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THE EFFECT OF GALα(1,3)GAL GLYCOPEPTIDE-CONJUGATES AND HUMAN SERUM ON HIV-1 INFECTION AND REPLICATION

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Cover Picture: CD4 derived-galα(1,3)gal conjugated glycopeptide bound to HIV chronically infected ACH-2 cells and visualized with isolectin B4- AF488.

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"Early on I had to stop my education in order to go to school"

George Bernard Shaw

ABSTRACT

The first step in the entry of HIV-1 is the binding of the viral envelope glycoprotein (gp120) to the CD4 molecule expressed mainly on T helper cells. Upon binding, gp120 undergoes a series of conformational changes that expose inner epitopes, which in turn facilitate binding to the viral co-receptors (CCR5 or CXCR4). Further rearrangement of gp120 enables the fusion peptide of gp41 to reach out towards the cell membrane and facilitate fusion. It is thus evident that antibodies blocking any of these events could successfully inhibit viral entry. However, the high variability and glycosylation of gp120 makes of it a very poor and evasive immunogen. Thus all strategies directed towards this molecule have so far proved less than satisfactory.

In this thesis I present evidence for a new approach that could block viral entry and viral spread. We used peptides corresponding to the region of CD4 that binds to gp120 and we tested them for their capability to inhibit HIV-1 infection by means of their competition with the actual receptor. Moreover, in order to increase their immunogenicity and possibly to increase their biodynamics, we coupled them to the galα(1,3)gal disaccharide. This sugar residue attracts naturally occurring antibodies present in the serum of all humans and launches an innate immune response. The hypothesis proposed was that the glycopeptides would function as adapters between the natural antibodies and the virus particles and/or virus infected cells. We were able to show that the CD4 derived-galα(1,3)gal-coupled glycopeptides could neutralize HIV-1 and that in the presence of human serum (containing the anti-gal antibodies) the infection was further inhibited owing to the activation of proteins of the complement system and to the role of NK cells in mediating lysis of infected cells (ADCC). This was first tested in *in vitro* assays using the T cell adapted virus HIV_{IIIB} (paper I). Following these studies we tested the efficacy of the glycopeptides on patient isolates in the context of PBMC infection in an attempt to mimic more closely an *in vivo* situation (paper II). We used six different primary isolates, belonging to different subtypes of HIV-1 and also with different co-receptor specificity. We found that now ~100 times higher concentrations of the glycopeptides were needed to neutralize the different isolates and yet the inhibition was only modest. We therefore designed new glycopeptides with the aim of increasing their binding affinity and we also synthesized three glycopeptides corresponding to the extracellular regions of CCR5. Again the

neutralization capacity of the single glycopeptides was not satisfactory but the combination of CD4 and CCR5-derived glycopeptides proved to be a useful strategy.

While in the process of testing the efficacy of the glycopeptides, we found that the human serum used as a source of the anti-gal antibodies, increased the viral production in a dose dependent manner (paper III). This enhancement was reproducible in different cell lines, as well as, on freshly isolated PBMCs. We used two single replication assays, TZM-bl and ACH-2 cells, to try to dissect the step in the viral replication cycle that was affected by human serum. We showed that a protein (~ 250-300 kDa) was responsible for the enhancement of infectivity. It was found to activate three members of the AP-1 family of transcription factors, which in turn can promote viral transcription at the LTR level. Point mutations in the AP-1 binding sites of the LTR confirmed the specific role of these proteins in the human serum induced- enhanced replication of HIV-1. Further studies on the effect of human serum on *in vitro* cultured TZM-bl cells, showed also a dose dependent increase in CD4 expression that would impact viral infection and cytopathicity in these cells (paper IV). We showed that human serum influences the steady state levels of CD4 by increasing its recycling to the cell surface from early endosomes.

LIST OF PUBLICATIONS

I. **Perdomo MF**, Levi M, Sällberg M, Vahlne A. Neutralization of HIV-1 by redirection of natural antibodies. *Proc Natl Acad Sci U S A. 2008 Aug 26;105(34):12515-20.*

II. **Perdomo MF,** Sällberg M, Vahlne A. HIV Inhibition by CD4 and CCR5-Derived Glycopeptides. *AIDS Res Hum Retroviruses. 2012 Sep 28(9): 852-858*.

III. **Perdomo MF**, Hosia W, Jejcic A, Corthals G, Vahlne A. Human serum protein enhances HIV-1 replication and up-regulates the transcription factor AP-1. *Proc Natl Acad Sci U S A. In press. Published online ahead of print October 9,2012.*

IV. **Perdomo MF**, Vahlne A. Human serum increases CD4 recycling to plasma membranes of *in vitro* cultured cells**.** *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

Aa	Amino acids
Ab	Antibody
ADCC	Antibody Dependent Cell- mediated Cytotoxicity
Ag	Antigen
AIDS	Acquire Immunodeficiency Syndrome
AP-1	Activator Protein-1
ART	Antiretroviral Therapy
CAT	Chloramphenicol acetyltransferase
CD	Cluster Differentiation
CMV	Cytomegalovirus
CRF	Circulating Recombinant Form
ECL	Extracellular loop
EGFP	Enhanced green fluorescent protein
Env	HIV-1 envelope Glycoprotein
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
gp120	Glycoprotein 120 (surface protein)
gp41	Glycoprotein 41 (transmembrane protein)
HA	Hemagglutinin
HAART	Highly Active Antiretroviral Therapy
HIV-1	Human Immunodeficiency virus type 1
HIV-2	Human Immunodeficiency virus type 2
HS	Human serum
kDa	Kilo Dalton
KO	Knockout
LTR	Long Terminal Repeat
Nab	Natural Antibody
Nef	Negative regulatory factor protein
NHS	Non- heat inactivated human serum
NSI	Non-syncytium inducing viruses
PBMC	Peripheral Blood Mononuclear Cells
PDBu	phorbol 12,13 dibutyrate
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
RT	Reverse Transcriptase
Rev	Regulator virion protein
RRE	Rev-response element
sCD4	Soluble CD4
SI	Syncytium inducing viruses
SIV	Simian Immunodeficiency virus
Tat	Viral transcriptional trans-activator protein
TCLA	T cell line adapted viruses
TfR	Transferrin receptor

URF	Unique Recombinant Form
Vpr	Viral protein R
Vpu	Viral protein U
WHO	World Health Organization
α1-3 GT	Galα1-3Galβ1-4GlcNAc-R glycosyltransferase

1 INTRODUCTION

By 2010 an estimated 34 million people were leaving with human immunodeficiency virus (HIV), of which 10% are children. The infection is un-evenly distributed around the globe with nearly 68% of the reported cases being concentrated in the sub-Saharan countries of Africa [1]. Here the burden of the disease has taken a quote of more than 20 million deaths since the discovery of the virus and has left more than 14 million orphans. Since most infected individuals are economically active, these numbers are translated into an important decrease in the labor productivity and on a shift on the sex and age distribution of the labor force. Moreover, it has had an impact on education (i.e. due to the decline of number of teachers and school enrollment as children are needed to work or to take care of ill relatives) and it has put a great load on the already frail health care systems [2].

The establishment of new politics, prevention and awareness programs, as well as, the increase in the treatment coverage has brought the incidence of new cases to a halt. However, in other regions such as Eastern Europe and Asia, the number of newly reported cases has more than tripled in the last years. This has set on the alarms as the potential for spread can be almost exponential in these areas and has underlined the fact that the epidemic is far from being controlled.

The pandemic has moved the scientific community to join efforts to develop new treatment strategies while facing the challenges of developing a vaccine. It is clear that new prevention technologies are needed and an HIV vaccine offers still the greatest hope for reverting the incidence of the disease.

In this thesis I present a novel approach to inhibit HIV infection by competing with the viral port of entry but most importantly by involving in the process the innate immune system. Also, during the course of these studies we discovered important *in vitro* effects of human serum on culture cells, which have a significant impact on the viral replication, infection and cytopathicity.

2 HUMAN IMMUNODEFIENCY VIRUS

HIV is a retrovirus belonging to the lentivirus group. It was first discovered in 1983 by Luc Montagnier´s group at the Pasteur Institute [3] and it was identified as the causative agent of AIDS by Robert Gallo´s group in 1984 [4-6].

The first known HIV antibody positive human sample was found retrospectively in a serum sample from 1959. The virus has an origin in Africa and it is a result of cross-species transmissions of lentiviruses from non-human primates to humans. In fact, old world monkeys are commonly infected with more than 40 types of lentiviruses, termed simian immunodeficiency viruses (SIVs), and it is accepted that HIV originated from the passage across species barriers possibly during hunting, butchering or taming of primates [7].

There are two strains of HIV: HIV-1 and HIV-2. HIV-1 has the oldest lineage in humans, with an estimated cross species presentation (from chimpanzees to human) occurring around 1900 [8, 9]. HIV-1 is responsible for almost all of the global infections. It is also more virulent and has a more rapid progression to disease than HIV-2. The latter, on the other hand, is mostly confined to West Africa and has an origin in SIV from sooty mangabey monkeys [10, 11].

The main routes of transmission of HIV are unprotected sexual intercourse, contact with contaminated blood products (e.g. blood transfusions, contaminated syringes), and vertical transmission perinatally, as well as, by breast-feeding [12, 13].

HIV infects cells bearing the surface molecule CD4 [14] , which is typically expressed on T helper cells, but also on monocytes, macrophages, dendritic cells and microglial cells [15-17]. The number of cells in these populations is severely affected during the course of infection as a result of direct virus killing, induction of apoptosis and pyroptosis (due to the accumulation of defective DNA transcripts), as well as, to the cytotoxic immune response against infected cells [18-21].

The natural history of the disease is characterized by an initial acute viral replication, which presents with mild and uncharacteristic clinical symptoms (up to 10 weeks) [22], followed by a latent phase with low viral replication but chronic T cell depletion (lasting approximately 10 years). As $CD4^+$ T cells orchestrate the cellular and humoral immune responses, their progressive depletion results in a general impairment to fight infections and malignancies. The end stage of the HIV infection is consequently

manifested by a broad range of pathologies, a condition known as acquired immunodeficiency syndrome (AIDS) [23].

The rate of disease progression varies greatly among individuals and it is determined by multiple variables, which are related to the virus, to the individual and to the route of transmission [24, 25]. However, the life expectancy has been improved thanks to early diagnosis and to the establishment of antiretroviral therapy (ART) [26, 27].

2.1 HIV-1

2.1.1 HIV-1 Genes and proteins

HIV-1 is an RNA virus containing two single strands of RNA. Each strand is approximately 9kb long and is flanked by two LTR promoters. The HIV-1 genome encodes 9 open reading frames that are translated into 15 different proteins. The HIV-1 genome can thus be subdivided into structural genes (*gag, pol* and *env*), regulatory genes (*tat* and *rev*) and accessory genes (*vif, vpr, vpu* and *nef*) [28]. Below are represented the proteins by them encoded, as well as, a brief annotation on their function.

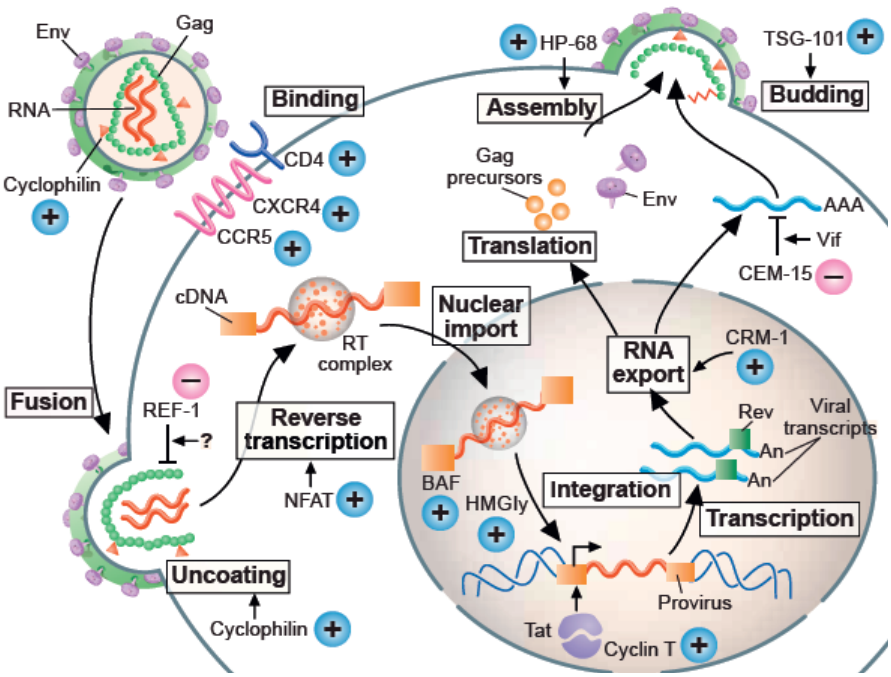
Structural genes	**Encoded proteins**		**Function**
	Gag polyprotein	**Pr55gag**	
gag	Matrix	MA p17	Targets gag and gag-pol precursors to the plasma membrane prior to particle assembly [29]
	Capsid	CA p24	Formation of viral core (~2000 molec/particle)[30]
	Nucleocapsid	NC p7	Binds to the packaging signal and delivers full length viral RNAs into the assembly virion [31]
	p6	p6	Incorporation of Vpr during viral assembly [32]
pol	**Gag-pol polyprotein**	**Pr160gag-pol**	
	Protease	PR p10	Cleavage of polypeptide precursors[33]
	Reverse Transcriptase	RT p51/66	Reverse transcription of viral RNA to DNA [34]
	Integrase	IN p32	Viral DNA integration into host chromosome [35]
env	**Envelope**	**env**	
	Surface glycoprotein	gp120	Binding to CD4 receptor and co-receptors [36]
	Transmembrane glycoprotein	gp41	Fusion [37]
Regulatory genes			
tat	Tat Transcription transactivator	p14	Transcription enhancer. Increases processivity of RNA polymerase II [38, 39]
rev	Rev Posttranscriptional transactivator	p19	Export of un-spliced and partially spliced viral mRNAs to cytoplasm by binding to RRE [40]
Accesory genes			
vif	Vif (viral infectivity factor)	p23	Enhances infectivity [41]
vpr	Vpr (virus protein r)	p15	Leads pre-integration complex to the nucleus [42]
vpu	Vpu (Virus protein u)	p16	CD4 degradation at the endoplasmic reticulum [43]
nef	Nef (Negative factor)	p27	CD4 and MHC-I internalization and induction of lysosome degradation [44-47]

HIV viral proteins and their function.

2.1.2 HIV-1 Replication cycle

The HIV-1 replication cycle can be divided into an early and a late phase. Among the early events, are the binding of the viral envelope glycoprotein (gp120) to the CD4 receptor and subsequently to the co-receptors (CCR5 or CXCR4) [48], followed by fusion of the virus particle to the cell membrane mediated by gp41[37].

Upon entry, the viral RT facilitates the reverse transcription of virion RNA to DNA, called proviral DNA. The RT switches back and forth between both templates of viral RNA, and as a result new recombinant DNA is obtained from the two parental RNA strains [49]. The RT activity lacks proof-reading and many mutations can be potentially introduced into the nascent DNA ($3x10^5$ per cycle of replication) [50]. Although many of the introduced mutations may not be fit, the generation of a heterogeneous viral population provides the virus with a clear advantage in developing drug resistance and immune escape.

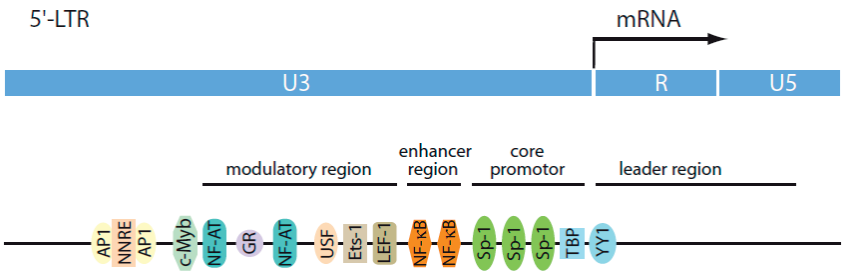


HIV-1 Pathogenesis. Figure adapted from [51]

The proviral DNA is subsequently transported into the nucleus as a pre-integration complex together with MA, IN and cellular proteins [52, 53]. Once in the nucleus it is integrated into the host cell chromosome by the viral integrase.

From this point on, the late events of the viral replication cycle, which include provirus expression, transport of the viral mRNA to the cytoplasm, assembly, budding and maturation take part.

Once the viral DNA is integrated into cellular genome, the virus uses the host cell machinery to initiate transcription at the LTR promoter. The LTR is divided into 3 regions U3, R and U5.



HIV-1 LTR and binding sites for the most important transcription factors. Adapted from [54]

Within U3 and R there are four several functional regions that regulate HIV transcription: U3 can be divided into the core promoter (nt -78 to -1), the core enhancer (nt -105 to -109) and a modulatory region (nt -454 to 104) [55]. To U3 binds the RNA polymerase II that initiates transcription. In R is found the transactivation response element (TAR) (nt +1-+60) which is the binding site for the viral protein Tat [55-58].

Several binding sites for cellular transcription factors have been mapped within the LTR and the combination of interactions between DNA and cellular proteins create a complex network through which HIV-1 can regulate its gene expression (reviewed in [59]). Several external stimuli can activate these interfaces. Moreover, the proteins that are activated, their coordination and their end effect are cell and virus specific [55, 56, 60, 61].

The basal transcription of LTR is low, yet still essential for the initiation of transcription of the viral DNA and for the expression of two important viral regulatory proteins, Tat and Rev. Tat greatly enhances viral transcription (~100 fold) by interacting with TAR in association with cyclin T and CDK9 [62-64]. Together they hyper-phosphorylate the carboxyl terminal domain of the large subunit of RNA pol II. This in turn liberates the polymerase from the promoter and provides the necessary switch from initiation to active elongation [65].

Rev binds to the Rev-response element (RRE) located in env and facilitates the export of un-spliced or partially spliced mRNA from the nucleus [40, 66]. This is critical as most viral structural proteins are encoded by un-spliced or partially spliced mRNAs and the new virus particle itself needs to contain the full length RNA. In the cytoplasm the translation of the precursor structural proteins p55Gag, p160 and gp160 occurs. While the first two are transported to the inner face of the plasma membrane, the latter (gp160) is transported to the endoplasmic reticulum where it undergoes glycosylation, folding, as well as, trimerization prior to its export to the Golgi [67]. In this organelle, gp160 is processed into the envelope glycoprotein gp120 and the transmembrane protein, gp41. Both proteins (which are non-covalently linked) are then transported to the cell surface were they are incorporated into the nascent viral particle together with p55Gag and p160 [68]. Budding occurs primarily at the level of the lipid rafts [69, 70] and the p6 protein of Gag has an important role in this event [32]. The nascent virus is non-infectious and the viral protease must cleave the precursor polyproteins gag and gag-pol to induce maturation of the budding particle. This results into a conformational rearrangement of the virus particle and it gives rise to the Matrix protein (MA, p17), the capsid protein (CA, p24), the NucleoCapsid protein (NC, p7) and the p6 protein (derived from Gag), as well as, PR, RT and IN (derived from Pol) [71].

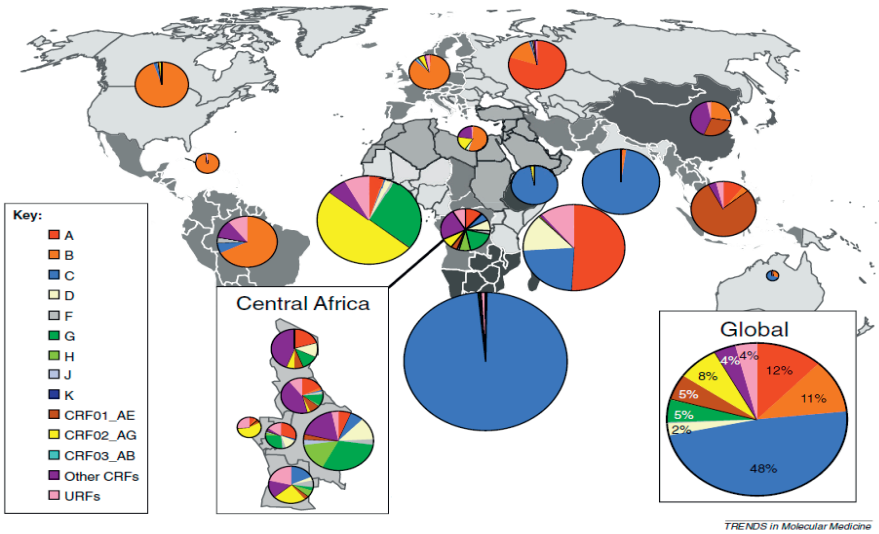
2.1.3 HIV-1 strains

As mentioned above, the great genetic diversity of HIV is mainly due to the high recombination and mutation rate generated by the reverse transcriptase. This, together with a high replication rate (~10^9-10^{10} particles/day) explains the remarkable genetic variability of this virus and stresses the difficulties met in vaccine design [72].

HIV-1 can be classified into three main groups based on the envelope sequence: M (main), N (non M, non O) and O (outlier). The M group is the most prevalent and it is

subdivided into nine subtypes (A-D, F-H, J and K) that can defer by 25-35% in env, by 20% in gag and by 10% in pol [73, 74]. Also within subtypes there is an important genetic variation (8-17%) and this has led to further classification of sub-subtypes (e.g A1-A4, F1-F2) [73, 75].

The subtype C accounts for nearly half of the global infections, followed by subtype A and B that represent 11% and 12% respectively [76]. Bellow can be observed the global distribution of the different subtypes.



Global distribution of HIV strains. Reproduced with permission from Elsevier [7]

An individual can be co-infected by more than one subtype and the recombination at the cellular level of RNAs from essentially different viruses can give rise to chimeric viruses, known as recombinant forms. Up to date have been recognized 48 circulating recombinant forms (CRFs) and multiple unique recombinant forms (URFs) [74]. By definition a CRF must have been detected in at least 3 unrelated individuals and should be able to establish an epidemic on its own. They are referred by number (according to the order of discovery) and by the subtypes involved (e.g. CRF02_AG). The URFs are found in one individual or in related individuals and are a localized phenomenon.

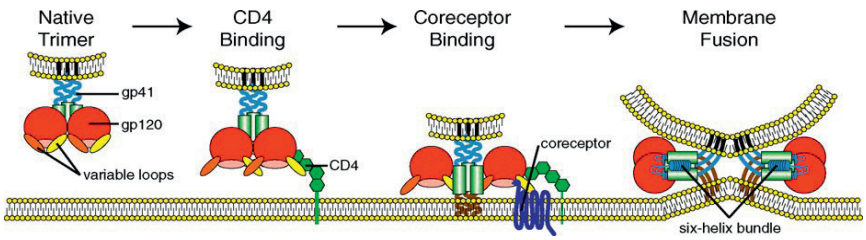
The subtypes may differ in their rate of disease transmission [77] and progression [78, 79] but also on their response to treatment [80] and on the emergence of drug resistance [81].

2.1.4 HIV-1 entry and tropism

The entry of HIV-1 is mediated by the viral envelope glycoproteins gp120 and gp41. These molecules are non-covalently linked and they are functionally active only when presented as trimers in the viral surface. Nearly half of their molecular weight is due to glycosylation and all the glycosylation sites are surface exposed [82]. Each gp120 molecule is composed of five variable regions (V1-V5). The first four form surface exposed loops with disulphide bonds at their bases. The conserved regions are discontinuous and they are mostly located in the core of the glycoprotein [83].

The first step in the entry of HIV-1 is the binding of gp120 to the CD4 molecule. However, other molecules such as heparan sulfate and DC-SIGN on dendritic cells have been described to provide ancillary binding to the virus by interacting with positively charged side chains and/or glycans on env [84].

Direct interatomic interactions are made by 22 amino acids in CD4 (located in the span of amino acids 25 to 64 of the D1/D2 region) [86] and 26 residues in gp120 which are distributed over 6 segments and located between the outer an inner domains and the bridging sheet of the envelope glycoprotein [36, 87].



Schematic representation of HIV binding to a CD4$^+$ cell. Reproduced with permission from Elsevier [85].

Upon binding to CD4 the gp120 molecule is stabilized and it undergoes a series of conformational changes that bring the V1/V2 loop to the proximity of the viral membrane thereby exposing inner epitopes and the co-receptor binding sites [86-88]. The main co-receptors for HIV-1 are CCR5 and CXCR4 [89-91]. They are integral membrane proteins with seven transmembrane helices, an extracellular N terminus and 3 extracellular loops (ecl). The N terminus of CCR5 binds to the base of the V3 loop of gp120 and to the bridging sheet, while the ecl2 binds to the tip of the V3 loop. The

dependence on the N terminus in regards to the ecl2 seems to be virus specific [92-94]. Upon binding to the co-receptor [95], further conformational changes in env occur which result in the release of the fusion peptide of gp41 and its insertion into the cell membrane. This glycoprotein refolds then into a 6 helix bundle that brings both membranes into proximity and thereby facilitates the fusion [96, 97].

New evidence has shown that the fusion and effective delivery of viral core and genome depends on endocytosis and that the endocytic route used by the virus is cell dependent (e.g. clathrin-mediated in Hela cells, macropinocytosis in macrophages) [98-101].

Historically, primary strains were subdivided into two categories: Macrophage-tropic (M tropic) and T cell line tropic (T tropic) because of their ability to infect macrophages and T cell lines respectively *in vitro*. M tropic viruses do not usually induce syncytia in cell lines, therefore they are classified as non-syncytium inducing (NSI) while T tropic viruses commonly induce syncytia in T cell lines and are subsequently also known as syncytium inducing viruses (SI) [102]. It was later found that these strains require different co-factors to enter $CD4^+$ cells [103]. While M tropic viruses use CCR5 as co-receptor, T tropic viruses use CXCR4.

CCR5 using viruses are usually found in early stages of the disease and they are most likely to be transmitted. They persist through the course of the disease but they may evolve into CXCR4 using viruses [104]. The latter are thus commonly found in later stages of the disease and they are normally associated with a poor prognosis. However, the co-receptor switch is only observed in 50% of the individuals indicating that the phenotype change is not essential for disease progression [96].

During sexual transmission, in 80% of the cases, only a single variant is transferred to the recipient [105]. Similar bottlenecks have also been described in cohorts of intravenous drug users [106] and during vertical transmission [107].

3 HIV THERAPY AND PREVENTION STRATEGIES

HIV, unlike many other viruses, is treated with drugs that have been tailor made to counteract specific steps in the viral replication cycle.

Type	Mechanism of action
Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)	Structurally similar to DNA nucleoside/nucleotide bases. They become incorporated into the proviral DNA chain, resulting in termination of proviral DNA formation.
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Bind the p66 subunit of RT and induces a conformational change in the enzyme that alters the active site and limits its activity.
Protease inhibitors (PIs)	Bind to the HIV protease and prevent cleavage of individual proteins from the gag and gag-pol polypeptide precursors
Integrase inhibitors (IIs)	Competitively inhibit the strand transfer reaction necessary for linking the proviral DNA to the cellular DNA by binding metallic ions into the active site.
Fusion inhibitors (FIs)	Bind to the HR1 region of gp41 and prevents its conformational change
Chemokine receptor antagonists (CRAs)	Selectively and reversibly bind to the CCR5 co-receptor blocking interaction with V3 loop interaction

HIV Antiretroviral Therapy. Drug classes and their mechanism of action.

The combination of drugs from different drug classes (highly active antiviral treatment or HAART, now commonly referred to as ART) has successfully been able to lower viral load to almost undetectable levels thereby improving the life expectancy and quality of life of infected individuals [108, 109]. It has had also a significant impact on the reduction of the rate of transmission. However, the efficacy of available treatments is limited by factors such as compliance, drug-drug interactions, drug toxicity giving side effects, as well as, the emergence and transmission of drug-resistant strains [110].

New therapeutic agents, within the existing drug classes, are being tested for similar efficacy but lower toxicity profiles [111]. Other novel agents, interacting at different levels of the viral life cycle (e.g tat-TAR antagonists, vpu inhibitors, zinc finger nucleases etc) are also currently under investigation [112]. New treatment strategies are also aiming to modulate immune responses to reduce HIV-related immune activation and to target latently infected cells [113].

One step in the viral replication cycle that is being targeted for drug development is the entry of HIV. This includes compounds that may interfere with the gp120/CD4

interaction, with the gp120/co-receptor interaction or with the fusion step. Currently only two drugs within this class are used for treatment: maraviroc, a molecule that binds to CCR5 and competes with gp120 for binding [114], and enfurvitide (T-20) a 36 amino acid peptide that has an identical sequence to a region of HR2 and competes for binding with the HR1 region of gp41, thereby preventing the six helix bundle formation and fusion [115, 116].

This group of compounds is collectively known as entry inhibitors. They will be given a special mention in this section, as they are related to the subject of this thesis.

Initial approaches to block the gp120/CD4 interaction were attempted using a soluble CD4 molecule (sCD4), which was found to inhibit HIV *in vitro* [117-120]. This molecule however did not have any effect in HIV infected individuals because the plasma level concentrations were not sufficient to neutralize primary strains of the virus [121, 122] and also due to its short plasma half-life (45 minutes in humans) [123]. However, the finding that CD4 could inhibit HIV entry has encouraged the development of multiple sCD4 derivatives and CD4 mimics [124-132].

Other small molecule inhibitors and peptides have also been studied for their ability to block the interaction between the envelope and the receptor or the co-receptors, as well as, to block gp41 [133-141].

Currently in phase II trials are also two antibodies that bind to CD4 or CCR5 but do not block their ligand activity: Ibalizumab binds to the D2 region of CD4 and interferes with the CD4-induced conformational changes in gp120 [142, 143] and PRO-140, a humanized mouse anti-CCR5 that prevents binding of env [144, 145].

Despite its effectiveness, current ART is not able to completely eradicate the virus from infected individuals. Therefore an important focus has been set on prevention strategies and the use of ART as pre-exposure prophylaxis [146-149].

Besides ART, there are a number of strategies to prevent or reduce transmission of HIV-1. Male circumcision has been shown to confer a 51-60% protection against HIV infection in three independent randomized clinical trials [150-152]. Possible reasons for this effect are: decreased trapping of HIV containing mucosal secretions under the foreskin, reduced surface exposure (i.e. lower number of macrophages, CD4+ and Langerhans cells) and decreased local inflammation due to uropathogens.

The most obvious form of prevention is a prophylactic vaccine. However, despite major efforts, no vaccine has yet shown to provide any significant protection [153]. Up to date four efficacy trials have been conducted.

Initial attempts were focused on the development of a vaccine that could induce neutralizing antibodies. The first two trials, VAX003 and VAX004, tested the efficacy of bivalent recombinant gp120 products derived from clades B and E, AIDSVAX B/E and AIDSVAX B/B. The results of these trials were discouraging as they failed to provide any protection in vaccinees in relation to placebo and the anti-gp120 antibodies generated had narrow specificity and thus were not broadly reactive [154]. The second trial, known as the STEP trial, used a replication-defective adenovirus type 5 (Ad5) vector expressing gag, pol and nef genes to test the ability of cell mediated immunity to protect against infection. Not only did this strategy fail to protect against HIV, but there was a relative increase in the risk of HIV-1 acquisition in those subjects who were un-circumcised and with high pre-existing levels of immunity against Ad5 [155]. The last trial, RV144, included a replication-deficient canary pox vector expressing HIV-1 CRF01_AE Env, clade B Gag and the protease-encoding portion of Pol together with AIDSVAX-B/E. The vaccine efficacy at 12 months post-vaccination was as high as 60% but only 31.2% after 3.5 years when the trial was completed [156, 157]. Although mild, these results have provided with a new optimism to vaccine development.

4 NATURAL ANTIBODIES

During B and T cell development, self-reactive cells are normally negatively selected to avoid autoimmune responses. However, there is a group of B cells that are positively selected for their self-reactivity [158]. They form a pool of long-lived, self-renewed cells that spontaneously secrete antibodies although, in the absence of a specific antigen stimulation. These cells contribute to most of the resting pool of IgM and IgA antibodies present in serum. These antibodies are called natural antibodies (NAb). They are broadly reactive and they can bind to proteins, nucleotides, polysaccharides or lipids [159-161]. Some of the NAbs may display a low affinity and poly-reactivity for several antigens while others display specificity for a particular ligand and they bind with higher affinity [162].

In the mouse, natural antibodies (NAb) are derived from a subset of mature B cells, called B1 cells, which appear early during development and reside principally in the peritoneum and pleural cavity. The B1 cells are phenotypically different from other B cells that may produce IgM (B2 cells) [163]. Contrary to follicular B cells which are responsible for T cell dependent-adaptive immune responses, the B1 cells are more likely to cooperate in innate immune responses [164]. The homolog of these cells in humans has been recently identified to be $CD20^{+}CD27^{+}CD43^{+}$ $CD70^{-}$ and can be found both in umbilical cord and peripheral blood [165].

The significance of NAbs lies in their innate role in the host defense against common microbiological agents. Owing to their poly-reactivity, NAbs are able to react against pathogens that have never been encountered and contribute to the microbe clearance by direct neutralization or by cooperation with the innate immune system (e.g complement binding, opsonization) while serving as a link to establish the more specific adaptive immune response [166]. They have been shown to recognize pathogen-antigens such as the lipopolysaccharide (LPS) of Gram negative bacteria [167] or the phosphorylcholine of Gram positive bacteria [168, 169]. Their role has also been documented as first line response in the infection by influenza, west nile virus, vesicular stomatitis virus, lymphocytic choriomeningitis vius, vaccinia virus among other viral [170] and parasitic infections [171].

Moreover, NAbs are important in the maintenance of tissue homeostasis. In fact, they can recognize self-antigens such as annexin V, oxidized lipids and phosphatidylcolin and can contribute to the clearance of apoptotic cells [172].

They are also gaining increasing importance as they have been recently associated with protection against Alzheimer´s disease, atherosclerosis, stroke, cancer development [173-177] and as immune-modulators in autoimmune diseases [178, 179].

5 GALα(1-3)GAL

Oligosaccharides are commonly presented on the cell surface or bound to extracellular proteins and lipids. They are known to modulate many biological processes and to participate in specific recognition events by lectin receptors (both endogenous and exogenous) [180, 181] as well as, to contribute to the physical properties of many proteins (e.g folding, stability, charge) [182-185].

The pattern and distribution of glycans in mammals is the result of glycosyltransferases, which give rise to disaccharides, oligosaccharides and polysaccharides. One of the products of these family of enzymes is the Galα1-3Galβ1-4GlcNAc-R tetrasaccharide (referred here as Galα(1-3)Gal) which is abundantly expressed as the terminal sequence of many glycolipids and glycoproteins on the cell surface of non-primate mammals [186-190]. In old world monkeys, apes and humans the Galα1-3Galβ1-4GlcNAc-R glycosyltransferase (α1-3 GT) is inactivated [191] and in contrast, these species express high levels of antibodies to this oligosaccharide [192, 193]. These antibodies will henceforth be referred to as anti-gal antibodies. It is estimated that 1% of the total human B cells are expressing natural antibodies towards Galα(1-3)Gal [194]. They represent approximately 1.8 to 8% of the total IgM and 1 to 2.4% of the total IgG in serum [195]. This pool, however, doesn´t necessarily correspond exclusively to natural antibodies. In fact, the Galα(1-3)Gal epitope is expressed by several strains of enterobacteria (e.g. E. Coli, Salmonella, Klebsiella), thus the levels of anti-gal antibodies might be maintained by continuous stimulation by the normal flora in the gut [196].

These antibodies have been described to play an important role in the early protection against dissemination of bacterial and viral infections (e.g. C-type retroviruses) [197-200] but their relevance was made clear in the field of transplantation.

The great demand for organ donors has expanded the search for suitable organs to other species and it was after xenotransplantation of kidneys from pigs to humans that these

antibodies were identified as an important part of the innate immune response [201]. In fact, the anti-Gal antibodies mediate rejection of pig xenograft organs in humans and monkeys by binding to the Galα(1-3)Gal epitope. They induce a hyper-acute reaction that involves complement activation and antibody dependent cell cytotoxicity [202]. In addition, binding of the anti-Gal antibodies induces also platelet activation and aggregation resulting in ischemia of the graft [202]. This chain of events was shown to be transiently delayed by infusion of the disaccharide Galα(1-3)Gal [203] or by removal of anti-Gal antibodies from the blood of monkeys by affinity columns [204].

The complete elimination of Galα(1-3)Gal epitopes was achieved by targeted disruption of the α1-3 GT gene [205-208]. The transplantation of hearts and kidneys from α1-3 GT knockout pigs to monkeys resulted in viable organs for several weeks to months [209, 210].

The ability of the Galα(1-3)Gal epitope to attract antibodies and to induce an immune response has promoted the development of technologies that aim to use its immunogenicity to enhance viral and autologous cancer vaccines.

Henion et al. added this epitope to the carbohydrate chain of the hemagglutinin (HA) glycoprotein and showed that upon injection, the immune complexes formed by the anti-gal antibodies enhance the uptake of the viral antigen by antigen presenting cells (APC) [211]. This resulted in ~100 fold higher anti-flu virus antibodies and T cell responses in α1-3 GT knockout (KO) mice injected with the modified glycoprotein as compared to those vaccinated with the common flu vaccine. The survival rates were also superior after intranasal challenge with the live virus [212]. This same principal was used to engineer gp120 molecules expressing the Galα(1-3)Gal epitope and also in this case the addition of this carbohydrate moiety proved to enhance immunogenicity and to increase anti-gp120 ab responses [213].

This approach has also been studied in melanoma, where B16 mouse melanoma cells were transfected with a plasmid containing the α1-3 GT gene [214] or transduced with a retrovirus [215] or an adenovirus vector [216] containing this gene. Vaccination of α1-3 GT KO mouse with irradiated B16-modified cells induced an immune response that protected the mice against challenge with B16 live cells.

In summary, the value of this epitope as an immunogen has been tested successfully in the context of various experimental models. In this thesis I describe how we made use of redirecting these anti-gal antibodies to new targets, in our case HIV-1, by use of HIV-binding peptides conjugated to the Galα(1-3)Gal epitope.

6 AIMS OF THIS THESIS

The main objective of this thesis was to evaluate the HIV-1 neutralizing capacity of CD4-derived- Galα1-3Gal-conjugated glycopeptides.

More specifically:

1. To test whether CD4-derived-Galα(1-3)Gal-conjugated glycopeptides could neutralize HIV-1 *in vitro*.

2. To test whether human serum, containing naturally occurring anti-Galα(1-3)Gal antibodies, could contribute to the virus neutralization by triggering the innate immune system.

3. To test the effectiveness of the glycopeptides and human serum in *in vitro* neutralization of primary isolates.

4. To study the *in vitro* effects of human serum on HIV-1 replication and infection.

7 METHODOLOGICAL CONSIDERATIONS

In this section, some of the methods used in this thesis will be discussed. For a detailed description of the different procedures used please refer to the corresponding papers.

7.1 NEUTRALIZATION ASSAYS

Virus neutralization assays are designed to measure a reduction in the virus titers after addition of a drug or an antibody.

The first assays established to measure HIV-1 infection monitored syncytia formation after infection with T-cell line adapted viruses (TCLA; e.g. HIV_{IIIB}) [217]. This method allowed the visualization and quantification of virus infection in both a sensitive and reproducible manner. Initial assays, however, were limited to SI viruses [218, 219] and other cell lines were introduced to reproduce the features of the original plaque assay. In paper I we used a syncytium assay developed by Shi et al. [220] that uses glioma derived U87 cells which are $CD4^+$ and express either CCR5 or CXCR4. The syncytia are visualized after staining with hematoxylin and can be quantified by light microscopy, making of this method readily accessible. It is also sensitive and reproducible. However the procedure of enumeration is time consuming. Also, since virus isolates can differ in their cytopathic ability, difficulties may arise when trying to compare different neutralization assays. In paper IV we developed a new syncytia assay that is based on the passive transfer of an intracellular dye from infected (ACH-2) to un-infected cells (TZM-bl), which can be conveniently quantified and analyzed by flow cytometry.

Other neutralization assays have been developed on PBMC and T cell lines (e.g H9 cells), which typically monitor viral growth by measuring extracellular levels of p24 or reverse transcriptase. However, the threshold for detection of these proteins require several rounds of replication and therefore the amount of protein detected might not necessarily reflect the actual number of infected cells [221]. In fact, factors such as cell death and release of viral antigens together with the presence of non-infectious viral particles can affect the total amount of cell free p24-Ag detected in culture supernatant [222]. This latter issue can be partially overcome by the measurement of the activity of the reverse transcriptase, which preferentially reflects viable virus particles.

The PBMC assay is still the most physiologically relevant to measure virus neutralization and in our experience it is also the most sensitive to detect inhibition of patient isolates. However there are several drawbacks that limit its use:

Several cycles of replication imply incubation times between 7 and 14 days plus the time needed to detect the chosen viral antigen in culture supernatant. Closely related to this is also the fact that primary isolates display different replication kinetics in PBMCs [223-225]. Thus the measurement of the viral antigen at specific days post-infection might be reflecting early phase of growth for some strains while several cycles for more rapidly growing viruses, making the comparison of neutralization assays more difficult.

This assay displays also a high intra-assay and inter-assay variability and in contrast to available cell lines, the results are highly dependent on the donor. To avoid this, the PBMC assays performed in this thesis (paper II) were run on pools obtained from four different donors, which were used at the initial virus titration and through the multiple biological replicates performed.

Newer PBMC based assays have tried to reproduce the positive features of these cells while increasing sensitivity, specificity and decreasing incubation times [226, 227].

In addition, many different reporter-cell lines [228-232], as well as, viral constructs expressing CAT, alkaline phosphatase, β-galactosidase, luciferase or GFP [233-236] have been developed in an attempt to quantify the number of infected cells in a more precise manner. Among these, an assay that is now widely diffused is the TZM-bl assay [237]. These Hela derived cells express CD4 and both HIV co-receptors and contain a tat-responsive luciferase reporter gene. The presence in the culture media of the protease inhibitor indinavir ensures that the luminescence levels measured are a reflection of only a single round of infection. This assay provides fast, convenient and reliable results and it is very sensitive when testing TCLA and env-pseudotype viruses.

However, in our experience these cells are hardly infected by primary strains even in the presence of DEAE-dextran, which promotes infection by increasing electrostatic interactions between the virus and the cell membrane. A lack of sensitivity of this assay when compared to PBMC was also reported by Mann et al [238].

The field of vaccine development has promoted the standardization of reagents and assays for evaluation of immune responses elicited by candidate vaccines. With this purpose NeutNet was created, a network of independent laboratories distributed worldwide, which aims to elucidate the prerequisites of accurate and reproducible measurement of HIV-1 functional antibodies. In 2004, a first study tested the efficacy of monoclonal antibodies and sCD4 against 11 HIV-1 isolates in 10 different

neutralization assays [239] and in 2008 a second study evaluated polyclonal antibodies against 8 viruses in 9 neutralization assays [240]. The conclusions drawn by both studies are similar and they highlight the fact that the assays sensitivities are dependent on both the neutralizing agent and the virus. Thus, none of the assays evaluated performed superiorly than others under all circumstances. The current recommendation is to run parallel testing of pseudoviruses (e.g. TZM-bl) and viral infectivity assays (e.g. PBMC).

7.2 CELL-MEDIATED CYTOTOXICITY

The gold standard for measurement of cell-mediated cytotoxicity is the Chromium (^{51}Cr) release assay [241]. This is based on the passive internalization and binding of ^{51}Cr to target cells and its posterior detection in cell culture supernatant where it is released as a result of lysis by effector cells. It is reproducible and relatively easy to perform but it has also various disadvantages such as: high inter and intra-assay variability, high background do to spontaneous release from some target cells, it provides only a bulk estimate of lysis and not information about single cells, poor labeling of some target cells, as well as, biohazard and disposable problems related to the radioisotope.

These considerations have driven the development of alternative methods to replace this assay (reviewed on [242]) some of which are based for example on the release of other radioactive (^{125}I or ^{3}H) or non-radioactive compounds, on the detection of enzymatic activity in target cells (LDH, calcein, alkaline phosphatase), or on the detection of specific effector molecules (IFNγ or granzymeB ELISpot) [243]) etc. Along with them several flow cytometry based methods have been developed [244] which have proven to have increased sensitivity and specificity while allowing the study of specific cell populations and simultaneous measurement of multiple parameters.

In this thesis (paper I) we developed a flow cytometry-based method for detection of ADCC where our target cells (ACH-2) were labeled with a specific anti CD5 ab and dead cells were identified by staining with propidium iodide, which binds to DNA. Thus we were able to precisely quantify the amount of target cells that have been lysed by the effector cells after treatment with our glycopeptides and human serum.

8 RESULTS

In this section, the results from papers I-IV are summarized. For figures and detailed description of the results please refer to the respective papers.

8.1 NEUTRALIZATION OF HIV-1 BY REDIRECTION OF NATURAL ANTIBODIES (PAPER I)

The first step in the entry of HIV-1 to susceptible cells is the binding of the viral envelope glycoprotein (gp120) to the CD4 molecule. Thus, blocking this interaction is an obvious target for any entry inhibitor. However, the great variability and high glycosylation of gp120 poses a great challenge for the development of a broadly reactive neutralizing antibody or molecule. The CD4 molecule, on the other hand, is conserved and only a limited amount of amino acids make direct contact with gp120 [36].

In this first paper we explored the possibility of blocking the entrance of HIV by synthesizing six peptides overlapping the region in CD4 that binds to gp120. They were 15 amino acids long and overlapped one-another by 70%. These peptides were able to inhibit the entrance of HIV_{IIIB} in viral neutralization assays.

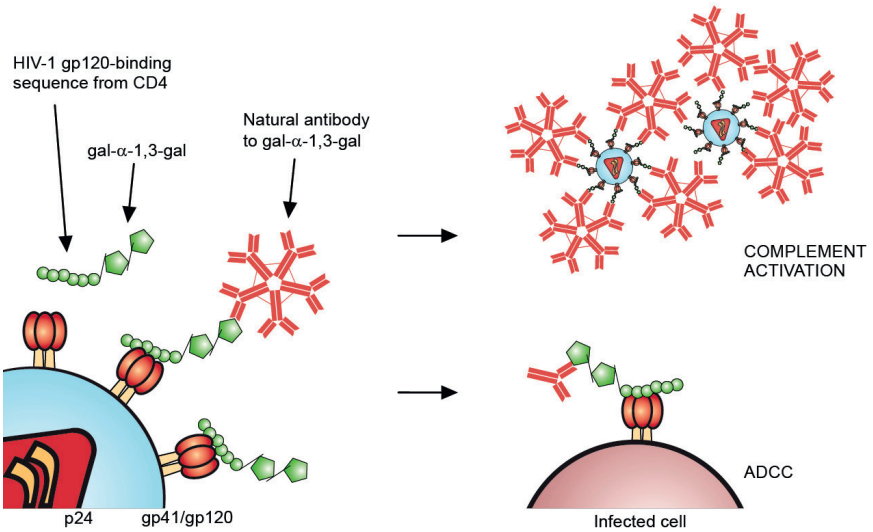
We then aimed to improve the immunogenicity of these peptides by conjugating them to the Galα(1-3)Gal disaccharide. This disaccharide is commonly exposed on the tip of many glycans expressed on the cell surface of epithelial cells and erythrocytes of all mammals except humans, apes and old world monkeys. In contrast, in humans the largest pool of natural antibodies is directed towards this epitope. These antibodies have been shown to launch an acute immune response in humans upon encounter with the epitope, which includes activation of the complement cascade and triggering of antibody dependent cell cytotoxicity (ADCC).

We then tested the hypothesis whether linking the CD4 derived peptides to Galα(1-3)Gal could attract the natural anti-gal antibodies and induce an innate immune response which could ultimately lead to clearance of the virus or of an infected cell.

We found that the glycopeptides had an improved neutralization capacity as compared to the single peptides. Moreover, when tested in the presence of heat inactivated human serum (as a source for the natural anti-gal antibodies), we found that the neutralizations increased by ~10-20% units. The efficiency of the inhibition was further improved when testing the glycopeptides together with non-heat inactivated human serum, in

which consequently both the anti-Gal antibodies and the active complement proteins were present. This suggested that the complement indeed played an important role in the clearance of the infected particles or cells.

To test ADCC, we treated chronically infected-PMA stimulated ACH-2 cells with the glycopeptides and human serum and we co-cultured them with freshly isolated NK cells. We found that the NK cells were able to induce cytotoxicity as assayed by the number of cells stained by propidium iodide, a probe that binds to the DNA of the lysed cells.



Schematic representation of the redirection of antibody specificity. CD4-derived-peptides can bind to the gp120 expressed on the virus surface or on the cell surface of an infected cell. By linking these peptides to the Galα(1-3)Gal disaccharide, specific anti-Gal natural antibodies can be attracted to the site of infection and form immune complexes that can activate the complement or that can be recognized by NK cells and launch ADCC.

Thus, in this paper we were able to show that CD4-derived-Galα1-3Gal conjugated glycopeptides in the presence of human serum were able to inhibit the entrance and spread of HIV_{IIIB} to susceptible cells *in vitro*, partly by competing with the receptor for interaction with gp120 but most importantly by activating the complement and promoting ADCC.

8.2 HIV INHIBITION BY CD4 AND CCR5-DERIVED GLYCOPEPTIDES (PAPER II)

The results presented in paper I were based on the neutralization of the T cell line adapted virus $HIV_{IIIB.}$ However, it is known that laboratory strains are more susceptible to neutralization than primary strains of the virus [121, 245, 246]. Therefore, in this paper we aimed to study the effectiveness of the glycopeptides on the neutralization of primary isolates with known phenotype and genotype [247].

Primary isolates belonging to different subtypes can differ in their envelope glycoprotein by as much as 30%, thus the results from neutralization assays can vary greatly among strains. Consequently we selected six isolates belonging to different clades (A-D) and also using different co-receptors to outline the breath of action of the glycopeptides.

Primary isolates are best expanded and tested on PBMCs and therefore they were the cells of choice for the neutralization assays.

We first tested three of the glycopeptides that showed the highest neutralization capacity in our previous work (glycopeptides 3, 4 and 5; paper I). We found that the neutralizations were in general low (below 50%) and that 100 to 1000 times higher concentrations of the glycopeptides (compared to those used in paper I) were needed. We then introduced a series of sequence and structural modifications to our existing peptides in an attempt to increase their binding avidity. Unfortunately, the modified glycopeptides did not drastically improve the neutralization capacity of the previous glycopeptides. However, the peptide conformation (e.g. by circularization) seemed to be beneficial for binding to the virus.

We tested these peptides both in the presence and absence of heat inactivated human serum and we confirmed that the presence of the anti-Gal antibodies in the culture medium did indeed contribute to neutralization.

We also included three new glycopeptides, corresponding to the extracellular loops (ecl) of the CCR5 co-receptor. Unfortunately, we were not able to synthesize a peptide corresponding to the N-terminal of the co-receptor due to instability of the disaccharide amino acid building block during amine de-protection conditions and resin cleavage. The neutralization by these glycopeptides did not reach IC50 levels even at the highest concentrations used, possibly due to the fact that these peptides can only bind to inner epitopes which are only transiently exposed after CD4 binding.

Thus, we explored the possibility of combining the CD4 and CCR5 derived glycopeptides. We found that this mixture improved the inhibition percentages of some of the strains, inclusive some that were shown to be resistant to neutralization in the previous assays. The combination of CD4 peptides also showed to positively affect the neutralization.

8.3 A HUMAN SERUM PROTEIN ENHANCES HIV-1 REPLICATION AND UP-REGULATES THE TRANSCRIPTION FACTOR AP-1 (PAPER III)

During the selection and optimization process of the neutralizations used in paper I and II, we found that the addition of increasing concentrations of heat inactivated human serum (used as a source of anti-Gal antibodies) to the cell culture directly correlated with an increase in viral output. This effect was reproducible on four different cell lines (TZM-bl, H9, CEM-GFP and U87), as well as, on PBMC, the latter cells cultured in the presence of autologous plasma. This result was also independent of the viral strain used (HIV_{IIIB}, 90SE364, BaL, A083M411), of the method used to quantify infectivity (p24, viral RNA load, RT activity, luciferase activity, GFP expression) and of the batch of human serum (HS) used (total of nine tested).

In this paper (III) we showed that the enhancement is specific for HS and that the effect is best seen when culturing the cells with non-heat inactivated HS (NHS).

We aimed to dissect the mechanism by which NHS was enhancing viral replication. For this purpose we used two cell lines, TZM-bl and ACH-2 cells, both of which encompass single replication assays but with different characteristics and outputs.

The TZM-bl cells are Hela derived cells that have been modified to express CD4 and both co-receptors. They have also a luciferase reporter gene whose expression is driven by the LTR promoter of HIV. Upon infection, the Tat protein of HIV increases transcription at the level of the LTR, thus, collaterally also inducing luciferase transcription, which can then be read as an indirect measure of viral infectivity. This assay therefore allows us to effectively study the early steps of the viral replication cycle (binding, fusion, reverse transcription and integration).

The ACH-2 cells, on the other hand, are chronically infected and they are CD4 negative. Thus the effects detected in this system include only the late steps of the viral replication cycle (transcription, translation, budding, maturation).

Through pre-infection and transfection of the infectious plasmid pNL4.3 in the TZM-bl cells, we were able to show that the effect of NHS is not due to an increased entry of the virus. Instead, we showed that the number of virus particles present in the supernatant of PMA stimulated ACH-2 cells directly correlated with the concentration of NHS in culture. This suggested an effect on already integrated viruses.

By means of infection with an HIV_{IIIB} with a deleted tat gene and through transfection of TZM-bl and 293T cells with LTR reporter genes we were able to show that NHS triggers the activation of the LTR promoter, thereby facilitating virus replication and infectivity. Further characterization of the transcription factors involved, showed that three members of the AP-1 family, c-Fos, JUND and JUNB, increased in a time and dose dependent manner in relation to the amount of NHS present in the culture of TZM-bl cells. Moreover, NHS was not able to increase the EGFP expression of an LTR driven plasmid when the promoter had point mutations in each of the three AP-1 binding sites.

Preliminary characterization of the HS factor(s) responsible for the effect described above indicated that it is a single protein with a molecular mass between 250 kDa and 300 kDa.

8.4 HUMAN SERUM INCREASES CD4 RECYCLING TO PLASMA MEMBRANES OF *IN VITRO* CULTURED CELLS (PAPER IV).

The expression of surface markers on cells of the immune system may change during the course of infection as a result of the cytokines encountered in the inflammatory milieu. Indeed, both the receptor and co-receptor levels of HIV have been described to be regulated by specific cytokines. Thus, the T cell subsets might have different susceptibility to infection with HIV-1 through the course of infection. This effect by different cytokines has been reproduced *in vitro*. Moreover, some of the culture techniques, as well as, some separation methods commonly used in the laboratory have been shown to modify the surface molecules of different cells.

We studied the effect that NHS could have on the pattern of expression of CD4 and co-receptors on the TZM-bl cells since any up-regulation could have a profound impact on the entry and infectivity of the virus.

We found that NHS up-regulates CD4 surface levels but down-regulates CXCR4 and CCR5 levels in a dose dependent manner in TZM-bl cells.

We proceeded to study the mechanisms by which NHS affects CD4 expression. By following the steady state levels of CD4 across time we found that NHS does not affect the *de novo* synthesis of this molecule and that instead the effect was owing to an increase in the presentation of already synthesized molecules.

In TZM-bl cells, the CD4 molecule is not anchored to the cell surface and undergoes continuous internalization and recycling to and from early endosomes [248]. In contrast, in T cells CD4 is anchored by $p56^{lck}$. We found that NHS does not affect the steady state levels of CD4 on the T-cell derived SupT1 cells, possibly as a result of the anchoring.

We then studied the role of NHS on recycling in this cell line by treating the cells with PDBu, a phorbol that induces internalization of surface molecules, and we followed the levels of CD4 after culture with NHS, FBS or serum-deprived medium. We found that indeed NHS enhances the recycling of CD4 to the cell surface as compared to the controls. This effect was also confirmed by measuring in parallel the expression of the transferrin receptor´s (TfR) levels, a receptor extensively studied and known to be constitutively recycled.

We also studied the impact that NHS would have on the expression levels in an infectious setting and on infected cells. The viral protein *nef* induces internalization of CD4 to early endosomes where it then forwards it to the late endosomes and triggers it for degradation by the lysosomes. We found that in *nef* transfected cells, NHS helped to maintain the normal levels of CD4, possibly by partially rescuing the CD4 molecules targeted for degradation. Also in infected cells, the NHS helped to keep the baseline levels of the HIV receptor.

The higher expression of CD4 on the surface correlated also with a higher cytopathicity. In fact, after co-culture of infected cells with uninfected cells, the number of syncytia was higher in the samples of NHS pre-treated cells as compared to those cultured with FBS.

9 DISCUSSION

The efficacy of ART is to some extent limited by drug resistance, drug-drug interactions and drug related side effects. This is driving the development of new compounds with new mechanisms of action and improved toxicity profiles. In this thesis, I present a novel mechanism based on CD4 derived-galα(1,3)gal coupled glycopeptides that can bind to the gp120 expressed on the surface of a virus or of an infected cell. This glycopeptides can effectively inhibit infection/transmission, by competing with the CD4 molecule but importantly, by activating the innate immune system. We showed that the glycopeptides effectively inhibit HIV_{IIIB} and that natural antibodies bound to the disaccharide can enhance the neutralization effect by attracting proteins of the complement system and facilitating ADCC. Thus we showed that the biological activity of the anti-gal antibodies can be efficiently redirected towards the virus or a virus-infected cell surface.

Other studies have shown an increased uptake by macrophages and antigen presenting cells of virus coated with complement proteins or antibodies [249]. Thus, our peptides might also be enhancing processes such as opsonization and phagocytosis. Besides the clearance of the virus, this can also be contributing to antigen presentation and priming of adaptive immune responses. This, however, remains to be shown.

The studies performed in paper I were based on infection by the T cell adapted virus HIV_{IIIB}. However, it is known that laboratory strains are easier to neutralize than primary strains, therefore we aimed to test the efficacy of the glycopeptides on patient isolates (paper II). Moreover, we included seven modifications to the previously published glycopeptides (length, sequence, structural conformation) to increase their binding affinity. We also tested three glycopeptides corresponding to the complete sequence of the extracellular loops of CCR5. We found that the single glycopeptides only modestly inhibited the primary isolates and that higher concentrations were needed to neutralize them. However, the combination of CD4 and CCR5 derived glycopeptides proved to be beneficial.

It is clear that peptides with higher binding avidities are needed if to be of use in the clinic. Therefore, peptides with higher affinities to the virus envelope proteins, e.g. derived from random libraries should be constructed and tested. We hope that peptides that bind to the virus are potentially less toxic than compounds that act by allosteric interaction with the receptor/co-receptors.

Worth of mentioning is the fact that CCR5 using viruses are present through the whole length of the HIV infection but that these viruses become more cytopathic over time. R5 using viruses collected from AIDS patients are more resistant to inhibition by RANTES, the natural ligand of the CCR5 receptor [250]. This molecule inhibits HIV-1 by competing for the binding to the N-terminal of CCR5, thus this evolution suggest that viruses from later stages of the disease might interact differently with the co-receptor [251]. In fact Karlsson et al. [252] showed that R5 using viruses adapt to bind to CCR5 in a more flexible way, one that possibly favors binding to the ecl2. The authors showed that this translated into an increased binding affinity and correlated with pathogenicity and disease progression.

Thus, our ecl2 derived glycopeptides might neutralize more efficiently viruses from AIDS patients.

While in the process of doing these studies, we found that the human serum, which was added to the cell culture as a source for natural anti-gal antibodies, greatly influenced the viral output. In fact, increasing amounts of heat inactivated human serum sharply enhanced the amount of virus detected. We found that this effect was independent of cell line, virus strain or batch of human serum used. We then tried to study the mechanism(s) by which human serum could be promoting viral infectivity. We found two possible explanations for this effect:

a. Human serum increases/activates three members of the AP-1 family of transcription factors, which can in turn bind to the viral LTR promoter and promote viral transcription (paper III).
b. In TZM-bl cells, the CD4 molecule is not anchored to the cell surface and undergoes continuous internalization and recycling to and from early endosomes. Human serum enhances the extracellular levels of CD4 on these cells by promoting the recycling of the receptor from early endosomes. This effect correlates with a higher viral infectivity and cytopathicity (paper IV).

Although these results are derived from studies in single replication assays, we found that human serum also increases viral replication on the H9, U87, CEM-GFP cell lines and on freshly isolated PBMCs. These assays, however, require several cycles of replication in order for a virus or viral antigen to be detected and therefore other mechanisms might also be operating in these cellular systems. Moreover, the transcription factors and the interaction between transcription regulatory proteins might vary between cell types.

We do not know whether these effects are taking place *in vivo*. However, the results on the increased transcription might warrant a closer evaluation of the protein profile of human serum from individuals with different disease progression (rapid progressors vs elite controllers).

In addition, as many of the current infectivity-cell based assays are performed in the presence of fetal bovine serum rather than human serum, these studies highlight the need for proper controls to avoid bias in the interpretation of results.

10 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In summary the main findings of this thesis were:

1. CD4-derived-Galα(1-3)Gal-conjugated glycopeptides effectively neutralize HIV_{IIIB} and the anti-Gal antibodies present in human serum enhance the inhibition by activating the complement and inducing ADCC.

2. The glycopeptides were only moderately effective in neutralizing primary isolates. However, the combination of both CD4 and CCR5 derived glycopeptides proved to be a useful strategy.

3. Human serum increases HIV transcription in *in vitro* culture cells by activating c-FOS, JUNB and JUND.

4. Human serum increases the CD4 levels of *in vitro* culture cells by enhancing the recycling of the receptor.

Importantly, as many of the current cell-based assays are supplemented with FBS instead of HS, the results from paper III and IV stress the need for proper controls to avoid unintended bias in the interpretation of results from virus pathogenesis studies and neutralization assays.

Future perspectives for the work on the glycopeptides (papers I and II) include:

a. Test the efficacy of the glycopeptides on CCR5 tropic viruses from AIDS patients.
b. The selection of other peptides from e.g. random libraries that may display high binding affinity to gp120.
c. Optimize a sensitive method for detection of the glycopeptides in serum (e.g. mass spectrometry).
d. Study bioavailability and kinetics of the glycopeptides in animals, including α1-3 GT KO mice.

The main future goal for the work on the HS effect on transcription (paper III) is to identify the effector protein. Several actions are under consideration to enable its identification and these involve:

a. Optimization of the sample preparation and fractionation using newer kits and columns for removal of abundant proteins, as well as, more powerful separation techniques such as HPLC SEC-columns, instead of gravity assisted flow columns.
b. Use of mass spectrometry for the detection of protein candidates and test of individual proteins on our cell assays.
c. Serum proteomics on sera from rapid progressors vs elite controllers.

11 POPULÄRVETENSKAPLIG SAMMANFATTNING

Human immunodeficiency virus (HIV) är det virus som orsakar AIDS. En HIV-partikel består av en bit genetisk information (i detta fall i form av RNA), som inkapslats i ett proteinskal och utanför detta en membranstruktur kallat hölje. Det första steget när en HIV-partikel skall infektera en cell är att virus höljeprotein gp120 binder till proteinet CD4, som finns framför allt på T-hjälparcellers yta. Efter bindning undergår gp120 en serie ändringar av sin struktur vilka exponerar inre epitoper, strukturer som kan ge upphov till och binda antikroppar. Strukturförändringen av gp120 möjliggör bindning av proteinet till de så kallade co-receptorerna (CCR5 or CXCR4). Denna sekundära bindning ger ytterligare förändring av gp120s struktur vilket gör att fusionsproteinet gp41, som till skillnad från gp120 är förankrat i virus hölje, nu kan sträckas ut och med sin andra ända binda till cellens plasmamembran och därmed starta en fusion mellan virus hölje och plasmamembranet. Det är således uppenbart att om man med antikroppar blockerar något av dessa steg hindrar man virus att ta sig in i cellen och infektera den. Emellertid gör såväl den stora variationen i virus höljeprotein som det faktum att detta protein är kraftigt glycosylerat (täckt med sockerstrukturer) att gp120 är ett dåligt och undflyende immunogen. Därför har ingen av alla ansträngningar att direkt använda detta protein som vaccin varit lyckosamma.

I denna avhandling presenterar jag experimentella resultat från en ny metod att blockera upptaget av HIV typ 1 (HIV-1) i celler och därmed förhoppningsvis dess spridning. Vi har använt peptider (syntetsikt framställda små bitar av protein) motsvarande den region av CD4 som binder gp120. Vi testade dessa peptiders förmåga att tävla med receptorn för bindning till gp120 och därmed hindra virus inbindning till och infektion av celler. För att öka dessa peptiders antivirala förmåga och möjligen deras biodynamik kopplade (konjugerade) vi till dem di-sackariden galα(1,3)gal. Denna di-sackarid attraherar naturliga antikroppar som är specifikt riktade mot denna di-sackarid och som finns i hög koncentration i blodet hos alla människor. Tanken var att antikropparna skulle binda till den ena ändan av peptiderna och den andra ändan skulle binda till virus eller virusinfekterade celler, det vill säga att de konjugerade peptiderna skulle verka som adaptorer mellan virus/virusinfekterade celler och de naturliga antikropparna och därmed sätta igång en immunologisk förstörelse av virus och/eller virusinfekterade celler. Vi kunde visa att de CD4-deriverade peptiderna kopplade till galα(1,3)gal kunde neutralisera HIV-1-infektionen och att i närvaro av humanserum som innehåller anti-

galα(1,3)gal-antikroppar hindrades infektionen ytterligare på grund av aktivering av komplementsystemet och så kallad antikroppsberoende cell-cytotoxicitet (ADCC). Vid den senare binder naturliga mördarceller (NK-celler) till de anti-galα(1,3)gal-antikroppar, som via de konjugerade peptiderna i sin tur bundit till HIV-1-infekterade celler. Dessa infekterade celler dödades då av NK-cellerna. Vi visade detta först med virusstammen HIV_{IIIB} som i laboratorium adapterats till T-celler (arbete I) och vi använde etablerade cell-linjer i dessa försök. Därefter testades de konjugerade peptiderna mot färska patientisolat och deras förmåga att hindra dessa virus att infektera T-celler från blodgivarblod (PBMC) för att mer efterlikna den faktiska *in vivo*-situationen (arbete II). Vi testade sex olika primära isolat, vilka alla tillhörde olika subtyper av HIV-1 och med olika co-receptorutnyttjande. Vi fann att cirka 100 gånger högre koncentration av peptiderna behövde användas för att få virusneutralisation och att trots det var inhibitionen modest. Vi konstruerade nya peptider i avsikt att öka deras inbindningsförmåga till gp120 och vi syntetiserade också tre peptider, som motsvarade den extracellulära delen av CCR5. Åter fann vi att neutralisationsförmågan hos enskilda peptider inte var tillräcklig men vid kombination av peptiderna motsvarande delar av CD4 och CCR5 gav bättre resultat och att detta är en framkomlig väg, framför allt om man med ny teknik kan ta fram peptider som binder starkare till gp120.

Under arbetet med att testa de konjugerade peptiderna fann vi att närvaro av humanserum, som källa för anti-galα(1,3)gal-antikroppar, i odlingsmediet i sig ökade HIV-1-produktionen på ett dos-beroende sätt (arbete III). Denna ökning var reproducerbar i såväl olika cell-linjer som i färskt isolerade PBMC från blodgivarblod. Vi använde två olika typer av modellsystem där virus bara tilläts en replikations-cykel, TZM-bl-celler och ACH-2 celler, för att utröna vilket steg i replikations-cykeln som var upp-reglerad av närvaron av humanserum. Vi fann att ett protein (~ 250-300 kDa stort) var ansvarigt för infektionsökningen. Proteinet aktiverar tre medlemmar av den så kallade AP-1 familjen av transkriptionsfaktorer, vilka i sin tur kan öka transkriptionen av virus provirus-DNA på dess promoter-nivå, det vill säga den del av virusgenomet som kallas LTR. Punktmutationer i de AP-1-bindande delarna av LTR konfirmerade den specifika rollen dessa proteiner har i den humanserum-inducerade ökningen av HIV-1-replikationen (arbete III). Vi kunde i ett ytterligare arbete (arbete IV) också visa att humanserum hos vissa celler odlade *in vitro* gav en dos-beroende ökning CD4-uttrycket vilket kan öka HIV-1s replikation och sjukdomsalstrande effekt i dessa celler.

Vi kunde visa att denna effekt på CD4 berodde på en ökad återvinning av proteinet från de tidiga endosomerna till cellytan.

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