Cloning and Characterization of Novel IgA Antibody Variable Heavy and Light Chains from HIV-1 Resistant Sex Workers from Nairobi, Kenya

by

Caitlin S. Sarna

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Department of Medical Microbiology
University of Manitoba
Winnipeg

Abstract

Heterosexual intercourse now accounts for the majority of HIV transmission within sub-Saharan Africa. The generation of microbicides and vaccines, therefore, requires a better understanding of the mucosal correlates of protection, including the role of HIV-specific IgA.

It is now accepted that not all individuals are equally susceptible to HIV-1 infection, as exemplified by the HIV Exposed Seronegative (HESN) women of the Pumwani Cohort in Nairobi, Kenya. To assess whether mucosal IgA responses contribute to this protection, 3 novel IgA variable genes were cloned from HESN cervical B-cell cDNA.

Nine monoclonal IgA Abs were produced, two of which were properly produced from cell culture. The HESN-derived A6/30L and A9/30L variants had a greater specificity for $gp120_{IIIB}$ than their A6/4L and A9/4L counterparts, while the A6 variant recognizes a distinct gp120 epitope compared to the broadly neutralizing antibody IgGb12. Further characterization of these IgA chains may suggest their suitability for use in microbicides or mucosal vaccines.

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Dedication

This thesis is dedicated to the women of the Pumwani research cohort. Their selfless generosity and continued participation in the study make this research possible and, any progress we make is direct result of their commitment and dedication.

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1.0 Introduction

1.1 Global impact and challenges of HIV

Since its discovery almost 30 years ago, HIV/AIDS (Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) has emerged to be one of the most catastrophic global health issues to plague mankind. In 2004, HIV/AIDS was the 6th leading cause of death worldwide and the main cause of adult mortality in Africa [1]. By December 2008, it was estimated that 33.4 million people were living with HIV and that the disease had already claimed up to 25 million lives. Despite prevention efforts and the availability of limited drug treatment options, 2.7 million new cases of HIV and 2.0 million AIDS deaths were reported in 2008 alone [2].

As serious as the HIV pandemic has become worldwide, no region has been hit harder than sub-Saharan Africa. Despite being home to roughly 10% of the global population [1], this region accounted for 22.4 million (67%) of people living with HIV and 1.4 million (72%) of AIDS-related deaths. The average prevalence of adult HIV infection for the region is 5.2% [2], ranging from ~2% in western and central Africa to an astounding 26% in Swaziland [1, 2]. Countries with a high prevalence of HIV in sub-Saharan Africa have witnessed a decline in life expectancy at birth to just below 50 years of age [1]; in 2007 the average life expectancy in Swaziland fell to just 37 years [1, 2]. The impact of HIV/AIDS on families, communities, public services and economies of these nations has been devastating. The high rate of HIV infection has eliminated many wage-earning adults from the population due to AIDS-related illness and mortality [3]. Furthermore, it is estimated that 14.1 million children in sub-Saharan Africa have lost one or both parents to AIDS [2]. Due to an already heavy burden on social welfare

services (where these services even exist), these orphaned children are more likely to drop out of school to care for the remainder of their families or to try to earn a wage; many of them end up on the streets to survive [3, 4]. This continues the vicious cycle of infection by deepening poverty and increasing the risk these children will contract the virus themselves.

Another major group of concern in the HIV/AIDS pandemic is women, as they currently make up the fastest growing demographic of people living with HIV worldwide. In sub-Saharan Africa, women now account for 60% of people infected with HIV [1]. This gender disparity is even more pronounced in young women and girls. In the nine southern African countries with the highest prevalence of HIV, women aged between 15-24 years are three times more likely to be infected compared with men of the same age [2]. Heterosexual transmission is the driving force of the epidemic in sub-Saharan Africa [2, 5], and the rate of HIV transmission from a male to a female sexual partner is estimated to be 2-4 times higher than from a female to a male partner. A number of reasons may account for this, including increased physiological susceptibility of women due to the larger anatomical surface area of the genital tract, the increased number of target cells, and unique hormonal changes that occur in the vagina as compared to the penis [6]. The presence of semen favours HIV persistence by raising the vaginal pH; semen also contains amyloid fibrils that have been shown to increase viral infectivity by capturing virions and promoting their attachment to target cells [7]. These factors also contribute to a higher incidence of other types of ulcerative or inflammatory sexually transmitted infections (STIs) in women, which themselves increase the likelihood of HIV infection up to 10 times by disrupting the integrity of the mucosal

epithelial barrier and activating susceptible target cells [8, 9]. In addition to physiological susceptibility, in many developing countries with a high prevalence of HIV, women experience gender inequality, relationship power imbalances and sexual subordination which also increase their vulnerability to HIV infection [10]. These women may have limited economic security, fall victim to sexual violence and have limited access to information about safer sexual practices. Many of these women have little control over the conditions of their sexual encounters and may be unable to negotiate abstinence or condom use (especially within the context of marriage since planned pregnancy necessitates unprotected sex) [3, 11]. Therefore, in the battle to prevent sexual transmission of HIV, particularly in sub-Saharan Africa, it will be important to develop discreet, female-controlled methods of HIV prevention.

To date, there is no cure for HIV/AIDS and few treatment options are available. Since the advent of AZT in 1987, 32 different antiretroviral drugs (ARVs) targeting various phases of the viral life cycle have been made available by the U.S. Food and Drug Administration [12]. Where available, Highly Active Antiretroviral Therapy (HAART), a combination therapy approach of three or more ARVS directed against different stages of viral replication, has largely transformed the diagnosis of HIV from a death sentence to a manageable chronic disease [13]. Antiretroviral therapy is widely available in the developed world, and drug coverage in sub-Saharan Africa has increased 10-fold from 2003-2008 [2]. By reducing viral load, these treatments prolong the lives of infected individuals and reduce the incidence of HIV transmission to others. However, antiretroviral drugs are by no means a cure for HIV/AIDS, as patients may eventually experience treatment failure due to the development of drug resistance. The complexity

of ARV drug regimens and high rates of undesirable side effects contribute to a poor rate of adherence to ARV therapy [14]. While ARV drug coverage is improving, less than half of the adults and children in need of antiretroviral therapy in resource-limited settings actually receive treatment [2].

Because there is no cure for HIV/AIDS, current interventions are focused on preventing infection. In some regions of sub-Saharan Africa, public education campaigns and HIV testing with counselling are slowly curtailing the rate of HIV infection. Prevention strategies such as the ABC approach (remaining abstinent until marriage, being faithful to one partner and using condoms) have partially contributed to the declining rates of HIV; however relying solely on abstinence-based interventions is impractical and ignores some of the realities of the HIV epidemic (such as the high rate of HIV acquisition in married, monogamous women) [10, 15]. A promising new approach to HIV prevention is male circumcision, which may decrease male susceptibility to sexually-acquired HIV infection by as much as 60% [16-18]. However, circumcision alone will not stop HIV transmission because it only provides partial protection to men and may not directly reduce HIV transmission to women.

In order to curtail the HIV/AIDS pandemic, we need to develop safe and effective biomedical interventions such as topical microbicides and vaccines. In the absence of a preventative vaccine, the development of an intravaginal topical microbicide could help limit sexual transmission of HIV. It has been suggested that a microbicide at 20% population coverage with only 60% effectiveness against HIV could prevent 2.5 million new infections over the course of 3 years [19]. When applied before intercourse, a microbicide would prevent or reduce the risk of HIV transmission by reinforcing the

natural physical barrier of the genital mucosa, by inhibiting viral fusion and replication, or by virucidal surfactant activity [20, 21]. To date, several initially promising microbicide candidates have failed. Microbicides containing the detergent nonoxynol-9 were shown to inhibit HIV-1 *in vitro* but also caused inflammation by disrupting the cell membranes of the genital mucosal epithelium and enhanced HIV infection *in vivo* [19]. Polyanionic substances such as PRO 2000 disrupt receptor-ligand interactions by masking viral envelope proteins but appear to be inactivated by seminal plasma *in vivo* [22]. Other strategies such as the Invisible Condom® polymer gel are well tolerated but their effectiveness against HIV transmission has not been fully assessed [23]. New microbicide strategies will need to more specifically target HIV without harming or disrupting natural genital mucosal defences.

An important goal in the development of future microbicides will be the generation of human HIV-specific monoclonal antibodies that can protect the genital mucosa from HIV entry by topical passive immunization [8]. As a naturally occurring protein, human monoclonal antibodies have low immunogenicity and produce no metabolic side effects and are thus likely to be safe and non-inflammatory for the genital mucosa [24]. Furthermore, this strategy has already proven to be very effective in an animal model [25].

By far the best hope for controlling HIV is the development of a safe and effective protective vaccine. Currently, no effective vaccine is available despite years of research. Since neutralizing antibodies (defined as antibodies that inhibit viral entry into cells in the absence of other antiviral functions [26]) are the best correlate of protection for virtually all successful vaccines [27-29], the original focus of HIV vaccine

development was to block HIV acquisition by eliciting a neutralizing antibody response against purified recombinant viral envelope proteins [30]. However, the incredible viral diversity and the inaccessibility of conserved neutralizing envelope protein epitopes complicated the generation of broadly neutralizing antibodies against HIV [28]. As such, the development of immunogens that elicit broadly neutralizing antibodies still remains one of the most important goals in HIV vaccine research [27, 30]. As a result of the difficulties in generating effective neutralizing antibody vaccines and because HIV-specific cytotoxic T lymphocytes (CTLs) are one of the best correlates of HIV control, focus shifted to the generation of T-cell vaccines that would not prevent HIV infection but would control viral replication to prevent disease progression and reduce transmission [27, 30]. However, after the failure of the adenovirus type 5 HIV-1 gag/pol/nef vaccine of the Step Study [31] this vaccine strategy is also being re-evaluated. The first glimmer of success in the 25 year history of HIV vaccine development was the RV 144 phase III clinical trial in Thailand [32], which combined four priming injections of the cell-mediated immunity-inducing ALVAC-HIV recombinant canarypox vaccine with two boosting injections of the antibody-mediated immunity-inducing AIDSVAX B/E recombinant gp120 vaccine. Neither of these vaccines was protective individually, but vaccination with both reduced the risk of HIV infection by a marginally statistically significant 31.2%. While the immune correlates of protection in this study have not been identified, the modest success of this strategy supports the suggestion that an effective HIV vaccine will need to elicit both cell-mediated and antibody-mediated immunity against diverse strains of HIV [30, 33-35]. In an effort to better understand the appropriate correlates of protection that must be achieved by an HIV vaccine, many

researchers are focusing on individuals who are naturally resistant to HIV infection. By defining the mechanisms by which HIV exposed seronegative (HESN) individuals resist HIV infection, it is hoped that a vaccine can be generated to elicit a similar immune response.

1.2 Origin and early history of HIV:

It is now generally accepted that HIV is of zoonotic origin. The three main groups of HIV-1 (M, N and O) and the main groups of HIV-2 (A and B) each originated from independent cross-species transmissions of Simian Immunodeficiency Viruses (SIVs) from non-human primates (NHPs) to humans, as evidenced by the close phylogenetic relationship between these viruses and that the range of infected NHPs corresponds to the earliest HIV outbreaks [36, 37]. Transmission most likely occurred by contact with SIV-infected blood from primates hunted for bushmeat, through animal bites, through the butchering process or the consumption of uncooked meat [37]. Each HIV-1 group arose from transmission of the SIVcpz virus carried by *Pan troglodytes troglodytes* chimpanzees in western equatorial Africa to humans, namely in Congo, Gabon, Equatorial Guinea and Cameroon [36, 38]. The exact date of introduction of the virus into humans is not known, but molecular analyses suggest the HIV-1 most recent common ancestor probably arose between 1884-1924 and no later than 1933 [39]. HIV-2 was derived from separate transmission events of SIVsm transmitted by *Cercocebus atys* sooty mangabeys in the west African nations of Liberia, Sierra Leone and Côte d'Ivoire [40-42]. The timing of these cross-species transmissions is unknown but is thought to be similar to that of HIV-1.

The oldest direct evidence of HIV-1 infection in humans comes from sequences from Léopoldville (now Kinshasa) from an adult male in 1959 (ZR59) and an adult female in 1960 (DRC60) [36, 37]. Although the AIDS epidemic was not yet recognized in equatorial Africa at this time, the ZR59 and DRC60 viral sequences were found to be highly divergent, indicating that HIV had already been circulating and diversifying within the human population for some time [37, 39]. As urban populations exploded amidst post-colonial unrest in the second half of the 20th century, the HIV epidemic quickly and silently began to spread [39]. Refugees from west equatorial Africa fleeing from pre-independence upheaval exported the virus to Congo, Uganda and Tanzania, where it spread throughout the rest of the continent along international trucking routes [43] and eventually to India and the rest of Asia. Around 1966, the virus was exported from the Congo to Haiti by returning UNESCO-based workers [43, 44]. After circulating for a few years within Haiti, a single subtype B "pandemic strain" migrated around 1969 to the United States of America, from where it spread to Europe, South America and Oceania [44].

By June 1981, an unusual syndrome was observed in a small cluster of homosexual men linked by sexual contact in Los Angeles and New York. These previously healthy individuals developed opportunistic *Pneumocystis carinii* pneumonia and/or Kaposi's sarcoma, accompanied by a dramatic depletion of CD4+ T-cells [45-47]. Before long, the same symptoms appeared in haemophiliacs and other blood transfusion recipients. As a result of the characteristic severe immune suppression observed in these patients, the acronym AIDS (Acquired Immune Deficiency Syndrome) was adopted to describe the disease. The causative agent of AIDS was unknown until 1983, when the

research team of Dr. Luc Montagnier at the Institut Pasteur isolated a retrovirus from a Caucasian patient with AIDS symptoms [45]. This virus appeared to be a novel member of the Human T-cell Leukemia Virus (HTLV) family and was named lymphadenopathy-associated virus (LAV). Almost simultaneously, Dr. Robert Gallo's lab at the National Cancer Institute published a description of an HTLV-related retrovirus isolated from an American AIDS patient, which they named HTLV-III [46]. It was subsequently determined by the International Committee on the Taxonomy of Viruses in May 1986 that HTLV-III and LAV were the same virus, which was given the name Human Immunodeficiency Virus to reflect its main pathogenic characteristic [48]. As awareness of HIV spread, cases were quickly identified around the world and the search for a cure began.

1.3 Modern Epidemiology & Distribution of HIV

The current HIV pandemic is widespread and diverse. While both HIV-1 and HIV-2 cause disease, the spread and pathogenicity of HIV-2 is comparatively limited. HIV-2 is subdivided into clades A-H, however only the A and B subtypes are prominent in humans [42, 49] and infections are primarily limited to western Africa. HIV-1 is the main cause of the global HIV/AIDS pandemic. Based on viral phylogeny, HIV-1 can be classified into three groups: M (main), N (new, or non-M, non-O) or O (outlier) [36, 50] which genetically differ by a minimum of 30% [51]. HIV-1 group N and group O infections are relatively rare; group O viruses account for about 100,000 HIV-1 infections [51] and are mostly limited to individuals residing in Cameroon, Equatorial Guinea & Gabon [49]. Group N infections are primarily restricted to Cameroon and only a handful

of cases have ever been identified [38, 50]. Group M, responsible for the main HIV pandemic, is genetically diverse and is divided into 9 different genetic subtypes or clades (A, B, C, D, F, G, H, J & K) [49, 50] with 15-20% diversity between strains [51]. Clades A and F are further subdivided into sub-subtypes A1 & A2 and F1 & F2 respectively [50]. In addition, almost 50 intersubtype recombinants called circulating recombinant forms (CRFs) have been described [52, 53]. Worldwide, clade C is the most prevalent subtype at 47%, followed by clade A (27.2%), clade B (12.3%), clade D (5.3%) and CRF01_AE (3.2%) [50]. The global distribution of the HIV-1 subtypes is varied and complex, however specific viral clades are more prevalent in various geographical regions, most likely due to the founder effect. In addition to being responsible for the majority of HIV infections worldwide, HIV-1 subtype C is predominant in southern Africa and south-east Asia where the pandemic is the most serious [50]. Subtype B is the major clade in developed regions such as the Americas, Japan, Oceania and Western Europe; as such many of the available treatments have been based on subtype B viruses [54]. Subtypes A and D are primarily found in east Africa; in Kenya clade A accounts for 70% of infections while clade D and CRF viruses account for the remainder [49, 54, 55]. It has been suggested that different HIV subtypes may display different characteristics in terms of transmissibility, response to ARV drug treatments, and disease progression to AIDS; however due to the regional distribution of these clades, it is difficult to determine whether these effects are due to viral genetic differences or to the population dynamics of the affected regions [56]. Indeed, the incredible diversity of HIV (as well as the genetic diversity of the populations affected by the virus) is a major obstacle for developing a broadly effective HIV vaccine.

1.4 HIV Structure & Replication

1.4.1 HIV Structure

HIV is a ~110nm spherical enveloped lentivirus belonging to the *Retroviridae* family [57]. As a retrovirus, HIV possesses two copies of a ~9.7kb single stranded positive-sense RNA genome that replicates through a DNA intermediate which can be integrated into the host chromosomal DNA. The HIV genome contains 9 open reading frames which encode a total of 15 viral proteins (Figure 1) [58, 59]. Like other retroviruses, HIV encodes three main structural polyproteins gag, pol and env. The gag polyprotein is post-translationally cleaved into 4 proteins which make up the internal structure of a virion. Two of these, the p6 and p7 proteins, form a protective complex around the two full-length ssRNA molecules of the viral genome called the nucleocapsid (NC). The p24 capsid (CA) proteins form a characteristic cone-shaped structure around the ribonucleoprotein and its associated enzymes to form the core of the virion. Just inside the viral envelope, the p17 matrix (MA) protein encircles and protects the viral core. The pol polyprotein is comprised of three products, all of which have enzymatic functions [58]. The protease (PR) enzyme cleaves the gag & pol polyproteins into their component viral proteins. The reverse transcriptase (RT) enzyme transcribes the single-stranded viral genomic RNA into double-stranded DNA in order for the integrase (IN) enzyme to incorporate the viral genetic material into the host chromosomal DNA. All three of these enzymes are also encapsulated within the viral core. The env gene product is a large glycoprotein precursor termed gp160 which is cleaved by a host cellular protease into the transmembrane protein gp41 and the surface glycoprotein gp120. Trimers of gp41 are embedded in the viral membrane and are non-covalently attached to

gp120 trimers to form the HIV viral spike [57]. These envelope proteins are responsible for cell tropism and viral entry and are also the major target for HIV-specific antibodies. In addition to its structural and enzymatic proteins, the HIV genome also encodes 2 regulatory proteins rev and tat, and 4 accessory proteins nef, vif, vpr and vpu [59]. Both rev and tat are transcriptional regulatory proteins; rev favours the production of full-length viral genomic RNAs by exporting them from the nucleus and preventing their splicing, while tat increases viral RNA transcription [59]. The accessory proteins nef and vpu are both involved in immune evasion by promoting the endocytosis and degradation of surface CD4 molecules (which also facilitates viral release); nef additionally downregulates surface MHC-I expression to limit the ability of cytotoxic T lymphocytes (CTLs) to identify infected cells. The roles of the vif and vpr accessory proteins are somewhat less understood. The vif protein is necessary for virions to be infectious [58]. Vif is known to counteract innate restriction factors such as APOBEC and may be involved in the proper assembly of the viral core. Vpr targets the viral preintegration complex to the host cell nucleus and appears to induce G_2 phase cell cycle arrest. The purpose of this arrest is unclear but may help to promote transcription from the HIV long terminal repeat (LTR) [59].

1.4.2 HIV Life Cycle

HIV primarily infects and replicates within CD4+ T-cells and macrophages [58, 59]. The viral life cycle begins with the adsorption of an HIV virion to the surface of a susceptible CD4+ target cell. The HIV envelope glycoprotein gp120 first attaches to the cellular CD4 receptor, which changes the conformation of the gp120 trimer and allows

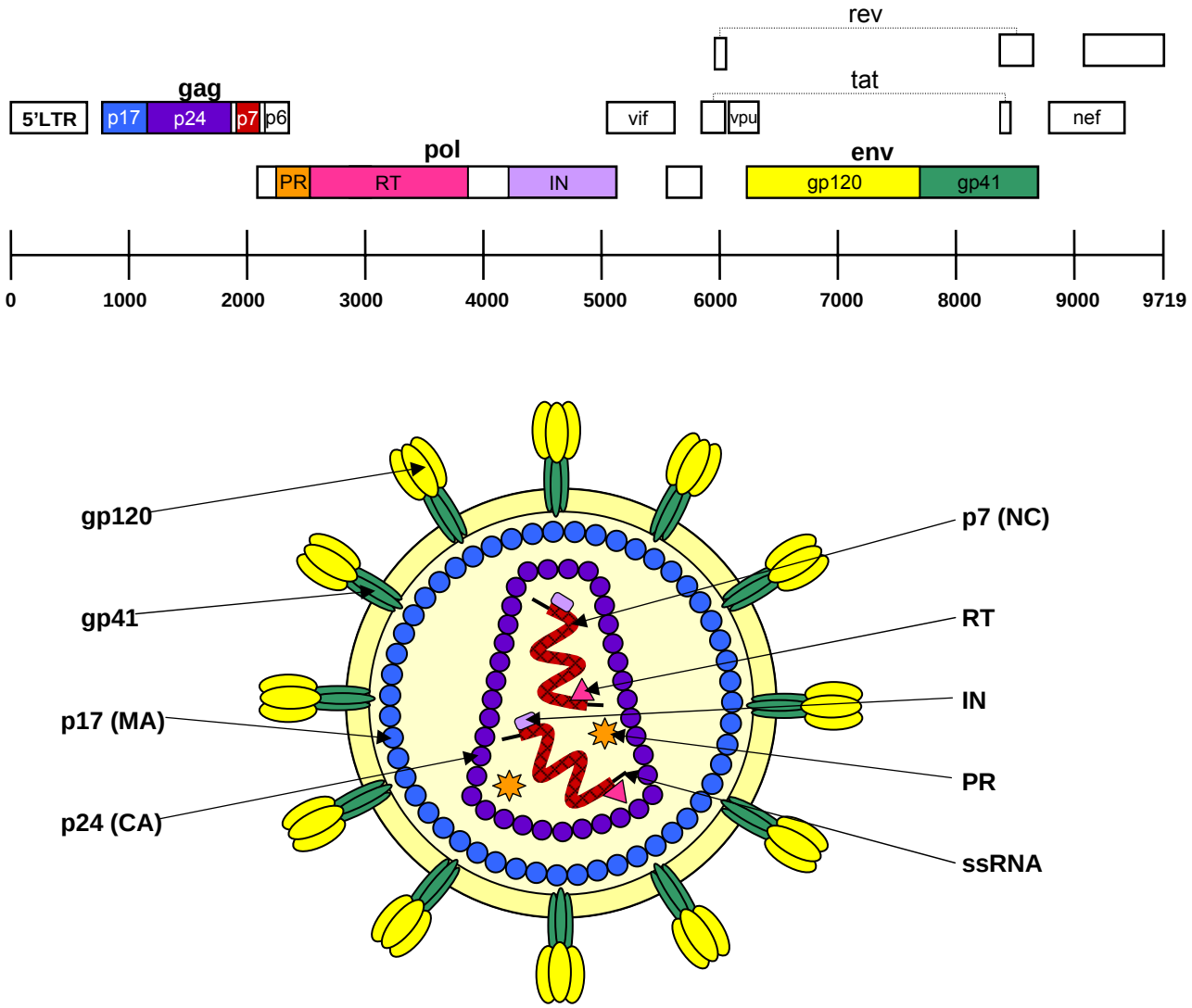

Figure 1. HIV-1 genome and structure. The genome map is drawn to scale and is based on the NCBI HIV sequence database (2010) and [51]. The virus cartoon is adapted from [58] and [57].

gp120 to bind to one of the main HIV co-receptors, most commonly the chemokine receptors CCR5 or CXCR4. This gp120-coreceptor interaction changes the conformation of the gp41 trimer which inserts into the target cell membrane and initiates direct fusion of the viral envelope with the target cell plasma membrane [60]. This fusion releases the viral core into the cytoplasm where it is further uncoated by the release of the capsid proteins. Uncoating reveals the reverse transcriptase complex, which is made up of viral RNA and the RT enzyme in conjunction with the IN, MA, NC and Vpr viral proteins as well as the host-derived factor $tRNA^{Lys3}$ [58]. Reverse transcription of the viral genomic RNA is initiated in the cytoplasm by $tRNA^{Lys3}$ annealing to the HIV LTR region and acting as a primer for RT-catalyzed cDNA synthesis [61, 62]. Much of the incredible diversity of HIV stems from its RT enzyme, which is error-prone (generating an average of 1 nucleotide error per genome) and has no proofreading activity [37, 56]. The resulting viral cDNA complexed with viral and host proteins (notably IN, MA, NC and Vpr) are referred to as the pre-integration complex (PIC) which is thought to be imported into the host cell nucleus under the direction of Vpr by cellular mechanisms [59].

The viral IN enzyme catalyses integration of the proviral DNA within the host cell chromosome by cleaving both the double-stranded linear viral cDNA and the host chromosomal DNA and catalyzing a strand transfer reaction; the integration process is completed by cellular DNA repair mechanisms [63]. Once integrated, the proviral DNA is effectively part of the host cell chromosome and its transcription is regulated by the host cell. As such, viral progeny are produced in actively transcribing cells, but the presence of latent provirus in non-dividing cells acts as a viral reservoir protected from drug interventions and the immune system [59]. Viral mRNAs are transcribed from the

LTR by host RNA Polymerase II and are transported to the cytoplasm to be translated by host ribosomes [58]. Most of the mRNA produced in a host cell is spliced before leaving the nucleus and HIV RNA is no exception; the initial transcripts produced are short splice variants encoding only a few viral proteins including regulatory Tat and Rev. The Tat protein combines with host RNA Polymerase II and other transcription factors such as NF-κB to dramatically increase viral transcription efficiency [59]. Meanwhile, the Rev protein also migrates back to the nucleus where it binds to full-length and partially spliced viral RNAs to prevent further splicing. Rev also helps to shuttle viral RNAs from the nucleus to the cytoplasm for synthesis of the structural and enzymatic viral proteins. The Env polyprotein gp160 is expressed in the endoplasmic reticulum, is heavily glycosylated and transported to the Golgi where host proteins mediate cleavage to gp120 and gp41. These two envelope proteins are trafficked to the host cell membrane where they assemble into heterotrimers and are anchored to the outer surface of the membrane by a hydrophobic tail of gp41 [59].

Virion assembly takes place in the cytoplasm near the host cell plasma membrane and is primarily directed by the Gag polyprotein. The N-terminal MA domain of the Gag and Gag-Pol proteins binds directly to the inner surface of the cell membrane while the CA and NC domains interact with neighbouring Gag proteins [64]. In this manner, a spherical shell of structural protein precursors form around two genomic copies of viral ssRNA, the HIV accessory proteins and certain host molecules such as $tRNA^{Lys3}$. Once the core of the immature virion has assembled, the virus buds from the host cell, acquiring a lipid envelope with gp120 & gp41 in the process. The newly formed immature virion is non-infectious until the Gag-Pol polyprotein is autocatalysed to

release PR, which cleaves the Gag and Gag-Pol components into mature structural proteins. These mature viral proteins rearrange to form a mature, infectious virion distinguishable by the conical viral core [58, 64].

1.5 HIV Transmission & Pathogenesis

HIV is spread through contact with infected bodily fluids, which includes unprotected sexual intercourse, sharing of contaminated injection drug needles, receipt of tainted blood products, or from mother to child during childbirth and breastfeeding. While transmission dynamics vary by region, globally more than 90% of new infections occur across a mucosal barrier [65] with heterosexual intercourse accounting for the majority of these infections [66].

The probability of heterosexual HIV transmission is dependent on both host and viral factors, such as the infectivity of the transmitted virus, the infectious dose of the infected bodily fluids, and the susceptibility of the uninfected partner [51]. The infectivity of the transmitted virus is influenced by viral tropism, as 90% of new HIV infections are caused by viruses that use the CCR5 co-receptor (R5 tropic viruses), rather than the CXCR4 co-receptor (X4 tropic viruses) [67]. Additionally, certain viral clades have genetic properties that may increase their pathogenicity, as in the case of clade C viruses, which demonstrate a replicative advantage over other clades due to the presence of an extra NF-κB binding site within the LTR promoter which increases viral responsiveness to pro-inflammatory cytokines such as TNF-α [68]. The likelihood of transmission is also tightly linked to the infectious dose of the infected partner. Systemic plasma viral load is an important correlate of virus shedding in the genital tract and is strongly predictive of

sexual transmission [51]. Individuals suffering from acute HIV infection as well as those in late-stage HIV disease have the highest viral loads and are therefore at highest risk to pass the infection to their sexual partners [69]. By contrast, individuals who control their viral loads either through immune mechanisms or with the use of ARVs are less infectious. One study of serodiscordant couples in Rakai, Uganda recorded zero HIV transmission events when the infected partner had a serum HIV RNA level below 1500 copies/ml [70]. The susceptible partner may also be at increased risk of HIV acquisition due to participation in risky sexual practices that compromise the integrity of the genital mucosa; however, host genetics and immune function also play a role in determining the likelihood of transmission [71].

In the event that free- or cell-associated HIV successfully penetrates the mucosal defences, the virus quickly disseminates to establish a systemic infection. Normally, the initial viral inoculum is quite small and may even be caused by a single virion [72]. During the 2-3 days following infection, the founder virus population expands by infecting susceptible CD4+ T cells, macrophages and dendritic cells present in the lamina propria at the point of entry [6, 67]. Dendritic cells transport HIV from the peripheral tissues to regional draining lymph nodes either by direct infection or by viral capture and internalization via the DC-SIGN C-type lectin receptor, known as *trans* enhancement [73, 74]. Due to the high concentration of activated target CD4+ target cells in the lymph nodes, HIV massively replicates and establishes the lymphatic tissue as the major viral reservoir of the body [75]. By ~3 weeks post-transmission, HIV has disseminated throughout the body via the bloodstream to other lymph nodes and to the gut-associated lymphoid tissues and genital tract as well as to non-immune organs. Latent viral

reservoirs are established in resting, long-lived immune cells that persist over the course of the infection [6, 75].

1.5.1 Clinical course of HIV infection

HIV disease progression can be classified into three clinical phases: acute infection, clinical latency, and AIDS. The acute phase is the initial stage of HIV infection and corresponds to the exponential production and systemic dissemination of the virus throughout the body. Regardless of the route of transmission, the circulating virus is predominantly R5-tropic and can be detected in the bloodstream by ~8 days post-infection [67]. Peak viremia occurs about 2-3 weeks post-infection, when plasma viral loads double every 0.3 days and reach a titre of up to 10^7-10^8 copies/ml. The increase in plasma viral RNA is echoed by a similar spike in viral shedding in the genital secretions which increases the risk of sexual transmission 10-20 fold [76, 77]. The exponential increase of viral replication also results in a dramatic decline of CD4+ effector memory T cells [78], causing two-thirds of patients to experience a sudden onset of flu-like symptoms such as fever, myalgia and lymphadenopathy [67]. After ~4 weeks of infection, the viral load begins to decrease due to the reduced availability of CD4+ target cells [66] and the emergence of host antiviral antibody and cytotoxic T lymphocyte (CTL) responses. Between 3-6 months post-infection, a robust antibody response can be measured in the bloodstream [67]. The appearance of these HIV-specific IgG antibodies is known as seroconversion and is a major diagnostic factor of HIV infection.

By approximately 6 months post-infection, patients enter a chronic, asymptomatic stage of infection known as clinical latency. This stage lasts approximately 10 years in a

typical course of HIV infection. Chronic infection is characterized by a drop in viral load to a relatively low and stable viral "set point", usually below 20,000 copies of viral RNA per millilitre of blood, and a corresponding partial recovery of CD4+ T cell levels [51]. Viral replication is partially controlled by the development of broader and more effective CTL and neutralizing antibody responses. However, the HIV-specific immune response exerts selective pressure on the virus which rapidly increases its diversity and drives the evolution of viral escape mutants [51, 79], which may also account for the emergence of directly cytopathic X4-tropic viral strains in the latter stages of HIV disease. The viral infection cannot be completely cleared due to the persistence of latent proviral reservoirs that are protected from host immune mechanisms [66], allowing low-level viral replication to persist in the host lymphoid tissues. Viral persistence ensures chronic generalized immune activation, the degree of which is a strong predictor of the rapidity of disease progression [80]. This chronic activation results in immune exhaustion accompanied by a gradual decline in CD4+ T cell levels and a corresponding upswing in viral load [81, 82].

By about 10 years post-infection, the majority of untreated patients develop signs of disease associated with the transition to AIDS. Symptoms begin to appear when the CD4+ T cell count drops between 500-350 cells/μl of blood [83]. A diagnosis of AIDS occurs once the CD4+ T cell count has fallen below a threshold of 200 cells/μl [84] as a result of the destruction of lymphoid tissues by direct viral cytopathogenicity and indirect mechanisms such as chronic activation-induced cell death and apoptosis [51, 82]. In addition to a low CD4+ T cell count, this stage is characterized by a dramatic increase in viral load and the emergence of opportunistic infections. Eventually the depleted host

immune system is no longer able to prevent the acquisition of AIDS-defining illnesses such as pneumonia and Kaposi's sarcoma and the patient succumbs to the infection.

1.5.2 Altered disease progression

Although the majority of HIV-infected persons follow a typical course of disease progression described above, some individuals display abnormally rapid or slow progression to AIDS. In addition to the average course of infection, three other classes of disease progression have been described [83]. Rapid progressors undergo an extremely short chronic infection and develop AIDS 2-3 years post-infection. Long-term survivors progress normally to AIDS but clinically stabilize thereafter. Long-term non-progressors (LTNP) have an extended clinical latency period and remain asymptomatic and healthy for longer than 10 years while maintaining a low viral load and a roughly normal CD4+ T cell count in the absence of treatment [83]. A subgroup of LTNP known as elite controllers maintain clinically undetectable plasma viral loads of less than 50 copies/ml [51]. The mechanisms responsible for altered HIV disease progression have not been fully elucidated, but viral fitness, host genetic factors and HIV-specific immune responses are all thought to play a role [71, 83].

1.6 Defences of the female genital tract to HIV

Since the discovery of HIV, research has primarily focused on understanding the immunopathogenicity of HIV within the systemic immune compartment. However, more than 90% of HIV infections occur across a mucosal barrier [85] with heterosexual intercourse accounting for 70-80% of these transmissions, especially in women [66, 86].

As such, the female genital mucosa is often the site of initial contact between HIV and the host immune system, making the mucosal immune response the body's first line of defence against HIV infection. Consequently, it is now believed that an effective method of preventing HIV infection will rely on a thorough understanding of the immune correlates of protection present at the female genital tract.

Despite the dominant contribution of heterosexual intercourse to the spread of HIV-1 infection, the risk of HIV transmission per coital act is surprisingly low. The probability of male-to-female HIV transmission per coital act has been estimated at 0.0005-0.0026 depending on the seminal viral load, the presence or absence of other STIs and the stage of HIV infection [76]. This low transmission rate suggests that the natural physical and immune barriers of the healthy female genital tract (FGT) are relatively effective at preventing the entry of HIV. The three lines of defence of the genital tract include the genital mucosal barrier, innate immune factors and the adaptive immune response, which act in a synergistic manner to resist infection by HIV and other sexually acquired pathogens [87].

1.6.1 Physical barriers to HIV

The genital mucosa consists of an epithelial cell layer covered by mucoid cervicovaginal secretions and natural vaginal microflora, both of which provide a physical barrier to HIV entry. The FGT is colonized by a diverse array of commensal bacteria that can adhere to the endothelial cell surfaces to physically exclude pathogenic microorganisms. The vaginal microflora of a healthy individual is dominated by *Lactobacillus* species, which metabolically produce lactic acid and lower the pH of the

vaginal lumen [88]; under these conditions the diffusion of HIV within cervicovaginal mucus is drastically reduced [89]. Additionally, many lactobacilli produce hydrogen peroxide, which has potent antimicrobial activity and is toxic to HIV-1 [90]. The FGT also produces cervicovaginal secretions which guard against infection by several different mechanisms. A thick mucus plug blocks the endocervical canal and prevents pathogens from entering the uterus; this may also prevent HIV from gaining access to the relatively vulnerable endocervical columnar epithelium [91]. Vaginal secretions are viscous and can capture microorganisms and prolong their exposure to antiviral compounds [92]. The chemical composition of cervicovaginal mucus is diverse and includes a number of compounds with broad antimicrobial activity: mucins (which are protective against proteolysis of defensive host proteins), lysozyme (which degrades bacterial peptidoglycan), lactoferrin (which has anti-bacterial properties and has been demonstrated to have an inhibitory effect on HIV replication), secretory leukocyte protease inhibitor (SLPI) and α/β-defensins [93-96]. The genital secretions also provide lubrication during sexual intercourse which helps to prevent epithelial tears and microabrasions that could otherwise allow HIV to penetrate the epithelium.

The mucosal epithelium itself provides the main physical barrier to infection. Two types of epithelial barrier exist within the female genitourinary tract: the stratified squamous epithelium which lines the vaginal walls and the ectocervix, and the columnar epithelium which lines the endocervical canal [87]. The squamous epithelium is made up of multiple layers of epithelial cells which provide excellent mechanical protection against HIV infection when intact; these cells do not express CD4 [97] and have not been shown to be directly infectable with HIV-1 *in situ* [20]. The columnar epithelium is made

up of a single layer of polarized cells connected by tight junctions [87]. While this single-layer epithelium is easier to breach, its location in the upper genital tract behind the endocervical mucus plug makes it relatively inaccessible to HIV infection [91]. Additionally, the epithelial cells of this barrier express Toll-like receptors as well as the polymeric immunoglobulin receptor (pIgR) on the basolateral side of the membrane which serve to transport immunoglobulins into the vaginal lumen and provide the vulnerable endocervix with additional protection [98].

The physical defences of the genital mucosa are sufficiently effective to ensure that, when HIV infection does occur, it's usually caused by a few or even a single virion [66]. This low viral inoculum provides the host with a short "window of opportunity" to eliminate the virus before it has a chance to disseminate beyond the mucosal compartment and establish a systemic infection [51]. In fact, if the HIV infection is cleared or contained in the periphery at this stage, the exposure might act as a local "minivaccination" and generate adaptive immune responses that could protect against subsequent infections [87].

1.6.2 Innate & Adaptive Immune Responses

In the event that HIV penetrates the mucosal barrier, a number of general innate and adaptive immune mechanisms may help to control or eliminate the infection. The invading microbe is initially detected by circulating complement proteins or by pattern recognition receptors (PRRs) expressed on innate immune cell surfaces. Complement proteins bind to free- or antibody-bound pathogens and cause direct lysis or opsonization to promote phagocytosis. PRRs, such as Toll-like receptors (TLRs) recognize conserved

molecular motifs common to pathogenic microorganisms (such as dsRNA or bacterial lipopolysaccharide) and initiate a number of intracellular signaling cascades, resulting in cytokine production [99]. Cytokines activate, recruit and direct the various types of immune cells at the site of infection. Natural killer cells detect and kill virally-infected cells based on the downregulation of human leukocyte antigen class I (HLA-I) marker expression on the cell surface. Macrophages and dendritic cells phagocytose and digest pathogenic microorganisms in order to present them to CD4+ T helper (T_H) adaptive immune cells in the context of surface HLA class II (HLA-II) molecules. Antigen binding activates the T_H cells to produce numerous cytokines which in turn initiate an adaptive immune response.

The adaptive immune response is comprised of B cell and T cell activation. B cells make up the humoral branch of the adaptive immune response and produce antigen-specific antibodies that neutralize, opsonize or lyse the invading microbe by direct mechanisms or by antibody-dependent cell-mediated cytotoxicity (ADCC). CD4+ T cells and CD8+ T cells, which are also known as CTLs when activated, make up the cell-mediated adaptive immune response. After activation by CD4+ T helper cells, CTLs interact with viral antigens presented by HLA-I molecules on the surface of virally infected cells and release cytotoxic enzymes to kill the infected cell by apoptosis or direct lysis. In addition to these effector cells, long-lived memory B and T cells are also produced to enable a quicker and more effective response to the pathogen in the event of a future exposure.

1.6.3 HIV invasion of the female genital tract

Despite the numerous defences present at the FGT, the ability of HIV to gain entry into the body can be facilitated by numerous host factors. In addition to infection with free virions, cell-associated HIV can also infect the genital mucosa, in which case the infected donor cell may help to shield the virus from antiviral host proteins and chemicals present in the vaginal lumen. While the presence of lactobacilli in the genital tract is beneficial, colonization with other types of microbiota can facilitate HIV transmission. Bacterial vaginosis, a condition characterized by the overgrowth of anaerobic bacteria such as *Gardnerella vaginalis* in the genital tract, is associated with an increased risk of HIV acquisition [88]. These organisms increase the pH of the vaginal lumen which improves HIV survival and migration through the squamous epithelium [100]. Infection with other STIs also promotes HIV transmission by activating Langerhans cells and recruiting target CD4+ T cells and macrophages to the site of infection [100]. Ulcerative STIs such as HSV-2 additionally facilitate HIV infection by disrupting the integrity of the epithelial barrier [101]. Other pathogens produce host protein-damaging enzymes such as the IgA1 protease secreted by *Neisseria gonorrhoeae* [92, 102].

Natural changes in the FGT during menstruation also affect HIV susceptibility. Although mucosal secretions are always present in the female genital tract to some degree, their pH and viscosity is affected by the menstrual cycle. In particular, the endocervical mucus plug is thinned in response to high levels of estrogen present during ovulation, which may result in the loss of its barrier function [91]. The stratified squamous epithelium is also affected by menstrual cycle hormones; progesterone reduces

the thickness of this barrier which can increase the risk of infection [20]. Despite the multiple cell layers and thickness of the squamous epithelium, it is prone to inflammation or injury. Epithelial barrier microabrasions are detectable in up to 60% of women following consensual sexual intercourse [97]. Small tears in the barrier can permit HIV with more direct access to susceptible intraepithelial CD4+ T cells and Langerhans cells, while serious breaches allow the direct passage of free virions and infected donor cells into the submucosa [91, 100, 103]. However, tissue damage is not necessarily a requirement for HIV entry; the virus may also be capable of interstitial diffusion through the intact squamous epithelium [103]. Additionally, Langerhans cell dendrites are capable of projecting around squamous epithelial cells and extending into the vaginal lumen to sample the microenvironment, which puts them at risk of HIV binding and infection. The transitional zone between the ectocervical squamous epithelium and the endocervical columnar epithelium is particularly enriched with HIV target cells. This zone is normally protected by the cervical mucus plug but in the event of cervical ectopy, a condition common in younger women, the transitional zone extends beyond the mucus plug and is exposed to the vaginal lumen [97, 102]. As a cell monolayer, the columnar epithelium is relatively fragile and is quite vulnerable to direct infection by HIV, to HIV transcytosis by viral endocytosis [104, 105] and to the epithelial transmigration of infected donor cells [97]. There is also evidence that the direct exposure to HIV-1 gp120 enhances TNF-α production and disrupts tight junction proteins, thereby allowing microbial translocation across the barrier [106]. Due to these factors, it is believed that cervical epithelial barrier is the most common site of HIV penetration within the female genital tract [102].

Once HIV gets past the mucosal barrier, there are a number of ways it can evade and exploit the immune defences. First, although only a low amount of virus is able to cross the epithelial layer, HIV replicates and disseminates very quickly. HIV reaches the peripheral draining lymph nodes within the first week of infection, by which point it is too late to avert a full-blown systemic infection [75]. This is too narrow a time frame for the generation of an effective primary immune response, which can take up to 2 weeks [107]. The broadly neutralizing antibodies and CTLs that are eventually generated have been shown to be very adept at killing virus in the bloodstream [67], but these responses emerge too late to be able to prevent the establishment of viral reservoirs and reverse the infection. Notably, since recall immune responses peak ~5 days after re-exposure, a vaccination that could generate an appropriate and rapid HIV-specific response might be able to halt viral expansion. The proinflammatory cytokines produced by innate immune cells in response to many pathogens may bolster HIV replication [108]; activating and recruiting the very cells targeted by HIV to the infection site where they become hosts for further viral replication. Furthermore, HIV mainly targets macrophages, dendritic cells and CD4+ T cells which are the main cell types involved in initiating and directing the downstream adaptive immune responses. As such, by invading and crippling these cells during early infection, HIV may further delay the development of an effective immune response until after a latent infection has been established.

Even if the adaptive immune responses are present, the HIV virus itself is very difficult to target due to both its genetic variability and the conformational mutability of its envelope glycoproteins [109]. The HIV RT enzyme is error-prone and without proofreading ability; on average one error occurs in every 10^4 nucleotides which

corresponds to one mutation in every replicated virion [56]. On average, 10.3 x 10^9 virions are produced in a chronically-infected individual daily; as such it is theoretically possible for every mutation to occur at every position along the viral genome every day [110]. Many of the resulting viral progeny are defective; however some of the random mutations generate escape mutants that can evade existing immunity and replicate freely until new HIV-specific responses are generated. Furthermore, the host cell-derived HIV envelope shields the viral core proteins and restricts the HIV-specific neutralizing antibody response to the two proteins that make up the viral envelope spike. Both gp120 and gp41 are heavily glycosylated (gp120 is 50% carbohydrate by weight [111]) which serves to shield these proteins from the immune response. The envelope spike is arranged in a manner which minimizes the exposure of any functionally constrained cell receptor-binding sites. The five highly variable regions loops of gp120 shield the functionally conserved regions and tend to be strain-specific and immunodominant [112], which may help to cause deceptive imprinting and ultimately facilitate viral immune evasion [113]. Furthermore, the gp120-gp41 heterotrimer is arranged so that important structurally conserved regions such as the CD4 and co-receptor binding sites are sterically inaccessible to most neutralizing antibodies. The CD4 receptor binding site is located within a deeply recessed pocket of the inner domain of gp120, which appears to restrict binding to antibodies such as b12 which possess unusually long heavy chain CDR3 loops [113]. The co-receptor binding site (which is located within the gp120 V3 loop) is only exposed after conformational rearrangement occurring after gp120-CD4 binding. Together, these factors make the generation of lasting, broadly HIV-neutralizing humoral responses to HIV-1 a difficult task.

1.7 Natural Immunity to HIV Infection

Despite the original assumption that all individuals are equally susceptible to HIV infection following exposure, considerable evidence has emerged over the past ~15 years to suggest that considerable variation exists in one's susceptibility to infection. Despite frequent, long term unprotected sexual exposures to HIV, some individuals do not become infected and are therefore considered to be relatively "resistant" to HIV-1 infection [114]. Examples of HIV-1 exposed seronegative (HESN) individuals have been identified in a number of high-risk cohorts including infants of HIV-infected mothers, intravenous drug users (IDUs), HIV-serodiscordant couples and commercial sex workers (CSWs) [55, 114-118]. However, the mechanisms that mediate protection from HIV infection are still poorly understood. While the presence of an adaptive HIV-specific immune response in these HIV uninfected individuals proves viral exposure occurred, it is not known for certain whether these responses are the cause or result of viral clearance and also whether they are protective upon subsequent exposure [119]. As a direct experimental HIV-1 challenge in human populations is not possible for obvious ethical and practical reasons, much of our knowledge of the correlates of HIV resistance stems from observational cohort studies and from animal NHP studies involving SIV/SHIV. So far, these studies have made it clear that no single factor is responsible for the HIV-resistant phenotype and that different correlates of protection exist in different individuals. Overall, a multitude of genetic, innate and acquired immune mechanisms may contribute to this phenomenon, and it is hoped that a thorough understanding of these mechanisms will inform the design of a protective vaccine or microbicide.

1.7.1 The Pumwani Cohort

One of the first descriptions of the phenomenon of natural immunity to HIV-1 infection arose from the Pumwani commercial sex worker (CSW) cohort based in Nairobi, Kenya. This cohort, originally established with 600 women in 1985, has been used to study risk factors for seroconversion to HIV-1 [114]. Between 1985 and 1994, 424 initially HIV-1 seronegative women were followed up for a period of 1-10 years. While the majority of the women quickly seroconverted, the study unexpectedly found that the risk of seroconversion was inversely proportional to the duration of viral exposure via sex work. Seroconversion was most likely to occur in the first 2 years of follow-up; thereafter the risk of becoming infected was greatly reduced. Most importantly, about 10% of the women remained HIV-1 seronegative after 3 or more years of follow-up despite experiencing an estimated average of 64 unprotected exposures per year. The absence of infection was not associated with differences in sexual behaviour (such as condom use or number of clients per day), suggesting a genetic or immune basis for protection from infection.

Since 1985, over 3500 women have been enrolled in the Pumwani CSW cohort, of which ~800 are actively followed up. Between 5-10% of the participants meet the updated HESN definition of 7 or more consecutive years of follow-up in the cohort without seroconverting to HIV-1 [114]. Resistance appears to be specific to HIV; these women do not appear to have a decreased susceptibility for other types of STIs. While HIV resistance in some other cohorts can be attributed to the CCR5Δ32 polymorphism (where an internal 32bp deletion on the CCR5 co-receptor gene prevents CCR5 expression on target cell surfaces) [120], or to the overexpression of beta-chemokine

CCR5 co-receptor ligands (RANTES, MIP-1α & MIP-1β) or CXCR4 ligands (SDF-1), this does not appear to be the case in the Pumwani cohort [121, 122]. Peripheral blood mononuclear cells (PBMCs) from these women are able to be infected with various R5- and X4-tropic isolates of HIV-1 under *in vitro* culture conditions; suggesting that resistance is not due to co-receptor polymorphisms or in response to beta chemokines [122]. Interestingly, resistance to HIV-1 is not absolute; seroconversion may occur after a HIV-resistant individual takes a break from sex work and is associated with a loss of HIV-specific CD8+ T cells which suggests that ongoing antigenic priming is required to maintain a protective CTL response [123, 124]. It should also be noted that the women of the Pumwani cohort are exposed to many diverse strains of HIV-1 endemic to Kenya, and the mechanisms that mediate their protection are likely to be broadly cross-protective [114].

1.7.2 Genetic Correlates of Resistance in the Pumwani cohort

The possibility of a genetic basis for resistance to HIV-1 infection was proposed after it was observed that HIV resistance clustered in families; relatives of HIV resistant women are more likely to remain HIV uninfected than the relatives of either HIV seropositive or new HIV negative cohort enrolees [125]. The first major set of genetic variants associated with the HIV resistance phenotype occurs in the HLA class I and II locus. This is not surprising, as HLA molecules delineate the molecular targets of the adaptive immune system by presenting specific viral antigens to CD4+ and CD8+ T cells. Alleles associated with HIV-1 resistance in the Pumwani cohort include the class I HLA-A2/6802 supertype as well as several class II alleles and haplotypes belonging to the

HLA-DRB1*01, HLA-DQB/DQA and HLA-DPA1 families [126-131]. The high proportion of resistance-associated HLA class II alleles suggests an important role for CD4+ T cell effector mechanisms in protection from infection. The second major genetic correlate of HIV- resistance in this cohort is a set of polymorphisms in the Interferon regulatory factor 1 (IRF-1) gene. IRF-1 is a host interferon- and T_H2 cytokine-transcriptional regulator that can also activate early proviral transcription from the HIV-1 LTR. A number of non-coding single nucleotide polymorphisms within IRF-1 lower its basal expression, reduce early HIV-1 LTR transcription and are significantly more prevalent among HIV resistant individuals [132-134].

1.7.3 Immune Correlates of Resistance in the Pumwani cohort

A number of unique innate and adaptive immune characteristics have been observed in HIV-1 resistant women of the Pumwani cohort. Recent studies show that at least some of these women overexpress antiviral antiproteases such as serpin B and cystatin A at the level of the cervical mucosa [93]. TLR expression also appears to be downregulated in the FGT of these women which may help to prevent localized inflammation [Lester, unpublished data].

Adaptive HIV-specific immune responses are also frequently observed in HESN women of the Pumwani cohort. The presence of broadly cross-reactive HIV-specific CTLs in the blood and especially in the FGT is a major correlate of protection from HIV-1 in the Pumwani cohort. A number of studies have shown that over half of the HIV-resistant women tested have circulating HIV-specific CTLs directed against multiple viral peptides (including Env, Gag, Pol and Nef) across several clades [135-137]. HIV-

resistant women with the longest follow-up in the cohort, and therefore the highest levels of viral exposure, are more likely to generate HIV-specific CTL responses, indicating that these responses are inducible rather than intrinsic [119]. Both HIV seropositive and HIV-resistant women also have detectable cervical CTL responses; however the HIV-specific CTLs of HIV-resistant women are enriched at the FGT as compared with the blood [138] and also target distinct viral epitopes [119, 139].

There is also evidence to suggest a role for CD4+ T cells in HIV resistance. Because CD4+ T_H cells play an important role in the activation and coordination of the CD8+ T cell response, it has been suggested that the presence of specific CD4+ T cell responses improve the "quality" of the CTL response [137, 140]. One study determined that p24-stimulated CD4+ T cells from HIV-resistant individuals had lower IFN-γ production but a 4.5-fold greater proliferative response compared to CD4+ T cells from HIV seropositive individuals [141]; this observation is significant, given that many studies have now shown that proliferative responses are more protective during HIV infection than IFN-γ responses [142].

In addition to the HIV-specific adaptive immune responses observed in the Pumwani cohort, the resistance phenotype correlates with a recently described phenomenon called immune quiescence [143], where the adaptive immune system exists in a lower baseline state of activation. CD4+ T cells isolated from HIV-resistant individuals express lower levels of immune activation markers HLA-DR, CD38 and CD69 compared to HIV-uninfected CSWs [141, 144]. Compared to high- and low-risk HIV negative controls, the CD4+ T cells of HIV-resistant women show a pattern of reduced mRNA expression of cellular genes involved in T cell activation, cytokine

signalling and HIV replication pathways, which is corroborated with lower levels of proinflammatory cytokines detected in resting cells *ex vivo* [143]. This state of low activation may be mediated by the high frequency of CD4+CD25+Foxp3+ regulatory T cells (Tregs) in the PBMC of HIV-resistant women [144], a cell subset which is known to suppress the activation of both CD4+ and CD8+ T cells [145, 146]. Taken together, there is strong evidence to suggest that HIV-resistant individuals have a lower level of basal immune activation than HIV-1 susceptible individuals. Since HIV-1 preferentially infects and replicates within activated CD4+ T cells, maintaining a lower state of immune activation could provide an advantage by reducing the number of target cells able to support productive infection.

Whether humoral responses such as HIV-specific IgA can contribute to HIV resistance remains somewhat unclear since evidence from the Pumwani cohort as well as from other HIV-resistant cohorts has been inconsistent (discussed in section 1.8.5 in detail). A recent study found no significant difference in HIV-specific IgA levels detected in a large sample of 41 HIV-resistant, 146 HIV seropositive and 85 HIV seronegative women [147]. These results conflict with an initial report that found higher HIV-specific IgA in HIV-resistant women [148]. Regardless of their conclusions, neither study assessed the capacity of these antibodies to inhibit HIV. Since antibody specificity does not necessarily imply functionality [149], there remains the distinct possibility that HIV-1 specific IgA recovered from HIV-resistant individuals have different functional characteristics than IgA recovered from control groups, and may therefore be better able to inhibit HIV-1 by viral neutralization or inhibition of transcytosis. Indeed, polyclonal HIV-specific IgA isolated from HIV-resistant women of the Pumwani cohort neutralized

HIV-1 and inhibited viral transcytosis *in vitro*, and single chain variable fragments (ScFvs) cloned from the cervical B cells of several of these women also had the capacity to neutralize HIV-1 *in vitro* [150], which supports the possibility that HIV-specific IgA in the FGT plays a role in protection. It is clear that IgA antibodies isolated from HIV-resistant women will need to be further characterized to determine whether and at what levels IgA is protective in this cohort.

1.8 Antibodies and HIV

1.8.1 The antibody response in the female genital tract

While a number of immune mechanisms are thought to be involved in mucosal protection from HIV infection, neutralizing antibodies are a critical host defence in providing sterilizing immunity [151]. Within the FGT, the humoral response is mainly mediated by antibodies of the IgA, IgG and occasionally IgM isotypes [152, 153]. While IgA is the main immunoglobulin isotype found in most external mucosal secretions (including saliva, tears, gastrointestinal fluid, milk & colostrum), IgG antibodies predominate within genitourinary tract secretions [153, 154]. Interestingly, HIV serostatus appears to strongly affect the relative abundance of HIV-specific antibody isotypes. In HIV seropositive individuals, HIV-specific IgG predominates in all external secretions and HIV-specific IgA levels, if present, are extremely low [153]. In contrast, HIV-specific IgA is commonly (but inconsistently) detected in the cervical fluid, serum and saliva of HIV-resistant individuals, while HIV-specific IgG is infrequently detected. This suggests that HIV-specific IgA, not IgG, responses may be most effective in preventing HIV infection at the FGT. Unfortunately, due to the relative ease of isolating

and characterizing HIV-specific IgG from HIV+ patients compared to HIV-specific IgA from HIV-resistant individuals, all animal model studies to date that demonstrate a protective effect of HIV-specific antibodies have focused on IgG clones. The results of these studies (discussed below) suggest that antibody-based microbicides may be an effective and viable means of HIV protection.

1.8.2 Structure and composition of IgA in female genital tract secretions

Like other antibody isotypes, IgA is composed of two identical heavy chains (designated as α chains for IgA) which are linked by disulfide bonds to two identical light chains (Figure 2) [155, 156]. The heavy and light chains are named for their respective molecular weights; each IgA heavy chain is ~60kDa (heavy glycosylation increases the molecular weight compared to IgG) while each light chain is ~25kDa in size. A different α gene product accounts for each of the two IgA subtypes IgA1 & IgA2; the major distinguishing feature between these subtypes is a 13 amino acid deletion in the hinge sequence of IgA2 [155]. IgA2 is further subdivided into two allotypic variants IgA2m(1) & IgA2m(2). All of these subtypes and variants of IgA differ slightly in their amino acid sequence and glycosylation pattern [156]. While the α heavy chains are specific to IgA antibodies, the light chains are shared across the other antibody isotypes and are designated as either κ- or λ-type [158].

Both the heavy and light chains are subdivided into variable and constant regions, made up of ~110 amino acid repeating folded domains. The antibody variable regions are made up of the N-terminal domains of both the heavy and the light chains (termed V_H & V_L respectively) and are responsible for antigen recognition. Each antigen binding site

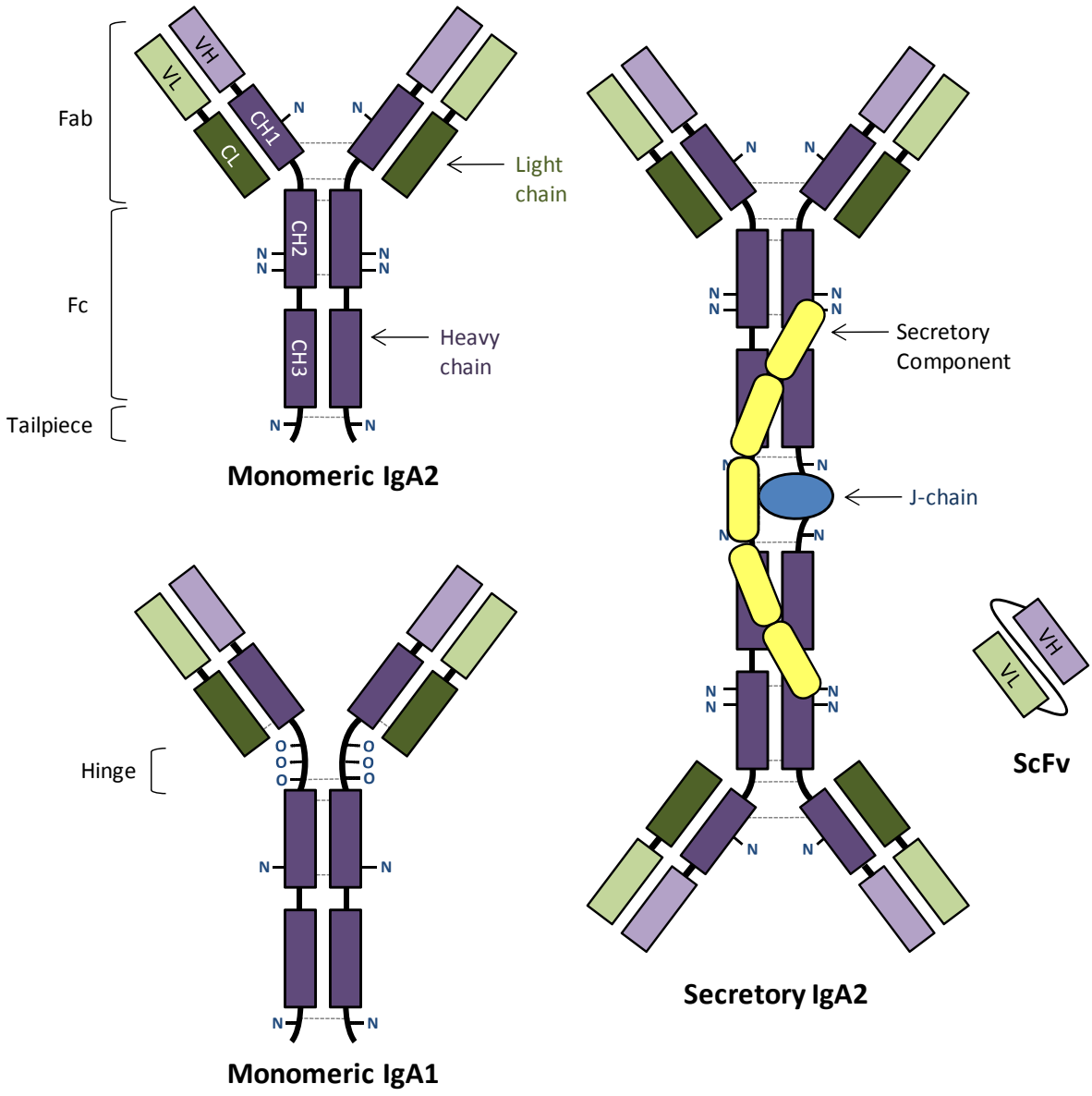

Figure 2. Structure of human monomeric IgA1 and IgA2, secretory IgA2 (s-IgA2) and a single-chain variable fragment (ScFv). Constant heavy chains are shown in dark purple, constant light chains in dark green, variable heavy chains in light purple and variable light chains in light green. The s-IgA2 diagram also shows the J-chain (in blue) and the secretory component (in yellow). N-linked oligosaccharides are represented as N, O-linked oligosaccharides as O, and disulfide bonds as dashed lines. The IgA2 structure is illustrated as the IgA2m(1) allotypic variant. Antibody diagrams are adapted from [156] and [163], and the ScFv diagram from [157].

forms a three-dimensional pocket where 6 short peptide loops (3 from the heavy chain and 3 from the light chain) make contact with the antigen. These short regions are known as the antibody complementarity determining regions (CDRs) and are interspersed with 4 relatively conserved framework sequences (FRs) [159]. The CDRs are hypervariable and in a typical IgG response undergo affinity maturation to increase antibody diversity and improve specificity upon subsequent exposures to an antigen [160]. The specificity of an antibody as well as its ability to bind antigen is in most cases determined primarily by the heavy chain CDRs and particularly by CDR3 [161]. The antibody constant region is made up of the C-terminal half of the L chains (C_L) and three additional H-chain domains (C_H1, C_H2 & C_H3). The constant region can interact with complement proteins and cell receptors to help determine the effector functions of the molecule (including ADCC and opsonisation) [156].

The full-length IgA molecule is arranged into a Y-shape of two N-terminal Fab regions, a flexible hinge region, and a C-terminal Fc region [156]. Monomeric IgA is ~160kDa in size and heavily glycosylated; N-linked oligosaccharides account for up to 10% of the molecular mass of IgA2 [155, 158]. Glycosylation is important to the function of the antibody and affects both antibody solubility as well as its ability to bind Fc receptors and mediate ADCC and complement activity [162, 163].

Unlike all types of IgG and the predominantly monomeric IgA found in serum, IgA recovered from the FGT is primarily secretory IgA (s-IgA), consisting of joined IgA polymers and a secretory component (SC), with the IgA2 subtype being overall more prevalent than IgA1 [164]. Although some serum-derived monomeric IgA can be recovered in the FGT, the majority of the IgA is locally produced as dimers by

endocervical B-lymphocytes [158, 164]. Dimeric (and tetrameric) IgA are cross-linked by disulfide bonding of a J-chain polypeptide to the cysteine 471 residue located within the Fc tail of each IgA monomer [158]. The SC component is added to s-IgA during transepithelial transport by the polymeric immunoglobulin receptor (pIgR). The pIgR is expressed on the basolateral side of the mucosal epithelial barrier and covalently binds to antibody-associated J-chain in order to transcytose polymeric antibodies to the luminal surface, at which point the antibody is released by pIgR cleavage, leaving an ~80kDa SC protein associated with the antibody complex [155]. The SC component has been shown to further stabilize IgA and increase its resistance to proteolysis [155, 158], ultimately allowing the antibody to remain resistant to degradation by pathogens present at the mucosal surface.

1.8.3 Potential mechanisms of antibody-mediated protection from HIV infection

There are a number of mechanisms by which HIV-specific IgA may be able to mediate protection from infection by HIV-1. To date, the majority of studies have focused on the generation or recovery of antibodies with CD4+ cell-based, *in vitro* HIV-neutralizing capacity. HIV neutralizing antibodies inhibit infection by directly binding to HIV and preventing its entry into susceptible target cells [24]. These antibodies generally target HIV envelope proteins to directly or sterically hinder binding to CD4 and/or an HIV coreceptor or to inhibit viral membrane fusion with target cells [165, 166]. Although most of the neutralizing antibodies described act upon cell-free HIV prior to viral attachment, certain antibody types have been shown to directly interact with intracellular HIV and inhibit viral replication and virion assembly in cultured epithelial cells.

Intracellular HIV replication was potently inhibited by IgA but not paired IgG antibodies, suggesting that vesicle-bound HIV-specific IgA antibodies undergoing pIgR-mediated transcytosis encounter and bind to newly synthesized HIV envelope proteins in post-Golgi secretory vesicles [167]. Transcytosing Gag- and RT-specific IgA has also been shown to reduce HIV replication, although the mechanism by which vesicle-bound antibodies disrupt cytoplasm-localized viral protein assembly is poorly understood [168]. Regardless, in all cases, the most effective HIV neutralizing antibodies target broadly conserved and functionally constrained viral epitopes, thus allowing the antibody to inhibit a diverse range of viral isolates across multiple viral clades.

Antibodies that do not directly neutralize HIV can also play a role in the prevention of HIV-1 infection. Polymeric IgA antibodies undergoing active pIgR-mediated transport through the epithelial cell barrier can capture transcytosing intracellular vesicle-bound HIV virions and redirect them to the lumen in a process known as viral excretion [169-171]. S-IgA but not IgG antibodies have also been shown to block viral transcytosis across the epithelial cell barrier by preventing viral uptake by the mucosal epithelium in the first place [172]. Anti-gp41 IgA (but not IgG) antibodies specific for the gp41 ELDKWA epitope have been shown to block HIV binding to Gal-Cer expressed on the apical surface of a cultured epithelial cell monolayer, inhibiting receptor-mediated viral endocytosis [105]. In a somewhat related phenomenon, luminal s-IgA antibodies may also non-specifically prevent viral attachment to the epithelial barrier by immune exclusion; multiple virions and antibodies become cross-linked and form immune complexes which are sterically unable to attach to the epithelial lining [158, 173].

Non-neutralizing IgA has a moderate capacity to trigger ADCC, especially in conjunction with NK cell activation [165]. ADCC has also been proposed as an eventual possible strategy towards eliminating viral reservoirs, although efforts thus far have not been successful [24]. While ADCC activity has been shown to be an important aspect of the anti-HIV activity of IgG [26, 174, 175], the contribution of ADCC activity to the HIV-inhibitory activity of IgA has not been well investigated. IgA has been shown to have ADCC activity in other retroviral models [173]. IgA isolated from the serum or colostrum of HIV infected individuals has been shown to lyse gp120-coated target cells in the presence of mononuclear effector cells *in vitro* [233]. In an unconventional twist on ADCC activity, an engineered bispecific anti-gp41/anti-CD89 (IgA Fc receptor) antibody has been shown to direct neutrophils to destroy HIV *in vitro* [176].

Finally, natural antibodies directed against HIV receptor molecules on uninfected target cells may also contribute to protection from HIV infection. This strategy has attracted considerable interest due to the comparable lack of variability of the host cell protein targets as compared to HIV envelope proteins. However, this strategy carries a risk that antibody binding to these receptors may disregulate the immune functions of these cells. Anti-CD4 antibodies were largely discontinued after they were found to induce immune suppression, while the CXCR4 coreceptor was too ubiquitous to be a good target. In contrast, most natural and engineered anti-CCR5 antibodies appear to be reasonably effective at blocking HIV attachment and produce no obvious adverse effects [24]. Breast milk samples from HIV seropositive and seronegative women have been found to contain CCR5-specific IgA & IgG antibodies that neutralize certain strains of clade B virus by up to 75% [177]. These antibodies have also been recovered from the

CVLs of HIV-resistant individuals; anti-CCR5 IgA, IgG and IgM antibodies purified from Central African Republic CSWs inhibited infection of macrophages and DCs with primary & lab-adapted strains of R5 virus [178], while anti-CCR5 IgA from discordant couples inhibited transcytosis of primary R5 viruses [170]. While moderately effective, the obvious drawback to this strategy is that the antibodies are only effective against R5 viral isolates. Finally, natural antibodies to the DC-SIGN carbohydrate recognition domain have also shown some promise; IgA & IgG-type antibodies recovered from breast milk and plasma have been shown to inhibit *trans* virus transfer between immature monocyte-derived DCs and CD4+ T cells by up to 60% [179].

1.8.4 Animal Models: Proof-of-concept for antibody-mediated protection

Several well-characterized human monoclonal IgG antibodies have been described that, in addition to demonstrating broadly HIV-neutralizing activity *in vitro*, also provide compelling evidence for the role of antibody-mediated protection from HIV infection based on SHIV/macaque infection models. These antibodies include IgG1b12, which recognizes an epitope overlapping the gp120 CD4 receptor binding site; 2G12, which is specific for a unique mannose-dependent conformational epitope within the C3-V4 region of gp120; and 2F5 & 4E10, which are respectively specific for the linear ELDKWA and NWFDIT epitopes within the membrane-proximal external region (MPER) of gp41 [24, 165]. All of these antibodies were originally derived from HIV clade B infected individuals and target highly conserved epitopes within the HIV envelope glycoproteins; interestingly all but 2G12 possess an unusually extended (18-22

amino acid) CDR3 loop which may enable antibody access to recessed viral binding sites [113].

A number of studies have demonstrated that the passive transfer of the above human HIV-neutralizing antibodies (administered individually or in combination) can protect macaques from various routes of SHIV challenge [34]. In the majority of these studies, the intravenous injection of HIV-neutralizing antibodies prior to intravenous [180, 181], oral [181], vaginal [182, 183] or rectal [184] SHIV challenge could completely prevent viral infection in the majority of macaques; in the few cases where SHIV transmission occurred, the infected macaques generally experienced lower viral loads and exhibited less severe clinical disease. Antibody-mediated protection was most effective when the 2F5, 2G12 & polyclonal HIV immune globulin (HIVIG) antibodies were administered in combination rather than individually [180, 182], and IgGb12-mediated protection was shown to be dose-dependent [183]. Interestingly, although antibodies were administered intravenously in these studies, it is known that the IgG detected in most mucosal secretions is partially of systemic origin, suggesting that the injected IgG may have migrated to the lumen of each mucosal compartment to prevent tissue uptake of HIV [164]. An additional study investigated whether synergistic combinations of neutralizing antibodies could protect against infection when administered after SHIV challenge; the intravenous infusion of IgG1b12, 2G12, 2F5 & 4E10 one hour after oral challenge protected 2/4 neonatal macaques and reduced disease severity in the others, demonstrating their utility even in cases of post-exposure treatment [185].

While these studies make it clear that pre-existing neutralizing antibodies can provide sterilizing immunity against HIV, long-lasting high-titre protective antibodies are difficult to generate though vaccination, and it is still unknown how to present these epitopes to the immune system in a native, immunodominant conformation [27]. The passive transfer of human HIV-neutralizing monoclonal antibodies is very effective at preventing SHIV infection in NHPs; however despite the fact that these antibodies appear to be well-tolerated in humans [24, 35, 186, 187], the high titres required for protection and the relatively short serum half-lives (4.3-21.8 days) of these antibodies make them impractical for general prophylactic use. A more practical approach would involve the addition of potent broadly-neutralizing antibodies to microbicides. This strategy is supported by a study in which b12 applied to the vaginal lumen 2 hours prior to high-dose intravaginal SHIV challenge protected 9/12 macaques from infection [25].

While these studies provide an important rationale for the follow-up of antibody-based microbicides and vaccines, many gaps in our current knowledge still remain. First, with the exception of two recently-identified monoclonal antibodies PG9 & PG16 (discussed in section 1.9.2), all of the HIV-neutralizing monoclonal antibodies discovered to date are primarily directed against clade B infecting viruses. While antibodies such as 4E10 are capable of neutralizing viral isolates belonging to all clades, others such as 2F5 and 2G12 are not as effective against non-B viruses [188, 189]. As such, it will be important to identify and characterize potent HIV-specific monoclonal antibodies directed against non-clade B viruses in order to address the bulk of HIV exposures worldwide. Furthermore, as previously mentioned, protective humoral immune responses in HIV-resistant seronegative individuals are more likely to be mediated by IgA rather

than IgG responses, yet the protective effects of IgA have not been evaluated in animal studies to date. Interestingly, there is some evidence to suggest that polymeric IgA and IgM antibodies may exhibit increased antiviral activity over IgG antibodies of the same specificity. Class-switching the IgG 2F5 and 2G12 antibodies to IgA or IgM isotypes revealed that the neutralization potency of 2G12 increased with antibody valence; furthermore the ability of polymeric IgA and IgM but not of IgG to prevent HIV entry across a cultured epithelial cell barrier afforded these isotypes with an additional means of preventing HIV infection above and beyond the IgG-mediated protection originally observed in the macaque model [190]. As such, IgA antibodies may actually be more effective than IgG at mediating mucosal protection from HIV infection. Consequently, further characterization of HIV-specific monoclonal IgA antibodies is required in order to assess their protective effectiveness, to develop new therapeutic antibodies and to identify novel HIV envelope epitopes for future vaccine targets.

1.8.5 Evidence for HIV-specific IgA in HIV-resistant cohorts

Despite evidence for antibody-mediated protection from HIV in animal models and from *in vitro* studies, the precise role of HIV-specific IgA in protecting HIV-resistant individuals from HIV infection cannot be directly tested in humans and therefore remains controversial. HIV-specific IgA was first suggested to be a correlate of protection in the mid-1990s after IgA1 purified from the serum of several HIV positive individuals was shown to neutralize HIV-1_{MN} in a human T-lymphoblast cell line-based *in vitro* assay [191]. Thereafter, HIV-specific IgA was also detected in the urine and cervical fluid of the majority of ESN partners enrolled in an Italian cohort of heterosexual discordant

couples [117]. This prompted a search for HIV-specific IgA in the serum, saliva and genital tract secretions of individuals involved in other HIV-resistant cohorts, with varying results. In addition to the Italian study, genital tract HIV-specific IgA in the absence of HIV-specific IgG was also elevated in HIV-resistant individuals of the Pumwani CSW cohort where 76% of HIV-resistant had HIV-specific IgA as compared to 26% of HIV seropositive CSWs and 11% of low-risk controls [148] and in Thai CSWs, where gp160-specific IgA was detected in 46% of HIV-resistant but not in low-risk controls [115]. Serum gp41-specific IgA was also detected in 37.5% of exposed seronegative partners enrolled in a Cambodian cohort of discordant couples [192]. While these initial cohort observations suggested that HIV-specific IgA could play a role in protection, the HIV inhibitory capacity of the antibodies was not determined.

The contribution of IgA antibodies to HIV resistance was less clear in other HIV-resistant cohorts [147, 193]. Several of these report the recovery of HIV-specific IgA from HIV-resistant individuals but not at levels different from HIV seropositive and seronegative controls. HIV-specific IgA was recovered from urethral swabs of 78% of ESN males but also from 71% of HIV positive males in an Italian cohort of discordant couples [193]. Furthermore, a larger and more recent investigation of the Pumwani CSW cohort contradicted earlier reports by not observing significant differences in cervical HIV-specific IgA levels between HIV-resistant and HIV positive or HIV negative controls [147]. However, because the properties of IgA isolated from HIV-resistant individuals versus HIV seropositive individuals were not characterized these studies do not preclude the possibility that HIV-specific IgA from HIV-resistant individuals could be functionally superior at preventing infection. In fact, Clerici *et. al.* demonstrated that HIV-specific

neutralizing IgA isolated from HIV positive and HIV-resistant individuals recognized different viral epitopes and may therefore exhibit distinct antiviral activities [194]; IgA antibodies from HIV positive patients were specific for immunodominant epitopes on both gp120 and gp41, while the IgA recovered from HIV-resistant individuals bound exclusively to a restricted epitope within the external region of gp41 [194].

Amidst these conflicting results, other cohorts fail to detect HIV-specific IgA in HIV-resistant individuals at all. A CSW cohort in Cote d'Ivoire could confirm cervical gp160-specific IgA, IgM or IgG in only 2.9% of individuals [195], while no HIV-specific IgA or IgG was detectable in the CVLs of any HIV-resistant individuals in a cohort of CSWs from the Gambia [196]. The absence of HIV-specific IgA responses in these cohorts may indicate that genital mucosal IgA does not mediate protection in all individuals. However, it has also been suggested that since the CSWs of the Gambian cohort had a much lower HIV exposure rate (~0.4 unprotected exposures/month) than CSWs of the Pumwani (~8.7 unprotected exposures/month) or Thai cohorts, the lower HIV infection pressure may select for different protective immune responses in these individuals [197], especially since IgA responses appear to rapidly decline in the absence of continued antigenic stimulation [198].

Adding to this controversy, not all cohort studies that report HIV-specific IgA have investigated the functionality of these antibodies, leaving it unclear in many cases whether this IgA is protective or is merely a marker of previous HIV exposure [149]. However, a number of studies have begun to characterize the anti-HIV functionality of HIV-specific antibodies in assays relevant to the function of antibodies in the mucosa using IgA purified from the serum, saliva or genital tract secretions of HIV-resistant or

occasionally HIV seropositive and seronegative individuals. HIV-specific IgA from a number of cohorts has been found to inhibit viral transcytosis across cultured epithelial cell barriers. IgA, but not IgG, purified from the genital tract of HIV seropositive Cambodian women directed against the gp41 ELDKWA epitope inhibited transcytosis of clade A, B and D viral isolates [105]. With regards to IgA recovered from HIV-resistant individuals, salivary HIV-specific IgA isolated from 5/6 Italian ESN partners of HIV seropositive individuals inhibited transcytosis of at least one out of two clade B primary viral isolates [65], while cervical HIV Env-specific antibodies from 5/6 women enrolled in a Cote d'Ivoire CSW cohort could inhibit transcytosis of a lab-adapted clade A viral isolate [199]. Viral transcytosis-blocking HIV-specific IgA has also been observed in the Pumwani CSW cohort. ~50% of IgA samples purified from the genital tract or serum of these HIV-resistant individuals inhibited transcytosis of at least one HIV-1 clade B primary isolate; interestingly, since clades A and D (rather than B) predominate in Kenya, this data suggests the HIV-specific IgA present in these individuals might be broadly cross-reactive [65, 152]. A number of studies have also provided evidence that HIV-specific IgA recovered from the serum, saliva and genital tract of HIV-resistant individuals is capable of neutralizing HIV-1 [152, 194, 198, 200-202]. In a study of HIV seropositive and seronegative CSWs in Kibera, Nairobi, genital tract HIV-specific IgA able to neutralize either clade A or clade C virus was detected in 81% of women who remained HIV-1 seronegative compared with only 46% of HIV-1 seroconverters and was therefore strongly correlated with protection [200]. HIV-specific IgA recovered from the serum of Italian ESN individuals had neutralization activity against a total of 5 different primary clade B isolates [194, 198]. The HIV-1 neutralization capacity of salivary IgA

purified from several ESN members of this cohort was also assessed; while 6/10 IgA samples neutralized clade B, a number of samples were also found to neutralize a clade A primary isolate [201]. Within HIV-resistant individuals of the Pumwani cohort, 73% of IgA samples purified from serum, 73% from saliva and 79% from CVLs neutralized a primary HIV-1 clade B primary isolate [152, 202]. HIV-specific IgA recovered from a number of these individuals could also neutralize clade A, B, C, D or CRF01_AE primary isolates (some of which are rare in Kenya) [201]. While these data confirmed that the detectible IgA had extensive cross-clade neutralization ability, the full extent of this cross-reactivity could not be determined due to insufficient sample volume recovery.

While the evidence supporting IgA-mediated protection is fairly compelling, the criticism remains that many of these studies test the HIV inhibitory effects of purified IgA fractions as opposed to IgA alone [147]; therefore despite purification other defensive molecules may persist in these samples which might be responsible for the observed anti-HIV activity. However, several of these studies have demonstrated that depleting IgA from the experimental IgA fractions collected from serum or mucosal secretions rendered the samples incapable of neutralizing HIV [191, 202, 203]; thereby convincingly demonstrating that HIV-specific IgA recovered from some individuals can directly inhibit HIV *in vitro*.

Regardless of data obtained from functional studies, the protective role of HIV-specific IgA in ESN individuals will likely remain controversial until HIV-resistant cohorts adopt standardized isolation and characterization protocols and report more consistent results. The variability in HIV-specific IgA detection in HIV-resistant cohorts may be due in part to the difficulty in detecting low concentrations of IgA from the FGT

and the variety of methods employed for antibody recovery, storage and processing [204, 205]. A blind study involving six research laboratories experienced in HIV-specific IgA purification inconsistently detected HIV-specific IgA in rectal washes, underscoring the difficulty of reliably evaluating this response [206]. The concentration of IgA in the FGT is low to begin with (total IgA levels are approximately 30μg/ml while HIV-specific IgA levels occur in the ng/ml range, with as little as 2-3mL of CVL collected per patient) and may be reduced even further depending on the stage of the menstrual cycle or the presence of concurrent STIs [153, 205]. Additionally, sample collection methods (such as CVL washes) further dilute the mucosal secretions, sometimes by up to 30-fold [195]. These factors also severely limit the scope of functional studies, since the amount of IgA recovered is often too low to be able to fully characterize the mechanisms by which they inhibit HIV. Instead, the development of monoclonal antibody libraries presents an attractive alternative in order to avoid IgA sample inconsistency. By generating such libraries, monoclonal antibodies can be produced in large quantities free of other host immune factors, thereby eliminating sample variability and recovery limitations.

1.9 Monoclonal antibodies

1.9.1 Methods of monoclonal antibody generation

An excellent alternative to purifying small quantities of antibodies from the mucosal secretions of HIV-resistant individuals is the large-scale generation of monoclonal antibodies. Monoclonal antibodies provide a renewable and reproducible source of homogenous product, which is ideal for performing in-depth characterization experiments. The two main methods used to generate monoclonal antibodies are classical

hybridoma fusion and recombinant antibody libraries [207, 208]. Developed in 1975, hybridoma fusion was the first technique developed to produce monoclonal antibodies. In this method, antibody-producing B-cells collected from the spleen of antigen-immunized animals are immortalized by fusion with drug-sensitive myeloma cells, and individual clones are selected through drug selection and screening against the desired antigen [209]. While simple and efficient, a major drawback to this technique is that human hybridoma cell lines cannot be generated owing to a lack of stable myeloma fusion partners for human B-cells; as such monoclonal antibodies generated by hybridoma fusion may be immunogenic and therefore without further modification are unsuitable for therapeutic use [207].

Recombinant antibody libraries take the approach of immortalizing antibody variable genes rather than the whole B cell [207] and has previously been used effectively to generate a number of important broadly neutralizing HIV-specific antibodies, including IgG1b12 [210]. In this method, immunoglobulin V_H and V_L genes are amplified from B cell mRNA and cloned into plasmid heavy and light chain libraries. V_H and V_L gene combinations producing the desired antigen specificity are generally selected by phage display, a technique in which the antibody gene library is fused to a bacteriophage surface protein, causing antibody variable gene fragments to be expressed on the surface of the phage for convenient antigen screening [208]. The selected monoclonal antibody construct can then either be produced by *E. coli* upon its infection with bacteriophage or, more commonly, the desired antibody variable genes are cloned into antibody expression vectors and transfected into animal cells. The major advantage of this approach is the ability to generate entirely human monoclonal antibodies; it is also

possible to generate isotype-specific libraries and easily perform isotype class-switching by altering the antibody expression vector used for cloning [207].

1.9.2 Novel and promising HIV-specific monoclonal antibodies

The use of monoclonal antibody-generating techniques have been recently used to generate several promising HIV-specific monoclonal antibodies from novel sources. PG9 and PG16 are the first broadly neutralizing monoclonal antibodies to be generated from a non-clade B-infected donor [189, 211]. Using a variation of the antibody library cloning technique, these IgG somatic variants were isolated from an HIV-1 clade A infected African donor and recognize a conformational epitope in the V2/V3 region of gp120 that preferentially occurs on the pre-CD4-bound trimeric envelope spike. Both antibodies were tested against an exhaustive multiclade pseudovirus panel; PG9 and PG16 could respectively neutralize 127 and 119 out of 162 viruses across multiple clades. Excitingly, while the neutralization breadth of these antibodies was comparable to b12, 2G12, 2F5 & 4E10; PG9 & PG16 were shown to be considerably more potent [189]. Another recent study used the combinatorial library and phage display technique to generate IgA Fabs to gp41 from the cervical B cells of Cambodian HIV-resistant individuals [212]. Four Fabs were generated that could potently neutralize HIV and block viral transcytosis at low antibody titres. Interestingly, Fab 43 was shown to have a long (22 amino acid) CDR3 loop, similar to other broadly neutralizing monoclonal antibodies. It was also observed that class-switching the C_H1 domain from IgA to IgG drastically diminished the neutralization capacity [212], providing further supporting evidence that IgA may provide better protection from HIV-1 than IgG.

A recombinant antibody library has also been generated from cervical B-cells collected from HIV-resistant individuals of the Pumwani cohort. Berry *et. al.* developed novel antibody single chain variable fragments (ScFvs) by pairing libraries of Kenyan IgA V_H or V_L antibody genes with the V_L or V_H genes of an affinity-matured IgG1b12 variant. The hybrid A6/4L ScFv neutralized clade B HIV-1 *in vitro*, while the exclusively HIV-resistant -derived A6/30λ ScFv was shown by competition assay with IgG1b12 to recognize a separate epitope [150]. While encouraging, these results are preliminary and require further characterization, as ScFvs completely lack antibody constant domains and therefore cannot be used in the investigation of isotype-specific and Fc-mediated effector functions such as transcytosis and ADCC activity. As such, the ScFv antibody variable regions need to be cloned into an antibody expression vector in order to express them as full-length monoclonal antibodies to fully characterize their function.

1.10 Project rationale

HIV-specific antibodies are a promising line of investigation in the search for both preventative and therapeutic treatments against HIV infection. Broadly neutralizing HIV-specific antibodies have shown promise both in NHP models and *in vitro*. Most antibodies tested to date, however, have been IgG antibodies generated from HIV seropositive individuals infected with clade B virus, while little attention has been given to antibodies of the IgA isotype, to antibodies generated from HIV-resistant individuals or to antibodies directed against non-B clades prevalent in the geographical areas hit hardest by the HIV pandemic. As such, the large scale expression, characterization and epitope mapping of HIV-1 specific IgA from HIV-1 resistant individuals has become an

attractive goal in understanding the mechanisms of HIV resistance and to influence vaccine design. Previous studies investigating the role of HIV-specific IgA in protecting HIV-resistant individuals from HIV-1 infection have been hindered due to the difficulty of isolating sufficient quantities of HIV-1 specific IgA from cervicovaginal fluids. To address this issue, we have employed a cloning strategy using the pSM102 IgA2 expression vector to produce full-length monoclonal IgA generated from cervical B-cells of HIV-1 resistant women of the Pumwani CSW cohort. This strategy will enable the *in vitro* production of large quantities of IgA needed to eventually fully characterize their anti-HIV functionality.

1.11 Hypothesis

In this study, we hypothesize that HIV-1 specific IgA cloned from the genital tract of HIV-1 resistant women of the Pumwani cohort will efficiently recognize epitopes of monomeric and/or trimeric HIV-1 envelope protein gp120 that are distinct from the well characterized HIV-neutralizing antibody, IgGb12.

1.12 Specific Objectives

In order to prove this hypothesis, we produced Kenyan-derived monoclonal IgA antibodies cloned from the genital tract of HIV-resistant women *in vitro* in order to characterize the ability of these antibodies to recognize HIV-1 gp120. With this in mind, we developed the following specific objectives:

1. Clone IgA variable heavy and light genes isolated from a cervical B-cell Fab library from the genital tract of HIV-resistant CSWs

2. Produce 9 full-length human monoclonal IgA2 antibody variants *in vitro* by means of CHO-K1 cell culture transfection
3. Purify the monoclonal IgA from the cell culture supernatants
4. Verify the proper integration of all IgA genes into the genome of each transfected monoclonal CHO-K1 cell line
5. Confirm the size and purity of the purified IgA samples
6. Characterize the ability of the engineered monoclonal IgA variants to bind to gp120

2.0 Materials

2.1 Sources of DNA

2.1.1 Variable heavy & light genes used for cloning

The Africanized variable heavy chain antibody genes "A6" and "A9" (GenBank accession numbers AY044149 and AY044150) and the variable light chain antibody gene "30L" (GenBank accession number AY044151) used in these experiments were obtained from Dr. Jody Berry in the form of pComb3x ScFv expression plasmids. The generation of these phagemid vectors is described in [150]. Briefly, cytobrush samples were collected from 12 Kenyan HIV-resistant sex workers from the Pumwani Cohort in Nairobi, Kenya: ML 767, 881, 889, 896, 1192, 1260, 1327, 1441, 1705, 1732, 1747, and 1832. All of these women were confirmed to be HIV-1 PCR- and IgG- seronegative. Cervical B-cell antibody cDNA gene pools were derived from the samples and antibody genes of interest were initially identified by phagemid selection against $gp120_{IIIB}$. These Africanized antibody genes were cloned into pComb3x for further experimentation.

The "H31" variable heavy gene and the "4L" variable light gene make up an affinity-improved version of antibody IgG1b12 (Figure 3). IgG1b12 is a broadly neutralizing monoclonal antibody generated from a variable heavy gene (GenBank accession number AAB26315.1) and a variable light gene (GenBank accession number AAB23607.1) originally selected from a Fab library cloned from bone marrow lymphocytes of a clade B-infected individual [210, 213]. These genes were provided in the IgA2 expression vector pSM102. The kappa-type constant light chain gene (Cκ) used to clone the $30LV_LC\kappa$ gene was also derived from pSM102. The lambda 2-type constant light chain gene (Cλ) used to clone the 30λ gene constructs was obtained from the

Heavy chains

```
                FR1              CDR1       FR2             CDR2
b12 LEQSGAEVKKPGASVKVSCQASGYRFS NFVIH WVRQAPGQRFEWMG WINPYNGNKEFSAK 60
H31 LVQSGAEVKKPGASVKVSCQASGYRFS NFVIH WVRQAPGQRFEWMG WINPYNGNKEFSAK 60
    * ************************ ***** ************** **************

                     FR3                              CDR3          FR4
b12 FQD RVTFTADTSANTAYMELRSLRSADTAVYYCAR VGPYSWDDSPQDNYYMDV WGKGTTV 120
H31 FQD RVTFTADTSANTAYMELRSLRSADTAVYYCAR VGPYSWDDSPQDNYYMDV WGKGTTV 120
    *** ******************************** ****************** *******


b12 IVSS 124
H31 IVSS 124
    ****
```

Light chains

```
               FR1              CDR1           FR2         CDR2
b12 --ELTQAPGTLSLSPGERATFSC RSSHSIRSRRVR WYQHKPGQAPRLVIH GVSNRAS GIS 58
4L  EIVLTQSPGTLSLSPGERATFSC RSSHSIRSRRVA WYQHKPGQAPRLVIH GVSNRAS GIS 60
      *** ***************** ***********  *************** ******* ***

                 FR3                CDR3        FR4
b12 DRFSGSGSGTDFTLTITRVEPEDFALYYC QVYGASSYT FGQGTKLERKRT 108
4L  DRFSGSGSGTDFTLTITRVEPEDFALYYC QVYGASSYT FGQGTKLERKRT 110
    **************************** ********* ************
```

Figure 3. Amino acid sequences of the variable heavy (V_H) and variable light (V_L) domains of IgG1b12 compared to the V_H and V_L domains of H31 and 4L included in the IgA2 expression vector pSM102. Sequences in non-bolded text denote framework (FR) regions, while sequences in bolded text indicate complementarity determining (CDR) regions. The IgG1b12 genes, FRs and CDRs are included as reported in [213]. Sequence alignments were performed using ClustalW2 [214].

pASK43 cloning plasmid provided by Dr. Morgane Bomsel, Institut Cochin, INSERM, Paris, France.

2.1.2 The pSM102 expression vector

The pSM102 human IgA2 expression vector was obtained from Dr. Nick Mantis, the Wadsworth Center, New York State Department of Health, Albany, NY. pSM102 is a 11,670bp expression vector containing a cytomegalovirus-derived promoter region as well as the ColE1 replicon for maintenance in *Escherichia coli* and SV40 promoters for replication in eukaryotic cells (Figure 4). The vector encodes the heavy and light chain genes for human IgA2 (allotype m1). Notably, the IgA2 constant heavy gene encoded in this vector includes the 18 amino acid antibody tailpiece bearing the asparagine 459 glycosylation site required to interact with J-chain and produce dimeric IgA molecules [215]. Despite its relatively large size, the inclusion of both the heavy and light chain genes on a single expression vector simplifies plasmid transfection and eliminates co-expression variability. pSM102 encodes the H31-V_H and 4L-V_L genes of an affinity-matured version of IgG1b12, and produces an IgA2 version of the IgG1b12 antibody when transfected into animal cells [216]. pSM102 is also intended as a convenient vector for the expression of other antibody genes; as such, the plasmid contains a number of unique restriction enzyme sites for easy cloning. The b12 V_H region and a V_H leader sequence is flanked by *XbaI* and *SstI* restriction sites to allow cloning new variable heavy genes in frame with the IgA2 constant heavy gene backbone. The b12 V_L region, a kappa-type constant light gene (Cκ), and a V_L leader sequence is flanked by a *HindIII* and an *EcoRI* restriction site. A *SalI* restriction site is included downstream of the IgA2

constant heavy gene in order to linearize the plasmid for transfection and increase the likelihood of producing a stable cell line. Additionally, pSM102 contains two selectable markers. The beta-lactamase gene confers ampicillin resistance to transformed *E. coli* cultures. The glutamine synthetase gene acts as a dominant amplifiable selectable marker for maintenance in transfected animal cell cultures.

2.2 Animal Cell Lines

Chinese Hamster Ovary (CHO-K1) cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. These cells were used as a transfection host for the experimental IgA2 plasmids.

An HIV-1-uninfected H9 T-cell line was obtained from Dr. Robert Gallo through the NIH AIDS Research & Reference Reagent Program, Germantown, MD, USA. These cells were used as a control for flow cytometry experiments to characterize gp120 binding.

The H9/HTLV-IIIB NIH 1983 T-cell line was also obtained from Dr. Robert Gallo through the NIH AIDS Research & Reference Reagent Program, Germantown, MD, USA. These cells were also used for flow cytometry experiments.

2.3 Reagents and Solutions

All commercial reagents and laboratory-prepared solutions are described in detail in Appendices A and B respectively.

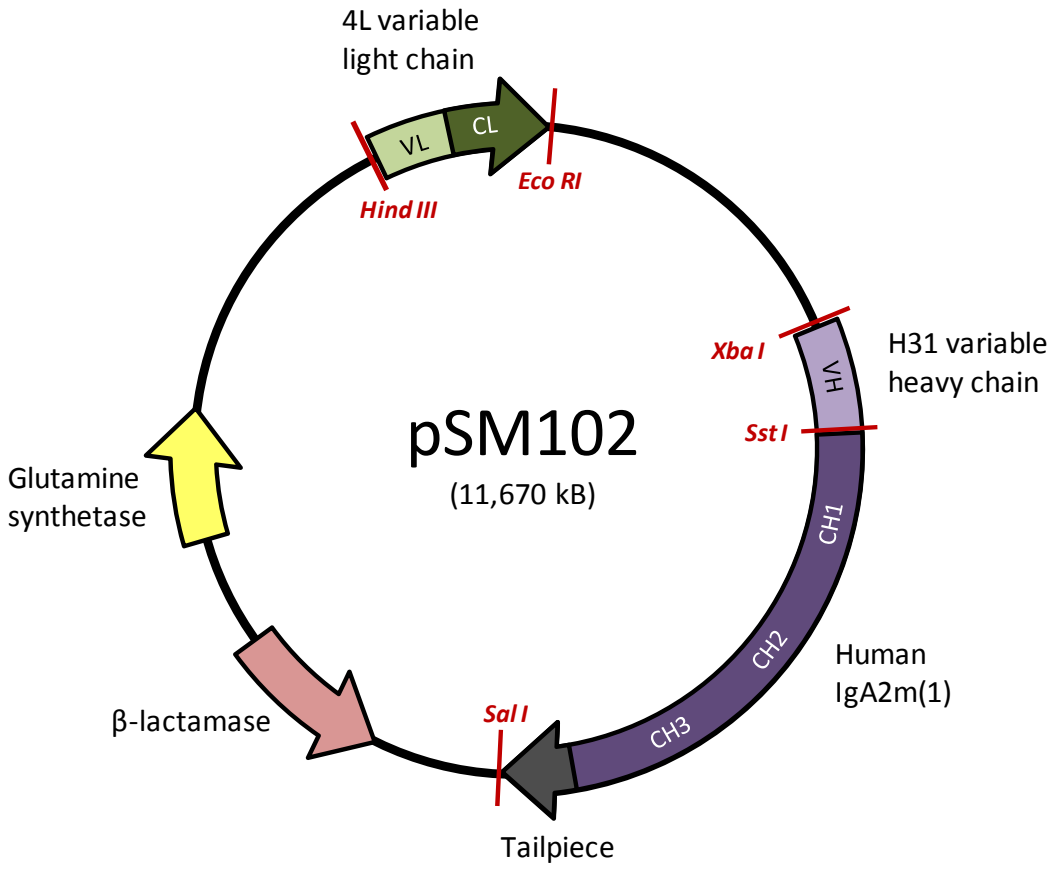

Figure 4. The pSM102 human IgA2 expression vector. Features of this plasmid include an IgG1b12-derived variable heavy gene, H31 (shown in light purple), an IgG1b12-derived variable light gene, 4L (shown in green), and a human IgA2m(1) constant heavy gene (shown in dark purple and in grey). This vector also contains a glutamine synthetase amplifiable marker (in yellow) for selection in mammalian cells, and β-lactamase (in pink) for selection in *Escherichia coli*. *HindIII*, *EcoRI*, *XbaI* and *SstI* directional cloning sites and a *SalI* restriction site for vector linearization are also present. This pSM102 schematic was adapted from [216].

3.0 Methods

3.1 Overall strategy of variable gene assembly by PCR

All antibody gene constructs were composed of an upstream DNA "leader" segment (see section 3.2.2) fused to the variable gene of interest by means of the polymerase chain reaction (PCR). The variable light chain genes were additionally fused to a downstream constant light chain gene to produce a full-length antibody light chain. The variable heavy chain genes were joined to the constant heavy genes directly by cloning into the pSM102 vector, not by the PCR-fusion process. In all cases, the variable gene of interest was amplified using semi-complementary primers in order to generate complementary regions on the variable gene. These sticky ends were used to facilitate fusing the variable genes to the upstream and downstream DNA segments by overlap PCR.

All PCR reactions performed consisted of the same basic master mix of: 10x Taq DNA Polymerase PCR Buffer (Invitrogen, Burlington, ON, Canada), 50mM $MgCl_2$ (Invitrogen, Burlington, ON, Canada), 10mM dNTP Mix (Invitrogen, Burlington, ON, Canada), 15 pmol each of the upstream and downstream primers, 0.5µl of DNA template, 2.5 units of *Taq* DNA Polymerase (Invitrogen, Burlington, ON, Canada) and sterile double-distilled water (ddH_2O) up to a final volume of 50µl. After amplification, 10µl of each PCR product was combined with 2µl 6x loading buffer and loaded onto a 1% UltraPure™ Agarose gel in TBE buffer containing 0.1µl/ml ethidium bromide (EtBr). Gel electrophoresis was preformed to ensure the PCR products were specific and of the correct size.

3.2 Development of the Variable Heavy Chain genes

As the cloning plasmid pSM102 already contained the b12-derived variable heavy chain (V_H) gene H31, only the B-cell derived variable heavy chain A6 and A9 genes needed to be constructed for cloning. These genes consisted of the V_H leader fragment containing an *XbaI* restriction site, the B-cell derived A6 or A9 variable gene and a downstream *SstI* restriction site for cloning into pSM102 (Figure 5).

3.2.1 PCR Amplification of the variable heavy gene

Both the A6 and A9 genes were amplified from template pComb3x phagemid vectors by PCR. 150ng of A6 or A9 template DNA was amplified and extended in preparation for overlap PCR using semi-complementary primers IgA6/9tail5 (5′- GGT GTC CAC TCC CAG GTG CAG CTG GTG -3′) and IgA6/9tail3 (5′- GAG GAG GAG GAG CTC GAG GAG ACG ATG AC -3′) which contained an *SstI* restriction site (see Table 1). The upstream portion of the IgA6/9tail5 primer was complementary to the 3′ end of the V_H leader DNA sequence (discussed below in section 3.2.2). The upstream portion of the IgA6/9tail3 primer contained an *SstI* enzyme restriction site to incorporate into the PCR product for cloning into pSM102. The PCR amplification program consisted of an initial denaturation step at 94°C for 2 minutes, followed by 3 cycles of a 94°C denaturation for 20 seconds, a 40°C primer annealing step for 30 seconds and a 90 second extension step at 72°C. After another 2 minute initial denaturation step at 94°C, the reactions were cycled 30 times at 94°C for 25 seconds, 68°C for 30 seconds and 72°C for 1 minute. There was a final 10-minute extension period at 72°C. This process generated the main A6 or A9 V_H gene product of the full-length cloning construct.

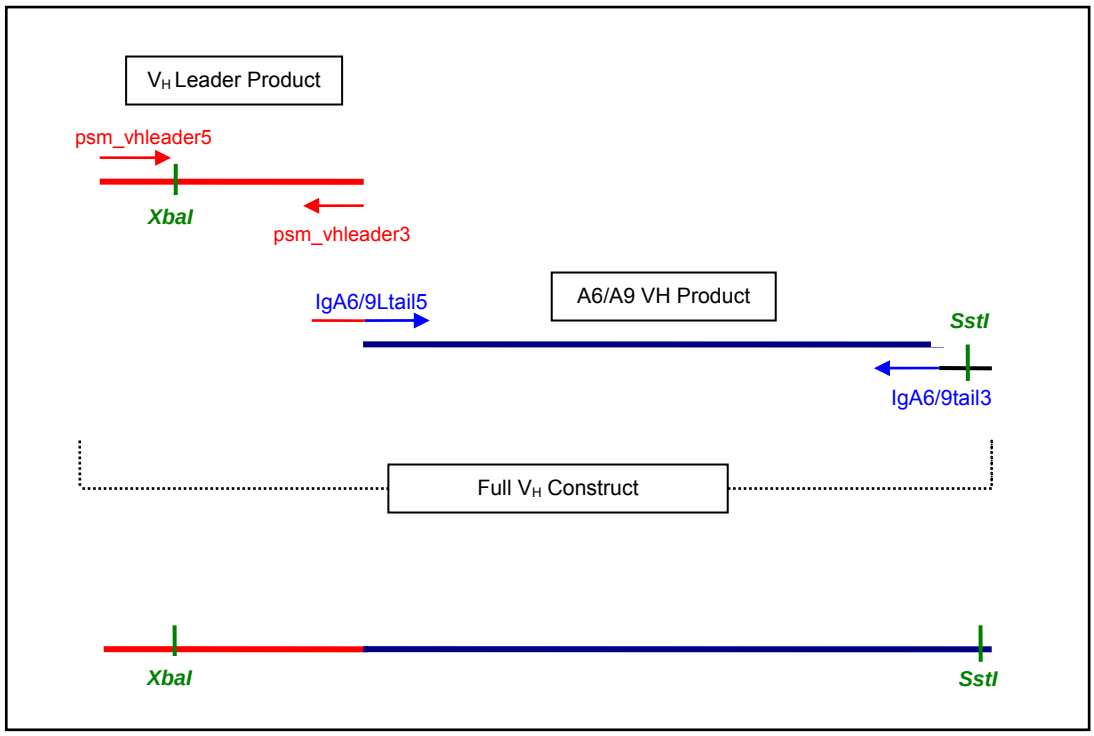

Figure 5. Amplification strategy for V_H gene products. The variable gene of interest is extended with semi-complementary primers to include an upstream sequence complementary to the 3′ end of the V_H leader and a downstream *SstI* cloning site. Thereafter, the V_H leader and V_H gene products are fused by overlap PCR.

Table 1. Primers used for V_H gene construction.

Name	Primer Sequence (5′ to 3′)	Orientation	Tm (°C)	Features
IgA6/A9tail5	GGTGTCCACTCCCAGGTGCAGCTGGTG	Forward	90	
IgA6/A9tail3	GAGGAGGAGGAGCTCGAGGAGACGATGAC	Reverse	94	*SstI site*
psm_vhleader5′	CTGCAGTCACCGTCCTTGAC	Forward	64	
psm_vhleader3′	GGAGTGGACACCTGTAGTTAC	Reverse	64	
b12VHup	CTCTAGAGCCGCCACCATGG	Forward	66	*XbaI site*

3.2.2 PCR Amplification of the V_H leader fragment

The A6 and A9 V_H genes next had to be fused to a gene segment termed the "V_H leader" fragment. This native IgA2 sequence encodes a 10 amino acid peptide immediately upstream of the antibody variable region for the purpose of directing the peptide to the endoplasmic reticulum for processing. The V_H leader PCR product was generated by amplifying 150ng of the pSM102 plasmid using the upstream psm_vhleader5′ primer (5′- CTG CAG TCA CCG TCC TTG AC -3′) and the downstream psm_vhleader3′ primer (5′- GGA GTG GAC ACC TGT AGT TAC -3′) (see Table 1). The PCR program consisted of a 2 minute initial denaturation step at 94°C, followed by 30 cycles at 96°C for 15 seconds, 57°C for 20 seconds, 72°C for 1 minute and a final 10-minute extension period at 72°C.

3.2.3 Assembly of the full-length V_H cloning construct

To assemble the full-length V_H cloning construct, 0.5µl of the A6 or A9 PCR product was combined with 0.5µl of V_H leader PCR product as a template. Initially, no primers were added prior to thermal cycling. The PCR program was comprised of a 94°C denaturation step for 2 minutes followed by 4 cycles of 94°C for 20 seconds, 40°C for 30 seconds and 72°C for 90 seconds. The temperature was lowered to 4°C and primers psm_vhleader5 and IgA6/9tail3 were added. The program continued with another 2 minute, 94°C denaturation step followed by 26 cycles of 94°C for 15 seconds, 57°C for 30 seconds and 72°C for 90 seconds and finishing with a 10 minute elongation period at 72°C.

Once the cloning construct was assembled and its size assessed by agarose gel electrophoresis, the V_H constructs were immediately cloned into the Invitrogen pCR®4-TOPO vector according to kit instructions for long-term storage at -20°C.

3.3 Development of the 30LV_λCκ Light Chain gene

The pSM102 expression vector contained the b12-derived variable light "4L" gene. As such, only the B-cell derived light "30L" gene was constructed and inserted into the vector. The 30L construct (30LV_λCκ) consisted of an upstream $V_L$leader fragment, the 30L variable lambda-type gene, and also a constant kappa-type light chain gene obtained from the pSM102 plasmid (Figure 6). The final PCR product was flanked by *HindIII* and *EcoRI* restriction enzyme sites for cloning into pSM102.

3.3.1 PCR Amplification of the 30L variable gene

The 30L variable lambda-type light gene was amplified from a template pComb3x storage vector by PCR. 150ng of template DNA was amplified with upstream primer 30λUpstream (5′- GAT GCC AGA TGT CAG GCG GCC GAG CTC GCC CTG -3′) and downstream primer 30λVL_short3 (5′- GGT CCC CTG GCC GAA AAC ACT ATC ACC -3′) (Table 2). The 30λUpstream primer was partially complementary to the $V_L$leader DNA sequence and the 30λVL_short3 primer was partially complementary to the constant kappa light chain from pSM102. The 30L variable light chain was amplified by an initial denaturation step for 2 min at 94°C, followed by 30 cycles of 94°C for 20 sec, 82°C for 15 sec and 72°C for 45 sec and a final elongation step of 72°C for 10 minutes.

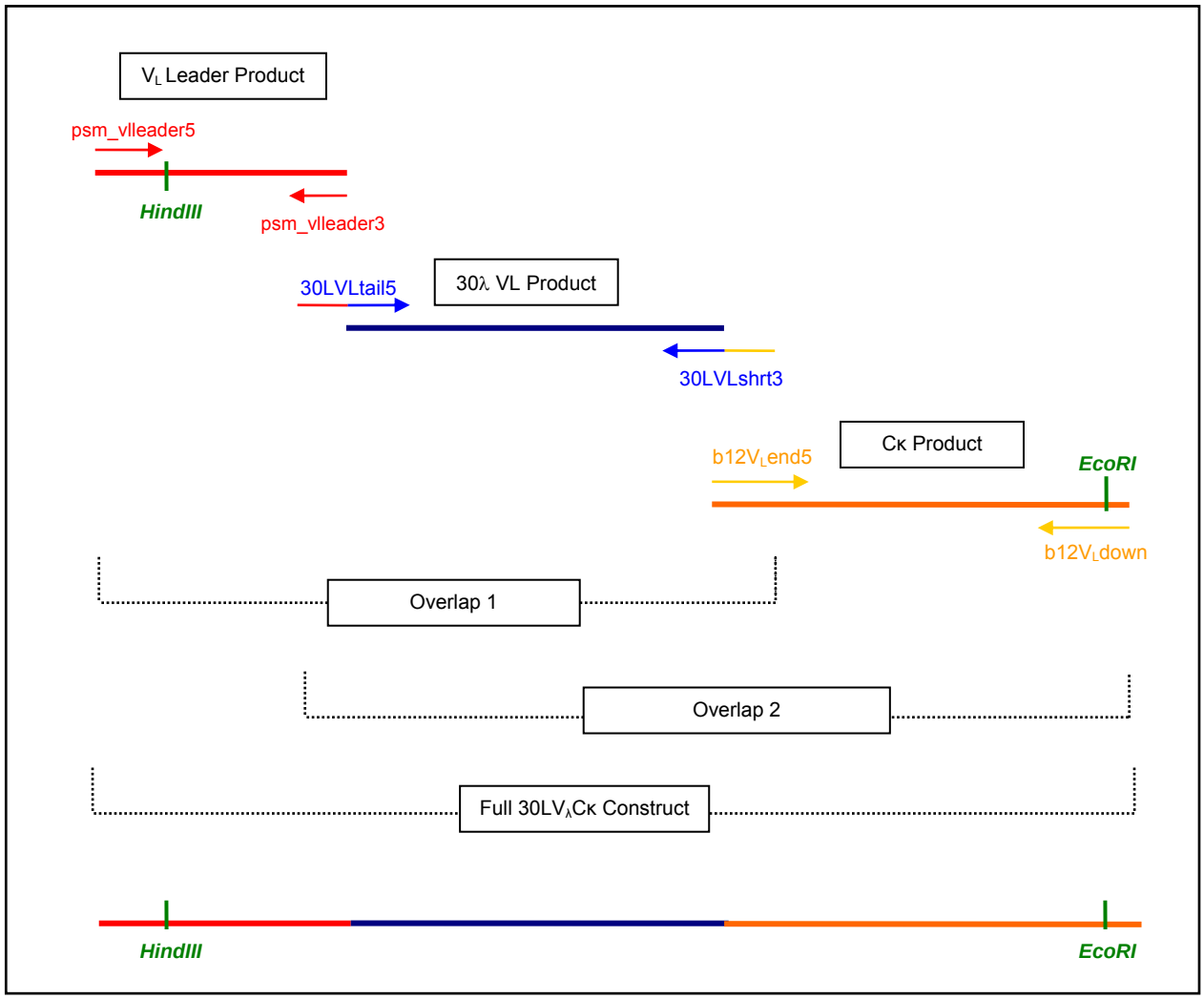

Figure 6. Amplification strategy for the 30LV$_\lambda$Cκ gene products. The 30λ variable gene is extended with semi-complementary primers to include an upstream sequence complementary to the 3′ end of the V$_L$ leader sequence and a downstream sequence complementary to the Cκ gene. The Cκ itself is also extended to include an *EcoRI* restriction site for cloning. Thereafter, the V$_L$ leader, the 30λ variable gene product and the Cκ gene are fused by overlap PCR.

Table 2. Primers used for 30LV$_\lambda$Cκ gene construction.

Name	Primer Sequence (5′ to 3′)	Orientation	Tm (°C)	Features
30λUpstream	GATGCCAGATGTCAGGCGGCCGAG CTCGCCCTG	Forward	112	longer version of 30lambdaVL_tail5
30λVL_short3	GATGCCAGATGTCAGGCGGCCGAGCTC	Reverse	86	
psm_vlleader5	CACCAGACATAATAGCTGACAG	Forward	64	
psm_vlleader3	ACATCTGGCATCTGTAAGCCAC	Reverse	66	
b12VLend5	GGCCAGGGGACCAAACTGG	Forward	64	
b12VLdown	GATCAATGAATTCATTAACACTCT	Reverse	62	*EcoRI site*
b12VLup	GAAGCTTACCATGGGTGTGC	Forward	62	*HindIII site*

3.3.2 PCR Amplification of the V_L leader fragment

The V_L leader region was amplified from 150ng of the pSM102 plasmid using the psm_vlleader5 primer (5′- CAC CAG ACA TAA TAG CTG ACA G -3′) and the psm_vlleader3 primer (5′- ACA TCT GGC ATC TGT AAG CCA C -3′) (Table 2). The reaction vessel was heated to 94°C for 2 min, followed by 30 cycles of 95°C for 20 sec, 59°C for 20 sec and 72°C for 60 sec, then a final incubation at 72°C for 10 min.

3.3.3 PCR Amplification of the Constant kappa V_L gene

The constant kappa-type light chain was obtained from the pSM102 plasmid. 150ng of plasmid template DNA was amplified using the complementary primer b12VLend5 (5′- GGC CAG GGG ACC AAA CTG G -3′) and the b12VLdown primer (5′- GAT CAA TGA ATT CAT TAA CAC TCT -3′), which added an *EcoRI* restriction site to the product (Table 2). The PCR product was generated by means of an initial denaturation of 94°C for 2 min, 30 cycles of 94°C for 20 sec, 60°C for 15 sec and 72°C for 45 sec and a final extension period of 72 for 10 min.

3.3.4 Assembly of the full-length V_L cloning construct

The full-length 30L$V_\lambda C\kappa$ light chain gene was constructed in three overlap PCR steps. In the first reaction, the 30L variable gene product was fused to the $V_L$leader fragment. Next, the 30L variable gene product was added to the kappa-type constant light gene. Lastly, the two partial overlap PCR fragments were annealed and extended into a single full-length construct comprising of the $V_L$leader fragment, the 30L variable gene and the kappa constant gene.

The first overlap PCR reaction template consisted of 5.0µl each of the $V_L$leader and the 30L variable gene PCR products. The reaction vessel was incubated at 94°C for 2 min, and then cycled three times at 94°C for 20 sec, 26°C for 30 sec and 72°C for 1 min. The reaction was then cooled to 4°C and the psm_vlleader5 and the 30λVL_short3 primers were added. Following an additional incubation at 94°C for 2 min, the vessel was cycled 30 additional times under the same conditions but increasing the primer annealing conditions to 59°C for 30 sec.

The second overlap PCR reaction was performed by combining 5.0µl each of the 30L and constant kappa PCR products. The reaction vessel was incubated at 94°C for 2 min and cycled three times at 94°C for 20 sec, 39°C for 30 sec and 72°C for 90 sec. Thereafter, the reaction was cooled to 4°C and the 30λUpstream and b12VLdown primers were added. The reaction was incubated for 2 min at 94°C, then cycled 30 times under the same PCR conditions but increasing the primer annealing temperature to 57°C for 30 sec.

The full-length 30LV_λCκ gene construct was assembled by means of overlap PCR using 5.0µl of overlap 1 PCR product ($V_L$leader & 30L) and 5.0µl of overlap 2 PCR product (30L & constant kappa). The full length PCR product was generated by an initial denaturation of 94°C for 2 min followed by three cycles of 94°C for 20 sec, 35°C for 35 sec and 72°C for 75 sec. The apparatus was cooled to 4°C for the addition of psm_vlleader5 and b12VLdown, then incubated for 2 min at 94°C and cycled 30 times at 94°C for 20 sec, 57°C for 30 sec and 72°C for 90 sec. Once assembled and verified by agarose gel electrophoresis, the 30LV_λCκ construct was immediately cloned into the

Invitrogen pCR®4-TOPO vector, according to kit instructions, for long-term storage at -20°C.

3.4 Development of the 30LV$_\lambda$C$_\lambda$ Light Chain gene

Antibody light chains are made up of a variable gene and a constant gene; in a naturally occurring antibody both of these are either kappa-type or lambda-type genes. However, our 30LV$_\lambda$Cκ construct was made up of a lambda-type variable gene and a kappa-type constant gene. As a result, we also constructed a different variant of the "30L" light chain composed of the upstream V$_L$leader fragment and the same the B-cell-derived 30L variable light gene described in section 3.3.1, but included a different downstream region: a lambda 2-type constant light chain gene. For this reason, the fully lambda-type light chain is referred to as "30 lambda" (30LV$_\lambda$C$_\lambda$) to distinguish it from the 30LV$_\lambda$Cκ construct. The final product was also flanked by *HindIII* and *EcoRI* restriction enzyme sites for cloning into pSM102. The amplification strategy is outlined in figure 7.

3.4.1 PCR Amplification of the 30LV$_\lambda$C$_\lambda$ gene

The first overlap PCR product, V$_L$leader-30L, was already available for direct amplification from the cloned 30LV$_\lambda$Cκ PCR protocol described in section 3.3.4. Briefly, 150ng of template V$_L$leader & 30L overlap in the pCR®4-TOPO vector was amplified using the psm_vlleader5 primer (5′- CAC CAG ACA TAA TAG CTG ACA G -3′) and the 30Ltail3-lambda-c primer (5′- GCC TTG GGC TGA CCG AAA ACA CTA TCA CC -3′) (Table 3). The 30Ltail3-lambda-c primer was designed so that the upstream portion of the primer was complementary to the lambda constant chain fusion target. The PCR reagents

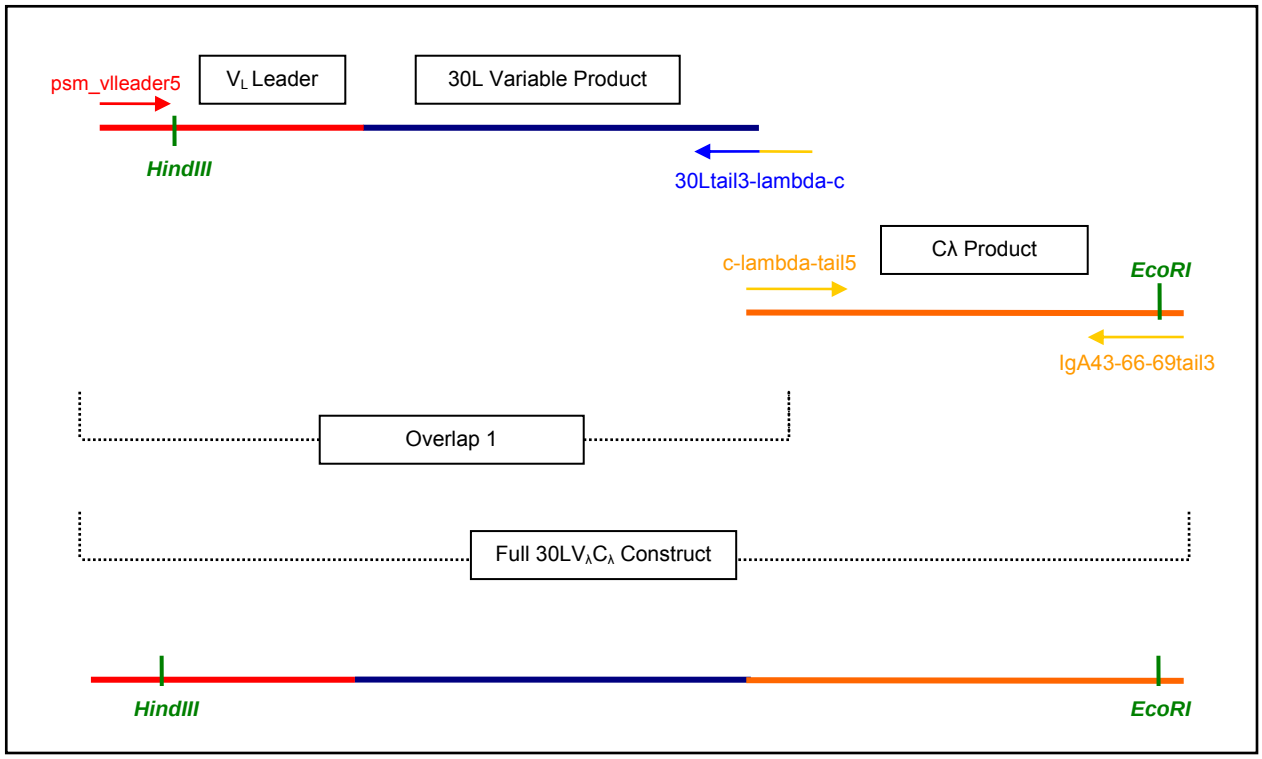

Figure 7. Amplification strategy for $30LV_{\lambda}C_{\lambda}$ gene products. The V_L leader is already fused with the 30λ variable gene. This fragment is extended by a downstream semi-complementary primer to generate complementarity with the Cλ gene. Thereafter, the V_L leader/30λ variable gene and the Cλ gene products are fused by overlap PCR.

Table 3. Primers used for 30LV$_\lambda$C$_\lambda$ gene construction.

Name	Primer Sequence (5′ to 3′)	Orientation	Tm (°C)	Features
psm_vlleader5	CACCAGACATAATAGCTGACAG	Forward	64	
30Ltail3-lambda-c	GCCTTGGGCTGACCGAAAACACTATCACC	Reverse	92	
c-lambda-tail5	GGTCAGCCCAAGGCTGCCCCCTCGG	Forward	88	
IgA43-66-69tail3	GATCAATGAATTCATTATGAACATTCTG	Reverse	64	*EcoRI site*
b12VLup	GAAGCTTACCATGGGTGTGC	Forward	62	*HindIII site*

were initially cycled 3 times at 94°C for 20 sec, 37°C for 30 sec and 72°C for 60 sec so that the semi-complementary primers could extend the template. Thereafter, the PCR reactions underwent an additional 30 cycles at 94°C for 20 sec, 60°C for 30 sec and 72°C for 60 sec to complete DNA amplification.

3.4.2 PCR Amplification of the Constant lambda gene

The constant lambda-type light chain gene was sequenced and obtained by PCR from the pASK43 plasmid. 150ng of the plasmid template was amplified with the complementary c-lambda-tail5 primer (5′- GGT CAG CCC AAG GCT GCC CCC TCG G -3′) and the semi-complementary IgA43-66-69tail3 primer (5′- GAT CAA TGA ATT CAT TAT GAA CAT TCT G -3′) (Table 3) which added an *EcoRI* restriction site to the product. The constant lambda chain was amplified by cycling the reaction 3 times at 94°C for 20 sec, 31°C for 30 sec and 72°C for 60 sec. Thereafter, the PCR reactions underwent an additional 30 cycles at 94°C for 20 sec, 60°C for 30 sec and 72°C for 60 sec to complete DNA amplification.

3.4.3 Assembly of the full-length 30LV$_\lambda$C$_\lambda$ cloning construct

The two PCR products were fused into a full-length lambda-type light chain by overlap PCR using 5µl each of the $V_L$leader-30VL and the constant lambda light chain PCR products as template. The PCR product was generated by an initial denaturation of 94°C for 2 min followed by three cycles of 94°C for 20 sec, 40°C for 35 sec and 72°C for 60 sec. The apparatus was cooled to 4°C for the addition of the psm_vlleader5 and IgA43-66-69tail3 primers. Thereafter, the PCR reactions underwent an additional 30

cycles at 94°C for 20 sec, 57°C for 30 sec and 72°C for 90 sec to complete DNA amplification. Since the PCR reaction produced some unspecific product, the desired PCR product was excised from a 1% UltraPure™ Agarose gel and isolated by means of the QIAquick® Gel Extraction Kit, as per kit instructions. DNA. Once assembled and verified, the $30LV_{\lambda}C_{\lambda}$ construct was immediately cloned into the Invitrogen pCR®4-TOPO vector, according to kit instructions, for long-term storage at -20°C.

In this manner, the complete variable heavy genes as well as full-length kappa-type and lambda-type light chain genes were generated by overlap PCR and sequenced for cloning purposes (Table 4).

3.5 Cloning the Variable genes into the IgA Expression Vector

3.5.1 Cloning the variable heavy chain gene constructs into pSM102

The A6 and A9 V_H gene constructs were cloned into the pSM102 IgA expression vector using the *SstI* restriction site in the 30L variable light chain DNA. 5µl of plasmid DNA was added to 1.0µl 10x REact 2 buffer (Invitrogen), 0.5µl *XbaI*, 0.5µl *SstI*, and 3.0µl ddH_2O. The samples were incubated at 37°C for 1 hour. After restriction, samples were electrophoresed in a 1% UltraPure™ agarose gel which was then stained with 25µl SYBR® Green I Nucleic Acid Gel Stain in 250ml TBE buffer for 30 minutes and visualized using the Typhoon Scanner (GE Biosciences) at 532nm. Bands corresponding to the pSM102 backbone (lacking the V_H fragment) and to the A6 and A9 V_H gene constructs were excised from the gel with a scalpel and purified using the QIAquick® Gel Extraction Kit (Qiagen, Mississauga, ON, Canada) as per kit instructions.

Table 4. PCR amplicons generated to produce the 9 monoclonal IgA variants.

Plasmid Name	V_H Chain	V_H Source	V_L Chain	V_L Source	Type
pSM102 (H31/4L)	H31	pSM102 (b12)	4L	pSM102 (b12)	IgAb12
A6/4L	A6	HIV-Resistant women	4L	pSM102 (b12)	Hybrid
A9/4L	A9	HIV-Resistant women	4L	pSM102 (b12)	Hybrid
H31/30LV$_\lambda$Cκ	H31	pSM102 (b12)	30LV$_\lambda$Cκ	HIV-Resistant women	Hybrid
A6/30LV$_\lambda$Cκ	A6	HIV-Resistant women	30LV$_\lambda$Cκ	HIV-Resistant women	Africanized
A9/30LV$_\lambda$Cκ	A9	HIV-Resistant women	30LV$_\lambda$Cκ	HIV-Resistant women	Africanized
H31/30LV$_\lambda$C$_\lambda$	H31	pSM102 (b12)	30LV$_\lambda$C$_\lambda$	HIV-Resistant women	Hybrid
A6/30LV$_\lambda$C$_\lambda$	A6	HIV-Resistant women	30LV$_\lambda$C$_\lambda$	HIV-Resistant women	Africanized
A9/30LV$_\lambda$C$_\lambda$	A9	HIV-Resistant women	30LV$_\lambda$C$_\lambda$	HIV-Resistant women	Africanized

Thereafter, the A6 and A9 V_H gene products were ligated into pSM102 by the following method: 5.0µl of pSM102 backbone DNA was combined with 5.0µl of either the A6 or A9 V_H insert, 3.0µl of 10x Ligation buffer for T4 DNA ligase (Roche), 1.0µl T4 DNA Ligase enzyme (Roche), and 16.0µl ddH_2O. The solution was incubated at 24˚C for 1 hour, then at 70˚C for 10 min to heat-inactivate the ligase enzyme. These manipulations produced three separate IgA2 expression vectors containing different V_H genes: the original b12-VH derivative from pSM102 (H31/4L) as well as two b12/KenyanV_H gene hybrids; pSM102-A6/4L and pSM102-A9/4L.

One Shot® TOP-10 Chemically Competent *E. coli* cells (Invitrogen) were transformed using 3.0µl of each cloned plasmid mixture, as described in the Invitrogen cloning manual. Thereafter, 50 and 100µl of each cell suspension was spread onto LB Miller agar plates supplemented with 100µg/ml ampicillin and incubated overnight at 37°C. Five colonies each of the pSM120-A6/4L and pSM120-A9/4L –transformed cells were identified by blue-white selection and cultured overnight at 37°C in 5ml LB Miller broth supplemented with 100µg/ml ampicillin. 1ml of each broth culture was mixed with sterile-filtered 40% glycerol and stored at -70°C as long-term glycerol stocks. The remaining 4ml of each broth culture was used for plasmid DNA isolations using the QIAGEN Plasmid Mini Kit (Qiagen), as per kit instructions.

The variable heavy region of each cloned plasmid sample was sequenced using 3.0µl plasmid template, 2.5µl Big Dye® 3.1 (v 1:1) (Applied Biosystems) and 1.5µl of either the b12VHup primer, the IgA6/9tail5 primer or the IgA6/9tail3 primer (Table 1). The sequencing program was comprised of a 96°C denaturation step for 5 minutes followed by 80 cycles of 96°C for 30 seconds, 55°C for 30 seconds (or 75°C for 30

seconds when using the IgA6/9tail5 or IgA6/9tail3 primers) and 60°C for 4 minutes before cooling to 4°C. Subsequently, the DNA was precipitated with 3M sodium acetate (pH 5.2) and 95% ethanol for 3 hours, centrifuged at 4000rpm for 1 hour, washed twice with 70% ethanol and denatured at 90°C in Hi-Di™ formamide (Applied Biosystems). The samples were sequenced using the ABI PRISM® 3100 Genetic Analyser system (Applied Biosystems). Sequence analysis was performed using Lasergene and GeneDoc Alignment software, version 7 (DNASTAR Inc., Madison, WI, USA).

3.5.2 Cloning the Variable Light Chain genes into pSM102

The $30LV_{\lambda}C\kappa$ and $30LV_{\lambda}C_{\lambda}$ antibody genes were then each cloned into the three heavy chain-switched versions of the IgA2 expression vector described above (pSM102, pSM102-A6/4L & pSM102-A9/4L) to produce six additional IgA2 expression vectors containing three different V_L genes: $H31/30LV_{\lambda}C\kappa$, $A6/30LV_{\lambda}C\kappa$ & $A9/30LV_{\lambda}C\kappa$ as well as $H31/30LV_{\lambda}C_{\lambda}$, $A6/30LV_{\lambda}C_{\lambda}$ & $A9/30LV_{\lambda}C_{\lambda}$. In all cases, 5µl of plasmid DNA was added to 1.0µl 10x REact 2 buffer (Invitrogen), 0.5µl *HindIII* and 3.0µl ddH_2O. The samples were incubated at 37°C for 45 min. Thereafter, 1.0µl 10x REact 3 buffer (Invitrogen) and 0.5µl *EcoRI* enzyme were added and the samples were incubated at 37°C for an additional 45 min. After restriction, samples were loaded onto an unstained 1% UltraPure™ agarose gel and run until bands were well separated, stained with SYBR® Green I Nucleic Acid Stain and purified as described in section 3.5.1. The $30LV_{\lambda}C\kappa$ and $30LV_{\lambda}C_{\lambda}$ light chain genes were each ligated into each of the three pSM102-V_H backbone variants. The samples were transformed into One Shot® TOP-10 Chemically Competent *E. coli* cells and plasmids were isolated for sequencing as using

the method described in section 3.5.1. The b12VLup primer, the b12VLdown primer the b12VL_end_tail5 primer or the 30λVL_short3 primer were used for sequencing the entire light chain gene (Table 2). Samples were sequenced at an annealing temperature of 55°C (75°C when using the 30λVL_short3 primer) and analysed in the same manner as section 3.5.1.

3.6 Production of human IgA2 antibody by means of Animal Cell Culture

3.6.1 CHO-K1 cell culture conditions

CHO-K1 cells were cultured in 12ml supplemented F12-K culture medium (see section 2.4) in 75cm^2 T-flasks at 37°C and 5% CO_2. Culture growth was monitored daily and cells were propagated by replacing culture media every 48 hours. Cell cultures were split when monolayer confluence approached 90%; culture medium was removed, 4ml TrypLE Express Stable Trypsin-Like Enzyme (Gibco) was added and the vessel was incubated at 37°C for 10 minutes. Cells were collected and gently pelleted by centrifugation at 1500 x g for 7 minutes, resuspended in a total volume of 1ml of medium and quantified using a haemocytometer. Cells were sub-cultured into additional T-flasks at a ratio of 1:8.

3.6.2 Preparation of IgA2 plasmid DNA for transfection

The nine IgA2 plasmid vectors (pSM102, pSM102-A6/4L, pSM102-A9/4L, H31/30LV$_\lambda$Cκ, A6/30LV$_\lambda$Cκ, A9/30LV$_\lambda$Cκ, H31/30LV$_\lambda$C$_\lambda$, A6/30LV$_\lambda$C$_\lambda$ & A9/30LV$_\lambda$C$_\lambda$) were linearized prior to CHO-K1 cell transfection in order to increase the chances of producing stable transfectants. Briefly, 20μg of each DNA sample was incubated

overnight at 37°C with 3.5µl REact10 buffer and 3.5µl *SalI* restriction enzyme (Invitrogen). The samples were precipitated at -80°C for 20 minutes with 30µl 3M sodium acetate and 1ml cold 95% ethanol and centrifuged at 14,000rpm at 4°C for 15 minutes. Samples were twice washed with 500µl cold 70% ethanol and centrifuged at 14,000rpm at 4°C for 5 minutes. The DNA was resuspended in sterile ddH_2O to a final concentration of 1µg/µl. A 1:50 diluted aliquot of each linearized IgA sample was run on a 1% UltraPure™ agarose gel to confirm plasmid size and linearization.

3.6.3 CHO-K1 cell transfection with IgA2 plasmid constructs

CHO-K1 cells were grown from frozen stocks in 6-well tissue culture plates at an initial concentration of $1x10^5$ cells/ml. One 6-well plate was seeded for every IgA transfection sample and an additional plate was seeded as a non-transfected negative control. The tissue culture samples were incubated in 2.5ml/well of supplemented F12-K medium overnight at 37°C and 5% CO_2, in order to reach 50-80% confluence for DNA transfection.

For each of the nine IgA transfection samples, 635µl of F12-K medium was pre-incubated with 21µl FuGENE 6 Transfection Reagent (Roche) for 5 minutes at room temperature. Thereafter, 9.8µl of linearized IgA DNA sample was added, and the sample was mixed by vortex and incubated at room temperature for an additional 15 minutes. Each IgA sample was inoculated into all six wells of an individual CKO-K1 6-well plate by adding 90µl of the transfection mixture to each culture well in a drop-wise manner. The negative control culture was not manipulated with FuGENE 6 transfection reagent.

All transfected CHO-K1 cultures and the non-transfected CHO-K1 control were incubated at 37°C and 5% CO_2 for 48 hours.

Both the transfected and untransfected CHO-K1 cultures were then switched to selective cell culture media in order to encourage the growth of stably transfected cells. The F12-K medium was aspirated from the 6-well plate cultures and replaced with glutamine-free supplemented GMEM medium containing 10% dialyzed fetal calf serum (as described in Appendix B). Furthermore, the selective agent L-methionine sulfoximine (MSX) was added to every 6-well culture plate at a final concentration of either 20μM, 40μM, 60μM, 80μM, 100μM or 120μM per well. The plates were incubated at 37°C and 5% CO_2 with media replacement every 48 hours. Cultures were maintained for approximately 2 weeks and compared to the non-transfected negative control culture daily until the negative control cultures died and growth of stable transfected cell clones was visually detected .

All cultured wells of each IgA sample were maintained in GMEM with the appropriate concentration of MSX until the cultures reached 70% confluence, at which point the culture supernatants were sampled and tested for IgA production. 1ml of cell culture supernatant was collected from each growing well of each IgA-transfected CHO-K1 cell culture. Culture supernatants were stored in sterile 1.5ml Eppendorf tubes at -20°C until use and IgA production was quantified by IgA-specific ELISA.

3.6.4 ELISA screening of culture supernatant for production of human IgA

Transfected CHO-K1 cell cultures were tested for IgA2 production in triplicate by means of ELISA. MaxiSorp Immuno 96 MicroWell™ Plates (NUNC) were coated with

12µg/ml AffiniPure Goat anti-human serum IgA, α-chain specific (Jackson) in PBS at a volume of 100µl/well. The plate was incubated overnight at 4°C. The coat solution was aspirated from the wells, 350µl/well of blocking solution (PBS+0.25%BSA) was added and the plate was incubated for 2 hours at 37°C. The plate was washed 10 times with ELISA wash buffer using a Wellwash 4 Mk 2 plate washer (Thermo Scientific, Waltham, MA, USA). The IgA cell culture supernatants and IgA antibody standard were added to the ELISA plate at 100µl/well. Human IgA kappa (Protos Immunoresearch) was used as a standard and applied at 2-fold dilutions ranging from 500-7.8ng/ml. Both the standard and the supernatant samples were serially diluted in PBS where applicable. The ELISA plate was incubated overnight at 4°C, washed 10 times, and then incubated for 1 hour at 37°C with 100µl/well of HRP-Conjugated AffiniPure Goat Anti-Human Serum IgA, α-Chain Specific (Jackson Immunoresearch) secondary antibody diluted 1:10,000 in blocking solution. This solution was removed from the plate by washing 10 times with wash buffer and an additional 3 times with ddH_2O. The ELISA plate was developed at room temperature using 100µl/well of 3, 3′,5 ,5′-Tetramethylbenzidine Liquid Substrate (TMB) solution (Sigma). Colour development was visually monitored for 30 minutes and the reaction was halted with 100µl/well of ELISA stop solution. The ELISA plate was read using a SpectraMax Plus spectrophotometer (Molecular Devices) and the Softmax® PRO software, version 3.1.2 (Molecular Devices).

For each IgA sample, the culture well that produced the greatest amount of IgA at the highest concentration of MSX inhibitor was selected for limiting dilution and monoclonal antibody generation.

3.6.5 Production of monoclonal IgA2-transfected CHO-K1 cell lines by limiting dilution

Human monoclonal IgA2 antibody cell cultures were generated by subjecting each of the 9 selected cell lines to multiple rounds of limiting dilution. The best IgA-secreting cell line of each IgA culture plate was detached by means of TrypLE as previously described in section 3.6.1. Cells were counted using a haemocytometer and diluted in supplemented GMEM with the applicable concentration of MSX to a final concentration of 2.5cells/ml. Each dilute cell suspension was added to the wells of a 48-well culture plate at a volume of 200µl/well, giving an average of 1 viable cell for every two plate wells. Each new IgA plate was cultured in GMEM + MSX at 37°C and 5% CO_2 and the medium was replaced every 48 hours.

Limiting dilution was considered to have been successful when one half to one third of the 48-wells of a culture plate were positive for cell growth. Once monoclonal cell colonies were visible under a microscope, the culture supernatant from positive wells were collected at every media change and stored at -20°C. The limiting dilution sample plates were cultured for approximately 30 days until cell confluence was reached. Thereafter, all cell culture supernatants were thawed and the best IgA-producing clone was identified by IgA-specific ELISA. The best IgA-producing clone of each IgA sample was used in a subsequent round of limiting dilution performed in the above manner.

3.6.6 Scale-up of monoclonal cell cultures

The best-producing clone of each of the nine IgA2 constructs was scaled up into ten 75cm^2 T-flasks and cultured as described in section 3.6.3 with GMEM + MSX at 37°C and 5% CO_2. The IgA content of each 48 –hour supernatant sample was assessed

by IgA-specific ELISA, and samples containing detectable IgA were retained. These 48-hour culture supernatants were combined, clarified by means of centrifugation at 3500xg for 30 minutes and either immediately used for purification or stored at -20°C until use. This yielded up to 500ml of supernatant per cell line to be used for antibody purification.

3.6.7 Storage of monoclonal antibody supernatants & cell lines

A total of $5x10^6$ cells of each of the nine monoclonal IgA cell lines used for experimental IgA production were resuspended in 90% FCS and 10% DMSO. These samples were placed in an isopropyl alcohol freezing container at -80°C overnight, then transferred to liquid nitrogen for long-term storage. $5x10^6$ cells of each of the nine IgA clones were set aside for genomic DNA extraction using the QIAamp DNA Mini Kit (Qiagen) to confirm plasmid incorporation.

3.7 Genetic Confirmation of CHO-K1 cell transfection

3.7.1 Genomic DNA Extraction from transfected CHO-K1 cells

In order to confirm the successful incorporation of the IgA2 heavy and light genes into each host CHO-K1 cell genome, transfected CHO-K1 genomic DNA was extracted using a QIAamp DNA Mini Kit, as per the "Cultured Cells" protocol. A total of $2\text{-}5x10^6$ cells were used for each DNA extraction, and genomic DNA was eluted in 200µl sterile ddH_2O. Samples were stored at -20°C until use.

3.7.2 Overall PCR amplification & sequencing strategy

All PCR reactions described below consisted of the same basic master mix of 22.75µl 2x PCR Mix, 16.0µl sterile ddH_2O, 3.0µl of the appropriate upstream and downstream primers and 0.25µl Expand High Fidelity^{PLUS} Polymerase Enzyme (Roche). 5.0µl of genomic DNA was used in each PCR reaction, giving a total reaction volume of 50µl per tube. In all cases, the 9 IgA2-transfected CHO-K1 cell genomic DNA samples were amplified along with an appropriate plasmid DNA positive control, a non-transfected CHO-K1 genomic DNA sample negative control, and a ddH_2O blank.

All PCR amplification programs used followed the same basic structure with the annealing temperature modified to suit the specific primers used. Samples were initially denatured for 2 minutes at 94°C, followed by 35 cycles of 94°C for 20 sec, primer annealing for 30 sec and template elongation at 72°C for 90 sec.

After amplification, 10µl of each PCR product was combined with 2µl 6x loading buffer and run on a 1% UltraPure™ agarose gel to ensure proper product amplification. Once the PCR products were confirmed to be the desired size, the remaining 40µl were purified for sequencing using Amicon Microcon PCR centrifugal Filter Devices (Millipore), as per kit instructions. PCR products were eluted in 20µl ddH_2O. All heavy and light gene products were sequenced as described in section 3.5.1.

3.7.3 PCR detection and sequencing of transfected V_H genes

The human full-length variable heavy chain transfected gene fragments were amplified from CHO-K1 genomic DNA by PCR as described above using one of two variable heavy chain-specific primer sets (Table 5). The b12-derived H31 gene was

detected using upstream primer psm_vhleader5′ (5′- CTC TAG AGC CGC CAC CAT GG- 3′) and downstream primer b12VHdown (5′- CAG GTC ACA CTG AGT GGC TC- 3′). The B cell-derived A6 and A9 genes were detected using upstream primer psm_vhleader5′ and semi-complementary downstream primer IgA6/A9tail3 (5′- GAG GAG GAG GAG CTC GAG GAG ACG ATG AC- 3′). Both PCR programs used a primer annealing temperature of 57°C. After PCR product purification as described, H31 genes were sequenced at an annealing temperature of 55°C using primers b12VHup & b12VHdown. The A6 & A9 genes were sequenced using the b12VHup primer at 55°C and the IgA6/9tail3 primer at 75°C.

3.7.4 PCR detection and sequencing of transfected V_L genes

Full-length variable light chain transfected gene fragments were also amplified using one of two primer sets (Table 6). The 4L gene and the 30LV$_\lambda$Cκ gene shared a light chain constant kappa region and therefore were able to be amplified with the same primer set. Both were amplified using the b12VLup upstream primer (5′- GAA GCT TAC CAT GGG TGT GC-3′) and b12VLdown downstream primer (5′- GAT CAA TGA ATT CAT TAA CAC TCT- 3′), with PCR conditions of an annealing temperature of 50°C. The 30λ gene was amplified using upstream primer b12VLup and semi-complementary downstream primer IgA43-66-69tail3 (5′- GAT CAA TGA ATT CAT TAT GAA CAT TCT G-3′), using a PCR program annealing temperature of 57°C. A second round of PCR amplification was required for the 30λ samples; the same PCR ingredients and conditions were used and 5µl of the first round 30λ PCR products were used as a template. After purification, the 4L & 30LV$_\lambda$Cκ genes were sequenced at an annealing

Table 5. Primers used for V_H gene confirmation & sequencing.

Name	Primer Sequence (5′ to 3′)	Orientation	Tm (°C)	Features	Target DNA
psm_vhleader 5′	CTGCAGTCACCGTCCTTGAC	Forward	64		H31, A6 & A9 V_Hs
b12VHdown	CAGGTCACACTGAGTGGCTC	Reverse	64		H31 V_Hs
IgA6/A9tail3	GAGGAGGAGGAGCTCGAGGAGACGATGAC	Reverse	94	*SstI site*	A6 & A9 V_Hs
b12VHup	CTCTAGAGCCGCCACCATGG	Forward	66	*XbaI site*	H31, A6 & A9 V_Hs

Table 6. Primers used for V_L gene confirmation & sequencing.

Name	Primer Sequence (5′ to 3′)	Orientation	Tm (°C)	Features	Target DNA
b12VLup	GAAGCTTACCATGGGTGTGC	Forward	60	*HindIII site*	4L, 30L$V_\lambda C_\lambda$, 30L$V_\lambda C\kappa$ V_Ls
b12VLdown	GATCAATGAATTCATTAACACTCT	Reverse	55	*EcoRI site*	4L & 30L$V_\lambda C\kappa$ V_Ls
IgA43-66-69tail3	GATCAATGAATTCATTATGAACATTCTG	Reverse	64	*EcoRI site*	30L$V_\lambda C_\lambda$ V_Ls

temperature of 55°C using primers b12VLup & b12VLdown. The 30λ gene was sequenced using the b12VLup primer at 55°C and the IgA43-66-69tail3 primer at 75°C.

3.7.5 PCR detection and sequencing of transfected IgA2 genes

Due to DNA sequence similarities between the transfected C_H3 domain of human IgA2 and the CHO-K1 cell genetic material, it was not feasible to PCR-amplify the full-length human IgA2 constant heavy gene product without also amplifying background CHO-K1 DNA. As a result, only the C_H1 & C_H2 domains of the transfected IgA2 samples were targeted to be detected by PCR and sequenced. The samples were amplified using upstream primer IgAFwd (5′- GCT CAT CCC CGA CCA GCC- 3′) and downstream primer IgAR2 (5′- GAC CTC GGG CCG GAA TG- 3′). The PCR reaction annealing temperature was 50°C. After purification, the PCR products were sequenced using the IgAFwd, IgAR2, IgAF3 (5′- GCA CCA CCA CCT TCG CTG- 3′) and IgAR3 (5′- TGG GCA GGG CAC AGT CAC -3′) primers. All sequencing reactions were performed at a primer annealing temperature of 55°C.

Because the transfected IgA2 constant heavy chain genes were not able to be sequenced in full, the entire IgA2 gene of each of the initial transfection plasmids were also confirmed by means of PCR amplification and sequencing. All 9 plasmid variants were amplified with the V_H fragments included in order to confirm that these variable genes were fused in-frame to the IgA backbone. For each PCR reaction, 1µl of plasmid DNA was used as a template, and the samples were amplified using the upstream primer psm_vhleader5′ and the downstream primer newIgARev (5′- CTT TCC CAA GTG CTG AGA CC- 3′) (Table 7). The PCR reaction annealing conditions were changed to 51°C

for 25 seconds. After purification, the PCR products were sequenced using the IgAFwd, newIgARev, b12vhup, and IgAF2 (5′- CCA CTA ACC GCC AAC ATC -3′) primers. All sequencing reactions were performed at a primer annealing temperature of 55°C.

3.8 Purification of Monoclonal IgA from Cell Culture Supernatants

The 9 experimental IgA samples were each purified from the cell culture supernatants by means of anti-IgA column chromatography.

3.8.1 Immunoaffinity Column Set-up

The gravity flow immunoaffinity column used for sample preparation was prepared by packing 8ml of goat anti-human IgA (α-chain specific), (Sigma-Aldrich, Saint Louis, MO, USA) coated agarose resin onto a BioRad glass Econo-Column (1.0 x 10cm). The column was allowed to settle at room temperature for 1 hour. 50ml of degassed room temperature binding buffer (PBS, pH 7.4) was then passed through the column. The column apparatus was stored in degassed PBS at 4°C when not in use; however during prolonged storage periods (greater than 1 week) the column was stored in PBS supplemented with 0.2% sodium azide.

The column apparatus was always equilibrated to room temperature and rinsed with 10 column volumes (50ml) of PBS, 10 volumes of low-pH elution buffer (0.1M glycine-HCl, pH 2.0), and an additional 10 volumes of PBS before use. All column chromatography buffers were filtered, degassed and brought to room temperature before use.

Table 7. Primers used for IgA2 gene amplification & sequencing.

Name	Primer Sequence (5′ to 3′)	Orientation	Tm (°C)	Target DNA
IgAFwd	GCTCATCCCCGACCAGCC	Forward	62	IgA2 C_H1-C_H3 (all plasmids)
newIgARev	CTTTCCCAAGTGCTGAGACC	Reverse	60	
b12vhup	CTCTAGAGCCGCCACCATGG	Forward	66	
IgAF2	CCACTAACCGCCAACATC	Forward	58	

3.8.2 Immunoaffinity Column Purification of IgA from Cell Culture Supernatant

For each purification run, the column was pre-rinsed with 10 volumes binding buffer, 10 volumes of low-pH elution buffer and 10 additional volumes of binding buffer as described above. Thereafter, the cell culture supernatant was diluted 1:1 with binding buffer and applied to the column. The culture supernatant flowthrough was collected and passed through the purification column one additional time. The column was rinsed with 10 column volumes of binding buffer prior to sample elution. Antibody was eluted in three column volumes of elution buffer (0.1M glycine-HCl, pH 2.5). Three 10ml fractions of antibody were collected and eluted directly into 533µl of neutralization buffer (1.5M Tris-HCl, pH 8.8). The immunoaffinity column was regenerated with a final 10 column volumes of binding buffer, capped and stored at 4°C.

Fractions of the sample flowthrough were collected throughout the entire purification process, stored at 4°C and immediately tested by anti-IgA ELISA (see section 3.6.4) to determine that the IgA samples were appropriately retained and eluted by the purification column. Thereafter, neutralized fractions found to contain IgA were concentrated and desalted using Macrosep® 30K Centrifugal Devices (Pall Corporation, Mississauga, ON, Canada), as per kit instructions. Antibody samples were eluted in PBS, aliquoted into single-use vials and stored at -70°C until use.

3.9 Quality control of purified IgA samples

3.9.1 Quantification of IgA

The concentration of the purified IgA samples was determined using the BioRad Protein Assay kit, as per the Microassay Procedure instructions (BioRad). Human IgA

kappa (Protos Immunoresearch) at a concentration range of 1mg/ml to 0.05mg/ml was used as a standard. IgA samples were incubated with diluted Dye Reagent in the wells of a MaxiSorp Immuno 96 MicroWell™ Plate (NUNC) for 10 minutes, at which time sample absorbance was measured at 595nm on a SpectraMax Plus spectrophotometer (Molecular Devices) with Softmax® PRO software, version 3.1.2 (Molecular Devices). Experimental IgA sample concentrations were extrapolated from the linear portion of the human IgA kappa standard curve.

3.9.2 IgA-specific ELISA

Experimental IgA samples with concentrations below the detectable range of the BioRad Protein Assay kit were estimated by IgA-specific ELISA, as described previously. Human IgA Purified Immunoglobulin from Colostrum (Sigma-Aldrich, Saint Louis, MO, USA) was used as standard. The sample concentrations were estimated from the linear portions of the IgA Colostrum standard curve (see section 3.6.4).

3.9.3 SDS-PAGE & Coomassie Staining

Antibody size and purity was verified by SDS-PAGE and Coomassie staining, with human IgA kappa and Human Colostrum-derived IgA as positive controls. In each case, 12.5µg (or 25µl of the low-concentration samples) of each IgA sample was combined with 5µl of 6X loading dye. All samples were boiled for 5 minutes to facilitate denaturation. The denatured samples were loaded onto a BioRad pre-cast gel, Tris-HCl with a 4-15% acrylamide gradient. 10µl of the Precision Plus Protein™ Kaleidoscope™ Standard protein ladder (Bio-Rad) was used as a molecular mass standard. The gel was

run at 200V for 45 minutes. Protein bands were visualized by staining the acrylamide gel in Coomassie Blue dye for 3 hours at room temperature. The gel was destained with Destain I solution (50% methanol, 10% glacial acetic acid) overnight, followed by Destain II solution (5% methanol, 7% acetic acid) for 1hour. The protein bands were photographed with a Bio-Rad Gel Doc 2000 apparatus using Quantity One software, version 4.2.1.

3.9.4 Western Blot Analysis

To confirm the identity of the protein bands, SDS-PAGE was performed as described in section 3.8.3. Briefly, 100ng, or 25µl, of the nine IgA samples and the human IgA kappa & human IgA colostrum controls were separated on Bio-Rad pre-cast gels as described above. Thereafter, the acrylamide gel was soaked in transfer buffer for 20 minutes at room temperature with gentle agitation; meanwhile, a nitrocellulose membrane and three sheets of filter paper were pre- soaked in methanol and then transfer buffer for 15 minutes. The protein samples were transferred to a nitrocellulose membrane pre-soaked in methanol and transfer buffer using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell and applying 15V of current to the apparatus for 30 minutes. The nitrocellulose membrane was then incubated in blocking solution (PBS + 0.1%Tween + 5% skim milk powder) for 1 hour with gentle agitation before being incubated with 1:10,000 anti-IgA detection antibody (HRP-Conjugated AffiniPure Goat Anti-Human Serum IgA, α-Chain Specific (Jackson Immunoresearch)) diluted in 10mL blocking solution at 4°C overnight. The following day, the blot was washed three times for a total

of 1 hour in wash buffer (PBS + 0.1% Tween). The GE ECL Detection kit was used as a substrate to develop the blot, and the fluorescence was detected by the FluorChem 8900.

3.10 Characterization of gp120 specificity of the IgA2 Experimental Samples

3.10.1 gp120 ELISA

A gp120-specific ELISA was performed to determine whether the nine experimental IgA constructs were capable of binding to monomeric gp120. Recombinant gp120 HIV-1_{IIIB} (ImmunoDiagnostics, Woburn, MA, USA) was diluted in PBS at a concentration of 2.5µg/ml. Coating solution was added to MaxiSorp Immuno 96 MicroWell™ Plates (NUNC) in triplicate at a volume of 100µl/well. The plates were incubated overnight at 4°C, then the coat solution was aspirated from the wells. 350µl/well of blocking solution (PBS+0.25%BSA) was added and the plate was incubated for 2 hours at 37°C. Thereafter, the plate was washed 10 times with ELISA wash buffer using a Wellwash 4 Mk 2 plate washer (Thermo Scientific). No gp120-specific IgA antibody standard was available for use in this ELISA protocol. 100µl/well of the experimental samples was added to the plate and serially diluted using block solution where applicable. The plates were incubated overnight at 4°C, then washed 10 times. 100µl/well of secondary antibody (HRP-Conjugated AffiniPure Goat Anti-Human Serum IgA, α-Chain Specific (Jackson Immunoresearch)) diluted 1:10,000 in block solution was added. After a 2-hour incubation at 37°C, the plate was washed 10 times, rinsed 3 times with ddH_2O and developed using 100µl/well of TMB solution (Sigma). The plate was incubated for 30 minutes at room temperature, then colour development

was halted using 100µl/well ELISA stop solution. The plates were read at 450nm using the SpectraMax Plus spectrophotometer and the Softmax® PRO software, version 3.1.2.

3.10.2 Flow Cytometry

The ability of the two properly-sized, highly-expressed IgA experimental constructs, H31/4L and A6/4L, to bind trimeric gp120 was assessed by flow cytometry. The H31/4L and A6/4L IgA antibodies were tested for binding to the gp120-expressing H9/HTLV-IIIB NIH 1983 cell line compared to the non-gp120 expressing control cell line, H9.

Both cell lines were cultured in RPMI-1640 cell culture medium with 2.05mM L-glutamine (HyClone) supplemented with 10% FCS and P/S/F. The cell cultures were sub-cultured twice weekly at a 1:4 ratio. Prior to cell culture use, a p24 ELISA was performed on the cell culture supernatants of both cell lines to ensure that the H9/HTLV-IIIB NIH 1983 cell line was producing viral antigen.

One million cells of the appropriate cell line were aliquoted into FACS tubes, washed twice with 1ml wash solution (PBS+2%FCS), and centrifuged at 1400rpm for 5 minutes. After resuspension of the cell pellet, 2µg, 1µg, 0.5µg, 0.25µg or 0.1µg of H31/4L or A6/4L antibody was added to the tube of infected H9 cells and to a tube of uninfected H9 cells. Samples were incubated at 4 degrees in the dark for 30 minutes. Thereafter, cells were washed in 2mL wash buffer, centrifuged as described above and resuspended. 2µl of secondary antibody (FITC-conjugated Goat F(ab')2 anti-human IgA (α), Invitrogen) was added to each experimental tube. Samples were covered and incubated at 4 degrees for 30 minutes. All cells were washed as before and resuspended

to a final volume of 300ul in wash solution. Control tubes were prepared with either no antibody, 1ug primary antibody only or 2ul secondary antibody only.

Cells were fixed with 1% paraformaldehyle prior to analysis. Sample data was collected using a FACSCalibur 4-Colour Flow Cytometer (Becton Dickinson) and analysed using CellQuest Pro software.

3.10.3 IgA Inhibition Assay with IgGb12 Competition

Two competition ELISAs were performed to compare the gp120 epitopes recognized by the experimental IgA constructs to the parental IgGb12. In the first assay, the concentration of the two properly-sized, highly-expressed IgA antibody samples (H31/4L and A6/4L) was kept constant while the concentration of IgGb12 was varied. Triplicate MaxiSorp Immuno 96 MicroWell™ Plates (NUNC) were coated with 100µl/well of gp120 capture antibody (Sheep anti-HIV-1 gp120env Antibody (Aalto Bio Reagents, Dublin, Ireland) at a concentration of 2.5 µg/ml. Plates were sealed and incubated overnight at 4°C. The capture antibody solution was aspirated from the plate wells and 350µl/well of filtered block solution (PBS + 1% BSA) was added. Plates were sealed and incubated at 37°C for 3 hours. Thereafter, the plates were washed 10 times with ELISA wash solution using a Wellwash 4 Mk 2 plate washer (Thermo Scientific). 1µg/ml of recombinant $gp120_{IIIB}$ (Immunodiagnostics) in block solution was then captured on the plates by adding 100µl of the solution per well, incubating for 1 hour at 37°C, and washing 10 times as described above. Meanwhile, the competition antibody samples were pre-combined in a separate NUNC plate. 50µl/well of IgGb12 was added to the setup plate at an initial concentration of 5µg/ml and serially diluted using 50µl of

block solution. The H31/4L IgA sample was added at a concentration of 0.140µg/ml to approximate its half-saturation of gp120. The lower-affinity A6/4L IgA sample was added at 6.5µg/ml. Both of these samples were added at 50µl/well. As a positive control, 50µl/well of both H31/4L and A6/4L IgA samples were added to the plate and serially diluted in block solution without any competing IgGb12, and as a negative control 50µl/well of IgGb12 was added and serially diluted in block solution without either competing IgA sample. The H31/4L sample was added at an initial concentration of 4.5µg/ml, the A6/4L sample was added at 208µg/ml and the IgGb12 sample was added at 5µg/ml. The pre-mixed antibody samples were added to the ELISA plates, sealed and incubated at 4°C overnight. After washing the plates, 1:1,000 secondary anti-IgA antibody (HRP-Conjugated AffiniPure Goat Anti-Human Serum IgA, α-Chain Specific (Jackson Immunoresearch)) in block solution was added at 100µl/well and incubated at 37°C for 1 hour. The ELISA plates were washed 10 times with wash buffer and an additional 3 times with ddH_2O, then developed for 15 minutes with TMB solution (Sigma). Colour development was stopped by adding 100µl/well ELISA stop solution. Plates were read using the SpectraMax Plus spectrophotometer and the Softmax® PRO software, version 3.1.2.

3.10.4 IgGb12 Inhibition Assay with IgA Competition

The IgA inhibition assay results were confirmed with an assay where the concentration of IgGb12 was kept constant and the effect of different concentrations of experimental IgA antibody on the gp120-binding ability of IgGb12 was determined. The triplicate anti-IgG competition ELISA plates were coated, blocked, captured and washed

using the same procedure as the anti-IgA competition ELISA plates. While the $gp120_{IIIB}$ solution was being captured, the competition antibody samples were pre-aliquoted on a separate NUNC plate. 50µl/well of H31/4L IgA antibody was added to the setup plate at an initial concentration of 4.5µg/ml and serially diluted using 50µl/well of block solution. Similarly, 50µl/well of A6/4L IgA antibody was added at an initial concentration of 208µg/ml and serially diluted with 50µl/well of block solution. The IgGb12 antibody sample was added at a concentration of 0.165µg/ml to approximate its half-saturation of gp120. Additionally, 50µl/well of IgGb12 was added to the plate and serially diluted in block solution without any competing IgA as a positive control, and as a negative control 50µl/well of H31/4L and A6/4L IgA was added and serially diluted in block solution without any IgGb12 sample. The positive control IgGb12 dilution curve initial concentration was 5µg/ml and the negative control H31/4L and A6/4L IgA dilution curves commenced at 4.5µg/ml and at 208µg/ml respectively. The pre-mixed antibody samples were added to the ELISA plates, sealed and incubated at 4°C overnight. After washing the plates, 1:1,000 secondary anti-IgG antibody (AffiniPure Goat anti-human IgG (Fcγ- specific)-HRP, Jackson ImmunoResearch) in block solution was added at 100µl/well and incubated at 37°C for 1 hour. The ELISA plates were washed 10 times with wash buffer and an additional 3 times with ddH_2O, developed using 100µl/well of TMB solution (Sigma), and halting the colour development with 100µl/well of stop solution after 15 minutes. Plates were read using the SpectraMax Plus spectrophotometer and the Softmax® PRO software, version 3.1.2.

4.0 Results

4.1 Cloning B-cell derived variable genes to produce antibody constructs

To construct the Kenyan B-cell derived variable antibody fragments for cloning into the pSM102 IgA2 expression vector, these variable genes were extended into full-length IgA heavy and light genes by means of overlap PCR. In addition to the IgGb12-derived affinity-matured variable heavy gene (H31) and light gene (4L) already encoded by the pSM102 plasmid, 4 additional B-cell derived antibody genes were initially generated.

The two B-cell derived heavy chain genes A6 and A9 were developed by fusing a DNA leader sequence containing the *XbaI* restriction site to each variable gene. PCR was also performed using a semi-complementary primer in order to add an *SstI* restriction site downstream of each variable gene. The resulting A6 variable fragment was 473bp in length while the A9 variable fragment was 476bp, as confirmed by gel electrophoresis. After restriction with *XbaI* and *SstI*, the A6 fragment was reduced to 426bp and the A9 fragment to 429bp. The pSM102 vector contained an in-frame *SstI* restriction site directly upstream of the IgA C_H1 constant heavy gene domain allowing full-length IgA heavy chain genes containing A6 & A9 variable heavy chains to be generated by directional cloning into pSM102.

Two versions of the B-cell derived light chain gene were also developed by overlap PCR. The 30L lambda-type variable light fragment was coupled to a constant kappa-type light gene, producing a full-length antibody light chain named $30LV_{\lambda}C\kappa$. Due to poor antibody production (described in section 4.2.3), the $30LV_{\lambda}C\kappa$ variable light fragment was later also fused with a constant lambda-type gene to produce a second full-

length light chain gene named 30LV$_\lambda$C$_\lambda$. Both light chains were developed by adding an upstream DNA leader sequence containing the *HindIII* restriction site and a downstream constant light chain containing an *EcoRI* restriction site to the 30L variable gene. Successful fusion by PCR resulted in two full-length antibody light gene products: a 30LV$_\lambda$Cκ product of 732bp and a 30LV$_\lambda$C$_\lambda$ product of 702bp. Restriction with *HindIII* and *EcoRI* resulted in 718bp and 688bp products respectively which were directionally cloned into the pSM102 vector. Once cloned, antibody constructs containing the 30LV$_\lambda$Cκ light chain gene product were henceforth abbreviated as "30L" clones, whereas constructs containing the 30LV$_\lambda$C$_\lambda$ light chain gene product were referred to as "30λ" clones for simplicity.

A total of nine experimental antibody constructs were produced by cloning different combinations of the Kenyan- and IgGb12-derived heavy and light genes into the pSM102 IgA2 expression vector. These 9 IgA constructs could be subdivided into three categories based on the origin of their variable genes. One IgGb12-like positive control (H31/4L) was produced without modification to the pSM102 plasmid and was comprised solely of antibody genes derived from an affinity-matured version of IgGb12. Four hybrid constructs (A6/4L, A9/4L, H31/30L and H31/30λ) contained one IgGb12-derived variable gene and one B-cell derived variable gene. Finally, four Africanized constructs (A6/30L, A6/30λ, A9/30L and A9/30λ) were produced which contained variable genes that were completely derived from cervical B-cell cDNA collected from Kenyan HIV-resistant women.

Each of the nine plasmid variants was sequenced prior to CHO-K1 cell transfection. All of the cloned heavy and light chain genes matched the expected template

sequence; however two base pair discrepancies were detected in all A6 variable heavy genes in the A6/4L, A6/30L & A6/30λ subclones. All A6 clones had a C residue at position 20 and a G residue at position 26, whereas the published IgA6 sequence indicated these bases should have been T and A respectively. Because these base pair discrepancies occurred in the framework region (and also due to time factors considered in the discussion) it was decided to proceed without modifying the A6 clones.

4.2 Production of human IgA2 antibody by Animal Cell Culture

4.2.1 Generation of the IgA2-transfected CHO-K1 cell lines

In order to generate full-length, glycosylated monoclonal IgA antibody *in vitro*, we utilized a stable DNA transfection and culturing approach. Chinese Hamster Ovary (CHO-K1) cells were cultured and transfected with one of the nine linearized IgA expression vectors described in section 4.1. Selection of successfully transformed CHO-K1 cells was performed by adding methionine sulfoximine (MSX) to the culture media at a concentration of 20μM, 40μM, 60μM, 80μM, 100μM or 120μM. All nine of the transfected CHO-K1 cell cultures were able to withstand a minimum 20μM of MSX inhibitor, indicating that IgA vector-encoded genes were successfully being expressed by the host cells. There was a correlation between the type of antibody variable light chain gene used in the transfection, the relative amount of IgA produced by the culture, and the maximum concentration of MSX at which a transfected cell culture could be sustained. Cell lines transfected with IgA 4L-type light chain genes produced the highest yields of IgA and were able to withstand the highest concentrations of MSX (80-100μM). The cell lines transfected with IgA-30L and IgA-30λ light chain genes produced very low

amounts of antibody as measured by IgA-specific ELISA and could only withstand MSX at concentrations ranging from 20-60μM.

In order to produce monoclonal IgA cell lines, the highest IgA-producing clone of each culture was selected and subsequently used in two rounds of limiting dilution. The highest producing clone was identified as the clone producing the most IgA (as measured by ELISA) at the highest possible concentration of MSX inhibition. Based on these criteria, H31/4L and A9/4L at 80μM MSX; A6/4L and A6/30λ at 60μM MSX; H31/30L, H31/30λ and A9/30λ at 40μM MSX and A6/30L and A9/30L at 20μM MSX were selected for limiting dilution. Cell cultures underwent two rounds of limiting dilution with the exception of the A9/30L sample, which underwent a third round due to a high number of positive wells in the first round, and the three IgA-30λ samples, which only underwent a single round of limiting dilution once it was apparent that the reconstruction of the 30λ light chain gene did not improve IgA production compared to the IgA-30L constructs (discussed in detail in section 4.2.3).

4.2.2 Optimization of CHO-K1 cell scale-up procedure

Once monoclonal CHO-KI cell cultures were established, they were scaled up to produce large amounts of spent supernatant from which experimental IgA could be purified. Cell cultures were initially scaled up as described in [227]. This method required the CHO-K1 cells to be cultured in 75cm^2 T-flasks with replacement of the selective medium every 48 hours until 100% confluence was reached; thereafter the cell lines were incubated for 6 days without media replacement and the spent supernatants harvested. Since the IgA-30L and IgA-30λ CHO-K1 cell lines were low-producing

clones, it was desirable to increase the efficiency of the culture supernatant scale-up procedure. We developed a culture method to improve both the efficiency and yield of the scale-up procedure by expanding the volume of recoverable IgA-containing supernatant by decreasing nutrient limitation.

We examined the IgA production of pre-confluent transfected CHO-K1 cultures to determine whether additional IgA could be recovered from the scale-up cultures. We conducted a pilot experiment to correlate CHO-K1 cell culture confluence with the concentration of monoclonal IgA secreted into the culture supernatant. The H31/4L, A6/4L and A9/4L CHO-K1 cell lines were cultured in 6-well plates as described in section 3.6.3. Every 24 hours, the confluence of each culture was visually assessed and the supernatants were sampled and assayed by IgA-specific ELISA. In the case of all three cell lines, IgA production was measurable well before CHO-K1 culture confluence was reached, as early as 25% confluence, and at moderate concentration by 50% confluence (Figure 8). Although the total amount of IgA produced varied by sample, the IgA production of all three cell cultures increased exponentially after ~80% confluence. While the highest concentrations of IgA were indeed recovered once the cell cultures reached 100% confluence, retaining sample supernatants collected between 25-90% culture confluence nearly doubled the overall yield of IgA. The overall IgA recovery was increased by 92.7% for the H31/4L sample, by 73.1% for the A6/4L sample and by 64.2% for the A9/4L sample (Table 8). Based on these results, we determined that the overall IgA yield could be dramatically increased by collecting all spent supernatants from cultures exceeding 25% confluence.

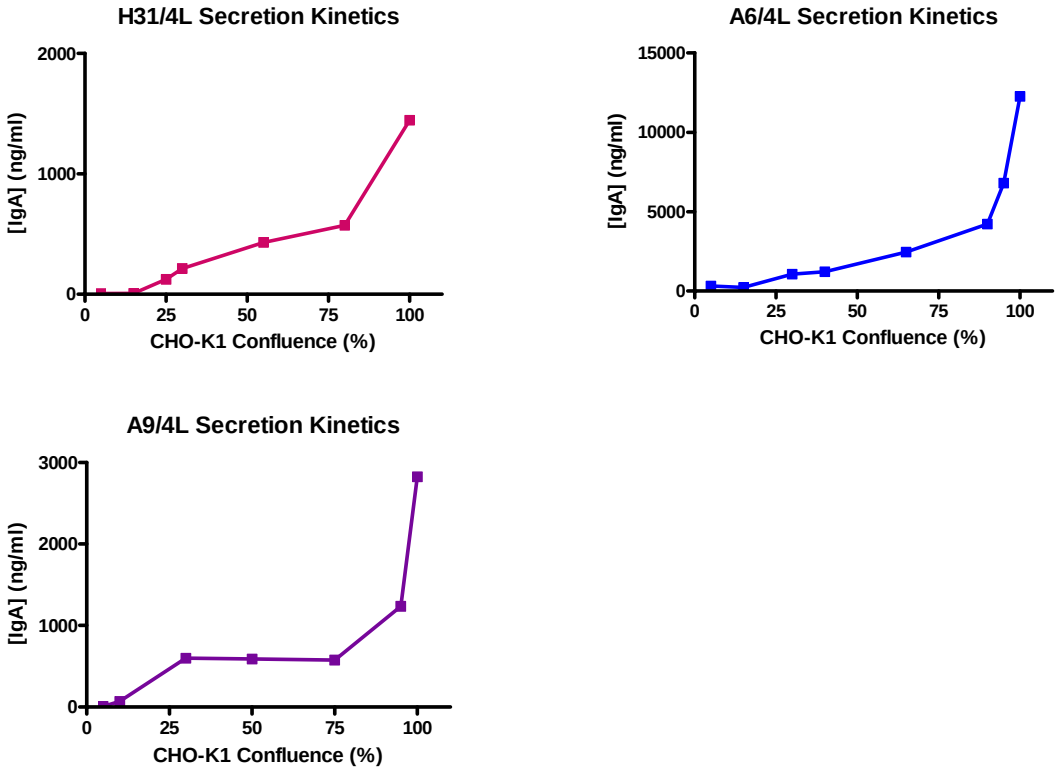

Figure 8. Production of human IgA2 by H31/4L, A6/4L & A9/4L transfected CHO-K1 cell lines at different stages of cell culture confluence. CHO-K1 cell lines were cultured as described in section 3.6.3. Culture supernatants were sampled every 24 hours and IgA production was measured by IgA-specific ELISA.

Table 8. Increase of total IgA yields from cell culture supernatant when retaining all supernatants collected between 25-90% confluence.

IgA Sample	Old IgA Yield (ng) 100% Confluence	IgA Yield (ng) 25-90% Confluence	IgA Yield (ng) Combined	% Increase
H31/4L	1445.6	1340.6	2786.2	92.7
A6/4L	12252.8	8954.3	21207.1	73.1
A9/4L	2827.2	1813.7	4640.9	64.2

Next, we investigated whether IgA production by the confluent transfected CHO-K1 cultures could be improved by avoiding nutrient limitation. To do so, we cultured two 25cm^2 flasks of CHO-K1 cells transfected with the H31/4L IgA variant as described in section 3.6.1. The first flask was cultured according to the original scale-up method; once the culture reached confluence it was left to incubate at 37°C and 5% CO_2 for 6 days. The second flask was cultured as above until confluence was reached, then the culture medium replacement continued every 48 hours for six days. Each of the post-confluence supernatant samples was tested for IgA concentration by IgA-specific ELISA.

It was observed that replenishing the culture medium throughout the 6 day culture period dramatically increased the yield of IgA antibody produced by the CHO-K1 cells (Figure 9). The flask that did not undergo any media changes produced 27.4μg of IgA at a titre of 5.48μg/ml. The pooled supernatants from the media-replenished flask yielded a total of 71.1μg of IgA with an average titre of 4.74μg/ml. While the original scale-up method produced a high titre of IgA, collecting and replenishing the culture media throughout the entire process generated 2.5 times more product. Furthermore, combined with collecting the pre-confluent cell culture supernatants, a total 96.69μg of IgA was generated (at a titre of 3.22μg/ml), which is approximately 3.5 times more IgA than was produced by the original scale-up method.

Based on the above findings, we modified the CHO-K1 cell culture scale-up procedure by continually changing the culture medium every 48 hours and by retaining all spent culture supernatant generated after cell cultures reached 25% confluence. In total, ~500ml of the H31/4L, A6/4L & A9/4L supernatants, ~300ml of the H31/30λ,

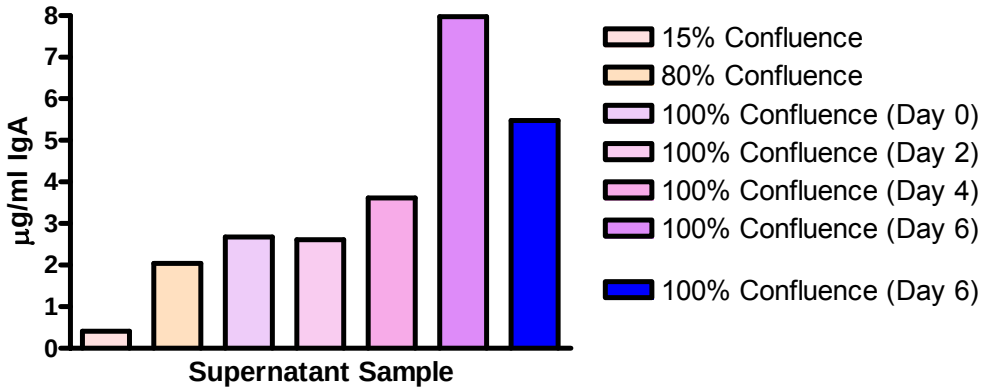

Figure 9. Effect of replenishing the cell culture supernatant on IgA production by the H31/4L-transfected CHO-K1 cell line. The pink bars represent the amount of IgA recovered from cultured cells maintained at 100% confluence for 6 days when the culture medium was exchanged every 48 hours. The blue bar represents the amount of IgA recovered from the culture supernatant when maintained at 100% confluence for 6 days without media exchange, as described in [227].

4.2.3 Antibody quantification

In order to compare the quantity of IgA produced by the nine transfected monoclonal CHO-K1 cell lines, the IgA levels in the scaled-up supernatants were quantified by comparison to known concentrations of human IgA kappa in an IgA-specific ELISA. This comparison revealed vast differences in IgA production across the transfected CHO-K1 cell lines that corresponded to the type of antibody variable light gene being expressed (Figure 10). The three CHO-K1 cell lines containing the 4L light chain produced the most IgA. The A6/4L [60μM MSX] cell line was the most productive clone, followed by the H31/4L [80μM MSX] "positive control" and the A9/4L [80μM MSX] cell lines. The three IgA-30L CHO-K1 cell lines exhibited much lower IgA productivity. Similar to the IgA-4L cell lines, the A6/30L [20μM MSX] cell line was the most productive, followed by the H31/30L [40μM MSX] and A9/30L [20μM MSX] samples.

Despite the reconstruction of the 30L gene to produce a fully lambda-type light chain, the CHO-K1 cell lines expressing the re-cloned 30λ light chain produced even less IgA than the 30L constructs. IgA production was not reliably identified in the raw supernatant and was only definitively quantified following supernatant concentration (see section 4.3).

The 9 supernatant samples were also evaluated by gp120-specific ELISA to determine if the secreted IgA samples recognized recombinant monomeric HIV-1 $gp120_{IIIB}$. Antibody binding was detected in only the H31/4L [80μM MSX] and A6/4L [60μM MSX] supernatant samples (Figure 11). The A6/4L sample seemed to exhibit a greater degree of gp120 reactivity than the H31/4L sample. However, since the A6/4L

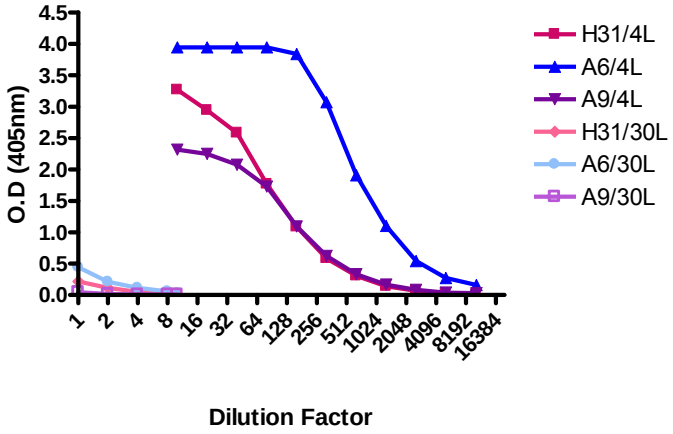

Figure 10. Human IgA content of transfected CHO-K1 cell line supernatants. Antibody production was detected by IgA-specific ELISA. IgA production by the H31/30λ, A6/30λ & A9/30λ cell lines IgA was too low to be accurately quantified and therefore is not included in this figure.

supernatant also contained the highest overall concentration of IgA, it was unclear whether this effect was due to the A6/4L sample having a greater affinity for gp120 or simply to an overabundance of gp120-specific IgA antibody relative to the other experimental samples. Additionally, gp120 specificity was not detected in the A9/4L [80μM MSX] supernatant despite relatively high concentration of IgA. gp120 specificity was also not detected in any of the IgA-30L or IgA-30λ supernatant samples, but given that the overall IgA concentrations of these samples were barely detectable by ELISA, it is possible that gp120-specific IgA was present in the unpurified culture supernatants but at concentrations too low to be detected.

4.3 Purification of Monoclonal IgA from Cell Culture Supernatants

The nine monoclonal IgA antibodies were purified from the cell culture supernatants by means of immunoaffinity gravity-flow column chromatography as described in section 3.8.2. All fractions eluted from the column throughout the purification process were collected and screened for IgA by IgA-specific ELISA.

This column chromatography method proved to be an efficient method to recover monoclonal IgA from cell culture supernatant. Prior to purifying the experimental supernatants, we validated the approach by performing a test purification using 15ml of H31/4L sample supernatant (Figure 12). The raw supernatant was determined by ELISA to contain 112.70μg monoclonal IgA. The majority of this antibody was recovered in the desired elution fractions; 88.07μg was recovered in glycine fraction 1, 5.30μg in glycine fraction 2 and 2.98μg in glycine fraction 3, giving a total of 96.35μg and a recovery of 85.5% of the H31/4L IgA input. A further 3.27μg (2.9%) of IgA remained in the

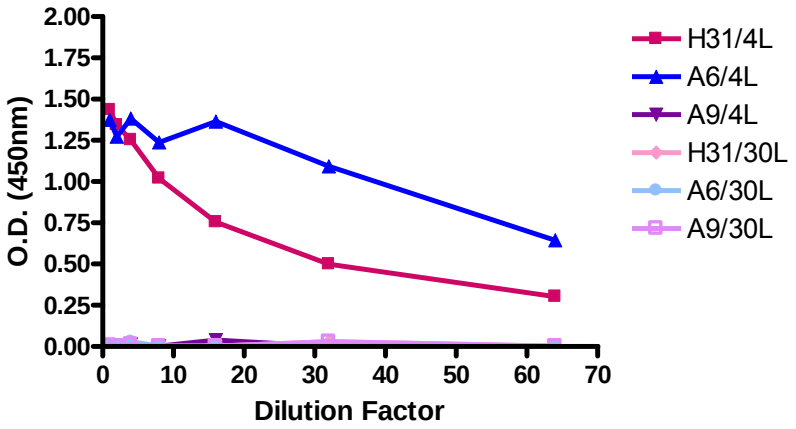

Figure 11. Detection of $gp120_{IIIB}$-specific human IgA2 in transfected CHO-K1 cell line supernatants. The antibodies were detected by means of $gp120_{IIIB}$-specific ELISA. $gp120_{IIIB}$-specific IgA from the H31/30λ, A6/30λ & A9/30λ samples was not detected and is therefore not shown.

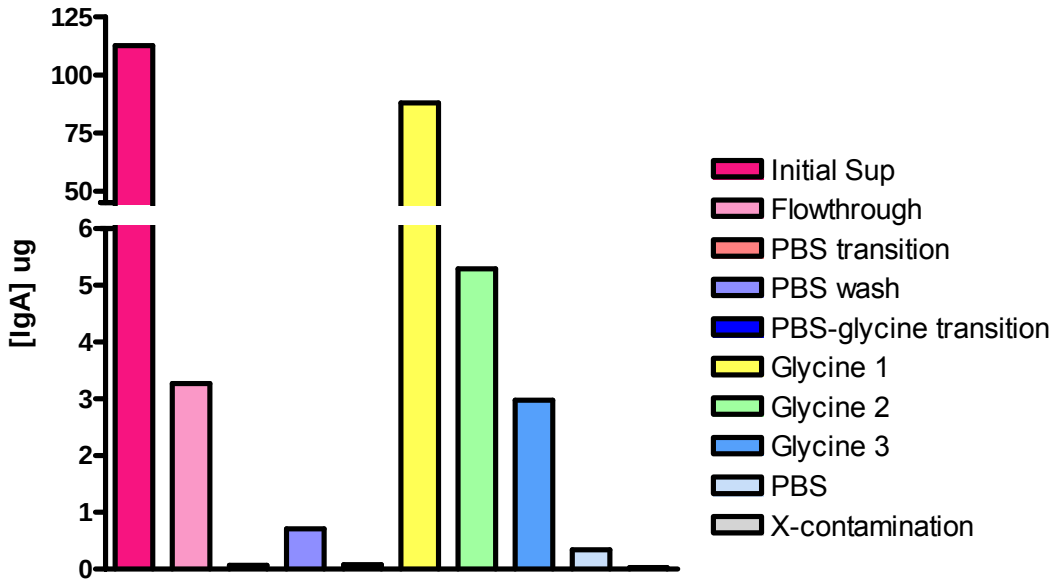

Figure 12. Detection of monoclonal IgA-H31/4L during purification from bulk cell culture supernatant by immunoaffinity gravity-flow column chromatography. Each bar indicates the concentration of IgA in µg recovered at each step of column chromatography. The IgA content of each fraction was quantified by means of IgA-specific ELISA.

supernatant flowthrough after being passed twice through the purification column. Low amounts of antibody were also detected in the intermediate PBS wash (0.71μg or 0.63%) and the post-elution PBS wash (0.34μg or 0.3%). Thus, the total amount of H31/4L IgA measured across all fractions was 100.67μg, or 89.3%.

In order to monitor the potential for IgA cross-contamination between samples undergoing purification, the column was rinsed with 10 volumes of low-pH elution buffer after the H31/4L sample test validation. This eluate was collected and assayed by IgA-specific ELISA. Only a trace amount of IgA was detected in this low-pH eluate (approximately 30ng/50ml), indicating that nearly all of the H31/4L antibody was removed from the chromatography column during the normal purification procedure. To further reduce the potential for IgA cross-contamination, samples were purified in order of ascending supernatant IgA concentration, with large-volume elution buffer rinses between all samples.

Following immunoaffinity column purification, the glycine elution fractions from each IgA sample were combined and the samples were applied to Macrosep 30K Centrifugal Devices for sample desalting and concentration. The Macrosep columns were first validated using a known concentration of 3.5μg/ml IgA Colostrum in neutralized glycine elution buffer. Based on IgA-specific ELISA measurements, of the 9976ng of input IgA Colostrum, 8984ng or 90.1% of the IgA was retained in the sample concentrate after two rounds of desalting. The trace amounts of IgA Colostrum detected in the Macrosep column filtrates were too dilute to be accurately quantified. Given the high recovery rate, the nine purified experimental IgA samples were concentrated and desalted in the same manner. In most cases, only trace IgA was detected in the sample waste

filtrates. However, a small amount of IgA was detected in the waste filtrates of the A6/4L and the A9/4L IgA samples (756.2ng and 705ng, respectively), although it accounted for less than 1% of the total antibody recovered.

The overall protein concentration of the desalted IgA concentrates was determined by means of the BioRad protein assay for the high-concentration IgA-4L samples and by means of IgA-specific ELISA for the low-concentration IgA-30L and IgA-30λ samples. Known concentrations of human IgA kappa antibody were used as a standard for the BioRad assay in order to best approximate the amino acid content of the experimental IgA samples. Using these quantification methods, we determined the IgA sample yields to be elevated for the IgA-4L samples: in total 0.99mg of H31/4L, 2.4mg of A6/4L and 0.724mg of A9/4L was recovered. The IgA-30L samples yielded lower amounts of antibody, yielding 148.8ng for H31/30L, 170.8ng for A6/30L and 591.7ng for A9/30L. In contrast, barely any of the IgA-30λ samples were recovered; yields were merely 11.6ng for H31/30λ, 13.4ng for A6/30λ and 12.5ng for A9/30λ. Overall, this purification approach was effective and provided acceptable antibody recover, and the low recovery rate of the IgA-30L and IgA-30λ samples was primarily due to the low initial concentration of antibody in these sample supernatants.

4.4 Genetic Confirmation of CHO-K1 cell transfection

In order to verify that the transfected IgA genes were properly integrated into the genome of the CHO-K1 monoclonal cell lines, genomic DNA was extracted from each culture and the transfected variable heavy, variable light and IgA2 constant heavy backbone genes were confirmed by PCR and DNA sequencing.

The variable heavy genes were detected as described in methods section 3.7.3. A single PCR product corresponding to 605bp for the H31 chains, 473bp for the A6 chains and 476bp for the A9 chains was detected in each of the transfected CHO-K1 genomic DNA samples, confirming that appropriately-sized variable heavy genes had been integrated into the CHO-K1 cell chromosomal DNA during transfection (Figure 13). The PCR heavy chain bands from the H31/30λ, A6/30λ and A9/30λ products were difficult to detect, suggesting these inserts were dropped from the genomic DNA. However, a second round of PCR was able to confirm the presence of correctly-sized products. No PCR product was detected in the non-transfected CHO-K1 genomic DNA control, indicating that the PCR primers used to detect the variable heavy chains were specific for the transfected human genes. Sequencing the purified PCR products confirmed the identity of each variable heavy gene, and no mutations were identified.

The variable light genes were detected as described in methods section 3.7.4. A single PCR product of 732bp was generated for both the 4L and 30L chains and a 702bp product was observed for the 30λ chain, confirming that these genes had also been integrated appropriately into the CHO-K1 cell genomic DNA during transfection (Figure 14). Similar to the variable heavy genes, the IgA-30λ light chain samples generated faint PCR products but performing a second round of PCR amplification on these products improved their detection. As before, no PCR product was detected in the non-transfected CHO-K1 genomic DNA control. All products were identified by sequencing and no mutations were detected.

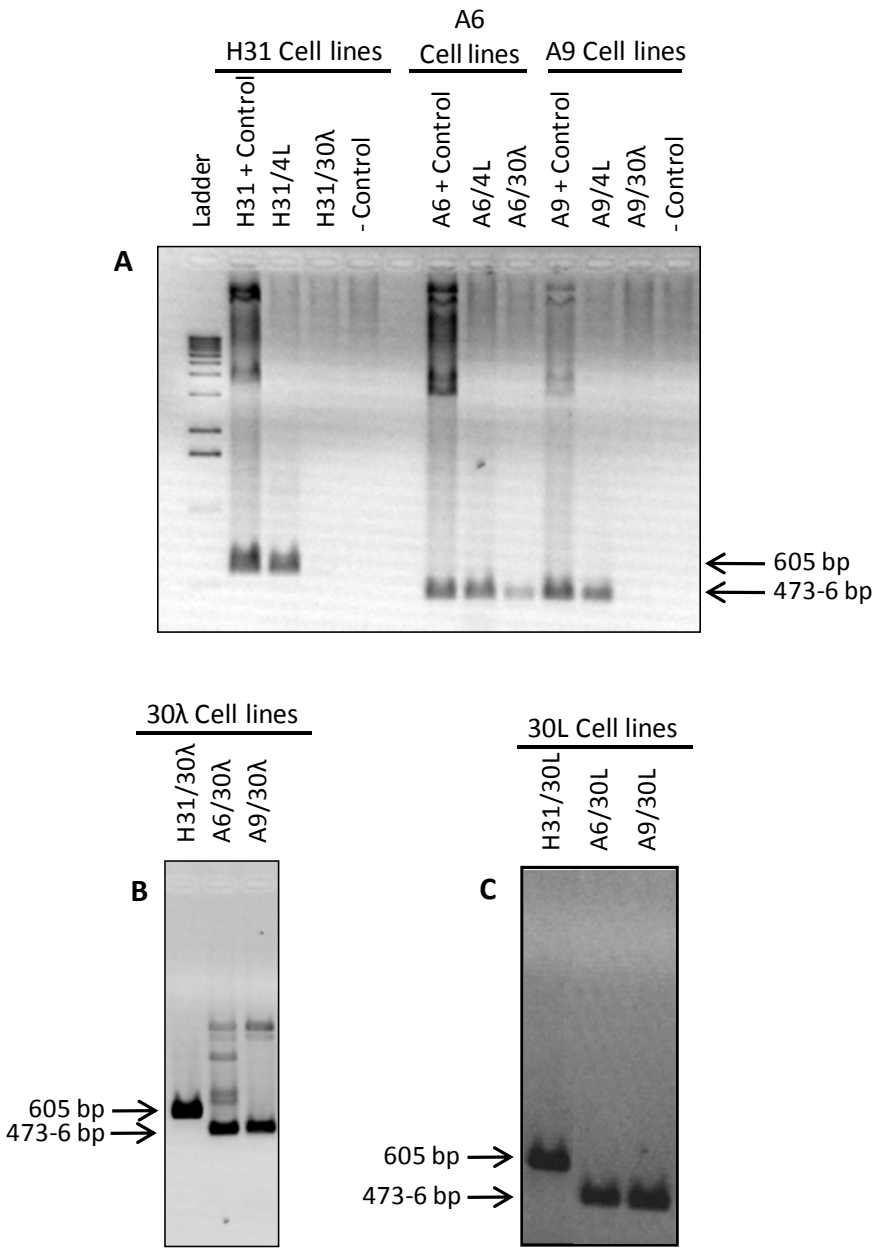

Figure 13. PCR detection of human immunoglobulin variable heavy genes from genomic DNA isolated from transfected monoclonal CHO-K1 cell lines. **A**) First-round PCR amplification of variable heavy gene products. The pSM102 plasmid was used as a positive control for the H31 products (expected size of 605bp), the A6/4L and A9/4L plasmids were used as positive controls for the A6 & A9 products (expected size of 473bp and of 476bp respectively). Genomic DNA from untransfected CHO-K1 cells served as a negative control. **B**) Second-round variable heavy gene products isolated from CHO-K1 cells transfected with $30\lambda V_L C_\lambda$ (30L) plasmids. **C**) Second-round variable heavy gene products isolated from CHO-K1 cells transfected with $30\lambda V_L C_\lambda$ (30λ) plasmids.

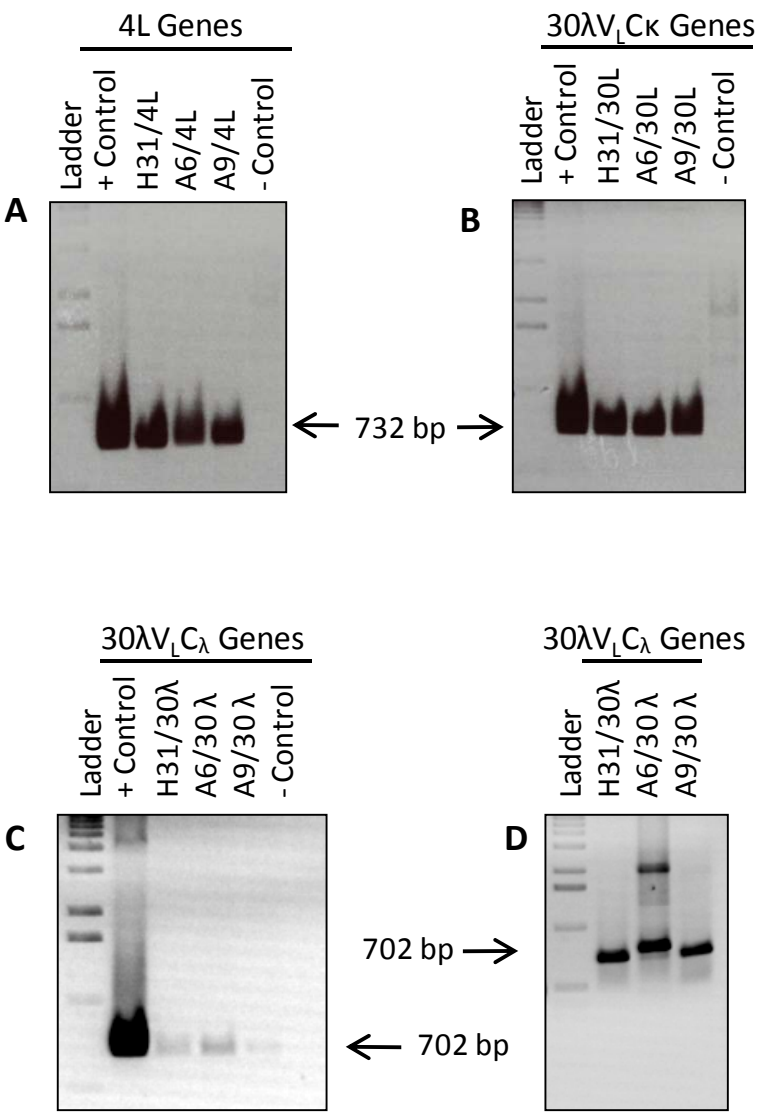

Figure 14. PCR detection of human immunoglobulin variable light genes in genomic DNA isolated from transfected monoclonal CHO-K1 cell lines. **A**) IgA-4L variable light gene PCR products. pSM102 was used as a positive control while genomic DNA from untransfected CHO-K1 cells served as a negative control. The expected product size was 732bp. **B**) IgA-30λV$_L$Cκ variable light gene PCR products. The H31/30λV$_L$Cκ plasmid construct was used as a positive control, untransfected CHO-K1 genomic DNA was used as a negative control, and the expected product size was also 732bp. **C**) First-round IgA-30λV$_L$C$_λ$ variable light gene products. The positive control was the H31/30λV$_L$C$_λ$ plasmid, the negative control was untransfected CHO-K1 genomic DNA, and the expected product size was 702bp. **D**) Second-round 30λV$_L$C$_λ$ variable light gene products. The expected product size was 702bp; although the A6/30λ product appeared larger on the gel, sequencing confirmed the correctly-sized gene was integrated without modification.

When we examined the complete constant chain to determine the proper sequence size, we found that detecting the integration of the IgA2 constant heavy gene into the transfected CHO-K1 genomic DNA proved difficult. Indeed, although it had been possible to detect the full-length IgA2 constant gene within the pSM102 vector, repeated attempts to detect the full-length IgA2 backbone within the genomic DNA of transfected CHO-K1 cultures generated products that could not be distinguished from bands amplified from an untransfected CHO-K1 negative control (data not shown). Unfortunately, since the unique *SalI* site used for vector linearization was located immediately downstream of the IgA2 open reading frame, it was not possible to generate a vector-specific downstream primer for sequencing. By designing several stringent primer sets located at various positions within the IgA2 backbone, we determined that non-specific amplification occurred when using a downstream primer located anywhere within the IgA2 C_H3 domain. As a result, we took the approach of amplifying and sequencing only the C_H1-C_H2 domains of the IgA2 gene from the transfected CHO-K1 genomic DNA samples. These sequences were compared to the full-length IgA2 heavy chain genes sequenced from each of the nine original transfection plasmids (Figure 15).

In this manner, we confirmed that all 9 of the original transfection plasmids used to generate the CHO-K1 cell lines contained full-length IgA2 heavy genes of the expected sizes (2051bp for products containing an H31 variable gene, 2027bp for products containing an A6 variable gene and 2030bp for products containing an A9 variable gene). Sequencing detected no mutations in any of the plasmids. In the case of the IgA2-transfected CHO-K1 genomic DNA samples, the expected 1089bp C_H1-C_H2 PCR product of the correct sequence was detected in the majority of the transfected

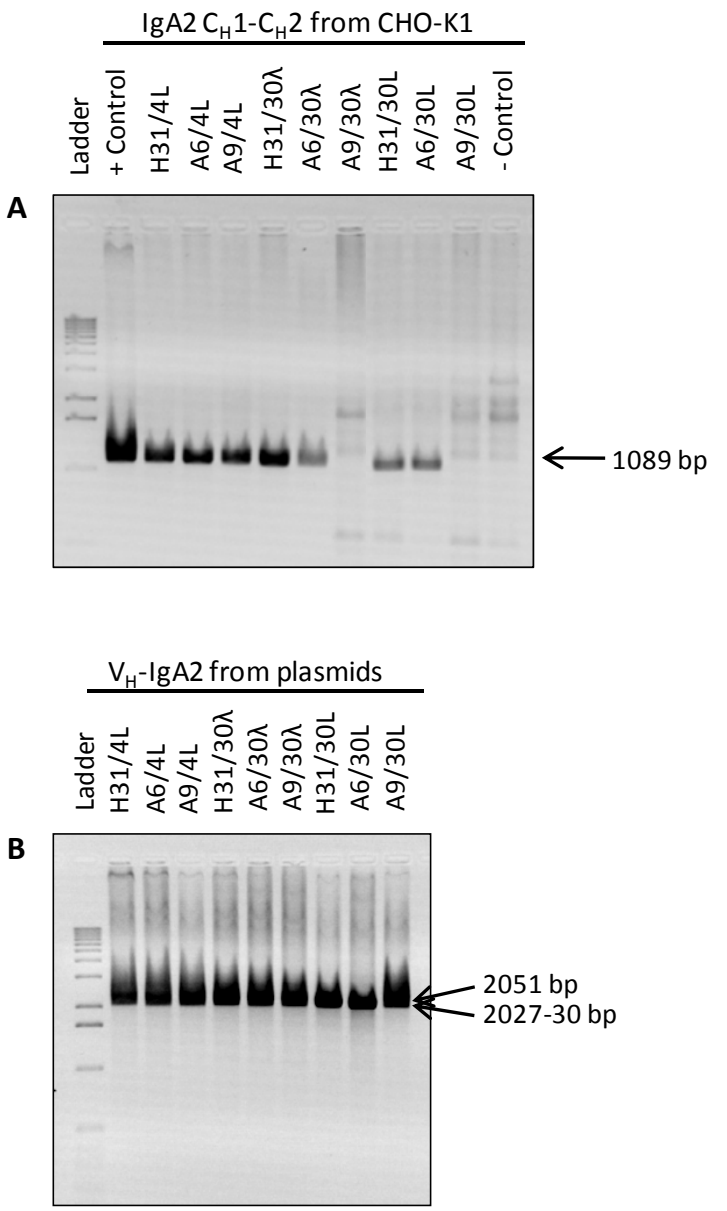

Figure 15. Human IgA2 constant gene PCR products from stable transfected CHO-K1 clones and from control plasmids. **A**) IgA2 C_H1 and C_H2 domains amplified from the genomic DNA of transfected CHO-K1 cell lines. PCR products containing only the IgA2 C_H1 and C_H2 domains were generated because attempts to detect transfected full-length human IgA2 genes generated products that were not distinguishable from untransfected CHO-K1 negative controls (see text for details). pSM102 was used as a positive control and untransfected CHO-K1 genomic DNA was used as a negative control. The expected product size was 1089bp. **B**) Full-length IgA2 constant heavy genes (corresponding to human IgA2 V_H, C_H1, C_H2 & C_H3 domains) amplified from the plasmid samples used to transfect each CHO-K1 cell line. The expected product sizes were 2051 for H31 products, 2027bp for A6 products and 2030bp for A9 products.

CHO-K1 genomic DNA samples. However, both the A9/30L and A9/30λ samples produced a multitude of non-specific PCR bands that were indistinguishable from the untransfected CHO-K1 negative control, indicating that the majority of the IgA2 constant heavy gene had not integrated into the genomic DNA. Notably, since these samples were able to be retained by a purification column that was specific for the α-heavy chain of human IgA, it is likely that these samples do express at least part of the constant heavy chain, but that the IgA2 backbone was interrupted somewhere before the end of the C_H2 domain. Interestingly, the transfected A9/4L IgA2 gene was complete despite evidence suggesting this IgA heavy chain was not being expressed in full (see section 4.5).

4.5 Quality control of purified IgA samples

To confirm that the purified IgA protein was composed of appropriately sized heavy and light chain fragments, we performed SDS-PAGE/Coomassie staining and IgA heavy chain-specific Western blots. Visible Coomassie staining of SDS-PAGE gels required a high quantity of protein, and despite running the maximum volume of the IgA-30L and IgA-30λ samples on the SDS-PAGE gel, only the high concentration IgA-4L samples were visible after Coomassie staining (Figure 16). Of these, the H31/4L & A6/4L samples showed clear bands corresponding to the expected sizes of IgA heavy and light chains (60kDa & 25kDa, respectively). In contrast, the A9/4L sample showed only a 25kDa band, despite sequencing confirmation that the majority of the IgA2 constant heavy gene had successfully integrated into the CHO-K1 genome.

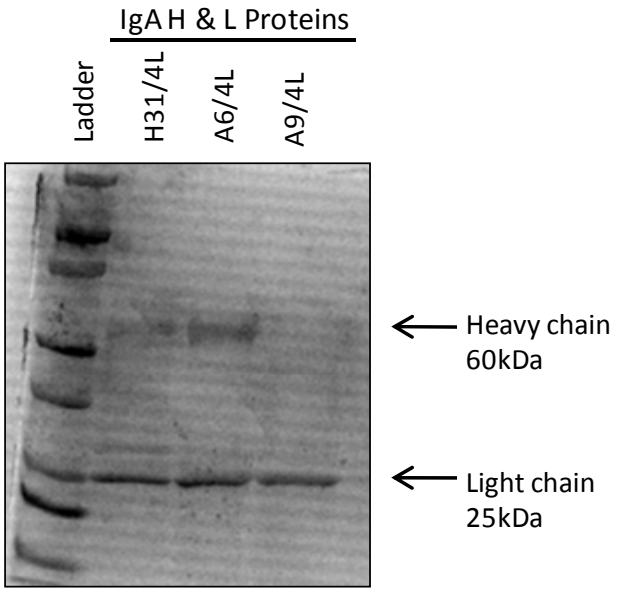

Figure 16. Coomassie stained SDS-PAGE gel of purified human IgA-4L samples secreted by stable transfected CHO-K1 cell lines. Staining was performed to confirm the presence of IgA light chains (which were not detectable by the antibody used for western immunoblotting). The expected size of the heavy chain (H) proteins was 60kDa and the expected size of the light chain (L) proteins was 25kDa.

For Western blot IgA heavy chain-specific analysis, 100ng of the IgA-4L samples and 25µl of the IgA-30L & IgA-30λ samples were separated by SDS-PAGE. The detection antibody utilized was IgA α-chain specific, allowing only IgA heavy chain fragments to be identified. The IgA-4L samples were clearly visible by Western blot (Figure 17a). The H31/4L and A6/4L samples showed a strong, dominant band of the expected 60kDa size. The A9/4L sample, however, exhibited two bands of approximately 45-50kDa in size, suggesting that this sample did not contain full-length IgA heavy chain. Despite the low concentration of the IgA-30L and IgA-30λ samples, a 60kDa band corresponding to the full-length IgA heavy chain was detected in all six of the samples (Figure 17b). However, an additional ~32kDa band was detected in the A6/30L, A9/30L & A9/30λ samples and another ~50kDa band was detected in the A9/30L, and to a lesser extent, H31/30L and A6/30L samples. This indicated that these samples contained both full-length and truncated IgA heavy chain.

4.6 Characterization of Experimental IgA Constructs

Once the nine IgA samples were generated, we were interested in determining the binding affinity and specificity of each by standard ELISA, by flow cytometry, and by competition against IgGb12.

4.6.1 Standardized gp120 ELISA

The specificity of the nine IgA samples for monomeric $gp120_{IIIB}$ was assessed by ELISA. In order to facilitate a direct comparison of the ELISA results, the total IgA concentration of the antibody samples was standardized in two different ways: First,

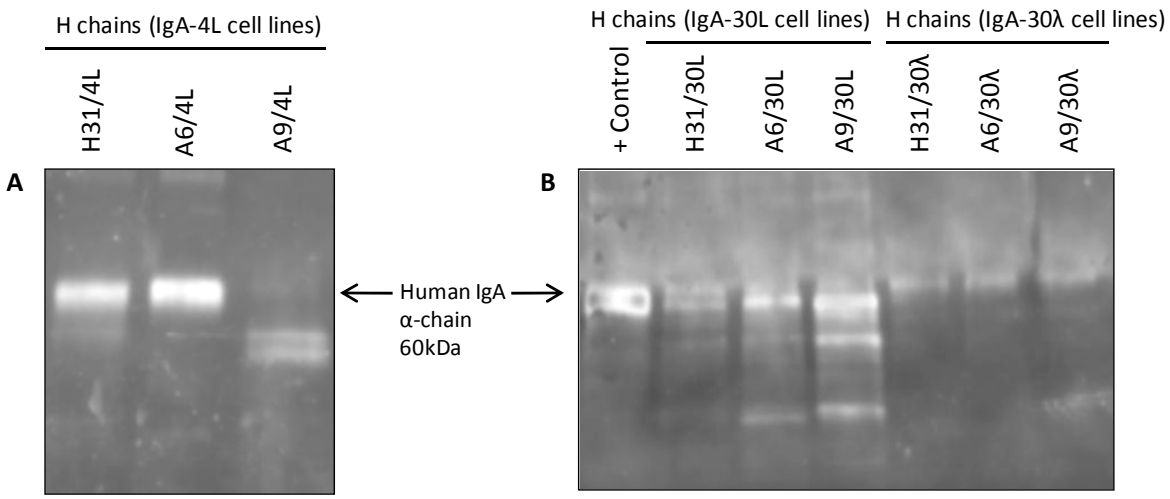

Figure 17. Western immunoblot analysis of purified human IgA2 secreted from stable transfected CHO-K1 cell lines. Immunoblotting was performed to verify the presence and size of the IgA2 heavy chains (H). A polyclonal rabbit HRP-conjugated IgG antibody specific for the 60kDa α-chain of human IgA was used to detect the heave chain proteins. **A**) H-chains from human IgA2 antibodies produced by IgA-4L CHO-K1 cell lines. Proteins were detected after incubation with ECL reagent for 40 seconds. **B**) H-chains from human IgA2 antibodies produced by IgA-30L and IgA-30λ CHO-K1 cell lines. Proteins were detected after incubation with ECL reagent for 90 seconds. Human IgA from Colostrum served as the positive control.

concentrations were standardized within groups of samples carrying the same variable light chain, and gp120 specificity was compared within the group. Second, IgA-4L and IgA-30L samples were diluted to the same total IgA concentration in order to permit a direct comparison of their $gp120_{IIIB}$ specificity.

Since the overall amount of IgA produced for each sample depended upon its variable light gene, the experimental IgA samples were first compared to the other samples sharing the same type of variable light chain. The high-concentration IgA-4L samples were diluted to 20μg/ml and were assayed using the gp120 ELISA protocol described in section (3.10.1) (Figure 18). Results indicated that all three of the IgA-4L samples were highly specific for monomeric recombinant $gp120_{IIIB}$. The H31/4L IgGb12-like positive control showed the greatest binding to $gp120_{IIIB}$. The A6/4L sample binding was intermediate. The A9/4L sample maintained its $gp120_{IIIB}$ specificity despite the truncation detected by the Western blot, although it was the poorest binder of the three IgA-4L samples. The lower-concentration IgA-30L samples were diluted to 150ng/ml prior to being assayed. A gp120 specificity was also detected in all of these samples. Interestingly, the fully Africanized A6/30L & A9/30L antibodies exhibited more specific binding to monomeric $gp120_{IIIB}$ than the H31/30L hybrid. The three IgA-30λ samples were assessed for gp120 specificity without undergoing any dilution due to very low antibody recovery. Despite the low IgA concentration of these samples, gp120 specificity above background was detectable.

The $gp120_{IIIB}$ specificity was also determined for the IgA-4L and IgA-30L samples after standardization of the IgA-4L samples to match the IgA-30L samples at 150ng/ml (Figure 19). Of the six samples, the H31/4L positive control still showed the

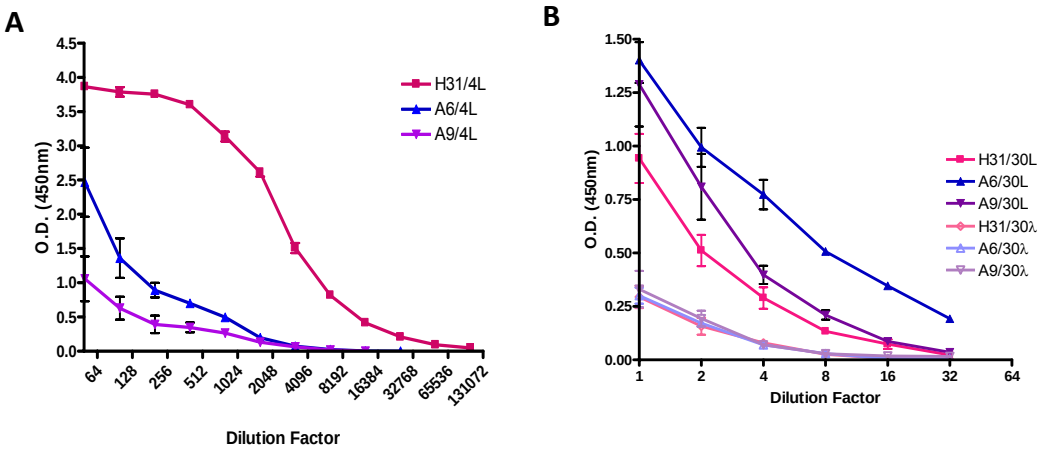

Figure 18. Specificity of the nine purified human recombinant IgA samples for monomeric $gp120_{IIIB}$ as measured by $gp120_{IIIB}$-specific ELISA. **A**) IgA-4L samples standardized to 20µg/ml. **B**) IgA-30L & IgA-30λ samples. IgA-30L samples were standardized to 150ng/ml whereas IgA-30λ samples were not diluted any further for standardization.

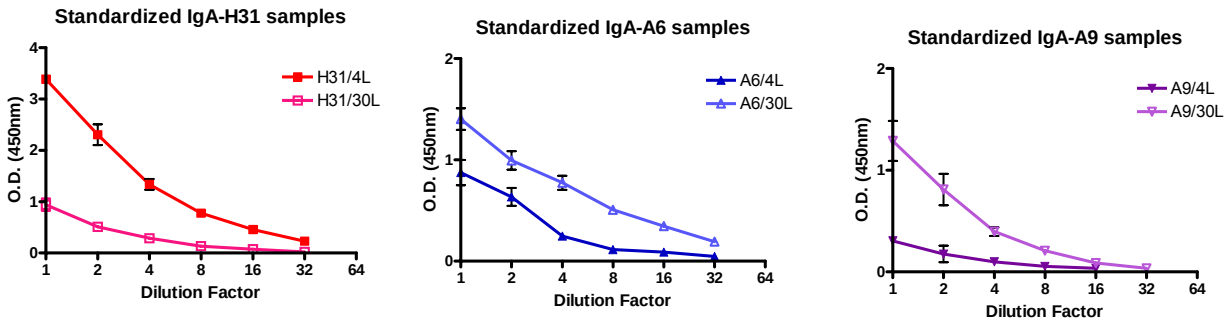

Figure 19. ELISA results showing a comparison of $gp120_{IIIB}$-specificity of the IgA-4L and IgA-30L samples after equalizing the concentration of IgA. IgA-4L samples were diluted to 150ng/ml to match the IgA-30L sample concentrations and were assayed by $gp120_{IIIB}$-specific ELISA.

highest binding of $gp120_{IIIB}$. Interestingly, the Africanized A6/30L & A9/30L antibodies bound more strongly to $gp120_{IIIB}$ than their hybrid A6/4L & A9/4L counterparts. Despite the fact that the IgA-4L light chain was capable of producing overall higher levels of antibody, the IgA-30L light chain antibodies from HIV-resistant women were more avid for $gp120_{IIIB}$.

Because the production and purification did not produce adequate amounts of IgA-30L or IgA-30λ samples, and because the A9/4L sample appeared to produce only truncated IgA heavy chain, the following characterization experiments were confined to the H31/4L and A6/4L antibody samples.

4.6.2 Flow Cytometry

The ability of H31/4L and A6/4L to bind trimeric $gp120_{IIIB}$ expressed on the surface of HIV-infected H9 cells was assessed by flow cytometry as described in methods section 3.10.2. The H31/4L and A6/4L primary antibodies were incubated at test concentrations of 2µg, 1µg, 0.5µg, 0.25µg and 0.1µg. The binding of the IgA antibodies to HIV-infected H9 cells was compared to the level of non-specific binding to uninfected H9 cells. Unfortunately, no $gp120_{IIIB}$-specific IgA positive control was available for inclusion in this experiment.

Under these experimental conditions, neither the H31/4L nor the A6/4L exhibited a high degree of binding to $gp120_{IIIB}$ expressed on infected H9 cells, as most samples had less than 4% of cells analysed in the positive gate. Analysis was confined to the population of H9 cells as determined by forward-scatter and side-scatter population

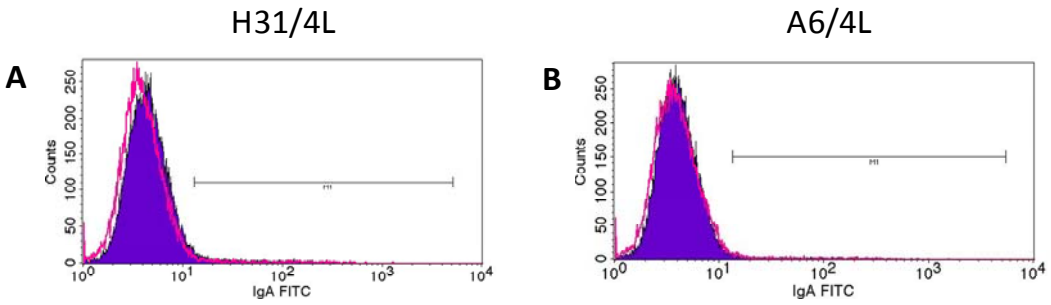

Figure 20. Binding of H31/4L and A6/4L to $gp120_{IIIB}$ trimers expressed on the surface of HIV-infected H9 cells as measured by flow cytometry. **A**) Assessment of H31/4L binding to HIV-infected H9 cells when applied at a concentration of 1µg. **B**) Assessment of H31/4L binding to HIV-infected H9 cells when applied at a concentration of 0.25µg. M1 represents the portion of cells considered to be positive for IgA binding (IgA+).

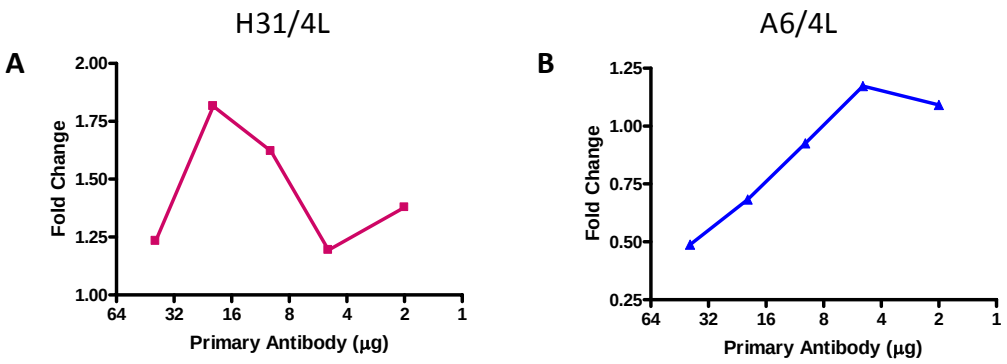

Figure 21. Fold-change analysis of the percent IgA+ population between HIV-infected and HIV-uninfected H9 cells. **A)** Fold-change binding of H31/4L to infected versus uninfected H9 cells. **B)** Fold-change binding of A6/4L to infected versus uninfected H9 cells.

gating. A minor population shift was observed when the H31/4L antibody bound to infected H9 cells, but this was not observed for the A6/4L samples (Figure 20).

Because total levels of binding were low for all samples, we examined the fold-change in the % IgA+ population between the infected and the uninfected H9 cells. For the H31/4L sample, a maximum fold-change of 1.82 was observed at an antibody concentration of 1μg (Figure 21). The A6/4L sample exhibited a much higher degree of non-specific binding as evidenced by the high degree of staining of the uninfected H9 cell line, particularly at elevated concentrations of A6/4L primary antibody. A maximum fold-change of 1.2 was observed at an A6/4L concentration of 0.25μg.

4.6.3 IgA & IgG Competition ELISA Analysis

To investigate whether the experimental antibodies bind to the same epitope as the parental IgGb12 sample, we tested whether IgGb12 could competitively inhibit binding of H31/4L or A6/4L to monomeric $gp120_{IIIB}$. First, we assessed whether varying concentrations of IgGb12 could inhibit H31/4L & A6/4L binding to $gp120_{IIIB}$ as described in methods section 3.10.3. It was observed that IgGb12 had little effect on A6/4L binding to $gp120_{IIIB}$, even at the highest concentrations tested (Figure 22). In contrast, increasing concentrations of IgGb12 steadily inhibited the binding of H31/4L to $gp120_{IIIB}$ as expected, suggesting that the H31/4L antibody shares its epitope with IgGb12.

These observations were confirmed with a reciprocal experiment to investigate the effect of increasing concentrations of H31/4L and A6/4L on IgGb12 binding to

$gp120_{IIIB}$. This assay was performed as described in methods section 3.10.4. The addition of elevated concentrations of H31/4L, but not of A6/4L, competitively inhibited the binding of IgGb12 to $gp120_{IIIB}$ (Figure 23).

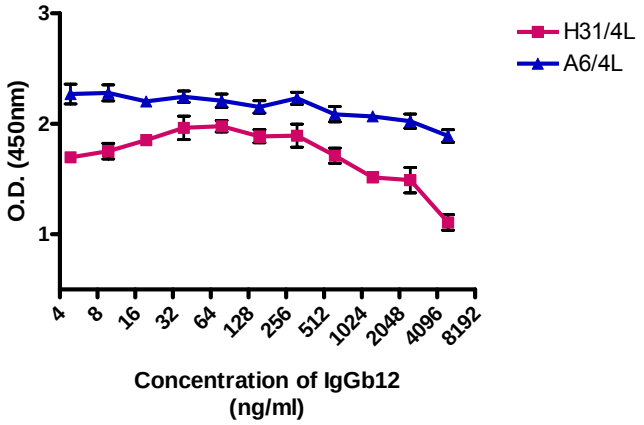

Figure 22. Competition ELISA results showing an effect of IgGb12 on the ability of IgA-H31/4L and IgA-A6/4L to bind recombinant monomeric $gp120_{IIIB}$. Increasing the concentration of IgGb12 partially inhibited H31/4L binding to gp120 but did not significantly affect A6/4L binding.

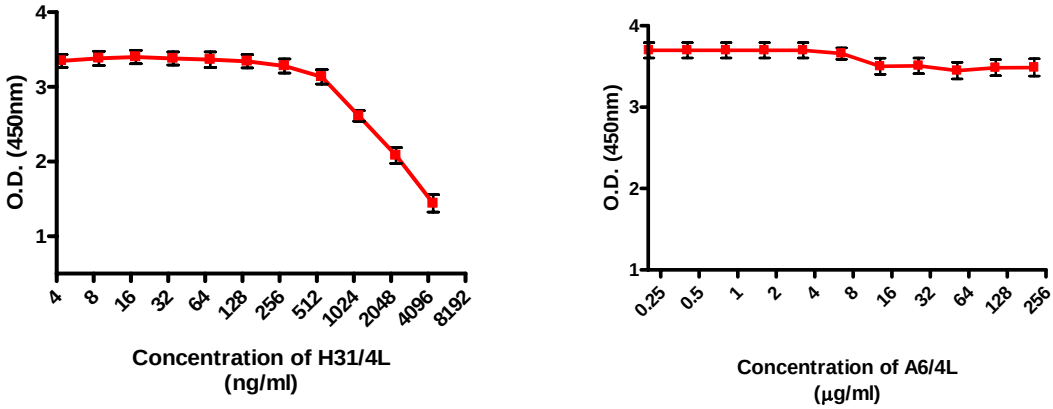

Figure 23. Inhibition ELISA of IgGb12 binding to recombinant monomeric $gp120_{IIIB}$ by increasing concentrations of IgA-H31/4L but not of IgA-A6/4L.

4.6.4 Summary of results

In summary, a total of nine IgA2 expression vectors comprising different combinations of three variable heavy genes (H31, A6 & A9) and three light chain genes (4L, 30LV$_\lambda$Cκ & 30LV$_\lambda$C$_\lambda$) were generated and subsequently transfected into CHO-K1 cells to create cell lines expressing nine different variants of monoclonal IgA2 (Table 9). Antibody production by the transfected CHO-K1 cells was extremely variable and depended on the type of antibody light gene incorporated within the CHO-K1 genomic DNA. All IgA-4L -transfected cell lines produced large quantities of IgA while the IgA-30L and IgA-30λ -transfected cell lines secreted limited quantities of antibody. Most of the transfected IgA genes were confirmed to have integrated into the CHO-K1 genomic DNA, however, the IgA2 constant heavy genes of the A9/30L and A9/30λ cell lines appeared to be incomplete despite the full-length IgA2 gene having been present in the transfection plasmid. Polyacrylamide gel electrophoresis and Western blot suggested that while most of the IgA constructs were being expressed in some form, only H31/4L and A6/4L were of the expected size. Despite this, all nine IgA antibodies were able to bind to recombinant monomeric gp120$_{IIIB}$ to some degree; the Africanized constructs in particular had high avidity despite low IgA concentration. The H31/4L antibody exhibited weak binding to trimeric gp120$_{IIIB}$ expressed on the surface of HIV-1 infected H9 cells. Also, H31/4L but not A6/4L also competed with IgGb12 to bind gp120, suggesting that IgA class-switching retained the parental epitope.

Table 9. Summary of the cloning, purification, protein structure and functional characterization of the nine IgA2 antibody constructs produced by cloning and transfection.

Antibody	Genetic Composition		Recovered Amount	Protein Confirmation		gp120-specificity	Competition
	Gene	PCR Confirmation		Coomassie	Western Blot		
H31/4L	H31	OK	990µg	V_H, V_L	60kDa (OK)	Yes	Competes with IgGb12
	4L	OK					
	IgA2	OK					
A6/4L	A6	OK	2400µg	V_H, V_L	60kDa (OK)	Yes	No competition with IgGb12
	4L	OK					
	IgA2	OK					
A9/4L	A9	OK	724µg	V_L only	~45kDa (Truncation)	Yes	-------
	4L	OK					
	IgA2	OK					
H31/30L	H31	OK	148.8ng	-------	60kDa, ~50kDa	Yes	-------
	30L	OK					
	IgA2	OK					
A6/30L	A6	OK	170.8ng	-------	60kDa, ~50kDa, ~32kDa	Yes	-------
	30L	OK					
	IgA2	OK					
A9/30L	A9	OK	591.7ng	-------	60kDa, ~50kDa, ~32kDa	Yes	-------
	30L	OK					
	IgA2	Incomplete					
H31/30λ	H31	OK (weak)	11.6ng	-------	60kDa (OK)	Yes (low)	-------
	30λ	OK (weak)					
	IgA2	OK					
A6/30λ	A6	OK (weak)	13.4ng	-------	60kDa (OK)	Yes (low)	-------
	30λ	OK (weak)					
	IgA2	OK					
A9/30λ	A9	OK (weak)	12.5ng	-------	60kDa, ~32kDa	Yes (low)	-------
	30λ	OK (weak)					
	IgA2	Incomplete					

5.0 Discussion

The HIV/AIDS pandemic is one of the most serious global health concerns of our time, with an estimated 60 million lives affected and no promising vaccine or microbicide candidates developed to date [2]. Because heterosexual transmission is the driving force of the HIV-1 epidemic in Sub-Saharan Africa [1], and since women in this region are becoming disproportionately affected with HIV-1 in comparison with men, it is crucial to understand the correlates of protection at the female genital mucosa. Exploration of the protective immune mechanisms observed in naturally HIV-1 resistant individuals have suggested that the presence of HIV-1 gp120-specific IgA produced at the female genital tract may provide protection against sexually acquired HIV-1 infection. As such, the goal of this project was to clone HIV-1-specific antibody genes isolated from cervical B-cells of HIV-1 exposed seronegative (HESN) women of the Pumwani sex worker cohort, to develop these genes into full-length monoclonal IgA2 antibodies by *in vitro* cell culturing, and to evaluate the gp120 specificity of these antibodies.

Berry *et. al.* [150] previously selected three novel antibody variable genes by phage display from the cervical B-cell cDNA libraries of Kenyan HEPS sex workers of the Pumwani cohort. When combined with affinity-matured variable genes derived from the HIV-1 neutralizing antibody IgGb12, these variable genes produced single-chain variable fragments (ScFvs) which bound to gp120. Therefore, we hypothesized that HIV-1 specific full-length IgA cloned from the genital tract of HIV-1 resistant women will efficiently recognize gp120. The ultimate goal of this project is to characterize the ability of these IgA constructs to neutralize HIV-1 isolates and to inhibit HIV-1 transcytosis *in*

vitro, in order to demonstrate the role of HIV-specific genital mucosal IgA in protective immunity.

5.1 Cloning B-cell derived variable genes to produce antibody constructs

To purify and characterize the gp120-specific IgA antibodies of interest, we took the approach of a recombinant antibody library cloning technique for several reasons. First, we needed to avoid the low recovery and poor reproducibility issues associated with directly obtaining IgA from cervical lavages to ensure adequate antibody recovery for functional studies [147, 206]. Second, the library method of monoclonal antibody development provides an advantage over the hybridoma fusion technique in that human IgA (rather than humanized IgA) was produced, providing a more accurate reflection of the IgA present in the female genital tract. At the time of cloning, the hybridoma fusion technique was mostly reserved for the production of rodent-derived antibodies since few myeloma fusion partners produced stable clones when paired with non-rodent species [207]. The major disadvantage of the recombinant library cloning technique was the randomization of the variable heavy and variable light genes within the library. Since the cloned IgA genes investigated in this thesis were generated from a pool of Kenyan B-cell genes isolated from 12 HESN women, there is no way to determine whether antibodies bearing the same combinations of V_H and V_L chains are actually produced *in vivo*. As such, the Africanized IgA variants described in this thesis are a theoretical representation of the gp120-specific IgA present at the genital tract of HEPS women.

The ScFvs produced by Berry *et al* [150] were reproduced as full-length human IgA2 by cloning into the pSM102 human IgA2 expression vector. IgA2 was an

appropriate choice for these experiments because IgA2 is the predominant IgA isotype present in the female genital tract [158]. The three novel gp120-specific antibody variable genes A6, A9 and 30L were identified by Berry *et al* from the recombinant antibody libraries described above. Before these variable genes were cloned, they were first extended into full-length IgA heavy and light chain genes by means of overlap PCR. First, both the A6 and A9 variable heavy genes and the 30L variable light gene were fused to an upstream leader DNA fragment to direct the antibody variable heavy chain peptide to the lumen of the endoplasmic reticulum of a transfected animal cell for proper post-translational modification, including glycosylation and appropriate protein folding [217]. The generation of full-length A6 and A9 heavy chains was relatively simple, as the incorporation of a downstream *SstI* restriction site allowed the variable genes to be fused directly to the IgA constant heavy chain domains of the pSM102 human IgA2 expression vector. The production of full-length $30LV_LC\kappa$ and $30LV_LC_\lambda$ light chain genes was more complex, as an additional overlap PCR fusion with an appropriate constant light gene was required prior to cloning into pSM102. Initially, the full-length $30LV_LC\kappa$ gene was constructed using a downstream kappa-type constant light chain gene derived from pSM102. However, all of the resulting IgA-30L vectors generated low IgA-producing cell lines when transfected into CHO-K1 cell cultures, and the IgA-30L antibodies were difficult to detect in the culture supernatants. Since the original 30L gene construct was composed of a lambda-type variable gene fused to a kappa-type constant gene (a combination that does not occur in natural human antibodies), we were concerned that the fusion construct might not produce a functional antibody chain. To address this possibility, a second version of the 30L gene was designed with the 30L variable gene

fused to a lambda 2-type constant light gene. The new version of the Kenyan light chain was termed "30LV_LC_λ" in order to highlight the fact that both the variable and constant light chains of this construct were lambda-type genes.

In addition to the 4 full-length antibody genes generated from Kenyan B-cell cDNA, an additional variable heavy gene, H31, and a kappa-type light chain, 4L, were available from the pSM102 expression vector. Together, the H31 and 4L antibody chains express an affinity-matured IgA version of the IgGb12 antibody. These b12-derived variable genes had previously been expressed as "hybrid" ScFvs with B-cell-derived cervical V_H and V_L libraries developed by Berry *et al* and were used to identify the A6, A9 and 30L genes of interest by means of guided affinity selection against $gp120_{IIIB}$. As such, the H31 and 4L antibody genes were of interest both for the purpose of expressing/characterizing an IgA version of HIV-1 neutralizing antibody IgGb12 and to further develop the promising "hybrid" ScFvs originally described in [150].

By cloning the H31, A6 and A9 variable heavy genes into pSM102 in different combinations with the 4L, 30L and 30λ light genes, a total of 9 distinct IgA expression vectors were produced. The resulting IgA antibody variants were comprised either of antibody genes derived from the IgGb12 antibody (the H31/4L "positive control"), of antibody genes derived from both the B-cell and the IgGb12 sources (the A6/4L, A9/4L, H31/30L and H31/30λ "hybrid" samples) and solely of Kenyan-derived antibody genes (the A6/30L, A6/30λ, A9/30L and A9/30λ "Africanized" samples).

Sequencing was performed on the variable heavy and variable light genes cloned into each plasmid to ensure the antibody gene constructs were amplified correctly without acquiring point mutations. Antibody genes are particularly sensitive to point mutations

because even relatively minor nucleic acid sequence modifications can greatly alter the binding properties of an antibody, especially if they occur in a CDR region. Most of the overlap PCR-generated cloning genes perfectly matched the expected sequences; however, all experimental clones of the A6 variable heavy gene had two base pair discrepancies compared to the published IgA6 sequence. The T to C mutation at position 20 and an A to G mutation at position 26 could have both been problematic since they were non-synonymous and would alter the amino acid codons included in the fragment, changing a phenylalanine (aromatic, TTT) into a serine (polar uncharged, TCT) and a glutamate (negatively charged, GAA) into a glycine (Non-polar aliphatic, GGA), respectively. This could potentially alter the structure of the mature protein and thus modify the binding motif of the expressed monoclonal antibody, changing or even eliminating its ability to bind to gp120.

In order to address this problem, the primers used to generate the original ScFvs (described in [150]) were compared to both the experimental and published A6 sequences. The last 23 bases (italicized) of the heavy chain forward primer "HSCVH1-FL" (5'-GGT GGT TCC TCT AGA TCT TCC TCC TCT GGT GGC GGT GGC TCG GGC GGT GGT GGG *CAG GTG CAG CTG GTG CAG T**C**T* GG-3'), used to generate the original A6 and A9 ScFvs, corresponded with the first 23 base pairs of the A6 sequence. The primer sequence confirmed that a C residue (in bold text) was expected at the position equivalent to base pair 20 of the A6 variable heavy gene, indicating that the mutation should not alter the binding activity of the reconstructed full-length IgA antibody compared to the original ScFv. Unfortunately, the HSCVH1-FL primer did not extend far enough to verify the base at position 26 of the A6 gene; because this base was

not located in a CDR region and should therefore have a minimal impact on antibody binding specificity, we decided to proceed with the cloning of the A6 gene with the G residue at position 26.

The remaining plasmid DNA of each of the nine IgA constructs was stored for use as a positive control to compare to the transfected CHO-K1 cell line genomic DNA.

5.2 Production of human IgA2 antibody by Animal Cell Culture

Following successful generation of the nine IgA constructs, we aimed to stably transfect Chinese Hamster Ovary (CHO-K1) cells to create nine cell lines that secrete large amounts of full-sized, functional human monoclonal IgA antibodies under standard cell culture conditions. The CHO-K1 cell line was selected as the transfection host for the experimental cell lines because it is one of the most widely used cell types for the expression of recombinant glycoproteins; this is largely due to its ability to stably maintain recombinant proteins and its reputation for producing a variety of recombinant proteins at high levels without undergoing toxicity and metabolic burden [218, 219]. CHO have also been confirmed to produce IgA2m(1) (the IgA2 allotype produced for our study) with the same covalent structure as is found naturally in human serum [220]. Furthermore, glycosylation is an important property of antibodies, and CHO cells produce protein glycoforms that are quite similar to human glycoproteins [221]. By means of transfection, the pSM102 plasmid DNA carrying the antibody genes of interest was integrated into the CHO-K1 cell genomic DNA so that the plasmid genes were expressed under the control of the CHO-K1 cells. The CHO-K1 cell line proved to be an effective transfection host for our purposes, as all nine IgA vector constructs were

successfully integrated into the CHO-K1 cells on the first attempt at transfection and all transfected cell lines grew rapidly and robustly. We would then assume that the IgA antibody peptides were assembled, glycosylated and then excreted as IgA2 monomers into the cell culture supernatant, which we collected and purified for further study. While the magnitude of IgA secretion varied between cell lines, all 9 could be scaled up to produce measurable amounts of IgA antibody for further experimental characterization. In this experiment, the CHO-K1 cells were not adapted from adherent to suspension cultures, though in the future, this technique could be a good way to increase culture productivity and yields of the low-producing 30L and 30λ variants (as in [226]).

In order to generate robust cell lines, it was important that stable, and not transient, transfection was achieved in the CHO-K1 cell cultures. This requires the transfected DNA to integrate via non-homologous recombination or "end joining" within random loci of the host chromosomal DNA, ensuring that the IgA antibody genes are not lost over multiple cell culture passages. To promote stable transfection, the plasmid DNA was linearized by means of *SalI* restriction enzyme digestion prior to transfection. Since terminal DNA ends are favoured over internal sites for non-homologous recombination, by introducing the plasmid DNA into the host cell in a linearized conformation, the odds of achieving plasmid DNA integration were improved. Furthermore, by pre-linearizing the vector DNA using a restriction site located within the non-essential plasmid framework region, it was less likely that any of the IgA genes or the glutamine synthetase selectable marker gene would be disrupted by a recombination event. The successful stable transfection of most of these plasmids was later confirmed by PCR and sequence-

based detection of the presence of transfected IgA genes within the CHO-K1 cell genomic DNA.

To further promote stable transfection and optimal gene amplification, the IgA-CHO-K1 cell lines were grown in glutamine-free medium under constant selective pressure from the inhibitory agent L-methionine sulfoximine (MSX). The pSM102 IgA expression vector contains a glutamine synthetase (GS) gene as an amplifiable selectable marker that converts glutamate and ammonium to glutamine, which is a necessary nutrient for CHO-K1 cell growth [222]. Selection is based on the principle that MSX can block the activity of GS by binding irreversibly to the enzyme active site [222]. In this manner, the endogenous GS activity of untransfected CHO-K1 cells is suppressed, but transfected cells expressing an excess of the GS enzyme are able to overcome an otherwise toxic level of MSX inhibition and produce the glutamine required for CHO-K1 cell survival. All CHO-K1 cell lines were cultured in glutamine-free GMEM media beginning 48 hours post-transfection. The GMEM medium was supplemented with glutamate to ensure that the GS enzyme substrate would not be limiting and with pre-dialyzed FCS to avoid contamination of the culture media with endogenous glutamine or with bovine IgA. Under these conditions, the pSM102 GS gene was an essential enzyme for CHO-K1 cell growth and could be used as a selectable marker to ensure the continued expression of the transfected antibody genes.

Based on published reports of MSX-based GS selection [223], each of the nine transfected CHO-K1 cell lines was cultured in 20μM 40μM, 60μM, 80μM, 100μM and 120μM MSX inhibitor, with the goal of identifying the optimal concentration of MSX inhibitor to use for subsequent scale-up, limiting dilution, and monoclonal antibody

generation. For each cell line, we chose the clone producing the most IgA at the highest possible concentration of MSX inhibition. Since a concentration of 20μM MSX has been shown suppress the wild-type endogenous activity of glutamine synthetase in nontransfected CHO-K1 cells [219], we chose this concentration as our lower limit of inhibitor. A non-transfected CHO-K1 control culture was unable to withstand a concentration of 20μM MSX, which confirmed that this lower selection limit was appropriate.

We observed experimentally that the three IgA-4L CHO-K1 cell lines were able to grow at the highest concentrations of MSX (60μM & 80μM) and produced high yields of IgA. Both the IgA-30L and IgA-30λ cell lines were only able to withstand moderate to low concentrations of MSX; IgA production was very low for the IgA-30L cell lines and barely detectable in the IgA-30λ cell lines. This correlation between IgA yield and MSX concentration was consistent with the principle of selective gene amplification. Transfected cell cultures that are able to withstand the highest concentrations of MSX inhibitor are able to do so by expressing the highest levels of GS. Furthermore, the genes on the transfected plasmid behave as an amplification unit. When the GS gene expression is amplified to withstand the toxicity of a high concentration of MSX, the IgA antibody gene expression is similarly co-amplified [223]. Conversely, low amplification of the GS gene results in low tolerance to MSX inhibition and low antibody yield.

In our experimental CHO-K1 cell lines, IgA production was high in all of the IgA-4L cultures and very low in all of the IgA-30L and IgA-30λ cultures. We were initially concerned that the low productivity of the IgA-30L clones was caused by a defect in the 30L light chain. We suspected the unusual combination of a lambda-type

variable gene and a kappa-type constant gene in this construct might not produce a functional antibody chain. To address this problem we developed the 30λ gene, composed of lambda-type variable and constant light chains. Unfortunately, re-cloning the 30L gene did not result in better IgA productivity by the CHO-K1 cell lines; in fact, the antibody productivity of the IgA-30λ cell lines was even lower than the productivity of the IgA-30Ls. This discrepancy may be partially explained by the fact that the IgA-30λ samples underwent less limiting dilution and therefore had less opportunity for selectable gene amplification and affinity maturation than the IgA-30L samples. In any case, these results show that factors other than the 30L light chain variable lambda/constant kappa gene fusion were responsible for the low IgA productivity of the IgA-30L CHO-K1 cell lines.

A number of additional factors can affect the productivity of transfected cell lines. Recombinant protein expression is mainly affected by transcriptional regulation [218], however transgene copy number, mRNA stability, post-translational processing, protein folding and protein secretion can all profoundly affect the expression levels of transfected genes [218, 224]. High-producing clones often contain high transgene DNA copy numbers as a result of numerous plasmid integration events and of gene translocation and duplication events during DNA replication [218, 223]. While an elevated DNA copy number often corresponds to a high level of protein production, too many insertions carry the risk of generating karyotypic instabilities and reducing the growth rate of the transfected cells [225]. Transcriptional efficiency is also an important determinant of recombinant protein production. Jiang *et al* [224] found that in addition to a 2-3 fold increase in transgene copy number; high-producing monoclonal antibody CHO cell

cultures also had a 5-7 fold increase of monoclonal antibody mRNA levels over low-producing cultures. The transcription rate of the integrated DNA is also profoundly affected by its position within the host chromosome, a phenomenon known as the "Position Effect" [218]. Transgene transcription can be enhanced if the DNA is integrated downstream of an active host cell promoter and can be limited if it is integrated into the tightly packed heterochromatin of the host cell. Gene expression is further regulated at the mRNA level both by the regulation of mRNA passage to the cell cytoplasm and by silencing the mRNA through degradation or RNA interference.

At the level of protein regulation, recombinant antibody chain peptides are post-translationally modified in the lumen of the endoplasmic reticulum (ER) and only correctly folded proteins are released [217]. Peptide misfolding can occur in high-producing cell lines by overloading ER chaperone proteins with recombinant peptides, or by the formation of inappropriate disulfide bonds between recombinant peptide cysteine residues. The misfolded peptides are either delivered to the endosomes for degradation or can accumulate in the ER as intracellular aggregates, causing a bottleneck in peptide secretion [217]. Furthermore, high levels of recombinant protein secretion can exert too much metabolic stress on the host cell line, resulting in transgene silencing by histone deacetylation or DNA methylation [218, 225]. To further complicate matters, monoclonal antibody expression levels are often affected by DNA characteristics of the antibody gene. It has been shown that single amino acid changes in the signal sequence, amino acid substitutions in the variable domains of λ1 and κ light chains, and a point mutation in the CDR2 region of the heavy chain can reduce or even block antibody secretion from transfected CHO cells [226].

The low IgA productivity of our experimental IgA-30L and IgA-30λ cell lines may have been influenced by a multitude of the aforementioned factors. Since these cell lines could only withstand low concentrations of MSX, it is clear that vector gene expression was lower in these samples than in the IgA-4L cell lines. However, since all CHO-K1 cell lines were transfected with identical amounts of vector DNA and were linearized using the same *Sal I* restriction site (thus generating the same linear ends for chromosomal integration), it is unlikely that the low IgA productivity of the IgA-30L and IgA-30λ clones was caused by the transgene copy number or the position effect. Instead, since both the IgA-30L and IgA-30λ cell lines were affected, low IgA productivity was most likely caused by an innate characteristic of the 30L variable light gene present in both of these samples.

Several approaches could be taken to improve the IgA-30L and IgA-30λ sample yields for future experiments. The specific causes of low productivity in the IgA-30L and IgA-30λ CHO-K1 cell lines could be identified and addressed. The relative copy number and position of the IgA transgenes could be detected using florescent *in situ* hybridization (FISH), transcription could be investigated using qRT-PCR to detect antibody mRNA levels, and intracellular protein aggregates could be visualized using electron microscopy. It is also possible that inserting these genes into a different vector and culturing system could improve the IgA yield of these samples.

In order to accurately determine the optimal culture conditions and MSX concentrations to use for monoclonal cell culture generation, IgA yield had to be measured as accurately as possible. We tested the cell culture supernatants by means of an IgA-specific ELISA rather than a bulk protein assay due to the presence of large

amounts of interfering protein from the FCS in the culture supernatant. Prior to quantifying the IgA in the culture supernatants, we confirmed that the only detectible IgA in the cell culture was the experimental IgA produced by the transfected CHO-K1 cells. To prevent the interference of bovine immunoglobulin in the culture media, the FCS supplement was pre-dialysed. Furthermore, IgA was neither detected in aliquots of the complete GMEM culture medium nor in spent culture supernatant collected from untransfected CHO-K1 cells. To compare the IgA production of different cell cultures as accurately as possible, the supernatant samples for ELISA were all taken immediately prior to replacement of the culture medium at confluence. Supernatant samples were stored at -20°C until assayed so that the IgA production of cell cultures growing at different rates could be directly compared at the same cell culture confluence levels. The IgA ELISA results were used to select cultures and MSX for limiting dilution and the resulting IgA production of the limiting dilution cultures was quantified in the same manner for scale up.

After limiting dilution, the nine resulting monoclonal IgA cell cultures were scaled up with the goal of producing large volumes of IgA-containing culture supernatant. The cell cultures were originally scaled up using the method outlined in Current Protocols in Immunology [227], described in section 4.2. This supernatant harvesting method was likely based on the presumption that the CHO-K1 cells would secrete the most IgA after the metabolic costs of log-phase growth abated. While this method was effective, it was less than ideal. In this scenario, each 75cm^2 T-flask would yield only 12-15 ml of spent culture supernatant, which we found to be prohibitively inefficient. Furthermore, we were concerned that leaving the cultures to incubate at

confluence until the medium was acidic would result in cell death and antibody-degrading protease release, a concern that was supported by visual evidence of cell death.

In light of these concerns, we investigated methods to improve both the efficiency and yield of the supernatant harvesting process. First, we investigated whether CHO-K1 cell cultures could produce high levels of IgA antibody before reaching confluence. Because all genes encoded by a transfected IgA vector are expressed as a single amplification unit [223], the low-confluence transfected CHO-K1 cell cultures that are able to withstand high concentrations of MSX inhibition by producing elevated levels of GS should also produce high levels of IgA. Based on this logic, we reasoned that large quantities of monoclonal IgA might be secreted into the culture supernatant earlier than described, and that because our initial scale-up method discarded all culture supernatants harvested before confluence, a significant portion of IgA was being discarded. To address this possibility, we conducted a pilot experiment to correlate CHO-K1 cell culture confluence with the concentration of monoclonal IgA secreted into the supernatant. Only the H31/4L, A6/4L and A9/4L CHO-K1 cell cultures were investigated as the IgA-30L and IgA-30λ clones produced too little IgA to be accurately quantified before confluence. We observed that a significant amount of IgA was being produced by the time each cell line reached 25% confluence, and that retaining cell culture supernatants harvested between 25-90% confluence nearly doubled the overall yield of IgA.

Next, we investigated whether changing the culture medium of the scaled-up confluent CHO-K1 cultures regularly would improve antibody yield using the H31/4L CHO-K1 cell line. Our rationale was that changing the culture medium would prevent nutrients in the media from becoming a limiting factor of recombinant antibody protein

synthesis and would also prevent the accumulation of toxic metabolic by-products and proteases in the culture flasks. Although we observed that replenishing the culture medium throughout the scale-up process reduced the titre of IgA in the supernatant, the overall IgA yield was more than doubled compared to the Current Protocols in Immunology method. If the pre-confluent supernatants were also retained, more than triple the amount of total IgA was recovered.

Taken together, these two experiments suggested a more efficient method for scaling up and harvesting IgA in the spent cell culture supernatants. We modified our scale-up protocol by changing the medium every 48 hours regardless of cell confluence, and by pooling all spent supernatants collected after approximately 25% confluence. This new method was more work intensive but allowed antibody to be produced more quickly and with less material waste. Further improvement of antibody yield might also be achieved by adapting the cells to suspension cultures in roller bottles.

In total, ~500ml of each IgA-4L supernatant, ~250ml of each IgA-30L supernatant and ~300ml of each IgA-30λ supernatant was collected, clarified and stored for IgA antibody purification. These supernatants were screened for total human IgA and gp120-specific IgA content by means of ELISA. These preliminary results indicated that the IgA-4L cell lines produced relatively high amounts of IgA antibody, whereas the IgA-30L antibody samples were produced at much lower titres and the IgA-30λ samples were virtually undetectable in the non-concentrated cell culture supernatant. Although it had previously been established that all IgA detected in the culture supernatant was produced by the CHO-K1 cell transfections, gp120-specificity was more difficult to detect. The discrepancies between the IgA-specific and the gp120-specific ELISA results may be due

in part to the different purpose of each assay. The IgA-specific ELISA targets the α-chain of human IgA; as such it is not a functional assay and its purpose is simply to detect any and all fragments of human heavy chain IgA peptide and/or full-length IgA. In contrast, the gp120-specific ELISA is a functional assay which assesses the capacity of full-length IgA to bind to monomers of recombinant $gp120_{IIIB}$ ligand. Experimentally, only the H31/4L and the A6/4L samples had any detectable gp120 specificity in the unpurified cell culture supernatants. gp120 specificity was not detected for the A9/4L supernatant sample despite a relatively high concentration of IgA detected in the unpurified supernatant. These results offered the first suggestion that the A9/4L CHO-K1 cell line was producing IgA that was attenuated in some manner. Similarly, gp120 binding was not observed in any of the IgA-30L or IgA-30λ supernatant samples, although this was reasonable given the nearly undetectable concentrations of IgA in these samples. Since both the A9 and the 30L genes were initially identified by Berry *et al* [150] based on their ability to bind gp120, we expected that gp120 specificity would become quantifiable at higher concentrations of IgA.

5.3 Genetic Confirmation of CHO-K1 cell transfection

To confirm that the transfected IgA genes had been correctly incorporated into each monoclonal cell line, we isolated genomic DNA from the nine CHO-K1 cell cultures to detect the variable heavy, variable light and IgA2 constant heavy chain "backbone" genes by PCR. In all nine genomic DNA samples, PCR products corresponding to the expected sizes of the designated variable heavy and light chain genes were detected. Interestingly, the PCR products of both the variable heavy and light

genes of all three IgA-30λ samples were difficult to detect, requiring a second round of PCR amplification for visualization. This might suggest the possibility that a comparatively low copy number of IgA-30λ plasmid DNA contributed to the low IgA productivity of these cell lines; however, the low amplification was not observed for any of the IgA-30λ backbone PCR products. Sequencing the variable heavy and light gene PCR products confirmed that all matched the gene sequences of the original transfected plasmids described in section 4.1.

Specific PCR amplification of the IgA2 constant heavy genes from CHO-K1 genomic DNA was much more difficult. We initially attempted to detect the entire length of the transfected backbone gene (corresponding to the C_H1, C_H2 and C_H3 domains of IgA2); however, PCR products generated from the transfected CHO-K1 cell lines were identical to a non-transfected CHO-K1 genomic control. Optimization of the PCR amplification using different combinations of IgA2-specific primers showed that the problem was due to non-specific primer binding within the C_H3 gene domain. Unfortunately, the genomic sequence information for the CHO cell parental species, *Cricetulus griseus*, was not available for comparison to our human IgA2 gene. However, comparative sequence analyses between species for which full genome data is available have shown that Chinese Hamster gene sequences are most similar to the mouse genome [219], allowing the *Mus musculus* genome to be frequently used as a model to predict CHO cell sequences [219-230]. Using this rationale, we performed a BLAST search to compare DNA sequence homology of the transfected human IgA2 constant heavy gene to the *Mus musculus* genome. We found that the IgA2 C_H3 domain had 78% identity to a region of the *Mus musculus* chromosome 12 which was predicted to code for an

immunoglobulin heavy chain. Therefore, given the similarity between CHO and *M. musculus* genomes, it is likely that the human IgA2-specific downstream primer also detects immunoglobulin-like genes in the CHO-K1 genome. Furthermore, it was not possible to alleviate this problem by designing a pSM102 vector-specific primer site downstream of the C_H3 domain due to the fact that the *Sal I* restriction site used for vector linearization was located immediately downstream of the IgA2 backbone gene.

Given this information, we decided to detect only the IgA2 C_H1-C_H2 domains from each CHO-K1 cell line and compare their sequences to the full-length IgA2 backbone genes from the original transfected plasmids. Each of the nine original plasmid samples contained a correctly-sized full-length IgA2 backbone product which perfectly matched the expected gene sequence. In contrast to the plasmid sequences, not all of the IgA2 backbone genes transfected into the CHO-K1 genomic DNA samples could be detected. While the majority of the transfected CHO-K1 cell lines contained the expected C_H1-C_H2 IgA2 backbone gene fragment, the A9/30L and A9/30λ products were indistinguishable from the non-transfected CHO-K1 negative control. This showed that despite being properly introduced into the CHO-K1 host cells, the IgA2 backbone genes in these cell lines were being disrupted prior to integrating into the CHO-K1 genome, or else dropped from the genome after integration. After transfection, the exposed ends of a molecule of linearized DNA vector are vulnerable to the effects of endo- and exonucleases [218]. Since the IgA2 backbone gene is located immediately upstream of the pSM102 vector *Sal I* linearization site, this gene may have been particularly susceptible to enzymatic degradation.

Because the Western blot results discussed in section 4.5 indicated that the A9/4L antibody sample did not contain any full-length 60kDa heavy chain, we were particularly interested in the size of the IgA2 bands amplified from the A9/4L genomic DNA. The Western blot estimated the length of the A9/4L heavy chain at ~45-50kDa, which suggested that the majority of the C_H3 domain was missing from this antibody. PCR confirmed that the C_H1-C_H2 IgA2 gene fragment of the A9/4L genomic DNA sample was intact; however, since we were unable to generate any C_H3 PCR product, the cause of the A9/4L antibody truncation could not be determined. It is possible that the IgA2 gene was degraded by nucleases prior to vector integration, or that a point mutation generated a premature stop codon in the C_H3 domain of the antibody chain. When stable clones are selected for antibody production at the industrial scale, several hundred are chosen to avoid selecting clones with deleterious mutations. Due to time and material restraints, it was not possible to scale up multiple clones of each of the nine IgA variants for this project. However, examining the other monoclonal A9/4L CHO-K1 cell lines generated during the limiting dilution steps may identify alternate, un-truncated A9/4L variants for future study.

5.4 Purification of Monoclonal IgA from Cell Culture Supernatants

Each of the nine experimental antibody samples were purified from cell culture supernatants using the same IgA-specific immunoaffinity column and then desalted and concentrated with single-use Macrosep 30K centrifugal devices. Both of these protocols were validated using control IgA from colostrum suspended in GMEM medium. Validation confirmed that IgA recovery was acceptable from both the immunoaffinity

column and the Macrosep centrifugal devices (>85% and 90% respectively), indicating that little antibody was being lost to the waste filtrates or adsorbed to the purification devices. After final desalting and concentration, a total of 0.99mg of H31/4L, 2.4mg of A6/4L, 0.724mg of A9/4L, 148.8ng of H31/30L, 170.8ng of A6/30L, 591.7ng of A9/30L, 11.6ng of H31/30λ, 13.4ng of A6/30λ and 12.5ng of A9/30λ were ultimately recovered. To reduce the possibility of antibody degradation prior to sample purification, the supernatants were stored at -20°C until use; where possible, supernatants were applied to the purification column directly from culture. To prevent the growth of protease-producing organisms, both the supernatants and the purification columns were stored aseptically until use.

One of the major considerations of the immunoaffinity purification column was that the agarose resin was coated with polyclonal antibody specific for the α-chain of human IgA, rather than for the $gp120_{IIIB}$ ligand. Since we had previously verified that all IgA in the culture supernatants was produced by the transfected CHO-K1 cultures, we expected that all IgA recovered by the immunoaffinity column would be gp120-specific, allowing us to use the less expensive and readily available IgA-specific agarose. However, a disadvantage of using an IgA-specific rather than a gp120-specific resin was that the immunoaffinity column was capable of retaining α-chain backbone fragments and non-biologically active IgA from the supernatant along with the desired gp120-specific functional IgA. This may have contributed to the recovery of the extra fragments of IgA heavy chain detected by Western blot in the A6/30L, A9/30L & A9/30λ samples. The 30kDa size exclusion membrane of the Macrosep 30K columns used to desalt and concentrate the IgA samples helped to eliminate some of the degraded IgA fragments

from the purified sample concentrates. However, this still allowed IgA antibody fragments of 30kDa or larger to be retained in the purified antibody samples. Therefore, while using a purification column based on antibody affinity for the gp120 ligand would have been more costly, it is likely that this method would have simplified the purification procedure and led to better quality antibody concentrates and should be considered for use in future experiments.

Another challenge involving the immunoaffinity column was the issue of sample cross-contamination, since using a new column for each samples was cost-prohibitive. Since all IgA samples were purified using the same immunoaffinity column, there was a small possibility of cross-contamination of each IgA sample with trace amounts of antibody that had previously been purified on the column. While trace antibody contamination would have little effect on the high-concentration IgA-4L samples, it could greatly influence the properties of the low-concentration samples such as the IgA-30Ls or especially the IgA-30λs. Traditional methods of column sterilization, such as the use of NaOH to sterilize protein A resin, could not be used in this case; any treatment capable of denaturing the contaminating IgA would also destroy the agarose-bound IgA-specific IgG column resin. To reduce the occurrence of IgA cross-contamination, the purification column was rinsed between sample runs with a large volume of glycine elution buffer at a lowered pH of 2.0 in order to promote the elution of residual IgA. As a further precaution, supernatant samples were purified in ascending order of supernatant IgA concentration to minimize the effect of any contaminating IgA on subsequent samples. Finally, sample cross-contamination was monitored before the application of each new supernatant sample by testing the pre-run glycine blank for the presence of eluted IgA.

By following these precautions, IgA cross-contamination between samples was virtually eliminated.

All fractions generated from the column purification procedure were tested for IgA content by means of IgA-specific ELISA, as opposed to more common screening methods such as A_{280} absorbance or the BioRad protein assay. The ELISA procedure is better suited to the detection of low concentrations of IgA, while the A_{280} absorbance and the BioRad protein assay are best suited to the detection of protein in the mg/ml and μg/ml range. Since six of the nine IgA samples that underwent column purification were produced in low amounts, it was essential to detect recoverable IgA as accurately as possible to reduce sample loss. The IgA-specific ELISA method was also able to estimate the relative amount of IgA present in the culture supernatants before sample purification, whereas A_{280} absorbance and the BioRad protein assay measurements would have detected FCS present in the supernatant. The downside to using IgA-specific ELISA to screen high-concentration IgA fractions was that these samples had to be highly diluted in order to be quantified, which introduced additional quantification error. Once the fractions of each sample were combined, desalted and concentrated, a final IgA concentration measurement was performed more accurately using the BioRad protein assay for the high-concentration IgA-4L samples and the IgA-specific ELISA for the low-concentration IgA-30L and IgA-30λ samples.

5.5 Quality control of the purified IgA samples

We assessed the heavy and light chain size and the purity of the nine IgA samples by performing SDS-PAGE with Coomassie staining as well as IgA α-chain specific

Western blots. A high quantity of protein was required for Coomassie staining to visualize the presence and the size of the IgA heavy and light chains; as such the IgA-30L and IgA-30λ samples were not detected due to the low protein yields of these samples. The H31/4L & A6/4L samples had clear protein bands at 60kDa and 25kDa, corresponding to the expected sizes of IgA heavy and light chains. However, only the 25kDa light chain was detected in the A9/4L sample. The absence of any visible heavy chain protein (or any fragments larger than 25kDa) was unexpected in light of the fact that the A9/4L CHO-K1 cell genome was confirmed to have integrated an intact IgA2 C_H1-C_H2 segment. Since Western blotting revealed a weak double band of heavy chain proteins in the A9/4L sample (as discussed below), it is likely that the fragmented A9/4L heavy chain was too faint to be visualized by the Coomassie stain.

In addition to the non-specific Coomassie staining, we performed Western blots to confirm the identity of the 60kDa protein bands as the heavy chains of human IgA. Due to the α-chain specificity of the detection antibody, the light chains of these samples were not able to be detected. Because Western blots are much more sensitive than Coomassie staining, IgA heavy chain protein was detected in all six of the low-concentration IgA-30L and IgA-30λ samples. We detected the presence of a 60kDa band in 8 of the 9 antibody samples which confirmed that these samples contained at least some full-length, correctly-sized IgA heavy chains. No 60kDa product was detected in the A9/4L sample; instead a weak 45kDa and 50kDa double band was observed, which indicated that the A9/4L sample was being truncated or perhaps digested in some manner. This result is consistent with the lack of gp120 binding of the raw culture supernatant as observed by ELISA (described in section 4.2). We also detected additional ~30kDa IgA heavy chain

bands in the A6/30L, A9/30L & A9/30λ samples and another additional ~45kDa IgA band in the A9/30L sample, indicating that a portion of the IgA heavy chain of these samples had been attenuated.

It is interesting that despite the fact that a number of the experimental antibody samples contained truncated heavy chains, all 9 purified IgA samples retained a degree of biological activity as shown by gp120-specific ELISA (discussed in section 5.6). Since the degraded samples were shown by gp120-specific ELISA to be capable of binding antigen, the variable regions of these antibodies are most likely intact, suggesting that the samples are missing a portion of the C-terminus. The presence of distinct bands of IgA heavy chain fragments on the Western blot (as opposed to a smear) also suggests that the antibody samples had been truncated or digested at a specific point along the peptide as opposed to being non-specifically degraded. The ~45kDa size of the shortened IgA heavy chain in the A9/4L sample showed this antibody to be relatively intact and only missing a part of the C_H3 domain and the antibody tailpiece. Because no full-length 60kDa heavy chain was detected in the A9/4L antibody sample, it is most likely that the IgA heavy chain protein was produced in a truncated form, rather than being produced correctly and then later digested with protease. Truncation of the IgA2 gene could have resulted from a recombination event within the plasmid or from a point mutation producing a premature stop codon within the C_H3 domain; however the true cause could not be confirmed due to the unavailability of C_H3 domain sequence info from the CHO-K1 genomic DNA. Alternately, since N-linked oligosaccharides account for up to 10% of the molecular mass of IgA2 [158], is could be possible that IgA heavy chain bands slightly smaller than the expected 60kDa are indicative of a glycosylation defect. The IgA heavy chain fragments

observed in the A6/30L, A9/30L & A9/30λs could have been generated by either truncation or by digestion. The ~30kDa heavy chain fragment detected in these samples suggested both the C_H2 and C_H3 domains were missing, accounting for half of the entire IgA backbone. Complete truncation of all of the genomic IgA2 gene copies is unlikely, as the Western blot demonstrated that at least some full-length 60kDa IgA heavy chain was produced; however, we were unable to detect any trace of the C_H1-C_H2 domains of the IgA2 gene in the A9/30L and A9/30λ CHO-K1 genomic DNA samples. This supports the possibility that the IgA2 gene was truncated somewhere upstream of the C_H2 region. Despite the precautions taken to avoid contact with proteolytic enzymes, antibody digestion during cell culture was still possible. Notably, most of the small heavy chain fragments detected in these samples had the same ~30kDa size, which could be consistent with papain digestion between the C_H1 and C_H2 protein domains to produce Fab fragments.

5.6 Characterization of the experimental IgA Constructs

Following production of the nine IgA samples, we aimed to characterize the monomeric $gp120_{IIIB}$ specificity of all IgA variants since the antibody purification procedure described in section 4.3 increased the IgA-30L and IgA-30λ sample concentrations to detectable levels. We also wanted to assess the ability of the experimental IgA to bind oligomeric $gp120_{IIIB}$ and to investigate whether these antibodies bind to the same $gp120_{IIIB}$ epitope as their parental IgGb12 antibody. However, since the A9/4L sample was truncated and the amounts of the IgA-30L and IgA-30λ samples were

very limited, these characterization experiments were confined to the H31/4L and A6/4L IgA samples.

5.6.1 Standardized gp120 ELISA

The gp120 specificity of the nine IgA variants was assessed by ELISA using monomeric recombinant $gp120_{IIIB}$. In addition to detecting whether the experimental antibodies could bind to gp120, we also wanted to directly compare the gp120 binding of the samples to one another. Since a gp120-specific IgA standard was not available for this assay, we chose to standardize the total IgA concentration between samples being compared. Therefore, with the total amount of IgA being equal across samples, differences in gp120 specificity would be attributable to the affinity of the antibodies rather than to their relative abundance. However, given the fragmented α-chain bands appearing in the western blots of most purified samples, it is important to note that the IgA quantification may have included these protein fragments and introduced some error into the concentration standardization.

Initially, IgA samples were only compared to other samples sharing the same type of variable light chain, since variable gene type correlated with the yield of IgA sample generated. The high-concentration IgA-4L samples were adjusted to 20μg/ml and the intermediate IgA-30L samples to 150ng/ml, while the low-concentration IgA-30λ samples were not standardized to avoid diluting them further. Of the IgA-4L samples, H31/4L exhibited the greatest affinity for monomeric $gp120_{IIIB}$. This was expected since both of the variable genes of this antibody were derived from IgGb12, a potent HIV-1 specific antibody with a high affinity for gp120. Furthermore, unlike the Kenyan B-cell

derived antibody genes (which were most likely generated by exposure to circulating clade A or D virus endemic to Kenya), IgGb12 was initially raised against a clade B strain of HIV-1. This may afford the H31/4L variant an advantage over the A6/4L and A9/4L hybrids with respect to $gp120_{IIIB}$ specificity. The A6/4L hybrid antibody had an intermediate gp120 specificity when standardized to the same concentration of the other IgA-4L samples. This was in contrast to the gp120-specific ELISA results obtained from screening the cell culture supernatants in section 4.2, indicating that the high degree of gp120 binding observed in the unpurified A6/4L culture supernatant was caused by a relative overabundance of IgA rather than a stronger gp120 affinity. The A9/4L sample had the lowest gp120 specificity of the IgA-4L samples, but retained a degree of biological activity despite its truncated conformation. As previously discussed, the Western blot results indicated that the A9/4L heavy chain backbone was ~15kDa smaller than expected. Since sample binding to gp120 was observed, it follows that the C-terminus rather than the N-terminus of the antibody was missing and that the antibody variable regions were likely intact. As such, despite the truncation of the A9/4L antibody sample, the observed gp120 binding is probably reflective of the specificity of a full-length antibody.

Comparison of the IgA-30L samples revealed that both Africanized IgA antibodies exhibited superior gp120 specificity compared to the H31/30L hybrid. The A6/30L sample had the highest affinity for gp120, followed by A9/30L and then H31/30L. This was particularly interesting because the H31 variable gene was raised against a clade B virus, yet the patient-derived A6, A9 and 30L genes were better able to bind $gp120_{IIIB}$. The gp120 specificity of the three IgA-30λ samples was also investigated.

The binding of these samples was barely above the limit of detection, and appeared to be roughly equal between all samples. Since the IgA-30λ samples were confirmed to be biologically active but remained inferior to the IgA-30L samples, they were excluded from the remainder of the characterization experiments.

5.6.2 Comparison of IgA-4L & IgA-30L samples

Next, we compared $gp120_{IIIB}$ specificity across all IgA-4L and IgA-30L samples by adjusting the IgA concentration of all samples to 150ng/ml. Interestingly, it became apparent that antibodies with variable heavy and light genes derived from the same source (i.e. both variable genes originated from either IgGb12 or from Kenyan B-cells) had greater specificity for gp120 as compared to hybrid samples: the H31/4L "b12" antibody exhibited the highest gp120 affinity of any sample, including the H31/30L hybrid, and the A6/30L and A9/30L were both able to bind gp120 better than the A6/4L and A9/4L hybrids. Although it is possible that the hybrid antibodies are intrinsically inferior to antibodies with variable genes derived from the same source, this association is likely a coincidence. Both the A6/30L and A9/30L samples were artificially constructed with a random pairing of B-cell derived gp120-specific variable heavy and light genes that are unlikely to belong to the same antibody *in vivo*. As such, since the A6 and A9 heavy genes are not naturally paired with either the 4L or the 30L light genes, the original source of the light chain gene used in the pairing is irrelevant. In fact H31/4L, the antibody construct with the highest avidity for gp120, is a variant of b12 which itself was generated from a random pairing of variable genes derived from an Fab library.

The gp120 binding disparity between the H31/4L and H31/30L IgA samples was also interesting because of conclusions made about the IgGb12 light chain binding in previous studies. Based on analysis of the crystal structure of a b12 antibody binding fragment (Fab) in complex with gp120, it was controversially reported that only the heavy chain of b12 interacts with gp120 [231]. Our H31/4L sample is an affinity-matured IgA version of the b12 antibody which has a variable heavy chain that is identical to the b12 antibody with the exception of a valine instead of a glutamic acid residue at position 2 of the FR1 region (Figure 3). As such, H31/4L should interact with gp120 in the same manner as b12 and, if only the heavy chain interacts with gp120, we would expect all samples with the H31 variable heavy chain to have the same gp120 specificity regardless of the variable light chain. Instead, we observed a significant difference in gp120 specificity between the H31/4L and H31/30L samples. This contradicts the earlier report of a heavy chain-only interaction with gp120 and suggests that light chain interactions are an important factor of gp120 specificity. This is further supported by the observation that the A6/30L and A9/30L samples exhibited greater gp120 binding than the A6/4L and A9/4L samples, respectively; once again, ligand binding appears to be determined at least partially by the type of light chain present.

The fact that gp120-specific binding was detected from the IgA-30L samples was very promising for future studies involving these antibody genes. When the IgA-30L cell culture supernatants were initially screened by ELISA (described in section 4.2) gp120 specificity was not detected, and it was unclear as to whether the IgA-30L samples lacked biological activity or whether the IgA concentrations in those supernatants were simply too low to be detected. Increasing the concentration of the IgA-30L samples showed that

these antibodies were not only capable of binding $gp120_{IIIB}$, but in the case of the Africanized A6/30L and A9/30L variants, actually bound $gp120_{IIIB}$ with greater affinity than their IgA-4L counterparts. As mentioned above, the antibody genes produced by Kenyan B-cells would most likely have been raised against HIV-1 clades A or D rather than the clade B gp120 used in the assay. The fact that the Africanized antibodies bind to $gp120_{IIIB}$ and that they do so with relatively high affinity may suggest cross-clade specificity. As such, if the productivity of the IgA-30L cell lines could be increased, these antibodies could be useful for further characterization of IgA-mediated protective immunity at the genital mucosa.

5.6.3 Flow Cytometry analysis of binding to trimeric gp120

While the above experiments proved that the experimental IgA antibodies were capable of binding to monomeric $gp120_{IIIB}$, we also wanted to investigate how successful these antibodies were at binding oligomeric gp120 presented in a more realistic context. In a natural infection, HIV-specific antibodies mostly encounter gp120 expressed as trimers with gp41 on the surface of the HIV virion or on the surface of an infected cell. Trimeric gp120 may present different epitope targets than monomeric gp120 due to its different conformation and association with gp41. Therefore, we opted to investigate the ability of the experimental IgA to recognize trimeric gp120 using flow cytometry. HIV-1 infected H9 cells should express gp120 on the cell surface and were therefore used as a model for assessing how the H31/4L and A6/4L IgA antibodies might bind to HIV-1 infected cells *in vivo*.

Overall, the extremely low binding measured by this assay suggests that the system was not sufficiently optimized to assess binding to trimeric gp120; additionally, the lack of a gp120-specific positive control IgA antibody made it difficult to interpret any binding observed. Neither IgA sample exhibited a high degree of binding to $gp120_{IIIB}$ expressed on infected H9 cells at any of the antibody concentrations tested. When comparing the antibody binding on infected H9 cells to the background non-specific binding observed on uninfected H9s, the H31/4L antibody showed greatest binding at a concentration of 1μg, where staining of the infected cells was 1.82 fold higher than background. A higher concentration of H31/4L antibody appeared to result in competitive inhibition. Although these results were extremely modest, the fact that staining of infected cells decreased with decreasing titrations of primary IgA antibody suggested that the observed H31/4L binding was genuine. Conversely, A6/4L gp120-specific staining was not detected; this was likely due to an excess of non-specific binding, especially in the uninfected H9 samples. Based on these results, we could not definitively determine whether the A6/4L sample was capable of binding to trimeric $gp120_{IIIB}$ expressed on the surface of infected H9 cells. However, since both the H31/4L and A6/4L ScFvs investigated by Berry *et al* [150] were able to bind to 293T cells infected with both clade A and clade B gp120, it is likely that improving our experimental protocol would result in observable IgA binding.

First, the results could likely have been improved by adjusting the titration of the primary IgA antibodies by adjusting the titration of the secondary anti-IgA-FITC antibody, and by increasing the washing during the staining procedure. We initially chose to titrate the primary IgA antibodies at concentrations ranging from 0.1- 2μg. These IgA

concentrations were estimated to be appropriate based on the recommended concentrations of other commercial pre-conjugated antibodies used for flow cytometry. However, since this range of IgA concentrations was not optimized prior to the experiment, it is possible that testing additional concentrations of primary IgA antibody would have given clearer results. Similarly, 2µl of secondary antibody was selected based on manufacturer recommendations, but alternate titrations of this antibody were not investigated. Second, the number of specific binding events may have been increased if the infected H9 cells had expressed more antigen. Prior to the experiment, modest p24 production in the infected H9 cell culture was detected by ELISA. This could either indicate that only a small proportion of the H9 cells were productively infected, or that infected H9 cells were only supporting low levels of HIV replication and, therefore, expressing low amounts of gp120 on the cell surface. Both of these scenarios would limit the ability of the H31/4L and A6/4L experimental antibodies to bind to the infected H9 cells.

A number of improvements could be made to this protocol for future experiments. First of all, due to the inefficiency of H9 cell infection and the high rate of cell death observed in the infected H9 cell culture, transfected (rather than infected) H9 cells should be used. In both the infected and uninfected H9 samples, cells determined to be dead based on forward scatter and side scatter discrimination appeared to have absorbed large amounts of primary and/or secondary antibody, which potentially prevented preferential staining of gp120 on live cells. The flow cytometry staining protocol could also be expanded to include additional wash steps, live/dead cell discrimination and intracellular p24 staining. Adding a live/dead cell discriminator would help ensure that only living H9

cells were being included in the gating strategy, and intracellular p24 staining would indicate which cells are productively infected with HIV (if infected, and not transfected, cells were analysed). This would allow more accurate determination of IgA binding to cells known to be alive and productively infected with HIV.

5.6.4 IgA & IgG Competition ELISA

We assessed whether the $gp120_{IIIB}$ epitopes targeted by the H31/4L and A6/4L antibodies differed from the epitope of the parental IgGb12 antibody by means of a competition ELISA. The ELISA protocol used for the competition assays were similar to the gp120-specific assay described in section 4.2, except that we chose to adsorb the $gp120_{IIIB}$ to the plate using a capture antibody in order to present it in as natural a conformation as possible. Prior to performing the competition assay, gp120 binding curves for both IgA variants and for IgGb12 were performed to determine the concentration of each that would give approximately 50% gp120 saturation in order to avoid confounding steric inhibition. We assayed the effect of IgGb12 on IgA sample binding to gp120 and also performed the reverse in order to confirm our findings and to control for the different gp120 affinities of the antibody samples (The A6/4L sample was of particular concern since it had a much weaker gp120 affinity than IgGb12).

The results indicated that at elevated concentrations of competing antibody, IgGb12 was able to inhibit H31/4L binding to $gp120_{IIIB}$ and vice versa. This was expected since the H31/4L antibody was an affinity-matured IgA version of the IgGb12 antibody, and as such should target the same $gp120_{IIIB}$ epitope. In contrast, $gp120_{IIIB}$ binding of the A6/4L antibody neither inhibited nor was inhibited by IgGb12. The A6/4L

sample contained a B-cell derived variable heavy chain but also an IgGb12-derived 4L variable light chain, so there was a possibility that its $gp120_{IIIB}$ epitope could have overlapped with the parental IgGb12 $gp120_{IIIB}$ epitope, or could have been sterically hindered from binding. As no binding interference was observed, the $gp120_{IIIB}$ epitope targeted by A6/4L must be primarily determined by the Kenyan B-cell derived heavy chain.

Because the heavy chain of an antibody generally contributes to antigen binding and specificity more than the light chain [161], it was expected that the A6/4L antibody would exhibit an altered gp120 epitope specificity compared to H31/4L. However, previous studies have shown that light chain switching can also alter epitope specificity [232]. Once the IgA-30L sample yields are increased, competition ELISAs could be performed between the H31/4L, H31/30L, A6/4L and A6/30L samples to assess whether these antibodies bind to the same gp120 epitope.

The competition ELISA method, while informative, is a crude method of comparing the epitopes between two different antibody samples. A much more accurate assessment of the gp120 epitopes of each antibody could be performed using matrix-assisted laser desorption ionization quadrupole time-of-flight (MALDI-qTOF) mass spectrometry to identify each epitope and to extrapolate the location of each epitope within gp120. The MALDI-qTOF mass spectrometry approach would allow a comparison of the gp120 epitopes between IgA samples composed of the same heavy chain but different light chains, which would clarify the effect of light chain interactions on gp120 ligand binding. It would also be possible to determine how different combinations of the heavy and light chain genes affect the gp120 epitope.

6.0 Summary & Future Directions

Understanding the protective immune responses at the site of viral exposure is the first step towards designing safe and effective treatments to block HIV-1 transmission. Mounting evidence suggests HIV-1 specific IgA antibodies in the female genital tract are important in the development and maintenance of immune-mediated protection from sexual acquisition of HIV-1. As such, we developed a cloning method to generate recombinant human monoclonal IgA2 antibodies by means of *in vitro* mammalian cell culture in order to address our hypothesis that HIV-1-specific IgA cloned from the genital tract of HESN women of the Pumwani cohort will efficiently recognize epitopes of monomeric and/or trimeric forms of HIV-1 envelope protein gp120 that are distinct from the well characterized HIV-neutralizing antibody, IgGb12. To investigate this hypothesis, we developed the following specific objectives:

1. To clone IgA variable heavy and light genes isolated from a cervical B-cell Fab library from the genital tract of HESN CSWs,
2. To produce 9 full-length human monoclonal IgA2 antibody variants *in vitro* by means of CHO-K1 cell culture transfection,
3. To purify the monoclonal IgA from the cell culture supernatants,
4. To verify the proper integration of all IgA genes into the genome of each transfected monoclonal CHO-K1 cell line,
5. To confirm the size and purity of the purified IgA samples, and
6. To characterize the ability of the engineered monoclonal IgA variants to bind to $gp120_{IIIB}$.

With regards to our objectives, four novel antibody variable heavy and variable light genes isolated from cervical B-cells of HESN sex workers were successfully combined with variable chains from the potent HIV-1-neutralizing monoclonal antibody IgG1b12 and cloned into the pSM102 human IgA2 expression vector. The successful incorporation of each variable heavy and variable light gene into pSM102 was confirmed by DNA sequencing. By using the pSM102 cloning vector and refining an *in vitro* CHO-K1 cell culture expression system, a total of 9 novel gp120-specific monoclonal IgA antibodies were produced; one IgGb12-like positive control (H31/4L), four hybrid constructs (A6/4L, A9/4L, H31/30L and H31/30λ), and four Africanized constructs (A6/30L, A6/30λ, A9/30L and A9/30λ). IgA sample purification from cell culture supernatants by IgA-specific immunoaffinity column chromatography was effective for all 9 IgA samples in that a quantifiable amount of each antibody was recovered with very little sample loss. Integrating the transfected human IgA genes into the CHO-K1 genomic DNA was achieved with mixed results. Each transfected CHO-K1 cell line was assessed by PCR and sequencing to verify the stable integration of all antibody genes into the CHO-K1 genomic DNA. While all IgA variable heavy and variable light genes were confirmed to have been integrated, we encountered issues detecting human IgA2 constant heavy genes in the context of the CHO-K1 genome and also found that two of the IgA backbone genes had been truncated. We also observed mixed results in terms of the size of the antibodies that were generated. While most of the antibodies were produced with at least some of the 60kDa IgA2 backbone intact, heavy chain degradation products were detected in five of the nine antibody variants and the A9/4L construct did not appear to have full-length heavy chains at all. Lastly, although only H31/4L and A6/4L were

produced at the proper size and in sufficient quantities for in-depth characterization, all 9 IgA variants produced in this thesis had detectable specificity for $gp120_{IIIB}$. The H31/4L variant, an IgA2 version of IgGb12 which exhibited the greatest gp120-specificity, appeared to be directed toward the parental b12 epitope and could modestly bind trimeric gp120 expressed on infected mammalian cells. The A6/4L construct was also highly specific for monomeric gp120 and recognized a distinct epitope compared to b12, but did not demonstrate detectable binding to trimeric gp120. The A9/4L clone showed affinity for gp120 despite constitutive truncation of its C-terminus. The three IgA-30L and IgA-30λ samples were not extensively characterized due to the low IgA productivity of these clones, however the fully Africanized A6/30L and A9/30L variants were shown to have a relatively high affinity for $gp120_{IIIB}$.

Overall, our experimental results confirmed our hypothesis that HIV-1 specific IgA cloned from the genital tract of HESN women of the Pumwani cohort would efficiently recognize gp120, and in the case of the A6/4L IgA variant, recognized a gp120 epitope that was distinct from IgGb12. The constructed antibodies demonstrate that variable chain genes isolated from the highly HIV-exposed commercial sex workers have high gp120-specificity and thus may potentially play an important role in HIV protection.

To fully elucidate the role of these monoclonal IgA antibodies in HIV-1 resistance, the functional capacity of these antibodies must be characterized. It will be necessary to scale up the expression of the low producing IgA-30L and IgA-30λ clones in order to generate sufficient quantities of IgA for extensive characterization. It will also be crucial to assess the ability of these antibodies to neutralize HIV-1 infection in an *in vitro* culture system as well as to perform transcytosis assays using an *in vitro* model of the

genital mucosal membrane barrier to identify non-neutralizing protective s-IgA. In both the neutralization and transcytosis systems, lab strains and primary HIV-1 isolates of multiple viral clades and tropisms should be assayed to determine whether the experimental antibodies possess cross-clade specificity. (Clade B HIV-1_{IIIB} and HIV-1_{BAL}would corroborate Berry *et. al.* [150], while clade A and D virus would be of particular relevance for the Kenyan-derived clones.) Epitope excision MALDI-qTOF mass spectroscopy epitope mapping should also be performed to identify and map the gp120 epitopes involved in eliciting protective IgA responses; particularly for the A6/4L construct, which appears to target an unidentified epitope, and to identify the as-yet unknown epitopes bound by the fully Africanized constructs. In light of the fact that the constant domains of an antibody appear to affect its antiviral activity, it would be interesting to express the variable heavy and variable light genes investigated in this thesis as IgA1 isotypes and compare their properties with IgA2 variants of the same specificity. Furthermore, since genetic polymorphisms of antibody constant genes have not yet been elucidated, the scope of the project could be expanded to investigate IgA constant heavy and light genes recovered from HESN women as well. Lastly, now that the methodology for *in vitro* production of human monoclonal IgA cloned from cervical B-cell cDNA has been established, it will be possible to generate new novel IgA antibodies from HESN women of the Pumwani cohort for further investigation. Characterizing the functionality of HIV-specific IgA cloned from the genital tract of HESN individuals will be important to the development of future protective vaccines and microbicides against HIV.

7.0 References

1. UNAIDS, *Report of the global AIDS epidemic*. 2008: Geneva.

2. UNAIDS, *AIDS epidemic update*. 2009: Geneva.

3. Buve, A., K. Bishikwabo-Nsarhaza, and G. Mutangadura, *The spread and effect of HIV-1 infection in sub-Saharan Africa.* Lancet, 2002. **359**(9322): p. 2011-7.

4. Kaul, R., T. Makadzange, and S. Rowland-Jones, *AIDS in Africa: a disaster no longer waiting to happen.* Nat Immunol, 2000. **1**(4): p. 267-70.

5. Hunter, D.J., *AIDS in sub-Saharan Africa: the epidemiology of heterosexual transmission and the prospects for prevention.* Epidemiology, 1993. **4**(1): p. 63-72.

6. Fox, J. and S. Fidler, *Sexual transmission of HIV-1.* Antiviral Res, 2010. **85**(1): p. 276-85.

7. Munch, J., et al., *Semen-derived amyloid fibrils drastically enhance HIV infection.* Cell, 2007. **131**(6): p. 1059-71.

8. Ndesendo, V.M., et al., *A review of current intravaginal drug delivery approaches employed for the prophylaxis of HIV/AIDS and prevention of sexually transmitted infections.* AAPS PharmSciTech, 2008. **9**(2): p. 505-20.

9. Risbud, A., *Human immunodeficiency virus (HIV) & sexually transmitted diseases (STDs).* Indian J Med Res, 2005. **121**(4): p. 369-76.

10. Higgins, J.A., S. Hoffman, and S.L. Dworkin, *Rethinking gender, heterosexual men, and women's vulnerability to HIV/AIDS.* Am J Public Health, 2010. **100**(3): p. 435-45.

11. Cates, W., Jr., *Review of non-hormonal contraception (condoms, intrauterine devices, nonoxynol-9 and combos) on HIV acquisition.* J Acquir Immune Defic Syndr, 2005. **38 Suppl 1**: p. S8-10.

12. *Antiretroviral drugs used in the treatment of HIV infection*. 2010, U.S. Food and Drug Administration.

13. Mahungu, T.W., A.J. Rodger, and M.A. Johnson, *HIV as a chronic disease.* Clin Med, 2009. **9**(2): p. 125-8.

14. Bhat, V.G., et al., *Factors associated with poor adherence to anti-retroviral therapy in patients attending a rural health centre in South Africa.* Eur J Clin Microbiol Infect Dis, 2010.

15. Murphy, E.M., et al., *Was the "ABC" approach (abstinence, being faithful, using condoms) responsible for Uganda's decline in HIV?* PLoS Med, 2006. **3**(9): p. e379.

16. Auvert, B., et al., *Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial.* PLoS Med, 2005. **2**(11): p. e298.

17. Gray, R.H., et al., *Male circumcision for HIV prevention in men in Rakai, Uganda: a randomised trial.* Lancet, 2007. **369**(9562): p. 657-66.

18. Bailey, R.C., et al., *Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomised controlled trial.* Lancet, 2007. **369**(9562): p. 643-56.

19. Shattock, R. and S. Solomon, *Microbicides--aids to safer sex.* Lancet, 2004. **363**(9414): p. 1002-3.

20. Lederman, M.M., R.E. Offord, and O. Hartley, *Microbicides and other topical strategies to prevent vaginal transmission of HIV.* Nat Rev Immunol, 2006. **6**(5): p. 371-82.

21. Moscicki, A.B., *Vaginal microbicides: where are we and where are we going?* J Infect Chemother, 2008. **14**(5): p. 337-41.

22. Ramjee, G., *Microbicide research: current and future directions.* Curr Opin HIV AIDS, 2010. **5**(4): p. 316-21.

23. Mbopi-Keou, F.X., et al., *A randomized, double-blind, placebo-controlled Phase II extended safety study of two Invisible Condom formulations in Cameroonian women.* Contraception, 2009. **81**(1): p. 79-85.

24. Huber, M., W.C. Olson, and A. Trkola, *Antibodies for HIV treatment and prevention: window of opportunity?* Curr Top Microbiol Immunol, 2008. **317**: p. 39-66.

25. Veazey, R.S., et al., *Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120.* Nat Med, 2003. **9**(3): p. 343-6.

26. Hessell, A.J., et al., *Fc receptor but not complement binding is important in antibody protection against HIV.* Nature, 2007. **449**(7158): p. 101-4.

27. Miedema, F., *A brief history of HIV vaccine research: stepping back to the drawing board?* Aids, 2008. **22**(14): p. 1699-703.

28. Walker, L.M. and D.R. Burton, *Rational antibody-based HIV-1 vaccine design: current approaches and future directions.* Curr Opin Immunol, 2010. **22**(3): p. 358-66.

29. Mascola, J.R. and D.C. Montefiori, *The role of antibodies in HIV vaccines.* Annu Rev Immunol, 2010. **28**: p. 413-44.

30. Fauci, A.S., et al., *HIV vaccine research: the way forward.* Science, 2008. **321**(5888): p. 530-2.

31. Buchbinder, S.P., et al., *Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial.* Lancet, 2008. **372**(9653): p. 1881-93.

32. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.* N Engl J Med, 2009. **361**(23): p. 2209-20.

33. Burton, D.R. and J.P. Moore, *Why do we not have an HIV vaccine and how can we make one?* Nat Med, 1998. **4**(5 Suppl): p. 495-8.

34. Ferrantelli, F., et al., *Do not underestimate the power of antibodies--lessons from adoptive transfer of antibodies against HIV.* Vaccine, 2002. **20 Suppl 4**: p. A61-5.

35. Ferrantelli, F. and R.M. Ruprecht, *Neutralizing antibodies against HIV -- back in the major leagues?* Curr Opin Immunol, 2002. **14**(4): p. 495-502.

36. Gao, F., et al., *Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes.* Nature, 1999. **397**(6718): p. 436-41.

37. Sharp, P.M., et al., *The origins of acquired immune deficiency syndrome viruses: where and when?* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1410): p. 867-76.

38. Keele, B.F., et al., *Chimpanzee reservoirs of pandemic and nonpandemic HIV-1.* Science, 2006. **313**(5786): p. 523-6.

39. Worobey, M., et al., *Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960.* Nature, 2008. **455**(7213): p. 661-4.

40. Hirsch, V.M., et al., *An African primate lentivirus (SIVsm) closely related to HIV-2.* Nature, 1989. **339**(6223): p. 389-92.

41. Chen, Z., et al., *Genetic characterization of new West African simian immunodeficiency virus SIVsm: geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically*

diverse viruses from a single feral sooty mangabey troop. J Virol, 1996. **70**(6): p. 3617-27.

42. Santiago, M.L., et al., *Simian immunodeficiency virus infection in free-ranging sooty mangabeys (Cercocebus atys atys) from the Tai Forest, Cote d'Ivoire: implications for the origin of epidemic human immunodeficiency virus type 2.* J Virol, 2005. **79**(19): p. 12515-27.

43. Vangroenweghe, D., *The earliest cases of human immunodeficiency virus type 1 group M in Congo-Kinshasa, Rwanda and Burundi and the origin of acquired immune deficiency syndrome.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1410): p. 923-5.

44. Gilbert, M.T., et al., *The emergence of HIV/AIDS in the Americas and beyond.* Proc Natl Acad Sci U S A, 2007. **104**(47): p. 18566-70.

45. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.

46. Gallo, R.C., et al., *Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 865-7.

47. Montagnier, L., *25 years after HIV discovery: prospects for cure and vaccine.* Virology, 2010. **397**(2): p. 248-54.

48. Coffin, J., et al., *Human immunodeficiency viruses.* Science, 1986. **232**(4751): p. 697.

49. Papathanasopoulos, M.A., G.M. Hunt, and C.T. Tiemessen, *Evolution and diversity of HIV-1 in Africa--a review.* Virus Genes, 2003. **26**(2): p. 151-63.

50. Takebe, Y., S. Kusagawa, and K. Motomura, *Molecular epidemiology of HIV: tracking AIDS pandemic.* Pediatr Int, 2004. **46**(2): p. 236-44.

51. Levy, J.A., *HIV pathogenesis: 25 years of progress and persistent challenges.* Aids, 2009. **23**(2): p. 147-60.

52. Thomson, M.M. and R. Najera, *Molecular epidemiology of HIV-1 variants in the global AIDS pandemic: an update.* AIDS Rev, 2005. **7**(4): p. 210-24.

53. *The Circulating Recombinant Forms (CRFs)*. 2010, Los Alamos National Laboratory.

54. Butler, I.F., et al., *HIV genetic diversity: biological and public health consequences.* Curr HIV Res, 2007. **5**(1): p. 23-45.

55. Murray, M.C., et al., *Effect of human immunodeficiency virus (HIV) type 1 viral genotype on mother-to-child transmission of HIV-1.* J Infect Dis, 2000. **181**(2): p. 746-9.

56. Requejo, H.I., *Worldwide molecular epidemiology of HIV.* Rev Saude Publica, 2006. **40**(2): p. 331-45.

57. Goto, T., M. Nakai, and K. Ikuta, *The life-cycle of human immunodeficiency virus type 1.* Micron, 1998. **29**(2-3): p. 123-38.

58. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. **67**: p. 1-25.

59. Freed, E.O. and M.A. Martin, *HIVs and Their Replication*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2001, Lippincott Williams & Wilkins: Philidelphia.

60. Pancera, M., et al., *Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility.* Proc Natl Acad Sci U S A, 2010. **107**(3): p. 1166-71.

61. Kleiman, L., *tRNA(Lys3): the primer tRNA for reverse transcription in HIV-1.* IUBMB Life, 2002. **53**(2): p. 107-14.

62. Kleiman, L., C.P. Jones, and K. Musier-Forsyth, *Formation of the tRNALys packaging complex in HIV-1.* FEBS Lett, 2010. **584**(2): p. 359-65.

63. Ciuffi, A., *Mechanisms governing lentivirus integration site selection.* Curr Gene Ther, 2008. **8**(6): p. 419-29.

64. Ganser-Pornillos, B.K., M. Yeager, and W.I. Sundquist, *The structural biology of HIV assembly.* Curr Opin Struct Biol, 2008. **18**(2): p. 203-17.

65. Devito, C., et al., *Mucosal and plasma IgA from HIV-1-exposed uninfected individuals inhibit HIV-1 transcytosis across human epithelial cells.* J Immunol, 2000. **165**(9): p. 5170-6.

66. Haase, A.T., *Perils at mucosal front lines for HIV and SIV and their hosts.* Nat Rev Immunol, 2005. **5**(10): p. 783-92.

67. Zetola, N.M. and C.D. Pilcher, *Diagnosis and management of acute HIV infection.* Infect Dis Clin North Am, 2007. **21**(1): p. 19-48, vii.

68. Montano, M.A., et al., *Elevated tumor necrosis factor-alpha activation of human immunodeficiency virus type 1 subtype C in Southern Africa is associated with an NF-kappaB enhancer gain-of-function.* J Infect Dis, 2000. **181**(1): p. 76-81.

69. Chan, D.J., *Factors affecting sexual transmission of HIV-1: current evidence and implications for prevention.* Curr HIV Res, 2005. **3**(3): p. 223-41.

70. Quinn, T.C., et al., *Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group.* N Engl J Med, 2000. **342**(13): p. 921-9.

71. Mothe, B., et al., *Virological, immune and host genetics markers in the control of HIV infection.* Dis Markers, 2009. **27**(3): p. 105-20.

72. Miller, C.J., et al., *Propagation and dissemination of infection after vaginal transmission of simian immunodeficiency virus.* J Virol, 2005. **79**(14): p. 9217-27.

73. Wilkinson, J. and A.L. Cunningham, *Mucosal transmission of HIV-1: first stop dendritic cells.* Curr Drug Targets, 2006. **7**(12): p. 1563-9.

74. Izquierdo-Useros, N., et al., *HIV and mature dendritic cells: Trojan exosomes riding the Trojan horse?* PLoS Pathog, 2010. **6**(3): p. e1000740.

75. Haase, A.T., *Targeting early infection to prevent HIV-1 mucosal transmission.* Nature, 2010. **464**(7286): p. 217-23.

76. Pilcher, C.D., et al., *Brief but efficient: acute HIV infection and the sexual transmission of HIV.* J Infect Dis, 2004. **189**(10): p. 1785-92.

77. Baeten, J.M. and J. Overbaugh, *Measuring the infectiousness of persons with HIV-1: opportunities for preventing sexual HIV-1 transmission.* Curr HIV Res, 2003. **1**(1): p. 69-86.

78. Picker, L.J., *Immunopathogenesis of acute AIDS virus infection.* Curr Opin Immunol, 2006. **18**(4): p. 399-405.

79. Frost, S.D., et al., *Neutralizing antibody responses drive the evolution of human immunodeficiency virus type 1 envelope during recent HIV infection.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18514-9.

80. Ford, E.S., C.E. Puronen, and I. Sereti, *Immunopathogenesis of asymptomatic chronic HIV Infection: the calm before the storm.* Curr Opin HIV AIDS, 2009. **4**(3): p. 206-14.

81. Paiardini, M., et al., *Mucosal immune dysfunction in AIDS pathogenesis.* AIDS Rev, 2008. **10**(1): p. 36-46.

82. Alimonti, J.B., T.B. Ball, and K.R. Fowke, *Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS.* J Gen Virol, 2003. **84**(Pt 7): p. 1649-61.

83. Pantaleo, G. and A.S. Fauci, *Immunopathogenesis of HIV infection.* Annu Rev Microbiol, 1996. **50**: p. 825-54.

84. CDC, *1993 Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults*. 1992, Centre for Disease Control: Atlanta.

85. Hirbod, T. and K. Broliden, *Mucosal immune responses in the genital tract of HIV-1-exposed uninfected women.* J Intern Med, 2007. **262**(1): p. 44-58.

86. Wira, C.R., et al., *Innate and adaptive immunity in female genital tract: cellular responses and interactions.* Immunol Rev, 2005. **206**: p. 306-35.

87. Eberhard, A., et al., *[Mechanisms of resistance to sexual transmission of HIV-1].* Med Mal Infect, 2005. **35**(11): p. 517-24.

88. Baeten, J.M., et al., *Prospective study of correlates of vaginal Lactobacillus colonisation among high-risk HIV-1 seronegative women.* Sex Transm Infect, 2009. **85**(5): p. 348-53.

89. Lai, S.K., et al., *Human immunodeficiency virus type 1 is trapped by acidic but not by neutralized human cervicovaginal mucus.* J Virol, 2009. **83**(21): p. 11196-200.

90. Klebanoff, S.J. and R.W. Coombs, *Viricidal effect of Lactobacillus acidophilus on human immunodeficiency virus type 1: possible role in heterosexual transmission.* J Exp Med, 1991. **174**(1): p. 289-92.

91. Hladik, F. and M.J. McElrath, *Setting the stage: host invasion by HIV*. Nat Rev Immunol, 2008. **8**(6): p. 447-57.

92. Belec, L., *[Defenses of the female genital tract against infection]*. J Gynecol Obstet Biol Reprod (Paris), 2002. **31**(6 Suppl): p. 4S45-4S59.

93. Burgener, A., et al., *Identification of differentially expressed proteins in the cervical mucosa of HIV-1-resistant sex workers.* J Proteome Res, 2008. **7**(10): p. 4446-54.

94. Puddu, P., et al., *Antiviral effect of bovine lactoferrin saturated with metal ions on early steps of human immunodeficiency virus type 1 infection.* Int J Biochem Cell Biol, 1998. **30**(9): p. 1055-62.

95. Schenkels, L.C., E.C. Veerman, and A.V. Nieuw Amerongen, *Biochemical composition of human saliva in relation to other mucosal fluids.* Crit Rev Oral Biol Med, 1995. **6**(2): p. 161-75.

96. Cole, A.M. and A.L. Cole, *Antimicrobial polypeptides are key anti-HIV-1 effector molecules of cervicovaginal host defense.* Am J Reprod Immunol, 2008. **59**(1): p. 27-34.

97. Shattock, R.J. and J.P. Moore, *Inhibiting sexual transmission of HIV-1 infection.* Nat Rev Microbiol, 2003. **1**(1): p. 25-34.

98. Russell, M.W. and J. Mestecky, *Humoral immune responses to microbial infections in the genital tract.* Microbes Infect, 2002. **4**(6): p. 667-77.

99. Madkan, V., K. Sra, and S.K. Tyring, *The Role of Mucosal Immunity in Protection against Viral Diseases*, in *Mucosal Immunology and Virology*, S.K. Tyring, Editor. 2006.

100. Broliden, K., et al., *Introduction: Back to basics: mucosal immunity and novel HIV vaccine concepts.* J Intern Med, 2009. **265**(1): p. 5-17.

101. Pala, P., et al., *An African perspective on mucosal immunity and HIV-1.* Mucosal Immunol, 2009. **2**(4): p. 300-14.

102. Kaul, R., et al., *The genital tract immune milieu: an important determinant of HIV susceptibility and secondary transmission.* J Reprod Immunol, 2008. **77**(1): p. 32-40.

103. Hladik, F. and T.J. Hope, *HIV infection of the genital mucosa in women.* Curr HIV/AIDS Rep, 2009. **6**(1): p. 20-8.

104. Bomsel, M., *Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier.* Nat Med, 1997. **3**(1): p. 42-7.

105. Alfsen, A., et al., *Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1.* J Immunol, 2001. **166**(10): p. 6257-65.

106. Nazli, A., et al., *Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation.* PLoS Pathog, 2010. **6**(4): p. e1000852.

107. *Medical Microbiology & Immunology*. 7 ed. Lange.

108. Alfano, M. and G. Poli, *Role of cytokines and chemokines in the regulation of innate immunity and HIV infection.* Mol Immunol, 2005. **42**(2): p. 161-82.

109. Karlsson Hedestam, G.B., et al., *The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus.* Nat Rev Microbiol, 2008. **6**(2): p. 143-55.

110. Perelson, A.S., et al., *HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time.* Science, 1996. **271**(5255): p. 1582-6.

111. Leonard, C.K., et al., *Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells.* J Biol Chem, 1990. **265**(18): p. 10373-82.

112. Sato, S. and W. Johnson, *Antibody-mediated neutralization and simian immunodeficiency virus models of HIV/AIDS.* Curr HIV Res, 2007. **5**(6): p. 594-607.

113. Lin, G. and P.L. Nara, *Designing immunogens to elicit broadly neutralizing antibodies to the HIV-1 envelope glycoprotein.* Curr HIV Res, 2007. **5**(6): p. 514-41.

114. Fowke, K.R., et al., *Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya.* Lancet, 1996. **348**(9038): p. 1347-51.

115. Beyrer, C., et al., *Epidemiologic and biologic characterization of a cohort of human immunodeficiency virus type 1 highly exposed, persistently seronegative female sex workers in northern Thailand. Chiang Mai HEPS Working Group.* J Infect Dis, 1999. **179**(1): p. 59-67.

116. Clerici, M., et al., *Cell-mediated immune response to human immunodeficiency virus (HIV) type 1 in seronegative homosexual men with recent sexual exposure to HIV-1.* J Infect Dis, 1992. **165**(6): p. 1012-9.

117. Mazzoli, S., et al., *HIV-specific mucosal and cellular immunity in HIV-seronegative partners of HIV-seropositive individuals.* Nat Med, 1997. **3**(11): p. 1250-7.

118. Barcellini, W., et al., *In vitro production of type 1 and type 2 cytokines by peripheral blood mononuclear cells from high-risk HIV-negative intravenous drug users.* Aids, 1995. **9**(7): p. 691-4.

119. Kaul, R., et al., *New insights into HIV-1 specific cytotoxic T-lymphocyte responses in exposed, persistently seronegative Kenyan sex workers.* Immunol Lett, 2001. **79**(1-2): p. 3-13.

120. Liu, R., et al., *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection.* Cell, 1996. **86**(3): p. 367-77.

121. Huang, Y., et al., *The role of a mutant CCR5 allele in HIV-1 transmission and disease progression.* Nat Med, 1996. **2**(11): p. 1240-3.

122. Fowke, K.R., et al., *HIV type 1 resistance in Kenyan sex workers is not associated with altered cellular susceptibility to HIV type 1 infection or enhanced beta-chemokine production.* AIDS Res Hum Retroviruses, 1998. **14**(17): p. 1521-30.

123. Kaul, R., et al., *Late seroconversion in HIV-resistant Nairobi prostitutes despite pre-existing HIV-specific CD8+ responses.* J Clin Invest, 2001. **107**(3): p. 341-9.

124. Laurence, J., *Repetitive and consistent cervicovaginal exposure to certain viral pathogens appears to protect against their sexual acquisition in some women: potential mechanisms.* J Reprod Immunol, 2003. **58**(1): p. 79-91.

125. Plummer, F.A., et al., *Resistance to HIV-1 infection among highly exposed sex workers in Nairobi: what mediates protection and why does it develop?* Immunol Lett, 1999. **66**(1-3): p. 27-34.

126. MacDonald, K.S., et al., *Influence of HLA supertypes on susceptibility and resistance to human immunodeficiency virus type 1 infection.* J Infect Dis, 2000. **181**(5): p. 1581-9.

127. MacDonald, K.S., et al., *The HLA A2/6802 supertype is associated with reduced risk of perinatal human immunodeficiency virus type 1 transmission.* J Infect Dis, 2001. **183**(3): p. 503-6.

128. MacDonald, K.S., et al., *Human leucocyte antigen supertypes and immune susceptibility to HIV-1, implications for vaccine design.* Immunol Lett, 2001. **79**(1-2): p. 151-7.

129. Lacap, P.A., et al., *Associations of human leukocyte antigen DRB with resistance or susceptibility to HIV-1 infection in the Pumwani Sex Worker Cohort.* Aids, 2008. **22**(9): p. 1029-38.

130. Hardie, R.A., et al., *A common human leucocyte antigen-DP genotype is associated with resistance to HIV-1 infection in Kenyan sex workers.* Aids, 2008. **22**(15): p. 2038-42.

131. Hardie, R.A., et al., *Human leukocyte antigen-DQ alleles and haplotypes and their associations with resistance and susceptibility to HIV-1 infection.* Aids, 2008. **22**(7): p. 807-16.

132. Ball, T.B., et al., *Polymorphisms in IRF-1 associated with resistance to HIV-1 infection in highly exposed uninfected Kenyan sex workers.* Aids, 2007. **21**(9): p. 1091-101.

133. Ji, H., et al., *Reduced HIV-1 long terminal repeat transcription in subjects with protective interferon regulatory factor-1 genotype: a potential mechanism mediating resistance to infection by HIV-1.* Scand J Infect Dis, 2010. **42**(5): p. 389-94.

134. Ji, H., et al., *Novel interferon regulatory factor-1 polymorphisms in a Kenyan population revealed by complete gene sequencing.* J Hum Genet, 2004. **49**(10): p. 528-35.

135. Rowland-Jones, S.L., *Survival with HIV infection: good luck or good breeding?* Trends Genet, 1998. **14**(9): p. 343-5.

136. Fowke, K.R., et al., *HIV-1-specific cellular immune responses among HIV-1-resistant sex workers.* Immunol Cell Biol, 2000. **78**(6): p. 586-95.

137. Rowland-Jones, S.L., et al., *How important is the 'quality' of the cytotoxic T lymphocyte (CTL) response in protection against HIV infection?* Immunol Lett, 2001. **79**(1-2): p. 15-20.

138. Kaul, R., et al., *HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi.* J Immunol, 2000. **164**(3): p. 1602-11.

139. Kaul, R., et al., *HIV-1 Env-specific cytotoxic T-lymphocyte responses in exposed, uninfected Kenyan sex workers: a prospective analysis.* Aids, 2004. **18**(15): p. 2087-9.

140. Alimonti, J.B., et al., *Characterization of CD8 T-cell responses in HIV-1-exposed seronegative commercial sex workers from Nairobi, Kenya.* Immunol Cell Biol, 2006. **84**(5): p. 482-5.

141. Alimonti, J.B., et al., *CD4+ T cell responses in HIV-exposed seronegative women are qualitatively distinct from those in HIV-infected women.* J Infect Dis, 2005. **191**(1): p. 20-4.

142. Julg, B., et al., *Enhanced anti-HIV functional activity associated with Gag-specific CD8 T-cell responses.* J Virol, 2010. **84**(11): p. 5540-9.

143. McLaren, P.X., et al., *HIV-Exposed Seronegative Commercial Sex Workers Show a Quiescent Phenotype in the CD4+ T Cell Compartment and Reduced Expression of HIV-Dependent Host Factors.* J Infect Dis, 2010. **202**(S3): p. S339-S344.

144. Card, C.M., et al., *Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells.* J Infect Dis, 2009. **199**(9): p. 1318-22.

145. Sempere, J.M., V. Soriano, and J.M. Benito, *T regulatory cells and HIV infection.* AIDS Rev, 2007. **9**(1): p. 54-60.

146. Keynan, Y., et al., *The role of regulatory T cells in chronic and acute viral infections.* Clin Infect Dis, 2008. **46**(7): p. 1046-52.

147. Horton, R.E., et al., *Cervical HIV-specific IgA in a population of commercial sex workers correlates with repeated exposure but not resistance to HIV.* AIDS Res Hum Retroviruses, 2009. **25**(1): p. 83-92.

148. Kaul, R., et al., *HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers.* Aids, 1999. **13**(1): p. 23-9.

149. Hirbod, T., K. Broliden, and R. Kaul, *Genital immunoglobulin A and HIV-1 protection: virus neutralization versus specificity.* Aids, 2008. **22**(17): p. 2401-2.

150. Berry, J.D., et al., *Development of functional human monoclonal single-chain variable fragment antibody against HIV-1 from human cervical B cells.* Hybrid Hybridomics, 2003. **22**(2): p. 97-108.

151. Pantaleo, G. and R.A. Koup, *Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know.* Nat Med, 2004. **10**(8): p. 806-10.

152. Broliden, K., et al., *Functional HIV-1 specific IgA antibodies in HIV-1 exposed, persistently IgG seronegative female sex workers.* Immunol Lett, 2001. **79**(1-2): p. 29-36.

153. Alexander, R. and J. Mestecky, *Neutralizing antibodies in mucosal secretions: IgG or IgA?* Curr HIV Res, 2007. **5**(6): p. 588-93.

154. Mestecky, J., et al., *Mucosal immunology of the genital and gastrointestinal tracts and HIV-1 infection.* J Reprod Immunol, 2009. **83**(1-2): p. 196-200.

155. Kerr, M.A., *The structure and function of human IgA.* Biochem J, 1990. **271**(2): p. 285-96.

156. Woof, J.M. and M.A. Kerr, *The function of immunoglobulin A in immunity.* J Pathol, 2006. **208**(2): p. 270-82.

157. Presta, L., *Antibody engineering for therapeutics.*Curr Opin Struct Biol, 2003. **13**(4): p. 519-25.

158. Woof, J.M. and J. Mestecky, *Mucosal immunoglobulins.* Immunol Rev, 2005. **206**: p. 64-82.

159. Tonegawa, S., *Somatic generation of antibody diversity.* Nature, **302**(5909): p. 575-81.

160. Manser, T., *Evolution of antibody structure during the immune response. The differentiative potential of a single B lymphocyte.* J Exp Med, 1989. **170**(4): p. 1211-30.

161. Arnaout, R.A., *Specificity and overlap in gene segment-defined antibody repertoires.* BMC Genomics, 2005. **6**: p. 148.

162. Nose, M. and H. Wigzell, *Biological significance of carbohydrate chains on monoclonal antibodies.* Proc Natl Acad Sci U S A, 1983. **80**(21): p. 6632-6.

163. Arnold, J.N., et al., *The impact of glycosylation on the biological function and structure of human immunoglobulins.* Annu Rev Immunol, 2007. **25**: p. 21-50.

164. Mestecky, J., *Humoral immune responses to the human immunodeficiency virus type-1 (HIV-1) in the genital tract compared to other mucosal sites.* J Reprod Immunol, 2007. **73**(1): p. 86-97.

165. Huber, M. and A. Trkola, *Humoral immunity to HIV-1: neutralization and beyond.* J Intern Med, 2007. **262**(1): p. 5-25.

166. Li, Y., et al., *Analysis of neutralization specificities in polyclonal sera derived from human immunodeficiency virus type 1-infected individuals.* J Virol, 2009. **83**(2): p. 1045-59.

167. Huang, Y.T., et al., *Intraepithelial cell neutralization of HIV-1 replication by IgA.* J Immunol, 2005. **174**(8): p. 4828-35.

168. Wright, A., et al., *Immunoglobulin A antibodies against internal HIV-1 proteins neutralize HIV-1 replication inside epithelial cells.* Virology, 2006. **356**(1-2): p. 165-70.

169. Bomsel, M., et al., *Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM.* Immunity, 1998. **9**(2): p. 277-87.

170. Bomsel, M., et al., *Natural mucosal antibodies reactive with first extracellular loop of CCR5 inhibit HIV-1 transport across human epithelial cells.* Aids, 2007. **21**(1): p. 13-22.

171. Wright, A., M.E. Lamm, and Y.T. Huang, *Excretion of human immunodeficiency virus type 1 through polarized epithelium by immunoglobulin A.* J Virol, 2008. **82**(23): p. 11526-35.

172. Hocini, H. and M. Bomsel, *Infectious human immunodeficiency virus can rapidly penetrate a tight human epithelial barrier by transcytosis in a process impaired by mucosal immunoglobulins.* J Infect Dis, 1999. **179 Suppl 3**: p. S448-53.

173. Kozlowski, P.A. and M.R. Neutra, *The role of mucosal immunity in prevention of HIV transmission.* Curr Mol Med, 2003. **3**(3): p. 217-28.

174. Hessell, A.J., et al., *Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques.* Nat Med, 2009. **15**(8): p. 951-4.

175. Nag, P., et al., *Women with cervicovaginal antibody-dependent cell-mediated cytotoxicity have lower genital HIV-1 RNA loads.* J Infect Dis, 2004. **190**(11): p. 1970-8.

176. Duval, M., M.R. Posner, and L.A. Cavacini, *A bispecific antibody composed of a nonneutralizing antibody to the gp41 immunodominant region and an anti-CD89 antibody directs broad human immunodeficiency virus destruction by neutrophils.* J Virol, 2008. **82**(9): p. 4671-4.

177. Bouhlal, H., et al., *Natural antibodies to CCR5 from breast milk block infection of macrophages and dendritic cells with primary R5-tropic HIV-1.* J Immunol, 2005. **174**(11): p. 7202-9.

178. Eslahpazir, J., et al., *Infection of macrophages and dendritic cells with primary R5-tropic human immunodeficiency virus type 1 inhibited by natural polyreactive anti-CCR5 antibodies purified from cervicovaginal secretions.* Clin Vaccine Immunol, 2008. **15**(5): p. 872-84.

179. Requena, M., et al., *Inhibition of HIV-1 transmission in trans from dendritic cells to CD4+ T lymphocytes by natural antibodies to the CRD domain of DC-SIGN purified from breast milk and intravenous immunoglobulins.* Immunology, 2008. **123**(4): p. 508-18.

180. Mascola, J.R., et al., *Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies.* J Virol, 1999. **73**(5): p. 4009-18.

181. Baba, T.W., et al., *Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection.* Nat Med, 2000. **6**(2): p. 200-6.

182. Mascola, J.R., et al., *Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies.* Nat Med, 2000. **6**(2): p. 207-10.

183. Parren, P.W., et al., *Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro.* J Virol, 2001. **75**(17): p. 8340-7.

184. Hessell, A.J., et al., *Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L.* J Virol, 2010. **84**(3): p. 1302-13.

185. Ferrantelli, F., et al., *Post-exposure prophylaxis with human monoclonal antibodies prevented SHIV89.6P infection or disease in neonatal macaques.* Aids, 2003. **17**(3): p. 301-9.

186. Trkola, A., et al., *Delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies.* Nat Med, 2005. **11**(6): p. 615-22.

187. Joos, B., et al., *Long-term multiple-dose pharmacokinetics of human monoclonal antibodies (MAbs) against human immunodeficiency virus type 1 envelope gp120 (MAb 2G12) and gp41 (MAbs 4E10 and 2F5).* Antimicrob Agents Chemother, 2006. **50**(5): p. 1773-9.

188. Binley, J.M., et al., *Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies.* J Virol, 2004. **78**(23): p. 13232-52.

189. Walker, L.M., et al., *Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target.* Science, 2009. **326**(5950): p. 285-9.

190. Wolbank, S., et al., *Characterization of human class-switched polymeric (immunoglobulin M [IgM] and IgA) anti-human immunodeficiency virus type 1 antibodies 2F5 and 2G12.* J Virol, 2003. **77**(7): p. 4095-103.

191. Burnett, P.R., et al., *Serum IgA-mediated neutralization of HIV type 1.* J Immunol, 1994. **152**(9): p. 4642-8.

192. Nguyen, M., et al., *HIV-specific antibodies but not t-cell responses are associated with protection in seronegative partners of HIV-1-infected individuals in Cambodia.* J Acquir Immune Defic Syndr, 2006. **42**(4): p. 412-9.

193. Lo Caputo, S., et al., *Mucosal and systemic HIV-1-specific immunity in HIV-1-exposed but uninfected heterosexual men.* Aids, 2003. **17**(4): p. 531-9.

194. Clerici, M., et al., *Serum IgA of HIV-exposed uninfected individuals inhibit HIV through recognition of a region within the alpha-helix of gp41.* Aids, 2002. **16**(13): p. 1731-41.

195. Ghys, P.D., et al., *Cervicovaginal anti-HIV antibodies in HIV-seronegative female sex workers in Abidjan, Cote d'Ivoire.* Aids, 2000. **14**(16): p. 2603-8.

196. Dorrell, L., et al., *Absence of specific mucosal antibody responses in HIV-exposed uninfected sex workers from the Gambia.* Aids, 2000. **14**(9): p. 1117-22.

197. Kaul, R., et al., *Mucosal IgA in exposed, uninfected subjects: evidence for a role in protection against HIV infection.* Aids, 2001. **15**(3): p. 431-2.

198. Mazzoli, S., et al., *Human immunodeficiency virus (HIV)-specific IgA and HIV neutralizing activity in the serum of exposed seronegative partners of HIV-seropositive persons.* J Infect Dis, 1999. **180**(3): p. 871-5.

199. Belec, L., et al., *Cervicovaginal secretory antibodies to human immunodeficiency virus type 1 (HIV-1) that block viral transcytosis through tight epithelial barriers in highly exposed HIV-1-seronegative African women.* J Infect Dis, 2001. **184**(11): p. 1412-22.

200. Hirbod, T., et al., *Upregulation of interferon-alpha and RANTES in the cervix of HIV-1-seronegative women with high-risk behavior.* J Acquir Immune Defic Syndr, 2006. **43**(2): p. 137-43.

201. Devito, C., et al., *Cross-clade HIV-1-specific neutralizing IgA in mucosal and systemic compartments of HIV-1-exposed, persistently seronegative subjects.* J Acquir Immune Defic Syndr, 2002. **30**(4): p. 413-20.

202. Devito, C., et al., *Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate.* Aids, 2000. **14**(13): p. 1917-20.

203. Granados-Gonzalez, V., et al., *Neutralizing inter-clade cross-reactivity of HIV-1 V1/V2-specific secretory immunoglobulin A in Colombian and French cohorts.* Aids, 2009. **23**(16): p. 2219-22.

204. Moldoveanu, Z. and J. Mestecky, *Mucosal antibody responses to HIV*. Methods Mol Biol, 2009. **485**: p. 333-45.

205. Donadoni, C., et al., *Setting of methods for analysis of mucosal antibodies in seminal and vaginal fluids of HIV seropositive subjects from Cambodian and Italian cohorts.* PLoS One, 2010. **5**(3): p. e9920.

206. Wright, P.F., et al., *Detection of mucosal antibodies in HIV type 1-infected individuals.* AIDS Res Hum Retroviruses, 2002. **18**(17): p. 1291-300.

207. Berry, J.D., *Rational monoclonal antibody development to emerging pathogens, biothreat agents and agents of foreign animal disease: The antigen scale.* Vet J, 2005. **170**(2): p. 193-211.

208. Steinitz, M., *Three decades of human monoclonal antibodies: past, present and future developments.* Hum Antibodies, 2009. **18**(1-2): p. 1-10.

209. Kohler, G., and C. Milstein., *Continuous culture of fused cells secreting antibody of predefined specificity.* Nature, 1975. **256**(5517): p. 495-7.

210. Burton, D.R., et al., *A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals.* Proc Natl Acad Sci U S A, 1991. **88**(22): p. 10134-7.

211. Kwong, P. D., J. R. Mascola, and G. J. Nagel, *Mining the B Cell Repertoire for Broadly Neutralizing Monoclonal Antibodies to HIV-1*. Cell Host Microbe, 2009. **6**(4): p. 292-4.

212. Tudor, D., et al., *HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4(+) cell infection: an IgA gene and functional analysis.* Mucosal Immunol, 2009. **2**(5): p. 412-26.

213. Barbas, C. F., et al., *Molecular Profile of an Antibody Response to HIV-1 as Probed by Combinatorial Libraries.* J Mol Biol, 1993. **230**(3): p. 812-23.

214. Chenna, R., et al., *Multiple sequence alignment with the Clustal series of programs.* Nucleic Acids Res, 2003. **31**(13): p. 3497-500.

215. Luton, F., et al., *Identification of a Cytoplasmic Signal for Apical Transcytosis.* Traffic, 2009. **10**(8): p.1128-42.

216. Mantis, N.J., et al., *Inhibition of HIV-1 infectivity and epithelial cell transfer by human monoclonal IgG and IgA antibodies carrying the b12 V region.* J Immunol, 2007. **179**(5): p. 3144-52.

217. Jenkins, N., L. Murphy, and R. Tyther, *Post-translational modifications of recombinant proteins: significance for biopharmaceuticals.* Mol Biotechnol, 2008. **39**(2): p. 113-8.

218. Chusainow, J., et al., *A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer?* Biotechnol Bioeng, 2009. **102**(4): p. 1182-96.

219. Kingston, R.E., et al., *Amplification using CHO cell expression vectors.* Curr Protoc Mol Biol, 2002. **Chapter 16**: p. Unit 16 23.

220. Chintalacharuvu, K.R., B. Gurbaxani, and S. L. Morrison, *Incomplete assembly of IgA2m(2) in Chinese hamster ovary cells.* Mol Immunol, 2007. **44**(13): p.3445-52.

221. Warner, T.G., *Enhancing therapeutic glycoprotein production in Chinese hamster ovary cells by metabolic engineering endogenous gene control with antisense DNA and gene targeting.* Glycobiology, 1999. **9**(9): p. 841-50.

222. Berlicki, L., *Inhibitors of glutamine synthetase and their potential application in medicine.* Mini Rev Med Chem, 2008. **8**(9): p. 869-78.

223. Vile, R., *Selectable Markers for Eukaryotic Cells*, in *Practical Molecular Virology: Viral Vectors for Gene Expression*, M. Collins, Editor. 1991, The Humana Press Inc.: Clifton, NJ.

224. Jiang, Z., Y. Huang, and S.T. Sharfstein, *Regulation of recombinant monoclonal antibody production in chinese hamster ovary cells: a comparative study of gene copy number, mRNA level, and protein expression.* Biotechnol Prog, 2006. **22**(1): p. 313-8.

225. Lattenmayer, C., et al., *Characterisation of recombinant CHO cell lines by investigation of protein productivities and genetic parameters.* J Biotechnol, 2007. **128**(4): p. 716-25.

226. Trill, J.J., A.R. Shatzman, and S. Ganguly, *Production of monoclonal antibodies in COS and CHO cells.* Curr Opin Biotechnol, 1995. **6**(5): p. 553-60.

227. *Current Protocols in Immunology*. 1994.

228. Wlaschin, K.F. and W.S. Hu, *A scaffold for the Chinese hamster genome.* Biotechnol Bioeng, 2007. **98**(2): p. 429-39.

229. Wlaschin, K.F., et al., *EST sequencing for gene discovery in Chinese hamster ovary cells.* Biotechnol Bioeng, 2005. **91**(5): p. 592-606.

230. Omasa, T., et al., *Bacterial artificial chromosome library for genome-wide analysis of Chinese hamster ovary cells.* Biotechnol Bioeng, 2009. **104**(5): p. 986-94.

231. Zhou, T., et al., *Structural definition of a conserved neutralization epitope on HIV-1 gp120.* Nature, 2007. **445**(7129): p. 732-7.

232. Zebedee, S.L., et al., *Human combinatorial antibody libraries to hepatitis B surface antigen.* Proc Natl Acad Sci U S A, 1992. **89**(8): p. 3175-9.

233. Black, K.P., et al., *Serum and Secretory IgA from HIV-Infected Individuals Mediate Antibody-Dependent Cellular Cytotoxicity.* Clin Immunol Immunopathol, 1996. **81**(2): p.182-90.

8.0 Appendices

Appendix A: Commercial Reagents

PCR Reagents & Equipment

All PCR reagents were obtained from Invitrogen (Burlington, ON, Canada), unless otherwise stated.

-10x *Taq* DNA Polymerase PCR Buffer

-50mM $MgCl_2$

-10mM dNTP Mix

- Recombinant *Taq* DNA Polymerase

- Expand High Fidelity^{PLUS} Polymerase Enzyme (Roche Diagnostics, Mannheim, Germany)

Agarose Gel Purification Reagents & Equipment

-UltraPure™ Agarose (Invitrogen, Burlington, ON, Canada)

-1kb Plus DNA Ladder (Invitrogen, Burlington, ON, Canada)

-UltraPure™ 10mg/ml Ethidium Bromide (Invitrogen, Burlington, ON, Canada)

-BioRad Gel Doc 2000 (Hercules, CA, USA)

-SYBR® Green I Nucleic Acid Gel Stain (Invitrogen, Eugene, Oregon, USA)

-Typhoon 9400 Scanner (GE Healthcare, QC, Canada)

-QIAquick® Gel Extraction Kit (Qiagen, Mississauga, ON, Canada)

Restriction Enzymes & Cloning Reagents

All restriction enzymes and their accompanying buffers were obtained from Invitrogen (Burlington, ON, Canada), unless otherwise stated.

-pCR®4-TOPO® Cloning Vector (Invitrogen, Burlington, ON, Canada)

-REact® 2. 3 & 10 Buffers

-*Xba I* Restriction Enzyme

-*Sst I* Restriction Enzyme

-*HindIII* Restriction Enzyme

-*EcoRI* Restriction Enzyme

-*Sal I* Restriction Enzyme

-10x Ligation buffer for T4 DNA ligase (Roche Diagnostics, Indianapolis, IN, USA)

-T4 DNA Ligase (Roche Diagnostics, Indianapolis, IN, USA)

-One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, Burlington, ON, Canada)

-Ampicillin (Invitrogen, Burlington, ON, Canada)

-QIAGEN Plasmid Mini Kit (Qiagen, Mississauga, ON, Canada)

Sequencing Reagents & Equipment

All DNA sequencing reagents and equipment were obtained from Applied Biosystems (Foster City, CA, USA).

- Amicon Microcon PCR centrifugal Filter Devices (Millipore, Bedford, MA, USA)

-Big Dye® Terminator v3.1 Cycle Sequencing Kit

-Hi-Di™ Formamide

-ABI PRISM® 3100 Genetic Analyzer

Animal Cell Culture Reagents

-FuGENE® 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA)

-Methionine Sulfoximine (MSX) solution (Sigma-Aldrich, Saint Louis, MO, USA)

-TrypLE™ Express Stable Trypsin Replacement Enzyme (Gibco, Burlington, ON, Canada)

-QIAamp DNA Mini Kit (Qiagen, Mississauga, ON, Canada)

Column Chromatography Reagents

-Anti-human IgA (α-chain specific)-Agarose antibody produced in goat (Sigma-Aldrich, Saint Louis, MO, USA)

-Econo-Column Chromatography Column, 1.0x10cm (Bio-Rad, Mississauga, ON, Canada)

-Macrosep® 30K Centrifugal Devices (Pall Corporation, Mississauga, ON, Canada)

Protein Gel Reagents

Protein gel reagents were from Bio-Rad (Hercules, CA, USA) unless otherwise noted.

-Bio-Rad Protein Assay Kit

-Protein Gel 5x loading dye

-Ready Gel Tris-HCl Gel, 4-15% precast linear gradient polyacrylamide gel

-Precision Plus Protein™ Kaleidoscope™ Standard

-Amersham™ ECL Advance™ Western Blotting Detection Kit (GE Healthcare, QC, Canada)

-Fluor Chem 8900 (Alpha Innotech/Cell Biosciences, San Leandro, CA, USA)

ELISA Reagents & Commercial Antibodies

-AffiniPure Goat Anti-Human Serum IgA, α-Chain Specific

(Jackson ImmunoResearch, West Grove, PA, USA)

-HRP-Conjugated AffiniPure Goat Anti-Human Serum IgA, α-Chain Specific

(Jackson ImmunoResearch, West Grove, PA, USA)

-Human IgA kappa

(Protos Immunoresearch, Burlingame, CA, USA)

-Human IgA Purified Immunoglobulin from Colostrum

(Sigma-Aldrich, Saint Louis, MO, USA)

-HRP-Conjugated AffiniPure Goat Anti-Human Serum IgG, Fcγ Fragment Specific

(Jackson ImmunoResearch, West Grove, PA, USA)

-Sheep anti-HIV-1 gp120env Antibody

(Aalto Bio Reagents, Dublin, Ireland)

-Bovine Serum Albumin (BSA), fraction V (Sigma-Aldrich, Oakville, ON, Canada)

-Recombinant gp120 HIV-1_{IIIB} (ImmunoDiagnostics, Woburn, MA, USA)

-3, 3′,5 ,5′-Tetramethylbenzidine Liquid Substrate (TMB) solution for ELISA

(Sigma-Aldrich, Saint Louis, MO, USA)

-Wellwash 4 Mk 2 plate washer (Thermo Scientific, Waltham, MA, USA)

Flow Cytometry Commercial Antibody & Equipment

-Goat $F(ab')_2$ Anti-Human IgA (α) conjugated to FITC (Invitrogen, Camarillo, CA, USA)

-FACSCalibur flow cytometer (Beckman Coulter, Mississauga, ON, Canada)

Appendix B: Laboratory-Prepared Solutions

General Lab Buffers

All chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise indicated.

-TBE electrophoresis buffer (10x)

- -108g Trizma® Base
- -55g Boric Acid
- -40ml 0.5M EDTA (pH 8.0)
- -ddH_2O to 1 litre

-6x Gel loading buffer

- -0.25% Bromophenol Blue
- -0.25% Xylene Cyanol
- -3ml glycerol
- -ddH_2O to 10ml

-2x PCR mix

- -15ml of 400mM Tris-HCl - 10mM $MgCl_2$.
- -1.5ml of 1M $(NH_4)_2SO_4$
- -0.4ml of 25mM dNTP mix
- -10ml of 1% gelatin
- -18.6ml ddH_2O

-3M Sodium Acetate (pH 5.2)

-40.83g sodium acetate

-80ml ddH_2O

-adjust pH to 5.2 with glacial acetic acid

-ddH_2O to 100ml

-Phosphate Buffered Saline (PBS) pH 7.4

-9.55g Phosphate-buffered saline powder

-ddH_2O to 1 L

-sterile filter by means of a vacuum apparatus with a 0.22μM filter membrane

-0.1M Glycine-HCl elution buffer (pH 2.5)

-7.51g glycine

-800ml ddH_2O

-adjust pH to 2.5 with HCl

-ddH_2O to 1 litre

*Note: for low-pH elution buffer, adjust pH to 2.0 with HCl.

-1.5M Tris-HCl neutralization buffer (pH 8.8)

-18.15g Tris base

-80ml ddH_2O

-adjust pH to 8.8 with HCl

-ddH_2O to 100ml

-ELISA wash buffer

-9.55g Phosphate-buffered saline powder

-ddH_2O to 1 L

-sterile filter by means of a vacuum apparatus with a 0.22µM filter membrane

-500µl Tween-20

-ELISA stop solution

-97ml ddH_2O

-3ml (1M) HCl

-Coomassie stain

-0.5g Coomassie Brilliant Blue R-250, dissolved in

-200ml methanol

-50ml glacial acetic acid

-ddH_2O to 500ml

-Destain I for protein gel

-500ml methanol

-100ml glacial acetic acid

-ddH_2O to 1 litre

-Destain II for protein gel

-50ml methanol

-70ml glacial acetic acid

-ddH_2O to 1 litre

-Running buffer for Western blot

-3.03g Tris base

-14.4g Glycine

-1.0g SDS

- ddH_2O to 1 litre

-Transfer buffer for Western blot

-2.42g Tris base

-11.25g Glycine

-200ml methanol

- ddH_2O to 1 litre

-Wash solution for Western blot

-2.0 litres PBS, pH 7.4

-1ml Tween-20

-Blocking solution for Western blot

-5g skim milk powder

-Wash solution for Western Blot to 100ml

Bacterial Cell Culture

-LB Miller culture broth

-10g Tryptone

-5g yeast extract

-10g NaCl

-ddH_2O to 1 litre

-autoclave at 121°C

-LB Miller agar plates

-15g to the above culture broth ingredients

-ddH_2O to 1 litre

-autoclave at 121°C

-25ml warm agar per sterile empty Petri plates

Animal Cell Culture

-Supplemented F12-K medium

-500ml F12 Nutrient Mixture, Kaighns Modification (Invitrogen, Burlington, ON)

-6ml L-glutamine

-6ml Penicillin-Streptomycin

-50ml Dialyzed, heat-inactivated FCS (Gibco)

-Supplemented GMEM medium

-500ml Glascow Minimum Essential Medium

-6ml Non-Essential Amino Acid Solution (100x)

-6ml Sodium Pyruvate solution, 100mM

-6ml Penicillin-Streptomycin

-7.5ml L-glutamic acid & L-asparagine solution in GMEM (1.2g/L)

-4.76ml 125x Nucleosides (A, G, C, U & T)

-50ml Dialyzed, heat-inactivated FCS (Gibco)

-Supplemented RPMI medium

-500ml RPMI-1640 Medium with 2.05mM L-glutamine (HyClone Laboratories Inc, South Logan, Utah, USA)

-50ml heat-inactivated FCS (Gibco)

-6ml Penicillin-Streptomycin

Appendix C: List of Abbreviations

A	Adenine
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
Amp	Ampicillin
APC	Antigen-presenting cell
bp	Base pairs
BSA	Bovine Serum Albumin
C	Cytidine
CDR	Complementarity determining region
C_H1	Heavy chain constant domain 1
C_H2	Heavy chain constant domain 2
C_H3	Heavy chain constant domain 3
Cκ	Kappa-type constant light chain gene
C_λ	Lambda-type constant light chain gene
CHO	Chinese hamster ovary
CRFs	Circulating recombinant forms
CSWs	Commercial sex workers
CTL	Cytotoxic T- lymphocyte
ddH_2O	Double-distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminescence
EtBr	Ethidium bromide
Fab	Fragment antigen binding
FCS	Fetal calf serum
FGT	Female genital tract
FISH	Florescent in situ hybridization
FR	Framework region
G	Guanosine
gp41	Glycoprotein 41
gp120	Glycoprotein 120
GS	Glutamine synthase
HESN	HIV-exposed seronegative
HIV	Human immunodeficiency virus
HIVIG	HIV immune globulin
HLA-I	Human leukocyte antigen class I
HLA-II	Human leukocyte antigen class II
HRP	Horse Radish Peroxidase
HTLV	Human T-cell leukemia virus
IDUs	Intravenous drug users
IRF-1	Interferon regulatory factor-1
kB	kilobase
kDa	kilodalton

LAV	Lymphadenopathy-associated virus
LB	Luria-Bertani broth
LTR	Long terminal repeat
MALDI-qTOF	Matrix-assisted laser desorption ionization quadrupole time-of-flight
MHC-I	Major histocompatibility complex I
MHC-II	Major histocompatibility complex II
MPER	Membrane-proximal external region
MSX	Methionine sulfoximine
NHP	Non-human primate
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIC	Pre-integration complex
pIgR	Polymeric immunoglobulin receptor
PRR	Pattern recognition receptor
s-IgA	Secretory IgA
SC	Secretory component
ScFv	Single-chain variable fragment
SIV	Simian immunodeficiency virus
STI	Sexually transmitted infection
T	Thymidine
TBE	Tris/Borate/EDTA
TMB	3, 3′, 5, 5′-Tetramethylbenzidine
TLR	Toll-like receptor
Tregs	Regulatory T-cells
U	Uridine
V_H	Variable heavy chain
V_L	Variable light chain