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Glycomic Approaches to Understanding HIV-1 Budding in T cells

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Dedication

This dissertation is dedicated to my parents for their love, support and constant encouragement in every aspect of my life.

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Glycomic Approaches to Understanding HIV Budding in T cells

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The causative agent of AIDS (acquired immune deficiency syndrome), HIV (human immunodeficiency virus), is one of the most extensively studied pathogens in modern history. The virus has multiple mechanisms of persisting in the host including evading host immune response. Since HIV-1 depends heavily on the host machinery for various aspects of its life cycle, unraveling the complex interplay between the host and HIV-1 could provide new clues to therapeutic avenues.

In T cells, HIV assembles and subsequently buds through the plasma membrane incorporating host derived proteins and lipids in the viral envelope. HIV is thought to utilize a pre-existing mechanism for the budding of normal cellular vesicles called microvesicles to exit host cells. The evidence for this theory comes from reports of similarities between HIV and microvesicles observed for a small subset of proteins and lipids, leading to controversies about its validity. To further test this hypothesis, we utilized lectin microarrays to obtain a comprehensive glycomic profile of HIV and microvesicles derived from a panel of T cell lines. Glycosylation is critical to protein

sorting and has a crucial role in HIV-1 biology, making it an ideal marker to compare the particles and the host cell membrane.

We observed similar glycomic profiles for HIV-1 and microvesicles strongly suggesting an analogous mode of egress. Glycosylation of both particles seems to vary based on the parent cell line, providing additional evidence for this hypothesis. Microvesicles are involved in immune response modulation; hence the incorporation of microvesicular proteins could influence interactions of HIV with the immune system. The differences in glycosylation between these two particles could be potentially explained by the heavily glycosylated viral envelope glycoprotein. I also demonstrated that these vesicles bud from particular glycan enriched domains of the plasma membrane. Additionally, this work sheds light on the potential mode of interaction between galectin, an immune lectin and HIV-1. This work strongly argues for a conserved mechanism of exocytosis for both particles and sets the stage for examining the role of glycosylation in trafficking of proteins to the sites of microvesicular and viral budding.

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Chapter 1: Introduction

1.1 Overview

The prevention and cure of AIDS (acquired immunodeficiency syndrome) has emerged as one of the most important challenges facing scientists and the medical community today. This syndrome is caused by HIV (human immunodeficiency virus), which is notorious for its extraordinary ability to survive and replicate successfully when subjected to both robust immune responses and intensive therapy [1] . Many aspects of viral replication including its exploitation of the host machinery are still a mystery. The mechanisms of viral egress from infected cells constitute an important therapeutic intervention step as they could be targeted to prevent formation of new virions [2]. In spite of extensive research in this area, the exact mechanism of viral exit from its main target cells, the T lymphocytes, is still not fully understood. In particular, the theory that HIV uses the exocytic machinery utilized in the release of normal cellular vesicles called microvesicles [3] is still being strongly debated [4].

This dissertation focuses on the use of glycomics to shed light on several aspects of HIV biology. To this end, we have utilized glycosylation as a marker to obtain critical information about the exit machinery utilized by the virus, lending strong support to the theory that HIV uses a pre-existing host machinery to bud from the plasma membrane of T cells. In my introductory chapter, I will provide background on the life cycle of HIV-1 with emphasis on the assembly and budding of the virus in T cells. Additionally, I will summarize the current knowledge on microvesicles and on glycans as sorting determinants. This background provides context for my work involving the use of glycosylation as a marker to study microvesicles and HIV.

1.2 HIV-1

HIV was first identified in 1983 by Francosise Barre-Sinoussi and Luc Montagnier [5]. In the past 25 years since its discovery, AIDS has been responsible for over 25 million deaths. It is currently estimated that there are about 33 million people worldwide who are infected by the virus [6]. HIV mainly infects cells of the immune system, with T lymphocytes being the major target. The onset of full-blown AIDS is characterized by a significant reduction in number of T lymphocytes. This results in susceptibility to secondary infections leading to death [7].

The success of HIV as a pathogen is attributed to multiple factors including its ability to infect immune cells that are involved in fighting pathogens and the establishment of a latent reservoir in these cells [8]. Additionally, HIV is known to functionally impair both uninfected and infected cells of the immune system, resulting in a slow, but steady weakening of the immune system [9]. The virus mutates at an alarming rate and can thus effectively persist in the body in the face of strong immune responses and anti-retroviral therapy used to control disease progression. Although current treatment options cannot cure the disease, they can delay the onset of full-blown AIDS in infected individuals. This is primarily attributed to the fact that these drugs can reduce viral titers in the blood, but cannot completely eliminate the virus from the body. The cost and pitfalls of existing treatment strategies have emphasized the need for a vaccine in eradication of this disease [10]. Unfortunately, attempts to develop a vaccine for this disease have not been successful.

In spite of the significant advances that have been made in understanding both the basic biology of HIV-1 and its interactions with the host machinery, there are many aspects of host-virus interactions that are still relatively unknown. In order to effectively combat this disease, we need more insights into the viral replication cycle and the

mechanisms that HIV utilizes to escape immune surveillance [11]. This knowledge will provide us with the much-needed ammunition to fight the spread of this modern day epidemic.

Structure and replication of HIV

HIV is a member of the *Lentivirus* class of viruses which are a part of the *Retroviridae* family. Retroviruses are enveloped viruses with a RNA genome and replicate via a DNA intermediate in the host. Other members of the *Retroviridae* family include rous sarcoma virus (RSV), murine leukemia virus (MLV) and simian immunodeficiency virus (SIV), which is closely related to HIV [12]. There are two subtypes of HIV, HIV-1 and HIV-2, which are about 50% identical to each other. HIV-1 (referred to as HIV in this report) is the main causative agent of AIDS globally, while HIV-2 is mainly restricted to West Africa [13].

The genome of HIV consists of two single-stranded RNA molecules, which are 9.2 kb in length and encode structural proteins, enzymes, regulatory and accessory factors (Fig 1.1). The *gag* gene encodes for the Gag precursor protein, p55, which is cleaved by the viral protease to yield the CA, MA, NC and p6 proteins [14, 15]. The *env* gene is translated to a 160 KDa precursor protein, which is glycosylated by the host cell machinery. This precursor is cleaved by a cellular protease to produce the surface subunit (SU), gp120 and the transmembrane subunit (TM), gp41. The *pol* gene encodes for proteases, reverse transcriptase and integrase enzymes.

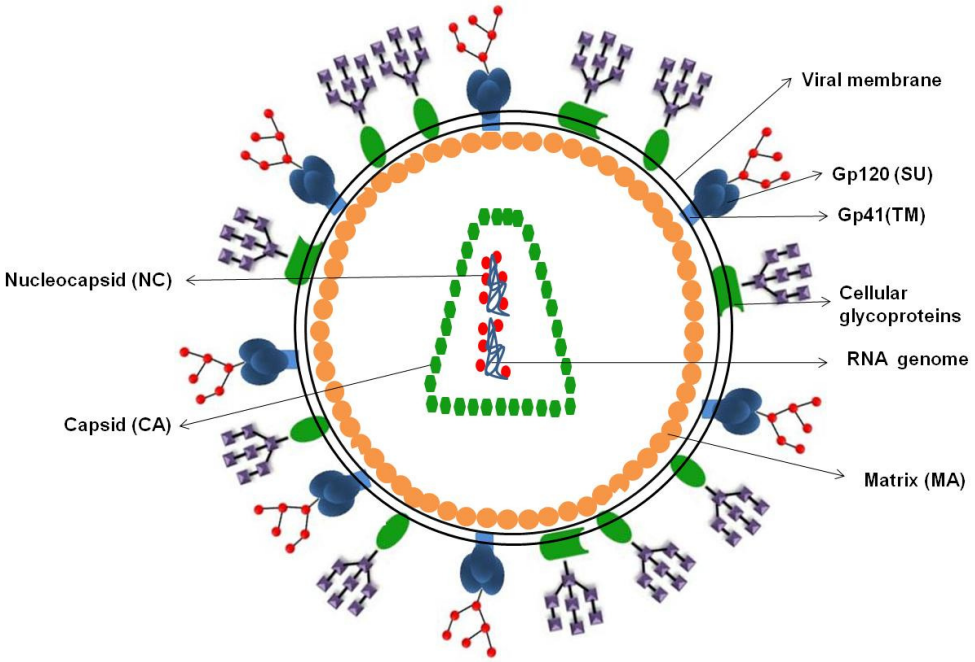


Figure 1.1. Schematic of structure of HIV. The viral envelope is derived from the host cell membrane and contains the envelope proteins, gp120 and gp41. The viral envelope is also coated with cellular glycoproteins. The mature HIV virion is about 100 nm and contains the viral genome which is encapsulated in a dense conical protein core. The RNA genome is tightly associated with the nucleocapsid proteins. The enzymes encoded by the viral genome are also found in the mature virion.

HIV infects cells of the immune system, namely dendritic cells, macrophages and T cells. Macrophages and T cells both function as sites of productive infection and thus are crucial to understanding mechanisms of viral dissemination [16]. The viral replication cycle begins with entry of the virus into the host cell followed by replication. The final step in viral replication is the assembly and egress of virus from host cells. The mechanisms of viral assembly and egress are currently not fully understood. HIV is heavily dependent on the host machinery for all aspects of its life cycle [17]. The involvement of about 250 host proteins in various stages of HIV life cycle was recently identified using a functional genomic screen [18], confirming the cadre of host proteins that are involved in this process. Given the intimate association between the host cell

machinery and HIV, the incorporation of wide array of host factors into the virus is not surprising [19]. Thus, understanding the complex interplay between HIV and host factors is crucial to controlling viral replication and could lead to new therapeutic avenues [6].

HIV entry and replication

Viral entry is initiated by binding of envelope glycoproteins to specific cellular receptors on the cell surface of immune cells [14]. T lymphocytes are major sites for HIV infection and formation of infectious virions [7]. Viral entry into CD4+ T cells is accomplished by two main mechanisms, fusion with the host cell plasma membrane and pH-dependent endocytosis [20]. The fusion of the viral envelope with the host plasma membrane is mediated by the envelope glycoproteins, gp120 and gp41, which interact with the T cell receptor CD4, and co-receptors CCR5 or CXCR4 respectively [14]. A less utilized mechanism involves the endocytosis of the virus by the host cell, followed by replication. This endocytosis is independent of the envelope proteins as HIV lacking the envelope protein can infect CD4- human epithelial cells and CD4+ T cells at low levels. This type of entry cannot be inhibited by neutralizing antibodies posing additional problems for effective control of viral replication by the host immune system [21, 22].

Following entry of the viral core, viral replication leads to production of viral proteins. The envelope glycoprotein, gp160, is extensively modified with glycans in the endoplasmic reticulum and Golgi by the host glycosylation machinery. These glycans are necessary for proper folding and transport of the envelope protein from the endoplasmic reticulum. Gp160 undergoes multimerization in the ER and cleavage by a host protease in the Golgi to form the subunits, gp120 and gp41 [15]. The assembly of the viral components, including proteins, and the RNA genome, into viral particles and budding from the cell membrane are the final steps in the production of infectious virions. The

budding and fission events necessary for generation of new virions are described in more detail in the next section [14, 19].

1.3 Assembly and budding of HIV in T cells

The proper assembly and budding of the virus is crucial to propagation of virions and subsequent infection of new target cells. Enveloped viruses like influenza, hepatitis C, herpes virus and HIV, are known to acquire their outer envelope by budding through the host cell membrane. Unlike non-enveloped viruses, which lyse their host cell during egress, the budding of enveloped viruses does not necessarily result in cell death contributing to regulated virus release [23]. These viruses have evolved to bud from the cell membrane through a succession of carefully coordinated budding events. The viral structural protein Gag must first be targeted to appropriate microdomains in the plasma membrane, followed by interaction of the viral machinery with the host machinery. The plasma membrane undergoes curvature at the site of budding followed by pinching of the membrane to allow the release of virus from infected cells [19]. The site of HIV budding in different cell types is still being heavily debated as there is evidence for budding of the virus into internal structures in some cells notably macrophages [24], while in other cells such as T lymphocytes, budding is known to occur exclusively at the plasma membrane [5].

HIV Gag is the main structural protein responsible for the formation of viral particles and is targeted to the plasma membrane for assembly of the virus in T cells [25]. The expression of Gag alone is sufficient to produce VLPs (virus like particles) [26]. This indicates that Gag can interact with components of the host machinery to facilitate budding and release of the virus. The Gag precursor, p55, is cleaved by viral protease to produce different polypeptides including matrix (MA), capsid (CA), nucleocapsid (NC)

domains. In addition, it is also cleaved to produce smaller proteins p6, p1 and p2. These domains have unique roles to play in the production of infectious virions.

The capsid domain is responsible for Gag-Gag multimerization, which influences membrane curvature at the budding site. The nucleocapsid domain is responsible for recruiting viral RNA to the site of budding. The matrix domain, MA, has a membrane targeting domain, and is modified with myristylation [19, 25]. The MA domain is responsible for binding to lipids, PIP2 (Phosphatidylinositol (4,5) bisphosphate) and PIP (Phosphatidylinositol phosphate). This binding is thought to be critical for targeting Gag to the cell membrane [27, 28]. Envelope glycoproteins are recruited to the membrane via interactions with the matrix domain (Fig 1.2). The matrix domain seems to be responsible for targeting viral assembly to distinct subcellular localizations such as the endosomal machimery or the plasma membrane. Thus, the interactions of Gag with the host cell machinery could be primarily responsible for determining the localization of budding [29].

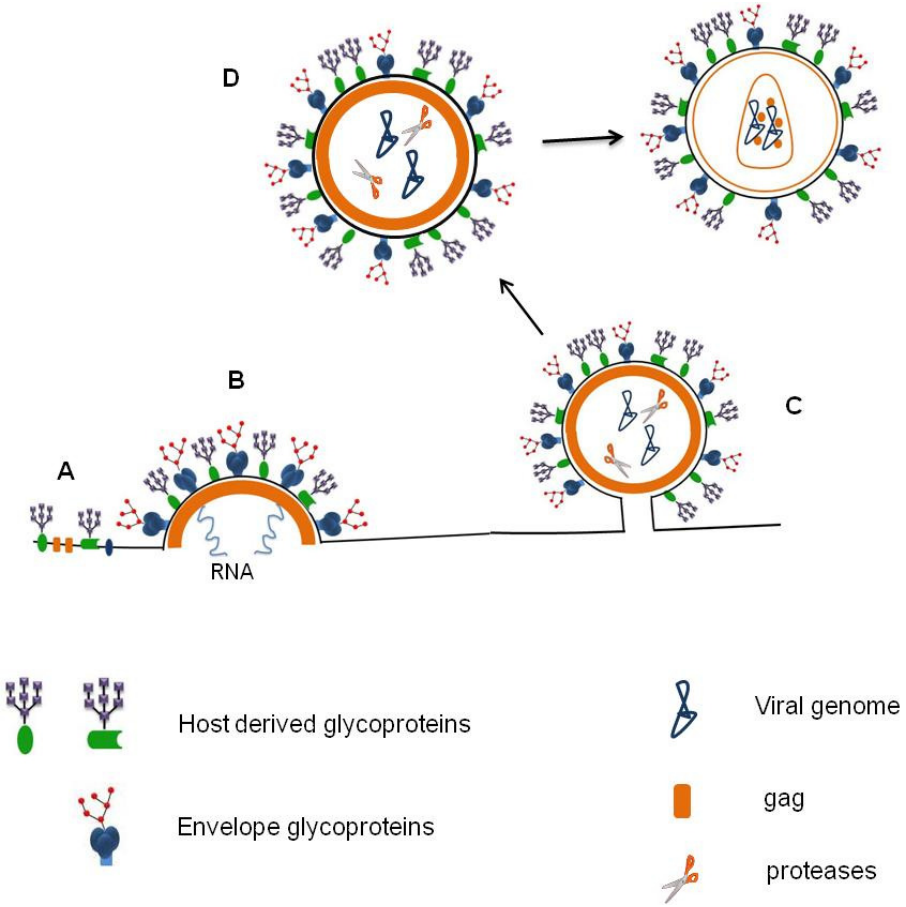


Figure 1.2 Assembly and budding of HIV in T cells. A) Viral Gag and envelope proteins are recruited to the plasma membrane. B) Oligomerization of Gag and its interaction with host proteins mediates membrane curvature. Gag recruits the rest of the viral machinery including RNA and proteins to the site of budding. The host membrane proteins, many of which are glycoproteins are also incorporated into HIV. C) Viral budding is completed and the virus is released from the host cell membrane by membrane fission. D) HIV undergoes further maturation to produce infectious virions.

The viral accessory protein, vpu, also plays a role in release of virus in a cell type dependent manner. Vpu has two major functions, enhancing degradation of CD4 in host cells and promoting release of progeny virions [30]. In T cells, it has been shown that vpu defective virons are not effectively released from the plasma membrane [31]. A recent study identified the cellular protein, tetherin as a vpu agonist in HeLa cells, a cell line in

which vpu expression is necessary for viral release [32]. Apart from these viral factors, various host components are also known to be involved in viral release.

Gag is primarily responsible for interacting with host proteins to mediate viral budding [25]. In particular, the p6 protein (also called the late domain or L domain) has a PTAP sequence that mediates interactions with cellular factors involved in the vacuolar protein sorting system. The L domain interacts with factors are members of the ESCRT family of proteins (endosomal sorting complexes required for transport) such as tsg101 (tumor susceptibility gene) [33] and Alix [34] for efficient assembly and release from certain cells. Reduced expression of tsg101 in 293T human embryonic kidney cells blocked the release of virus particles from the plasma membrane, highlighting the role of these factors in viral life cycle [35].

Proteins belonging to the ESCRT family are involved in the formation of multi vesicular bodies (MVBs). MVBs are formed from the late endosomes by inward budding of the endosomal membrane into the lumen of the endosome, resulting in the formation of intra luminal vesicles. These vesicles are then released into the extracellular milieu by fusing with the plasma membrane and are called exosomes or microvesicles [36]. This process is analogous to outward budding of the virus at the plasma membrane and thus similar machineries are thought to be involved in both processes. This mechanism is thought to be involved in HIV biogenesis in both macrophages and T cells. The budding of HIV from macrophages was previously shown to be mediated by the endosomal pathway [37]. However, recent reports have indicated that the plasma membrane may be sites of biogenesis in macrophages as well [38]. Thus, the mechanisms of viral budding

in the two cell types that are productively infected by HIV i.e macrophages and T cells are still relatively unknown [16].

The plasma membrane is the site of budding in T cells and viral budding seems to occur at specific microdomains [39, 40]. The specificity of incorporation of some proteins in HIV strongly argues for budding at specialized sites in the plasma membrane rather than random budding events [40-42]. There are two current models of HIV biogenesis, both of which are based on the involvement of specific microdomains [14]. These are the microvesicular sites/tetraspanin enriched microdomains (TEM) model [41] [40] and lipid raft model [39] (Fig 1.3). Whether these microdomains are separate entities that can both be utilized by the virus or these domains interact with each other in the context of HIV budding is still unknown. The targeting of the viral machinery especially Gag to these microdomains has been observed to both domains [39, 40].

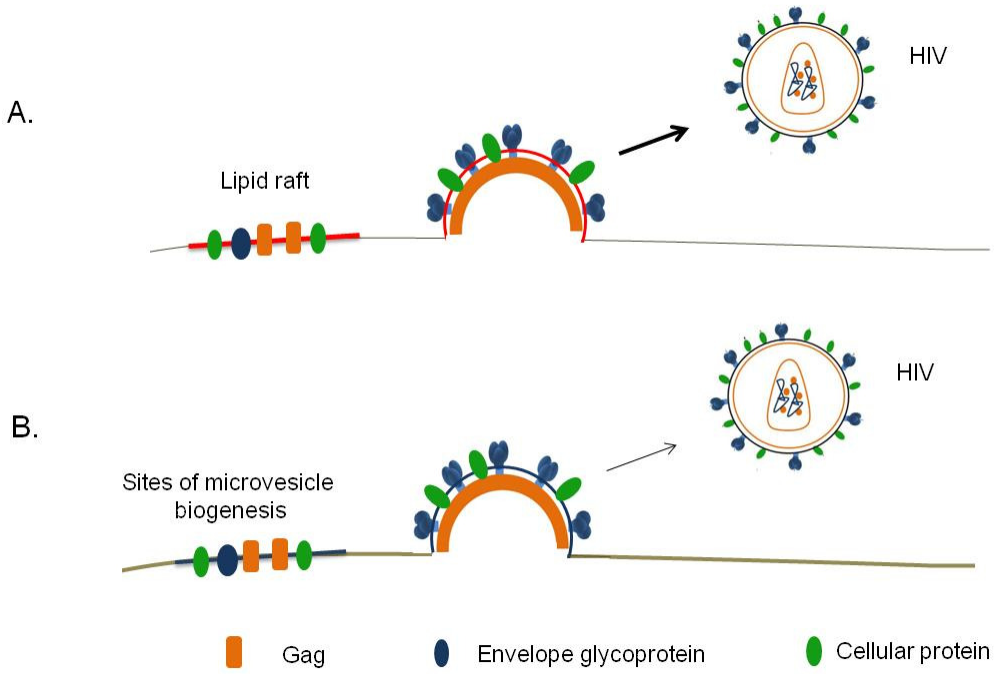


Figure 1.3.Models of HIV budding from T cells. The viral structural protein, Gag, is targeted to microdomains on the plasma membrane. These microdomains could be either sites of A) lipid raft regions (B) sites of microvesicular biogenesis.

Models of HIV budding

Lipid raft model

Lipid rafts are unique membrane microdomains that have differential lipid and protein composition from the rest of the membrane and are involved in signaling events [43]. There is considerable data to implicate lipid raft regions in the egress of HIV from host T cells. The incorporation of specific lipid raft protein markers Thy-1, CD59 and the lipid GM1 in HIV provided the first line of evidence for lipid raft association of HIV. The co-localization of these markers with viral proteins was shown to occur in specific regions of the membrane. Viral proteins were also shown to co-localize with a fluorescent

lipid analogue that partitioned specifically to lipid rafts in these cells. Moreover, majority of myristylated Gag was found to concentrate in lipid raft regions that were isolated using Triton-X-100 followed by sucrose density centrifugation [39].

Subsequent work has strengthened the proposed role of lipid rafts in HIV budding by providing several pieces of evidence. These include the association of Gag with detergent resistant membranes [44-48], though some discrepancies have been observed [49] and similarities in lipid compositions of HIV and lipid rafts [50, 51]. The key role played by cholesterol in HIV budding has been used as additional evidence for the role of lipid rafts [52-54]. Moreover, lipid rafts regions are also crucial for transmission of HIV through the virological synapse in regions of T cell contacts [55]. A detailed discussion of this model is provided in chapter 4.

Microvesicular model of HIV biogenesis

The microvesicular model for HIV release from T cells has been proposed by several groups [56] [57], but the idea gained momentum after the Trojan exosome hypothesis was proposed by Hildreth and colleagues in 2003 [3]. This model attempted to explain some unique aspects of HIV biology as a byproduct of usurping the microvesicular pathway. These included proteomic and lipidomic similarities observed between HIV and microvesicles, envelope independent entry of HIV into cells and certain aspects of the immune evasive capacity of HIV. The use of a pre-existing microvesicular pathway would provide HIV an armor of cellular proteins normally found in microvesicles, allowing HIV to masquerade as a pathogenic microvesicle [3].

The microvesicular biogenesis theory has been proposed as a mechanism for viral release in both T cells and macrophages [3, 16, 37]. Prior evidence for this theory comes from the comparison of a limited set of protein markers (less than 10 proteins) in both microvesicles and HIV derived from T cells [40, 56, 58]. More evidence for this theory came from work that demonstrated the localization of a microvesicular lipid marker, N-Rh-PE with HIV Gag in T lymphocytes. In addition, N-Rh-PE was sorted into both microvesicles and Gag VLPs suggesting a common origin for these particles [40]. Several of the proteins enriched in both HIV and microvesicles belong to the tetraspanin family of proteins, prompting the suggestion that the TEMs function as sites of microvesicular biogenesis [16].

The microvesicular theory of HIV biogenesis is controversial, with many prominent experts questioning the validity of this hypothesis [42, 59]. Minor differences in protein and lipid composition between the two particles are cited as proof for the existence of different pathways for both particles [4, 60]. A detailed discussion on the controversial aspects of this theory is presented in Chapter 2.

In view of the existing evidence for the involvement of both microvesicular sites and lipid raft regions in HIV biogenesis, the mutual exclusivity of their contributions to HIV biogenesis has not been examined. These two domains could function independently in HIV biogenesis. On the other hand, it is quite possible that a particular subset of lipid rafts could serve as sites of assembly during the processes of microvesicular and HIV biogenesis. Thus, a better definition of the compositions of both these microdomains would shed light on HIV and microvesicular budding. Furthermore, the evidences that supports the lipid raft model of HIV budding as well as the microvesicle model of viral

biogenesis warrants further investigation into any links between these plausible mechanisms. The contributions of this understudied area of research could be significant in understanding viral budding. Considering that the surface molecules of the viral envelope could directly influence immune responses to HIV, this area of research could result in therapeutic targets aimed at both inhibiting its replication and undermining the immune evasive capabilities of HIV. It is important to understand the origin and functions of microvesicles for a better appreciation of the involvement of this pathway in HIV biogenesis. In addition, the role of microvesicles in wide variety of cellular processes and diseases necessitates a thorough understanding of its biogenic modes.

1.4. Microvesicles

Microvesicles are small membrane bound vesicles that can shuttle biomolecules between cells, thus emerging as crucial players in the field of cell-cell communication. These vesicles primarily mediate transfer of proteins and RNA between cells. Microvesicles are released by a wide variety of cell types including cells of the immune system (T lymphocytes, B cells, dendritic cells and macrophages), tumor cells, and epithelial cells among others. These are also commonly found in body fluids including blood, urine, and breast milk. Microvesicles are heterogenous in size often ranging from 0.03-1 μm and are typically isolated from cell culture supernatants [61]. They were first discovered by Rose Johnstone who isolated them from sheep reticulocytes. These vesicles were enriched in proteins that were eliminated from the cell during reticulocyte maturation [62].

The nomenclature of these membrane vesicles is somewhat controversial and the terms membrane microparticles, microvesicles, exosomes and ectosomes are used to refer to the vesicles. These terms are sometimes used interchangeably in literature, while others use these terms more specifically. For instance, microvesicles and membrane microparticles are used to describe vesicles that are derived from the plasma membrane, while the term exosome is commonly used to refer to 30-100 nm vesicles that originate from the late endosome [61]. Recent reports also indicate the existence of plasma membrane derived exosomes, to indicate similarities between these vesicles and classical endosomally derived vesicles [40]. The term microvesicle used in this thesis refers to membrane microvesicles encompassing all of the above particles.

Although the biological functions of microvesicles are still largely unknown, there has been renewal of interest in these bioactive vesicles due to their role in various disease states such as cancer, retroviral infections, and prion diseases. Microvesicles are involved in a range of biological functions such as development, immune activation and suppression, and tumor biology [61, 63]. Antigen presenting cells like dendritic cells [64] and EBV-transformed B cells release microvesicles that can activate T cells [65]. Mast cells are known to release microvesicles that can activate B and T cells [66]. Dendritic cells secrete microvesicles which are known to stimulate anti-tumor responses [65]. Microvesicles secreted by T cells could carry the Fas ligand which induces apoptosis in target cells. Thus, these microvesicles possess important immune modulation properties [63].

Microvesicles are characterized by the presence of certain proteins that have been found in almost all preparations, suggesting the existence of common biogenesis

mechanism among different cell types. The shared protein markers include tetraspanin proteins like CD63, CD81, cytoskeletal proteins, metabolic enzymes, and proteins involved in membrane fusion and trafficking [67]. Their cargo is often reflective of the host cell from which they are derived and thus they contain some cell-type specific protein markers. For example, microvesicles isolated from T-cells include HLA class II molecules while microvesicles from ovarian and breast cancer cells contain receptor protein kinase, Erb2. Thus, the biological activities of these microvesicles are determined by their molecular composition, which is dependent on the cell of origin [61]. Although, it is not clear at the present time what homes these particles to their respective target cells, it has been shown that there is some selectivity in this process. This is most likely influenced by the molecular cargo present on the microvesicular surface [68].

The currently accepted model for microvesicular biogenesis begins with endocytosis at the cell surface resulting in the formation of early endosomes. The early endosomes further mature into late endosomes after acidification. Multi vesicular bodies (MVBs) are formed from the late endosomes by inward budding of the endosomal membrane into the lumen of the endosome. MVBs can further fuse with lysosomes resulting in the degradation of its constituents. Alternatively, they can fuse with the plasma membrane resulting in release of these vesicles into the extracellular milieu [69]. Microvesicular biogenesis is also known to occur in the plasma membrane of certain cell types such as T cells [56] [58] . The hijacking of this pathway for budding and exit from the cell would be an ideal route for viruses in general including retroviruses. This provides them with an excellent exit strategy, given that these viruses typically have very small genomes and have to rely extensively on the host machinery for every aspect of

their life cycle [3]. Utilizing this pathway would also allow HIV to incorporate proteins which could influence the interaction of the virus with the host immune system.

1.5 Immune evasion by HIV

The unique ability of HIV to escape the immune system in the face of strong immune response generated by the body has thwarted any attempts so far to successfully eliminate the virus from the body of an infected individual. The failure of a recent HIV vaccine trial has necessitated a thorough understanding of the mechanisms of HIV infection and immune evasion before more clinical trials can be conducted [70]. Some key unique features of HIV infection distinguish it from other viral infections to which vaccines have been successfully produced. These include the enormous genetic diversity of the virus and the latency of infection which seem to maintain a reservoir for viral replication. Considering that the main targets of HIV are the immune cells, it is not surprising that the virus has had tremendous success in persisting in infected individuals [6]. The virus also mutates at a rapid rate allowing it to escape from anti-retroviral therapy as well as from constant immune pressure [71]. Apart from these, the virus has multiple immune avoidance mechanisms including down regulation of MHC class II molecules (the proteins that required for recognition and targeting of infected cells by other T cells), masking of epitopes on envelope glycoproteins by both mutations and modulation of glycans on envelope glycoproteins [6].

Several host derived proteins enhance infectivity of the virus or play a role in HIV pathogenesis by influencing its interaction with the host immune system. Host derived proteins on the viral envelope such as ICAM-1 play a role in enhancing infectivity of the virus, while HLA-class II (human leukocyte antigen) proteins play a role in down-modulation of immune responses to the viral infection [42]. Lipid rafts marker proteins

like CD55 and CD59, which are enriched in HIV, are known to prevent complement mediated lysis of the virus [72]. MHC class I and class II proteins on the surface of infectious virions can trigger apoptosis in T cells, thereby reducing effective cellular immunity in response to HIV. Interestingly, all of these proteins are also known to be enriched in microvesicles from T cells [57]. The role of these proteins in HIV pathogenesis stresses the need for understanding the contributions of the microvesicular pathway to HIV biogenesis.

The genetic diversity in HIV also provides it with the unique ability to survive the strong cellular and humoral immune responses that occur during the course of infection. Cellular immunity involves the proliferation of cytotoxic T lymphocytes which recognize and destroy cells infected with HIV [73]. Humoral immunity is mediated by neutralizing antibodies which are generated in response to specific antigens [74]. The function of a neutralizing antibody is to bind infectious virions and effectively prevent its entry into target cells. One of the major problems with generation of effective neutralizing antibodies to HIV arises from the structure of the envelope glycoprotein, gp120. The gp120 and gp41 proteins are found on the viral surface as homotrimers. Since the epitopes that are targeted by the neutralizing antibody are generated against the monomeric protein, most of the epitopes that the antibody recognizes are structurally masked by the conformation of the gp120 trimer [75].

In addition, the glycosylation of gp120 is known to influence the ability of neutralizing antibodies to bind HIV. Gp120 is a heavily glycosylated protein with carbohydrates accounting for about 50% of its molecular weight. Gp120 contains about 18-30 potential sites for *N*-linked glycosylation depending on the strain of the virus and the host that is used for viral propagation. *N*-linked glycosylation of proteins occurs on Asparagines in the consensus sequence Asn-X-Thr [76, 77]. Gp120 is also known to

contain O-linked sugars which occurs on Ser-Thr repeat regions in proteins [78]. Thus, on an average, gp120 contains about 24 N-linked sites and an as yet unknown number of O-linked sites. Out of the 24 N-linked sites, about 13 of those are modified with complex N-linked sugars and the other 11 are a mixture of hybrid and high mannose structures [76, 79]. An overview of representative N-linked and O-linked structures is given in Figure 1.4. All the N-Linked structures share the trimannosyl core and vary with respect to substitutions on the core mannose residues. High mannose structures contain terminal α-1,2 mannose residues, while complex N-linked structures can vary considerably based on substituents. Some of the potential linkages found in complex structures include poly LacNAc (Gal-β-1,4-GlcNAc) , sialic acid linked to the trimannosyl core. Hybrid N-linked contain features of both high mannose and complex sugars and hence contain terminal α-1,3 mannose and α-1,6 mannose residues [80].

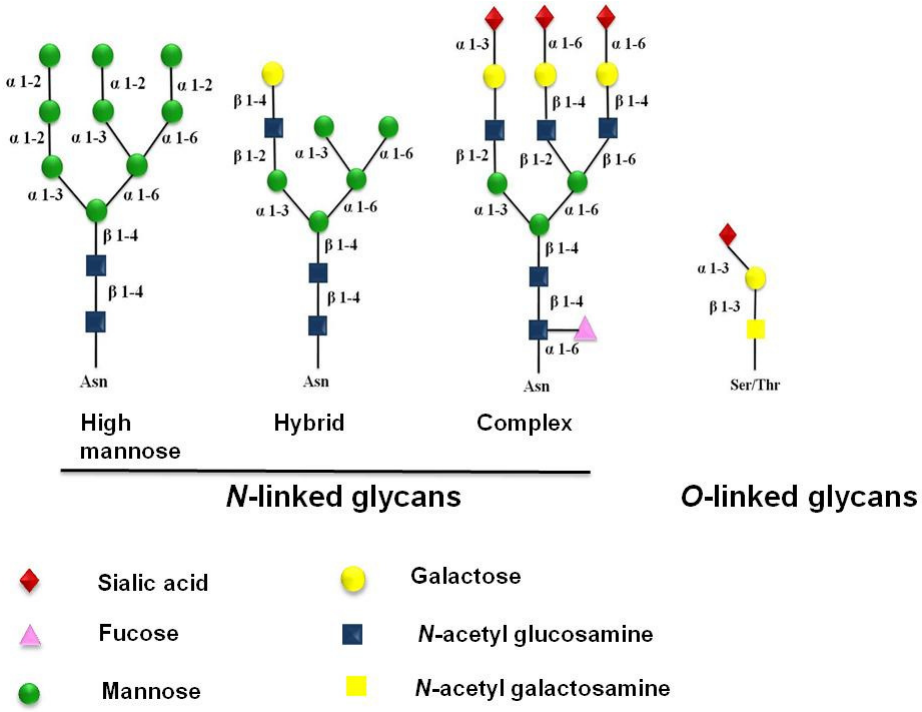


Figure 1.4. Representative structures of various carbohydrate modifications found in gp120. The *N*- linked glycans include high mannose, hybrid and complex structures. The high mannose structures contain terminal α-1,2-mannose residues. The hybrid and complex structures consist of various diversifications of the trimannosyl core. Gp120 is also modified with several O-linked glycans.

The role of these glycans in HIV immune evasion has been studied extensively. The high mannose glycans in gp120 are found in a cluster and the sites that are modified with high mannose are relatively more conserved than the other glycosylation sites. It was first demonstrated that HIV can modulate the glycans on gp120 by mutations at the consensus sites, resulting in deletions of glycosylation sites. These changes in position of the glycans can mask the virus from neutralizing antibodies that were generated in response to the earlier versions of gp120. This hypothesis is referred to as the glycan shield theory which states that HIV rapidly changes the glycans on gp120 in response to selective pressure exerted by neutralizing antibodies, effectively using the glycans on gp120 to shield itself from recognition by antibodies [81, 82].

The glycans on gp120 are known to exist in an unusual cluster of oligomannose structures which are not commonly found on the host cell surface. This cluster is specifically recognized by the antibody, 2G12, which is known to neutralize HIV entry into cells [83]. Unfortunately, the neutralizing ability of 2G12 along with other antibodies is confined to certain lab strains of HIV and not primary isolates. The genetic diversity of this virus is a major hindrance to the neutralizing antibody approach as changes in carbohydrate coat of the virus could render neutralizing antibodies ineffective [84].

The unusual cluster of high mannose and the neutralizing ability of 2G12 antibody have brought to forefront the potential of carbohydrate binding agents to function as anti-viral agents [79]. Carbohydrate binding proteins called lectins have been used extensively to study glycosylation of proteins and lipids. In the context of HIV, the role of few antiviral plant and bacterial lectins has been examined closely as an alternative to the neutralizing antibody strategy. These antiviral lectins include plant lectins like GNA, ConA, HHL and bacterial lectins like Cyanovirin, Scytovirin and griffithssin share a common affinity for mannose residues. The specificity of these lectins for mannose residues is varied. For example, Con A binds all mannose residues while CVN is specific for α-1,2-mannose residues. These antiviral lectins have been shown to bind gp120 in the context of HIV [85, 86].

The exposure of HIV to selective pressure of these antiviral lectins has been shown to lead to deletions in glycosylation sites. The use of carbohydrate binding agents to neutralize HIV has several advantages. These include the high genetic barrier needed to develop resistance to them (greater than 5 mutations), no cross resistance to other anti-viral drugs being used, ability of these agents to reduce cell to cell transmission as well as cell free transmission of the HIV. The deletions in carbohydrates epitopes upon exposure to the anti-viral agents could result in exposure of sites that might be recognized by

neutralizing antibodies [87]. Thus, the carbohydrate-binding agents constitute a novel class of anti-viral agents that might be used in conjunction with other therapies. However the systemic use of these lectins may result in immune reactions to these foreign proteins. Hence, the use of these lectins in microbicides as prophylactics rather than systemic drugs is currently being pursued [85]. Given the importance of glycans in HIV biology, a detailed analysis of the glycans on HIV and microvesicles could yield valuable information about the biology of HIV. The glycans on HIV and microvesicles could also provide clues about the trafficking of proteins into these particles, as glycans are known protein sorting determinants.

1.6 Glycans in trafficking

The role of glycosylation in protein trafficking has been well studied in classical secretion pathways as well as targeting of proteins to specific sites. Glycosylation of proteins with *N*-linked glycans plays a critical role in protein folding and transport through the endoplasmic reticulum into the Golgi network [88]. Proteins are further modified extensively with glycans in the Golgi network before being transported to their destinations inside and outside the cell. The transport of lysosomal resident proteins into lysosomes requires the mannose-6-phosphate moiety, one of the best characterized glycans in trafficking. This is recognized by the mannose-6-phosphate receptor which is present in trans Golgi network and enables the specific targeting of these proteins to lysosomes [89].

The role of both N-glycans and O-glycans in protein trafficking has been studied extensively in polarized epithelial cells. Epithelial cells have apical and basolateral cell surfaces which require targeting of specific proteins to either surface. Rat growth hormone (rGH) in Madin-Darby Canine Kidney cells (MDCK) was targeted to the apical

surface upon addition of N-linked glycans and subsequently secreted from this surface [90] [91]. Addition of N-glycans was also shown to be sufficient to mediate apical targeting and secretion of three different proteins in MDCK cells [92]. Removal of a single N-glycan is known to drastically alter the trafficking of the protein, membrane dipeptidase [93].

O-glycans can also function as apical targeting tags for secretory and transmembrane proteins. Inhibition of O-glycosylation reduces apical targeting of glycoproteins such as MUC1 and CEA (carcinoembryonic antigen) [94]. In addition O-glycans have been shown to function as sorting signals in yeast for the protein, Fus1p. The protein accumulates in the Golgi in the absence of O-glycosylation, instead of being targeted to the cell surface [95]. The role of N-linked and O-linked glycans in the targeting of proteins may be due to multiple mechanisms. The glycans could facilitate the interaction of these proteins with lectins, which then delivers the protein to the appropriate location. The glycans could also affect conformational changes in the protein allowing them to interact with different partners to facilitate their transport. At present, it is not clear which of the mechanisms is at play or if it is a combination of both mechanisms [96]. The proposed role of glycans in trafficking makes them an attractive candidate for evaluating sorting mechanisms of glycoproteins into exocytic particles.

The remainder of my thesis focuses on utilizing glycomics as a tool to probe the relationship between HIV and microvesicles and to shed light on question of HIV biogenesis. Subsequent chapters focus on other aspects of HIV biology including its potential mode of interaction with the immune system lectin, galectin-1 and the glycomic characterization of lipid raft regions. The final chapter summarizes the controversies associated with the microvesicular theory and the contributions of our system based

glycomic approach in support of this theory as well other novel aspects of HIV and microvesicular biogenesis that were uncovered using this approach.

Chapter 2: HIV and microvesicles derived from T cells share glycomic profiles

2.1 Introduction

Overview

The budding of HIV is intimately associated with the host cell machinery and incorporates a wide variety of host cell proteins both on its viral envelope as well as inside the virions [97]. The observation that HIV and microvesicles share select protein markers led to the theory that HIV and microvesicles bud from similar microdomains and utilize the same machinery for egress. Even though, this theory was first proposed in 1997 by Sattentau and co-workers [56], followed by Esser *et al.,* [57], it has gained prominence only in recent years after the proposal of the Trojan exosome hypothesis [3]. The sharing of exocytic machinery would result in the incorporation of microvesicular proteins in the viral envelope. The role of microvesicles in immune modulation is not clear yet, but current data suggests important roles in immune system processes. Given the emerging roles of microvesicles in immune responses, the usurping of this biosynthetic pathway could influence the interactions of HIV with the host immune system and contribute to immune evasion by HIV.

Evidences for the microvesicular theory of HIV biogenesis

The microvesicular theory of HIV biogenesis originates in the observation that cellular vesicles were often found to contaminate HIV preparations. This was reported in 1997 by two independent groups, when they discovered that these contaminating

microvesicles bud from the plasma membrane of T cells [56, 58]. Since it was known that HIV also buds from the plasma membrane of T cells, it led these groups to hypothesize that there could be a common mechanism for particle exit that may be utilized by both particles. Upon isolation of cell culture supernatants from infected and uninfected cells, they observed the presence of cellular vesicles in both supernatants, revealing that these vesicles are released by cells under regular growth conditions. Closer examination of transmission electron micrographs of infected cells indicated that both particles bud from overlapping regions of the plasma membrane. In addition, both HIV and microvesicles contained increased amounts of protein markers such as CD5, HLA-DR, CD30 and CD44 in comparison to the parent cell. Interestingly, they also noticed differences such as enrichment of CD63 and CD43 in the virus alone [56]. They suggested that these vesicles could be similar to the immunologically relevant microvesicles released by B cells [98].

Bess and colleagues also reported similar observations when they detected cellular material in viral preparations. These microvesicles shared some similarities with the virus including presence of HLA-DR [58]. Further studies yielded more similarities between microvesicles and HIV in terms of protein composition. These included both inclusion and exclusion of common proteins in these particles relative to the cell membrane they were derived from. Proteins that were enriched in both these particles included immunologically relevant molecules such as MHC-class I and II, CD86, while CD80 was excluded by both particles. CD45 was identified as a protein that was excluded from HIV but not microvesicles. Thus, with the exception of CD45, proteomic similarities between HIV and microvesicles were observed. This led the authors to propose that HIV could be using the microvesicle exit mechanism to egress host cells. It

was also proposed that these microvesicular proteins could have a role in the pathogenesis of HIV [57]. The pathogenesis of HIV relies on the loss of function as well the decline in number CD4+ positive T cells with disease progression [9]. MHC class I and II molecules and CD86 could bind to T cell receptors (TCR) and trigger T cell apoptosis, thereby contributing to declining T cell levels. Usurping this pathway enables the incorporation of these immune modulatory proteins in the viral envelope, and therefore could further the pathogenesis of HIV [99].

As discussed previously (chapter 1, section 1.3), Hildreth and colleagues put forth the Trojan exosome hypothesis which states that retroviruses including HIV exploit the microvesicular biogenesis pathway to exit cells. The theory emphasized that HIV is essentially a pathogenic microvesicle and uses this disguise to further its infectivity and pathogenesis [3]. Subsequent studies revealed the enrichment of CD63 and CD81 in microvesicles isolated from T cells. Using HIV Gag VLPs as a model system, it was shown that these particles also demonstrated similar enrichments [40]. Additional evidence comes from the fact that proteins that are normally not sorted to microvesicles were shown to be secreted in microvesicles upon oligomerization. These oligomerized proteins were also sorted to HIV Gag VLPs, further strengthening the hypothesis that HIV and microvesicles share exocytic mechanisms [100]. Moreover, based on co-localization with a microvesicle lipid marker, N-Rh-PE, with the proteins CD63 and CD81, it was evident that microvesicles bud from specific domains of the plasma membrane. Fluorescence microscopy analysis of T cells expressing Gag showed the co-localization of HIV Gag with the microvesicle lipid marker. These cells also sorted the fluorescent lipid into secreted microvesicles and HIV Gag VLPs. Even though this

observation was confirmed using multiple methods such as western blot analysis, fluorescence and electron microscopy, there was no comparison to authentic HIV [40]. Although, this work lent great support to the microvesicular biogenesis theory, it is yet to be proven in the context of HIV virions. The authors of this seminal paper conclude with the following statement. “Although many predictions of the Trojan exosome hypothesis remain to be tested, the available data indicate that it is at least a viable, parsimonious hypothesis of retroviral biogenesis” [40].

Evidences against the microvesicular theory of HIV biogenesis

The microvesicular model of HIV biogenesis is still an active area of debate as shown by two papers published this year, which dispute the validity of this theory. In addition to enrichments in similar proteins, the lack of CD45 in the virions and Gag VLPs was used as evidence in favor of the microvesicular model [40]. Recent work by Ott and colleagues examining the presence of CD45 in microvesicles produced contradictory results. Their experiments revealed that all microvesicular populations contain CD45, while CD45 is excluded from virions suggesting that the mechanisms of viral and microvesicular biogenesis are different [4]. This interpretation does not account for other proteins that are enriched and excluded by both microvesicles and HIV and instead relies on this one protein marker to disprove the microvesicular model. It is possible that certain proteins may be differentially present in either particle due to specific protein-protein interactions mediated by the viral components. Thus, further studies are needed before results based on presence or absence of single protein or lipid species can be correctly interpreted.

A recent study of lipid compositions of HIV derived from H9 cell line and HIV and microvesicles derived from macrophages showed that the HIV samples and microvesicles have very similar lipid profiles displaying enrichments in several lipid species including ceramides, GM3, and cholesterol. The key difference between the microvesicles and HIV derived from both infected macrophages and H9 was in the levels of the lipids, PIP2 and PIP, with significant enrichments in HIV alone. The matrix domain of Gag is responsible for binding to these lipids and deletion in this domain resulted in incorporation of similar levels of PIP and PIP2 in both HIV and microvesicular particles. The authors use this difference as evidence in favor of the theory that HIV and microvesicles do not share an exocytic pathway. The simple explanation for this observation would be that microvesicles lack Gag and hence do not accumulate these particular lipids [60]. Even though the authors claim otherwise, however based on the overall similarities in lipid species, it seems likely that HIV and microvesicles share exit mechanisms.

The proteomic evidences that links HIV and microvesicular biogenesis in T cells are based on select panel of proteins (~10) and lipid species. Mechanisms of microvesicle biogenesis in T cells are yet to be elucidated and the sorting signals that recruit proteins to these microdomains are currently being investigated. Ubiquitination of proteins has been shown to target proteins into microvesicles [101]. In addition, ubiquitin is known to play a role in HIV egress from cells [102]. Higher order oligomerization of proteins is another factor that may be involved in the targeting of these proteins to the sites of microvesicle biogenesis [100, 103]. Given that glycosylation of proteins plays a critical role in the targeting and sorting of proteins, it seems likely that glycans could also

function as sorting determinants to these particles. It is also known that interactions of proteins and lectins like the immune lectin, galectin-1 could lead to protein clustering [104]. Thus, exploring the roles of glycans in this sorting mechanism could yield new insights into microvesicular biogenesis.

Lectin microarray technology

The development of lectin microarrays has enabled the high-throughput screening of carbohydrate epitopes on proteins [105]. Lectins are carbohydrate binding proteins that are not antibodies or enzymes and have been used to characterize glycans in techniques such as affinity chromatography, western blots, and histology. Lectin microarrays consist of a panel of lectins covalently linked to a glass slide in an array format. The use of this technology enables the examination of multiple binding events to different carbohydrate epitopes on the fluorescently labeled analyte simultaneously. Lectin microarrays have been used for detecting glycans in mammalian and bacterial cell surfaces by our lab and several other groups .Their utility has been demonstrated in identifying unique carbohydrate patterns in differentiated cells, tumor cells and different strains of pathogenic bacteria [106-109].

The analysis of glycan epitopes on samples involves fluorescent labeling of samples with Cy3 or Cy5 dyes, followed by hybridization with the lectin microarray. The slide is rinsed to get rid of unbound samples, and then scanned, enabling us to decipher the glycan epitopes present in the sample. A single color lectin microarray format involves the binding of a single analyte to the array and examining the resultant lectin pattern (Fig 2.1) [107, 110].

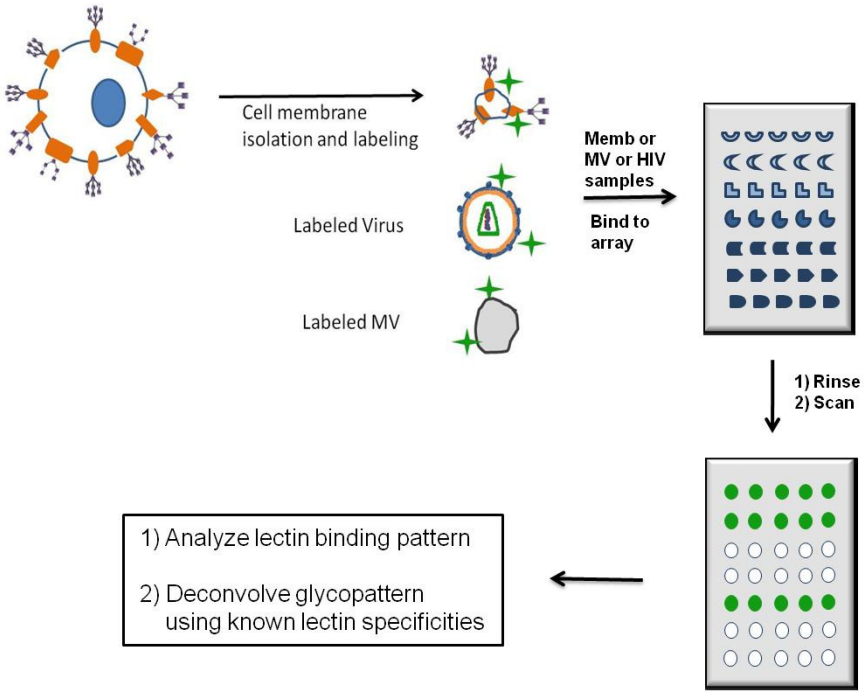


Figure 2.1. Schematic of single color lectin microarray format. The glycopatterns of a fluorescently labeled analytes (cell membrane preparations, microvesicles (MV) or HIV) can be deciphered using this format.

Though single color analysis gives us a good method for analysis of glycans, it relies heavily on both consistent lectin activity and prints across multiple days and consistent labeling of samples, making comparison between arrays more difficult. Our lab has demonstrated the sensitivity and reliability of the ratiometric approach in comparison to the single color microarray analysis. Ratiometric analysis utilizes a common biological reference to enable the comparison of different samples directly. The dual color analysis is more sensitive than the single color analysis as any changes in lectin activity would affect binding to both the reference and the sample, making it ideal for comparing multiple samples [107]. A schematic of the ratiometric approach is illustrated in Figure 2.2.

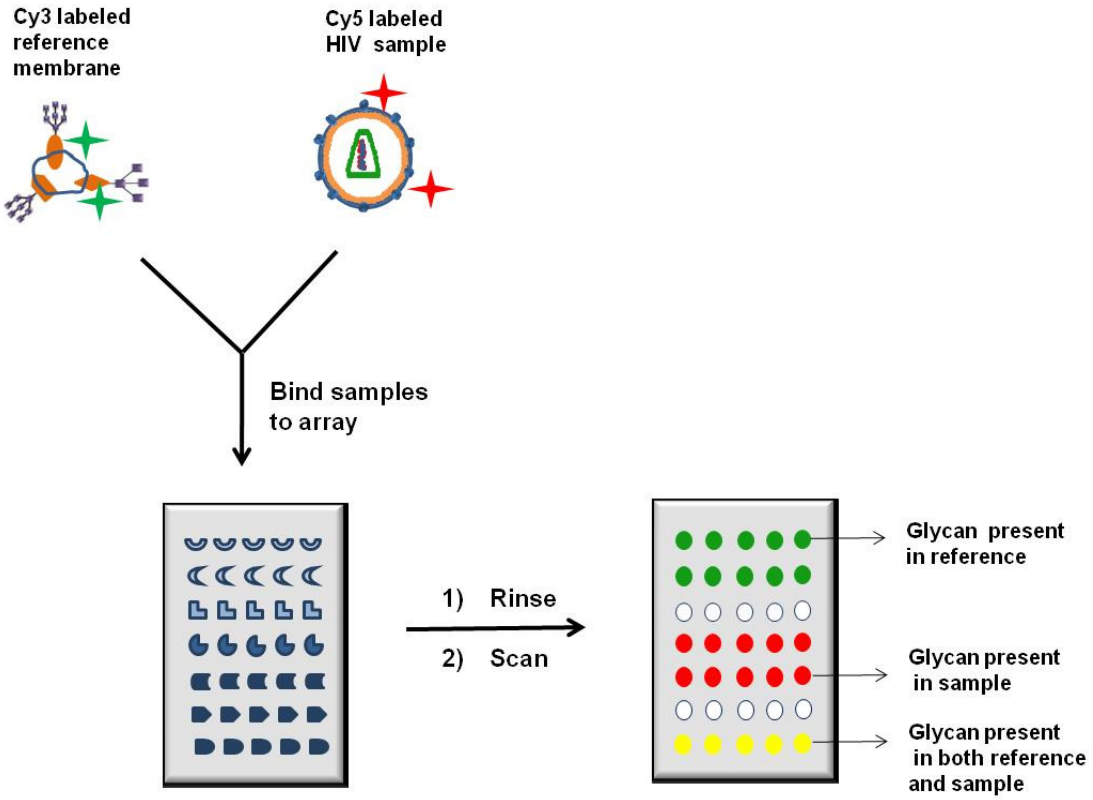


Figure 2.2. Schematic of dual color lectin microarray format. Ratiometric dual color analysis is ideally suited for direct comparison of multiple samples and involves the use of orthogonally labeled samples on the array.

Briefly, samples are individually labeled with Cy3 and Cy5 dyes, generating an orthogonally labeled sample set for each sample. A dye-swapped pair of arrays is hybridized for each sample. The $\log_2$ ratio of fluorescent intensities of each dye swapped pair is averaged to account for any dye-bias observed in sample labeling. A more detailed explanation of the data analysis is provided in the following section [107]. Thus, lectin microarrays provide a high-throughput method for comparative glycomic analysis of microvesicles, HIV and the parent cell membrane, enabling the comprehensive profiling of glycomic signatures of these particles. This will provide us with additional means of

examining similarities between these particles and re-examine the Trojan exosome hypothesis in view of this data.

2.2 Results and discussion

Microvesicles and HIV derived from T cells share a glycomic profile

HIV has been hypothesized to utilize the microvesicular pathway for exit from host cells by budding from plasma microdomains that are enriched in specific microvesicular markers [3, 40]. Based on current literature [40, 57], it seemed highly likely that microvesicles and HIV would share similar glycomic signatures if both particles utilize the same exit mechanism. If on the other hand, these two particles exit via separate mechanisms, then we should obtain distinct glycomic signatures for both particles.

Initial data from our lab indicated that microvesicles from uninfected cells and HIV derived from T cells might share a glycome that is distinct from the cell membrane [111]. Upon a thorough examination of the initial data, we realized that there were some inaccuracies in the data set. This was notably due to the unequal concentrations of microvesicles and HIV used for this comparative analysis. Additional concerns about the purity of the virus led us to re-examine this earlier work. To this end, I began this project by characterizing the viral samples provided by our collaborators. Microvesicular and viral samples from a panel of T cells, including H9, SupT1 and Jurkat-Tat-CCR5 were initially characterized using western blot analysis to detect the presence of viral antigen, p24 in the viral samples alone (Fig 2.3).

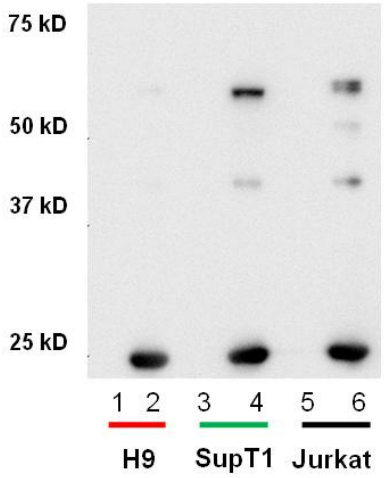


Figure 2.3.Western blot analysis confirms the presence of HIV in viral samples. Presence of the viral antigen, p24, in the HIV samples derived from H9, SupT1 and Jurkat T cell lines (lanes 2, 4, 6 respectively) was confirmed by western blot analysis. The viral antigen is absent in the microvesicle samples from H9, SupT1 and Jurkat T cell lines (lanes 1, 3, 5 respectively). Red line indicates samples from H9 cells, green line indicates samples from SupT1 cells and black line indicates samples from Jurkat cells.

Assessment of viral purity of different HIV preparations from a panel of T cell lines

The contamination of viral preparations by cellular microvesicles has been observed for viral preparations from both primary and cultured T lymphocytes [56, 58] as well as macrophages [112]. The difficulty in separating these two particles arises from the population of microvesicles that have similar densities to viral particles. The extent of contamination has been shown to vary greatly based on the clone of virus used for generating virions as well as the amount of time that the cells were cultured for generation of virions. For instance, infection of the H9 T cell line with highly infectious HIV-1(MN) CL.4 [113] yielded relatively less contaminations when compared to HIV-1(MN) parental strain [58].

The differences in the level of contaminations in these preparations could have a huge effect on the glycomic pattern observed. Initial data that indicated similar glycomic

patterns obtained for microvesicles and HIV made it critical for us to estimate the relative purity of the viral samples. We estimated the relative purity of the virion preparations using a combination of the two methods. We obtained crude estimates of viral purity based on SDS-PAGE analysis from our collaborators at the National Cancer Institute (Fig 2.4). This was calculated by comparing the relative amounts of viral proteins with non viral proteins as visualized by coomassie blue staining of proteins. The total amount of the viral proteins (p24, p17, p6/7) was expressed as a ratio of the total protein as estimated by SDS-PAGE (Table 1). This method is relatively conservative as it does not take into account the other viral proteins including the enzymes as well as envelope glycoproteins. This method also does not factor the amount of cellular proteins that are contained in the virions (Julian W. Bess Jr, NCI, personal communication).

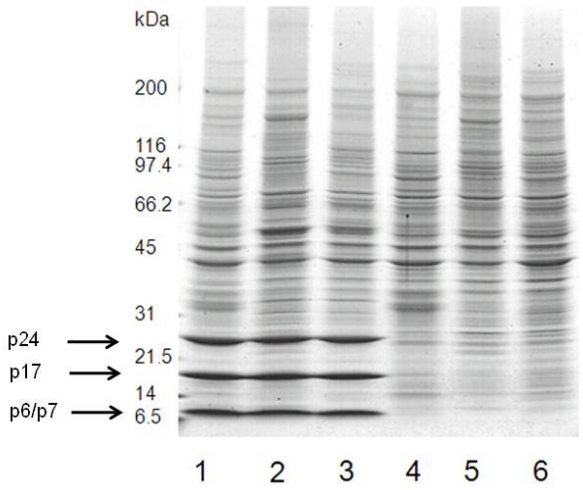


Figure 2. 4. SDS-PAGE analysis of microvesicles and HIV. The SDS-PAGE analysis shown above is courtesy of Julian Bess (NCI). 100 µg of protein sample per lane was loaded and the gel was stained with Gelcode blue. The lanes are as follows: 1. HIV-1(MN) CL.4/ H9 (P3944) 2. HIV-1(MN) CL.4/SupT1 (P4062) 3. HIV-1(MN) /Jurkat-Tat-CCR5 (P4066) 4. H9 MV 5.SupT1 MV 6. Jurkat-Tat-CCR5 MV.

Table 1. Relative estimates of viral purity based on SDS-PAGE analysis (Courtesy of Julian W.Bess. NCI)

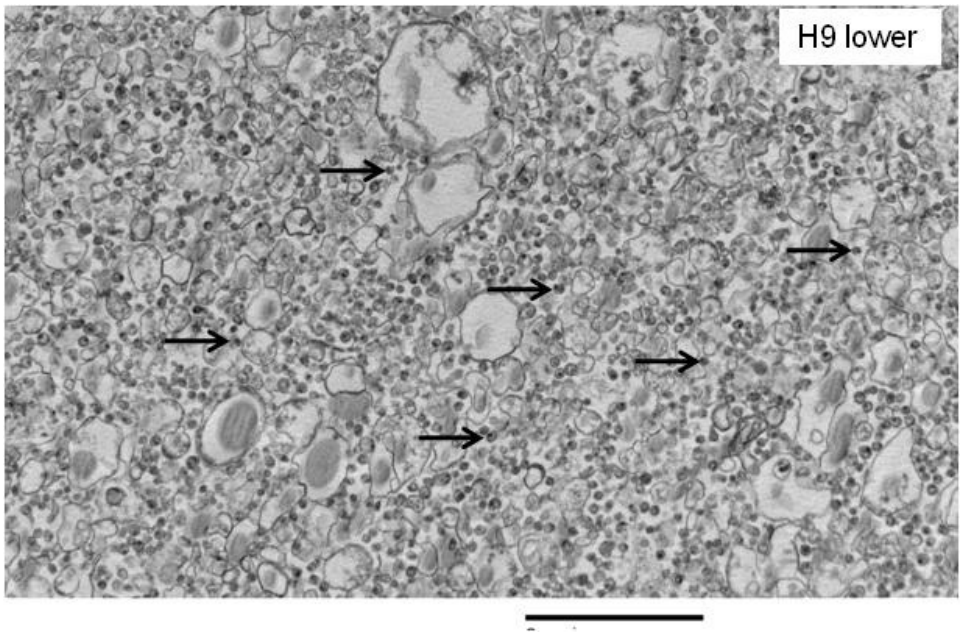
Agent	Cell Line Number	Replicate	Product Lot	Purity Estimate*
HIV-1(MN)/H9 CL.4	CLN71	1	P3935	80%
		2	P3945	80%
		3	P3944	80%
HIV-1(MN) CL.4/SUPT1	CLN219	1	P4095	85%
		2	P4092	90%
		3	P4098	80%
SIV NC-MAC/SUPT1	CLN130	1	P3700	95%
SIV CP-MAC/SUPT1	CLN131	1	P3866	90%
HIV-1(MN)/Jurkat-TAT-CCR5	CLN284	1	P4066	70%
		2	P4067	70%
		3	P4068	70%

The second method of estimating viral purity is based on transmission electron micrographs of the viral samples from the three T cell lines, H9, SupT1 and Jurkat-Tat-CCR5. This method was mostly used to corroborate the estimates of viral purity obtained with SDS-PAGE analysis and were obtained from our collaborators. The H9 and SupT1 cells were infected with the HIV-1(MN)CL.4 strain that is known to yield a relatively pure preparation compared to the HIV-1(MN) parental strain of HIV [113]. The electron micrographs consisted of different sections of the sample, imaged at three different planes (upper, middle and lower). One biological replicate per cell line was used for this analysis. A representative series of three different planes per sample is shown below (one of two data sets obtained from our collaborators).

We carefully examined a series of 6 images per sample to verify the relative purity of the samples. The HIV particles are very similar in size ranging from about 100-120 nm and contain a dense viral core. Thus, it was easy to identify virions which

contained a visible dense core and the larger contaminating microvesicles. It was harder to classify the smaller particles which lacked a viral core as they could be either viral particles or small microvesicles depending on the sectioning of the sample (Kunio Nagashima and Julian W. Bess, personal communication). The extent of microvesicular contamination varied significantly with the plane of sectioning, further complicating our analysis. Keeping these factors in mind, we realized that the estimation of viral purity using electron micrographs would be subjective. We based our assessment of viral purity on the percent area of the image that we attributed to virion particles identified using the above criteria. Using this method of analysis, we estimated slightly different purities for the samples, when compared to SDS-PAGE analysis (H9 derived HIV-70-80% (Fig 2.5), SupT1 derived HIV-50-60% (Fig 2.6) and Jurkat derived HIV-50-60% (Fig 2.7)).

A.



B.

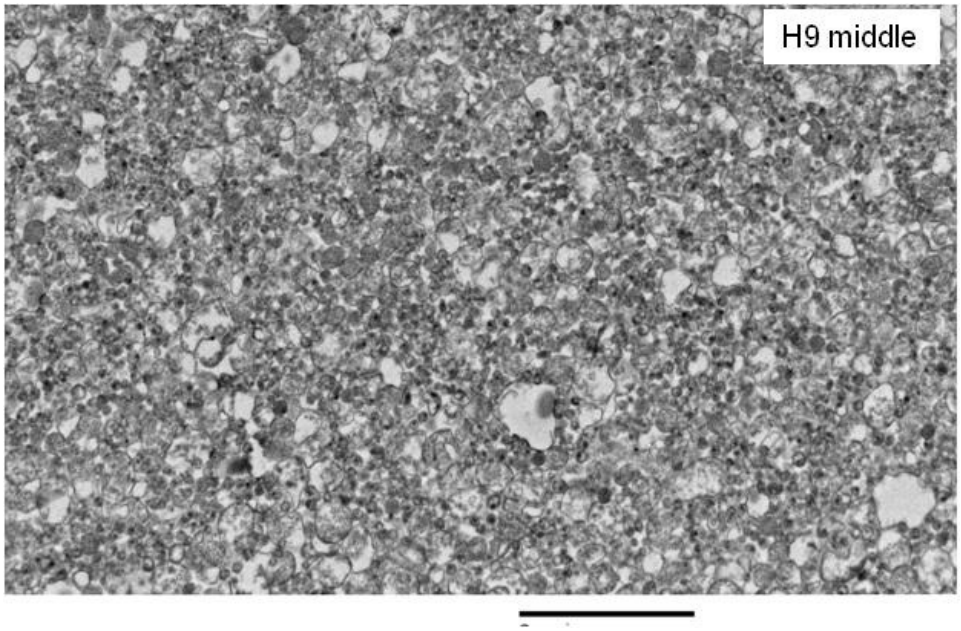


Figure 2.5. **Transmission electron micrographs of H9 derived HIV samples.** Representative images of different sections of images are shown. Images shown are courtesy of Kunio Nagashima (Image analysis Lab, NCI). The bar represents 2 microns. A) Lower plane of section for H9 derived HIV. B) Middle plane of section for H9 derived HIV. Arrows indicate some of the virions in field of view.

C.

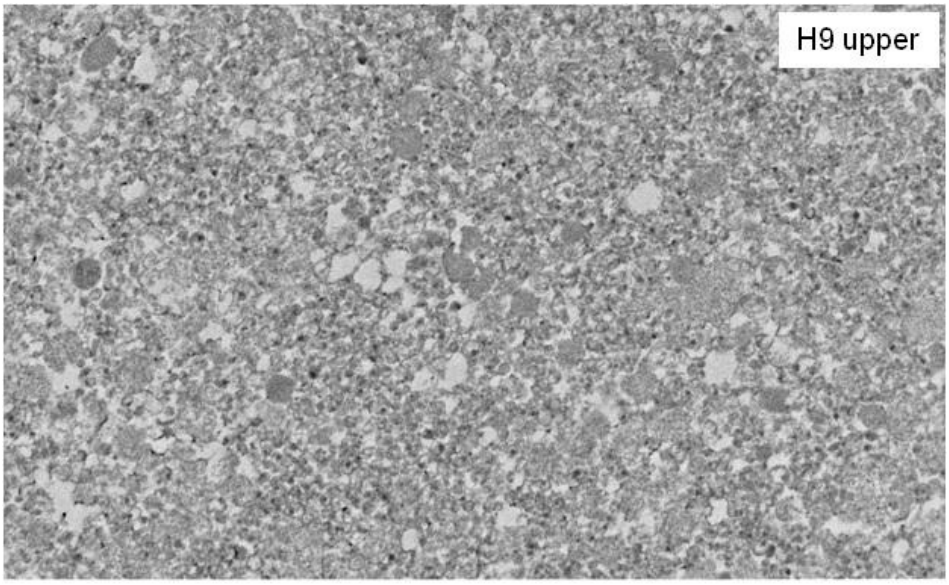
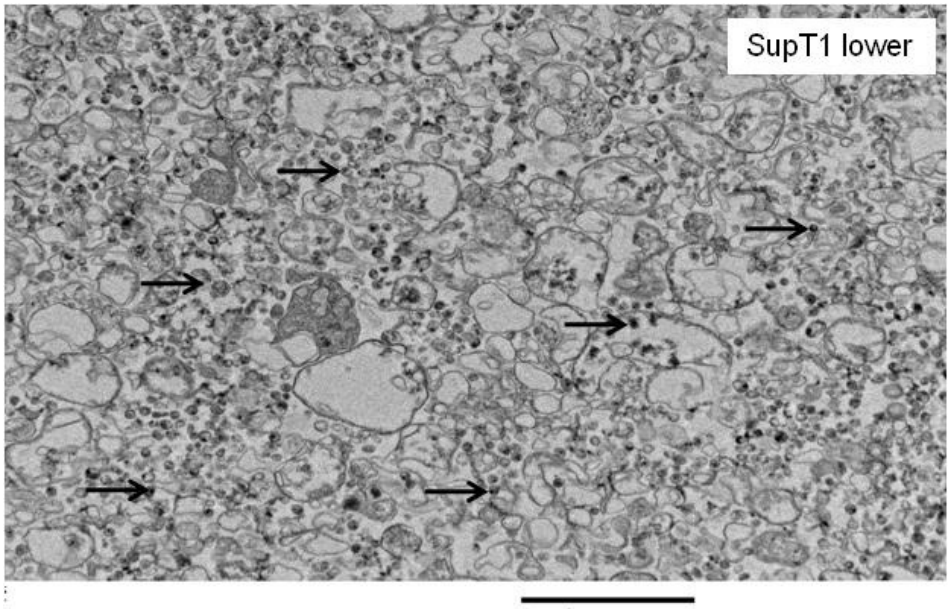


Figure 2.5. **Transmission electron micrographs of H9 derived HIV samples.** Representative images of different sections of images are shown. Images shown are courtesy of Kunio Nagashima (Image analysis Lab, NCI). The bar represents 2 microns.C) Upper plane of section for H9 derived HIV.

A.



B.

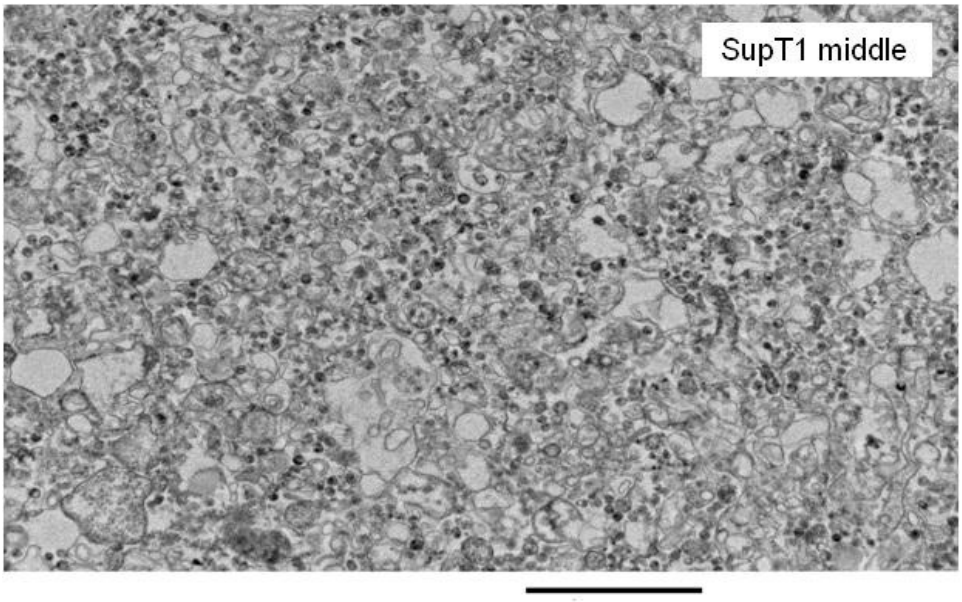


Figure 2.6. **Transmission electron micrographs of HIV samples.** Representative images of different sections of images are shown. Images shown are courtesy of Kunio Nagashima (Image analysis Lab, NCI). The bar represents 2 microns. A) Lower plane of SupT1 derived HIV (P4092) B) Middle plane of section for SupT1 derived HIV. Arrows indicate some of the virions in the field.

C.

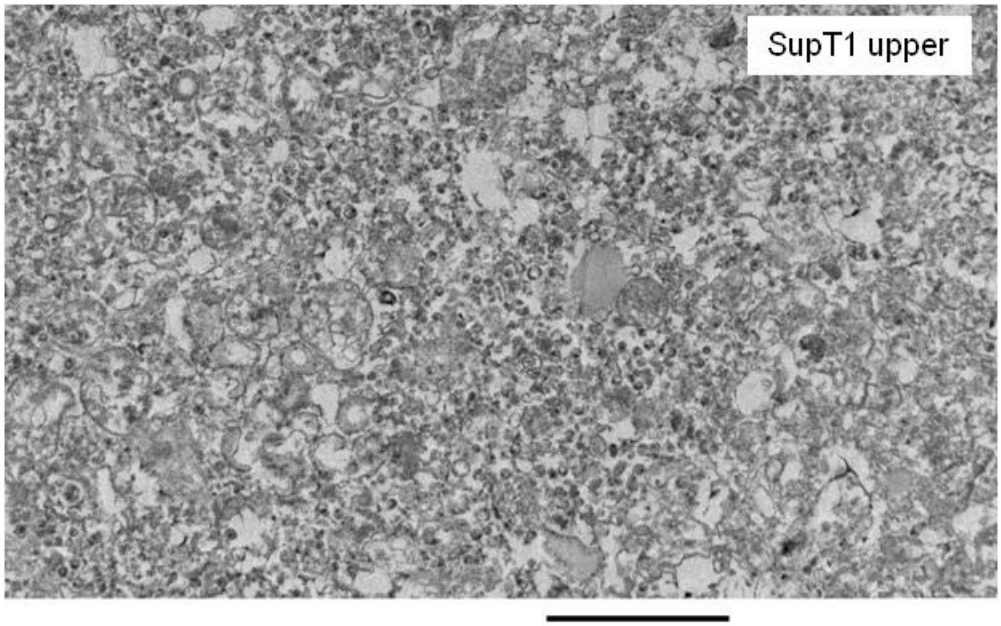
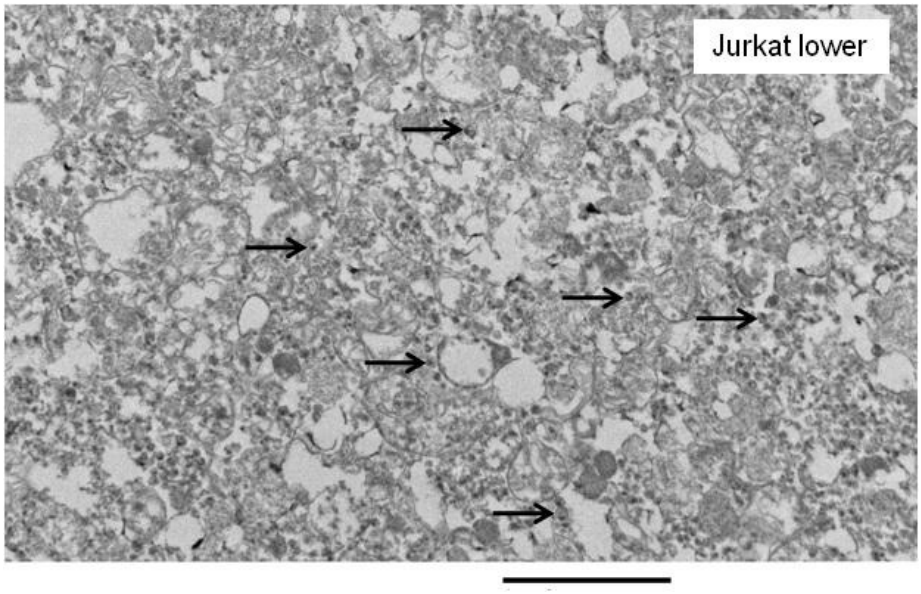


Figure 2.6. **Transmission electron micrographs of HIV samples.** Representative images of different sections of images are shown. Images shown are courtesy of Kunio Nagashima (Image analysis Lab, NCI). The bar represents 2 microns. C) Upper plane of section for SupT1 derived HIV.

A.



B.

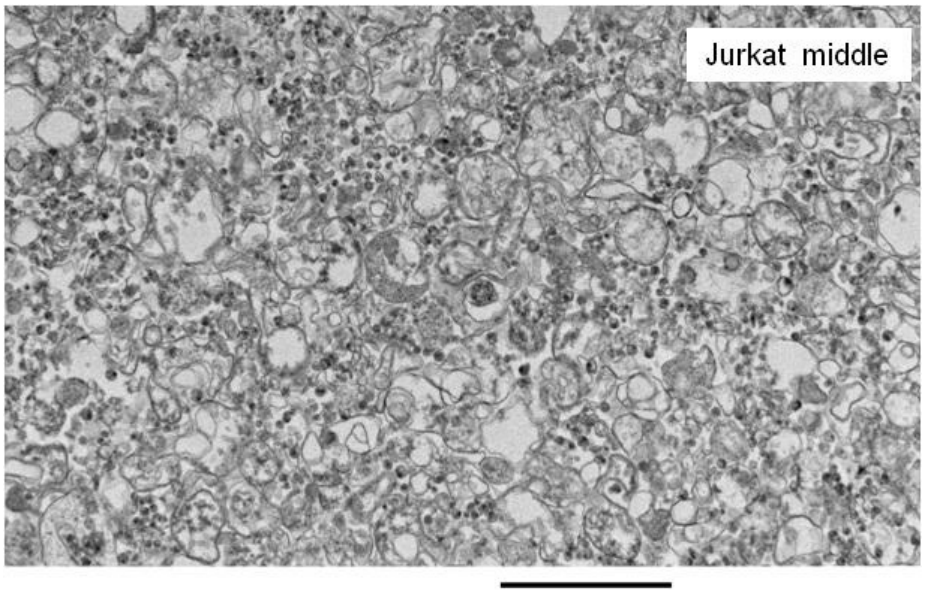


Figure 2.7. **Transmission electron micrographs of HIV samples.** Representative images of different sections of images are shown. Images shown are courtesy of Kunio Nagashima (Image analysis Lab, NCI). The bar represents 2 microns. A) Lower plane of section for Jurkat derived HIV. B) Middle plane of section for Jurkat derived HIV. Arrows indicate virions in the field of view.

C.

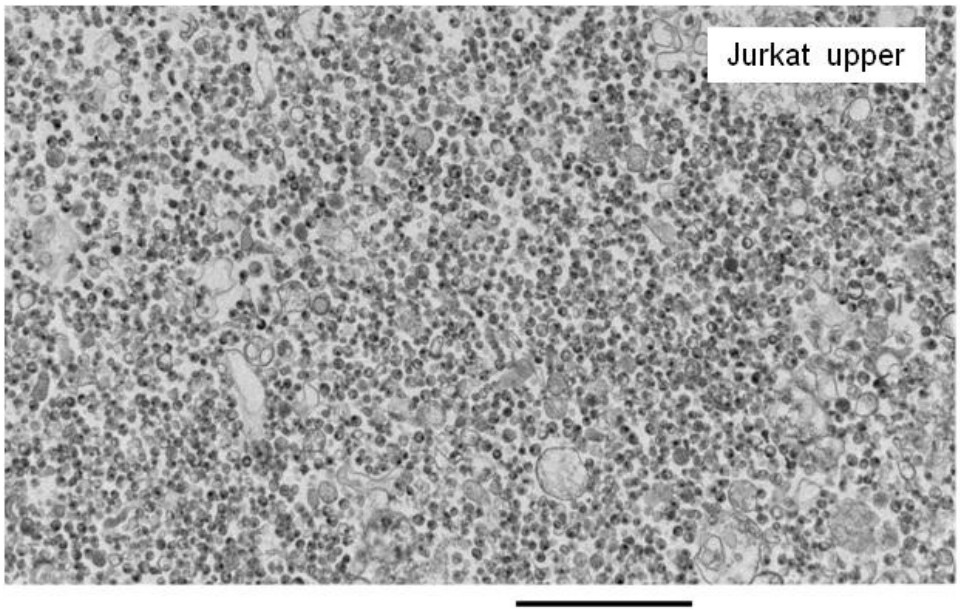


Figure 2.7. **Transmission electron micrographs of HIV samples.** Representative images of different sections of images are shown. Images shown are courtesy of Kunio Nagashima (Image analysis Lab, NCI). The bar represents 2 microns. C) Upper plane of section for Jurkat derived HIV.

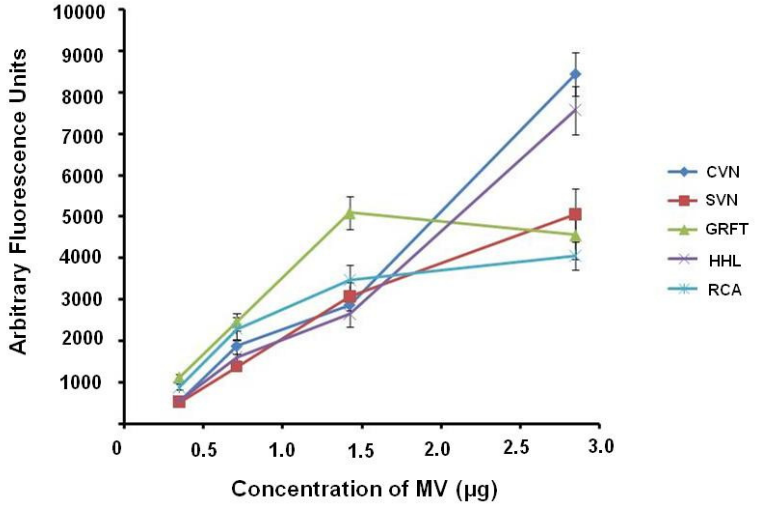
Upon closer examination of HIV literature, I could not find any instances of quantitation of viral sample purity. Instead, amounts of viral antigen (p24) and infectivity assays are commonly used to compare virions from different sources [57, 58]. The lack of reported quantization of HIV viral purity is indicative of the inherent difficulties in this type of quantification.

Due to the concerns with viral purity, it is feasible to imagine that the glycomic signatures observed for HIV could result solely from the contaminating microvesicles. In order to rule out microvesicular contamination as a source of glycomic pattern of HIV, I performed titration experiments with HIV derived from H9 cell line. The H9 derived HIV sample seems to be approximately 70-80% pure, based on SDS-PAGE analysis (Fig 2.4) and electron microscopic examination (Fig 2.5.A-C). Thus, the contaminating

microvesicular contribution would only account for 20-30% of the observed signals, resulting in a huge reduction in signals observed for H9 derived HIV.

In order to validate these results, it was critical to examine if the amounts of samples used on the array would give rise to signals that are in the linear range for majority of the lectins on our array. I performed titration experiments using different amounts of microvesicles and HIV derived from H9 using single color lectin array format. Titration experiments showed that the amounts of both microvesicles and HIV used on the arrays were indeed in the linear signal range (Fig 2.8). This makes it highly unlikely that the glycopatterns are derived from only from the contaminating microvesicles, since I would observe a huge reduction in the fluorescence intensity if microvesicles were the source of the glycopatterns observed. Similar results were also obtained with HIV derived from Jurkat T cell line and microvesicles from matched uninfected Jurkat cells (data not shown).

A.



B.

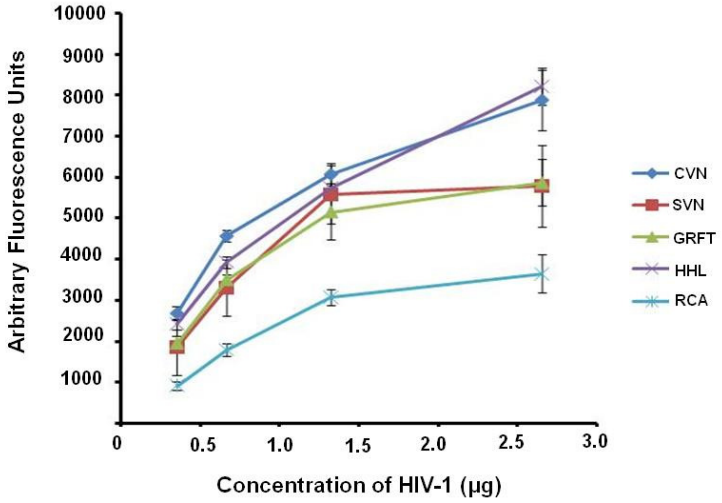


Figure 2.8. Titration experiments confirm that the glycopattern of HIV does not solely derive from the contaminating microvesicles. A) H9 derived microvesicles from matched uninfected cells and B) H9 derived HIV were analyzed using single color lectin microarray analysis. Briefly, different amounts of Cy3 labeled microvesicles and HIV were hybridized to the array and resulting fluorescence intensities were plotted as a function of amounts of total proteins. Results obtained for a select panel of lectins are shown. The bars represent standard deviations from the experiment. The fluorescence intensity for the samples is in the linear range for the amount of samples used on the array (1 µg) for majority of the lectins.

HIV samples were treated with 2,2'-dithiodipyridine (aldrithiol-2, AT-2) before the labeling procedure to inactivate the virus for safety concerns. AT-2 inactivates the virus by modification of cysteine residues in the nucleocapsid zinc finger proteins and thus effectively prevents these proteins from retaining zinc. The inactivated virus is still capable of entry into host cells, but replication is prevented before reverse transcription is initiated. Since AT-2 only affects zinc finger proteins, it preserves the conformation and integrity of the viral envelope glycoproteins [114]. The inactivated virus samples were also demonstrated to induce activation and apoptosis in CD4+ and CD8+ T cells, further confirming the integrity of surface proteins in the virions [99].

For accurate comparison, the microvesicle samples derived from uninfected samples were treated with AT-2 at similar concentrations. Initial work from our lab indicated that the glycomic profiles of AT-2 treated and untreated microvesicles are similar [111]. I repeated the experiments using similar protein concentrations again confirming that AT-2 treatment does not alter the glycomic profile of AT-2 treated and non-treated H9 derived microvesicle samples (Fig 2.9).

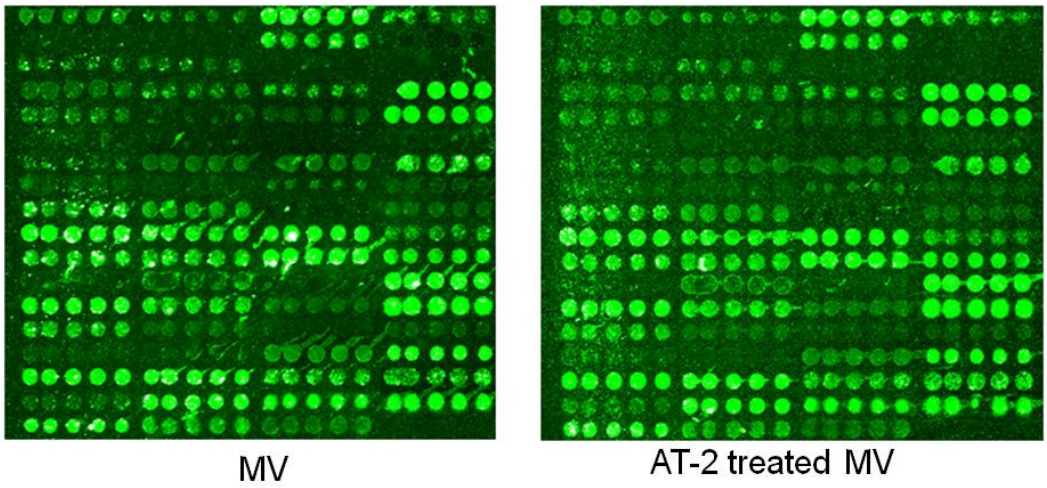


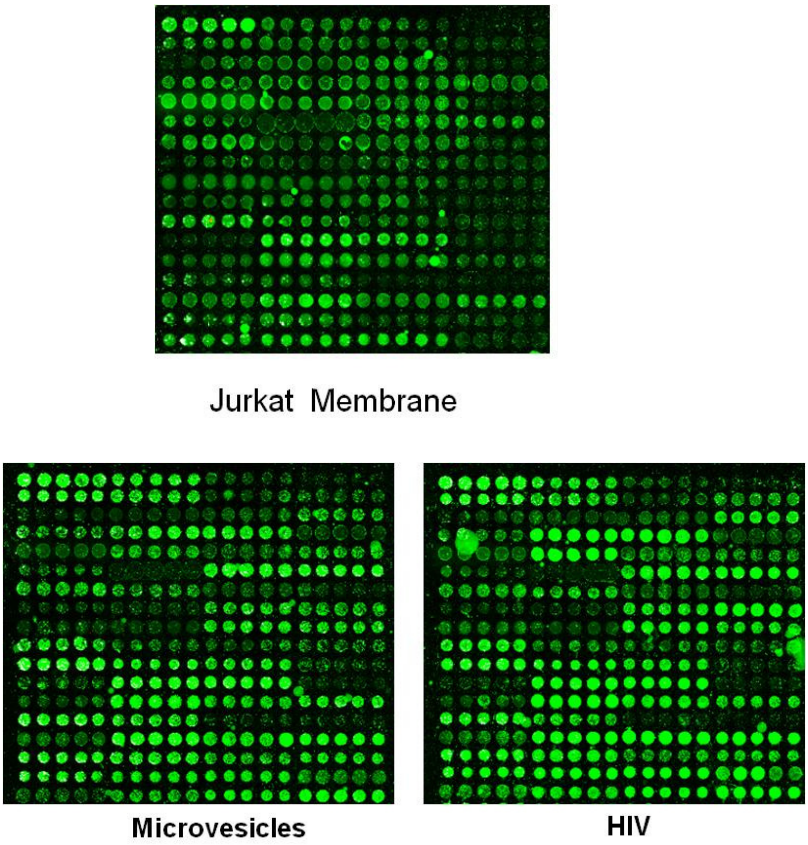
Figure 2.9. Single color lectin array analysis reveals that AT-2 treated and untreated microvesicles have similar glycomes. Equal amounts of –Cy3 labeled microvesicles (MV) (1 μg) were hybridized to the array.

As discussed previously, evidence for the microvesicular theory of HIV biogenesis has mainly relied on select protein and lipid markers. In order to compare the glycomes of the two particles with their parent cell membrane, fluorescently labeled microvesicles and cellular micellae from uninfected cells and HIV isolated from Jurkat cells were analyzed using single color lectin microarray format. This cellular membrane preparation includes membrane from cellular organelles as well. It has been previously shown that the glycopatterns obtained for this preparation are representative of cell surface glycosylation [107]. Equal amounts of fluorescently labeled cell membrane and microvesicles from uninfected Jurkat cells and HIV (based on total protein concentrations) were hybridized to the lectin microarray. In line with initial work from our lab for particles obtained from other T cell lines [111], the microvesicular and viral glycomes from Jurkat cells exhibited remarkable similarity (Fig 2.10). Lectin

microarray analysis also revealed differences between the glycomes of uninfected cell membrane and the particles that bud from the cell membrane.

In comparison to the cell membrane, both samples are enriched in high mannose epitopes as revealed by enhanced binding to known anti-viral lectins including NPA, HHL, PSA, and cyanovirin-N (CVN). In addition, microvesicles and HIV are enriched in complex *N*-linked glycans (PHA-L, PHA-E), *N*-acetyllactosamine (LacNAc, lectins: DSA, MAL-I, RCA, STA, WGA), sialic acid (SNA, MAA, MAL-II), fucosylated (AAL) and GalNAc (HPA, BPA, VVA) epitopes. Compared to the cell membrane, the exclusion of blood group antigens A/B (EEA, LBA) was observed for both HIV and microvesicles. Similar enrichment of glycan epitopes in HIV and microvesicles, coupled with the exclusion of certain glycan epitopes present in the cell membrane indicates that these exocytic particles do not bud non-specifically from any region of the cell membrane. Rather, this data strongly suggests that both these particles bud from specific domains of the membrane resulting in similar glycomic signatures, and share exit mechanisms.

A.



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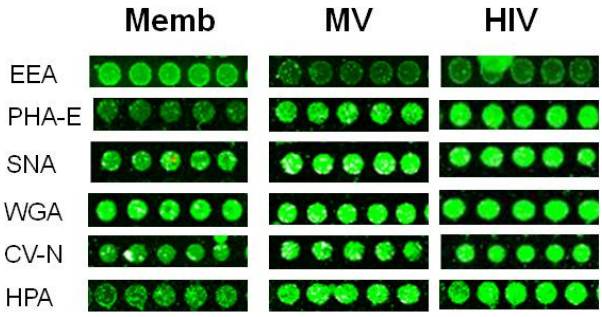
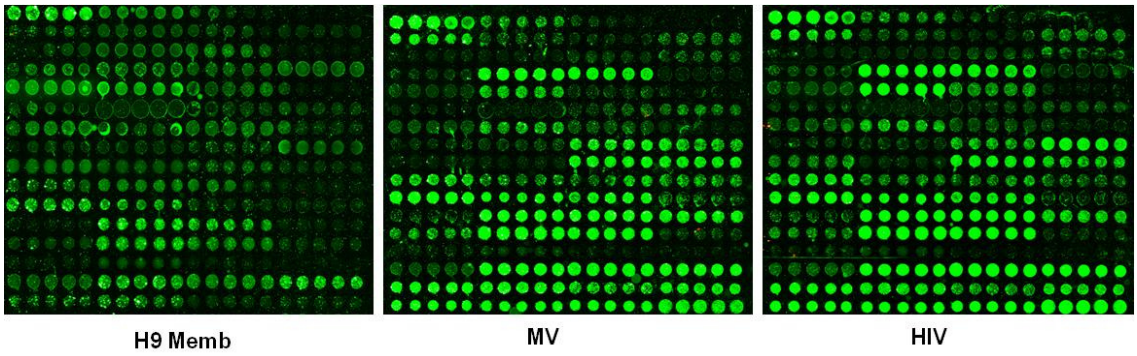


Figure 2.10. Single color analysis reveals glycomic similarities between microvesicles and HIV derived from Jurkat cells. A) Lectin microarray analysis of Jurkat membrane and MV derived from uninfected cells and HIV from Jurkat cells. Glycomic profiles of HIV and MV exhibit similarity to each other and are different from the glycome of Jurkat cell membrane. B) Direct comparison of a select panel of lectins (from Fig 2.8.A) highlights the similarities between the two particles.

Initial results from our lab indicated that HIV from H9 and SupT1 cells likely share a glycome with the microvesicles derived from matched infected cells [111]. However due to concerns about protein concentrations of the samples, I re-examined the glycomic profiles of HIV and microvesicles isolated from H9, SupT1 and the respective parent cell membranes. In line with the initial data obtained, HIV and microvesicles from a given T cell line exhibited a similar glycome and differed from the respective cell membrane (Fig 2.11).

A.



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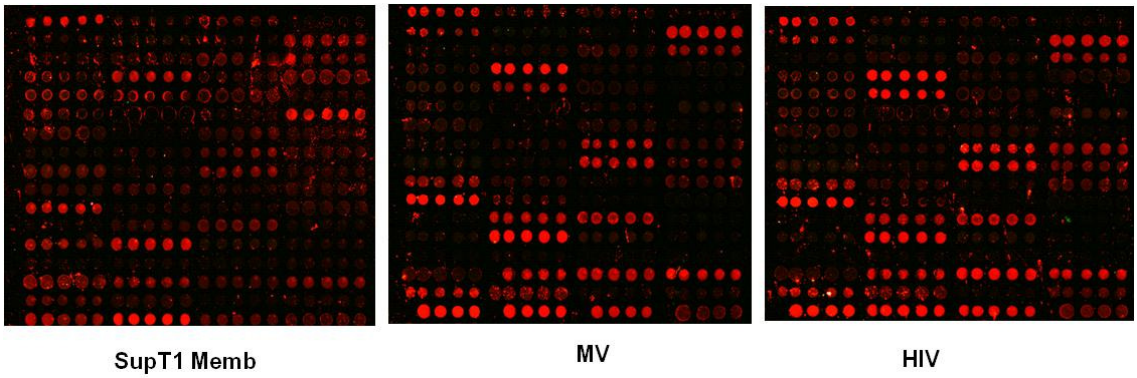


Figure 2.11. Single color lectin microarray analysis reveals similarities between microvesicles and HIV from T cells. A) Equal amounts of Cy3-labeled H9 derived samples were hybridized to array. B) Equal amounts of Cy5-labeled SupT1 derived samples were hybridized to array. Memb refers to cellular membrane sample, while MV refers to microvesicles. The microvesicles and HIV from a given cell line exhibit similar glycomic profiles and are different from the cell membrane.

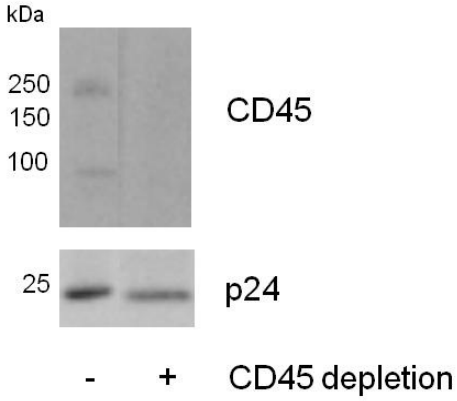
Glycopattern observed for HIV is reflective of the glycans present on viral envelope

Given the concerns with viral purity, we decided to perform additional experiments to ensure that the glycomic similarities between HIV and microvesicles from uninfected cells are indeed an accurate reflection of their glycans. An additional method to unequivocally confirm whether the glycomic signature emanates from the virus is to

examine ultra-pure virus samples that are virtually devoid of contaminating microvesicles. Since microvesicles and HIV share many protein markers with the exception of CD45, immunodepletion using CD45 antibodies is a useful technique to prepare ultra-pure virus samples [112]. We obtained labeled CD45 immunodepleted H9 derived HIV samples from our collaborators and compared the glycomic profiles of undepleted and CD45 depleted HIV samples.

The absence of CD45 in the immunodepleted samples was confirmed using western blot analysis. The presence of the viral antigen, p24, in both depleted and non-depleted samples was confirmed using western blot analysis (Fig 2.12.A). The CD45 depleted and un-depleted samples were analyzed using single color lectin microarray analysis (Fig 2.12.B). The samples exhibited remarkable similarities in the glycopatterns observed. This confirms that the glycomic signature observed for HIV is an accurate reflection of the viral and host derived glycoproteins present on the viral envelope. This again lends strong credence to the theory that HIV utilizes the microvesicular machinery for exit from host cells.

A.



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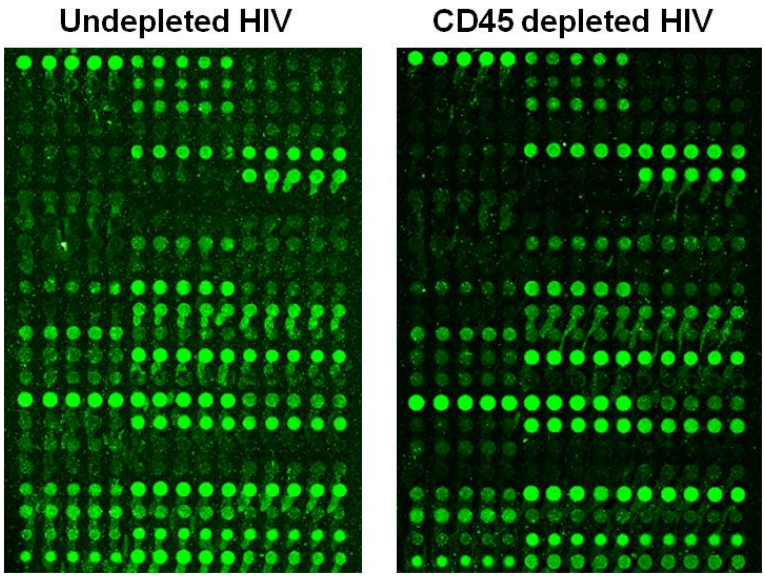


Figure 2.12. Single color analysis of CD45 depleted and non-depleted viral samples reveals similar glycomic profiles. A) Western blot analysis confirms the presence of p24 and the absence of CD45 in the CD45 depleted samples.B) Single color lectin microarray analysis confirmed that CD45 depleted and non-depleted H9 derived HIV samples share common glycomic patterns.

Inhibition experiments confirm specificity of carbohydrate based interactions with lectin microarray

An important quality control for lectin microarray analysis involves the use of inhibitory sugars to inhibit lectin activity. The carbohydrate based interactions of lectins with the samples should be diminished in the presence of appropriate sugars [110]. The use of a panel of sugars for inhibition experiments provides clues about any non-specific inhibition that results from the excessive concentrations of sugars used. The lectin microarray is pre-incubated with 100 mM sugar and hybridized with the sample in single color microarray format. The resultant data is normalized to the un-inhibited control signal which is set to 100%. Inhibition experiments were performed using Jurkat derived HIV samples (Fig 2.13) and microvesicles and membrane from uninfected cells (data not shown). Results for a select panel of lectins are shown.

The inhibitions experiments confirmed the specificity of the lectin microarray. The Gal-β-1,3-GalNAc binders, APA and ABA, were greatly inhibited by lactose and to a much lesser extent by the other sugars, showing that the carbohydrate based interactions detected by the lectin microarray are specific (Table 3). Lactose was also the better inhibitor for the terminal β-GalNAc binders BPA, CSA, although they are significantly inhibited by the other sugars as well. This could be due to the high concentrations of inhibitory sugars used, resulting in some non-specific inhibitons. Similarly, GNA and NPA, the mannose binding lectins exhibit greater inhibition upon binding to mannose, although the other sugars seem to inhibit it as well.

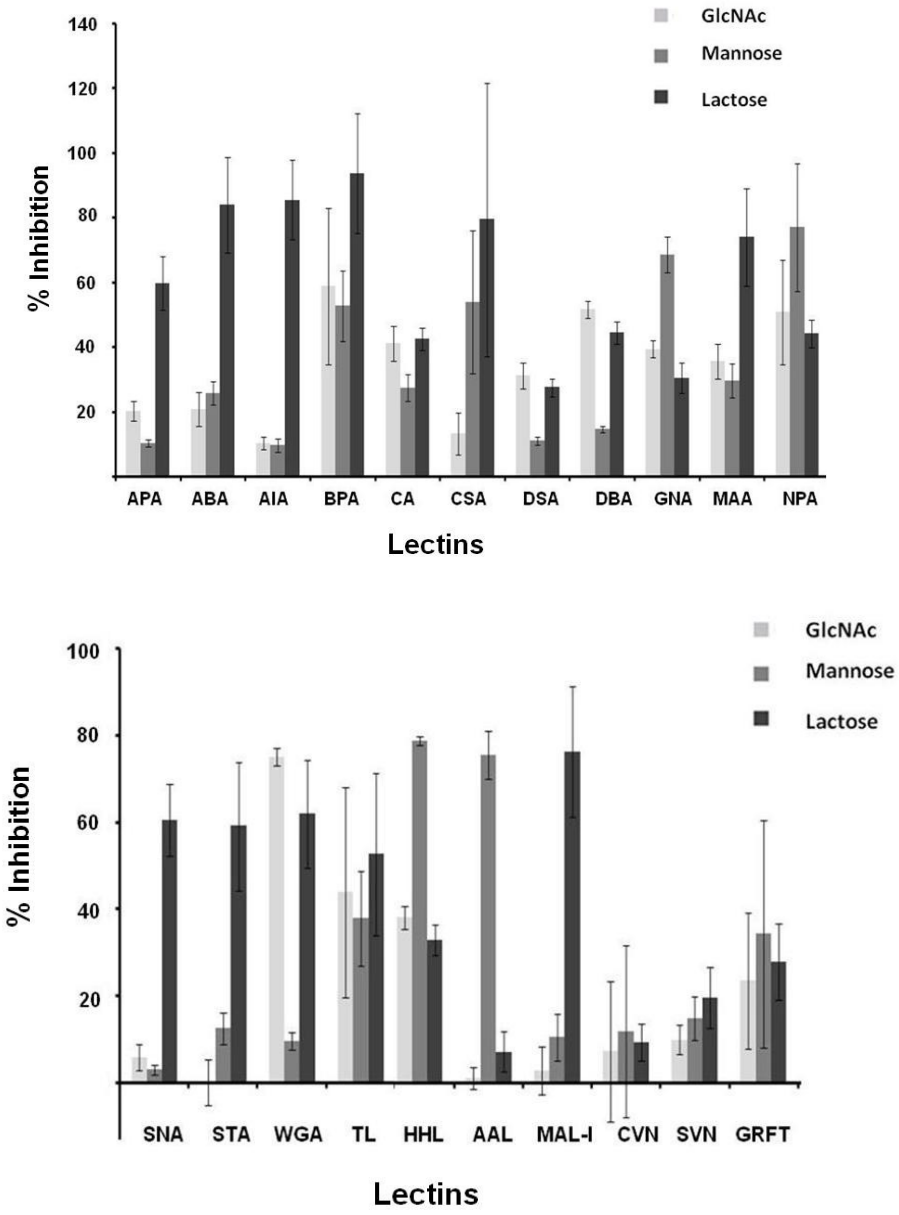


Figure 2.13. Inhibition with small panel of carbohydrates confirms that the interactions are carbohydrate based. Lectin microarrays were preincubated with either 200 mM of the appropriate carbohydrate (N-acetylglucosamine, GlcNAc, light grey; lactose, medium grey; mannose, dark grey) or PBS (control), followed by addition of Cy3-labeled Jurkat-derived HIV samples. The inhibition profile for a representative subset of lectins is shown. The graph depicts the percentage of signal inhibition for each lectin which was calculated as described in methods. The errors were propagated using the standard deviations and standard propagation of error equations [106].

The LacNAc binders, STA and MAL-I (Table 3) are very strongly inhibited by lactose. WGA binds GlcNAc as well as galactose and is inhibited equally by both GlcNAc and lactose but not mannose. CVN, which binds high mannose structures, is not inhibited by significantly by any of the sugars. This agrees with literature reports, as CVN is only inhibited by addition of high mannose ligands [115]. Thus, inhibition experiments provide good evidence for the carbohydrate dependence of the interactions observed using the lectin microarray.

Glycan epitopes enriched in microvesicles and HIV-1 localize to specific domains of the plasma membrane

Microvesicular proteins such as CD63 and CD81 have been previously shown to localize with HIV Gag in specific domains of the plasma membrane, indicating that these regions could function as sites of budding for both particles. Labeling of cells with N-Rh-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[lissamine rhodamine B sulfonyl]), a fluorescent lipid which is known to be enriched in microvesicles, was used to demonstrate co-localization of this lipid with HIV Gag, thereby strongly supporting the existence of common budding sites for both HIV and microvesicles in T cells [40].

Lectin microarray analysis showed that microvesicles and HIV were enriched in certain glycans when compared to the cell membrane as discussed previously. If this were the case, it strongly argues for the existence of certain glycan enriched regions that will serve as budding sites for both microvesicles and HIV. Therefore, I used Jurkat cells that were previously labeled with N-Rh-PE, to examine the colocalization of specific fluorescently labeled lectins with the lipid marker. Unpermeabilized Jurkat cells were used to examine the cell surface localization of these markers.

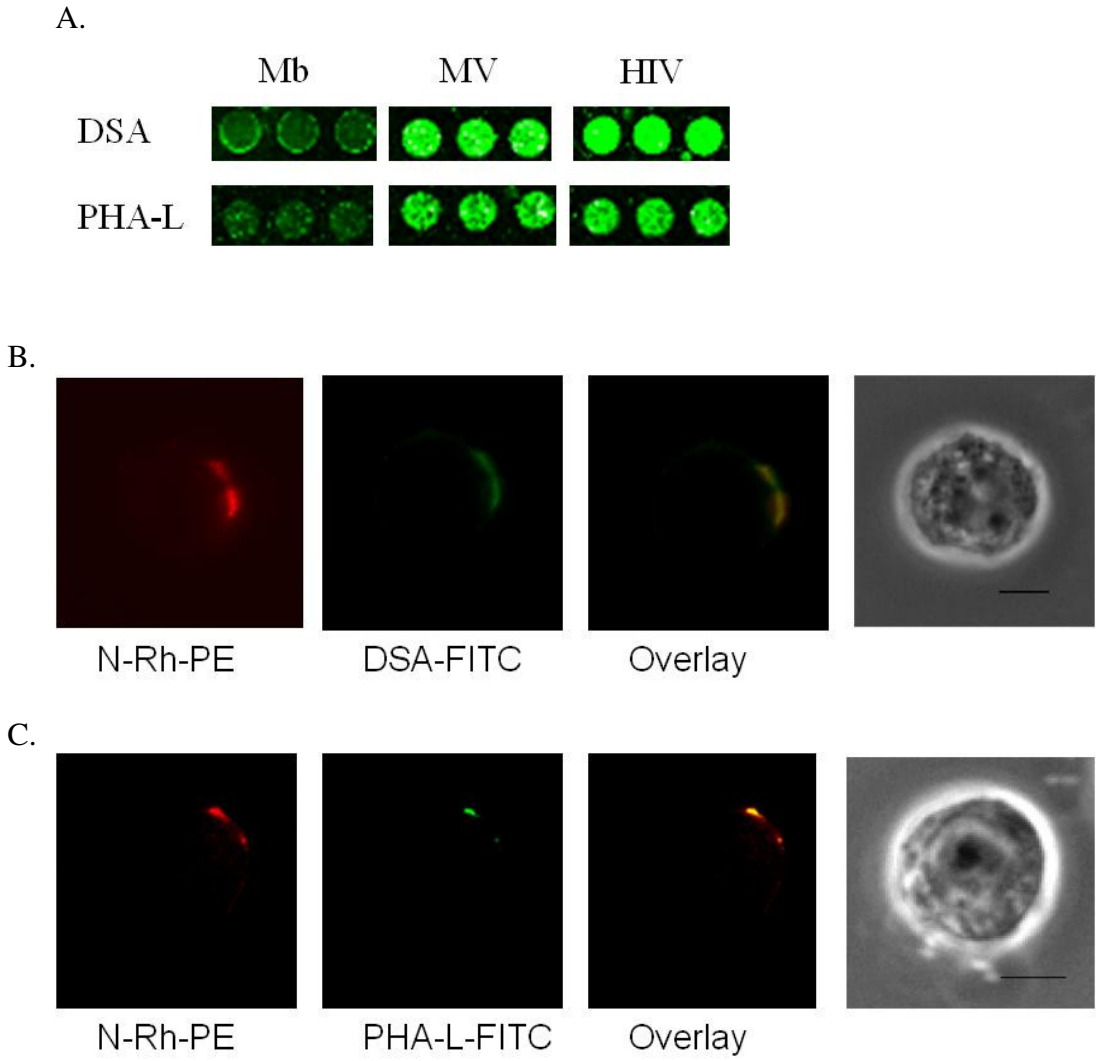


Figure 2.14. N-Rh-PE enriched domains co-localizes with lectins on Jurkat cell surface. A) DSA and PHA-L exhibit increased binding to microvesicles (MV) and HIV derived from Jurkat cells in comparison to cell membrane (Mb). Jurkat cells were pulse labelled with N-Rh-PE for 1 hour and then allowed to grow for ~20 hrs before fixation. Unpermeabilized cells were then stained with FITC-conjugated lectins and examined using fluorescence microscopy. B) N-Rh-PE domains (red) colocalize with domains that are enriched in glycans recognized by DSA (green). C) N-Rh-PE domains (red) colocalize with domains that are enriched in glycans bound by PHA-L (green). The bar represents 5 μm.

D.

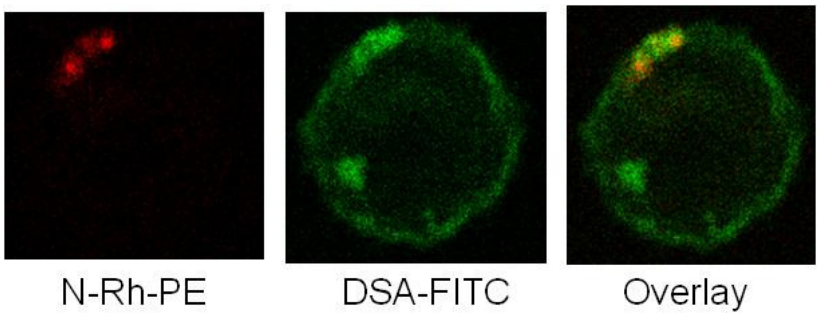


Figure 2.14. N-Rh-PE enriched domains co-localizes with lectins on Jurkat cell surface. D) Confocal microscopic images of DSA stained Jurkat cells confirms the co-localization of the fluorescent lectin and N-Rh-PE to microdomains on the cell surface. The bar represents 5 μm.

Unpermeabilized, labelled cells were fixed and stained with either FITC-conjugated DSA or PHA-L to allow for visualization of cell surface glycans. These lectins bind poly-*N*-acetyl lactosamine and α-1,6-branched *N*-linked glycans respectively and demonstrated increased binding to HIV-1 and microvesicles when compared to Jurkat cell membrane by microarray analysis (Fig 2.14.A). In line with data obtained from the microarrays, both lectin staining were enriched in certain regions of the plasma membrane. These enriched glycan epitopes exhibited co-localization with N-Rh-PE in specific microdomains of the membrane, validating the lectin microarray data (Fig 2.14.B,C). To further corroborate our results, confocal microscopic images of cells labeled with N-Rh-PE and FITC-conjugated DSA were obtained (Fig 2.14.D). The specificity of the lectin staining was observed by treating the cells with PNGase F, an enzyme that specifically cleaves N-linked sugars. This should result in reduced binding of the lectin, DSA, to the cell surface (Fig 2.15) when compared to cells treated with buffer. As expected, there was a significant reduction in the fluorescence intensity of the images due to reduced DSA binding to the cells.

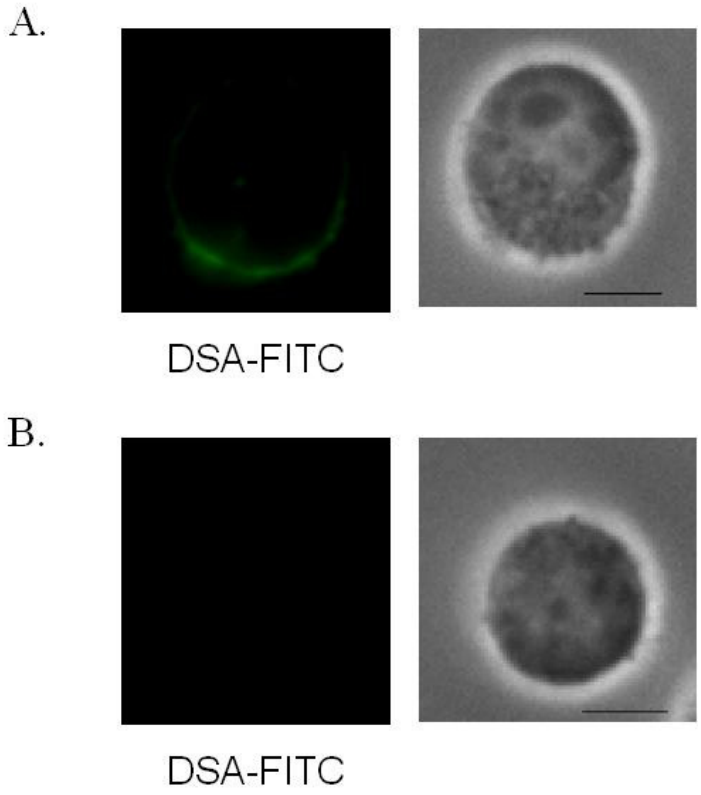


Figure 2.15. DSA-FITC interaction with Jurkat cell surface is carbohydrate dependent. Jurkat cells were adhered and fixed as descried previously. The cells were then treated with A) Buffer or B) PNGase F for 1 h to remove N-linked glycans, followed by incubation with FITC-DSA. The observed loss in DSA-FITC staining confirms the specificity of the lectin interaction with the glycans on the cell surface.

Given the high degree of similarity between the glycomes of HIV and microvesicles, our data provides strong evidence for the existence of specific microdomains, which are sites of microvesicular and HIV budding. Specific glycans can be mapped to these sites, pointing to glycosylation as a potential sorting determinant for this domain. Taken together with the existing data, it seems highly likely, that these particles likely bud from specific microdomains that are characterized by enrichments in

proteins, lipids and glycan epitopes, thus making a stronger case for the utilization of similar budding machineries by both particles.

Microvesicles and HIV-1 derived from multiple T-cell lines cluster closely based on cell line of origin

Glycosylation of the envelope glycoproteins of HIV-1 is known to vary based on the cell line that the virus is propagated. This is due to the reliance of HIV-1 on the host glycosylation machinery to glycosylate its proteins [116]. It has also been shown that the cellular protein component of HIV-1 largely reflect its cell line of origin [117, 118]. Given that the glycosylation studies on HIV-1 have only examined glycans on gp120, it could be expected that the glycans on the host glycoproteins incorporated in the viral surface will also vary in a manner reflective of the cell line of origin.

Previous studies in our lab provided some preliminary evidence regarding the cell line dependent glycosylation of HIV and microvesicles [111]. For accurate and direct comparison of HIV and microvesicles derived from panel of T cells, I extended these observations by performing a large scale comparative analysis of microvesicles and HIV derived from three T cell lines, Jurkat, H9 and SupT1 (Table 2) at equal protein concentrations. We chose the ratiometric lectin microarray approach to facilitate direct comparison of the membranes, microvesicles and HIV from all three cell lines (Fig 2.2). Our lab has previously demonstrated the sensitivity and reliability of the ratiometric approach in comparison to the single color microarray analysis [107]. Briefly, all samples are individually labeled with Cy3 and Cy5 dyes, generating an orthogonally labeled sample set for each sample. We chose H9 cell membrane as the common biological

reference to examine the glycosylation of all the relevant samples. For each sample, two sets of arrays (dye-swapped pairs) consisting of 1 μg of each sample (Cy3-labeled H9 membrane and Cy5-labeled sample in one array , Cy5-labeled sample and Cy3-labeled H9 membrane in corresponding dye-swapped array) were hybridized. The $\log_2$ ratio of fluorescent intensities of each dye swapped pair was averaged to account for any dye-bias observed in the sample labeling. This approach was utilized by Yang and co-workers in DNA microarray analysis [119] and has been successfully used by our lab for lectin microarray analysis. The resulting Yang correlation values for the data set were used to generate hierarchically clustered heat maps using Pearson correlation coefficient as the distance metric for the samples, and average linkage analysis [107].

For each of the three cell lines, three biological replicates consisting of samples from different viral (HIV) or microvesicular (MV) preparations were analyzed with the exception of SupT1 derived microvesicles (one sample). HIV-1(MN) CL.4 strain was used to infect H9 and SupT1 cells, while the parental strain HIV-1(MN) was used to infect Jurkat-Tat-CCR5 cells. The Jurkat and SupT1 cell membranes were also analyzed using the dual color approach. The data obtained from the ratiometric experiment was analyzed as described previously. Only lectins that were positive for at least one of the samples (as determined by single color analysis) were considered for this analysis. The heat map generated as a result of the hierarchical clustering was examined and several interesting observations could be made from the heat map. First, HIV and microvesicles derived from a specific T cell line clustered more closely with each other (Jurkat-Tat-CCR5, R=0.81; H9, R=0.86; SupT1, R= 0.9). The same strain of HIV propagated through two different cell lines did not cluster relatively well when compared to microvesicles

from the same cell line. This is probably due to differences in the glycosylation machinery of the three cell lines, which are reflected in the glycome of both particles. The cell line dependent glycans seem to have a profound influence on the clustering of these particles and could be accounted for by differences in a subset of glycans including β-GalNAc (BPA, CSA, VVA), which is higher in Jurkat-Tat-CCR5-derived HIV/MV, and α-1,2 fucosylated LacNAc (UEA-I, PTA, PTL-II) which is higher in H9-derived HIV/MV (Fig 2.16.A). In addition, this data concords with data obtained from the single color analysis, as the glycopatterns of the particles is different from that of the parent cell membranes ($R=0.47$, $P<0.001$). This data is also reflective of the huge repertoire of common proteins that are enriched in both particles. The conservation of this slight glycomic variation between microvesicles and HIV from any given T cell line strongly argues for the theory that similar machineries are involved in the exocytosis of both particles in these cells.

It is interesting to note that our samples have varying amounts of viral purity (ranging from 70% for Jurkat derived samples, 80% for H9 derived HIV to 95% for SIV samples from SupT1, as determinded by SDS-PAGE analysis), and yet the glycomic similarities between microvesicles and viral particles (SIV and HIV) from a given cell line are observed by both single and dual color analysis for all these samples, strengthening the case for sharing of exocytic pathways by the two particles.

This also raises the possibility that HIV may vary its glycosylation based on the type of host cell it targets. Considering that HIV infects dendritic cells, macrophages and T cells, this provides the virus with an additional means of changing its glycosylation and potentially could contribute to immune evasion by HIV. These changes in glycosylation

may also result in changes in the infectivity of the virus. Differences in gp120 and gp41 glycosylations are known to affect binding interactions between the envelope glycoproteins and receptors such as DC-SIGN [120]. Variations in the glycosylation of whole viral particles could also infectivity of the virus. This was shown in SIV where changes in sialic acid and mannose residues on whole virus influenced viral infectivity [121]. It will be interesting to examine the glycome of HIV derived from different types of cells such as macrophages and dendritic cells and compare the glycomes to their respective microvesicles and cell membranes. Based on our results, we would expect HIV and microvesicles from a given cell type to have the same glycomic coat.

Despite the fact that HIV and microvesicles from the same cell line clustered very closely and were distinct from the parent cell membrane, we also observed a metapattern for all exocytic particles from the three T-cell lines (Figure 2.16.A, R=0.66, n=48, P <0.0001), thereby showcasing the existence of a conserved set of glycans that are present in both particles. Given that these are all T cell lines, the existence of a common pattern for exocytic particles from the three T-cell lines (Figure 2.16.A, R=0.66, n=48, P <0.0001) is not unexpected. The conserved set of glycans includes LacNAc, sialic acid and complex and hybrid *N*-linked glycans on both HIV and microvesicles and the exclusion of blood group A/B antigen binding from these samples.

This data implies that these conserved glycans may constitute core glycans that are required for sorting of proteins into the exocytic particles regardless of the cell line of origin. It is also possible that the glycome is simply a reflection of the proteome and that protein-protein or lipid-protein interactions are responsible for the sorting of proteins into these vesicles. Dissecting the role of glycans in this sorting mechanism could lead to

methods to modulate the cargo that is sorted into microvesicles and HIV. A portion of the cellular cargo present on the viral envelope is known to enhance infectivity and hence knowledge of the sorting mechanism could lead to novel ways of reducing HIV infectivity.

A.

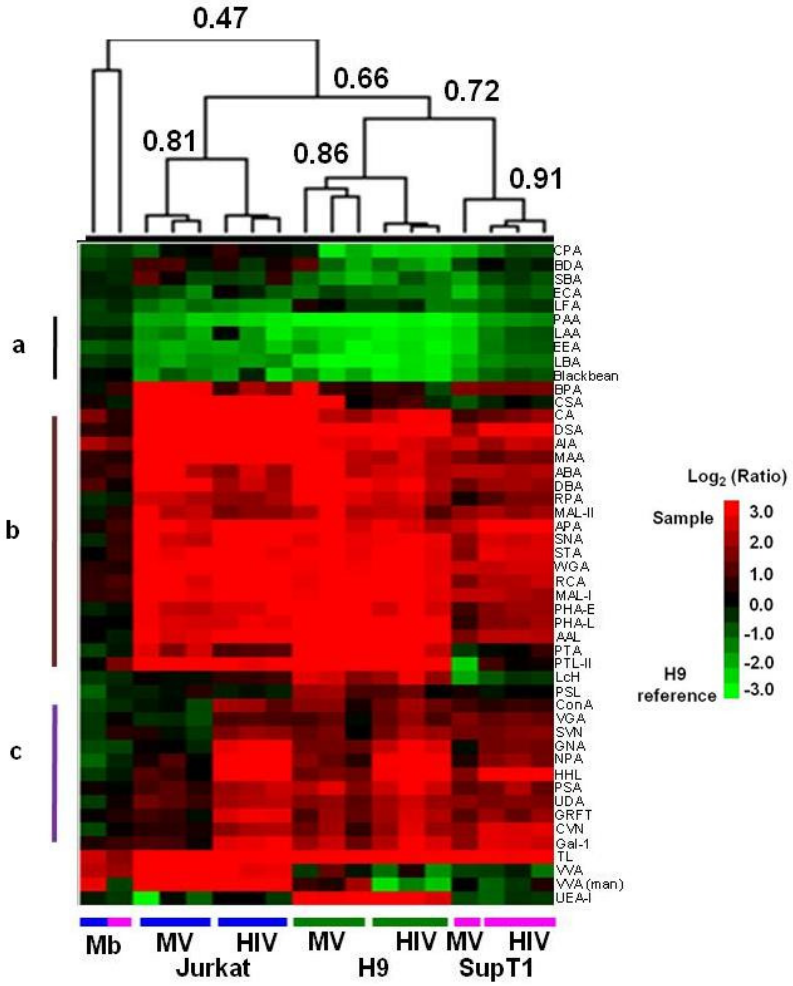


Figure 2.16. Ratiometric dual color comparison of microvesicles, cell membrane from uninfected cells and HIV-1 from panel of three T-cell lines. A) Glycomic comparison of HIV, MV and membrane samples derived from multiple T-cell lines. H9 membrane was used as the common biological reference for comparison of MV and HIV derived from different T-cell lines (H9 (green line), Jurkat-Tat-CCR5 (Jurkat, blue line), and SupT1 (pink line)) and their respective cell membranes (Mb). Different biological replicates of HIV and microvesicles (MV) were analyzed for each of the three cell lines. Yang correlation values [107] were obtained for the data set and used to generate heat maps using Cluster 3.0 and Java TreeView with the Pearson correlation coefficient as the distance metric for the samples, and average linkage analysis. To facilitate visualization, lectins were clustered using the Euclidean distance metric. The Pearson correlation coefficients are indicated at each point. Red indicates enhanced binding to the sample while green indicates enhanced binding to the reference (H9 membrane). Groups a and c refer to Blood group antigens, and high mannose respectively, while group b indicates sialic acids, LacNAc, complex and hybrid N-linkages.

B.

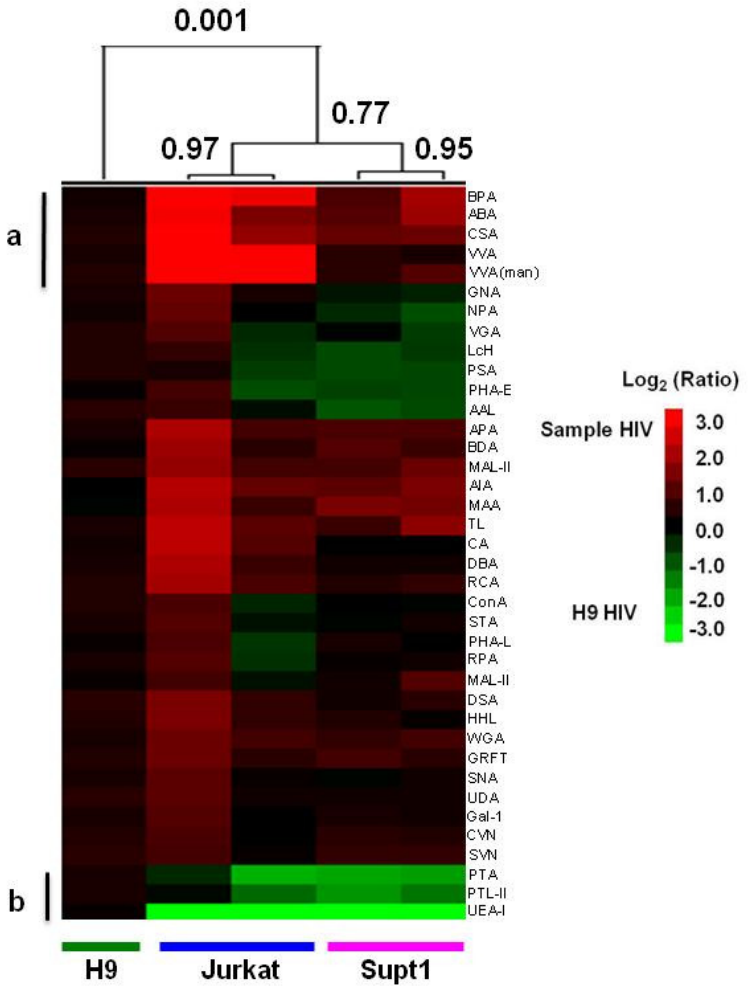


Figure 2.16. Ratiometric dual color comparison of microvesicles, cell membrane from uninfected cells and HIV-1 from panel of three T-cell lines. **(c)** H9-derived HIV was used as the biological reference to directly compare the glycome of HIV derived from two other T cell lines. Equal amounts of biological replicates of H9-, Jurkat- and SupT1-derived HIV were hybridized against the reference, with two arrays (dye-swapped pair) run for each sample to generate Yang correlations as before. The hierarchical cluster map is shown. Red indicates enhanced binding to the varying HIV samples while green indicates enhanced binding to the biological reference sample (H9-derived HIV). Groups a, b refer to β-GalNAc and α-1,2 –fucosylated LacNAc respectively.

To further confirm the cell line dependent differences in the HIV samples, I chose one of the biological replicates of H9 derived HIV as biological reference. Two

biological replicates of HIV derived from Jurkat and SupT1 were hybridized against the reference in ratiometric dual color approach. As expected, the hybridization of a second biological replicate of H9 derived HIV against the reference HIV sample showed little differences in their glycan compositions. The cell line based differences such as increased β-GalNAc in Jurkat cells and increased α-1,2-fucosylated epitopes in H9 cells were confirmed by this approach and further verified the cell line dependent differences in the glycans on HIV derived from distinct T cell lines (Figure 2.16.B). This data implies that the sharing of exocytic mechanisms by HIV and microvesicles is reflected in the cellular glycomic fingerprint that parent cells imprint on both HIV and microvesicles which exit these cells.

Viral gp120 accounts for the glycomic differences between microvesicles and HIV-1

If the theory that HIV and microvesicles utilize similar exit machinery and these particles share similar proteins, glycans and lipids is accurate, then the only difference between these two particles should emanate from the viral components in HIV which are absent in the microvesicles. The differences observed in the concentration of lipids, PIP and PIP2, between HIV and microvesicles seems to be a direct result of the binding of these lipids by viral Gag [60]. Along similar lines, the extensively glycosylated gp120 is the only viral protein present in the viral envelope along with the whole cadre of host derived proteins, hence it is possible that gp120 could account for some variations that are observed between HIV and microvesicles. Gp120 is heavily glycosylated and contains about 24 sites that are modified by complex, hybrid and high mannose N-linked structures [77]. Even though the mature virion only has about 20-40 copies of gp120 [122], one would expect to see differences between HIV and microvesicles in the

mannose binding lectins that bind to gp120. We observed that the biological replicates of HIV clustered very tightly (Jurkat-Tat-CCR5, R=0.96; H9, R=0.98, SupT1, R=0.97) and are different from microvesicles derived from the same cell line based upon increased high mannose levels (ConA, CVN, GNA, GRFT, HHL, NPA, PSA, SVN, UDA, Figure 2.14.A). Both microvesicles and HIV contained higher amounts of mannose residues when compared to the cell membrane (Figure 2.16.A).

We chose to investigate whether the highly glycosylated gp120 could account for differences observed between microvesicles and HIV from a given cell line. For a valid comparison, we used two strains of the related virus, simian immunodeficiency virus (SIV) SIVmac-NC and SIVmac-CP. The two strains differ in the amounts of gp120 present in the viral envelope with SIVmac-cp having approximately 10-fold higher levels (~200 copies per virion) when compared to SIVmac-nc (~ 20 copies per viron) [122, 123]. The SIV was obtained from SupT1 host cells and previous data from the lab indicated that microvesicles, SIV and HIV from SupT1 cells cluster very closely to one another [111].

To better delineate the difference between microvesicles and HIV, SIV from SupT1 cells, I used microvesicles from uninfected SupT1 cells as a biological reference in dual color microarray experiments (Fig 2.17.A). If the differences between microvesicles and HIV were only due to gp120, then lectins that have enhanced affinity for gp120 should discriminate between the SIV samples. The lectins that demonstrate increased affinity to SIVmac-cp include LacNAc binders such as DSA, RCA and WGA as well as the fucose binder, AAL. This increase in affinity could be explained by the increase in complex N-linked structures on gp120 found in SIV cp (Fig 2.17.A). I observed higher binding to the gp120 enriched SIVmac-CP than to SIVmac-NC for the anti-viral lectins that exhibited differential affinities to microvesicles and HIV. A select

subset of these lectins is shown in Figure 2.17.B. The anti-viral lectin, AIA, was an exception as it exhibited no differences in binding to microvesicles and SIVmac-NC, thus this effect is not attributable to differences in sample labeling (AIA, Figure 2.17.B). The SIV strains provide an excellent comparison to highlight the effect of gp120 glycosylation on the glycopattern observed for HIV and SIV. Since the difference between the SIV strains is attributable to gp120, it strongly suggests that gp120 accounts for the discrepancies observed between HIV and microvesicles from any given T cell line.

A.

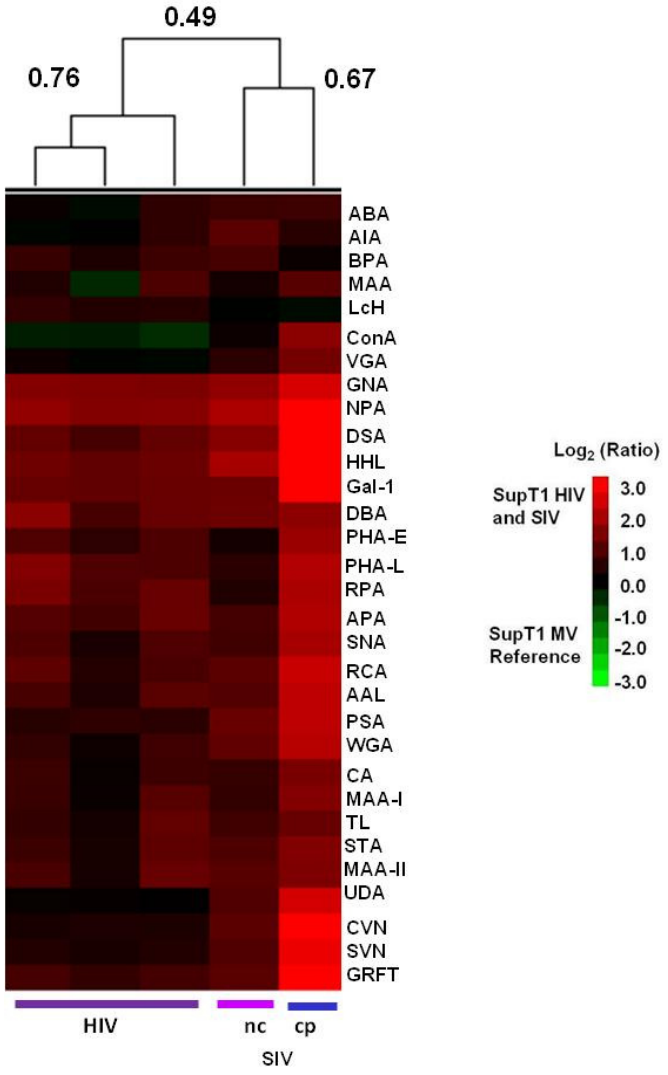


Figure 2.17. The glycans on gp120 could account for differences between glycopatterns of HIV and microvesicles. A) Direct comparison of the glycomes of SIVmac-NC, SIVmac-CP and HIV. Equal amounts of SupT1-derived HIV and SIV samples were hybridized against microvesicles derived from uninfected SupT1 cells (biological reference), with two arrays (dye-swapped pair) run for each sample to generate Yang correlations as described previously.

B.

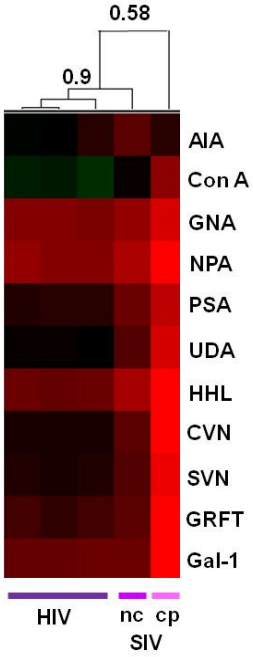


Figure 2.17. The glycans on gp120 could account for differences between glycopatterns of HIV and microvesicles. B) The hierarchical cluster map of a select group of anti-viral lectins with Pearson correlation coefficients is shown. Data used is the same as used in Fig 2.17.A. Red indicates enhanced binding to the sample while green indicates enhanced binding to the SupT1-derived MV reference.

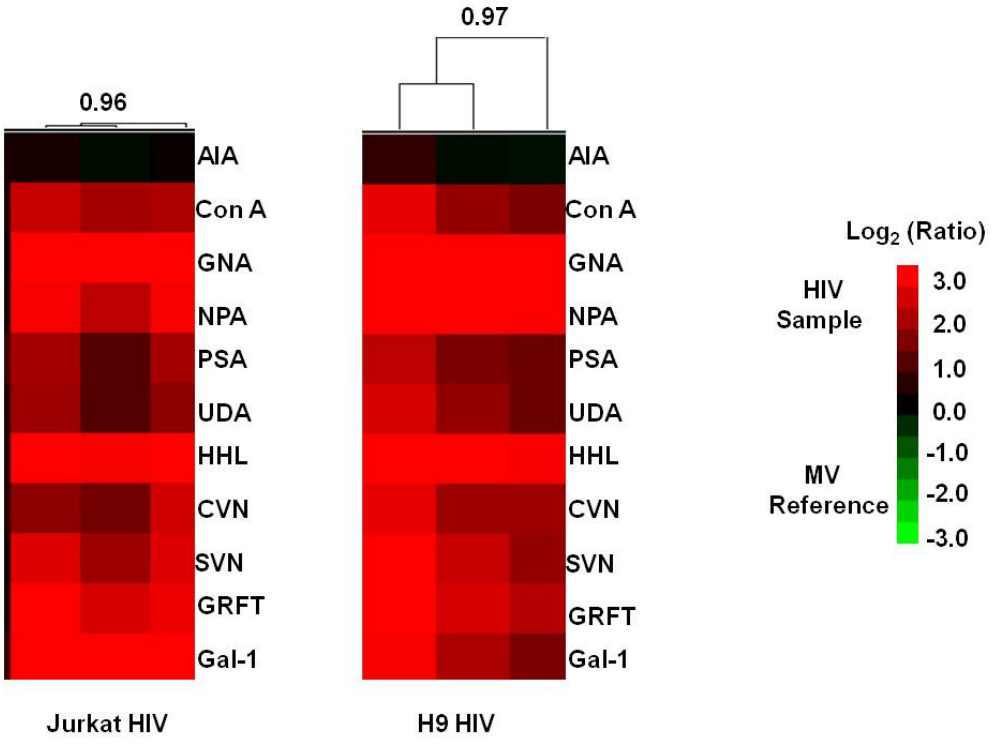


Figure 2.18. Mannose specific lectins exhibit enhanced binding to HIV samples over microvesicles derived from both Jurkat and H9 cells. Equal amounts of H9- and Jurkat-Tat-CCR5-derived HIV samples were hybridized against microvesicles derived from their respective uninfected cells (biological reference), with two arrays (dye-swapped pair) run for each sample to generate Yang correlations as before. The hierarchical cluster map of a select group of mannose-binding lectins is shown. Jacalin, AIA, which binds O-linked sugars has anti-viral activity and is included for comparison with binding patterns of the anti-viral mannose binding lectins. Red indicates enhanced binding to the sample while green indicates enhanced binding to the microvesicles. HIV clearly shows 4-8-fold better binding to mannose lectins than the matched microvesicles in both cell lines.

Similar data was also obtained when I compared microvesicles and HIV derived from both Jurkat and H9 cells using their respective microvesicles as a biological reference for ratiometric dual color analysis. A subset of the lectins which constitute the anti-virals lectins are shown in Figure 2.18. These lectins demonstrated increased affinities for the HIV samples as compared to their respective microvesicles. This again

strongly argues for the theory that the glycans on gp120 could account for the differences observed between HIV and normal cellular microvesicles. Taken together with the data obtained for the SIV variants, this data strongly suggests that HIV and microvesicles share an exit mechanism.

Clusters of high mannose epitopes were thought to be unique to the extensively glycosylated gp120 in comparison to host cell surface glycosylation. The absence of similar high mannose clusters on the surface of the host cell made these high mannose epitopes an excellent therapeutic target [79]. Surprisingly, both microvesicles and HIV-1 were enriched in these glycans when compared to the cell membrane. This enrichment of high mannose in both of these particles is a concern, as this epitope is a potential target for both anti-viral lectin based therapies and vaccine development [79, 85]. Recent attempts have included the synthesis of oligomannose dendrons as potential immunogens for generating a HIV vaccine [124]. This work also raises the possibility these mannose epitopes may play role in protein sorting to microvesicles. This, coupled with the proposed role of microvesicles in cellular processes and cell to cell communication, implies that targeting of these high mannose residues may have deleterious consequences. This work argues for a thorough study of the possible side effects prior to the use of high mannose binders for therapeutics.

2.3 Conclusions

The complex interactions of the host and HIV during viral replication have been of considerable interest to the scientific community due to their potential as emerging therapeutic avenues. Sharing of protein and lipid markers is highly indicative of common biogenesis mechanisms; however there are still debates about the validity of this hypothesis. The similarities between glycomic profiles of both microvesicles and HIV derived from T cell line argues that these particles likely bud from specific microdomains that are characterized by enrichments in proteins, lipids and glycan epitopes, thus making a stronger case for the utilization of similar budding machineries by both particles. Given the large number of host derived proteins in HIV, the mechanisms that are at play in recruiting individual proteins to microvesicles and HIV are still being investigated. This work opens up the possibility that glycan dependent protein sorting could play a role in microvesicular and HIV biogenesis. Given the role of microvesicles in wide variety of cellular processes and disease states, this work sets up the stage for dissecting potential cargo sorting mechanisms into these particles.

2.4 Materials and methods

Cell culture

Cell lines H9 and SupT1 were obtained from the AIDS Vaccine Program at NCI (Frederick, MD). Jurkat-Tat-CCR5 cells were obtained from Dr. Quentin Sattentau (University of Oxford, Oxford, England). Cells were cultured in RPMI 1640 (Hyclone, Logan, UT), supplemented with 2 mM L-glutamine, 10% fetal bovine serum (Mediatech, Herndon, VA) and Penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37 ºC and 5 % CO_2.

Cellular membrane preparations

Cell membrane samples from uninfected cells were prepared and labeled using published protocols [107]. Briefly, cells were pelleted, washed once in PBS (pH 7.4) and resuspended in ice-cold PBS. Cells were sonicated on ice (three times for 5 seconds each) to disrupt cell membranes. Membranes were isolated by pelleting for 1 h at 50,000 rpm using a TLA 100.3 rotor in Beckmann Optima L-80 ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet was resuspended in carbonate buffer (0.1 M Na_2CO_3, pH 9.3) and homogenized using a 20 gauge needle for about 10 times. The homogenization was repeated using a 27 gauge needle until the sample was completely homogenized. Protein concentration was determined using DC protein assay (Bio-Rad, Hercules, CA) and subsequently fluorescently labeled with –Cy3 or –Cy5 dyes (GE Life Sciences, Piscataway, NJ). One mg of protein was labeled with 60 μg of dye for 45 min at room temperature on a shaker. Excess dye was removed by dialysis (Spectrapor regenerated cellulose membrane, 6000-8000 Da) into PBS (pH 7.4) in the cold room overnight. Protein concentrations were determined after dialysis using DC protein assay.

Preparation of microvesicle and virus samples

Labeled microvesicles from matched uninfected cells and virus samples were obtained from our collaborators at the National Cancer Institute (Table 2). Briefly, virus samples were collected from the culture supernatants of infected cells. Both H9 and SupT1 cells were infected with HIV-1(MN) CL.4 virus strains while Jurkat-Tat-CCR5 cells were infected with HIV-1(MN) strain. SupT1 cells were also used to generate

infectious SIV strains; SIVmac-nc and SIV mac-cp. Microvesicles were isolated from matched uninfected T cells [58]. All samples were treated with Aldrithiol-2 [114]. Samples were resuspended in TNE buffer (0.01 M Tris pH 7.2, 0.1 M NaCl and 1 mM EDTA) and stored at -70 ºC prior to use.

Fluorescent labeling of the viral and microvesicular samples were performed by our collaborators. Briefly, samples were pelleted (60,000 rpm, 6 min, Beckman TLA-100 centrifuge, TLA100.3 rotor), resuspended in Cy-buffer (0.1 M Na_2CO_3/$NaHCO_3$ in H_2O, pH 9.3) and then incubated at room temperature for 30 min with Cy3- or Cy5-NHS. Excess free dye was removed from samples by centrifugation through a 20 % sucrose pad (25,000 rpm, 1 h, 4 ºC). The pellet was then resuspended in PBS, pelleted (60,000 rpm, 6 min) and diluted in 1 mL of PBS. Labeled samples were stored at -70ºC prior to hybridization to the lectin microarrays. The concentration of the samples were obtained using DC protein assay. Samples were solubilized in PBS with 0.2 % sarkosyl reagent to obtain protein concentrations.

Table 2 – Cell lines, Microvesicles and HIV-1 used in this study.

Agent	Cell Line Number	Replicate	Product Lot
H9 MV	CLN283	1	P4075
		2	P4076
		3	P4077
HIV-1(MN) CL.4/ H9	CLN71	1	P3935
		2	P3945
		3	P3944
HIV-1(MN) CL.4/SUPT1	CLN219	1	P4095
		2	P4092
		3	P4098
SUPT1 MV	CLN52	1	P3772
SIVmac-NC/SUPT1	CLN130	1	P3700
SIVmac-CP/SUPT1	CLN131	1	P3866
HIV-1(MN)/Jurkat-Tat-CCR5	CLN284	1	P4066
		2	P4067
		3	P4068
Jurkat-Tat-CCR5 MV	CLN259	1	P4058
		2	P4060
		3	P4061

CD45 immunodepletion of HIV-1 virions

Fluorescently labeled CD45 immunodepleted H9 derived HIV samples were obtained from our collaborators at the National Cancer Institute. Briefly, H9 derived HIV virions were immunodepleted using anti-CD45-conjugated paramagnetic microbeads (Miltenyi Biotech, Auburn, CA) as previously described [112].

Manufacture of Lectin microarrays

Lectin microarrays were manufactured as described previously with minor modifications [107]. All lectins were purchased from EY laboratories (San Mateo, CA) with the following exceptions: AAL, HHL, RCA, MAL-I, MAL-II, PTL-I and PTL-II

were obtained from Vector Labs (Burlingame, CA), cyanovirin [125], syctovirin [126], and griffithsin [127] were gifts from Dr. Barry O'Keefe (NCI-Frederick, Frederick, MD) while Galectin-1 [128] was a gift from Dr. Linda Baum (UCLA Medical School, Los Angeles, CA). Briefly, lectins were appropriately diluted in PBS containing 0.5 mg/mL BSA and 1 mM of appropriate carbohydrates (lactose, N-acetyl glucosamine (GlcNAc), mannose, galactose, or fucose). The print concentrations and the specificities of the lectins are given in Table 3.

Lectin microarrays were fabricated on Nexterion Slide H (Schott Nexterion) in a 16 sub-array format. The microarrays were printed using a Spotbot ArrayIt personal microarrayer (Telechem, Sunnyvale, CA). The appropriate humidity range (45-55%) and the temperature (8°C) were maintained throughout the print. SMP3B pins were used to print the arrays. After printing, the arrays were incubated at room temperature for 1 h before storage at -20°C until further use. The arrays could be stored at -20°C for two weeks with no appreciable loss in lectin activity.

Table 3. Panel of lectins used in microarray.

Lectin	Abbreviation	Print Concentration (µg/mL)	Rough Specificity*
Abrus precatorius	APA	500	Gal β-1,3GalNAc (TF antigen) > Gal,
Agaricus bisporus	ABA	500	Gal β-1-3GalNAc
Aleuria aurantia	AAL	1000	Fuc
Anguilla anguilla	AAA	1000	α-Fuc
Arachis hyogaea	PNA	500	Terminal Gal β-OR
Artocarpus intergrifolia (Jacalin)	AIA	500	α-GalNAc not substituted at C-6 (i.e. core 1, 3, T-antigen but not core 2).
Bauhinia purpurea	BPA	500	Primarily Gal β-1,3 or 1,4 but will also bind β-GalNAc more weakly
Black bean crude	Blackbean	1000	GalNAc
Bryonia dioica	BDA	500	GalNAc
Canavalia ensiformis	Con A	500	branched and terminal mannose, terminal GlcNAc
Cancer antennarius	CCA	500	9-*O*-Acetyl Sia and 4-*O*-Acetyl Sia
Caragana arborescens	CAA	500	GalNAc/Gal (monosaccharides best)
Cicer arietinum	CPA	1000	Complex
Colchicum autumnale	CA	500	Terminal Gal β-OR
Cystisus scoparius	CSA	500	β-GalNAc, terminal
Cyanovirin	CVN	500	a-1,2 mannose
Datura stramonium	DSA	500	GlcNAcβ-1,4GlcNAc oligomers and LacNAc
Dolichos biflorus	DBA	500	GalNAcα-OR
Erythrina cristagalli	ECA	1000	LacNAc and GalNAc

Lectin	Abbreviation	Print Concentration (µg/mL)	Rough Specificity*
Euonymus eurpaeus	EEA	1000	Blood Groups B and H
Galanthus nivalis	GNA	500	terminal α-1,3 mannose
Galectin-1	Gal-1	2500	LacNAc
Glycine max	SBA	500	terminal GalNAc
Griffithsin	GRFT	1000	Mannose, GlcNAc
Griffonia simplicifolia I	GS-I	500	α-galactose
Griffonia simplicifolia II	GS-II	500	terminal GlcNAc
Helix pomatia	HPA	500	α-GalNAc terminal
Hippeastrum Hybrid	HHL	1000	α-1,3 mannose and α-1,6 mannose
Homaris americanus	HMA	500	sialic acid
Iberis Amara	IAA	500	GalNAc
Laburnum alpinum	LAA	500	GlcNAc oligomers
Lens culinaris	LcH	1000	Complex (Man/GlcNAc core with α-1,6 Fuc)
Limax flavus	LFA	500	α-Sia
Limulus polphemus	LPA	500	α-Sia
Lotus tetragonolobus	LTL	500	Terminal α-Fuc, Lex
Lypersicon esculentum	LEA	1000	β-1,4GlcNAc oligomers
Maackia amurensis	MAA-I	1000	LacNAc
Maackia amurensis	MAA-II	1000	α-2,3 sialic acid
Maackia amurensis	MAA	500	α-2,3 sialic acid
Narcissus pseudonarcissus	NPA	1000	Terminal and internal Man
Persea Americana	PAA	500	Unknown
Phaseolus lunatus	LBA	1000	GalNAcα-1,3[Fucα-1,2]Gal
Phaseolus vulgaris-L	PHA-E	500	complex
Phaseolus vulgaris-L	PHA-L	500	β-1,6 branched trimannosyl core N-linked glycans
Pisum sativum	PEA, PSA	1000	Man

Lectin	Abbreviation	Print Concentration (µg/mL)	Rough Specificity*
Polyporus Squamosus	PSL	500	α-2,6 sialic acid
Psophocarpus tetragonolobus	PTA galactose	500	Gal
Psophocarpus tetragonolobus	PTL-I	1000	α-GalNAc
Psophocarpus tetragonolobus	PTL-II	1000	a-1,2 fucosylated LacNAc
Ricin B chain	RCA	1000	b-Gal/GalNAc
Robinia pseudoacacia	RPA	1000	Complex
Sambucus nigra	SNA	500	α-2,6 sialic acid on LacNAc
Scytovirin	SVN	500	α-1,2 mannose
Solanus tuberosum	STA	500	GlcNAc oligomers, LacNAc
Sophora japonica	SJA	1000	GalNAc
Trichosanthes kirilowii	TKA	500	β-Gal, LacNAc but Sia-α-2,3 or 2,6 inhibits best.
Trifolium repens	RTA	500	2-deoxy-Glu
Tritiicum vulgare	WGA	1000	β-GlcNAc, sialic acid, GalNAc
Tulipa sp.	TL	1000	GlcNAc
Ulex europaaeus I	UEA	500	α-fucose
Ulex europaaeus II	UEA-II	500	GlcNAc oligomers
Uritica dioica	UDA	500	GlcNAc β-1,4GlcNAc oligomers and high mannose epitopes
Vicia fava	VFA	500	Man>Glc>GlcNAc
Vicia graminea	VGA	500	O-linked Gal β-1,3GalNAc clusters
Vicia villosa	VVA	500	GalNAc
Vicia villosa	VVA (man)	500	Man
Wisteria floribunda	WFA	500	GalNAc

* Specificity data was obtained from a variety of sources including the Consortium for Functional Glycomics Carbohydrate Array Analysis and the Handbook of Plant Lectins (1998, Wiley and Sons).

Hybridization of samples to the microarray

The printed slides were brought to room temperature and then blocked using 50 mM Ethanolamine in 50 mM Sodium Borate solution for 1 h. The slides were then rinsed in PBST (PBS+0.05% Tween) for three times, followed by final rinse in PBS. The slides were then dried using a slide spinner (ISC Bio-express, Kaysville,UT) to remove any excess liquid. The microarray slides were fitted to the 16-well FAST frame (Schleicher & Schuell, Keene, NH) to create a separate well for each array resulting in a total of 16 sub-arrays per slide. Two of the sub-arrays were hybridized with 10 μg of glycoproteins with known lectin binding patterns such as Ovalalbumin and Porcine mucin as a quality control measure for each slide [107].

For single color experiments, 1 μg of labeled sample in a final volume of 100 μL (PBS+ 0.05%Tween) was added to each sub-array. Samples were hybridized to the arrays for 2 h at room temperature with gentle rocking. The individual sub-arrays were then rinsed five times with PBST (PBS, 0.5% Tween) for 3 min each. The slide was rinsed with PBS and dried using a slide spinner, prior to scanning. For ratiometric two-color experiments, 1 μg of each orthogonally labeled sample in a total volume of 100 μL (PBS+0.05% Tween) was used for a single array. Dye-swapped pairs were hybridized for each sample set using the respective Cy3- and Cy5- labeled pairs [107].

Inhibition experiments

The specificity of the lectin interactions were confirmed by carbohydrate inhibitions [110]. The slides were pre-incubated with 200 mM of each of the carbohydrates (Lactose, Mannose and *N*-Acetylglucosmaine (GlcNAc)) for 30 min. The

carbohydrates were diluted in PBS and 50 μL of carbohydrate solution was added to each array and incubated for 30 min with gentle rocking. 1 μg of Jurkat derived HIV-1 samples were diluted in 50 μL (PBS+0.1% Tween) and added to the arrays and incubated for an additional 2 h. The slides were processed as described before. The resultant data for five replicate spots of each lectin was Q-tested and the percentage inhibiton was calculated with respect to the uninhibited control using the following formula: Percentage of signal inhibited = 100*[((Average Signal of control array)-(Average signal of inhibited array))/Average signal of control array. The errors were propagated using the standard deviations for the signals [106]. Similar experiments were performed for Jurkat derived microvesicles and cell membrane.

Analysis of Microarray Data

Slides were analyzed using a GenePix 4000B fluorescent slide scanner (gain = 400, 5 μM scan, Molecular Devices, Sunnyvale, CA) with GenePix Pro 5.1 software. Statistical analysis was done as described previously [107]. To obtain ratiometric data, the background-subtracted median intensity for each spot was used. The data from five replicate spots per lectin was Q-tested using the Grubs Outlier test. Microsoft Excel was used for further data analysis. Data from a pair of dye-swapped arrays was used to generate the yang correlation [119]. Briefly, the $\log_2$ value for the ratio of binding intensities for the two samples were generated using Microsoft excel. Hierarchical clustering of the resultant data was generated using cluster analysis and visualized using Java tree View [129] with Pearson co-relation co-efficient as the distance metric and average linkage analysis [107].

For the cluster analysis, only lectins that were considered positive for one or more samples were used. Single color microarray data for individual samples was used to determine the set of positive lectins for a given sample. For each lectin, the median fluorescence intensity of the signal was divided by the local median background for five replicate spots. The resultant signal to noise ratios (S/N) for each lectin, were Q-tested using the Grubbs outlier test and averaged. Lectins which exhibited S/N + 1 standard deviation of 5 or greater were considered positive for a given sample.

Labeling of cells with N-Rh-PE

Jurkat cells were labeled with N-Rh-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-[lissamine rhodamine B sulfonyl]) (Avanti Polar Lipids, Alabaster, AL) for 1 h at 4°C . The labeling was performed in sterile glass test tubes which were pre-cooled to 4°C. N-Rh-PE was dissolved in ethanol and added to cold RPMI media (with no additives) at a final concentration of 5 μM using a Hamilton syringe. The cells were then washed extensively in cold RPMI media for 6 times by repeated pelleting at 150 x g and resuspension in fresh cold media. Labeled cells were cultured in regular media for an additional 20 h at 37 °C [40].

Fluorescence microscopy

For fluorescence microscopy, Jurkat cells were adhered to poly-lysine coated glass-bottomed dishes (In Vitro Scientific, Sunnyvale, CA) for 5 min at 37 °C. The adhered cells were further incubated with 5 % BSA in modified PBS (mPBS, 3.8 mM KCl, 1.18 mM KH_2PO_4, 1.39 mM NaCl, 3.15 mM Na_2HPO_4, 1 mM $MgSO_4$) for 1 h.

Following this incubation, the cells were fixed in 3% paraformaldehyde (Sigma Aldrich, St.Loius, MO) in mPBS for 15 min at room temperature. The cells were then washed with mPBS for three times, 5 min each. The unpermeabilized cells were labeled with either FITC-labeled DSA (Datura stramonium Lectin) or FITC-labeled PHA-L (Phytohemagglutinin-L, EY Labs) at a concentration of 4 μg/100 μL (total volume of 100 μL) for 15 min at room temperature. The cells were subsequently washed with mPBS (3 x 5 min) with gentle shaking to wash away unbound lectins. The cells were imaged using an inverted microscope (Nikon Eclipse TE 2000-U; Photometrics CoolSNAP ES monochrome camera) and MetaMorph image analysis software (Version 6.2r6; Molecular Devices) with a 60X oil immersion lens (NA 1.4). FITC and Rhodamine images were obtained using the same dichroic mirror (86012bs, Chroma Technologies, Rockingham Vermont) and separate excitation (ex.) and emission (em.) filters (FITC: ex. S501/16, em S534/30; Rhodamine: ex. S568/24, em. S610/40). For confocal microscopy, cells were prepared as previously described and stained with FITC conjugated DSA.. Confocal images were obtained using the Leica confocal system TCS4D with a 63X oil immersion lens (NA 1.4; FITC: ex. 503 nm, em. 552 nm; Rhodamine: ex. 561 nm, em. 624 nm; Core Facility, Institute for Cell and Molecular Biology, University of Texas, Austin, TX).

Western blot analysis

Equal amounts (1 μg) of unlabeled microvesicles from matched uninfected cells and HIV derived from Jurkat-tat-CCR5 cells were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked for 1 h in block

buffer (5% BSA in TBS, 0.1% Tween) and then incubated with anti-p24 antibody (1:50,000, AIDS Vaccine Research Program, NCI) in blocking buffer for 1 h. The membrane was then washed (6 x with TBST) and incubated with goat-anti-mouse-HRP (1:10,000, BioRad, Hercules, CA) for 1 h. The membrane was then rinsed 6 x with TBST and developed using chemiluminscent substrate (West Femto substrate, Pierce, Rockford, IL) and imaged using a Kodak IS4000R imaging station (Carestream Health, Rochester, NY).

Equal amounts of CD45 immunodepleted and non-depleted virus samples (based on p24 viral antigen amounts) were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in block buffer (5% BSA in TBS, 0.1% Tween) and then incubated with either anti-p24 antibody (1:50,000, AIDS Vaccine Research Program, NCI) or anti-CD45 antibody (1:500, Clone 69, BD Transduction Laboratories, San Jose, CA) in blocking buffer for 1 h. The membrane was then washed (6 x with TBST) and incubated with goat-anti-mouse-HRP (1:10,000, BioRad, Hercules, CA) for 1 h. The membrane was then rinsed 6 x with TBST and visualized as described previously.

Electron microscopy

Electron microscopy images of the HIV samples were collected and processed by our collaborators at the National Cancer Institute [130].

Fluorescence microscopy of PNGase F-treated Jurkat cells

Jurkat-Tat-CCR5 cells were adhered to poly-Lysine coated glass bottom dishes and fixed as described previously. The fixed cells were then treated with 100 U of PNGase F in PBS or PBS alone at 37°C for 1 hour [131]. Cells were washed with PBS (3x for 5 min each), stained with FITC-conjugated DSA and fluorescent images were obtained as described previously.

Chapter 3: Galectin-1 binds HIV via mannose residues in addition to LacNAc epitopes

3.1 Introduction

The glycomic similarities between microvesicles and HIV were revealed using our systems based lectin microarray approach, supporting the theory that HIV and microvesicles share exocytic machinery in T cells. The lectin array consists of lectins with varied specificities, some of which are overlapping while others are more distinct (Table 1). Upon closer examination of the hierarchical clustering of HIV and microvesicles derived from three T cell lines (Figure 2.16.A) , we were surprised to note the presence of Galectin-1, a β-galactoside binding lectin, in the midst of a cluster of mannose binding lectins. This was particularly intriguing as several of these lectins are known anti-viral lectins [85].

Galectins are a family of immune lectins which are known to bind β-galactosides. Different members of the family are involved in a variety of processes including cell proliferation, cellular differentiation and death, tumor cell metastasis and trafficking of lecukocytes [132]. There are about 14 members of this family, which share a conserved carbohydrate-recognition domain (CRD) and a common structural fold [133]. Galectins bind preferentially to *N*-acetyllactosamine (Gal-β-1, 4-GlcNAc) units, which are mainly found in the termini of complex *N*-linked glycans and to poly *N*-acetyllactosamine which are found on *O*-linked and *N*-linked chains. In spite of the similarities in structure, members of the galectin family have also been shown to bind different ligands and thus effect specific functions [134].

Members of the galectin family can exist in as monomers (gal-5,-7), or can multimerize as either homodimers (gal-1), or chimera type (gal,-3). This multivalency allows galectins to form glycoprotein lattices by binding multiple ligands simultaneously.

Galectins are often released into the extracellular space and thus can mediate bridging interactions between glycoproteins expressed on different cells. Apart from its cellular functions, galectins are also known to play in host pathogen interactions. For example, gal-3 is known to affect binding of bacteria to host cell surface [135] while gal-9 enhances binding of the protozoan parasite, *Leshmania major* to macrophages [136] .

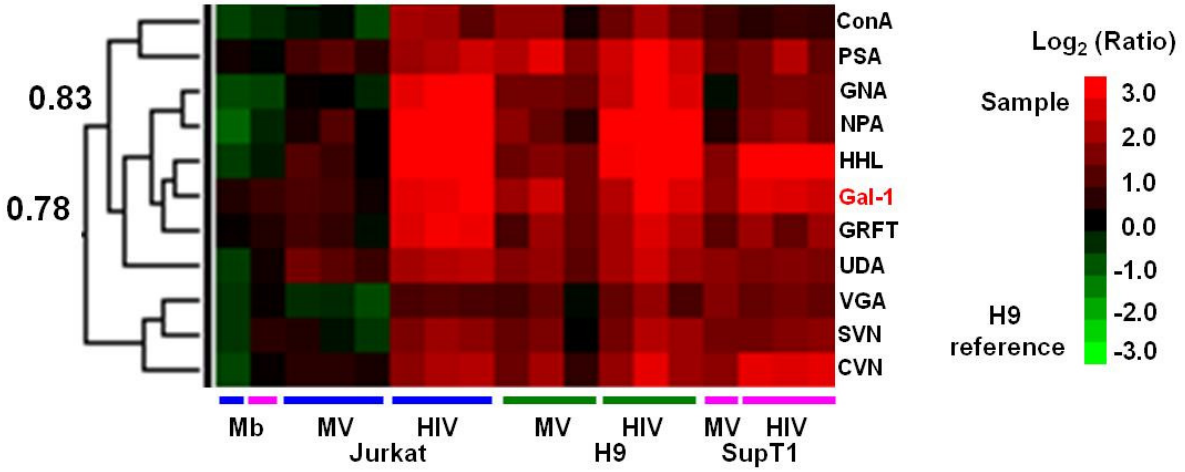
The role of Galectin-1 in HIV biology has been examined in both macrophages and T cells. It was first demonstrated that galectin-1 but not galectin-3 stabilizes HIV interactions with the host cell by forming stable interactions with the host cell and the virus. This bridging interaction was inhibited by addition of lactose, confirming the carbohydrate dependent interaction of galectin-1 with HIV [137]. Galectin-1 was also shown to enhance viral adsorption on macrophages in a lactose dependent manner [138]. During the course of both these experiments, lactose seems to the only carbohydrate that was examined for its inhibitory effects on galectin mediated HIV binding. Our systems based approach points to mannose binding as a contributory factor in galectin mediated interactions and warrants further investigation.

3.2 Results and discussion

Ratiometric analysis of HIV and microvesicles derived from panel of T cells lines revealed that galectin-1 was at the center of a cluster of lectins that binds to high-mannose epitopes (GNA, NPA, HHL, CVN, SVN, GRFT R=0.78, N=18, P=0.0001, Fig 3.1.A). Contrary to expectations, galectin-1 did not cluster with other lectins that bind to LacNAc epitopes (R=0.36, n=18, P=0.14), although these lectins clustered closely together (R=0.87, P<0.0001, lectins: MAL-I, RCA, WGA, STA, DSA, Fig. 3.1.B). This was surprising as there are no reports that indicate galectin-1 can bind to mannose

residues. Our data indicates that galectin-1 may bind to high mannose epitopes in certain contexts, such as high cluster of mannose residues found in gp120. This could represent an additional mode of binding apart from its usual binding partner, LacNAc [139].

A.



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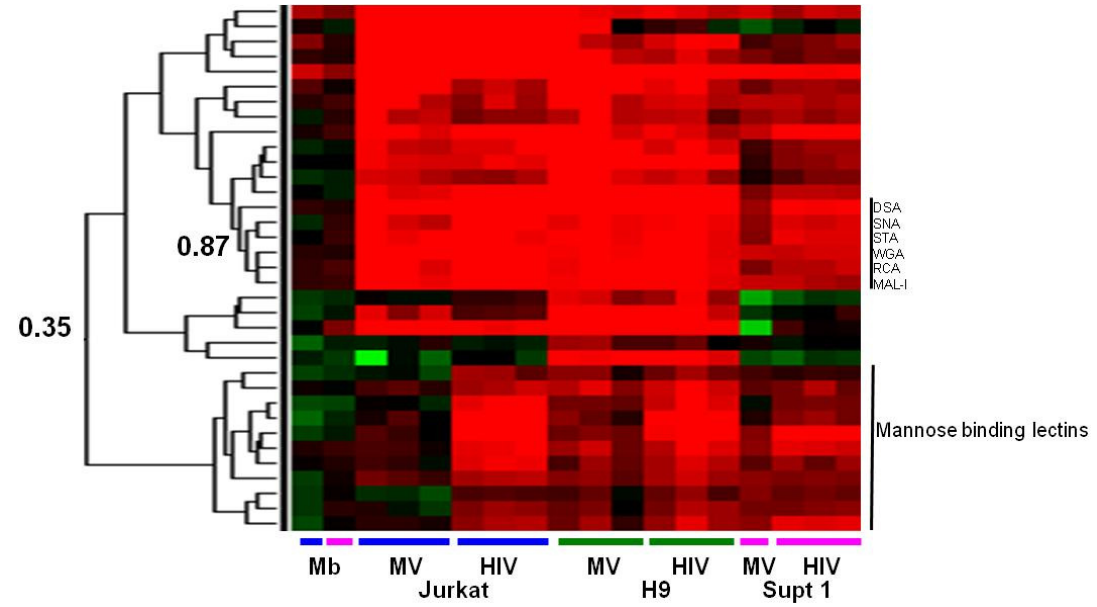


Figure 3.1. Galectin-1 clusters with mannose binding lectins rather than LacNAc binders. A) Galectin-1 (Gal-1) clusters tightly with mannose binding lectins (R = 0.78, n=18, P = 0.0001) B. Galectin-1 does not cluster with other LacNAc binding lectins (R = 0.35, P = 0.142). Data used for the hierarchical clustering is identical to that in Figure 2.16.A. Lectins were clustered using the Pearson correlation coefficient as the distance metric and average linkage analysis. A portion of the heat map is displayed with the Pearson correlation coefficients indicated for selected branch points.

We then examined the inhibition of H9 derived HIV and microvesicles using both lactose and mannose. Both mannose and lactose seemed to significantly inhibit galectin-1 binding to microvesicles. The inhibition of galectin-1 binding with lactose indicates that galectin-1 on our array is active. This is important due to concerns that immobilization of galectin-1 on the array could adversely affect the activity of galectin-1. Mannose greatly inhibited HIV binding to galectin-1, indicating that galectin-1 may bind to high mannose in the context of certain glycoproteins such as gp120 (Fig 3.2).

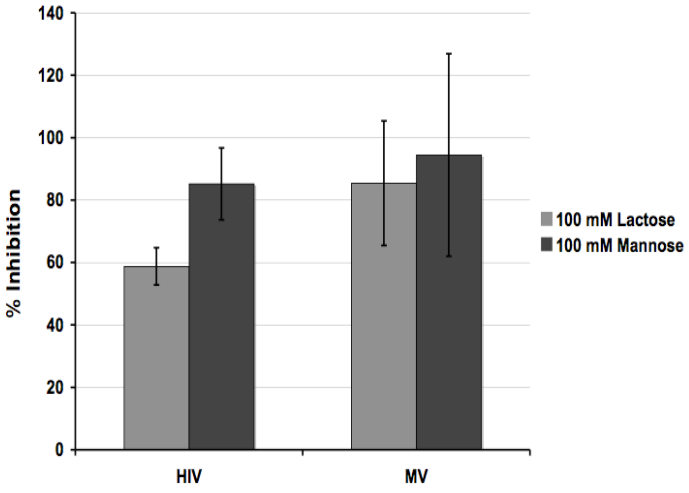


Figure 3.2. Lactose almost completely inhibits H9-derived MV binding to Galectin-1 but not to H9-derived HIV. In contrast to mannose which causes >85% inhibition of galectin-1 binding to both MV and HIV-1, lactose inhibits HIV-1/galectin-1 interactions only 59% but inhibits the MV signal 85%. The graph depicts the % inhibition of each lectin as discussed previously. The errors were propagated using the standard deviations and standard propagation of error equations.

To further evaluate this potential mode of interaction using our lectin microarray technology, I performed inhibition experiments with differing amounts of lactose and mannose to compare the inhibitory effects of the two carbohydrate motifs on the binding

of H9 derived HIV by galectin-1. Mannose is a better inhibitor of galectin-1 binding to HIV than lactose over a range of inhibitory sugar concentrations (Fig 3.3.A). The inhibition of the GalNAc binding lectin, Jacalin with lactose (Fig 3.3.B) and the mannose binding lectin, Concanavalin A with mannose (Fig 3.3.C) confirms the specificity of inhibitions observed.

A.

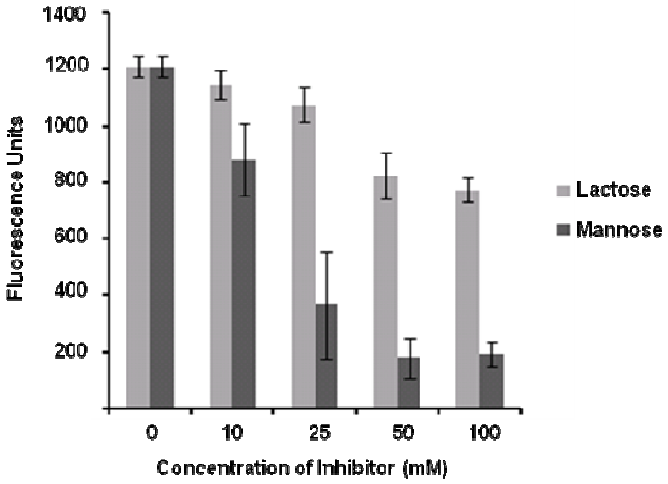
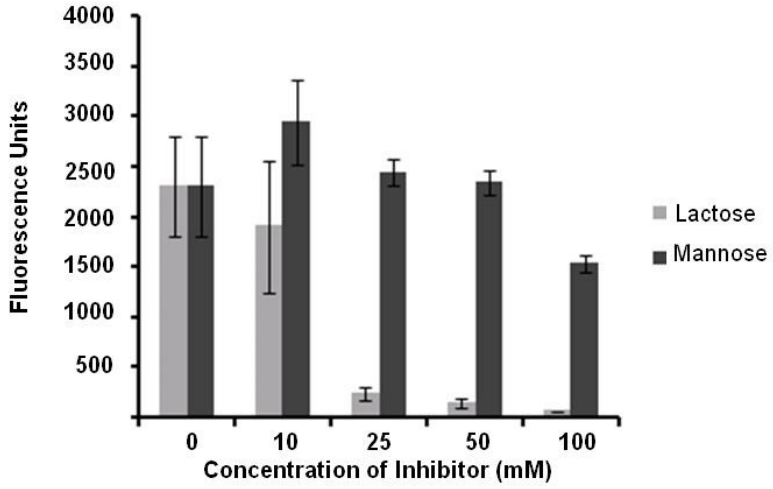


Figure 3.3. Mannose is a better inhibitor of galectin-1 binding to HIV in comparison to lactose. A) Inhibition of Galectin-1 interaction with HIV is displayed as a function of final concentration of inhibitor (lactose, light grey; mannose, dark grey). Error bars represent the standard deviation for the 4 spots.

B.



C.

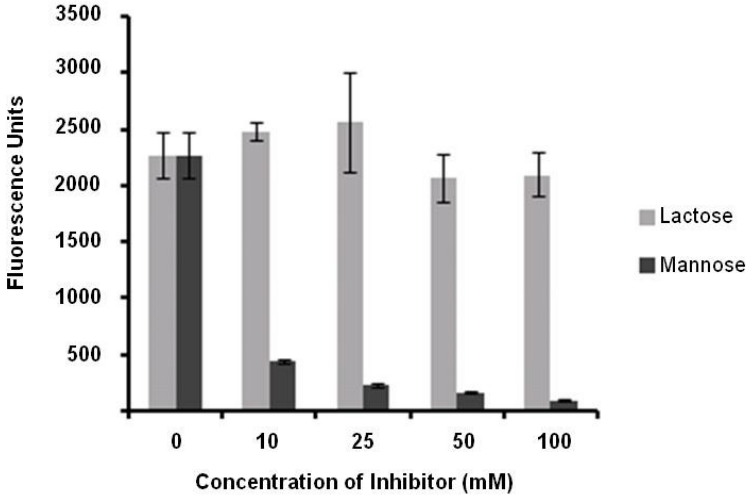


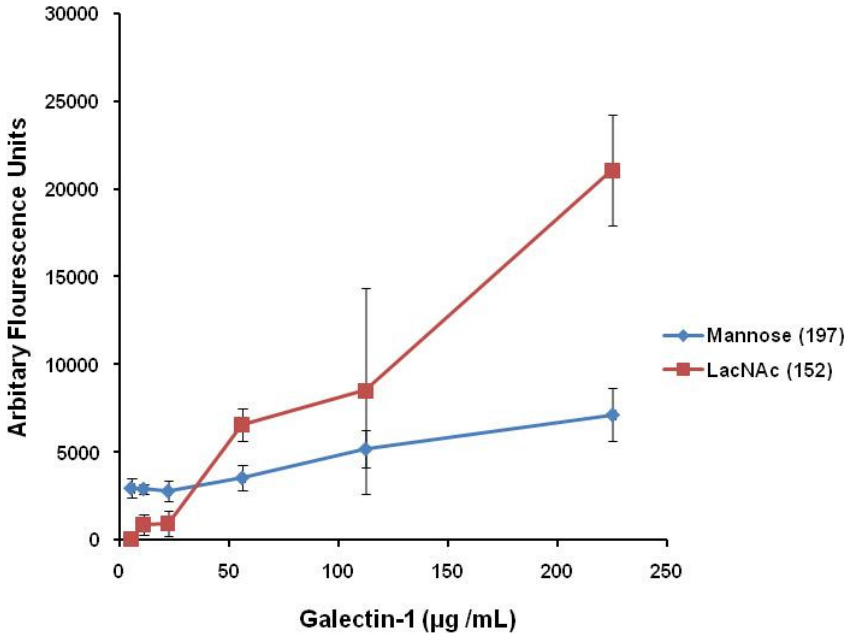
Figure 3.3. Mannose is a better inhibitor of galectin-1 binding to HIV-1 in comparison to lactose. A B) Inhibition of Jacalin binding to HIV is displayed as a function of final concentration of inhibitor (lactose, light grey; mannose, dark grey). Mannose does not seem to inhibit this binding with the exception of the 100 mM Lactose. C) Inhibition of Concanavalin A binding to HIV is displayed as a function of final concentration of inhibitor (lactose, light grey; mannose, dark grey). Lactose does not seem to inhibit this binding even at 100 mM.

A closer examination of the reported binding motifs of galectin family revealed that even though they can all bind LacNAc, some galectins have been known to bind mannose residues. Galectin-3 is known to bind β-1,2 mannans present in yeast cell surface in addition to LacNAc [140, 141]. Another member of the galectin family, galectin-10, has been previously demonstrated to exhibit greater affinity to mannose when compared to LacNAc [141].

We also examined the publically available database of the Consortium for Functional Glycomic (CFG) to determine if there are any reports of galectin-1 binding to mannose on the glycan array. The CFG (www.functionalglycomics.org) as a part of its initiative investigates the binding of lectins to a wide variety of immobilized glycoprotein and glycolipid glycans (~300) [139, 142]. The data for galectin-1 binding was obtained from experiments performed by the laboratory of Dr. Richard Cummings [134], indicates that galectin-1 binds to high mannose epitope (Glycan 197) at low but significant levels. This was further confirmed by examining the glycan array data for a range of concentrations of galectin-1 (225 μg/mL to 5.625 μg/mL) from the database. We compared the affinities of galectin-1 to LacNAc ligand (Galβ-1,4GlcNAcβ-Sp0 – Glycan 152) and this particular high mannose epitope over a range of galectin-1 concentrations (Fig 3.4). Even though, there were other high mannose epitopes on the glycan array, this particular glycan was bound by galectin-1 even at low concentrations of the lectin, strongly indicating that this interaction is specific. Thus, it seems likely that galectin-1 may bind high mannose in the context of certain ligands such as gp120 in HIV. This

mode of binding is probably in addition to the LacNAc binding exhibited by galectin-1 to proteins in the viral envelope.

A.



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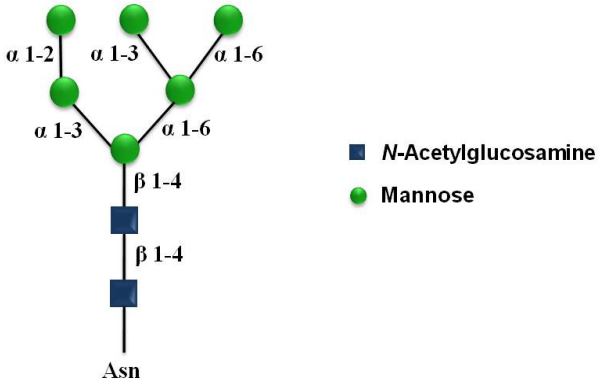


Figure 3.4. Comparison of dose–dependent binding of galectin-1 to mannose ligand (197) and LacNAc (152). A) Publically available data from the Consortium for Functional Glycomics (CFG) reveals that galectin-1 binds to high mannose epitope at low levels [134]. Binding of galectin-1 to LacNAc is plotted for comparison. B) Galectin-1 binds the high mannose epitope (197).

3.3 Conclusions

Our systems based approach of analyzing carbohydrate epitopes provides us with a large data set, which may reveal many potential interesting interactions. One such interaction, the binding of galectin-1 to high mannose, was further explored to give us insights into its mode of binding, which appears to have been overlooked so far. Traditional experiments involving galectin seem to only test the known inhibitor LacNAc to inhibit galectin binding. Discovery of this interesting mode of action was a consequence of our experimental design, in which inhibitions of the lectin on the array are typically performed with a small panel of sugars. Thus, it seems likely that galectin-1 can bind certain high mannose epitopes and this may play a critical role in the interaction of HIV with this immune lectin. The relevance of this mode of interaction in HIV entry and infection needs to be further examined.

3.4 Materials and methods

Inhibition experiments with titrations of different amounts of carbohydrates

Lectin microarrays containing galectin-1 (printed at 1.0 mg/mL) [128] were preincubated with varying concentrations of either lactose or mannose (50 μL in PBS) for 30 min, followed by addition of Cy3-labeled H9-derived HIV samples (1 μg, 50 μL in PBS with 0.1 % Tween). Samples were incubated for 2 h and slides were processed as previously described.

Chapter 4: Initial lectin microarray analysis of lipid rafts and microvesicles from Jurkat Tat-CCR5 T cells

4.1 Introduction

Taken together with previously reported proteomic and lipidomic similarities, the observed glycomic similarities between HIV and microvesicles strongly supports the theory that microvesicles and HIV bud from specific regions of the plasma membrane i.e. membrane microdomains which can be defined by specific protein, lipid and glycan compositions and are distinct from the rest of the plasma membrane [40, 60]. It is now well accepted that the plasma membrane consists of different microdomains which are dynamic and are modulated by lipid and protein mediated interactions [143]. Lipid rafts are a type of membrane microdomain, which are characterized by unique compositions of lipids including cholesterol, glycosphingolipids and saturated acyl chain phospholipids. These cholesterol rich regions also contains GPI (glycosylphosphatidylinositol) anchored proteins and proteins modified with palmitoylation and myristylation. Proteins modified with unsaturated acyl chains such as prenylated chains are excluded from these regions. The saturated chain modifications likely allow for tight packing of these proteins into raft regions in the plasma membrane [144]. The unique composition of lipid raft regions render them insensitive to detergent extraction at low temperatures, thus enabling their isolation. Raft components are typically isolated by lysing cells with detergents, followed by sucrose density centrifugation to separate the detergent insoluble fractions [145].

Lipid rafts are known to be involved in signal transduction and cellular processes such as T cell activation. Lipid rafts provide an attractive platform for cell signaling as they can facilitate the physical proximity of key signaling molecules thus enabling their interactions [43, 146] . The size and composition of lipid rafts are known to vary

dynamically based on the activation state of the cell. The size of lipid rafts has been proposed to range from 30-750 nm and is thought to account for 15-50% of the plasma membrane [147] [144].

Pathogens such as bacteria *(Escherichia coli, neisseria gonorrhoease,* and *Pseudomonas aeruginosa)* and viruses (Simian Virus 40 (SV40), influenza, measles virus and HIV are known to use raft regions to mediate their entry into mammalian cells [148, 149]. Viruses also are known to utilize raft domains to exit host cells (Fig 4.1A) [150]. The role of plasma membrane rafts in budding of HIV from T cells has been demonstrated by two independent groups [39, 52] (Fig 4.1.A).

The HIV viral protein, Gag, is myristylated and is targeted to lipid rafts [46, 47]. The envelope glycoprotein is palmitoylated and targeted to raft regions of the cell membrane [151] . Gag is also known to be involved in the targeting of the envelope glycoproteins (gp120 and gp41) to lipid raft regions [152, 153]. Both HIV and lipid rafts from infected cells exclude CD45 and are enriched in raft markers such as CD59 and Thy-1 [39]. Cholesterol depleting agents such as methyl-β-cyclodextrin are routinely used to confirm the association of proteins with these cholesterol rich raft regions [154]. Treatment with methyl-β-cyclodextrin is known to affect the stability of virion structures, thus implicating raft regions in the budding of HIV [155]. Similarities between the lipid composition of HIV and lipid rafts derived from T cells further implicated lipid rafts as key players in HIV biogenesis [50]. Thus, lipid rafts are crucial to HIV budding and egress in T cells.

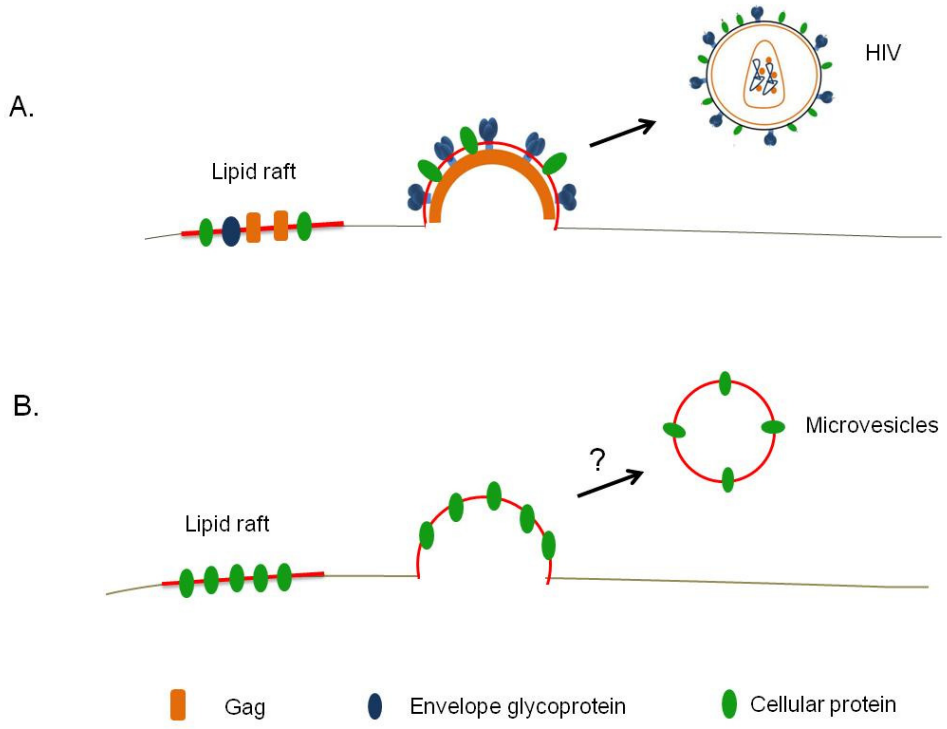


Figure 4.1.Role of lipid rafts in HIV and microvesicular biogenesis. A) Lipid raft regions are involved in biogenesis of HIV in T cells. B) The role of lipid raft regions in biogenesis of microvesicles in T cells has not been examined so far.

There is some data to indicate that lipid raft microdomains may also be involved in microvesicular biogenesis (Fig 4.1.B). B cells were found to release microvesicles which contain lipid rafts domains, implying that these microvesicles were formed from lipid raft regions of the membrane [156]. Proteins such as tissue factor [157], and transferrin 2 [158] were both shown to localize to lipid raft regions and subsequently secreted in microvesicles, strongly advocating a role for these regions in microvesicular biogenesis. In addition, protein oligomerization has been shown to target proteins to microvesicles [100] and lipid rafts in epithelial cells [159]. Moreover, cells treated with methyl-β-cyclodextrin did not display domain specific enrichment of N-Rh-PE in T cells. Taken together, this data points to a possible role for lipid rafts in microvesicles

biogenesis in T cells as well [40], though this has never been carefully examined. Considering that the glycome of lipid rafts has never been examined, we chose to use lectin microarray technology to determine if lipid rafts from T cells have similar glycomic signatures as microvesicles and HIV. This study would provide initial evidence that lipid rafts are the likely sites of budding of both these particles and potentially link the two proposed mechanisms that HIV uses to bud from the plasma membrane of T cells.

4.2 Results and discussion

Isolation of lipid rafts

Jurkat-Tat-CCR5 cells were grown in microvesicle depleted media for 48 h before isolation of microvesicles from the culture supernatant. Cell membrane samples were prepared from the cells as previously described. Matched cells were also used for isolation of lipid rafts using the Optiprep (Iodixanol) gradient method. This particular method was chosen as it was previously used for performing a comparative lipid analysis of lipid rafts and HIV from T cells [50]. Moreover, Gag has been shown to localize to lipid rafts using Optiprep gradient separation [47]. The second reason for using method is that it is relatively convenient and less time consuming when compared to traditional sucrose density centrifugations [160].

We chose the two detergents (1% Triton X-100 and 0.5% Brij 96) that were used to demonstrate similarities between lipid rafts and HIV from T cells. Lipid raft fractions were isolated by lysing cells in the presence of 1% Triton X-100, followed by ultracentrifugation using Optiprep gradient to separate the fractions based on density. Lipid raft fractions were taken from top to bottom of the gradient and analyzed by western blot for the presence of a known raft marker ,flotillin-1 and absence of a non-raft

marker, transferrin. These markers are commonly used to assay lipid raft fractions [50, 147].

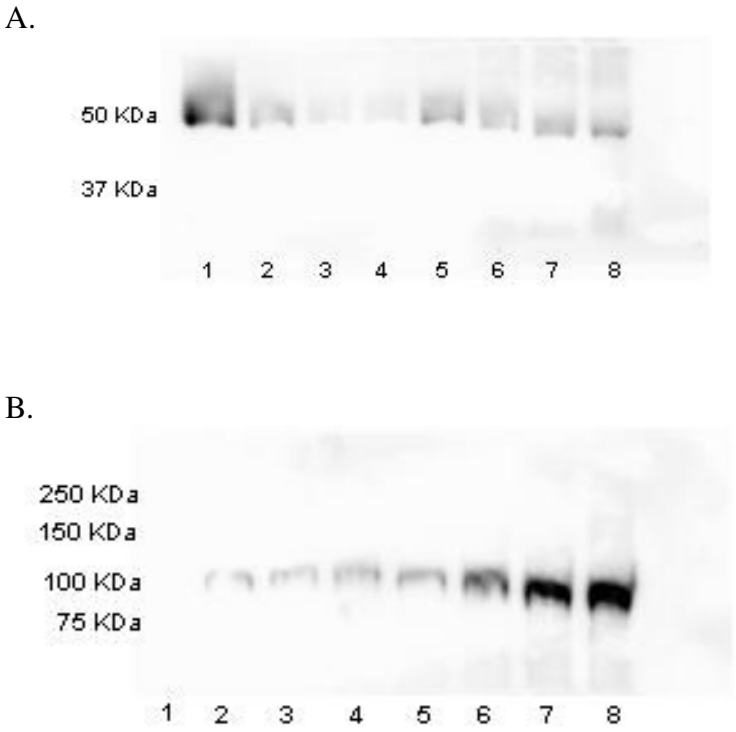


Figure 4.2. Western Blot analysis of fractions from lipid raft isolation. Lipid rafts were isolated form T cells using lysis buffer containing 1% Triton X-100, followed by separation using Optiprep density gradient. Lanes 1-8 indicate the number of fractions with lane 1 starting at the top and lane 8 at the bottom of the Optiprep gradient isolation. Equal volumes of lipid raft fractions were separated on SDS-PAGE and transferred to nitrocellulose membrane. A) Western blot analysis with anti-Flotillin-1 reveals the presence of flotillin-1 in the lighter fraction (Lane 1). B) Western blot analysis with anti-transferrin reveals the absence of transferrin in lighter fraction (Lane 1) and presence of transferrin in heavy fractions (7-8).

Western blot analysis revealed the concentration of flotillin-1 in fraction 1 (Figure 4.2.A). The presence of flotillin-1 was also detected in heavy fractions indicating possible contamination of the non-raft fractions. Most raft isolations tend to have a clean

distribution of flotillin-1, hence this could be an indication of some technical difficulties associated with the raft isolation protocol. The absence of non-raft marker, transferrin, in fraction 1, indicates that fraction 1 represents a clean raft fraction. This fraction was used as the lipid raft fraction for further analysis. Biological replicates of microvesicles, lipid rafts and cellular membrane preparations were prepared from Jurkat-Tat-CCR5. The samples were labeled with Cy3 and Cy5 dyes and used for lectin microarray experiments.

Lipid raft isolations can be performed using multiple detergents and Brij 96 has been used previously in the same report to demonstrate similarities in lipid compositions between HIV and lipid rafts. As an alternate method of isolating lipid rafts, I used 0.5% Brij 96 in the procedure instead of Triton X-100. A broader distribution of flotillin-1 was observed in the fractions when compared to those isolated by Triton X-100 [50]. Using the same protocol, I obtained a very broad distribution of flotillin-1 and transferrin, underlining the technical difficulties associated with these isolations. Based on these markers, I judged the lipid raft fractions obtained using Brij 96 to be unsuitable for the glycomic analysis of lipid rafts. Given these variations observed with different protocols, future studies should include lipid raft isolation using traditional sucrose density gradients along with the protocol discussed above. In addition, lipid rafts can be also isolated using detergent free methods [161]. A comparative analysis of the glycome of lipid rafts isolated using different protocols combined with their proteomic analysis (using a broader panel of known lipid raft markers) could provide us with another biochemical characteristic which could be potentially used to better define lipid raft regions.

Lectin microarray analysis reveals similarities between microvesicles prepared by different isolation protocols

The glycomic analysis of microvesicles and HIV derived from Jurkat revealed a similar glycome for the exocytosed particles, strongly suggesting that these particles share an exocytic mechanism. The glycomic signatures of microvesicles and lipid rafts isolated from matched Jurkat cells would be expected to be similar if lipid rafts function as sites of microvesicular release in T cells. Microvesicles used for the initial studies in this thesis were obtained from our collaborators at the National Cancer Institute. Their procedure involves flotation of cell culture supernatant through a sucrose density gradient followed by isolation of vesicles at the densities used to isolate HIV [58]. For accurate comparison, it was necessary to obtain microvesicles, lipid rafts and cellular membrane from matched cells. Given that the FBS used in cell culture contains bovine microvesicles, cells were grown in microvesicle-depleted media for 48 h and microvesicles were isolated from the culture supernatant by differential centrifugation [162]. The cells were then used for the isolation of lipid rafts and cellular membranes. The samples were fluorescently labeled prior to hybridization on the array.

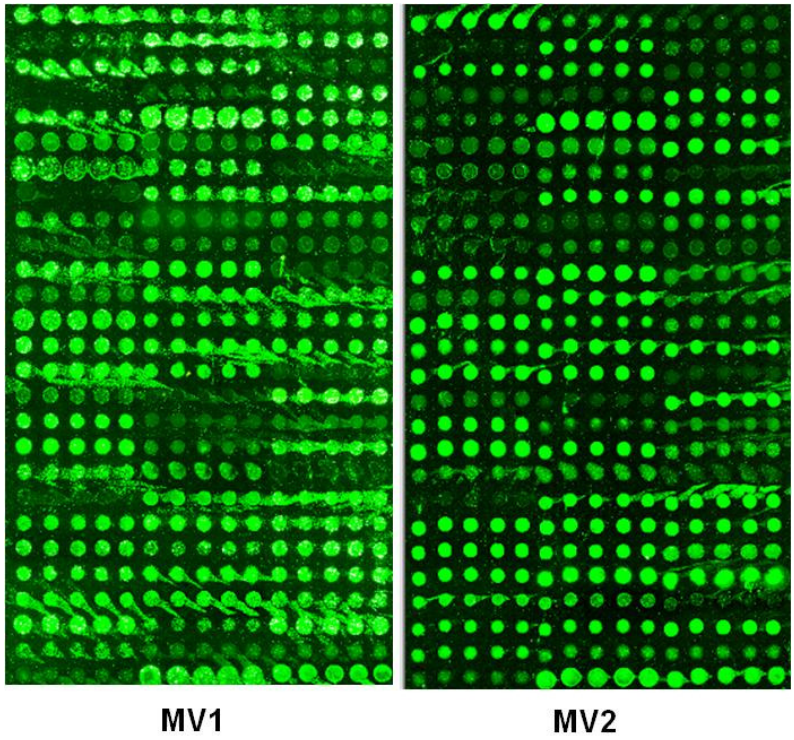


Figure 4.3. Jurkat derived microvesicles isolated by different protocols exhibit similar glycomic profiles. Equal amounts of Cy3- labeled samples (1 µg each) were hybridized to the array. MV1 represents microvesicles which were separated by sucrose density gradient (obtained from collaborators) and MV2 represents microvesicles isolated by differential centrifugation.

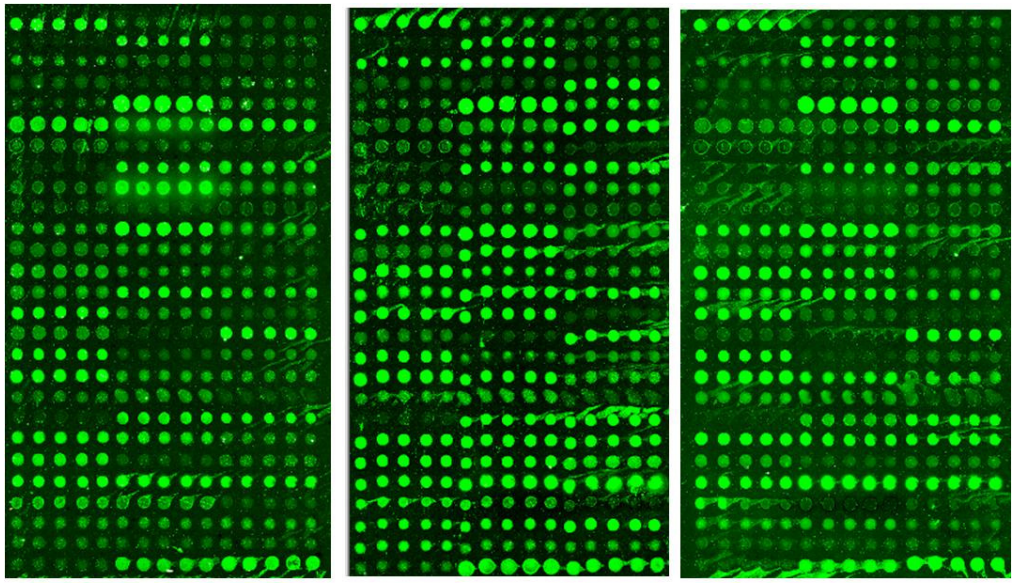
The glycomic signatures of microvesicles obtained by the two protocols were compared by single color lectin microarray analysis. Equal amounts of samples (based on protein concentrations) were hybridized to the lectin microarrays. The single color analysis revealed that the microvesicles isolated by the different protocols were almost identical (Fig 4.3). One exception was found, the lectin, BPA, which exhibited significantly increased binding to microvesicles obtained from our collaborators. The reasons for the difference in the binding of this lectin are currently unknown and could be due to several reasons. This procedure required the growth of cells in microvesicle depleted media as opposed to regular media used by our collaborators for their

microvesicle isolation. Thus, BPA may actually be binding to contaminating bovine microvesicles. This is unlikely considering that we do not observe BPA binding for microvesicles obtained from other T cell lines by our collaborators. The more likely possibility is that during the depletion of bovine microvesicles, we could be depleting specific growth factors which may influence this glycan specifically. Further analysis of this small difference is required.

Single color lectin microarray analysis of lipid rafts, microvesicles and cellular membrane isolated from Jurkat cells

Glycomic comparison of lipid rafts, microvesicles and cell membrane isolated from matched Jurkat cells was performed using single color lectin microarray analysis. Equal amounts of Cy3-labeled samples were hybridized to the array. The resultant fluorescence intensity of the samples was used to obtain the signal to noise ratio (S/N) as described in the methods.

A.



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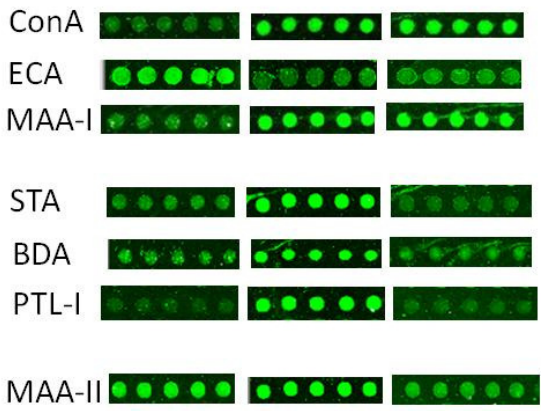
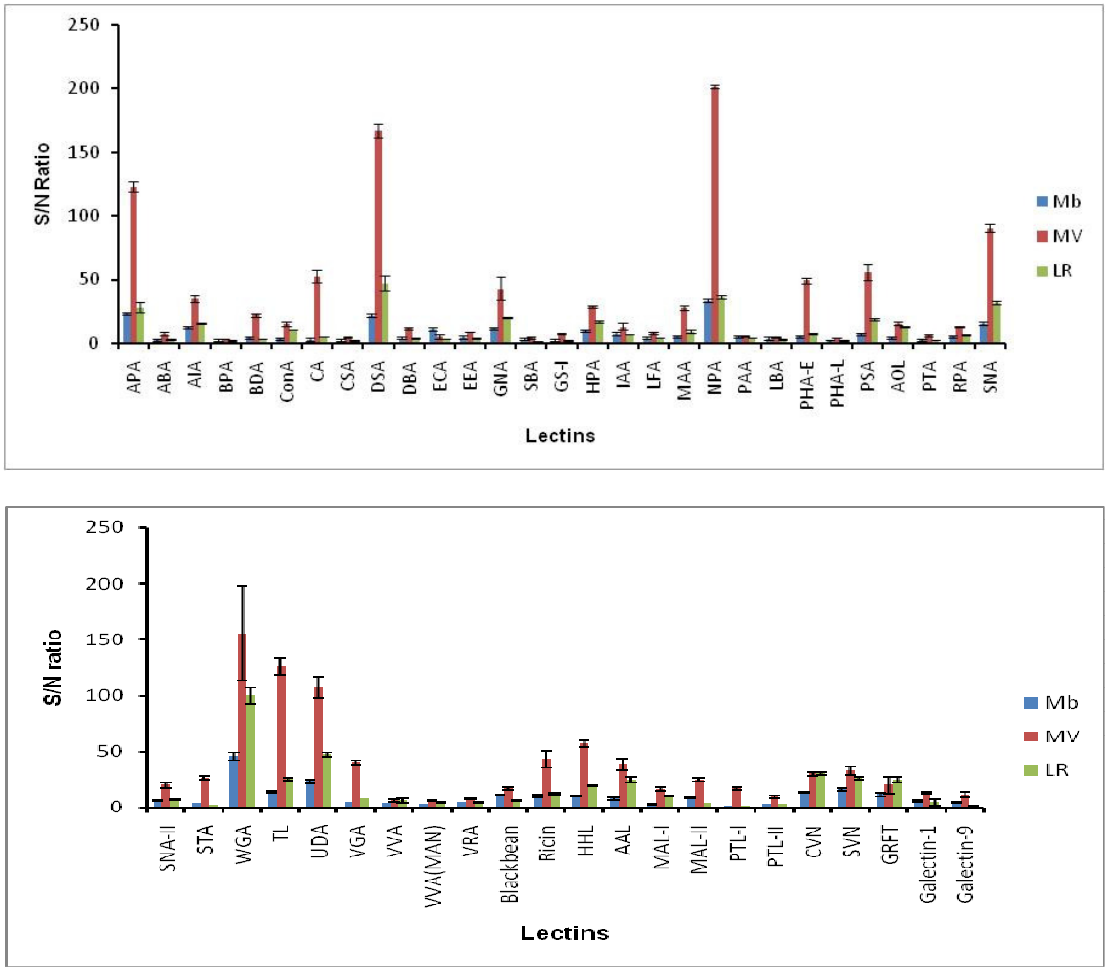


Figure 4.4. Glycomic analysis of lipid rafts, microvesicles and cellular membrane derived from Jurkat cells. Equal amounts of Cy3-labeled samples (1 μg of protein) were hybridized to the array. A) Glycomic signature obtained for the rafts exhibited similarity to the glycomic profile of membrane. There were few notable similarities between the glycomic profiles of microvesicles and lipid rafts. There were also differences in the lectin binding patterns between lipid rafts and microvesicles samples. B) Binding patterns for a select panel of lectins from Fig 4.4.A.

C.



D.

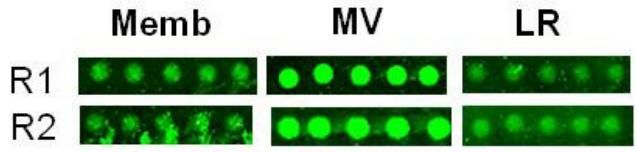


Figure 4.4. Glycomic analysis of lipid rafts, microvesicles and cellular membrane derived from Jurkat cells. Equal amounts of Cy3-labeled samples (1 µg of protein) were hybridized to the array. C) Graphical representation of the Signal to Noise ratio (S/N) obtained for the single color analysis for membrane (Mb), microvesicle (MV) and lipid raft (LR). Only positive lectins were used for this analysis. The error bars represent standard deviations. D) The binding of cholera toxin on the array to two biological replicates of Jurkat cell membrane, MV and LR (R1 and R2) is illustrated.

Lectin microarray analysis revealed more similarities between lipid rafts and cell membrane when compared to microvesicles (Fig 4.4). Interestingly, both raft and microvesicle samples exhibited increased binding to mannose binders (ConA, Cyanovirin-N, SVN, GRFT), LacNAc binders (SNA, WGA, DSA) and the fucose binding lectin, AAL. These lectins are of particular interest as they were among the lectins that exhibited increased binding to HIV and microvesicles (Chapter 2, Fig 2.10). Microvesicles and lipid rafts also demonstrated exclusion of certain epitopes such as blood group A/B (ECA) when compared to the cell membrane. Lipid raft fractions are known to be enriched in the ganglioside, GM1 and the presence of the GM1 binding protein, cholera toxin on the array would provide us with an additional means of assessing the raft fractions [147]. However, I noticed that the binding of cholera toxin to the two biological replicates of lipid raft isolations was significantly reduced compared to the microvesicles. The cell membrane and lipid raft fractions did not seem significantly different in terms of cholera toxin binding, creating doubts about the accuracy of the isolation protocols. In spite of the presence of flotillin-1 and the absence of transferrin in the lipid raft fractions, the low binding of cholera toxin to the raft fractions is a major source of concern. In the future, these isolations should be checked for the presence of GM1 using western blot analysis for better characterization. Thus, in spite of the problems associated with this isolation protocols, it seems likely that lipid raft regions share some glycomic characteristics with microvesicles and HIV, even though their overall glycopatterns is similar to that of the cell membrane.

There are many possible reasons for the glycomic differences observed between microvesicles and lipid rafts. First, the relevance of these detergent resistant membrane fractions to lipid rafts *in vivo* is still an area of active debate [163, 164]. In addition, detergent extraction at low temperatures could lead to artificial aggregation and may not

be representative of lipid raft structures *in vivo* [164]. Clustering of the ganglioside, GM1, with cholera toxin is routinely used for the visualization of lipid rafts in cells using fluorescence microscopy [165]. Thus, microscopic techniques should be used to examine co-localization of lipid raft marker such as GM1 with lectin staining, thereby providing another method to verify the enrichments as well as lack of specific glycans that were observed using the lectin microarray. Furthermore, the co-localization of the microvesicular lipid marker, N-Rh-PE with specific lectin (for example DSA) and cholera toxin staining will lend strong credence to the theory that microvesicles emerge from lipid raft regions in T cells. It will be interesting to examine if the microvesicular marker will be enriched in the biochemical isolations of lipid rafts from N-Rh-PE labeled cells. It has been reported that the distribution of the microvesicular lipid marker, N-Rh-PE, is disrupted upon treatment with methyl- β-cyclodextrin [40]. It will be interesting to examine if the lectin staining pattern is dependent on cholesterol as well, indicating the involvement of lipid raft regions in the process of microvesicular budding in T cells.

Part of the controversy surrounding lipid rafts is based on the observation that use of different detergents for raft isolation often yields different compositions of proteins. There are different scenarios that can be used to explain this phenomenon. First, lipid rafts could be homogenous in nature but the different solubilities of its protein and lipid constituents could lead to differences observed upon extraction with various detergents. The second scenario is that different types of rafts are present in cells accounting for the differences in raft associated components observed for different isolation procedures [166]. The heterogeneity of lipid rafts could contribute to the differences observed in the glycomic signatures of lipid rafts and microvesicles.

The existence of heterogeneous lipid rafts has been shown using both microscopy and immunoprecipitation. It has been previously shown that T cells have distinct raft

domains which segregate the proteins, Lck and linker of activation (LAT) [167]. Immunoprecipation experiments with lipid raft marker proteins also revealed compositional heterogeneity in the raft populations [168]. Thus, it is quite possible that HIV buds out of a specific type of raft microdomain. Consequently, glycomic analysis of all lipid rafts derived from T cells might result in lower levels of certain glycans due to their presence only in a particular subset of raft population.

The third explanation for this observation could be due to the activation status of these T cells. It is quite possible that this result may indeed be an accurate representation of the differences between lipid rafts and microvesicles in these cultured T cells. This implies that microvesicles and HIV do not bud from lipid raft regions in cells grown in regular culture conditions. HIV replication and budding is known to occur in activated T cells [169]. It is also known that activated T cells release more microvesicles than resting cells [170] . Since, these lipid rafts were isolated from T cells that were not exogenously activated, it will be interesting to examine if the lipid rafts from activated T cells have identical glycopatterns to the microvesicles isolated from these cells. This will be extremely relevant to HIV budding since replication and budding of HIV widely occurs in activated T cells [169].

In addition, recent studies have shown that the comparison to lipid composition of HIV and microvesicles to the lipids isolated from the plasma membrane revealed significant similarities between the lipid compositions of HIV and microvesicles and plasma membrnae [60]. These results were contradictory to those obtained by Brugger and colleagues, in which they observed significant differences between the lipid constituents of HIV and plasma membrane [50]. Chan and colleagues attributed the differences between the two results to the source of membrane lipids. Brugger and coworkers had isolated lipids from whole cell membrane while Chan and coworkers

isolated lipids only from the plasma membrane. The contamination of cell surface lipids with lipids from cellular organelles was cited as the reason for this discrepancy [60]. It has been previously shown that the glycosylation pattern observed for the cellular membrane preparation used in this report is representative of cell surface glycosylation. This cellular membrane preparation includes membrane from cellular organelles as well [107]. Hence, it is possible that a similar scenario could be at play here and direct comparison of glycoproteins isolated from plasma membrane to lipid raft glycoproteins could yield more accurate results.

4.3 Conclusions

Lipid rafts are known to play a critical role in HIV biogenesis and proteomic analysis and lipid analysis has lent strong support to this theory [39]. In spite of the considerable work in the field of proteomics and lipidomics, no attempt has been made to examine the glycans present in these microdomains. Our initial work in this area indicates that raft regions could be potentially characterized by glycan epitopes. Though the lipid rafts and microvesicles shared certain glycans, overall the glycome of the raft regions seemed to resemble the parent cell membrane. A more careful and detailed analysis of the glycome of lipid rafts and microvesicles is needed before any conclusions can be drawn. Lipid rafts isolated from other T cell lines could be compared to their respective microvesicles and membranes to extend these observations to more than one cell line. Fluorescence microscopy analysis for these visualizations of these domains is essential to the accurate interpretation of these results. Thus, this work represents the first steps towards understanding the role of lipid rafts in HIV and microvesicular biogenesis using a novel glycomics approach.

4.4 Materials and methods

Cell culture

Jurkat-Tat-CCR5 cells were obtained from our collaborators at the AIDS vaccine program and cultured as described previously.

Isolation of microvesicles from culture supernatants

Microvesicles were isolated from Jurkat-Tat-CCR5 cells using the following protocol [162]. Briefly, cells were grown in normal media and then pelleted gently to remove the supernatant. The cells were then resuspended in RPMI media with microvesicle free 10% FBS. The media with FBS was centrifuged overnight at 100,000 xg using a 45 Ti rotor and Optima LE ultracentrifuge to remove any contaminating bovine microvesicles. The cells were seeded at 0.6 million cells per mL in a total volume of 200 mL. The cells were then cultured for an additional 48 hrs in the media. The cells were pelleted at 300 xg for 10 min and the supernatant was separated through differential centrifugation to isolate microvesicles. The supernatant was centrifuged at 2000 x g or 20 minutes. The pellet was discarded and the supernatant was centrifuged again for 30 min at 10,000 xg using a type Ti rotor in an Optima LE ultracentrifuge.

In the final step, the supernatant was centrifuged for 70 min at 100,000 xg using the ultracentrifuge. The pellet from this step containing the microvesicles was resuspended in PBS to remove any media contaminants. This was centrifuged at 100,000 xg for 1 h in a Optima table top ultracentrifuge using a TLA 100.3 rotor. The final pellet was resuspended in carbonate buffer (pH 9.3) for subsequent labeling step.

Cell membrane preparation

Jurkat cells used for microvesicle isolation in the previous step were used to prepare the matched membrane preparation. Briefly, 100 million cells were used to sonicated and cellular membrane was isolated as previously described [107].

Labeling of microvesicle and cell membrane preparations

The protein concentration of both microvesicle and cell membrane preparations were determined using a DC-protein assay. Both samples were labeled with Cy3 and Cy5 dyes as described previously. The unreacted dyes in the samples were quenched using 500 mM Ethanolamine for 45 minutes. The samples were then aliqouted and stored at -80°C until further use.

Isolation of lipid rafts

Matched Jurkat cells were also used for isolation of lipid rafts using the following protocol [50]. For the isolation of lipid rafts, 100 million cells were resuspended in 0.9 mL of lysis buffer (50 mM Tris.HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Tritin X-100 or 0.5% Brij 96) on ice. The cells were further lysed using a dounce homogenizer and dounced for 10 times on ice. The lysate was then mixed with 1.8 mL of Optiprep solution (Axis-shield), transferred to ultra-clear ultracentrifuge tubes and overlaid with 7.5 mL of 28% Optiprep solution in their respective lysis buffer. This was centrifuged for 4 hr using a SW41 Rotor at 28,000 rpm in an Optima ultracentrifuge. Eight fractions of 1.35 mL each were collected from the top and further analyzed using western blot analysis for presence of flotillin-1 (lipid raft marker) and transferrin (non-lipid raft marker).

Western blot analysis

Equal volumes of lipid raft fractions were boiled in sample buffer and separated using SDS-PAGE analysis. The gel was then transferred to nitrocellulose membrane followed by incubation in blocking buffer (5% milk in TBS+0.1% Tween, pH 7.4). The membrane was then incubated with the following primary antibodies: anti Flotillin-1 (1:500, BD biosciences) and anti Transferin (1:1000, BD biosciences). The membranes were then washed for 6 times in TBST and then incubated with goat-anti-mouse HRP for 1 h. The blots were then washed 6 times in TBST and developed using chemiluminescence (West Femto substrate, Pierce Technology) and visualized using a Kodak IS4000R imaging station.

Labeling of lipid rafts

Lipid raft fractions which contained the lipid raft marker protein, flotillin and had miminal levels of non-raft marker, transferrin, were combined to generate the lipid raft fractions. Raft fractions were concentrated and dialyzed into carbonate buffer (pH 9.3) using the Amicon ultra concentrators (10 kDa cut-off, Amicon). The concentrated fractions were then assayed using DC protein assay (Bio-Rad) and then labeled with –Cy3 or –Cy5 for 45 minutes at room temperature. The excess dye in the samples was then reacted with 500 mM ethanolamine for 45 minutes, aliquoted and stored at -80°C until further use.

Lectin microarray experiments

Single color lectin microarray experiments were performed as described before. Briefly, 1 μg of Cy3 labeled samples were hybridized to the lectin microarray and analyzed as

before. For comparison, Jurkat derived microvesicles and HIV samples obtained from our collaborators, were also examined using this method.

Analysis of microarray data

Data analysis for the lectin microarray analysis was performed as previously described. For any given sample, lectins that gave S/N+1 SD values of 5 and above were considered positive.

Chapter 5: Conclusions

The prevailing hypothesis behind this body of work is that HIV exploits the microvesicular mechanism for its biogenesis and egress from host cells; consequently these particles should share biochemical characteristics including glycan epitopes. HIV is known to bud from the plasma membrane of T cells, the major sites of HIV replication, and current data indicates that the viral machinery is targeted to specific microdomains, instead of budding randomly from the cell surface [39-41]. The mechanistic details of HIV biogenesis in these cells are yet to be completely elucidated. In the absence of conclusive mechanistic evidence implicating this pathway in HIV biogenesis, several prominent retrovirologists including Mark Marsh (University College London, London) [59] and David Ott (AIDS Vaccine Program, NCI, Frederick) [4] have raised doubts about the validity of this theory.

To this end, we have taken advantage of a new technology, lectin microarray [110], to add crucial glycomic evidence to support the theory that HIV and microvesicles share an exit pathway (Fig 5.1.A). Additionally, I will discuss the potential role of these glycans as sorting determinants. Given the role of glycans in protein sorting to microdomains, glycomic analysis sheds light on both glycan composition as well as potential sorting mechanisms. In addition, this work also presents new evidence for the need of a careful examination of the use of specific glycan epitopes in HIV vaccine strategies. We have also begun to further characterize the microdomains that these particles emerge from during the course of this body of work.

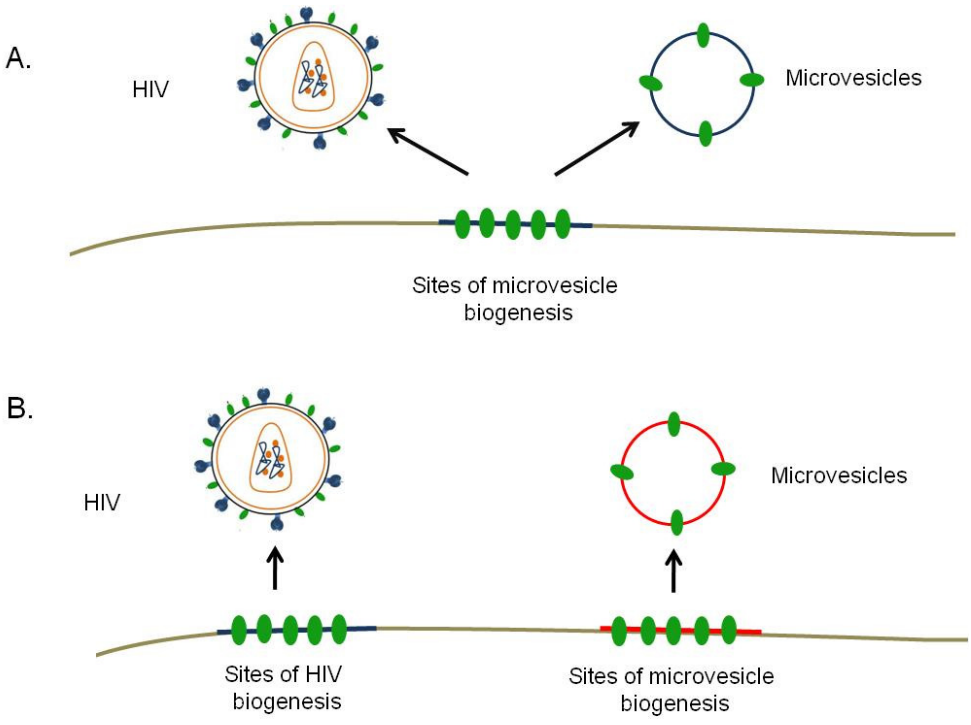


Figure 5.1. HIV and microvesicular budding models. A) Combination of existing proteomic and lipidomic data with our glycomic data strongly suggests that microvesicles and HIV bud from the same sites, thus they share biochemical characteristics and potentially utilize the same exit machinery. B) The alternative theory proposes the existence of separate exit machineries for microvesicles and HIV biogenesis.

Evidences against microvesicle biogenesis theory

The theory that HIV usurps microvesicular biogenesis to exit host cells sheds light on some key aspects of HIV biology including immune evasion. Given that microvesicles have immune modulation roles, use of this particular exit mechanism will enable the incorporation of specific proteins, lipids and glycans that aid viral infectivity and immune evasion. In addition, the use of a pre-existing host machinery for exit would be extremely advantageous for HIV since it has a very small genome which encodes for limited set of proteins [3]. Previous evidence for this theory relies on localization and

incorporation of a small set of proteins and lipids in both particles. Despite mounting evidence, there is some controversy about the validity of this hypothesis. This controversy stems from the differential exclusion or inclusion of an even smaller panel of protein (1 protein) or lipid markers (two lipids) in the two particles derived from T cells [4, 60].

Evidences against the microvesicular biogenesis theory primarily derive from recent reports of differential incorporation of the lipids, PIP2 and PIP [60] and protein marker, CD45 [4], in HIV and microvesicles. The presence of CD45 in microvesicles and its absence in HIV has been used as evidence to suggest that these particles do not share a common mechanism. The authors make the following statement in the paper. "Thus, our data provided here do not support this type of a distinct, specialized and shared release pathway for HIV-1 and microvesicles " [4].

Another study reported that HIV and microvesicles display similarities in lipid profiles with enrichments in ceramides, GM3, and cholesterol. The exceptions were the lipid species, PIP and PIP2, which were enriched in HIV alone. Their data also points to the binding of these lipids by viral Gag accounting for the increased incorporation of thee lipid species in virus as compared to microvesicles [60]. Both these authors use these differences as evidences to point to different biogenesis routes, while they seem to ignore reported similarities between these particles [40, 57]. Their reliance on presence or absence of couple of markers has underlined the need for a systems based approach to examining these particles. A comprehensive profiling of both particles to examine similarities and differences is necessary to test the validity of this hypothesis. If the alternative theory that HIV and microvesicular biogenesis pathways are distinct is

accurate [4, 59] [60] (Fig 5.1.B), then we would expect the glycomic patterns of both particles to be significantly different.

Glycomic analysis of HIV and microvesicles strongly supports the microvesicle theory of HIV biogenesis

Proteomic similarities (~10 proteins) between HIV and microvesicles have been reported with similar enrichments and exclusions providing initial evidence for the microvesicular theory of HIV biogenesis [40, 56, 57]. Further extension of proteomic studies using mass spectrometry is necessary for detailed profiling of microvesicles and ultrapure virus from T cells. Our systems based glycomic approach provides a comprehensive analysis of the glycan epitopes present in HIV and microvesicles.

Glycomic analysis of microvesicles from uninfected cells and HIV derived from a panel T cell lines revealed remarkable similarities in the glycomic signatures of HIV and microvesicles which is distinct from the parent cell membrane (Fig 2.10 and 2.11). This strongly suggested that these particles bud from a specific microdomain in the plasma membrane, characterized by enrichment in specific glycan epitopes. We validated this using fluorescence microscopy to study the distribution of glycans that were enriched in both microvesicles and HIV on our lectin microarrays. We observed regions of enrichment of these glycans on the cell surface. These regions exhibited co-localization with sites of microvesicular biogenesis, characterized by sorting of a fluorescent microvesicular lipid marker, N-Rh-PE, to these microdomains (Fig 2.14). We chose this lipid marker instead of reported protein markers due to complications in using lectins in combination with antibodies for immunofluorescence. Antibodies are heavily

glycosylated and could themselves be bound by the FITC-lectin used for the microscopy, rendering them unsuitable for this analytical technique [171]. This microvesicular lipid marker has previously been reported to co-localize with HIV Gag in T cells. In addition, this lipid was enriched in both microvesicles and Gag VLPs secreted by T cells [40]. Take together, our data that glycan epitopes are enriched in microvesicles, HIV and membrane microdomains presents compelling evidence for the convergence of these two exit pathways via a specific portion of the membrane.

Additional evidence for this theory comes from the cell line dependent clustering of microvesicles and HIV derived from a panel of T cells. It is known that presence or absence of some host proteins in the viral envelope can be accounted for by changes in expression levels of these proteins in the cell line of origin [117]. It is also known that the glycosylation of gp120 can vary with the host cell line used to propagate the virus [116]. In line with these observations, we expected to see changes in HIV glycosylation derived from a panel of T cells. These glycomic variations were conserved in both microvesicles and HIV from a given cell line, lending further support to the theory that both microvesicles and HIV utilize the same pathway for exit (Fig 2.16). Thus, the similarities in glycome between HIV and microvesicles coupled with the evidence that cell line dependent differences are reflected in both particles, provides a strong case for the existence of a shared exocytic mechanism. In addition, ratiometric glycomic analysis of SupT1 derived SIV variants which differ in the amounts of gp120, strongly suggests that in terms of glycans, the key distinguishing feature between HIV and microvesicles is the enrichment of high mannose epitopes in HIV (Fig 2.17). Given that gp120 is extensively modified with these epitopes [77], this data strongly suggests that expression

of gp120 could account for the differences in the glycan compositions of HIV and microvesicles. Taken together with the earlier data, this work strengthens the theory that microvesicles and HIV share and exocytic mechanisms.

Glycans could play a role in trafficking to exocytic particles

In spite of the differences observed in the glycopatterns of exocytic particles from different cell lines, they were also characterized by conservation of a core set of glycans in all the exocytic particles. In particular, lectins that recognize complex, hybrid N-links, sialic acids and LacNAc exhibited enhanced binding to both particles when compared to the parent cell membrane. There was also exclusion of certain glycan epitopes such as blood group A/B antigens in both particles (Fig 2.16). The reasons for enrichment of specific glycans in these particles could be due to either increased concentrations of specific proteins resulting in a concomitant increase in glycans associated with those proteins or the role of specific glycans as sorting signals for these domains. At the present time, we cannot distinguish between the two possible reasons. Glycans are known to be involved in protein sorting to apical and basolateral surfaces in epithelial cells. Both *N*-linked and *O*-linked glycans have been shown to play a role in this sorting process. In view of the role of glycans in trafficking, this work opens up the possibility that there could be a core set of conserved glycans that influences trafficking to these particles.

Upon closer examination of the conserved glycans, we observed enhanced affinities of LacNAc binding lectins such as DSA, STA, MAL-I, RCA to both particles (Fig 2.16). In addition, the lectin PHA-L, which binds to β-1,6-branched complex N-glycans, demonstrated increased binding to both microvesicles and HIV. Interestingly, these glycans function as some of the major ligands for members of the galectin family of immune lectins [134, 172, 173]. Members of the galectin family are known to

multimerize and interact with multiple ligands simultaneously, resulting in the formation of glycoprotein lattices [132]. Galectin-3 is known to be involved in apical sorting of proteins [174, 175]. Thus, enrichment in these particular ligands could have implications for sorting of proteins to these particles. It has also been shown that protein oligomerization could function as sorting signals for incorporation into microvesicles and Gag VLPs ([100]. This work opens up the possibility that protein oligomerization by lectins could influence targeting of proteins to these microdomains and their subsequent incorporation into these exocytic particles. This observation could have critical implications for understanding mechanisms of microvesicular biogenesis.

One interesting piece of additional data to support the role of glycans in protein sorting to these particles comes from a recent genomic screen to identify host dependent factors in HIV replication. Among several other genes, the *mgat1* gene was identified as a cellular factor that is involved in late stage of HIV replication i.e assembly and budding. The *mgat1* encodes for mannosyl (α-1,3-)-glycoprotein β-1,2-N-acetylglucosaminyltransferase, a glycosyltransferase, which is involved in the initiation of complex *N*-linked glycan formation and is crucial for conversion of high mannose to hybrid and complex *N*-glycans [18]. The deletion of *mgat1* gene results in loss of galectin-1 ligands in mouse neuronal cells [176]. The transferase encoded by *mgat1* could play a role in HIV assembly and budding by influencing protein sorting to these domains, since its deletion will presumably give rise to defects in the formation of specific glycans. Given the type of glycans that are generated by this enzyme, future studies are necessary to determine if glycan dependent sorting to these domains is essential for microvesicular and HIV biogenesis. Future studies are needs to determine if genetic manipulation of this gene could result in alterations in either the type or amounts of microvesicular protein

cargo. This would indicate that these specific glycans play a role in sorting proteins sorting to these domains.

Presence of high mannose epitopes in microvesicles is a potential concern for anti-viral therapy

High mannose epitopes are key epitopes in HIV biology in terms of both prophylactic therapies based on anti-viral lectins and more importantly, as a basis for vaccines [79, 87]. The presence of high mannose residues in microvesicles was demonstrated by enhanced binding of anti-viral lectins to these particles, when compared to the parent cell membrane. The basis of targeting these high mannose epitopes for viral therapy stems from the relatively lower amounts of these ligands on the cell surface in comparison to the viral envelope glycoprotein [87]. Given the presence of these ligands on microvesicles, the administration of high mannose based therapeutic agents could result in targeting these microvesicles, thereby creating a self antigen. Given that, we are still uncovering the roles of microvesicles in immune modulation, a negative effect on microvesicular biogenesis and functioning is probably undesirable.

The neutralizing antibody, 2G12, has been a key player in the fight against this disease, as it is recognizes the unusual cluster of high mannose epitopes in gp120 [83]. This antibody is unique as it is one of the few naturally isolated antibodies that can function as a neutralizing antibody [75]. An interesting extension of this preliminary work would be to examine whether 2G12 would also exhibit enhanced binding to microvesicles compared to the cell membrane from which these particles are derived. Given that 2G12 binds to similar epitopes as the anti-viral lectin, cyanovirin, which exhibits increased binding to microvesicles, it seems very likely that it would have increased affinity towards microvesicles, when compared to the cell membrane [177].

Examination of this binding sets up the stage for a more thorough examination of the possible side effects of targeting high mannose epitopes.

Glycomic characterization of lipid rafts

Lipid rafts constitute an important class of microdomains which are known to be involved in HIV budding [150]. In view of data strongly implicating the sites of microvesicle biogenesis in HIV budding, it is feasible to hypothesize that lipid rafts could serve as budding platforms for both HIV and microvesicular biogenesis. In order to test this hypothesis using glycomic characterization of lipid rafts, we isolated lipid rafts and microvesicles from T cells and compared their glycomes to the cell membrane. Preliminary data indicates that even though certain glycans are enriched in both lipid rafts and microvesicles compared to the cell membrane, the lipid rafts seem to mostly resemble the cell membrane in terms of glycan compositions (Fig 4.4). Given that there has been no glycomic characterization of lipid rafts reported, our initial attempts at such characterization opens up the possibilty that the glycome might be used to define lipid raft regions.

Even though this initial result is interesting, the interpretation of these results is complicated by several factors. First, we do not know whether the detergent resistant membrane isolated as lipid rafts accurately reflect the composition of lipid rafts in live cells [160]. Second, cells are believed to contain a heterogeneous population of lipid rafts [167, 178]. It is quite possible that the microvesicle sites arise from a particular subset of lipid rafts during the processes of microvesicular and HIV biogenesis. The activation status of T cells is known to play a crucial role in determining lipid raft composition in these cells[179] and also leads to increases in microvesicular release [170]. In addition, HIV replication is known to occur in activated T cells [7]. Given that the cells used for

isolation of lipid rafts are not exogenously activated, it is possible that lipid rafts in activated cells could function as sites of microvesicular and HIV biogenesis. Thus, a more detailed analysis of the glycome of lipid rafts coupled with the use of microscopic techniques to examine the specific enrichment of glycan epitopes and microvesicular lipid marker in lipid raft regions could shed new light on the ongoing debate about the relevance of classically defined lipid rafts in live cells, provide another method of characterizing these regions and dissect possible links between lipid rafts and sites of HIV and microvesicular biogenesis in T cells.

Summary

The theory that HIV exploits the microvesicular mode of biogenesis for its egress from the host T cells is an active area of research that has primarily involved using small panels of lipid and protein markers to either support or oppose this theory. Glycosylation is a ubiquitous modification found on proteins and lipids and hence is an ideal candidate to characterize the cadre of host cell derived components present in HIV and compare it to its proposed natural cellular counterparts, the microvesicles. This work provides us with a comprehensive comparison between the two particles in terms of glycomic signatures and strongly supports the utilization of microvesicular biogenesis pathways by HIV. The use of this type of systems based approach revealed many interesting aspects of microvesicles and HIV from the same data set. This work also includes our initial attempts to perform glycomic characterization of lipid rafts using lectin microarrays and future lectin array experiments combined with proteomic and fluorescence microscopy approaches could lead to further resolution of modes of HIV biogenesis in T lymphocytes. Even though, this work does not conclusively prove that HIV and microvesicles utilize the same machinery for exit, taken together with the existing

proteomic and lipidomic data, this work argues strongly for the sharing of exocytic machinery by these particles (Fig 5.1.A).

Future work

More detailed analysis on the role and mutual exclusivity of these models of viral egress is definitely the first step towards any attempt at targeting these microdomains for therapeutic measures. An understanding of the role of glycans in protein sorting to microdomains could help to better classify these microdomains. The creation of a synthetic microvesicular cargo has already been attempted based on protein oligomerization [100]. The identification of conserved glycan epitopes among these microdomains can be exploited to create a glycan based synthetic microvesicular cargo. It will be interesting to examine if a non glycosylated, non-microvesicular protein, will be sorted to microvesicles and potentially to lipid rafts, upon modification with specific glycans. More conclusive proof for the theory that HIV is a pathogenic microvesicle could be obtained upon electron microscopic examination of budding sites in infected and uninfected cells. This method allows us to examine the localization of specific glycan epitiopes to sites of budding in the plasma membrane and in mature virions and microvesicles. Understanding the mechanistic details of microvesicular biogenesis is crucial to the establishment of this theory as the effects of genetic manipulation of microvesicle biogenesis can be examined on viral biogenesis and vice versa, offering confirmation of the role of microvesicle biogenesis in HIV budding and release. Given the emerging roles of microvesicles, this work may have implications far beyond the world of HIV and virology.

References

1. Geeraert, L., G. Kraus, and R.J. Pomerantz, *Hide-and-seek: the challenge of viral persistence in HIV-1 infection.* Annu Rev Med, 2008. **59**: p. 487-501.
2. Li, F. and C. Wild, *HIV-1 assembly and budding as targets for drug discovery.* Curr Opin Investig Drugs, 2005. **6**(2): p. 148-54.
3. Gould, S.J., A.M. Booth, and J.E. Hildreth, *The Trojan exosome hypothesis.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10592-7.
4. Coren, L.V., T. Shatzer, and D.E. Ott, *CD45 immunoaffinity depletion of vesicles from Jurkat T cells demonstrates that exosomes contain CD45: no evidence for a distinct exosome/HIV-1 budding pathway.* Retrovirology, 2008. **5**: p. 64.
5. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.
6. Walker, B.D. and D.R. Burton, *Toward an AIDS vaccine.* Science, 2008. **320**(5877): p. 760-4.
7. Stevenson, M., *HIV-1 pathogenesis.* Nat Med, 2003. **9**(7): p. 853-60.
8. Peterlin, B.M. and D. Trono, *Hide, shield and strike back: how HIV-infected cells avoid immune eradication.* Nat Rev Immunol, 2003. **3**(2): p. 97-107.
9. Forsman, A. and R.A. Weiss, *Why is HIV a pathogen?* Trends Microbiol, 2008.
10. Johnston, M.I. and A.S. Fauci, *An HIV vaccine--evolving concepts.* N Engl J Med, 2007. **356**(20): p. 2073-81.
11. Johnston, M.I. and A.S. Fauci, *An HIV vaccine--challenges and prospects.* N Engl J Med, 2008. **359**(9): p. 888-90.
12. Demirov, D.G. and E.O. Freed, *Retrovirus budding.* Virus Res, 2004. **106**(2): p. 87-102.
13. Reeves, J.D. and R.W. Doms, *Human immunodeficiency virus type 2.* J Gen Virol, 2002. **83**(Pt 6): p. 1253-65.
14. Gomez, C. and T.J. Hope, *The ins and outs of HIV replication.* Cell Microbiol, 2005. **7**(5): p. 621-6.
15. Freed, E.O., *HIV-1 replication.* Somat Cell Mol Genet, 2001. **26**(1-6): p. 13-33.
16. Carter, C.A. and L.S. Ehrlich, *Cell biology of HIV-1 infection of macrophages.* Annu Rev Microbiol, 2008. **62**: p. 425-43.
17. Pornillos, O., J.E. Garrus, and W.I. Sundquist, *Mechanisms of enveloped RNA virus budding.* Trends Cell Biol, 2002. **12**(12): p. 569-79.
18. Brass, A.L., et al., *Identification of host proteins required for HIV infection through a functional genomic screen.* Science, 2008. **319**(5865): p. 921-6.
19. Bukrinskaya, A.G., *HIV-1 assembly and maturation.* Arch Virol, 2004. **149**(6): p. 1067-82.
20. Bedinger, P., et al., *Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4.* Nature, 1988. **334**(6178): p. 162-5.
21. Pang, S., et al., *Human immunodeficiency virus Env-independent infection of human CD4(-) cells.* J Virol, 2000. **74**(23): p. 10994-1000.
22. Chow, Y.H., et al., *gp120-Independent infection of CD4(-) epithelial cells and CD4(+) T-cells by HIV-1.* J Acquir Immune Defic Syndr, 2002. **30**(1): p. 1-8.
23. Welsch, S., B. Muller, and H.G. Krausslich, *More than one door - Budding of enveloped viruses through cellular membranes.* FEBS Lett, 2007. **581**(11): p. 2089-97.

24. Pelchen-Matthews, A., B. Kramer, and M. Marsh, *Infectious HIV-1 assembles in late endosomes in primary macrophages.* J Cell Biol, 2003. **162**(3): p. 443-55.
25. Scarlata, S. and C. Carter, *Role of HIV-1 Gag domains in viral assembly.* Biochim Biophys Acta, 2003. **1614**(1): p. 62-72.
26. Shioda, T. and H. Shibuta, *Production of human immunodeficiency virus (HIV)-like particles from cells infected with recombinant vaccinia viruses carrying the gag gene of HIV.* Virology, 1990. **175**(1): p. 139-48.
27. Ono, A., et al., *Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane.* Proc Natl Acad Sci U S A, 2004. **101**(41): p. 14889-94.
28. Chukkapalli, V., et al., *Interaction between the human immunodeficiency virus type 1 Gag matrix domain and phosphatidylinositol-(4,5)-bisphosphate is essential for efficient gag membrane binding.* J Virol, 2008. **82**(5): p. 2405-17.
29. Ono, A. and E.O. Freed, *Cell-type-dependent targeting of human immunodeficiency virus type 1 assembly to the plasma membrane and the multivesicular body.* J Virol, 2004. **78**(3): p. 1552-63.
30. Nomaguchi, M., M. Fujita, and A. Adachi, *Role of HIV-1 Vpu protein for virus spread and pathogenesis.* Microbes Infect, 2008. **10**(9): p. 960-7.
31. Klimkait, T., et al., *The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release.* J Virol, 1990. **64**(2): p. 621-9.
32. Neil, S.J., T. Zang, and P.D. Bieniasz, *Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu.* Nature, 2008. **451**(7177): p. 425-30.
33. VerPlank, L., et al., *Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag).* Proc Natl Acad Sci U S A, 2001. **98**(14): p. 7724-9.
34. Strack, B., et al., *AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding.* Cell, 2003. **114**(6): p. 689-99.
35. Garrus, J.E., et al., *Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding.* Cell, 2001. **107**(1): p. 55-65.
36. Stoorvogel, W., et al., *The biogenesis and functions of exosomes.* Traffic, 2002. **3**(5): p. 321-30.
37. Nguyen, D.G., et al., *Evidence that HIV budding in primary macrophages occurs through the exosome release pathway.* J Biol Chem, 2003. **278**(52): p. 52347-54.
38. Welsch, S., et al., *HIV-1 buds predominantly at the plasma membrane of primary human macrophages.* PLoS Pathog, 2007. **3**(3): p. e36.
39. Nguyen, D.H. and J.E. Hildreth, *Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts.* J Virol, 2000. **74**(7): p. 3264-72.
40. Booth, A.M., et al., *Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane.* J Cell Biol, 2006. **172**(6): p. 923-35.
41. Nydegger, S., et al., *Mapping of tetraspanin-enriched microdomains that can function as gateways for HIV-1.* J Cell Biol, 2006. **173**(5): p. 795-807.
42. Ott, D.E., *Cellular proteins detected in HIV-1.* Rev Med Virol, 2008. **18**(3): p. 159-75.
43. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-9.

44. Holm, K., et al., *Human immunodeficiency virus type 1 assembly and lipid rafts: Pr55(gag) associates with membrane domains that are largely resistant to Brij98 but sensitive to Triton X-100.* J Virol, 2003. **77**(8): p. 4805-17.
45. Yang, L. and L. Ratner, *Interaction of HIV-1 gag and membranes in a cell-free system.* Virology, 2002. **302**(1): p. 164-73.
46. Halwani, R., et al., *Rapid localization of Gag/GagPol complexes to detergent-resistant membrane during the assembly of human immunodeficiency virus type 1.* J Virol, 2003. **77**(7): p. 3973-84.
47. Lindwasser, O.W. and M.D. Resh, *Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains.* J Virol, 2001. **75**(17): p. 7913-24.
48. Feng, X., N.V. Heyden, and L. Ratner, *Alpha interferon inhibits human T-cell leukemia virus type 1 assembly by preventing Gag interaction with rafts.* J Virol, 2003. **77**(24): p. 13389-95.
49. Ding, L., et al., *Independent segregation of human immunodeficiency virus type 1 Gag protein complexes and lipid rafts.* J Virol, 2003. **77**(3): p. 1916-26.
50. Brugger, B., et al., *The HIV lipidome: a raft with an unusual composition.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2641-6.
51. Aloia, R.C., H. Tian, and F.C. Jensen, *Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes.* Proc Natl Acad Sci U S A, 1993. **90**(11): p. 5181-5.
52. Ono, A. and E.O. Freed, *Plasma membrane rafts play a critical role in HIV-1 assembly and release.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13925-30.
53. Pickl, W.F., F.X. Pimentel-Muinos, and B. Seed, *Lipid rafts and pseudotyping.* J Virol, 2001. **75**(15): p. 7175-83.
54. Ono, A., A.A. Waheed, and E.O. Freed, *Depletion of cellular cholesterol inhibits membrane binding and higher-order multimerization of human immunodeficiency virus type 1 Gag.* Virology, 2007. **360**(1): p. 27-35.
55. Jolly, C. and Q.J. Sattentau, *Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity.* J Virol, 2005. **79**(18): p. 12088-94.
56. Gluschankof, P., et al., *Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations.* Virology, 1997. **230**(1): p. 125-33.
57. Esser, M.T., et al., *Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation.* J Virol, 2001. **75**(13): p. 6173-82.
58. Bess, J.W., Jr., et al., *Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations.* Virology, 1997. **230**(1): p. 134-44.
59. Pelchen-Matthews, A., G. Raposo, and M. Marsh, *Endosomes, exosomes and Trojan viruses.* Trends Microbiol, 2004. **12**(7): p. 310-6.
60. Chan, R., et al., *Retroviruses human immunodeficiency virus and murine leukemia virus are enriched in phosphoinositides.* J Virol, 2008. **82**(22): p. 11228-38.
61. Simpson, R.J., S.S. Jensen, and J.W. Lim, *Proteomic profiling of exosomes: current perspectives.* Proteomics, 2008. **8**(19): p. 4083-99.

62. Pan, B.T. and R.M. Johnstone, *Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor.* Cell, 1983. **33**(3): p. 967-78.
63. Schorey, J.S. and S. Bhatnagar, *Exosome function: from tumor immunology to pathogen biology.* Traffic, 2008. **9**(6): p. 871-81.
64. Thery, C., et al., *Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes.* Nat Immunol, 2002. **3**(12): p. 1156-62.
65. Li, X.B., et al., *Role of exosomes in immune regulation.* J Cell Mol Med, 2006. **10**(2): p. 364-75.
66. Skokos, D., et al., *Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes.* J Immunol, 2001. **166**(2): p. 868-76.
67. Fevrier, B. and G. Raposo, *Exosomes: endosomal-derived vesicles shipping extracellular messages.* Curr Opin Cell Biol, 2004. **16**(4): p. 415-21.
68. Pap, E., et al., *T lymphocytes are targets for platelet- and trophoblast-derived microvesicles during pregnancy.* Placenta, 2008. **29**(9): p. 826-32.
69. Keller, S., et al., *Exosomes: from biogenesis and secretion to biological function.* Immunol Lett, 2006. **107**(2): p. 102-8.
70. Ledford, H., *HIV vaccine developers battle on, despite high-profile failures.* Nat Biotechnol, 2008. **26**(6): p. 591-2.
71. Karlsson Hedestam, G.B., et al., *The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus.* Nat Rev Microbiol, 2008. **6**(2): p. 143-55.
72. Stoiber, H., et al., *Complement-opsonized HIV: the free rider on its way to infection.* Mol Immunol, 2005. **42**(2): p. 153-60.
73. Kalams, S.A., *Cellular immunity for prevention and clearance of HIV infection.* Curr Mol Med, 2003. **3**(3): p. 195-208.
74. Huber, M. and A. Trkola, *Humoral immunity to HIV-1: neutralization and beyond.* J Intern Med, 2007. **262**(1): p. 5-25.
75. Pantophlet, R. and D.R. Burton, *GP120: target for neutralizing HIV-1 antibodies.* Annu Rev Immunol, 2006. **24**: p. 739-69.
76. Mizuochi, T., et al., *Diversity of oligosaccharide structures on the envelope glycoprotein gp 120 of human immunodeficiency virus 1 from the lymphoblastoid cell line H9. Presence of complex-type oligosaccharides with bisecting N-acetylglucosamine residues.* J Biol Chem, 1990. **265**(15): p. 8519-24.
77. Balzarini, J., *Targeting the glycans of gp120: a novel approach aimed at the Achilles heel of HIV.* Lancet Infect Dis, 2005. **5**(11): p. 726-31.
78. Corbeau, P., J.L. Pasquali, and C. Devaux, *Jacalin, a lectin interacting with O-linked sugars and mediating protection of CD4+ cells against HIV-1, binds to the external envelope glycoprotein gp120.* Immunol Lett, 1995. **47**(1-2): p. 141-3.
79. Scanlan, C.N., et al., *Exploiting the defensive sugars of HIV-1 for drug and vaccine design.* Nature, 2007. **446**(7139): p. 1038-45.
80. Marth, J.D. and P.K. Grewal, *Mammalian glycosylation in immunity.* Nat Rev Immunol, 2008.
81. Wei, X., et al., *Antibody neutralization and escape by HIV-1.* Nature, 2003. **422**(6929): p. 307-12.
82. Mascola, J.R. and D.C. Montefiori, *HIV-1: nature's master of disguise.* Nat Med, 2003. **9**(4): p. 393-4.

83. Sanders, R.W., et al., *The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120.* J Virol, 2002. **76**(14): p. 7293-305.
84. Manrique, A., et al., *In vivo and in vitro escape from neutralizing antibodies 2G12, 2F5, and 4E10.* J Virol, 2007. **81**(16): p. 8793-808.
85. Balzarini, J., *Inhibition of HIV entry by carbohydrate-binding proteins.* Antiviral Res, 2006. **71**(2-3): p. 237-47.
86. Botos, I. and A. Wlodawer, *Proteins that bind high-mannose sugars of the HIV envelope.* Prog Biophys Mol Biol, 2005. **88**(2): p. 233-82.
87. Balzarini, J., *Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy.* Nat Rev Microbiol, 2007. **5**(8): p. 583-97.
88. Hebert, D.N., S.C. Garman, and M. Molinari, *The glycan code of the endoplasmic reticulum: asparagine-linked carbohydrates as protein maturation and quality-control tags.* Trends Cell Biol, 2005. **15**(7): p. 364-70.
89. Helenius, A. and M. Aebi, *Intracellular functions of N-linked glycans.* Science, 2001. **291**(5512): p. 2364-9.
90. Scheiffele, P., J. Peranen, and K. Simons, *N-glycans as apical sorting signals in epithelial cells.* Nature, 1995. **378**(6552): p. 96-8.
91. Benting, J.H., A.G. Rietveld, and K. Simons, *N-Glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells.* J Cell Biol, 1999. **146**(2): p. 313-20.
92. Gut, A., et al., *Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins.* EMBO J, 1998. **17**(7): p. 1919-29.
93. Urquhart, P., S. Pang, and N.M. Hooper, *N-glycans as apical targeting signals in polarized epithelial cells.* Biochem Soc Symp, 2005(72): p. 39-45.
94. Huet, G., et al., *Involvement of glycosylation in the intracellular trafficking of glycoproteins in polarized epithelial cells.* Biochimie, 2003. **85**(3-4): p. 323-30.
95. Proszynski, T.J., K. Simons, and M. Bagnat, *O-glycosylation as a sorting determinant for cell surface delivery in yeast.* Mol Biol Cell, 2004. **15**(4): p. 1533-43.
96. Potter, B.A., R.P. Hughey, and O.A. Weisz, *Role of N- and O-glycans in polarized biosynthetic sorting.* Am J Physiol Cell Physiol, 2006. **290**(1): p. C1-C10.
97. Cantin, R., S. Methot, and M.J. Tremblay, *Plunder and stowaways: incorporation of cellular proteins by enveloped viruses.* J Virol, 2005. **79**(11): p. 6577-87.
98. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* J Exp Med, 1996. **183**(3): p. 1161-72.
99. Esser, M.T., et al., *Partial activation and induction of apoptosis in CD4(+) and CD8(+) T lymphocytes by conformationally authentic noninfectious human immunodeficiency virus type 1.* J Virol, 2001. **75**(3): p. 1152-64.
100. Fang, Y., et al., *Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes.* PLoS Biol, 2007. **5**(6): p. e158.
101. Raiborg, C., T.E. Rusten, and H. Stenmark, *Protein sorting into multivesicular endosomes.* Curr Opin Cell Biol, 2003. **15**(4): p. 446-55.
102. Martin-Serrano, J., *The role of ubiquitin in retroviral egress.* Traffic, 2007. **8**(10): p. 1297-303.

103. Vidal, M., P. Mangeat, and D. Hoekstra, *Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation.* J Cell Sci, 1997. **110 (Pt 16)**: p. 1867-77.
104. Camby, I., et al., *Galectin-1: a small protein with major functions.* Glycobiology, 2006. **16**(11): p. 137R-157R.
105. Pilobello, K.T. and L.K. Mahal, *Deciphering the glycocode: the complexity and analytical challenge of glycomics.* Curr Opin Chem Biol, 2007. **11**(3): p. 300-5.
106. Hsu, K.L., K.T. Pilobello, and L.K. Mahal, *Analyzing the dynamic bacterial glycome with a lectin microarray approach.* Nat Chem Biol, 2006. **2**(3): p. 153-7.
107. Pilobello, K.T., D.E. Slawek, and L.K. Mahal, *A ratiometric lectin microarray approach to analysis of the dynamic mammalian glycome.* Proc Natl Acad Sci U S A, 2007. **104**(28): p. 11534-9.
108. Angeloni, S., et al., *Glycoprofiling with micro-arrays of glycoconjugates and lectins.* Glycobiology, 2005. **15**(1): p. 31-41.
109. Tateno, H., et al., *A novel strategy for mammalian cell surface glycome profiling using lectin microarray.* Glycobiology, 2007. **17**(10): p. 1138-46.
110. Pilobello, K.T., et al., *Development of a lectin microarray for the rapid analysis of protein glycopatterns.* Chembiochem, 2005. **6**(6): p. 985-9.
111. Preston, A.B., *Carbohydrate cloak disguises HIV as normal cellular product*, in *Chemistry and biochemistry*. 2007, University of Texas at Austin: Austin. p. 53.
112. Trubey, C.M., et al., *Quantitation of HLA class II protein incorporated into human immunodeficiency type 1 virions purified by anti-CD45 immunoaffinity depletion of microvesicles.* J Virol, 2003. **77**(23): p. 12699-709.
113. Ott, D.E., et al., *The majority of cells are superinfected in a cloned cell line that produces high levels of human immunodeficiency virus type 1 strain MN.* J Virol, 1995. **69**(4): p. 2443-50.
114. Rossio, J.L., et al., *Inactivation of human immunodeficiency virus type 1 infectivity with preservation of conformational and functional integrity of virion surface proteins.* J Virol, 1998. **72**(10): p. 7992-8001.
115. Bolmstedt, A.J., et al., *Cyanovirin-N defines a new class of antiviral agent targeting N-linked, high-mannose glycans in an oligosaccharide-specific manner.* Mol Pharmacol, 2001. **59**(5): p. 949-54.
116. Liedtke, S., R. Geyer, and H. Geyer, *Host-cell-specific glycosylation of HIV-2 envelope glycoprotein.* Glycoconj J, 1997. **14**(7): p. 785-93.
117. Bastiani, L., et al., *Host cell-dependent alterations in envelope components of human immunodeficiency virus type 1 virions.* J Virol, 1997. **71**(5): p. 3444-50.
118. Lawn, S.D., et al., *Cellular compartments of human immunodeficiency virus type 1 replication in vivo: determination by presence of virion-associated host proteins and impact of opportunistic infection.* J Virol, 2000. **74**(1): p. 139-45.
119. Yang, Y.H., et al., *Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation.* Nucleic Acids Res, 2002. **30**(4): p. e15.
120. Lin, G., et al., *Differential N-linked glycosylation of human immunodeficiency virus and Ebola virus envelope glycoproteins modulates interactions with DC-SIGN and DC-SIGNR.* J Virol, 2003. **77**(2): p. 1337-46.

121. Gaskill, P.J., et al., *Macrophage-derived simian immunodeficiency virus exhibits enhanced infectivity by comparison with T-cell-derived virus.* J Virol, 2008. **82**(3): p. 1615-21.
122. Chertova, E., et al., *Envelope glycoprotein incorporation, not shedding of surface envelope glycoprotein (gp120/SU), Is the primary determinant of SU content of purified human immunodeficiency virus type 1 and simian immunodeficiency virus.* J Virol, 2002. **76**(11): p. 5315-25.
123. LaBranche, C.C., et al., *Biological, molecular, and structural analysis of a cytopathic variant from a molecularly cloned simian immunodeficiency virus.* J Virol, 1994. **68**(9): p. 5509-22.
124. Wang, S.K., et al., *Targeting the carbohydrates on HIV-1: Interaction of oligomannose dendrons with human monoclonal antibody 2G12 and DC-SIGN.* Proc Natl Acad Sci U S A, 2008. **105**(10): p. 3690-5.
125. Boyd, M.R., et al., *Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development.* Antimicrob Agents Chemother, 1997. **41**(7): p. 1521-30.
126. Bokesch, H.R., et al., *A potent novel anti-HIV protein from the cultured cyanobacterium Scytonema varium.* Biochemistry, 2003. **42**(9): p. 2578-84.
127. Mori, T., et al., *Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga Griffithsia sp.* J Biol Chem, 2005. **280**(10): p. 9345-53.
128. Pace, K.E., H.P. Hahn, and L.G. Baum, *Preparation of recombinant human galectin-1 and use in T-cell death assays.* Methods Enzymol, 2003. **363**: p. 499-518.
129. Eisen, M.B., et al., *Cluster analysis and display of genome-wide expression patterns.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14863-8.
130. Mariner, J.M., et al., *The HIV-inactivating protein, cyanovirin-N, does not block gp120-mediated virus-to-cell binding.* Biochem Biophys Res Commun, 1998. **248**(3): p. 841-5.
131. Chu, V.C. and G.R. Whittaker, *Influenza virus entry and infection require host cell N-linked glycoprotein.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 18153-8.
132. Brewer, C.F., M.C. Miceli, and L.G. Baum, *Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions.* Curr Opin Struct Biol, 2002. **12**(5): p. 616-23.
133. Rabinovich, G.A., et al., *Functions of cell surface galectin-glycoprotein lattices.* Curr Opin Struct Biol, 2007. **17**(5): p. 513-20.
134. Stowell, S.R., et al., *Galectin-1, -2, and -3 exhibit differential recognition of sialylated glycans and blood group antigens.* J Biol Chem, 2008. **283**(15): p. 10109-23.
135. John, C.M., et al., *Galectin-3 binds lactosaminylated lipooligosaccharides from Neisseria gonorrhoeae and is selectively expressed by mucosal epithelial cells that are infected.* Cell Microbiol, 2002. **4**(10): p. 649-62.
136. Pelletier, I., et al., *Specific recognition of Leishmania major poly-beta-galactosyl epitopes by galectin-9: possible implication of galectin-9 in interaction between L. major and host cells.* J Biol Chem, 2003. **278**(25): p. 22223-30.
137. Ouellet, M., et al., *Galectin-1 acts as a soluble host factor that promotes HIV-1 infectivity through stabilization of virus attachment to host cells.* J Immunol, 2005. **174**(7): p. 4120-6.
138. Mercier, S., et al., *Galectin-1 promotes HIV-1 infectivity in macrophages through stabilization of viral adsorption.* Virology, 2008. **371**(1): p. 121-9.

139. Stowell, S.R., et al., *Galectins-1, -2 and -3 exhibit differential recognition of sialylated glycans and blood group antigens.* J Biol Chem, 2008.
140. Kohatsu, L., et al., *Galectin-3 induces death of Candida species expressing specific beta-1,2-linked mannans.* J Immunol, 2006. **177**(7): p. 4718-26.
141. Swaminathan, G.J., et al., *Selective recognition of mannose by the human eosinophil Charcot-Leyden crystal protein (galectin-10): a crystallographic study at 1.8 A resolution.* Biochemistry, 1999. **38**(42): p. 13837-43.
142. Blixt, O., et al., *Printed covalent glycan array for ligand profiling of diverse glycan binding proteins.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17033-8.
143. Laude, A.J. and I.A. Prior, *Plasma membrane microdomains: organization, function and trafficking.* Mol Membr Biol, 2004. **21**(3): p. 193-205.
144. Thomas, S., et al., *Analysis of lipid rafts in T cells.* Mol Immunol, 2004. **41**(4): p. 399-409.
145. Brown, D.A., *Lipid rafts, detergent-resistant membranes, and raft targeting signals.* Physiology (Bethesda), 2006. **21**: p. 430-9.
146. Kabouridis, P.S., *Lipid rafts in T cell receptor signalling.* Mol Membr Biol, 2006. **23**(1): p. 49-57.
147. Pike, L.J., *Lipid rafts: bringing order to chaos.* J Lipid Res, 2003. **44**(4): p. 655-67.
148. Manes, S., G. del Real, and A.C. Martinez, *Pathogens: raft hijackers.* Nat Rev Immunol, 2003. **3**(7): p. 557-68.
149. van der Goot, F.G. and T. Harder, *Raft membrane domains: from a liquid-ordered membrane phase to a site of pathogen attack.* Semin Immunol, 2001. **13**(2): p. 89-97.
150. Luo, C., et al., *The functional roles of lipid rafts in T cell activation, immune diseases and HIV infection and prevention.* Cell Mol Immunol, 2008. **5**(1): p. 1-7.
151. Rousso, I., et al., *Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13523-5.
152. Wyma, D.J., A. Kotov, and C. Aiken, *Evidence for a stable interaction of gp41 with Pr55(Gag) in immature human immunodeficiency virus type 1 particles.* J Virol, 2000. **74**(20): p. 9381-7.
153. Bhattacharya, J., A. Repik, and P.R. Clapham, *Gag regulates association of human immunodeficiency virus type 1 envelope with detergent-resistant membranes.* J Virol, 2006. **80**(11): p. 5292-300.
154. Shaw, A.S., *Lipid rafts: now you see them, now you don't.* Nat Immunol, 2006. **7**(11): p. 1139-42.
155. Graham, D.R., et al., *Cholesterol depletion of human immunodeficiency virus type 1 and simian immunodeficiency virus with beta-cyclodextrin inactivates and permeabilizes the virions: evidence for virion-associated lipid rafts.* J Virol, 2003. **77**(15): p. 8237-48.
156. de Gassart, A., et al., *Lipid raft-associated protein sorting in exosomes.* Blood, 2003. **102**(13): p. 4336-44.
157. Del Conde, I., et al., *Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation.* Blood, 2005. **106**(5): p. 1604-11.
158. Calzolari, A., et al., *TfR2 localizes in lipid raft domains and is released in exosomes to activate signal transduction along the MAPK pathway.* J Cell Sci, 2006. **119**(Pt 21): p. 4486-98.
159. Paladino, S., et al., *Protein oligomerization modulates raft partitioning and apical sorting of GPI-anchored proteins.* J Cell Biol, 2004. **167**(4): p. 699-709.

160. Lingwood, D. and K. Simons, *Detergent resistance as a tool in membrane research.* Nat Protoc, 2007. **2**(9): p. 2159-65.
161. Macdonald, J.L. and L.J. Pike, *A simplified method for the preparation of detergent-free lipid rafts.* J Lipid Res, 2005. **46**(5): p. 1061-7.
162. Thery, C., et al., *Isolation and characterization of exosomes from cell culture supernatants and biological fluids.* Curr Protoc Cell Biol, 2006. **Chapter 3**: p. Unit 3 22.
163. Munro, S., *Lipid rafts: elusive or illusive?* Cell, 2003. **115**(4): p. 377-88.
164. Hancock, J.F., *Lipid rafts: contentious only from simplistic standpoints.* Nat Rev Mol Cell Biol, 2006. **7**(6): p. 456-62.
165. Delaguillaumie, A., et al., *Tetraspanin CD82 controls the association of cholesterol-dependent microdomains with the actin cytoskeleton in T lymphocytes: relevance to co-stimulation.* J Cell Sci, 2004. **117**(Pt 22): p. 5269-82.
166. Pike, L.J., *Lipid rafts: heterogeneity on the high seas.* Biochem J, 2004. **378**(Pt 2): p. 281-92.
167. Schade, A.E. and A.D. Levine, *Lipid raft heterogeneity in human peripheral blood T lymphoblasts: a mechanism for regulating the initiation of TCR signal transduction.* J Immunol, 2002. **168**(5): p. 2233-9.
168. George, S., et al., *A novel approach to examining compositional heterogeneity of detergent-resistant lipid rafts.* Immunol Cell Biol, 2006. **84**(2): p. 192-202.
169. Greene, W.C., *Denying HIV safe haven.* N Engl J Med, 1996. **334**(19): p. 1264-5.
170. Blanchard, N., et al., *TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex.* J Immunol, 2002. **168**(7): p. 3235-41.
171. Chen, S., et al., *Multiplexed analysis of glycan variation on native proteins captured by antibody microarrays.* Nat Methods, 2007. **4**(5): p. 437-44.
172. Patnaik, S.K., et al., *Complex N-glycans are the major ligands for galectin-1, -3, and -8 on Chinese hamster ovary cells.* Glycobiology, 2006. **16**(4): p. 305-17.
173. Karmakar, S., et al., *Galectin-1 signaling in leukocytes requires expression of complex-type N-glycans.* Glycobiology, 2008. **18**(10): p. 770-8.
174. Delacour, D., et al., *Requirement for galectin-3 in apical protein sorting.* Curr Biol, 2006. **16**(4): p. 408-14.
175. Delacour, D., et al., *Apical sorting by galectin-3-dependent glycoprotein clustering.* Traffic, 2007. **8**(4): p. 379-88.
176. Ye, Z. and J.D. Marth, *N-glycan branching requirement in neuronal and postnatal viability.* Glycobiology, 2004. **14**(6): p. 547-58.
177. Esser, M.T., et al., *Cyanovirin-N binds to gp120 to interfere with CD4-dependent human immunodeficiency virus type 1 virion binding, fusion, and infectivity but does not affect the CD4 binding site on gp120 or soluble CD4-induced conformational changes in gp120.* J Virol, 1999. **73**(5): p. 4360-71.
178. Mishra, S. and P.G. Joshi, *Lipid raft heterogeneity: an enigma.* J Neurochem, 2007. **103 Suppl 1**: p. 135-42.
179. Kobayashi, M., et al., *Global analysis of dynamic changes in lipid raft proteins during T-cell activation.* Electrophoresis, 2007. **28**(12): p. 2035-43.

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