgGs from colostrum were incubated for 2 hrs at RT. After washing unbound antibody, biotinylated recombinant FcR dimers were added and incubated for 1 hr at 37°C. Detection of dimers was done by using streptavidin-HRP-conjugated molecule. Data points show binding of IgG from 16 animals representing one of the 5 groups from phase 1: AD8 Unc 100 (blue open circles), KNH1/BG505 SOSIP 100 (green open squares), AD8 Unc 500 (blue closed triangles), PSC89 Unc 500 (orange closed triangles) or MW Unc 500 (red closed diamonds). Dotted line indicates threshold signal which corresponds to double the signal observed in negative control. Bars in each condition indicate average plus/minus SEM of the group.

Together with results from section 3.4, a negative correlation was observed between binding to antigen and FcγRIIa R131, with values from AD8 Unc 500 group clustered in the top left corner of the graph showing high binding to both antigen and receptor (figure 4-2). On the contrary, values from groups such as KNH1/BG505 SOSIP 100 and PSC89 Unc gp140 clustered in the bottom right corner, indicating high endpoint titre to HIV Env (low binding) and low binding to FcγRIIa R131. Overall, these results demonstrate that bovine IgGs bind to human FcγRIIa R131 in an affinity-dependent

manner, with IgGs from AD8 Unc 500 group showing the highest binding to this receptor, followed by AD8 Unc 100 and MW Unc 500.

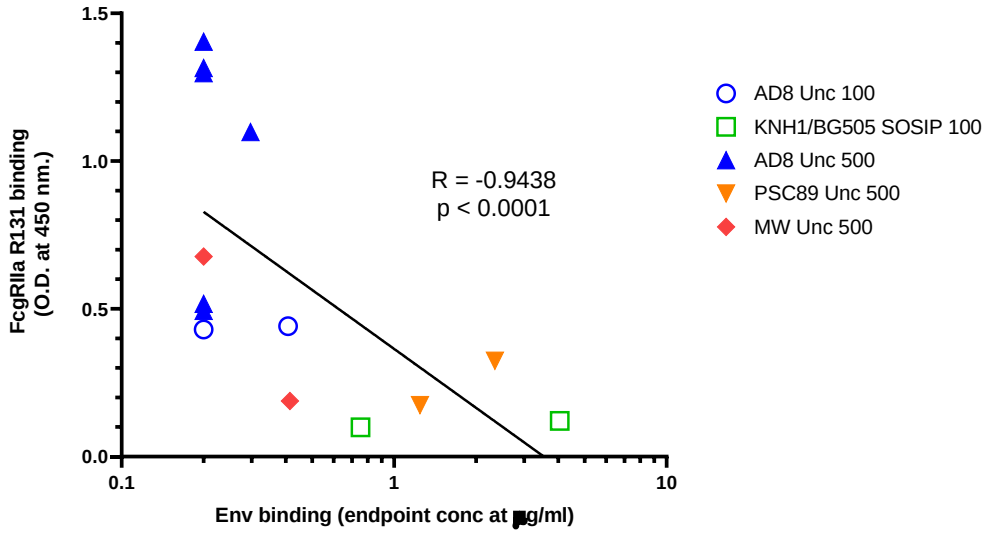


Figure 4-2: Pearson correlation between binding against cognate antigen and binding to FcRs. Endpoint concentrations (µg/ml) of IgGs from colostrum of 16 animals were plotted against the optical density (O.D.) of FcγRIIa R131 at 450 nm. for the same samples. Animals selected represent one of 5 groups in phase 1 of study: AD8 Unc 100 (blue open circles), KNH1/BG505 SOSIP 100 (green open squares), AD8 Unc 500 (blue closed triangles), PSC89 Unc 500 (orange closed triangles) or MW Unc 500 (red closed diamonds). Linear regression was calculated, as shown in the graph, with an R of -0.9438 (p value < 0.0001).

4.3 IgGs from AD8 Unc 500 and KNH1/BG505 SOSIP 100 groups induced phagocytosis

Activation of phagocytosis depends on binding of IgGs to FcγRIIa in monocytes, macrophages and dendritic cells which results in elimination of infected targets and free virions (Karupiah et al., 1996; Nagl et al., 2002; Hashimoto et al., 2007; Nainu et al., 2017). Because of the high affinity interaction of IgGs from AD8 Unc 500 group with human FcγRIIa, an adapted ADP-SHIP assay was performed to investigate the

induction of phagocytosis by these IgGs (Ana-Sosa-Batiz et al., 2016), using streptavidin-coated FITC-fluorescent beads with AD8 Unc gp140 bound to the surface as target and U937 cells as effector cells.

As shown in figure 4-3, phagocytosis was elicited only by IgGs from AD8 Unc 500 group using AD8 Unc gp140 as a probe (phagocytic score of 4377.33). Although IgGs of this vaccinated group showed similar binding affinity to BG505 SOSIP in ELISA (section 3.4), no phagocytosis was induced using BG505 SOSIP gp140 probe. This could indicate that antibodies reaching the same endpoint concentration against these two vaccines are different and are likely binding to different epitope, thus allowing the activation of ADP when bound to AD8 Unc gp140 but not to BG505 SOSIP gp140.

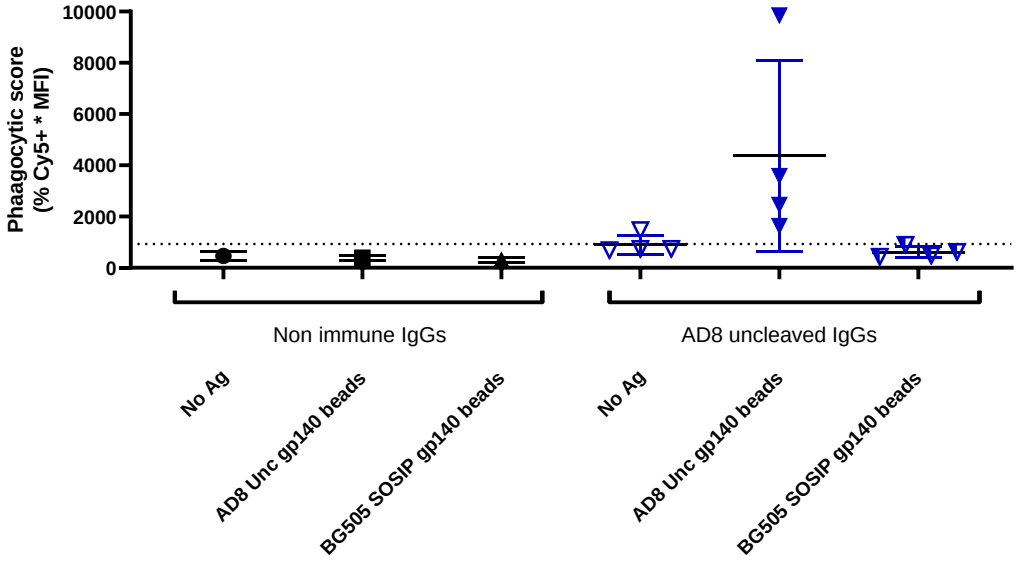


Figure 4-3: Assessment of activation of phagocytosis of Env coated beads, mediated by bovine IgGs. Streptavidin-coated FITC-fluorescent beads were prepared with biotinylated AD8 Unc gp140 or BG505 SOSIP gp140 and a Cy5-fluorescent internalisation probe (Cy5-FIP), incubated with purified IgGs from groups AD8 Unc 500 and co-incubated with U937 cells for 16 hrs at 37°C. Phagocytic score was calculated as percentage of Cy5 positive signal in the FITC+ group, multiplied by its mean fluorescence intensity (MFI) in the FITC channel. In addition, cut-off value of phagocytic score was calculated based in the signal observed for non-immune IgGs, which was 930.35 in average. Error bars: average ± SEM.

4.4 Effects of structural changes of HIV-1 Env in elicitation of phagocytosis

The fact that IgGs from AD8 Unc 500 group presented the same endpoint concentration against both AD8 Unc gp140 and BG505 SOSIP gp140, but phagocytosis was elicited only with beads coated with AD8 Unc gp140, raises the question whether the loss of phagocytosis against BG505 SOSIP was due to change of virus clade or Env structure in the assay. To address this question, ADP-SHIP assay was performed using multiple forms of AD8 Env including AD8 SOSIP gp140 and AD8 gp120 as targets (figure 4-4). Important to note is that Env gp120 is commonly used for phagocytosis assays, whereas trimeric structures are not used, most probably due to their difficulties of production and purification. Change into AD8 SOSIP gp140 or AD8 gp120 resulted in a drop in phagocytic activity even with using high amount of IgGs (100 µg/ml). No significant changes were observed for the negative control (non-immune IgGs from colostrum).

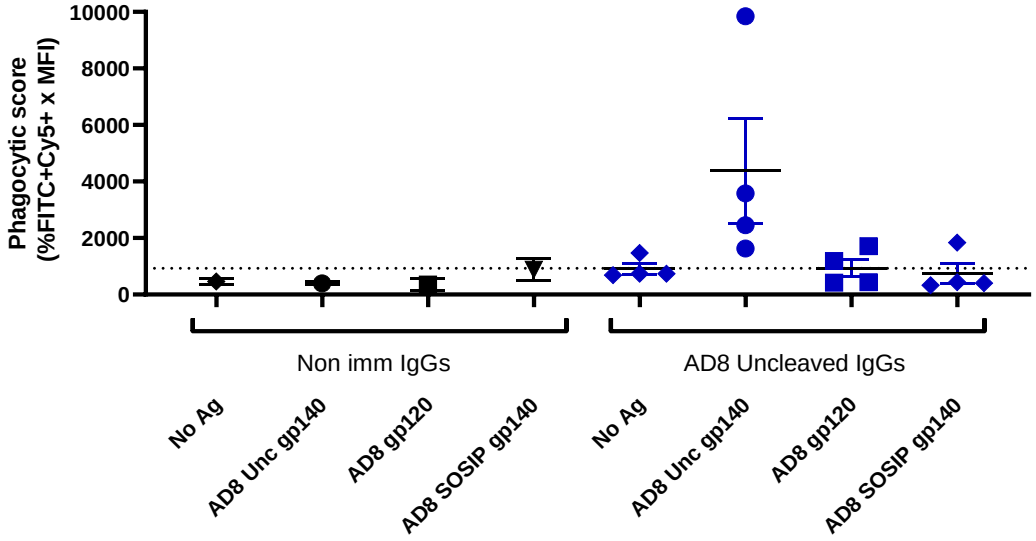


Figure 4-4: ADP-SHIP assay mediated by IgGs elicited by AD8 Unc gp140 vaccinated cows, against different Env antigens. Streptavidin-coated FITC-fluorescent beads were prepared with biotinylated AD8 Unc gp140, AD8 SOSIP gp140 or AD8 gp120 and a Cy5-fluorescent internalisation probe (Cy5-FIP), then incubated with purified IgGs from AD8 Unc 500 group, and later incubated with U937 cells for 16 hrs at 37°C. Phagocytic score was calculated as percentage of Cy5 positive signal in the FITC+ group, multiplied by its mean fluorescence intensity (MFI) in the FITC channel. In addition, cut-off value of phagocytic score was calculated based in the signal observed for non-immune IgGs, which was 930.35 in average. Error bars: average ± SEM of group.

To assess whether loss of phagocytosis was due to lack of binding of IgGs to AD8 gp120 and AD8 SOSIP gp140, we performed a capture ELISA and detected a reduced binding to the mentioned Envs compared with AD8 Unc gp140 Env (figure 4-5). This indicates that epitopes responsible for eliciting phagocytosis are not present in gp120 alone or in gp41 ectodomain and could be quaternary epitopes within uncleaved gp140 that could not be exposed on AD8 SOSIP gp140 Env.

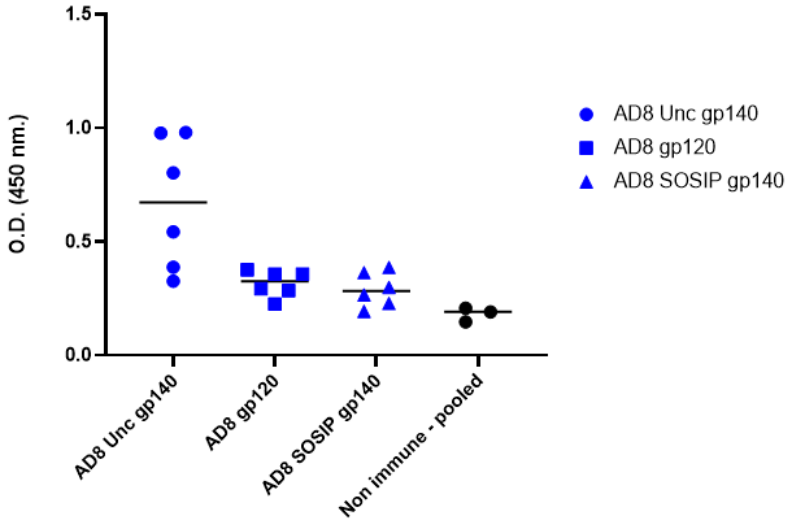


Figure 4-5: Binding of purified IgGs from colostrum to AD8 Unc gp140, AD8 gp120 and AD8 SOSIP gp140. Biotinylated Env proteins (100ng per well) were captured on an ELISA plate, and 100 µg/ml of purified IgGs from colostrum of animals from AD8 Unc 500 group were loaded and incubated for 2 hrs at RT. After wash, bovine IgGs bound to Env proteins were detected with an anti-bovine IgG-HRP-conjugated antibody. As negative control, same binding was measured for non-immune samples, against all three proteins. Figures indicate different proteins used in ELISA (circles: AD8 Unc gp140, squares: AD8 gp120; triangles: AD8 SOSIP gp140).

4.5 Assessment of complement activation mediated by bovine IgGs from colostrum

Antibodies bound to their antigen and forming ICs can activate classical pathway of complement. As mentioned in section 1.2.5, activation of complement occurs when 6 IgGs bind to the soluble protein C1q, which once activated initiates the complement cascade. C1q is associated with serine proteases C1r and C1s. Once IgGs bind to C1q, conformational changes within C1q allow the self-activation of both C1r and C1s, thus allowing the cleavage of other complement proteins such as C2 and C4, binding of C2a and C4b, formation of the enzyme C3 convertase, and finally forming the

membrane attack complex (MAC) in charge of cell lysis. MAC is formed in infected cells and is recruited by the complement protein C3b, a product of cleavage of the protein C3 mediated by C3 convertases. C3 binds covalently to infected cells near the IgGs forming such IC and acts as an opsonin (Janeway Jr, Travers, Walport et al., 2001); therefore, formation of MAC is initiated by binding of C3, which occurs only when C1q activation happens.

Since contact residues to activate human complement cascade are present in Fc region of bovine IgGs, we aimed to assess activation of complement by bovine IgGs using human plasma. CEM.NKR.CCR5 cells, a lymphocyte cell line expressing CD4 and CCR5 receptors and resistant to NK cell activation, was used as a target by coating cells with cognate Env vaccines. Then, purified IgGs from all vaccine groups were incubated with cells opsonised with cognate Env vaccines, in order to test complement deposition of C3b in presence of human plasma using anti-C3b-FITC specific antibody (Figure 4-6). Among the vaccinated groups, AD8 Unc 500 group induced deposition of C3b with an average relative fluorescence of 357.15, and one animal reaching 805.03. IgGs from AD8 Unc 100 was the second highest group, but relative fluorescence values achieved by this group did not surpass background levels. Overall, the results suggest that AD8 Unc gp140 was the only vaccine inducing activation of complement by bovine IgGs.

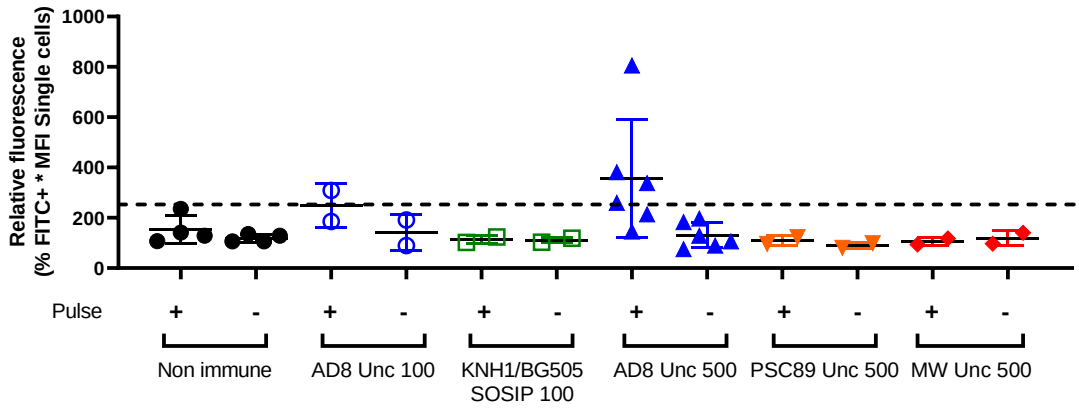


Figure 4-6: Assessment of complement activation by bovine IgGs, using human plasma. CEM.NKR.CCR5 cells were either pulsed (+) with recombinant Env trimers used in vaccination study or not pulsed (-), incubated with IgGs from colostrum of 16 cows representing each one of the five groups from phase 1: AD8 Unc 100 (blue open circles), KNH1/BG505 SOSIP 100 (green open squares), AD8 Unc 500 (blue closed triangles), PSC89 Unc 500 (orange closed triangles) or MW Unc 500 (red closed diamonds). Bars in each condition indicate average plus/minus SEM of the group.

4.6 Effects of HIV-1 Env structural on activation of complement

Results from section 4.4 showed the effects of changing structure of AD8 Env in eliciting phagocytosis. When using AD8 gp120 and AD8 SOSIP gp140, epitopes needed to elicit this Fc-effector function are missing, producing a decrease in antibodies bound to each protein compared to AD8 Unc gp140. Since this reduction was observed with phagocytosis, we wanted to evalulate whether the same effect occurs with complement activation. Same as in section 4.5, complement deposition assay was performed using AD8 gp140 and gp120 and IgGs from both groups vaccinated with AD8 Unc gp140 were used. As shown in figure 4-7, replacement of AD8 Unc gp140 by AD8 gp120 as target decreased significantly the activation of complement mediated by all samples, with 7 out of 8 samples reducing their relative

fluorescence values to background levels. These results suggest that IgGs responsible for complement activation, as well, are not targeting epitopes on gp120, and instead could target epitopes only available in Unc gp140 trimers.

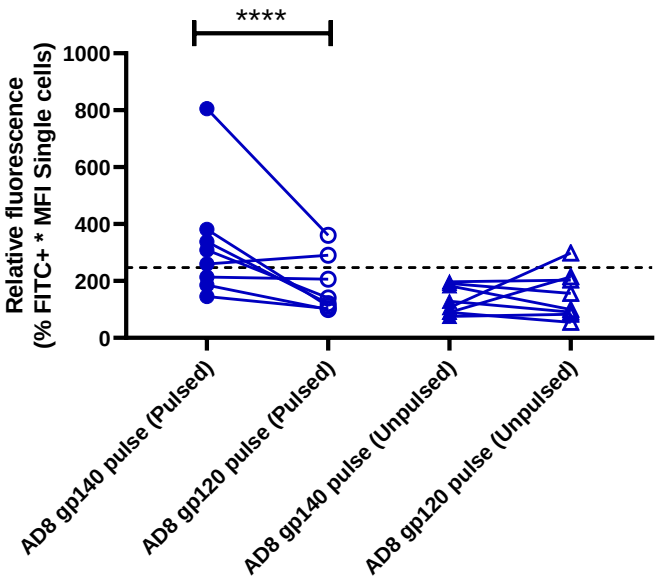


Figure 4-7: Complement deposition assay mediated by IgGs elicited by AD8 Unc gp140 group, against different targets. CEM.NKR.CCR5 cells were either pulsed (+) with AD8 Unc gp140 or AD8 gp120, or not pulsed (-), and opsonised with 500 µg/ml of purified IgGs from colostrum of cows from AD8 Unc 500 group. After incubating mixture with human plasma for 20 minutes, cells were washed and incubated with an anti-C3b FITC-conjugated antibody and analysed through flow cytometry. Data shows results in duplicate for n = 3 separate experiments.

4.7 Epitopes available in AD8 Unc gp140 eliciting Fc-effector functions

According to the results from section 3.8, both BG505 SOSIP gp140 and AD8 Unc gp140 could expose neutralising epitopes such as CD4bs and V3-glycan loop while differing in the exposure of the V1/V2 apex epitope. BG505 SOSIP gp140 presented the latter epitope but AD8 Unc gp140 was not able to expose it. As mentioned before

(Hessell et al., 2007; Bournazos and Ravetch, 2015), CD4bs Abs can elicit Fc-effector functions, while V3-glycan Abs like PGT121 do not (Parsons et al., 2019). It is possible that other epitopes, not characterised in section 3.8, could be involved in eliciting phagocytosis and complement activation. Non-neutralising epitopes such as Co-Rbs and cluster A are involved in mediating strong ADCC responses (Ding et al., 2016; Richard et al., 2015, 2016, 2017, 2018). Hence, we assessed the presence of both Co-Rbs and cluster A epitopes in AD8 Unc gp140 and BG505 SOSIP gp140 (Figure 4-8). ELISA was performed to evaluate the binding of 17b (binding to Co-Rbs receptor) and A32 (binding to cluster A epitope) as well as a few more human BrNAbs to AD8 unc gp140 and BG505 SOSIP gp140. 3 As expected (Binley et al., 2000, Sanders et al., 2002), BG505 SOSIP gp140 did not present epitopes of 17b and A32, whereas AD8 Unc gp140 could expose both epitopes.

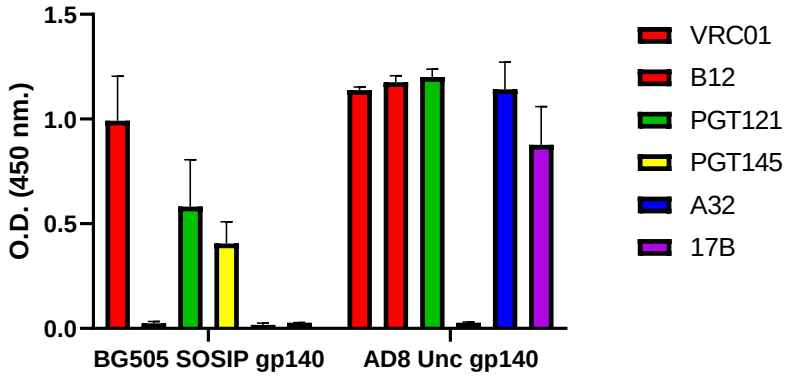


Figure 4-8: Assessment of epitope availability on BG505 SOSIP gp140 and AD8 Unc gp140. Antibodies targeting specific epitopes were used to assess presence of specific epitopes within BG505 SOSIP gp140 and AD8 Unc gp140: VRC01 and b12 for CD4bs (red), PGT121 for V3-glycan loop (green), PGT145 for V1/V2 apex (yellow), A32 for cluster A epitope (blue) and 17b for Co-Rbs (purple). For AD8 Unc gp140, 0.6ug/ml of BnAb was used, whereas for BG505 SOSIP gp140, 2ug/ml were used.

After confirming the presence of Co-Rbs and cluster A epitopes in AD8 Unc gp140, competition ELISAs using mAbs A32 or 17b mAbs were performed. As mentioned in section 3.8, a reduction in binding of these mAbs compared to a negative control without bovine Abs would indicate blockade of that epitope by the polyclonal mixture (Figure 4-9)

The result showed a significant competition between bovine IgGs and 17b antibody against Co-Rbs epitope with a reduction of AUC from 176.3 to 97.8. This indicates the presence of antibodies directly targeting the Co-Rbs or its vicinity, blocking the binding of 17b. No significant difference was found in binding for A32 (AUC of 175.3 in negative control v/s 153.5 with IgGs from AD8 Unc 500 group). In conclusion, the results show that AD8 Unc gp140 elicited antibodies targeting the Co-Rbs and the fact that BG505 SOSIP gp140 does not present this epitope supports the hypothesis that bovine IgGs targeting Co-Rbs epitope were responsible for eliciting Fc-effector functions

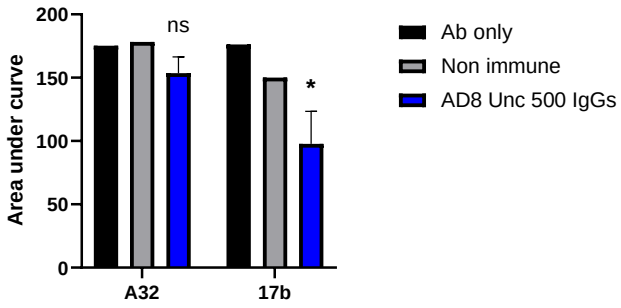


Figure 4-9: Epitope characterisation for polyclonal IgGs from Unc 500 groups by using non-neutralising mAbs. Competition ELISAs were performed in order to block binding of mAbs A32 and 17b by previously binding polyclonal IgGs from vaccinated cows. Binding curves were plotted and area under each curve (AUC) was calculated using GraphPad Prism v7. Blue bars show decrease by IgGs from AD8 Unc 500 group, with an average of AUC calculated, and error bars showing SEM from the whole group.

4.8 Discussion

Among tested HIV-1 Env vaccines, only AD8 Unc gp140 was able to activate Fc-effector functions by using human cells and proteins. Although KNH1/BG505 SOSIP gp140 demonstrated the highest neutralisation activity, it was unable to induce antibody dependent effector functions. This was unexpected as KNH1/BG505 vaccine induced V1/V2 apex binding antibodies, which have been linked to Fc-effector functions in previous studies of human HIV infection (Karasavvas et al., 2012; Zolla-Pazner et al., 2014; Bonsignori et al., 2012; Chung et al., 2015; Perez et al, 2017; Mujib et al., 2017). Elicitation of Fc-effector functions is dependent on the formation of ICs which itself is related to IgGs bound to the antigen surface (Smalls-Mantey et al., 2012). Therefore, absence of activation of effector functions in this group could be due to low titre of V1V2 apex binding antibodies. Formation of ICs depends on the amount of IgGs bound to the antigen surface, which is directly associated with the elicitation of Fc-effector functions (Smalls-Mantey et al., 2012). In addition to the titre of antibodies, their binding arrangement to HIV-1 Env also plays a critical role in formation of ICs. Frequency of ICs is important and needs to be higher than monomeric IgGs in binding to FcRs. A lower frequency of ICs versus monomeric IgGs will activate ITAM-mediated inhibitory pathways (ITAMi), therefore inhibiting Fc-effector functions mediated by Fc receptors (Boruchov et al., 2005).

V1/V2 loop epitope is not the only neutralising epitope related to induction of Fc-effector functions. CD4bs antibodies such as VRC01, b12, 3BNC117 and 10-1074 have activated ADCC against different Envs (Pollara et al., 2013; von Bredow et al., 2016; Bruel et al., 2017) and have conferred protection through ADCC in SHIV challenges (Hessell et al., 2007, 2009; Bournazos et al., 2014). Although binding titres against HIV-1 Env of such mAbs is higher than titres observed in polyclonal mixtures, these epitopes should also be considered as targets for eliciting Fc-effector functions.

For instance, AD8 Unc gp140 induced Abs targeting CD4bs in section 3.8. It is possible that Abs targeting this site are both neutralising as well as activators of Fc-effector functions. In addition, AD8 Unc gp140 also presented the Co-Rbs epitope, a non-neutralising epitope related to eliciting strong ADCC responses (Ding et al., 2016; Richard et al., 2015, 2016, 2017), and bovine antibodies produced in with this vaccine showed strong competition against 17b, a mAb that targets Co-Rbs. In consequence, both epitopes, CD4bs and Co-Rbs, could be responsible for activating Fc-effector responses with antibodies elicited in AD8 Unc gp140 group. To evaluate this, functional assays could be performed in presence of F(ab')2 fragments of mAbs VRC01, b12 and 17b, which block CD4bs and Co-Rbs epitopes and could impede the activation of phagocytosis in ADP-SHIP assays or complement activation in C3b deposition assays by bovine antibodies.

Other factors were not evaluated when assessing phagocytosis, like presence of FcγRIIb receptors on the surface of U937 cells or glycosylation levels in bovine IgGs. Binding of IgGs to FcγRIIb activates ITIM-mediated pathways, which will then inhibit Fc-effector functions (Baerenwaldt et al., 2011). Furthermore, the glycosylation of antibody Fc region can modulate binding of CH2 domain to FcR therefore affect the elicitation of FcR-mediated effector functions, (Niwa et al., 2004; Suzuki et al., 2007; Li et al., 2017; Dekkers et al., 2017). Due to the absence of interaction between anti-HIV bovine IgGs and human FcγRIIIa, we did not assess ADCC activation.

Results from sections 4.4 and 4.6 showed that changes in structure of Env affected the activation of Fc-effector functions. When AD8 SOSIP gp140 or AD8 gp120 were used instead of AD8 Unc gp140, both phagocytosis and complement activation were lost. It is known that the conformation of Env influences its vulnerability to Fc-effector functions. According to the three smFRET (single-molecule fluorescence resonance energy transfer) states Env undergo before fusion (Munro et al., 2014), Envs on state

1 are susceptible to ADCC by BnAbs targeting CD4bs and V1/V2 apex, whereas Envs in state 2 are susceptible to CD4-induced (CD4i) antibodies, such as the Co-Rbs and cluster A antibodies (Prevost et al., 2018; Richard et al., 2018). Downregulation of CD4 receptor maintains Env on state 1 (Hershhorn et al., 2016), which is regulated by the viral proteins Nef and Vpu (Pham et al., 2014), and protects cells from Fc-effector functions mediated by CD4i antibodies (Veillette et al., 2014 a,b). Once Env engages CD4, it exposes CD4i epitopes and produces conformational changes known as state 2 and 3 (Hershhorn et al., 2016). Based on structural analysis, SOSIP trimers are more close to state 1 Envs, whereas uncleaved trimers are more similar to Envs on state 2. This could explain the susceptibility of AD8 Unc gp140 to activate phagocytosis and complement, and the absence of both activities when AD8 SOSIP gp140 was used instead. In addition, since all AD8 Unc gp140, AD8 SOSIP gp140 and AD8 gp120 present the CD4bs epitope, but only AD8 Unc gp140 presents the Co-Rbs and cluster A epitopes suggest that antibodies responsible for eliciting Fc-effector functions in the AD8 Unc 500 group were directed against the Co-Rbs and not to CD4bs, otherwise the same functions would have been observed with all proteins. Recently, a new asymmetric Env conformation, called state 2A, has been described, which is present when soluble CD4 (sCD4) or CD4 mimic small compounds (CD4mcs), plus Co-Rbs and anti-cluster A antibodies are co-incubated. This state requires opening of Env, and primary CD4 T cells that present this conformation become susceptible to ADCC (Alsahafi et al., 2019). It would be of interest to analyse the formation of state 2A with AD8 Unc gp140, and to assess whether this state generates the activation of phagocytosis and complement.

As mentioned in the previous paragraph, the structure presented to study Fc-effector functions can affect activation of such functions. In this chapter, the analysis of both phagocytosis and complement activation was performed with recombinant Env trimers. It is possible that these trimeric structures do not resemble native trimers in infected

cells or virions in terms of structure, but also in terms of concentration. In order to elicit these functions, amount of surface Env on infected cells must be high enough to be recognised by antibodies. In HIV-1 infected cells, the expression of Env is downregulated by a conserved signal sequence in the cytoplasmic tail of gp41 (Rowell et al., 1995; Boge et al., 1998) leading to protection of infected cells from ADCC (von Bredow et al., 2015). Additionally, viral protein Vpu aids to maintain surface Env levels to the minimum by blocking host protein tetherin (Arias et al., 2014; Alvarez et al., 2014). Considering all these factors, future studies are required to investigate the level of surface Env used in our assays to ensure that it reflects physiological levels observed on infected cells, therefore presenting the conditions at which Fc-effector functions can be activated. Another alternative would be using primary cells infected with HIV-1. In addition, differences in HIV-1 Env strains can also affect the assay. For example, phagocytosis of BaL-coated virions has been detected (Tay et al., 2016) but not against JRFL- or NL4-3-coated virions (Gach et al., 2017). Absence of glycans can also affect the binding of antibodies eliciting Fc-effector functions, for example BnAb PG9 cannot induce Fc-effector functions against SF162 Env, as it lacks the N160 glycan needed for PG9 binding (Mayr et al., 2017). The usage of lab-adapted strains also favours the elicitation of Fc-effector functions, which can be activated by both BnAbs and non-neutralising antibodies. The latter is, however, not observed in cells infected with transmitter-founder viruses (Bruel et al., 2016, 2017; Mayr et al., 2017).

In conclusion, AD8 Unc gp140 was the only vaccine from this study eliciting Fc-effector functions such as phagocytosis and complement activation. This could be due to the presence of antibodies targeting epitopes involved in the activation of such functions, like the Co-Rbs and CD4bs, in AD8 Unc gp140 vaccinated groups. For KNH1/BG505 SOSIP gp140 group, low titre in autologous Abs along with absence of Co-Rbs epitope within BG505 SOSIP gp140 could explain the absence of Fc-effector functions mediated by this vaccine. Together with results from chapter 3, it is apparent that

eliciting both neutralisation and Fc-effector functions with the same vaccine is not possible, due to Ab titres and epitopes targeted to elicit each protective function.

5. Analysis of affinity maturation and levels of somatic hypermutations achieved by antibodies elicited by KNH1/BG505 SOSIP gp140 and AD8 Unc gp140 vaccines

5.1 Background

Analysis of neutralising activity elicited by all four vaccines in this study showed that KNH1/BG505 SOSIP gp140 induced the strongest and broadest neutralising response during phase 1 and 2 of the vaccination course, while AD8 Unc gp140 produced Abs with high binding, but mild neutralisation profile. On the other hand, analysis of Fc-effector functions elicited by Abs from all groups showed that only AD8 Unc gp140 vaccine could activate phagocytosis in U937 cells and complement in CEM cells. Abs elicited by these two vaccines targeted different epitopes: while IgGs elicited against AD8 Unc 500 and AD8 Unc 100 bound to CD4bs and Co-Rbs, animals from KNH1/BG505 SOSIP 100 group elicited antibodies against the V1/V2 apex. Differences in structure and clade affected the antibody profiles elicited, which could also reflect the level of BCR engagement for each antigen prior to differentiation into plasma cells.

As explained in section 1.2.1, Ig gene diversity occurs through V(D)J recombination, and through presence of polymorphisms in germline genes within H and L gene loci between individual animals. In addition, as explained in section 1.2.2, activation and further production of IgGs requires engagement of B cell precursors through their BCR to their cognate antigen, which will first release IgM molecules, and further stimulation of T-helper cells, which will cause CSR and further circulation of IgG-secreting plasma cells (Allman and Pillai, 2008; MacLennan et al., 2003). Once plasma cells get into circulation, affinity maturation occurs by formation of germinal centres, re-ingress of plasma cells into lymph nodes, and engagement with Tfh cells (Nutt and Tarlinton, 2011; Shlomchik and Weisel, 2012). For phase 1, it was unclear how further vaccine boosts during bovine pregnancy affected on SHM levels and, thus, improvement of HIV Env binding. Furthermore, in phase 2, it is unknown whether changes in the HIV Env immunogen used could affect the level of SHM and hence binding and neutralising activity of antibodies elicited in vaccinated animals.

To address these questions, we studied the antibody repertoire generated by AD8 Unc gp140 and KNH1/BG505 SOSIP gp140 by next-generation sequencing (NGS). First, we analysed the antibody repertoire elicited before any event of affinity maturation and then, we compared it with the antibody repertoire elicited after phase 1 and phase 2 of the vaccination scheme. For the first part, analysis of IgM repertoire in cows at different timepoints provided background information, needed to deduce the unmutated antibody repertoire. For the second part, analysis of the IgG repertoire at week 33 (after phase 1) and week 59 (phase 2) was performed to investigate SHM rates induced after each vaccination phase.

Several groups have followed this strategy by using two sequencing techniques: 5' rapid amplification of cDNA ends (5'-RACE) and Multiplex PCR, (Corcoran et al., 2016; Vigdorovich et al., 2016; Waltari et al., 2018; Vazquez Bernat et al., 2019). Each technique has some limitations, but a combination of them could deliver a robust analysis. Both approaches require production of cDNA libraries prepared from mRNA of bovine PBMCs, extracted at different vaccine timepoints, and both libraries were prepared using specific primers for the Ig constant region of either IgM or IgG in the 3' end. Each method, however, uses different 5' primers for library preparation. 5' RACE PCR libraries were prepared using primers that target a poly-C tail produced during 5' RACE protocol (Figure 5-1). On the other hand, 5' primers for Multiplex PCR libraries were designed to target leader sequences observed in 5'RACE PCR libraries (Figure 5-2). Finally, by adding adapters for Illumina sequencing methods, each cDNA strand could be sequenced by pair-end reads of around 300 bp each, and corresponding reads could be merged, processed, and analysed using bioinformatic tools.

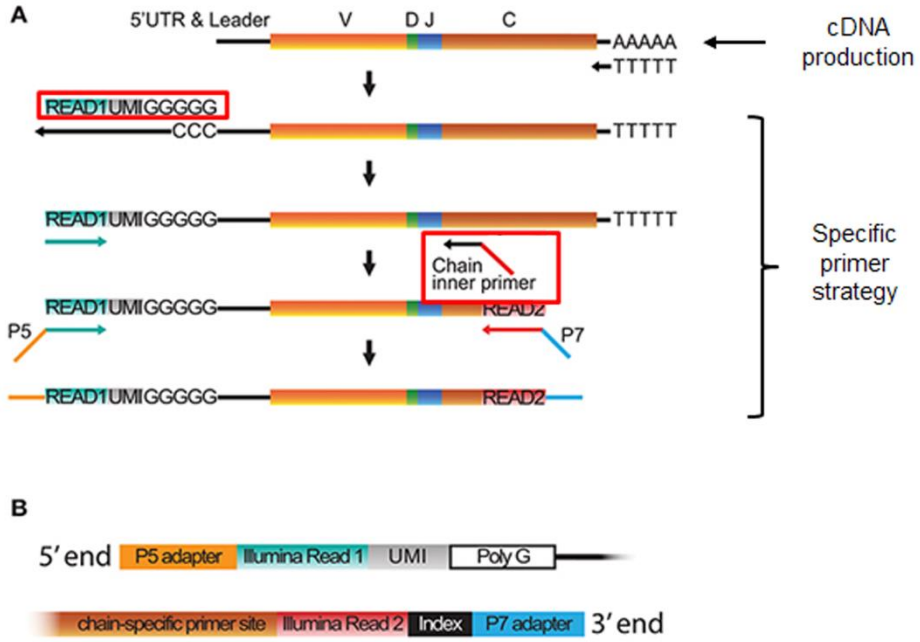


Figure 5-1: PCR strategy for 5' RACE PCR library production. A) Production of cDNA was performed using OlidoDT as primer to target poly-A tail in mRNAs. Next, primers targeting a poly-C tail in the 5' end and the IgM/IgG constant region of each sequence (red boxes) were used to prepare PCR libraries. Each of these primers carry Read1 and Read2 adapters for Illumina sequencing (Cyan and red lines, respectively). Such sequences were later used to amplify libraries with new primers carrying P5 and P7 adapters (orange and blue lines, respectively), which are directly used for sequencing. B) Structure of amplicons used for NGS, which once merged, are of around 550 bp (Image adapted from Vasquez-Bernat et al., 2019).

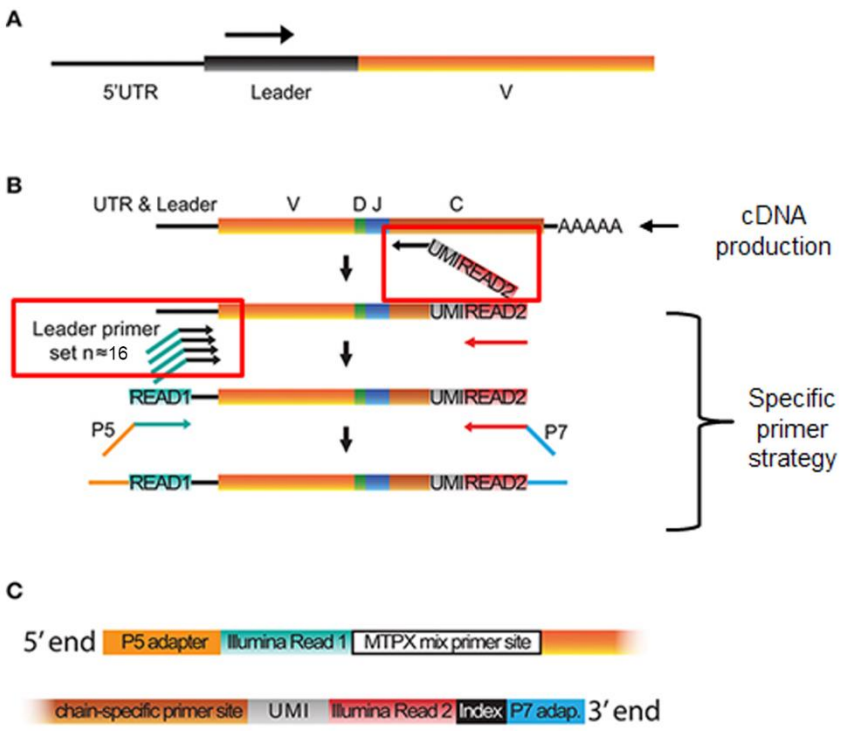


Figure 5-2: PCR strategy for Multiplex PCR library production. A) Information of 5' UTR and leader sequence for sequences isolated from bovine PBMCs was obtained after analysis of 5' RACE library NGS experiments. This information was used to design specific primers targeting leader sequence region. B) PCR libraries were prepared with a set of primers (n = 16) targeting leader sequences in 5' end and the IgM/IgG constant region in 3' end (red boxes). Each of these primers carry Read1 and Read2 adapters for Illumina sequencing (Cyan and red lines, respectively). Such sequences were later used to amplify libraries with new primers carrying P5 and P7 adapters (orange and blue lines, respectively), which are directly used for sequencing. C) Structure of amplicons used for NGS, which once merged, are of around 450 bp. (Image adapted from Vasquez-Bernat et al., 2019).

5.2 Evaluation of different bioinformatic tools to analyse bovine antibody sequences

In order to distinguish Ig gene diversity, it is first necessary to identify polymorphic germline sequences for the individual host, and then measure the adaptations that

arise by the vaccine used. To do this, a novel bioinformatic tool developed by Karlsson Hedestam's laboratory, called IgDiscover (Corcoran et al., 2016), was used to merge Illumina reads, annotate V, D and J germline genes, and detect polymorphisms (novel alleles) for each species. It enables calculation of the level of SHM and affinity maturation reached in each animal, and reduces the variability found by incomplete gene databases. To evaluate how IgDiscover works with bovine sequences, a training dataset was formed with 3 publicly available bovine sequences as query, along with a bovine database with germline genes provided by IMGT (http://www.imgt.org). Bovine mAbs 6A and 8C were isolated in our lab from a cow previously vaccinated with AD8 uncleaved gp140 (Heydarchi et al., 2017); respectively, they present a CDRH3 length of 23 and 16 residues, and germline genes were identified as a consensus sequence in the V segment, IGHD1-8 and IGHJ1-1 in their publication. NC-Cow 1 (Sok et al., 2017), on the other hand, was isolated from a bovine vaccinated with BG505 SOSIP gp140, presents a CDRH3 of 62 residues and the germline genes of IGHV1-7, IGHD8-2 and IGHJ2-4.

Sequences were first analysed through IgDiscover. The program analysed the training dataset in a unique fasta file, meaning that no merging was needed, and only one iteration of the program was performed in order to assign genes without searching for novel alleles. Gene annotation of V, D and J genes was successful for antibodies 6A and 8C. Both sequences used the J germline gene IGHJ2-4*01, while differed in genes used for regions V and D (IGHV1-27*01 and IGHD6-2 for 6A, and IGHV1-17*01 and IGHD1-1*01 for 8C) (table 5-1 A). Both sequences presented a V_covered value above 97, indicating that germline genes assigned by IgDiscover covered above 97% of the V segment found in query. J_covered value was different between both sequences, 93.8 for 6A and 68.8 for 8C. Same as in a BLAST search, coverage values can be compared with the expected (E) values offered by IgDiscover (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_T

YPE=FAQ#expect). High coverage values usually result in low E-values, which implies that results are significant and reliable. For both 6A and 8C, assigned V gene E-values were below 10^{-99}, and between 10^{-15} and 10^{-22} for the assigned J gene (table 5-1 A). Conversely, D gene coverage was low in both cases (15.5 for 6A and 32.3 for 8C), which affected their E-values for D gene, with 0.73 for 6A and 0.17 for 8C2. In framework regions, FR2 presented the highest level of SHM (13.2 for 6A and 9.6 for 8C), followed by FR3 (10.5 for 6A and 5.3 for 8C) and FR1, with the lowest SHM (2.7 for both sequences). In CDRs, CDR2 presented the highest SHM rates (36.8 for 6A and 23.8 for 8C) compared to CDR1 (20.8 for 6A and 8.7 for 8C) (table 5-1 B). Lastly, CDR3 regions for 6A and 8C were recognised in agreement with sequences published in Heydarchi et al., 2017, with the highest contribution to diversity in this region by D segment (table 5-1 C). An alignment of 6A and 8C with putative germline genes can be found in figures 1A and 1B.

For NC-Cow1, only genes V and D genes were annotated. Ninety-three percent of the query V region was covered by IGHV1-7*01, and only 6.1% of the D region was covered by IGHD8-2*01. In consequence, V region E-value was around 10^{-111}, while D region E-value was 1. Overall, both FR and CDR presented lower SHM levels compared to 6A and 8C, with FR having the lowest percentage of SHM (1.3 for FR1, 3.9 for FR2 and 9.6 for FR3), and with CDRs having lower values than mAb 6A (16.7 for CDR1 and 19 for CDR2) (tables 5-1 A and 5-1 B). No CDR3 was recognised, as shown in table 2C, despite that residues involved in the start and end of the region are present in the sequence.

Table 5-1: Analysis of bovine antibodies by IgDiscover. Heavy chain sequences from monoclonal antibodies 6A, 8C (8C2 on table) and NC-Cow1 were analysed by IgDiscover, using a bovine germline gene database provided by IMGT. Table A shows an analysis of germline gene usage, coverage and expected values (E-values) based on their BLAST score. Table B shows percentage of somatic hypermutations (SHM) within framework regions (FRs) and complementarity determining regions (CDRs) compared to the V, D and J genes assigned in A. Finally, table C presents the sequence where CDR3 is located.

A

Name	V_gene	D_gene	J_gene	V_covered	D_covered	J_covered	V_evalue	D_evalue	J_evalue
6A	IGHV1-27*01	IGHD6-2*01	IGHJ2-4*01	98	15.5	93.8	5.7E-99	0.73	2.6E-22
8C2	IGHV1-17*01	IGHD1-1*01	IGHJ2-4*01	97.6	32.3	68.8	3E-110	0.17	3.6E-15
NC-Cow1	IGHV1-7*01	IGHD8-2*01		99.3	6.1		1.8E-111	1	

B

Name	FR1_SHM	CDR1_SHM	FR2_SHM	CDR2_SHM	FR3_SHM	V_SHM	J_SHM
6A	2.7	20.8	13.2	36.8	10.5	11.7	0
8C2	2.7	8.7	9.6	23.8	5.3	7	0
NC-Cow1	1.3	16.7	3.9	19	9.6	8.4	

C

Name	CDR3_aa
6A	ARCASNIGVSWGCGEHTGNYVDA
8C2	ESWVCVGGRDDVCHSD
NC-Cow1	

According to Sok et al., 2017, germline genes involved in the generation of NC-Cow1 were IGHV1-7, IGHD8-2 and IGHJ2-4. While the first two genes match the results offered by IgDiscover, IGHJ2-4 was not suggested. In order to confirm or discard the presence of this gene as the germline sequence leading to NC-Cow1 sequence, we aligned NC-Cow1 with each germline gene individually using SnapGene. The results showed alignment with IGHV1-7 and with IGHJ2-4, but not with IGHD8-2. In order to simulate a full unmutated germline sequence, further alignment of these sequences together in frame revealed that IGHV1-7 and IGHJ2-4 aligned over 95%, and IGHD8-2

aligned with NC-Cow1 in the last third of its D segment sequence (Figure 5-3 C). Based on these results, it appears that IgDiscover can assign germline genes and recognise CDRs and FRs in sequences with average CDRH3 size, but not in sequences with ultralong CDRH3.

The problem of gene annotation for ultralong CDRH3 sequences could be caused by IgBLAST, the program that recognises germline genes within IgDiscover. An analysis of the IgBLAST pipeline showed that it first searches a putative CDR3 before annotating V and J genes, and such search proceeds based on files stored in the program, where FR and CDR regions are indicated for V genes of different species including human, rhesus macaque and mouse. Later in the IgDiscover pipeline, the script in charge of detecting CDR3 regions, called “species.py”, overrides the putative CDR3 found by IgBLAST, but it searches a CDR3 based on the germline genes assigned by IgBLAST. This implies that if IgBLAST is unable to find a putative CDR3 due to absence of a species FR and CDR file, it will not identify certain germline genes. In consequence, this will affect the detection of CDRH3 region by the script “species.py” in IgDiscover. Fortunately, both J gene assignment and CDR3 detection, can be done using other softwares.

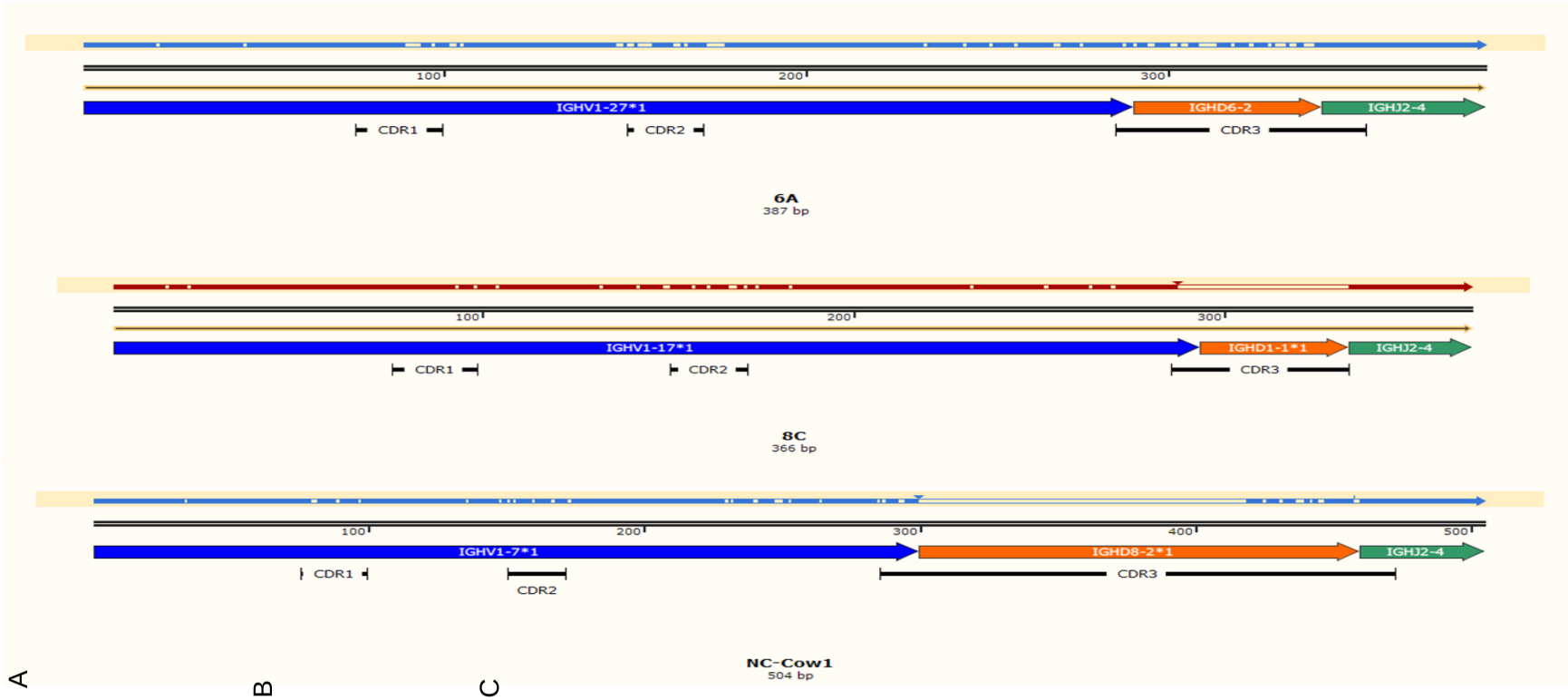


Figure 5-3: Sequence alignment and map of bovine antibodies 6A, 8C and NC-Cow1. Based on information given by IgDiscover and previous publications with these sequences (Heydarchi et al., 2017; Sok et al., 2017), H chain sequences of antibodies were analysed using the program SnapGene to compare with germline genes for mAbs 6A (A), 8C (B) or NC-Cow1 (C). CDRs were mapped based on the locations offered by IgDiscover or by a regular expression used to detect CDR3 (D'Angelo et al., 2014) (black segments below sequence), while germline genes were located by individual alignment with their corresponding antibody sequence (blue: V gene; orange: D gene; green: J gene). Finally, V, D and J germline genes assigned for each antibody were merged in frame, to simulate an unmutated germline sequence, and aligned with its corresponding antibody sequence (blue and red segments above sequence). In this last part, filled segments are a match, whereas blank segments are mismatches.

Gene annotation and CDR3 detection of ultralong sequences could be performed by IMGT V-quest (Brochet et al., 2008), an online platform offered by the International Immunogenetics Information System (IMGT). When the sequences in test_seqs.fasta were uploaded onto the server, output summary showed assignment of V, D and J genes in all three sequences (table 5-2). Compared to IgDiscover, assignment of V genes was identical in all cases, as well as assignment of J genes for mAbs 6A and 8C. J segment of NC-Cow1 was correctly assigned, with 88.1% of identity compared to germline gene IGHJ2-4*01. For D gene assignment however, only mAb 8C presented same germline gene annotated between IgDiscover and IMGT V-quest. In the case of 6A, the assigned germline gene was IGHD3-1*01 and not IGHD6-1*01, whereas in the case of NC-Cow1 it was IGHD5-2*01 instead of IGHD8-2*01. Interestingly, IMGT V-quest was also able to detect the ultralong CDRH3 present in NC-Cow1, and in total all CDR3 values were in concordance with information in the literature (Heydarchi et al., 2017; Sok et al., 2017).

Table 5-2: Output result from IMGT V-quest analysis of known bovine sequences. Table indicates V, D and J gene assignment with their respective identity from germline gene. Additionally, lengths of CDR1, CDR2 and CDR3 are indicated for each sequence.

Sequence ID	V-GENE and allele	V-REGION identity %	J-GENE and allele	J-REGION identity %	D-GENE and allele	CDR1-IMGT length	CDR2-IMGT length	CDR3-IMGT length
6A	Bostau IGHV1-27*01 F	88.42	Bostau IGHJ2-4*01 F	97.87	Bostau IGHD3-1*01 F	8	7	23
8C2	Bostau IGHV1-17*01 F	92.98	Bostau IGHJ2-4*01 F	78.72	Bostau IGHD1-1*01 F	8	7	16
NC-Cow1	Bostau IGHV1-7*01 F	92.28	Bostau IGHJ2-4*01 F	88.1	Bostau IGHD5-2*01 F	8	7	62

Unfortunately, IMGT V-quest works as an online tool, meaning that libraries and mAb sequences need to be uploaded onto a server for use. We decided to not use this program as it risks sharing information that is protected by intellectual property. Additionally, no other bioinformatic tool working as a command-line tool with customised databases and local execution (no need to upload information on the

internet) uses the IMGT framework and gene assignment, so we were unable to present complete data of J gene annotation for our sequences. A positive observation, however, from results in table 5-2 is that missing information of J gene annotation and CDR3 length can be obtained by using other bioinformatic tools.

Another program available to exclusively detect CDR3s in antibody sequences is AbMining ToolBox (D'Angelo et al., 2014), a command-line tool that uses a regular expression to detect both start and end of a CDR3 within sequences. In simple terms, a "string" is an array of characters, and a "regular expression" (or "regex") is a pattern that matches a string. Regular expressions are used in informatics to search for specific strings and are used with a specific language, which includes or excludes characters within a string in order to match the search. Therefore, in this case, the regular expression is used to search for the start and the end of a CDR3 within the Ab sequence, based on the residues observed at both sites (D'Angelo et al., 2014). The original script was adapted for IgDiscover by Corcoran et al. and used in the script species.py, and its result overrides the CDR3 sequence offered by IgBLAST during gene assignment. As mentioned before, the script searches for the start of CDR3 at the end of the previously annotated V gene, and the end of the CDR3 at the start of the previously annotated J gene, meaning that using this regular expression within IgDiscover will have issues finding ultralong CDRH3 sequences. In contrast, AbMining ToolBox works without previous gene annotation, making it easier to adapt.

Training dataset was used as input for the script cdr3_pipeline.py. After using the default settings, AbMining ToolBox annotated CDR3 sequences only for mAbs 6A and 8C (table 5-3 A). A revision of cdr3_pipeline.py, the script used to search for CDR3 sequences, allowed to identify the regex used by this script:

```
cdr3_pattern =
r'(TT[TC]|TA[CT])(TT[CT]|TA[TC]|CA[TC]|GT[AGCT]|TGG)(TG[TC])(([GA][AG
CT])|TC)[AGCT]([ACGT]{3}){5,32}TGGG[GCT][GCT]'
```

The section within apostrophes is the regex, and it is divided into two sections. The first one, from `(TT[TC]|TA[CT])` to `([ACGT]{3})` comprises the regex searched at the start of the CDR3, while the second one, `TGGG[GCT][GCT]`, comprises the regex searched at the end of the CDR3. The section `{5,32}` presents a length limit where the end of the regular expression will be searched. This implies that only CDR3s up to 35 residues will be detected. Because bovine CDR3s can be beyond 60 residues, the length values were changed to {5,72}, creating a new script which was called cdr3UL_pipeline.py. Indeed, usage of cdr3UL_pipeline.py allowed the detection of CDR3 in NC-Cow1 and provided similar sequences as the one detected by IMGT V-quest (table 5-3 B).

Table 5-3: Output result from AbMining ToolBox analysis of known bovine sequences. Sequences from mAbs 6A, 8C (8CA on table) and NC-Cow1 were submitted as query using original script “cdr3_pipeline.py” (A) or the modified script “cdrUL_pipeline.py” (B).

A

Name	CDR3 sequence	Frequency
6A	CARCASNIGVSWGCGEHTGNYVDAW	1
8C2	CESWVCVGGRDDVCHSDW	1

B

Name	Sequence	Frequency
6A	CARCASNIGVSWGCGEHTGNYVDAW	1
8C2	CESWVCVGGRDDVCHSDW	1
NC-Cow1	CITAHQKTNKKECPEDYTYNPRCPQQYGWSDCDCMGDRFGGYCRQDGCSNYIHRSTYEWYVSAW	1

Modification of the script allowed detection of ultralong CDRH3 in NC-Cow1. Together with the usage of IgDiscover, analysis of ultralong sequences can be made. IgDiscover is able to annotate correctly V and D genes, and AbMining Toolbox helps obtaining the CDR3 sequences that IgDiscover was unable to find. For J genes, we will assume that almost all sequences used IGHJ2-4*01, as described previously in the literature (Ma et al., 2016; Deiss et al., 2017).

5.3 Analysis of IgM expression in vaccinated cows by 5' RACE PCR library

After deciding which programs to use and test the information they can provide, analysis of larger datasets was performed. Four animals, two from AD8 Unc 500 group and two from KNH1/BG505 SOSIP 100 group, were selected for NGS analysis (see table 5-4 for identities). Cows from AD8 Unc 500 group were vaccinated with AD8 Unc gp140 on phase 1, and receive a different vaccine on phase 2, either AD8 SOSIP gp140 or BG505 SOSIP gp140. On the other hand, cows from KNH1/BG505 SOSIP 100 group received the same vaccine during phase 1 and phase 2. Initially, a cDNA library was prepared by Dr. Shuo Li following the 5' RACE PCR protocol, using mRNA extracted from bovine PBMCs, and sequencing was performed by paired-end reads (300 bp each read) in a Illumina MiSeq platform.

Table 5-4: Animals used for NGS analysis of cows immunised with HIV-1 Env vaccines. Two animals representing groups AD8 Unc 500 (blue) and KNH1/BG505 SOSIP 100 (green) were selected for next-generation sequencing analysis. Table shows vaccinations received during phase 1 (week 33) and phase 2 (week 59) of vaccination scheme.

Cow ID#	Group (amount vaccine received)	gp140 Env received after calving
	Phase 1 (Wks 0, 7, 16 and 32)	Phase 2 (week 54)
35	AD8 Unc 500 (4 x 500µg)	100µg BG505 SOSIP gp140
8434		100µg AD8 SOSIP gp140
609	KNH1/BG505 SOSIP (3 x 100µg + 1 x 100µg)	100µg BG505 SOSIP gp140
617		50µg BG505 SOSIP gp140

First, data was processed by IgDiscover. According to the program pipeline, both 5' and 3' reads were evaluated in terms of read quality and merged, discarding un-merged reads for further steps. Between 1.5 x 10^6 and 2 x 10^6 merged reads were analysed through IgBLAST and germline genes were annotated. CDR3 was detected and annotated, and lastly, after 2 iterations, variants of V genes were annotated as draft alleles in each animal. The results showed that gene usage for IgM sequences was similar in each animal between weeks 33 and 59, but different between animals. All animals presented high usage of germline genes IGHV1-10*01, IGHV1-17*01 IGHV1-20*01, IGHV1-21*01 and IGHV1-27*01 (between 15.2% and 39.1%) (figure 5-4). D genes in all animals presented high expression frequency (around 10%) of genes IGHD1-1*01, IGHD1-3*01, IGHD3-1*01, IGHD4-1*01, IGHD5-2*01, IGHD6-2*01, IGHD7-3*01 and IGHD8-2*01, and low frequency (around 2%) for genes IGHD1-2*01, IGHD2-1*01, IGHD5-3*01 and IGHD9-1*01. J genes on the other hand, had a gene assignment of almost 100% for IGHJ2-4*01 in all animals, which has been previously mentioned in the literature (Deiss et al., 2017; Ma et al., 2016) (figure 5-4 and 5-5).

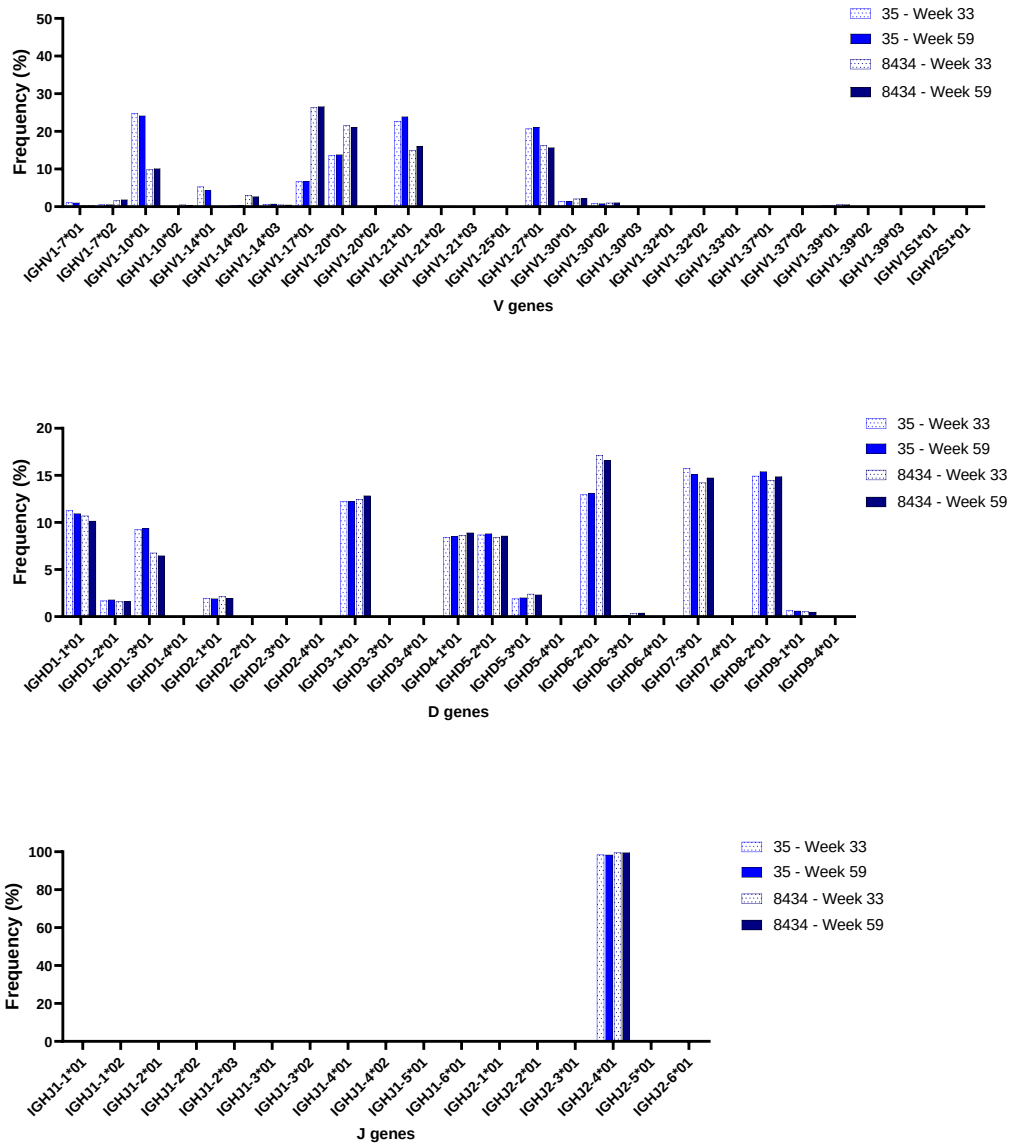


Figure 5-4: Gene assignment in 5' RACE libraries for animals vaccinated with AD8 Unc gp140. Analysis of sequences from cows 35 (light blue) and 8434 (dark blue) was performed using IgDiscover. Data from phase 1 of vaccination (week 33, open bars) and phase 2 (week 59, filled bars) was analysed.

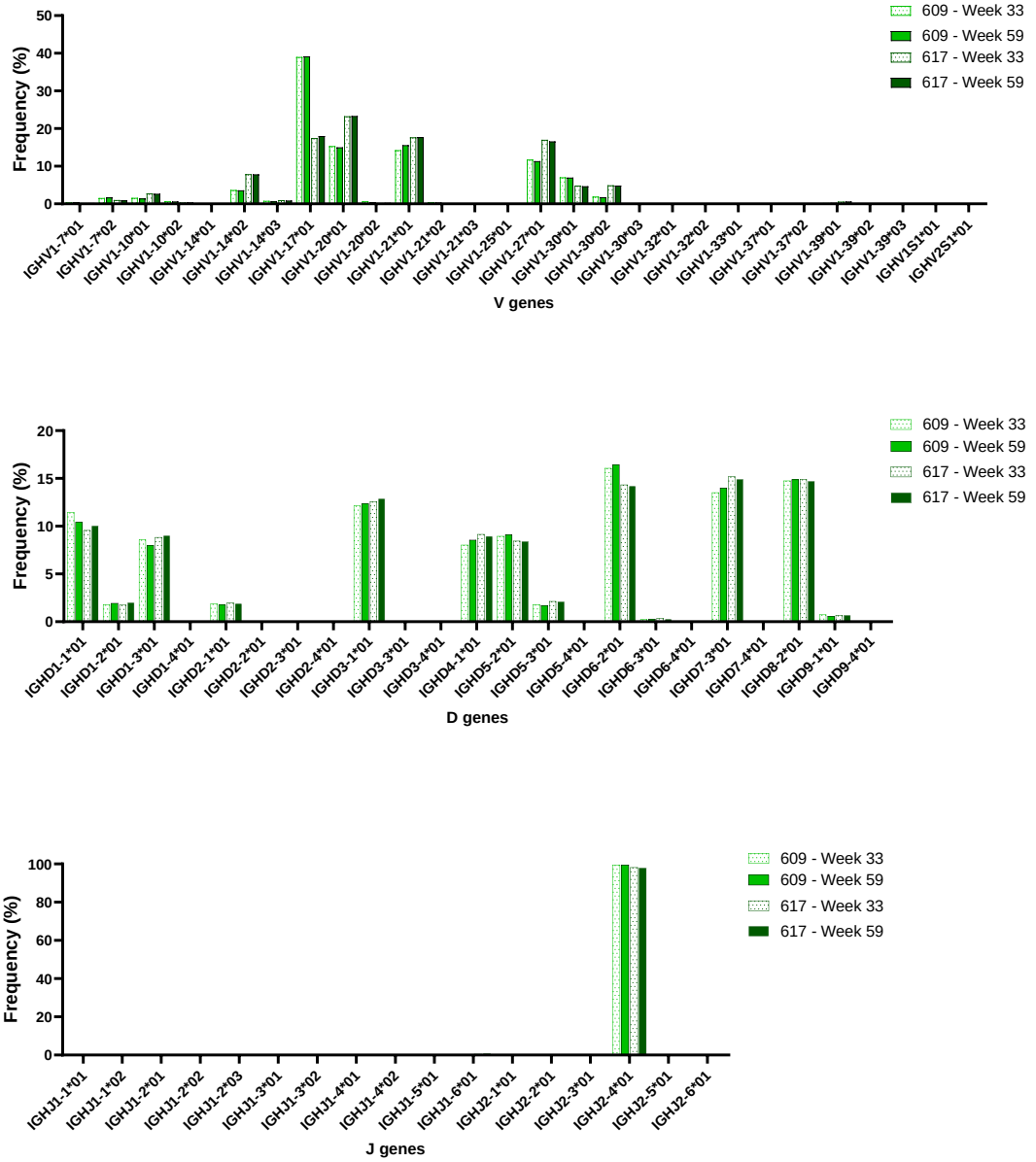


Figure 5-5: Gene assignment in 5' RACE libraries for animals vaccinated with KNH1/BG505 SOSIP gp140. Analysis of sequences from cows 609 (light green) and 617 (dark green) was performed using IgDiscover. Data from phase 1 of vaccination (week 33, open bars) and phase 2 (week 59, filled bars) was analysed

Next, merged sequences were analysed by AbMining ToolBox, since the longest CDR3 detected by IgDiscover was of 37 residues. Sequences up to 74 residues were detected by the program in all samples, nevertheless they represented a negligible

percentage of all CDRH3 lengths compared to sequences under 40 residues (below 0.5%, Figure 5-6). Comparing sequences at weeks 33 and 59, animals presented no significant differences in CDRH3 length between timepoints, but every animal presented a different distribution. The lack of ultralong CDRH3 sequences in 5' RACE libraries could be caused by limitations of the technique itself. It is possible that library preparation for Illumina sequencing using 5' RACE PCR is unable to pair sequences with ultralong CDRH3s, simply because these fragments are too long for this technique, meaning that most of sequences above 40 residues are lost in the un-merged reads. Finding ultralong sequences was expected because it has been observed in previous studies (Walther et al., 2013; Heydarchi, Salazar-Quiroz and Purcell, 2016; Liljavirta et al., 2014). We used NC-Cow1 in our training dataset because it presents a whole sequence of 504 bp without the inclusion of leader sequence and CH1 region, meaning that amplicons generated from this template using the same primers utilised in the 5' RACE library preparation will be of above 550 bp. Because sequencing limit for Illumina is 300 bp for each read, paired-ends would require, at least, 10 bp overlap to merge two ends, meaning that the top limit for paired-end reads would be 580 bp. In previous works, it has been estimated that an accurate top limit for paired-end reads using Illumina would be of 550 bp, a number surpassed by bovine sequences as shown before (Vazquez-Bernat et al., 2019). In conclusion, sequencing by 5' RACE PCR is unable to detect ultra-long CDRH3 sequences, therefore a new technique for library preparation must be used.

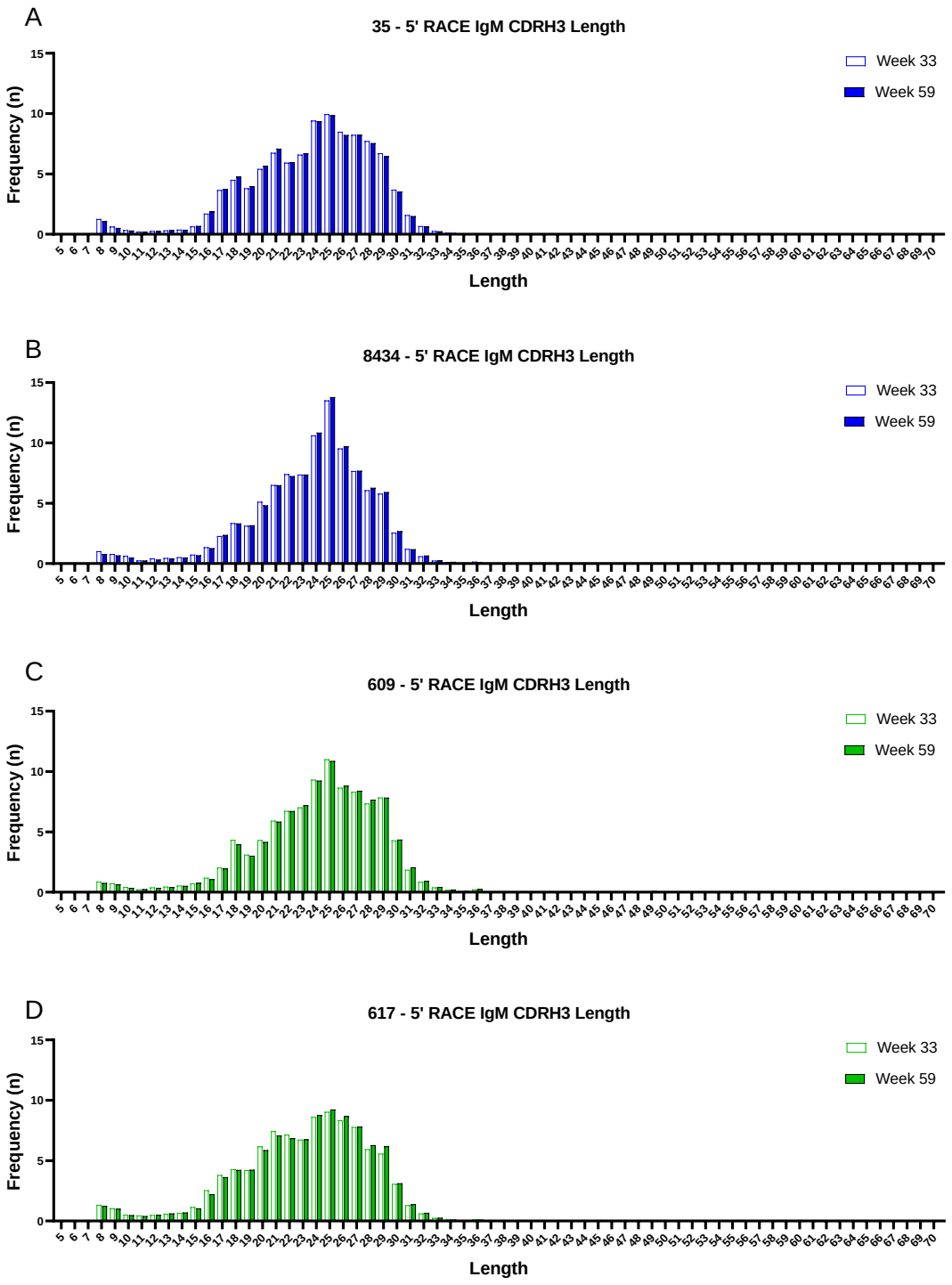


Figure 5-6: CDRH3 length distribution in 5' RACE libraries for vaccinated cows. Analysis of sequences from cows vaccinated with AD8 Unc gp140 (blue, A and B) or with KNH1/BG505 SOSIP gp140 (green, C and D) was performed using AbMining ToolBox. Bars show length frequency at week 33 (open bars) and week 59 (filled bars).

5.4 Analysis of IgM expression in vaccinated cows by Multiplex PCR library

The data obtained by 5' RACE library allowed recognition of leader sequences present in each expressed germline gene. These sequences were taken for each sample and clustered using MUSCLE (Edgar, 2004), in order to design primers that target specifically all clusters of leader sequences (see section 2.13.14). With these primers, a Multiplex library was produced for each sample in duplicate, reducing the amplicon size to less than 550 bp and thus increasing the likelihood of obtaining paired-end reads from sequences with ultralong CDR3s.

Sequences were first analysed with IgDiscover. After filtering results for sequences that have a complete open-reading frame (over 95% of total reads) and belonged to a VH chain (over 80% of reads with open reading frames), gene assignment was analysed for all V, D and J genes. Gene usage observed for each cow was similar to the results observed with 5' RACE library, with the exemption that between 3 to 5% of V segments were now assigned to either IGHV1-7*01 or IGHV1-7*02 using Multiplex library, while no more than 1% of V segments were assigned to any of the two germline genes in the 5' RACE library (figure 5-7 and 5-8). Again, each cow presented a unique pattern of gene usage, indicating that their IgM repertoire is different between each other, and additionally, gene usage at week 33 presented no significant differences with gene usage at week 59.

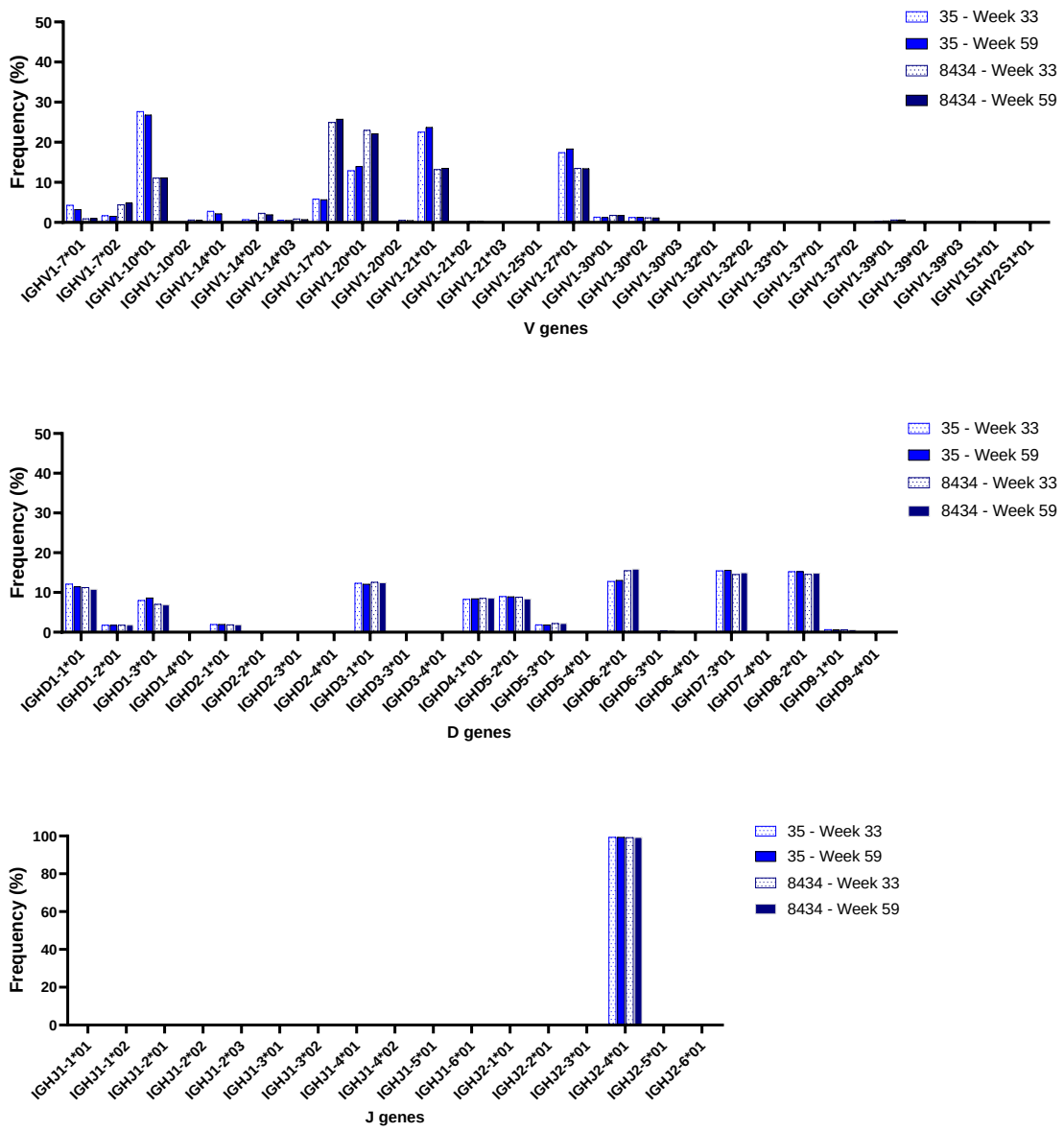


Figure 5-7: Gene assignment in Multiplex libraries for animals vaccinated with AD8 Unc gp140. Analysis of sequences from cows 35 (light blue) and 8434 (dark blue) was performed using IgDiscover. Data from phase 1 of vaccination (week 33, open bars) and phase 2 (week 59, filled bars) was analysed

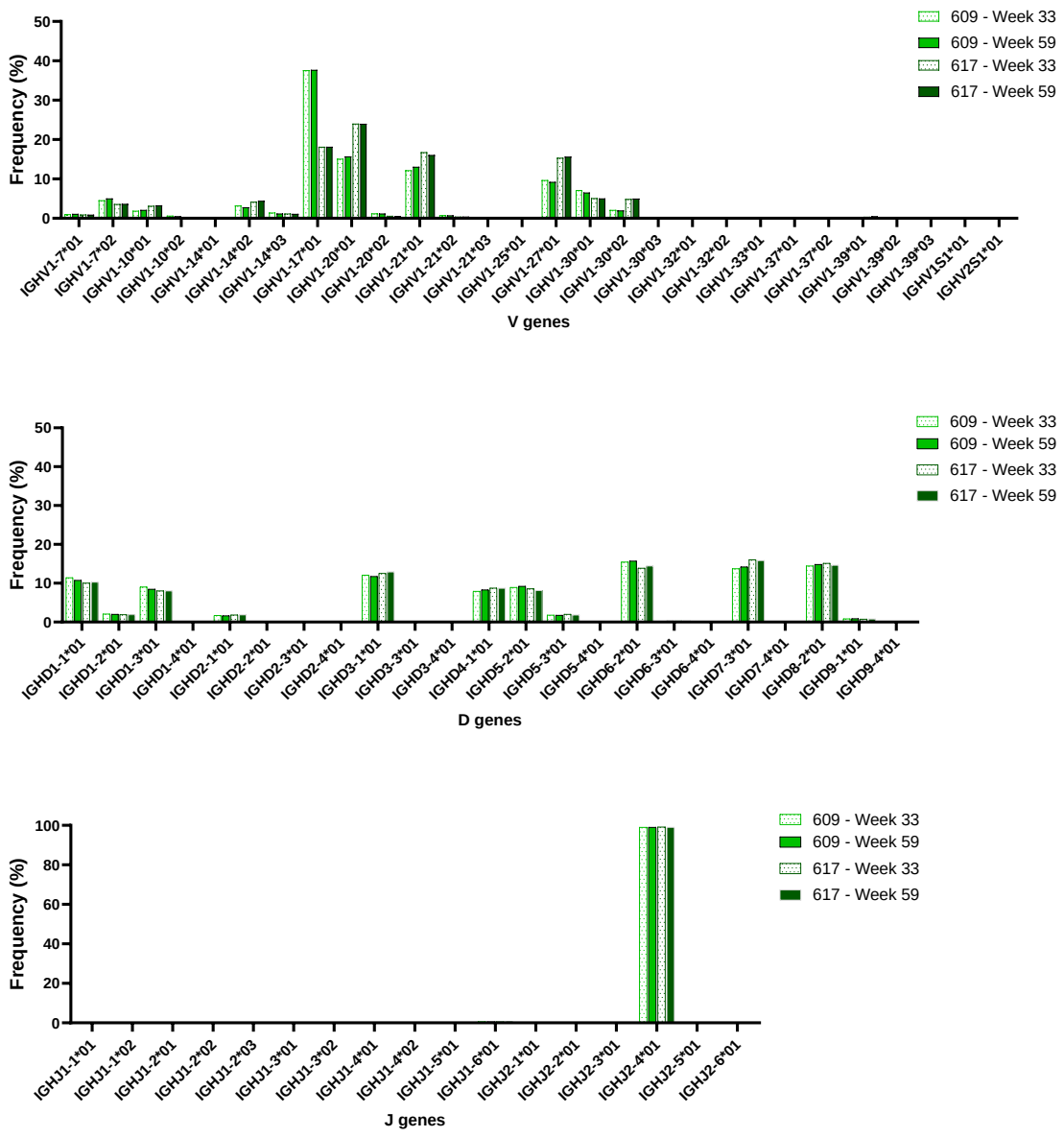


Figure 5-8: Gene assignment in Multiplex libraries for animals vaccinated with KNH1/BG505 SOSIP gp140. Analysis of sequences from cows 609 (light green) and 617 (dark green) was performed using IgDiscover. Data from phase 1 of vaccination (week 33, open bars) and phase 2 (week 59, filled bars) was analysed

Next, paired-end sequences were analysed through AbMining ToolBox in order to evaluate CDRH3 length distribution and number of unique sequences. The number or unique CDRH3 detected by AbMining ToolBox are listed in table 5-5. Unlike with 5'

RACE library, the program was able to now detect ultra-long CDRH3 sequences in Multiplex libraries from all samples (figure 5-9). Compared to the profiles observed in 5' RACE library, sequences with CDRH3 below 40 residues presented the same pattern than Multiplex library.

Table 5-5: Summary of sequences obtained in Multiplex PCR libraries. Number of total assembled reads, CDRH3 detected and unique CDRH3 detected in Multiplex PCR library for IgM sequences are indicated on table for week 33 (phase 1 of vaccination) and week 59 (phase 2 of vaccination).

Group: AD8 Unc 500

Cow: 35	week 33		week 59	
total CDRH3	173486	176813	163258	116215
Unique CDRH3	57270	58026	50601	42987
Assembled reads	209801	215296	203804	150972

Cow: 8434	week 33		week 59	
total CDRH3	174174	164660	199379	153301
Unique CDRH3	62806	61291	60413	52835
Assembled reads	203695	193326	237114	181980

Group: KNH1/BG505 SOSIP 100

Cow: 609	week 33		week 59	
total CDRH3	164449	143380	189649	170633
Unique CDRH3	61892	61042	66220	61946
Assembled reads	193800	171716	220954	198477

Cow: 617	week 33		week 59	
total CDRH3	180164	171130	167292	173877
Unique CDRH3	68530	67087	54627	55323
Assembled reads	224903	215368	201898	211579

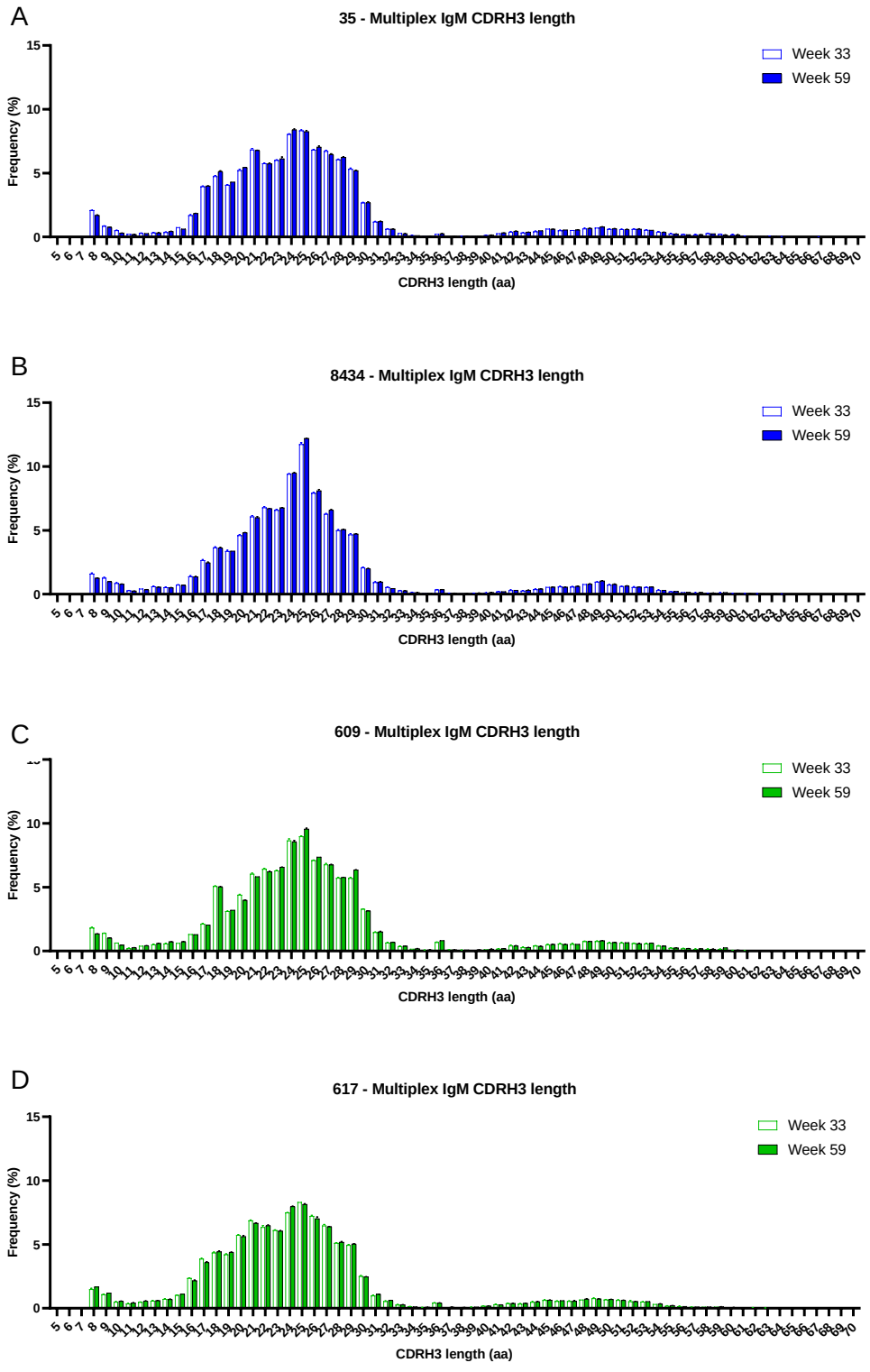


Figure 5-9: CDRH3 length distribution in IgM Multiplex libraries for vaccinated cows. Analysis of sequences from cows vaccinated with AD8 Unc gp140 (blue, A and B) or with KNH1/BG505 SOSIP gp140 (green, C and D) was performed using AbMining ToolBox. Bars show length frequency at week 33 (open bars) and week 59 (filled bars).

The percentage of sequences with CDRH3s equal and over 40 residues for all samples were around 8% of total reads, independent of timepoint, animal vaccinated or vaccine received (Figure 5-10). This is in concordance with the literature (Deiss et al., 2017; Wang et al., 2013).

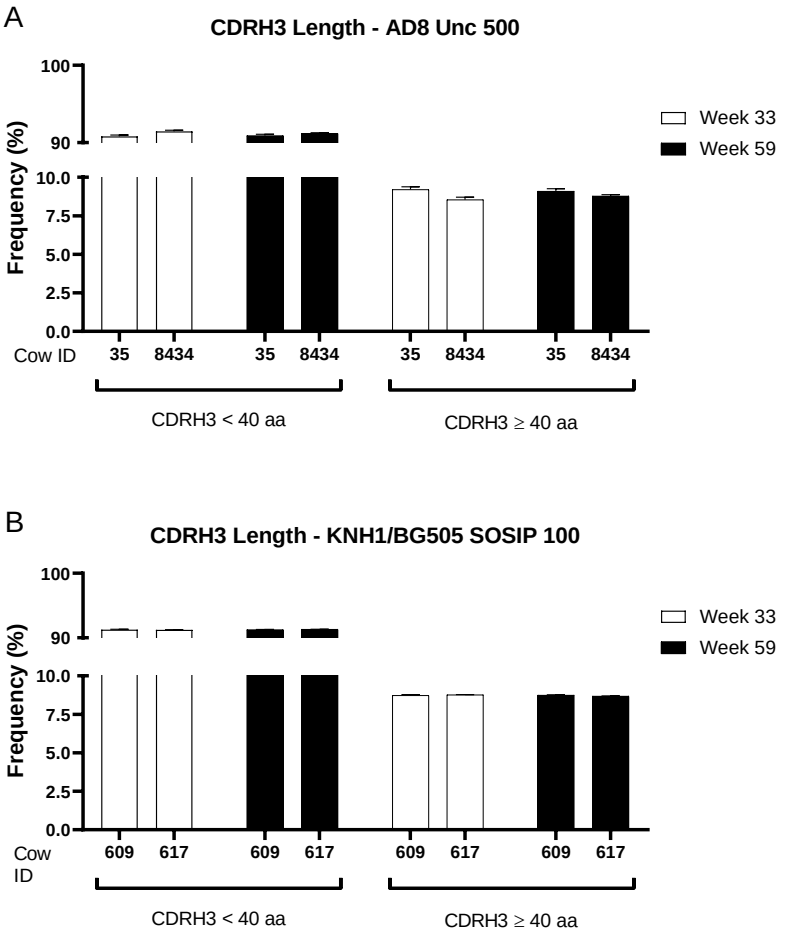


Figure 5-10: CDRH3 length observed for IgM Multiplex library. Total frequency of CDRH3 lengths observed in average-sized (< 40 amino acids) and ultra-long CDRH3 sequences (> 40 amino acids) were plotted as bar graph. Sequences from phase 1 (week 33, open bars) and phase 2 (week 59, filled bars) were counted in all animals analysed.

Together, these results indicate that IgM repertoire has not changed over the course of vaccination, and that none of the animals favoured the expression of ultra-long CDRH3 before the production of IgGs. In conclusion, each animal possesses a unique IgM repertoire, which might present a different affinity against either AD8 Unc gp140 or KNH1/BG505 SOSIP gp140 during phase 1, and against AD8 SOSIP gp140 or BG505 SOSIP gp140 during phase 2.

5.5 Identification of novel alleles of V germline genes in vaccinated cows

After analysis of IgM libraries produced by 5' RACE and Multiplex using IgDiscover, several novel alleles of V germline genes were inferred by the program (table 5-6). Their names indicate their closest germline gene in sequence identity, and the identifier number indicates the differences between the novel germline gene and its relative. Some of these alleles were found in both libraries (indicated in bold on table 4x), while most of the alleles were found exclusively by either 5' RACE or Multiplex. In order to progress with the analysis, all new alleles were included in the V germline gene database for each animal, making a custom database for each cow.

Table 5-6: Alleles inferred by IgDiscover in PCR libraries produced by 5' RACE and Multiplex PCR. Libraries prepared from cows vaccinated with either AD8 Unc gp140 (blue) or KNH1/BG505 SOSIP gp140 (green) were analysed through IgDiscover. Sequences found in both libraries are indicated in bold.

		5' RACE	Multiplex
AD8 Unc 500	Cow: 35	**IGHV1-17*01** IGHV1-14*01 IGHV1-39*01_S9680 IGHV1-7*01 IGHV1-7*02_S0302	**IGHV1-17*01**
	Cow: 8434	IGHV1-14*02 IGHV1-17*01_S3945 IGHV1-25*01_S4414 IGHV1-27*01_S2303 IGHV1-27*01_S8725 IGHV1-7*02_S0302	IGHV1-17*01_S5870 IGHV1-27*01 IGHV1-39*01_S5610 IGHV1-7*02_S0302 IGHV1-7*02_S8117
KNH1BG505 SOSIP 100	Cow: 609	IGHV1-14*02 **IGHV1-20*01_S0271** IGHV1-20*01_S9028 IGHV1-27*01_S8725 **IGHV1-30*01** **IGHV1-39*01_S3000** IGHV1-7*02_S0302	**IGHV1-20*01_S0271** IGHV1-27*01_S9385 **IGHV1-30*01** IGHV1-7*02_S0302 IGHV1-14*02 **IGHV1-39*01_S3000** IGHV1-17*01_S3945 IGHV1-21*01_S0648
	Cow: 617	**IGHV1-14*02** **IGHV1-17*01_S3945** IGHV1-17*01_S4207 **IGHV1-20*01** IGHV1-27*01_S0764 **IGHV1-30*02_S0628** **IGHV1-39*01_S3000** **IGHV1-39*01_S8451** **IGHV1-7*01_S2237** **IGHV1-7*02_S4274**	**IGHV1-14*02** **IGHV1-17*01_S3945** **IGHV1-20*01** IGHV1-27*01_S9890 IGHV1-30*01 **IGHV1-30*02_S0628** **IGHV1-39*01_S3000** **IGHV1-39*01_S8451** **IGHV1-7*01_S2237** **IGHV1-7*02_S4274**

Additionally, a phylogenetic analysis was done for each animal using germline genes used in the initial database plus the novel alleles inferred by IgDiscover (Figure 5-11 and 5-12). The appearance of unique alleles in each animal changed the distribution of distances between known sequences. Cow 35 presents a main cluster and a small one, formed by members of the family IGHV1-32 and IGHV1-7, plus two genes unrelated to each other: IGHV1-27*01_S3164 and IGHV2S1*01 (figure 5-11 A). On the other hand, cows 8434, 609 and 617 present a more complex tree, with smaller clusters which members present a close distance from each other. For unknown reasons, the analysis did not pair IGHV1-27*01_S3164 with its most related gene,

IGHV1-27*01; instead, it was paired separated at a distance scale of 10% from the main cluster. Same issue was observed with cows 8434, 609 and 617 with this gene (figure 5-11 B and figure 5-12 A and B, respectively). IGHV1-27*01_S3164 was found in all four animals but the phylogenetic analysis did not pair it with IGHV1-27*01, even though IgDiscover found these were related to it. For the other novel alleles found in all four cows, each one was paired in a small cluster along with all other genes from the same family.

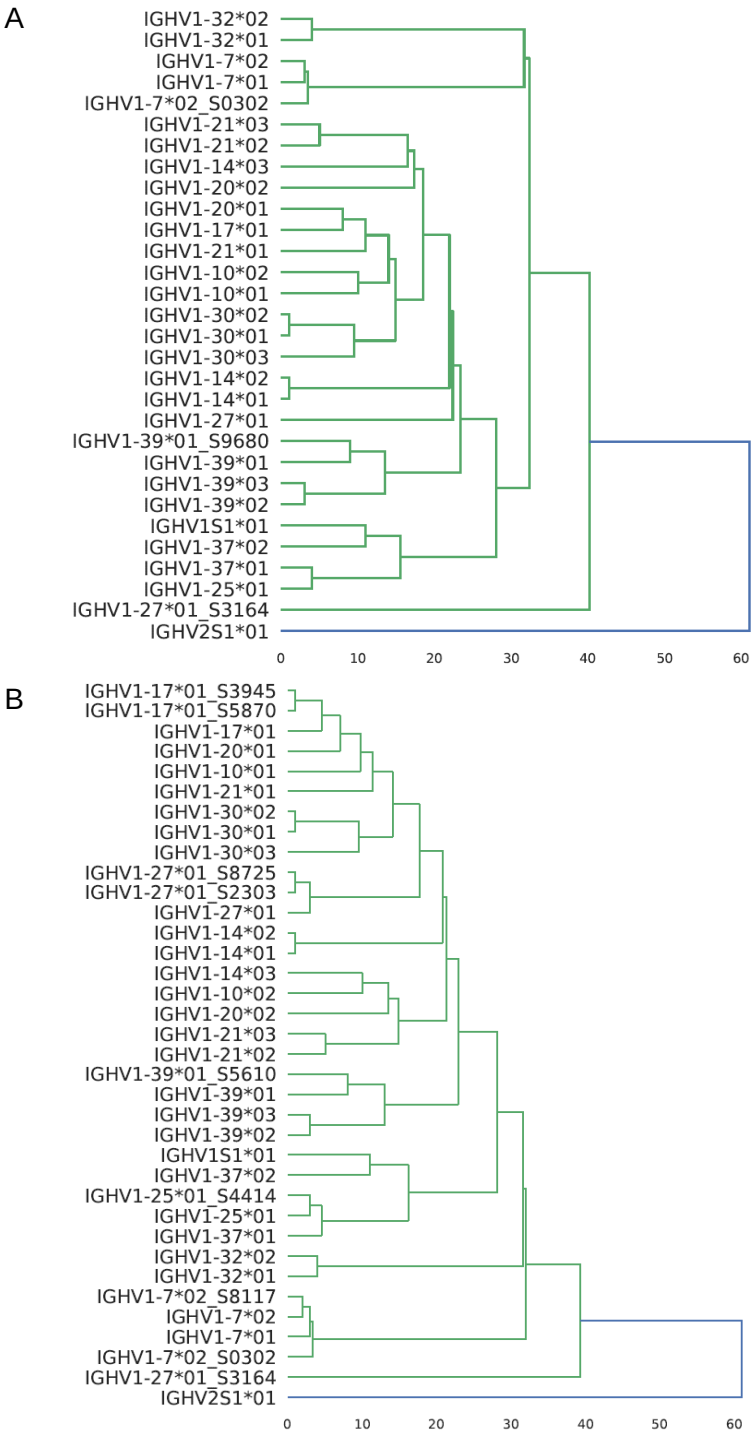


Figure 5-11: Dendrogram for V genes in cows vaccinated with AD8 Unc gp140. Sequence alignment was performed by MUSCLE and dendrogram plotted by IgDIscover. Sequences include genes in the initial V gene database and novel alleles found for cow 35 (A) and cow 8434 (B).

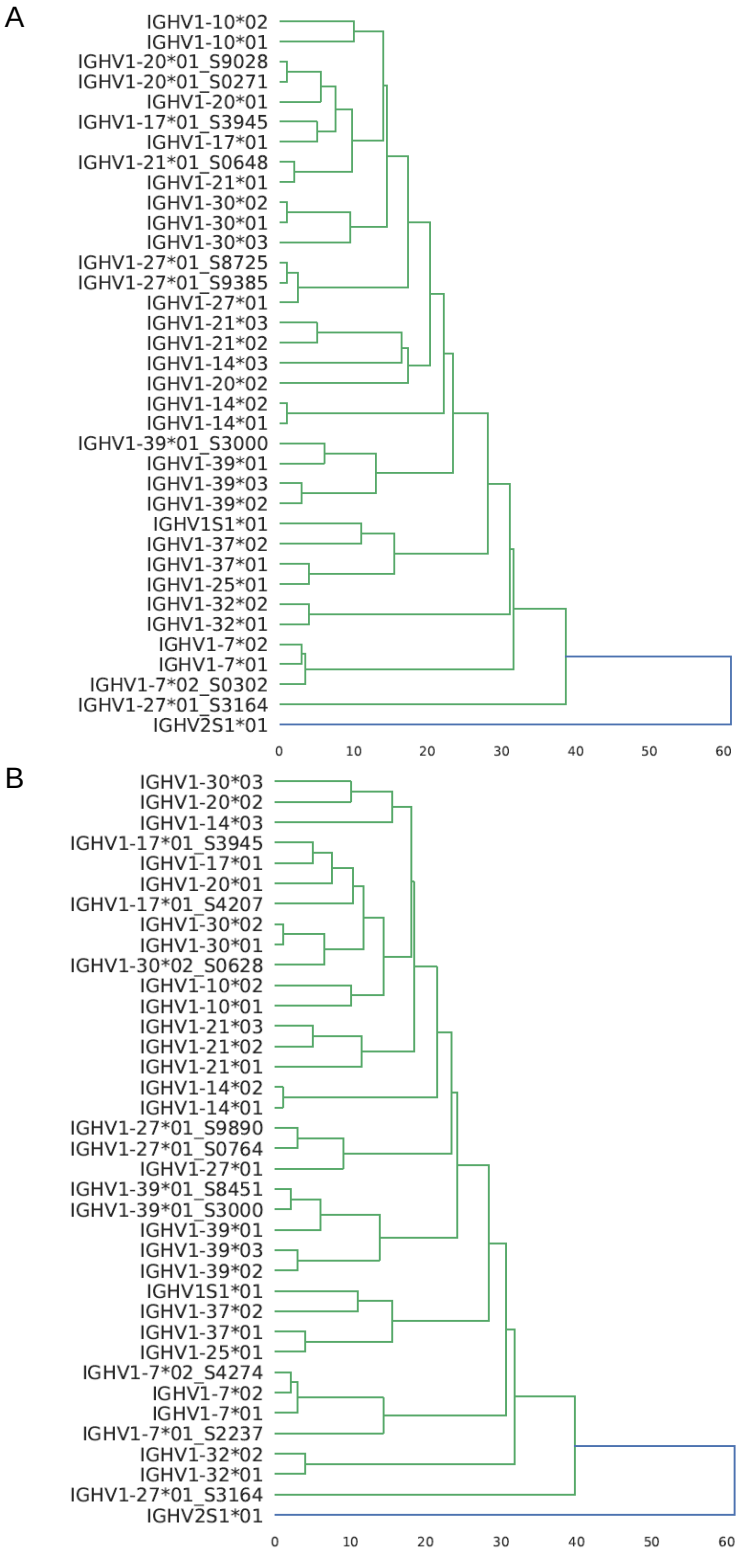


Figure 5-12: Dendrogram for V genes in cows vaccinated with KNH1/BG505 SOSIP gp140. Sequence alignment was performed by MUSCLE and dendrogram plotted by IgDIscover. Sequences include genes in the initial V gene database and novel alleles found for cow 609 (A) and cow 617 (B).

5.6 Analysis of SHM levels in IgM libraries after discovery of novel alleles

To study the levels of somatic hypermutation (SHM) found in IgM sequences, custom databases for each animal were created to re-analyse the Multiplex IgM library. New databases include novel alleles found by IgDiscover, which allows to calculate SHM rates in a more accurate manner. As described in Corcoran et al., 2016, germline genes in IgM libraries should present a low rate of SHM, if not close to zero. High rates of SHM in IgM libraries indicate the absence of alleles which, when present in the database, reduce the mutation rates detected. When new alleles were included in databases used for analyses, all animals presented mean SHM levels above 5%, which could indicate that initial database is incomplete (Figures 5-13 and 5-14). Germline genes presented mean SHM values between 5 and 10%, In cow 35, IGHV1-32*01 presented a mutation rate of 10%, with no inferred allele (figure 5-13 A, red box). For cow 609, however, gene IGHV1-27*01 presented SHM rate of 10% even when novel allele IGHV1-27*01_S8725 (figure 5-14 A, blue box). It is possible that multiple rounds of sequencing are needed to obtain all novel alleles, thus reducing the observed SHM rates. For the purpose of this analysis, however, a threshold of 5% will be considered to determine SHM in IgG libraries.

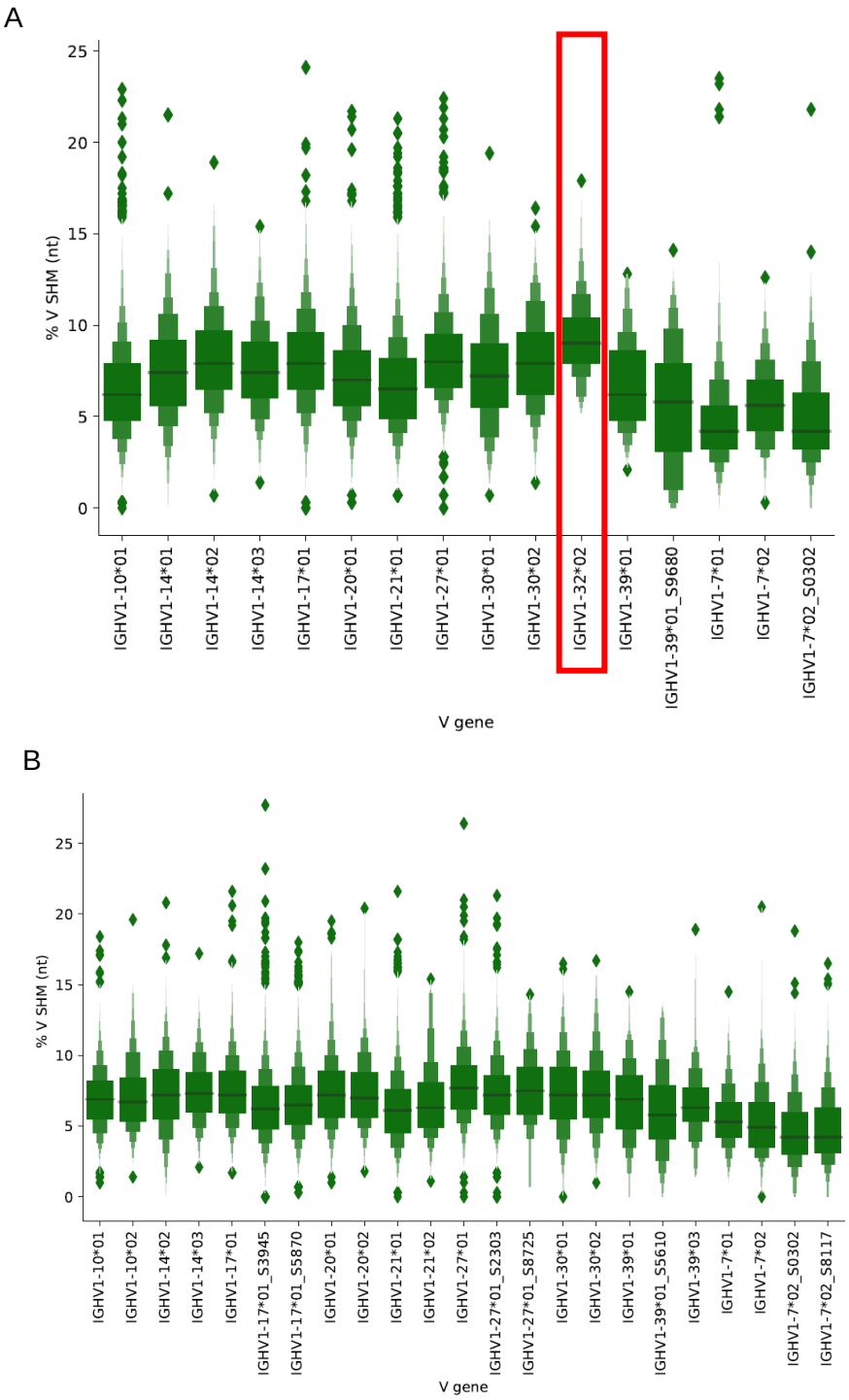


Figure 5-13: SHM rates observed in IgM libraries from bovines from AD8 Unc 500 group trimers. Sequences from cows 35 (A) and 8434 (B) were analysed through IgDiscover, and mutations found in all sequences were plotted in R. Most sequences presented SHM rates between 5 and 10%. Cow 35, however, presented SHM levels of 10% in gene IGHV1-32*01 (red box).

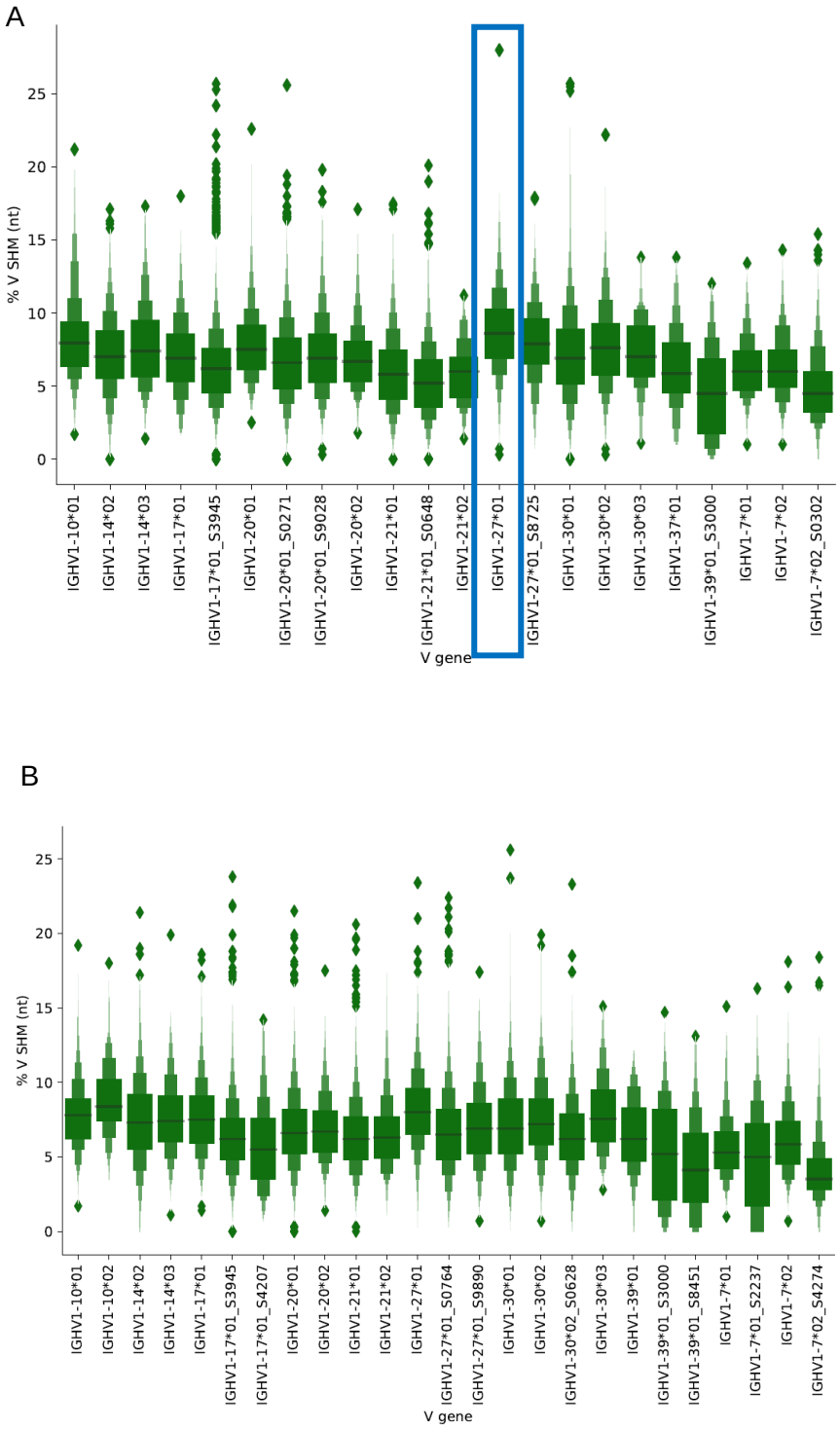


Figure 5-14: SHM rates observed in IgM libraries from bovines from KNH1/BG505 SOSIP 100 group. Sequences from cows 609 (A) and 617 (B) were analysed through IgDiscover, and mutations found in all sequences were plotted in R. Most sequences presented SHM rates between 5 and 10%. Cow 609, however, presented SHM levels of 10% in gene IGHV1-27*01 (blue box).

5.7 Analysis of IgG expression in vaccinated cows by Multiplex PCR library using custom databases

Using a custom database for each animal, IgG Multiplex libraries were firstly analysed through IgDiscover. After merging sequences, results from gene assignment were filtered and quantified. In order to observe changes in the usage of V, D and J genes, a direct comparison between IgM and IgG repertoires at weeks 33 and 59 was performed. Overall, gene expression in IgG libraries was different from IgM libraries, with particular differences observed between week 33 and week 59.

Cow 35 was initially vaccinated with AD8 Unc gp140 during phase 1 and revaccinated with BG505 SOSIP gp140 during phase 2. At week 33, a significant increase was observed in the frequency of V genes IGHV1-7*01, IGHV1-7*02, IGHV1-7*02_S0302, IGHV1-14*01 and IGHV1-27*01. At week 59, however, only genes IGHV1-7*01, IGHV1-7*02 and IGHV1-14*01 presented an increased frequency in IgG libraries compared to IgM at same timepoint. Interestingly, both IgG libraries presented a decrease of genes IGHV1-10*01 and IGHV1_21*01, two of the most expressed genes in IgM libraries (figure 5-15 A). D gene assignment presented most of the frequency differences (figure 5-15 B). At week 33, IgG libraries presented an increase in frequencies of genes IGHD1-3*01, IGHD7-3*01 and IGHD9-1*01, while at week 59 only gene IGHD7-3*01 was in common with the previous timepoint. In addition, week 59 IgG library for cow 35 had an increase in sequences with genes IGHD4-1*01, IGHD5-3*01 and IGHD6-2*01. A significant decrease of gene IGHD5-2*01 was observed at week 33, while at week 59 genes IGHD1-1*01, IGHD1-3*01 and IGHD8-2*01 decreased. Genes IGHD1-2*01 and IGHD5-2*01 in particular had a dissimilar trend between timepoints; at week 33, their frequency decreased compared to IgM, while at week 59 it increased significantly. Lastly, both timepoints showed a decrease

in frequency of gene IGHJ2-4*01 from IgM libraries, while an increase in the presence of IGHD1-6*01 was found (figure 5-15 C).

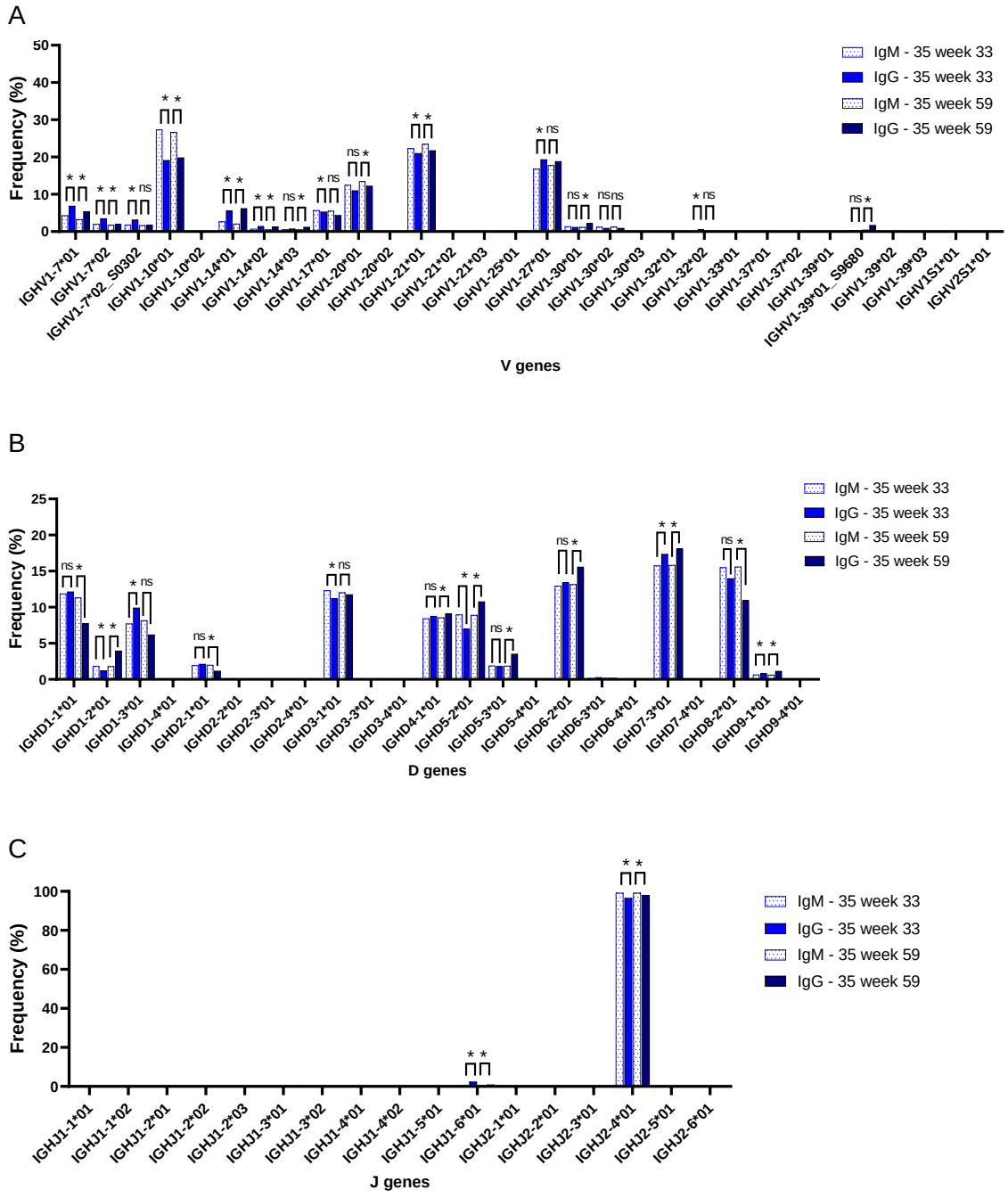


Figure 5-15: Gene assignment in Multiplex libraries for cow 35 (phase 1: AD8 Unc gp140; phase 2: BG505 SOSIP gp140). Analysis of sequences was performed using IgDiscover. Data from IgM libraries (open bars) and IgG libraries (filled bars) at week 33 (light blue) and at week 59 (dark blue) was done for V (A), D (B) and J (C) genes. Statistical analysis was performed by a 2-way ANOVA on GraphPad Prism v7.

Cow 8434 was initially vaccinated with AD8 Unc gp140 during phase 1 and revaccinated with AD8 SOSIP gp140 during phase 2. Increased frequencies were observed at week 33 in V genes IGHV1-7*02_S8117, IGHV1-14*01 and IGHV1-27*01_S2303, and at week 59 an increase was observed in genes IGHV1-7*01, IGHV1-7*02 and IGHV1-27*01 in addition to the previous genes mentioned at week 33. Decreased presence of genes IGHV1-10*01, IGHV1-17*01_S3945 and IGHV1-17*01_S5870 was observed at both timepoints compared to IgM libraries. Again, the last two genes mentioned were also the most expressed genes in IgM libraries at both timepoints (figure 5-16 A). When D genes were analysed, a similar trend was observed only for gene IGHD8-2*01, which decreased. Genes IGHD5-3*01 and IGHD6-2*01 showed an increase at week 33, while decreased at week 59, while gene IGHD3-1*01 decreased at week 33 and increased at week 59. Individually, at week 33 gene IGHD4-1*01 showed an increase and gene IGHD5-2*01, a decrease. In addition, genes IGHD1-1*01 and IGHD1-2*01 exhibited a decrease at week 33 (figure 5-16 B). Finally, same as in cow 35, gene IGHJ2-4*01 showed a decrease in frequency at both timepoints while an increase of gene IGHJ1-6*01 was observed (figure 5-16 C).

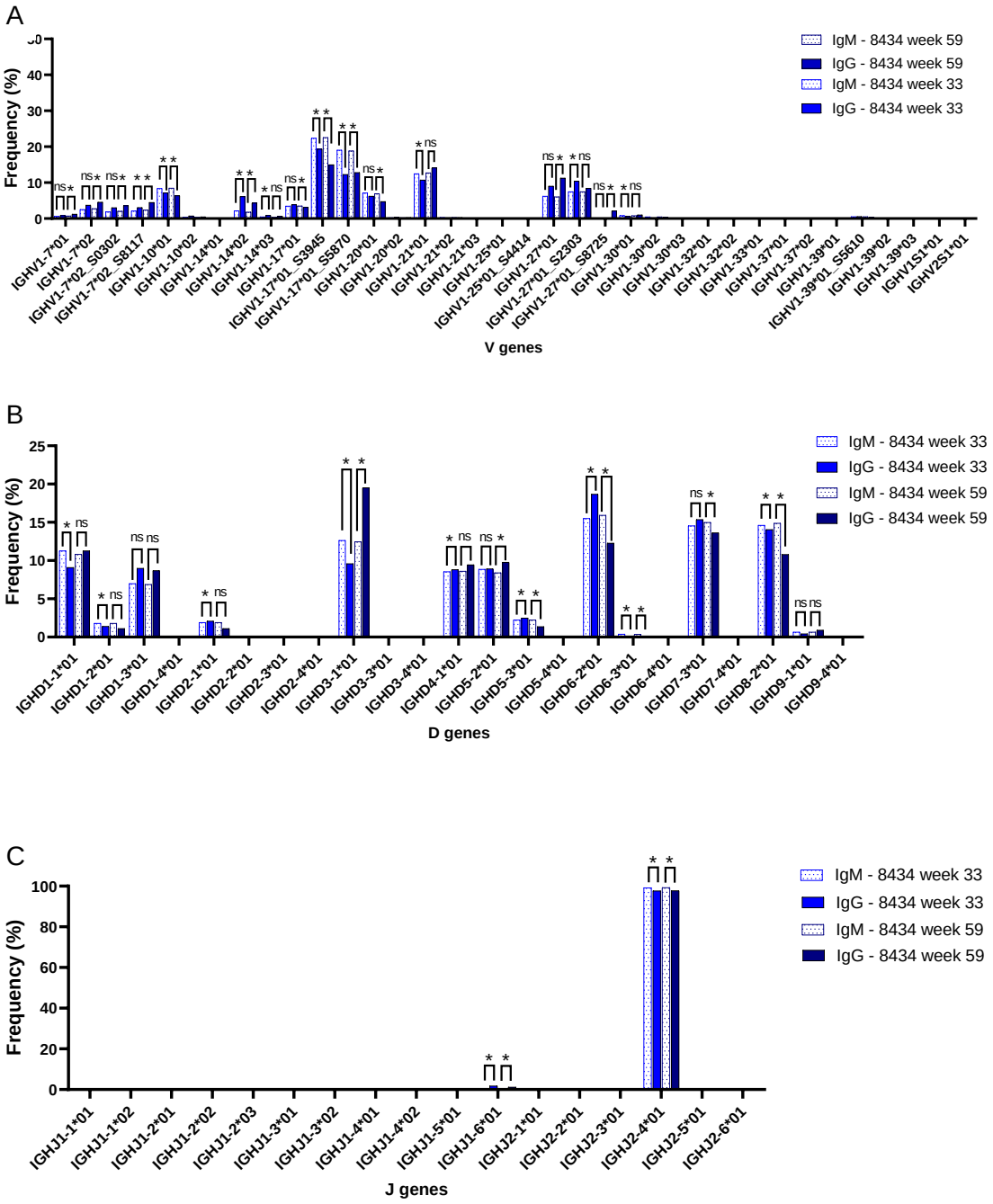


Figure 5-16: Gene assignment in Multiplex libraries for cow 8434 (phase 1: AD8 Unc gp140; phase 2: AD8 SOSIP gp140). Analysis of sequences was performed using IgDiscover. Data from IgM libraries (open bars) and IgG libraries (filled bars) at week 33 (light blue) and at week 59 (dark blue) was done for V (A), D (B) and J (C) genes. Statistical analysis was performed by a 2-way ANOVA on GraphPad Prism v7.

Cows 609 and 617 were vaccinated during phase 1 with KNH1/BG505 SOSIP gp140 and during phase 2 with BG505 SOSIP gp140. Because there was no change in immunogen at week 33 and week 59 for these animals, no significant differences in gene usage were expected when comparing IgG libraries at both timepoints. Indeed, gene frequencies for V, D and J genes were similar at both timepoints and displayed a similar behaviour. Both cows showed a decrease in presence of genes IGHV1-7*01, IGHV1-7*02 and its related alleles at weeks 33 and 59 compared to IgM, along with gene IGHV1-17*01_S3945, while an increased frequency of genes IGHV1-14*02, IGHV1-20*01 and IGHV1-27*01 plus related alleles was observed at both timepoints compared to IgM (figures 5-17 A and 5-18 A). In D genes, increase of gene IGHD1-1*01 was observed at week 33, and of IGHD1-3*01 at both timepoints, while frequency of genes IGHD5-3*01, IGHD7-3*01, IGHD8-2*01 and IGHD9-1*01 decreased (figure 5-17 B and 5-18 B). Lastly, as observed in cows 35 and 8343, a decrease of frequency in gene IGHJ2-4*01 was detected, while a significant increase of gene IGHJ1-6*01 was reported (figure 5-17 C and 5-18 C).

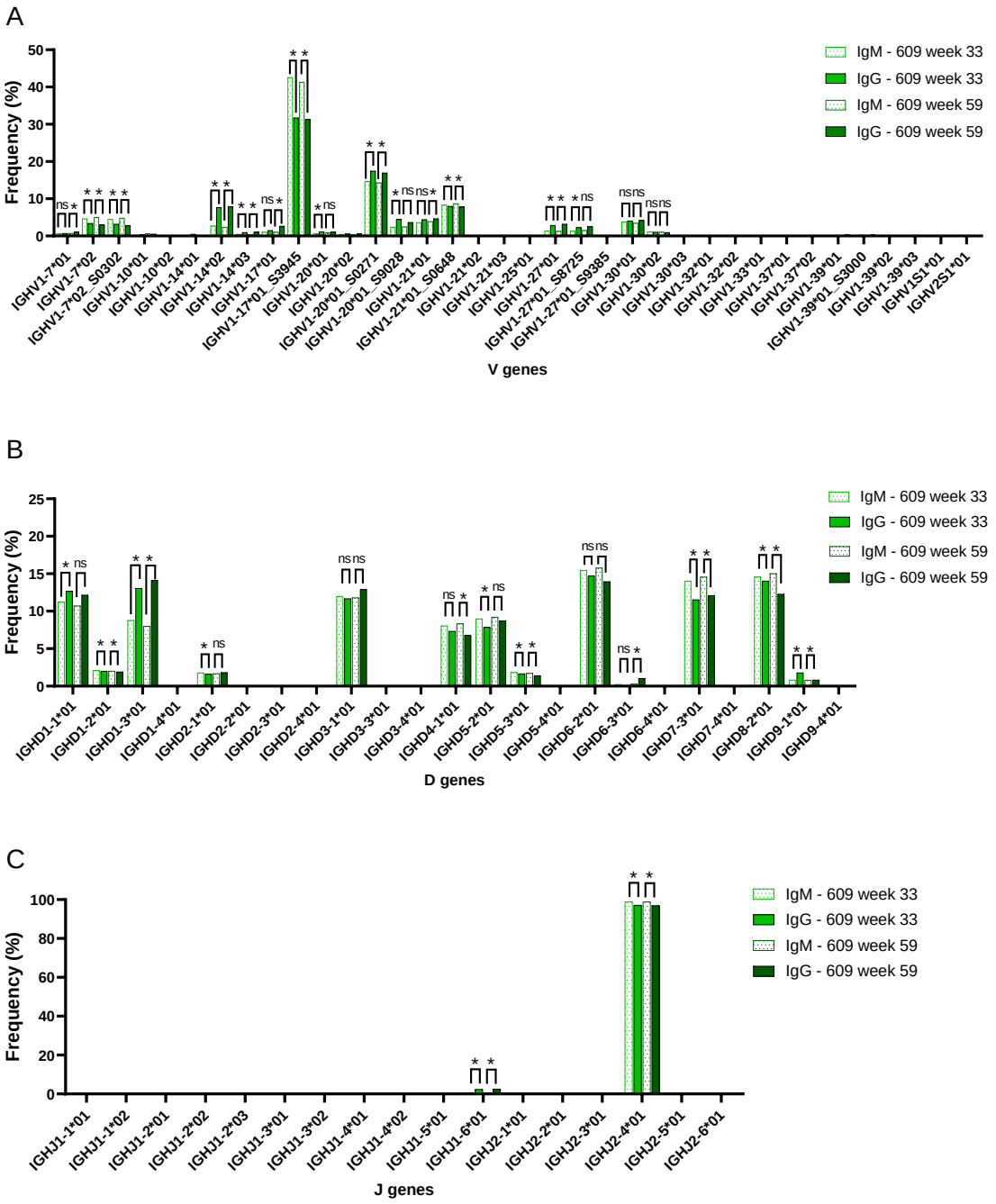


Figure 5-17: Gene assignment in Multiplex libraries for cow 609 (phase 1: KNH1/BG505 SOSIP gp140; phase 2: BG505 SOSIP gp140). Analysis of sequences was performed using IgDiscover. Data from IgM libraries (open bars) and IgG libraries (filled bars) at week 33 (light green) and at week 59 (dark green) was done for V (A), D (B) and J (C) genes. Statistical analysis was performed by a 2-way ANOVA on GraphPad Prism v7.

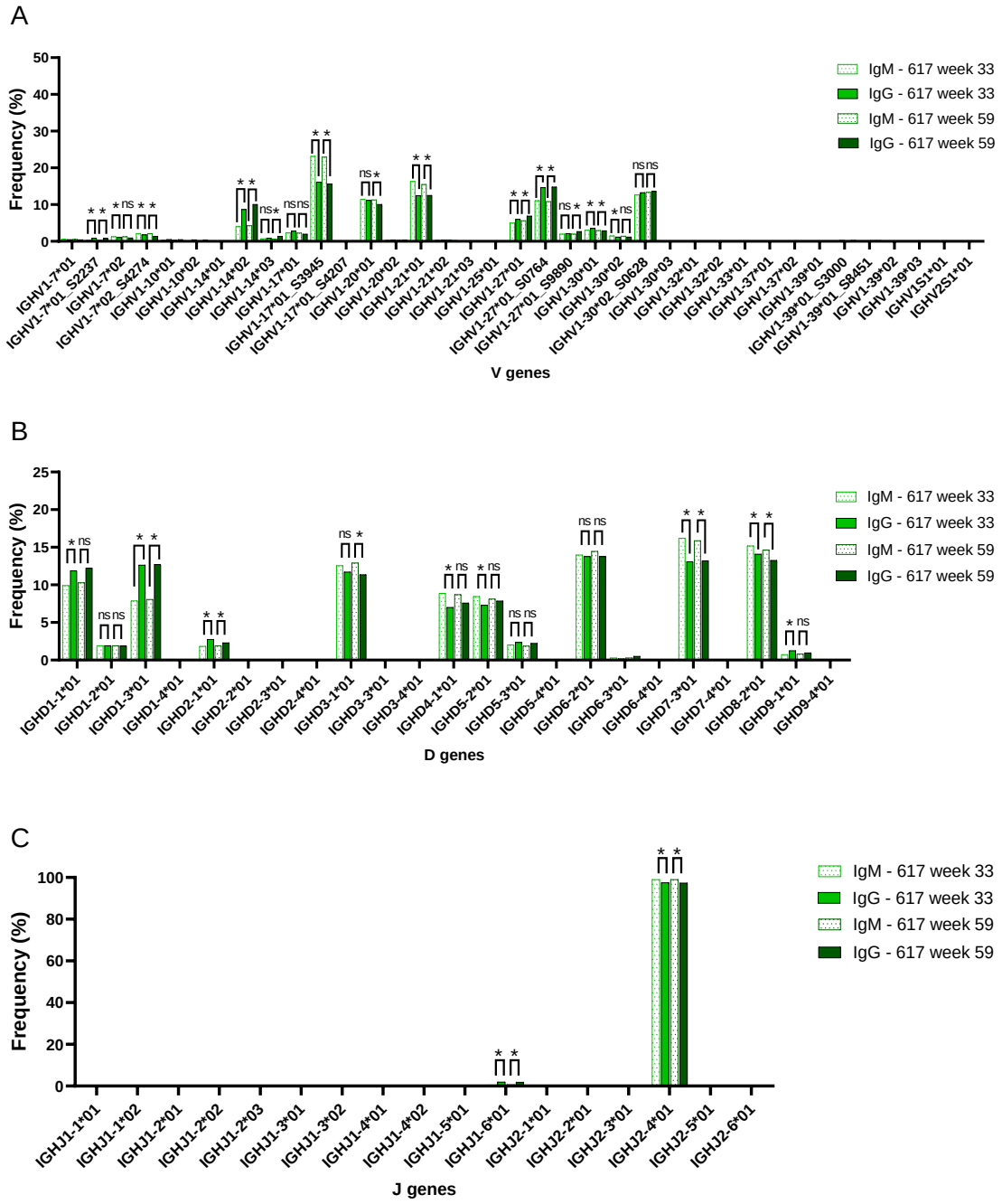


Figure 5-18: Gene assignment in Multiplex libraries for cow 617 (phase 1: KNH1/BG505 SOSIP gp140; phase 2: BG505 SOSIP gp140). Analysis of sequences was performed using IgDiscover. Data from IgM libraries (open bars) and IgG libraries (filled bars) at week 33 (light green) and at week 59 (dark green) was done for V (A), D (B) and J (C) genes. Statistical analysis was performed by a 2-way ANOVA on GraphPad Prism v7.

An important difference between gene assignments observed in cows from groups AD8 Unc 500 group and KNH1/BG505 SOSIP 100 group was the trend observed in genes from the IGHV1-7 family. While in AD8 Unc 500 group these genes presented a higher frequency after vaccination in IgG libraries compared to IgM, a decreased frequency of these genes was observed in animals from KNH1/BG505 SOSIP 100 group. This is important considering that genes from the IGHV1-7 family participate in the formation of ultralong CDRH3s and thus could be implicated in the production of antibodies with ultralong CDRH3, which was higher in animals from AD8 Unc 500 group compared to KNH1/BG505 SOSIP 100 group.

In order to evaluate the production of ultralong IgGs, merged sequences were analysed through AbMining ToolBox. First, sequences were analysed in terms of total CDRH3 sequences (table 5-7). While the total amount CDRH3 sequences obtained by this library was similar to the number obtained from Multiplex IgM library, the amount of unique CDRH3 in the IgG library was only around 10% of all sequences, in contrast with the 25% observed in IgM library (compare with table 5-5). This indicates the presence of clonal expansion, but it additionally shows that library depth is not enough to find unique CDRH3s. It is, however, useful to determine clonal families within the IgG repertoire at different timepoints.

Table 5-7: Summary of sequences obtained in IgG Multiplex PCR libraries. Number of total assembled reads, CDRH3 detected and unique CDRH3 detected in Multiplex PCR library for IgG sequences are indicated on table for week 33 (phase 1 of vaccination) and week 59 (phase 2 of vaccination).

Group: AD8 Unc 500				
Cow: 35	week 33		week 59	
total CDRH3	164295	155242	211305	140451
Unique CDRH3	24348	22131	19856	11786
Assembled reads	207770	195362	255990	180237
Cow: 8434	week 33		week 59	
total CDRH3	169861	169647	163732	200839
Unique CDRH3	16962	16808	16064	19000
Assembled reads	198278	197409	194868	239193
Group: KNH1/BG505 SOSIP 100				
Cow: 609	week 33		week 59	
total CDRH3	199100	179642	186666	161690
Unique CDRH3	24446	23602	23624	21404
Assembled reads	239973	219500	231815	201686
Cow: 617	week 33		week 59	
total CDRH3	188295	188116	218930	196056
Unique CDRH3	24980	25629	29161	28171
Assembled reads	243551	246842	267763	244708

When unique sequences were analysed by CDRH3 length, distributions at week 33 were different to the ones observed at week 59, unlike in IgM libraries (figure 5-19). Both groups of animals presented a population with ultralong CDRH3 ranging between 40 and 55 residues. Interestingly, a new population of ultralong CDRH3 over 55 residues appeared in cows from AD8 Unc 500 group at both timepoints, which is not present in KNH1/BG505 SOSIP 100 group. An analysis of the distribution frequencies according to CDRH3 length can be found in figure 5-20, and shows that around 2.5% of all sequences in both groups presented CDRH3 between 40 and 55 residues, and around the same percentage of sequences over 55 residues was found in the AD8 Unc 500 group, while a significant decrease was observed in the population of sequences with CDRH3 over 55 residues in the KNH1/BG505 group.

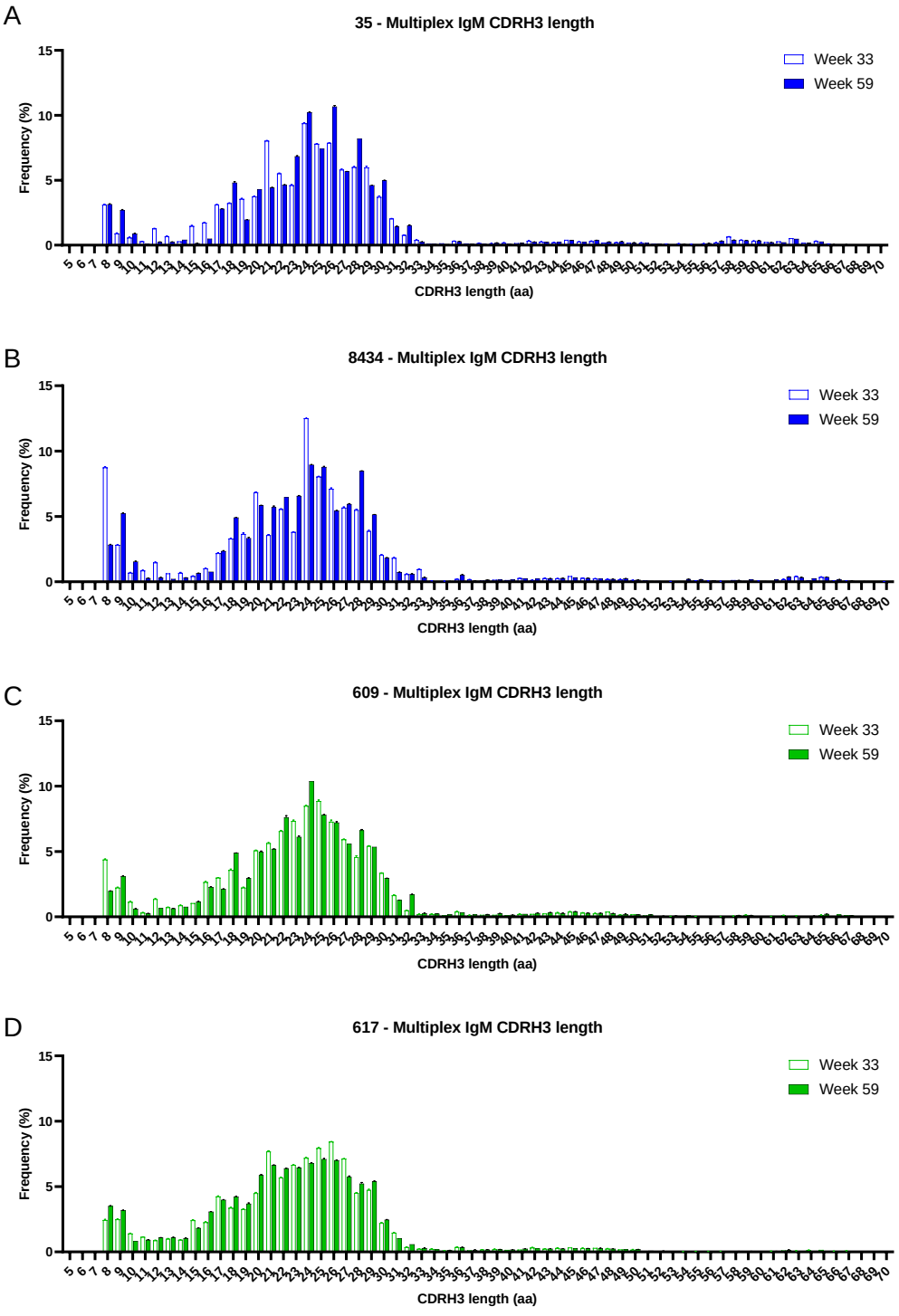


Figure 5-19: CDRH3 length distribution in IgG Multiplex libraries for vaccinated cows. Analysis of sequences from cows vaccinated with AD8 Unc gp140 (blue, A and B) or with KNH1/BG505 SOSIP gp140 (green, C and D) was performed using AbMining ToolBox. Bars show length frequency at week 33 (open bars) and week 59 (filled bars).

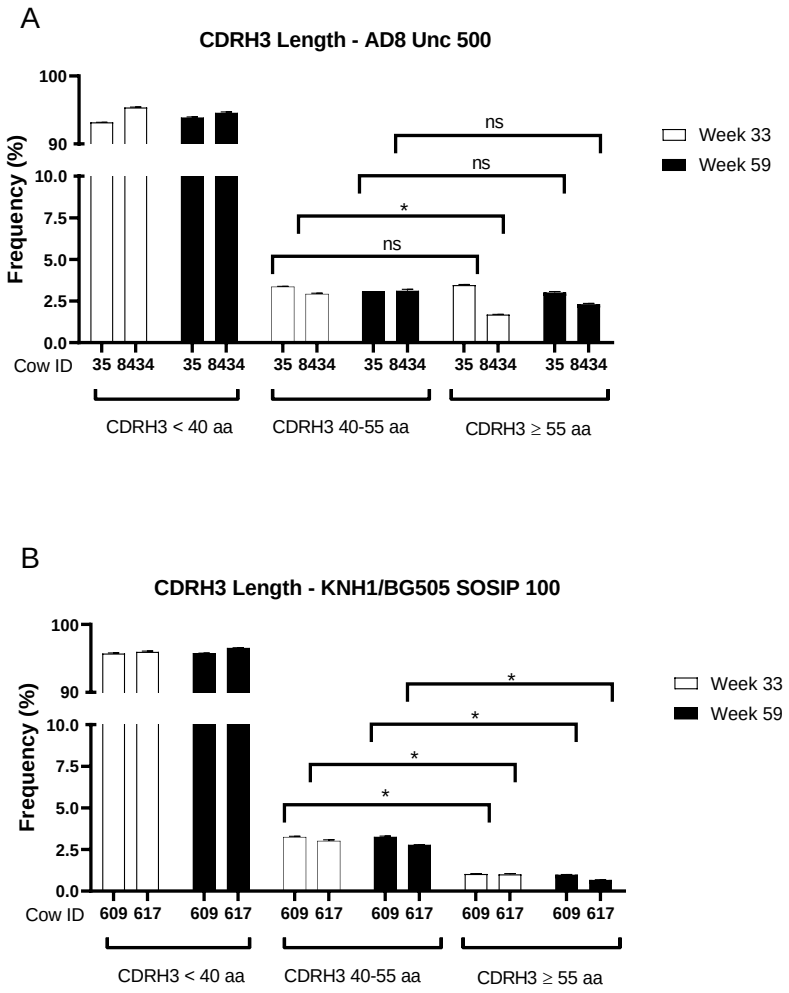


Figure 5-20: CDRH3 length observed for IgG Multiplex library. Total frequency of CDRH3 lengths observed in average-sized (< 40 amino acids) and ultra-long CDRH3 sequences (between 40 and 55 amino acids and over 55 aminoacids) were plotted as bar graph. Sequences from phase 1 (week 33, open bars) and phase 2 (week 59, filled bars) were counted in all animals analysed. Ns: not significant. *: p value below 0.05.

Together with the information obtained by IgDiscover, these results suggest that both AD8 Unc gp140 and KNH1/BG505 SOSIP gp140 induced the production and later expansion of IgGs with ultralong CDRH3. However, cows vaccinated with AD8 Unc gp140 and revaccinated with AD8 SOSIP gp140 or BG505 SOSIP gp140, induced the formation of antibodies with ultralong CDRH3 above 55 residues at the same rate as

antibodies with shorter CDRH3 (40-55 aa), whereas KNH1/BG505 SOSIP gp140 did not. This does not imply the absence of antibodies with CDRH3 above 55 residues in cows from KNH1/BG505 SOSIP 100 group. Indeed, generation of ultralong CDRH3 bovine antibodies with neutralising activity against HIV-1, using BG505 SOSIP gp140 as vaccine, has been proven, since NC-Cow1 presented 60 residues (NC-Cow1). It is possible that likelihood of obtaining antibodies with neutralising activity against broad strains of HIV-1 would be high among few candidates from this small group.

5.8 Analysis of SHM levels in IgG libraries using custom databases

In order to determine the level of affinity maturation induced by each vaccine, SHM levels were analysed in V segments of all animals at week 33 and week 59 by IgDiscover. Since gene expression measurements used different primers for IgG and IgM libraries, it is not possible to perform a direct comparison between SHM levels found in IgM and IgG libraries, but comparisons between timepoints can be done using genes expressed in both libraries. Nevertheless, a threshold of 5% was used to determine mutation rates between animals. Overall, all animals with genes in common between IgM and IgG libraries presented a higher percentage of SHM in IgG libraries for both timepoints. It is important, however, to note that binding specificity of these antibodies is unknown.

Cow 35 presented SHM levels above 5% in all sequences. IGHV1-32*01 presented the highest degree of genetic difference at week 33 (12.5%) and at week 59 (15%). Additionally, IGHV1-39*01_S9680 showed the second highest level of SHM at week 59 (10.5%). On the contrary, gene with the lowest level of gene difference was IGHV1-7*01 at week 33 (5%) and at week 59 (6%) (Figure 5-21 A and B).

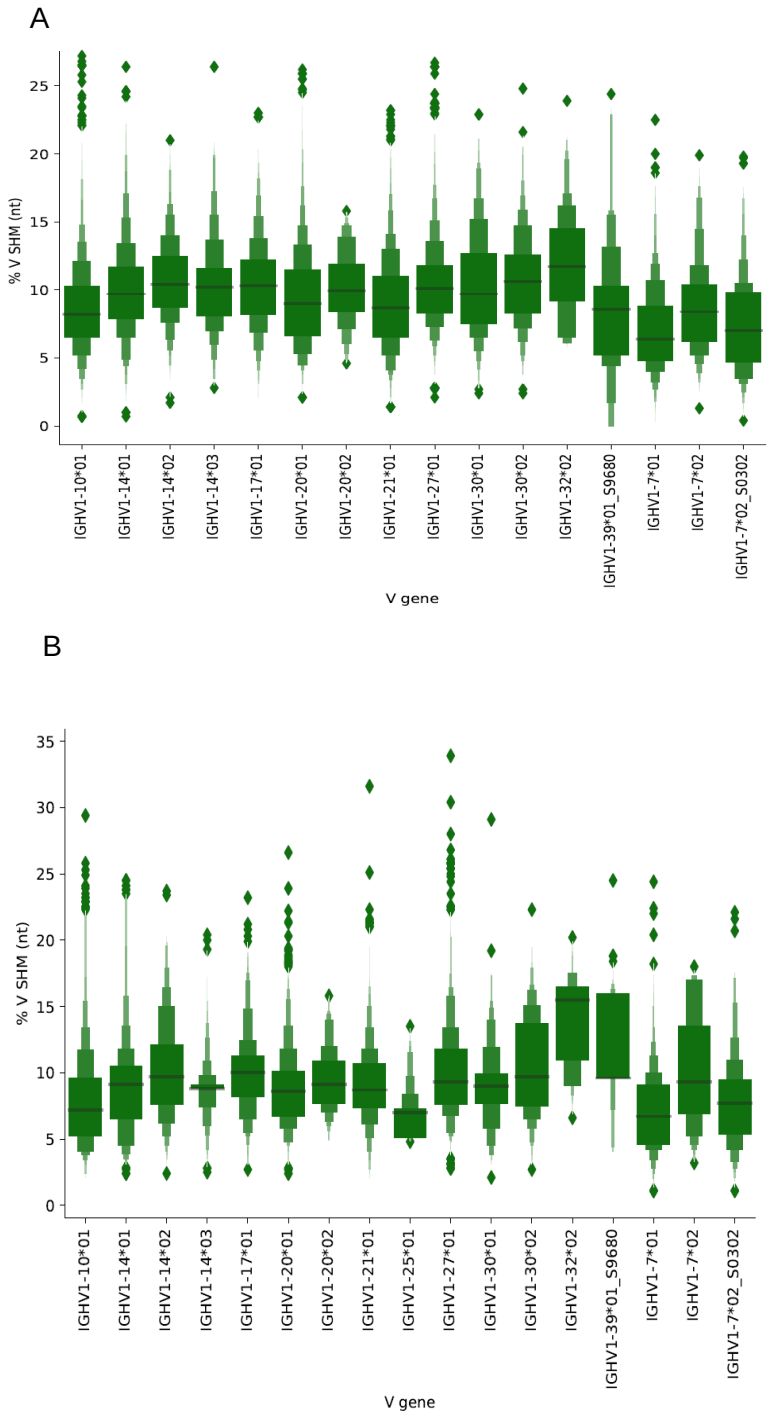


Figure 5-21: SHM rates observed in IgG libraries from cow 35 (phase 1: AD8 Unc gp140; phase 2: BG505 SOSIP gp140). Sequences at week 33 (A) and at week 59 (B) were analysed through IgDiscover, and mutations found in all sequences were plotted in R.

Cow 8434 had levels of SHM higher than 5% in all sequences at week 33, and almost in all sequences at week 59 except for gene IGHV1-7*02_S8117 (4.5%), which presented a higher mutation rate at week 33 (6%). The decrease observed at week 59 might indicate that sequences that expanded during phase 2 of vaccination, formed by this germline gene, were those with low levels of SHM, thus indicating that BCRs formed using this gene already presented high affinity for AD8 SOSIP gp140 when phase 2 of vaccination occurred. In contrast, genes with the highest mutation rates were IGHV1-39*01_S5610 (11%) and IGHV1-27*01_S8725 (11%). Compared to cow 35, cow 8434 presented sequences with lower mutation rates (figure 5-22 A and B).

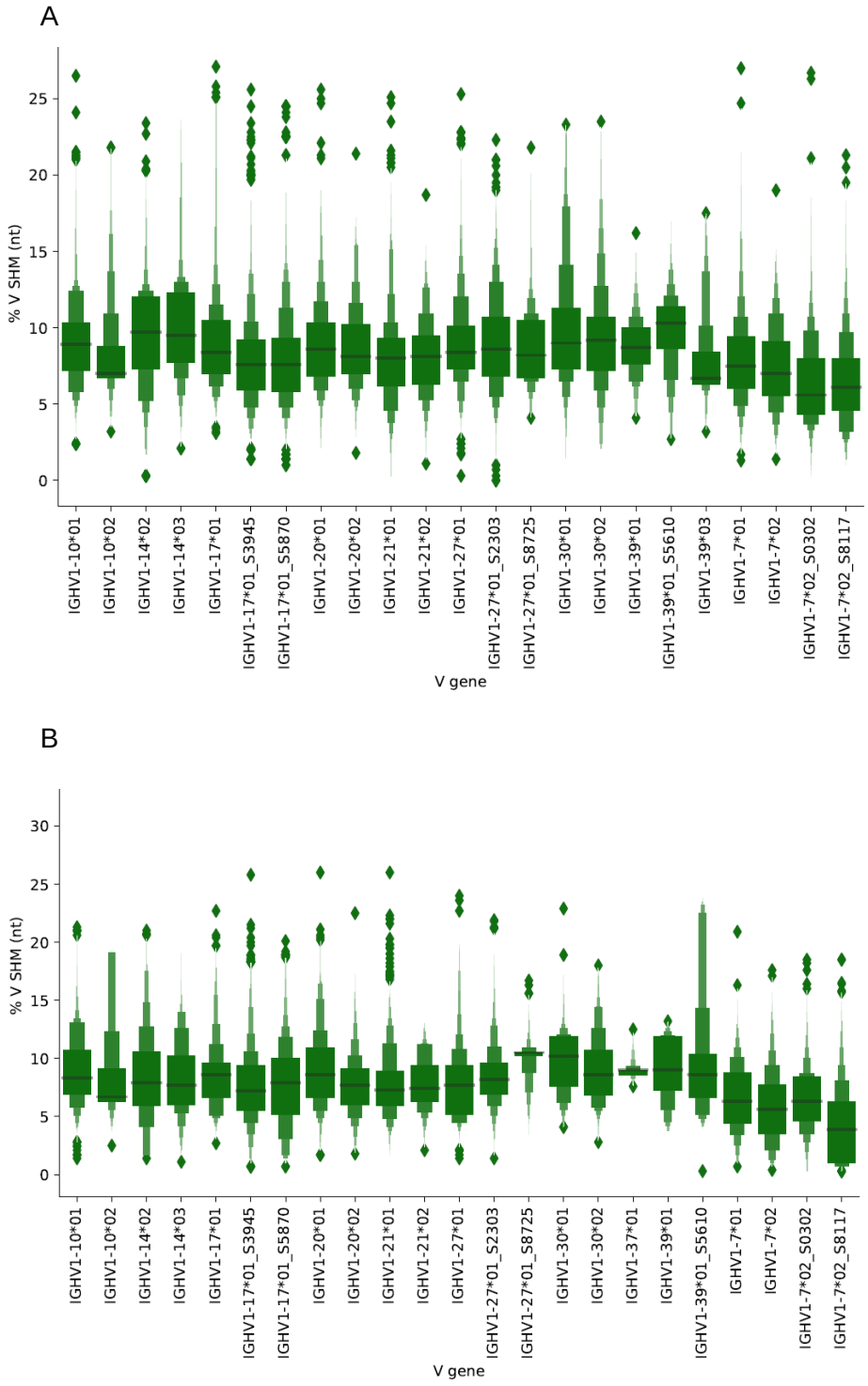


Figure 5-22: SHM rates observed in IgG libraries from cow 8434 (phase 1: AD8 Unc gp140; phase 2: AD8 SOSIP gp140). Sequences at week 33 (A) and at week 59 (B) were analysed through IgDiscover, and mutations found in all sequences were plotted in R.

Cow 609 presented SHM rates higher than 10% in half of the germline genes at week 33, and in almost all germline V genes at week 59. IGHV1-10*02 presented the highest mutation rate at week 33 (14%) and decreased at week 59 to 12.5%, showing the same levels of SHM than genes IGHV1-10*01, IGHV1-14*03 and IGHV1-7*01 at this timepoint. At both timepoints, the gene with lowest mutation rate was IGHV1-7*02_S0302 (7.5% at both week 33 and week 59). Similarly, low levels of SHM were observed in gene IGHV1-39*03 at week 33 (8%) and IGHV1*39_S3000 at week 59 (7.5%) (figure 5-23 A and B).

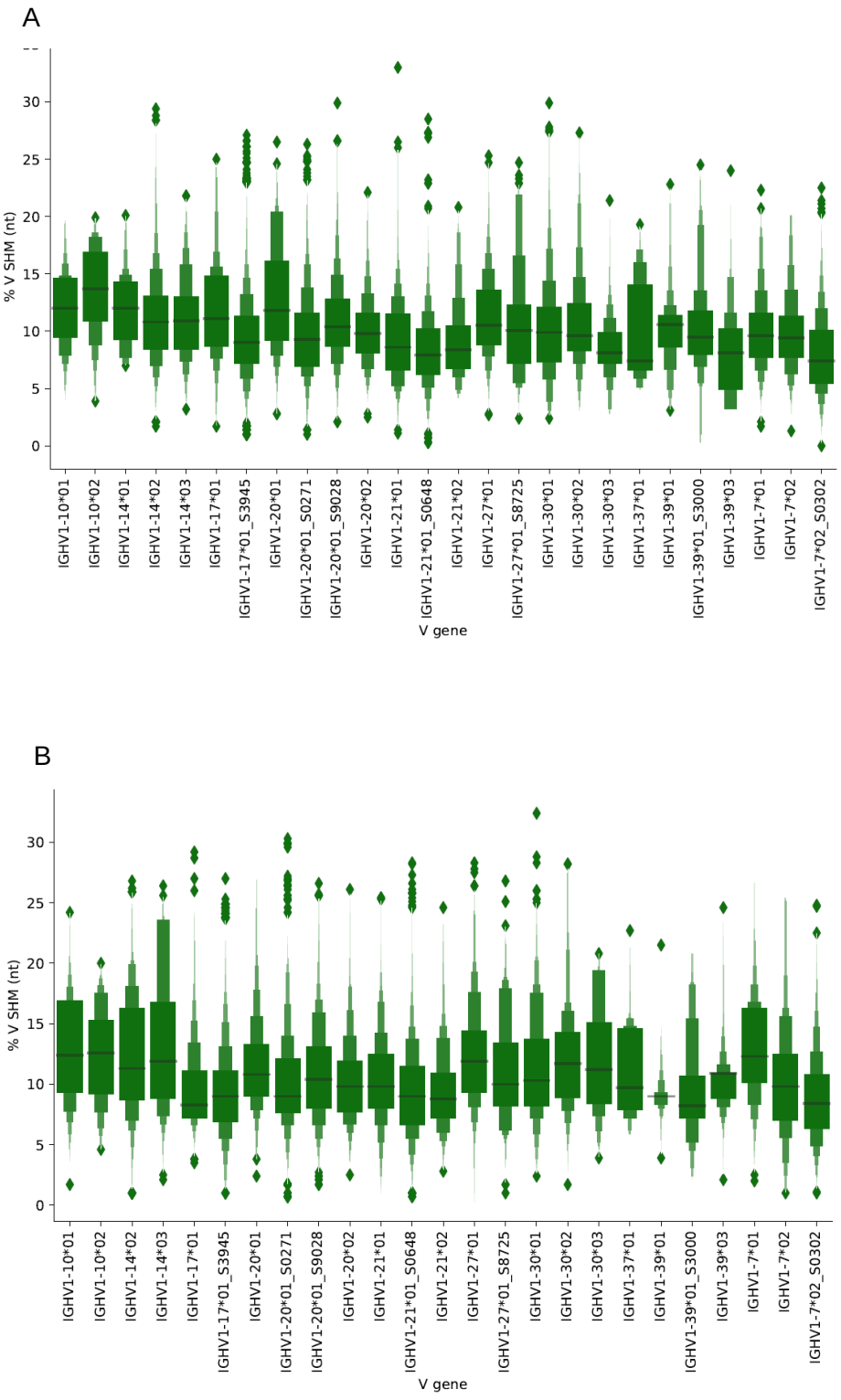


Figure 5-23: SHM rates observed in IgG libraries from cow 609 (phase 1: KNH1/BG505 SOSIP gp140; phase 2: BG505 SOSIP gp140). Sequences at week 33 (A) and at week 59 (B) were analysed through IgDiscover, and mutations found in all sequences were plotted in R.

Cow 617 also showed SHM rates above 10% in half of V germline genes at week 33, and almost all genes at week 59. IGHV1-39*01 showed the highest mutation rate at week 33 (12.5%), while IGHV1-30*03 showed the highest mutation rate at week 59 (16%), followed by IGHV1-39*03 (15%). On the contrary, the gene with the lowest SHM rate was IGHV1-7*02_S4274 at both timepoints (7.5% at both week 33 and week 59). Compared to cow 609, cow 617 presented higher rates of mutation in its sequences but achieved the same mutation rates at their members of the IGHV1-7 family (figure 5-24 A and B).

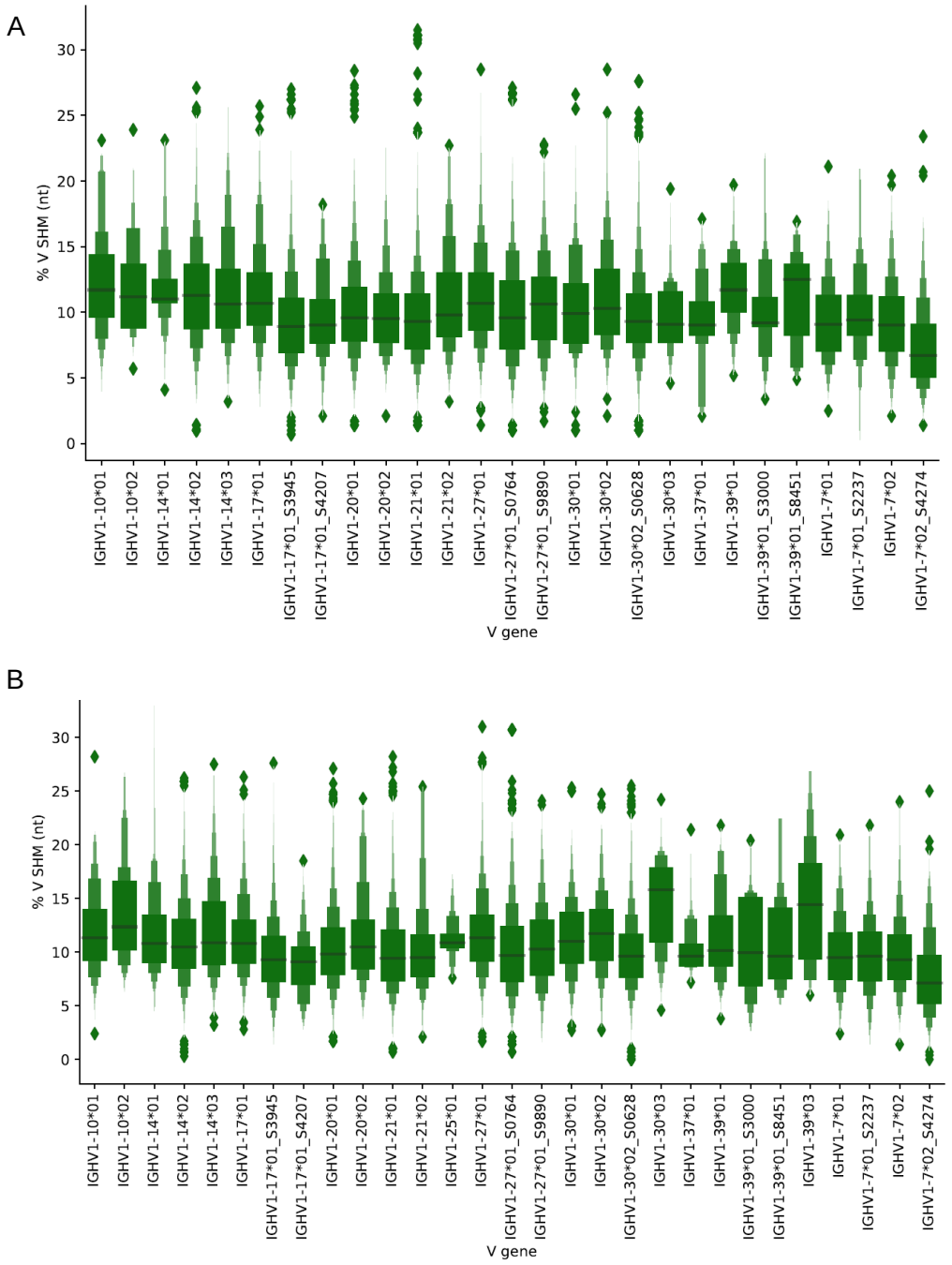


Figure 5-24: SHM rates observed in IgG libraries from cow 617 (phase 1: KNH1/BG505 SOSIP gp140; phase 2: BG505 SOSIP gp140). Sequences at week 33 (A) and at week 59 (B) were analysed through IgDiscover, and mutations found in all sequences were plotted in R.

Based on these results, we conclude that KNH1/BG505 SOSIP gp140 generated a higher degree of SHM than AD8 Unc gp140. Moreover, SHM levels observed were higher at week 59 compared to week 33 in animals from KNH1/BG505 SOSIP 100 group, while animals from AD8 Unc 500 group presented no significant difference of SHM in almost all genes between timepoints. The latter could indicate that change of immunogen did not increase the amount of SHM observed in sequences at week 59. Finally, genes with the highest level of SHM in almost all animals were part of the family IGHV1-39 (except cow 609), while genes with the lowest level of SHM in all animals were members of the family IGHV1-7.

5.9 Analysis of bovine monoclonal antibodies isolated from animals vaccinated with different HIV-1 Env gp140 vaccines

To recapitulate at this point, IgM libraries were produced for cows from groups AD8 Unc 500 and KNH1/BG505 SOSIP 100, which allowed the discovery of novel alleles in each animal. Then, IgG libraries were produced from same animals and gene usage and levels of SHM were characterised. The characterisation of each cow's IgG repertoire after phase 1 and phase 2 of vaccination gives important information about the unique IgGs formed and expanded during vaccination, which allows the analysis of clonal families and allows the deduction of unmutated common ancestor (UCA) sequence for mAbs from vaccinated animals.

In parallel to this project, our lab isolated several monoclonal antibodies (mAbs) from cows 35, 8434 and 617, and their binding and neutralising activity against a diverse panel of HIV Env pseudoviruses has been characterised (Heydarchi et al., in preparation). A summary of mAbs isolated can be found in table 5-8. Same peripheral blood mononuclear cells (PBMCs) used for IgM and IgG library preparation were used for B cell sorting, from which these mAbs were isolated, meaning that if IgG libraries

had enough depth, then mAbs sequences should be found in such libraries. Moreover, a phylogenetic analysis can expose families where mAbs sequences belong to, helping to elucidate the previous events of affinity maturation that led to their formation. Lastly, information regarding germline genes used, CDRH3 length and levels of SHM can be inferred by using both IgDiscover and AbMining Toolbox.

Table 5-8: List of monoclonal antibodies isolated from animals from AD8 Unc 500 and KNH1/BG505 SOSIP 100 groups. B cells were sorted from PBMCs from cows 35, 8434 (blue) and 617 (green) at weeks 33 and 59, and antibody sequences were cloned and produced (Heydarchi et al., in preparation). Binding against AD8 SOSIP gp140 and neutralising activity against heterologous Env are indicated for each mAb on the table, with positive activity (Y) indicated in red, while negative activity (N) indicated in white.

Group	Cow	Week	Name	Binding	Neutralisation
AD8 Unc 500	35	33	H563	Y	Y
			H512	N	N
			H529	N	N
			H594	N	N
			H680	N	N
		59	H663	Y	Y
			H782	Y	Y
			H699	N	N
	8434	33	H33	Y	Y
			H29	N	N
			H52	N	N
			H54	N	N
			H25	N	N
			H34	N	N
			H36	N	N
			H79	N	N
			H82	N	N
		59	H130	Y	Y
			F2	Y	Y
			D1	Y	Y
			H198	Y	Y
			H105	N	N
			H128	N	N
			H134	N	N
			H135	N	N
			H5	N	N
			H10	N	N
			H135	N	N
			H144	N	N
			H150	N	N
			H167	N	N
KNH1/BG505 SOSIP 100	617	59	H1967	Y	Y
			H2000	Y	Y
			H2114	Y	Y
			H1905	Y	Y
			H2010	Y	Y
			H2129	Y	Y
			H2028	Y	Y
			H1872	Y	Y
			H1842	Y	Y
			H2023	N	N
			H1796	N	N
			H1851	N	N
			H1887	N	N
			H2087	N	N

Using both IgDiscover and AbMining ToolBox, germline genes for each mAb and CDRH3 sequence and length were calculated. For ultralong CDRH3 sequences, J gene assignment was missing as described before. In those cases, sequences were aligned with germline J genes to confirm origin using SnapGene. The results showed that novel alleles discovered in cows 8434 and 617 were used as germline genes assigned by IgDiscover, whereas cow 35 did not use any novel allele to produce the mAbs isolated from this animal. A diversity of CDRH3 lengths were found in both neutralising and non-neutralising antibodies, with the shortest sequence of 9 residues and the longest of 69, both found in cow 8434 at week 33 (Table 5-9). Frequency of gene usage was also calculated for all mAbs. Comparing neutralising and non-neutralising antibodies, there was no significant difference in expression of gene families between both groups. Both groups presented high frequency of genes of the families IGHV1-7, IGHD8-2 and IGHJ2-4 (table 5-10).

Table 5-9: Gene assignment and CDRH3 sequences of mAbs isolated from cows vaccinated with different HIV-1 Env vaccines. Sequences from monoclonal antibodies isolated from cows 35, 8434 (vaccinated with AD8 Unc gp140, blue) and 617 (vaccinated with KNH1/BG505 SOSIP, green) were evaluated using custom databases constructed by IgDiscover and AbMining ToolBox. Table informs gene assignment, CDRH3 sequence and CDRH3 length. Sequences in red: neutralising antibodies.

Group	Cow	Week	name	V_gene	D_gene	J_gene	CDR3 sequence	CDR3 length
AD8 Unc 500	35	33	H563	IGHV1-7*01	IGHD8-2*01	IGHJ2-4*01	CTTVHQETKHTCPDDYTSDHACACDGCCGGGYLCYFSCYANVAGGCSGSRGRNYTYTYGFHVDDW	65
			H680	IGHV1-10*01	IGHD5-3*01	IGHJ2-4*01	CAKGTYGAAVSACFSYSSGYIDAW	24
			H512	IGHV1-10*01	IGHD8-2*01	IGHJ2-4*01	CAKHGTDDYSYWWYATTNGRSKYIDSW	27
			H529	IGHV1-7*01	IGHD8-2*01	IGHJ2-4*01	CTTVHQKTTKTCPDGSSDECGCGCISGCEWICSHCCPYARSGRYGVKTTNYIEAYEHHIEAW	62
			H594	IGHV1-7*01	IGHD8-2*01	IGHJ2-6*01	CTTVHQQTETTRNCPDGWTRGYDCGNGCDCSAHVCSVYRTRPVWRDRGCRAYSDRYTYEWYVETW	65
		59	H782	IGHV1-10*01	IGHD6-2*01	IGHJ2-4*01	CAKLYSRAFYTGDGCASYLTSDGWGLQVDAW	31
			H663	IGHV1-7*01	IGHD8-2*01	IGHJ2-4*01	CVTVHQKTVEICPDGYIDVSSCSRRGLCCSGDCCNSRCCAGNVVYAVGAPYSYTYEYHVDAW	62
			H699	IGHV1-7*02	IGHD8-2*01	IGHJ2-4*01	CTTVHQKTNTQKNCPDDRSHECTRPCPEGSDVCCCRFPCNWNDVTSCSSHIYTSTYEFRVDAW	63
	8434	33	H33	IGHV1-21*01	IGHD6-2*01	IGHJ2-4*01	CGAWYGNDYCTTLFVEGNVDPW	22
			H34	IGHV1-17*01_S3945	IGHD8-2*01	IGHJ2-4*01	CTKDRALAW	9
			H36	IGHV1-14*02	IGHD1-3*01	IGHJ2-4*01	CGSCESSNVYDSDCNYNW	18
			H54	IGHV1-17*01_S3945	IGHD7-3*01	IGHJ2-4*01	CVKSFQTSNGWRTDGCYGVASTSNYVDAW	29
			H79	IGHV1-17*01_S3945	IGHD8-2*01	IGHJ2-4*01	CGSCESSNVYDSDCNYNWGQGLLVTVSSSXHQGPIGLPPGTLLQEHLW	48
			H82	IGHV1-17*01_S3945	IGHD3-1*01	IGHJ2-4*01	CVKGSKTYVYYGGCPFATTYYIDAWGQGLLVTVSQLAPRAHRSSPWHPPPRAPLGAQRPW	60
			H25	IGHV1-27*01_S2303	IGHD4-1*01	IGHJ2-4*01	CAKFTRSICWRRYSPSSYNKNYLDAWGRGLLVTVSQLAPRAHRSSPWHPPPRAPLGAQRPW	61
			H52	IGHV1-7*02_S0302	IGHD8-2*01	IGHJ1-2*01	CGTVYQKTTQSCPDGWRASDYCGSWISACSPCICCGRGSYPGYEDCADWSCGGHTSTHELYLEAW	65
			H29	IGHV1-7*02_S0302	IGHD5-2*01	IGHJ2-4*01	CTTVHQKTQTGECDCRDYYSLTYIYGCSNGCLCATSGCCDYIRNGADGGSGRRCATTAFHKYEFHVDSW	69
		59	H130	IGHV1-21*01	IGHD7-3*01	IGHJ2-4*01	CAKEVYGGYGYGGANCHGYGYGSDAW	26
			F2	IGHV1-21*01	IGHD7-3*01	IGHJ2-4*01	CVKYGHRNRGCYLNSWGGGQTYVDAW	26
			D1	IGHV1-7*02	IGHD8-2*01	IGHJ1-6*01	CTTVHQKTKKSCPDDYRGVYECSRPYGTGADACCRALSWGRYDCDCTNTYTYTYTYEFHVDAW	63
			H198	IGHV1-7*02_S0302	IGHD8-2*01	IGHJ2-4*01	CTSVHQKTISGCPDGTVARDTCSRPDSCCSGNDCCGRSCCAGNYAWSGGASSTNTYQWYVDSW	63
			H144	IGHV1-27*01	IGHD3-1*01	IGHJ2-4*01	CVRCVRGYYLTCAANVDAW	19
			H10	IGHV1-27*01_S2303	IGHD1-3*01	IGHJ2-4*01	CVKRVGPKVYNCGNFHHSYGYVDAS	25
			H167	IGHV1-17*01	IGHD5-2*01	IGHJ2-4*01	CGAVRHLYGGLDCFQNGIGGGCYVDAW	27
			H105	IGHV1-7*02	IGHD8-2*01	IGHJ2-4*01	CTTVHQKTNTQKNCPDDRSHERTRPCPEGSDVCCCRFPCNWNDVTSCSSYIYTSTYEFRVDAW	63
			H128	IGHV1-7*02	IGHD8-2*01	IGHJ2-4*01	CTTVHQKTNTQKNCPDDRSHECTRPCPEGSDVCCCRFPCNWNDVTSCSSYIYTSTYEFRVDAW	63
			H135	IGHV1-7*02	IGHD8-2*01	IGHJ2-4*01	CTTVHQETQKSCPEGYKHGFDCCCNYDCAIDDCGDERDCGRSVYGECASQRYIYSYEFYIAAW	63
			H134	IGHV1-7*02_S0302	IGHD8-2*01	IGHJ2-4*01	CSSLYQKRIRSCPVGYTQSANCDRRTSNNCRRSDCCRDRCCSAISCSSVDYSSYEYTYEFYVDTW	65
			H5	IGHV1-7*02	IGHD8-2*01	IGHJ2-4*01	CATVYQKTHEKRTCPDGYTLEHECDYGYDCEFRECCSYWTGMNLPRRCSGDDVSTYNYEFHVDAW	65
			H150	IGHV1-27*01	IGHD1-3*01	IGHJ2-4*01	CVRVLCVDSDAHSVGCDYYLWDQDYHYVDVWGQGLLVTVPQLAPRAHRSSPWHPPPRAPLGAQRPW	66
KNH1/BG505 SOSIP 100	617	59	H2114	IGHV1-20*01	IGHD1-3*01	IGHJ2-4*01	CGRCMYGTCWADVDTW	16
			H1905	IGHV1-7*02_S4274	IGHD6-2*01	IGHJ2-4*01	CGAVHQQTNRRKETCWGGRDTGFGCAWGDTVQPYTYIDTYELHVEAW	47
			H1967	IGHV1-7*02_S4274	IGHD8-2*01	IGHJ1-6*01	CSTVHQKIERSCPEDFTISSFCGSDGCCPLGWGGCRGYYFQCRKIATTTYQFHIDAW	57
			H2000	IGHV1-7*02_S4274	IGHD8-2*01	IGHJ2-4*01	CSTVHQRTEETCPKDYIRSSRCGSRGCCPLGLGDCSWTMHMCTDAATTLEEFHVDAW	57
			H2010	IGHV1-7*02	IGHD4-1*01	IGHJ1-5*01	CTTVHQKTTITCPDNYSGRDGWNSWCTGRCSGYGCCNYYAFPSAWCKAGSSTYEHHVDAW	60
			H1872	IGHV1-7*02_S4274	IGHD8-2*01	IGHJ2-4*01	CSTVHQKTRRSCPSDYSFICDCEGTGSHCCRASNCPYYCNYGRDRMCTGRSNIHEWHVDAW	61
			H1842	IGHV1-7*02_S4274	IGHD8-2*01	IGHJ2-4*01	CSTVHQKTLRSCPSDYPYICDCEDTGSHCCRATNCPYYCNHGRDRMCTGRTYTYEWHVEAW	61
			H2129	IGHV1-7*02_S4274	IGHD8-2*01	IGHJ2-4*01	CSTVHQKTRRSCPVDYYYSCDCEGSGSHCCSASNCPYYCKYGRDRVCTDKHTYSYEWYVDAW	62
			H2028	IGHV1-7*02_S4274	IGHD8-2*01	IGHJ2-4*01	CATVYQKTKKSCPENYEDRCGCVRGLGDDCGCYDCRSVILGIPWTYNRRRTASYTYASDFHVDAW	65
			H1796	IGHV1-7*02	IGHD6-2*01	IGHJ2-4*01	CGAVNQKATRRPCPPDYIDRGSCCAQWGWPCGSSMSIDITYTYEFWVEAW	50
			H1851	IGHV1-7*02_S4274	IGHD8-2*01	IGHJ2-4*01	CTTVHQKTKKGCPDGYDYDSGCGNTDSYGCSAWPCYRVGGGSIRGCCTGTYTYTYEFHVDAW	62
			H1887	IGHV1-7*02	IGHD8-2*01	IGHJ1-3*01	CTTVHQKTNPTGRCPDGYNECVNWSWYGCDCSSWDCYKRASIGDCTKYTSTTYTYEFHVDAW	62
			H2087	IGHV1-7*02	IGHD8-2*01	IGHJ2-4*01	CTTVHQKTNPTGRCPDGYNECVNWSWYGCDCSSWDCYKRASIGDCTKYATTTYTYEFHIDAW	62
			H2023	IGHV1-7*02	IGHD4-1*01	IGHJ2-4*01	CTNVHQETHETTYCPNGEGFRCETSGGTDGLCRCRWGICMLGFRRFRWGDDSGSCTLTHDLHVEAW	66

Table 5-10: Frequency of gene assignment in mAbs isolated from cows vaccinated with HIV-1 Env vaccines. Gene assignment for V, D and J genes was performed by IgBLAST under IgDiscover program pipeline and total frequencies were calculated by Microsoft Excel. Nabs: Neutralising antibodies, nNabs: non-neutralising antibodies.

V genes	Frequency	Freq. Nabs	Freq. nNabs
IGHV1-7	27	12	15
IGHV1-10	3	1	2
IGHV1-14	1	0	1
IGHV1-17	5	0	5
IGHV-20	1	1	0
IGHV1-21	3	3	0
IGHV1-27	4	0	4
D genes	Frequency	Freq. Nabs	Freq. nNabs
IGHD1-3	4	1	3
IGHD3-1	2	0	2
IGHD4-1	3	1	2
IGHD5-2	2	0	2
IGHD5-3	1	0	1
IGHD6-2	4	3	1
IGHD7-3	3	2	1
IGHD8-2	25	10	15
J genes	Frequency	Freq. Nabs	Freq. nNabs
IGHJ1-2	1	0	1
IGHJ1-3	1	0	1
IGHJ1-5	1	1	0
IGHD1-6	2	2	0
IGHJ2-4	38	14	24
IGHJ2-6	1	0	1
Total	44	17	27

Next, SHM rates were analysed for all mAbs sequences. In general, the region with highest mutation rate was CDR2, while the region with lowest SHM was framework 1 (FR1) (Figure 5-25 A). Some differences were noticeable when mutation rates between sequences where compared based on their CDRH3 length. Both neutralising and non-neutralising monoclonal antibodies with ultralong CDRH3 (equal of above 40 residues) presented lower SHM levels in CDR1 and CDR2 compared to short sequences (below 40 residues) (compare Figure 5-25 B and C). In addition, when only ultralong CDRH3 antibodies are analysed, mAbs with neutralising activity presented significantly lower SHM than non-neutralising ultralong CDRH3 mAbs in CDR1 and CDR2 (Figure 5-25 C). A list of SHM rates found in all mAbs isolated can be found on table 9x.

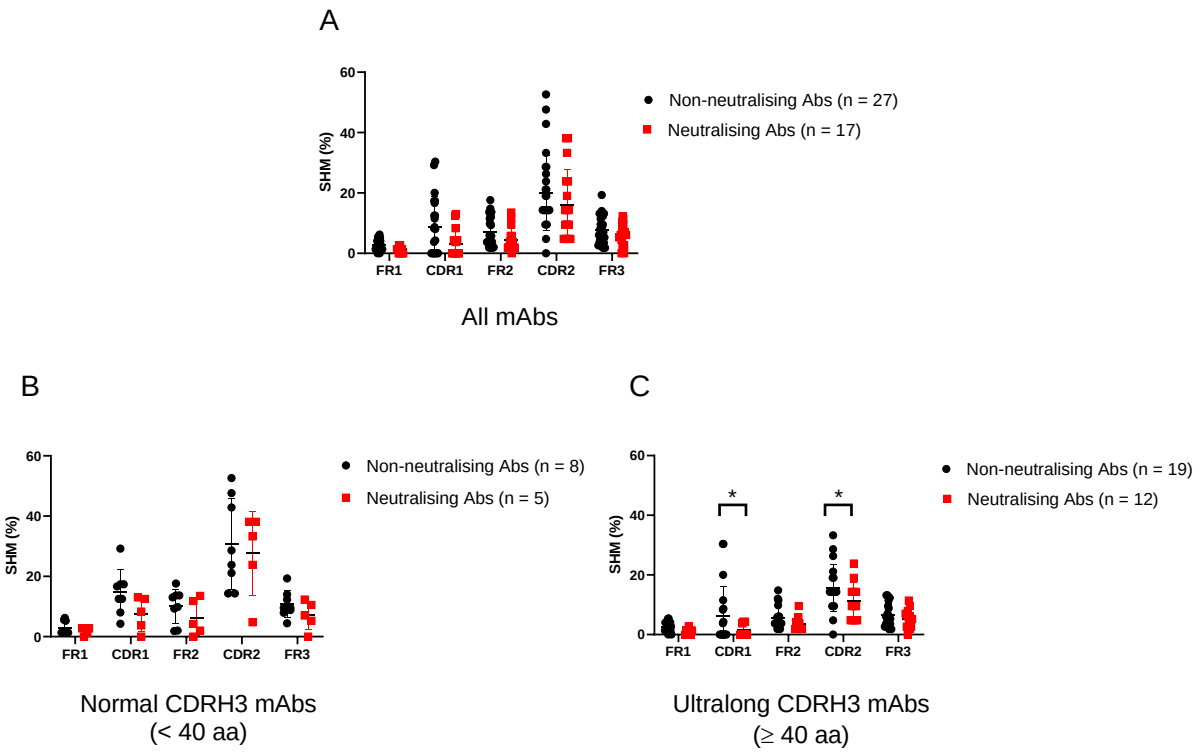


Figure 5-25: Effect of CDRH3 length in SHM rates observed in monoclonal antibodies isolated from HIV-1 Env vaccinated cows. Sequences from monoclonal antibodies isolated from cows 35, 8434 and 617 were evaluated using custom databases constructed by IgDiscover. Sequences were divided into neutralising mAbs (red) or non-neutralising mAbs (black). A) Total sequences isolated. B) Sequences with average size CDRH3. C) Sequences with ultralong CDRH3.

Table 5-11: SHM rates found in monoclonal antibodies isolated from cows vaccinated with HIV-1 Env vaccines. Sequences from monoclonal antibodies isolated from cows 35, 8434 (vaccinated with AD8 Unc gp140, blue) and 617 (vaccinated with KNH1/BG505 SOSIP, green) were evaluated using custom databases constructed by IgDiscover. Sequences in red: neutralising antibodies. Bold: sequences with ultralong CDRH3.

Group	Cow	Week	name	V_gene	CDR3 length	FR1_SHM	CDR1_SHM	FR2_SHM	CDR2_SHM	FR3_SHM	VH_SHM
AD8 Unc 500	35	33	**H563**	**IGHV1-7*01**	**65**	**1.3**	**0**	**1.9**	**9.5**	**2.6**	**3**
			H680	IGHV1-10*01	24	1.4	17.4	9.3	21.1	8.8	8.2
			H512	IGHV1-10*01	27	1.3	4.3	1.9	23.8	9.6	6.6
			H529	**IGHV1-7*01**	**62**	**1.4**	**30.4**	**3.7**	**21.1**	**12.3**	9.4
			H594	**IGHV1-7*01**	**65**	**0**	**0**	**1.9**	**19**	**2.6**	3
		59	H782	IGHV1-10*01	31	2.7	3.7	4.2	33.3	5.3	6.6
			H663	**IGHV1-7*01**	**62**	**1.3**	**0**	**3.8**	**19**	**11.4**	**7.4**
			H699	**IGHV1-7*02**	**63**	**1.4**	**0**	**5.8**	**14.3**	**3.5**	**4**
	8434	33	H33	IGHV1-21*01	22	2.7	13	13.5	38.1	7	9.8
			H34	IGHV1-17*01_S3945	9	5.6	12.5	9.8	14.3	14	8.3
			H36	IGHV1-14*02	18	1.3	17.4	13	52.6	9.6	8
			H54	IGHV1-17*01_S3945	29	1.4	8	2	28.6	4.4	5.5
			H79	**IGHV1-17*01_S3945**	**48**	**4.1**	**8.3**	**9.8**	**14.3**	**13.2**	**8.2**
			H82	**IGHV1-17*01_S3945**	**60**	**5.3**	**11.5**	**6.1**	**33.3**	**6.1**	**10.4**
			H25	**IGHV1-27*01_S2303**	**61**	**4**	**20**	**12**	**9.5**	**6.1**	**11.9**
			H52	**IGHV1-7*02_S0302**	**65**	**1.6**	**0**	**5.9**	**28.6**	**8.8**	**10.1**
			H29	**IGHV1-7*02_S0302**	**69**	**1.4**	**0**	**11.5**	**14.3**	**11.4**	**8.6**
		59	H130	IGHV1-21*01	26	0	0	0	4.8	0	0.3
			F2	IGHV1-21*01	26	1.4	8.3	2	38.1	10.5	8.6
			D1	**IGHV1-7*02**	**63**	**2.7**	**0**	**1.9**	**4.8**	**0**	**1.3**
			H198	**IGHV1-7*02_S0302**	**63**	**0**	**4.3**	**1.9**	**9.5**	**2.6**	**3**
			H144	IGHV1-27*01	19	6.2	12.5	13.7	14.3	7.9	4
			H10	IGHV1-27*01_S2303	25	1.6	16.7	17.6	47.6	12.3	4.4
			H167	IGHV1-17*01	27	5.3	29.2	13.7	42.9	19.3	3.7
			H105	**IGHV1-7*02**	**63**	**2.7**	**0**	**3.8**	**14.3**	**3.5**	**4**
			H128	**IGHV1-7*02**	**63**	**4.1**	**0**	**3.8**	**14.3**	**3.5**	**5**
			H135	**IGHV1-7*02**	**63**	**1.3**	**0**	**1.9**	**14.3**	**5.3**	**13.4**
			H134	**IGHV1-7*02_S0302**	**65**	**1.4**	**4.3**	**3.8**	**4.8**	**1.8**	**9.1**
			H5	**IGHV1-7*02**	**65**	**5.4**	**8.7**	**3.8**	**9.5**	**2.6**	**13.5**
			H150	**IGHV1-27*01**	**66**	**4.1**	**30.4**	**14.8**	**26.3**	**13.2**	**17.1**
KNH1/BG505 SOSIP 100	617	59	H2114	IGHV1-20*01	16	2.7	12.5	11.8	23.8	12.3	10.5
			H1905	**IGHV1-7*02_S4274**	**47**	**0**	**0**	**5.8**	**14.3**	**1.8**	**4.3**
			H1967	**IGHV1-7*02_S4274**	**57**	**0**	**4.3**	**1.9**	**4.8**	**6.1**	**4.4**
			H2000	**IGHV1-7*02_S4274**	**57**	**1.4**	**3.7**	**4.2**	**23.8**	**7.9**	**6.7**
			H2010	**IGHV1-7*02**	**60**	**1.4**	**0**	**1.9**	**14.3**	**5.3**	**3.7**
			H1872	**IGHV1-7*02_S4274**	**61**	**1.4**	**0**	**9.6**	**14.3**	**6.1**	**5.7**
			H1842	**IGHV1-7*02_S4274**	**61**	**1.4**	**0**	**3.8**	**9.5**	**7**	**4.7**
			H2129	**IGHV1-7*02_S4274**	**62**	**0**	**4.3**	**1.9**	**4.8**	**4.4**	**3**
			H2028	**IGHV1-7*02_S4274**	**65**	**1.3**	**0**	**5.8**	**4.8**	**9.6**	**6.3**
			H1796	**IGHV1-7*02**	**50**	**1.4**	**3.8**	**6.1**	**14.3**	**9.6**	**7.7**
			H1851	**IGHV1-7*02_S4274**	**62**	**0**	**0**	**1.9**	**0**	**1.8**	**1.7**
			H1887	**IGHV1-7*02**	**62**	**2.7**	**0**	**3.8**	**19**	**5.3**	**4.7**
			H2087	**IGHV1-7*02**	**62**	**2.7**	**0**	**3.8**	**14.3**	**7**	**5**
			H2023	**IGHV1-7*02**	**66**	**4.1**	**0**	**3.8**	**9.5**	**6.1**	**5.4**

Since most of the sequences isolated were generated by using germline genes from the IGHV1-7 family, a phylogenetic analysis was made using the most used gene of the IGHV1-7 family as root for sequences in each animal. In cow 35, neutralising antibodies H563 and H663 were the only ones using gene IGHV1-7*02. Monoclonal antibody H563 was found at week 33, whereas H663 was isolated at week 59, and

both share a common ancestor as sequences clustered together (figure 5-26 A). On the contrary, neutralising antibodies H198 and D1 from cow 8434 did not cluster together. Instead, mAb H198 which was isolated at week 59, was the second closest mAb from germline gene IGHV1-7*02_S0302 and clustered with a non-neutralising antibody, H29, which was isolated at week 33. D1 on the other hand, clustered together with two non-neutralising antibodies, H128 and H105, raised at week 59 (figure 5-26 B). Lastly, two main clusters were formed with sequences from cow 617. The closest one from germline gene IGHV1-7*02_S4274 contains the neutralising mAbs H2010, H2000 and H1967, together with non-neutralising antibody H1796, while the farthest one divides into two branches, with neutralising mAb H1905 and non-neutralising mAbs H2087 and 1887 in one branch, and with neutralising mAbs H2129, H1842 and H1872 along with non-neutralising mAbs H1851 and H2023 in another. Monoclonal antibody H2028 was not included in any of the previously mentioned clusters. From all neutralising antibodies isolated from this animal, mAbs H2129, H1842 and H1872 which presented the highest neutralising activity (Heydarchi et al., in preparation), clustered together in the farthest branch from germline gene IGHV1-7*02_S4274. Interestingly, this branch also contains the most mutated mAb isolated from this animal, H2023, which was non-neutralising (Figure 5-26 C).

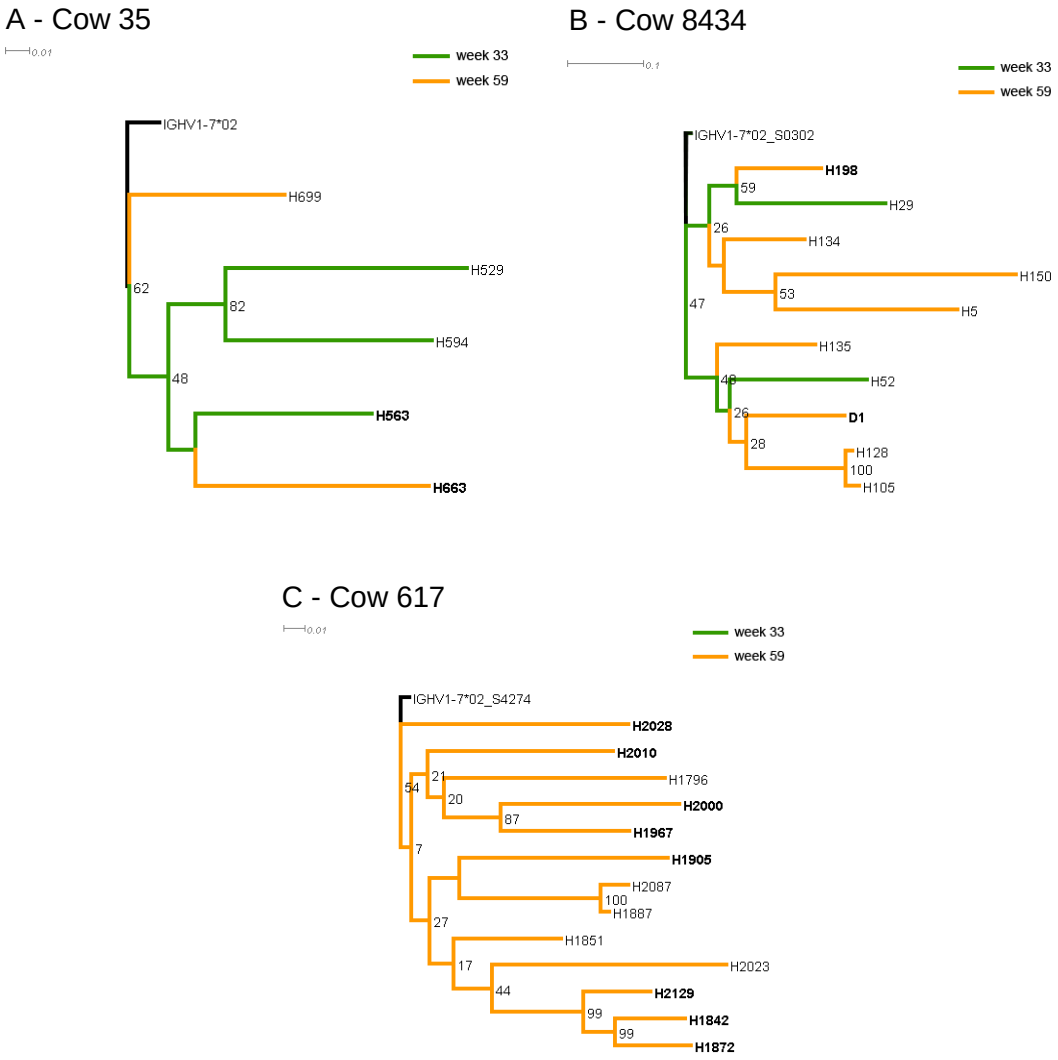


Figure 5-26: Phylogenetic analysis of monoclonal antibodies isolated from cows vaccinated with HIV-1 Env vaccines. Sequences from monoclonal antibodies isolated from cows 35, 8434 and 671 were evaluated using custom databases constructed by IgDiscover. Antibodies with neutralising activity are indicated in bold, while sequences from phase 1 and phase 2 of vaccination scheme are color coded (green: week 33; orange: week 59).

Overall, most of the neutralizing antibodies isolated from vaccinated cows presented ultralong CDRH3 sequences and used germline genes from the family IGHV1-7, IGHD8-2 and IGHJ2-4. Neutralising antibodies with CDRH3s shorter than 40 residues

used, on the other hand, genes from the families IGHV1-10, IGHV1-20 and IGHV1-21. Phylogenetic trees using genes from the IGHV1-7 family showed clustering of neutralising antibodies for cow 35, different families for neutralising antibodies from cow 8434, and high mutation rates from germline gene in the case of antibodies from cow 617. The analysis of SHM levels for antibodies with ultralong CDRH3s revealed that sequences with neutralising activity presented the closest phylogenetic distance from the root, while non-neutralising antibodies presented the highest distance from their determined germline gene.

5.10 Discussion

While *in vitro* plate based assays can characterise antibody functions of antigen binding, such as virus neutralisation or activation of Fc-effector functions, these assays cannot assess the beneficial contribution of antibodies that lack any measurable functionality. Antibodies with similar binding sites typically differ in their sequence, and plate-based assays cannot study how in a polyclonal mixture each of those antibodies interact with the same binding site. On the other hand, computational analysis of large datasets generated through NGS presents the evolution of all antibodies regardless of their functionality, and thus, can characterise the impact that certain vaccines have on directing the whole antibody repertoire.

Analysis of antibody responses against HIV-1 through bioinformatic tools has been used extensively in the last decade, either to characterise antibody families binding to important epitopes within HIV-1 Env (Zhu et al., 2013; Kwong et al., 2017; Wu et al., 2019) or co-evolution between humoral immune response and HIV-1 (Carter et al., 2015; Bonsignori et al., 2017). Computational analysis allows processing of large datasets, making it possible to interpret NGS data. Nevertheless, the programs for data processing must be accurate and adequate for the type of sample used. IgBLAST

for example (Ye et al., 2013), a program sub-routine included in IgDiscover for gene annotation, is a program that recognises antibody sequences in certain species where the structure of FRs and CDRs within each V gene are defined, as well as the start and end of the CDRH3 according to the Kabat and IMGT numbering schemes. However, not all species have this reference library information available at this time. While these files exist for human, mouse, rat, rabbit and rhesus macaque repertoires they do not exist for bovine (Dondelinger et al., 2018). During this project, we corresponded with the IgBLAST support team, who confirmed that their program does not support cattle antibody repertoires. To work with bovine Ig sequences, we would have to manually construct the correctly annotated FRs and CDRs files that included the ultralong CDR3 needed for IgDiscover to work with our bovine Ig sequences, which was beyond the reference data available to us and our expertise.

The success of NGS for antibody repertoire analysis relies heavily on the read length and sequence depth of the amplicon libraries prepared. Illumina paired-end reads are well suited to cover the ~500 bp variable region of typical human or mouse antibody sequence from start to end. However, the length of bovine Ig variable sequence is significantly longer for sequences with ultralong CDRH3 and prevents end-pairing of ultralong sequences from bovine 5' RACE libraries, made by following methods for human and mouse. It was only possible to study bovine antibody repertoires by using Multiplex PCR libraries which positions the primers more closely to each other. The Multiplex approach revealed between 8 and 10% of sequences that belonged to the ultralong CDRH3 lineage, meaning that a tenth of the whole antibody repertoire was missing when only 5' RACE was used. Length constraints for 5' RACE libraries have led others to recommend first characterising the leader sequences within an antibody repertoire from an uncharacterised species, then use this information to prepare Multiplex PCR libraries (Vazquez Bernat et al., 2019).

Despite the disadvantages with 5' RACE libraries compared to Multiplex libraries, both techniques inferred different novel alleles in IgM libraries by using IgDiscover. This may be due to differences in amplicon size between 5' RACE and Multiplex, or from differences in sequences amplified in these different libraries. In the case of 5' RACE, it amplifies sequences from one 5' end primer. On the other hand, Multiplex PCR library construction requires a set of 5' end primers with different affinity towards their complementary target. This could affect the end result in terms of sequences amplified and, thus, novel alleles inferred by these two techniques.

Another aspect upon which successful NGS analysis relies, is sampling depth. The number of total unique sequences in a library, called "species richness" (Chaudhary and Wesemann, 2018), depends on sampling depth. The results obtained by AbMining ToolBox showed that, unfortunately, our Multiplex IgG libraries were not deep enough to capture all species present in the sample. As an example, none of the neutralising mAbs isolated from cow 617 were present in the list of unique CDR3s generated by AbMining ToolBox. It is estimated that a minimum depth of 10^6 unique CDR3s are required to capture enough species richness (Corcoran et al., 2016; Fantini et al., 2017). Therefore, our results indicate that isolated mAbs with neutralising activity are rare species within the whole antibody repertoire.

A noticeable feature of analysed IgM libraries was the high SHM rates observed in such libraries. Despite discovery of novel alleles, no unmutated common ancestor germline gene with 0% SHM was evident after 2 iterations of IgDiscover. This may be explained by previous studies that indicate an increased activity of *de novo* AID-mediated mutation in Peyer's patches and spleen during B-cell ontogeny in bovines, which expands their antibody repertoire in absence of exogenous antigens (Liljavirta et al., 2013). In support of this mechanism, repertoire expansion through AID activity prior to antigen encounter has also been observed in rabbits (Mehr et al., 2004). Thus, a baseline of 0% mutation from the unmutated germline sequence cannot be reached

using the SHM analyses employed here with B-cells from bovines. Another interesting feature observed in IgM libraries is the usage of few VH genes in the formation of naïve repertoires. Despite having 16 functional VH genes (Ma et al., 2016), only 8 of them were used in all animals. Skewing of VH repertoire usage has been observed in other species and is related to chromosomal position of certain genes, which might make them more prone to expression (Lu et al., 2014; Rettig et al., 2018). This, however, is the first time such a skewed expression has been observed in bovines.

In IgG libraries, noticeable differences were observed in D gene annotation at week 33 and week 59. While the KNH1/BG505 SOSIP 100 group presented no significant differences in gene usage frequency between phases, cows from AD8 Unc 500 group showed dissimilar results for genes IGHD5-2*01 and IGHD1-2*01 in cow 35, and for genes IGHD3-1*01 and gene IGHD6-2*01 in cow 8434. Such differences may be due to changing the immunogen for phase 2 vaccination. The CDRH3 of bovine IgG is mostly formed by the D gene segment and due to its variability, it has a crucial role in BCR engagement to a specific antigen, and therefore linked to B cell activation and expansion (Rosner et al., 2001). Changing antigen during phase 2 of our vaccination scheme could have altered the high affinity epitopes presented within the vaccine that drive B cell activation at the start, including those in germinal centres. In consequence, a new immunogen would change the repertoire of plasma cells activated for the first time and those that would undergo affinity maturation and further expansion.

Analysis of mAbs showed high mutation rates of CDRH2 in neutralising mAbs, regardless of CDRH3 size. Although previous works have mentioned that CDRH1 and CDRH2 in ultralong bovine antibodies do not interact with antigen based on their mutation pattern and Shannon entropy values (Deiss et al., 2019), antibodies from macaques vaccinated with clade C Env trimers presented neutralising antibodies with high SHM levels (around 10% from baseline of 0%) in CDR2 and FR3 (Phad et al., 2020). In bovines, AID activity shows preference for inserting mutations in CDR

regions over FR regions (Liljavirta et al., 2013), which could explain the mutations observed on CDRH2. Mutations outside of CDRH3 are also important for Ig binding. As an example, D'Angelo et al. have shown that sorted B cells against human protein CDK2 presented identical CDRH3 and a full sequence homology between 91.6% and 97.8%, indicating affinity maturation in areas outside of CDRH3. However, when unsorted samples were analysed, the same CDRH3 was found in sequences with an overall sequence homology between 37.7% and 73.2%. This implies that even some antibodies presenting the same CDRH3 were unable to bind to CDK2, highlighting the importance of other regions in the production of affinity towards a specific antigen (D'Angelo et al., 2018).

Another important observation made from our AD8 SOSIP gp140-isolated bovine mAbs, was the surprising low levels of SHM observed in neutralising Abs. Genes from the IGHV1-7 family in bovines have lower levels of SHM compared to other genes (Deiss et al., 2019), and may be related to the stability needed for the long disulphide bonded stalk-like region to support the globular head structure of ultralong CDRH3 IgGs, which in contrast is highly flexible for shorter CDRH3 IgGs (Dong et al., 2019). Additionally, high levels of SHM in heavy and light chain do not always assure higher binding or neutralisation. As an example, in the PGT121 family of BnAbs, mAbs 22H and 6H present higher SHM rates than PGT121 but reduced neutralisation when paired with the same light chain (Sok et al., 2013). Taking into account that non-neutralising antibodies isolated from cows presented higher SHM levels than neutralising antibodies, the results might indicate that binding against HIV-1 requires a fine balance between the amount of mutations introduced in a germline sequence, and the conservation of key residues to maintain constrained structure together with affinity for a certain epitope within HIV-1 Env.

In summary, our results indicate that bovine antibodies with ultralong CDRH3 already present high affinity towards HIV-1 Env trimers, and that they do not require the same

high levels of affinity maturation as human in order to acquire neutralising activity. When considered together with the observation that KNH1/BG505 SOSIP gp140 induced higher rates of SHM in germline genes compared to AD8 Unc gp140, and the low rates achieved with later phase 2 stimulation with of AD8 SOSIP gp140 or BG505 SOSIP gp140, our conclusion is that high neutralising antibody responses observed in KNH1/BG505 SOSIP 100 group is a product of two factors: a high-SHM-inducer antigen, and an animal model that requires low SHM rates to produce Abs with neutralising activity.

6. General Discussion

6.1 Thesis Discussion

Vaccination of bovines with HIV-1 Env gp140 trimers had previously proven effective for both heterologous neutralising activity against HIV-1 (Kramski et al., 2012; Heydarchi et al., 2016; Sok et al., 2017) and activating Fc-effector functions (Kramski et al., 2012b). Before this work, however, mechanisms of Fc-effector activation by bovine Abs were poorly understood. Moreover, levels of affinity maturation required for bovine antibodies to engage HIV-1 Env were unknown. This work characterised vaccine effectiveness in terms of neutralising activity and Fc-effector function activation, as well as the level of affinity maturation required by these animals to develop neutralising antibodies against HIV-1.

From all HIV-1 Env vaccines studied in this work, choosing one that presents the best profile is complicated. Despite showing KNH1/BG505 SOSIP gp140 as a better promoter of affinity maturation than AD8 Unc gp140 in chapter 5, results from chapters 3 and 4 showed that no vaccine was able to elicit all protective functions: AD8 Unc gp140 and KNH1/BG505 SOSIP gp140 presented either strong neutralising activity or strong activation of Fc-effector functions.

6.1.1 Affinity maturation required for strong neutralising responses in bovines

Most BnAbs against HIV-1 Env have their origin in patients who have lived with HIV-1 for years (Sather et al., 2009; Liu et al., 2015) and common features found in human BnAbs are long CDRH3 and high levels of SHM: CD4bs antibodies present SHM levels of around 30% (Wu et al., 2010), while V1/V2 apex antibodies present CDRH3 lengths between 24 and 32 residues (Pejchal et al., 2010; McLellan et al., 2011), and V3-glycan antibodies present SHM rates between 15 and 23% within CDRH3s between 18 and 24 (Walker et al., 2011). Antibodies with these features are not

common in healthy patients, in fact they are rare in human IgG repertoires (Johnson and Wu, 1998; Wu et al., 2012). Therefore, it takes years to produce them using the human antibody repertoire as base. As explained in Chapter 1, advantages of bovines over humans is a capacity to produce antibodies with long CDRH3s residing within their germline antibody repertoire. Bovines initially produce antibodies with longer than average human CDRH3 sequence lengths, together with approximately 10% of antibodies with ultralong CDRH3s (Heydarchi, Salazar-Quiroz and Purcell, 2016). This dissimilarity places bovines in a more advantageous position to produce Abs with high affinity against HIV-1 Env compared to humans, and reduces the level of affinity maturation required for bovines to increase their affinity against HIV-1 Env, with beneficial neutralising activity.

If we use as baseline a 5% mutation rate to calculate SHM rates from the bovine IgM unmutated germline repertoires, it is evident that both AD8 Unc gp140 and KNH1/BG505 SOSIP gp140 required much lower levels of SHM to reach neutralisation than BnAbs such as VRC01 (40 to 48% SHM), PGT121 (21 to 27% SHM) or PGT145 (21 to 29% SHM) (Mascola and Haynes, 2013). Animals from both groups reached SHM rates of less than 10% on average with genes from the family IGHV1-7, which was the precursor for all ultralong neutralising mAbs. Additionally, mAbs isolated with the most potent neutralising activity had ultralong CDRH3 presenting less than 10% of SHM across the total V region, with a maximum of 23% of SHM in CDR2 region (mAb H2000 from cow 617 isolated at week 59). This indicates two things: ultralong CDRH3 IgGs do not require high levels of SHM to improve binding against HIV-1 Env, and compared to human antibodies, they require much less SHM in order to gain affinity.

In addition to the low SHM rates observed, the timeframe needed to develop these Abs in bovines is also surprising. Bovines required only one year of vaccination to produce Abs with high neutralising activity in both groups, a shorter period of time compared to the 3-to-5 years needed in humans to develop BnAbs. During that year,

maximum breadth was achieved by the end of week 33, and by week 59, neutralisation potency was increased. It is important to mention that breadth measured in this work only covered heterologous neutralising activity in pseudoviruses from clades A, B and C, and that others with access to more comprehensive arrays of pseudoviral Env have studied neutralisation breadth with greater numbers of Env from all clades. Nevertheless, such works have shown that humans require a long period of time to increase neutralisation breadth. For instance, Abs from a patient giving rise to the CH235 mAb lineage, a CD4bs mAb, presented 18% of breadth by week 41 and only by week 157, maximum neutralisation breadth was achieved (Bonsignori et al., 2016). In another work, antibodies from the DH270 mAb lineage, a V3-glycan antibody, reached maximum breadth by week 186 (Bonsignori et al., 2017). It would have been interesting to study neutralisation breadth and potency by bovine Abs with a larger set of viruses, in order to evaluate whether maximum neutralisation breadth was indeed achieved by week 33.

Among the tested HIV-1 Env vaccines, KNH1/BG505 SOSIP gp140 was the most favoured for initiating production of BnAbs, because it induced higher levels of SHM compared to AD8 Unc gp140, an observation from chapters 3 and 5. It is possible that KNH1/BG505 SOSIP gp140 induces a higher engagement of bovine BCR repertoires than AD8 Unc gp140. However, it is unclear how the AD8 SOSIP gp140 would compare and the original antigenic stimulus, because this regimen was not examined. Previous analysis of antibody responses in macaques with BG505 SOSIP.664 gp140 showed that top neutralisers presented higher degree of SHM than non-neutralisers or low neutralisers (Francica et al., 2015), which also correlated with formation of germinal centres, with a high frequency of antigen specific B cells and Tfh cells in them and high expression of activation markers (Havenar-Daughton et al., 2016). Another important observation is that binding affinity did not correlate with neutralisation in these studies (Francica et al., 2015; Havenar-Daughton et al., 2016).

Based on the results observed in animal models, the capacity of BG505 SOSIP gp140 to elicit strong neutralising responses in humans is currently being tested in a phase 1 clinical trial (Clinical Trial identifier: NCT03699241). One aspect of interest will be the level of SHM achieved by this vaccine in humans, and whether this correlates with higher, if any, neutralising activity. A valid concern is that human unmutated germline precursors of VRC01-like BnAbs are known to not bind to wild-type circulating HIV Envs (Hoot et al., 2013; Jardine et al., 2013; McGuire et al., 2013), and PG9/PG16 BnAbs bind poorly (Doria-Rose et al., 2014; Andrabi et al., 2015; Bhiman et al., 2015; Gorman et al., 2016). It is therefore possible that BG505 SOSIP gp140 may not produce BnAbs akin to the VRC01 or PG9/PG16 antibody family lineages in humans, but may still elicit others binding to CD4bs, V2-apex or other neutralising epitopes.

6.1.2 Engagement of bovine BCRs to HIV-1 Env vaccines and implications in epitopes targeted by bovine antibodies

Cows from groups KNH1/BG505 SOSIP 100 and AD8 Unc 500 elicited antibodies targeting different binding sites. While antibodies from KNH1/BG505 SOSIP 100 mostly focused on V1/V2 apex epitopes, antibodies from AD8 Unc 500 produced antibodies targeting the Co-RBS. Both epitopes are spatially located at the upper side of each vaccine, which is curious. While Co-RBS antibodies are common in infected patients (Pollara et al., 2013; Ding et al., 2016; Richard et al., 2015, 2016, 2017), apex antibodies appear rarely in humans (Briney et al., 2012), probably due to the low frequency of epitope binding precursors, which usually require long CDRH3 sequences (Pejchal et al., 2010; McLellan et al., 2011; Julien et al., 2013; Doria-Rose et al., 2014). The unusual long CDRH3 of bovine antibodies might have conferred advantages in producing antibodies targeting V1/V2 apex epitopes. Structural analysis has shown that apex mAbs such as PG9, PG16 and CAP256.09 require a glycan at

position N160 and a lysine-rich positively charged patch located at the strand C of V2 loop for binding. These antibodies also present a conserved YYD motif in their CDRH3 which interacts with V2 loop (Andrabi et al., 2015). Unfortunately, none of the isolated mAbs from cow 617 bound to apex epitopes, but a quick search in our NGS data revealed that this YYD motif was frequent in ultra-long CDRH3. It would have been interesting to study if Env mutants lacking either the N160 glycan site or the lysine-rich patch would demonstrate diminished binding or neutralisation by the polyclonal antibodies from cows of KNH1/BG505 SOSIP 100 group.

In addition to V1/V2 loop and Co-RBS epitopes, all isolated mAbs from cows of both groups displayed neutralising activity targeting CD4bs. It is possible that the appearance of antibodies with neutralising activity in early stages of the vaccination course might have determined an increase in the appearance of other lineage antibodies, targeting and defining other important epitopes. For instance, favourable changes of host antibody responses have been observed when BnAbs have been administered through passive immunisation (Ng et al., 2010; Schoofs et al., 2016). Thus, early production of neutralising antibodies may have fostered the production of an antibody repertoire targeting the epitopes mentioned before.

Another hypothesis to explain the effectiveness of bovines in producing BnAbs against HIV-1 is their naïve state to HIV-1-like antigens and differences found in their CD4 receptor. For instance, infants with high viremia (Muenchhoff et al., 2016) develop strong neutralising antibodies targeting CD4bs, V2-loop, V3-glycan loop or the gp120-gp41 interface (Ditse et al., 2018). The effective neutralising response observed in infants can be explained by their differential expression of CCR5 coreceptor compared to adults (Shalekoff et al., 2004). Although CCR5 is highly expressed in bovines, there are key differences with human CCR5 (Widdison et al., 2010). Furthermore, CD4 receptor shows only 60.9% of identity between bovine and human sequences and 76.3% similarity in the first domain, which is the domain responsible for interaction with

gp120 (Silberman et al., 1991). It is clear that bovine CD4 does not bind to HIV-1 Env trimer vaccines, replicating the conditions observed in infants where low engagement between HIV-1 Env and CCR5 is associated with an increased production of BnAbs.

6.1.3 Correlates of protection and the need to analyse complete antibody responses

Measuring the efficacy of an antibody-based vaccine requires observation from different points of view. The most common one is the analysis of neutralising activity elicited by a certain vaccine against a virus. Less common approaches, however, include an assessment of the activation of Fc-effector functions, by engagement of neutralising or non-neutralising antibodies with other components of the immune system such as Fc-receptors on immune cells or complement proteins. For each infectious disease, the relationship between antibody titre, the epitopes targeted by these antibodies, and the Fc-effector functions elicited by them are different, and it is imperative to analyse which features are covered by a vaccine candidate. In diseases like Hepatitis A and B, Human Papillomavirus or Measles, antibody binding is enough as a correlate of protection. For other pathogens such as Influenza, Japanese Encephalitis, Polio, Rabies or Yellow Fever, direct neutralisation of virions is required for vaccine efficacy. For HIV-1, both direct neutralisation of virions and activation of Fc-effector functions have demonstrated relevance in clinical studies (Tomaras and Plotkin, 2017).

Special attention should be taken for Fc-effector functions. While they were identified as correlates of protection in the RV144 vaccine trial (Rerks-Ngarm et al., 2009; Pitisuttithum et al., 2012; Bonsignori et al., 2012; Chung et al., 2015; Perez et al, 2017), vaccine trials VAX004 and STEP showed increased risk of infection via the activation of these same functions (Gilbert et al., 2005; Forthal et al., 2012; Huang et

al., 2014). A phenomenon observed in natural infections as well as in vaccine trials is the activation of antibody-dependent enhancement (ADE), which instead of conferring protection through activation of Fc-effector functions, instead promotes infection of new cells (Kulkarni, 2020). It is a well-studied event in infections with flaviviruses such as Dengue and Zika (Dejnirattisai et al., 2016) and MERS-Coronavirus (Wan et al., 2020), and it has also been observed for HIV-1.

ADE occurs when antibodies bind to a virus but fail to neutralise it (Halstead, 2003). This allows engagement of Fc-receptors at sub-optimal concentrations to trigger an Fc-effector function, and instead stabilise the virus near the cell membrane enabling trans-infection (Taylor et al., 2015). in HIV-1, ADE has been activated by both Fc-receptors (Takeda et al., 1988; Homsy et al., 1989) and complement (Robinson et al., 1987; Yu et al., 2010). For FcR-mediated ADE, all three FcγRs can facilitate infection of suitable bystander cells (Homsy et al., 1989; Takeda et al., 1988,1990; Laurence et al., 1990), however FcγRI and FcγRII require previous CD4 engagement, while FcγRIII does not (Kulkarni, 2020). On the other hand, complement-mediated ADE occurs by virus opsonisation of C3 and recognition of receptor CR2 in target cells followed by CD4 engagement (Lund et al., 1995) or by interaction between C1q and IgGs bound to virus in the N-terminal portion of gp41 (Prohaszka et al., 1997). A third mechanism has been described, which is independent of FcRs and complement proteins, but requires binding of antibodies targeting CD4bs, V3-loop or CD4i epitopes in non-neutralising conditions (Sullivan et al., 1998). Because it is uncertain whether a vaccine could or could not elicit ADE, it is proposed to produce vaccines without gp41 or epitopes linked to activation of ADE, plus to test neutralisation with complement proteins and multiple rounds of replication, in order to evaluate whether the IgGs in samples also aid infection (Beck et al., 2008). Our results showed activation of Fc-effector functions, but because inhibition or complete clearance of virus was not measured under these conditions, we cannot discard the possibility of eliciting ADE at sub-optimal

concentrations of neutralising antibodies. In particular, AD8 Unc gp140 is at risk of eliciting ADE under conditions where generation of neutralising antibodies is low, but engagement of FcRs or activation of complement is still observed. Therefore, the analysis of virus clearance mediated by Fc-effector functions is needed for a complete understanding of a vaccine response, and the current methods to assess Fc-effector functions might need adaptation, because they are unavailable to provide this information.

6.2 Concluding Remarks

The main goal of this study was to profile antibody responses against different HIV-1 Env trimer vaccines in bovines. Our results show that differences in the stabilising mutations and clade of Env vaccines affected binding, neutralisation, Fc-effector function activation and affinity maturation in IgG repertoires.

From all vaccine regimens used, KNH1/BG505 SOSIP gp140 induced the highest and broadest neutralising activity. It induced significant responses against the V1/V2 apex and CD4bs epitopes, and IgG repertoires from animals in this group exhibited the highest degree of affinity maturation. Cows in this group, however, presented low binding titres against BG505 SOSIP gp140 and antibodies from this group were unable to activate Fc-effector functions, such as phagocytosis or complement deposition.

On the other hand, cows from AD8 Unc gp140 groups presented the highest binding titres and induced antibody responses against Co-RBS and CD4bs epitopes. In consequence, antibodies from this group demonstrated heterologous neutralising activity and activation of Fc-effector functions. This was the only vaccine regimen in the study that activated both phagocytosis and complement deposition responses. However, antibodies elicited against AD8 Unc gp140 did not achieve autologous

neutralisation of AD8 pseudovirus, and rates of SHM observed were lower compared to those observed KNH1/BG505 SOSIP 100 IgGs.

Results from other uncleaved-stabilised trimers used in this study showed that reduction of the glycosylation site density on Env trimers decreases the Env-specific immunogenicity. This was demonstrated with pre-seroconversion strain, PSC89 Unc gp140, that only induced low binding titres and, thus, did not elicit antibodies with any functionality. The chronic Clade C isolate with high levels of glycan, MW Unc gp140, on the other hand, did induce strong binding and neutralising activity, however lower compared to AD8 Unc gp140.

Finally, changes of immunogen during the vaccination course improved neutralisation, but not all changes improved responses. Cows initially vaccinated with AD8 Unc gp140 improved neutralisation when antigen was changed for either AD8 SOSIP gp140 or BG505 Unc gp140, but not for BG505 SOSIP gp140. These changes of immunogen were reflected in IgG repertoires, as changes in gene usage were observed between phase 1 and phase 2 of vaccination. These changes could also affect the level of SHM observed in AD8 Unc 500 group, as both animals studied in chapter 5 were vaccinated with a different antigen than the original one. Nevertheless, the regimen AD8 Unc gp140 - AD8 SOSIP gp140 was shown to elicit high titres of antibodies with desirable functions, despite having lower SHM rates than its KNH1/BG505 SOSIP 100 counterparts.

6.3 Future Directions

Vaccination of bovines with HIV-1 Env trimers allows rapid elicitation of antibodies with strong neutralising potency and breadth. Because these animals are favoured into producing superior antibodies compared to other animal models including humans,

they can be used to develop strong BnAbs using better HIV-1 Env immunogens. These can be further isolated and used for passive immunisation, or alternatively used in other platforms for activation of immune responses such as chimeric antigen receptor T cells (CAR-T cells). Additionally, polyclonal mixtures from colostral milk or serum can serve as topical microbicides, especially due to their ability to activate phagocytosis and complement.

The results shown in this work also highlight the challenges of eliciting antibodies with both neutralising activity and the ability to activate Fc-effector functions when utilising either uncleaved-modified Env trimers or SOSIP-modified trimers as immunogens. The lack of dual functionality must be related with the constraints of each structure: while SOSIP-modified trimers expose neutralising epitopes, they occlude epitopes important for Fc-effector function yet while uncleaved-modified trimers expose the latter, this comes at the expense of disassembly of the former. Since the current experience from human trials indicates an efficacy benefit from eliciting both antibody functions together, new Env stabilising methods are required to find a more flexible and dynamically “breathing” structured Env trimer which might result in a superior antigen.

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