TARGETING GP41 AS A STRATEGY TO INDUCE MUCOSAL AND HUMORAL IMMUNITY AGAINST HIV-1.

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-- ABSTRACT –

Majority of new HIV-1 infections world-wide occur via the genital tract. Therefore, achieving effective mucosal immunity will be a critical component of vaccine strategies in the prevention and control of infection during early stages of transmission itself. Rigorous efforts have been made to identify conserved epitopes and develop rational design of immunogens, in order to elicit broadly-reactive protective Abs against HIV. Newly emerging data have highlighted the significance of Ab effector functions other than classical IgG-mediated neutralization in HIV infection. In the studies contributing towards this thesis, an optimized vaccine model is described that successfully elicits potent systemic and mucosal Abs against the highly conserved epitopes of the membrane-proximal external region and the coiled coil region of gp41. Intriguing observations are reported on the IgA-inducing capacity of the coiled coil epitope, QARVLAVERY, which highlight the potential of this epitope as an attractive candidate for mucosal vaccines. Most importantly, the epitope-specific Abs proved to be functional in neutralizing HIV in a standardized assay. With particular relevance to mucosal protection, epitope-specific IgA also effectively inhibited the transcytosis of HIV in an optimized transwell assay. The effect of gp41-specific Abs was also assessed against a novel panel of HIV-1 viral clones, and exhibited significant protection. These clones selectively express envelopes from viruses that would be desired targets of prophylactic immune responses, the earliest founder population in the mucosa after virus transmission.

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Ab – antibody

ADCC – antibody-dependent cellular cytotoxicity

ADCVI – antibody-dependent cell-mediated virus inhibition

AID – activation-induced cytidine deaminase

AIDS – acquired immunudeficiency syndrome

APC – antigen presenting cell

ARVs – Anti-retroviral therapies

AZT – azidothymidine

bNAb – broadly neutralizing antibodies

CA – capsid gag protein or p24

CCR5 – chemokine co-receptor 5

CD – cluster of differentiation

CR – complement receptor

CTL – cytotoxic T-cell lymphocytes

CXCR4 – chemokine co-receptor 4

CVL – cervico-vaginal lavages

DC – dendritic cell

DC-SIGN – dendritic cell specific intracellular adhesion molecule-3 grabbing non-integrin

DNA – deoxyribose nucleic acid

ELISA – enzyme linked immunosorbent assay

ELISPOT – enzyme linked immunospot assay

Env – HIV envelope glycoprotein

EU – exposed infected

Fc – crystallizable fragment region of an antibody

FcR – Fc binding receptor

Gag – group specific antigen

GalCer – galactosyl ceramide

gp41/ gp120/ gp160 – glycoprotein 41 or 120 or 160

HAART – highly active anti-retroviral therapy

HEPS – highly exposed persistently seronegative

HIV-1 – human immunodeficiency virus type 1

HLA – human leukocyte antigen

HSV-2 – herpes simplex virus type 2

HVTN – HIV Vaccine Trials Network

Ig – immunoglobulin

IL – interleukin

IFN-γ – interferon gamma

LTNP – long term non-progressor

MA – matrix gag protein or p17

MHC – major histocompatibility complex

MMR – macrophage mannose receptor

MSM- men who have sex with men

nAb – neutralizing antibodies

NC – nucleocapsid gag protein or p7

Nef – HIV negative factor

NK cell – natural killer cell

NRTI – nucleoside reverse transcriptase inhibitor

NNRTI – non-nucleoside reverse transcriptase inhibitor

PBMC – peripheral blood mononuclear cells

pDC – plasmacytoid dendritic cell

pIgA – polymeric IgA

pIgR – polymeric immunoglobulin receptor

Pol – HIV polymerase gene

Rev – regulator of expression of viral proteins

RNA – ribonucleic acid

SHIV – hybrid virus encoding the SIV envelope and HIV viral core

SIV – simian immunodeficiency virus

SLPI – secretory leukocyte protease inhibitor

STI – sexually transmitted infection

Tat – transactivator of transcription

TLR – toll-like receptor

T-reg –regulatory T-cells

UNAIDS – The joint United Nations programme on HIV/AIDS

V(D)J – variable, diverse and joining gene segments

Vif – viral infectivity factor

V1/V2 – variable loop 1 and 2

VLP – virus-like particle

Vpr – viral protein R

Vpu – viral protein U

-- CHAPTER 1 --

INTRODUCTION

1. The Need for an HIV Vaccine

Since the discovery of the human immunodeficiency virus (HIV) in 1981, the HIV/AIDS pandemic has claimed more than 25 million lives. Despite advances in anti-retroviral (ARV) treatment, which has dramatically reduced AIDS related mortality, consistent and easy access to such treatment is still limited in parts of the world where it is needed most, namely Sub-Saharan Africa and South-East Asia. Although there are two strains of HIV, HIV-1 largely dominates the epidemic while HIV-2 is mostly restricted to Western Africa [1]. As of 2009, global UNAIDS statistics estimate that 33.3 million people are living with the disease and that there are approximately 2.6 million new HIV-1 infections each year [2]. The most ideal means to limit and retract this global pandemic would be a prophylactic therapy, in the form of a vaccine or microbicide, which could effectively prevent HIV entry and/or control its initial spread upon exposure.

HIV-1 has the highest degree of global diversity, unprecedented by any other infectious agent. It has evolved into eleven genotypically distinct subtypes or clades, A1, A2, B, C, D, F1, F2, G, H, J and K [3], and into at least 43 major circulating recombinant forms worldwide [4] that result from a genetic cross between two distinct clades. Its high mutation rate allows HIV-1 to rapidly evolve in a host, days after transmission, making it a constantly moving target for host immune responses [5]. The HIV/AIDS vaccine is often referred to as 'elusive' since all vaccine approaches against it have thus far been unsuccessful in clinical trials, largely due to the overall diversity and rapid evolution of the virus within the host [6,

7]. Current vaccines against other viral and bacterial pathogens rely primarily on the induction of antibodies (Abs). Prophylactic vaccination aims to generate long lasting sterilizing immunity, where pathogen-specific Abs would block and clear the pathogen from the host, i.e. 'neutralize' it [8]. Successful Ab-based vaccination should ideally lead to the production of memory B-cells that would proliferate into antibody secreting plasma cells within hours of subsequent exposure to a specific pathogen, producing a potent antiviral response. In contrast, a natural primary Ab response takes 10-14 days to be generated, occurring too late for HIV control [9]. Hence, a form of prophylactic intervention is required to respond quickly against HIV-1 transmission and infection.

The mucosal surface is the predominant site of transmission of the virus and it is now well acknowledged that mucosal intervention may be essential for effective prophylaxis. Great efforts have been made to characterize mechanisms of virus passage, infection and submucosal cellular interactions in order to identify points of viral vulnerability to aid in the design of mucosal vaccines against HIV-1. Thus, in addition to the induction of broadly reactive immunity to address its sequence diversity, an ideal vaccine against HIV-1 must induce potent mucosal responses to target the virus at the site of entry itself.

1.1. *The course of HIV infection*

As a lentivirus belonging to the Retroviridae virus family, the pathogenesis of HIV-1 is characterized by a long interval between initial infection and the progression to acquired immunodeficiency syndrome or AIDS [10]. The clinical hallmark by which HIV-1 disease

progression is monitored is the CD4 T-cell count [11]. Figure 1 illustrates a typical course of HIV-1 infection and reflects the change in CD4 T-cell count in correlation with the fluctuating viral load in circulation indicated by plasma RNA levels. Primary HIV infection is associated with a burst of HIV viremia, followed by an asymptomatic phase indicative of viral latency in infected cells [12, 13]. Peak viremia is accompanied by a rapid decline in CD4 T-cell count which stabilizes during proviral latency [13, 14]. More recently, it has been shown that even though systemic CD4 T-cell levels stabilize as the viral set point is established, their loss at the gut mucosa throughout the course of infection is much more profound, even with ARV treatment [15, 16]. Viral set point levels and patterns of CD4 T-cell decline during clinical latency is variable, the duration of which can range from months to several years [9, 12, 17].

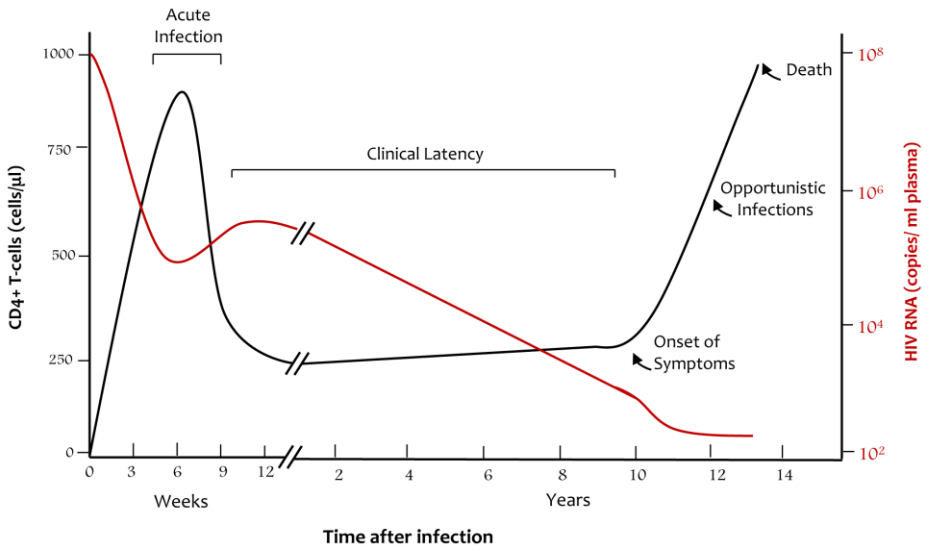

Figure 1. A typical course of HIV infection. Primary infection is characterized by a peak in viremia accompanied with a decline in systemic CD4 T-cell counts. After a period of clinical latency, circulating viral loads spike, CD4 T-cell counts drop dramatically and AIDS-related symptoms appear. *Figure adopted from Pantaleo et al, New England Journal of Medicine 328, 1993* [18].

Although multiple innate and adaptive immune mechanisms strive to control and maintain viremia at a low steady set point, without intervention, HIV-1 rebounds quickly as latently infected cells release bursts of virus [19]. Concomitant with subsequent virus spread is the rapid decline of CD4 T-cells which severely impairs the proper functioning of the immune system below 220 counts per mm^3 of blood [20]. CD4 T-cells play a critical role in the immune system and offer signaling support to CD8 T-cells, B-cells, natural killer (NK) and regulatory cells [21, 22]. Therefore, their loss leads to onset of AIDS related symptoms making the patient susceptible to fatal opportunistic infections or tumours [23, 24].

1.2. *The human immunodeficiency virus type 1*

HIV-1 consists of two strands of RNA that encode the following genes: 1. The *gag* (group specific antigen) gene produces p55, a multimeric protein comprised of the matrix (MA, p17) capsid (CA, p24), nucleocapsid (NC , p7), p1 and p6 components, which collectively constitute the core virus structure; 2. The *env* gene encodes the surface glycoprotein gp160 that consists of gp120 and gp41 subunits; 3. Accessory proteins encoded by the *nef*, *rev, tat, vpr, vpu* and *vif* genes, serve an activating or regulatory function; 4. The *pol* gene encodes the viral polymerase, which consists of the reverse transcriptase, integrase and protease enzymes [25-29].

Virus entry into target cells is a complex and multipart process. For productive binding and infection to occur, the surface glycoprotein must be in its native trimeric conformation consisting of three associated gp160 subunits, of which the gp120 trimer is

exposed and the gp41 trimer is half embedded within the virus membrane [26]. HIV-1 gp120 binds to the primary human CD4 receptor, found on CD4 T-cells and macrophages, causing a conformational shift exposing adjacent glycoprotein domains that subsequently bind to a chemokine co-receptor CXCR4 or CCR5 [30]. Co-receptor usage determines the tropism of the virus: typically, CXCR4-tropic viruses infect CD4 T-cells with efficiency and CCR5-tropic viruses infect macrophages [31]. The final virus entry steps are mediated by gp41. Briefly, the N- and C- termini of gp41 undergo conformational changes, where they disassociate to 'spring open' to form a bridge between virus and host membranes, subsequently leading to fusion, as demonstrated in Figure 2 [32, 33]. Membrane fusion allows viral RNA entry into host cell where viral reverse transcriptase (RT) converts viral RNA to DNA using host cell machinery prior to its integration into the host genome, by which the virus subsists latently within the infected cell. The high error rate of the reverse transcriptase and the lack of any corrective transcription enzyme are responsible for the enormous genetic diversity of HIV-1.

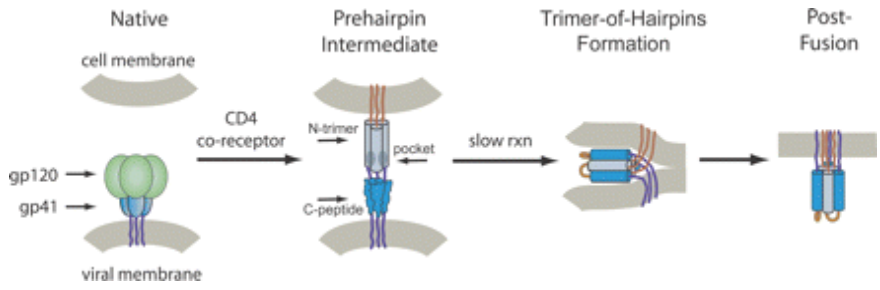

Figure 2. The HIV entry pathway. Upon recognition and attachment of HIV gp120 to CD4 and a coreceptor on the host cell membrane, gp120 and gp41 undergo conformational changes. This results in the exposure of the N-trimer and its hydrophobic pocket forming the prehairpin intermediate. This transient formation provides a short but effective window for fusion inhibitors to block the virus/ host cell membrane fusion process. The process continues as the N- and C- termini of gp41 form the trimer-of-hairpins and leads to the fusion of both cell and virus membranes. *Figure from Welch et al, PNAS 43, 2007* [32]. *Copyright 2007, National Academy of Sciences, USA. Permission to re-print granted, please see communication in Appendix III.*

1.3. *Highly active anti-retroviral therapy (HAART)*

Administration of HAART or the multiple drug 'cocktail' was a strategy employed to add pressure and to challenge the viral 'fitness' of HIV-1, in order to counteract its rapid mutation rate. By co-administering drugs of 2 or more classes, a combination therapy can target independent mechanisms so that newly emerging viral resistance against one drug can be simultaneously overcome by another in the 'cocktail' [46]. Thus, when administered appropriately, HAART can significantly lengthen the treatment window prior to the emergence of ARV resistant strains compared to single drug regimens [47, 48]. However, other than regular monitoring of a patient's immune function, persistent issues such as adverse drug reactions, long term drug side effects [49, 50], regimen adherence [51] and co-treatment of other acute and chronic health conditions [52, 53], make the management of HIV/AIDS a highly complex matter. Some serious chronic side effects of HAART include hepatotoxicity [54, 55], lipodystrophy [56], insulin resistance [57] and the development of cardiovascular disease [58, 59], which require monitoring.

Life-long ARV treatment and long-term health monitoring is not always accessible in regions highly affected by the pandemic, given high drug costs and poor health care infrastructure. The average annual cost to treat one patient with single drug therapy ranges from US$ 55-400 and is about US$ 230 for combination therapy when purchased through the Global Funds to Fight AIDS, TB and Malaria initiative, despite the initiative's efforts to increase accessibility to treatment [60]. These factors represent an urgent need to develop a

safe, affordable and effective prophylactic vaccine, which could significantly reduce the occurrence of new HIV-1 infections worldwide and eventually curb the pandemic.

1.4. *Generation of pathogen-specific adaptive immunity*

Vaccines are a modality used to induce a pre-existing immune response against a specific pathogen to prevent manifestation of disease or to curb disease progression upon actual exposure, in order to maintain host health.

Induction of innate immunity is the primary response to first-time pathogen exposure. It is triggered by the recognition of conserved, non-specific pathogen-associated molecular patterns that are often abundant in microbial or viral pathogens and consist of repeating subunits [61]. Hence, they are recognizable by the host as 'foreign' motifs even in the absence of pathogen-specific immunity. Innate activation generally occurs in non-lymphoid tissues and triggers the production of inflammatory cytokines, thereby recruiting and activating innate cells that mediate pathogen clearance by phagocytosis (macrophages, DCs) or by killing compromised host cells (NK cells) [61-63].

Antigen presentation of pathogen-specific peptides by innate cells, such as DCs and macrophages, creates the bridge from innate immunity to adaptive immunity. Pathogen capture, internalization and degradation in antigen-presenting cells (APCs) produces short peptide sequences within lysosomal vesicles that are loaded onto and subsequently displayed by major histocompatibility class II antigens (MHC II) in exogenous antigen processing [64]. Alternatively, in endogenous processing antigens are synthesized within the host cell, as in

the case of virus infection, and degraded to short peptides by proteasomes to subsequently by presented by MHC class I antigens (MHC I) [64]. Activated APCs, primed with antigen, traffic to lymphoid tissues to activate naïve B-cells and T-cells and generate effector clonal populations that are specifically responsive to the antigen. Generally, MHC I presentation stimulates CD8 T-cells to become cytotoxic lymphocytes (CTL) that induce the death of cells infected by the pathogen, whereas MHC II presentation stimulates type 1 (Th1) and type 2 (Th2) helper CD4 T-cells that modulate cell-mediated and humoral immunity, respectively, within a specific cytokine environment supported by co-stimulatory signaling [62].

Naïve B-cells also play a key role as an APC for the generation of Abs against a pathogen. The binding of an antigen to the B-cell receptor activates resting B-cells, which engulf the antigen to process it into peptides that are displayed by MHC II molecules on the B-cell surface [62]. The loaded MHC II molecules bind their counterpart T-cell receptor on helper cells that are sensitized to the same antigen, for complete signaling in order to produce antigen-specific Abs [62]. Activated antigen-specific B-cells further differentiate into Ab-secreting plasma cells or memory B-cells. The generation of pathogen-specific Abs by B-cells upon primary exposure takes approximately 12-14 days, and in the case of HIV infection, this occurs too late after virus dissemination and establishment of productive systemic infection.

Acquired immunity by natural exposure protects the host in subsequent exposures to the same pathogen strain by rapid generation of effective and robust pathogen-specific responses. Acquired immunity induced by vaccination can also protect the host at primary

exposure to the pathogen and prevent disease, particularly in cases where host immunity may be unable to control and clear infection by innate or primary adaptive immune responses.

1.5. *Preclinical vaccine testing of HIV vaccine candidates*

An appropriate animal model to test a vaccine is critical for accurate determination of its efficacy. Felines and simian/ non-human primates (NHP) are the only other natural host of HIV-like immunodeficiency viruses. The course of the simian immunodeficiency virus (SIV) infection and the arms of immunity activated by it closely mimic HIV-1 [65]. Indeed, much of our knowledge of HIV-1 transmission and pathogenesis comes from NHP studies [66]. SIV models have also been extensively developed to evaluate the safety and efficacy of new vaccine candidates [67].

SHIV is a hybrid recombinant infectious virus consisting of an HIV-1 surface glycoprotein and a SIV viral core [68]. SIV has an approximate50% sequence homology to HIV-1 [69], therefore SHIV hybrid viruses are used in simian 'challenge' models to assess HIV-specific responses induced by vaccines. Models of SIV/SHIV immunization and challenge are diverse, ranging in the immunization regimens (vectors and adjuvants used, frequency and routes of immunization), the routes of virus challenge (parenteral [70], intravenous [71], oral [72], vaginal [73] or rectal [67]) and dose distribution [74]. Low dose, repeated vaginal challenge of virus is the most clinically representative model for sexual transmission of HIV-1 [74, 75].

Once a vaccine candidate exhibits pathogen-specific efficacy in proof-of-concept animal studies, it enters clinical testing which consists of four consecutive phases. If a vaccine enters phase I clinical trials, its safety, tolerability and dosage options are closely evaluated in a small testing group (20-100 individuals) [76]. Phase II testing expands into a larger study group (100-300 individuals) where the initial efficacy of the vaccine is determined. If a vaccine candidate proves to elicit effective immunity and is successful in curbing infection rates, it enters phase III to undergo multicenter randomized control trials for more definitive assessment of its efficacy and possible long-term side-effects prior to public use [76]. Monitoring the efficacy or side-effects long term and conducting any post approval studies constitute Phase IV. No HIV vaccine candidate has ever progressed past phase III testing and majority do not succeed past phase I [77]. Section 4 of the introduction further explores the key clinical trials conducted thus far, with a particular focus on Ab-inducing vaccine strategies.

2. Mucosal Transmission of HIV

HIV-1 is a sexually transmitted infection and approximately 80% of the epidemic is fuelled by heterosexual transmission via the genital mucosa [2]. Infected semen or vaginal fluids can be transferred via the rectal and oral mucosa, infected plasma or the virus may be transmitted intravenously, and in the case of vertical transmission, infected breast milk can transmit the virus from a mother to her baby in-utero [78]. Statistically, HIV affects both men and women in equal proportions globally [2], however, women are a highly vulnerable group as they are 4-5x more likely to contract infection than men and can vertically transfer infection to their children during pregnancy. Much current knowledge of HIV mucosal transmission exists in the context of the female genital tract. The mucosal landscape is complex and affected by various factors in healthy and disease states. The following sections summarize the critical aspects of the female and male genital tracts involved in HIV-1 transmission and infection, and review key factors affecting the probability and rate of infection.

The earliest events involved in mucosal HIV-1 transmission are complex and their precise unfolding still remains unclear. Non-human primate models of infection have shown that a narrow window of opportunity exists to control virus passage across the epithelium lasting merely a few hours [79]. Figure 3 is a simple illustration of the major events in female mucosal transmission and the time frame in which they occur. As shown, there is a window of opportunity, within initial days of exposure, for mucosal prophylactic therapies to

intervene in viral transmission at the female genital mucosa and subsequent infection events leading up to systemic dissemination.

Despite its expansive global spread, HIV has a surprisingly low natural rate of transmission. In a cohort of discordant heterosexual couples, the reported rate of transmission ranged from 0.0001-0.0040 per unprotected coital act [80]. This low rate of transmission is suggestive of some form of natural protective barrier or immune control at the mucosa. Although other factors may be involved, one suggested correlate of mucosal protection against HIV is IgA [81]. However, the strategic induction of IgA and its protective functions have been undermined in vaccine studies, relative to IgG. The lack of knowledge of IgA-inducing HIV epitopes along with limited assessment and characterization of protective IgA functions in simian and human vaccine trials, are a considerable drawback in the field for the development of prophylactic therapies for mucosal protection.

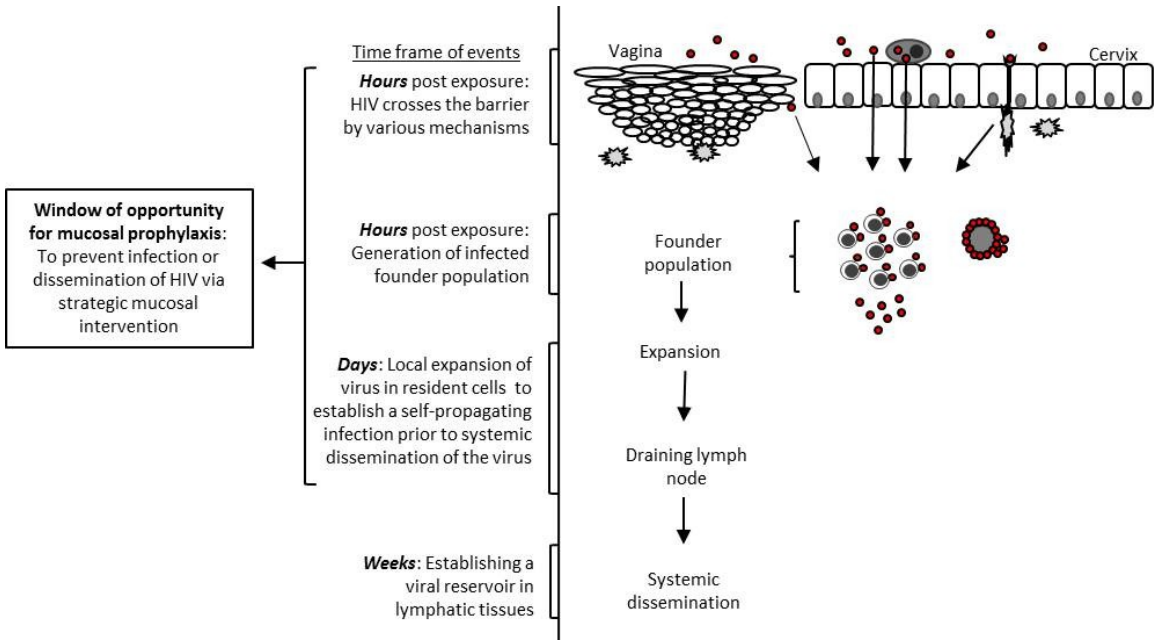

Figure 3. Illustration of the time frame of the major events post vaginal transmission of HIV-1. Virus entry may occur at the epithelium within hours and a founder population expands by infecting local resting CD4 T cells in the subsequent days. The founder population disseminates systemically to establish a self-propagating infection within weeks of initial exposure. *Figure is loosely adopted from Haase AT, Nature Reviews 464, (2010) [82].*

2.1. *Crossing the barrier: female genital tract*

Exposure to STIs occurs at the lower female genital tract which is comprised of 3 distinct regions based on physiology and function: the vagina, ectocervix and endocervix. Whereas the mucosal surface at the vagina and ectocervix consists of a multilayered stratified epithelium, the endocervix mucosa is lined with a single layered columnar epithelium similar to the gut mucosa [83]. The transformation zone is a transitional region at the anatomical cervix, where the epithelial lining transitions from thick squamous epithelium of the ectocervix to the thinner columnar layer of the endocervix [84]. Despite physiological differences, HIV-1 renders all three regions of the female genital tract prone to infection [83-86]. The ectocervix region of the female genital tract is the first site of exposure to the HIV-1. Its epithelial lining expresses HIV receptors, CD4 and CCR5, but not CXCR4 [87], by which R5-tropic viruses may bind and enter cells [88]. However, mechanisms such as transcytosis, receptor-mediated endocytosis and dendritic cell capture are thought to be the predominant mechanisms of infectious viral passage across the epithelium. Micro-abrasions are detected in as many as 60% of women following intercourse which compromises the integrity of the physical barrier, thus, significantly increasing exposure of HIV-1 to its target cells submucosally.

Infection initiated by cell-associated virus has been shown to be more rapid and efficient than cell-free virus infection [89]. It is hypothesized that donor HIV-infected cells or cells that have endocytosed HIV-1 can form viral synapses with the host epithelial cells for direct transfer of the virus so that it traverses the epithelial layer by transcytosis [90]. Transcytosis is a mechanism by which trafficking across a tight polarized epithelial monolayer occurs via selective and rapid transcellular vesicular transport from the apical to the basolateral pole [91, 92]. Transcytosis primarily occurs across the columnar epithelium of the endocervix, rectum and small intestine. Several *in vitro* models of transcytosis have been designed to test the effectiveness of antiviral agents in preventing the passage of HIV-1 across a simulated mucosal monolayer [93-96]. Epithelial cells are grown as monolayers in transwells that are incubated in a deeper well, entrenched in media, creating a basolateral and apical compartment [95]. Trans-epithelial resistance is measured across the monolayer to ensure formation of tight junctions and adjacent cell-to-cell impermeability [94]. IgA, the primary functional immunoglobulin isotype at mucosal surfaces, has been shown to inhibit transcytosis of HIV-1 in such *in vitro* models [93-95].

HIV may also traverse multilayered stratified mucosa by simple interstitial diffusion. Stratified squamous epithelium consists of five zones which become progressively flattened and keratinized from the basal to the top layer [97]. Along with the formation of desmosomes and aggregation of intercellular lipoidal material, squamous epithelium has long been considered to have limited permeability to viruses [97]. However, recent models using human tissue explants from genital tissue [98, 99] and fluorescently tagged HIV-1 *in vitro* [100] have

demonstrated interstitial diffusion of HIV, even in the absence of tissue abrasions and with no evidence of loss of integrity of tight intercellular junctions [99]. Interestingly, and quite simply, acidic mucus secreted by epithelial cells can effectively trap the virus and block HIV diffusion in similar explant models [101].

2.2. *Crossing the barrier: male genital tract*

Due to the relative inefficiency of female-to-male HIV-1 transmission (~0.001%) compared to the male-to-male via the rectum (~0.005%) or male-to-female scenarios (~0.003%) [102], transmission across the male genital tract is far less characterized or described compared to the female genital tract. The route of sexual transmission of HIV-1 in men is either through the penis or the rectal mucosa. Circumcision or removal of the penile foreskin has been the first prophylactic measure to significantly reduce heterosexual HIV transmission rates thus far [103-105]. Although there has been evidence implicating compartmentalization of HIV-1 within the male genital tract upon infection [106, 107], questions about the exact location of the viral reservoir or the origin of HIV-1 in semen still remain unanswered [108].

Recent studies have attempted to decipher mechanisms and the functional role of immune cells at the male genital tract using tissue explants *in vitro*. The adult foreskin consists of a multilayered stratified squamous epithelium with an inner and outer layer, where the latter is more heavily keratinized. Thus far, there are conflicting results on the relative target cell densities in the inner and outer foreskin compared to the glans penile

tissue, with respect to langerhan cells and CD4 T-cells, although they are present in both tissues [106, 107]. Macrophages and DCs are typically present in the dermis region below the stratified epithelial barrier. The success of circumcision in preventing HIV transmission is attributed to the change in microbiome that results from the removal of the outer foreskin [109]. The presence of anaerobic bacteria under the foreskin in uncircumcised men was found to create a pro-inflammatory environment that increased the numbers and proximity of HIV-1 target cells at the epithelium [109]. Circumcision has also effectively shown a decreasing trend in the transmission of other STIs at the male genital tract, in turn decreasing rates of HIV-1 infection and transmission [110-113].

2.3. *Virus capture and mucosal spread*

Other than CD4 T-cells, which are the primary cellular target of HIV-1, dendritic cells and macrophages also play an important role in initial virus capture, local infection and transport of virus to the draining lymph nodes for dissemination. Dendritic cells are the primary APC at the mucosa that provide a signaling link between the innate and adaptive immunity. Of the two classes, myeloid or plasmacytoid (pDCs), pDCs are relatively abundant in mucosal tissues of the genital tract, rectum and small intestine, even in the absence of inflammation. pDCs are infectable by R5- tropic HIV as they express CD4 and CCR5 [114]. In addition, the C-type lectin DC-SIGN expressed by pDCs is also able to bind gp120 with high affinity and can transfer HIV to other target cells *in trans* [115]. pDCs are considered to be

the 'trojan horses' or sentinels of HIV since they can directly capture the virus in the lumen by squeezing their dendrites across the epithelium between cell junctions [116].

Langerhan cells are a specialized subset of DCs normally found at the skin, mucosa and lymphoid tissues. In addition to expressing CD4 and CCR5, their intraepithelial positioning makes them one of the earliest targets of HIV-1 at the genital mucosa [117]. Their frequency is higher at the ectocervix than the vaginal epithelium under non-inflammatory conditions [117], however, under inflammatory conditions, they can migrate within the female genital tract and to the draining lymph nodes (LN) in response to pathogen-associated molecular patterns (PAMPs) [118]. Langerhan cells also express a C-type lectin receptor, langerin, which binds gp120 which can also mediate *trans*-infection of HIV-1 [119]. It has also been shown that at low virus concentrations, langerin bound HIV-1 is in fact efficiently internalized into Birbeck granules to be degraded [120], however, at high viral concentrations the protective effect of langerin-mediated clearance is kinetically inhibited [121] and it serves to transport the virus to target cells instead.

Macrophages reside submucosally and interact with HIV-1 only after its passage across the genital epithelium. Although they express both co-receptors CCR5 and CXC4 and have long been thought to be exclusively infected by R5 viruses, results reporting the efficiency of macrophage infectivity by X4 viruses are contradictory [122-124] and may be dependent on local cytokine environment [125]. They also express a receptor of the C-lectin family, macrophage mannose receptor (MMR), which binds mannose residues of the

naturally glycosylated gp120 trimer and assists with *trans*-infection of HIV-1 [69, 126]. Similar to langerin, MMR is an endocytic and phagocytic receptor, binding to which may lead to the uptake and eventual lysosomal degradation of the virus [127]. Interestingly MMR expression has been shown to decrease *in vivo* in chronically infected individuals [128], suggesting a possible method of innate immune modulation and evasion by HIV-1 during the course of infection.

2.4. *Genetic bottleneck*

Once past the mucosal barrier, transmitted virus replicates in resting or activated CD4 T-cells to generate a founder population, which facilitates effective dissemination of infection. Interestingly, during local expansion, only a single viral clone is propagated in approximately 80% of infections despite the heterogeneity of the donor virus quasi-species [129], and in about 20% of cases between two to five variants constitute the founder population [130]. Simian models [131-133] and multiple African cohort studies [134-136] have confirmed that the homogeneity of the transmitted/ founder population (T/F) is a result of the genetic bottleneck phenomenon, which is responsible for dramatically reducing viral diversity right after transmission. However, in the presence of other STIs, due to resultant local inflammation, the severity of the genetic bottleneck is lessened, increasing heterogeneity among founder viruses [130, 137]. Whether the observed bottleneck phenomenon is a result of a selective pressure on the transmitted viruses [138-140], of specific innate immune responses [141-143], or an entirely random process due to defective virus

transmission, competing virus replication rates, virus compartmentalization, or a combination of such events, is yet unknown and remains to be determined. However, importantly, evidence thus far implicates that the trends in glycosylation patterns and sensitivity to inhibitory Abs may be distinct between acute infection viruses and chronic infection viruses [129, 135, 144-147].

Identification of acute infection and acquisition of mucosal samples has been challenging in the past, but has been made easier due to the use of rapid testing kits and improved methodologies in mucosal sampling [148]. Recent advances in single genome amplification by PCR have allowed for accurate sequencing and phylogenetic analysis of transmitted viruses. These studies have also given a better understanding of the relationship between donor and recipient viral quasi-species. Despite the fact that transmitted viruses may be X4 and R5 tropic, the founder virus propagated in the host upon transmission almost exclusively relies on the R5 coreceptor for infection [136, 149]. Novel reagents such as replication-competent infectious molecular clones (IMC) have also been developed, which encode the R5-tropic T/F virus *env* sequences within an isogenic NL4.3 viral backbone [150, 151] referred to as the T/F Env-IMC. Such reagents can play a key role in the screening of HIV-specific mucosal responses since the T/F envelope glycoproteins more accurately represent the target of the earliest immune responses, and particularly since chronic and acute strains may exhibit differential sensitivities to Abs [136].

Viral transmission and local expansion of T/F virus represent a short but highly opportune window for intervention. Mucosal antibodies could ideally prevent the establishment of the founder population at the site of transmission itself as demonstrated in a proof of principle rhesus macaques study, where prophylactic vaginal application of the broadly neutralizing Ab, b12, protected against SHIV challenge in a dose dependent manner [152]. Furthermore, non-neutralizing Abs may also confer protection via alternative mechanisms that are elaborated in section 4.

2.5. *Other factors influencing HIV transmission*

The clinical reality is that multiple factors influence the transmission of HIV across the mucosa. For example, virus shedding is higher during the acute phase than in chronic infection [153] and higher in the presence of other STIs [154] or in the absence of treatment [155], all of which significantly increase the incidence of HIV transmission.

In addition, the thickness of the vaginal tract, its barrier integrity and the local immune environment are specifically influenced by a woman's hormonal cycle. High progesterone levels decrease the thickness of the stratified epithelium making the trans-migration of HIV across the mucosa relatively easier [156, 157]. Non-human primate models of HIV infection have shown that pre-treatment of rhesus macaques with progesterone enhanced the risk of infection more than 7-fold [158]. In contrast, estrogen application vaginally prior to virus challenge showed a strong protective effect, where none of the pre-treated animals became infected [159]. Although specific mechanisms of immune modulation

are not known, progesterone treatment can suppress the induction of an adaptive immune response [160] and regulate the production of pro-inflammatory cytokines such as TNF-α [161]. An inflammatory milieu attracts CD4 T-cells to the tissue, increasing the probability of infection. Estrogen, however, inhibits inflammatory cytokine secretion by inhibiting the transcription factor NF-κβ [162]. This implicates that prophylactic microbicides and mucosal vaccines need to be effective despite hormonal variation and the widespread use of contraception, given the influence of such factors in HIV acquisition.

The presence of other STIs can be a direct cause of tissue irritation, inflammation or ulcer formation at the genital mucosa [154, 163]. This significantly enhances the risk of infection by compromising barrier integrity and by recruiting target cells to the site due to local immune activation [164, 165]. Activation of langerhan cells and pDCs by viral RNA/DNA or viral surface proteins can directly upregulate the expression of the HIV receptors CD4 and CCR5 and initiate an inflammatory cytokine cascade [166, 167]. There is a great body of evidence supporting the causal relation between herpes simplex virus type 2 (HSV-2) infection and HIV-1 replication in co-infected individuals [168, 169]. Not only does pre-existing HSV-2 infection increase susceptibility to HIV-1 infection, but it also increases HIV-1 shedding in co-infected individuals [170]. Further, subclinical HSV-2 reactivation causes local inflammation which increases the availability of target cells for HIV infection submucosally, even in the absence of ulcer formation or tissue disruption [169, 171]. Other STIs that result in vaginitis or cervicitis (chlamydia [172, 173], gonorrhea [172, 173], trichomoniasis [174]) and genital ulcerative disease (chancroid [175, 176], syphilis [177, 178])

or genital warts (human papilloma virus [179, 180]) have also been associated with increasing the risk HIV-1 infection and viral shedding [154, 181].

2.6. *Immune-quiescence or immune activation?*

It is noteworthy that the production of cytokines and the upregulation of interferon stimulated genes (ISGs) are much greater in response to HIV-1 than other infections such as avian influenza and severe acute respiratory syndrome (SARS), in which increased cytokine levels are associated with enhanced immunopathology [182, 183]. There is a continuing debate on the role of innate immunity in early modulation of HIV-1 infection and it is hypothesized that a fine balancing act may be required for the 'right response at the right time' in order to prevent infection. It is postulated that the antiviral cascade initiated by innate responses may in fact enhance target cell availability, fuel viral replication and generation of the founder population.

Comparative infection studies between natural (sootey mangabees) and unnatural (rhesus macaques) SIV have shown that the distinct immune-modulatory responses are responsible for subtle viral control by which natural hosts do not succumb to similar immunopathology [63]. HIV-1 infection mimics the pathology and course of SIV infection in unnatural hosts. Interestingly, although robust innate immunity is associated with both pathogenic and non-pathogenic SIV infection, it is observed that innate responses subside in nonpathogenic infection during transition from the acute to the chronic phase [63]. These studies have also demonstrated that chronic immune activation is primarily responsible for

associated pathology and disease progression [184, 185]. At the gut mucosa, chronic activation leads to a significant loss in TH17 CD4 T-cells in pathogenic hosts but not in nonpathogenic hosts, disrupting the balance between CD25+ regulatory CD4 T-cells (T-regs) and TH17 cells, thereby disrupting local immune regulation [186, 187]. The subsequent translocation of microbial products into the gut, such as lipopolysaccharides, activate innate sensors like TLR-4 among other cellular components which sustain local inflammation, fuel infection and severely deplete mucosal CD4 T-cells [184]. In elite HIV controllers, however, the mucosal T-reg/ TH17 balance is not disrupted and is considered to be a central feature of virus control observed in these individuals.

It is an intriguing possibility that immune-quiescence at mucosal surfaces, upon transmission (genital) and in latent infection (gastro-intestinal), may be critical in quenching the immunopathology associated with HIV-1. Mucosal inflammation caused by STIs has also been associated with increased susceptibility to HIV acquisition, as have microbicides, such as nonoxymol-9, that contributed to significant local inflammation [188]. Therefore, the immune-modulatory effects of prophylactic therapies and their particular influence on mucosal inflammation merit serious consideration.

3. The Effect of Antibodies on HIV

HIV-1 infection induces an array of Ab responses primarily against env, gag and pol. Only recently, the functions of both neutralizing and non-neutralizing Ab responses have been investigated in vaccine studies. In addition, other than IgG, HIV-specific IgA may be of significance particularly in mucosal defense against HIV. Ab class switch recombination (CSR) is a process by which activated Ag-specific B-cells differentiate from IgM producing to IgA, IgD, IgG or IgE producing cells, by re-arranging the variable, diverse and joining (V(D)J) gene segments [189]. Each isotype confers protection by distinct mechanisms largely dependent on their microenvironment. In the following sections, the unique characteristics of systemic and mucosal Abs pertaining to HIV infection and their antiviral functions are described.

3.1. *Protective functions of IgG*

Neutralization has long been viewed as the main effector function of humoral immunity and has been considered to be the primary correlate of Ab protection in HIV infection. The antigen-binding site (Fab) of neutralizing Abs (nAbs) binds, blocks and inactivates the pathogen, inhibiting its ability to propagate infection in a host [190]. In neutralization, the pathogen is not only inactivated but subsequently cleared by opsonization and phagocytic degradation of the Ab-pathogen complex, or by the activation of the classical or alternative complement pathways [191]. The most commonly used assays utilize pseudoviruses or replicating HIV with reporter TZM-bl cell lines or PBMCs to assess Ab

mediated neutralization of HIV [192-194]. However, the binding of the Fc region of Abs to the Fc receptor (FcR) or complement receptor (CR) found on effector immune cells can also trigger clearance of HIV by alternate mechanisms that are summarized below.

IgG subtypes: IgG has 4 subclasses, each with distinct protective roles. IgG1 is the dominant class in both acute and chronic infection of HIV-1, exhibiting the broadest response to env, gag and pol proteins [195]. Further, in addition to neutralizing HIV, the IgG1 isotypes of bNAbs b12 and 2F5 can bind FcR and can mediate antibody-dependent cellular cytotoxicity (ADCC) of HIV-1 infected cells [196, 197]. IgG2 plays a role in classical HIV clearance by opsonization [198] and is generated against carbohydrate-dense regions of the surfaceglycoprotein [199]. IgG3 is the second most predominant subclass of Abs in HIV-1 infection, that may exhibit neutralizing function and the capacity to mediate ADCC [200]. It is suggested that IgG3 has greater *in vitro* neutralizing ability than IgG1 due to the enhanced flexibility of its hinge region [201]. HIV-specific IgG4 is rarely found as it is normally generated by chronic antigenic stimulation and is more readily observed in chronically infected and hemophiliac patients [202, 203].

Complement receptor mediated IgG function: The complex of serum proteins known as 'complement' mediate the lytic and inflammatory properties of antibodies. HIV activates complement throughout infection and the ultimate actions of the resulting cascade are opsonization of the pathogen or lysis of the pathogen or infected cell through the formation of a membrane attack complex [204]. Evidence suggests that the expected clearance of HIV as

a result of complement fixation is in fact inhibited by intrinsic resistance mechanisms developed by the virus [205]. Rather, HIV opsonized with IgG and complement significantly enhances virus infectivity by the process of complement-mediated Ab-dependent enhancement (C'-ADE), particularly when CRs are expressed on CD4 T-cells and macrophages, [206, 207]. CR3 is expressed on DCs and Fc-CR3 interactions have been implicated in enhancing *in trans* infection of HIV-1 by DC-SIGN [207]. Complement activation is inflammatory and can recruit target cells which would be undesirable in mucosal transmission due to the resulting immune activation [208].

Fc-receptor mediated IgG functions: ADCC responses link the innate and adaptive immune systems. HIV-infected CD4 T-cells that display HIV-specific Ab are killed by innate cells such as NK-cells, macrophages, dendritic cells, neutrophils or γδ T-cells, upon direct activation of a IgG Fcγ receptor (FcγR) on their cell surface [209]. These could be either FcγRI (CD64), FcγRIIa/b (CD32a/b) or FcγRIII (CD16) [209]. As mentioned above, bNAbs b12 and 2F5 have been shown to also confer protection by ADCC via FcγRI [197] in addition to their neutralizing function. Functionally, *in vitro* assessment of ADCC function is primarily assessed by target CD4 T-cell lysis by NK cells [210]. A caveat of this assay is that it only utilizes ones of the various effector cell type, NK cells, which express FcγRIII [209], thereby neglecting the role of other innate cells and FcRs in ADCC.

Antibody-dependent cell-mediated virus inhibition (ADCVI) is another mechanism which similarly relies on the interaction between an infected target cell, an effector cell and

IgG Fc. Instead of measuring the killing of infected cells, ADCVI assays determine the degree to which NK cells, or other innate cells, inhibit virus production and release from the infected cells in the presence of Ab. The success of this assay relies on stringent controls, however, multiple effector cell types can be used: NK cells, monocytes/ macrophages, and even unfractionated PBMCs [211]. Improvements and standardization of the ADCC/ ADCVI assays pertaining to HIV-1 infection are still underway [212], however, the relevance and potential importance of ADCC and ADCVI functions in HIV inhibition, particularly in early infection, are now strongly supported.

3.2. *Protective functions of IgA*

IgA subtypes: Humans, chimpanzees, gorillas and gibbons have two IgA heavy chain constant region (Cα) genes whereas other species (notably mouse and rhesus macaques) have just one Cα gene, changes in which give rise to the distinct IgA subclasses, IgA1 and IgA2 [213]. Evidence suggests that IgA1 is primarily generated in adaptive immunity as it is the dominant Ag-specific subtype, whereas IgA2 has a relatively broad albeit non-specific recognition pattern, and may be an innate Ab subtype [213, 214]. Furthermore, IgA1 is 80-90% dominant in nasal and male genital secretions, and 60% in saliva relative to IgA2, whereas IgA2 is 60% dominant in colonic and female genital secretions relative to IgA1 [214]. Differences in the hinge region between the two IgA subtypes may play a role in determining their distinct functions. The IgA1 hinge is longer and offers greater flexibility, allowing it to recognize Ag with higher avidity, especially distantly spaced antigenic regions, however, this

feature also makes it more prone to proteolytic cleavage than IgA2 [213, 215, 216]. This vulnerability of IgA1 may explain its lower proportion in the relatively acidic female genital tract and enzyme-rich environment of the gut.

Transcytosis or HIV entry inhibition at the mucosa: As mentioned earlier, mucosal IgA may inactivate HIV-1 by blocking its surface glycoproteins and preventing virus passage across the epithelial barrier. This can be mediated by secretory IgA (sIgA) or monomeric IgA (mIgA), with poly-specific or HIV-specific activity. The function of such IgA is primarily measured by transcytosis assays in vitro, which have been briefly discussed earlier and are further elaborated in chapters 3 and 4. Monomeric IgA found in genital secretions primarily diffuses into the lumen from the systemic circulation, whereas dimeric IgA is generally locally produced in humans [214]. In addition, mIgA is more prone to preoteolytic degradation and it has a shorter half-life than sIgA [214].

Fc-mediated function of IgA: IgA also has a unique non-inflammatory Fc function. By binding to the FcαRI (CD89) found on macrophages/monocytes, DCs and neutrophils, under specific physiological conditions, it can play an essential anti-inflammatory role. Monomeric IgA binds to FcR and by association with the immunoreceptor tyrosine-based activation motif (ITAM) can induce an inhibitory state, down-regulating cellular activation signals [217]. Such FcR binding of non-specific IgA at the mucosa can down-modulate inflammation and immune activation which is desirable for healthy mucosal tissue [218]. This effect is reversed in an exacerbated immune state however, where IgA forms complexes at the ITAM signaling

cellular activation, generally followed by the release of pro-inflammatory cytokines [219]. IgA mediates pathogen clearance upon FcαR binding by mechanisms of phagocytosis, cytokine release and ADCC [214, 220, 221]. However, in contrast to IgG, it does not activate complement which could be detrimental in HIV-1 infection due to enhancement of local inflammation [208]. When compared directly, target opsonization at similar IgG1, IgA1 and IgA2 levels led to significant enhancement of complement activation with IgG1, whereas IgA1/2 reduced complement binding. Further, this effect was consistently more pronounced for IgA2 than IgA1 which may be due to its relative resistance compared to IgA1 [220].

3.3. *Mucosal antibodies*

The genital mucosa exhibits certain unique characteristics compared to the lung or GI mucosa, particularly in the ratio of predominant isotype in local secretions. There is a significantly higher contribution of circulating Abs in genital secretions which is evidenced in the genital IgG:IgA ratio which closely mimics systemic levels [222]. In contrast, IgA is typically the abundant isotype at the lung and intestinal mucosa, the source of which are primarily local B-cells and not systemic Abs that have diffused into local tissue [223].

Antibody transport: Secretory IgA (sIgA), found distinctly at the mucosa, is dimeric and consists of two monomers joined in an end-to-end configuration by a joining (J)- chain, stabilized by disulfide bridges [224]. sIgA is transported to the mucosal lumen by its association with the polymeric Ig receptor (pIgR) at the basolateral pole, where the J-chain binds to the secretory component of the pIgR [224, 225]. The dimeric IgA-pIgR complex is

endocytosed for transportation across the epithelium to the lumen where sIgA is released upon cleavage of the secretory component and the pIgR is recycled. This mechanism only allows the passage of mucosal dimeric IgA and not monomeric IgA, which is predominant in the serum and lacks the J-chain by which it cannot associate with the secretory component or pIgR. In addition to dIgA, pentameric IgM (pIgM) also utilizes pIgR transport [225]. Generally, mucosal sIgA and pIgM are considered to be polyspecific with low affinity but broad reactivity against microbial antigens and are primarily produced by innate B1-cells [226, 227]. Ab transport via pIgR is dependent on local innate and environmental factors that modulate pIgR expression. For example, enhanced presence of microbial factors such as lipopolysaccharide or ssRNA, higher estradiol levels, or the presence of pro-inflammatory cytokines INF-γ and TNF-α, can all upregulate pIgR expression basolaterally to enhance sIgA transport to the lumen [224]. Functionally, the pIgR-pIg structure may even inhibit HIV within endosomes by intraepithelial neutralization since they form stabilized structures that are protected from proteolytic degradation [224, 228].

As an alternate mechanism, trans-epithelial transport of IgG across intestinal and genital epithelia has been demonstrated in mouse models, mediated by neonatal FcR [229, 230]. However, the biological relevance of this mode of transport in humans is still questionable.

3.4. *Role of Abs in HEPS cohorts*

Additional insights about the potential role of Abs, particularly IgA, has come from cohort studies involving high risk individuals that remain uninfected despite exposure to HIV-1, referred to as exposed uninfected (EU) or highly exposed persistently seronegative (HEPS) individuals. The data reviewed below comes from two types of cohorts; commercial sex workers that maintain a seronegative status despite being in a high risk environment and discordant couples, in which one partner is infected while the other remains seronegative.

Significantly higher serum IgA levels in HEPS individuals have been associated with resistance to HIV-1 in multiple cohort studies [81, 231, 232]. In addition, HIV-specific IgA has been detected in the serum and cervico-vaginal lavage fluids (CVL) of HEPS women that has demonstrated neutralizing ability against the virus [81, 233-235]. With greater relevance to mucosal protection, HEPS serum and CVL-derived IgA can inhibit virus transcytosis across epithelial cells *in vitro*, in transwell assays [235, 236]. Interestingly, in other HEPS/EU cohorts, IgA function was found to be associated with resistance rather than virus specific cellular immunity [237] or IgG [238]. It is noteworthy however, that these IgA trends are not consistently observed in all HEPS/EU cohorts and despite the long-standing association between HIV-specific IgA and the observed resistance, there are no implications of a direct causal link between the two.

Thus far, only a single epitope of the virus surface glycoprotein has been identified as a unique target of serum IgA that was specifically obtained from an EU cohort of discordant

couples [232, 239]. This epitope, QARILAVERY, is found at the coiled region of the N-terminus of gp41, which is critical in the virus fusion process [232]. It is further reviewed in chapter 3.

4. Evolution of Vaccine Strategies against HIV-1

Effective vaccine design relies on accurate knowledge of the correlates of protection associated with a pathogen, in order to induce relevant and effective immune responses against it. In the case of HIV-1, however, our understanding of which correlates of protection are critical and how they may be effectively exploited by vaccination is still evolving. An effective HIV vaccine would induce durable immunity for the prevention of disease acquisition or would control virus replication to slow down disease progression [240-242]. In the following sections, various strategies for induction of HIV-specific immunity are summarized as well as the largest HIV vaccine efficacy trials that have been conducted thus far. Although no vaccine has demonstrated effective protection against the virus, results from these trials have served to fill gaps in knowledge and reveal limitations that remain to be addressed by the research field for successful vaccination.

4.1. *T-cell mediated control of HIV-1*

The potential roles of antibodies in immune protection against HIV have already been reviewed in section 3. Although prophylactic mucosal Abs may contain virus at the site of transmission, as seen in the natural course of infection, once systemic infection is established, neutralizing and non-neutralizing protective Abs are insufficient in controlling viremia [18, 243]. T-cell mediated responses however, have demonstrated efficacy in suppressing virus replication and in the clearance of infected cells from the host [244].

The CTL response is the key immune correlate mediating control of viral replication and maintenance of viral set-point during latency. Ag display and presentation induce T-cell immunity, subsequent to which Ag-specific effector T-cells clear HIV-infected CD4 T-cells by various mechanisms that ultimately lead to cell death [245]. Support for T-cell based strategies strengthened once a clinical correlation was made between CTL function and suppression of HIV-infected PBMCs and by the observation that early emergence of effector T-cells correlated with long-term viral control in infected individuals [246]. Perhaps the most striking support for T-cell mediated immunity comes from the identification of seropositive individuals that exhibit natural 'elite' control of HIV-1 [247]. Long-term non-progressors (LTNPs) maintain normal CD4+ T-cell counts for 10-15 years even in the absence of ARV [247], whereas elite controllers, a subset of LNTPs, maintain plasma viral RNA levels of less than 50 copies per ml of blood during latency [248]. Extensive analysis has implicated unique host genetics (HLA type) as the key factor for robust CD8+ T-cell activation and proliferation, to which virus control is attributed. Specifically, host HLA class I alleles are associated with greater efficiency of Ag-presentation and processing of gag and pol [249]. There is also evidence supporting heightened innate immune control mediated by NK cells [250], plasmacytoid dendritic cells [251] and possibly regulatory T-cell subsets, in association with lower viral set points in LNTPs [252], although exact mechanisms are unknown. Attenuated or less virulent forms of the virus that contain mutations or deletions in key regulatory proteins such as nef [253] have also been detected in LNTPs, which would contribute to slower viral evolution [254].

Various SIV infection models have shown that CTL responses are critical for control of systemic viremia [244, 255, 256], however T-cell based vaccines presenting viral subunits or immunodominant epitopes have thus far been unsuccessful [257-259]. Despite initial control of virus replication, hosts succumb to infection due to the emergence of viral 'escape mutants' that evade Ag-specific responses and continue proliferating to propagate infection [260]. The dominant epitope-specific CTL responses are targeted against env and nef in acute infection [261], however, gag p24- and pol-specific responses carry virus control during the latency period [262]. It is now well accepted in the field that an ideal HIV vaccine would induce both mucosal Ab immunity for maximal local containment of the virus, as well as effective cell-mediated immunity for systemic control of virus replication.

4.2. *Whole-killed and live-attenuated vaccines*

In 1796, Edward Jenner introduced the concept of a 'vaccine' for the first time by using infected materials from cowpox lesions to immunize against smallpox [263]. A century later, when microbes were identified as the causative agent for infections, Louis Pasteur established the earliest basic tenets for an effective vaccine, that one should "isolate, inactivate and inject the microorganism" that caused the disease [264]. This approach led to the development of live attenuated vaccines, which are live but less virulent forms of an infectious virus, and whole killed vaccines that are non-infectious 'dead' viral components [265]. Several infectious diseases such as polio and measles are still prevented by these traditional approaches that induce both cellular and sterilizing immunity [265-268]. Polio

vaccines, for example, have been clinically administered in both forms, as the orally administered live-attenuated vaccine and the parenterally administered inactive whole-killed form [267].

Live attenuated vaccination has only recently been explored in the context of HIV, in simian models. SHIV-immunized animals were effectively protected from SIV vaginal challenge due to effective control of virus replication, mediated primarily by gag-specific CD8+ T-cell immunity [269]. However, the possibility of live attenuated vaccines to re-assort and revert to the wild-type infectious virus poses a high risk for HIV-1 vaccination and therefore is not considered as a safe option for clinical practice. In addition, the clinical trial of a whole killed HIV-1 vaccine (*Remune®*) unfortunately induced poor levels of Ab and T-cell immunity and was ineffective in significantly reducing HIV infection rates in multicenter studies [270, 271].

Live attenuated viral vaccines maintain the ability to infect host target cells, however, due to deletions of key genes, the virus loses the ability to efficiently replicate and propagate infection. Maintenance of low grade infection continuously primes the immune system yet the loss of pathogenicity of the attenuated virus protects the host from disease manifestation. *Nef*-deleted SIV mutants have been tested in simian models of infection and have demonstrated effective generation of long lasting CTL immunity [272-274]. *Nef*- deleted SIV vaccination was able to establish a low-grade persistent infection and the resulting immunity was able to protect against vaginal challenge, however, the precise immune correlates of

protection still remain unclear [275]. Although this approach demonstrated efficacy in the simian model, the high risk of such therapy has dampened its progress, particularly in light of the possibility that the attenuated strain may revert to wild-type by recombination over time.

4.3. *Env subunit vaccines*

Soluble env-based immunogens were the first candidate vaccines to be assessed in clinical trials with the hypothesis that they would elicit nAbs for sterilizing immunity. Antigens delivered as whole or partial soluble subunit vaccine are processed exogenously by activating MHC II pathways. Since the goal of earlier vaccines against HIV-1 was to induce sterilizing immunity by Abs, immunization regimens were primarily based on the glycoprotein subunit.

The very first env-based immunogen to be tested for safety and efficacy in Phase I/II trials was a monomeric recombinant gp160, which entered clinical trials in 1987 [276]. However, in simian challenge studies, gp120 proved to be superior in eliciting nAbs capable of protection against high dose systemic viral challenge [277]. Various forms of the surface glycoprotein have been tested in animal models, derived from diverse strains, such as monomeric gp160 [277], monomeric gp120 [278-281], bivalent gp120 [282], dual gp120 and gp160 co-administration [283] and a trimeric gp140ΔV2, where the highly variable V2 region was deleted from gp160 [284]. However, none of these forms have proven to be potent immunogens capable of efficient prevention of HIV infection.

Many of the earliest Ab-inducing subunit vaccines used gp160/gp120 sequences that were genotyped from lab-adapted HIV-1 strains or from clade B strains that are widespread in North America and Europe. The Abs they generated showed variable and sometimes potent neutralization against lab-adapted virus strains, however, failed to exhibited poor activity against primary clinical strains *in vitro* when assessed in later studies [285, 286]. This highlights the technical limitations of preclinical vaccine testing at the time and the significance of using clinically relevant virus panels and standardized testing to assess Ab function. The International AIDS Vaccine Initiative (IAVI) formed a 'neutralizing antibody consortium' in 2002 to standardize preclinical assessment of nAbs and to develop panels representative of viral diversity, that are divided and tiered by their difficulty to be neutralized [287]. A new standard for advancement of nAb-inducing vaccines past phase I is that they must at least exhibit neutralizing ability against tier 2 and 3 virus panels [288].

4.4. *The Vaxgen gp120 phase III efficacy trial*

The first large HIV phase IIb/III trial (VAX 004) was conducted from 1997 to 2002, across the Netherlands, United States and Canada, assessing a monovalent clade B gp120 vaccine developed by VaxGen referred to as AIDSVAX® B/B, since clade B was the predominant infectious strain in those global regions [289, 290]. VAX 004 tested the vaccine in a total of 5417 test subjects consisting of two subgroups: men who have sex with men (MSM) and heterosexual women. The study was expanded to a larger randomized, double-blind, phase III efficacy trial (VAX 003) when an additional recombinant gp120 immunogen

was added to the vaccine, derived from a clade A and E circulating recombinant form (CRF01_AE), which was the predominant strain in Thailand, after the safety and immunogenicity of the CRF01_AE rgp120 was assessed in Phase I and II trials among Thai populations [282]. VAX 003 tested the bivalent gp120 vaccine referred to as AIDSVAX® B/E in Thailand between 1999 and 2003 among a test sample of 2546 male and female intravenous drug users. In both trials, vaccinees received intramuscular immunizations of the recombinant protein adsorbed onto alum at 0, 1, 6, 12, 18, 24 and 30 months [290].

Neither of the VAX 004 or 003 trials demonstrated significant efficacy in lowering infection rates compared to placebo control groups in similar test populations [291, 292]. A preliminary study conducted in chimpanzees formed the basis of this trial, which showed that the administration of recombinant gp120 derived from the clade B MN strain protected animals against a heterologous viral challenge consisting of lab-adapted viruses MN, IIIB and SF2 [293]. The assessment of the Abs induced in this study, published in 1996, relied on virus panels and neutralization assays that limited the determination of broadly protective antiviral Ab responses. Despite demonstrating efficacy in the simian challenge model, MN-gp120 failed to elicit robust immunity in humans and most likely, did not elicit the appropriate breadth of responses to protect against primary transmitted viruses in a large population [243, 290]. Thus the IAVI panel of primary viruses has been developed as a tool to screen breadth of neutralizing ability and to appropriately examine the efficacy of a vaccine candidate prior to its entry into clinical trials, especially one that primarily relies on the induction of nAbs.

The failure of gp120 as an immunogen in clinical trials dampened efforts supporting Ab-based vaccines and shifted to the development of T-cell based strategies. Further analysis and crystallographic characterization of the surface glycoprotein also revealed that majority of its neutralizable regions are sterically hindered by heavy glycosylation or are inaccessible due to their location in recessed pockets of the inner core [294]. It is also known that the glycoprotein exists as a trimer in its native form which itself is covered by a glycosylated sheath and subunit-subunit interactions of the trimer further conceal neutralizable domains [295, 296]. Therefore, even if protective Abs were elicited by vaccination, the vulnerable regions of the glycoprotein in its native glycosylated form may remain inaccessible to the Abs. This may explain why recombinant gp120-based immunogens as monomers, or as trimers stripped of glycosylation, fail to elicit functional Abs against HIV. Unfortunately, glycosylated forms of gp120 have been found to be significantly weaker immunogens than the protein subunit alone [297].

4.5. *HIV and neutralizing antibodies: after the Vaxgen gp120 trials*

Despite humbling results of past clinical trials, Ab-based vaccine approaches were revived upon the identification of highly potent IgG-Abs from seropositive individuals, that were protective against a broad range of viral clades: 1b12, 2G12, 2F5, 4E10, Z13 [298]. These broadly reactive nAbs (bNAbs) were naturally generated and were unique in their affinity and breadth of effector function, as determined *in vitro* against various panels of HIV-1 lab and primary strains [299-301]. The ultimate support for bNAbs came from studies in which

passive immunization of neonatal macaques with a bNAb 'cocktail' successfully protected against a subsequent pathogenic SHIV challenge [302, 303]. In addition, bNAb cocktails have conferred protection post-exposure in a SHIV 89.6 infection model in neonatal macaques, postulating that effective nAbs may be able to prevent peri- and postnatal HIV transmission [304]. Phase I trials have shown that 2F5, 2G12 and 4E10 Abs are safely tolerated in clinical administration [302], however no clinical trials have been conducted to determine passive immunization efficacy of the bNAbs against HIV-1 infection in humans.

Monoclonal forms of the bNAbs have been generated to determine binding specificity and binding properties. 1b12 targets a gp120 pocket which spans the CD4 binding site [305]; 2G12 recognizes mannose clusters located on the surface of the 'silent' gp120 glycan shield [306]; 2F5 binds the linear ELDKWA epitope at the C-terminus of gp41 whereas 4E10 and Z13 both bind the NWFDIT epitope also at the C-terminus of gp41 [307]. Together, the ELDKWA and NWFDIT epitopes span the membrane-proximal external region (MPER) of gp41, which is highly conserved and has been extensively characterized as a potent immunogen in mice, rabbits and simian models, as well as in human studies [299-301, 308, 309]. Importantly, the MPER region and its epitopes offer greater flexibility for incorporation in vaccine strategies since they are not conformation dependent [310]. In addition to inducing nAbs, the ELDKWA epitope of the MPER also induces protective IgA, capable of preventing HIV transcytosis *in vitro* [309, 311].

The fact that broadly reactive nAbs could indeed be generated naturally despite the scarcity of accessible neutralizable glycoprotein targets was optimistic news. However, it is now known that bNAbs are only found in approximately 25% of chronically infected individuals and rarely ever in acute infection [312, 313]. Furthermore, independent groups have shown that both serum-derived and monoclonal forms of the 2F5 and 4E10 Abs can bind to ubiquitous auto-antigens such as membrane-bound phospholipids and cardiolipin *in vitro* [314, 315]. It has been hypothesized that the clearance of MPER-specific B-cells due to auto-reactivity of the Abs may be cause for the rare occurrence of 2F5 and 4E10-like Abs in infected individuals [316]. In contrast, studies have also implicated that anti-cardiolipin Abs are independently associated with MPER-specific Abs in chronic infection [317]. An alternate proposed explanation for the rare occurrence of MPER-specific Abs is that since MPER is only transiently exposed during the formation of the intermediate hairpin [44], it is a relatively poor immunogen in natural infection, although MPER epitopes are indeed immunogenic in vaccines. The possible auto-reactivity of the MPER-specific Abs has raised concerns over their passive administration in clinical trials.

Another concern for nAb-based approaches arises from new evidence suggesting that Abs may need to undergo affinity maturation to acquire broadly neutralizing capacity. More recently, a new set of bNAbs have been identified that exhibit exceptional potency and breadth of protection against HIV: PG9, PG16, VRC01 and HJ16. Interestingly, PG9 and PG16 are remarkably potent and could prevent infection of more than 70% of a panel of 162 strains tested *in vitro*, at relatively minute concentrations [318]. Crystallographic analysis

shows that they bind regions of the gp120 variable loops in the native trimeric spike conformation, but do not bind monomeric gp120 [318]. VRC01 and HJ16 recognize regions in the CD4 binding site of gp120 [319, 320]. Interestingly, the PG9, PG16 and VRC01 Abs undergo exceptional affinity maturation which is indeed a requisite for their function [319, 321]. The bNAbs PG9/16 and VRC01 differ from their germline version by more than 20% and 30% respectively at the variable loop binding region, and their germline-reverted forms show poor or no affinity against gp120. The bNAbs b12, 2F5 and 2G12 also differ from their germline versions by approximately 20%, and their reverted forms also lose measurable binding capacity to gp120 [322]. The need to undergo affinity maturation explains why bNAbs are not generated till about two years after infection. This new finding implies that multiple, long-term immunizations may be required for the effective generation of bNAbs.

4.6. *New directions for env-based immunogens*

The simian and clinical trials mentioned earlier, testing recombinant gp120 based immunogens, failed for three key reasons: 1) the overall response did not protect from infection, 2) the neutralization breadth of the antibodies elicited was poor, which can be attributed to the diversity of the variable loops of gp120, its most immunogenic region, and 3) the potency of antibody induction was insufficient [278, 281, 289, 323, 324]. Thus, novel approaches have been explored to enhance the immunogenicity of the envelope glycoprotein and to better characterize epitope-Ab interactions [243]. Subunit immunization generates a vast polyclonal response consisting of both nAbs and non-nAbs [323, 324] which

hypothetically could also have been a contributing factor for the lack of efficacy of the Abs elicited by the gp120 Vaxgen vaccine. One strategy to enhance the generation of nAb over non-nAbs is to develop immunogens that can focus the immune response towards specific and desirable neutralizable regions instead of the entire subunit [325, 326].

Crystallographic structure studies of the glycoproteinin monomeric and trimeric forms have revealed that conserved regions of gp120 are found within the CD4/ co-receptor binding domain and that Abs against them are conformation dependent in addition to being covered by glycan sheaths [327, 328]. Furthermore, Ab function can be dependent on its anchoring or binding to sugar residues surrounding the epitope for effective neutralization [306]. Interestingly, relative to gp120, the immunogenic dominas of gp41 are highly conserved [329], probably due to its critical involvement in the membrane fusion process. Even though the conserved MPER epiotpes lie within the hydrophobic trans-membrane region [307, 330], the MPER is indeed exposed during a transient window during fusion [44], making it accessible and vulnerable to Ab-binding, by which it could block the fusion process. Various MPER-based immunogens have been tested with the goal to induce potent MPER-specific protective responses [299-301, 308, 309]. As reviewed earlier, the observation that the 2F5 monoclonal bNAb which targets the MPER also binds autologous lipids, has raised some concerns about MPER vaccine approaches due to possible autoimmunity in the host [315].

The overall challenges in eliciting Abs against neutralizable regions of the glycoprotein have led to the development of various innovative immunogen designs to target select regions or to overcome the structural attributes that limit its immunogenicity. These include 'mimicry' strategies where immunogens are designed to display neutralizing epitopes in native conformation [331] or where they mimic the native trimeric viral spike [332]; the removal or masking of immunodominant regions of the glycoprotein to generate glycan inclusive and excusive versions of immunogens [333]; the use of polyvalent and consensus sequences of *env* to increase breadth and coverage of the immune response [334]; methods of stabilization of shorter glycoprotein epitopes to improve their immunogenicity, like their incorporation in a virus-like particle (VLP) [325, 335].

4.7. *Virus-like particles*

The gag or p55 subunit of HIV-1 constitutes its viral core and independently generates VLPs that are highly immunogenic due to their particulate nature compared to soluble antigens [336]. Constituting the core of HIV itself, gag-VLPs are similar in size to the virus and are efficiently taken up by APCs for presentation to cells of the adaptive immune system [337]. Further, lacking internal regulatory proteins, they are non-infectious and replication-deficient, and therefore are relatively safer options than whole-killed or live attenuated vaccines [338]. Purified VLPs of the human papilloma virus have proven to be a highly effective vaccine against the virus [339].

A new generation of chimeric VLPs has emerged serving as a platform to display glycoprotein epitopes or subunits, within or on the surface of VLPs [336-338]. Although VLPs also induce gag-specific T-cell immunity, the primary goal of chimeric VLPs is to induce Ab responses. Co-expression of both gag and env components in insect cells spontaneously produce particulate VLPs that are released and purified prior to administration *in vivo* [337]. This approach releases VLPs with the glycoprotein component displayed in the surface which is ideal for B-cell priming, however, the caveat lies in the variability of the number of surface glycoprotein spikes per VLP. Although delivery of chimeric VLPs has induced systemic and mucosal Ab-responses against the envcomponents in various animal models, these approaches have not been successful in generating robust Ab responses in simian studies thus far, even with the use of adjuvants and alternate routes of delivery [340-343].

Alternative design of chimeric VLPs, such as cloning tandem repeats of an Ab-inducing epitopes within full-length gag, can be employed as a strategic means to enhance the immunogenicity of the glycoprotein epitopes/ subunit [325, 344]. VLPs can also be delivered to hosts by recombinant vectors such as DNA or viral vectors, for *in vivo* production of particles that could present to APCs via both endogenous and exogenous pathways. However, the relative immunogenicity of particulate VLP administration versus vector delivery of VLPs has not been directly evaluated thus far.

4.8. *New generation of vaccine platforms*

Recombinant DNA and viral vectors constitute a newer generation of vaccine modalities that offer greater options for immunization. Strategic selection of immunogens and vectors platforms along with adjuvants can assist in the activation of multiple arms of immunity to provide the host with optimal immune protection against the antigen.

Recombinant vectors emerged as an alternative to subunit vaccines primarily for the elicitation of T-cells responses. They are useful modalities to deliver select immunogenic epitopes or partial subunits by genetic delivery into host cells, thus, antigens delivered via DNA or viral vectors are generated endogenously in host cells that are available at the site of immunization, which may be APCs or non-APCs. Endogenous antigen production and processing in an APC primarily promotes CTL activation by MHC I, which is an ideal strategy for T-cell based vaccines [64]. However, vector delivery of immunogens to non-APCs, by intradermal or intramuscular delivery, can process the antigen endogenously for extra-cellular release or secretion, making it available for cross presentation to other APCs for exogenous processing and activation of MHC II pathways [62].

Employing prime-boost immunization regimens with the same vector (homologous) or different vectors (heterologous), is another strategy that can significantly boost the immune response against the immunogen. Although its immunological basis is still poorly understood, it has been consistently observed that mixed-modality heterologous vaccine

administration elicits significantly greater immunity compared to the administration of each component vaccine alone in single or multiple doses [345].

Although the use of recombinant vectors, along with heterologous prime-boost regimens, have primarily been employed in HIV-1 clinical trials to elicit adaptive T-cell immunity, such regimens also enhance HIV-specific Ab responses [346]. Viral vectors used to express rgp120, that have been approved by the Food and Drug Administration (FDA), are the canarypox, adenovirus (serotype 5: rAd5, serotype 35: rAd35), vaccinia (ALVAC, NYVAC and modified vaccinia Ankara-MVA) [347-349] and the Venezuelan equine encephalitis (VEE) vectors. Newer vaccine platforms must acquire a licensure prior to its entry into market for widespread use. Pre-marketing (pre-license) vaccines are tested rigorously in animal models and subsequently in phase I clinical trials in a small number of closely monitored patients to assess efficacy, safety, tolerability, immunization site specific reactions or other side effects. *DNA vectors*: Plasmid or DNA vectors serve as an effective tool for gene delivery *in vivo* and can be administered via various routes, intramuscular, intra-dermal, intra-nasal or intravenous [352]. The DNA backbone can be manipulated in multiple ways to influence transgene expression: by choice of promoter to direct ubiquitous or tissue-specific expression, inclusion of Kozak sequences to enhance expression levels, insertion of CpG motifs, optimization of vector/ transgene sequences specific to codon usage in the host [353-356]. Naked DNA is quickly degraded in vivo, therefore innovative measures have been employed to enhance its uptake and immunogenicity in a host, such as electroporation of the injection site, adsorption onto gold beads for gene gun blasting of the DNA coated particles,

Nanopatch™ microprojection delivery or needleless Biovector™ delivery [357-359]. Although, plasmid vectors can be optimized for immunogenicity, the best results of DNA vaccination have been observed in prime-boost strategies in combination with recombinant viral vectors or protein vaccines [241, 360].

Recombinant adenovirus 5 (rAd5) vector: Ad5 is a non-enveloped DNA virus that has an icosahedral structure with large fiber proteins protruding from surface penton molecules [361]. The tip or knob of the fiber protein interacts with receptors to initiate entry into host cells, however, the virus must bind the coxsackie-adenovirus receptor (CAR) and a coreceptor, like integrins α5β5, αvβ3, αvβ5, αMβ2, for internalization via endocytosis [362]. Replication-defective Ad5, which has the early gene E1 deleted, enhances the safety of the vector without affecting its ability to deliver the transgene. The deletion of both E1 and E3 genes allows a large insertional capacity for foreign genes, of up to 7-8 kilobases to generate recombinant vectors [361]. Ad5 can infect a wide range of cell types but does not infect APCs efficiently via CAR [361]. Upon infection, the internalized Ad5 genome would use host machinery to synthesize the transgene for release to be processed exogenously by neighboring APCs for MHC II presentation of antigens. rAd5 vectors are also widely implemented in immunization strategies to induce mucosal immunity since adenoviruses cause the common cold at the respiratory mucosa and so they are naturally interactive at mucosal surfaces.

CpG as an adjuvant: In mammals, 70-80% of adjoining cytosine (C) and guanine (G) residues in DNA are methylated. However, bacterial DNA lacks methylation between adjoining C-G, referred to as CpG, where the 'p' denotes phosphate or the phosphodiester DNA backbone sans methylation [363]. CpG is recognized as foreign by innate sensors and interacts with the TLR-9 receptor which is primarily expressed in APCs such as pDCs and B-cells, on the cell surface or within endosomal compartments [364, 365]. CpG mediated activation of innate responses promotes multiple APC functions that enhance humoral, cell-mediated and CTL immunity. Specifically, it enhances activation of B-cells, their expression of MHC II, the expression of co-stimulatory molecules required for CTL activation, and the stimulation of inflammatory cytokine production (interleukins-1, -6, -12, TNF-α and IFN-γ) to promote Th1-dominated responses [224, 363, 365-367].

4.9. *The phase IIb STEP trial*

The failure of the gp120 based Vaxgen trials prompted the field to shift focus from pursuing sterilizing immunity based approaches against HIV to T-cell based vaccines. Although the emergence of viral escape mutants had been demonstrated in simian immunization models, suggesting that T-cell based vaccines may be ineffective in controlling HIV long-term [5, 7, 257], this trial was conducted as a proof-of-concept to test the capacity of the vaccine to potentially reduce the viral set-point (circulating viral load throughout infection, particularly during clinical latency), by which it would reduce infection, pathogenesis and related pathology in the infected individual.

A large multicenter trial was organized to test the first T-cell based vaccine manufactured by Merck: a trivalent replication-competent Ad5 vaccine expressing HIV proteins gag, pol and nef [368]. The STEP trial was conducted by the HIV vaccine trials network (HVTN), in a test population of 3000 heterosexual men and women as well as homosexual males, from North America, the Caribbean, South America and Australia between 2004-2009, and in addition, the Phambili trial enrolled 801 heterosexual men and women in South Africa between 2006-2009; both trials tested the same vaccine [290, 368]. This randomized, double-blind multicenter trial was intended to be a phase III efficacy trial of the trivalent Ad5 gag/pol/nef vaccine, however, preliminary results emerged in September 2007 indicating that the vaccine was not only ineffective in lowering HIV acquisition, but indeed, it marginally increased the incidence of infection in vaccinees compared to the placebo group, which resulted in early termination of the trial [364].

Although the vaccine had elicited cell-mediated immunity against HIV in safety and immunogenicity trials [369], it unfortunately failed to protect at large [364]. After thorough analysis to determine the reason for increased infection rates among vaccinees, correlation was made between Ad5 sero-prevalence and enhanced infection rates. It is hypothesized that pre-existing immunity to the rAd5 vector itself contributed to systemic inflammation that resulted in increased availability of activated target CD4 T-cells for enhanced HIV infection in vaccinees [370]. This hypothesis received conflicting support initially [371, 372], however it is now widely acknowledged now that multiple rAd5 immunizations in clinical trials may be counter-productive due to its wide sero-prevalence in the population at large. Sero-

epidemiological studies have revealed that there is significantly lower pre-existing immunity against alternative rAd vectors such as rAd26, rAd35, rAd48 vectors [373], the use of which may circumvent the issues associated with rAd5 vector use as observed in the STEP trial.

Further, reducing the number of rAd5 immunizations and avoiding boosters of the same vector may also circumvent the issue. Patients in the STEP/ Phambili trials received three intramuscular injections of the vaccine at 0, 3 and 6 months. In an ongoing phase III efficacy trial, HVTN 505, vaccinees are being immunized with a mulitclade DNA prime via Biojector® delivery, three times, (encoding HIV clade B *gag*, *pol*, and *nef*, and *env* from HIV clades A, B, and C) followed by a single rAd5 booster immunization (encoding clade B *gag*, *pol*, and *env* from clades A, B, and C) [374]. The immunogens used are modeled after and optimized from the ones used in the STEP trial and will desirably induce balanced T-cell and Ab based protective responses against HIV [374]. The outcomes of this trial will offer many useful insights, and also shed light on the use of rAd5 as a single booster in a heterologous immunization regimen with DNA.

4.10. *The phase III RV144 efficacy trial*

The largest multicenter phase III trial conducted to date is the Thai RV144 trial, led by the US Military Research Program, to test a multi-component HIV vaccine in a heterologous prime-boost regimen, in a randomized test group of 16,402 Thai heterosexual men and women, from 2003-2009. Vaccinees received a recombinant canarypox vector, ALVAC vcp1521, developed by Sanofi Aventis, expressing HIV-1 gag and protease (strain

LAI) as well as HIV-1 gp120 (clade CRF01_AE) linked to the transmembrane-anchoring portion of subtype B gp41 with a deletion in the immunodominant region, followed by a booster of the bivalent Vaxgen AIDSVAX® B/E rgp120 vaccine [375]. In this randomized, double-blinded trial, vaccinees received a placebo or the ALVAC vcp 1521 vaccine as a prime intramuscularly at 0, 1, 3 and 6 months, followed by an intramuscular rgp120 boost at 3 and 6 months [290].

Overall, the resulting outcome showed varying efficacy and durability of the immune responses generated. However, within a 1-year duration of protection, it exhibited modest efficacy of 31.2% in the general test population but had no significant effect on early viral load set point or CD4 count of infected individuals [347]. Results from preclinical and phase I/II testing of the ALVAC and Vaxgen vaccines generated mixed reviews on the decision to proceed to a phase III trial, since reports showed that it induced poor CTL immunity, merely 44 – 64% neutralization efficacy against a CRF01_AE strain, albeit high Ab-binding to gp120 from MN (100%) and a clade A strain, A244 (96%) [375, 376].

The first detailed report on the correlates of immune protection contributing to the modest success of this trial has been published very recently [377]. The study design and data analysis relied heavily on statistical methodologies to highlight correlates of infection risk, in order to generate hypotheses that could be tested for further characterization and determination of causation, and for improved strategic vaccine design. Briefly, the results reported two correlates for infection risk: 1) risk inversely correlated with IgG-binding to the

variable loops 1 and 2 (V1/V2) of gp120, and 2) risk correlated with *env*-specific IgA. However, there is no indication that these observations had any association with actual enhancement of infection, since low levels of V1/V2-specific Abs or high levels of *env*-specific IgA were not associated with higher rates of infection. Intriguingly, although CTL immunity and neutralizing Abs were induced, they were not the primary effector functions that correlated with protection, however, ADCC function and high avidity *env*-specific IgG did correlate with protection. Further, secondary analysis showed that vaccines that generated low levels of IgA, four other variables (nAbs, ADCC, IgG avidity and *env*-specific CD4+ T-cells) were inversely correlated with infection. However, in vaccinees with high env-specific IgA, these variables did not correlate with infection, leading to the hypothesis that high IgA may impede or overshadow other protective functions. Although this hypothesis and its true impact remains to be verified, for the first time in a clinical trial, a significant interaction of IgA with other immune variables has been highlighted, implicating it in a potentially important role in influencing HIV protection. Thus, an improved understanding of IgA effector functions and characterization of IgA-inducing immunodominant epitopes that elicit protective Abs can be very important in the design of future vaccines, even if they are not primarily mucosa-targeting.

It is also important to note that this trial was conducted among a test population that was at low to medium risk of infection and therefore posed some inherent challenges in statistical analysis [290, 347, 377]. Generally, vaccine efficacy is defined as the "complement of the relative risk among vaccinated and unvaccinated persons", however since the incidence

of infection was so low in the entire pool, estimates of efficacy between specific time-points can be highly variable, making patterns difficult to perceive, thus limiting accuracy [378]. In addition, the response to the vaccine was highly variable among infected and uninfected individuals, in either the placebo or vaccine group. Therefore, it should be noted that the degree of correlation of immune function with risk varied, based on the relative immune response generated by the individual (high, medium, low). It was primarily individuals with high responses that displayed the correlation trends described in the main outcomes of the study, which constitutes a relatively small test population, particularly the group exhibiting high immune responses among infected individuals, since the overall incidence of infection was low to begin with in the study [377]. It remains to be known how such a vaccine would fare among populations that would be at high risk of contracting HIV, and if the correlation trends observed for low/ medium risk populations would be reflected in a high risk environment.

Most importantly, IgA-binding reported in this study may not have been a true representation of IgA function. Just as env-based subunit vaccines elicit polyclonal IgG consisting of a pool of protective and non-protective Abs, it is quite likely that a polyclonal IgA response was also generated against the glycoprotein in this trial. Further, thus far, there are no known functional IgA inducing domains in gp120 and the immunodominant gp41 MPER region, the only gp160 domain known to elicit functional IgA, was not present in the ALVAC vcp 1521 vaccine, only the gp41 transmembrane region was, which served to anchor gp120. Therefore, evaluation of IgA binding capacity without any measure or assessment of

IgA function adds little value, even though it is a step ahead of previous phase III trials that never even reported IgA responses. There was also no assessment of mucosal IgA function or comparison of systemic versus mucosal *env*-specific IgA, since mucosal samples were not collected. This highlights the lack of understanding and the under-appreciation of the role of IgA, particularly with relevance to mucosal responses as evaluated in major HIV clinical trials.

4.11. *Lessons learned: moving forward with Ab-based vaccines*

Despite the robust efforts that have been dedicated towards the development of a vaccine, over three decades since the discovery of HIV, our understanding of the potentially critical correlates of protection against the virus is still evolving. As evidenced by the latest report on the outcome of the RV144 trial, the previous assumption that nAbs and CTL immunity would be paramount for an effective prophylactic vaccine was challenged by data which highlighted ADCC function instead.

A critical component that is missing in all three efficacy trials highlights a major gap in vaccine strategies thus far: the induction and assessment of mucosal immunity. It is widely known and accepted that HIV is a mucosal infection that is primarily transmitted via mucosal surfaces and propagates at the gastro-intestinal mucosa during latency. Given that intervention by prophylactic therapies within the window of opportunity during acute HIV infection (Figure 3) would be optimal and ideal [82], the development and testing of mucosal vaccines for the induction of mucosal immunity should be a serious consideration of vaccine

efforts. Many questions remain unaddressed and unanswered due to the lack of collection of clinical mucosal data. The type of immunogen and the various components of an immunization regimen, that would promote and optimize mucosal Abs and/or T-cell immunity, remain to be evaluated in larger clinical trials. Standardization of mucosal sample collection from clinical trials and the optimization of assays for assessment of mucosal Ab functions, of both IgG and IgA, yet represent another important gap that must be filled before we can accurately decipher the efficacy of future mucosal intervention strategies.

Mucosal responses will also need to address unique events that occur specifically at the mucosa to offer optimal protection, such as targeting the uniquely homogeneous founder virus population during its expansion locally prior to systemic dissemination, when it is highly vulnerable to immune pressure. The development of transmitted/ founder (T/F) viral clones offers a means to additionally evaluate Ab responses against the env glycoprotein phenotype that succeeds in forming the primary infectious pool at the mucosa. Results from the Vaxgen trial prompted the development of virus panels that could be used to assess systemic nAb function more accurately, since the lab-adapted and limited viruses from chronic infection did not constitute an apt panel at that time. Furthermore, to assess breadth of Ab responses, viral strains that are distant from the immunogen strain must also be included to diversify the test panel, since achieving broadly protective responses is an important requisite for an effective HIV vaccine. The nAb results from the RV144 phase I/II trials received mixed reviews since the test panel used to assess neutralization consisted of a limited number of virus strains, which were similar to the viral strains that the vaccine

immunogens were derived from, despite which they yielded only modest neutralizing results [375, 376]. Even in the recently published study on the immune correlates of the phase III RV144 trial, only four viruses constituted the panel to test for neutralization in the TZM-bl assay: two clade B pseudotypes, MN and SF162, a clade C strain and a CRF01_AE virus strain [377]. This panel is again closely associated to the strains that the vaccine immunogens were derived from, except for the clade C virus, and does not test for breadth of neutralizing response. Therefore, ideally, a virus panel should not only be diverse to assess breadth of Ab function, but it should also include T/F virus clones to better assess mucosal effectiveness of the Abs.

In the RV144 study, serum IgG targeting the V1/V2 gp120 region inversely correlated with risk, and this region has therefore been highlighted as an attractive candidate for Ab-based vaccines against HIV [377]. However, sequence conservation analysis of V1/V2 using alignment tools of the Los Alamos HIV database showed that this region is poorly conserved across HIV clades [329], which suggests that efficacy of aV1/V2 based vaccine would likely be clade restricted and not cross-reactive. Elicitation of broadly reactive immune responses by a vaccine, particularly of broadly protective Abs, will require the identification and characterization of highly conserved immunodominant regions of the virus.

Furthermore, till very recently, the role of Abs against HIV has been largely dominated by one effector function: neutralization, and in particular nAbs targeting gp120. In comparison, gp41 has been largely overshadowed despite having multiple immunodominant

and highly conserved regions that are accessible during the fusion process and that are known to induce both functional IgG and IgA [44, 379]. The gp120 Vaxgen trials lacked in the generation of robust nAbs which can be attributed to multiple factors, however, one emerging hypothesis has been that functional Abs may be overshadowed by competing non-functional ones [323, 324]. To counter the effect of the env-specific polyclonal burst, immunogens can be designed to specifically target and focus the immune response on select functional regions of the glycoprotein rather than the entire subunit. In subsequent studies that constitute the body of this thesis, we have selected highly conserved regions of gp41 and presented them in a chimeric VLP model to immune-focus and enhance the Ab response towards the epitopes.

5. Thesis Objective

My thesis objective was to develop a vaccine model with the goal to generate protective Abs against highly conserved regions of gp41, particularly at the mucosa to target early stages of infection. To elicit high titre, cross-reactive Abs, appropriate target epitopes were selected to be presented via a chimeric virus-like particle platform that was administered to mice via strategic immunization regimens employing recombinant DNA and Ad5 vectors, with the goal to induce potent mucosal immunity. The systemic and mucosal Ab responses were characterized and assessed for function against HIV *in vitro*, where assays and viral reagents were strategically implemented to assess mucosal response.

5.1. *Selection of viral targets*

As discussed earlier, the development of gp120-based immunogens has previously been met with certain challenges. However, the highly immunogenic and relatively conserved gp41 MPER contains within it two linear epitopes that are ideal for incorporation into a vaccine model, since they are not conformation restricted or glycan coated and hence do not require structural mimicry to elicit functional Abs. More importantly, numerous studies have shown that the MPER epitopes elicit Abs of neutralizing potential [95, 310, 380, 381].

Our particular interest in eliciting mucosal immunity brought the QARVLAVERY epitope to the forefront, which was shown to be the target of systemic IgA obtained from a cohort of discordant couples of which one partner remains exposed yet uninfected. The

immunogenicity of QAR*I*LAVERY and the antiviral effects of QAR*I*LAVERY-specific IgG and IgA have been characterized in a study demonstrating suppression of HIV infection in a syncytia-inhibition assay [232], however, no published study had incorporated the epitope in a vaccine model since. Please note the distinction between the QAR*I*LAVERY epitope [232] and QAR *V*LAVERY, the latter being the sequence used in our studies as it exhibits higher conservation across the HIV clades according to the alignment analysis on the Los Alamos HIV database [329]. In QAR *V*LAVERY, the valine (*V*) amino acid replaces isoleucine (*I*) while conserving function of the amino acid, since both valine and isoleucine have branched side chains differing in only a single carbon residue. Our expanded analysis of sequence conservation using the Los Alamos HIV database also showed that the QARVLAVERY epitope is indeed very highly conserved and therefore could be a good candidate to elicit broadly reactive protective responses, as further elaborated in chapter 3.

Hence, the MPER epitopes, ELDKWA and NWFDIT, as well as QARVLAVERY were selected to be the key virus glycoprotein targets in our vaccine for the elicitation of gp41-specific Abs. We hypothesized that these epitopes would induce effective Abs against HIV, which would protect against infection and inhibit viral transcytosis. *in vitro*. To test this, the epitopes were incorporated within a full-length HIV-1 clade A *gag* gene in multiple tandem repeats, to be expressed in a chimeric VLP, designed with the goal to immuno-focus the response on the chosen epitopes. We hypothesized that the multiple tandem repeat insert design would significantly enhance the generation epitope-specific Abs. Selective vectors and

immunization regimens were utilized to elicit both systemic and mucosal humoral immunity, which are further elaborated in chapter 2.

5.2. *Selection of platform and vectors for antigen delivery*

In the following studies, we have administered chimeric VLPs expressing select gp41 epitopes in multiple tandem repeats at the gag C-terminus, via recombinant DNA and Ad5 vectors. In vivo delivery of DNA and Ad5 offers greater flexibility and effectiveness in stimulating both systemic and mucosal immunity. Further, both approaches deliver the genetic component (DNA or Ad genome) within a target cell where the chimeric VLP can be continuously produced intracellularly, to either be processed for MHC I presentation by an APC or to be released and become available for exogenous uptake and processing for MHC II presentation [382, 383]. Any carrier or platform used to display epitopes triggers innate and adaptive immunity and gag itself is known to have various epitopes that induce potent CTL responses and promote helper T-cell generation [383, 384]. Therefore it is an ideal platform to present Ab-inducing epitopes, as it balances the humoral response with an effective CTL and cellular response against HIV [336].

5.3. *Mucosal immunization*

Studies evaluating immune responses at mucosal surfaces have identified the existence of a common mucosal immune system (CMIS) that consists of the gastrointestinal, respiratory and genitourinary mucosae. It has been shown that one mucosal immunization route can induce responses at distal mucosal effector sites as well. For example, intranasal

immunization of humans with cholera toxin B (CTB) induced CTB-specific IgG and IgA Abs in vaginal secretions in women, and in urine in men [385]. NHP models have primarily been used for the assessment of preclinical CTL-based vaccines and of these, only few have employed mucosal immunization routes and sampled mucosal specimens. However, in the few relevant studies, direct comparison between systemic versus mucosal immunization routes has demonstrated that for mucosa effector function immunization via a mucosal route is superior. For example, rectal immunization elicited significantly higher rectal HIV-specific CTL immunity and protected against a rectal SHIV challenge compared to parenteral immunization of a gag and pol based vaccine [386]. In addition, nasal rather than intravenous administration of non-pathogenic SHIV was able to protect from a subsequent pathogenic SHIV challenge [387]. Therefore, given our goal to induce optimal epitope-specific responses in genital secretions of immunized mice, the intranasal mucosal immunization route has been employed in the following studies. We hypothesized that intranasal administration of the recombinant Ad vector, expressing our chimeric VLP, would significantly enhance the generation of mucosal Abs in an optimized heterologous immunization regimen, along with systemic administration of recombinant DNA vectors.

5.4. *Following chapters*

This introduction has reviewed the major concepts and the most relevant background to place the following studies in context of the field. Chapter 2 consists of the study describing the design and construction of chimeric virus-like particles expressing the MPER

epitopes, ELDKWA and NWFDIT, in tandem copies within HIV gag. The chimeric gag+epitope construct was administered to mice in various immunization regimens to determine the optimal method to induce potent epitope-specific mucosal immunity.

The study outlined in chapter 3 focuses on the QARVLAVERY epitope and is the first published study to have incorporated this epitope in a vaccine model. The epitope was administered to mice using vectors and an optimized immunization route for mucosal Ab induction, by delivering it in the gag+epitope construct described in chapter 2. Intriguingly, it was found that QARVLAVERY elicits unique immunogenic properties which are of particular mucosal interest.

The study in chapter 4 utilizes purified subsets of anti-ELDKWA and anti-QARVLAVERY IgG and IgA, from systemic and mucosal compartments to assess Ab function against a panel of acute infection viruses. As reviewed in previous sections, surface glycoproteins from transmitted/founder virus populations, obtained during acute infection display distinct patterns of sensitivity to antiviral agents, compared to chronic infection viral glycoproteins. Given that the intent behind mucosal responses is to target virus entry at the earliest stages of transmission and infection, such a panel of viruses is critical for screening mucosal Ab function.

-- CHAPTER 2 --

MULTIPLE TANDEM COPIES OF CONSERVED GP41 EPITOPES INCORPORATED IN GAG VIRUS-LIKE PARTICLES ELICIT SYSTEMIC AND MUCOSAL ANTIBODIES IN AN OPTIMIZED HETEROLOGOUS VECTOR DELIVERY REGIMEN.

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The manuscript in this chapter describes the rationale and the methodology for developing chimeric VLPs that express highly conserved gp41 epitopes of the MPER, ELDKWA and NWFDIT. Immunization regimens utilizing DNA and rAd5 vectors were assessed to determine the optimal strategy to induce potent epitope-specific Abs at the mucosa. Our results showed that electroporation delivery of DNA with CpG as an adjuvant significantly enhanced DNA immunogenicity, and that intra-muscular DNA-priming followed by intra-nasal rAd5 boosters optimally elicited high titer systemic and mucosal MPER-specific Abs. Lastly, we showed how a chimeric VLP model approach can be used to immune-focus on a specific epitope induced response by including multiple tandem repeats of the epitope within *gag*.

I was responsible for the primary research work contributing to this study and for the writing of this manuscript, under the supervision of Dr. K. L. Rosenthal. Technical assistance was provided by Amy Patrick for all of the immunization studies.

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Multiple tandem copies of conserved gp41 epitopes incorporated in gag virus-like particles elicit systemic and mucosal antibodies in an optimized heterologous vector delivery regimen

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ABSTRACT

Induction of neutralizing antibodies to prevent HIV infection, especially at the mucosa, is a critical goal of future vaccines. In this study, we have designed chimeric HIV-gag virus-like particles (VLPs) that contain multiple copies of the two highly conserved gp41 membrane-proximal external region (MPER) epitopes, ELDKWA and NWFDIT, with the objective of generating high titers of MPER-specific antibodies. We have shown that the implementation of optimized vector design, delivery regimens and appropriate delivery methods is critical to significantly increase epitope-specific antibody titers. One goal of the methods that were tested and employed was to generate high levels of mucosal MPER-specific antibodies, as mucosal immune induction could play a key role in preventing HIV infection. We also tested a design strategy that incorporated multiple repeats of the MPER epitopes within gag, which significantly increased specific antibody titers, systemically and mucosally. This alternative design strategy and the implementation of optimized heterologous immunization regimens can serve to 'immuno-focus' and significantly increase epitope-specific titers.

1. Introduction

After years of research, the quest to develop an effective vaccine against HIV-1 is still being met with challenges. It is well known that the mucosa is the primary site of viral transmission and persistence of HIV. Therefore, it has become increasingly clear that a prophylactic vaccine must not only generate long-lasting, broadly reactive immunity but also provide mucosal protection against HIV, particularly at the urogenital tract [1]. Given the limited window of time before the virus disseminates from the mucosa to the systemic lymphoid tissue, ideally, a mucosal vaccine would need to prevent virus transmission across the epithelium, prevent viral entry into host target cells and suppress viral replication. This optimal defense can best be achieved by the induction of both cellular and humoral mucosal immunity against HIV.

One mechanism by which effective protection can be imparted at the mucosa, is by the induction of broadly neutralizing virus-specific antibodies (nAbs) that could inhibit HIV infection at the entry site itself. Appropriate immunization vectors and delivery regimens are required for effective induction of mucosal antibodies. Furthermore, in order to generate broadly reactive antibodies against HIV, relevant highly conserved target epitopes need to be expressed in a highly immunogenic manner. Past antibody-based vaccines against HIV have been unsuccessful in their attempt to mount potent nAbs against the viral envelope antigens [2,3] and were focused predominantly on gp120-targeting approaches. Although it provides the largest surface area for neutralization, some targets of gp120 are hidden by heavy glycosylation or steric hindrance and few epitopes have notable sequence conservation across clades [4–7].

An alternative to the gp120 subunit or its epitopes is the gp41 membrane-proximal external region (MPER). The MPER consists of two distinct, well conserved, linear and accessible antigenic regions, the ELDKWA and NWFDIT, which were identified as the targets of 2F5 and 4E10 human monoclonal antibodies (mAbs), respectively [8]. These mAbs were among a panel (2F5, 4E10, b12, 2G12) that were able to broadly neutralize HIV, individually and in combinations, and were largely responsible for the resurgence of the field of Ab-inducing vaccines [9,10]. Subsequent studies in macaque models showed that passive administration of a mixture of these nAbs could in fact prevent SHIV89.6 infection via

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different routes [11,12]. The mechanism by which the 2F5 and 4E10 Abs work is thought to be similar to that of fusion inhibitor drugs, which block virus–host membrane fusion during the intermediate steps when gp41 epitopes are exposed and accessible [13,14]. Of the mAb panel, 2F5 and 4E10 are the two Abs that bind to well characterized, highly conserved, linear epitopes of gp41 that are unhindered by glycans [15,16]. These characteristics make the MPER epitopes ideal candidates to be incorporated in our proposed chimeric virus-like particle (VLP) design, with the objective of generating nAbs against HIV to prevent infection at the mucosa.

VLPs have been used as antigen delivery platforms in several *in vivo* models as well as successful commercial vaccines for the hepatitis B and human papilloma viruses [17,18]. Being particulate with similar size and conformation to the parent virus, they make good immunogens and offer a safer alternative to live attenuated vaccines due to their self-assembling and non-replicating nature [19]. They are favorably taken up by antigen-presenting cells such as dendritic cells (DCs) [20–22] and can also induce innate immunity through the activation of patter-recognition receptors. The effectiveness VLPs alone as antigens has also been tested and they elicit strong CD4 helper and CTL responses in non-human primate models [23,24]. These characteristics make it an ideal immunogenic carrier for other HIV antigens. Here, we have developed a model where the MPER epitopes have been incorporated at different permissive sites within gag [25–28], as a strategy to present them effectively *in vivo*.

In order to optimize the antigenicity of the MPER epitopes, they were inserted in multiple tandem copies just prior to the C-terminus of p55 Gag. In an HSV-1 immunization model, it has previously been shown that incorporating up to four tandem copies of a linear neutralizing epitope of the surface glycoprotein gD, linked to a β-galactosidase carrier, correlated with an increase in antibody titers against gD [29]. Furthermore, the titer raised against four copies of peptide was equivalent to that raised against the entire gD protein in the same construct and delivery system. This strategy is of particular interest when seeking to 'immuno-focus' or optimize the immune response elicited against a desired neutralizing Ab-inducing target [30]. 'Immune-focusing' may generate a more relevant and functionally potent response against a desired, immunogenic epitope instead of a polyclonal burst against the entire subunit which would also be comprised of non-neutralizing Abs [31]. Recent studies characterizing the occurrence of natural nAbs in HIV-infected patients have suggested that the polyclonal burst of antibodies against non-neutralizing regions of the envelope might be an impeding factor in the generation of potent and functional nAbs [32,33]. This may be an important consideration for future vaccine design which could gain more from driving responses against specific Ab-inducing envelope epitopes rather than entire viral subunits [31].

Generating effective titers of protective Abs against HIV, systemically and particularly at the mucosa, is a major goal for future vaccine strategies. In order to achieve higher titers of MPER-specific Abs in our model, we cloned the chimeric VLP construct (Gag + MPER) into DNA and recombinant adenovirus (rAd) vectors, which were delivered to mice and expressed the VLPs *in vivo*. Here, we have tested several vector delivery methods and combinations with the goal to determine an optimal immunization strategy that would raise significantly high titers of antibodies in serum and mucosal secretions. Using a separate set of DNA and rAd vectors, we also independently assessed and verified the original HSV gD hypothesis in our model: that increasing the number of tandem repeats of an Ab-inducing epitope such as ELDKWA could indeed increase the level of Ab raised against it significantly.

2. Materials and methods

2.1. Construction of DNA and recombinant adenovirus vectors

An HIV-1 clade A gag gene cloned from a Ugandan patient was kindly obtained from Dr. Greg Dekaban (Robarts Institute, Univ., Western Ontario, London, ON). The gag gene was modified using the following primers to insert a specific restriction enzyme digest site (Nhe-BamH1) just prior to the stop codon, to allow for the insertion of the gp41 epitopes within gag (forward primer: 5′-TGG AAT TCG CCC TTA CGC GAT ATC CGT TAA, reverse primer: 5′-GCC CCG GGA CAA AAA TTA GGA TCC AAT CGC TAG CTG TGA CAA GAG GTC GTT GCC). The epitope strings, encoding the MPER epitopes or the ELDKWA repeats for their respective vectors, were codon optimized for expression in mice and designed with flanking restrictions enzyme sites for insertion into the Nhe-BanH1cloning site. They were synthesized at the MOBIX facility (McMaster University, Hamilton, ON) and their complete nucleotide sequences can be found in Table 1.

The entire [Gag + epitope] gene construct was cloned into (i) a pcDNA3.1(+) vector driven by the CMV promoter (Invitrogen, Burlington, ON) and (ii) an intermediate shuttle plasmid (obtained from the Robert E. Fitzhenry Vector facility, McMaster University, Hamilton, ON) to be used to generate the rAd vectors (Fig. 1). All vectors were designed using standard molecular biology techniques. Large-scale DNA stocks were prepared under endotoxin-free conditions for safe use in animals (QIAGEN, Plasmid Giga kit, Mississauga, ON). In order to generate replication-defective recombinant adenovirus vectors (rAd), the shuttle plasmid containing the [Gag + epitope] gene construct was co-transfected with a human Ad5 genomic plasmid (BHG10) to generate virus plaques in HEK293 cells (ATCC, Manassas, Virginia). The selected shuttle-genomic plasmid combination takes advantage of the controlled Cre-lox recombination to increase chances of recovery of the desired virus construct. The methods for co-transfection, verification of virus colonies, viral plaque purification and large-scale stock generation were described previously [34,35]. An additional rAd construct expressing wild-type (WT) myristoylated clade A Gag was also generated in the manner described above, to serve as a control unaltered WT VLP.

2.2. Western blots and cesium-chloride (CsCl) density centrifugation for VLP verification

To assess the proper generation and cellular release of particles from the recombinant DNA constructs, 293T cells (ATCC) were transfected with either an empty mock pcDNA3.1 vector or the recombinant pGag-MPER construct using the Lipofectamine 2000 reagent (Invitrogen, Burlington, ON). A549 cells (ATCC) were adsorbed with either a replication-defective empty mock rAd5 vector or the rAdGag-MPER construct, to determine particle formation and release by the rAd constructs. Supernatants from all cultures were collected at 48 h and concentrated using Amicon Ultra 50 kD filters (Millipore). The concentrated samples were boiled in a reducing buffer for SDS-PAGE containing dithiothreitol (DTT, Invitrogen), to reduce VLPs to gag monomers. The gel was transferred to a PVDF membrane to generate a blot and the VLP-p55 bands were detected upon incubation with a mouse monoclonal antibody to p24 (Abcam, ab19054, Cambridge, MA), followed by a secondary HRP-labeled goat anti-mouse antibody. The blot was developed using the Pico chemiluminescent substrate (Pierce/Thermo Scientific, Rockford, IL) and bands were visualized by X-ray exposure.

In CsCl sedimentation, the VLP samples were centrifuged through a continuous CsCl density gradient, allowing the particles to sediment along the gradient according to their buoyant density. Proper particulate formation was determined for all four chimeric VLPs (Gag-MPER, Gag-ELDx1, Gag-ELDx3, Gag-ELDx5) generated

Table 1
List of the peptide and corresponding nucleotide sequences for each epitope string inserted at the Gag C-terminus to generate a chimeric VLP construct.

VLP construct	Epitope insert (number of repeats)	Nucleotide sequence
VLP-MPER	NEQELLELDKWASLWNLWNWFDIT (×2)	AATGAACAGGAGCTGCTGGAACTCGATAAATGGGCCTCCCTCTGGAATCTGT GGAACTGGTTTGACATCACAAACGAGCAGGAACTGCTCGAGCTGGACAAGTG GGCTAGCCTGTGGAACCTCTGGAATTGGTTCGATATTACC
VLP-ELDx1	NEQELLELDKWASLWN (×1)	AACGAGCAGGAACTGCTCGAGCTCGATAAGTGGGCCAGCCTGTGGAACGGATCC
VLP-ELDx3	NEQELLELDKWASLWN (×3)	AATGAACAGGAGCTGCTGGAACTCGATAAATGGGCCTCCCTGTGGAATAACGAGC AGGAACTGCTCGAGCTCGATAAGTGGGCCAGCCTGTGGAACAATGAGCAGGA ACTCCTGGAACTGGACAAATGGGCTTCCCTCTGGAAT
VLP-ELDx5	NEQELLELDKWASLWN (×5)	AATGAACAGGAGCTGCTGGAACTCGATAAATGGGCCTCCCTGTGGAATAACG AGCAAGAACTGCTCGAGCTCGATAAGTGGGCCTCTCTGTGGAACAATGAA CAAGAACTGCTAGAACTGGATAAGTGGGCATCTCTCTGGAACAACGAACA GGAGCTCCTAGAGCTCGACAAGTGGCGCTTCCCTGTGGAACAATGAGC AGGAACTCCTGGAACTGGACAAATGGGCTTCCCTCTGGAAT

in vitro by the rAd constructs, in comparison to WT-VLPs generated by rAdWT-Gag. A549 cells were adsorbed with each of the rAd constructs and supernatants containing the VLP were collected at 48 h. Supernatants were centrifuged to pellet any debris and ultra-centrifuged (35,000 rpm, 2 h, 4 °C) on a 20% sucrose cushion to pellet the particles. The pellet was resuspended in 100 μl PBS and ultra-centrifuged through the 8-step CsCl overlay gradient (top – 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% – bottom) at 35,000 rpm for 4 h. Fractions were collected in 1 ml aliquots from the top of the gradient and run through reducing SDS-PAGE. The presence of p55 was detected by western blot using an anti-p24 antibody for detection (Abcam) and developed after incubation with a HRP-labeled secondary antibody using the Pico chemiluminescent reagent. Bands were visualized by X-ray exposure.

2.3. Animal immunizations and sample collection

Eight-to-ten week-old female C57BL/6 mice (Charles River, Quebec, Canada) were used for all of the immunization experiments. The DNA vector was delivered intra-muscularly (i.m.) in each

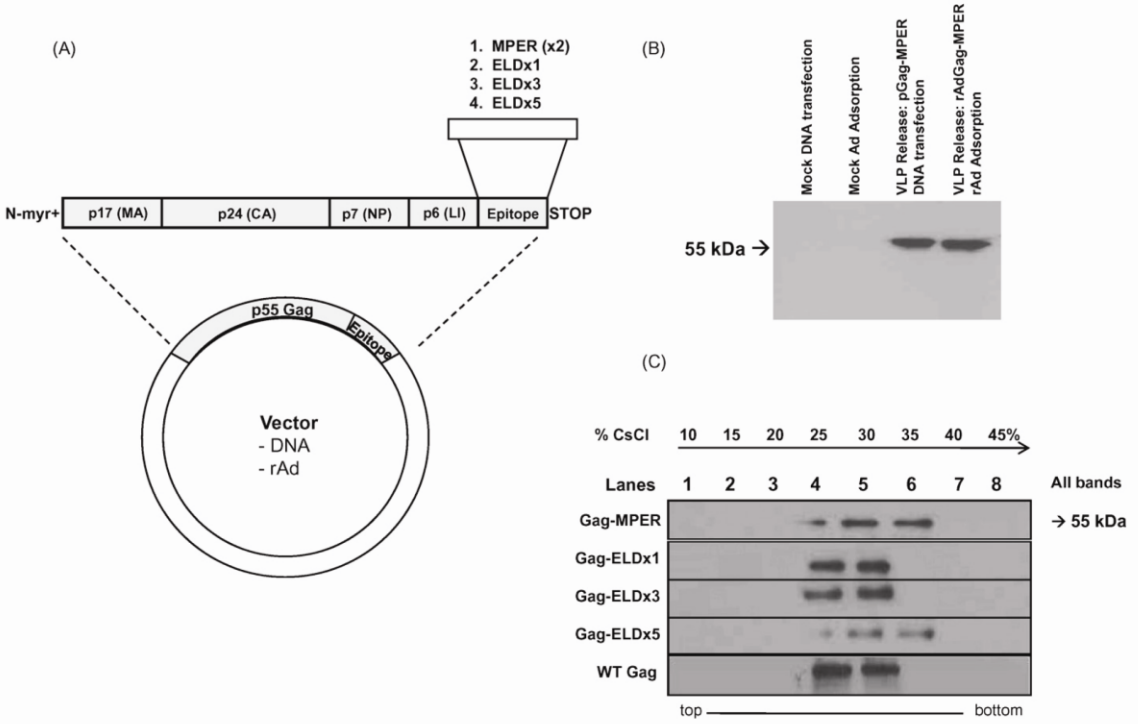

Fig. 1. Recombinant DNA and adenovirus vectors expressing Gag-MPER generate chimeric VLPs. (A) Schematic overview of the construction of the recombinant vectors. The epitope string was inserted prior to the STOP codon of the full-length Clade A gag gene. (B) Western blot visualization of the VLPs released *in vitro*. 293T cells were transfected with a mock pcDNA3.1 or the pGag-MPER DNA vector, and A549 cells were infected with a mock or the recombinant Ad vector, rAdGag-MPER. Supernatants collected at 48 h were concentrated and run through reducing SDS-PAGE. The p55 Gag band derived from reduced particles is representative of VLPs generated and released by both Gag-MPER constructs. (C) Verification of the particulate integrity of the chimeric VLPs. Particles were obtained from supernatants of cells infected with each of the rAd vectors (rAdGag-MPER, rAdGag-ELDx1, rAdGag-ELDx3, rAdGag-ELDx5) and their density was compared with that of WT-VLP generated by rAdWT-Gag. Particles were concentrated and pelleted by centrifugation on a 20% glycerol cushion. Subsequently, they were run through a continuous 10–45% CsCl density gradient where they equilibrated relative to their buoyant density along the gradient. Eight continuous fractions (lanes 1–8) were collected. Each lane represents a sample from each fraction that was run through a reducing SDS-PAGE and analyzed for the presence of p55 Gag in a western blot, to compare chimeric and WT particle density sedimentation.

quadriceps, immediately followed by electroporation of the area to optimize DNA uptake and minimize its degradation *in vivo*, while keeping the mice anesthetized in a nose cone. Fifty micrograms of endotoxin-free plasmid DNA was delivered in a total volume of 50 μl per leg, diluted in 1× endotoxin-free PBS (GIBCO), with or without 50 μg CpG (ODN 1826, 5′-TCCATGACGTTCCTGACGTT, MOBIX, Hamilton, ON) as per the experiment. The pulses were generated by a BTX ECM830 square wave generator at the following setting: 6 pulses, each at 100 mV, 20 ms long, 1-s apart. The rAd vectors were diluted in saline and administered intranasally (i.n.) at 10^8 pfu/ml in a total volume of 15 μl. Different immunization regimens have been employed and described in the results or figure captions. DNA immunizations were always performed 3 weeks apart, whereas the rAd immunizations were 2 weeks apart in the repeated homologous and heterologous regimens. Mice immunized intravaginally (IVAG) with the rAd vectors received 2 mg of Depo-Provera subcutaneously (Depo, Upjohn, Don Mills, Ont., Canada) for 5 days before the vaginal delivery of rAd at 10^8 pfu/ml in a maximum volume of 30 μl. I.vag. delivery methods have been described previously [36].

Blood was collected from mice via retro-orbital bleeds using heparinized capillary tubes and centrifuged at room temperature (RT) to obtain serum. Vaginal washes were collected by pipetting in and out of the vagina several times with 30 μl of PBS, twice in succession. Washes were pooled over a 5-day period to compensate for shedding differences during the mouse estrus/diestrus cycle. Fecal pellets were collected over a 3-day period and soaked in a PBS buffer containing 0.05% sodium azide (NaAz) (100 μl buffer/1 g pellet) at 4 °C overnight to allow for antibody diffusion into the buffer. The antibody sample was obtained after centrifuging twice to remove any fecal matter. All samples were stored at −20 °C prior to use in assays and moved to −80 °C for long-term storage.

2.4. ELISAs

The MPER peptides were used to coat Amino-Immobilizer 96-well plates (NUNC, #436006, Mississauga, ON) which allow the shorter peptides to conform more naturally in solution, be more accessible to the Abs and give significantly lower background than other plates (tested against the NUNC MaxiSorp plates). Separate ELISAs were conducted to determine ELDKWA-specific or NWFDIT-specific antibodies. Wells were coated with either the ELLELDKWASL peptide or NLWNWFDITN (synthesized by CPC Scientific, San Jose, CA) at 5 μg/well in 100 μl of 0.1 M carbonate buffer pH 9.6 and left for overnight incubation at 4 °C. Plates were subsequently blocked with 5% BSA for 4 h at RT and washed (three times with 0.05% Tween-PBS and three times with PBS) in an automated plate washer, prior to addition of serial dilutions of the serum or mucosal samples that were allowed to incubate overnight at 4 °C. Samples were washed from wells (3× 0.05% Tween-PBS, 3× PBS), biotinylated anti-mouse IgG or IgA antibody (Southern Biotech, Birmingham, Alabama) was added (1:1000 dilution, 100 μl/well) and incubated for 1–1.5 h at RT, followed by the addition of extravidin peroxidase (SIGMA, 1:10,000 dilution, 100 μl/well) and incubation for 45 min at RT. The SureBlue TMB substrate (KPL, Guelph, ON) was used to develop the ELISA for 20 min prior to the addition of H_2SO_4 to stop the reaction. Plates were read at 450 nm to determine OD values. Antibody titers were reported as the reciprocal end-point of the Ab-sample dilution, relative to the OD of the same sample from a naïve, non-immunized mouse. The end-point titer was considered to be the last dilution at which the OD of the sample reached at least 1.5 times the background OD of the non-immunized sample on the same assay plate. The protocol described here was optimized to give minimal background OD in un-immunized sample, to increase the accuracy of end-point titer determination.

The specificity of the assay was tested by adding dilutions of the 2F5 mAb and the 4E10 mAb (NIH AIDS Reference Reagent Program, #1475 and #10091) to wells coated with either ELDKWA, NWFDIT or a mock gp41 peptide (QARVLAVERY), following the protocol described above. We confirmed that 2F5-IgG bound only to ELDKWA-coated wells and not to NWFDIT or the mock peptide, and 4E10-IgG showed specificity to NWFDIT-coated wells alone. Serum samples from previous C57BL/6 mouse experiments that received recombinant vaccinia virus (rVV) Clade A Gag immunizations [37] and were positive for anti-gag IgG, were tested against the NWFDIT and ELDKWA epitopes separately to determine if anti-gag Abs cross-reacted with the epitopes. We confirmed that non-specific binding of anti-gag IgG to ELDKWA or NWFDIT in our peptide-specific ELISA did not occur.

2.5. Neutralization assays

The neutralization assays were performed at Duke University by the David Montefiori lab. Antibody samples were sent in PBS supplemented with 5% anti-protease cocktail (SIGMA). Samples were heat-inactivated prior to neutralization against the SF162.LS pseudovirus in the TZM-bl luciferase indicator assay. The TZM-bl assay protocol has been outlined previously [38]. Values reported are sample dilutions at which relative luminescence units (RLUs) were reduced 50% compared to virus infection control wells that contained no Abs. HIV hyperimmune-globulin (HIV-IG) served as a positive control for infection inhibition.

2.6. Statistical analysis

All epitope-specific Ab titers were determined relative to the background OD of representative samples from age-matched, un-immunized female mice. In homologous immunization groups, significant differences in epitope-specific Abs generated after each additional immunization were determined relative to the Ab titer measured after the previous immunization, for the same group of mice, using the paired Student's *t*-test. In other cases, where significance was measured between different immunization groups, statistical analysis was performed using unpaired Student's *t*-test analyses. Significance (*) is indicated by the following *p*-values: $^{*}p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$.

3. Results

3.1. Generation and release of chimeric VLPs by the DNA and rAd vectors in vitro

In order to generate MPER-specific antibodies, the sequence was inserted in tandem copies just prior to the stop codon at the C-terminus of myristoylated full-length Ugandan clade A HIV-gag (Fig. 1A). Although there are multiple permissive sites within the full-length p55 gag gene (p24, p7, p6), it has been shown that the C-terminus is best for the insertion of up to 200 amino acids without affecting the generation and release of chimeric particles compared to wild-type VLPs [25]. Here, we have verified the cellular release of chimeric VLPs generated by our DNA and rAd constructs and their proper formation as particles *in vitro*. In addition to the set of DNA and rAd vectors expressing the Gag-MPER construct, three other sets of vectors were produced that express either one, three or five copies of the ELDKWA epitope, which were used to test the hypothesis that multiple tandem copies of an epitope inserted at the gag C-terminus would significantly increase the levels of specific Abs against it. Each epitope insert for the individual vectors was codon optimized and their full sequence is listed in Table 1.

Chimeric particle release from cells was determined by verifying the presence of VLPs in supernatants from cells transfected

with plasmid DNA or adsorbed with the rAd vectors expressing the Gag-MPER construct. The supernatants were collected, concentrated and run through reducing SDS-PAGE prior to western blot analysis to detect p55 Gag monomers derived from reduced VLPs. Our results confirm the production of VLPs by both recombinant DNA and Ad vectors *in vitro* and VLP release from the cells by detecting p55 in cell supernatants from Gag-MPER expressing constructs compared to the mock DNA transfection and Ad adsorption (Fig. 1B). Under reducing conditions the VLPs were broken down into monomers of p55 Gag which are represented by the band seen at 55kDa, detected by a gag-specific antibody; as expected, the mock vectors did not yield any bands. To further verify that the size of the epitope insert incorporated within gag in each chimeric construct did not affect the structural integrity of the particles released, VLP supernantants underwent CsCl density centrifugation to compare the density of the chimeric particles relative to WT-Gag VLPs. Cells were adsorbed with each of the chimeric rAd constructs as well as a rAdWT-Gag to generate VLPs *in vitro*. Supernatants containing the particles were collected, concentrated and centrifuged along a CsCl density gradient. Eight continuous gradient fractions ranging from 10 to 40% CsCl were serially collected and each fraction was run through a reducing SDS-PAGE prior to visualizing the gag-specific bands in a western blot. The fractions in which the gag-specific bands were detected are shown (Fig. 1C) and are representative of the density range in which the particles reached buoyancy. The density range of the chimeric VLPs would be similar but not identical to the density WT-VLP since they have added epitope string within gag. The position of the bands indicates that the chimeric VLPs have a similar buoyant density range as the WT-Gag in the CsCl fractions. Therefore, the MPER string insertion in our constructs did not impede proper VLP assembly *in vitro*.

3.2. Administration of three intra-muscular (i.m.) pGag-MPER immunizations with CpG significantly increased MPER-specific Ab titers compared to other homologous DNA delivery regimens, with and without CpG

Various methods and regimens were tested in preliminary experiments to determine the best DNA delivery protocol that would elicit MPER-specific Abs in our peptide-specific ELISA. Serum IgG against each of the MPER epitopes was measured on separate plates, which were coated with either peptide to characterize the anti-ELDKWAS or anti-NWFDIT Ab response individually, relative to representative samples from un-immunized mice. The specificity of the epitope-specific ELISAs was tested and is briefly described in the methods. We found that up to three DNA immunizations i.m., without electroporation, did not yield any detectable MPER-specific IgG in the serum from blood samples collected at various time-points (days 7, 14, 21, 28, 35 after the first immunization, data not shown). In mice that received immediate electroporation subsequent to DNA delivery i.m., a minimum of three DNA immunizations were required for detectable serum IgG against the MPER epitopes compared to un-immunized control samples (data for one or two DNA-only immunizations is not shown). Therefore, a minimum total of three DNA immunizations were administered to groups of mice, in either the presence or absence of CpG, to determine the effectiveness of CpG as an adjuvant with DNA administration i.m. (Fig. 2A). Our results clearly show that significantly higher MPER-specific IgG was generated at each corresponding time-point in mice that were co-administered CpG with DNA compared to the mice that received DNA alone. Based on these findings, in the following experiments, all DNA immunizations at the hind limb quadriceps were followed by immediate electroporation of the muscle and co-administered with CpG.

Next, we determined the optimal number of [DNA + CpG] immunizations that would most significantly increase MPER-specific Ab titers. The same group of mice was given pGag-MPER with CpG i.m., 3 weeks apart, up to four times. Serum IgG was measured in a peptide-specific ELISA, from blood collected at day 21 after each immunization, prior to receiving the next immunization. Our results show that there was a significant increase in MPER-specific Ab titers with each additional booster, up to three total immunizations, but a fourth one did not contribute to a significant rise in titers (Fig. 2B; [DNA + CpG] $\times 4$ versus $\times 3$: not significant, [DNA + CpG] $\times 3$ versus $\times 2$: $^{***}p < 0.001$, [DNA + CpG] $\times 2$ versus $\times 1$: $^{**}p < 0.05$). Mucosal IgG or IgA titers were not detectable in peptide-specific ELISAs from vaginal washes and fecal pellets collected between days 14 and 18 or days 21 and 25, from the same groups of mice after the third or fourth immunization, respectively (data not shown). A separate group of mice were immunized with an empty control DNA vector up to four times with CpG followed by electroporation and did not yield detectable MPER-specific Abs in serum (Fig. 2B, 'Mock (DNA + CpG)$\times 4$'), nor in vaginal washes and fecal pellet samples collected at the same time-points mentioned above (data not shown).

3.3. Intranasal (i.n.) delivery of rAdGag-MPER elicits epitope-specific mucosal IgA and IgG

rAdGag-MPER was administered to mice i.n. with the goal to induce MPER-specific Abs in mucosal compartments, particularly at the genital mucosa. To determine the optimal number of immunizations that would offer a significant increase in epitope-specific Ab titers, groups of mice were immunized with an empty rAd vector or the rAdGag-MPER i.n. up to three times, each immunization 2 weeks apart. MPER-specific serum IgG was measured by peptide-specific ELISA, from blood collected at day 14 after each immunization, prior to receiving the subsequent one. The second delivery of rAd i.n. significantly raised the specific Ab titers relative to a single rAd administration (Fig. 3A, $^{***}p < 0.001$), however, a third delivery only raised titers with modest significance ($^{*}p < 0.05$). Further examination of the kinetic profiles of groups of mice that received a total of two times or three rAdGag-MPER immunizations, showed that there was no benefit to a third rAd delivery over time, since the epitope-specific serum IgG titers reached a plateau with no significant differences between groups after day 21 (Fig. 3B). Epitope-specific mucosal IgG and IgA were detected in the vaginal washes and fecal pellets collected from the $2\times$ rAdGag-MPER group between days 14 and 18 following i.n. delivery (Fig. 3C), confirming that i.n. rAd administration can indeed elicit mucosal Abs specific to the MPER. Vaginal lavage titers were reported between days 14 and 18 since that is when they were higher compared to other collection periods (days 7–11 and 21–25, data not shown). Mice that were immunized with a control mock rAd vector up to three times i.n. did not yield any MPER-specific Abs in the serum (Fig. 3A, 'Mock $3\times$rAd'), vaginal washes or fecal pellets (data not shown).

3.4. Comparison of different heterologous [DNA + rAd] and [rAd + DNA] prime-boost immunizations to determine the optimal regimen for generating high titers of MPER-specific Abs

In order to generate potent Ab titers against the MPER epitopes, it is not only important to design optimal immunogens but also to implement an effective immunization strategy. Heterologous immunization has been shown to significantly increase immune responses compared to homologous immunization of either of the component vectors [39]. To verify this, separate groups of mice were administered different total numbers of DNA and rAd immunizations: $2\times$(DNA + CpG) and $1\times$rAd, or $2\times$(DNA + CpG) and $2\times$rAd, or $3\times$(DNA + CpG) and $2\times$rAd, where either the (DNA + CpG)

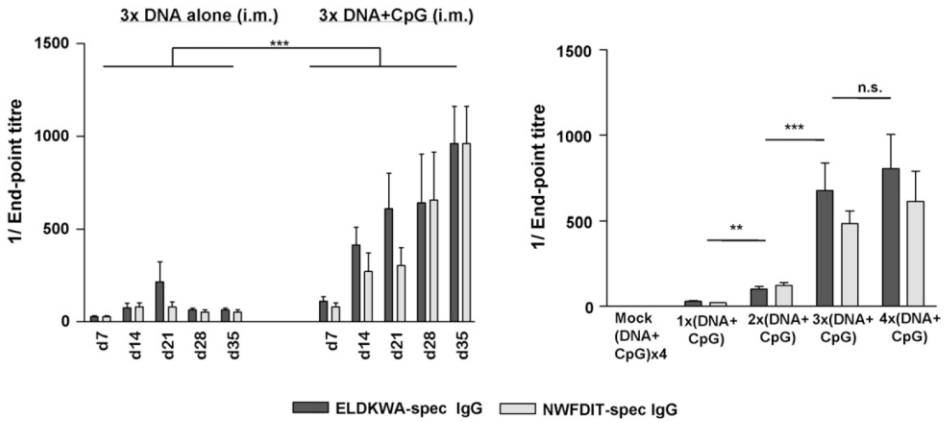

Fig. 2. Three DNA intra-muscular (i.m.) immunizations, with CpG, were optimal in eliciting significantly higher MPER-specific Ab titers compared to other combinations. (A) C57BL/6 mice were immunized with pGag-MPER i.m., with or without CpG, at the hind limb quadriceps followed by immediate electroporation of the muscle. DNA was administered a total of three times 3 weeks apart and serum samples were collected every 7 days for up to 35 days after the last immunization. Serum IgG was measured by epitope-specific ELISAs against the ELDKWA or the NWFDIT epitopes individually. Significant differences in serum MPER-specific IgG between the DNA alone versus the DNA + CpG group were determined by unpaired *t*-test at each of the time-points (***$p < 0.001$). (B) Mice were immunized with an empty pcDNA3.1 vector or the pGag-MPER, i.m. with CpG and electroporation, up to four times 3 weeks apart. Serum IgG was measured by ELISA from blood collected at day 21 after each pGag-MPER immunization, prior to receiving the subsequent one. Paired *t*-test (***$p < 0.001$, **$p < 0.05$) was used to determine significant increases in Ab titer after each additional immunization of the [pGag-MPER + CpG] relative to the titer after the previous immunization. All results are representative of two independent experiments ($n = 5$ for each experiment).

i.m. was delivered first followed by rAd i.n. boosts (Fig. 4A), or vice-versa (Fig. 4B). In both cases, we observed that the [3×(DNA + CpG) + 2×rAd] and the [2×rAd + 3×(DNA + CpG)] regimens significantly increased the MPER-specific titers compared to the other groups. The titers raised by these two heterologous regimens were up to 10-fold higher compared to the homologous immunizations of their component vectors: 3×(DNA + CpG) alone shown in Fig. 2B and 2×rAd alone shown in Fig. 3A. Therefore, our

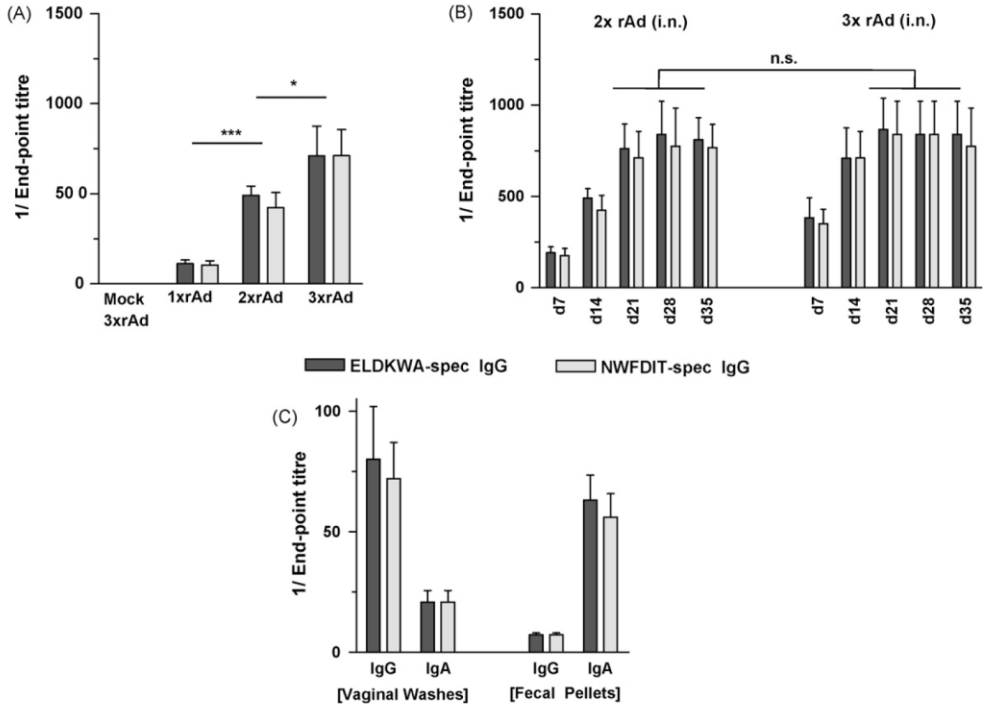

Fig. 3. Intranasal (i.n.) delivery of rAdGag-MPER elicits mucosal and systemic MPER-specific Abs. (A) C57BL/6 mice were administered a mock, empty rAd vector or the rAdGag-MPER up to three times i.n. Serum IgG was measured from blood collected 14 days after each immunization using epitope-specific ELISAs. Significant differences in Ab titers after each subsequent immunization relative to the previous one was determined by paired *t*-tests (***$p < 0.001$, *$p < 0.01$). (B) MPER-specific ELISA in the serum of mice immunized with rAdGag-MPER two or three times, 2 weeks apart, after the last immunization. No significant differences were observed between the groups at the later time-points as indicated (n.s. = non-significant). (C) Mucosal Abs in mice administered 2×rAdGag-MPER i.n.; fecal pellets were collected at day 14 and vaginal washes over 5 days, during days 14–18 after the last immunization. MPER-specific IgG and IgA were measured using peptide-specific ELISAs ($n = 5$ for each group of mice).

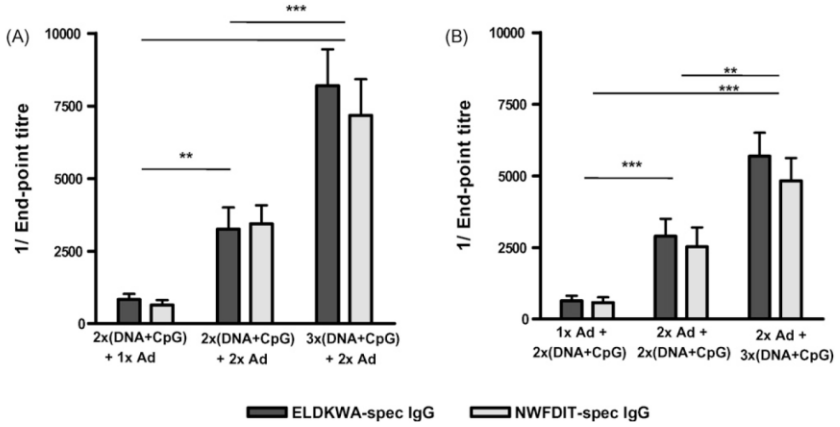

Fig. 4. The comparison of different prime-boost regimens with (DNA + CpG) and rAd vector delivery expressing Gag-MPER. ELDKWA- and NWFDIT-specific IgG was measured by ELISA in the serum of mice that were administered: (A) DNA + CpG i.m. as a prime followed by a rAd i.n. boost. (B) rAd i.n. as a prime followed by DNA + CpG i.m. boost. Total number of DNA and rAd immunizations that were administered to the different groups of mice is indicated. DNA + CpG was delivered 3 weeks apart each time whereas rAd was administered 2 weeks apart. Serum was collected at day 14 after the last immunization for each group ($n = 5$). Significant differences in ELDKWA and NWFDIT-specific Ab titers between the different prime-boost groups were determined by *t*-test and indicated by $^{**}p < 0.05$, $^{***}p < 0.001$.

results show that the total number of component vector immunizations, DNA or rAd, in heterologous regimens significantly affects the titers of MPER-specific Abs, and also clearly increases the Ab titer compared to homologous vector immunizations.

The serum titers reported are from blood collected at day 14 after the last immunization, since that is when two of the groups had reached their peak titers: [2×(DNA + CpG) + 1×rAd] and [1×rAd + 2×(DNA + CpG)]. Serum sampling at days 21 and 28 for the other groups showed the same trend of significant differences (data not shown). From our results, we concluded that the heterologous 3×(DNA + CpG) followed by 2×rAd and the reverse prime-boost regimens elicited the highest titers of epitope-specific serum IgG. The specific differences in the systemic and mucosal Ab titers generated by these two delivery strategies were characterized next.

3.5. The heterologous 3×(DNA + CpG) prime followed by the 2×rAd-boost significantly increased the titer of systemic and mucosal IgG and IgA against the MPER compared to the reverse prime-boost regimen

A key objective of our study was to drive potent Ab responses against the MPER epitopes, particularly at the mucosa, by strategic delivery of the DNA or rAd vectors expressing MPER-VLPs. Here, we characterized the systemic and mucosal IgG and IgA titers generated by the heterologous immunization combinations: [3×(DNA + CpG) + 2×rAd] and [2×rAd + 3×(DNA + CpG)]. We also questioned if the route and order of vector delivery would make a significant difference in eliciting mucosal Abs. Specifically, we compared systemic DNA-prime i.m. followed by a mucosal rAd-boost i.n. [DNA + rAd], with the mucosal rAd-prime followed by a systemic boost (i.m.) [rAd + DNA].

Fig. 5 compares the difference in Ab titers generated in the serum, vaginal washes and fecal pellet secretions obtained from mice that were immunized by each of the prime-boost regimens. Our results show that the [DNA + rAd] group induced significantly higher serum IgG and IgA against the MPER epitopes compared to reverse regimen, [rAd + DNA] (Fig. 5A, IgG: $^{**}p < 0.05$, IgA: $^{*}p < 0.05$). The titers are shown for blood collected at day 14 after the last immunization representing peak titers, Ab titers from day 21 and 28 samples were not significantly higher (data not shown). Significantly higher MPER-specific IgG and IgA titers were observed in vaginal washes from the [DNA + rAd] group compared to the [rAd + DNA] group (Fig. 5B, IgG and IgA: $^{**}p < 0.05$). However, there were no significant differences in the fecal pellet secretions (Fig. 5C). Results for vaginal washes are reported for the day 14–18-period after the last immunization and from the day 14 collection of fecal pellets since that is when epitope-specific titers were the highest (compared to sample titers from the collection period of days 21–25, data not shown). Therefore, we have shown that it was indeed the [3× DNA + CpG + 2×rAd] regimen that elicited significantly higher titers of MPER-specific IgG and IgA at the urogenital tract compared to the [2×rAd + 3×(DNA + CpG)].

3.6. Multiple tandem repeats of an epitope at the C-terminus of p55 gag significantly increased the epitope-specific antibody response compared to a single copy

With the goal to optimize epitope antigenicity in our constructs, we strategically incorporated multiple tandem copies of the MPER epitopes within the Gag VLP. In light of a previous study which also applied the multiple tandem copy strategy in an HSV model [29], we hypothesized that this approach would significantly increase the MPER-specific Ab titer in our chimeric VLP immunization model. To test this hypothesis, three additional sets of DNA and rAd vectors were generated, which expressed VLPs containing either one (Gag-ELDx1), three (Gag-ELDx3) or five (Gag-ELDx5) copies of the ELDKWA epitope. Groups of mice were either given three repeated (DNA + CpG) i.m. or two repeated rAd i.n. immunizations in a homologous delivery scheme, or 3×(DNA + CpG) i.m. followed by 2×rAd i.n. immunizations in a heterologous delivery scheme (Fig. 6A). There was significantly higher serum IgG raised against the ELDKWA epitope in mice immunized with the Gag-ELDx3 or Gag-ELDx5 constructs compared to the Gag-ELDx1 construct, independent of the vector or immunization regimen used. However, there were no differences between mice immunized with Gag-ELDx3 versus Gag-ELDx5. Results for serum Abs are shown from blood collected at day 21 after the last immunization since that is when the greatest significance differences between groups were observed. At the mucosa, however, there was significantly higher ELDKWA-specific IgG and IgA in mice that received Gag-ELDx3 construct versus Gag-ELDx1 or Gag-ELDx5, via the heterologous delivery 3×(DNA + CpG) followed by 2×rAd (Fig. 6B). These results confirm that increasing the number of tandem copies of an epitope in our chimeric VLP design is an effective strategy to increase the

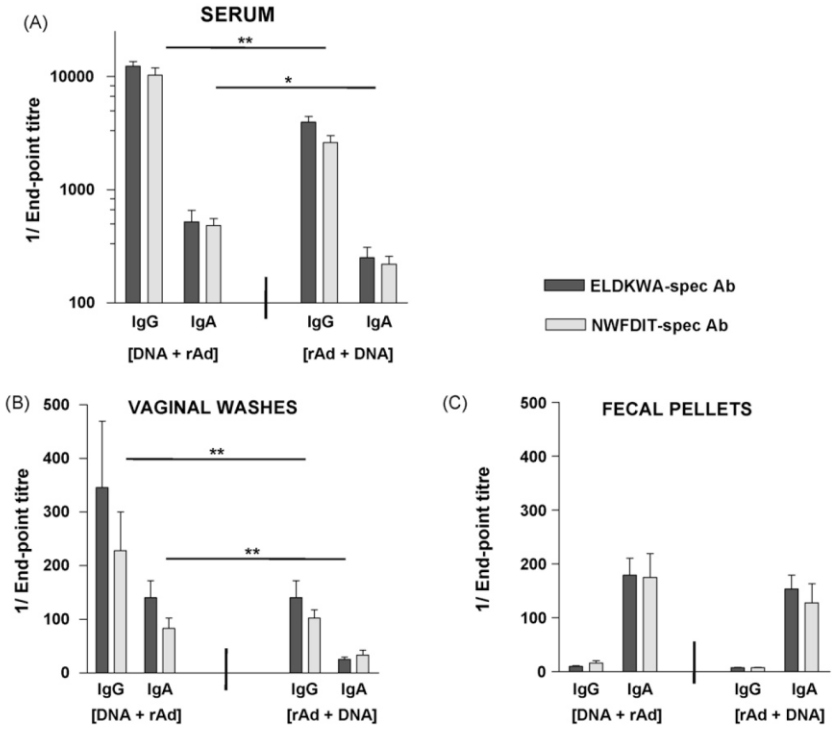

Fig. 5. Comparison of the MPER-specific IgG and IgA in the serum and mucosal compartments of mice administered 3×(DNA + CpG) i.m. followed by 2×rAd i.n. versus the reverse regimen. Mice were immunized under both regimes and epitope-specific IgG and IgA were measured in each group, by ELISA, in the following samples obtained at day 14 after the last immunization: (A) serum, (B) vaginal washes collected over 5 days from the day 14 mark, and (C) fecal pellets. Results are indicative of two independent experiments ($n = 5$ each) and statistically significant differences in Ab titers between the two groups are shown by $^{*}p < 0.01$, $^{**}p < 0.05$.

epitope-specific Ab titer in both systemic and mucosal compartments.

4. Discussion

The generation of a potent neutralizing Ab response by vaccines against HIV, especially at the mucosa, could play a critical role in preventing viral infection. However, the induction of such a response by various vaccine strategies thus far has come with its limitations and challenges. In this study, we have developed a model that elicits high titers of both systemic and mucosal Abs against the MPER epitopes incorporated within HIV-Gag VLPs in multiple tandem repeats. By expressing the chimeric Gag + MPER construct *in vivo* via DNA and rAd vectors, employing strategic design, optimal delivery routes, adjuvants and prime-boost combinations, we were able to generate high titers of epitope-specific Abs, notably in mucosal compartments.

The multiple tandem repeat design is a unique aspect of our VLP construct. We showed that increasing the total number of ELDKWA repeats in our HIV-VLP construct, from a single copy to three or five tandem copies, could significantly increase the ELDKWA-specific serum IgG titer, by either the DNA or rAd vector alone or their combination. Mucosal Ab titers also increased with a greater number of tandem repeats compared to a single copy, however, Gag-ELDx3 induced significantly higher mucosal titers (Fig. 6B). Therefore, in our model, a total of three tandem copies of ELDKWA was the optimal epitope insert that could significantly increase epitope-specific Ab titers in serum and mucosal compartments of mice. Our results confirm the findings of a previous HSV study that also reported enhanced immunogenicity of a linear gD epitope when expressed in multiple copies [29]. This has an important implication in future Ab-inducing vaccine design, since including multiple tandem repeats of linear epitopes could indeed contribute to a significant increase in epitope-specific Abs.

The immunogenicity of our Gag + MPER expressing DNA and rAd vectors was greatly enhanced by the utilization of optimal delivery methods. Muscle electroporation was critical in our DNA delivery scheme for effective MPER-specific Ab generation since it is known to increase the efficiency of DNA uptake into cells. Clinical trials employing electroporation with DNA immunization in immunotherapy for various conditions have supported its benefit in enhancing antigen immunogenicity [40]. CpG co-administration with DNA also had a significant benefit in our model and it has previously been shown to increase the magnitude of both cellular and humoral responses, as well as the rate at which the response is generated and sustained [41,42]. More specifically, B-cells and DCs express the Toll-like receptor 9 (TLR-9) which recognizes CpG motifs, and they undergo activation/maturation and proliferation upon CpG exposure [43–45].

Mucosal induction of MPER-specific Abs, however, was only achieved upon i.n. immunization with the rAdGag-MPER vector (Fig. 3C) since homologous (DNA + CpG) immunizations i.m. were unable to elicit detectable mucosal IgG or IgA against the MPER. Furthermore, only two i.n. rAd immunizations were required to elicit specific Abs in serum and vaginal lavage samples since a third i.n. immunization did not significantly boost the epitope-specific titers over time (Fig. 3B). This could possibly be explained by pre-existing immunity elicited against the Ad vector itself from the previous two rAd immunizations, which would impede the booster efficacy of the antigen when delivered i.n. a third time. In preliminary experi-

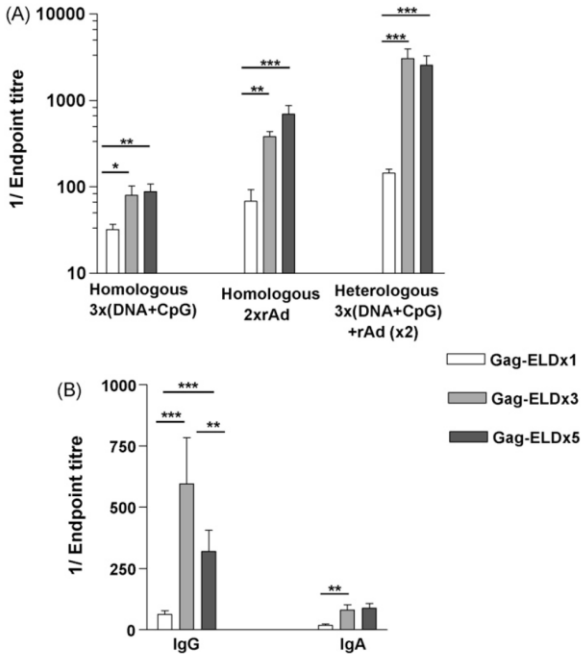

Fig. 6. Increased number of tandem repeats of the ELDKWA epitope at the p55 Gag C-terminus significantly increases systemic IgG raised against the epitope. (A) Mice were administered (DNA + CpG) i.m. three times or rAd i.n. two times in homologous immunization regimen or with 3×(DNA + CpG) i.m. followed by 2×rAd i.n. in a heterologous prime-boost. Three different groups of mice were immunized for each regimen, with DNA or rAd vectors expressing Gag containing a single copy of ELDKWA versus three or five tandem copies at the C-terminus (VLP-ELDx1, VLP-ELDx3, VLP-ELDx5). ELDKWA-specific IgG was measured by ELISA from serum obtained 21 days after the last immunization. (B) The mucosal IgG and IgA in the vaginal washes of mice administered the heterologous [3× DNA + 2×rAd] prime-boost. Vaginal washes were collected between days 17 and 21 and pooled. Significant differences in ELDKWA-specific Abs titers are indicated by the following *p*-values: $^{*}p < 0.01$, $^{**}p < 0.05$, $^{***}p < 0.001$, for $n = 5$ in all groups.

ments, intravaginal (IVAG) delivery of rAd was also tested to assess the effectiveness of other mucosal delivery routes in the generation of mucosal antibodies (data not shown). However, given the same number of two rAd deliveries, IVAG administration did not elicit mucosal IgG or IgA and generated one fifth of the MPER-specific IgG in the serum. Therefore, rAd delivery i.n. was optimal in our model for MPER-specific mucosal Ab elicitation.

We also confirmed with our model that heterologous prime-boost regimens offer a significant advantage in boosting immune responses compared to their component vectors alone [39]. Furthermore, administration of different vectors allows the use different delivery routes which can be used to an advantage to obtain a balance between systemic and mucosal responses, as we have shown in our study. Specifically, we found that systemic priming with DNA immunization was more effective at eliciting significantly higher mucosal Ab responses compared to rAd-priming when comparing reverse heterologous prime-boost strategies (Fig. 5).

The generation of mucosal Abs, especially IgA, against the MPER was an important target of our vector delivery strategy. In the past Ab-based vaccines have largely focused on IgG induction since it is the predominant Ab subtype in the serum and because IgG is largely responsible for classical 'sterilizing' immunity and clearance of HIV. However, there is substantial evidence supporting the role of HIV-specific IgA in preventing virus transmission at the mucosal epithelium [46,47]. The ELDKWA epitope of the MPER has been implicated as a domain capable of binding galactosylceramide (Gal-Cer) on cell membranes, a mechanism by which it aids in attachment and transmission of HIV [48]. Indeed, ELDKWA-specific IgA can effectively prevent transcytosis of HIV across epithelial cells (ECs) by blocking the binding of HIV to Gal-Cer [49].

As supplementary table shows, unfortunately, systemic and mucosal Abs generated by the heterologous DNA and rAd immunizations in mice did not significantly neutralize HIV infection *in vitro*. Serum, vaginal lavage and fecal secretion samples as well as concentrated serum antibody samples were screened in a TZM-bl reporter assay against the SF162 virus strain. No inhibition of virus infection was observed compared to the positive control of HIV hyper-immune globulin (HIV-IG) in the neat samples and only modest neutralization was observed for the concentrated serum samples. Although other studies have reported the generation of MPER-specific Abs in animal models and shown modest to effective neutralization of HIV lab strains [50–53], potent neutralization has yet to be achieved in a manner similar to that of the broadly neutralizing 2F5 or 4E10 mAbs.

Structure-based design approaches have also been used to present the MPER epitopes similar to their native gp41 conformation however, thus far, they too have not been able to induce potent Abs [53]. Implementing current knowledge of the crystallographic structure of the mAbs and how they bind to native MPER may be required to elicit 2F5- or 4E10-like nAbs, by designing and testing novel structure-based antigens [54–56]. One possible explanation for the challenge in eliciting MPER-targeting nAbs is offered by the fact that these Abs cross-react with self-antigens such as cardiolipin and membrane-bound lipids [57,58]. Therefore, B-cells producing Abs against the MPER would subsequently be eliminated from circulation, accounting for the scarcity of 2F5 or 4E10 Abs in HIV-positive serum. Although the MPER is a highly conserved and well characterized target, such limitations may hinder its use in vaccines that aim to generate protective neutralizing Abs against HIV.

There are considerable ongoing efforts seeking to identify and profile naturally occurring nAbs against HIV with the goal to identify new targets capable of inducing nAbs [7,59–62]. These naturally occurring Abs, however, do not bind to accessible viral envelope regions but have more complex targets such as epitopes associated with sugar moieties or quaternary structures of the gp41 trimer conformation [4,63–64]. Structural knowledge and crystallographic analysis will be required to develop structure-based designs to mimic such antibodies [65–66]. Although our model serves as a suitable platform to elicit Abs against linear epitopes, it may not be ideal for epitopes that require high conformational integrity in the immunogen design to maintain their neutralizing function. It is, however, ideal for the incorporation of multiple copies of linear epitopes capable of generating nAbs against the virus. Several computational tools are available to identify linear HIV epitopes [67] that could be characterized and selected for insertion into gag to form chimeric VLPs as shown in our model.

The previous HSV study that reported enhanced immunogenicity of a gD epitope when expressed in multiple copies interestingly noted that the level of Ab raised against four copies fused to β-galactosidase was comparable in titer to that raised against the entire gD protein [29]. Furthermore, mice immunized with Ad vectors expressing 4-copies of the gD epitope versus the entire gD protein offered comparable protection against an i.p. HSV-2 challenge. It has been suggested that the polyclonal burst of Abs against entire HIV antigens such as gp120 or gp160 could contribute to the reduced production and efficacy of neutralizing Abs, due to a dominance production of non-neutralizing polyclonal Abs [32]. Immunizing with multiple copies of an Ab-inducing epitope offers a method to 'immuno-focus' the response against known immunogenic epitopes rather than generating a broad polyclonal response

against a larger antigen. This could be an important consideration for future immunogen design. Such a strategy could also be employed for other viruses such as influenza, where mucosal vector delivery of 'immuno-focused' Gag expressing multiple copies of immunogenic conserved linear flu epitopes would be applicable [68]. Furthermore, i.n. delivery of the chimeric VLP construct via rAd vectors would be ideal to target the respiratory mucosa.

Alternative VLP designs have also been developed and characterized that display selective Ab-inducing epitopes on the surface of VLPs [69] or by fusion to the hepatitis B surface antigen or by fusion to surface loops of the human rhinovirus [53,70]. Although these particles can now be generated in a stable fashion with consistency, they vary in the number of epitope copies displayed on the surface and are largely restricted to protein delivery in animal models [71]. Even though our chimeric VLPs do not display the epitopes on the VLP surface in a classical B-cell stimulating manner, we have still been successful in generating high titers of epitope-specific Abs. The chimeric VLP design used here does not rely on protein display at the surface by which it would be largely limited to protein delivery and therefore offers the possibility to exploit other delivery routes or strategies to optimize and increase epitope-specific Ab titers.

Overall, this study shows that MPER-specific IgG and IgA were elicited in the serum as well as the mucosal secretions of mice immunized with DNA and rAd vectors expressing chimeric Gag-MPER-VLPs. MPER-specific Ab induction was significantly enhanced by the optimization of vector delivery in both homologous and heterologous prime-boost scenarios. Furthermore, multiple tandem repeats of the ELDKWA epitope in our chimeric VLP design significantly enhanced its immunogenicity. Such strategies can be employed to significantly increase specific Ab titers and in particular, for efficient induction of mucosal IgG and IgA, which would provide a crucial first-line defense against the virus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.08.009.

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Supplementary Table. TZM-bl cell based neutralization assay to test the efficiency of Abs in neutralizing the SF162 lab strain. Listed are the ID50 values for the systemic (serum) and mucosal samples (vaginal washes, fecal pellets) obtained from mice immunized with Gag-MPER (DNAx3 + rAdx2) and Gag-ELDx5 (DNAx3 + rAdx2). Samples were collected 14 days after the last immunization.

	Gag-ELDx5			Gag-MPER			
SAMPLE	**Serum**	**Vaginal washes**	**Fecal pellet**	**Serum**	**Vaginal washes**	**Fecal pellet**	**Conc. Serum**
ID50 (n=3)	< 45	<20	<20	<45	<20	<20	<45
	< 20	<20	<20	<20	<45	<20	<25
	< 20	<20	<20	<20	<20	<20	<45

-- CHAPTER 3 --

THE GP41 EPITOPE, QARVLAVERY, IS HIGHLY CONSERVED AND A POTENT INDUCER OF IGA THAT NEUTRALIZES HIV-1 AND INHIBITS VIRAL TRANSCYTOSIS.

Jain S. and Rosenthal K.L.

Mucosal Immunology 4(5):539-553

The study outlined in this chapter characterizes the unique immune response elicited by the QARVLAVERY epitope, upon delivery in a chimeric VLP construct, utilizing an optimized immunization regimen to elicit potent mucosal Abs in mice. The results of the study show that the QARVLAVERY epitope is a potent inducer of IgA in the serum and mucosal secretions of immunized mice. Furthermore, the ratio of QARVLAVERY-specific IgG to IgA in the serum was found to be 1:1 which is a unique characteristic. QARVLAVERY-specific IgA was also found to be generated before epitope-specific IgG in the serum; another unusual characteristic. Most importantly, QARVLAVERY-specific IgG exhibited modest neutralization ability against lab-adapted X4 and R5 virus strains, while QARVLAVERY-specific IgA inhibited transcytosis of HIV across an epithelium in a transwell assay which is of particular interest for mucosal protection against the virus.

I was solely responsible for the work done to acquire the data for this study and for the writing of the manuscript, under the supervision of Dr. Ken Rosenthal. Some technical assistance was provided by Jen Newton and Amy Patrick.

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The gp41 epitope, QARVLAVERY, is highly conserved and a potent inducer of IgA that neutralizes HIV-1 and inhibits viral transcytosis

S Jain[1] and KL Rosenthal[1]

Mucosal surfaces are the predominant site of human immunodeficiency virus (HIV)-1 transmission. For prophylactic approaches to effectively prevent HIV infection and subsequent dissemination, the induction of mucosally relevant protective immunity will be critical. Here, we have characterized the antibody (Ab) response generated by a highly conserved gp41 epitope, QARVLAVERY, in an optimized immunization model that elicits potent epitope-specific Abs in the serum, vaginal washes, and fecal secretions of immunized mice. Our results show that QARVLAVERY is indeed a potent inducer of IgA and importantly, QARVLAVERY-specific IgA was effective in neutralizing HIV and inhibiting viral transcytosis. Intriguingly, QARVLAVERY also generated an approximate 1:1 ratio of IgG:IgA in the serum of immunized mice, independent of the delivery regimen and produced early systemic IgA, even before IgG. In light of the significantly high IgA induction by QARVLAVERY and the functionality of epitope-specific Abs in the inhibition of HIV infection and transcytosis, QARVLAVERY is an attractive epitope to be considered in mucosal vaccination strategies against HIV.

INTRODUCTION

The development of a prophylactic vaccine that generates potent cross-clade immunity against HIV is critical for effectively curbing the global human immunodeficiency virus (HIV)/AIDS (acquired immunodeficiency syndrome) pandemic. HIV is primarily a sexually transmitted infection, and in women, the majority of new infections occur through the urogenital tract. It is well accepted that strengthening mucosal defenses against virus entry, viral transmission, and infection will need to be addressed by prophylactic approaches. Thus far, this goal has met with several challenges, the greatest being the enormous diversity of HIV-1, which makes the generation of long-term immunity against it a constantly moving target.

At large, antibodies (Abs) generated during acute and chronic HIV infection are not effective in neutralizing the virus.[1,2] In addition, a majority of the rare neutralizing Abs (nAbs) that are detected in natural infection tend to target non-conserved regions of the envelope and are quickly rendered ineffective because of rapid mutation of the virus.[3,4] Clinical vaccines based on envelope antigens have thus far been unsuccessful in inducing long-term cross-reactive Ab responses.[5,6] T cell-based vaccine approaches in simian models and in clinical cohorts have also eventually succumbed to the emergence of viral escape mutants.[7–10] These past lessons have stressed the importance of eliciting effective and broad immunity against conserved targets of HIV, to prevent and control its infection despite the genetic diversity.

The identification of broadly reactive human monoclonal Abs such as 2F5, 4E10, 2G12, 1b12, VRC01, HJ16, and PG9/16,[11–15] against gp120/gp41 motifs or the envelope trimer, has been the basis of renewed and ongoing focus on nAbs. 2F5 and 4E10 are the only nAbs that bind to linear and accessible viral targets located in the membrane-proximal external region (MPER) of gp41, ELDKWA, and NWFDIT. Several vaccine approaches incorporating MPER epitopes have attempted to mimic 2F5- or 4E10-like Abs but have only induced modest responses *in vivo*.[16–19] Unfortunately, because of the partial homology of MPER epitopes with autoantigens such as cardiolipin, nAbs of 2F5/4E10-like potency and neutralizing activity are not elicited in the course of natural infection or upon vaccination.[20–22] Therefore, the identification of novel immunogenic and conserved viral targets that can be incorporated into vaccine models to generate broadly reactive immunity against HIV is still of great interest.

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Although the role of virus-specific Abs has largely focused on the neutralizing ability of IgG in the past, there has been ample emerging evidence supporting the role of alternate mechanisms of protection by non-nAbs, such as Ab-dependent cellular cytotoxicity[23–26] and Ab-dependent cell-mediated virus inhibition.[27,28] In addition to the role of IgG, several studies have also reported the importance of IgA against HIV. IgA has proven to be functional in protecting against HIV infection by direct neutralization,[29–34] by inhibiting transcytosis of HIV across the mucosa,[29,31,35–40] by intracellular virus neutralization,[41,42] and by preventing HIV entry past the epithelial lumen.[37] Furthermore, virus-specific IgA has been independently identified in the serum and cervicovaginal lavage of exposed uninfected (EU) cohorts of European, African, and Asian origins.[30,43–45] Thus far, there is no firm evidence of an association or causal relationship between virus-specific IgA and natural resistance to HIV in EU individuals.[46] However, HIV-reactive IgA from such cohorts has exhibited the ability to neutralize HIV[29,31,32] and inhibit syncytia formation.[30] With particular interest to mucosal protection, cervicovaginal lavage-derived secretory IgA from EU can inhibit virus transcytosis *in vitro*.[38,39]

Only one epitope has been specifically identified as a target of functional IgA obtained from EU discordant couples, QAR*I*LAVERY, which is found within the coiled-coil region of gp41.[30,34] In a study conducted by Clerici *et al.*,[30] epitope-specific IgA was purified from the sera of EU individuals and showed effective virus-neutralization capacity. In addition, serum obtained from mice immunized with the QAR*I*LAVERY peptide inhibited syncytia formation. Older studies have also reported immunogenicity of coiled-coil region epitopes when delivered in the peptide form,[30,47,48] and showed that peptide-specific Abs could inhibit syncytia formation[30,47,49] and neutralize HIV *in vitro*.[50] The coiled-coil pocket of gp41 (HIV-1 env 583–599)[51] is indeed a highly conserved region and has been reported as such in studies as early as 1988.[52] It is a relatively stable structure that has proven to be critical in the host–virus membrane fusion process.[53] Interestingly, serum samples obtained from chronically infected individuals[49,50,54] and hosts immunized with gp120/gp41[54] have also shown cross-reactivity to the coiled-coil region, which proves that it is naturally immunogenic. However, since these early studies, the IgA-inducing capacity of the coiled-coil region and assessment of the IgA function against HIV, have not been explored further.

We have previously designed and tested an immunization model that generates chimeric Gag virus-like particles (VLPs) *in vivo*, with the goal to elicit potent Abs against the MPER. The [Gag + MPER]-VLP constructs were expressed in mice through strategic delivery of heterologous DNA and recombinant adenovirus (rAd) vectors in an optimized prime-boost regimen, which elicited potent systemic and mucosal epitope-specific Abs.[16] The study also independently showed that the expression of multiple tandem copies of an Ab-inducing epitope within a Gag-VLP could significantly increase epitope-specific IgG and IgA titers compared with a single copy.[16]

In this study, we characterized the immunogenicity of the highly conserved gp41epitope, QARVLAVERY. Different lengths and slight amino-acid (aa) variations of this coiled-coil epitope have been used in previous studies;[30,55,56] however, here, we specifically characterized the QAR*V*LAVERY epitope. QARVLAVERY-specific Abs were induced systemically and mucosally by immunizing C57Bl/6 mice with DNA and rAd vectors that generate chimeric [Gag + QARVLAVERY]-VLPs, using previously optimized regimens.[16] We assessed the immunogenicity of QARVLAVERY in our model, particularly with regard to IgA induction, and in comparison with the ELDKWA-specific IgA induced in similar head-to-head immunization regimens. Furthermore, the functional capacity of QARVLAVERY-specific IgG and IgA from systemic and mucosal samples has been assessed for their HIV infection inhibition or viral transcytosis-inhibition ability, *in vitro*.

RESULTS

QARVLAVERY is a highly conserved epitope

Figure 1 summarizes the overall sequence homology of the QARVLAVERY epitope (aa 577–586) against all the listed HIV-1 sequences in the Los Alamos HIV immunology database. This first-glance analysis is a preliminary indication of the remarkable conservation of this epitope determined using the QuickAlign tool.

Figure 1a summarizes the percentage of total HIV sequences that have differences of 0, 1, 2, 3, or > 4 aa in the alignment results of the input sequence "QARVLAVERY," against 2,230 of the total listed HIV-1 sequences (T_{seq}). As shown, 44.3% of all listed viral sequences show 100% alignment with QARVLAVERY (zero changes in aa sequence), and in fact, 79.4% of all listed viruses only show a difference of 1 aa (44.3 + 35.1%). **Figure 1b** shows similar alignment results for the gp41 ELDKWA epitope (aa 622–667) to compare the relative conservation of both epitopes with all listed sequences. Alignment results for QARVLAVERY illustrated in **Figure 1c** are specific to individual clades, the most prevalent ones worldwide. The global distribution of HIV-1 clade prevalence in **Figure 1d** was obtained from the geography search interface of the Los Alamos HIV sequence database. It is noteworthy that 73.6% of all listed clade B sequences have 100% homology with the QARVLAVERY epitope as it has the highest global prevalence of 62.4%. Furthermore, the percentage of clade-specific HIV sequences that differ from the QARVLAVERY alignment by only 1 aa, ranges from 52% (clade C: 48.9 + 3.1%) up to 99.1% (clade 01_AE: 94.6 + 4.5%), i.e., > 50% of 7 of the most globally prevalent clades only differ from the QARVLAVERY sequence alignment by 1 aa. These results highlight the remarkable sequence conservation of this coiled-coil epitope, potentially making it an attractive immunogenic target to elicit broadly reactive immune responses against HIV-1. Next, we characterized its effectiveness as an antigen in generating epitope-specific Abs, especially IgA, in comparison with ELDKWA.

QARVLAVERY elicits significantly higher IgA compared with ELDKWA in both systemic and mucosal compartments

To evaluate the immunogenicity of the QARVLAVERY epitope, DNA and rAd vectors expressing the Gag + QARVLAVERY

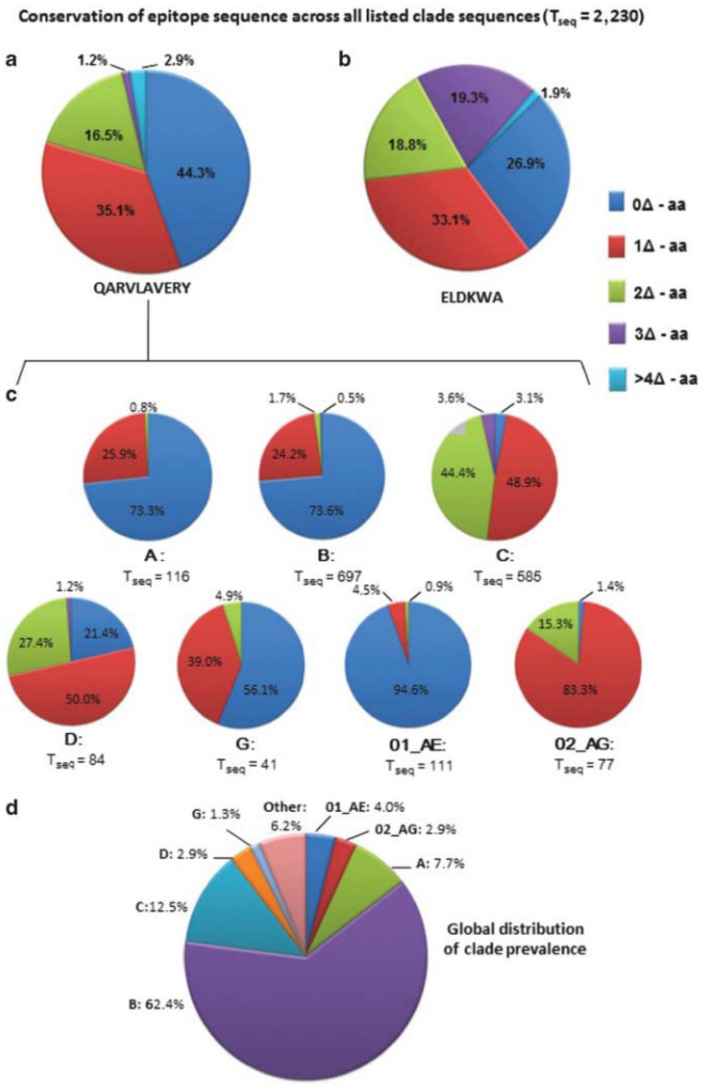

Figure 1 The gp41 epitope QARVLAVERY is highly conserved, as determined by epitope alignment across the listed HIV-1 sequences in the Los Alamos HIV database (dated 10 August 2010). (**a**) The percentage of sequences that have 0, 1, 2, 3, or >4 point changes in amino-acid sequence (Δ0 aa, Δ1 aa, Δ2 aa, Δ3 aa, >Δ4 aa, respectively) of the epitope QARVLAVERY or (**b**) ELDKWA. The alignment was performed against all listed HIV sequences (T_{seq}=2,230). (**c**) The percentage of sequences that have 0, 1, 2, 3, or >4 changes in amino acids for the QARVLAVERY epitope alignment, divided by the most prevalent HIV clades. The total number of sequences per clade from which the data were derived is indicated. (**d**) Global distribution of HIV-1 indicating the relative prevalence of the most widespread clades. Data were obtained from the HIV Los Alamos Geography search interface. HIV, human immunodeficiency virus.

construct were generated for immunization in mice. In a previous study,[16] we have shown that inserting multiple copies of an epitope, such as ELDKWA, within Gag served as an effective design strategy to generate chimeric VLPs that elicited high titers of ELDKWA-specific Abs. The Gag+QARVLAVERY construct was designed in a similar manner, with three tandem copies of the epitope inserted just before the C terminus of the gag. The proper formation and release of VLPs from each of the vectors were verified as described before[16] (data not shown). To contrast the immunogenicity of QARVLAVERY, separate groups of mice were immunized with DNA and rAd vectors expressing [Gag+ELDKWA]-VLPs. QARVLAVERY and ELDKWA

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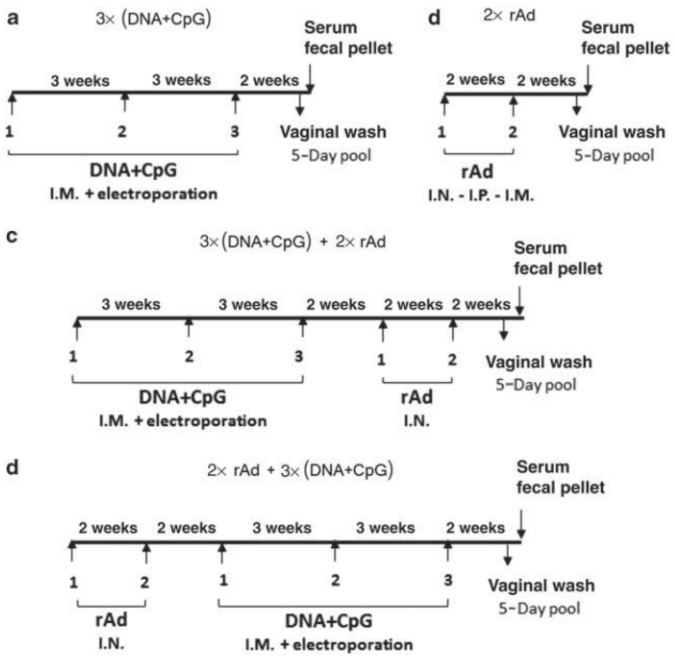

Figure 2 An illustration of the immunization regimens used in subsequent experiments: homologous prime-boost delivery of (**a**) 3× DNA I.M. or (**b**) 2× rAd, I.N., I.M., or I.P., the heterologous prime-boost delivery of (**c**) 3× (DNA + CpG) I.M. + 2× rAd I.N. or (**d**) 2× rAd I.N. + 3× (DNA + CpG) I.M. Serum samples and fecal pellets were collected on day 14 after the last immunization, whereas vaginal wash samples were pooled over 5 consecutive days per mouse ending on day 14, to account for any estrus cycle-dependent Ab shedding over the 5-day hormonal cycle of female mice. rAd, recombinant adenovirus; I.M., intramuscular; I.N., intranasal; or I.P., intraperitoneal.

expressing VLP constructs each had three tandem copies of the respective epitope, inserted at the same site within the gag, delivered by the same vectors and delivery regimen to mice, to be able to directly compare the IgA-inducing ability of both epitopes. Groups of C57Bl/6 mice were given the optimized prime-boost regimen that elicited the best titers of systemic and mucosal epitope-specific Abs,[16] 3× (DNA + CpG) administered intramuscularly (I.M.) with immediate local electroporation, followed by 2× rAd intranasally (I.N.). **Figure 2c** illustrates the 3× (DNA + CpG) + 2× rAd immunization scheme. Each set of DNA/rAd constructs expressed either Gag + QARVLAVERY or Gag + ELDKWA.

Results in **Figure 3a** illustrate an intriguing observation: significantly higher IgA was generated against QARVLAVERY compared with ELDKWA in the serum of immunized C57Bl/6 mice, as well as in mucosal samples: vaginal washes (VWs) and fecal pellets (FPs). As strains of mice have been shown to exhibit unique immunogenic responses to epitopes that may not be reflective of their true immunogenicity,[57] the same delivery regimen was administered to Balb/c mice to verify that this phenomenon was not solely specific to C57Bl/6 mice. Once again, significantly higher IgA was elicited against the QARVLAVERY epitope compared with ELDKWA in the serum, VWs, and FPs, of immunized mice (**Figure 3b**). Epitope-specific Ab titers were determined by peptide-specific enzyme-linked immunosorbent assays (ELISAs). All representative samples (serum, VW, FP) obtained from C57Bl/6 and Balb/c mice that were immunized with mock DNA and rAd vectors did not exhibit any non-specific reactivity against either epitope in the ELISAs compared with baseline (data not shown).

These results show that QARVLAVERY is indeed a potent inducer of IgA systemically and mucosally. In fact, it generated significantly higher epitope-specific IgA than did ELDKWA when delivered by similar constructs administered by the same delivery regimen, in two independent strains of mice.

QARVLAVERY elicits significantly higher IgA than does ELDKWA independent of the vector-delivery regimen

Recent evidence has suggested that factors such as mucosal delivery of antigens,[58] use of adjuvants,[59,60] certain viral antigens,[61,62] or VLP administration[63,64] may influence IgA induction and can directly or indirectly modulate Ab class-switch recombination. Here, we determined whether the significantly higher IgA generated against QARVLAVERY was a property of the epitope alone, independent of such factors in our immunization model. Here, QARVLAVERY vs. ELDKWA IgA titers were compared between groups of mice that were immunized with the following

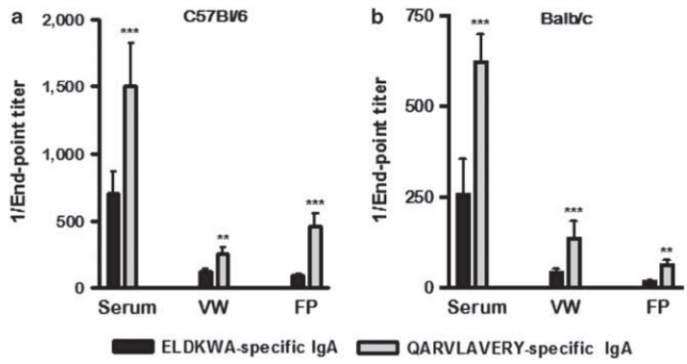

Figure 3 QARVLAVERY induces significantly higher IgA in immunized mice compared with ELDKWA-immunized mice. Epitope-specific IgA titers measured in the serum, vaginal washes (VWs), and fecal pellets (FPs) of mice that were immunized by the following heterologous prime-boost regimen: (3× (DNA + CpG) + 2× rAd). Separate groups of C57Bl/6 mice ($n = 7$, **a**) or Balb/c mice ($n = 5$, **b**) were administered DNA and rAd vectors expressing either the Gag + ELDKWA or the Gag + QARVLAVERY constructs, through the prime-boost regimen. Serum samples were obtained at day 14 and VWs were collected over 5 days starting at day 9, after the last immunization. Results are representative of three independent experiments. $^{**}P < 0.05$, $^{***}P < 0.001$ as determined by the Mann–Whitney test. rAd, recombinant adenovirus.

regimens: (i) 3× (DNA + CpG) I.M. with electroporation, (ii) 2× rAd I.N., and (iii) 2× rAd followed by 3× (DNA + CpG). **Figure 2** illustrates the different immunization regimens, which were the same, to compare epitope-specific Ab titers head to head.

This served to determine whether QARVLAVERY-specific IgA was uniquely raised against the epitope or whether the use of CpG as an adjuvant, any viral antigens from rAd or the mucosal vs. systemic priming order, were possible factors influencing the observations made in **Figure 3**. As the results show, regardless of the immunization regimen, mice immunized with QARVLAVERY induced significantly higher epitope-specific IgA in their serum (**Figure 4a**) and VWs (**Figure 4b**) than did mice immunized with ELDKWA. Hence, we can deduce that this characteristic is attributable to the epitope and is independent of CpG, the vectors used or any specific prime-boost regimen.

QARVLAVERY elicits ~1:1 ratio of IgG:IgA in the serum of mice administered homologous DNA, rAd, or heterologous DNA + rAd

In characterizing the Ab profile generated by QARVLAVERY, another unique attribute of its IgA-inducing capacity was observed. As shown in **Figure 5a**, the ratio of IgG:IgA titers elicited against the epitope in the serum of immunized mice is ~1:1. This was independently observed in all groups of C57Bl/6 mice that received any of the four different immunization regimens. The ratio of IgG:IgA in the VWs of immunized mice was also ~1:1, whereas fecal samples exclusively contained epitope-specific IgA (data not shown). **Figure 5b** shows the IgG:IgA ratio in the serum of mice immunized with the rAd vector twice, intraperitoneally (I.P.) and I.M., to determine whether the ~1:1 ratio was independent of the route of immunization. Indeed, the ~1:1 ratio was observed in these groups as well. In all of the immunization groups, no significant difference was observed in the serum IgG:IgA titers, which is unusual as systemically induced HIV-specific responses typically consist of higher IgG than IgA. This unique property of QARVLAVERY further adds to its appeal as a highly conserved IgA-inducing epitope.

QARVLAVERY-specific IgA is induced before IgG induction in the serum

To determine at which point upon immunization the ~1:1 IgG:IgA ratio emerges, serum was sampled over days 12, 18, 23, 32, 37, 45, 51, and 60 from mice administered rAdGag-QARVLAVERY, two times through two independent routes, I.N. and I.P. The profile of epitope-specific IgA and IgG induced over time is shown in **Figure 6a** and **b**. Interestingly, QARVLAVERY-specific IgA emerged earlier than did epitope-specific IgG in the serum of mice delivered rAd I.N. or I.P., even before the booster rAd immunization administered on day 14. In fact, epitope-specific IgA was significantly higher ($P < 0.001$) than specific IgG at the earlier time points, as indicated in **Figure 6a** and **b**. As these observations were made in groups of mice administered the same vector systemically (I.P.) and mucosally (I.N.), we showed that they are not dependent on the immunization route. This is an unusual observation as antigen-specific IgG responses typically dominate among systemic humoral responses, as shown in **Figure 6c** with ELDKWA-specific IgG and IgA responses in mice, which were immunized with rAdGag-ELDKWA, twice, I.N. In fact, these mice elicited significantly higher IgG against ELDKWA at the later time points of days 45, 51, and 60, and no early peptide-specific IgA was detected.

Mice were immunized with a mock rAd vector I.N. and serum samples were obtained on days 12, 18, and 23 to test for possible cross-reactivity of anti-adenovirus IgA with QARVLAVERY in the peptide-specific ELISA. No cross-reactivity against QARVLAVERY was detected; hence, the sensitivity of the assay was confirmed to be specific to the peptide (data not shown). Furthermore, to ensure that QARVLAVERY-specific IgG was not being masked by peptide-specific IgA in the samples because of possible avidity differences between Ab isotypes, IgG fractions

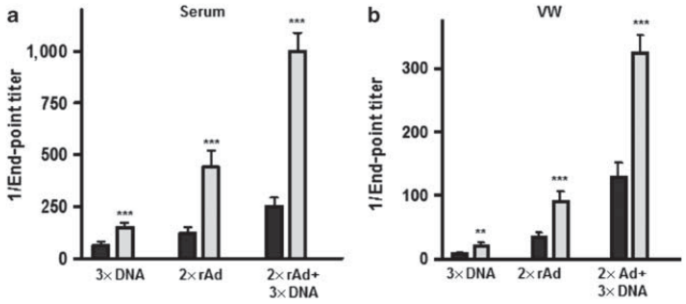

Figure 4 QARVLAVERY induces significantly higher IgA compared with ELDKWA regardless of the vectors or delivery regimen used. Epitope-specific IgA titers in the (**a**) serum and (**b**) VWs of C57Bl/6 mice ($n=5$ for each group) that were immunized via the following immunization regimes: (3× (DNA+CpG)+2× rAd) or (2× rAd+3× (DNA+CpG)). For each regimen, mice were immunized with vectors expressing either the Gag+QARVLAVERY construct or the Gag+ELDKWA construct. Serum samples were obtained at day 14 and VWs were collected over 5 days starting at day 9, after the last immunization. $^{**}P<0.05$, $^{***}P<0.001$. rAd, recombinant adenovirus; VW, vaginal wash.

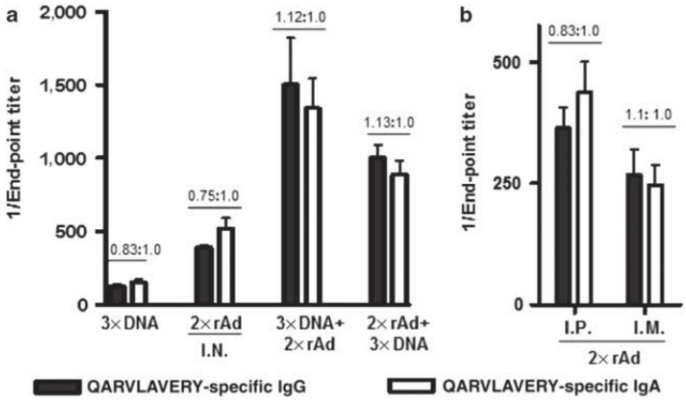

Figure 5 The ratio of serum IgG:IgA raised against QARVLAVERY. (**a**) Groups of mice ($n=5$ each) were immunized by the following immunization regimens: 3× (DNA+CpG) I.M., or 2× rAd I.N., or 3× (DNA+CpG), followed by 2× rAd, or 2× rAd, followed by 3× (DNA+CpG), with constructs expressing Gag+QARVLAVERY. (**b**) QARVLAVERY-specific IgA and IgG in the serum of mice immunized with rAd twice I.P. or I.M. Serum samples were collected at day 14 after the last immunization for all groups. Results are representative of three independent experiments. There was no significant difference between the IgG and IgA titers in any of the groups. rAd, recombinant adenovirus; I.M., intramuscular; I.N., intranasal; or I.P., intraperitoneal.

were purified from serum obtained on days 12, 18, and 23 and re-tested by peptide-specific ELISA. However, purified serum IgG showed no difference in reactivity to QARVLAVERY (data not shown) compared with IgG detected in neat samples at earlier time points as shown in **Figure 6a** and **b**. The emergence of early IgA, even before IgG, uniquely against the QARVLAVERY epitope, further highlights the intriguing IgA-inducing capacity of this epitope.

QARVLAVERY-specific IgG is capable of neutralizing X4-tropic IIIB virus but not the R5-tropic ADA viral strain

The relative functional capacity of epitope-specific IgG vs. IgA in the direct neutralization of IIIB and ADA virus strains was assessed using the TZM-bl indicator cell line. Furthermore, the efficiency of QARVLAVERY- vs. ELDKWA-specific Abs in neutralizing IIIB and ADA was also evaluated. Purified IgG- and IgA-enriched fractions were obtained from the serum, VWs, and FPs of mice immunized with either empty vectors or vectors expressing Gag+QARVLAVERY or Gag+ELDKWA. Samples from both immunization groups underwent similar processing that involved elimination of Gag-specific Abs, followed by IgG purification and volume reduction to concentrate both IgG- and IgA-enriched (original sample minus IgG) fractions. Both fractions were equalized for their epitope-specific Ab content, such that the final working samples contained equal concentrations of QARVLAVERY and ELDKWA specific IgG or IgA, as determined by peptide-specific ELISA. Control serum samples were obtained from mice immunized with mock vectors and were processed in the same manner, whereas control mucosal samples from mock-immunized mice were used neat. The neutralization

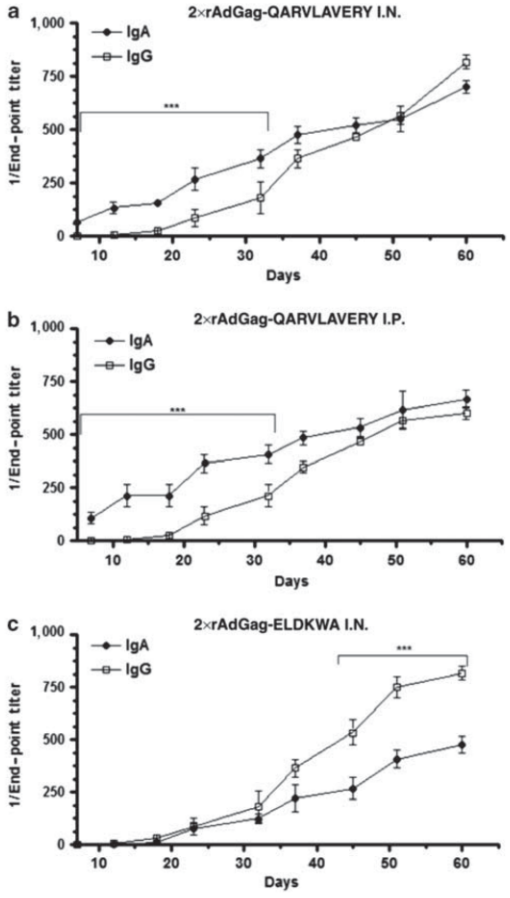

Figure 6 Early detection of QARVLAVERY-specific IgA and IgG in the serum of mice immunized with rAd expressing Gag + QARVLAVERY through the (**a**) I.N. and (**b**) I.P. routes, or by rAd expressing Gag + ELDKWA (**c**) I.N., twice, 2 weeks apart. Serum was obtained starting day 7 and subsequently on days 12, 18, 23, 32, 37, 45, 51, and 60. ***$P < 0.001$. Results are representative of at least two independent experiments. rAd, recombinant adenovirus; I.N., intranasal; or I.P., intraperitoneal.

efficiency of Ab fractions is reflected by the percentage infection inhibition of IIIB or ADA upon pre-incubation with IgG or IgA, relative to direct virus infection in the same assay, in the absence of any inhibitory agent. Positive controls of infection inhibition used were 2F5-IgG, 2F5-IgA, and T20 (Fuzeon, Trimeris, kindly obtained from the NIH AIDS Research and Reference Reagent Program, Germantown, MD), and the concentrations that conferred 90, 70, or 50% neutralization (IC90/IC70/IC50) of IIIB and ADA were determined in standardizing the assay.

As shown in **Figure 7a** and **b**, serum-derived QARVLAVERY-specific IgG and IgA are capable of inhibiting IIIB and ADA virus infection at the indicated Ab dilutions. Although ELDKWA-specific serum IgG exhibits significantly greater neutralizing ability toward the X4-tropic IIIB virus than does QARVLAVERY-specific IgG, it shows no difference in the neutralizing ability against R5-tropic ADA virus. Furthermore, when equal concentrations of IgG and IgA were used under the same assay conditions, as determined by epitope-specific ELISA, IgG against either epitope was significantly more effective in inhibiting virus infection of TZM-bl cells compared with epitope-specific IgA, except for QARVLAVERY-specific IgG against ADA. Mucosally derived IgG fractions also showed greater neutralizing ability against IIIB compared with IgA (**Figure 7c**); however, there was no significant difference between IgG and IgA infection inhibition of ADA (**Figure 7d**). Interestingly, QARVLAVERY-specific IgA from FPs was significantly more effective in neutralizing IIIB than was ELDKWA-specific IgA.

QARVLAVERY-specific IgA effectively inhibits transcytosis of cell-associated IIIB virus

Here, the ability of epitope-specific Ab fractions to inhibit virus transcytosis upon pre-incubation with IIIB-infected peripheral blood mononuclear cells (PBMCs) was assessed. Ab fractions used were equalized by epitope-specific Ab content. Infected PBMCs were delivered to the apical compartment of a transwell system, in the presence or absence of purified Abs, over a polarized HEC-1A cell layer. Subsequently, the passage of infectious virus particles to the basolateral compartment was evaluated by the TZM-bl luciferase reporter assay. Basolateral supernatants were concentrated to a final volume of 100 µl, the entire amount of which was used to quantitatively detect total infectious virus passage through the epithelial layer using the TZM-bl reporter cell line. This method was adopted in lieu of measuring p24 levels, which is not an accurate representation of infectious particles. Percentage reduction in transcytosis efficiency conferred by the Ab samples was determined relative to total transmission of virus from IIIB-infected PBMCs alone, in the absence of inhibitory Abs, under similar conditions.

As seen in **Figure 8a**, epitope-specific IgA was significantly more efficient in reducing transcytosis efficiency than was epitope-specific IgG (IgA vs. IgG: S-QARV, **$P < 0.01$; S-ELD, *$P < 0.05$). Furthermore, QARVLAVERY-specific IgA was significantly more effective in inhibiting transcytosis than was ELDKWA-specific IgA (**Figure 8b**, S-IgA: QARV vs. ELD, **$P < 0.01$). This was also observed in FP-derived IgA (FP-IgA: QARV vs. ELD, *$P < 0.05$). Although epitope-specific IgA from VWs was significantly more efficient in reducing virus transcytosis than was specific IgG (**Figure 8b**, IgA vs. IgG: VW-QARV, **$P < 0.01$; VW-ELD, *$P < 0.05$), there was no significant difference in transcytosis inhibition mediated by QARVLAVERY- vs. ELDKWA-specific IgA from VW samples.

HIV-specific systemic and mucosal IgA, obtained from EU cohorts, have previously been shown to inhibit virus transcytosis *in vitro*. Here, we compared the ability of purified IgG- and IgA-enriched fractions obtained from Gag + QARVLAVERY- vs. Gag + ELDKWA-immunized mice, to reduce virus transcytosis efficiency upon pre-incubation of IIIB-infected PBMCs with either Ab fraction.

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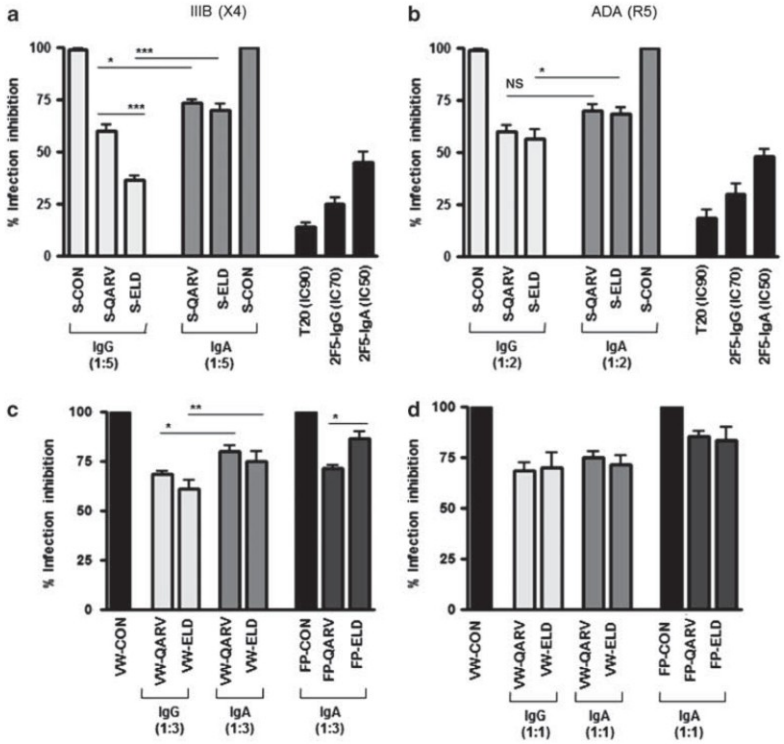

Figure 7 Neutralization efficiency of purified IgG- and IgA-enriched samples obtained from serum (S), vaginal wash (VWs) and fecal pellet (FPs) samples assessed by the TZM-bl reporter against the (**a**: serum, c: VW, FP) IIIB and (**b**: serum, **d**: VW, FP) ADA virus strains. Samples were obtained from control mice (CON) or mice immunized with the (3 × (DNA + CpG) + 2 × rAd) regimen, expressing either Gag+QARVLAVERY (QARV) or Gag+ ELDKWA (ELD). The aliquots of purified IgG- and IgA-enriched fractions were equalized for their peptide-specific Ab content determined by epitope-specific ELISA. Equivalent dilutions of the IgG and IgA fractions were used, as indicated, for direct comparison between QARVLAVERY and ELDKWA-specific IgG and IgA. Percentage inhibition of infection of TZM-bl cells was determined by the reduction in relative luminescence units (RLUs) upon pre-incubation of virus with mouse IgG and IgA Abs, the 2F5 mAbs or T20 (Fuzeon, Trimeris), relative to direct virus infection of TZM-bl cells alone in the same assay. The average of a minimum of five samples per group is shown. ***P< 0.001, **P<0.01, *P<0.05. Ab, antibody; mAb, monoclonal antibody; NS, not significant.

Infection inhibition and transcytosis inhibition of IIIB by total IgG and IgA fractions

In the previous figures, Ab fractions used were equalized by peptide-specific content to be able to compare the efficacy of epitope-specific Abs elicited by immunization. Although epitope-specific ELISA used to quantitate the Ab content has been previously optimized for sensitivity and specificity,[16] Abs of lower avidity or conformational dependence may not bind efficiently to linear epitopes used in the ELISA. Here, we assessed the efficiency of serum and mucosal Ab fractions that were equalized at the final step according to their total IgG or IgA content, instead of epitope-specific content. Only the raw data are shown, as statistical comparison between IgG and IgA efficacy or between samples obtained from mice immunized with ELDKWA- vs. QARVLAVERY-expressing constructs cannot be made. The assays were performed by the same method, using all appropriate controls as described above.

DISCUSSION

Despite its expansive global spread, HIV has a surprisingly low natural rate of transmission. In a cohort of discordant heterosexual couples, the reported rate ranged from 0.0001 to 0.0040.[65] This low rate of transmission is suggestive of some form of natural protective barrier or immune control at the mucosa. IgA is one suggested correlate of mucosal protection against HIV.[29]

In this study, we evaluated the immunogenicity of the highly conserved QARVLAVERY epitope in an optimized prime-boost vaccination model, and assessed the function of QARVLAVERY-specific IgG and IgA elicited in systemic and mucosal compartments. Our collective results highlight an intriguing property of QARVLAVERY: that it is a potent inducer of IgA. This was demonstrated by its ability to consistently generate significantly higher IgA in comparison with ELDKWA, using different immunization regimens and vectors. In addition, it elicited an unusual ~1:1 ratio of IgG:IgA, and QARVLAVERY-specific IgA was detected even before IgG in the serum. With

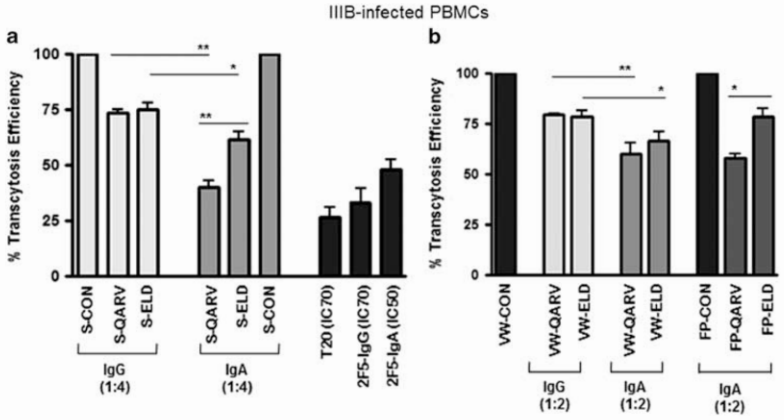

Figure 8 Transcytosis inhibition of purified IgG- and IgA-enriched fractions from the (**a**) serum (S), (**b**) VWs, and FPs of control mice (Con) or mice immunized with Gag+QARVLAVERY (QARV) or Gag+ELDKWA (ELD). Final Ab fractions equalized by their epitope-specific IgG and IgA content and appropriate dilutions based on titrations curves were used as indicated (1:4 or 1:2). Transcytosis of virus from IIIB-infected PBMCs across a polarized epithelial layer of HEC-1A cells was measured by detecting passaged infectious virus particles in the basolateral transwell compartment, in a TZM-bl luciferase reporter assay. Percentage reduction in virus passage across the epithelium upon pre-incubation of infected PBMCs with the diluted Ab fractions from immunized mice, mAbs (2F5-IgA, 2F5-IgG) or T20 (Fuzeon, Trimeris), was determined by direct comparison of relative transcytosis efficiency of virus alone in the same manner. The average of a minimum of five samples per group is shown. $^{**}P<0.01$, $^{*}P<0.05$. Ab, antibody; FP, fecal pellet; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; VW, vaginal wash.

particular importance and relevance to inducing mucosal immunity against HIV, QARVLAVERY-specific IgA was found to be functional and effective in inhibiting HIV infection and viral transcytosis across an epithelial barrier *in vitro*.

Our primary analysis of overall sequence conservation of the QARVLAVERY epitope against all listed HIV-1 sequences in the Los Alamos HIV database reflected the striking conservation of this epitope, particularly among the most prevalent clades worldwide. Earlier studies have also implicated high reactivity of serum from HIV-seropositive individuals against the coiled-coil region of gp41.[47,50,54] The QARVLAVERY epitope is located at the α-helix of the gp41 coiled-coil region, which is involved in the critical cell-fusion process.[30] Therefore, investigation to identify the critical residues that are involved in Ab recognition and possible conformational constraints required for proper Ab function should be pursued to further characterize this epitope, which could be an ideal candidate immunogen to elicit broadly reactive mucosal immunity against HIV.

IgA is known to be the dominant functional isotype at the mucosa; however, contrary to other mucosal sites, female genital tract secretions predominantly consist of IgG rather than IgA.[66,67] This is attributed to the diffusion of circulatory Abs into genital secretions. Various immunization regimens have attempted to elicit mucosal immunity in simian models of Simian immunodeficiency virus infection but were unable to detect antigen-specific B cells at the genital tract, even though HIV-specific CD8 T-cell induction has been shown.[68–71] Therefore, as antigen-specific Abs in genital secretions are likely to be derived from systemic circulation, it is intriguing that QARVLAVERY generates unusually high IgA in the serum; in fact, ~1:1 IgG: IgA. Interestingly, the ~1:1 ratio of QARVLAVERY-specific IgG: IgA that we observed has been reflected by data presented in a previous study (gp41 coil aa 578–592), by an different group.[72] Although a direct comparison of epitope-specific IgG and IgA against the coiled-coil region was not made, their reported data for Abs directed against the MPER and coiled-coil regions correlate with our findings.

Ab-based vaccine strategies have been heavily dominated with the goal to induce neutralizing IgG against HIV, and there are several ongoing efforts to identify novel neutralizable virus targets.[73] However, there is ample evidence supporting the role of non-neutralizing HIV Abs in conferring protection as well.[23–27] A recent immunization study in rhesus macaques has demonstrated that HIV-specific Abs could control infection by Ab-dependent cellular cytotoxicity, Ab-dependent cell-mediated virus inhibition, and transcytosis-inhibition mechanisms.[74] Indeed, there was a strong correlation between viremic control and inhibition of transcytosis by rectal IgA, whereas the nAb response did not correlate with control of viral load.[74] Furthermore, unlike IgG, high titers of systemic IgA are not as concerning as it is non-inflammatory by nature and does not activate complement by acting through the inhibitory Fcα receptor.[75,76] There is ample evidence implicating the importance of "immune quiescence" in maintaining HIV resistance in highly exposed persistently seronegative individuals,[77–80] and in fact, systemic or mucosal inflammation as a result of immune activation may increase the risk of HIV transmission and pathogenesis.[81,82] Therefore, an IgA-mediated protective, yet non-inflammatory response is a desirable goal to be achieved by prophylactic mucosal vaccines.

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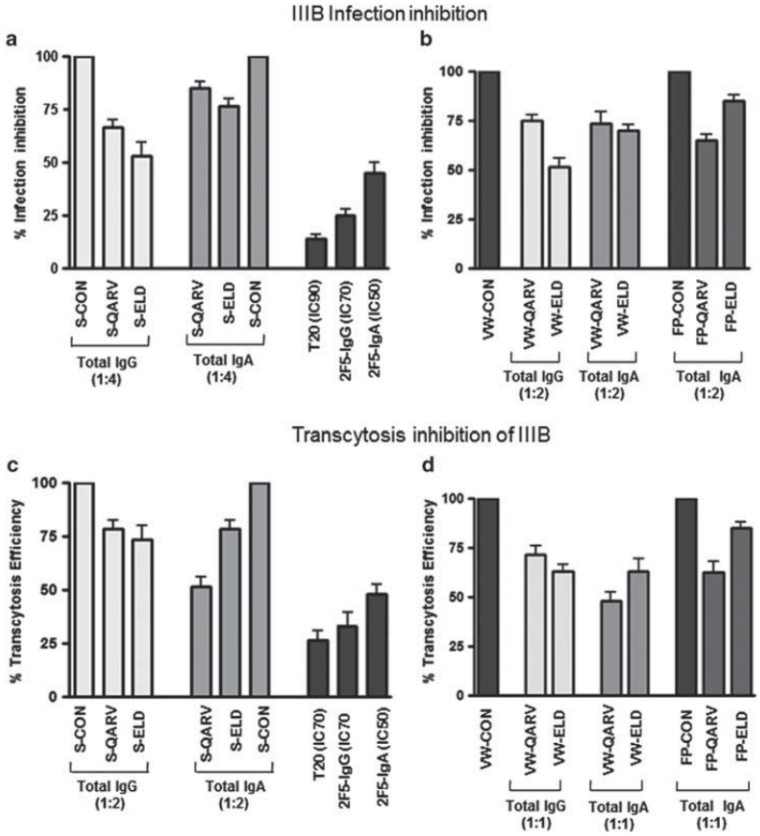

Figure 9 Ab fractions from mice immunized with Gag+QARVLAVERY (QARV) or Gag+ELDKWA (ELD) were enriched, fractionated, and equalized by total IgG or IgA content determined by IgG/IgA ELISA. Serum-derived fractions were assessed for their ability to inhibit direct infection of TZM-bl cells with the IIIB (X4) virus (**a**) and to inhibit transcytosis of cell-associated IIIB across a HEC-1A cell monolayer in a transwell assay (**c**), whereas VW and FP samples were assessed in respective assays in (**b**) and (**d**). The TZM-bl luciferase assay was used to quantify their direct infection in panel **a** and **b** or to quantify the total infectious virus that traversed into the basolateral supernatant in panels **c** and **d**. The percentage reduction in infection or transcytosis efficiency is shown relative to virus infection or virus passage across the transwell, in the absence of an inhibitory agent, in the same assay. The average of a minimum of four samples per group is shown. ELISA, enzyme-linked immunosorbent assay; FP, fecal pellet; VW, vaginal wash.

Our results illustrate that QARVLAVERY-specific IgG and IgA exhibit modest neutralization of HIV and are capable of inhibiting virus transcytosis *in vitro*. In particular, QARVLAVERY-specific IgA was significantly more effective at inhibiting IIIB transcytosis than was ELDKWA-specific IgA, with Ab fractions equalized by epitope-specific Ab content. Overall, our results show similar trends: systemic IgG was significantly more effective at direct IIIB virus neutralization compared with IgA, whereas IgA was significantly more efficient at inhibiting cell-associated IIIB transcytosis relative to IgG. Mucosally derived IgG and IgA, directed at either epitope, showed no difference in the neutralizing ability against the R5 virus. However, QARVLAVERY-specific IgA from fecal samples was significantly more efficient both in neutralizing IIIB and in inhibiting virus transcytosis, than was ELDKWA-specific IgA. It is noteworthy, however, that the hepatic pump in rodents is a mode of passage of circulating polymeric IgA into the gastrointestinal tract.[83] Therefore, IgA measured in the FPs from immunized mice consists of both mucosally produced and systemic IgA.

Rigorous efforts are being made to profile the early dynamics of systemic Ab induction to better understand the role of Abs in acute HIV-1 infection.[84–86] Although overall knowledge is still sparse, it has been shown that anti-gp41 Ab responses, in particular IgM, are among the first to be generated systemically, followed by class switching to IgG and IgA.[1,85] It has also been reviewed[87] that the earliest HIV-specific IgA responses in mucosal secretions appear within 3 weeks after infection and are also gp41 specific. So far, the MPER is the only identified target of the early gp41-specific IgA.[88] Our results indicate

that IgA was detected against QARVLAVERY within 7 days of immunization. Therefore, it would be intriguing to determine whether early IgA isolated from acutely infected individuals may be reactive to the gp41 QARVLAVERY epitope. Our data show that QARVLAVERY-specific IgA from immunized mice could confer protection against HIV by direct virus neutralization or by transcytosis inhibition. However, the biological implications of these findings for humans are yet to be determined, especially in light of the differences between human IgA subtypes IgA1 and IgA2, and murine IgA.[67] Isolating QARVLAVERY-specific IgA in natural HIV infection and acute infection cohorts to determine its function in virus neutralization, transcytosis inhibition, or Fc receptor-mediated mechanisms, would provide additional information on the implications of this epitope in vaccine design.

The unusually early systemic IgA generated against QARVLAVERY, within 7 days of primary immunization, is another intriguing observation of our study. IgA was induced in the serum before IgG, uniquely in response to QARVLAVERY and in contrast to ELDKWA, which displayed an expected antigen-specific Ab profile. Recently, there has been a growing understanding of the role of innate-like B1 cells, which suggests that they may have a greater role than producing low-affinity polyreactive "natural" Abs as first-line defense at the mucosa.[89–92] B1 cells can also respond to antigens and generate Abs independent of T-cell help,[93] in extrafollicular compartments.[89,94,95] An influenza study initially showed that B1 and B2 subsets exhibit distinct, independent, and critical Ab functions in response to virus infection.[96] Subsequently, they showed that influenza-reactive Abs of B1 origin were those responsible for direct virus neutralization and clearance.[97] In addition, B1 cells have been reported to switch to IgA more promptly than B2 cells in response to antigen.[98] Given our data, we hypothesize that the early IgA induced against QARVLAVERY may be a result of direct or indirect stimulation of B1 cells and that the additional priming of B1 cells may be responsible for the unusually high IgA observed against QARVLAVERY.

With respect to mucosal protection, innate mechanisms and local cytokines are among the first in line to defend against pathogens. Type 1 interferons (IFNs), and particularly IFN-α, have been associated with antiviral activity against HIV.[99–101] Interestingly, sequence similarity between the gp41 coiled-coil region, R*I*LAVERY—YLKD, and two domains of human[102,103] and murine IFN-α (--LAV--Y) and IFN-β (R-----RYLK), within regions that constitute the IFN-α/β receptor-binding site has been reported.[103] IFN-α has been implicated in stimulating class switching to IgM, IgG2, and IgA,[104] and may be required to generate mucosal IgA.[105] However, there are concerns that immune activation led by innate sensors at the mucosa may result in inflammation and an influx of HIV target cells, thereby enhancing HIV.[81,82,106] It would be intriguing for future studies to determine whether anti-QARVLAVERY Abs could prevent IFN signaling at the mucosa and have a role in enhancing innate protection, in addition to its function in inhibiting transcytosis at the mucosa.

In this study, we have shown that a highly conserved HIV epitope, QARVLAVERY, located in a critical α-helix region of the gp41 coiled-coil, is a potent IgA inducer. The epitope showed two interesting properties in our model, that it elicited ~1:1 ratio of IgG:IgA in the serum of immunized mice and that epitope-specific IgA was detected within days, even earlier than IgG. These findings can have important implications for future vaccines seeking to induce mucosal immunity against HIV. Interestingly, QARVLAVERY-specific IgA exhibited modest HIV neutralization capacity and was an effective inhibitor of HIV transcytosis *in vitro*, especially in comparison with ELDKWA. This makes QARVLAVERY a very attractive target for the induction of mucosally relevant IgA responses in prophylactic approaches against HIV.

METHODS

Epitope sequence alignment. The QuickAlign tool found under the Los Alamos HIV immunology database (http://www.hiv.lanl.gov/content/immunology) was used to determine the percentage sequence conservation of the QARVLAVERY epitope that had 0, 1, 2, 3, or >4 aa changes from the input sequence, across all listed HIV-1 sequences. The data indicating global distribution of the predominant HIV clades were obtained from the geography search interface under the "HIV Sequence" database (http://www.hiv.lanl.gov/). All data were recorded as of 10 August 2010.

DNA and rAd vector construction. Recombinant DNA and rAd vectors expressing the [Gag + QARVLAVERY] construct were constructed in the manner described previously.[16] In brief, three tandem copies of the following gp41 sequence [GIKQLQARVLAVERYLKDQQLLG x3] (HIV-1 envelope: 572→594) were inserted before the stop codon of a HIV-1 clade A gag gene. The nucleotide sequence of the epitope string insert is: 5′-(GGCATCAAACAGCTGCAGGCCAGAATCCTGGTGGA ACGGTATCTGAAAGATCAGCAGCTGCTGGGCGGGATTAAACA GCTCCAGGCTAGGGTCCTGGTCGAGAGATACCTGAAGGACCA ACAGCTCCTGGAAGGAATCAAGCAACTGCAGGCCCGGGTGCT CGTGGAAAGGTATCTCAAAGATCAGCAACTGCTCGGC). Proper generation and formation of Gag + QARVLAVERY chimeric VLPs were verified by western blot characterization, as described previously.[16]

Immunizations and sample collection. Female C57Bl/6 mice, 8–10-weeks of age, were used in all the experiments, except for Balb/c mice where mentioned (Charles River, Quebec, Canada). DNA constructs were scaled-up as endotoxin-free preparations for safe use in animals. In all, 50 μg DNA was co-administered with 50 μg CpG (ODN 1826, 5′-TCCATGACGTTCCTGACGTT, MOBIX, Hamilton, ON, Canada) I.M. in the hind limbs of mice, immediately followed by electroporation, whereas rAd immunizations were administered I.N. at 10^8 p.f.u. ml^{-1}, as described previously.[16] Repetitive DNA and rAd immunizations were given 3 or 2 weeks apart, respectively. Where mentioned, rAd was also administered I.P. and I.M. at 10^8 p.f.u. ml^{-1} in a total volume of 30 μl, diluted in saline. Samples were collected as described previously.[16] In brief, serum was obtained from retro-orbital bleeds, VWs were collected over 5 days to account for the estru/diestrus shedding differences, and Abs from collected FPs were left to diffuse out into a 0.05% sodium azide buffer overnight at 4°C. All samples were stored at −80°C in the long term.

ELISA. Detailed procedures for detecting epitope-specific IgG or IgA by peptide-specific ELISA have been described previously.[16] In brief, amino-immobilizer plates (Nunc, Mississauga, ON, Canada) were coated either with the QARVLAVERY peptide (full sequence: KQLQARVLAVERYLK, CPC Scientific, San Jose, CA) or with the ELLELDKWASL epitope, at 5 μg ml^{-1} per well. Plates were subsequently blocked with 5% bovine

serum albumin and washed before addition of sample dilutions. After overnight incubation at 4°C, a secondary biotin-conjugated anti-mouse IgG or IgA Ab was added to be detected by the colorimetric 3,3′,5,5′-tetramethylbenzidine reagent (KPL, Guelph, ON, Canada). The end point of for each test sample was determined as the dilution at which its OD was at least 1.5 times that of the sample representative sample from a naive, unimmunized mouse of the same strain. The protocol for determining total IgG and IgA titers has also been described before.[16]

Ab fractionation. Gag-specific Abs were pulled out of serum, VW, and FP samples obtained from immunized mice before IgG purification and IgA enrichment. This allowed for a more accurate assessment of the epitope-specific Ab function in neutralization and transcytosis assays by increasing its relative proportion in the samples. The function of epitope-specific Abs could be assessed without saturating the assays with non-functional Abs and with greater accuracy as purification and fractionation significantly reduced the background. Recombinant p24 (Genway, San Diego, CA) was covalently linked to activated cyanogen-bromide beads (Sigma, Oakville, ON, Canada) and subsequently blocked following the manufacturer's recommendations. Overall, 50 $\mu g\,ml^{-1}$ of p24 per 10 ml of bead slurry was sufficient for saturation of available p24-binding sites. The p24-conjugated beads were used to capture anti-p24 Abs present in individual Ab samples from immunized mice. Individual serum, VW, or FP samples were diluted 1:3 in a binding buffer, containing 1% protease inhibitor (Sigma), before addition of the beads to allow for adsorption of p24-specific Abs, over two rounds, once at room temperature for 1 h followed by an overnight incubation at 4°C with fresh beads, in a rotary apparatus. Unbound fractions containing ELDKWA- or QARVLAVERY-specific Abs were collected and the absence of p24 Abs was confirmed by ELISA. The eluates were applied to Protein A beads (Thermo Scientific, Rockford, IL) for IgG purification as per the manufacturer's recommendations, and the remaining non-IgG fraction is referred to as the IgA-enriched fraction. Aliquots were tested for purity of IgG fractionation by ELISA and dot blots. Final purified IgG and enriched IgA fractions were dialyzed against phosphate-buffered saline and concentrated using 5-K Amicon filter units (Millipore, Burlington, ON, Canada). Two separate sets of Ab fractions were used in the functional assays: fractions were either normalized by their epitope-specific IgG and IgA content by ELDKWA/QARVLAVERY-specific ELISA or by total IgG and IgA content as determined by a total IgG/IgA ELISA. Serum samples equalized by epitope-specific IgG or IgA concentration at a final average OD of 3.5 and 1:5, 1:4, or 1:2 dilutions of the concentrated stock were used. VW and fecal secretion samples equalized by epitope-specific content at an average OD of 3.0, and neat (1:1), 1:2, or 1:3 dilutions were used in the assays as indicated. Serum samples equalized by total Ab content were equalized at a final average OD value of 3.5 in a total IgG or IgA ELISA, and 1:4. 1:2, or 1:1 dilutions were used in the assays. The standard peptide-specific ELISA procedure with appropriate controls has been described previously.[16] Samples were stored with 1% anti-protease cocktail (Sigma) at −80°C in the long term.

HIV stock preparation. The lab-adapted IIIB virus strain (X4-tropic) was scaled-up in PBMCs after a 3-day activation with 7 $\mu g\,ml^{-1}$ phytohemagglutinin (Sigma) and 35 Units ml^{-1} of recombinant interleukin-2 (R&D, Burlington, ON, Canada). The ADA virus (R5-tropic) was passaged and scaled-up in adherent macrophages purified from human peripheral blood.[107] Supernatants of infected cells were collected at various time points and concentrated using 30-K Amicon filter tubes (Millipore).The median tissue culture infectious dose (TCID50) for each final stock was determined using the Reed-Muench method in TZM-bl cells, which was 5.00×10^3 for the IIIB virus and 2.77×10^4 for the ADA stock. The virus transcytosis assay was performed with IIIB-infected PBMCs prepared from PBMCs isolated from a single donor for all experiments. PBMCs were activated with 7 $\mu g\,ml^{-1}$ phytohemagglutinin and 35 Units ml^{-1} interleukin-2 for 3 days at which point cells were supplemented with fresh interleukin-2 and infected with 300 TCID50 of IIIB, per 10^6 cells, for an additional 5 days before use in the assay. Cell cytotoxicity was monitored and not observed in the preparation of stocks.

Neutralization assays. TZM-bl cells (JC53-BL) cells produce luciferase under tat control upon HIV infection and were used to determine infection using the standard protocol.[108] In brief, assays were set up in 96-well plates with 5×10^4 TZM-bl cells and 25 $\mu g\,ml^{-1}$ diethylaminoethyl-dextran (Sigma) per well, which was determined to be optimal for significant enhancement of infection in our system. Luciferase production was developed by Bright-Glo reagents (Promega, Sunnyvale, CA) following the manufacturer's protocols, and luminescence was read in the Veritas microplate luminometer (Promega). From the infection curves generated for each virus with increasing TCID50, 175 TCID50 of IIIB and 130 TCID50 of ADA per well were determined to be optimal for >95% infection of TZM-bl cells. Assay conditions were standardized by incubation with known infection-inhibiting agents, T20 (Fuzeon, Trimeris), 2F5-IgG (NIH AIDS Research and Reference Reagent Program, Germantown, MD), or monomeric 2F5-IgA (Duke Human Vaccine Institute, Durham, NC). The infection dose of the virus was chosen such that RLUs showed at least a 1.5-log reduction in luminescence in the presence of T20 or the 2F5-monoclonal Abs to accurately determine the neutralization effect. The concentration of monoclonal Abs that conferred 90, 70, or 50% reduction (IC90, IC70, IC50) of luciferase production in TZM-bl cells was also determined. IC90 for T20 (Fuzeon, Trimeris) was 2 $ng\,ml^{-1}$ against IIIB and 5 $ng\,ml^{-1}$ against ADA. IC70 of 2F5-IgG against IIIB was 30 $ng\,ml^{-1}$ and 100 $ng\,ml^{-1}$ against ADA. 2F5-IgA only showed 50% reduction in RLUs; its IC50 was determined to be 45 $ng\,ml^{-1}$ against IIIB and 125 $ng\,ml^{-1}$ against ADA. Ab titration curves against IIIB were performed; 1:5 dilutions of the serum-derived IgG/IgA and 1:3 dilutions of mucosal IgG/IgA were optimal in neutralization and were used in reported data. Titration curves against ADA showed that 1:2 dilutions of serum IgG/IgA and neat mucosal samples gave the highest detectable neutralization. Reducing the TCID50 of ADA did not affect the neutralization capacity. Ab samples were incubated with IIIB or ADA for 1 h at 37°C, in triplicate, before TZM-bl cell addition. Final neutralization efficiency was determined based on the average RLU reduction in the presence of Ab fractions or T20 or the 2F5-monoclonal Abs, relative to average baseline infection, per plate, and is reported as % infection inhibition.

Transcytosis-inhibition assay. HEC-1A cells (ATCC, Manassas, VA) were grown as tight polarized monolayers in 0.4-μm permeable polycarbonate cell culture inserts or transwells for 24-well plates (Nunc, BD, Burlington, ON, Canada)[109] in 10% McCoy's media (Invitrogen, Burlington, ON, Canada). Transcytosis-inhibition assay was performed on cells that had reached a minimum trans-epithelial resistance of 330–420 $\Omega\times cm^2$ (EVOM, World Precision Instruments, Sarasota, FL) after being in culture for a minimum of 3 days to form a polarized epithelial layer. IIIB-infected PBMCs were added at 7.5×10^5 per well to the apical transwell compartment over polarized HEC-1A cells, and virus passage to the basolateral compartment was measured at 2, 4, and 8 h. Basolateral supernatants were collected and concentrated in 30-K Amicon microcentrifugation tubes (Millipore) down to 100 μl. They were analyzed quantitatively for the content of infectious virus particles by testing the entire 100 μl against TZM-bl cells in the luciferase reporter assay. The transcytosis period of 4 h was determined as optimal for detection of virus particles in basolateral supernatants. Transcytosis-inhibition assay conditions were first standardized by titrating T20, 2F5-IgG, and 2F5-IgA against IIIB-infected PBMCs. On the basis of titration curves, a maximum of 70% reduction in transcytosis was achieved upon incubation of 7.5×10^5 IIIB-infected PBMCs with 5 $ng\,ml^{-1}$ T20 (Fuzeon, Trimeris) and 65 $ng\,ml^{-1}$ of 2F5-IgG per well, and 50% reduction with 100 $ng\,ml^{-1}$ 2F5-IgA, for 2 h at 37°C before the addition of infected-PBMCs apically. The effectiveness of mouse-derived Ab fractions in inhibiting virus transcytosis was determined by the percentage decrease in the average RLU, relative to the average RLU of basolateral supernatants from IIIB-infected PBMCs that were not incubated by any inhibitory agent. Ab titrations from the working aliquots of purified IgG and IgA-enriched fractions

were performed; 1:4 dilutions of serum-derived IgG and IgA fractions and 1.2 dilutions of mucosal IgG and IgA fractions were determined to be optimal in the reduction of transcytosis.

Statistics. All significance data were obtained using the Mann–Whitney test to directly compare two groups, using the GraphPad Prism 4 software (Graphpad, LaJolla, CA). Significance values are indicated by $***P<0.001$, $**P<0.01$, and $*P<0.05$.

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DISCLOSURE

The authors declared no conflict of interest.

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-- CHAPTER 4 --

ANTI-GP41 ANTIBODIES INHIBIT INFECTION AND TRANSCYTOSIS OF HIV-1 INFECTIOUS MOLECULAR CLONES THAT EXPRESS TRANSMITTED/ FOUNDER ENVELOPES.

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This study characterizes the protective response of anti-gp41 Ab fractions against a new generation of HIV reagents that express envelope sequences isolated from acute stage transmitted/ founder (T/F) virus populations. Abs obtained from the serum and mucosal secretions of mice immunized by the ELDKWA or QARVLAVERY expressing chimeric VLP constructs were fractionated to obtain purified IgG and enriched IgA. The ability of epitope-specific IgG/IgA to neutralize T/F viral clones was assessed in a TZM-bl reporter assay and the transcytosis-inhibiting ability of gp41-specific IgA was characterized in a transwell assay. Further we reported a new method for a more accurate measurement of transcytosis efficiency compared to previously used methods.

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Anti-gp41 antibodies inhibit infection and transcytosis of HIV-1 infectious molecular clones that express transmitted/founder envelopes

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Abstract

Effective intervention at mucosal surfaces will be critical for early control and inhibition of HIV since it is the primary site of infection. Recent identification of transmitted/ founder (T/F) HIV-1 genomes has demonstrated a severe genetic bottleneck during transmission and suggests that T/F viruses may exhibit distinct phenotypes. HIV-1 Env gp41-specific responses are among the first to be generated in natural HIV infection. We have previously demonstrated that two remarkably conserved gp41epitopes, ELDKWA and QARVLAVERY, that are critical for efficient virus entry, elicit protective antibodies against HIV-1. QARVLAVERY is of particular mucosal interest as it is a potent inducer of IgA. In this study, we address the effectiveness of mucosal and systemic mouse antibodies elicited against these epitopes to inhibit T/F Env function. For this, we employed recombinant infectious molecular clones (Env-IMC) of HIV-1 that encode mucosally transmitted/ founder *env* genes. We found that the gp41-specific IgG and IgA fractions effectively prevented the infection of TZM-bl cells and in inhibited transcytosis in an assay measuring the passage of infectious virus across an epithelial monolayer. Interestingly, the T/F Env-IMC we tested were more sensitive to the antibodies than the R5 lab-adapted strains included as controls. This highlights the potential of gp41-based immunogens to impart effective mucosal protection in the early stages of HIV transmission and infection.

Introduction

Prophylactic strategies against HIV must effectively prevent its transmission, infection and cell-to-cell spread during the earliest stages of acute infection. Since the genital mucosa is the primary site of entry, a mucosal defense is critical for early control of the virus. Based on non-human primate models of SIV infection, it is estimated that the window of opportunity to block virus passage is merely a few hours [1,2] and that, in less than 10 days post-exposure, transmitted viruses establish a founder population which is expanded submucosally prior to systemic dissemination [3,4].

In natural infection, gp41-directed IgA are the earliest Abs to be detected in mucosal secretions, arising within three weeks post-exposure [5]. Systemically, the earliest IgM, IgG and IgA Abs are also generated against gp41 within three weeks of infection [6]. Unfortunately, natural Abs arise too late after systemic infection is already established, and therefore, vaccine-induced mucosal antibodies (Abs) could be critical in protection at the earliest stages of infection. Immunogens delivered via several immunization regimens have been shown to elicit HIV-specific mucosal Abs [7,8,9,10,11,12,13,14] that can inhibit infection and replication via multiple mechanisms such as: virus neutralization, inhibition of virus transcytosis, Ab-dependent cellular cytotoxicity (ADCC) or Ab-dependent cell-mediated virus inhibition (ADCVI) [7,8,9,11].

Previously, we have developed chimeric virus-like particle (VLP) immunogens that elicit potent systemic and mucosal Abs against two highly conserved regions of gp41, by employing an optimized immunization strategy in mice [8,9]. The ELDKWA and QARVLAVERY epitopes are found within the membrane proximal external region (MPER) and the coiled coil region of

gp41 respectively. Importantly, the epitope-specific IgG and IgA fractions derived from immunized mice were effective in neutralizing HIV lab-adapted strains and in preventing viral transcytosis *in vitro* [9]. In particular, the QARVLAVERY epitope is remarkably conserved and is an unusually high inducer of IgA in comparison to ELDKWA, making it an attractive candidate to be used for the generation of broadly reactive mucosal Abs [9].

Rigorous efforts are being made to decipher events that unfold during acute infection and to characterize transmitted or founder (T/F) virus populations [15,16,17]. Whether phenotypic attributes are distinct between chronic infection and T/F viruses remains unclear and may be clade specific, however, distinct sensitivity to certain standard inhibitory Abs has been reported for T/F and acute infection viruses compared to chronic viruses [15,17,18,19,20,21] . Clade D and A acute infection virus envelopes were also reportedly shorter and have distinct glycosylation patterns [22]. It has been demonstrated for clade B mucosal transmission that the founder virus population is remarkably homogeneous, originating from a single or a few transmitted virus clone [19] which preferentially utilize the CCR5 coreceptor, but not CXCR4 [23]. The extreme genetic bottleneck phenomenon has been confirmed in other SIV [24,25,26] and HIV studies [16,23,27], however which factors contribute to transmission of some variants but not others is still under investigation [28].

Since mucosal transmission is the primary route of virus entry and since T/F viruses represent the earliest target of the immune responses against HIV-1, it is logical to functionally assess vaccine-induced immunity against T/F virus panels. Thus, in this study, we evaluated the ability of the above-mentioned ELDKWA- or QARVLAVERY-specific IgG and IgA fractions, obtained from previous immunization studies [9], to inhibit the infection and transcytosis of recombinant HIV-1 infectious molecular clones, which express T/F *env* genes in an isogenic

backbone (Env-IMC) [29]. To our knowledge, this is the first study in which the potency of mucosally induced IgG and IgA Abs directed against two highly conserved gp41 epitopes is tested against the most relevant target, *bona fide* T/F virus Env.

Results

Anti-gp41 antibodies effectively inhibit T/F Env-IMC infection of TZM-bl cells

To assess the ability of anti-gp41 antibodies directed against the ELDKWA- or QARVLAVERY-epitoped to inhibit T/F HIV, we utilized recombinant HIV-1 infectious molecular clones which express T/F *env* genes in an isogenic backbone (Env-IMC) [29]. In parallel to three T/F Env-IMC, NL-CH040.ecto, NL-CH058.ecto, and NL-6240.TA5.ecto, we included two lab-adapted R5-tropic reference strains, ADA and MN. We first determined the sensitivity of T/F Env-IMC to anti-gp41 inhibitory agents T20, 2F-5-IgG and 2F5-IgA in a TZM-bl neutralization assay [30] since those reagents served as controls for assessing IgA and IgG from our immunization studies. Table 1 lists the IC70 and IC90 values indicating the concentration of inhibitor required to inhibit infection by 70% or 90% respectively. The reduction in infection mediated by the inhibitor was measured relative to control wells that were indicative of 100% infection in the absence of any inhibitor. All inhibitors reached IC90, except for 2F5-IgA which could not achieve 90% reduction in infection by NL-CHO40.ecto and NL-CHO58.ecto.

Anti-gp41 Ab fractions were obtained from previously described immunization studies [8,9]. Briefly, C57BL/6 mice were immunized with DNA and recombinant adenovirus vectors (rAd) that expressed the chimeric VLPs in vivo, using a previously optimized heterologous prime-boost immunization regimen [8,9]. Mice were primed with three intramuscular DNA immunizations with CpG as an adjuvant, followed by immediate electroporation of the immunized muscle, and subsequently received two intranasal rAd immunizations. We have shown that this regimen successfully elicits systemic and mucosal IgG and IgA against the

respective gp41 epitope expressed by the constructs, either ELDKWA or QARVLAVERY. The Ab fractions obtained from the serum and vaginal washes of immunized mice were assessed for their ability to inhibit infection by the T/F Env-IMC, as well as ADA and MN, using the standard TZM-bl assay [30]. For this, the mouse Ab samples were first processed to enhance the epitope-specific Ab content and to obtain purified IgG and IgA-enriched fractions. The final Ab fractions were equalized for their epitope-specific IgG or IgA content using an epitope-specific ELISA as described previously [9] and in Materials and Methods. Optimal dilutions of the equalized samples were used in the assays as determined by titration curves (data not shown).

Our data, shown in Figure 1, indicates that the T/F Env-IMC were more sensitive to the neutralizing effect of the Ab fractions compared to ADA and MN, given the same infectious dose for each virus and the same IgG and IgA concentration across the board for each sample. In addition, NL-CHO40.ecto was the least sensitive to the neutralizing effects of the serum-derived Ab fractions compared to NL-CHO58.ecto or NL-6240.TA5.ecto. Both IgG and IgA fractions exhibited a neutralizing effect against the T/F IMCs, however, ELDKWA-specific IgG was significantly more effective at neutralizing the T/F IMCs compared to QARVLAVERY-specific IgG.

Anti-gp41 antibodies effectively inhibit the transcytosis of T/F Env-IMC across a HEC-1A monolayer

Secretory IgA mediates critical protection against mucosal pathogens by preventing their transcytosis across the epithelium, from the lumen to the submucosa [31,32,33]. Growing polarized HEC-1A cells in a transwell allows us to model an epithelial barrier *in vitro* to monitor

HIV-1 passage from the apical to the basolateral compartment across a monolayer that has formed tight junctions, determined by measuring trans-epithelial resistance (TER). We have previously shown in this model that extent of passage of infectious HIV-1 virions can be determined by quantitating the infectious virus particles in the basolateral supernatant, using TZM-bl cell infection read-out [9].

Figure 2 reports the effectiveness of ELDKWA- and QARVLAVERY-specific IgA in inhibiting the transcytosis of infectious T/F Env-IMC, and ADA and MN viruses. This was measured as the relative reduction in passage of infectious virus into the basolateral compartment, upon pre-incubation with the IgA fractions relative to their absence. As the results indicate, the T/F Env-IMCs were more sensitive to the IgA fractions derived from the serum (Figure 2A), vaginal washes (Figure 2B) and fecal pellets (Figure 2C) relative to the lab-adapted strains ADA and MN. Equal titers of ELDKWA- and QARVLAVERY-specific IgA were used in all assays and significant differences in the transcytosis-inhibiting efficiency of each fraction were determined. Where indicated (Figure 2), QARVLAVERY-specific IgA was found to be significantly more effective at inhibiting transcytosis compared to ELDKWA-specific IgA. This is noteworthy since this trend was not observed in the TZM-bl neutralization assay illustrated in Figure 1, suggesting that while neutralization of transcytosed virions cannot be excluded, it does not appear to be the only operative mechanism.

2F5-IgG and 2F5-IgA inhibit transcytosis of infectious T/F Env-IMCs by two distinct mechanisms

To begin to address by which mechanism the reduction of infectious virus particles in the presence of antibody was achieved, we used 2F5-IgA and 2F5-IgG as model antibodies. Here, we assessed their capacity to effectively inhibit HIV transcytosis across a HEC-1A cell monolayer, using two distinct read-outs: We quantified the amount of p24 transported into the basolateral supernatant in the absence or presence of serially diluted antibody and compared this to the presence of infectious virus in the basolateral compartment under the identical conditions. While quantitating p24 is the most commonly reported method for reflecting transcytosis efficiency [34,35,36,37], we believe that measuring the passage of actual infectious virus particles is more biologically relevant. The basolateral supernatant aliquots used to determine p24 levels by ELISA were taken from the same sample sets used to measure infectious virus particle content in the TZM-bl assay.

As illustrated in Figure 3, for all three T/F Env-IMCs, a dose dependent effect on the efficiency of transcytosis is observed for each antibody. The overall trend shows that at any given concentration of each antibody, the degree of reduction in transcytosis as measured by p24 levels is lower than the degree of inhibiton observed for presence of infectious virus. Also, the maximum protection achieved by the inhibitors was reflected to be higher when reporting passage of infectious virus versus p24 levels. This suggests that both IgG and IgA can in fact block the translocation of virion-associated p24 into the basolateral compartment. However, in addition the transcytosed virions appeared to be at least partially neutralized and thus rendered less or non-infectious, highlighting an important inhibitory mechanism that is missed when only p24 translocation is measured.

DISCUSSION

The processes involved in HIV-1 transmission at mucosal surfaces are complex, and thus far, not well understood. However, recent advances in identifying the partial or whole genomes of transmitted HIV-1 strains demonstrated a strong genetic bottleneck in sexual transmission resulting from only one or few virus clones being transmitted across the mucosa, and the nearly exclusive transmission of R5 strains [15,17,19,23]. Whether the transmitted envelopes and viruses have a genetic advantage or whether components of mucosal host defense play a role in virus selection is currently not known. Nevertheless, T/F HIV-1 constitute highly relevant reagents for the assessment of protective immune function, since they best represent what the immune system encounters at the earliest stages of HIV-1 infection, when virus replication is still locally contained prior to systemic dissemination.

Here, the inhibitory function of gp41-targeting Abs was assessed against three T/F Env-IMC and two lab-adapted R5 strains. While it would be desirable to test a larger panel of T/F HIV-1, the limited availability of immunization-derived samples, especially from mucosal sites, restricted the number of viruses that were tested. Our results showed that the systemic and mucosal IgG or IgA fractions, specific to the gp41epitopes ELDKWA or QARVLAVERY, exhibited effective protection against the infection of TZM-bl cells (Figure 1) and against transcytosis of infectious virus (Figure 2). The results further indicate that the IgG and IgA Ab fractions inhibit the T/F Env-IMC more effectively than the lab-adapted R5 strains in both assays.

It should be noted, however, that for the neutralization experiments using TZM-bl cells, cell-free virus stocks for T/F Env-IMC were produced in PBMC, while ADA and MN were

efficient at inhibiting transcytosis of the T/F Env-IMC compared to ELDKWA-specific IgA (Figure 2). Although IgG-based Ab approaches have dominated vaccine efforts against HIV-1, IgA may be a critical component of protection especially at the mucosa. Not only is it the main Ab secreted via transcytosis to form a key barrier against HIV entry at the mucosa [32,34,36,39], but IgA is uniquely non-inflammatory since it does not activate complement [40]. There is ample evidence indicating that inflammation at mucosal tissues can exacerbate the risk of HIV infection and pathogenesis [41]. Indeed, genital inflammation has been attributed to compromising barrier integrity which mitigates the genetic bottleneck phenomenon [16].

We also showed an interesting difference in the level of transcytosis inhibition when determined by two different output measures (Figure 3): quantitating the passage of infectious virus particles versus measuring the p24 levels in the basolateral supernatants. In the HIV-1 replication process, several steps precede the formation and release of fully mature infectious virus particles. Proviral or immature virus forms and multimeric cytosolic gag are also released [42], which can undergo transcytosis and traverse into the basolateral compartment. These non-infectious p24 forms but are not detected by the TZM-bl assay, since the latter relies on productive virus infection for luciferase reporter gene expression [30]. Our results show that by measuring p24 levels the effectiveness of inhibitory Abs is underestimated and therefore, we propose that measuring infectious virus particles is a more accurate reporting method for transcytosis efficiency.

Prophylactic Ab-based vaccines must not only generate potent, non-inflammatory and broadly-reactive antiviral responses at the mucosa but must also be effective against the relevant viral target: transmitted virus variants. Here we have shown that gp41-specific Ab fractions, that target two highly conserved epitopes, are functionally effective against the T/F Env-IMC in both

the TZM-bl neutralization assay and a transcytosis-inhibition assay. Gp41 is an attractive immunogen for the elicitation of mucosal Abs as demonstrated by these results. In addition, we support the notion that T/F Env-IMC, or full-length T/F IMC, represent the most biologically relevant viral reagents when assessing immune functions at the earliest stages of infection that may be critical for effective HIV prevention.

Materials and Methods

Generation of HIV stocks

We utilized replication-competent recombinant HIV-1 infectious molecular clones which encode transmitted/ founder virus *env* sequences in an isogenic NL4.3 HIV backbone (referred to as T/F Env-IMC), which we validated and described previously [29]. Briefly, the Env-IMC express a full-length Env protein in which all of gp120 and the ectodomain and membrane spanning domain is encoded by the heterologous env gene while the cytoplasmic tail is derived from NL4-3, a cloning strategy that avoids affecting overlapping reading frames of vpu, tat, and rev. The three T/F Env-IMC, pNL-CH040.ecto, pNL-CH058.ecto, and pNL-6240.TA5.ecto, expressed the single transmitted variant from patients, 700010040, 700010058 and 6240-08, respectively. 293T cells were transfected with the proviral plasmids using Fugene 6 (Roche), following manufacturer's recommendations. Supernatants from transfected cells were collected at 72 hours and used to infect fresh peripheral blood mononuclear cells (PBMCs) to generate a large-scale stock. PBMCs were isolated by ficoll-paque centrifugation and stimulated with 7ug/ml phytohemagglutinin (PHA, SIGMA) and 35 U/ml of recombinant IL-2 (R&D, Burlington) for 72 hours prior to infection with supernatants. Virus supernatant was collected on day 7, 9, 11 and pooled prior to determination of the stock TCID50 using the Reed-Muench method in TZM-bl cells [43] (TCID50/ml for NL-CH040.ecto: $1.58x10^6$/ml, NL-CH058.ecto: $1.32x10^6$/ml and NL-6240.TA5.ecto: $2.2x10^8$/ml).

The R5-tropic lab-adapted viruses, ADA and MN, were grown in adherent monocyte-derived macrophages as described [44] and virus supernatants were pooled from multiple collections. All pooled virus supernatants were concentrated using 30K Amicon filter tubes

(Millipore, Burlington, ON, Canada) and the TCID50 for each final concentrated stock was determined using the Reed-Muench method in TZM-bl cells (TCID50/ml for ADA: $2.77x10^4$/ml and MN: $5.5x10^4$/ml).

Gp41-specific antibody fractions

The antibody fractions used to inhibit HIV infection and transcytosis in the respective assays were derived from mice immunized with chimeric VLPs expressing either the ELDKWA or QARVLAVERY epitopes [8]. The chimeric VLP design and immunization strategies employed to elicit potent systemic and mucosal antibodies have been described previously [8,9]. Briefly, mice were administered a prime-boost regimen consisting of DNA and recombinant Ad5 vectors, expressing the [Gag+ELDKWA] or [Gag+QARVLAVERY] constructs. Serum, vaginal washes and fecal pellet samples were collected on day 14 after the last immunization from mice that were administered (DNA+CpG) intra-muscular immunizations with immediate electroporation three times, three weeks apart, followed by two consecutive rAd intra-nasal immunizations, two weeks apart [3x(DNA+CpG)i.m. + 2x rAd i.n.]. Briefly, antibody fractions were enriched for the epitope-specific Ab content by selective extraction of gag-specific Abs, followed by IgG purification and IgA enrichment. The purified IgG and IgA enriched fractions were concentrated and equalized by epitope-specific Ab content in an optimized epitope-specific ELISA. All protocols have been previously described in detail [9]. The IgG and IgA-enriched fractions from the serum, vaginal washes and fecal pellets were equalized to the same OD values based on ELISA, and equivalent OD values across all samples, across all viruses, were used in each assay.

Infection inhibition assay

As previously described [9], TZM-bl cells were used to measure virus infection by measure of the luciferase production upon infection [30]. Virus stocks were titrated and 130 TCID50 of ADA, 145 TCID50 of MN, 75 TCID50 of NL-CH040.ecto, 67 TCID50 of NL-CH058.ecto and 60 TCID50 of NL-6240.TA5.ecto gave >95% infection of TZM-bl cells, seeded at $5x10^4$/well in 25ug/ml of diethylaminoethyl-detran (DEAE-dextran, SIGMA, Oakville, ON, Canada). To determine the average concentration at which 50%, 70% or 90% of infection inhibition (IC50, IC70, IC90) was achieved, virus aliquots were incubated with serial dilutions of the inhibitors T20, 2F5-IgG and 2F5-IgA for 1 hour at 37^OC, in triplicate, prior to the addition of TZM-bl cells. To assess the sensitivity to the IgG and IgA fractions obtained from the immunization studies, dilutions of the serum (1:3) and vaginal wash (1:2) samples were incubated with each virus, in triplicate, for 1 hr at 37^o C, prior to the addition of TZM-bl cells. Luminescence readings were made after 48 hours of incubation, using the Veritas microplate luminometer as described [9]. The reduction in infection mediated by the inhibitor was measured relative to control wells that were indicative of 100% infection in the absence of any inhibitor.

Transcytosis-inhibition assay

Transcytosis assays were conducted in a transwell system by growing a monolayer of HEC-1A cells (ATCC, Manassas, VA) in 24-well culture plates (NUNC, BD, Burlington, ON, Canada) in conditions as previously described [9]. Infected PBMC were used as a source for cell-associated. Briefly, PBMCs were isolated and activated with 7ug/ml of phytohemagglutinin and 35 Units/ml of interleukin-2 for 3 days prior to infection with 300 TCID50 of virus per 10^6 PBMCs, to

generate either NL-CH040.ecto, NL-CH058.ecto, NL-6240.TA5.ecto, ADA or MN cell-associated stocks. 7.5 x 10^5 of infected PBMCs were added apically on HEC-1A monolayers seeded in transwells, once the monolayer reached a trans-epithelial resistance of >330 Ω/cm^2. Virus passage from the apical to the basolateral compartment was optimal at 4hrs which is when the basolateral supernatant was collected [9]. To determine p24 titers, a 100ul aliquot of the basolateral supernatant was analyzed in a p24 antigen capture assay (Advanced BioScience Laboratories, Inc., Kensington, MD) and virions in the remaining volume were concentrated using 50-K Amicon filters to a final volume of 100ul. The concentrated basolateral supernatant was used to infect TZM-bl cells in the standard protocol described previously [9] to quantitate the infectious virus particles that passaged across the HEC-1A epithelial barrier. To assess the effectiveness of the inhibitory agents, serial dilutions of each were pre-incubated with 7.5x10^5 infected PBMC, for 2 hours at 37^{O}C in triplicate, prior to being transferred onto the HEC-1A cells in the transwell system. To assess the sensitivity to inhibition by the IgA fractions obtained from the immunization studies, 1:3 dilution of the original serum fraction and 1:2 dilution of the vaginal wash/ fecal pellet samples were used for pre-incubation with the cell-associated virus stocks. All protocols have been previously described in detail [9].

Statistical Analysis

Significance was determined by the Mann-Whitney test between selected data sets, using the Graph Pad Prism 4 software (La Jolla, CA).

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Tables and Figures

Table 1. The concentration of the inhibitory agents T20, 2F5-IgG and 2F5-IgA, required for the inhibition of infection of TZM-bl cells by 70% (IC70) or 90% (IC90).

T/F Env-IMC	% Protection	T-20 (ng/ml)	2F5-IgG (ug/ml)	2F5-IgA (ug/ml)
CHO40	IC70	5.5	0.17	0.45
	IC90	15.0	0.35	NR
CHO58	IC70	5.0	0.14	0.35
	IC90	10.0	0.28	NR
6240.TA5	IC70	3.0	0.05	0.51
	IC90	5.5	0.10	0.87

IC: Inhibitory concentration
NR: Not reached

Figure 1

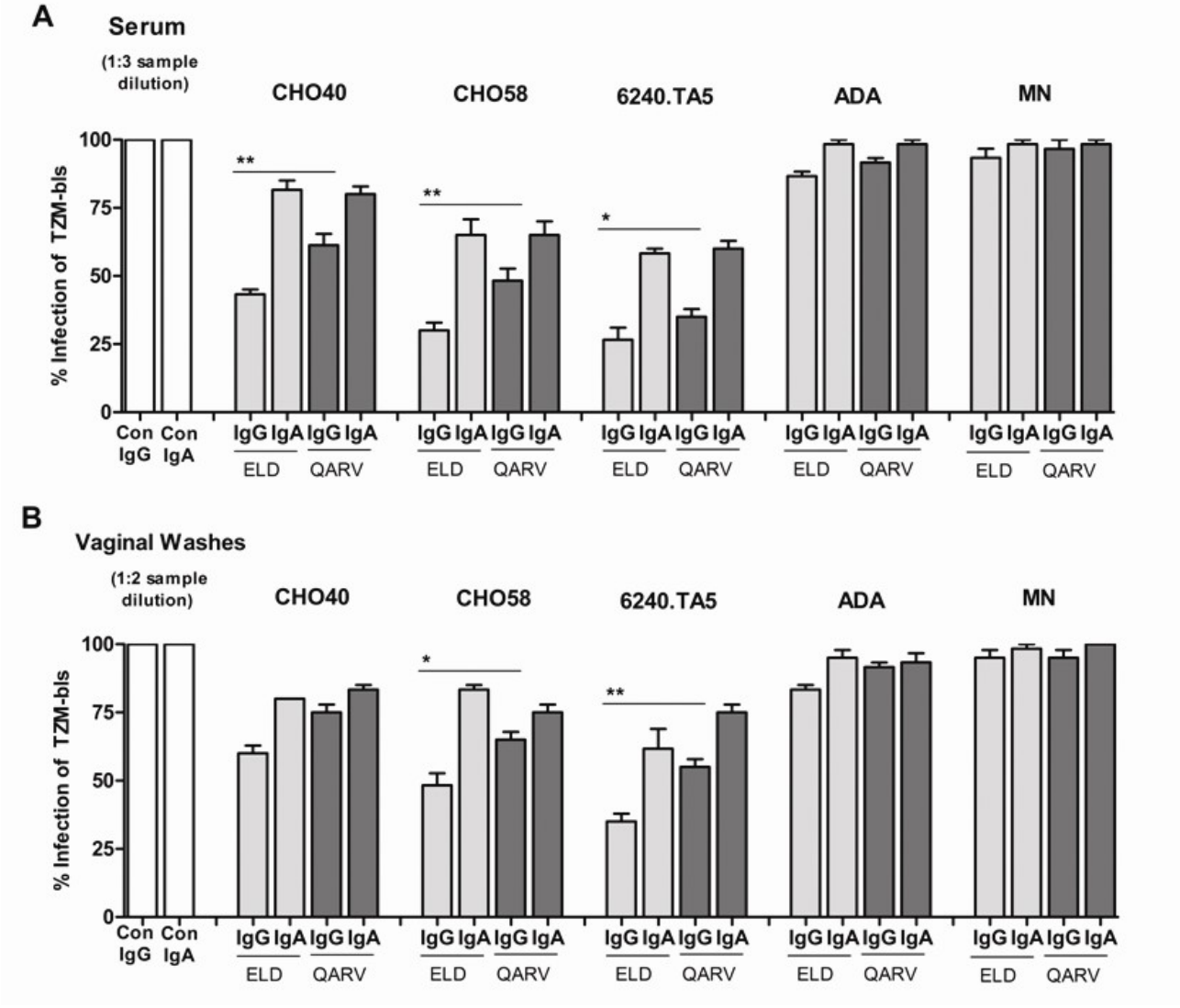
A
Serum
(1:3 sample dilution)
CHO40
CHO58
6240.TA5
ADA
MN
% Infection of TZM-bls
100
75
50
25
0
**
**
*
Con IgG
Con IgA
IgG IgA IgG IgA
ELD
QARV
B
Vaginal Washes
(1:2 sample dilution)
CHO40
CHO58
6240.TA5
ADA
MN
% Infection of TZM-bls
100
75
50
25
0
*
**
Con IgG
Con IgA
IgG IgA IgG IgA
ELD
QARV

Figure 1**.** Neutralization efficiency of ELDKWA- and QARVLAVERY-specific IgG and IgA fractions obtained from the serum (Panel A) or vaginal washes (Panel B) of immunized mice. Ab effectiveness was assessed against a panel of three T/F Env-IMCs (NL-CH040.ecto, NLCH058.ecto, and NL-6240.TA5.ecto and two lab-adapted R5 strains (ADA and MN). Percent reduction in infection mediated by the Abs was determined by the reduction in relative luminescence units upon incubation of the virus with the IgG or IgA fractions, compared to infection of TZM-bl cells in the absence of inhibitors. The control IgG or IgA samples were processed in the same manner but were obtained from naïve unimmunized mice. Data plotted is the average represented by three or more independent samples per condition. Significant differences in the neutralization capacity of ELD-specific versus QARV-specific IgG is shown, $^{**}P<0.01$ and $^{*}P<0.05$.

Figure 2

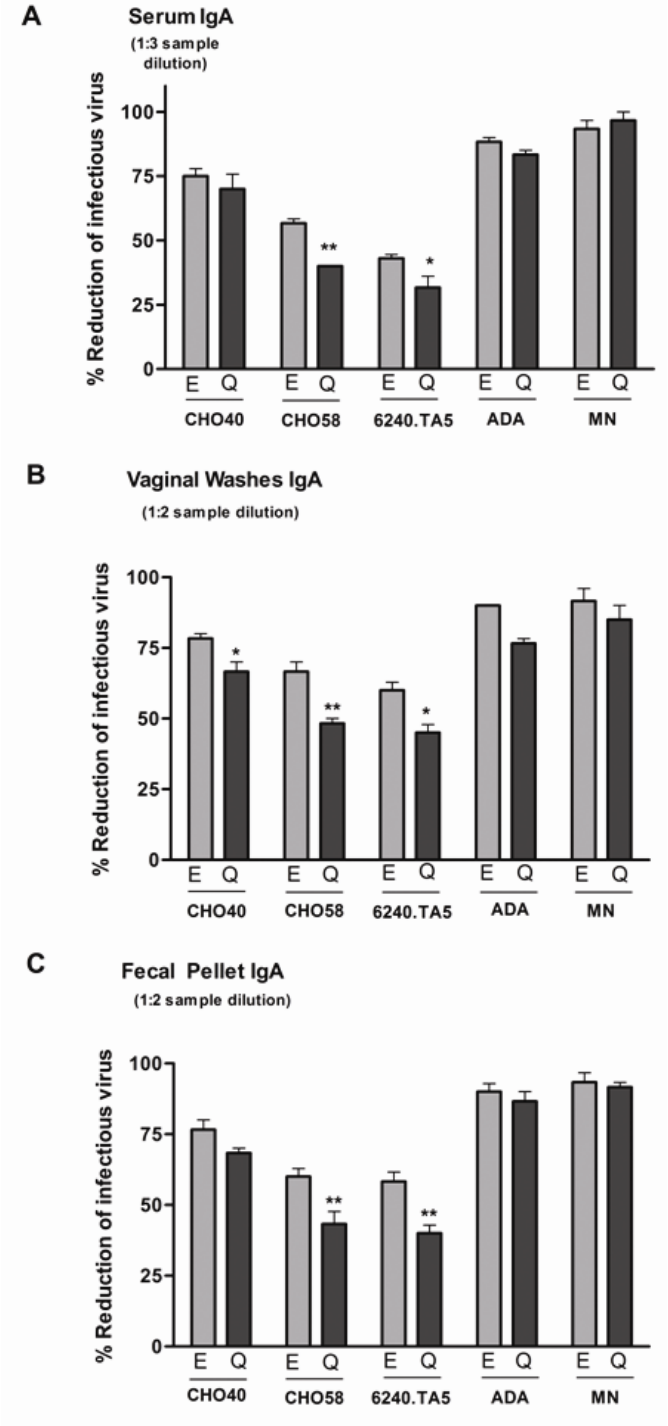
A
Serum IgA
(1:3 sample dilution)
% Reduction of infectious virus
100
75
50
25
0
E Q E Q E Q E Q E Q
CHO40 CHO58 6240.TA5 ADA MN
B
Vaginal Washes IgA
(1:2 sample dilution)
% Reduction of infectious virus
100
75
50
25
0
E Q E Q E Q E Q E Q
CHO40 CHO58 6240.TA5 ADA MN
C
Fecal Pellet IgA
(1:2 sample dilution)
% Reduction of infectious virus
100
75
50
25
0
E Q E Q E Q E Q E Q
CHO40 CHO58 6240.TA5 ADA MN

Figure 2. Transcytosis-inhibition of T/F Env-IMCs and lab strains mediated by the purified IgA samples obtained from the serum (Panel A), vaginal washes (Panel B) and fecal pellets (Panel C) of mice immunized with either the Gag+ELDKWA (E) or Gag+QARVLAVERY (Q) constructs. All IgA samples, from both immunization groups, were equalized by their epitope-specific Ab content determined by peptide-specific ELISA, in order to directly compare the efficacy of IgA in inhibiting virus transcytosis. Final samples were diluted prior to use in the assays as indicated. Data are plotted as the average of four or more samples per condition. Statistically significant differences in the ability of QARVLAVERY-specific IgA versus ELDKWA-specific IgA to inhibit transcytosis for any of the viruses (NL-CH040.ecto, NL-CH058.ecto, NL-6240.TA5.ecto, ADA or MN) are indicated: **$P<0.01$ and *$P<0.05$.

Figure 3

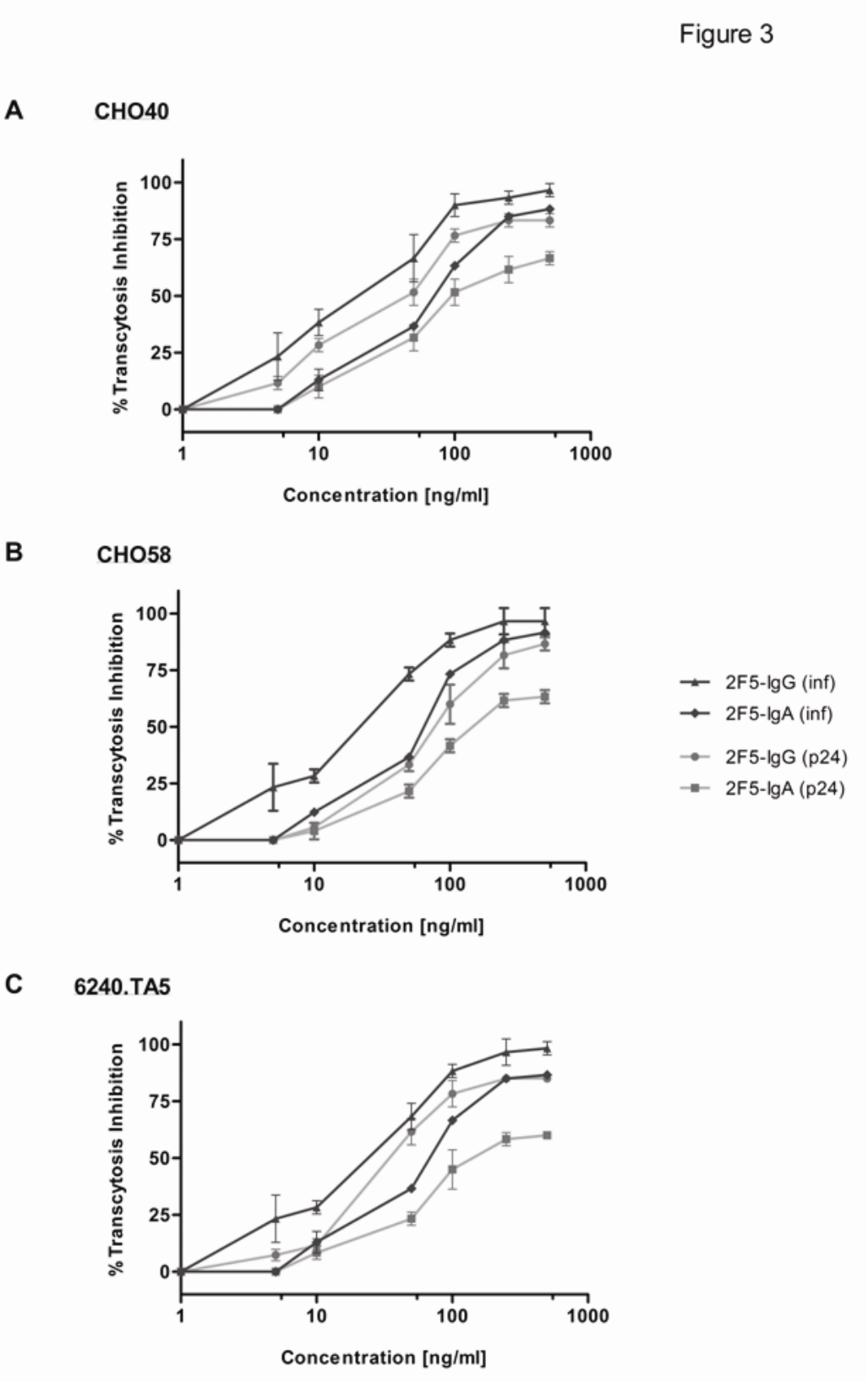
A
CHO40
% Transcytosis Inhibition
100
75
50
25
0
1
10
100
1000
Concentration [ng/ml]
B
CHO58
% Transcytosis Inhibition
100
75
50
25
0
1
10
100
1000
Concentration [ng/ml]
2F5-IgG (inf)
2F5-IgA (inf)
2F5-IgG (p24)
2F5-IgA (p24)
C
6240.TA5
% Transcytosis Inhibition
100
75
50
25
0
1
10
100
1000
Concentration [ng/ml]

Figure 3. The effect of monoclonal Abs 2F5-IgG and 2F5-IgA on the transcytosis of cell-associated HIV (NL-CH040.ecto: 3A, NL-CH058.ecto: 3B or NL-6240.TA2.ecto: 3C) across an epithelial monolayer, measured either as the reduction of infectious virus or the reduction of p24 in the basolateral chamber. Serial dilutions of the 2F5-IgG and 2F5-IgA (1, 5, 10, 35, 75, 100, 250, 500 ng/ml) were incubated with cell-associated HIV for 1 hour at 37^{o}C prior to their addition onto the HEC-1A transwells. The passage of infectious virus particles into the basolateral supernatants was quantified at 4 hours, by concentrating the supernatant and subsequently measuring the presence of virions able to infect TZM-bl cells. The passage of virion-associated p24 into the basolateral compartment was determined by a p24 ELISA. The percent reduction in transcytosis was determined relative to the average number of particles or the average amount of p24 that passaged across the epithelium in the absence of inhibitory agents. Data is representative of the average of four or more replicates per condition.

-- CHAPTER 5 --

DISCUSSION

1. Implications of Key Findings

The preceding chapters described studies that were conducted to address the primary objective of this thesis: to design and implement a vaccine model with the goal of eliciting mucosal and systemic Abs against highly conserved regions of gp41, that demonstrated protective function against HIV *in vitro*, particularly against viral clones from acute infection.

Undeniably, the most significant roadblock in achieving effective immune control of HIV can be attributed to its high mutation rate and enormous sequence diversity [5]. This has been demonstrated by the clinical practice of combination HAART and first/ second generation regimens that are required to maintain immune pressure on competing and newly emerging viral strains [47]. Further, despite the efficacy of T-cell based vaccines in controlling virus replication short-term [256], long-term studies have failed to control infection due to the continuous emergence of viral escape mutants [260].

1.1. *HIV intervention at the mucosa*

The field of vaccine development against HIV has faced various challenges as evidenced by the major phase IIB/III clinical trials that have been reviewed in chapter 1. However, the outcomes of these trials have also highlighted gaps in knowledge that remain to be explored and have forced the field to expand its concept of effective vaccination, which now includes mucosa-targeting approaches. Most importantly, our understanding of the correlates of immune protection has evolved and assumptions of which Ab functions contribute to protection have been re-evaluated. Culminating data from our studies and

current understanding of the mucosal immune system strongly suggest that targeting the mucosa may require a distinct approach from systemic vaccination strategies. The primary aim of most studies characterizing mucosal immunity has been to generate and assess neutralizing Ab responses in mucosal compartments. However, that alone may not be sufficient for effective mucosal vaccines since the quality of the response must also be relevant to the mucosal environment and directed towards the events that unfold during virus transmission.

Non-neutralizing IgG: As reviewed in chapter 1, historically, nAbs and CTL response generation have been the hallmark to measure vaccine efficacy. Although non-neutralizing protective Abs have been overlooked in the past, their importance is clearly demonstrated by recent characterization of ADCC/ADCVI effector functions in HIV infection. Assessment of ADCC kinetics in a SIV infection model revealed that responses were detected as early as 3 weeks post-infection, prior to nAbs, and the magnitude of the response was inversely associated with circulating viral loads [388]. A similar SIV infection study assessing ADCVI kinetics showed that Ab-dependent mechanisms inhibited virus production by 100-fold within 3 weeks of infection, prior to the detection of nAbs [389]. Further, ADCVI continued to confer protection even after the virus quasi-species evolved and adapted to evade the neutralizing response [389]. Interestingly, the modest success of the Thai RV144 trial was not found to be associated with NAbs or CTL responses, but correlated with ADCC function of env-specific IgG [211, 377, 390, 391]. In light of these results, identifying regions of the glycoprotein that would induce effective ADCC/ADCVI responses is an important next step

in order to develop immunogens that would elicit such Abs. The contribution and relevance of ADCC/ADCVI responses at the mucosa would also be important to determine, in natural infection and by vaccine induction in simian models of vaginal challenge.

Mucosal landscape: The landscape of the genital mucosa is complex and encompasses many factors that affect HIV transmission and immunopathogenesis. The humoral response is one aspect of mucosal immunity that can be exploited by prophylactic vaccination. Clearly, the greatest outcome of our studies has been the development of a vaccine model to elicit potent mucosal IgG and IgA against highly conserved gp41 epitopes that were indeed functional against HIV. It should be appreciated however, that other factors such as co-existing STIs, hormonal cycles, host immune fingerprints and host response to local stress or activation of innate signaling pathways, can all affect the environment of the female genital tract and may influence the local Ab response [154, 157, 161, 185, 392, 393]. Despite these confounding factors and various caveats arising from the field's compartmentalized view of the mucosa, a common thread has certainly emerged: that mucosal inflammation can increase the probability of contracting HIV or exacerbate infection. A highly attractive quality of IgA-mediated blocking and clearance of HIV is that it does not activate complement and therefore is non-inflammatory in comparison to IgG [208].

Window of opportunity: Evidence from simian mucosal infection studies also suggests that it may be possible to target HIV in early stages of transmission when it is most vulnerable to immune pressure [82]. This is due to the 'genetic bottleneck' phenomenon resulting in a

homogeneous post-transmission viral population, greatly reducing the challenge of genetic diversity [130], and due to the local containment of the virus population in early stages of mucosal transmission. Generating effective mucosal immunity will be an essential component of Ab-based prophylactic strategies seeking to prevent or clear infection at the site of transmission itself. It may be critical to achieve this prior to the dissemination of infection to the lymph nodes after which virus control could get significantly more challenging given the extent of possible virus spread.

1.2. *Optimized immunization strategy for mucosal immune induction*

It has been shown in various models, that employment of efficient mucosal immunization enhances the induction of mucosal immunity relative to systemic immunization alone [394, 395]. No HIV vaccine that has reached phase II/III trials thus far has ever been administered mucosally, which will be an important goal for HIV vaccine strategies to achieve in the future.

The immunization regimens employed in our studies utilized an optimized delivery strategy of three intramuscular deliveries of DNA and CpG with electroporation, followed by two intranasal deliveries of a booster rAd5 vector. This combination generated significantly higher Abs against the gp41 epitopes expressed by our chimeric VLP construct compared to other regimens that were tested in our study in chapter 2. Furthermore, as a proof-of-principle, our study showed that the use of CpG as an adjuvant with electroporation delivery of DNA significantly increased epitope-specific Ab generation [325]. Phase I/II studies using

electroporation delivery of DNA via intra-muscular and dermal routes [396, 397] have demonstrated that electroporation is safely tolerated in humans and significantly enhances DNA immunogenicity [398]. Furthermore, CpG is known to be a potent activator of innate responses at the mucosa [399]. Studies conducted in our lab have demonstrated the unique effects of CpG as a mucosal adjuvant in a murine HSV-2 challenge model. Upon intranasal CpG co-administration with HSV-2 surface antigen gB, animals generated significantly elevated levels of mucosal gB-specific IgA and induced significantly higher gB-specific antibody-secreting cells mucosally, in response to intravaginal HSV-2 challenge [400]. Mice were completely protected from HSV-2 infection and CpG co-immunization significantly reduced viral replication in the genital tract within the first few days of challenge [400]. Although it was not used as an adjuvant in mucosal delivery in our immunization model, evidence supports the use of CpG in mucosa-targeting vaccination strategies, by which it may significantly enhance the overall induction of mucosal immunity.

1.3. *Chimeric VLP design to immuno-focus epitope-specific Ab responses*

In chapter 2, our study demonstrated that a strategic immunogen design and vector delivery utilizing optimized prime-boost combinations could indeed generate high titer epitope-specific Abs against select conserved glycoprotein env regions [325]. As reviewed in chapter 1, polyclonal Abs against whole subunits leads to competitive production of functional and non-functional Abs, while exhausting B-cells and effective Ab generation [400], therefore a vaccination strategy that focuses the Ab response is important to consider.

The novel aspect of our vaccine construct was the incorporation of multiple tandem repeats of an epitope within a gag VLP as a delivery platform, to successfully 'immuno-focus' the host response towards an epitope of interest [324, 325]. Such chimeric VLPs have the dual benefit of eliciting gag-specific cell-mediated immunity, balanced with Ab generation, which is an idealistic goal for HIV vaccination to acquire effective control. Figure 4 in appendix I shows that splenocytes from mice, immunized with our constructs were reactive to gag peptide pools, demonstrating that gag-specific T-cell immunity was also induced.

The gag+epitope VLP construct itself also has the flexibility of being administered *in vivo* as a protein immunogen, in a DNA vector or via alternate viral vectors, while still producing functional and properly formed particles if an optimal insert length is maintained, as shown in our study [325]. The ability to choose and optimize the delivery method allows us to target specific arms of the immune system and desired compartments such as mucosal tissues. Emerging data from systems vaccinology, a field that characterizes the effects of viral vectors on the innate and adaptive immunity, implicates that vectors may indeed affect the host's response against the transferred immunogen since the vector itself interacts with the host, particularly with their innate immune 'fingerprints' [401, 402]. Basic DNA vaccines are 'inert' compared to viral vectors since they interact minimally with host immunity and are sought as a relatively safer option [403]. With a greater understanding from systems biology, however, information on how hosts may respond to viral vectors may open opportunities to exploit specific arms of immunity by strategic use of delivery platforms to induce desirable responses [401, 404].

1.4. *Functional Abs induced against gp41*

In preceding chapters, we have highlighted highly conserved domains of gp41, such as the MPER at the C-terminus and the coiled coil at the N-terminus, that encode immunogenic epitopes: QARVLAVERY, ELDKWA and NWFDIT, in their order of sequence conservation [329]. The fusion process during virus-host interaction is an absolute requisite for infection and is dependent on the proper configuration of the N- and C-termini during intermediate steps, as shown in figure 2, chapter 1. This is perhaps why these key epitopes of gp41 have not been subjected to diversification compared to the immunodominant variable loop regions of gp120 [284].

Interestingly, studies assessing immune responses during acute infection have shown that Abs specific to gp41 are identified as the earliest virus-specific response generated, both in the serum and cervico-vaginal secretions of women [203, 405]. We propose that gp41 is a superior viral immunogen since it encodes highly conserved immunodominant regions that can be incorporated in rational immunogen design to induce epitope-specific Ab responses that are cross-reactive and protective.

Functional IgA generated against MPER and QARVLAVERY: We have successfully demonstrated that our chimeric gag+epitiope VLP constructs elicit epitope-specific IgG and IgA in mice. Further, ELDKWA- and QARVLAVERY-specific IgG and IgA neutralized lab-adapted HIV in the gold standard TZM-bl neutralization assay [406], and epitope-specific IgA effectively inhibited virus transcytosis across mucosal epithelial cells in a transwell assay.

Intriguingly, the QARVLAVERY epitope was found to be an unusually potent inducer of IgA and generated IgA with significantly greater transcytosis-inhibiting ability than the ELDKWA epitope. Thus far, minimal attention has been placed on identifying glycoprotein regions that uniquely elicit IgA with potential mucosal function.

Since the identification of QARILAVERY as the target of serum IgA from discordant EU cohorts [232], ours was the first study to incorporate the epitope in a vaccine study to specifically assess its immunogenicity and function [406]. ELDKWA has been the only other HIV epitope that has been characterized as an inducer of IgA with transcytosis-inhibiting capacity [309]. IgA isotypes of 2F5, the ELDKWA targeting bNAb, have also been developed in monomeric and dimeric forms and were shown to block HIV transcytosis *in vitro* [407]. Other reports on the potential role of IgA in antiviral protection have emerged from serological studies of HEPS or EU individuals. Although a causal relation between HIV-specific IgA levels and protection is still unclear [408], there have been multiple reports demonstrating effective IgA-mediated neutralization [81, 231-233, 235, 239, 409] and transcytosis inhibition of HIV [231, 236, 410] *in vitro*. We have shown that virus-specific IgA induced by our vaccine model elicits antiviral functions and propose that this may be a critical component of mucosal vaccines to block HIV at the site of entry itself.

1.5. *Potential of gp41 as a mucosal immunogen*

The role of gp41 as an effective immunogen has long been undermined, primarily due to prior aims of inducing sterilizing immunity, for which gp120 is indeed better suited.

However, we now know that nAbs are not a primarily correlate of Ab protection, particularly in early stages of infection. Further, as we have shown in our studies, gp41 contains highly conserved immunodominant regions capable of inducing functional IgG and IgA against HIV.

Just last year, the first vaccine study utilizing a gp41-based immunogen in a SIV vaginal infection model was published, with the goal to primarily and specifically assess mucosal Ab function. Animals were administered gp41 virosomes, four times prior to thirteen repeated low-dose vaginal SHIV challenges, via the intramuscular (i.m.) route alone or were given two i.m. followed by two intranasal (i.n.) immunizations [379]. Interestingly, two gp41 regions were selected to be fused on the surface of an influenza virosome, one containing the MPER epitopes ELDKWA and NWFDIT, and the other containing the coiled coil region with the QARILAVERY epitope. Five of the six animals given the i.m. immunizations alone were protected from infection, however, animals given i.m. and i.n. were all protected and had significantly lower viral RNA at all monitored time-points, exhibiting superior protection with the use of mucosal immunization [379].

Although vaccination induced both systemic and mucosal Ab responses, it was Abs from mucosal secretions and not the plasma that exhibited antiviral function, in the form of neutralization ability, transcytosis inhibition and ADCC activity. Notably, the transcytosis function of mucosal IgA was significantly higher in the i.m. + i.n. group compared to the i.m. group alone, suggesting that mucosal immunization may be required to elicit IgA and optimize transcytosis protection. It was also the first simian study to determine correlation

between the efficiency of three distinct mucosal Ab functions (transcytosis-inhibition, ADCC, neutralization) and virus levels in immunized hosts, measured by systemic SIV RNA copies. Interestingly, for cervico-vaginal lavage Abs, the correlation between transcytosis blocking efficiency and reduction in viremia ($p<0.001$) was significantly higher than the correlation between neutralizing ability and reduction in viremia ($p>0.02$) [379]. IgA or IgG depletion of cervico-vaginal secretions also proved that transcytosis inhibition was mediated by IgA and not IgG.

The immunogen and immunization strategy of this study were different from the chimeric VLP design and the DNA+rAd5 prime-boost employed in our studies, however, they both sought to induce mucosal immunity and assessed mucosal Ab function. The results from this simian vaccine study still support our key finding: that gp41, in particular the MPER and coiled coil region are suitable immunogens for the elicitation of Abs with multiple effector functions. Furthermore, mucosal immunization may key for optimal induction of mucosal immunity. Gp41-based immunogens and the use of immunization platforms and regimens to optimize mucosal immune response induction, is an intriguing approach to elicit selective, broadly reactive protective Ab responses, with particular relevance for mucosal protection.

1.6. *Gp41-specific Abs exhibit protection against T/F viral clones*

Emerging data also supports the notion that determining efficiency of mucosal Abs may require different virus panels than those typically used to assess nAb function. The latter consist of commonly used R5/X4-tropic lab-adapted and chronic infection viruses or the

specialized 'tiered' virus panels developed by the IAVI that are cloned from global clinical strains to test cross-clade reactivity and extent of Ab potency [288]. As reviewed in chapters 1 and 4, the development of recombinant T/F virus clones was a critical step in the accurate assessment of mucosal Ab function against their actual target in mucosal HIV transmission, *bona fide* T/F virus env glycoproteins. Our data in chapter 4 shows that the gp41 specific Ab fractions from mice immunized with ELDKWA- or QARVLAVERY-expressing VLP constructs, efficiently protected against the T/F virus clones. Particularly, epitope-specific IgG fractions were significantly more effective in neutralizing T/F clones compared to the lab-adapted R5 strains MN and ADA. This trend was similarly observed for epitope-specific IgA which efficiently inhibited transcytosis of T/F viruses but not of MN or ADA.

1.7. *Assays for the assessment of non-neutralizing Ab function*

In addition to the appropriate selection of suitable virus targets, developing standardized assays to determine mucosal Ab function will be critical for reproducible assessment of Ab function and for accurate interpretation and comparison of results from different groups. Neutralization assays have been thoroughly characterized and applied in various studies, however, the key assays used to assess non-neutralizing Ab functions, transcytosis inhibition of HIV by IgA and ADCC-mediated killing of HIV by IgG-Fc, remain to be optimized.

Transcytosis assays: Thus far, p24 quantification has been used to determine 'virus' transcytosis across a polarized epithelium in a transwell, despite the fact that p24 production

and passage may occur regardless of the production or passage of infectious virus [94, 96, 309, 410]. It is possible for HIV to translocate across the polarized epithelium via vesicles while bound to virus-specific Ab that renders it non-infectious upon passage [228]. This inhibitory mechanism involving Ab-blocking of HIV during transcytosis would not be reflected in p24 quantification but would be accounted for in the TZM-bl based assay quantifying infectious virus, as evidenced by the different interpretation of Ab effectiveness by both methods reported in our study in chapter 4. We propose that quantifying infectious virus is a superior method for accurate determination of Ab function in the current transcytosis model.

ADCC/ADCVI assays: Recent studies have greatly emphasized the potential role of ADCC effector function of IgG, however, the singular assay used to determine this function relies solely on NK cell mediated clearance, which only express the FcγRIII receptor of all the others that also bind to IgG-Fc to mediate ADCC [209, 210, 377, 379]. This undermines the assessment of ADCC/ADCVI function mediated by alternate FcγRs and neglects the role of macrophages, which are not only targets of HIV infection themselves but also are abundant at the genital mucosa relative to NK cells [411].

As a future direction, the ADCC/ADCVI functions of the epitope-specific Abs generated in our model could also be assessed given that the Fc portion of murine IgG does cross-link human FcR. Furthermore, testing the epitope-specific Ab fractions against an expanded panel of T/F virus clones could further help to characterize the sensitivity profiles of the T/F env glycoproteins s to mucosal Abs in standardized assays. Reporter T/F virus

panels that co-express renilla luciferase or green fluorescence protein have also been generated for use in ADCC/ADCVI assays and for HIV tracking and detection, methods for which are also being developed [150, 412].The use of appropriate testing panels for mucosal Abs will be crucial for accurate and translatable assessment of function.

2. Characterizing the IgA-Inducing Ability of QARVLAVERY

As shown in chapter 3, QARVLAVERY exhibited a unique property with regards to IgA induction: the epitope consistently induced an approximate 1:1 ratio of IgG:IgA in both systemic and mucosal compartments by various regimens. In addition, QARVLAVERY-specific IgA was exclusively generated prior to IgG in the serum, within days of immunization, suggesting the possibility of innate B-cell activation by the epitope. This observation has not been reported for any other immunogenic epitope of HIV and further highlights QARVLAVERY in a field where strategies to induce mucosal effector Abs have been relatively scarce till now. Data has also been reported in an independent study supporting our finding that the coiled coil region induces an approximate 1:1 ratio of IgG:IgA, although this observation was not commented on by the authors [413]. However, this phenomenon must be verified in simian or human studies to conclusively determine if it can be useful in vaccination.

The fact that QARVLAVERY-specific IgA was detected prior to IgG prompted the hypothesis that innate B1 cells or extrafollicular mechanisms of activation may be involved in the process. Mature murine B-cells are heterogeneous and belong to four subsets: follicular, marginal zone, B1a (CD5+) and B1b (CD5-) [414, 415]. Follicular B-cells are what we know as the traditional B2 cells that circulate in the blood through lymphoid follicles and respond to antigens via T-cell dependent mechanisms, whereas B1 cells can undergo stimulation by both T-cell dependent and T-cell independent mechanisms [416]. The latter are located in the

pleural and peritoneal cavities in mice and they have been found to circulate mucosal tissues [415, 417]. B1a cells have long been viewed as the primary source of broadly-specific, low avidity antibodies that occur spontaneously in naïve 'antigen-free' mice and play a crucial role in protection against bacterial pathogens [415]. Although this is the main function of innate B-cells, studies are now implicating that their role may extend beyond this paradigm.

In preliminary experiments, we tested our hypothesis of the involvement of B1 cells in the early generation of QARVLAVERY-specific IgA and sought to determine if naive B1 cells could indeed be directly stimulated by QARVLAVERY *in vivo*, in an extrafollicular site. As described in appendix II, mice were administered short peptides of ELDKWA and QARVLAVERY with CpG intraperitoneally (i.p.), following which B2, B1a and B1b subsets were purified from the i.p. flushes obtained at different times after peptide delivery. Our data shows that within days, IgA secreting cells were identified in a gp41-specific ELISPOT assay. Interestingly, the B1b subset was the prime producer of gp41-specific IgA against both epitopes, and significantly higher IgA-secreting B-cells were generated in response to QARVLAVERY compared to ELDKWA. This initial data supports the notion that innate B-cells can be stimulated by the gp41 peptides and demonstrates again that a significantly higher IgA is generated against QARVLAVERY than ELDKWA. However, various future experiments are required to conclusively determine if innate B-cell signaling is a critical for and directly linked with the induction of early IgA, and to shed light on underlying mechanisms by which significantly higher and early IgA is induced against QARVLAVERY.

Old paradigms that assumed a passive role of innate B-cells are now being challenged by various models of infection, highlighting ways in which their function may be exploited as part of therapeutic strategies. In an influenza model, B1a cells were found to be the source of virus-reactive neutralizing IgM, the first study to show that B1a cells do not solely produce broad, non-specific responses but can generate Abs responsive to a specific virus antigen [417]. B1b cells have also shown some intriguing properties contrary to convention. Adaptive B-cell memory development is classically dependent on T-cells and develops after initial exposure to antigen in germinal centers. Unlike T-cell dependent antigens, T-cell independent antigens do not typically generate B-cell memory and are assumed to have short-term responses. A *B. hermsii* infection model showed that follicular B-cells were in fact dispensable for clearance whereas B1b cells, that were responsive to the pathogen, were sufficient for bacterial clearance and exhibited long-lasting T-cell independent memory, generating IgM only after bacterial exposure [418]. Further exploration of the alternative roles of innate B1a/B1b subsets and understanding of the mechanisms by which they may exhibit antigen-specificity would be especially intriguing for mucosal protection of HIV, where generating a quick local response in the absence of lymphoid involvement, bypassing inflammatory immune mechanisms would be highly ideal.

1.3. Concluding Statement

The cumulative data shown in chapters 2, 3 and 4 demonstrate that gp41 is indeed an effective immunogen that can elicit protective immunity against HIV. Furthermore, via strategic immunogen design and vaccine delivery regimens, the induction of mucosal epitope-specific Abs can be optimized. We propose that the development of mucosal vaccines will be a key component for effective prevention and control of HIV at the site of transmission itself, given that HIV is primarily contracted at mucosal surfaces. Most intriguingly, QARVLAVERY was found to be a unique and potent IgA inducer, which generated IgA capable of inhibiting HIV transcytosis *in vitro*, of both lab-adapted strains and novel T/F viral clones. Further investigation is still required for better characterization and a deeper understanding of innate and adaptive mechanisms modulating the mucosal environment of the genital tract. Such studies will be critical in highlighting the immune correlates of protection that are relevant to the mucosa and to develop specific strategies that exploit such responses. Recently, the roles of IgA and non-neutralizing Abs in HIV clearance have been highlighted in SIV models [379, 388, 389]. Indeed, these mechanisms were more significant in early and mucosal control of HIV compared to the previously assumed correlates of protection, cell-mediated immunity and nAbs. The MPER and QARVLAVERY epitopes have proven to elicit functional IgA against HIV in our studies, as well as functional non-neutralizing Abs in a recent simian study [379]. Thus, we propose that gp41 is a highly attractive immunogen that can be strategically utilized to induce Abs with multiple effector functions against HIV, with specific relevance to the genital mucosa.

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-- APPENDIX I --

GAG-SPECIFIC RESPONSES GENERATED AGAINST THE CHIMERIC VLPS IN OUR IMMUNIZATION MODEL

An advantage of utilizing the Gag-VLP platform to display the gp41 epitopes is that such a construct will elicit both Gag-specific cellular responses along with epitope-specific humoral responses. Here, gag-specific responses were measured in the splenocytes of mice immunized with the heterologous DNAx3 + rAdx2 regimen (refer to chapter 2). The results below show that significantly greater IFN-γ was produced by splenocytes of immunized mice after stimulation with gag-peptide pools, compared to stimulation with concanavalin A (ConA – an artificial, non-specific stimulant) or by a mock peptide.

Briefly, spleens were harvested from immunized mice and splenocytes were incubated in 96-well plates at $5x10^5$ cells/well. In triplicate for each condition, cells were stimulated with ConA (at 5ug/ml, positive control), mock peptide (5ug/well of HSV-2 gB peptide, negative control) or with a mixture of overlapping 15-mer gag peptides (10ug/well). The HIV-1 Consensus A Gag (15-mer) peptide set was obtained from the NIH AIDS Reference Reagent program and an equal parts mixture of peptides #7781 – 7816 was used to generate the peptide pool. Splenocytes were also isolated from non-immunized mice which were stimulated with the gag-peptide pool as described above. Supernatants were collected at

36 hours and used as neat samples in an IFN-ɣ ELISA (R&D Systems, Duo set) to determine IFN-ɣ levels which would indicate the presence of gag-specific lymphocytes in the spleen.

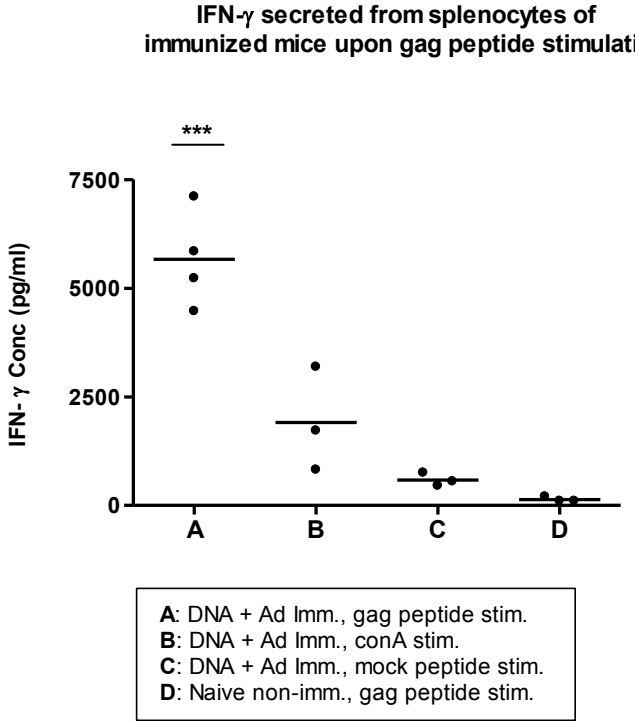

Figure 4. Gag-specific cellular responses induced in immunized mice upon stimulation with (A) gag peptide pool, (B) ConA and (C) mock peptide. (D) represents splenocytes from naïve mice stimulated with the gag peptide pool. Significance was determined by t-test, ***$p<0.001$

-- APPENDIX II --

INNATE B1B CELLS SPECIFICALLY RESPOND TO THE HIGHLY CONSERVED HIV-1 QARVLAVERY EPITOPE

The QARVLAVERY epitope exhibited a unique property in our chimeric VLP immunization model (refer to figure 6, chapter 2). Early epitope-specific IgA was observed systemically in mice administered the rAd vector via both the I.N. and I.P. routes within 7 days of the first immunization prior to a booster immunization and prior to epitope-specific IgG. As discussed in chapter 5, innate B1 cells can induce antigen-specific Abs and such responses are typically generated without the involvement of germinal centers and T-cell dependent mechanisms. Here, we have tested the hypothesis that the gp41 epitopes, in particular QARVLAVERY, can directly stimulate innate B1 cells in the peritoneal compartment of mice, and induce IgA in response to gp41 stimulation.

Briefly, C57Bl/6 mice were administered 75ug of purified peptide (ELLELDKWADL or KQLQARVLAVERYLK) with CpG (50ug) in a 75ul total volume into the peritoneal cavity (PerC). Intra-peritoneal (IP) flushes were obtained from mice on days 7, 11 and 13 and B-cell subsets were sorted using the strategy shown in figure 4, to obtain B1a, B1b or B2 subsets. The B-cell subsets were individually cultured on ELISPOT plates that were pre-coated with linear gp41 that had accessible MPER and QARVLAVERY regions (Genway). The B-cell subsets were incubated for 48 hours in these plates, after which they were developed using the

BCIP/NBT reagent to quantify the number of Ab-secreting cells (ASCs), particularly, IgM and IgA secreting cells. Preliminary experiments showed that CpG co-administration was required for the quantifiable detection of ASC and day 5 flushes did not consistently show epitope-reactive B-cell responses by ELISPOT. The marginal zone of spleens is also rich in B1 cells and in proximity to the site of peptide delivery. Therefore, splenocytes were also harvested, sorted and stimulated with gp41 in an ELISPOT assay to characterize innate B-cell responses in an alternative extrafollicular region.

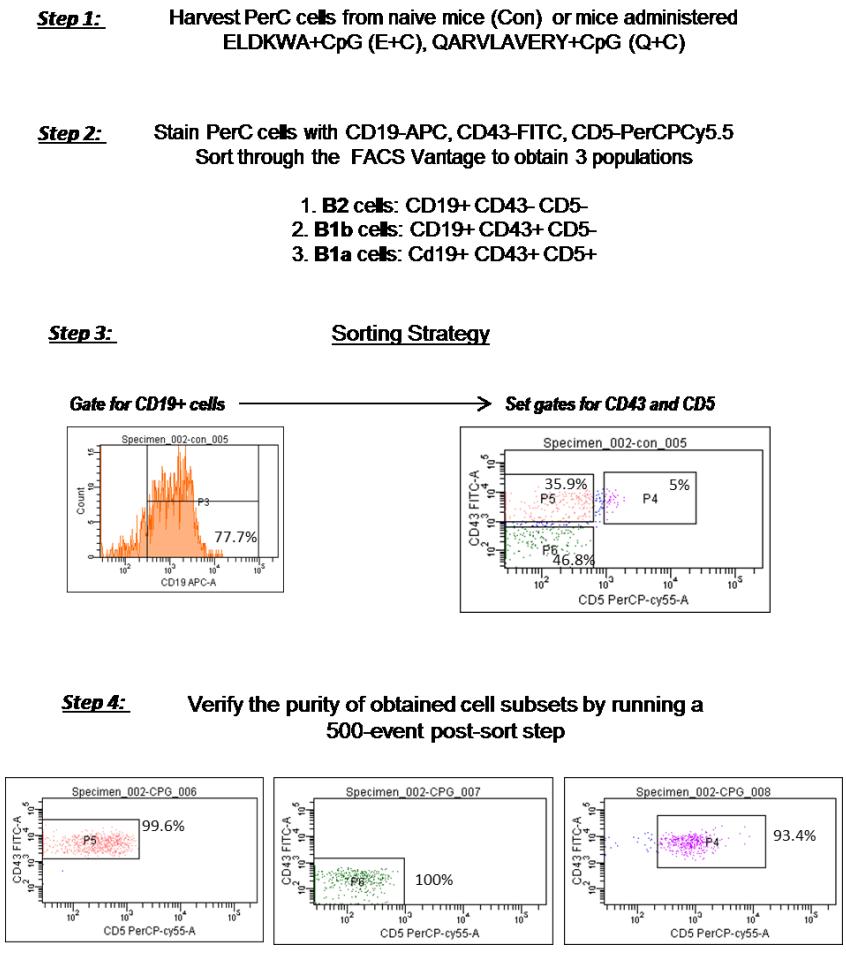

Figure 5. Strategy and method of purification of stained B-cell subsets by flow cytometry

The results in figure 5 show the number of IgA and IgM secreting spots obtained from the B1b cell subset from mice exposed to the ELDKWA and QARVLAVERY epitope IP, on day 11. The B1a subset only generated IgM spots on days 7, 11 and 13, with no significant differences in numbers (data not shown), whereas the B2 subset did not show significant IgM/IgA spots on any day (data not shown). However, as shown below in figure 6A, the IP-B1b subset secreted both IgA and IgM in response to gp41 stimulation. (Con) represents control ELISPOT wells were coated with a HSV-2 gB mock protein and (C) represents the number of spots generated upon gp41-specific stimulation of sorted B1b cells from mice that received CpG alone without peptide. Cultured B1b cells from splenocytes responded significantly less than IP-B1b cells to gp41 stimulation.

Interestingly, for the same number of cultured cells, significantly more IgA producing spots were formed in response to gp41, from mice that received QARVLAVERY+CpG compared to ELDKWA+CpG. This demonstrates, in an independent system, that QARVLAVERY is a significantly more potent stimulator of innate B1b cells and induces significantly greater IgA than ELDKWA. Mechanisms behind this observation however, are yet unknown.

In figure 5B, the phenomenon of direct stimulation of B1b cells to produce both IgM and IgA in response to QARVLAVERY was verified in an alternate strain of mice, Balcb/c. Figure 5C shows the total number of CD19+ cells in the IP flushes, which would include all 3 subsets, that respond to gp41 stimulation in both groups, receiving either ELDKWA+CpG or

QARVLAVERY+CpG. Significantly higher gp41-specific ASCs were detected in animals that received QARVLAVERY than ELDKWA, suggesting that there may be an unknown mechanism by which it specifically and uniquely stimulated B-cells of the peritoneal cavity. Understanding such mechanisms could delineate alternate ways of inducing early virus-specific Ab responses by vaccination.

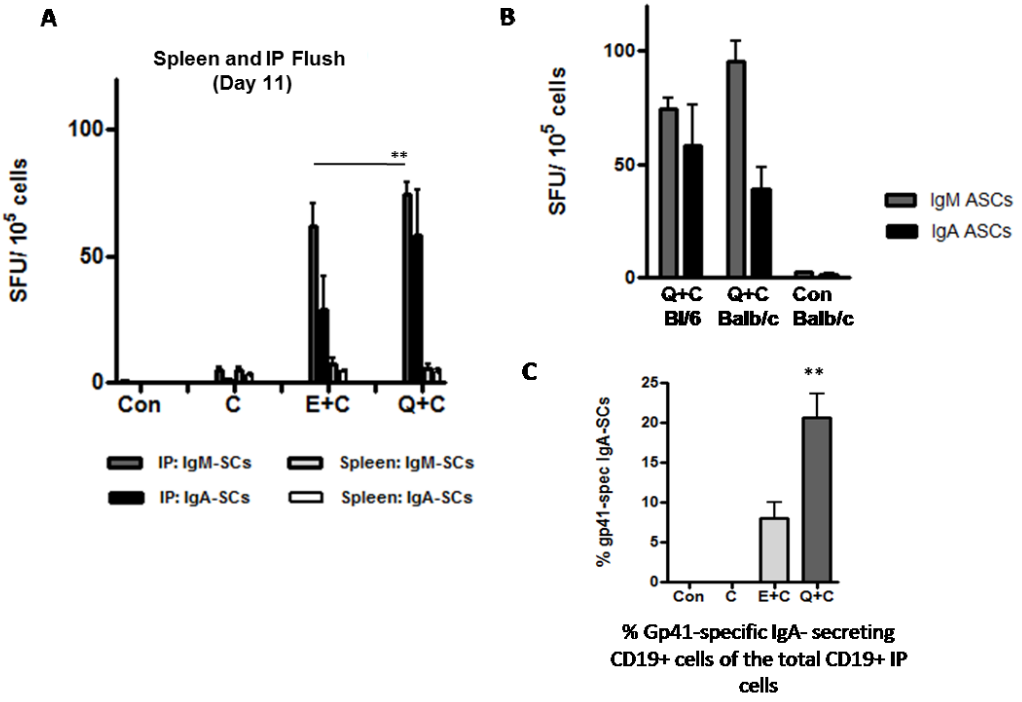

Figure 6. Quantification of the number of antibody-secreting cells generated in the ELISPOT assay upon gp41 stimulation, of the B1b subset obtained from the spleen or the PerC on day 11 after peptide delivery. (Con) represents cells that were added on control ELISPOT wells coated with a mock protein and (C) represents groups of mice that received CpG alone. Significance was determined by t-test, $^{**}p<0.01$

-- APPENDIX II --

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