CHAPTER THIRTEEN

GALECTIN-1 AND HIV-1 INFECTION

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Abstract

Initial binding of human immunodeficiency virus-1 (HIV-1) to its susceptible CD4+ cells is the limiting step for the establishment of infection as the avidity of viral envelope gp120 for CD4 is not high and the number of viral envelope spikes on the surface is found to be low compared to highly infectious viruses. Several host factors, such as C-type lectins, are listed as being able to enforce or facilitate the crucial interaction of HIV-1 to the susceptible cell. Recent works suggest that a host soluble β-galactoside-binding lectin, galectin-1, also facilitates both virion binding and the infection of target cells in a manner dependent on lactose but not mannose, suggesting that this soluble galectin can be

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considered as a host factor that influences HIV-1 pathogenesis. In this chapter, we describe methods used to investigate the potential role of the galectin family in HIV-1-mediated disease progression.

1. Overview

1.1. Human immunodeficiency virus

Human immunodeficiency virus-1 (HIV-1) is a lentivirus that belongs to the retrovirus family and causes acquired immunodeficiency syndrome (AIDS). Over 100 million people have been infected with this retrovirus and more than 25 million people have already died of AIDS. The impact of HIV-1 infections has been particularly devastating in the developing world. In some countries, as high as 25% of the adult population is reported to be infected with HIV-1, eliminating a large proportion of the working force that could support the development of financial and intellectual infrastructures in the near future (Greene, 2007). Since HIV-1 mainly infects cells of the immune system, namely CD4+ T lymphocytes and macrophages, and cripples the adaptive immune system, the impact of HIV-1 infection on emergence or spread of other infectious diseases is enormous (Weiss, 2001). First, HIV-1 infected individuals in poor settings, where access to medication is difficult, rapidly develop AIDS and thus become especially sensitive to infection by pathogens, opportunistic or not, present in the environment. Secondly, the ensuing immunocompromised status leads to high loads of coinfecting pathogens, including tuberculosis, a phenomenon increasing the chances of transmission to immunocompetent hosts. Thirdly, immunosuppression of millions of people in a population can also reduce the effectiveness of immunization campaigns and even make hazardous the use of live “attenuated” vaccines. When imagining the worst possible scenario, one can hypothesize that bacteria, fungi, and protozoa in the environment or zoonotic pathogens now have ~30 million immunocompromised people in which to learn and adapt as human pathogens. Thus, the control of the HIV/AIDS pandemics is urgent and a serious matter not only for the most afflicted countries, but also for the industrialized countries to reduce the risk of pandemics by other emerging infectious agents.

Despite the relentless progression toward AIDS in HIV-1 infected individuals, it is also known that HIV-1 is a relatively inefficient virus in regards to its potential of transmission and its overall infectivity. Epidemiological studies suggest that the possibility of transmission from a sexual intercourse ranges from 1 in 2000 to 1 in 200 depending on the type of sexual intercourse and the viral load of the infected individual. Simple preventative measures such as condom usage further reduce drastically the risk of HIV-1 transmission but cultural reasons hinder its acceptance in
many countries. Education about HIV/AIDS prevention and use of efficient physical or chemical barriers to sexual transmission of HIV-1 are certainly the most practical approaches to control the spread of the epidemic and to address the cost and availability issues associated with antiretroviral therapy in infected individuals. Very recent works indicate that at the port of entry in mucosal membranes, a very small proportion of HIV-1 reaches the lamina propria where resident dendritic cells can interact with the virus and transmit it \textit{in cis} or \textit{in trans} to a small number of “resting yet previously activated” effector memory CD4$^+$ T lymphocytes, establishing a founder HIV-1$^+$ cell population (Haase, 2005; Hel \textit{et al}., 2006; Mehandru \textit{et al}., 2004). Once this founder population initiates viral replication, a significant number of CD4$^+$ memory T lymphocytes found in the gut–associated lymphoid tissue are destroyed within 4–10 days. By 3 weeks postinfection, close to 80% of CD4$^+$ T lymphocytes are depleted and the virus has already spread to the whole organism while entering the chronic stage of the infection (Brenchley \textit{et al}., 2004; Li \textit{et al}., 2005b; Mattapallil \textit{et al}., 2005; Mehandru \textit{et al}., 2004). Since viral spread and memory CD4$^+$ T lymphocytes depletion arise from infection of such a limited number of susceptible cells initially, this port of entry also represents a major vulnerability for HIV-1. Thus, information related to the very early stage of HIV-1 infection could provide a possible means of prevention at this bottleneck of viral transmission.

To infect CD4$^+$ cells, HIV-1 must first stably attach to its target cells. This is mediated by the interaction between the viral envelope gp120 and the host integral membrane protein CD4, leading to a conformational change of gp120, which allows its interaction with a chemokine receptor, most notably CCR5 or CXCR4. This complex formation is crucial for viral entry through membrane fusion, which is initiated by insertion of the viral transmembrane glycoprotein gp41 into the target cell membrane (Chan and Kim, 1998; Gallo \textit{et al}., 2003). Importantly, however, the avidity of oligomeric gp120 for CD4 appears to be much lower than originally expected (Fouts \textit{et al}., 1997; Moore and Sweet, 1993; Moore \textit{et al}., 1992; Sattentau and Moore, 1995). It is suggested that the equilibrium binding at 37 °C is only achieved after 1–2 h (Moore and Sweet, 1993). The very low avidity and the slow binding kinetics displayed by oligomeric gp120 suggest that the gp120–CD4 interaction alone could not be sufficient to initiate fusion, especially in cells expressing low surface levels of CD4 (Ugolini \textit{et al}., 1999). In addition, HIV–1 carries less than 30 envelope spikes on its surface (Chan and Kim, 1998; Gallo \textit{et al}., 2003), which is in contrasts to the highly infectious influenza virus, which surface contains about 350 viral spikes (Karlsson Hedestam \textit{et al}., 2008). Indeed, even under optimal \textit{in vitro} condition, less than 0.5% of HIV-1 associates with target cells, suggesting that the initial interaction occurs under suboptimal conditions (Bobardt \textit{et al}., 2003; Cantin \textit{et al}., 2005; Mondor \textit{et al}., 1998; Tremblay \textit{et al}., 1998; Ugolini \textit{et al}., 1999).
Thus, viral attachment represents a rate-limiting step for virus entry and requires additional reinforcement especially at the beginning of HIV-1 pathogenesis (Mondor et al., 1998; Ugolini et al., 1999). Thus, attention has been drawn to identify other virus–cell interactions that could prompt efficient attachment and entry in vivo since disruption of such host factor-mediated interactions in the early stage of infection could greatly reduce the initial infection of susceptible cells and further limit the expansion of HIV-1 at the mucosal site (Bobardt et al., 2003; Cantin et al., 2005; Mondor et al., 1998; Tremblay et al., 1998; Ugolini et al., 1999).

To date, information concerning those host factors has been relatively limited. At least two types of host membrane proteins, that is, \(\beta\)-integrins and C-type lectins, have been shown to facilitate viral attachment (Bobardt et al., 2003; Cantin et al., 2005; Mondor et al., 1998; Saphire et al., 2001; Tremblay et al., 1998; Ugolini et al., 1999). For example, it has been shown that CD4\(^{+}\) T lymphocytes express a \(\beta\)-integrin, LFA-1, that promotes HIV-1 attachment by binding to ICAM-1, a host molecule acquired by HIV-1 into its envelope during the budding process (Cantin et al., 1997; Fortin et al., 1997; Hildreth and Orentas, 1989; Tremblay et al., 1998). Dendritic cells, which can transmit virus to permissive cells, express C-type lectins such as DC-SIGN, dendritic cell immuno-receptor (DCIR), CD207, and CD206. Those membrane C-type lectins capture many pathogens, including HIV-1, by binding to surface protein oligomannose glycans (Feinberg et al., 2001; Geijtenbeek et al., 2000; Kwon et al., 2002; Lambert et al., 2008; Lin et al., 2003; McDonald et al., 2003; Turville et al., 2003). In the case of HIV-1, this can result in dissemination of the virus to permissive cells (Pope and Haase, 2003). In addition to these molecules, we recently found that at least one member (galectin-1) of another type of lectin family, galectin, which has affinity for \(\beta\)-galactoside, can also contribute to HIV-1 binding to CD4\(^{+}\) T cells and macrophages.

1.2. Galectins

1.2.1. Structures of galectins

Galectin-1 belongs to the galectin family, which is defined by conserved peptide sequence elements in the carbohydrate recognition domain (CRD), consisting of \(\sim\)130 amino acid (Barondes et al., 1994a). Up to 14 galectins (galectin-1–14) have been found in mammals so far, as well as in many other phyla including birds, amphibians, fish, nematodes, drosophila, sponges, and fungi (Leffler, 2002). While all galectins share a core sequence in their CRD, galectins exhibit interesting structural differences in the presentation of their CRD (Hirabayashi and Kasai, 1993). Some galectins contain one CRD (prototype), and exist as monomers (galectin-5, -7, -10) or dimers (galectin-1, -2, -11, -13, -14) while other galectins, such as galectin-4, -6, -8, -9, -12 contain two CRD connected by a short linker region (tandem
repeat) (Hirabayashi and Kasai, 1993). In contrast, galectin-3 uniquely occurs as a chimeric protein with one CRD and an additional non-CRD domain, which is involved in its oligomerization. Upon binding to its glycan ligands at the cell surface, the conformation of galectin-3 appears to be altered, and galectin-3 oligomerizes by self-assembly of its N-terminal regulatory domain. This oligomerization results in the formation of galectin-3 molecules with multivalent CRDs (Ahmad et al., 2004; Nieminen et al., 2007).

1.2.2. Glycan-binding specificity
The minimum binding unit recognized by galectins is the galactose residue (Gal), linked to an adjacent saccharide in the β configuration (called β-galactoside), such as lactosamine residues (Galβ1-4N-acetylg glucosamine, Galβ1-4GlcNAc; Barondes et al., 1994b; Rabinovich et al., 2002; Sato, 2002; Sato and Nieminen, 2004; Sparrow et al., 1987). While this type of glycans (i.e., β-galactoside-containing glycans) is often found in N-linked “complex-type” glycans attached to proteins (glycoproteins), each galectin binds to a relatively limited set of ligands.

1.2.3. Expression of galectin-1
Galectin-1 is expressed in thymus and lymphoid parenchymal epithelial cells, lamina muscularis mucosae, endothelial cells, trophoblasts, activated T lymphocytes, macrophages, activated B cells, and follicular DCs (Baum et al., 1995; Blaser et al., 1998; Dettin et al., 2003; Jeschke et al., 2004; Rabinovich et al., 1996; Stillman et al., 2006; Zuniga et al., 2001). Similar to human tonsil lymphoid tissue, gut-associated lymphoid tissues could contain as high as ~20 μM of galectin-1 (Ouellet et al., 2005).

1.2.4. Release/secretion of galectins
With the unique exception of galectins, all the other mammalian lectins are synthesized in the lumen of the endoplasmic reticulum and Golgi apparatus and delivered to the extracellular space through the classical secretory pathway. In contrast, all galectins are synthesized as cytosolic proteins, even though their glycan ligands are found in the extracellular space (Hughes, 1999). The biological significance of this localization remains speculative (Sato, 2002; Sato and Nieminen, 2004; Sato et al., 2009; Vasta, 2009). It has, however, been clearly established that extracellular release of galectins is a highly regulated process that involves active secretion through an “alternative” secretory pathway, which is also used by fibroblast growth factors and IL-1 (Bianchi, 2007; Cooper et al., 1991; Hughes, 1999; Nickel, 2003, 2005; Oppenhein et al., 2007; Sato and Hughes, 1994; Sato et al., 1993). Although the alternative secretory pathway is still poorly understood, an original report on the observation that differentiated myoblasts can secrete massive amounts of galectin-1 suggests that (rather than direct transport through a hypothetical transporter) galectin-1 is first
accumulated below the plasma membrane, and is then exported by a mechanism involving ectocytosis (Cooper and Barondes, 1990). This has been confirmed by other reports on other galectins (Hughes, 1999; Nickel, 2003; Sato et al., 1993). The exported galectins in vesicles are then passively released to the extracellular space (Hughes, 1999; Mehul and Hughes, 1997). Galectin-1 is secreted by activated B cells, activated (but not quiescent) T lymphocytes, activated macrophages, and certain epithelial cells (Baum et al., 1995; Blaser et al., 1998; Rabinovich et al., 1998; Zuniga et al., 2001). In addition, galectin-1 can be passively released from necrotic cells (Sato, 2002; Sato and Nieminen, 2004; Sato et al., 2009).

2. Experimental

Our previous reports suggest that galectin-1, but not galectin-3, drastically increases the kinetics of HIV-1 binding to CD4$^+$ cells (T lymphocytes and macrophages; Mercier et al., 2008; Ouellet et al., 2005). This enhancement in binding thus greatly facilitates infection of susceptible cells and leads to a more robust viral replication. In the following sections, the various methods that were used to investigate the role of galectins in HIV-1 infection and new set of data related to the activity of galectin-1 in HIV-1 infection, will be described.

2.1. General precautions for HIV-1-related studies (biosafety level for HIV-1 research; Byers et al., 2004)

Selection of an appropriate biosafety level for work with a particular pathogen depends on a number of factors, such as the virulence, the pathogenicity, the route of spread, manipulations involving the agent and the availability of effective therapeutic measures. In the case of HIV-1, routine diagnostic work with clinical specimens to detect the presence of antibodies against HIV-1 can be performed in a biosafety level 2 (BSL2) facility using practices and procedures relevant to this biosafety level (Jackson and Balfour, 1988). However, the majority of experimental procedures involved in fundamental research of HIV-1, including infection of susceptible cells, replication and harvest of the virus, or the use of concentrated viral preparations must be carried out using BSL3 practices and procedures. Further, medium- to large-scale viral production and concentration of viral preparation by ultracentrifugation also require a BSL3 facility and use of BSL3 practices and procedures (Delenda et al., 2002). It is strongly recommended to strictly follow the laboratory safety and biosecurity measures recommended by both your country and institution. Some information can be found on web sites such as http://www.cdc.gov and http://www.phac-aspc.gc.ca.
2.2. HIV-1 production

Human embryonic kidney (HEK) 293T cells are normally used to obtain highly concentrated and infectious HIV-1 preparations, as this cell line consistently produces higher virus yields compared to methods using primary CD4\(^+\) cells. However, when compared to primary target cells such as CD4\(^+\) lymphocytes and macrophages (Brockhausen, 1999; Garcia et al., 1991), it is possible that established cancerous cell lines introduce different glycosylation patterns on both the viral envelope glycoproteins and the host glycoproteins that are being acquired by the virus. Thus, it is recommended to verify that any effects observed by a host lectin on HIV-1 infection are consistently found regardless of producing cells, or at least by the most physiological cellular targets. In the case of galectin-1 and -3, our previous works confirmed that galectin-1, but not galectin-3, enhances both HIV-1 attachment and infection, regardless of the virus producer cells (Mercier et al., 2008; Ouellet et al., 2005). The method to purify or maintain cells will be described in Section 2.3.

2.2.1. HIV-1 production in HEK 293 T cells

HEK cells are seeded at \(2 \times 10^6\) cells in a ventilated 75 cm\(^2\) cell culture flask (T-75) for 16 h prior to a transient transfection with infectious HIV-1 molecular clones, such as pNL4-3 (X4-tropic; Adachi et al., 1986) or pNL4-3 Bal env (R5-tropic; Cantin et al., 1996; Dornadula et al., 1999). For the preparation of this calcium phosphate transfection, all solutions should be at room temperature. The plasmid DNA (30 \(\mu\)g) is suspended in 500 \(\mu\)l of 0.25 M calcium chloride in sterile distilled water. This DNA solution is slowly added dropwise into 500 \(\mu\)l of 2\%/\% HEPES-buffered saline (280 mM NaCl supplement with 10 mM KCl, 1.5 mM Na\(_2\)HPO\(_4\), 12 mM dextrose, 50 mM HEPES (pH 7.05–7.12)) under continuous mild agitation. Once these reagents are mixed, they should be incubated for 20 min at RT (milky cloudiness should appear during the incubation). The culture medium of HEK cells in the flask is replaced with 9 ml of fresh DMEM supplemented with 10\% FBS, and the premixed transfection reagent is slowly added to cells. The flask is moved gently back and forth to distribute the transfection solution evenly. Rotation movements should be avoided, as this would increase the possibility of accumulation of the DNA–calcium phosphate precipitate in the center of the flask, which would affect the yield of virus production. After 4–16 h at 37 °C, the medium is removed and cells are washed gently with PBS and then 10 ml of fresh DMEM–10\% FBS is added. Two days posttransfection, virus-containing cell-free medium is collected, filtered through a 0.22-\(\mu\)m sterile syringe filter, and aliquoted to prepare frozen stocks at \(-80\) °C. If necessary, this viral preparation can be further concentrated and/or purified (see the sections
below). Quality control of prepared virus is routinely carried by measuring both p24 capsid protein and infectivity (see below for detail).

2.2.2. HIV-1 production in primary cells such as peripheral blood mononuclear cells, CD4+ T lymphocytes and monocyte-derived macrophages

In most cases, it is important to confirm observations made using cell line-derived viruses with a viral preparation produced by human primary target cells. In the case of HIV-1, however, it is important to mention that the expected yield in primary CD4+ cells such as CD4+ T lymphocytes and macrophages is often several orders of magnitude less than with HEK cells. For CD4+ T lymphocytes, cells (1–2 × 10^6 cells/ml) are first activated with 1 μg/ml of phytohemagglutinin-L (PHA-L, Sigma-Aldrich, Cat. # L4144) and 50 U/ml of recombinant human IL-2 (NIH AIDS Research & Reference Reagent Program) for 3 days in RPMI1640 supplemented with 10% FBS. Cells are then infected with HIV-1 (NL4-3, X4-tropic virus) at a concentration of 10 ng of p24 equivalent per 1 × 10^5 cells for 4–10 days. Virus-containing cell-free medium is then collected at 4, 7, and 10 days postinfection, and replaced by fresh culture medium to continue viral harvest. Supernatants can be concentrated by ultracentrifugation and further purified by Optiprep™ gradient (see below for the methods). For macrophages (monocyte-derived macrophages, MDMs), cells are plated at a density of 5–10 × 10^6 cells per T-75 flask and infected with HIV-1 (NL-4-3 Bal env) at a dose of 20 ng of p24 per 1 × 10^5 cells for 2–4 h at 37 °C. After this short exposure, MDMs are washed two times with PBS, followed by a brief wash with RPMI1640–5% FBS. Then, MDMs are incubated with RPMI1640–5% FBS for the production of virus. Half of the cell-free medium is collected at day 7, 14, 21, and 28 postinfection and replaced with fresh medium. Peak of virus production (based on p24 levels in the medium) is between 10 and 21 days postinfection. From this cell-free supernatant, virus is further purified or concentrated as described below.

2.2.3. p24 ELISA

Our previous observations indicate that the majority of the p24 capsid protein (>90%) is associated with HIV-1 particles (Fortin et al., 1997). Thus, virus stocks are normalized using p24 levels as estimated by an in-house p24 test. In brief, an ELISA 96-well plate (high binding, Immuno Plate MaxiSorp, NUNC) is first coated with a monoclonal anti-p24 antibody (183-H12-5C, 2.5 μg/ml, 100 μl/well, NIH AIDS Research & Reference Reagent Program) either overnight at 4 °C or for 1 h at 37 °C. After three washes with 300 μl PBST (PBS with 0.05% Tween-20), free surface sites are blocked by incubation with 200 μl of 1% bovine serum albumin (BSA) in PBST at RT for 30 min. Following another round of three washes, viral preparations are added to the wells (100 μl/well) at
various dilutions in 1% BSA-PBST. Recombinant purified p25gag/SF2 (31.25–2000 pg/ml, NIH AIDS Research & Reference Reagent Program) is used as a standard. To lyse viral particles, 25 μl/well of disruption buffer (2.5% Triton X-100, 0.05% Tween-20, thimerosal 0.02% in PBS) is added. After incubation for 1 h at RT followed by three washes, 100 μl/well of 0.5 μg/ml biotinylated anti-p24 antibody (clone 31-90-25, ATCC #HB-9725) is added and incubation is carried out for 1 h at RT. After removing unbound antibody by three washes, streptavidin-conjugated horseradish peroxidase (100 μl/well, 66 ng/ml, HRP40-streptavidin, Fitzgerald industries international) is added and incubated at RT for 20 min. Following the last series of wash, the wells are incubated for 10 min with a peroxidase substrate, TMB-S (100 μl/well, Fitzgerald industries international), and the reaction is terminated by adding 50 μl of 1 M H₃PO₄. The absorbance is measured using a microplate reader at 450 nm with a reference at 630 nm and p24 values are estimated based on regression analysis of p24 standards over a linear range (Bounou et al., 2002).

2.2.4. Concentration of HIV-1 preparation by ultracentrifuge
The cell-free supernatant containing HIV-1 can be further concentrated to obtain higher titer viral preparations by ultracentrifugation at 58,000xg (28,000 rpm with Beckman 70Ti rotor) for 45 min at 4 °C without brake. As the virus pellet is not visible at the end of the run, it is recommended to mark the expected pellet position. Discard supernatant by pouring it off in one continuous motion, being careful not to shake or blot off any drop that may remain hanging. The virus pellet is then thoroughly resuspended with 1 ml of PBS (the virus pellet can be located both at the bottom and on the side of the tube). The concentration of virus is then reevaluated with a p24 ELISA.

2.2.5. Purification of HIV-1 with Optiprep™
For highly purified HIV-1, an Optiprep™ gradient is used to separate virus particles from cell-released exosomes. Optiprep™ is a 60% (w/v) iodixanol solution in water (Axis-shield Cat. # 1030061) with a density of 1.32 g/ml. The gradient is composed of 11 fractions (900 μl each) of iodixanol in PBS with concentrations starting from 6% to 18% with 1.2% increments, that is, 6.0%, 7.2%, 8.4%, 9.6%, 10.8%, 12.0%, 13.2%, 14.4%, 15.6%, 16.8%, 18.0%. Under a laminar flow hood, stock solutions of these fractions are prepared and can be kept at 4 °C. Using a serological 1 ml pipette, 18% iodixanol–PBS is placed at the bottom of an Optiseal™ tube (Beckman Cat. # 362181). Then, the tube is tilted at 45° and 900 μl of the 16.8% solution is slowly but continuously deposited onto the 18% iodixanol layer. The end of the pipette should be maintained in a position so it barely touches the 18% layer. After the addition of a new layer, the interface of the layers becomes visible for a short period of time. The position of each
layer should be marked to facilitate the collection of a desired fraction after centrifugation. Nine more layers are placed in a similar manner to obtain a 6–18% gradient of iodixanol. Four hundred microliters of virus suspension, which was concentrated by ultracentrifugation, is slowly applied to the top of the iodixanol gradient (6%). Aluminum caps are inserted and firmly closed on Optiseal™ tubes to seal them in the NVT65 rotor and caps are then screwed over the filled positions of the rotor by applying pressure between 80 and 100 psi using the provided tool (Allen key with a dynamometer). The gradient is ultracentrifuged for 1 h 15 min at 219,000 x g (52,000 rpm) at 4 °C with slow acceleration and deceleration. With a 1-ml pipette, each gradient fraction is gently removed. HIV-1 is normally found between fractions 14.4% and 16.8%. Pooled fractions (~3 ml) are diluted ~10-fold with PBS followed by ultracentrifugation at 58,000 x g as above to obtain purified HIV-1. Quality control of prepared virus should be carried out following each purification using both the p24 ELISA described above and infectivity assays with reporter cell lines described below.

2.2.6. AT-2 inactivation of HIV-1
For some studies that do not require infectious HIV-1 virus particles, such as virion structure studies, a treatment with 2-aldrithiol (2,2'-dithiodipyridine or AT-2, Sigma-Aldrich, Cat. # D5767) can be used to inactivate HIV-1, thereby giving the possibility to perform experiments in a BSL2 facility using BSL2 practices and procedures. AT-2 covalently modifies the essential zinc fingers in the viral nucleocapsid proteins, reverse transcriptase and integrase, thereby inactivating HIV-1 while preserving the integrity of viral envelope proteins (Chertova et al., 2003; Rossio et al., 1998). AT-2 powder should be kept at 4 °C and protected from moisture and the stock solution (100 mM in methanol) should be prepared under a chemical hood (this solution can be stored at −20 °C for 1 month). After filtration of the viral solution using a 0.22-μm syringe filter, the AT-2 stock solution (10 μl/1 ml of virus solution, final concentration 1 mM) is added and incubated overnight at 4 °C and protected from light. Virus particles are then purified by ultracentrifugation at 58,000 x g (Beckman rotor type 70Ti at 28,000 rpm) at 4 °C for 45 min without brake. After carefully removing the supernatant, the virus pellet is resuspended in PBS and the virus concentration is estimated by monitoring the p24 content. It is important to remove the AT-2-containing supernatant as much as possible since concentrations of more than 8 μM have been observed to induce apoptosis of peripheral blood mononuclear cells (PBMCs) after 24 h of treatment. Lack of infectivity of each viral preparation treated with AT-2 has to be confirmed if the experiments with AT-2-treated HIV-1 are to be performed outside a BSL3 facility using a sensitive luciferase-based HIV-1 infection assay involving reporter cell lines, such as LuSIV or TZMBL cells. Typically, concentrations of HIV-1 between 1 and 100 ng of p24
per $1 \times 10^5$ cells are used to evaluate inactivation. Luciferase activity is checked after 24–48 h to be sure that viral particles do not carry any residual infectivity. It is only after this verification that the viral preparations can be used outside a BSL3 facility.

2.3. Cell lines and primary cells isolation for HIV-1 infection

2.3.1. LuSIV

The LuSIV cell line (NIH AIDS Research & Reference Reagent Program) is derived from the CEMx174 cell line, which stably expresses a firefly luciferase reporter gene driven by the SIVmac 239 long terminal repeat (LTR) region. This cell line is highly sensitive to infection by primary and laboratory T-cell-tropic strains of HIV-1 and SIV, resulting in Tat-mediated expression of luciferase correlating with viral infectivity. The LuSIV system is a powerful tool to analyze HIV/SIV infectivity, providing a unique assay system that can detect virus replication as soon as 24 h, due to the initial presence and early expression of Tat in infected cells (Wu, 2004). Since primate lentiviruses, such as HIV-1 and SIV, require an average of 24 h to complete one cycle of replication, LuSIV enables measurements after a single round of infection (Roos *et al*., 2000). Cells are cultured in RPMI1640 supplemented with 10% FBS, 300 $\mu$g/ml of hygromycin B at a cellular concentration between $2.5 \times 10^5$ and $2 \times 10^6$ cells/ml. Hygromycin B is used to maintain cells containing the episomal reporter plasmid (Yates *et al*., 1985).

2.3.2. TZM-bl

The TZM-bl cell line (NIH AIDS Research & Reference Reagent Program), also called JC53BL-13, is a CXCR4 positive HeLa cell clone that is engineered to express both CD4 and CCR5 (Platt *et al*., 1998). These cells have been further modified to contain integrated reporter genes for firefly luciferase and $\beta$-galactosidase under the control of the HIV LTR sequence (Wei *et al*., 2002). TZM-bl cells are permissive to infection by a wide variety of HIV-1, SIV, and SHIV strains, including primary HIV isolates and molecularly cloned Env-pseudotyped viruses. These adherent cells are grown in DMEM supplemented with 10% FBS. For the passage of this cell line, a 0.25% trypsin solution is used (Li *et al*., 2005a).

2.3.3. Peripheral blood mononuclear cells

PBMCs are purified from blood of healthy donors by Ficoll–Hypaque centrifugation. Fresh blood is collected and treated with anticoagulants, heparin or citrate depending on the system used to collect blood. Fresh blood (25 ml) is carefully applied over 20 ml of Ficoll–Hypaque (GE Healthcare Cat. # 17-1440-02) in a 50-ml conical tube and then centrifuged at 400$x$g for 30 min at RT without brake. After centrifugation,
the upper layer, containing diluted autologous plasma, is removed by aspiration to leave the interphase layer, rich in mononuclear cells, undisturbed. The cells at the interphase are then carefully transferred to a new conical tube, which is filled with Hank’s balanced salt solution (HBSS), and cells are centrifuged at 300×g for 10 min at RT. Supernatant is removed and two additional washing steps are carried out by resuspending cells with 50 ml of HBSS and centrifugation at 200×g for 10 min at RT. Cells are resuspended at 1 × 10^6 cells/ml in RPMI1640 with 10% FBS. PBMCs can be used immediately or activated (see below for conditions).

2.3.4. CD4+ T lymphocytes
CD4+ T lymphocytes are purified from PBMCs using the human CD4+ T lymphocytes enrichment kit (Stemcell Technologies Inc., EasySep CD4+ T cell enrichment kit Cat. # 19052). Following the manufacturer’s protocol, PBMCs are resuspended at a concentration of 5 × 10^7 cells/ml in PBS supplemented with 2% FBS and placed in a 5-ml polystyrene tube. The enrichment cocktail (50 µl/ml) is added, mixed, and incubated at RT for 10 min. EasySep D magnetic particles are vortexed for at least 30 s to prevent aggregation, and then the particles (100 µl/ml) are added to resuspended PBMCs, mixed and incubated at RT for 5 min. Then, PBS supplemented with 2% FBS is added to the cell suspension to obtain a total volume of 2.5 ml. The entire suspension is mixed gently and placed in a magnet stand, without cap, for 5 min. The cell suspension is then poured into a new tube by inversion of the magnet stand using a continuous motion. The CD4+ enriched cells can be used immediately or activated.

2.3.5. Monocyte-derived macrophages
PBMCs (1.25 × 10^8 cells per T-75 flask) are plated and incubated at 37 °C for 2 h. Nonadherent cells are then removed and adherent monocytes are washed twice with RPMI 1640–5% FBS and further cultured for a week in medium supplemented with 100 ng/ml macrophage colony-stimulating factor (M-CSF; GenScript Corporation) for differentiation into MDMs. MDMs are then removed from the flask by briefly incubating with Accutase® (Sigma-Aldrich, Cat. # A6964) followed by gentle scraping with a cell scraper. Cells are replated in a 48-well plate at 5 × 10^4 cells/well for infection studies (Mercier et al., 2008).

2.3.6. Cell activation
It is known that resting CD4+ T lymphocytes are only weakly permissive to productive HIV-1 infection due to host factors affecting different steps of the virus life cycle (Tremblay et al., 1998). Thus, to maximize infection, CD4+ T lymphocytes are activated with 1 µg/ml of PHA-L and 50 U/ml of recombinant human IL-2 (Scott and Nahm, 1984). Alternatively, PBMCs or CD4+ T lymphocytes can be activated through the T cell
receptor with the antibody OKT3 (1 μg/ml, ATCC) and a costimulation signal with an anti-CD28 antibody (clone 9.3 at 1 μg/ml) provided by J.A. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute), followed by cross-linking with goat anti-mouse IgG 5 μg/ml (Tardif and Tremblay, 2005).

2.4. Purification of galectin-1 and -3

To get consistent data obtained from experiments with galectins, it is essential to be fully aware that galectins are not necessarily stable (Hirabayashi and Kasai, 1991; Hsu et al., 1992) (unpublished observations in the laboratory of SS). Thus, constant monitoring of the quality of purified galectins is extremely important.

2.4.1. Purification

Human recombinant galectins are produced using BL21 Escherichia coli transformed with an expression plasmid containing the galectin gene as previously published (Mercier et al., 2008; Nieminen et al., 2005; Ouellet et al., 2005; Pelletier and Sato, 2002; Pelletier et al., 2003; Sato et al., 2002). E. coli are grown at 37 °C in LB culture medium with 100 μg/ml of ampicillin until optical density reaches 0.7 at 600 nm. Once in their exponential growth phase, bacteria are incubated with IPTG (1 mM) for 3 h at 37 °C. Then, bacteria are collected by centrifugation at 4 °C. The following steps are done on ice or at 4 °C and all buffers used for the preparation are required to be ice-cold. The bacteria pellet is resuspended with lysis buffer (22 mM Tris–HCl (pH 7.5)) supplemented with 5 mM EDTA, 1 mM DTT and a protease inhibitor cocktail (Sigma-Aldrich Cat. # P8465). We generally use at least 15 ml of lysis buffer to suspend bacteria derived from a 1 l culture to ensure sufficient disruption of membranes. The suspension is subjected to sonication in an ice-bath at 120 W for 30 s every 2 min eight times to disrupt bacterial membranes. Cell lysates are then centrifuged at 84,000×g (Beckman rotor 70.1Ti, 35,000 rpm) for 30 min at 4 °C to obtain a soluble fraction. Since galectins have high affinity for β-galactoside, lactosyl-agarose, or asialofetuin-agarose is used for purification of galectins. The soluble fraction is applied onto 2 ml of lactosyl-agarose column (Sigma-Aldrich Cat. # L7634), which is equilibrated with 50 mM Tris–HCl (pH 7.2) supplemented with 105 mM NaCl (TBS). A 25 column volumes wash with TBS is then carried out to remove unbound materials as well as EDTA and DTT in the lysis buffer. Since any trace of reducing agents such as DTT (as low as 1 μM) inhibits HIV-1 infection assays (unpublished data, CSP, SS) (Koken et al., 1994), it is important to completely remove DTT during purification. Active galectins are eluted from lactosyl-agarose with TBS supplemented with 150 mM lactose. The galectin-containing fractions (1 ml per fraction) are monitored by the
presence of protein with the Bradford reaction. Typically, the first 5–7 fractions (5–7 ml) contain galectin. Eluted galectin fractions are pooled and either ultrafiltration or gel filtration is used to remove lactose from the galectin preparation and to replace TBS with PBS. For galectin-3, we typically use gel filtration with a Hiprep 26/10 desalting column (53 ml column volume, GE Healthcare), which is equilibrated with PBS. Galectin-containing fractions (maximum volume to apply for this column is 15 ml) are applied and the fractions (1 ml/fraction) are collected. The presence of galectin is tracked by Bradford. Typically, the 15th to 30th fractions (~15 ml) contain galectin. To avoid dilution of purified lectin, only the peak fractions (5–7 ml, corresponding to ~80% of applied galectin) are pooled. For galectin-1, ultrafiltration is employed because it gives better yield and higher quality galectin than the gel filtration method. Galectin-1-containing fractions are placed in a dialysis tube (Spectrum, MWCO 6-8000) and extensively dialyzed against PBS at 4 °C for at least four rounds (1:500/dialysis, which theoretically reduces the lactose concentration from 150 mM to less than 2 pM). All purified galectins are then passed through Acticlean ETOX endotoxin-removing gels following manufacturer recommendations (Sterogene, Carlsbad, CA). Briefly, 1 ml of Acticlean ETOX is placed in a poly-prep chromatography column (Bio-Rad, #731-1550). Any other type of column can be used as long as the flow rate is well controlled to enable maximal interaction of samples with Acticlean. Endotoxin-binding capacity of this matrix is 20,000 EU/ml. Prior to use, Acticlean column must first be resuspended with 5 ml of 1 M NaOH and incubated overnight at 4 °C. The column is washed extensively with endotoxin-free sterile water until a neutral pH is reached in the eluate. After extensive washes with PBS, galectin solution is applied slowly, and the flowthrough fraction is collected. When necessary, this step is repeated. Galectin preparations are then sterilized by filtration through a 0.22 μm filter and kept at 4 °C until use. For both galectin-1 and -3, we found that freeze-thaw cycles significantly reduce the quality (specific lectin activity per protein, see the section on quality control for details). Mock preparations are also prepared when necessary using E. coli that does not express galectins by using the same purification protocol (Mercier et al., 2008; Nieminen et al., 2005). In our laboratory, these purification protocols yield 2–5 mg of galectin from every 3 l of bacteria culture medium.

2.5. Quality control of galectins

2.5.1. SDS-PAGE

Several procedures are used to ensure the quality of purified galectins. Purified galectins are subject to SDS-PAGE with a 12% or 15% reducing gel to confirm that galectin-1 and -3 migrate at their respective position of 14 and 30 kDa, without any additional protein band.
2.5.2. Endotoxin
The endotoxin level is measured using Limulus amebocyte lysate (LAL) assay kit (Associates of Cape Cod Incorporated). Protein preparations that exceed 10 EU/mg of protein are rejected or passed through the Acticlean ETOX endotoxin-removing gel.

2.5.3. Lectin activity (specific activity per weight of protein)
Specific lectin activity per protein concentration is then evaluated using a hemagglutination assay. To prepare the suspension of red blood cells (RBCs) for hemagglutination assay, heparine-treated human peripheral blood (10 ml) is first centrifuged at 2000×g for 5 min. Buffy coat is removed as much as possible and RBCs are washed three times with 50 ml PBS. Then RBCs are diluted with PBS to obtain 100 ml of the 8% cell suspension. RBC suspension is treated with glutaraldehyde (at a final concentration of 3%) under rotation for 1 h at RT, followed by washing with 0.0025% NaN3 in PBS. Fixed RBCs are resuspended at 3–4% in PBS–NaN3. Calibration of RBCs is required to obtain the appropriate concentration for lectin-mediated hemagglutination. Series of RBCs dilutions (2–20-fold) are distributed into a U-shape 96-well plate (100 µl/well) and incubated at 37 °C for 30 min. When RBCs are aggregated (hemagglutination), they are spread out like a sheet covering the entire surface of the well. In contrast, RBCs form very tight button-like precipitation at the bottom of the well when there is no aggregation. For lectin hemagglutination, we then use the minimum concentration of RBCs that results in this very tight button-like precipitation. RBC suspension can be kept at 4 °C for more than 3 months, although a routine check of the quality of RBCs is necessary. Serial dilutions of galectins (from 10 to 0 µM) are mixed with the appropriate quantity of RBCs in the wells of a U-shape 96-well plate and incubated at 37 °C for 30 min. Galectin-1 and -3 induce hemagglutination at concentrations of around 15 and 10 µg/ml, respectively (Butler, 1963; Giguere et al., 2006).

2.6. S-Carboxyamidomethylation of galectin-1
Among members of the galectin family, galectin-1 is a unique lectin that is sensitive to oxidation due to a cysteine residue proximal to its dimerization site (Cho and Cummings, 1996). Oxidation leads to its inactivation as a lectin and therefore loss of its hemagglutinin activity. Thus, some groups have been using relatively high concentrations of thiol-reducing agents such as DTT to prevent its oxidative inactivation and to maintain carbohydrate-binding activity upon purification and storage. However, treatment of live cells with DTT could seriously hamper cell viability and make interpretations about the effects of galectin-1 difficult as previously pointed out by the
group of Cummings (Stowell et al., 2007). Indeed, as little as 5 μM of DTT was sufficient to halt virtually all natural migration and movement of T lymphocytes (time-lapse live cell imaging, unpublished observations by CSP and SS). Moreover, the presence of 1 μM of DTT inhibited HIV-1 infection (unpublished observations by CSP and SS). Thus, it is highly recommended to avoid any reducing agents in assays related to galectins. It also raises some concerns over data obtained using commercially available galectins, as some of those galectins (galectin-1 as well as galectin-3) contain high levels of 2-mercaptoethanol (a reducing agent) as preservative. Other commercial sources provide preservative-free lyophilized form of galectin-1, although the state of oxidation of these reagents remains unknown. As recent works suggest, the oxidated form of galectin-1 also possesses some biological functions but they are independent of its lectin-binding activity (Horie et al., 2004; Scott et al., 2009). Thus, cautions must be applied for the analysis of data obtained with commercially available galectin-1. Recently, Stowell and colleagues found that S-carboxymidomethylation of galectin-1 by mild treatment with iodoacetamide makes galectin-1 resistant to atmospheric oxygen in absence of disulfide-reducing reagents and yet preserves some of its biological functions (Stowell et al., 2009). Our data (Fig. 13.1) also suggest that S-carboxymidomethylated galectin-1 facilitates HIV-1 infection to similar levels than freshly purified galectin-1. Thus, in some cases, we have begun to use this method to stabilize galectin-1, although it is highly recommended that each laboratory verifies whether this treatment has an impact on their own assays. Carboxymidomethylation of galectin-1 is achieved using the method published by Stowell et al. (2009). First, galectin-1 is resuspended in a 100 mM lactose–PBS solution to protect the CRD of the protein. Then, galectin-1 (2–5 mg/ml) is incubated overnight at 4 °C with iodoacetamide at a final

![Graph](Image)

**Figure 13.1** Carboxymidoacetylated galectin-1 facilitates HIV-1 infection. LuSIV cells were infected with HIV-1 (NL4-3) for 24 h in different concentrations of either galectin-1 (Gal-1) or carboxymidoacetylated galectin-1 (Gal-1\text{\textsubscript{IODO}}). Levels of infection were estimated by luciferase activity.
concentration of 100 mM. Free iodoacetamide and lactose are extensively removed by series of dialysis (ultrafiltration) against PBS, followed by sterilization as described above. The quality of galectin-1 is tested by hemagglutination assay. The treated protein remains stable over prolonged period at 4 °C (Stowell et al., 2008b; Whitney et al., 1986).

2.7. Inhibition of lectin activity of galectin in physiological condition

2.7.1. Sugar antagonists to inhibit galectin’s lectin activity

Being a $\beta$-galactoside-binding protein, the lectin activity of galectins can be readily inhibited by relatively small $\beta$-galactoside-containing sugars, such as lactose (Gal$\beta$1-4glucose) or other appropriate $\beta$-galactoside-containing oligosaccharides. While lactose is one of the most used inhibitors for galectins, affinity of galectins for lactose is often low and thereby high concentrations of lactose are necessary to inhibit their lectin activity. In our laboratory, 50–150 mM lactose is routinely used to ensure inhibition. The isotonicity of the medium has to be taken into account when such high concentrations of saccharide are used for cell assays as cells exposed to hypertonic solutions would become fragile to any successive treatment. To overcome this problem, the concentration of sodium chloride in PBS is reduced for the accommodation of high doses of lactose so that the lactose-containing PBS has the same osmolarity as saline (i.e., 317 mOsm/l).

Recently, Krishnamoorthy and coworkers reanalyzed the NIH consortium glycan array data (http://www.functionalglycomics.org), which the group of Cummings originally produced (Stowell et al., 2008a), in regard to the affinities of galectin-1 for oligomannose glycans (Krishnamoorthy et al., 2009), suggesting that galectin-1 is potentially a mannose-binding lectin. This suggestion is not consistent with previously published data concerning the specificity of galectin-1. In addition, our published data and Fig. 13.2 also indicate that galectin-1 promotes HIV-1 binding to the target cells in a $\beta$-galactoside-dependent manner, and cannot be inhibited by mannose (Fig. 13.2). Since the previous reports consistently suggest that binding of galectin-1 requires an intact nonreducing pyranose ring of GlcNAc residue that is linked to Gal residue, the same set of glycan array data are reanalyzed based on the number of those nonreducing lactosamine residues in the glycans used for this array (Fig. 13.3). Indeed, galectin-1 shows a weak affinity specific for only one mannose-containing glycan (#197; Man6) among five different oligomannose type glycans (#192, 193, 194, 197, 198; Man5, 6, 7, 8, and 9). However, galectin-1 binding to glycans containing one or two nonreducing lactosamine residues exhibits a 6.2-fold increase compared to those without, suggesting the superior binding preference of galectin-1 for $\beta$-galactoside over $\alpha$-mannoside. A modification of lactosamine with $\alpha$2–6 sialylation completely abolishes its binding,
consistent with the previous report of the group of Rabinovich (Toscano et al., 2007). This analysis, together with previously published data, confirms that galectin-1 is an almost exclusive \(\beta\)-galactoside-binding protein.

### 2.7.2. Dominant-negative form of galectins

The possibility to use the dominant-negative form of galectin molecules, which binds to similar ligands but lack cross-linking activity, has been put forward. For galectin-1, a modified protein unable to dimerize could be created by performing a point mutation of the valine residue at the amino position 5 (galectin-1-V5D) which is crucial for dimerization (Cho and Cummings, 1996). However, our unpublished observations suggest that this monomeric galectin-1 binds to lactosyl-agarose at 4 °C but not asialo-fetuin-agarose, which also contains \(\beta\)-galactoside (unpublished observation by J. Nieminen and SS). Furthermore, the monomeric form of galectin-1 could not inhibit dimeric galectin-1-induced hemagglutination (unpublished observation by JN and SS), suggesting that lack of avidity for \(\beta\)-galactoside prevents the dominant-negative galectin-1 from persistently binding to its ligand and from inhibiting binding by native galectin-1. For galectin-3, a potential dominant negative could be a truncated lectin that lacks the N-terminal repeating domain involved in its oligomerization. Truncated galectin-3 is easily prepared using collagenase VII (C0773; Sigma-Aldrich) (Nieminen et al., 2005, 2007). This truncated galectin-3 binds stably to the cell surface at 4 °C. However, immediately after increasing the temperature of the cell culture medium to 37 °C, the majority of truncated galectin-3 is released from the endothelial cell layer (Nieminen et al., 2007), suggesting that this form of truncated galectin-3 does not bind stably to the cell membrane at physiological temperatures. Indeed, the

![Graph showing the effect of galectin-1 on HIV-1 infection](image-url)
truncated form of galectin-3 could not compete with full-length galectin-3 binding, also suggesting that this form may not be considered as a dominant negative in certain conditions when stable binding of galectin-3 is necessary for its function.

2.8. Virus attachment assays

In physiological settings, binding kinetics of virion-associated gp120 to cellular CD4, the first step for virus infection, is known to be often slow. Further, in the early stages of HIV-1 infection, the majority of permissive
cells express low levels of CD4 (Ugolini et al., 1999). Thus, it has been proposed that a number of distinct interactions with cellular proteins may influence and facilitate virus attachment (Arthur et al., 1992). Indeed, even in vitro, binding of HIV-1 particles to CD4+ cells is found to be <1% after 60 min of binding at 37 °C. Our previous reports suggest that galectin-1 facilitates HIV-1 infection by increasing the kinetics of HIV-1 binding to its target cell (Mercier et al., 2008; Ouellet et al., 2005).

To study whether a lectin could increase HIV-1 binding to target cells or not, HIV-1 permissive cells, such as LuSIV cells, primary PBMC, or primary CD4+ cells are incubated with HIV-1 virions in the presence or absence of a lectin. Several approaches can be used to estimate the levels of HIV-1 attached to the target cells; for LuSIV, luciferase activity is evaluated while direct measurement of cell-associated p24 can be performed when primary cells are used.

### 2.8.1. Indirect evaluation of HIV-1 binding (luciferase activity)

Cells are first pretreated with various concentrations of galectins (0–4 μM) for 1 h at 4 °C. In some cases, a galectin antagonist, lactose (50 mM), is included to verify specificity. Then HIV-1 (virus suspension containing 10 ng of p24) is added to the well with 1 × 10^5 target cells and binding assays are performed by incubating for various times (0–2 h) at 4 °C. In some cases, higher temperatures such as RT or 37 °C can be used, especially when the dynamics or the tertiary structures of membrane proteins on the viral surfaces are a concern, although those temperatures also induce both viral–host membrane fusions and endocytosis, which may make the analysis of the impact of a lectin on binding difficult. Fusion inhibitors, such as enfuvirtide (T–20) (NIH AIDS Research & Reference Reagent Program, 100 ng/ml) can be included in such assays. After the incubations, cells are washed three times with PBS to remove both unbound lectin and virus. Cells are then resuspended in RPMI1640–10% FBS (100 μl/well) and cultured at 37 °C for 24 h. The initial binding of HIV-1 to LuSIV followed by incubation at 37 °C leads to membrane fusion, reverse transcription, integration, and production of the viral Tat protein. This viral transactivator binds to the TAR region of the integrated LTR located upstream of the luciferase gene, thereby increasing by 1000-fold the biosynthesis of the luciferase protein within 24 h. Since LuSIV cells are highly permissive to HIV-1 infection, the level of HIV-1 binding is directly correlated with the level of infection and therefore to the level of luciferase activity. To measure the activity of luciferase, cells in 100 μl/well are directly lysed by adding 25 μl of 5 × luciferase lysis buffer (Tris–HCl, pH 7.8 supplemented with 10 mM DTT, 5% Triton X-100, and 50% glycerol) and incubated for 30 min under agitation. The cell lysate (20 μl/well) is added to a 96 wells luminometer plate and the plate is placed in a Dynex MLX microplate luminometer. The luminometer injects 100 μl/well of luciferase assay buffer (20 mM tricine.
supplemented with 1.07 mM $(\text{MgCO}_3)_4\cdot\text{Mg(OH)}_2$, 2.67 mM $\text{MgSO}_4$, 0.1 mM EDTA, 220 $\mu$M coenzyme A, 4.7 $\mu$M d-luciferin potassium salt, 530 $\mu$M ATP, and 33.3 mM DTT). After a 2-s delay, the luciferase activity is monitored for 20 s per well (Ouellet et al., 1999).

2.8.2. Direct evaluation of HIV-1 binding (p24)

When natural cellular reservoirs, PBMCs, CD4$^+$ T lymphocytes, or MDMs as well as LuSIV cells, are used, HIV-1 binding to target cells is estimated by measuring cell-associated p24 with a p24 ELISA. Thus, a similar type of viral binding at 4 °C is carried out as described above and then, after extensive washing to remove unbound virus particles, the cells lysate is prepared for p24 ELISA. For example, MDMs at 5 $\times$ 10$^4$ cells/well are plated in a 48-well plate and incubated with R5-tropic virus (the viral input is usually 10 ng of p24) with or without galectin-1 for 1 h. After having been washed, cells are lysed with 25 $\mu$l/well of disruption buffer (see Section 2.2.3) and p24 levels are measured (Ouellet et al., 2005).

2.9. HIV-1 infection assay

For the function of a lectin in HIV-1 infection, it is recommended to initiate the study with a sensitive method like the LuSIV reporter cell system. This assay allows the quantitative evaluation of single-cycle infection events through activation of integrated LTR sequences driving the luciferase reporter gene following the production of the viral protein Tat by de novo viral infection. Once the lectin has been observed to influence HIV-1 infection in this reporter cell system, it is recommended to verify whether these effects can be observed in other HIV-1 susceptible cells, as different glycosylation patterns expressed by different cells may have an influence on the role of the lectin. For galectin-1, we have observed that galectin-1, but not galectin-3, facilitates HIV-1 infection in LuSIV cells, PBMC, both activated and naive CD4$^+$ T lymphocytes and macrophages (Mercier et al., 2008; Ouellet et al., 2005 and manuscript in preparation by CSP, MO, MJT, SS). We have also verified that this effect could be reproduced using HIV-1 produced in various cellular settings such as HEK cells, PBMCs, or macrophages. Thus, at least for galectin-1 activity in the context of HIV-1 infection, the involved glycan ligands appear to be similarly glycosylated, regardless of the cell type used for virus production and/or infectivity assays (manuscript in preparation by CSP, MO, MJT, SS). This further suggests that, in the case of the galectin-1 ligands involved in HIV-1 infection, the dominant glycosylation pattern of surface proteins could be mainly determined by their structure, rather than by the type of cells that produced the proteins.
2.9.1. LuSIV cells
Infectivity assays are performed in a similar manner to the HIV-1-binding assay described above with some modifications. LuSIV cells (1 × 10^5 cells/well) in RPMI1640–10% FBS (100 μl/well) are infected with HIV-1 (virus suspension containing 10 ng of p24) with various concentrations of galectins (0–4 μM) at 37 °C for 24 h to initiate a single round of viral replication. No washes are thus performed for infectivity assays. Lactose (50 mM) can be added to the cell culture medium (see 2.7.1 for some caution related to osmolarity) as a specificity control. To evaluate HIV-1 replication, cells are directly lysed with 20 μl of 5× luciferase lysis buffer, as above, and luciferase activity is monitored (Ouellet et al., 1999).

2.9.2. Primary cells
For infection in primary cells, PBMCs, CD4^+ T lymphocytes, and MDMs, the amount of HIV-1 capsid protein p24 in the culture medium is utilized to estimate viral replication as papers previously published by others and we have shown that the amount of p24 in the culture medium is closely correlated with the amount of HIV-1 produced by infected cells. Typically, for PBMCs or CD4^+ T lymphocytes, 1 × 10^5 cells/well are exposed to HIV-1. The levels of p24 in the culture medium collected at day 3, 6, and 9 posttreatment with HIV-1 are measured by p24 ELISA as described above. This multiround of infection assay allows us to study the role of a lectin in a more physiological context, since it provides an opportunity to address not only the role in a single viral replication (LuSIV cell system) but also to examine whether a lectin is involved in later steps of viral replication or in further rounds of infection.

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