Effects of galectins and antibodies in HIV infection; Novel Assays

Sheik-Khalil, Enas

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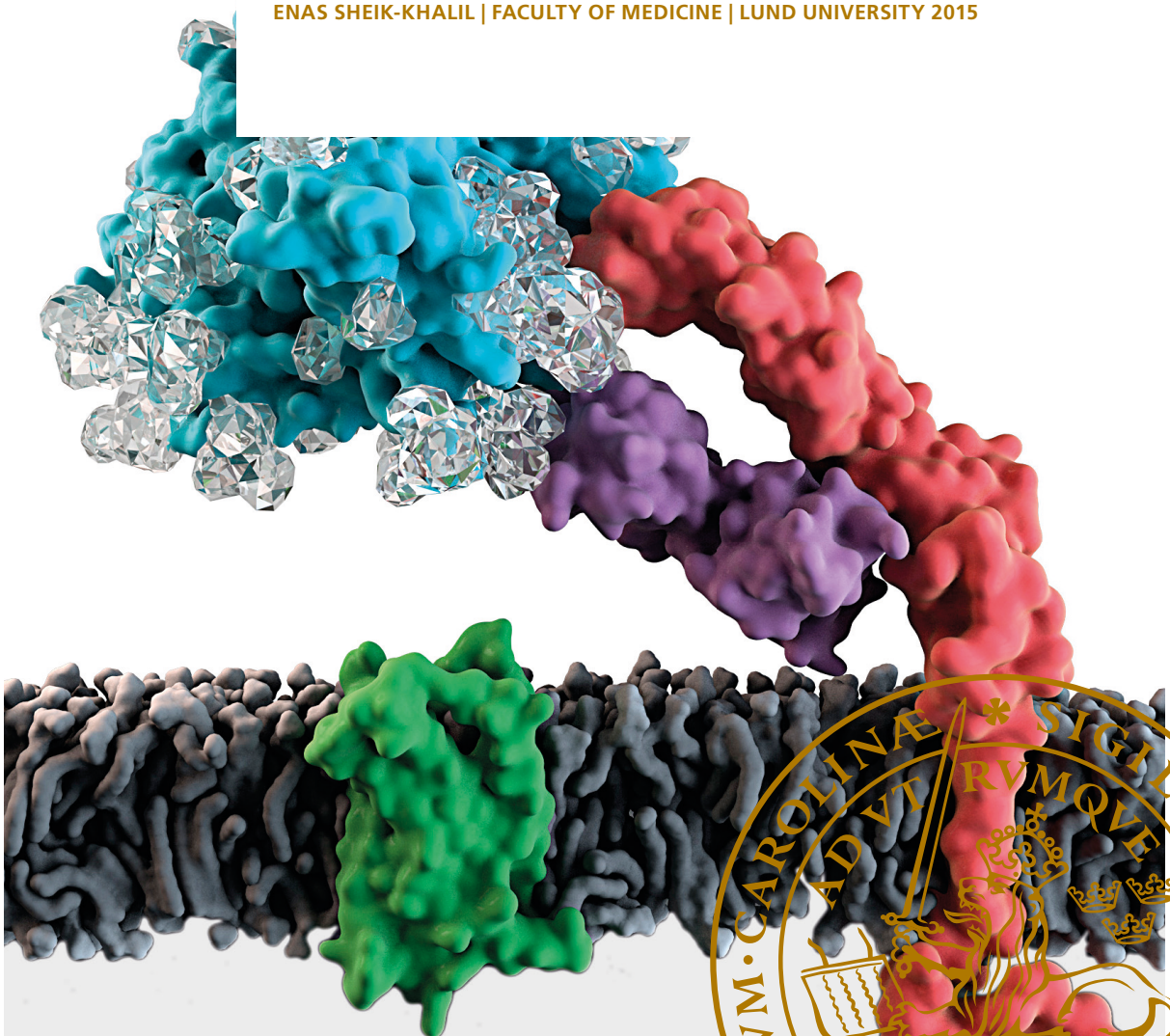

Effects of galectins and antibodies in HIV infection;

Novel Assays

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The high variability of the HIV envelope glycoproteins (Env), and their heavy glycan coating, contributes to the limited host immune control. Still, broadly neutralizing antibodies (bnAbs) are found in some chronically infected HIV-infected individuals, which has spurred the research on antibody-based vaccines. An important tool in detecting and studying bnAbs, are neutralization assays. Here we developed an image-based, high-content automated version of a plaque reduction (PR) assay, which uses green fluorescent protein expression as a reporter of HIV infection. This permitted simultaneous detection of antibodies mediating neutralization and inhibition of virus induced cell-cell fusion. In a multicenter study, Neutnet II, the assay compared well with other neutralization assays and was suggested to be an alternative to the traditional peripheral blood monocyte (PBMC)-based assay and the TZMbl assay. The glycans of Env can also take part in HIV host cell adhesion and infection, via host glycan-binding protein, such as galectins. To explore this, we examined the interaction of gp120 (the surface Env protein) with a panel of galectins, by adapting the fluorescent anisotropy (FA) assay to microscale. Galectin-8, a galectin with two carbohydrate recognition domains (CRDs), had high affinity for gp120 as well as the HIV receptor CD4. The N-terminal CRD mediated the strongest interaction with gp120. The results of the FA-assay correlated well with binding of whole virions screened in another assay against the same panel of galectins, now immobilized on beads. In the PR assay described above, here used as an infectivity assay, added intact galectin-8 enhanced infectivity of some HIV-1-strains, while this was not seen with the N-CRD, demonstrating that both CRDs of galectin-8 are required for the effect on infectivity. The enhancement effect mediated by galectin-8 was most pronounced with HIV-1 isolates obtained during the relative immune competent chronic phase, as compared to viruses isolated after AIDS onset. Hence, galectin-8 binding carbohydrate motifs on Env appear to be altered at severe immunodeficiency, adding to the knowledge on the evolution of Env glycosylation patterns related to HIV pathogenesis. These results add to the basic knowledge of virus-host interactions, which hopefully could be used for identification of antibodies and galectin-inhibitors effective in HIV prophylactic interventions.

Key words HIV-1, galectin-1, galectin-8, neutralizing antibodies, cell-cell fusion, Microscale anisotropy assay, virus binding assay, glycosylation, gp120, Automated plaque reduction assay, CellProfiler, NeutNet,

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I've learned that people will forget what you said, people will forget what you did, but people will never forget how you made them feel.

Maya Angelou

Now, as you awaken…

Now, as you awaken, remember the swan's
last dance. Did you dance with young angels
while you were dreaming? Did the butterfly
light you up when it burned with the eternal
light of the rose? Did the phoenix appear clearly
before you and call you by your name?
Did you see the morning dawn from the fingers
of the one you love? Did you touch
the dream with your hand or did you
leave it to dream alone, aware suddenly
of your own absence? Dreamers don't abandon
their dreams, they flare and continue
the life they have in the dream…tell me
how you lived your dream in a certain place
and I'll tell you who you are.

And now,
as you awaken, remember if you have wronged
your dream. And if you have, then remember
the last dance of the swan.

Mahmoud Darwish

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This dissertation summarizes and complements the following papers indicated with the Roman numerals I-IV.

I. Sheik-Khalil E1, Bray MA, Özkaya Şahin G, Scarlatti G, Jansson M, Carpenter AE, Fenyö EM

Automated image-based assay for evaluation of HIV neutralization and cell-to-cell fusion inhibition.

BMC Infect Dis. 2014 Aug 30;14:472.

II. Heyndrickx L1, Heath A, Sheik-Khalil E, Alcami J, Bongertz V, Jansson M, Malnati M, Montefiori D, Moog C, Morris L, Osmanov S, Polonis V, Ramaswamy M, Sattentau Q, Tolazzi M, Schuitemaker H, Willems B, Wrin T, Fenyö EM, Scarlatti G

International network for comparison of HIV neutralization assays: the NeutNet report II.

PLoS One. 2012;7(5):e36438.

III. Enas Sheik-Khalil, Barbro Kahl-Knutson, Ulf J. Nilsson, Marianne Jansson, Hakon Leffler

Galectin-8 binds with high affinity to HIV gp120 and promotes virus binding and infectivity

Manuscript

IV. Enas Sheik-Khalil, Hakon Leffler, Marianne Jansson

Galectin-8 enhancement of HIV infectivity correlates with patient CD4 levels

Manuscript

Other Related Papers by the Author

Özkaya Şahin G, Holmgren B, Sheik-Khalil E, da Silva Z, Nielsen J, Nowroozalizadeh S, Månsson F, Norrgren H, Aaby P, Fenyö EM, Jansson M
Effect of complement on HIV-2 plasma antiviral activity is intratype specific and potent.
J Virol. 2013 Jan;87(1):273-81

Ruffin N, Borggren M, Euler Z, Fiorino F, Grupping K, Hallengärd D, Javed A, Mendonca K, Pollard C, Reinhart D, Saba E, Sheik-Khalil E, Sköld A, Ziglio S, Scarlatti G, Gotch F, Wahren B, Shattock RJ.
Rational design of HIV vaccines and microbicides: report of the EUROPRISE annual conference 2011.
J Transl Med. 2012 Jul 11;10:144

Brinckmann S, da Costa K, van Gils MJ, Hallengärd D, Klein K, Madeira L, Mainetti L, Palma P, Raue K, Reinhart D, Reudelsterz M, Ruffin N, Seifried J, Schäfer K, Sheik-Khalil E, Sköld A, Uchtenhagen H, Vabret N, Ziglio S, Scarlatti G, Shattock R, Wahren B, Gotch F.
Rational design of HIV vaccines and microbicides: report of the EUROPRISE network annual conference 2010.
J Transl Med. 2011 Apr 12;9:40.

Ozkaya Sahin G, Bowles EJ, Parker J, Uchtenhagen H, Sheik-Khalil E, Taylor S, Pybus OG, Mäkitalo B, Walther-Jallow L, Spångberg M, Thorstensson R, Achour A, Fenyö EM, Stewart-Jones GB, Spetz AL.
Generation of neutralizing antibodies and divergence of SIVmac239 in cynomolgus macaques following short-term early antiretroviral therapy.
PLoS Pathog. 2010 Sep 2;6(9):e1001084.

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ADCC	Antibody-dependent Cellular Cytotoxicity
ADCVI	Antibody-Dependent Cell-Mediated Viral Inhibition
APC	Antigen-Presenting Cells
ART	Antiretroviral Treatment
bnAbs	Broadly Neutralizing Antibodies
CA	Capsid
CBA	Carbohydrate-binding Agents
CRD	Conserved Recognition Domain
C-CRD	C-terminal CRD
CD4	(cellular receptor) Cluster of Differentiation 4
CD4 cell+	CD4+ T helper cells
CD4bs	CD4-Binding Site
CTL	Cytotoxic T cells
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
EC	Elite Controllers
Env	HIV envelope glycoprotein $gp120_3gp41_3$
FA	Fluorescence Anisotropy
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetylgalactosamine
Glc	Glucose
GlcN	Glucosamine
GlcNAc	*N*-acetylglucosamine
GBP	Glycan-Binding Protein
GPI	Glycosyl Phosphatidyl Inositol
GIT	GastroIntestinal Tract
HAART	Highly Active AntiRetroviral Therapy
HCS	High-Content Screening
HIV	Human Immunodeficiency virus
HSV	Herpes Simplex Virus
HTS	High-Throughput Screening

ICpar50	Inhibitory Concentration in Plaque Area Reduction (50%)
IN	Integrase
IPCs	Interferon-Producing cells
KIR	Killer cell-immunoglobulin-like receptors
LacNAc	*N*-acetyllactosamine
LC	Langerhans cells
LTNP	Long-Term NonProgressor
MA	Matrix
Man	Mannose
MBL	Mannose-Binding Lectins
MPER	Membrane Proximal External Region
MS	Mass Spectrometry
Nabs	Neutralizing antibodies
NC	Nucleocapsid Protein
N-CRD	N-terminal CRD
NK	Natural killer cells
KIR	Killer-immunoglobulin receptors
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
PRR	Pathogen Recognition Receptors
PBMC	Peripheral Blood Mononuclear Cells
PNGS	Potential N-Glycosylation Sites
PIC	Pre-Integration Complex
PR	Protease
PSV assays	Pseudo-Type Virus Assays
RT	Reverse Transcriptase
SA	Sialic Acid
SIV	Simian Immunodeficiency Virus
SU	Surface glycoprotein
TM	Transmembrane
TLR	Toll-Like Receptor
µFA-assay	Microscale Fluorescence Anisotropy Assay
VI-assays	Virus Infectivity Assays
Xyl	Xylose

Abstract

The high variability of the HIV envelope glycoproteins (Env), and their heavy glycan coating, contributes to the limited host immune control. Still, broadly neutralizing antibodies (bnAbs) are found in some chronically infected HIV-infected individuals, which has spurred the research on antibody-based vaccines. An important tool in detecting and studying bnAbs, are neutralization assays. Here we developed an image-based, high-content automated version of a plaque reduction (PR) assay, which uses green fluorescent protein expression as a reporter of HIV infection. This permitted simultaneous detection of antibodies mediating neutralization and inhibition of virus induced cell-cell fusion. In a multicenter study, Neutnet II, the assay compared well with other neutralization assays and was suggested to be an alternative to the traditional peripheral blood monocyte (PBMC)-based assay and the TZMbl assay. The glycans of Env can also take part in HIV host cell adhesion and infection, via host glycan-binding protein, such as galectins. To explore this, we examined the interaction of gp120 (the surface Env protein) with a panel of galectins, by adapting the fluorescent anisotropy (FA) assay to microscale. Galectin-8, a galectin with two carbohydrate recognition domains (CRDs), had high affinity for gp120 as well as the HIV receptor CD4. The N-terminal CRD mediated the strongest interaction with gp120. The results of the FA-assay correlated well with binding of whole virions screened in another assay against the same panel of galectins, now immobilized on beads. In the PR assay described above, here used as an infectivity assay, added intact galectin-8 enhanced infectivity of some HIV-1-strains, while this was not seen with the N-CRD, demonstrating that both CRDs of galectin-8 are required for the effect on infectivity. The enhancement effect mediated by galectin-8 was most pronounced with HIV-1 isolates obtained during the relative immune competent chronic phase, as compared to viruses isolated after AIDS onset. Hence, galectin-8 binding carbohydrate motifs on Env appear to be altered at severe immunodeficiency, adding to the knowledge on the evolution of Env glycosylation patterns related to HIV pathogenesis. These results add to the basic knowledge of virus-host interactions, which hopefully could be used for identification of antibodies and galectin-inhibitors effective in HIV prophylactic interventions.

Guide to the Thesis

It is with great pleasure that I hereby present my doctoral thesis. The focus of this thesis is the HIV envelope glycoprotein complex (Env) and the opposite roles of two host molecules, neutralizing antibodies and galectins, in response to the HIV infection. Here the dense coating of glycans on Env plays dual roles: the glycans help virus to evade binding of and neutralization by antibodies, but they are also recognized by certain host glycan-binding proteins, such as galectins, that may promote HIV infection and transmission. In this thesis these two host molecule classes, antibodies and galectins, and their opposite impacts on the HIV infection are examined and discussed.

The Introduction first gives a background to the HIV-virus structure, replication, life cycle, pathogenesis and therapy. Then immune responses to the HIV infection are summarized with special focus on broadly neutralizing antibodies and their role in designing HIV vaccines. The potential role and importance of antibodies in inhibiting HIV mediated cell-cell fusion are also discussed.

In Part I, the need for efficient standardized infectivity assays to screen neutralization potency of sera (neutralization assays) is discussed, and a background to current assay types given. Finally, image-based high-content and high-throughput technologies are described.

In Part II an introduction is given to the glycan coating of HIV Env and its ability to be recognized by host cellular glycan-binding proteins (GBPs). Then, the galectins, their various roles in immune homeostasis, recognizing pathogens and finally their role in HIV pathogenesis are described, with a special part about galectin-8 as it is a main topic here. Finally, various tools that measures affinity of protein-protein interactions are discussed, with more detailed background to the mechanism behind the Fluorescence Anisotropy Assay used in our studies.

Under Present investigation, the overall Aims of the studies and the Results are summarized and discussed.

Sammanfattning på svenska

Humant immunbristvirus (HIV) är ett virus som orsakar AIDS, det vill säga förvärvat immunbristtillstånd. Idag uppskattas det att över 35 miljoner människor är infekterade med HIV och att över 35 miljoner redan avlidit i AIDS. HIV smittar via blod, sexuell kontakt och från barn till mor och det är bland annat de så kallade CD4+ T-hjälpar-cellerna som viruset attackerar inom immunförsvaret, vilket leder till att de sjunker i antal och AIDS uppkommer. Virusets arvmassa infogas också i den infekterade individens celler och orsakar kronisk infektion, och till skillnad från vid många andra virus sjukdomar, lyckas inte immunsvaret att eliminera HIV. Detta beror också på att HIV ständigt muterar samt det stora antalen HIV partiklar som produceras av målcellerna. De antikroppar och det immunförsvar, som ändå utvecklas även vid HIV-infektion blir oftast för svaga och hinner inte med att kontrollera nya virusvarianter. Det yttre HIV höljeproteinet, gp120 är även starkt glykosylerat (det vill säga täckt av många komplexa sockermolekyler) vilket hindrar en effektiv bindning och blockering av antikroppar. Trots detta uppkommer brett neutraliserande antikroppar hos vissa infekterade individer, som neutraliserar virus isolat från andra patienter samt hindrar infektion i djurförsök. Dessa har rönt mycket uppmärksamhet som ledtråd till hur man skulle kunna utveckla vaccin mot HIV, som kan inducera liknande antikroppar. Mycket forskning fokuserar därför på att upptäcka och analysera brett neutraliserande antikroppar. Detta kräver effektiva tester för att mäta hur väl sera från olika personer har förmåga att hindra infektion av olika HIV-virus stammar. Vi har därför vidareutvecklat ett tidigare helt manuellt sådant s.k. neutralisationstest, vilket bygger på att infektion mäts i odlade celler genom att virus orsakar uttryck av ett grön-fluorescerande protein. Om mängden gröna celler minskar i närvaro av serum är det tecken på att serum neutraliserar virus. Nu har vi genom datorbaserad bild analys (mjukvaran CellProfiler) utvecklat metoden så att antalet gröna celler (dvs indirekt graden av neutralisation) kan avläsas automatiskt. Vidare kan testet avläsa om celler smälter ihop, vilket är en annan effekt av HIV-infektion som kan vara viktig för spridning i kroppen. Våra testresultat har jämförts med andras på samma serum-virus kombinationer i en s.k. multicenter studie, NeutNet studien som inkluderade 13 laboratorier, och vårt automatiserade test visar på en känslighet motsvarande andra manuella tester.

De många glykanerna på gp120 hjälper inte bara virus att undgå immunsvaret, utan kan också påverka infektion genom att binda till värdens s.k. lektiner (glykan-bindande proteiner). Därför har vi studerat rollen för en familj humana lektiner vid HIV-infektion, de s.k. galektinerna. Bland annat har tidigare studier visat att glykanerna på HIV gp120 känns igen av ett sådant, galektin-1, som samtidigt kan binda värdcells-receptorn CD4 och därmed bilda en brygga mellan de två som ökar infektionen. Först ville vi mäta hur strakt renat gp120 band olika andra galektiner. Eftersom gp120 inte är tillgängligt i stora mängder, utvecklade vi en särskilt känslig metod för detta byggd på s.k. fluorescens anisotropi. Vi undersökte därefter affiniteten mellan olika galektiner och gp120 samt CD4 och upptäckte att galektin-8 binder med hög affinitet till både gp120 och CD4. I ett annat test visade vi att också hela virus-partiklar binder galektin-8 väl, nu fastsatt på små kulor. Slutligen, genom att använda vårt utvecklade neutralisations/infektionstest som beskrivs ovan, fann vi att närvaro av galektin-8 ökar infektionsbenägenhet för vissa HIV stammar. Dessa resultat understryker att glykosyleringen av HIV spelar ytterligare en roll vid infektionen. Genom att HIV använder cellulära mekanismer för glykosylering av gp120, är det möjligt att glykan-bindande proteiner misstar glykanerna på gp120 för värdglykaner och stabiliserar bindningen mellan HIV och värdcellen. Vi fann avslutningsvis, att effekten av galektin-8 på infektion var mycket lägre eller obefintlig för virus isolat från patienter med svår immunbrist (AIDS-stadiet) jämfört med tidigare från patiener med visserligen kronisk infektion men ännu ej utvecklad AIDS. Detta betyder att galektin-8 förstärker infektionsförmågan hos HIV från tidigare stadier, när immunbristen inte är fullt utvecklad, i större utsträckning än virus från AIDS stadier.

Dessa studier hoppas vi kan användas för bättre förståelse av funktionen hos antikroppar och galektiner, samt i förlängningen leda till att spridning av HIV kan stoppas.

Introduction

During the early 1980s, the world was faced with a rapidly transmissible and deadly disease called acquired immunodeficiency syndrome (AIDS) [1]. The syndrome symptoms, *Pneumocystis carinii* pneumonia [2, 3], Kaposi's sarcoma [4] and generalized lymphadenopathy, all related to severe immunodeficiency, were first reported in men who have sex with men and later intravenous drug users but came soon to include people who had blood transfusions [5] and finally the general population. Many theories came up, however, a couple of laboratories started their research on the hypothesis that the cause of AIDS was a retrovirus. This retrovirus was first isolated at the Institute Pasteur, Paris, France, in 1983, from a lymph node biopsy sample of a patient with generalized lymphadenopathy [6]. The isolation of the virus, later named human immunodeficiency virus (HIV), led to intense research on its pathogenesis and its interplay with the host aiming at treatment and prevention of HIV infection. Sero-epidemiological studies could confirm that HIV caused AIDS, and made diagnostic tests for HIV infection possible. Within two years, molecular cloning of HIV and subsequent nucleotide sequencing elucidated further knowledge about the viral infection. A few years later, another related retrovirus, HIV-2 exhibiting slower disease progression and lower transmission, was detected in West Africa. HIV, also known as HIV-1, and HIV-2 proved both to be the result of cross-species transmission of simian immunodeficiency virus (SIV) [7].

There are four groups of HIV representing three separate SIV transmission events from chimpanzees (M, N, and O) and one from gorillas (P) [8]. New HIV infections decreased from 3.3 million in 2002 to 2.3 million in 2012, largely due to reductions in heterosexual transmissions [9]. However the global prevalence of HIV has been estimated to increase from 31 million in 2002 to 35.3 million in 2012, where Africa has the highest global burden (70.8 %). Group M consists of nine subtypes (A-D, F-H, J and K) and is the cause of the global HIV pandemic. The dominating subtype in Africa is subtype C, whereas subtype B predominates in Western Europe, America and Australia.

HIV genome and structure

HIV is a lentivirus belonging to the *Retroviridae* family because of its particle associated reverse transcriptase [10, 11]. It is a 100 μm spherical enveloped virion composed of a lipid bilayer envelope acquired from the cellular membrane during the process of budding from an infected cell (Figure 1). The membrane contains approximately 4-35 spikes, trimers of the surface envelope glycoprotein gp120 (SU), non-covalently connected to the trimers of the transmembrane envelope glycoprotein gp41 (TM), and is called the envelope glycoprotein complex (Env).

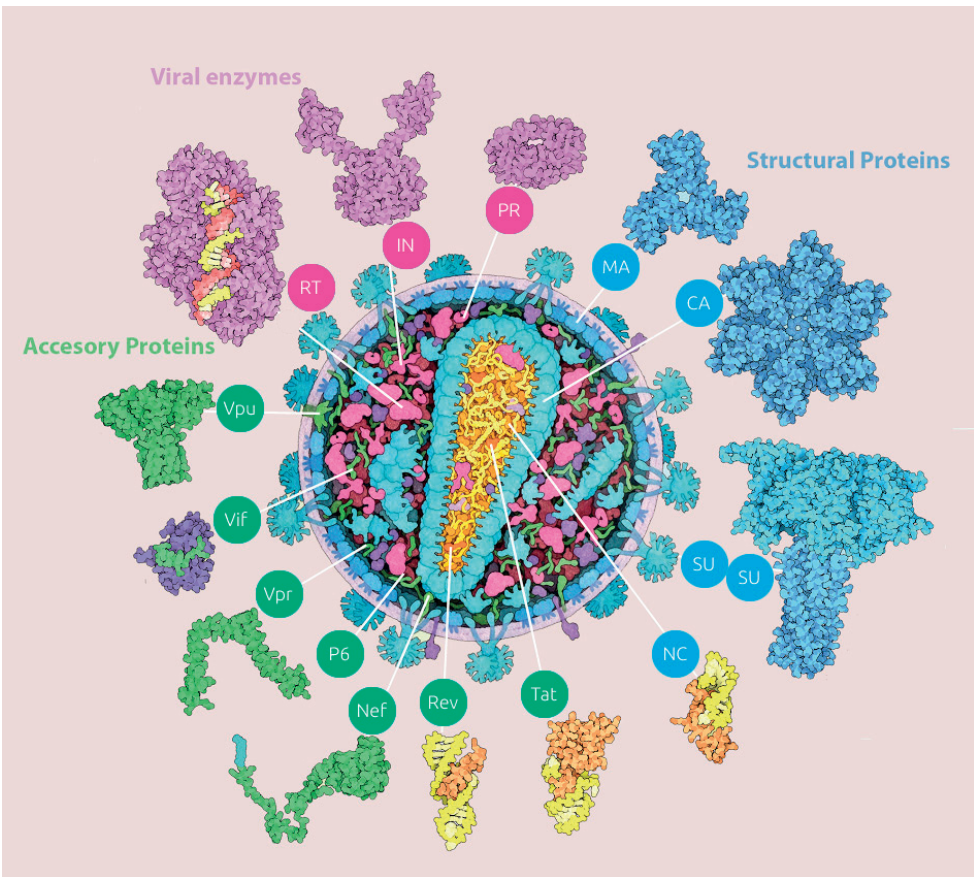

Figure 1.
A schematic view of the HIV virion and the virally encoded proteins. Reprinted from PDB 101, David Goodsell http://www.pdb.org/pdb/education_discussion/educational_resources/struct_bio_hiv_hires.pdf with persmission.

The matrix protein (MA) p17 lies just beneath the viral membrane whereas the capsid protein (CA) p24 makes up the conical icosahedral capsid or viral core, enclosing all viral proteins and enzymes as well as the two viral ssRNA genomes. The HIV genome comprises nine genes encoding the viral proteins (Figure 2). The three major coding genes are *gag*, *pol* and *env,* which encode the structural proteins, including MA, CA, p6, nucleocapsid protein (NC) and the three enzymes reverse transcriptase (RT), integrase (IN) and protease (PR), as well as the envelope glycoproteins, respectively. The other six genes encode multifunctional molecules playing roles in viral pathogenesis. The regulatory proteins Tat and Rev are crucial for viral replication and the four remaining accessory proteins Nef, Vif, Vpr and Vpu are responsible for enhancing viral replication and immune evasion. Finally, on both sides of the genes, the LTR, long terminal repeats, are the promoters for the transcription by cellular enzymes occurring after integration of the provirus into the host DNA.

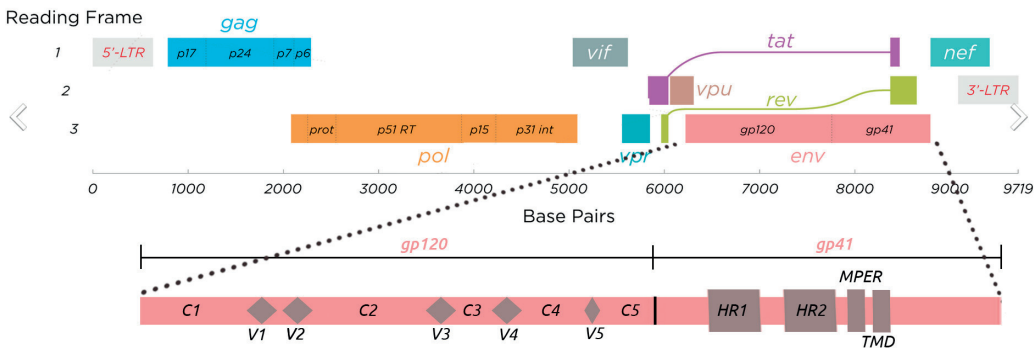

Figure 2.
HIV genome with a close up on *env* coding for gp120 and gp41. Adapted from Thomas Splettstoesser www.scistyle.com with persmission

HIV envelope glycoproteins

The HIV Env is responsible for receptor recognition and viral fusion with CD4+ T helper cells (CD4+) and is the sole target for neutralizing antibodies. Thus understanding its molecular architecture will enable us to identify immunologically sensitive sites for neutralization.

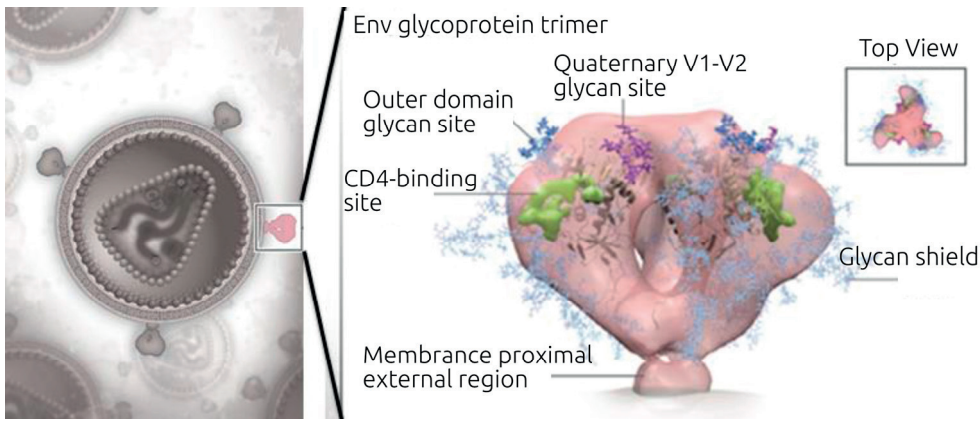

Figure 3.
Structure of HIV-1 and Expanded View of the Envelope Glycoprotein Trimeric complex (Env).
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The HIV glycoprotein is expressed as a gp160 precursor, that is proteolytically cleaved by host cell furin in the ER-Golgi into gp120, the surface protein and gp41, the transmembrane glycoprotein molecule [12]. Gp120 and gp41 are non-covalently associated and three such heterodimers assemble into a final trimeric *Env* spike (Figure 1). The overall conformation of gp120 is identical, where the "inner" domain refers to portions of gp120 closer to the inner regions of the trimeric spikes and the "outer" domain to the outer regions. In the gp120, five conserved domains (C1-C5) are interspersed with five variable domains (V1-V5) [13]. Furthermore, about 18 cysteine residues occur both in the conserved and variable domains to stabilize the structure by forming intramolecular disulfide bonds. When the trimeric Env is in the unliganded state, it constitutes a "closed" conformation where V1, V2, and V3 are located on the trimer apex and the V4 and V5 project outward from the gp120 surface (Figure 3).

In addition, the Env is heavily glycosylated and approximately half of the molecular mass of gp120 is contributed by glycans, which serve as potential epitopes and may shield gp120 from immune recognition (Figure 3). The gp41 subunits are more conserved in sequence as they house the fusion machinery. The extracellular domain

of gp41 contains the fusion peptide and the heptad regions HR1 and HR2 as well as the conserved membrane proximal external region (MPER) (Figure3).

HIV replication

HIV sexual transmission commonly starts through infection of the Langerhans cells (LC), a specialized dendritic cell (DC) enriched at genital mucosal surfaces. Alternatively, direct viral infection of CCR5+ CD4+ T-cells, located near mucosal surfaces, may occur [14]. LCs bearing virions subsequently migrate from mucosal sites to lymph nodes and transfer HIV directly to CD4+ T cell.

The virus replication cycle is initiated when HIV binds to the host CD4 receptor via gp120 (Figure 4). The finding that the CD4 cell surface molecule was the main receptor for HIV and that HIV was cytopathic, introduced the rationale to monitor CD4+ T cell counts in HIV infected patients [15]. The gp120 binding to the CD4 receptor triggers conformational changes in the gp120 upon which the trimer spike receives an "open" conformation and the V1 and V2 loops move to the periphery of the trimer. This reveals the co-receptor binding site [16, 17] and gp120 subsequently binds to the co-receptor, usually CCR5 or CXCR4 [18]. The CCR5 or CXCR4 binding preference of a HIV strain is commonly referred to as R5- or X4 tropism, respectively, and depends on the genetic variation within HIV *env* gene. The initial infection is dominated by R5 viral strains, which is a consequence of the strong CCR5 expression on cells first encountered at transmission. Over the course of disease progression, both in the chronic and late phase, the virus may evolve to CXCR4 use. This is significantly correlated with the onset of AIDS where co-receptor switching occurs relatively late in infection [19].

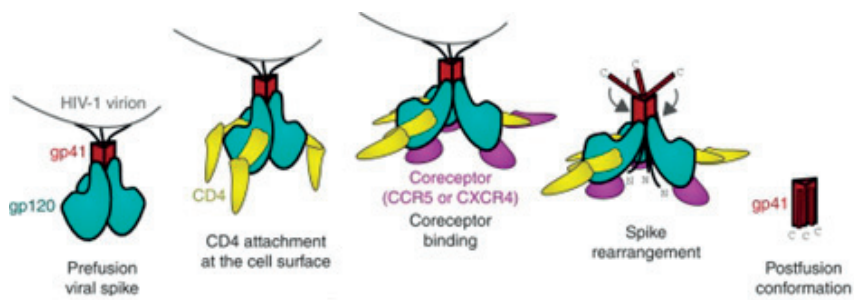

Figure 4.
An illustration on events taking place during HIV infection, involving the conformational changes of Env, i.e gp120 and gp41, during interactions with CD4 and coreceptor CCR5 or CXCR4. Reprinted from "Rational Design of Vaccines to Elicit Broadly Neutralizing Antibodies to HIV-1", Peter Kwong et al, 2011, Cold Spring harbor Perspectives in Medicine, with persmission.

The coreceptor binding further triggers a conformational change in gp120, exposing gp41, upon which the fusion peptide is inserted into the cell membrane. The HR1 and HR2 regions of gp41 then interact with each other to form a six-helix bundle in a hairpin structure. The viral membrane is brought into close proximity to the cellular membrane and a fusion pore is formed allowing the insertion of the viral core as well as the genomic material into the target cell [17]. It is thought that five to six Env spikes are required to initiate viral fusion with the cell membrane, creating an “entry claw” [20] (Figure 5).

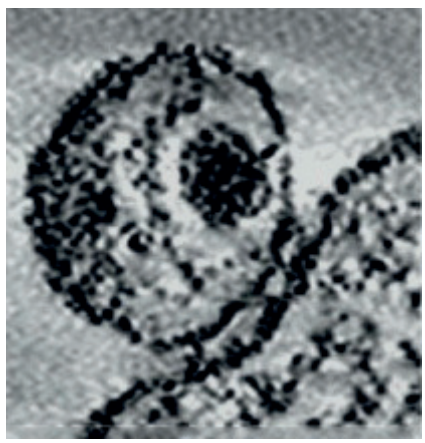

Figure 5.
Electron microscopy (EM) image of HIV virion attachment to target cell, creating an entry claw. Reprinted from ”Catching HIV 'in the act' with 3D electron microscopy”. Earl, L.A. et al, Trends Microbiol, 2013 with persmission

Shortly after infection, the capsid is partially uncoated into the cytoplasm where the reverse transcriptase initiates transcription of the viral RNA genome, from RNA to dsDNA (Figure 6). Since the reverse transcription is highly error prone this translates into a high mutation rate of approximately one point mutation per 10^4 amplified base pairs, i.e. one mutation for each replication cycle. This in addition to the rapid turnover of HIV, about 10^9- 10^{10} virions per day, results in a highly diverse pool of viruses present in one individual and is referred to as the quasi-species. This is thought to be the major driving force for the variability together with the immune response.

The double-stranded viral DNA is then transported into the nucleus as a component of a pre-integration complex (PIC) containing a subset of the Gag and Pol proteins and also including the viral integrase (IN). This full-length linear viral DNA is then integrated by the viral integrase as a provirus into the host genomic DNA of the infected cell (Figure 6). Once integrated, the provirus can stay latent until the host

cell is activated, and then make use of the cellular transcription machinery for initiation of the virus transcription.

These integrated proviral DNA subsequently serve as templates for DNA-dependent RNA polymerase II (pol II) that leads to the production of messenger RNA (mRNA). Early viral proteins constituted by Tat and Rev regulate the expression of the late viral proteins. The mRNAs are subsequently spliced and exported into the cytoplasm where they are translated into viral proteins. The HIV Env, Gag and Pol proteins, [21], are initially synthesized as precursor proteins. The produced viral proteins and the viral RNA genome assemble close to the cell membrane where processed Env is expressed and where the budding process of new virions is initiated. Subsequently the virion is budded from the target cells and the released virus particles turn mature and infectious after cleavage of the Gag-Pol precursors by the viral protease (Figure 6).

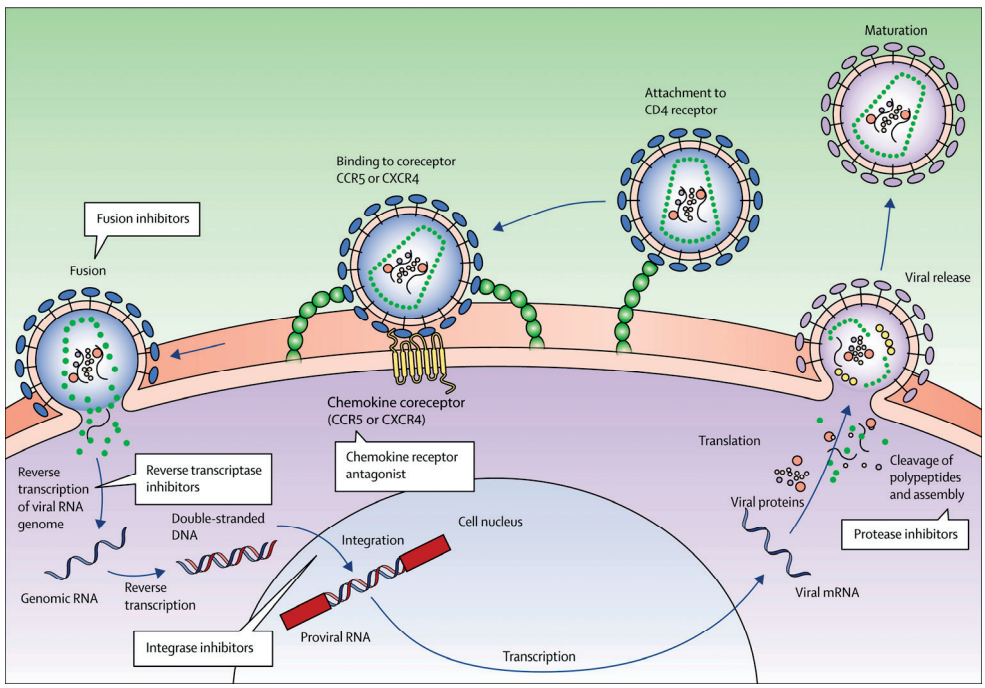

Figure 6.
The HIV replication cycle with antiretroviral targets. Reprinted from "HIV infection: epidemiology, pathogenesis, treatment, and prevention", Maartens G et al, 2014 with persmission from Elsevier.

HIV mediated cell-cell fusion

HIV glycoproteins on virions interacting with receptors on the target cells, result in infection of the cell. However, this HIV cell-free infection has disadvantages, including immunological barriers (innate and adaptive immune defenses) as well as biophysical barriers (mucus membranes and virion instability that results in progressive loss of infectivity) [22]. Viruses that spread directly from infected to uninfected cells can thus avoid many obstacles.

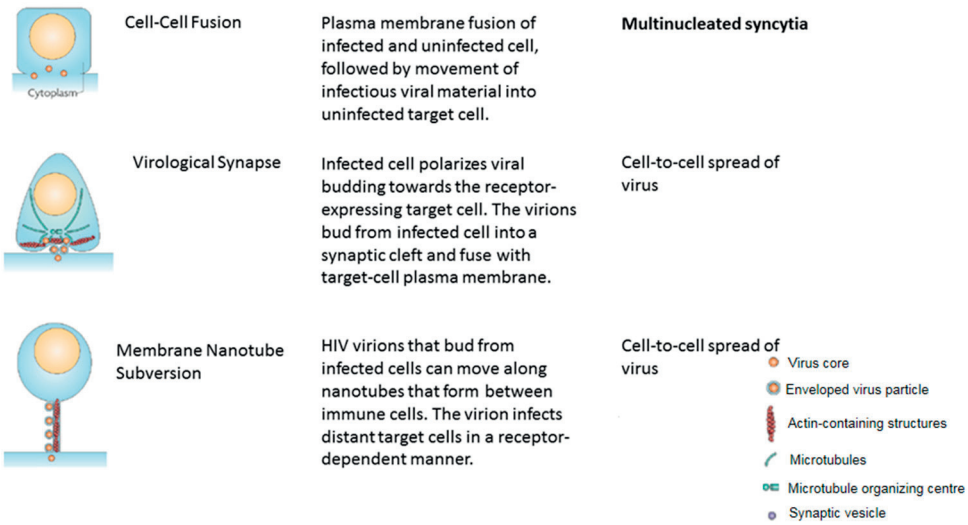

Figure 7.
Different mechanisms of HIV cell-to-cell spread [23]. Reproduced from Avoiding the void: cell-to-cell spread of human viruses , Quentin Sattentau Nature Reviews Microbiology 6, 2008 with persmission from Elsevier.

There are diverse mechanisms for HIV to spread from cell to cell (Figure 7). The simplest mechanism is the fusion of infected and uninfected cells, which has been shown to occur in vitro. Here, the infected cell expresses HIV Env on its surface, and interactions between Env and receptors expressed by a neighboring uninfected cell result in syncytia formation. This cell-cell fusion is distinct from the other mechanisms of HIV cell-cell spread. HIV frequently induces cell-cell fusion resulting in syncytia formation in CD4+ T cell cultures, and HIV has also been shown to induce syncytia in macrophage cultures [22] (Figure 8). Syncytia have also been observed in *ex vivo* samples from HIV infected patients from myeloid-derived cells in the brain. These syncytia have been proposed to serve as virus reservoirs, since they are detected in both asymptomatic as well as AIDS patients. Interestingly, significant

differences in cell–cell fusion between different HIV strains have been shown [24], suggesting that the level of HIV-mediated cell-cell fusion is strain specific.

Macrophages and dendritic cells can also spread the HIV infection to T cells by cell-cell contact zones called virological synapses or through nanotubes that form between cells [22] (Figure 7). Lymph nodes are important sites for HIV replication, where the formation of virological synapses between infected and uninfected T cells increases the efficiency of viral transfer significantly [22]. However these two mechanisms are outside the scope of this thesis.

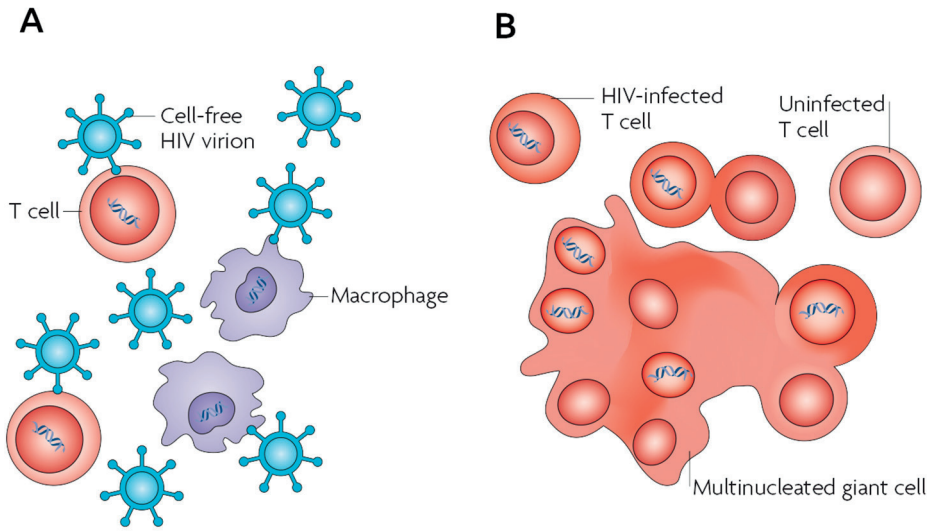

Figure 8.
HIV infection by (A) cell-free virion and via (B) cell-cell fusion resulting in syncytia [25]. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy, Jan Balzarini, 2007.

Finally, there are several assays to measure cell-cell fusion where a brief description is given under “Cell-cell fusion assays” below.

HIV pathogenesis

Acute phase

The HIV infection is divided into three stages. During the acute phase, HIV rapidly spreads to lymph nodes in the gastrointestinal tract (GIT), where a massive CD4+ T cell depletion occurs within the first week of infection [8]. This is due to direct depletion as well as innate immunity where natural killer (NK) cells attempt to control the infection through viral recognition by killer-immunoglobulin receptors (KIRs). This acute phase infection typically lasts two-four weeks where viremia results in viral loads reaching 10^6 copies/mL plasma, upon which the permanent infection is established (Figure 9A). An anti-viral immune response reduces the virus levels in the plasma to a so-called "viral set point", where a low level set point correlates with milder disease progression. The acute HIV infection is manifested by flu-like symptoms, including fever, body ache and swollen lymph glands.

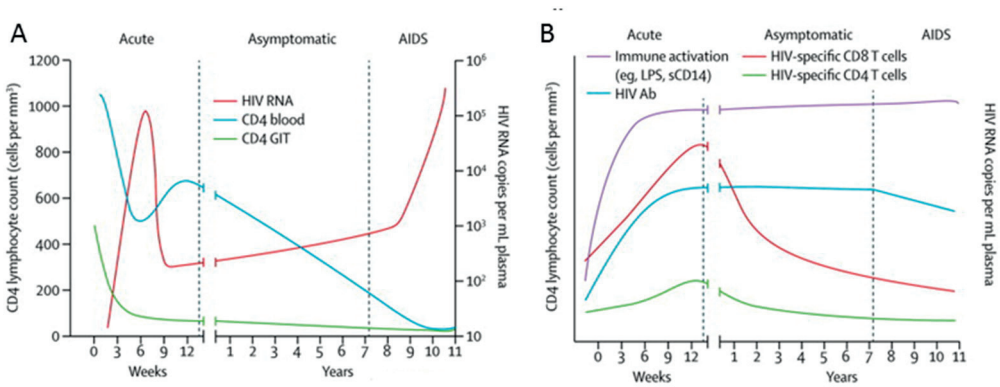

Figure 9.
A typical course of the untreated HIV infection, illustrating (A) kinetics of plasma viral load, CD4+ T cell loss in blood and gut intestinal tract (GIT), and (B) the HIV immune responses and markers of chronic immune activation. Reprinted from "HIV infection: epidemiology, pathogenesis, treatment, and prevention", Maartens G et al, 2014 with persmission from Elsevier.

Chronic phase

The immune response reduces the viral load to a viral set point of approximately 3 x 10^4 copies/mL plasma [26]. However, during the chronic phase CD4+ T cells are gradually reduced in peripheral blood, partly due to the constant turnover of T cells and a decaying regenerative capacity (Figure 9A). This T cell death also affects

uninfected T cells (called bystander effect) and whether they are truly uninfected and what causes this death has been widely debated. Furthermore, a viral reservoir of quiescent pools of infected cells is established. These cells are long-lived, resistant to eradication by drug treatment and continually re-infect the host. They primarily consist of memory T cells, as well as macrophages and DCs [27]. The chronic, or latent phase typically last a couple to no more than 10 years, where host genetics as well as viral factors affect the time span. Clinically, the individual experiences minimal symptoms, however an increase in chronic immune activation, such as elevated levels of activated immune cells, inflammatory cytokines and microbial translocation, is seen.

AIDS

Finally, the AIDS phase is reached when the patient's CD4+ T cell counts decline below 200uL/blood (Figure 9A). The AIDS development is characterized by a progressive generalized lymphadenopathy, opportunistic infections (for example pneumocystis jiroveci pneumonia and cytomegalovirus-associated retinitis) as well as unusual cancers (non-Hodgkin's lymphoma and Kaposi's sarcoma). The virus infects key cells of the adaptive immune response explaining the clinical manifestations of the severe immune deficiency that ultimately, in the absence of treatment, leads to the death of the patient within a couple of years after AIDS onset.

Although these stages are rather similar in all HIV infected patients, the course of disease varies enormously among infected individuals, where the time from acute infection to the development of AIDS may be as rapid as 6 months [28] or as slow as 25 years or more. A small subset of individuals does not progress in the absence of antiviral therapy, and are referred to as HIV controllers. Individuals who maintain CD4+ T cell counts (>500 cells/μL) for over 10 years without therapy and remain asymptomatic are denoted long-term-non-progressors (LTNP) [29]. In that category, a subset of individuals who control virus replication to less than 400 copies/mL for > 5 years are called viremic controllers, and those who have viral loads below 75 copies/mL for at least one year are called elite controllers (EC) [30].

Antiretroviral therapy and latency

In the mid-80´s the first HIV mono-therapy, Azidothymidin (AZT) was introduced. AZT, originally synthesized as an anticancer treatment, it was found to block the reverse transcription step of the HIV life cycle [31, 32]. However, viral resistance quickly developed. About ten years later, the breakthrough in HIV treatment was

made possible by the introduction of a therapy that combined several antiretroviral drugs to limit resistance development [33, 34], that both increased life expectancy as well as prevented mother-to-child transmission [35].

The combination therapy of at least three antiretroviral drugs, also termed highly active antiretroviral therapy (HAART) has resulted in a significant reduction in HIV-associated morbidity and mortality. There are today more than 25 licensed drugs that block HIV replication at various steps of the HIV lifecycle. The standard antiretroviral therapy combines two nucleoside reverse transcriptase (RT) inhibitors with a non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI) or integrase inhibitor [36]. Antiretroviral therapy usually decreases the viral load to concentrations below the lower detection limit of available commercial assays within three months.

Despite the great success of HAART, antiretroviral treatment is unable to cure HIV, leading to a need of life-long treatment. Latency is defined as integration of HIV DNA into the host genome in absence of virus production. Accordingly, latently infected cells may persist in patients on HAART [37]. New studies with the aim to cure HIV has therefore focused on eliminating latently infected T cells by activating latent HIV with histone deacetylase inhibitors [38].

Innate immunity in HIV infection

The innate immunity is the first line of defense against invading pathogens, and provides time for the subsequent development of the adaptive immune responses. It is characterized by speed and absence of memory. There are several anti-HIV innate components that have been linked to lower risk of HIV infection and enhanced progression to disease. Among the HIV-soluble components of the innate system with anti-HIV activity are the mannose-binding lectins (MBLs), complement, cytokines, chemokines and defensins. The MBLs bind to HIV and either lyse the virus directly or help phagocytosis by macrophages [39, 40]. The complement system as well as the production of cytokines participates and induces both innate and adaptive cellular responses to HIV [41]. The chemokines recruit natural killer (NK) cells, macrophages and T cells to increase the cytotoxic function of these cells [42].

The cellular components of the innate immune system comprise the dendritic cells (including LC), macrophages, NK cells, neutrophils, CD8+ T cells with non-cytotoxic antiviral activity and B1 cells [43-45]. The DCs are professional antigen presenting cells that recognizes microbes through various pathogen-associated molecular patterns (PAMPs) via pathogen recognition receptors (PRRs), e.g. DC-

SIGN and toll-like receptors [46]. DCs also bridge and contribute to both innate and the adaptive immune responses [47].

The innate immune system also regulates the selection, activation and expansion of B cells through complement receptors CD21/CD35 [48], where the elucidation of the mechanisms regulating the generation of memory B cells and long-lived plasma cells may benefit vaccine strategies [49].

Adaptive immune responses: HIV cellular immunity

The T-cell responses to the acute HIV infection are rapid and appear in blood before seroconversion and peak of viral load [50]. The HIV-specific CD4+ and CD8+ T cells expand rapidly [51] (Figure 9B), however, the early HIV-specific CD8+ T cell responses are narrowly focused on a few immunodominant epitopes, facilitating viral escape [52]. During the course of the infection, CD4+ T cell death outnumbers the thymic output of new cells, resulting in a progressive depletion (Figure 9A). As HIV infection primarily infects and destroys CD4+ T cells this results in a dysfunction of the help to cytotoxic T cells (CTLs). The HIV infection also causes dysfunction of the CD4+ T cell subsets [53]. The loss of control and elevated virus levels have been correlated with expression of exhaustion markers, for example PD-1, on both CD4+ and CD8+ T cells. Interestingly, HIV controllers commonly express a select group of HLA-B alleles [54] as well as polyfunctional CD8+ T cells [55].

Adaptive immune responses: HIV humoral immunity

To mediate HIV neutralization, antibodies must recognize the functional epitopes on the HIV Env and prevent virus attachment and entry into host cells. Initial antibody response to HIV can been detected as early as a few weeks post infection as immune complexes (Figure 9B). Circulating anti-gp41 antibodies are detected in the following days, followed by anti-gp120 antibodies a few weeks later [56].

However, the large number of antibodies generated during HIV infection are mostly highly strain specific or non-neutralizing. The non-neutralizing antibodies bind surfaces accessible on monomeric gp120 that has detached from the viral surface. These surfaces are inaccessible on the intact gp120 trimer. The strain specific antibodies bind trimeric gp120 but target nonconserved parts of the glycoprotein.

Autologous neutralization (NAbs against the infecting strain) only appears several months post infection. Although these antibodies does not exhibit heterologous

neutralization (neutralization of divergent viruses isolated from other individuals) they cause viral escape suggesting they do play a role in neutralization [57, 58]. At mucosal surfaces, high affinity anti-gp41 IgA is transiently produced at high levels during the first two weeks of infection in a T-dependent manner [59]. The massive depletion of gut CD4+ T cells during the early stage of infection, causing germinal center dysfunction, has been suggested to impair the T-cell dependent IgA production.

HIV infection also causes major perturbations in B cell development where the B-cell immune dysfunctions contribute to an inadequate humoral response to HIV. One virus-dependent mechanism underlying this is the direct interaction of B cells with gp120 and Nef that induces an inhibition of B cell activation and proliferation. The HIV infection also negatively impacts follicular T helper cells that in turn diminishes B cell immunity.

It is unclear whether antibodies can effectively neutralize virus spread from cell to cell [22]. Monoclonal antibodies that reportedly inhibit infection by a large number of HIV isolates had little or no inhibitory effect on cell-cell fusion. This was partly explained by the fact that significantly higher levels of gp120/41 are expressed on the surface of infected cells, and thus a higher concentration of antibody is necessary to inhibit fusion between infected and target cells [60, 61]. However, sera from HIV infected individuals containing IgG and IgM did show inhibitory activity of cell fusion [62].

Vaccines and microbicides

"That which does not kill us, makes us stronger".

……..Nietzsche

Historically, vaccines that induce antibodies have been the most effective strategy to fight viral diseases [63]. It is also thought that vaccination against HIV resulting in sterilizing immunity (preventing the person to become infected in the first place) requires stimulation of a humoral immune response. Indications supporting a protective role for antibodies comes from studies reporting that IgA from mucosa of highly exposed uninfected women may neutralize HIV [64]. Furthermore, vaccination studies performed in the macaque model have provided proof of concept that antibodies could be protective against viral infection [65]. Passive immunization with bNAbs has also been considered, however, since antibodies decay with a half-life spanning between one to three weeks, passive administration offers no permanent immunity and would be more like potential long-lasting anti-HIV drugs [66].

Vaccine strategies that exclusively stimulate T-cell immunity would at best delay or prevent the progress of illness by generating persistent and broadly reactive T-cell responses but not prevent infection. Still, one of the major challenges in HIV vaccine design is the extreme antigen variability.

HIV vaccine efficacy trials attempted to elicit protective antibodies against the viral Env and used two forms of recombinant glycoprotein 120 (rgp120) as the immunogen. VAX004 began recruitment in 1998 and derived from two subtype B strains, however it did not prevent disease acquisition nor impact level of viremia [1]. VAX003 contained two rgp120 HIV Env antigens from two different subtypes but again the vaccine did not prevent HIV infection or delay HIV disease progression [67]. These are referred to as the first generation of HIV vaccines, where monomeric Env antigens were intended to elicit an antibody response.

The second generation vaccines were designed as a proof of concept study for the efficacy of a cell-mediated immunity vaccine to protect against HIV infection and to test whether T cells could stimulate anti-HIV CD8+ cells and elicit a cellular immune response. The STEP trial, based on the adeno 5 viral vector including HIV clade B Gag/Pol/Nef, showed that the vaccine was ineffective and even suggested a trend towards increased HIV infection in subgroups who had antibodies to adenovirus type 5 at enrolment [68].

A third generation of HIV vaccines, tried a combination of two vaccines which resulted in the RV144 trial: ALVAC® HIV vaccine (the prime) and AIDSVAX® B/E vaccine (the boost). The vaccine combination was based on HIV strains that commonly circulate in Thailand and consisted of a viral vector containing genetically engineered versions of three HIV genes (env, gag and pol). The ALVAC vector is an inert form of canarypox, and AIDSVAX B/E is composed of genetically engineered gp120 [69]. It was the first trial to yield a moderate (31%), and yet statistically significant reduction in HIV acquisition and provided a proof of concept that it is possible for a vaccine to elicit protective immunity that blocks infection [70]. Interestingly, the limited protection was correlated with non-neutralizing IgG antibody responses, including antibody-mediated mechanisms such as binding antibodies and antibody-dependent cell-mediated cytotoxicity (ADCC) directed against the gp120 V1V2 domain.

Other promising preventive strategies include a pre-exposure prophylaxis designed as a microbicide containing 1% tenofovir. Here the CAPRISA004 microbicide trial gave a 39% reduction in risk of infection [71].

Part I- High-content PR assays in search for a bnAb-based HIV vaccine

Broad neutralizing antibodies

Crossreactive antibodies typically develop two-four years after seroconversion, and in a rather small fraction of chronic HIV infected individuals, so called broadly neutralizing antibodies (bnAbs), active against hundreds of different virus variants from various HIV subtypes, develop [72-74] . These gp120-specific bnAbs are mostly IgG1 [72, 75] and have been shown to share common features, e.g they express unusually long CDRH3 loops that are considered important to reach the region of their epitope and to penetrate the glycan shield or reach epitopes located close to the lipid membrane [76]. Another common characteristic of bnAbs is the high degree of somatic hypermutations involved in their maturation [77-80], however newly developed methods to extract bnAbs have also found bnAbs with little or no somatic hypermutation [81]. Furthermore, although not all bnAbs are polyreactive, a large fraction of Env antibodies are polyreactive, reacting with a number of host and other non-HIV antigens [82].

The bnAbs do not appear to provide substantial clinical benefit to the infected individuals in whom they occur [83]. However, bnAbs can function to prevent cellular entry by HIV in vitro and have been shown to prevent infection by passive infusion in non-human primate studies [65, 84]. As for the mode of action, these bnAbs may not need to cover the entire surface of an HIV virion, instead it appears to be enough that one of the spikes of the “entry claw” is neutralized for blocking the fusion [74, 85]. Thus, the generation of such bnAbs in humans by active or passive immunization may protect against HIV infection.

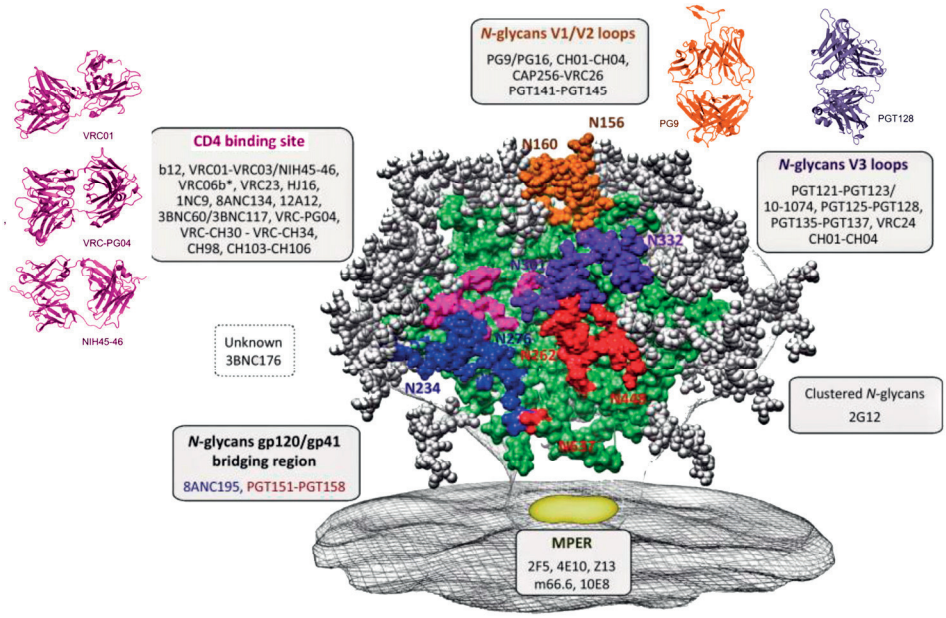

Figure 10.
HIV Env model where broadly monoclonal antibodies and their epitopes are illustrated. Modified from "Antibody B cell responses in HIV-1 infection", Volume 35/Issue 11, Hugo Mouquet (2015), and "Human Antibodies that Neutralize HIV-1: Identification, Structures, and B Cell Ontogenies", Volume 37, Issue 3, Peter D. Kwong,John R. Mascola (2012) with permission from Elsevier.

There are several sites of vulnerability on the HIV Env spike where the four major targets for bnAbs are the CD4 binding site (CD4bs), the N-glycan-associated epitopes on the V1/V2 loops, the V3 loop, and finally the membrane proximal external region (MPER) on gp41 [56, 86] (Figure 10). Many of the newly identified bnAbs, such as PG9, PGT128, VRC01, 8ANC195, PGT151, and PGDM1400 partially recognize glycans located on the outer domain of gp120, a domain previously considered devoid of binding epitopes and named the immunologically "silent face" because of the glycan shield surrounding it [56, 81]. Furthermore, the variable loops previously thought to be highly variable between patients and within patients, have been shown to contain substructures within the variable loops that are highly conserved and can serve as targets for bnAbs [87]. When combined, some bnAbs have shown synergistic effects, i.e. substantially improved neutralization breadth, suggesting utility in HIV prevention and treatment [81, 88].

In addition to the above mentioned bnAbs, a few antibodies were found to recognize O-glycan structures, mainly GalNAc, the Tn antigen[89], and sialylated GalNAc, the sialyl-Tn antigen. Others bound terminal histoblood group carbohydrate epitopes like

Le^y, Fucα1-2Galβ1,4(Fucα1,3)GlcNAc-R and A1-determinats, Fucα1,2 (GalNAcα1-3)Galβ1-3GlcNAc-R [90]. For virus coming from cells expressing such epitopes, these antibodies were shown to neutralize infection and syncytia formation [90, 91].

It has recently been suggested that Env conformations are rather dynamic prior to ligand binding and that the CD4 binding stabilizes the open conformation [92, 93]. Neutralization sensitive virus isolates opened relatively frequently, whereas more neutralization resistant rarely opened and as a consequence fewer antibodies had access to the coreceptor stabilized state [92, 93].

As cell-cell transmission of HIV is considered one of the major mechanisms contributing to viral spread, an obvious question is whether these bnAbs also act against HIV cell-cell spread and fusion in vitro [22]. Interestingly, it has been shown that merely a subset of bnAbs do inhibit viral cell-cell spread [94]. Another desirable feature of bnAbs is to mediate antibody effector functions mediated via the Fc region. Such functions include antibody-dependent cell-mediated cytotoxicity (ADCC) and Antibody-dependent cell-mediated viral inhibition (ADCVI), and have been associated with the reduction of risk of mother-to-child HIV transmission and protection in individuals, naturally controlling HIV infection. Moreover, it has also been suggested that ADCC and ADCVI contribute to the partial protective effect of the RV144 vaccine [56, 95].

Generation of bnAbs and Reverse vaccinology

Neutralizing antibodies must contain several features, including high magnitude, be broad, and have a long duration, with long lasting kinetics. Furthermore, they must have the right epitope specificity, should not allow viral escape, and be present systemically as well as in the mucosa and correlate to protective immunity. Uncovering the epitope of a bnAb will reveal sites of vulnerability on HIV Env; information that can serve as the basis for vaccine design. Structures that mimic the bnAb epitope should in theory be useful vaccines because antibodies generated against the mimetic constructs would focus on the bnAb epitope and thus neutralize in a broad manner. As appealing as this logic may be, there are several challenges along the way: the heterogeneity of HIV glycosylation may make it difficult to define the structures that comprise the epitope for bnAb that bind carbohydrate epitopes. Epitope mimicking by chemical synthesis is difficult. The constructs must also be evaluated in terms of two criteria: (i) antigenicity, i.e. their binding affinity for the bnAb, and (ii) immunogenicity, i.e. their ability to stimulate a bnAb-like antibody response in vivo. High antigenicity does not guarantee a desired immunogenicity, and one does not know whether an antigen is a good immunogen until it has been tested in animal immunogenicity studies. Finally, if they are immunogenic the antibodies

may still not neutralize the virus. However, the fact that bnAbs can neutralize viruses from different subtypes, and studies showing prevention of infection by passive infusion in non-human primate studies [65, 84] implies that the Env variation in sequence is not limitless and can be overcome.

The first step in designing bnAbs requires their identification by screening sera of HIV-infected individuals. Between 10% and 25% of HIV-infected subjects make nAbs that cross react with diverse HIV strains [96-100] where a small fraction have been found to neutralize the majority of circulating HIV isolates [72, 98]. An alternative way to find broad neutralizing antibodies involve screening of thousands of unselected individual memory B cells stimulated to secrete IgG. Here, high-throughput neutralization assays have permitted the subsequent screening for HIV neutralization from the supernatants of the B-cell cultures using microneutralization [73, 101].

There are several strategies for the following step, comprising the attempt to elicit an identical bnAb-like response. Here, understanding B-cell maturation pathways on how to induce these antibodies with the desired specificity is crucial. For example, by maximizing the exposure of the CD4bs through masking irrelevant regions of the protein that may distract, and by designing alternative forms of the gp120 protein, the VRC01-like bnAbs were identified [102]. Another approach has been to design modified gp120s with high affinity for B cells expressing early forms of bnAbs [69]. Recently, soluble trimeric Env molecule (BG505 SOSIP.664 gp140) was used to select quaternary-specific antibodies and appeared more effective than B-cell–culturing methods [81].

Obtaining an efficient vaccine against HIV will most likely require a multivalent vaccine. Targeting several bnAb specificities may conceivably elicit a sufficient breadth of protection. bnAbs have already been proven in the non-human primate models to have a good potential as prophylactic and therapeutic agents, and it may only be a matter of time before their efficacy is evaluated in human clinical trials [75].

NeutNet study; assessing neutralization and cell-cell fusion

HIV neutralization assays are important tools for HIV vaccine development. The detection of new bnAbs has initiated screening of sera of hundreds of HIV infected individuals. Furthermore, this type of research requires assays for detection and evaluation of the quality, specificity, magnitude and breadth of antibodies elicited by a potential vaccine. The analysis of the antiviral activity of HIV antibodies is typically done *in vitro* by measuring the inhibition of free virus binding to cellular receptors and/or of viral fusion and by measuring inhibitory concentrations i.e. IC50, IC75 and

IC90. Neutralization assays are commonly divided in virus infectivity (VI)-based and pseudo virus (PSV)-based neutralizing antibody assays.

The *VI assays* are based on adding the mixture of antibodies and HIV to target cells and measuring the potency of antibodies to inactivate virus infectivity with regard to control. Readout is measurement of either p24 (produced from the cell or intracellular) or a genome-triggered protein such as GFP or luciferase. The most commonly used VI assay is the PBMC assay where peripheral blood mononuclear cells (PBMC) from HIV-seronegative donor are used as target cells for infection with patient viruses (primary or clinical isolates). This approach is thought to be more physiologic, however PBMC from different donors display different susceptibility to HIV infection partly due to host genetics, CD8-factors, number of CD4+ cells, expression levels of CD4 molecules on host cells, host genetic polymorphism in chemokine and chemokine coreceptors.

PSV-based neutralizing antibody assays exist in a number of variant assay formats, mainly differing in the use of target cells. Env-pseudo typed plasmids are produced from cloned or uncloned virus and (co-)transfected with Δenv-containing genomes into 293T cells. The resulting pseudo viruses are then tested in a single round assay with luciferase readout. Cell lines may be engineered to express luciferase when triggered by the infecting PSV, or the incoming viral genome brings in luciferase. Since the PSV particle is non-infectious due to the use of clonal plasmids (in contrast to VI that utilizes quasispecies), PSV assays require merely BSL 2 laboratory conditions, whereas VI assays require BSL 3. The main endpoint neutralization assay used for assessment of pre-clinical and clinical trial samples is the TZMbl assay [103]. Here, psudoviruses incorporate molecularly cloned HIV Env into defective virus particles capable of only a single round of infection. The pseudo viruses are generated in 293 T cell lines by co-transfection of an env-mutated viral backbone with the env clone of choice. The epithelial HeLa-derived cell line (TZMbl) carries a luciferase reporter gene triggered upon the presence of the HIV tat protein. The manual TZMbl assay has also been optimized and qualified to a high-throughput, automated 384-well format [103], a possibility that has been lacking until now with VI assays. It has also been validated in compliance with Good Clinical Laboratory Practice (GCLP) guidelines [103].

Another, less commonly used neutralization assay is the cell-cell *fusion assay*. All fusion assays are based on two different cell lines where Env or HIV is constitutively or transiently expressed in one cell line, and the other cell line expresses the CD4 and CCR5 or CXCR4 receptor and fusion is detected within hours. Fusion assays comprising fusion index based on counting of nuclei has been turned down due to the tedious work of cell counting. [104]. Flow cytometry also possesses a difficulty to distinguish between fused cells and aggregates of cells. Instead most cell-cell fusion assays today are based on either photochemical, biochemical or genetic interactions

[61, 105-108]. Cell-cell fusion signals may result from lipid mixing or cytoplasm mixing where the detection involves a signal that depends on direct interaction between two components. The fusion assay used in the NeutNet I (described below) study was based on fusion of effector cells expressing the native HIV Env on their surface (PM1 persistently infected with HIV) with target cells expressing the appropriate receptors (initially NIH-3T3 mouse fibroblasts or HeLa human epithelial cells stably expressing human CD4, CCR5 and/or CXCR4). The readout is measurement of ß-galactosidase activity.

Recognizing the importance of assay standardization, several organizations initiated studies to help identify and reduce the variability across the assays in use and made significant efforts directed towards the generation of standardized neutralization assays [109]. As a result, a study entitled NeutNet I was conducted involving a variety of assay formats, where study participants assessed test reagents against a panel of HIV isolates or a sub panel of pseudo viruses [110-112]. The conclusions from Phase I, using monoclonal antibodies and soluble CD4, was that the PSV assays are more sensitive than VI assays, however the variation was dependent on both the reagents and the virus used. Most importantly, no single assay was capable of detecting the entire spectrum of neutralization activities, and utilizing a range of assays was recommended [113]. There is a long list of confounding variables attributable to all components of a neutralization assay: the virus, the antibodies and cells. The neutralization assay outcome may therefore be determined by several assay parameters; target cell used, cell density, host cell used for virus propagation, duration of infection with/without infection and length of culture time.

Nevertheless, the advent of neutralization assays have enabled for the screening of sera from relatively large cohorts of HIV-infected donors to detect neutralizing antibodies. In the absence of a “gold standard” in neutralization assay, the studies on passive antibody transfer and protection studies in animal models followed by assessment of antibodies in different neutralization assays may be the only way to determine which assay has the greater correlative value.

Image analysis in assay development

Visual analysis can reveal biological mechanisms not possible with other types of analyses. Cellular functions have long been studied by examining cells in the microscope. Advanced microscopes are capable of collecting thousands of high-resolution cellular images as part of a large-scale screen. Image cytometry yields many informative measures of cells, including the intensity and localization of each fluorescently labeled component as well as number, size and shape of the subcellular compartments. Image cytometry individually measures each cell rather than

producing a score for the entire image. Compared to flow cytometry, image cytometry can handle thousands of samples more conveniently, as well as adherent cell types, time-lapse samples and intact tissues.

However, human-scored image analysis is inherently subjective and qualitative, only able to categorize samples as "hits" or "non-hits". Automated image analysis overcomes this limitation by detecting and measuring cellular features undetectable by the human observer, such as intensity, area and shape. Image cytometry therefore provides a less-labor-intensive, as well as high-throughput, alternative to manual scoring. Furthermore, when enabled by appropriate software tools, it provides reliable results from large-scale experiments within minutes or hours.

High content- and High throughput screening

High-throughput screening (HTS) assays with only a single, population-averaged readout yield fast readouts, but are unable to discern subtle phenotypes within heterogeneous cell populations. High-content screening (HCS) refers to an automated platform for quantitative image analysis by multiple parameters integrated at the level of single cells via fluorescent microscopy [114]. Cellular changes are therefore determined on a per-cell basis, and quantification is possible through image analysis software, either operated in real time or after the samples have been scanned and the images stored.

There are five components to successful, reproducible HCS: sample preparation, image acquisition, data handling, image analysis and data mining [115] (Figure 11). To sustain a rapid flow of work and data, all these steps need to be tightly coordinated.

Figure 11.
The five components to reproducible HCS: sample preparation, image acquisition, data handling, image analysis and data mining. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, "High-throughput fluorescence microscopy for systems biology", Pepperkok et al, 2006

The biggest advantage of HCS over other cell-based assays is that 1) each cell is screened for whether it conforms to the characteristics chosen such as size, shape and staining intensity, 1) analysis of cellular sub-populations, and 3) the ability to interrogate multiple readouts (i.e., fluorophores) simultaneously.

This eliminates noise such as artifactual fluorescence from particulates and cellular debris. Historically, HCS was limited for its intrinsically expensive technology and not being particularly high-throughput. However, advancements in imaging instrumentation have significantly enhanced assays quality and facilitating more sensitive and higher resolution measurements from cells. HCS automates image acquisition and analysis of microtitre-plate assays by applying robotic instrumentation together with information processing and storage strategies. Furthermore, HCS consider individual cells and consider subpopulations of cells based on their phenotypes and/or secondary markers. In general, HCS assays are considered to provide significantly more information, and of higher quality, than equivalent non-imaging assays

There exist a number of automated fluorescence-microscope-based image acquisition systems. However, commercial systems are often designed and optimized for highly specific applications and may be limited in their use and adaptation to new assays and/or complex cellular phenotypes. Despite the revolutionary development in HCS and the more advanced fluorescence-microscopy techniques, image based HCS is not the primary technique used in industry or academia, probably due to the requirement of specialized expertise to carry out HCS [116]. To overcome these limitations, new automated software systems with an open architecture (allowing the user to fine-tune all parameters) have been developed that allow users to add their own modules [117].

CellProfiler Image analysis Tool

The field of HCS has flourished with advancements in sophisticated image analysis software. Typically, commercial imaging hardware is supplied with software that includes some form of image analysis capability. In most cases, “canned” modules that perform analysis for a specific assay is supplied with the software. There exist several commercial image software tools, such as Cellomics, TTP LabTech, Evotech, Molecular Devices and GE Healthcare. However, the high cost, inability to modify the algorithms or know the proprietary nature of the code make them practical only in some screens but limiting in others. As HCS assays become more complex, some packages offer an open environment where programmers can access the code and commands of the algorithm. Multi-purpose image analysis software (Adobe Photoshop, MetaMorph, ImagePro, and ImageJ) is usually geared to interactive analysis and processing of individual images rather than high-throughput analysis.

While functions can often be combined into a macro to process multiple images, researchers are often unable to determine which functions are necessary to accomplish a particular goal. ImageJ is a wide-used open-source image processing tool but is more geared towards single image analysis, and the user needs to learn programming to create macros for high-throughput work.

One software package that overcomes these limitations is CellProfiler, a freely available and commonly-used image analysis software, capable of handling hundreds of thousands images (Figure 12 A) [118]. The code is also open-source, allowing the user access to the underlying methodology so it is no longer a "black box" from the researcher's standpoint [119]. The software uses the concept of a "pipeline" of modules, with each module performing a single image processing step, e.g. object identification, tiling etc. All pipelines can be saved and also shared with other users which allows for the same settings and reproducibility of the analysis regardless of microscope used.

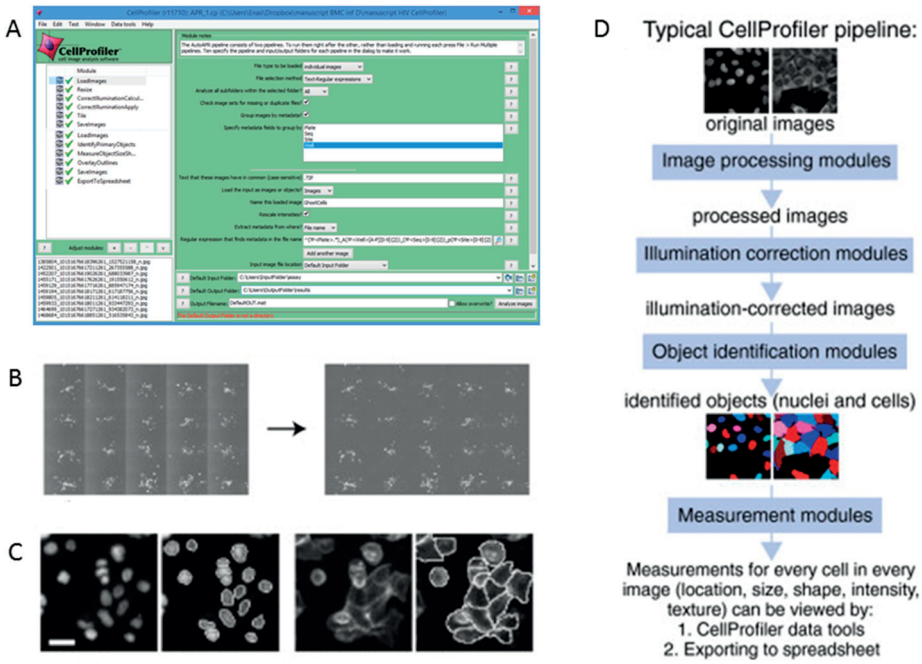

Figure 12
CellProfiler overview (A) CellProfiler interface software, with an analysis pipeline displayed. (B) uneven illumination at the left, and corrected by CellProfiler. (C) Identification of nuclei and identification of cell edges made by CellProfiler in a typical CellProfiler pipeline. (D) A typical pipeline in CellProfiler. Adapted from "CellProfiler: image analysis software for identifying and quantifying cell phenotypes", Genome Biology, Carpenter A.E. et al, 2006.

There are a number of different modules in CellProfiler, and they are all electable, where the user builds their pipeline by choosing the modules, and choosing at which turn-taking to build them after each other (Figure 12D). Within each module, there are several electable features for each identified compartment. Some of the modules are explained in detail below as they represent image processing concerns common to most pipelines apart from the easily understood "LoadImages" and "ExportToSpeadsheet".

- *Illumination correction* (Figure 12B) is one of the most critical steps in image analysis. The optics of the microscope may add illumination heterogeneities to the image characteristics; this aberration may vary more than 1.5-fold across the field of view that adds a level of noise, and could prevent experiments that rely on accurate fluorescence intensity measurements [120]. The estimation of the illumination pattern and its correction are handled by the "CorrectIlluminationCalculate" and "CorrectIlluminationApply" modules.

- *Cell Identification,* also called segmentation, is handled by the "IdentifyPrimaryObjects", "IdentifySecondaryObjects" and "IdentifyTertiary Objects" modules (Figure 12C). Primary objects are often nuclei identified from DNA-stained images; however they can also be cells, beads and so on. The algorithms chosen from cell identification depend on whether the objects are well-separated, non-confluent and appear bright on a darker background. The primary objects are recognized and separated, the dividing lines are subsequently found, and the resulting objects are either removed or merged together based on the settings. The secondary objects are more easily found, as well as subcellular compartments including cytoplasm, i.e., tertiary objects. CellProfiler's accuracy in separating and determining clumped nuclei is twice that reported for commercial software packages [118].

- *Cell* measurements are handled by the Measure modules, where a large number of features for each object can be measures, including morphology, intensity, correlation and texture.

A typical pipeline requires approximately 20 seconds per image on a standard desktop computer.

Part II – The role of galectins in HIV infection

Introduction to glycans

Glycans are one of the four basic components of cells, and may be the most abundant and diverse of nature's biopolymers. Glycomics refer to the study of the glycan structures that compose an organism's glycome. Glycans exist as covalent linkages of saccharides (carbohydrates) attached to proteins or lipids. Virtually all bacterial and eukaryotic cells, including some viruses, display surface carbohydrates. Cells are densely decorated with carbohydrates (Figure 13) and when we discuss carbohydrates in the cell, we mean carbohydrates attached to proteins (glycoproteins) and to lipids (glycolipids) on the plasma membrane. What looks like a fuzzy rug on the plasma membrane is rather the carbohydrates, the glycocalyx that allows the cell to interact and communicate with another cell by binding carbohydrates on the opposing cell surface (Figure 13).

Glycans are formed by a stepwise process where activated monosaccharides are added one by one to a growing chain. Each step is catalyzed by a specific enzyme (glycosyltransferase). In mammals, nine different monosaccharides exist and can be linked to each other in different (yet specific) ways, creating a wide diversity of glycans (Figure 14A). There are about 250 different glycosyltransferases. Glycans on the cell surface and extracellular proteins and lipids are formed in the classic secretory ER-Golgi pathway, as described below for the pathways most relevant for HIV Env.

A brief overview of monosaccharides and a disaccharide is given in Figure 14, and overviews of N- and O- glycans are given below.

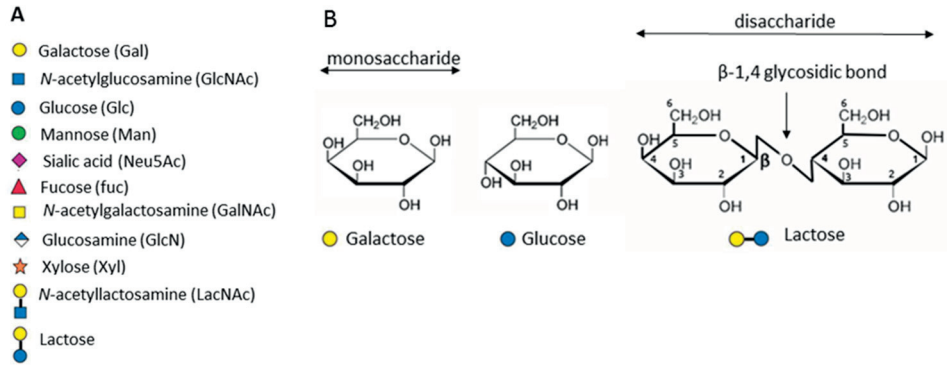

Figure 14.
The nine most common mammalian monosaccharides (A) and a lactose (B). A monosaccharide can bind the next monosaccharide at several positions, and with a different linkage configuration (α or β). This would theoretically create an immense glycan diversity, allthough not all of them occur in nature. An example of a disaccahride, lactose is shown, written as Galβ1,4Glc meaning that the 1st position of a galactose is bound via oxygen (glycosidic linkage) to position 4 of a glucocose, and that the linkage is in β configuration.

The precise linkages between monosaccharides create eptiopes or binding sites for carbohydrate binding proteins, like galectins of relevance here and described below. As an example, galactose fits in the core binding-site of galectins, whereas addition of sialic acid to the galactose at position 6 would block this binding due to steric conflict with the protein. In contrast, addition of sialic acid to the 3-position of galactose may enhance binding for some galectins, or prevent it for others. Sialic acid contains the same six membered ring as most other mammalian monosaccharides, but also contain a carboxyl group making it acidic and a few extra carbons (total of 9). It is often found as the last modifying sugar (cap) of a glycan.

Glycosylation of viruses

Many virus proteins are glycosylated by the host cell machinery, as most viruses do not encode enzymes for the glycosylation of their proteins. This may include N-linked, or O-linked glycans, or glycosylphosphatidyl inositol (GPI-) anchors. Although the mechanism of glycosylation is very similar to the way that cellular proteins are glycosylated, the pattern of glycosylation may be highly variable and depend on how a specific virus protein is processed by the host cell during infection. Many of these virus glycoproteins have been found to play essential roles during the virus replication cycle. In most cases, the viruses are glycosylated at relatively few sites (e.g. dengue virus E protein), however some virus glycoproteins, like HIV Env,

display very high levels of glycosylation. The effect of glycosylation has not been characterized in all viruses, but possible roles include ability of a virus to attach to and enter its target cell, which in turn could be a major determinant of host range specificity.

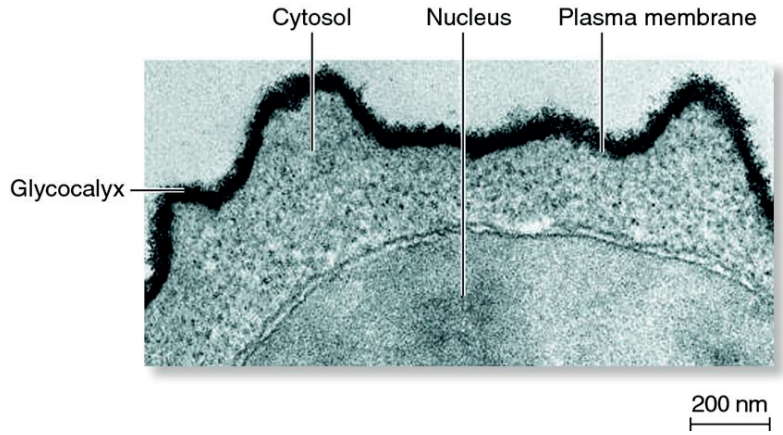

Figure 13.
A lymphocyte covered with a dense layer of carbohydrates (glycocalyx). Molecular Biology of the Cell, 4th Edition with permission

N-linked and O-linked Glycosylation

N-linked glycosylation occurs by linking a glycan to an asparagine residue (N), and requires the sequence motif NXS/TX', where X and X' are any amino acid with a few exceptions. This motif is often called a potential N-glycosylation site (PNGS), and can be used to predict the potential number of N-glycans in a protein, like gp120, just based on the amino acid sequence, even if all PNGS are not always glycosylated.

N-glycosylation starts co-translationally in the ER when the nascent peptide chain enters the ER, by transfer of the pre-made glycan core en bloc from a lipid (Dol-P) bound precursor (Figure 15). The N-glycan is then processed in the ER and Golgi, where additional mannose residues are added subsequently forming high mannose structures, and optionally trimming and adding GlcNAc, Gal, NeuAc, and Fuc residues to form so called complex N-glycans. The relative proportion of high mannose, hybrid (mixed), and the different types of complex structures can vary between glycoprotein specific PNGS, and cell type, by mechanisms that are not fully understood.

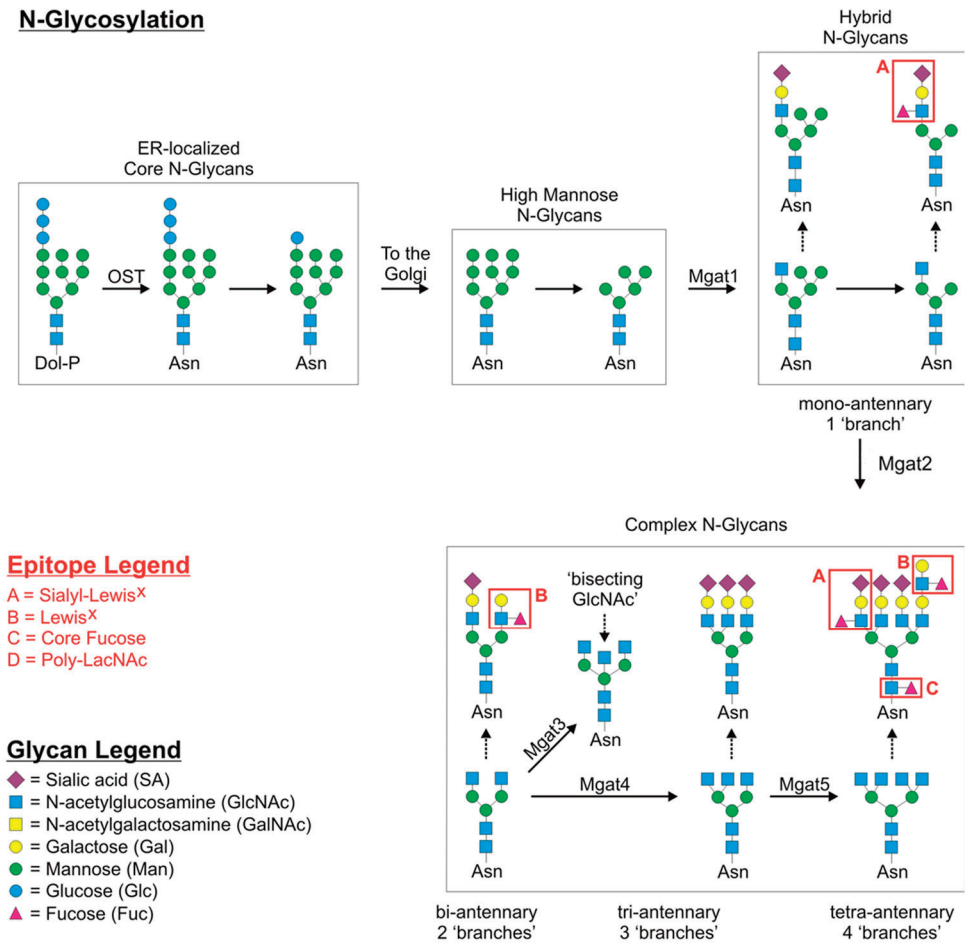

Figure 15.
Biosynthesis of mammalian N-glycans. The formation of N-glycans from the lipd linked precursor (Dol-P) in the ER to different types of N-glycans in the Golgi is shown, with examples of saccharide based epitopes in red squaraes.

O-glycosylation occurs in the Golgi, by stepwise addition of monosaccharides to S or T residues of already folded, N-glycosylated and oligomerized glycoproteins (Figure 16). Unlike the N-glycans, no amino acid sequence motif easily predicts O-glycosylation, and prediction attempts are instead based on modelling of surface accessibility, estimation of secondary structure and distance constraints and gives an accuracy of merely 76% [121]. Biochemical methods are needed to establish O-

glycosylation. The most common O-glycosylation on extracellular proteins starts with GalNAc linked to S or T, as found in e.g. mucins. Then additional sugars such as Gal, GlcNAc, and further out Fuc and NeuAc can be added. Other types of O-glycosylation exist but are beyond the scope of this thesis as they have not been found on Env.

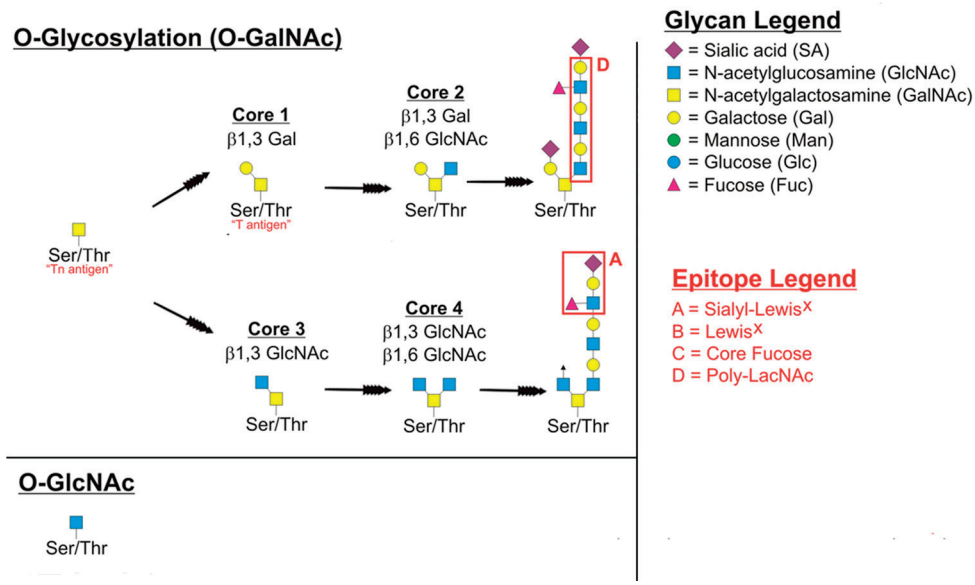

Figure 16.
Biosynthesis of mammalian O-glycans. The formation of O-glycans beginning with addition of GalNAc to a Ser or Thr residue to different more complex types is shown, with examples of saccharide based epitopes in red squares.

Glycosylation of Env

Glycans on the gp120 are predominantly N-glycans, with a small and variable contribution of O-linked glycans [122]. N-glycans [123] together with the small additional contribution of O-linked glycans [124] make up half of the molecular mass of gp120. There are typically around 20-35 N-linked glycosylation sites in gp120 and 3-5 N-linked glycosylation sites in gp41 [16, 122, 125, 126]. However, not all N-glycosylation motifs are occupied by glycans, thus it is more accurate to designate these motifs as potential N-glycosylation sites (PNGS) as mentioned above [127, 128]. The number and position of PNGS, as read from extensive Env-gene sequencing, varies between different virus strains and for the same strain with disease stage within one patient [129, 130].

Defining which PNGS are actually glycosylated, and which of the many types of glycans they carry require biochemical methods such as mass spectrometry (MS). The total glycans cleaved off the trimeric gp120 from viral particles are largely high mannose type glycans (i.e. 62-79 % Man5-9GlcNac2) with the remaining 21-38% being complex or hybrid type glycans [131]. The same study revealed that recombinant monomeric gp120 indicates a higher percentage (up to 71%) of complex and hybrid-type glycans. The lower ratio of complex type glycans in the trimer was explained by inaccessibility of the densely glycosylated regions to the glycosidase responsible for processing the immature high-mannose glycoforms to the more mature complex and hybrid type [131]. Moreover, the number, type and composition of glycans and ultimately the Env properties may vary significantly depending on cell expression system, HIV strain, the stage of HIV infection and the oligomeric form, i.e. whether the protein was obtained as recombinant cell-secreted gp120 monomer or gp140 monomer from pseudovirions or actual virus particles [122, 127, 128, 131, 132]. The HIV gp120 N-linked glycosylation also differ between plasma and leukocyte compartments, and there are significant differences in the number of glycosylation sites between plasma and cellular compartments [133].

Assessing O-glycan contribution to the biology of Env is more difficult. Although potential O-glycosylation sites cannot be easily read from the sequence, the CBS NetOGlyc prediction server (http://www.cbs.dtu.dk/services/NetOGlyc) has suggested that most Env sequences have one or two potential O-glycans [121]. The existence of the O-glycans on gp120 has been confirmed by binding of specific antibodies that neutralize HIV infectivity [89, 90, 134] and mass spectrometry.

Glycans have recently been modelled into a gp120 core and an Env trimer spike [135]. However, the 3D-structure and function of glycans in Env remain largely unknown, since crystallographic studies typically require removal of glycans.

Glycan-dependent cellular receptors for HIV

The binding of the HIV cellular receptors CD4, CCR5 and CXCR4 can be indirectly affected by glycans, as glycans contribute to Env folding [136, 137]. Furthermore, Env is a ligand for several classes of glycan-dependent cellular receptors for HIV, including glycan-dependent C-type lectins [136]. These glycan-dependent cellular receptors for HIV are mainly involved in *trans*-infection of the target cells, i.e. transmission and spread but not virus replication. E.g. the host integrins LFA-1 stabilize the binding of HIV to target CD4 positive in a glycan-independent manner. The ligands on HIV are of cellular origin where the LFA-1 ligand ICAM-1 is integrated into the viral envelope from host cellular phospholipid membrane. Other

examples of glycan-dependent cellular receptors for HIV is Langerin, Mannose-binding-lectin (MBL) and DC-SIGN.

Reading the glycans

The interaction between different cell types and their environment relies in part on their differential glycosylation profiling on the cell membrane. These protein-carbohydrate recognitions are crucial for intracellular processes, e.g. protein folding and transport, cell differentiation, adhesion and migration. Glycans play a wide variety of biological roles by virtue of their mass, shape and charge, and many of these biological roles are mediated via specific recognition by glycan binding proteins (GBPs), lectins. Mammalian lectins are classified into different families based on similarity of their carbohydrate recognition domains (CRDs) regarding sequence, structure and other properties. The largest and most extensively studied family are the C-type lectins that requires Ca2+ for sugar binding and include e.g. the selectins involved in leukocyte traffic, and DC-SIGN involved in intracellular traffic and immune regulation. The focus here is on another animal lectin family, namely the galectins [138].

Galectins

The galectins are small, soluble, glycan-binding proteins. They are defined by a CRD of about 130 amino acids with a conserved ~7 amino acid structure motif and affinity for beta-galactosides [139-141]. To date, there are 15 identified mammalian members of the galectin family, galectin-1 to 15 which have been numbered according to the order of their discovery and where 11 are known in humans. The ~14 kDa CRD contains two β-sheets of 5 and six strands with a ligand-binding groove possessing key features that define its specificity[139]. Within this groove, the conserved ~7 amino acids make up the defining galactose-binding site. Sub sites on either side of this differ among the galectins, and determine the distinct fine specificity for each one [139] [142].

On the basis of their molecular architecture, galectins are classified into three main types (Figure 17):

(i) *Prototype galectin*s (galectins -1, -2, -5, -7, -10, -11, -13, -14 and -15) have one CRD that is a monomer, non-covalent dimer or higher complex depending on ligand density,

(ii) *Tandem-repeat-type galectins* (galectins -4, -8, -9 and -12) (MW ~35 kDa) have two different CRDs joined by a linker within the same peptide chain. The two CRDs have different fine specificities, and this heterobifunctional property may allow them to crosslink a wide variety and combinations of glycoconjugates.

(iii) *Chimera-type galectins* (galectin-3) (MW ~30 kDa) consists of a C-terminal CRD linked to an N-terminal part with different structure. Also galecin-3 oligomerizes upon ligand encounter, and, hence, may cross-link ligands like the other galectins.

Figure 17.
Galectins are classified into three main types, prototype, chimer and tandem-repeat.

Galectin specificity

As mentioned above, the minimal structure recognized by galectins is a β-galactoside, found on complex N-glycans and O-glycans. One example is LacNAc that can be found on cell surface glycoproteins and be presented as poly-LacNAc. Galectins typically do not have specific individual glycoconjugate receptors, instead each galectin can bind to a set of cell-surface or extracellular matrix glycoproteins containing suitable oligosaccharides [143] (Figure 18). The functional differences in individual galectins' biological activities is largely due to the glycan-binding preferences. These variations are mainly associated with the extent of N-glycan branching, multiplicity of LacNAc residues and/or modification of terminal saccharides (e.g. sialylation or fucosylation) where e.g. complex N-glycans are the major ligands for galectin-1, -3 and -8 [142, 144, 145]. Moreover, specificity binding variations between galectins can result from spatial orientation of individual CRDs and the unique glycoprotein topologies determined by the number of attached glycans [144]. The specific binding of the neighboring glycans to the galectins enhances the overall affinity [143]. All galectins are either bivalent, or can multimerize into dimers and high-order oligomers. This multivalency allows the galectins to bind multiple glycan ligands on either the same cell or on opposing cells, as well as on microbes and host cells [146-148].

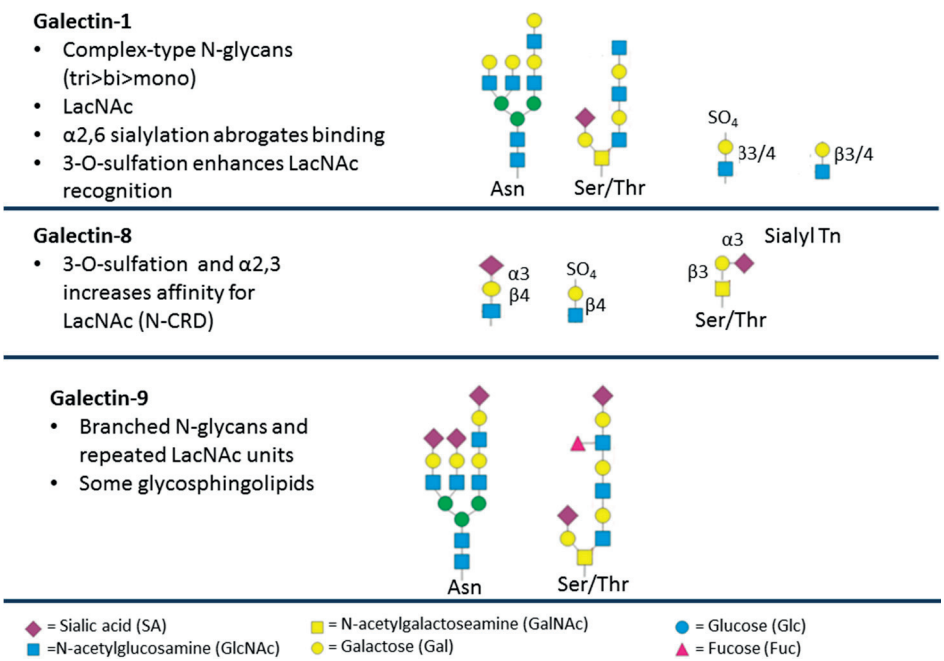

Figure 18.
Galectin 1, -8, and 9 specificity to different glycans.

Galectin expression intra-and extracellularly

As glycans occur extracellularly and on the cell membranes, lectins were first expected to also occur primarily in the extracellular compartment. However, galectins are the only glycan-binding proteins (GBPs) in animals that are found both in cytoplasm and on the plasma membrane and extracellular matrix, a property they share with a few other proteins like annexins, IL-1 and others [149]. They are synthesized on cytoplasmic ribosomes after which some are then secreted through an as yet unknown pathway.

Intracellularly, galectins are selectively targeted to subcompartments of the cytosol as well as to membranes and membrane-bound vesicles. Interestingly, some of their functions inside cells may be independent of their carbohydrate activities, such as roles in regulation of cell growth and apoptosis, regulation of the cell cycle and in pre-mRNA splicing [150]. There is little information on an endogenous carbohydrate ligand for the intracellular galectins and the significance of the carbohydrate-binding activity of any particular intracellular lectin remains as a challenge for future investigations.

Outside the cell, galectins bind to cell-surface and extracellular matrix glycans and thereby affect a variety of cellular processes discussed below.

Galectins in immune homeostasis

Galectins play important roles in diverse physiological and pathological processes, and have recently been suggested as the key mediators in innate and adaptive immune responses. Galectins are expressed and distributed in most cells of the innate and adaptive immune systems where they mediate communication between immune cells through the recognition of a preferred set of cell surface glycoconjugates [143]. Apart from participating in several physiological processes, they have also been studied in several pathological processes [151, 152]. Overall, they seem to have both overlapping and different functions in different cells. Given the regulatory roles they play in both innate and adaptive immune responses, their effects have been shown to be beneficial some times, but detrimental other times as in pathological conditions with exacerbated or depressed immune functions.

In innate immunity, galectins play roles in cell adhesion and transmigration through endothelial cell surfaces, as well as in the initiation and resolution phases of inflammatory responses by induction of pro- and anti-inflammatory cytokines [153]. The galectins may also induce indirect effects through regulation of innate and adaptive immunity, e.g. promoting dendritic cell maturation and migration, enhancing cytokine production and initiating release of intracellular mediators such as histamine [146, 148, 154, 155]. The same galectin may promote anti- or pro-

inflammatory effects depending on the concentration reached in inflammatory foci, the target cells and the extracellular microenvironment [156].

With regard to adaptive immune responses, galectins function as regulators of the immune cell development and homeostasis, best analyzed regarding T-cells [144, 151, 155, 157]. Galectins also modulate B cell maturation and differentiation both at the central and peripheral immune compartments [158, 159].

In adaptive immunity, galectin-1 is present at site of immunological synapse both in primary and secondary lymphoid organs and interferes with early T-cell signaling processes [151]. In the thymus, galectin-1 can shape the T cell repertoire by differentially modulating positive or negative selection. However there are ambivalent studies regarding apoptosis triggered by galectin-1 in the periphery [160-164]. Once inside secondary lymphoid organs, the galectin-1 regulates T cell fate by defining appropriate T cell activation thresholds. Most importantly, galectin-1 also has the ability to blunt Th1- and Th17-mediated response and skew the balance towards a Th2 polarized cytokine profile by inducing apoptosis in terminally differentiated T cells expressing specific carbohydrate ligands and/or by modulating cytokine production [153].

Furthermore, T-cell susceptibility to galectin-1 induced cell death is influenced by expression of cell-surface glycoprotein receptors (CD45, CD42, CD2 and CD7), the activity of some glycosyltransferases creating or masking specific cell-surface glycans and the activation of particular intracellular pathways. Administration of galectin-1 in vivo favored the recruitment of a population of uterine dendritic cells with a regulatory cell-surface phenotype. [165]

Within the bone marrow compartment, galectin-1 has a clear contribution on the formation of immune developmental synapse between human pre-B and stromal cells through binding to several integrins α4β1, α5β1 and α4β7 (same integrin involved in HIV transmission). Once in the periphery, galectin-1 is upregulated by activation signals and contributes to the differentiation of activated B cells into antibody-secreting plasma cells. Augmented expression of galectin-1 instead facilitates the death of memory B cells [151, 166].

Galectins in host-pathogen interactions

Lectins that recognize glycans that are typically found on pathogens are named pathogen-recognition receptors (PRRs). These glycans are termed pathogen-associated molecular patterns (PAMPs). There are however several lectins that recognize self glycans in addition to PAMPs. These include some C-type lectins, e.g. DC-SIGN, and the macrophage mannose receptor [167]. The β-galactosides recognized by the galectin family are mainly found on host cells but are also expressed

as saccharide ligands on some microorganisms. All galectins have thus the potential to act as PRRs and recognize both self-glycans and glycans on microbes (non-self). Interactions between animal lectins and ligands on microbial surfaces are weak compared with antibodies, however high avidity is reached when multiple CRDs interact with the ligand simultaneously [168]. From an evolutionary point of view the microorganism evolved PAMPs to avoid recognition by host PRRs. The Janeway and Medzhitov model for non-recognition does not seem to apply on galectins which bind to self-patterns [169]. This suggests that the pathogens, rather than avoiding recognition by the host, instead have evolved their glycans to mimic their hosts in a "Trojan horse" model where they rely on the host's self-recognition molecules, such as galectins, to attach to their vector for host invasion [168]. Well inside the host, the parasites have diverse mechanisms to evade intracellular killing by the host or down regulate downstream immune responses. This underscores the oversimplification in the PRR terminology, as well as the gaps in our knowledge of recognition of the host galectin repertoire along with the structural aspects of their interaction with microbial glycans. The galectins thus do not recognize true PAMPs, instead, they recognize glycans on microbes that are mimicking self-glycans. The ability to express such glycans by the microbial pathogens represent an interesting target for the development of novel strategies for intervention in human disease.

As galectins seem to play key roles in the host development and immunoregulation, a strong functional constraint should prevent galectins from undergoing changes in carbohydrate specificity, which is supported by the apparent conserved structure within this family. An interesting question arises as to whether the microbes, by mimicking-self-glycans, disturb the regular physiological role of galectins or whether they instead result in augmented physiological processes pathways by hijacking the galectins cellular activities. Nevertheless, the galectins offer promising possibilities for treatment and intervention of a wide variety of pathological processes, including inflammation and HIV intervention.

The carbohydrate ligands for most galectin-microorganism interactions remain largely unknown. As to date, several interaction between the glycans on host and microbes have been elucidated. Interestingly, these interactions may have three different outcomes: either enhancing the microbial infection, blocking the microbial infection or having a microbicidal activity.

Enhancing microbial infection

Galectins, in interactions with certain microbes, can promote pathogen attachment to or entry into host cells. As galectins are typically multivalent the galectins can directly interact with microbial pathogens by binding to glycan ligands on both the microbial pathogen and the host plasma membrane to "bridge" the microbe to the target cell.

This effect has been shown for galectin-1 that promotes viral attachment of HIV to human T-cells and is more thoroughly discussed under the section "Galectins in HIV pathogenesis". In short, it is suggested that galectin-1 bridges the glycoprotein receptors gp120 on the virus and CD4 on the host cells. As CD45 is also recognized by galectin-1 and exist on the viral envelope (as well as on the host cells), this cellular receptor may also contribute to the glycan interaction stabilized by galectin-1.

Another virus utilizing galectins for direct bridging and enhancement of infection is the herpes simplex virus 1 (HSV-1) where galectin-3 is specifically bound to HSV-1 in a carbohydrate dependent manner [170]. Added galectin-3 promoted infection of human corneal epithelial cells by HSV-1 and reduction of galectin-3 expression clearly reduced HSV-1 binding and infection. Direct bridging of host target cells to bacterial- as well as parasitic glycans by galectins leading to enhanced infection has also been found for Neisseria meningitidis, Leishmania major and Trypanosoma cruzi [167] .

Galectins blocking microbial infection

Galectin binding does not always lead to a pro-microbe effect, and may instead prevent microbial infection. Galectins are proposed to participate in the organization of mucins into a protective layer that impedes access of microbial pathogens to the host epithelium, suggesting an indirect role for galectins in reducing microbial invasion. Galectins may thus have a pro-viral or anti-microbial effect depending on the timing and context of galectin binding to viral glycoproteins [171].

Galectins' microbicidal activity

In addition to enhance or block microbial infections, galectins may also act as "danger receptors" and enhance cytokine production and phagocytic clearance of pathogens or by having direct microbicidal activity. Recently, a specific role for phagocytic clearance of *Salmonella typhimurium* by galectin-8 was described. Here, galectin-8 was found a critical component for activation of autophagy in cells infected by *S.typhimurium* [172].

In addition to promoting phagocytic uptake or autophagic destruction of bacteria, fungi and parasites, galectins also have a direct microbicidal activity. Galectin-3 binding to intact, live C.albicans with β1,2 oligomannan expression had a direct fungicidal effect, resulting in cell shrinkage and increased intracellular granularity [173].

Galectin-8 has also demonstrate microbicidal strength; addition of galectin-8 to susceptible E.coli strains resulted in direct killing of the bacteria [174].

Galectins in HIV pathogenesis

Current research has established roles for several galectins in HIV pathogenesis [175]. One of these galectins is galectin-1, being a dimer that can cross-link ligands expressed on different cells and by this mediate adhesion between cells or between cells and pathogens. Galectin-1 is abundantly expressed in the thymus and lymph nodes, organs that represent major reservoirs for HIV. Moreover, galectin-1 is secreted by activated CD8+ T lymphocytes, which are found in high numbers in HIV-positive patients. As a direct promotor of HIV infection, galectin-1 has been shown to facilitate viral adsorption to human T cells and macrophages by cross-linking HIV and target cells thereby augmenting the efficiency of the infection process [176]. Here galectin-1 was shown to promote the initial adsorption of HIV to CD4+ cells through its binding to viral gp120 and facilitate HIV infection in a manner that is dependent on its recognition of β-galactoside residues [177]. Experiments performed with the fusion inhibitor T-20 established that galectin-1 is primarily affecting HIV attachment. The complex glycans on gp120 seem to offer specificity for galectin-1, whereas another member of the same family, galectin-3, is unable to bind gp120 or enhance HIV infection. Since viral adhesion is a rate-limiting step for HIV entry, drug discovery approaches targeting galectin-1 would thus present an interesting candidate as antiretrovirals [175].

Furthermore, plasma levels of galectin-9 have been reported to be elevated in HIV infection [178]. Measuring the levels of galectin-9 in three patients with acute HIV before and after antiretroviral treatment showed also that the plasma levels of galectin-9 decreased rapidly after treatment. This suggests that the plasma levels of galectin-9 may be a sign of a severe inflammation during the acute phase of HIV infection and could be a potential biomarker during acute infection [179]. Other studies have shown that galectin-9 could increase HIV infectivity but via an indirect mechanism [180]. Here it was suggested that galectin-9 regulates the redox environment at the T cell surface by retaining the protein disulphide isomerase (PDI) on the cell surface. This reducing environment created by galectin-9 retention of PDI was suggested to enhance the HIV infection as reduced forms of gp120 and CD4 promote viral entry.

Moreover, galectin-3 was suggested to be translocated to immunological synapses in T cells upon T-cell receptor engagement, and promote human immunodeficiency virus (HIV)-1 virion release by associating with ALG-2-interacting protein X (Alix) [181]. Alix is known to coordinate with the endosomal sorting complex required for transport (ESCRT) to the cell membrane. However, *in vitro* studies have not shown enhanced HIV infection by galectin-3 [175].

Galectin inhibitors as potential anti-retroviral strategies

High-mannose N-linked glycans recognized by carbohydrate-binding agents (CBAs), a special class of HIV-binding plant lectins, are potential targets for topical microbicides. CBAs interact directly with the viral envelope glycans and block viral infection, and may present good microbicide candidates to prevent HIV transmission.

While galectins share relatively similar CRDs, their functions differ significantly, underscoring the importance of developing specific inhibitors for each galectin. There are a number of strategies for inhibiting the function of the galectins. The anti-sense interfering RNA (siRNA) is another powerful tool that work by suppressing gene expression. Small-molecule inhibitors (either synthetic or natural) bind in competition with endogenous ligands to prevent normal galectin function. Natural saccharides act by binding competitively with natural ligands to galectins thus disrupting galectin-glycan formation. One example is lactose that is known to inhibit galectins' activities, however requires high concentrations and is not galectin specific. In a study by St-Pierre et al 2011, three synthetic inhibitors, derived from lactoside molecules were shown to exhibit an anti-galectin-1 mediated HIV infection [182]. Galectin inhibitors blocking galectin-mediated increase in HIV-binding would thus enable their use as promising anti-retroviral strategies. However, potency as well as specificity studies are needed together with analysis of potential cytotoxicity given the importance of galectins in various physiological pathways.

Galectin-8

Galectin-8 (34kDa) is a tandem-repeat galectin with an N-terminal CRD (N-CRD) and a C-terminal (C-CRD), which share 38% sequence identity with each other (Figure 19). They are joined by a linker peptide of variable length, which is susceptible to proteolysis [183]. Six isoforms of galectin-8 harboring either one of two CRDs are encoded by one gene as a results of alternate splicing. The prototype galectin-8 isoforms however have never been isolated. The galectin-8 cellular localization seems to be a function of cellular type and cellular time, where galectin-8 is found accumulated on the plasma membrane, in cytoplasm or in nucleus at various time points [184]. When galectin-8 is secreted to the extracellular space, it exerts its function as paracrine/autocrine effector [185].

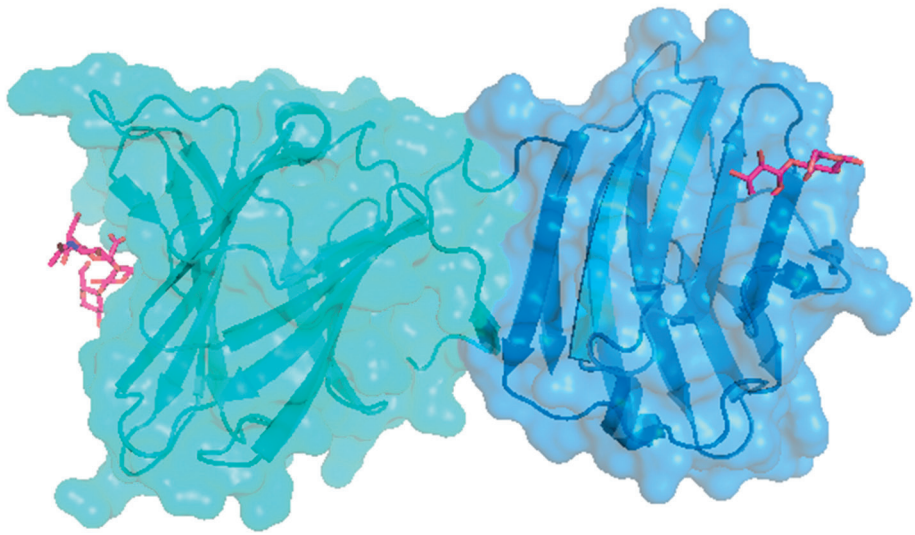

Figure 19.
Galectin 8 N terminal (cyan) binding a sialyllactose and C terminal (marine) binding lactose. PDB 3VKM.

The N-terminal CRD of galectin 8 (galectin-8N) has a particularly high affinity for 2-3 sialylated β-galactosides, a unique feature among galectins. Furthermore, galectin-8N prefers sialylated β-galactosides that are Galβ1-4Glc, Galβ1-3GlcNAc and Galβ1-3GalNAc. The preference of galectin-8N for Galβ1-3GalNAc over Galβ1-4GlcNAc suggests that galectin-8N favors O-linked glycans over N-linked glycans. This preference for O-linked glycans was further established by studies showing galectin-8 binding to IgA1 containing a mucin-like domain with O-glycans, but not IgA2 lacking the mucin domain. This affinity is not shared by the C-terminal of

galectin 8 (galectin-8C) which generally displays weaker affinities towards most glycans however with a slight preference for GalNAcα1-3(Fucα1-2)Galβ1-3/4GlcNAc [186, 187].

Galectin-8 is expressed in normal tissue such as brain, breast, colon, retina, kidney, pancreas, placenta, spleen, testis, cytotrophoblast, vascular, esophagus and heart [184, 188, 189]. Furthermore, galectin-8 has been found in thymocytes and splenocytes [190, 191].

Receptors that have been found to be the primary target for galectin-8 are members of the α-integrin family, LFA and the CD45 receptors. They are all abundantly expressed on the surface of leucocytic cells and have diverse effects when studied with galectin-8. E.g., treating cells that express α-integrin with galectin-8 induced adhesion, indicating that galectin-8 can behave as cell adhesion-inducing molecule for functional leucocytes in vivo. The second counter-receptor of galectin-8, β2 integrin LFA-1 [192], is composed of αL and β2 subunits, where β1 integrins carry predominantly α2-3-sialylated glycans (high-affinity ligands for galectin-8N) [193] and where ICAM-1 is the major ligand [194, 195]. LFA-1 is ubiquitously expressed on most leukocytes and endothelial cells where it is upregulated during inflammation. Galectin-8 has been shown to bind to LFA-1 in a carbohydrate-dependent manner, endocytosed after binding to the cell [196], thereby removing LFA-1 from the cell surface, inhibiting its interaction with ICAM-1 and blocking the LFA-1 function. CD45 is another major counter-receptor to galectin-8 [190]. CD45 is an abundant and highly glycosylated protein located at the surface of different cells from the hematopoietic lineage, including lymphocytes. Here, the galectin-8 binds to CD45, leading to antigen-dependent co-stimulation of T cells. In naïve T-cells, galectin-8 induces strong antigen-independent proliferation of resting CD4 T cells in the absence of antigenic stimulus when used at relatively high concentrations [190]. In the presence of antigen at low concentration, galectin-8 co-stimulates T cells interaction of APCs [197]. These activities were found mediated by the T cell surface glycoprotein CD45 interaction, involving the activation of ZAP70- and Erk signaling pathways [190]. For co-stimulation, galectins need to bind on APCs and T cells at the same time during the Ag-presenting process. As galectin-8 is able to co-stimulate T cells, even at low concentrations, this has been suggested to give a functional activity, not only under normal conditions, but also in altered responses such as in the induction of autoimmunity where the lowering of the threshold of T cell activation seems crucial [190].

Galectin-8 also exhibits apoptotic functions in several immune cells. Galectin-8 induces apoptosis in Jurkat T cells as well as in a subpopulation of T cells in activated PBMC cells [198]. Interestingly, autoantibodies against galectin-8 found in patients with systemic lupus erythematosus (SLE), a prototypic autoimmune disorder, impeded both its binding to integrins and cell adhesion [199] as well as the apoptotic

effect of galectin-8 [198]. The galectin-8 also induces apoptosis in $CD4^{high}CD8^{high}$ cells by binding to TCR. Thus galectin-8 by inducing apoptosis in activated T cells [198] may exert an immunosuppressive action. [196].

Conversely, galectin-8 has a pro-inflammatory role in other cellular systems, and orchestrates the interaction between leukocytes, platelets and endothelial cells. In endothelial cells, galectin-8 induces several pro-inflammatory molecules: CXCL1, GM_CSF, IL-6, CCL5, and in to lower degree CCL2, CXCL3 and CXCL8.

Furthermore, galectin-8 is prevalently expressed in mature B cells where galectin- 8 can promote stimulus-mediated plasma cell differentiation. In CD4+ T Cells, galectin-8 stimulates the cells to produce IL-2, IFN-y and IL-14, suggesting a Th1 as well as Th2 proliferation. As several integrins are targets for galectin-8, they could very well be the potentially responsible for the proliferative outcome.

The galectin-8 expression has been studied in neoplastic transformation and found to correlate with several types of tumor, where the level of galectin-8 expression was either elevated (breast), unchanged (lung, bladder, kidney, prostate, stomach) or decreased (colon, pancreas, liver, skin, larynx) [184].

Galectin-8 additionally plays an indirect role as a PRR by binding host glycans exposed in damaged vacuoles, endosomes or lysosomes to target them for autophagy. This involves the recruitment of NDP52 to invading bacteria by the bound galectin-8, and as previously shown by an ubiquitin-dependent pathway [172].

Measuring carbohydrate-protein interaction

There are a number of methods for determining carbohydrate-lectin affinities, e.g. Surface Plasmon Resonance and Isothermal Titration Calorimetry. The method we have relied on in our studies is fluorescence polarization (FP assay) also called fluorescence anisotropy (FA).

Florescence Anisotropy Assay

Fluorescence Polarization (FA) is a rapid and quantitative method that allows analysis of diverse molecular interactions in solution. The major advantage of FA is that is does not require the separation of bound and unbound phases, and presents a measure of true equilibrium. The principle of FA derives from the fact that fluorescent molecules (fluorophores) emit polarized light when they are excited with polarized light, and that the molecule will become heavier and as a result, experience less depolarization (higher polarization) when bound to a receptor [200].

In our case fluorescein tagged-saccharides are used as probes to measure interaction with galectins. The free probe will tumble in solution at a certain rate determined by its size and solvent viscosity. This combined with the fluorophore excited life time (4 ns for fluorescein) will determine the loss of fluorescence polarization. When a galectin is added, the bound probe will tumble slower, and hence less fluorescence polarization is lost. The measured FA value is an average of the values for the bound and free probe, weighted by their respective concentrations.

In the practical measurement, anisotropy is derived as the difference of the emission light intensity parallel (I||) and perpendicular (I⊥) to the excitation light plane normalized by the total fluorescence emission intensity where

$$\text{Polarization (F)} = (I_{||}-I_{\perp})/(I_{||}+I_{\perp}) \text{ or Anisotropy (A)} = (I_{||}-I_{\perp})/(I_{||}+2I_{\perp})$$

The polarization definition was used originally, but the anisotropy definition is now more common as it is linearly proportional to each contributing species, making calculations simpler. Thus, from the measured anisotropy value, the concentration of unbound and bound probe is obtained. Since all initial concentrations are known (probe, galectin and possible inhibitor), the concentration of each bound or unbound can be calculated and from this the K_d values for interactions.

In practice, a series of galectin concentrations and a fixed probe concentration is added to a 96-well microtitre plate and anisotropy measured as described above. Anisotropy will rise with galectin concentration from a low value for free probe (A_0) to approach a maximum (A_{max}) when all probe is bound by galectin. Our lab has a library of fluorescein tagged saccharides (probes) so the optimal one can be chosen for each galectin, when inhibitors, e.g. gp120, are tested [201, 202]. An optimal probe shall bind galectin with as high affinity as possible (binding curve shifted to the left), and give sufficiently high A_{max}. This is to permit choosing a fixed galectin concentration for inhibition that is as low as possible, but still gives a reasonably high anisotropy to give a sufficient measuring range above A_0. Having chosen these condition, the fixed probe and galectin are titrated with an increasing range of concentrations of inhibitor, in this case e.g. gp120. Should the inhibitor take the place of the probe in the galectin-binding pocket, the result will be decreased anisotropy due to increased amount of fluorophores in the solution, and from this affinity of the inhibitor for the galectin can be calculated (Figure 21).

Aims

One major effort in HIV research is to find an efficient antibody-based vaccine. As a guide to this, both the profile of natural antibodies occurring during HIV infection, and antibodies elicited in vaccine trials can be used. In either case there is a need for standardized assays to test the potency of antibodies or sera that neutralize HIV. Furthermore, the role of antibodies may be equally important in inhibiting virus cell-to-cell spread, but this has so far been difficult to evaluate. To address these issues we aimed to answer the following questions:

1. How do we automatize the readout of a manual HIV neutralization/plaque reduction assay, and how does it compare to other neutralization assays?
2. Can we study HIV neutralization and cell-cell fusion inhibition simultaneously?
3. Is it possible to detect simultaneously in the same assay antibodies that both neutralize and inhibit HIV-mediated cell-cell fusion in plasma of HIV-infected individuals?

The importance of glycosylation of HIV Env is not limited to immune evasion. Since HIV Env is glycosylated by the host cellular machinery, it may be recognized as self by glycan-binding proteins. Galectin-1 has previously been shown to enhance HIV infection, and has been suggested to stabilize CD4 and gp120 interactions, but other galectins have been studied much less or not at all in this regard. Our work aimed to answer the following questions:

1. What is the binding affinity of different galectins for gp120 and CD4 in solution? To make this possible, can we adapt a previously established fluorescence polarization assay to microscale?
2. To what extent can whole virus particles from different HIV strains bind the different galectins?
3. What is the effect of galectins on HIV infectivity, and can we apply our infectivity/plaque reduction assay to study this?
4. What is the impact of galectins on the infectivity of HIV isolates from different disease stages, the chronic and AIDS phases?

Present investigation

Automation of the GHOST(3) plaque reduction assay

Fluorescence-based imaging assays can be applied to large-scale analysis to solve biological problems in a standardized manner. GHOST(3) cells, which express the green fluorescent protein (GFP) upon HIV infection, have been used for measuring HIV neutralization using a plaque reduction (PR) read-out. Until now the read-out had been applied by microscopic reading in a manual manner. For standardization and development of a high through-put assay, it was mandatory to automate the read-out. Accordingly, we developed an image-based automated plaque reduction (APR) assay, with the aim to detect HIV neutralization, but also to be used in other applications including infectivity studies. The GHOST(3) PR assay is a virus infectivity based assay with a readout performed three days post-infection, i.e. allowing limited rounds of replication. The HIV infected cells often form syncytia (nuclei of two or more cells in the same cytoplasm). These and single infected cells showing fluorescence are counted as plaques. To adapt the previously manual PR assay into an automated assay, we used the wide field, inverted microscope AxioObserver Z1 using a motorized stand able to automatically acquire images from an entire 96-well plate. Unfortunately, most automated microscopes are limited in their flexibility to new assays and cell phenotypes. To overcome these limitations, we searched for an open software system that is specialized on HTS.

CellProfiler pipeline

Through a collaboration with researchers at the Broad Institute of Harvard and MIT, we developed a pipeline, or software workflow, to quantify the number of syncytia using the freely available image analysis software CellProfiler:

http://www.cellprofiler.org

Detailed protocol describing object type, exposure type and APR workflow are described in Paper I and is downloadable from:

http://www.cellprofiler.org/published_pipelines.shtml

Moreover, this workflow can be applied on any image formats and gives the user the freedom to choose any automated microscope while ensuring the use of the same parameters for cell and nuclei counting of GHOST(3).CCR5 and

GHOST(3).CXCR4 cells (Figure 20A and B). We thus developed an image-based automated assay that allows us to detect cell-cell fusion as well as HIV neutralization in one single assay. Although the APR assay is fine-tuned to detect GFP in GHOST(3) cells, it can easily be adapted to other cell lines.

The results are exported to an Excel sheet as well as a comma-delimited file (.csv). This gives the opportunity for TZMbl assay users to implement the TZMbl assay macro to calculate HIV infectivity [203]. In Paper I, we refer to our assay as an automated plaque reduction assay (APR assay), however, the assay can easily be used as an infectivity assay that measures both increase and decrease in syncytia. Thus, this assay has been used as an infectivity assay in Papers III and IV.

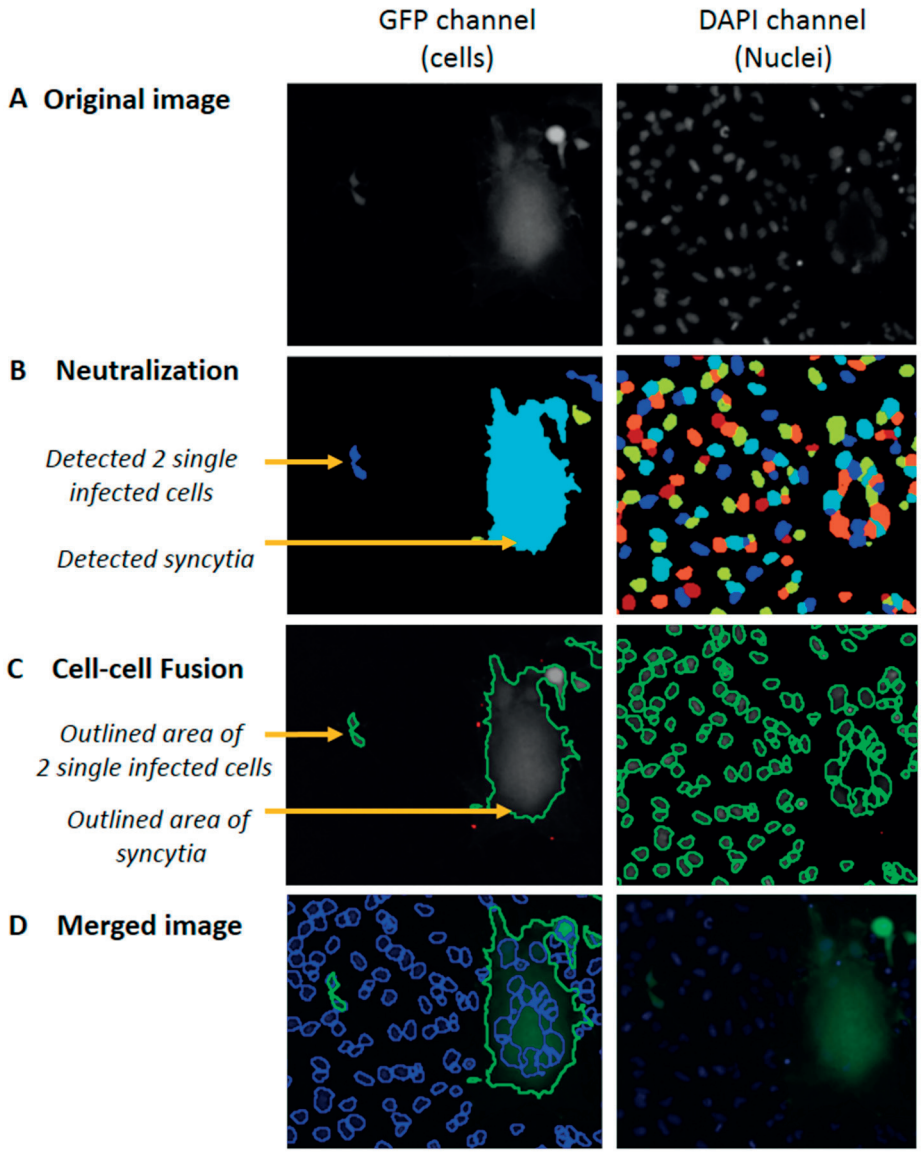

Figure 20.

An example of the microscopic image of infected cells (syncytia and single infected cells) in two channels, GFP and DAPI. (A) Original image acquired by the microscope (B) Image where neutralization is measured as the number of infected cells whereas (C) image where cell-cell fusion is measured as the area of each HIV infected cell. (D) Merged image of both nuclei and cells. The merged image shows that cell-cell fusion could also be detected by aggregated nuclei as they typically take on a ring-like structure.

The Automated plaque reduction assay compared to other assays

Our APR assay was conducted in the framework of NeutNet II, a network study between 13 laboratories around the world to compare the sensitivity of different neutralization assays [204]. The study included 72 virus-plasma combinations, including eight combinations of HIV-negative plasma. We utilized this material to compare our APR assay with the manual readout results in regard to sensitivity. As a second step we compared our assay with other assays in the framework of the NeutNet study II where several pseudo virus (PSV) based assays and virus infectivity based assays were compared along with one fusion assay. The TZMbl assay and the PMBC assay are the two most common assays today, with the TZMbl assay having recently been validated [103]. By comparing our APR assay with the PBMC assay, we could show that no statistical differences existed when considering either virus neutralization sensitivity or plasma neutralization capacity. When comparing the APR and the PSV assays, virus neutralization sensitivity was statistically similar for seven out of eight viruses. Overall, the sensitivity of our automatic PR assay was within the range of the other assays.

The overall conclusion from the NeutNet Phase II study, using polyclonal reagents, showed that sensitivities were dependent on both plasma and virus. No single assay was capable of detecting all neutralizing antibodies against HIV. It was thus recommended that multiple assays be used when evaluating vaccine-elicited neutralizing antibody responses.

Cell-cell fusion

Even though one of the major mechanisms contributing to HIV spread is cell-cell transmission [23], few studies have focused on whether bnAbs also act against HIV cell-cell spread and fusion *in vitro* [94]. It could be anticipated that an antibody with a broad range of effector functions, including the ability to both neutralize the virus and also prevent cell-cell fusion, would be effective in preventing HIV infection. Interestingly, only a subset of the studied bnAbs efficiently prevented HIV cell-to-cell transmission [94]. Thus it could be important to study the effect of newly developed bNAbs for their capacity to neutralization, as well as inhibition of cell-cell fusion. During the development of the APR assay we soon realized that we could utilize this assay to simultaneously measure the mean area of syncytia in each well and as a result detect the antibody capacity to inhibit cell-cell fusion.

The first fusion assays were based on counting nuclei, making them unsuitable for high-throughput studies [104]. The newer FRET or β-galactosidase based fusion assays utilize a donor and an acceptor cell line, with Env or HIV are constitutively expressed on the donor cell and where the acceptor cells are transfected with CD4 and coreceptors [61, 105-108]. Fusion is detected within hours, thus these assays measure

instant HIV cell-cell transmission rather than cell-cell transmission after HIV integration and newly produced gp120.

Here we added CellProfiler modules to not only count the number of syncytia (i.e. neutralization) but also to measure the mean syncytia area (i.e. HIV cell-cell transmission) (Figure 20C). As the measured fusion occurs *after* cell-free infection, where the virus gp120 is synthesized and assembled on the cell surface, we suggest that the APR assay most accurately mimics the natural course of cell-cell spread.

In GHOST(3) cells, the spread of HIV is a spread to adjacent cells occurs through cell-cell fusion resulting in syncytia and a larger cytoplasm due to the cell fusion. In the *IdentifyObject* module, the area of identified GFP object is measured, making it possible to distinguish between plaques (singly infected cells) with an area between 4-10 μm and syncytia with an area above 10μm (Figure 20C).

Antibody inhibition of HIV mediated cell-cell fusion

We developed an image-based automated assay that allowed the detection of antibodies capable of inhibiting cell-cell fusion, as well as HIV neutralization, in one single assay. By the use of the reagents and viruses provided through the NeutNet II study, we asked whether antibodies may affect cell-cell fusion and analyzed the antibody-mediated reduction of mean area in syncytia. By introducing a new nomenclature, inhibitory concentration for plaque area reduction (ICpar), we aimed to analyze the effect of antibodies in inhibiting HIV mediated cell-cell fusion. ICpar indicates the concentration at which the antibody causes a decrease in plaque area, e.g., ICpar50 = 1:25 means that a 50% reduction in plaque area is achieved by a 1:25 dilution of sera.

By the use of the APR assay we could show that the level of neutralization seems to be important for inhibition of virus-mediated cell-cell fusion. The decrease in plaque area was statistically significant only when neutralization reached IC90. The antibodies in our assay that neutralized the virus showed varied capacity to inhibit cell-cell fusion. Interestingly, in four reagent-virus combinations both decreased the number of syncytia as well as the area of the syncytia observed, and 11 combinations that reached IC50 neutralization showed a trend towards decreased plaque area, but could not be statistically confirmed. However, in other inhibitory reagent-virus combinations where neutralization was observed no reduction in mean plaque area was noted. Thus the ability of antibodies mediating neutralization and inhibition of cell-cell fusion does not always go hand in hand. Our findings confirmed earlier studies showing that elevated concentrations of antibody are necessary to inhibit fusion between infected and target cells [94]. This observation has been suggested to be due to higher levels of Env expressed on the surface of infected cells as compared to the viral envelope.

The ability of viruses to elicit cell-cell fusion measured by the area of syncytia

We next asked whether different viruses elicited cell-cell fusion with different potency by measuring the mean area of virus-induced syncytia. We detected that the size of the plaque area in relation to the different virus isolates yielded a characteristic size distribution regardless of virus titration. These characteristics did not seem to correlate to coreceptor use or subtype of HIV since both X4 and R5 viruses and subtype B and C viruses were found across the size distribution. The viruses could be divided into two groups yielding large or small plaques. Here the large plaques are the result of fusion of a higher number of cells than small plaques. The mechanisms behind differences between viruses in this regard are not known, but it has been suggested that virus ability to mediate cell-cell fusion, i.e. syncytia, depends on the strength of the interaction of viral envelope proteins with CD4 and coreceptors [205, 206].

Conclusions from the development of the automated plaque reduction assay

Automation offers many advantages: it is higher throughput, standardized, and inexpensive compared both to the manual PR assay and the classic virus infection assay performed in PBMC. Automated image analysis also increases the objectivity of the plaque counting demonstrated by the very low intra-well variation among triplicate wells as well as low background in the negative controls. Cell-cell fusion inhibition capacity of an antibody may play a role in HIV protective responses and may also be required for an effective HIV vaccine. This high-content assay may thus be used as a tool for evaluation of multiple antibody effector functions, by ways of quantifying the reduction in number of plaques and mean plaque area, respectively.

Galectin-gp120/ CD4 interactions

Galectin-1 has been shown to play an enhancing role in HIV infection and transmission by the recognition of glycans on gp120 and CD4 [175-177]. By promoting CD4-gp120 interaction and stabilization in a cross-linking manner, the galectin-1 enhanced the HIV infection. We set to study whether other galectins also could bind to gp120 and CD4 respectively, and what role they might play in HIV infection. For this purpose we adapted a fluorescent anisotropy assay to microscale.

Development of a Microscale fluorescent anisotropy assay

As a first step we set out to study the direct affinity between different galectins and gp120 and CD4 respectively. A fluorescent anisotropy (FA) assay previously established to detect interactions in solution is described above under Part II. However, it typically requires volumes of 100-200 microliter, making analysis of purified gp120 and CD4 expensive. Therefore, our first aim was to adapt the assay to

smaller volumes, i.e. into a microscale fluorescence anisotropy assay (μFA-assay). The Polar Star (BMG LabTech) fluorescence polarization microplate reader used is highly sensitive and permits use of 1536-well plates (Figure 21). However, these plates are usually used for high throughput screening in robotic systems. Here we wanted to use them manually to minimize the amount of glycoproteins consumed while maintaining reproducibility, sensitivity and ensuring no false positive/false negative results. The sample volume was reduced to 6μl. Besides careful pipetting, a key step to achieve high reproducibility was to centrifuge the plate after sample loading to bring sample to the bottom of the narrow wells and remove bubbles.

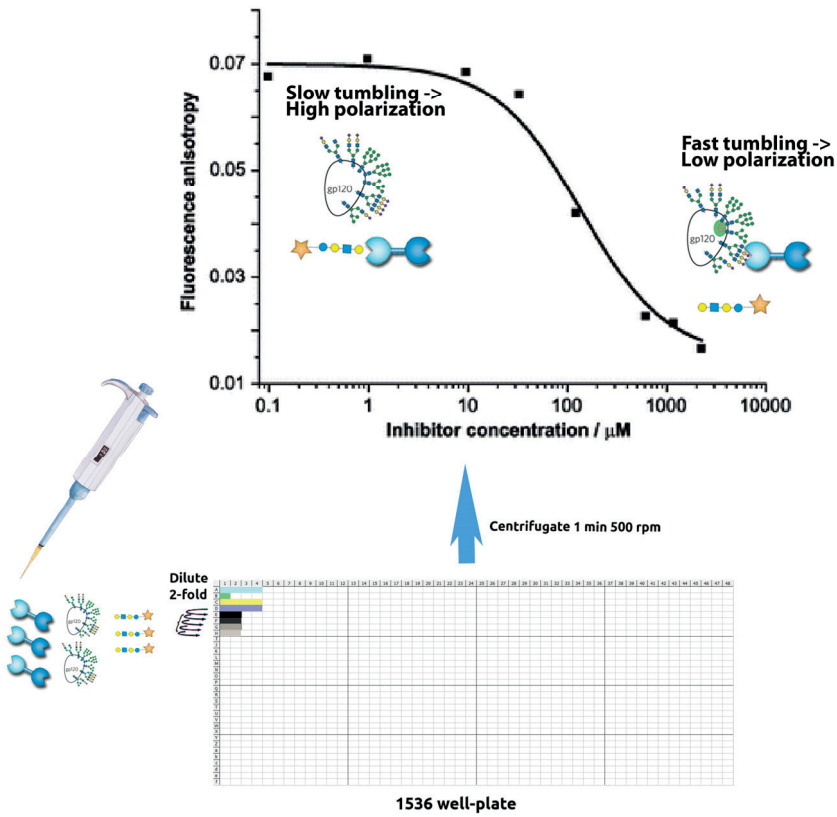

Figure 21.
Here we introduce a Microscale Fluorescence Anisotropy assay (μFA) for measuring interaction of galectins with limited amounts of glycoproteins in solution, allowing for the determination of the affinity between various galectins and gp120 or sCD4. A fluorescein-tagged saccharide (probe) binds the galectin with high affinity yielding high fluorescene anisotropy. As gp120 (here acting as an inhibitor) takes the place of the probe in the galectin-binding pocket, the result will be decreased anisotropy. From this, affinity of the inhibitor for the galectin can be calculated.

Galectins that bind to gp120 and CD4

By the use of the microscale anisotropy assay, we could determine the affinity of various galectins for gp120 and soluble CD4 (sCD4) (Figure 22). Recombinant gp120 derived from the reference HIV-1 strains with either X4 (IIIB) or R5 (BaL and SF162) coreceptor tropism were analyzed for binding to galectins (and galectin CRDs) 1, 2, 3, 4C, 4N, 8C, 8N, 8, 9C and 9N. Results showed that Galectin-8N and galectin-8 had the highest affinities among the tested galectins for all gp120 glycoproteins. Galectins-1, -3 and each CRD of galectin-9 also bound the three gp120, but weaker compared to galectin-8, whereas galectins-2, 4C and 4N did not interact with gp120. In the analysis of sCD4-galectin interactions we found that both N-CRD and C-CRD of galectin-8 bound sCD4, either by themselves or as part of intact galectin-8. Results also showed that sCD4 bound galectin-1 as well as galectin-9N and galectin-9C, but not galectin-2, -3, 4N and 4C.

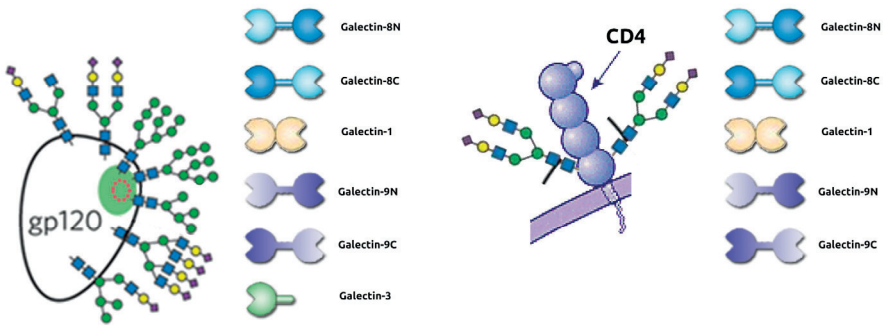

Figure 22.
Galectins and their subcomponents found to bind to (A) gp120 and CD4 (B).

Galectin-8 - HIV interactions

A key unexpected finding was that galectin-8N had the highest affinity for gp120 of all the galectins tested, with k_d < 1 μM, compared to for example galectin-1 that bound gp120 with k_d 1-2 μM. We therefor set out to study this interaction in more detail. Interestingly, we found that the gp120-galectin-8 interaction is due to initial binding of the N-CRD, as C-CRD did not bind unless N-CRD was both existent and functional. Galectin-8 also showed a preferential binding to α2-3 sialylated and 3'sulphated β-galactosides.

Development of virus binding assay and the detection of galectin-virion binding

In order to further study the interaction between HIV and galectins we developed an assay to screen binding of whole HIV virions to galectins immobilized onto beads (Figure 23). The results from these experiments revealed that virions of the the reference HIV-1 strains IIIB, BaL and SF162, showed similar binding to galectin-1 and galectin-8. The galectin-8N subunit also bound the virions but to lesser extent. Thus, using this assay we detected that gp120 binding established in μFA-assay also translated to whole virion binding.

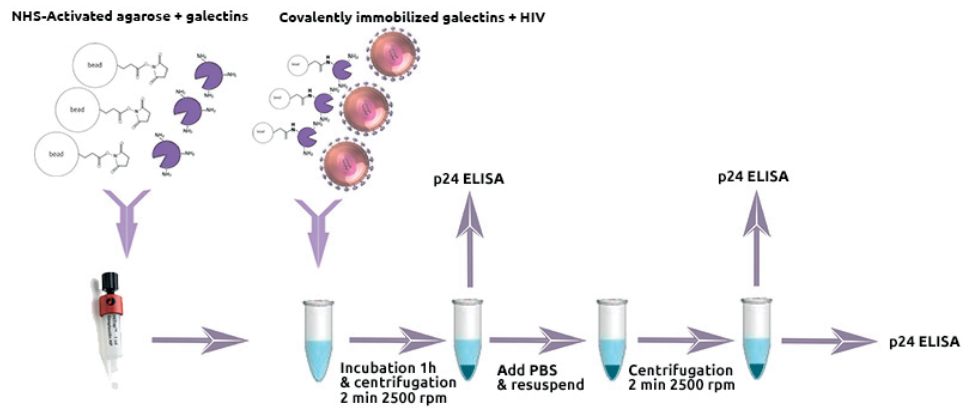

Figure 23.
HIV virion galectin binding assay. Galectins are coupled to a 1-ml NHS-activated Hi-Trap column as described [207] and in more detail in paper III. To measure virus-binding, bead slurry is mixed with HIV and incubated 1 h at 4 C end-over-end. After centrifugation for 2 min at 2500 rpm, supernatant 1 is replaced by fresh PBS, incubation and centrifugation repeated, and supernatant 2 recovered. Triton-X100 is added to bead slurry and the two supernatants, mixed end-over end for 1 h, and centrifuged, and all three constituents retrieved for p24 analysis. High % p24 associated with the washed beads indicates high virus binding of virions to the galectin.

Galectins-8 mediate enhancement of HIV infection

Next we asked what the functionality of galectins in HIV infections could be. This since results from both the gp120 affinity studies, using the μFA assay, as well as the virion binding assay had suggested that galectin-8 interacted with the virus. We set out to study the effect of galectin-8 on HIV infectivity using our newly developed APR assay, here adopted for the use as an HIV infectivity assay. We chose to incubate the GHOST-cells with galectin-8, galectin-8N or galectin-1, and subsequently infect these cells with reference HIV-1 strains, from which the gp120 originated. Indeed, galectin-8 was found to elevate the infectivity of HIV-1 BaL, by 3-fold at 5μM galectin-8 compared to control virus cultures without added galectin, which was more than that seen with galectin-1. Galectin-8N did not affect the HIV infection, suggesting that the HIV enhancement requires both the N-and C-terminal of galectin-8. Similar results were obtained when we included a panel of primary HIV-1 isolates representing R5, X4 and multitropic viruses. For most, but not all strains in the panel, galectin-8 enhanced infectivity to a larger extent than galectin-1 did.

Galectin-8 enhancement of HIV infection in relation to disease stages

To see if the impact of galectins on the infectivity of isolates differed depending on disease stage at isolation, we studied the effect of galectin-8 on the infectivity of several clinical isolates, sequentially obtained from patients in the chronic and AIDS phases. We here observed that galectin-8 preferentially enhanced infectivity of isolates obtained during the chronic phase, but much less or not all isolates from patients with severe immunodeficiency, i.e. low CD4 counts and AIDS. These findings suggest HIV circulating at the chronic phase benefit more from the interaction with galectin-8, than viruses emerging later, after AIDS onset. These results also suggest that galectin-8-binding carbohydrate motifs on Env are altered at severe immunodeficiency, adding to the knowledge on the evolution of Env glycosylation patterns related to HIV pathogenesis.

Discussion and Conclusions on the galectin-8-HIV interaction

As previous studies have shown that galectin-8N preferentially binds O-glycans, the N-CRD binding to gp120 could be through O-linked glycans. A recent publication identified an O-glycan at the conserved threonine residue T499 in gp120 [208]. Thus, is it plausible that the N-CRD of galectin-8 might bind to this site. One possibility is that galectin-8 enhances binding of virions to the host cell by binding with the N-CRD to gp120 and the C-CRD to CD4, similar as the cross-linking proposed for galectin-1 [175]. Our affinity studies suggest a scenario where the initial gp120 binding is via the N-CRD, and subsequently the C-CRD either comes to a correct confirmation or comes close enough for binding to bind CD4 (Figure 24A). However, since the T499 site was conserved in all isolates tested it cannot explain the differences in the galectin-8 enhancement effects seen between the isolates.

Other alternative mechanisms behind the observed galectin-8 mediated enhancement could be considered. Since the conserved glycosylated T499 is near the gp41 interaction site in the closed pre-fusion form of Env [209], binding of galectin-8 to that site may instead lock gp120 in an open conformation or stabilize the open confirmation by binding to gp120 and CD4 respectively (Figure 24B). The N-CRD might also bind to glycoconjugates in the virion, such as gangliosides [187] that recently were shown to be important in HIV-infection [210] (Figure 24C). As we incubated the galectins with the cells prior to HIV infection, we cannot exclude that the enhancement effect could be through some unknown signaling pathway that for instance increased the amount of CD4 receptors at the cell membrane (Figure 24D).

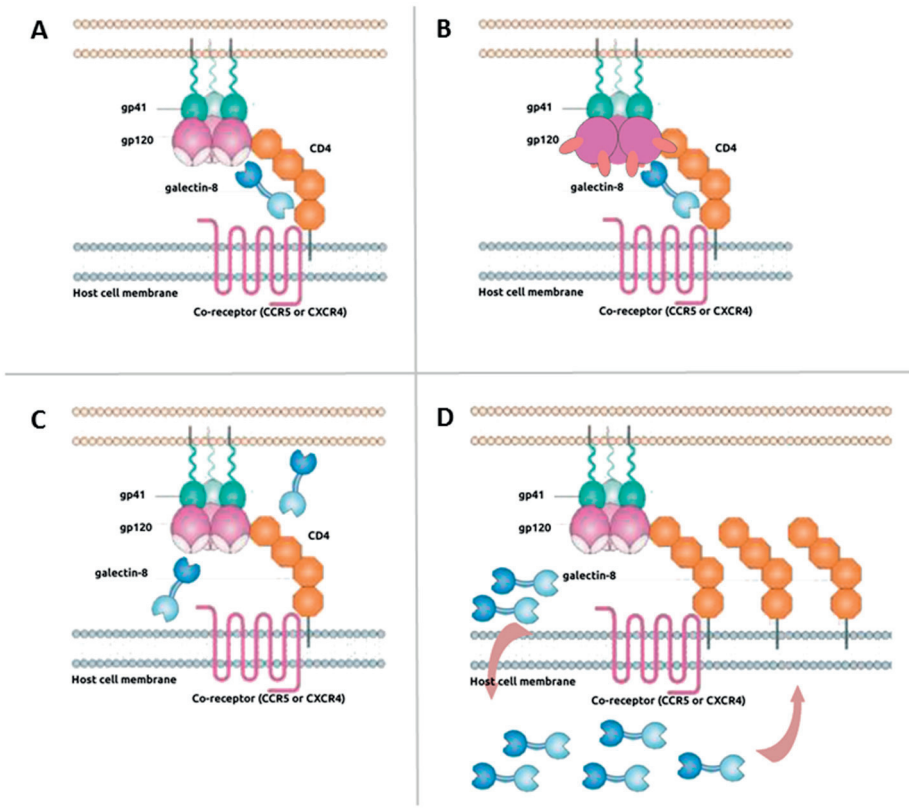

Figure 24.
Possible mechanims by which galectin-8 enhances HIV infection. Adapted from "The design of drugs for HIV and HCV", Erik De Clercq, Nature Reviews Drug Discovery, 2007, with persmission

Galectin-8 expression exist at high concentrations in the endometrium during the lutheal phase [189]. As the Langerhans cells and T cells are considered the primary cells for HIV transmission, also are located in the endometrium [211], this suggests that galectin-8 may play role in the in vivo HIV infection. Moreover, galectin-8 is

expressed in lymphatic endothelial cells (LEC) that is involved in the transport of immune cells to lymph nodes. This could indicate that galectin-8 is available and plays a role at the primary site of infection and throughout the disease.

As we show in our study, galectin-8 binds with high affinity to CD4. However, previous studies have also shown galectin-8 binding to LFA-1. LFA-1 is composed of αL and β2 subunits, where ICAM-1 is the major ligand [194, 195]. It is ubiquitously expressed on most leukocytes and endothelial cells where it is upregulated during inflammation. Wild-type LFA-1 enhances the initial processes of HIV infection, as well as the subsequent replication and transmission from cell to cell [212]. Galectin-8 might thus bind to LFA-1, and subsequently enhance HIV infection through LFA-1/ICAM-3 binding. Another glycan-dependent cellular receptor for HIV that binds to galectin-8 is α4β7 integrin, also expressed at high levels in CD4 T-cells present in vaginal and anal mucosa. Thus, there are numerous cellular receptors that galectin-8 may bind to and potentially use for modulation of HIV infection, which makes studies on the galectin-8-HIV interaction well worth pursuing.

Galectin-8 has also a pro-inflammatory role and induces several pro-inflammatory molecules in endothelial cells, T cells and B cells. Apart from playing a role in direct HIV infection/transmission as described above, this suggests that galectin-8 might enhance HIV infection by attracting more T cells to the site of infection subsequently helping the spread of virus. Interestingly, galectin-8 is also involved in apoptosis and autophagy, where it has been shown to induce apoptosis in Jurkat T cells and in a subpopulation of T cells among activated PBMC cells [198]. It has also a role in targeting damaged vacuoles, endosomes as well as lysosomes for autophagy by binding host glycans exposed on damaged vesicles. Galectin-8 recruits NDP52 in response to invading bacteria by triggering ubiquitin recruitment [172]. Galectin-8 may thus affect the HIV pathogenesis by other mechanisms equally important to binding, enhancing or spreading the HIV infection.

Future directions

The human sera of HIV infected individuals, including elite controllers, are actively screened for bnAbs that could provide templates for HIV vaccines. Potent neutralization as well as inhibition of cell-cell fusion, are two important effector functions of HIV-specific antibodies. In order to detect and evaluate such antibodies, standardized, reproducible as well as high-content, high-throughput assays are needed. Our APR assay is high-content as it simultaneously measures neutralization and inhibition of cell-cell fusion. With regard to analysis tools, the CellProfiler software offers powerful capacity in analyzing images in a high-throughput manner. However, the integration of image acquisition with a simultaneous image analysis in a high-throughput microscope would provide the possibility of screening thousands instead of hundreds of sera. On another note, our assay simultaneously measures cell-cell fusion by measuring the area of the resulting nucleus aggregate and surrounding cytoplasm. In addition, our developed CellProfiler pipeline has the potential to insert a module that calculates aggregated nuclei that would provide additional strength to the assay (Figure 20D).

It would be interesting to extend studies on bnAbs to include analyses of their effects on cell-cell fusion using our assay. This since no studies so far have analyzed cell-cell fusion inhibition in direct parallel with neutralization by the newly discovered bnAbs.

The role of galectin-8 in HIV enhancement has just been discovered and carries a large number of questions and roads for future research, including: Will we be able to produce galectin-8 inhibitors and will they block the infection without introducing cell-toxicity? Does the galectin-8 play a role *in vivo* and is it induced upon HIV infection? What mechanism lies behind the galectin-8 mediated enhanced HIV infection and what glycans on the gp120 and CD4 does the galectin-8 actually bind to? Why does the galectin-8 enhancement correlate with the CD4 count at the time of virus isolation, and does galectin-8 play an even stronger role during the acute infection? It is tempting to speculate that studies on galectin-8 and galectin-1 interactions with HIV could spur the development of new therapeutic or prophylactic interventions.

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References

1. Barre-Sinoussi, F., A.L. Ross, and J.F. Delfraissy, *Past, present and future: 30 years of HIV research.* Nat Rev Microbiol, 2013. **11**(12): p. 877-83.
2. Gottlieb, M.S., et al., *Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency.* N Engl J Med, 1981. **305**(24): p. 1425-31.
3. Masur, H., et al., *An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction.* N Engl J Med, 1981. **305**(24): p. 1431-8.
4. Borkovic, S.P. and R.A. Schwartz, *Kaposi's sarcoma presenting in the homosexual man - - a new and striking phenomenon!* Ariz Med, 1981. **38**(12): p. 902-4.
5. Centers for Disease, C., *Possible transfusion-associated acquired immune deficiency syndrome (AIDS) - California.* MMWR Morb Mortal Wkly Rep, 1982. **31**(48): p. 652-4.
6. 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.
7. Peeters, M., et al., *Isolation and Partial Characterization of an Hiv-Related Virus Occurring Naturally in Chimpanzees in Gabon.* Aids, 1989. **3**(10): p. 625-630.
8. Maartens, G., C. Celum, and S.R. Lewin, *HIV infection: epidemiology, pathogenesis, treatment, and prevention.* Lancet, 2014. **384**(9939): p. 258-71.
9. http://www.unaids.org/.
10. Coffin, J.M., S.H. Hughes, and H.E. Varmus, *The Interactions of Retroviruses and their Hosts*, in *Retroviruses*, J.M. Coffin, S.H. Hughes, and H.E. Varmus, Editors. 1997: Cold Spring Harbor (NY).
11. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. **67**: p. 1-25.
12. Land, A. and I. Braakman, *Folding of the human immunodeficiency virus type 1 envelope glycoprotein in the endoplasmic reticulum.* Biochimie, 2001. **83**(8): p. 783-90.
13. Starcich, B.R., et al., *Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS.* Cell, 1986. **45**(5): p. 637-48.
14. Shaw, G.M. and E. Hunter, *HIV transmission.* Cold Spring Harb Perspect Med, 2012. **2**(11).
15. Dalgleish, A.G., et al., *The Cd4 (T4) Antigen Is an Essential Component of the Receptor for the Aids Retrovirus.* Nature, 1984. **312**(5996): p. 763-767.

16. Kwong, P.D., et al., *Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody.* Nature, 1998. **393**(6686): p. 648-59.
17. Liu, J., et al., *Molecular architecture of native HIV-1 gp120 trimers.* Nature, 2008. **455**(7209): p. 109-13.
18. Bjorndal, A., et al., *Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype.* J Virol, 1997. **71**(10): p. 7478-87.
19. Karlsson, I., et al., *HIV biological variability unveiled: frequent isolations and chimeric receptors reveal unprecedented variation of coreceptor use.* AIDS, 2003. **17**(18): p. 2561-9.
20. Earl, L.A., J.D. Lifson, and S. Subramaniam, *Catching HIV 'in the act' with 3D electron microscopy.* Trends Microbiol, 2013. **21**(8): p. 397-404.
21. Fiorentini, S., et al., *Functions of the HIV-1 matrix protein p17.* New Microbiol, 2006. **29**(1): p. 1-10.
22. Sattentau, Q., *Avoiding the void: cell-to-cell spread of human viruses.* Nature Reviews Microbiology, 2008. **6**(11): p. 815-826.
23. Sattentau, Q., *Avoiding the void: cell-to-cell spread of human viruses.* Nat Rev Microbiol, 2008. **6**(11): p. 815-26.
24. Diaz-Aguilar, B., et al., *Significant differences in cell-cell fusion and viral entry between strains revealed by scanning mutagenesis of the C-heptad repeat of HIV gp41.* Biochemistry, 2013. **52**(20): p. 3552-63.
25. Balzarini, J., *Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy.* Nat Rev Microbiol, 2007. **5**(8): p. 583-97.
26. Geskus, R.B., et al., *The HIV RNA setpoint theory revisited.* Retrovirology, 2007. **4**: p. 65.
27. Chomont, N., et al., *HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation.* Nat Med, 2009. **15**(8): p. 893-900.
28. Markowitz, M., et al., *Infection with multidrug resistant, dual-tropic HIV-1 and rapid progression to AIDS: a case report.* Lancet, 2005. **365**(9464): p. 1031-8.
29. Buchbinder, S.P., et al., *Long-term HIV-1 infection without immunologic progression.* AIDS, 1994. **8**(8): p. 1123-8.
30. Deeks, S.G. and B.D. Walker, *Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy.* Immunity, 2007. **27**(3): p. 406-16.
31. Fischl, M.A., et al., *The Efficacy of Azidothymidine (Azt) in the Treatment of Patients with Aids and Aids-Related Complex - a Double-Blind, Placebo-Controlled Trial.* New England Journal of Medicine, 1987. **317**(4): p. 185-191.
32. Mitsuya, H., et al., *3'-Azido-3'-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus in vitro.* Proc Natl Acad Sci U S A, 1985. **82**(20): p. 7096-100.
33. Hammer, S.M., et al., *A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team.* N Engl J Med, 1997. **337**(11): p. 725-33.

34. Palella, F.J., Jr., et al., *Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators.* N Engl J Med, 1998. **338**(13): p. 853-60.
35. Connor, E.M., et al., *Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group.* N Engl J Med, 1994. **331**(18): p. 1173-80.
36. Thompson, M.A., et al., *Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel.* JAMA, 2012. **308**(4): p. 387-402.
37. Finzi, D., et al., *Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy.* Nat Med, 1999. **5**(5): p. 512-7.
38. Lewin, S.R. and C. Rouzioux, *HIV cure and eradication: how will we get from the laboratory to effective clinical trials?* AIDS, 2011. **25**(7): p. 885-97.
39. Garred, P., et al., *Susceptibility to HIV infection and progression of AIDS in relation to variant alleles of mannose-binding lectin.* Lancet, 1997. **349**(9047): p. 236-40.
40. Pastinen, T., et al., *Contribution of the CCR5 and MBL genes to susceptibility to HIV type 1 infection in the Finnish population.* AIDS Res Hum Retroviruses, 1998. **14**(8): p. 695-8.
41. Sullivan, B.L., et al., *Susceptibility of HIV-1 plasma virus to complement-mediated lysis. Evidence for a role in clearance of virus in vivo.* J Immunol, 1996. **157**(4): p. 1791-8.
42. Berger, E.A., *HIV entry and tropism: the chemokine receptor connection.* AIDS, 1997. **11 Suppl A**: p. S3-16.
43. Lennert, K., E. Kaiserling, and H.K. Muller-Hermelink, *Letter: T-associated plasma-cells.* Lancet, 1975. **1**(7914): p. 1031-2.
44. Siegal, F.P., et al., *The nature of the principal type 1 interferon-producing cells in human blood.* Science, 1999. **284**(5421): p. 1835-7.
45. Soumelis, V., et al., *Depletion of circulating natural type 1 interferon-producing cells in HIV-infected AIDS patients.* Blood, 2001. **98**(4): p. 906-12.
46. Germain, R.N., *An innately interesting decade of research in immunology.* Nat Med, 2004. **10**(12): p. 1307-20.
47. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
48. Reid, R.R., et al., *Functional activity of natural antibody is altered in Cr2-deficient mice.* J Immunol, 2002. **169**(10): p. 5433-40.
49. Pulendran, B. and R. Ahmed, *Translating innate immunity into immunological memory: implications for vaccine development.* Cell, 2006. **124**(4): p. 849-63.
50. Huber, M. and A. Trkola, *Humoral immunity to HIV-1: neutralization and beyond.* J Intern Med, 2007. **262**(1): p. 5-25.
51. Borrow, P., et al., *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection.* J Virol, 1994. **68**(9): p. 6103-10.
52. Goonetilleke, N., et al., *The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1253-72.

53. Munier, C.M., et al., *The role of T cell immunity in HIV-1 infection.* Curr Opin Virol, 2013. **3**(4): p. 438-46.
54. Tang, J., et al., *Human leukocyte antigen variants B*44 and B*57 are consistently favorable during two distinct phases of primary HIV-1 infection in sub-Saharan Africans with several viral subtypes.* J Virol, 2011. **85**(17): p. 8894-902.
55. Pereyra, F., et al., *Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy.* J Infect Dis, 2008. **197**(4): p. 563-71.
56. Mouquet, H., *Antibody B cell responses in HIV-1 infection.* Trends Immunol, 2014. **35**(11): p. 549-61.
57. Overbaugh, J. and L. Morris, *The Antibody Response against HIV-1.* Cold Spring Harb Perspect Med, 2012. **2**(1): p. a007039.
58. Albert, J., et al., *Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera.* AIDS, 1990. **4**(2): p. 107-12.
59. Yates, N.L., et al., *HIV-1 gp41 envelope IgA is frequently elicited after transmission but has an initial short response half-life.* Mucosal Immunol, 2013. **6**(4): p. 692-703.
60. Purtscher, M., et al., *A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1.* AIDS Res Hum Retroviruses, 1994. **10**(12): p. 1651-8.
61. Yee, M., et al., *Inhibition of HIV-1 Env-Mediated Cell-Cell Fusion by Lectins, Peptide T-20, and Neutralizing Antibodies.* Open Virol J, 2011. **5**: p. 44-51.
62. Huerta, L., et al., *Human immunodeficiency virus 1 (HIV-1) envelope-dependent cell-cell fusion modulation by HIV-positive sera is related to disease progression.* Journal of General Virology, 2005. **86**: p. 1961-1966.
63. Burton, D.R., *Antibodies, viruses and vaccines.* Nat Rev Immunol, 2002. **2**(9): p. 706-13.
64. Devito, C., et al., *Mucosal and plasma IgA from HIV-exposed seronegative individuals neutralize a primary HIV-1 isolate.* AIDS, 2000. **14**(13): p. 1917-20.
65. Shingai, M., et al., *Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques.* J Exp Med, 2014. **211**(10): p. 2061-74.
66. Chun, T.W., et al., *Broadly neutralizing antibodies suppress HIV in the persistent viral reservoir.* Proc Natl Acad Sci U S A, 2014. **111**(36): p. 13151-6.
67. van Gils, M.J. and R.W. Sanders, *Broadly neutralizing antibodies against HIV-1: Templates for a vaccine.* Virology, 2013. **435**(1): p. 46-56.
68. Gray, G., S. Buchbinder, and A. Duerr, *Overview of STEP and Phambili trial results: two phase IIb test-of-concept studies investigating the efficacy of MRK adenovirus type 5 gag/pol/nef subtype B HIV vaccine.* Curr Opin HIV AIDS, 2010. **5**(5): p. 357-61.
69. Kwong, P.D., J.R. Mascola, and G.J. Nabel, *The changing face of HIV vaccine research.* J Int AIDS Soc, 2012. **15**(2): p. 17407.
70. Rerks-Ngarm, S., et al., *Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand.* N Engl J Med, 2009. **361**(23): p. 2209-20.
71. Mayer, K.H., et al., *Safety and tolerability of tenofovir vaginal gel in abstinent and sexually active HIV-infected and uninfected women.* AIDS, 2006. **20**(4): p. 543-51.

72. Simek, M.D., et al., *Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm.* J Virol, 2009. **83**(14): p. 7337-48.
73. Walker, L.M., et al., *Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target.* Science, 2009. **326**(5950): p. 285-9.
74. Mascola, J.R. and D.C. Montefiori, *The Role of Antibodies in HIV Vaccines.* Annual Review of Immunology, Vol 28, 2010. **28**: p. 413-444.
75. Klein, F., et al., *Antibodies in HIV-1 vaccine development and therapy.* Science, 2013. **341**(6151): p. 1199-204.
76. West, A.P., Jr., et al., *Structural insights on the role of antibodies in HIV-1 vaccine and therapy.* Cell, 2014. **156**(4): p. 633-48.
77. Mouquet, H., et al., *Memory B cell antibodies to HIV-1 gp140 cloned from individuals infected with clade A and B viruses.* PLoS One, 2011. **6**(9): p. e24078.
78. Scheid, J.F., et al., *Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals.* Nature, 2009. **458**(7238): p. 636-40.
79. Liao, H.X., et al., *Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus.* Nature, 2013. **496**(7446): p. 469-76.
80. Sok, D., et al., *The effects of somatic hypermutation on neutralization and binding in the PGT121 family of broadly neutralizing HIV antibodies.* PLoS Pathog, 2013. **9**(11): p. e1003754.
81. Sok, D., et al., *Recombinant HIV envelope trimer selects for quaternary-dependent antibodies targeting the trimer apex.* Proc Natl Acad Sci U S A, 2014. **111**(49): p. 17624-9.
82. Mouquet, H. and M.C. Nussenzweig, *Polyreactive antibodies in adaptive immune responses to viruses.* Cell Mol Life Sci, 2012. **69**(9): p. 1435-45.
83. Kwong, P.D. and J.R. Mascola, *Human Antibodies that Neutralize HIV-1: Identification, Structures, and B Cell Ontogenies.* Immunity, 2012. **37**(3): p. 412-425.
84. Barouch, D.H., et al., *Therapeutic efficacy of potent neutralizing HIV-1-specific monoclonal antibodies in SHIV-infected rhesus monkeys.* Nature, 2013. **503**(7475): p. 224-8.
85. Pantophlet, R. and D.R. Burton, *GP120: Target for neutralizing HIV-1 antibodies.* Annual Review of Immunology, 2006. **24**: p. 739-769.
86. Julien, J.P., P.S. Lee, and I.A. Wilson, *Structural insights into key sites of vulnerability on HIV-1 Env and influenza HA.* Immunol Rev, 2012. **250**(1): p. 180-98.
87. Zolla-Pazner, S. and T. Cardozo, *Structure-function relationships of HIV-1 envelope sequence-variable regions refocus vaccine design.* Nat Rev Immunol, 2010. **10**(7): p. 527-35.
88. Kong, R., et al., *Improving Neutralization Potency and Breadth by Combining Broadly Reactive HIV-1 Antibodies Targeting Major Neutralization Epitopes.* J Virol, 2015. **89**(5): p. 2659-71.
89. Hansen, J.E., et al., *Broadly neutralizing antibodies targeted to mucin-type carbohydrate epitopes of human immunodeficiency virus.* J Virol, 1991. **65**(12): p. 6461-7.
90. Hansen, J.E., et al., *Inhibition of human immunodeficiency virus (HIV) infection in vitro by anticarbohydrate monoclonal antibodies: peripheral glycosylation of HIV envelope*

glycoprotein gp120 may be a target for virus neutralization. J Virol, 1990. **64**(6): p. 2833-40.
91. Arendrup, M., et al., *Antibody to histo-blood group A antigen neutralizes HIV produced by lymphocytes from blood group A donors but not from blood group B or O donors.* AIDS, 1991. **5**(4): p. 441-4.
92. Munro, J.B., et al., *Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions.* Science, 2014. **346**(6210): p. 759-63.
93. Munro, J.B. and W. Mothes, *Structure and dynamics of the native HIV-1 Env trimer.* J Virol, 2015.
94. Malbec, M., et al., *Broadly neutralizing antibodies that inhibit HIV-1 cell to cell transmission.* J Exp Med, 2013. **210**(13): p. 2813-21.
95. Kim, J.H., J.L. Excler, and N.L. Michael, *Lessons from the RV144 Thai phase III HIV-1 vaccine trial and the search for correlates of protection.* Annu Rev Med, 2015. **66**: p. 423-37.
96. Dhillon, A.K., et al., *Dissecting the neutralizing antibody specificities of broadly neutralizing sera from human immunodeficiency virus type 1-infected donors.* J Virol, 2007. **81**(12): p. 6548-62.
97. Doria-Rose, N.A., et al., *Frequency and phenotype of human immunodeficiency virus envelope-specific B cells from patients with broadly cross-neutralizing antibodies.* J Virol, 2009. **83**(1): p. 188-99.
98. Li, Y., et al., *Broad HIV-1 neutralization mediated by CD4-binding site antibodies.* Nat Med, 2007. **13**(9): p. 1032-4.
99. Sather, D.N., et al., *Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection.* J Virol, 2009. **83**(2): p. 757-69.
100. Stamatatos, L., et al., *Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine?* Nat Med, 2009. **15**(8): p. 866-70.
101. Walker, L.M., et al., *Broad neutralization coverage of HIV by multiple highly potent antibodies.* Nature, 2011. **477**(7365): p. 466-70.
102. Kwong, P.D., J.R. Mascola, and G.J. Nabel, *Rational design of vaccines to elicit broadly neutralizing antibodies to HIV-1.* Cold Spring Harb Perspect Med, 2011. **1**(1): p. a007278.
103. Sarzotti-Kelsoe, M., et al., *Optimization and validation of the TZM-bl assay for standardized assessments of neutralizing antibodies against HIV-1.* Journal of Immunological Methods, 2014. **409**: p. 131-146.
104. Shinn-Thomas, J.H., V.L. Scranton, and W.A. Mohler, *Quantitative assays for cell fusion.* Methods Mol Biol, 2008. **475**: p. 347-61.
105. Bradley, J., et al., *Development and automation of a 384-well cell fusion assay to identify inhibitors of CCR5/CD4-mediated HIV virus entry.* Journal of Biomolecular Screening, 2004. **9**(6): p. 516-524.
106. Broder, C.C., et al., *The block to HIV-1 envelope glycoprotein-mediated membrane fusion in animal cells expressing human CD4 can be overcome by a human cell component(s).* Virology, 1993. **193**(1): p. 483-91.

107. Kramer, S., et al., *Imaging-based assay for identification and characterization of inhibitors of CXCR4-tropic HIV-1 envelope-dependent cell-cell fusion.* J Biomol Screen, 2011. **16**(6): p. 668-75.
108. Lusso, P., et al., *Cryptic nature of a conserved, CD4-inducible V3 loop neutralization epitope in the native envelope glycoprotein oligomer of CCR5-restricted, but not CXCR4-using, primary human immunodeficiency virus type 1 strains.* J Virol, 2005. **79**(11): p. 6957-68.
109. Polonis, V.R., et al., *Recent advances in the characterization of HIV-1 neutralization assays for standardized evaluation of the antibody response to infection and vaccination.* Virology, 2008. **375**(2): p. 315-320.
110. Fenyo, E.M., et al., *International Network for Comparison of HIV Neutralization Assays: The NeutNet Report.* Plos One, 2009. **4**(2).
111. Scarlatti, G., et al., *Standardization of HIV Neutralization Assays for Use in Vaccine Research and Clinical Trials: Results from the NeutNet Working Group.* Aids Research and Human Retroviruses, 2008. **24**: p. 44-44.
112. Scarlatti, G., et al., *Comparison of HIV neutralization assays for use in vaccine research and clinical trials, phase II: results from the NeutNet working group.* Retrovirology, 2009. **6**.
113. Fenyo, E.M., et al., *International network for comparison of HIV neutralization assays: the NeutNet report.* PLoS One, 2009. **4**(2): p. e4505.
114. Haney, S.A., et al., *High-content screening moves to the front of the line.* Drug Discov Today, 2006. **11**(19-20): p. 889-94.
115. Pepperkok, R. and J. Ellenberg, *High-throughput fluorescence microscopy for systems biology.* Nat Rev Mol Cell Biol, 2006. **7**(9): p. 690-6.
116. Singh, S., A.E. Carpenter, and A. Genovesio, *Increasing the Content of High-Content Screening: An Overview.* Journal of Biomolecular Screening, 2014. **19**(5): p. 640-650.
117. Carpenter, A.E., L. Kamentsky, and K.W. Eliceiri, *A call for bioimaging software usability.* Nat Methods, 2012. **9**(7): p. 666-70.
118. Carpenter, A.E., et al., *CellProfiler: image analysis software for identifying and quantifying cell phenotypes.* Genome Biol, 2006. **7**(10): p. R100.
119. Bray, M.A., M.S. Vokes, and A.E. Carpenter, *Using CellProfiler for Automatic Identification and Measurement of Biological Objects in Images.* Curr Protoc Mol Biol, 2015. **109**: p. 14 17 1-14 17 13.
120. Singh, S., et al., *Pipeline for illumination correction of images for high-throughput microscopy.* J Microsc, 2014. **256**(3): p. 231-6.
121. Julenius, K., et al., *Prediction, conservation analysis, and structural characterization of mammalian mucin-type O-glycosylation sites.* Glycobiology, 2005. **15**(2): p. 153-64.
122. Zhu, X., et al., *Mass spectrometric characterization of the glycosylation pattern of HIV-gp120 expressed in CHO cells.* Biochemistry, 2000. **39**(37): p. 11194-204.
123. Allan, J.S., et al., *Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III.* Science, 1985. **228**(4703): p. 1091-4.
124. Bernstein, H.B., et al., *Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides.* J Virol, 1994. **68**(1): p. 463-8.
125. Korber, B., et al., *Evolutionary and immunological implications of contemporary HIV-1 variation.* Br Med Bull, 2001. **58**: p. 19-42.

126. Zhang, M., et al., *Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin.* Glycobiology, 2004. **14**(12): p. 1229-1246.
127. Go, E.P., et al., *Glycosylation site-specific analysis of HIV envelope proteins (JR-FL and CON-S) reveals major differences in glycosylation site occupancy, glycoform profiles, and antigenic epitopes' accessibility.* J Proteome Res, 2008. **7**(4): p. 1660-74.
128. Irungu, J., et al., *Comparison of HPLC/ESI-FTICR MS versus MALDI-TOF/TOF MS for glycopeptide analysis of a highly glycosylated HIV envelope glycoprotein.* J Am Soc Mass Spectrom, 2008. **19**(8): p. 1209-20.
129. Borggren, M., et al., *Increased sensitivity to broadly neutralizing antibodies of end-stage disease R5 HIV-1 correlates with evolution in Env glycosylation and charge.* PLoS One, 2011. **6**(6): p. e20135.
130. van Gils, M.J., et al., *Evolution of human immunodeficiency virus type 1 in a patient with cross-reactive neutralizing activity in serum.* J Virol, 2011. **85**(16): p. 8443-8.
131. Horiya, S., I.S. MacPherson, and I.J. Krauss, *Recent strategies targeting HIV glycans in vaccine design.* Nat Chem Biol, 2014. **10**(12): p. 990-9.
132. Go, E.P., et al., *Characterization of host-cell line specific glycosylation profiles of early transmitted/founder HIV-1 gp120 envelope proteins.* J Proteome Res, 2013. **12**(3): p. 1223-34.
133. Krishnamoorthy, L., et al., *HIV-1 and microvesicles from T cells share a common glycome, arguing for a common origin.* Nat Chem Biol, 2009. **5**(4): p. 244-50.
134. Hansen, J.E., et al., *Sensitivity of HIV-1 to neutralization by antibodies against O-linked carbohydrate epitopes despite deletion of O-glycosylation signals in the V3 loop.* Arch Virol, 1996. **141**(2): p. 291-300.
135. Kong, L., I.A. Wilson, and P.D. Kwong, *Crystal structure of a fully glycosylated HIV-1 gp120 core reveals a stabilizing role for the glycan at Asn262.* Proteins, 2015. **83**(3): p. 590-6.
136. Raska, M. and J. Novak, *Involvement of envelope-glycoprotein glycans in HIV-1 biology and infection.* Arch Immunol Ther Exp (Warsz), 2010. **58**(3): p. 191-208.
137. Ward, A.B. and I.A. Wilson, *Insights into the trimeric HIV-1 envelope glycoprotein structure.* Trends Biochem Sci, 2015. **40**(2): p. 101-107.
138. Barondes, S.H., et al., *Galectins. Structure and function of a large family of animal lectins.* J Biol Chem, 1994. **269**(33): p. 20807-10.
139. Leffler, H., et al., *Introduction to galectins.* Glycoconj J, 2004. **19**(7-9): p. 433-40.
140. Bidon, N., et al., *Galectin-8: a complex sub-family of galectins (Review).* Int J Mol Med, 2001. **8**(3): p. 245-50.
141. Gray, C.A., et al., *Galectin-15 in ovine uteroplacental tissues.* Reproduction, 2005. **130**(2): p. 231-40.
142. Hirabayashi, J., et al., *Oligosaccharide specificity of galectins: a search by frontal affinity chromatography.* Biochim Biophys Acta, 2002. **1572**(2-3): p. 232-54.
143. Yang, R.Y., G.A. Rabinovich, and F.T. Liu, *Galectins: structure, function and therapeutic potential.* Expert Rev Mol Med, 2008. **10**: p. e17.
144. Rabinovich, G.A. and M.A. Toscano, *Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation.* Nat Rev Immunol, 2009. **9**(5): p. 338-52.

145. 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.
146. Chen, H.Y., et al., *Galectins as bacterial sensors in the host innate response.* Curr Opin Microbiol, 2014. **17**: p. 75-81.
147. Garner, O.B. and L.G. Baum, *Galectin-glycan lattices regulate cell-surface glycoprotein organization and signalling.* Biochem Soc Trans, 2008. **36**(Pt 6): p. 1472-7.
148. Vasta, G.R., et al., *Galectins as self/non-self recognition receptors in innate and adaptive immunity: an unresolved paradox.* Front Immunol, 2012. **3**: p. 199.
149. Wang, J.L., et al., *Nucleocytoplasmic lectins.* Biochim Biophys Acta, 2004. **1673**(1-2): p. 75-93.
150. Liu, F.T., R.J. Patterson, and J.L. Wang, *Intracellular functions of galectins.* Biochim Biophys Acta, 2002. **1572**(2-3): p. 263-73.
151. Liu, F.T. and G.A. Rabinovich, *Galectins: regulators of acute and chronic inflammation.* Ann N Y Acad Sci, 2010. **1183**: p. 158-82.
152. Elola, M.T., et al., *Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival.* Cell Mol Life Sci, 2007. **64**(13): p. 1679-700.
153. Toscano, M.A., et al., *Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death.* Nat Immunol, 2007. **8**(8): p. 825-34.
154. Cedeno-Laurent, F. and C.J. Dimitroff, *Galectins and their ligands: negative regulators of anti-tumor immunity.* Glycoconj J, 2012. **29**(8-9): p. 619-25.
155. Liu, F.T., R.Y. Yang, and D.K. Hsu, *Galectins in acute and chronic inflammation.* Ann N Y Acad Sci, 2012. **1253**: p. 80-91.
156. Toscano, M.A., et al., *Dissecting the pathophysiologic role of endogenous lectins: glycan-binding proteins with cytokine-like activity?* Cytokine Growth Factor Rev, 2007. **18**(1-2): p. 57-71.
157. Tribulatti, M.V., et al., *Redundant and antagonistic functions of galectin-1, -3, and -8 in the elicitation of T cell responses.* J Immunol, 2012. **188**(7): p. 2991-9.
158. Gauthier, L., et al., *Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering.* Proc Natl Acad Sci U S A, 2002. **99**(20): p. 13014-9.
159. Rossi, B., et al., *Clustering of pre-B cell integrins induces galectin-1-dependent pre-B cell receptor relocalization and activation.* J Immunol, 2006. **177**(2): p. 796-803.
160. Blaser, C., et al., *Beta-galactoside-binding protein secreted by activated T cells inhibits antigen-induced proliferation of T cells.* Eur J Immunol, 1998. **28**(8): p. 2311-9.
161. Ilarregui, J.M., et al., *The coming of age of galectins as immunomodulatory agents: impact of these carbohydrate binding proteins in T cell physiology and chronic inflammatory disorders.* Ann Rheum Dis, 2005. **64 Suppl 4**: p. iv96-103.
162. Pace, K.E., et al., *CD7 delivers a pro-apoptotic signal during galectin-1-induced T cell death.* J Immunol, 2000. **165**(5): p. 2331-4.
163. Perillo, N.L., et al., *Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes.* J Exp Med, 1997. **185**(10): p. 1851-8.
164. Rabinovich, G.A., et al., *Activated rat macrophages produce a galectin-1-like protein that induces apoptosis of T cells: biochemical and functional characterization.* J Immunol, 1998. **160**(10): p. 4831-40.

165. Blois, S.M., et al., *A pivotal role for galectin-1 in fetomaternal tolerance.* Nat Med, 2007. **13**(12): p. 1450-7.
166. Bonzi, J., et al., *Pre-B cell receptor binding to galectin-1 modifies galectin-1/carbohydrate affinity to modulate specific galectin-1/glycan lattice interactions.* Nat Commun, 2015. **6**: p. 6194.
167. Baum, L.G., et al., *Microbe-Host Interactions are Positively and Negatively Regulated by Galectin-Glycan Interactions.* Front Immunol, 2014. **5**: p. 284.
168. Vasta, G.R., *Roles of galectins in infection.* Nat Rev Microbiol, 2009. 7(6): p. 424-38.
169. Medzhitov, R. and C.A. Janeway, Jr., *Decoding the patterns of self and nonself by the innate immune system.* Science, 2002. **296**(5566): p. 298-300.
170. Woodward, A.M., J. Mauris, and P. Argueso, *Binding of transmembrane mucins to galectin-3 limits herpesvirus 1 infection of human corneal keratinocytes.* J Virol, 2013. **87**(10): p. 5841-7.
171. Levroney, E.L., et al., *Novel innate immune functions for galectin-1: galectin-1 inhibits cell fusion by Nipah virus envelope glycoproteins and augments dendritic cell secretion of proinflammatory cytokines.* J Immunol, 2005. **175**(1): p. 413-20.
172. Thurston, T.L., et al., *Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion.* Nature, 2012. **482**(7385): p. 414-8.
173. 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.
174. Stowell, S.R., et al., *Innate immune lectins kill bacteria expressing blood group antigen.* Nat Med, 2010. **16**(3): p. 295-301.
175. Sato, S., et al., *Glycans, galectins, and HIV-1 infection.* Ann N Y Acad Sci, 2012. **1253**: p. 133-48.
176. 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.
177. Ouellet, M., et al., *Effect of galectins on viral transmission.* Methods Mol Biol, 2015. **1207**: p. 397-420.
178. Tandon, R., et al., *Galectin-9 is rapidly released during acute HIV-1 infection and remains sustained at high levels despite viral suppression even in elite controllers.* AIDS Res Hum Retroviruses, 2014. **30**(7): p. 654-64.
179. Saitoh, H., et al., *Rapid decrease of plasma galectin-9 levels in patients with acute HIV infection after therapy.* Tohoku J Exp Med, 2012. **228**(2): p. 157-61.
180. Bi, S., et al., *Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry.* Proc Natl Acad Sci U S A, 2011. **108**(26): p. 10650-5.
181. Wang, S.F., et al., *Galectin-3 promotes HIV-1 budding via association with Alix and Gag p6.* Glycobiology, 2014. **24**(11): p. 1022-35.
182. St-Pierre, C., et al., *Galectin-1-specific inhibitors as a new class of compounds to treat HIV-1 infection.* Antimicrob Agents Chemother, 2012. **56**(1): p. 154-62.
183. Nishi, N., et al., *Development of highly stable galectins: truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins.* FEBS Lett, 2005. **579**(10): p. 2058-64.

184. Bidon-Wagner, N. and J.P. Le Pennec, *Human galectin-8 isoforms and cancer.* Glycoconj J, 2004. **19**(7-9): p. 557-63.
185. Zick, Y., et al., *Role of galectin-8 as a modulator of cell adhesion and cell growth.* Glycoconj J, 2004. **19**(7-9): p. 517-26.
186. Carlsson, S., M.C. Carlsson, and H. Leffler, *Intracellular sorting of galectin-8 based on carbohydrate fine specificity.* Glycobiology, 2007. **17**(9): p. 906-12.
187. Ideo, H., et al., *Galectin-8-N-domain recognition mechanism for sialylated and sulfated glycans.* J Biol Chem, 2011. **286**(13): p. 11346-55.
188. Kolundzic, N., et al., *Galectin-8 is expressed by villous and extravillous trophoblast of the human placenta.* Placenta, 2011. **32**(11): p. 909-11.
189. Nikzad, H., et al., *Expression of galectin-8 on human endometrium: Molecular and cellular aspects.* Iran J Reprod Med, 2013. **11**(1): p. 65-70.
190. Tribulatti, M.V., et al., *Galectin-8 provides costimulatory and proliferative signals to T lymphocytes.* J Leukoc Biol, 2009. **86**(2): p. 371-80.
191. Tribulatti, M.V., et al., *Galectin-8 induces apoptosis in the CD4(high)CD8(high) thymocyte subpopulation.* Glycobiology, 2007. **17**(12): p. 1404-12.
192. Yamamoto, H., et al., *Induction of cell adhesion by galectin-8 and its target molecules in Jurkat T-cells.* J Biochem, 2008. **143**(3): p. 311-24.
193. Diskin, S., et al., *The role of integrin glycosylation in galectin-8-mediated trabecular meshwork cell adhesion and spreading.* Glycobiology, 2009. **19**(1): p. 29-37.
194. Hogg, N., I. Patzak, and F. Willenbrock, *The insider's guide to leukocyte integrin signalling and function.* Nat Rev Immunol, 2011. **11**(6): p. 416-26.
195. Springer, T.A., *Adhesion receptors of the immune system.* Nature, 1990. **346**(6283): p. 425-34.
196. Vicuna, L., et al., *Galectin-8 binds to LFA-1, blocks its interaction with ICAM-1 and is counteracted by anti-Gal-8 autoantibodies isolated from lupus patients.* Biol Res, 2013. **46**(3): p. 275-80.
197. Cattaneo, V., M.V. Tribulatti, and O. Campetella, *Galectin-8 tandem-repeat structure is essential for T-cell proliferation but not for co-stimulation.* Biochem J, 2011. **434**(1): p. 153-60.
198. Norambuena, A., et al., *Galectin-8 induces apoptosis in Jurkat T cells by phosphatidic acid-mediated ERK1/2 activation supported by protein kinase A down-regulation.* J Biol Chem, 2009. **284**(19): p. 12670-9.
199. Carcamo, C., et al., *Galectin-8 binds specific beta1 integrins and induces polarized spreading highlighted by asymmetric lamellipodia in Jurkat T cells.* Exp Cell Res, 2006. **312**(4): p. 374-86.
200. Checovich, W.J., R.E. Bolger, and T. Burke, *Fluorescence polarization--a new tool for cell and molecular biology.* Nature, 1995. **375**(6528): p. 254-6.
201. Oberg, C.T., et al., *Efficient and expedient two-step pyranose-retaining fluorescein conjugation of complex reducing oligosaccharides: galectin oligosaccharide specificity studies in a fluorescence polarization assay.* Bioconjug Chem, 2003. **14**(6): p. 1289-97.
202. Sorme, P., et al., *Fluorescence polarization to study galectin-ligand interactions.* Methods Enzymol, 2003. **362**: p. 504-12.
203. Laboratory, D.c.i., *Luminescence Macro User Guide.*
204. assays, N.I.n., *http://www.europrise.org/neutnet_sops.html.*

205. Mulampaka, S.N. and N.M. Dixit, *Estimating the threshold surface density of Gp120-CCR5 complexes necessary for HIV-1 envelope-mediated cell-cell fusion.* PLoS One, 2011. **6**(5): p. e19941.
206. Watkins, B.A., et al., *Syncytium formation induced by human immunodeficiency virus type 1 isolates correlates with affinity for CD4.* J Gen Virol, 1997. **78 (Pt 10)**: p. 2513-22.
207. Cederfur, C., et al., *Different affinity of galectins for human serum glycoproteins: galectin-3 binds many protease inhibitors and acute phase proteins.* Glycobiology, 2008. **18**(5): p. 384-94.
208. Yang, W.M., et al., *Glycoform Analysis of Recombinant and Human Immunodeficiency Virus Envelope Protein gp120 via Higher Energy Collisional Dissociation and Spectral-Aligning Strategy.* Analytical Chemistry, 2014. **86**(14): p. 6959-6967.
209. Pancera, M., et al., *Structure and immune recognition of trimeric pre-fusion HIV-1 Env.* Nature, 2014. **514**(7523): p. 455-61.
210. Izquierdo-Useros, N., et al., *Sialyllactose in viral membrane gangliosides is a novel molecular recognition pattern for mature dendritic cell capture of HIV-1.* PLoS Biol, 2012. **10**(4): p. e1001315.
211. Kaldensjo, T., et al., *Detection of intraepithelial and stromal Langerin and CCR5 positive cells in the human endometrium: potential targets for HIV infection.* PLoS One, 2011. **6**(6): p. e21344.
212. Hioe, C.E., et al., *LFA-1 expression on target cells promotes human immunodeficiency virus type 1 infection and transmission.* J Virol, 2001. **75**(2): p. 1077-82.

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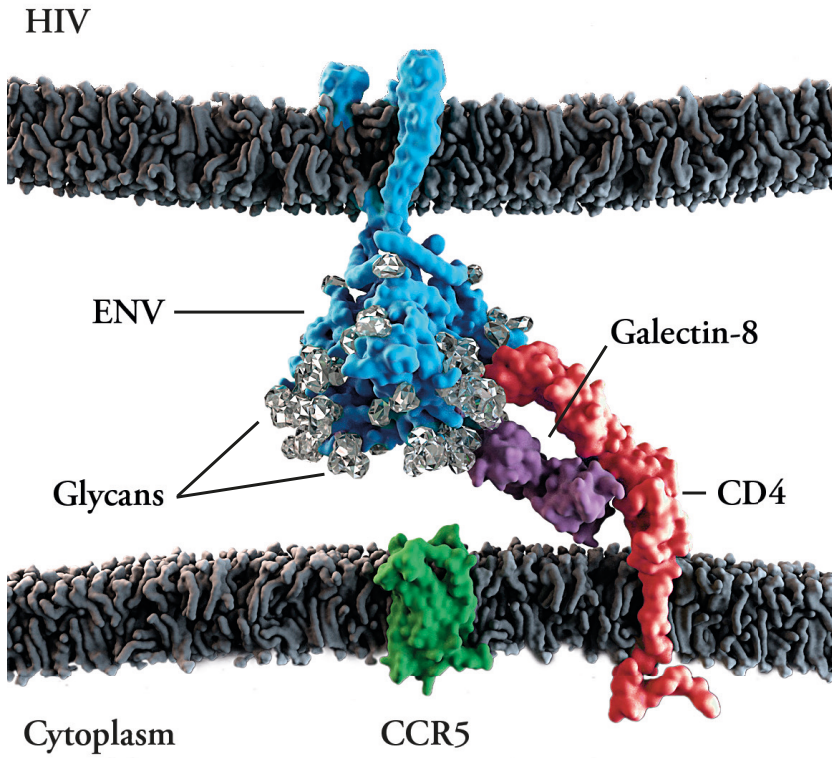

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