Herpesvirus-induced glycans

Selectin ligands and related carbohydrate structures on the surface of the infected cell

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To my family

ABSTRACT

Human herpesviruses are usually acquired early in life and are widely distributed in the population. A common feature of all human herpesviruses is that they persist in the host after the primary infection. Thus, the host immune system resolves the acute stage of the infection but these viruses have evolved means to remain in a state of latency in some cells from which they occasionally reactivate into a state of replication. A functional immune system will clear these episodes and the clinical manifestations are therefore usually mild or absent. On the other hand, when the immune system is dysfunctional the herpesviruses pose a serious threat. Especially cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are associated with severe infections in transplant patients and other immunosuppressed patients, where infiltration of virus-infected leukocytes into organ tissue can give rise to pneumonia, hepatitis and renal failure.

The mechanism behind organ colonization of herpesvirus-infected leukocytes is not clear. However, the normal pathway for leukocyte transmigration over the endothelial wall is well characterized and involves interaction between carbohydrate binding proteins, selectins, and selectin ligands, including the Lewis antigen sialyl Lewis X (sLeX). The selectin ligands are therefore potential targets in viral pathogenesis and we have previously demonstrated that several herpesviruses can in fact activate the cellular pathway for synthesis of sLeX and related structures. In this work we aimed at defining the mechanism behind herpesvirus-induced selectin-ligand expression using herpes simplex virus type 1 (HSV-1) as a model virus. Moreover, we aimed at establish a model system for studying the effects of CMV and EBV infections on selectin ligand synthesis in leukocytes.

We determined that sLeX expression in HSV-1 infected fibroblasts depends on viral RNA transcription and the cellular protein kinase R, an antiviral protein complex that detects small double stranded RNA fragments generated by transcription of HSV-1 genes. We also found that the mechanism for HSV-1-induced expression of sLeX in T-lymphocytes was dependent on viral early protein synthesis, contrary to the situation in fibroblasts. Selectin ligands are expressed on glycoproteins in the cell and we found that sLeX can also be displayed on virus-encoded glycoproteins in fibroblasts. Preliminary data suggests that CMV and EBV also can manipulate the cellular machinery for selectin-ligand synthesis in leukocytes.

Patients with supressed immune system are always at risk of developing severe CMV or EBV disease and are therefore carefully monitored for viral DNA in the blood. Unfortunately the viral load does not always correlate to disease progression and the patients risk severe complications. It is possible that selectin-ligands comprise a new set of diagnostic tools that can be used in parallel with traditional PCR based methods for better prediction of CMV/EBV disease progression. It is also possible that selectin-ligands are new targets for antiviral treatment and several substances, which block interaction with selectins, are already in clinical trials for evaluation of their anti-metastatic potential.

SAMMANFATTNING PÅ SVENSKA

Herpesvirus är en grupp av DNA-virus av vilka åtta stycken infekterar människor. Dessa delas in i tre underfamiljer, dels alfaherpesvirus herpes simplex virus typ 1 och typ 2 (HSV-1 och HSV-2) samt varicella zoster virus (VZV), dels betaherpesvirus cytomegalovirus (CMV) och Herpesvirus 6 och 7 (HHV6 och HHV7), och slutligen Epstein-Barr virus (EBV) och Kaposi´s sarcoma-associerat herpesvirus (HHV8) vilka är gammaherpesvirus. De flesta människor infekteras tidigt i livet och så många som 70-90 procent av befolkningen bär på ett eller flera herpesvirus då dessa inte lämnar kroppen efter den primära infektionen. I vanliga fall innebär detta inte några komplikationer då ett fungerande immunförsvar håller dessa herpesvirus i schack och symptomen vid den primära infektionen så väl som vid eventuell återaktivering av viruset är då milda eller obefintliga. Då immunförsvaret är funktionellt nedsatt, t.ex. vid transplantationer, utgör dessa virus, speciellt CMV och EBV, däremot ett potentiellt livsfarlig hot. Den delikata balansen mellan virus och immunceller rubbas och viruset får möjlighet att föröka sig i kroppen med ökade virusnivåer i vita blodceller som följd. Dessa infekterade vita blodceller kan transportera viruset till olika organ där det kan föröka sig och orsaka potentiellt livsfarliga skador.

Det är inte klarlagt hur virusinfekterade celler kan lämna blodbanan för att infiltrera olika organ. Den normala mekanismen som vita blodceller använder för att påbörja transporten över blodkärlsväggen är däremot välstuderad och innefattar att cellen uppvisar speciella kolhydrater (selektin-ligander) på cellytan, vilka binder till proteiner (selektiner) som sitter på kärlväggens celler. Kontakten mellan dessa påbörjar processen som leder till att blodcellen kan tränga ut i vävnaden som omger blodkärlet. Detta är normalt en välreglerad mekanism vilken förhindrar ospecifikt läckage av celler och endast tillåter aktiverade vita blodceller att lämna blodcirkulationen. Som många andra cellulära processer kan även denna kopplas till olika sjukdomsförlopp, det är t.ex. välkänt att cancerceller kan uttrycka selektin-ligander vilket korrelerar med förmågan att orsaka metastaser och därmed försämrar prognosen avsevärt. Det är möjligt att herpesvirus också kan utnyttja selektin-ligander för att via blodceller sprida sig i kroppen.

Uttrycket av selektin-ligander regleras framförallt av en typ av enzymer som katalyserar den sista överföringen av monosackariden fukos vilket genererar en komplett kolhydratstruktur. Generna som kodar för dessa enzym uttrycks normalt sparsamt i kroppens celler men det är tidigare visat att herpesvirus kan aktivera dem vid infektion i bindvävsceller. I detta arbete definierade vi några

av mekanismerna bakom HSV-1- aktivering av selektin-ligander både i bindvävsceller men även i speciella vita blodceller, T lymfocyter. Dessutom etablerade vi nya cellmodeller för studier av CMV- och EBV-infektion och visade att även dessa virus har förmågan att aktivera systemet för selektin-ligand uttryck vid infektion i kliniskt relevanta celler.

CMV- och EBV-infektioner är vanliga hos personer med nedsatt immunförsvar, så som transplantationspatienter. Dessa patienter kontrolleras därför regelbundet för nivåer av CMV- och EBV-DNA i blodet. Tyvärr överensstämmer inte nivåerna av virus alltid med det kliniska förloppet vilket innebär att det är svårt att förutsäga vilka patienter som riskerar allvarliga komplikationer av sina virusinfektioner. Det är möjligt att selektin-ligander utgör en ny metod som kan komplettera de traditionella mätningarna för att bättre förutse sjukdomsförloppet hos CMV och EBV infekterade patienter. I förlängningen är selektin-ligander potentiellt ett nytt mål för antivirala läkemedel. För närvarande finns det flera substanser vilka blockerar bindningen mellan selektin-liganden och dess receptor. Det är möjligt att de här substanserna kan användas för att hindra spridning av herpesvirus-infekterade vita blodceller hos patienter med nedsatt immunförsvar. Flera av dessa är redan under klinisk prövning för blockering av metastaser hos cancer-patienter.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I.   Nyström K, **Nordén R**, Muylaert I, Elias P, Larson G, Olofsson S. Induction of sialyl-Lex expression by herpes simplex virus type 1 is dependent on viral immediate early RNA-activated transcription of host fucosyltransferase genes. *Glycobiology* 2009 Aug;19(8):847-59.

II. **Nordén R**, Nyström K, Olofsson S. Activation of host antiviral RNA-sensing factors necessary for herpes simplex virus type 1-activated transcription of host cell fucosyltransferase genes FUT3, FUT5, and FUT6 and subsequent expression of sLe(x) in virus-infected cells. *Glycobiology* 2009 Jul;19(7):776-88.

III. **Nordén R**, Nyström K, Aurelius J, Brisslert M, Olofsson S. Virus-induced appearance of the selectin ligand sLex in herpes simplex virus type 1-infected T cells: Involvement of host and viral factors. *Glycobiology*. 2013 Mar;23(3):310-21.

IV. **Nordén R**, Nyström K, Adamiak B, Halim A, Nilsson J, Larson G, Trybala E, Olofsson S. Involvement of viral glycoprotein gC-1 in expression of the selectin ligand sialyl-Lewis X induced after infection with herpes simplex virus type 1. *APMIS*. 2013 Apr;121(4):280-9.

CONTENT

ABBREVIATIONS

2-AP – 2-amino purine
6´sulfo-sLeA – 6´sulfo sialyl Lewis A
6´sulfo-sLeX – 6´sulfo sialyl Lewis X
ATF – activating transcription factor
BMT – bone marrow transplant
CHX - cycloheximide
CLM - calicheamicin
CMV - cytomegalovirus
CNS – central nervous system
CREB – CRE-binding protein
C_T – cycle threshold
CTL – cytotoxic T cells
dsRNA – double stranded RNA
EBV – Epstein-Barr virus
ESL – E-selectin ligand
FucT- Fucosyltransferase
GMK – green monkey kidney cell
gC-1 – glycoprotein C-1
gI-1 – glycoprotein I
HELF – human embryonic lung fibroblast
HEV – high endothelial venules
HIV – human immunodeficiency virus
HHV6 – human herpesvirus 6
HHV7 – human herpesvirus 7
HSV-1 – herpes simplex virus type 1
HSV-2 – human herpesvirus type 2
HTLV-1 – human T-lymphotropic virus 1
ICP0 – infected cell polypeptide 0
ICP4 – infected cell polypeptide 4
IKK-2 – IkappaB kinase 2
IL-6 – interleukin 6
IRF-1 – interferon regulatory transcription factor
JNK – c-Jun N-terminal kinase
KSHV – Kaposi´s sarcoma-associated herpesvirus
LAT – latency associated transcripts
LeA – Lewis A
LeB – Lewis B
LeX – Lewis X
LeY – Lewis Y

LPS - lipopolysaccharide
MOI – multiplicity of infection
mRNA – messenger RNA
NF-kB – nuclear factor kB
ORF – open reading frame
PBMC – peripheral blood mononuclear cell
PFU – plaque forming units
PHA – Phaseolus vulgaris phytohemagglutinin
PKR – protein kinase R
PMN - polymorphonuclear
PSGL-1 – P-selectin glycoprotein ligand 1
PTLD – post transplant lymphoproliferative disease
qPCR – real time PCR
rRNA – ribosomal RNA
RT-qPCR – reverse transcription real time PCR
sLeA – sialyl Lewis A
sLeX – sialyl Lewis X
SOT – solid organ transplant
STAT - Signal Transducers and Activators of Transcription
T-bet – T-cell-specific T-box transcription factor
TEL – transcripts expressed in latency
TGF – tranforming growth factor
Th – T helper cells
TLR3 – toll like receptor 3
TNF-α - tumour necrosis factor – alpha
VZV – varicella zoster virus

1 PREFACE AND AIMS

Human herpesviruses (formally *Herpesviridae*) constitute a family of large enveloped DNA viruses whose major hallmark is that they after symptomatic or asymptomatic primary infection establish a life-long persistent infection, called latency, in the infected subject [1]. The latent herpesvirus constantly challenge the immune system during the life-time of the infected individual and occasionally reactivate into a replicative phase, which may or may not cause clinical symptoms [2]. Thus, the state of latency is maintained by an intricate interplay between the herpesvirus and the host immune system, controlling the infection and suppressing reactivation [3]. The human herpesviruses only rarely cause severe symptoms in individuals with a functional immune system [4].

In contrast, owing to the significance of an intact immune system for battling primary as well as recurrent infections, herpesviruses often pose a serious threat for the many categories of immunocompromised individuals, such as transplant or cancer patients. In this context, especially two herpesviruses, Epstein-Barr virus and cytomegalovirus (EBV and CMV respectively) entail great risks for the immunocompromised individual [5]. Both of these viruses are considered to be blood borne as they are found in white blood cells, leukocytes, during the latent phase as well as after reactivation. CMV disease in transplant patients usually presents with fever and related symptoms but in 10 to 30% of the cases these patients risk severe end organ disease, including hepatitis, pneumonitis and renal failure [6]. For EBV infection in individuals with compromised immune system the most feared consequence is post transplant lymphoproliferative disorder (PTLD), which is a potentially life-threatening neoplasm [7].

End organ disease caused by CMV and EBV in immunocompromised patients occurs in patients with high viral load detectable in the blood and can affect all organs [8, 9]. However, as high levels of virus alone is not sufficient for development of end organ disease it appears that some other confounding factor is required for colonization of the target organ by circulating virus or virus-containing leukocytes [10]. The nature of any such factor has for long been unknown, but data from our laboratory suggests that herpesvirus infection of a cell stimulates exposure of signal molecules identical with those used by activated leukocytes of different types for leaving the blood stream via transmigration.

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These signals can comprise of carbohydrates expressed on proteins, which interact with carbohydrate-binding proteins known as selectins on the endothelium, which is the first and essential step during transmigration. The induction of ligands for selectin binding on the infected leukocyte would be an efficient way to manoeuvre the cell out of the circulation.

We hypothesize that herpesviruses can manipulate the normal tools utilized by leukocytes for targeting peripheral organs and thereby facilitate viral dissemination.

The aims of the present thesis are to:

1. Define molecular mechanisms -with respect to host as well as viral effectors- by which herpesviruses can induce selectin ligands in the virus-infected cell

2. Identify possible viral glycoproteins that may serve as additional carriers for selectin ligands.

3. Characterize virus-induced selectin ligands and related glycoepitopes in different types of leukocytes infected/immortalized by herpes simplex virus type 1 (HSV-1) and blood-borne herpesviruses.

4. To determine whether herpesviruses-induced expression of selectin ligands and related structures, as observed in cell culture, also are occurring in clinical specimens from herpesviruses-infected, immunosuppressed patients.

5. To explore the possibility that measurements of selectin ligands or related structures can be used as a laboratory diagnostic complement to current monitoring of viral DNA levels for improved handling of CMV and EBV infections.

2 BACKGROUND

2.1 Herpesviruses

2.1.1 Human herpesviruses: Structure and basic properties

Human herpesvirus (*Herpesviridae*) is a family of large enveloped double stranded DNA (dsDNA) viruses of which eight members have humans as their natural host (Table 1). The herpesvirus particle has a diameter of about 200 nm and consists of an icosahedral nucleocapsid of 162 capsomers that is surrounded by a double phospholipid bilayer envelope (Fig. 1) [11]. All members have a relatively large genome size ranging from 120kb to 250kb and encode between 70 and 200 genes (Table 1). Herpesvirus gene expression is strictly regulated starting with expression of immediate early alpha (α) genes that encode proteins that activate subsequent viral gene expression as well as proteins that interfere with the cellular antiviral response. This is followed by expression of early (β) genes encoding proteins important for viral replication (i.e. DNA synthesis) and thereafter, transcription of leaky late (γ_1) genes and finally true late (γ_2) genes that encode structural proteins including glycoproteins [1, 12, 13]. Of special relevance for the present study are the ten or more different glycoprotein species that are located in the envelope of the viral particle, and later in the infectious cycle also at the cell surface and other membranes of the infected cell [11, 14]. The organization of these glycoproteins resemble that of normal cellular glycoproteins, but the genetic information for the polypeptide sequence is derived from the viral genome [1].
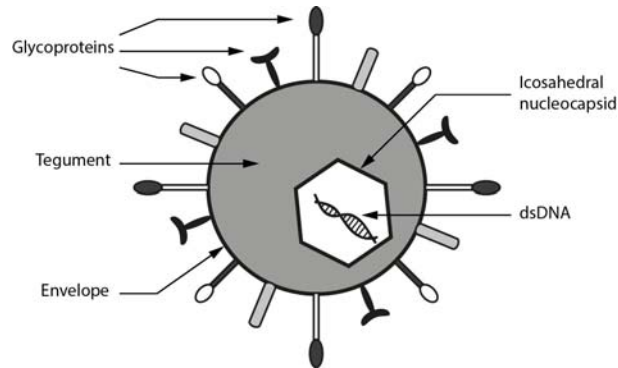
Figure 1. Structure of a herpesvirus particle. The linear dsDNA is enclosed in an icosahedral capsid that is assembled in the nucleoplasm of the infected cell. The nucleocapsid and the proteins that constitute the tegument are assembled at vesicles in the cytoplasm and finally surrounded by an envelope that carry the glycoproteins. The final enveloped viral particle is transported inside a vesicle and is released upon fusion of the vesicle with the plasma membrane [11].

2.1.2 Human herpesviruses, similarities, differences and tropism

Herpesviruses are divided into three subfamilies, alpha- (α), beta- (β) and gamma- (γ) *herpesvirinae* on the basis of their biological properties (Table 1) [1]. A characteristic feature common to all herpesviruses is that after primary infection – often during childhood- a latent phase is developed that persists lifelong in the infected host. The host reservoir for latent viruses differs dependent on the subfamily belonging of a herpesvirus (Table 1). Occasionally, latent virus is reactivated resulting in recurrent symptomatic or non-symptomatic productive infections. One explanation behind this coexistence between the herpesviruses and their host is a profound ability of these viruses to modulate the host immune response and, in fact, the function of the majority of proteins encoded by each of the large herpesvirus genomes is to interfere and interact with different immune effectors, thereby promoting viral persistence in its host [15-21]. There are several common characteristics shared by human herpesviruses; utilization of the same strategy for replication with a strictly controlled transcription program during a productive infection, they encode different glycoproteins that are abundantly expressed both in the viral particle and at the surface of cells during a productive infection, and they can establish and maintain a latent infection by expressing dedicated latency associated transcripts (LATs). Despite these similarities, important differences can be found between the subfamilies and also within each subfamily.

The alpha (α) herpesvirus subfamily contains herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV). All three cause a primary infection of mucoepithelial cells and later establish a persistent residence in sensory neuronal cells (Table 1). They are therefore referred to as neurotropic viruses, a characteristic distinct from the beta (β) and gamma (γ) herpesviruses. Upon reactivation the α-herpesviruses assemble new viral particles that are transported anterograde through the neuronal cell to infect dermal cells [22]. The most striking difference between the individual alpha herpes viruses is that HSV-1 and HSV-2 cause a local primary infection with a few blisters, cold soars, and with episodes of reactivation occurring essentially at the same location as the first infection, while during VZV primary infection the virus is consecutively spread to local immune cells or cells of the lymphoid system (i.e. white blood cells, leukocytes) [23, 24]. Trafficking of infected T lymphocytes to the skin enables VZV to cause a secondary infection in the form of pox lesions that can cover the entire body [24, 25]. Reactivation of VZV causes a dermatomal-distributed herpes zoster commonly referred to as shingles. Occasionally the α-herpesviruses can also cause severe infection of the central nervous system (CNS). HSV-2-infection can cause primary and

recurrent lymphocytic meningitis, while VZV and HSV-1 primarily cause encephalitis leading to severe neurological complications [26, 27].

The members of the beta (β) herpesvirus subfamily address a wide range of target cells, including fibroblasts, endothelial cells and leukocytes, in which the virus can replicate and thereby cause a lytic infection (Table 1). The primary infection by cytomegalovirus (CMV) appears to occur in mucoepithelial cells of the oral cavity although the exact location is not known [28, 29]. Further viral spread is dependent on white blood cells, leukocytes, and vascular endothelial cells. A subset of leukocytes, myeloid hematopoietic cells (progenitor cells to monocytes), as well as salivary and kidney epithelial cells are believed to be the main reservoirs for harbouring CMV during latency [3, 30]. Infections with CMV are asymptomatic or mildly symptomatic, characterized by fever and malaise both during primary and recurrent infection [9, 31]. The remaining herpesviruses of the beta subfamily share the target cell promiscuity with CMV but the entry pathway for human herpesvirus 6 and 7 (HHV6 and HHV7) is not known. The preferred cell types for establishing a latent infection by these viruses are CD4+ T-lymphocytes, salivary epithelial cells or myeloid lineage hematopoietic cells [3].

The members of the gamma (γ) herpesvirus subfamily include Epstein-Barr virus (EBV) and Kaposi´s sarcoma-associated herpesvirus (KSHV). The former is believed to initially infect mucoepithelial cells in the oral cavity while it is not known for the latter (Table 1). In contrast to the α- and β-herpesviruses, EBV and KSHV favour B-lymphocytes for further spread in the host. A distinguishing feature of the primary EBV-infection is that the virus enters the lymphoid tissue of the pharynx and infects resting B cells, which become dividing lymphoblasts. This situation is referred to as infectious mononucleosis (IM) and is characterized by a high number of infected B cells and manifested as fever and swollen lymph nodes [32]. The lymphoblasts then differentiate into resting memory B cells, viral transcription is suppressed and low numbers of latently infected cells continue to circulate in the blood [3, 33]. Both EBV and KSHV are associated with lymphoma originating in B cells, after a prolonged period of immune suppression, which also separates them from the α- and β-herpesviruses [7].

*Table 1. Human herpesviruses: basic properties *

Type	Common name	Genome size	Primary target	Site of latency	Seroprevalence Adults worldwide **
α-herpesvirinae					
HHV-1	Herpes simplex virus type 1 (HSV-1)	~152kb (~90 genes)	Mucoepithelial cells (predominantly orofacial tract)	Sensory and cranial nerve ganglia	50-90%
HHV-2	Herpes simplex virus type 2 (HSV-2)	~154kb (~90 genes)	Mucoepithelial cells (predominantly genital tract)	Sensory and cranial nerve ganglia	20-60%
HHV-3	Varicella zoster virus (VZV)	125kb (>70 genes)	Mucoepithelial cells and T cells	Sensory and cranial nerve ganglia	50-95%
β-herpesvirinae					
HHV-5	Cytomegalovirus (CMV)	~235kb (~213 genes)	Epithelial cells, monocytes, fibroblasts and more	Monocyte progenitor cells, kidney epithelial cell and others	40-80%
HHV-6 (A and B)	Roseolovirus	~160kb (~88 genes)	Epithelial cells, monocytes, fibroblasts and more	Mainly monocytes and macrophages	60-100%
HHV-7	Roseolovirus	~160kb (~97 genes)	Epithelial cells, monocytes, fibroblasts and more	CD4+ T cells	40-100%
γ-herpesvirinae					
HHV-4	Epstein-Barr virus (EBV)	172kb (~85 genes)	Mucoepithelial cells, B cells	Memory B cells	80-100%
HHV-8	Kaposi´s sarcoma-associated herpesvirus (KSHV)	~145kb (>87 genes)	n.d. [‡]	B cells	3-50%[‡‡]

* Adapted from [1] and [34])

** Differences in seroprevalence occur between different socioeconomic populations and geographical areas

[‡] Not determined

[‡‡] No approved assays are currently available

2.1.3 Interplay between the herpesvirus and a functional immune system

The infectious course of herpesviruses can be divided into three different stages starting with acute primary infection, which is followed by establishment of latency and later episodes of reactivation. The acute infection is usually resolved by the concerted action of the innate and adaptive immune response. However, the virus is not cleared from the body because of the viral ability to persist in the host despite a functional immune system, which results in a life long symbiotic relationship [3, 35]. For example, CMV and EBV as well as HSV-1/2 all have evolved means to interfere with the processing steps of major histocompability complex (MHC) class I and II antigen presentation, thereby protecting the infected cell from CD8+ cytotoxic T cells (CTL) and CD4+ T helper cells (Th) respectively [36]. With a functional immune system this complex interplay between the virus and the cells of the immune system ensure that the virus pool is maintained but also confined [16, 32, 37]. Consequently, herpesviruses alter their gene expression when establishing a latent infection, suppressing genes important for lytic replication and inducing latency associated transcripts (LATs) also called transcripts expressed in latency (TELs) [2, 30, 38]. For a long time a latent herpesvirus infection was regarded as totally dormant, with a quiet viral genome and a resting immune system [39]. This view is being abandoned as recent reports indicate actively on-going immune responses to various herpesviruses also in asymptomatic individuals [3, 40]. For example, a large proportion of the T cell pool is directed towards herpesviruses regardless of clinical symptoms and as much as 30% of the total amount of CD8+ T cells have a phenotype towards CMV, EBV and HSV-1 even in the absence of an active infection [3, 41-43]. Also, virus-specific CD4+ T cells are important not only for resolving the acute infection but also for controlling latent infection, and this pool of CD4+ and CD8+ T cells are constantly surveying the infected cells for markers of infection, specifically epitopes derived from TELs [7, 18, 32, 44-48]. It is now clear that in a subset of the "latently" infected cells the virus frequently reactivates and re-enters a replicative state. Contrary to the picture of herpesviruses being quiet viruses that only rarely give episodes of reactivation it now becoming more and more evident that they actually are constantly probing the immune system, causing subclinical infections that are eventually cleared by a functional immune system.

2.1.4 Herpesvirus infections in immunocompromised patients

In individuals with a suppressed immune system the herpesviruses ability to interact with and partially circumvent the immune response becomes a deadly threat. In this respect CMV and EBV are especially problematic, and

associated with severe complications primarily as a consequence of primary infection but also during viral recurrence [10, 34, 49, 50]. The seroprevalence for herpesviruses is high already early in childhood, increasing with age and reaching almost 100% for EBV and 70% for CMV in adults in developing countries, although large differences occur between socioeconomic populations and also between geographical areas (Table 1) [51-54]. As the herpesviruses are so widely spread within the human population the risk of either reactivation or primary infection is extremely high in patients with a defective immune system.

2.1.5   CMV infections in immunocompromised patients

In patients undergoing transplantation procedures or cancer treatment and in patients with other immune deficiencies (e.g. infection with human immunodeficiency virus - HIV) CMV is a constant menace resulting in frequent episodes of viremia, characterized by a high number of circulating infected leukocytes [10]. In solid organ transplant (SOT) patients invasive CMV disease usually occurs within the first year and is most often characterized by fever, weakness, myalgia and myelosuppression [9]. In some patients the infected leukocytes leave the circulation, causing secondary infections that lead to the development of life-threatening end-organ disease, which can affect several organs leading to pneumonitis, hepatitis, carditis, colitis, encephalitis, retinitis or nephritis [55]. Infection with CMV also has indirect effects associated with allograft injury and rejection, increased risk for additional infections and increased risk of EBV-associated post transplant lymphoproliferative disorder (PTLD) [56].

Due to high incidence of CMV infection during SOT (up to 75%), the patients are regularly monitored for CMV load in the blood [10]. This is usually done by qPCR with standardized thresholds and once the level of viral genomes rises above the limit, treatment with antivirals (valgangciclovir and ganciclovir) is started [57]. Antiviral treatment, both with ganciclovir and valganciclovir, is effective and the intervention strategy is aimed at only capturing the patients who are at risk of developing CMV disease instead of administering universal antiviral treatment, thereby lowering the risk of emergence of drug resistant CMV strains and reducing the risk of adverse effects of the medication [58-60].

2.1.6   EBV infections in immunocompromised patients

In most cases EBV infections in patients with a suppressed immune system will progress with mild symptoms including fever, malaise and infectious mononucleosis (IM). However, during the first year after transplantation some

patients will develop PTLD, a potential life-threatening neoplasm [49]. PTLD is a collective name for a diverse group of lymphoproliferative disorders that occur in 0.3-12.5% of allogenic bone marrow transplantations (BMT) patients and SOT patients, based on the type of organ transplanted and amount and type of immune suppression employed [61]. PTLD histopathologies include polyclonal lymphoid infiltration where EBV infected leukocytes can be detected in extravasal tissue [62]. Development of PTLD is highly associated with EBV, and viral genomes are found in over 90% of transformed B cells during PTLD in the first year after solid organ transplantation [61]. Also, the incidence of PTLD is higher in adolescent and young transplant recipients than in adults, which is largely explained by their 60-80% sero-negative status towards EBV [7].

In patients with a suppressed immune system EBV also greatly enhances the risk of developing various types of lymphoma that are dependent on expression of transcripts expressed in latency (TELs) and small nuclear RNAs (e.g. EBER1 and 2) [63, 64]. EBV associated lymphomas have a particularly high incidence in HIV infected individuals: in HIV patients with Hodgkin´s lymphoma (HL) almost all cases are associated with EBV and in patients with AIDS and Burkitt lymphomas the association is 40% [7]. The clinical outcome of PTLD varies; some lesions diminish after decrease of immune suppression, whereas more aggressive treatment might be required, especially after bone marrow transplantation the disease most often follows an aggressive course that in many cases is fatal [65].

In contrast to the situation for CMV infections, no general antiviral treatment is available for EBV although circumstantial reports suggest that ganciclovir and even ribavirin could be effective [66, 67]. Replication of latent EBV in proliferating B cells does not rely on viral DNA polymerase rendering the ganciclovir type of drugs ineffective. In the absence of effective antiviral treatment several immune modulatory and immune cell therapies have been tried and the most successful seem to be infusion with EBV-specific cytotoxic T lymphocytes (CTLs) [68-70].

2.1.7 Herpesvirus colonization of extravasal organs – unanswered questions

The most severe complications in both CMV and EBV infections are associated with virus-infected leukocytes leaving the circulation for infiltration of extravasal tissue [5, 71]. Despite this, the mechanisms behind organ colonization of virus-infected leukocytes remain largely unknown. However, the normal mechanism for leukocyte recruitment to extravasal tissue during

inflammation is well characterized and it is possible that herpesviruses utilize the same pathway for colonizing organs. Our group recently published evidence that HSV-1, CMV as well as VZV all have the capacity to induce genes relevant for leukocyte transmigration, at least in fibroblasts, supporting this notion [72, 73].

2.2 Normal and pathological colonization of organs by circulating leukocytes

2.2.1 Licensing of leukocytes to pass the endothelial wall

Occasionally, certain leukocytes of the blood stream have to cross the endothelial wall to perform tasks in adjacent tissue, i.e. to combat invading bacteria or viruses. However, the process by which circulating leukocytes may cross the endothelial wall to access adjacent tissue is strictly regulated, not least to prevent uncontrolled and unspecific leakage of white blood cells from the blood stream [74, 75]. This means that only activated or "authorized" leukocytes will be equipped with the necessary tools that enable them to penetrate the endothelial wall to perform their tasks.

Passage of activated leukocytes across the endothelial wall is initiated by the interactions between two actors: Special carbohydrate-binding protein molecules, *selectins*, that reside at the inner endothelial wall, and *selectin ligands*, surface carbohydrate structures that appear selectively in appropriately activated leukocytes and the direct binding target for selectins [76, 77]. Thus, most types of circulating leukocytes cannot leave the circulation until they are stimulated (except for many granulocytes that are constitutively activated), because in their resting state they do not express selectin ligands [75]. Therefore, priming of leukocytes for extravasal tasks must imply activation of the normally switched off mechanism for selectin ligand formation to make them competent for crossing the endothelial wall.

2.2.2 Pathological take-over of selectin-mediated functions

Hostile use of selectin function is an important pathogenic factor in tumour metastasis. Thus, by activation of "false" expression of selectin ligands on circulating tumour cells, several types of tumours succeed in passing endothelial wall for colonization of extravasal tissue [78], a phenomenon that contributes strongly to the metastatic potential of tumour cells. For several tumour types there is a direct correlation between the intensity of cell surface selectin ligand expression and the metastatic potential [79-82]. This type of hijacking of selectin functions has also been found to enhance the tissue invasiveness of a virally induced tumour, adult T-cell leukaemia that is caused by a retrovirus, human T-lymphotropic virus type 1 (HTLV-1). Thus, during viral transformation of virus-infected T cells to tumour cells, HTLV-1 activates constitutive expression of selectin ligands thereby promoting spread of circulating tumour cells across the endothelium for colonization of skin tissue targets [83-85]. Interestingly, the mechanism by which HTLV-1 induces expression of selectin ligands on virus-transformed cells and corresponding virus-induced expression in CMV-infected cells share many similarities [73, 84, 85], supporting the notion that this phenomenon may have implications for spread of herpesvirus-infected leukocytes in immunocompromised patients (Fig. 2).
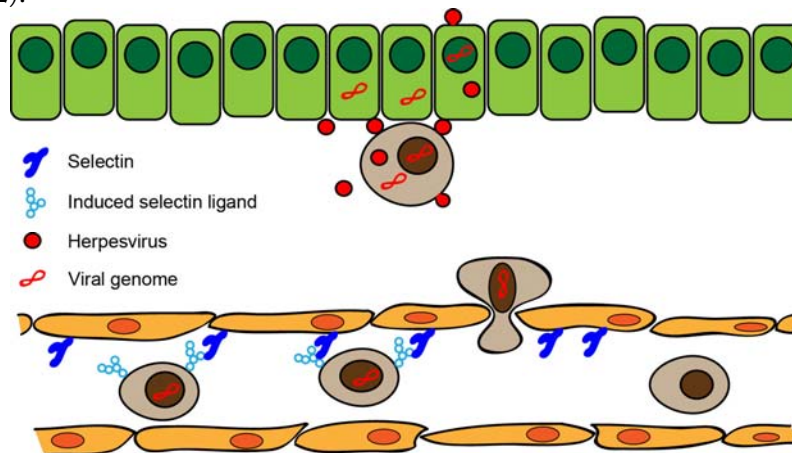
Figure 2. Hypothetical mechanism for spread of herpesvirus-infected leukocytes, based on published models for how human tumour viruses induce spread of virus-transformed leukocytes by fraudulent activation of the natural mechanism by which normal, activated leukocytes transmigrate across the endothelial wall [85]. The herpesvirus induces selectin ligands on the cell surface of infected leukocytes (See present study (III)). The endothelium (orange cells) carries selectins that bind the selectin ligands on the "authenticated" virus-infected leukocyte. This interaction enables further contact between the leukocyte and the endothelial cells and subsequent transmigration across the endothelial wall. The herpesvirus-infected leukocyte may then migrate further into extravasal tissue (green cells) where the virus replicates and infects new cells.

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2.3 Leukocyte migration over the endothelial wall

2.3.1 Selectins

Selectins are carbohydrate-binding proteins that mediate reversible interactions with glycoconjugates enabling tethering and rolling of the leukocytes along the endothelium, a prerequisite for subsequent passage across the endothelial wall. [76, 77]. E-selectin is expressed mainly on endothelial cells and P-selectin, which can be rapidly displayed on platelets and endothelial cells upon stimulation, are the most important selectins for this process [75, 86]. L-selectins are expressed at the surface of many leukocytes, with the capacity to induce tethering of blood cells to each other. L-selectins are also important in a variety of important selectin-dependent activities of circulating leukocytes, e.g. homing to peripheral lymph nodes [87, 88]. E- and P-selectins are expressed at the endothelial wall only after inflammatory stimulation [89]. Thus, upon activation by mediators of inflammation including histamines, tumour necrosis factor α (TNF-α) and lipopolysaccharide (LPS) resting endothelial cells can rapidly mobilize P-selectin to the cell surface from secretory granules [90]. Expression of E-selectin on resting endothelial cells also has to be activated by stimulation with TNF-α, LPS, interleukin-1 or other pro-inflammatory factors [91]. Both E- and P-selectin can support recruitment of appropriately "authorized" T cells, monocytes, dendritic cells and neutrophils to the stimulated endothelium [92-96], priming their transmigration over the endothelium.

2.3.2 Selectin ligands – versatile carbohydrate epitopes

The main ligands for selectin binding expressed on activated leukocytes are carbohydrate epitopes, glycans, belonging to the Lewis family of glycoepitopes (Fig. 3)[97]. The sialyl Lewis X (sLeX) glycoepitope and structural relatives, i.e. sulphated variants, are the most important selectin ligands [98, 99], but hereafter mainly sLeX will be considered for reasons of brevity. Like many other glycoepitopes sLeX can be associated with several types of glycoconjugates, including glycolipids and glycans of surface glycoproteins. There are two major classes of glycans associated with viral as well as host cell membrane proteins, designated N- or O-linked glycans owing to the nature of the linkage between the innermost glycan monosaccharide and the polypeptide backbone (Fig. 4) [100]. Both of these classes may express sLeX [76], but owing to its special relevance for the present study, the present review will focus on sLeX as a constituent of O-linked glycoprotein glycans.
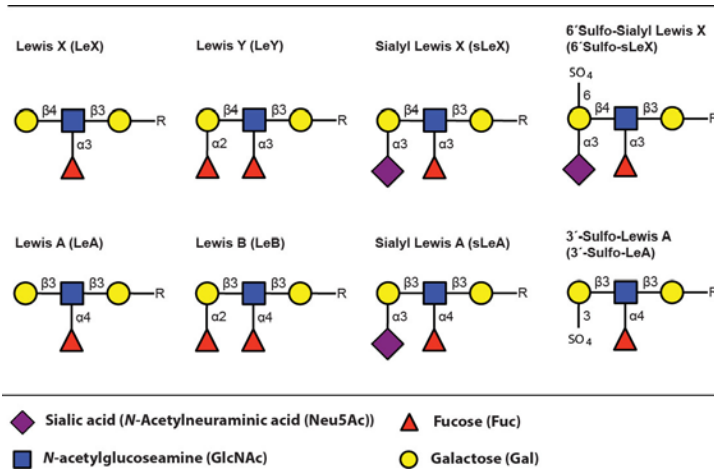
Figure 3. The Lewis glycoepitope family, showing only a subset of possible sulphated variants. The Lewis glycoepitopes are a related set of glycans that carry fucose in an α1–3 (Lewis X, Y) or an α1–4 (Lewis A,B) linkage to the GlcNAc monosaccharide. Sialyl Lewis X (sLeX) and its 6-O-sulfated GlcNAc variants are important for leukocyte recruitment by selectins expressed on the endothelium, both during inflammation and routine homing to lymph nodes.
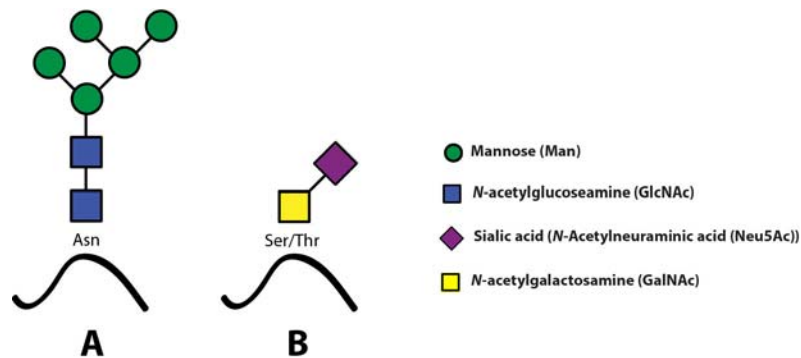
Figure 4. N-linked and O-linked type of glycans. (A) N-linked high mannose structure. N-glycans are associated with the polypeptide chain via a covalent linkage between the innermost N-acetylglucosamine (GlcNAc) of the glycan and a **nitrogen** atom of an aspargine (Asn) residue of the polypeptide. The minimal sequence requirement is Asn-X-Thr/Ser, where X can be any amino acid except for Pro. (B) O-linked sialyl Tn antigen. O-glycans are α-linked to the polypeptide via an **oxygen** atom of the hydroxyl groups of serine (Ser) or threonine (Thr) residues and the innermost N-acetylgalactosamine (GalNAc) of the glycan.

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Table 2. Macromolecules for display of selectin ligand

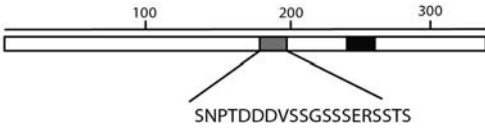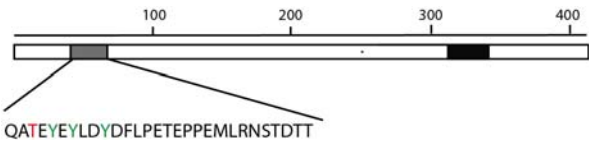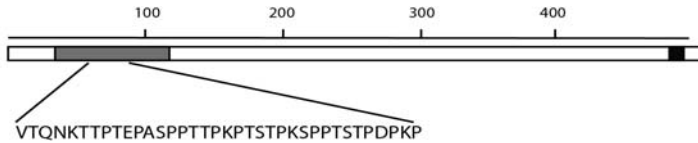| Selectin ligand carrier	Type of glycans	Carbohydrate structure	Selectin binding	Model system
Cellular				
PSGL-1 (P-selectin glycoprotein ligand 1)	O-linked, mucin-like domain, N-linked [101-103]	sLeX [101-103]	E-, L- and P-selectin [91, 104-107]	mouse and human
CD44 (CD44 molecule Indian blood group)	scattered O-linked, N-linked [108-110]	sLeX, sLeA and LeY [111, 112]	E-selectin [108, 113]	mouse and human
ESL-1 (E-selectin ligand-1)	N-linked [114]	Fucose [114, 115]	E-selectin [114, 116]	mouse
CD43 (leukosialin)	n.d. *	n.d. *	E-selectin [117]	mouse and human
Lipids	glycosphingolipid	sLeX, sLeA [118-120]	E-selectin [120]	mouse and human
Herpes simplex virus type 1				
gC-1 (glycoprotein C)	O-linked in mucin-like domain, N-linked	sLeX (present work)	n.d. *	human

* Not determined

2.3.3 Glycoproteins that harbour selectin ligands

Although more than 50% of the human proteome represents proteins that carry glyco-modifications [121], only a handful of proteins (Table 2) are known to present selectin ligands in a way that promotes leukocyte transmigration [122-124]. It is evident that the ability to express the particular carbohydrate epitopes (sLeX or related structures) as constituents of O-linked glycans is a common feature of these glycoproteins (Table 2). For one such protein, P-selectin glycoprotein ligand 1 (PSGL-1), the precise location of the relevant sLeX-carrying O-glycan has been determined (Table 3) [101-103].

Table 3. O–linked glycan–carrying leukocyte and viral glycoproteins with capacity to express sLeX.

Glycoprotein	Graphic Characteristics*	Comment
CD44	SNPTDDDVSSGSSSERSSTS GenBank Access ACI46596.1	E-selectin ligand
PSGL-1	QATEYEYLDYDFLPETEPPEMLRNSTDTT GenBank Access: NP_002997.2	Major ligand carrier for P selectins. Sulphated tyrosine indicated by green letters; sLeX-carrying threonine by red letter.
HSV-1 gC-1	VTQNKTTPTEPASPPTTPKPTSTPKSPPTSTPDPKP Genbank Access: AAA45779.1	

* The amino acid sequence denotes the mucin-like motif of each peptide

Some of the glycoproteins presenting functional selectin receptors contain special mucin-like domains, allowing expression of multiple O-linked glycans along a short peptide stretch (Table 3; detailed below).

The selectin ligands bind to different selectins depending on the macromolecule it is displayed on, i.e. PSGL-1 associated sLeX facilitates binding to all selectins while CD44 carrying sLeX only mediates E-selectin ligand interaction (Table 2 and 3). Hence, the sLeX structure is essential for selectin binding but additional factors also regulate the interaction.

2.3.4 Biosynthesis of selectin ligands associated with O-linked glycans

Posttranslational glycosylation of proteins or sphingolipids requires the synchronous action of roughly 150 enzymes, glycosyltransferases, to a large extent located in the golgi compartment where they catalyse the transfer of activated sugar nucleotide donors to glycoconjugate acceptors, and approximately ten glycosyltransferases are needed for sLeX synthesis [125-127]. The glycosyltransferases have three specificities (i) sugar specificity, i.e. the type of monosaccharide it can transfer, (ii) acceptor specificity and (iii) linkage specificity (Fig. 5) [128]. The "one enzyme – one linkage" hypothesis which postulated that each glycosyltransferase only generates one type of carbohydrate structure is an oversimplification but can help us understand the basics of glycan biosynthesis [128].

The most important steps in the synthesis of an O-linked glycan carrying the sLeX epitope is depicted in Fig 6. Although single O-linked glycans may be scattered along the peptide sequence, several glycoproteins with peptide stretches enriched in Ser, Thr and proline (Pro) residues, referred to as mucin-like proteins, may contain multiple, clustered O-linked glycans. Thus, while the clustered Ser and Thr units serve as glycan carriers, the Pro residues enable access to the Ser and Thr units by "bending" the polypeptide backbone in an appropriate manner for the O-glycosylation machinery [129, 130]. Table 3 presents partial mucin domain peptide sequences of two important human and one viral selectin ligand carriers of glycoprotein nature.

After the first O-linked GalNAc unit is connected to the peptide, the remainder of the O-linked glycan is assembled by the concerted and coordinated actions of sequentially acting glycosyltransferases, each adding a unique monosaccharide in a unique position to the growing glycan [131]. The mode of action and specificities of these glycosyltransferases are illustrated in Fig. 6. Important for the regulation of sLeX synthesis is that essentially all of the glycosyltransferases operating in O-linked sLeX synthesis, except for the last one, are constitutively expressed [132-135], resulting in an accumulation of the direct precursor to sLeX, "the sialylated core 2-precursor" (Fig 6). In contrast, the genes encoding fucosyltransferases carrying out the last step are normally switched off [132, 133]. Consequently, the rate limiting step and hence also the switch mechanism for inducing sLeX formation is activating one or more of these critical fucosyltransferase genes.

$$\text{UDP-Gal} + \text{GlcNAc-R} \longrightarrow \text{Gal}(\beta1\text{-}4)\text{GlcNAc-R}$$

A   **B**   **C**

Figure 5. Specificities of glycosyltransferases. The one enzyme – one linkage [128] postulates that glycosyltransferases have three distinct specificities. (A) Donor sugar specificity, the type of activated monosaccharide the glycosyltransferase is able to transfer. Thus, a given glycosyltransferase can only add one type of monosaccharide. Exemplified here by a uridine diphosphate galactose (UDP-Gal). (B) Acceptor specificity, represented here by a N-acetylglucosamine (GlcNAc) of the growing glycan chain. Sometimes the acceptor specificity includes larger portions of the acceptor glycans than its terminal monosaccharide (Relevant for the present study; see Table 4). (C) Linkage specificity, represented by a beta (β) 1–4 linkage between the donated Gal and the acceptor GlcNAc. In most cases the linkage specificity is absolute but a few exceptions are known, some of which relevant for the present study (See Table 4).
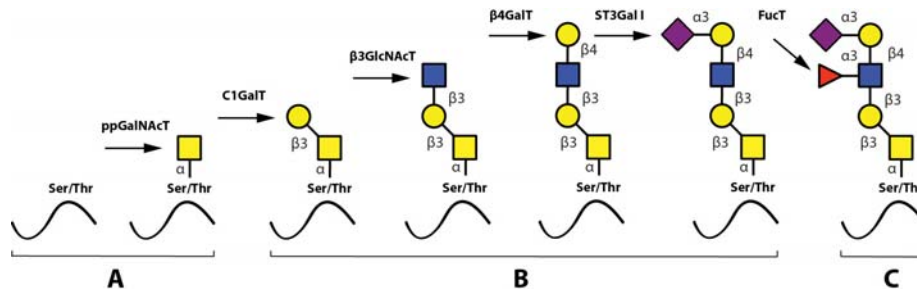

Figure 6. Sequential synthesis of complex O-linked glycans carrying the sLex glycoepitope, adapted from [136]. (A) The initial linkage between an N-acetylgalactosamine (GalNAc) monosaccharide and a serine (Ser) or threonine (Thr) residue is catalysed by any of twenty polypeptide GalNAc transferase (ppGalNAcT) isoenzymes. (B) Subsequent elongation can generate eight different core structures with core 1 and core 2 being the most common. For brevity only a core 1 structure is displayed, which is generated by the addition of a galactose (Gal) by the core 1 β1–3 Galactosyltransferase (C1GalT-1). This reaction generates a specific acceptor for the subsequently acting glycosyltransferase. Thus addition of an N-acetylglucosamine and a galactose by a β1–3 N-acetylglucosaminyltransferase (β3GlcNAcT) and a β1–4Galactosyltransferase (β4GalT) respectively further elongate the chain. The addition of a sialic acid (N-acetylneuraminic acid (Neu5Ac)) by an α2–3 sialyltransferase (ST3GalT) terminates the elongation by creating the direct precursor for sLeX. (C) Under some circumstances a specific fucosyltransferase (FucT) is activated and this opens for the final addition of a fucose (Fuc), resulting in formation of a complex type O-glycan decorated with the sLeX glycoepitope. This glycoepitope are in many cases associated with larger O-linked glycans; the one depicted here is the smallest one with capacity to express sLeX.

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2.3.5　　Regulating the rate-limiting step for sLeX synthesis

Initiation of sLeX formation means activation of the very last rate-limiting addition of fucose to the sialylated core 2-precursor. The human genome contains 13 genes, each encoding an enzyme, fucosyltransferases (FucT), with the capacity to add fucose residues to glycoprotein glycans or peptides (Table 4) [132, 137]. The nomenclature of genes and their products are as follows: The genes are enumerated FUT1-FUT13, whereas the corresponding gene products (enzymes) are designated FucT-I to FucT-XIII. Of these enzymes, only FucT-III, FucT-V, FucT-VI and FucT-VII (encoded by FUT3, 5, 6 and 7) are able to add fucose in the specific alpha 1,3 linkage to the sialylated type 2 precursors, which is a prerequisite to create sLeX [132]. The fine specificities of the FucT:s with the capacity to generate the sLeX-characteristic alpha 1-3-fucosidic linkage varies from enzyme to enzyme (Table 4). For example FucT-V is a promiscuous enzyme, which accepts all four variants of sialylated or nonsialylated core 1 or core 2-precursors. Moreover, this fucosyltransferase can form alpha1-3 as well as alpha 1-4 linkages, should the appropriate precursor be available. Hence, this enzyme is able to synthesize not only sLeX but also Lewis X (LeX), Lewis Y (LeY), sialyl Lewis A (sLeA), Lewis A (LeA) and Lewis B (LeB), depending on the identity of particular precursors available in the tissue expressing FucT-V (Table 4). In contrast, fucosyltransferase VII (FucT-VII) is highly sLeX-specific since this enzyme is able to address only the sialylated core 2-precursor, preventing synthesis of any other structures of the Lewis family of glycoepitopes, provided that no other relevant fucosyltransferase-encoding genes are expressed. Thus, FucT-VII has a narrow specificity and can only synthesize sLeX [132, 137, 138]. FUT4 and FUT7 encode the only fucosyltransferases expressed in leukocytes, determined so far, and both are important for proper selectin ligand display [87, 125, 137, 138]. Loss of FUT4 and/or FUT7 functional gene products significantly affects E- L- and P-selectin dependent binding, at least in mice [124, 139-142].

Normally an inflammatory stimuli of the leukocyte by interleukin 12 (IL-12) and transforming growth factor-β1 (TGF-β1) activate expression of FUT7 and this functions as an on-switch for the synthesis of sLeX in resting leukocytes [143]. Activation-dependent transcription factors, including T-cell-specific T-box transcription factor (T-bet) and CRE-binding protein (CREB)/activating transcription factor (ATF), induce FUT7 transcription upon the stimulation. This process is reversible in normal situations and loss of external stimuli results in down modulation of FUT7 expression and loss of sLeX [87, 144].

Table 4. Genomic location of fucosyltransferase–encoding genes relevant for this study, the linkage specificity, glycan structures synthesised* and cellular expression pattern of each fucosyltransferase [73, 132, 137]

Chromosome location	Gene	Enzyme/ Linkage	Synthesized Type 1 structures	Synthesized Type 2 structures	Expression pattern
19q13.3	FUT1	FucT-I / Fuc α(1,2)	H type 1 structure	H type 2 structure	Mesenchymal cells, Erythroid cells
19q13.3	FUT2	FucT-II / Fuc α(1,2)	H type 1 structure	H type 2 structure	Epithelial cells (Saliva and mucous)
19p13.3	FUT3	FucT-III / Fuc α(1,3/4)	Lewis A, Sialyl Lewis A, Lewis B	Sialyl Lewis X, Lewis X, Lewis Y	Epithelial cells (gastrointestinal tissue and kidney)
11q21	FUT4	FucT-IV / Fuc α(1,3)		Lewis Y, Lewis X	Myeloid cells, embryonic cells, ubiquitous
19p13.3	FUT5	FucT-V / Fuc α(1,3/4)	Lewis A, Sialyl Lewis A, Lewis B	Sialyl Lewis X, Lewis X, Lewis Y	Low level tissue expression; spleen and gastrointestinal
19p13.3	FUT6	FucT-VI / Fuc α(1,3)		Sialyl Lewis X, Lewis X, Lewis Y	Gastrointestinal tissue, kidney, liver and plasma
9q34.3	FUT7	FucT-VII / Fuc α(1,3)		Sialyl Lewis X	Leukocytes, spleen
6q16	FUT9	FucT-IX / Fuc α(1,3)		Lewis X, Lewis Y	Leukocytes, brain, stomach

◯ Galactose (Gal)   ▨ N-acetylglucoseamine (GlcNAc)   ▲ Fucose (Fuc)   ◆ Sialic acid (N-Acetylneuramiinic acid (Neu5Ac))

* The structures preferably synthesized by each fucosyltransferase indicated by coloured symbols. The uncoloured structures may also be synthesized by the fucosyltransferase but to a lesser extent [132, 145].

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2.3.6   Related glycoepitopes of the Lewis family

The fucosyltransferases are capable of generating a large collection of carbohydrate epitopes related to sLeX, many of them differing only in linkage specificity between the monosaccharides (Fig. 3 och Table 4). Of particular interest for this thesis is the Lewis Y (LeY) epitope, which is dependent on expression of the H type 2 precursors and FucT-I (FUT1) for its synthesis. LeY can be found in CD34+ hematopoetic precursor cells but is absent in mature lymphocytes isolated from both the blood and from the tonsils [146]. The LeY glycan can also be found in certain leukemic cell lines and abnormal expression is, like sLeX, highly associated with malignancy and strongly correlates to poor prognosis [147, 148]. Recently it was shown that LeY expressed on tumour cells mediates spread to the lung via interaction with Srfs proteins displayed on lung endothelial cells [149]. This indicates that other carbohydrate structures related to sLeX can function as mediators of transmigration and thereby act as tags for leukocyte homing.

It is well established that the modified sLeX structure, 6-sulfo sialyl Lewis X (6´Sulfo-sLeX) (Fig. 3), is important for L-selectin mediated homing by lymphocytes to high endothelial venules (HEV) in peripheral lymph nodes (PLN) and it is also expressed by subsets of T lymphocytes destined for routine homing to the skin [150, 151]. Lymphocytes obtained from healthy individuals mainly express this sulphated derivate of sLeX, which they use for routine migration in and out of tissue through interaction with E- and P-selectin expressed at the lining of dermal blood vessels [98]. This contrasts to the situation in patients with inflammatory disorders where activated T lymphocytes largely express the standard sLeX epitope [152].

Sialyl Lewis A (sLeA) is Lewis structure that is similar to sLeX, differing only in the specific linkage between the distal galactose and its neighbouring GlcNAc (Fig. 3). This epitope is atypically expressed in tumours of various origins and contributes to the metastatic potential [153-155]. It was recently shown that sLeA can be displayed on glycoprotein CD44, mediating polymorphonuclear leukocyte (PMN) transmigration over the intestinal epithelium [111]. Altogether this suggests that other Lewis structures can function as "address tags" during leukocyte homing, guiding the cells to specific areas in the body.

2.3.7   Molecular mechanisms behind tumour and viral hijacking of selectin functions

Various tumour cells can induce expression of selectin ligands, e.g. sLeX and sLeA, by activating FUT-genes and this contributes to their potential for colonizing new tissue [78]. It is generally not known how the activation of fucosyltransferase-encoding genes is accomplished in cancer cells, only that the ability to induce selectin ligands strongly correlates to metastatic capacity and poor prognosis. The retrovirus HTLV-1 triggers transformation of the infected lymphocyte, causing lymphoma. The virus induces FUT7 transcription, which leads expression of sLeX on the cell surface and this contributes to the skin infiltrating capacity of the transformed cells [138]. The mechanism behind transcriptional activation of FUT7 is well described for HTLV-1 infected leukocytes. The virus-encoded protein Tax carries out transactivation of FUT7, via association with CREB, in HTLV-1 infected leukocytes [85], bypassing the need for IL-12 and TGF-β1 stimulus in normal activation. One important difference compared with the normal situation is that Tax confers irreversible activation of sLeX synthesis, leading to the strong tissue invasive nature of adult T cell leukemic cells [84, 85].

Our group demonstrated that CMV can activate expression from several fucosyltransferase genes including FUT1 upon infection in human embryonic lung fibroblasts (HELF), leading to expression of LeY as well as sLeX, albeit not simultaneously, on the surface of the infected cell [73]. Also, VZV can induce sLeX in fibroblasts [73] and HSV-1 can activate expression of FUT5 that encodes fucosyltransferase V (FucT-V) in fibroblasts [72]. It appears that different types of human herpesviruses can interfere with the cellular machinery for Lewis antigen synthesis, and that the different herpesviruses can induce diverse fucosyltransferases for this purpose. Only two mammalian viruses encode glycosyltransferase of their own and none of these viruses infect humans [156], implying that any human virus strategy to induce novel glycoepitopes must be based on viral modification of host-encoded glycosyltransferase gene expression.

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3 METHODOLOGICAL CONSIDERATIONS

3.1 General workflow

The complete details regarding the methods and cell types used for the work in (**I, II, III, IV**) are described in detail in the corresponding article. The general workflow for infecting cell cultures with herpesviruses starts with isolation or growth of cells and subsequent attachment of viral particles to the respective cells, after which the residual particles are washed off. The infected cells are then incubated for the desired time in a controlled humid atmosphere with carbon dioxide to mimic the physiological situation. Thereafter the cells are harvested and prepared for respective analysis. There are several considerations when preparing an experiment, especially when working with different types of viruses and different cell types. In this section a brief overview of the workflow and some important aspects of the main methods used for this work are described.

3.2 Cell culture systems

Peripheral venous blood was obtained from anonymous healthy donors at the Department of Transfusion Medicine (Sahlgrenska University hospital, Göteborg, Sweden) and peripheral blood mononuclear cells (PBMCs) were isolated using ficoll separation as described in (**III**). For infection protocol with CMV the PBMCs were washed and CD14+ monocytes were isolated by negative selection. The isolated monocytes were cultured in a low adherence plate to avoid differentiation.

The method of isolating specific cellular components from PBMC using magnetic particles conjugated to antibodies is a powerful tool. It enables experimentation on primary cells instead of having to rely on transformed cell lines with an inherent bias due to alterations in the genome of haploid cells. On the other hand, PBMCs are considerably harder to infect *in vitro*, which render the H9 cell line more advantageous for establishing a synchronous infection. In **(III)** we compared the effect of HSV-1 infection in the H9 T cell line and in CD3+ T cells isolated from PBMCs. This revealed differences in the expression of fucosyltransferase encoding genes that may reflect genetic aberrations frequently occurring in cell lines utilized for *in vitro* experiments. In (**I, II, IV**) we utilized human embryonic lung fibroblasts (HELF), a diploid cell type with no alterations in the genome, which is used at a low passage

number. By using these unaltered cells it is possible to avoid an inherent source of bias that comes with cell lines originating from cancer cells. Also, by using a high number of infectious viral particles we could establish simultaneous infection with a high reproducibility in this cell type.

3.3 Herpesvirus infections

For infection of monocytes with CMV we used cell free viral particles, which were prepared by ultra centrifugation to obtain a sufficient concentration for a multiplicity of infection (MOI) of 5 plaque forming units (PFU)/cell, and the viral particles were allowed to bind to the cells for 3 hours. The cells were washed in PBS and fresh growth medium was added. The infection was allowed to proceed for 72 hours after which the cells were harvested for DNA or RNA content, described below.

Two separate methods were employed for HSV-1 infection were employed in (**III**), for H9 cells cell free virus was added to the cells but for infection of CD3+ T cells a cell-to-cell infection protocol was necessary to obtain a sufficient amount of infected cells (>70%) [157]. The cell-to-cell infection relies on a primary round of infection in HELFs after which the T cells are allowed to co-incubate with infected HELFs to allow the virus to cause a secondary infection via the formation of a virological synapse between the cells. In (**I, II, IV**) we used HELFs for studying HSV-1-infection. The viral particles were added to the cell culture at a high MOI in order to establish infection in all cells simultaneously. In all cases the HSV-1 viral particles were allowed to attach for 1 hour before the inoculum was removed. The cells were incubated and harvested for DNA or RNA content as described below, or prepared for analysis by immunofluorescence.

There are several methodological considerations when infecting a cell culture. We used virus particles only (cell free virus) when infecting monocytes with CMV. This differs from the method used for infecting CD3+ T cells where cell-to-cell spread was employed. The rationale for this is simply a matter of generating a productive infection and does not reflect any attempts to create an *in vitro* situation that resembles the *in vivo* milieu. The same reasoning applies for the synchronous infection of fibroblasts, where the method is a tool for transcriptional analysis early after infection.

3.4   DNA and RNA isolation and analysis

For isolation of the DNA the cells were lysed in a buffer with detergents and extracted using silica gel columns, then the amount of CMV or HSV-1 DNA was assessed by real time PCR (qPCR) (**I, II, III, IV**) designed to quantify genomic CMV DNA [10] or HSV-1 DNA [158]. For isolation of viral and cellular RNA, the infected cells were disrupted using a solution with detergents and RNA stabilizing agents. The RNA was extracted on a RNA specific silica gel column, and the concentration of RNA determined using a spectrophotometer. Transcription of the respective gene was determined for the total RNA fraction using reverse transcription real time PCR (RT-qPCR) with systems previously published [73, 159, 160]. The human 18S ribosomal RNA (rRNA) or human RPL4 messenger RNA (mRNA) was used as an internal "house keeping" control and the relative concentrations of transcripts from the different genes were determined using the ΔC_T method, optimized to compensate for bias due to sample preparation [72, 161]. As fibroblasts (HELFs) down regulate expression of most cellular genes we used 18S rRNA as internal reference in (**I, II, IV**) [162, 163]. However, monocytes are not as sensitive to viral modulation of cellular mRNA levels as the fibroblasts congruent to the situation observed for T cells in (**III**) [164]. The RPL4 mRNA system was preferred for determining fluctuations in low-expressing genes in the CMV-monocyte model system using the ΔC_T method.

Depending on the type of experiment, the expression data is normalized either against the gene with lowest expression or against the detection limit of the system [72]. For comparison between infected and uninfected cells it can be useful to normalize against the gene with the lowest expression to visualize relative changes. For analysis of residual expression of cellular genes before infection it is more useful to normalize against the detection limit of the system. Dilution series of plasmid with an insertion of the analysed PCR fragment was included in all qPCR runs, which enables comparison between the runs.

3.5   Detection of cell surface associated carbohydrates

Two main methods were used for detection of carbohydrate structures on the surface of the cells; either immunofluorescence detection by confocal microscopy or flow cytometry. Both methods rely on the detection of carbohydrates by antibody binding either to adherent cells or cells in suspension respectively.

To define the carbohydrate content of leukocytes, the isolated PBMC fraction from immunocompromised patients with CMV viremia was incubated with primary mouse IgM antibodies towards sLeX (clone CSLEX) and LeY (clone F3) essentially as described for the T cells used in **(III)** [164]. After washing the cells in PBS a secondary IgG goat anti mouse antibody conjugated to Alexaflour 647 was applied. Finally a series of washing steps and fixation of the cells with paraformaldehyde were applied prior to analysis by flow cytometry. In **(I, II and IV)** we used the same carbohydrate binding IgM antibodies but confocal microscopy was used for the immunofluorescence detection. The fibroblasts were grown on glass slides and infected with HSV-1 before they were fixated using cold acetone. The antibodies were applied and the cells were covered with mounting fluid and a cover glass.

The most obvious reason for choosing one immunofluorescence method over another depends on cell type used, the leukocytes are cultured in suspension that favour analysis by flow cytometry whereas the adherent fibroblasts favour microscopy. However, the flow cytometry method is also preferred when analysing large cell populations while confocal microscopy enables higher resolution for qualitative analysis of single cells and allows for distinct definition of subcellular compartments.

Irrespective of detection method, one major concern regarding experiments with carbohydrates is the low affinity IgM antibodies that depend on multivalent interactions with their antigen. One IgM antibody can bind multiple epitopes, which enhances the strength of the interaction. In mucin-like regions of a glycoprotein the O-linked glycans are densely packed together and this may strongly impact the binding properties of the IgM antibodies. However, there are a large fraction of glycoproteins, many of which are known to harbour selectin ligands, that only express dispersed single O-linked glycans and these might not be sufficient for detection. Moreover, different IgM antibodies towards the same antigen can have slightly different specificity and also variable affinity, e.g. clone KM93 and CSLEX towards sLeX. The KM93 clone recognizes sLeA in addition to sLeX (Gustaf Rydell personal communication) and also has higher affinity for sLeX in our hands (unpublished observations). To compensate for the dual recognition by KM93 we included CSLEX or a strict sLeA recognizing clone in the majority of the experiments, especially when a strong induction of FUT3 could be observed since the product of this gene preferably synthesis sLeA [165].

4 RESULTS

4.1 HSV-1 early transcription induces cellular fucosyltransferases

Most herpesvirus genomes encode a multitude of genes, whose products may be engaged in interactions with host gene expression. Defining the detailed mechanisms behind such complex interactions is possible only if an appropriate model virus is used. We choose HSV-1 as a suitable model herpesvirus because of the short time required to execute its replicative cycle, the great number of possible target cells that permit experiments under single step growth curve conditions, and the general availability of mutant viruses defective in defined genes, relevant for the present study.

The first aim was to define at what step in the viral infection cycle viral activation of host FUT genes took place. First we showed that HSV-1 induced transcription of FUT5, as previously determined [72], as well as FUT3, FUT6 and to some extent FUT9 in infected fibroblasts, whereas expression of FUT1, FUT2, FUT4 and FUT7 was only marginally altered (**I**). Next we used FUT5 as a model gene for defining the early mechanism of HSV-1 activated transcription, and found that elevated levels of FUT5 RNA as early as 90 minutes post infection (p. i.), suggesting that viral immediate genes were involved in the activation process. Two important immediate early HSV-1 proteins are infected cell polypeptide 0 and 4 (ICP4 and ICP0), encoded by the viral genes of RS1 and RL2, respectively. We detected transcription of RS1 and RL2 already at 30 minutes p. i., thus immediately before the elevated levels of FUT5 at 90 minutes p. i. To further characterize the significance of these viral factors we analysed viral mutants defective in ICP0 and ICP4. Indeed, ICP0 and ICP4 are vital for completing the HSV-1 infection cycle but residual viral transcription remains in HSV-1 mutants defective in these proteins. Surprisingly, we found that FUT5 transcription is induced in cells infected with both these HSV-1 mutants. To investigate the possibility that mere binding and uptake of HSV-1 particles was the only viral factor needed, FUT5 transcription was analysed using UV-irradiated virus and virus treated with genome-destroying agents (**I**). However, neither of these virus variants was able to activate FUT5 transcription. Moreover, HSV-1-induced FUT5 transcription took place also when protein synthesis was blocked with cycloheximide (CHX) in the infected cells. Altogether these data indicate that execution of the virus infectious cycle up to transcription of viral immediate early RNA is necessary for induction of cellular fucosyltransferase-encoding

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genes in the HSV-1-infected fibroblast, whereas later steps are not needed for this process.

Next, we pursued the RNA trail and assayed several cell signalling inhibitors, blocking cellular complexes responsive to viral RNA, using the HSV-1-fibroblast model system (**II**). We found that the cellular antiviral protein kinase R (PKR) complex was required for transcriptional induction of FUT3, FUT5 and FUT6 in the HSV-1 infected HELF cells. The PKR protein complex senses double stranded RNA (dsRNA) with a size of around 80 base pairs, which can be generated during transcription of viral open reading frames (ORFs) running in the opposite direction [166, 167]. Two inhibitors of PKR, C16 and 2-AP [168-171], both blocked induction of fucosyltransferase transcription in HSV-1 infected fibroblasts in a dose dependent manner. Addition of the highly specific PKR-inhibitor C16 to HSV-1 infected cells also precluded the display of sLeX on the cell surface. No involvement of other cellular dsRNA-sensing factors were found, indicating that PKR is the main mediator of transcriptional activation of FUT3, 5 and 6 during HSV-1 infection in fibroblasts. The antiviral PKR complex regulates several downstream pathways that inhibit viral replication of both DNA and RNA viruses [166, 172]. Though we excluded eIF-2α, NF-κB or JNK in activation of FUT3 and 5 other PKR activated signalling intermediates, e.g. IRF-1, STAT or p53, may be involved.

4.2 HSV-1 activation of host FUT genes – a highly selective process

The present study includes unpublished preliminary data indicating that the viral activation of host FUT genes is a highly selective process with respect to the cellular genes addressed. The genes encoding FucT-V, FucT-III and FucT-VI are located in tandem in this order on chromosome 19 (Fig. 7A) and are all induced in a PKR dependent fashion in the HSV-1-infected cell (**II**). On intriguing question was if only the FUT genes were activated by HSV-1 or if the viral activation process was a more general process affecting a larger region of chromosome 19 that includes additional human genes adjacent to the FUT gene complex. Thus, we found that the NDUFA11 gene, encoding a NADH dehydrogenase located next to FUT5 was down-modulated rather than induced by HSV-1, and the NRTN gene situated next to FUT6 was found to be transcriptionally silent in HSV-1-infected as well as uninfected cells (Fig. 7B and not shown). Altogether, these data indicate that viral addressing of the FUT gene triplex in chromosome 19 is strongly selective with no effect on immediately adjacent genes.
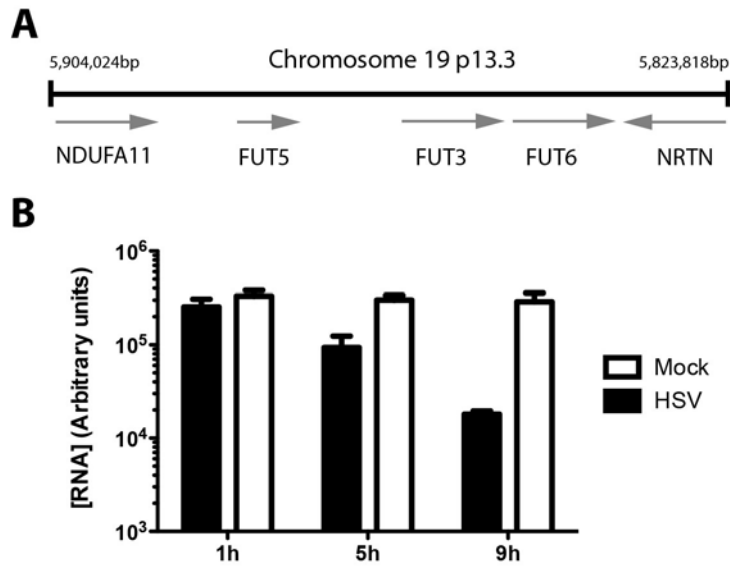
Figure 7. Transcription from the genes flanking the FUT-gene triplet on chromosome 19 are not specifically targeted by HSV-1 during infection. (A) Part of the short arm of chromosome 19 where FUT5, FUT3 and FUT6 are located in tandem, flanked by NDUFA11 and NRTN (II). (B) RNA expression of NDUFA11 RNA is down modulated by HSV-1 infection as compared to mock infected in HELF cells (Preliminary data from the present study).

4.3   HSV-1 induced sLeX expression in T cells

Leukocytes play an important role during herpesvirus viremia, which has been outlined in great detail for CMV, EBV and VZV [10, 173, 174]. Evidence of T cell dependence during HSV-1 and HSV-2 viremia also exists [157, 175]. We therefore wanted to expand the HSV-1 infection model to include these cells in order to study the natural target cells for blood borne spread of herpesvirus. Thus, we examined the selectin ligand expression in HSV-1-infected T cells, both in a transformed cell line as well as in primary CD3+ T cells isolated from blood from anonymous donors (**III**). We found that HSV-1-infection of the H9 T cell line is followed by increased display of sLeX and increased transcription of FUT3 and FUT6 but also of increased FUT7 and FUT1 expression, which is different from the situation in fibroblasts (**I**). The H9 T cell line proved to be a valuable model for studying early kinetics of HSV-1 infection. At variance with the results obtained for FUT5 in HSV-1-infected HELF cells (**I,II**), we found that transcription of RL2 encoding the viral immediate early protein ICP0 was necessary for induction of FUT7 transcription in the H9 T cell line. This conclusion is based on the finding of

no FUT7 transcription in cells infected with the RL2-deficient viral mutant. Moreover, no FUT7 transcription was found in HSV-1-infeted cells treated with protein translation inhibitor CHX. In H9 T cells, the mechanism for HSV-1-induced FUT3 and FUT6 transcription differs from that of FUT7 although the results here were not as clear-cut (**III**). The influence of ICP0 appeared to be minor; FUT3 and FUT6 may be regulated by some other viral factor, possibly ICP4.

By co-cultivation of HSV-1-infected fibroblasts with Phaseolus vulgaris phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMC) we could efficiently infect CD3+ T cells (**III**). These were subsequently analysed for FUT gene expression and for sLeX expression by flow cytometry. We found that HSV-1 induced expression of FUT1, FUT3, FUT5 and FUT6 in CD3+ T cells, whereas FUT7 expression remained unaltered. Also, the display of sLeX is increased in the infected cell population; both the total number of sLeX expressing cells and the intensity of sLeX fluorescence signal is enhanced. Altogether the data shows that HSV-1 can indeed manipulate the cellular mechanism for selectin ligand synthesis also in T cells with a potential role during viremia as an initiating factor for leukocyte transmigration.

4.4   CMV and EBV-induced sLeX and LeY in infected leukocytes (preliminary data)

The present study includes unpublished data indicating that CMV may induce Lewis-related glycoepitopes in infected leukocytes in a way that resembles that described for CMV infection of HELF cells [73]. Thus, after having established the ability of HSV-1 to induce sLeX in cells isolated from blood donors we wanted to verify that CMV could induce selectin ligands in monocytes, as this cell type is one of the main reservoirs for CMV latency and plays an important role in pathogenesis [10, 176, 177]. Blood was obtained from healthy anonymous donors and the CD14+ monocyte fraction was isolated from the PBMCs by negative selection using magnetic beads. The isolated CD14+ monocytes were subsequently infected with CMV at a MOI of 1-5 PFU/cell and kept in a non-adherent plate to minimize cell differentiation. A preliminary experiment indicated that active CMV replication in monocytes, is accompanied by elevated levels of FUT1 transcription and also increased transcription of FUT6 and FUT7 (Fig. 8). This suggested that CMV is able to induce both LeY and sLeX in monocytes similarly to the situation in fibroblasts [73].
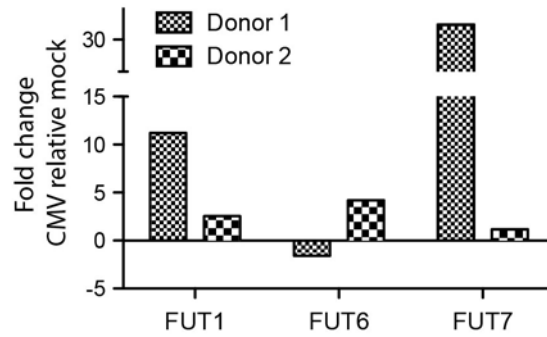
Figure 8. Induction of fucosyltransferases in monocytes by CMV infection. Expression of FUT1, FUT6 and FUT7 RNA in mock infected and CMV infected CD14+ monocytes was assessed 3 days post infection. Fold change as a result of CMV infection is showed. Fold change is calculated by dividing FUT RNA in CMV–infected cells with FUT RNA in respective mock–infected cells.

In addition to CMV, we also wanted to investigate the ability of EBV to induce Lewis-related glycoepitopes in transfected/immortalized B cells. Initial experiments with CD19+ B-lymphocytes isolated from healthy blood donors transfected with EBV indicated that also a representative for γ-Herpesviruses may enhance the display of selectin ligands on the surface of the infected cells, when compared with freshly isolated CD19+ B cells from the same individual. This phenomenon appears to be specific for each individual, as we observed a strong induction of sLeX and related epitopes in B cells from one subject when they were transfected with EBV, while in the other no induction was observed (Table 5).

Table 5. Selectin ligand expression‡‡ in EBV-transfected CD19+ cells (preliminary data)

Donor 1	LeX	sLeX	sLeX / sLeA‡	LeY
CD19+ *	0.7%	1.2%	1.2%	1.9%
CD19+ (EBV) **	34.5%	6.9%	18.6%	2.1%
Donor 2				
CD19+ *	2.3%	3.1%	1.1%	0.9%
CD19+ (EBV) **	3.4%	1.4%	3.9%	0.6%

‡‡ Percentage of cells expressing selectin ligand above threshold as determined by flow cytometry

‡ Antibody recognizing both epitopes (clone KM93)

* CD19+ B lymphocytes isolated from blood donor

** CD19+ B lymphocytes isolated from blood donor transfected with EBV DNA

Finally, in a pilot study we also analysed leukocytes, from patients with a suppressed immune system, regarding the display of Lewis antigens on the cell surface. We found that patients with an ongoing CMV viremia as determined by qPCR also had circulating leukocytes expressing both elevated levels of LeY and in some cases sLeX compared to healthy controls corroborating the results from the in vitro CMV-infected CD14+ monocytes. There was no correlation between viral load and the intensity or frequency of sLeX/LeY-positive leukocytes from the patients. Specimens with high sLeX expression were low in LeY-expression and vice versa (Table 6).

Table 6. Expression of sLeX and LeY ‡ in leukocytes from immunosuppressed patients with CMV and/or EBV viremia

CMV DNA copies / ml (log)*	EBV DNA copies / ml (log)*	LeY intensity**	sLeX intensity**
3.43	-	++	-
2.07	-	+++	-
3.99	-	+	+++
2.51	-	++	-
2.87	-	++	-
2.43	-	++	-
2.4	3.8	-	+++
2.9	2.77	++	-

‡ Expression of sLeX and LeY was determined by flow cytometry

* Copy numbers of viral genomic DNA in the blood determined by qPCR

** Intensity was graded (+) > 2 arbitrary log units (++) 2.5 – 3 arbitrary log units (+++) > 3 arbitrary log units

4.5 Identification of a viral glycoprotein that serves as a "professional" sLeX presenter

We also aimed at defining the sLeX carriers in HSV-1-infected human fibroblasts, a cell type that normally scarcely expresses any mucin-like proteins. The HSV-1 genome encodes 15-17 potential glycoproteins of which two, glycoprotein C (gC-1) and glycoprotein I (gI-1), have relatively long mucin-like domains associated with O-linked glycans [178-180]. We infected fibroblasts with viral mutants lacking either the entire gC-1 protein or the mucin-like domain of gC-1 and found that sLeX expression was totally abrogated. Both mutants still induced transcription from FUT3, FUT5 and FUT6 as determined by RT-qPCR and processed FUT3 and FUT5 proteins could also be detected with immunoblot. Co-localization studies with gC-1 and gI-1 showed that both proteins where displayed at the surface of the infected cell although not completely overlapping. This suggests that gI-1 is transported through the normal trans-golgi network and therefore has access to the glycosyltransferases residing there. This together with the observation that mutant viruses devoid of gC-1 fail to express sLeX indicates that gI-1 only acquire the short Tn antigen and cannot harbour longer Lewis structures in its mucin-like domain as suggested before [178]. Accordingly, gC-1 can act as a presenter of selectin ligands and other O-linked glycans in infected fibroblasts and other infected cells and tissues that lack endogenous glycoproteins optimized for presenting sLeX-carrying O-glycans.

5 DISCUSSION

Human viruses practise a total form of intracellular parasitism in the sense that they have to totally rely on the host cell metabolic machinery for gene expression, protein synthesis, genome replication, and assembly of progeny virus [181]. This includes also the genetically large human herpesviruses with genomes that encode a multitude of effectors engaged in focussing cell metabolism towards viral production. Until recently, it was believed that viral shutdown of host cell gene expression was complete, at least for lytic human herpesviruses such as HSV-1, implying that permissive cells shortly after virus infection become virus factories without any other metabolism than basic supply functions such as energy and raw materials for virus multiplication. During the last few years several lines of evidence have challenged this model, and contrary to previous models, it is now clear that replication of all herpesviruses takes place in parallel with expression of several host genes during the course of the infectious cycle [162]. This phenomenon does not necessarily represent incomplete viral suppression of host gene expression. In contrast, data from the present study demonstrates that there is an intricate interplay between viral and host gene expression. Thus, we demonstrate that specific herpesvirus gene products, like guided missiles, may address and activate the transcription of selected silent host genes in the infected cells, resulting in surface expression of novel potential virulence factors.

These virus-induced structures belong to the large family of Lewis-related glycoepitopes of which sLeX and derivatives thereof are the most well characterized owing to their significance as selectin ligands for homing of various types of leukocytes. Data is now accumulating that sLeX-related structures such as LeY, sLeA and others, although not constituting selectin ligands, may play roles in homing and organ colonization of different types of circulating cells [111, 149]. It is therefore not surprising that these functions have been hijacked in different pathological processes, not least for spread of various tumour cells, some of which are virally induced. There is now evidence suggesting that the hostile use of selectin ligands may not only be restricted to tumours and tumour viruses but also to the family of human herpesviruses. The present study aims at revealing details of the mechanisms involved in herpesvirus-induced expression of selectin ligands and related structures in the infected cell. Although other herpesviruses such and CMV and EBV may be more relevant from the clinical point of view, these viruses are difficult to use in mechanistic studies that require synchronously infected cell cultures. Therefore, it was considered advantageous to use the HSV-1-infected cell as a

model system owing to a short replicative cycle and other HSV-1 features that promote molecular studies (**I, II, III, IV**).

The first aim was to identify the different host genes that were activated by different herpesviruses for expressing Lewis glycoepitopes in virus-infected cells and how different activation patterns may induce formation of different structures. Our data indicates that the direct precursors to sLeX, "the sialylated Type 2 structure" is available in uninfected cells owing to presence of all the necessary glycosyltransferases needed to produce this precursor (**I, II, III**). Synthesis of Lewis epitopes must therefore be triggered by activation of one or more of a number of genes encoding critical fucosyltransferases that are rate limiting for formation of each of these structures. Dependent on the specificities of the particular FucT activated, different structures may be formed. If FUT7, encoding the strictly sLeX-specific FucT-VII, is activated, as is the situation for HSV-1-infected H9 cells **(III)** only sLeX can be formed (Table 4). Should FUT3 and FUT6 be activated, as is the situation for HELF cells infected with VZV or HSV-1 (**I, II** and [73]), then sLeX, LeX, LeA or sLeA could be formed (Table 4). However, no evidence for other virus-induced glycoepitopes than sLeX are found in these cells, when infected by HSV-1 or VZV (**I** and [73]), most likely because the balance of the previously acting glycosyltransferases in HELF cells does not permit formation of the critical precursors to LeY, LeA, LeX, LeB or sLeA (Table 4). In conclusion, alphaherpesvirus-induced expression of Lewis glycoepitopes appears to be restricted to sLeX due to lack of precursors for alternative Lewis glycoepitopes or because the virus-activated FUT genes are compatible only with sLeX synthesis even if alternative precursors were available.

The situation for CMV is more complex owing to its capacity to activate FUT1 in addition to FUT3, FUT6, and FUT7 ([73] and present study). FUT1 encodes an enzyme, adding fucose in an alpha 1-2 linkage rather than the sLeX-characteristic alpha 1-3 linkage, and this enzyme is responsible for formation of H Type 2, the direct precursor to LeY. This equips CMV with a switch between formation of either sLeX or LeY at the cell surface, dependent on the balance between H Type 2 and "the sialylated Type 2 structure", which in turn reflects the balance between the CMV-induced FUT1 gene product and the sialyltransferases engaged in sLeX formation (Fig. 6; Table 4) ([73] and present study). Thus, viral control of Lewis glycoepitope expression in herpesvirus-infected cells is a combination of the specificities of the enzymes encoded by the virus-activated FUT-genes and the balance of the host cell levels of the other glycosyltransferases engaged in synthesis of Lewis glycoepitopes (**I, II, III** and [73]).
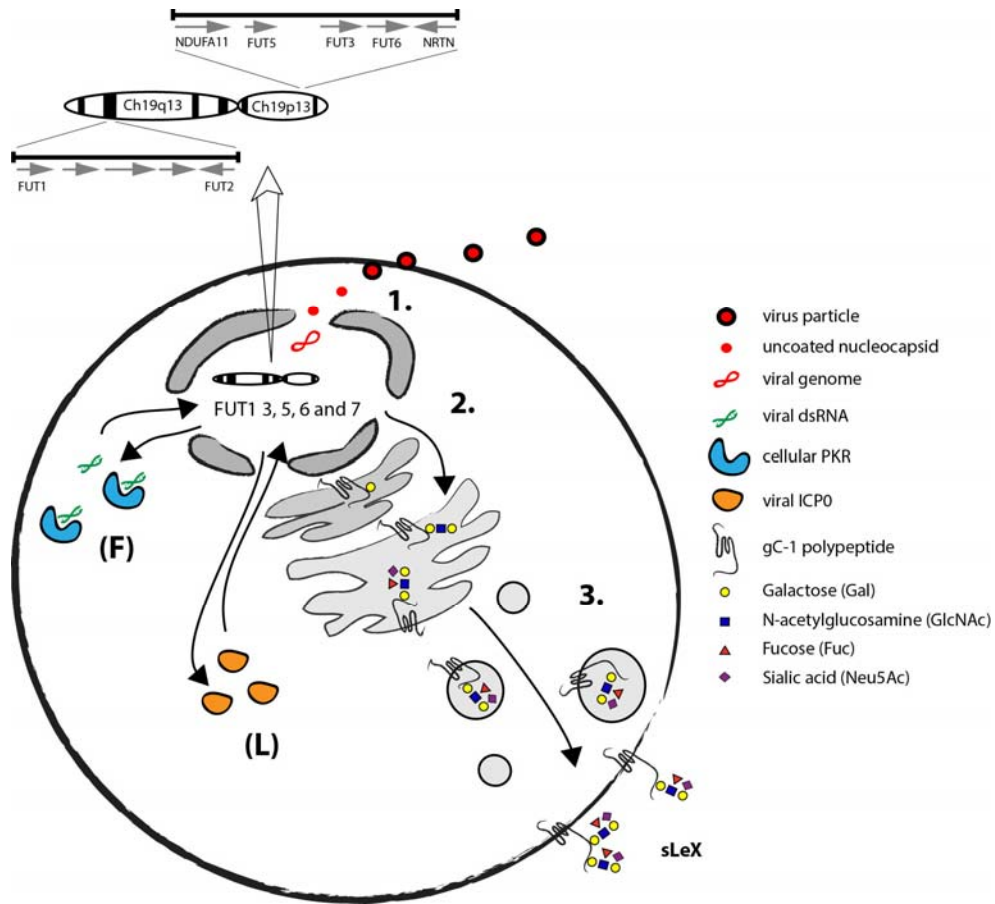
Figure 9. Pathway for HSV-1 induction of selectin ligands in fibroblasts and leukocytes. (1) Entry of HSV-1 viral particles with subsequent release of viral DNA in the nucleus and start of viral transcription. (2) Synthesis of selectin ligands (Based on viral activation of FUT genes via any of mechanisms "F" or "L") on glycoproteins (represented by HSV-1 glycoprotein C) in the golgi compartment. (3) Transport of glycoproteins or viral particles to the cell membrane in vesicles, for subsequent display at the cell surface. Activation mechanism details: (F) Activation of the cellular antiviral complex PKR by dsRNA, generated by active viral transcription, is necessary for induction of FUT3, 5 and 6 in fibroblasts. (L) Induction of FUT7 is dependent on the viral immediate early protein ICP0 in a T cell line. Details of both activation mechanisms are presented in I,II, and III. Enlarged is chromosome 19 that accommodates FUT1 and FUT2 on the long arm. The short arm of chromosome 19 accommodates the triplet of FUT5, FUT3 and FUT6 flanked by NDUFA11 and NRTN.

One pertaining question regards the level of specificity that characterizes the herpesvirus-induced transcriptional activation of selected FUT-genes. A number of glycosyltransferases with possible relevance for formation of O-linked glycans have been analysed by us in this respect. Thus, transcription of human genes encoding two sialyltransferases (ST3Gal-III and ST3Gal-IV), the "Core 2 GlcNA-transferase", engaged in formation of large O-linked sLeX-expressing glycans, and at least ten of the twenty ppGalNAc-T:s engaged in initiation of O-linked glycosylation (Fig. 6), are either down-regulated by herpesvirus infection or the RNA levels are remaining constant (**II**, [73], present study and preliminary data). The human FUT5, FUT3, and FUT6 genes are placed in tandem on the chromosome 19 short arm (Fig. 7A) (**II**), and one intriguing question is whether herpesvirus-induced activation is a part of a broad activation of this domain of chromosome 19. However, the finding that one of the immediately adjacent gene to the FUT5,3 and 6 cluster, NDUFA11, in fact is down-regulated by HSV-1 infection and the other adjacent gene, NRTN, is silent, in infected as well as uninfected cells, indicates a high degree of specificity in the herpesvirus control of FUT-genes (Fig. 7B). Hence, herpesvirus gene products may specifically address a few Lewis-related FUT-genes out of the around 150 different glycosyltransferase genes encoded by the human genome.

A striking feature of HSV-1 activation of FUT-genes in fibroblasts is that this process is extremely rapid: already 90 minutes after HSV-1 has attached significant levels of virus-induced FUT5 transcripts are detectable (**I**). However, we found that the mere binding of the virus to the cell surface was insufficient to activate the host FUT-genes (**I, II**). In contrast, available data indicates that viral RNA rather than virus-specific proteins are the viral gene products that address and activate FUT5 (**I, II**). There are several host cell factors that sense and response to foreign RNA [172, 182], but there is no evidence that any other RNA sensing protein than PKR is addressed in the HSV-1 activation of FUT5, FUT3, and FUT6 in HELF cells (**II**). Activated PKR may affect host gene transcription in several ways [166].The present data exclude that some of the most well characterized PKR-dependent mechanisms for influencing host gene expression are engaged in HSV-1 activated FUT transcription, such as NF-κB, JNK, p38 and eIF-2α (**II** and unpublished observations). However, PKR is a multifunctional RNA-sensing factor with several more or less characterized down-stream regulatory activities [166, 183], one or more of which may be engaged in HSV-1 activation of FUT transcription. The HSV-1 mode of FUT activation in HELF cells is indicated as pathway F in Fig. 9. The notion of PKR as a target for viral early RNA-dependent transcriptional induction of FUT-genes is attractive as PKR is constitutively expressed in the cytoplasm of low active cells such as confluent

HELF [166, 172, 183]. Hence, the PKR complex should be available for HSV-1 dsRNA transcripts early upon infection, permitting rapid induction of fucosyltransferases.

There are important differences between the mechanisms behind HSV-1 induction of sLeX in leukocytes compared with HEL cells, both with respect to the host FUT-genes addressed and the viral factors engaged (**I, II, III**). This was most evident for the situation in HSV-1 infected H9 cells, an established T cell line, where the transcriptional induction of FUT7 was found to be dependent on immediate early viral protein, identified as ICP0, rather than viral RNA, which was the case for the HSV-1-infected fibroblasts (Pathway "L" in Fig. 9) (**III**). In the corresponding situation in HTLV-1-transformed cells it was found that the HTLV-1 transactivator Tax was the only viral factor engaged in activation of FUT7 transcriptional activation [138]. The HSV-1 genome encodes several immediate early factors that often act in concert with ICP0 and the present data do therefore not exclude that ICP4 or any other HSV-1 immediate early factor is engaged in FUT7 induction in addition to ICP0. On the other hand, FUT6 and FUT3 transcription could be activated in HSV-1 infected leukocytes of different types, but the data did not permit detailed analysis as to the nature of the viral factors engaged (**III**). The HSV-1-induced activation of FUT7 is in line with the notion that activation of this gene is the cause of sLeX appearance on leukocytes during priming of these cells for crossing the endothelial wall [184, 185]. HSV-1-induced FUT activation was also analysed in a number of CD3+ T cells from blood donors, and surprisingly a more heterogeneous pattern of different FUT-genes were observed (**III**), indicating a significant role for FUT6 for virus-induced sLeX appearance in these latter cells.

It is proved that HTLV-1-induced sLeX indeed is a colonisation factor for addressing virus-transformed cells to the skin; a phenomenon of relevance for viral pathogenesis [85, 138]. But can we be sure that the herpesvirus-induced activation of FUT-genes and subsequent sLeX expression really is a viral pathogenic factor also in the progress of herpesvirus disease? In contrast, can this phenomenon represent a cellular defence mechanism against an imminent virus infection; does the virus or the host benefit from expression of sLeX or LeY? There are reasons to believe that both answers are applicable. On one hand, considering the engagement of a classical innate immune factor such as PKR in virus-induced FUT transcription in HELF cells it difficult to totally ignore the possibility of a cellular defence mechanism. One such possible benefit for the host, supported by the present data, could be that sLeX expressed from herpesvirus-infected cells in affected organs may attract circulating T lymphocytes and neutrophils. These cells are actively expressing

surface L-selectin with pronounced affinity for sLeX [186]. On the other hand, it is easier to envision herpesvirus-induced FUT activation and subsequent sLeX expression as a viral colonisation factor in the leukocyte situation. Since, (i) induced sLeX expression is the normal activation mode for permitting normal leukocytes to access extravasal tissue, (ii) it is the mechanism used by another human virus, HTLV-1, for hostile colonisation of extravasal tissue, (iii) and aberrant expression of sLeX and related structures is an important metastatic tool for tumours of several origins.

Leukocytes express several glycoproteins with mucin domains that may carry sLeX attached to O-linked glycans (Table 3), but in case the expression of these glycoproteins is low, the level of endogenously expressed O-glycan carriers may be suboptimal for display of sLeX. Most herpesviruses encode more than ten distinct glycoproteins each, some of which contain mucin-like domains and these glycoproteins are often synthesized at high rates in the infected cell [187]. One important question is whether any of these viral glycoproteins can support sLeX expression. The present study compared two different HSV-1 glycoproteins with large mucin domains in this context **(IV)**: One monotonous mucin-like domain with repeats of densely packed Ser and Thr stretches (glycoprotein gI-1) and one heterogeneous mucin-like motif more sparsely decorated with Ser and Thr units (gC-1) (Fig. 10). Our finding that only, the gC-1 mucin-like domain was associated with the sLeX epitope, emphasizes that specific sequence detail requirements of a mucin-like motif most be satisfied to permit sLeX expression. This conclusion is further supported by evidence that the O-glycan crowdedness of the gI-1 mucin motif is not compatible with elongation to larger glycans [178].
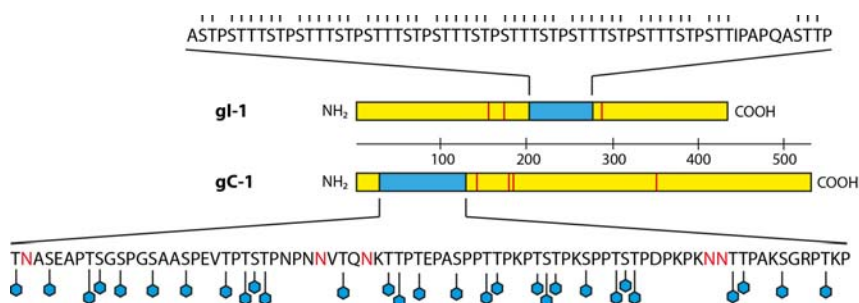
Figure 10. Glycosylation sites of HSV-1 glycoprotein C (gC-1) and glycoprotein I (gI-1). Schematic representation of gC-1 and gI-1 with their respective mucin-like domain indicated in green. The blue lollipops represent putative sites for larger O-linked glycan structures in the mucin motif of gC-1. The short black lines represent putative sites for the short Tn antigens in the mucin motif of gI-1. Sites for potential N-linked glycans are represented in red.

Most of the mechanistic studies in the present work have been carried out using HSV-1 as a model virus. It is evident that infections by other herpesviruses, such as CMV and EBV, have greater clinical impact in the perspective of immunocompromised patients. One pertinent question is therefore to what extent the results from the model systems involving HSV-1 are applicable for infections with other herpesviruses. So far, the present study only contains preliminary data that relates to CMV and EBV, but it is evident that CMV is able to induce a prominent increase in FUT1, FUT6 and FUT7 transcription in CD14+ monocytes isolated from blood donors (Fig. 8), which is in line with the results from the model systems described above. Of special interest for the CMV situation is the profound induction of FUT1 as well as FUT7 (Fig. 8), which is compatible with a switch between sLeX and LeY synthesis reported for fibroblasts [73]. No transcription data is available for EBV-transfected CD19+ B cells from blood donors. However, in one case transfection with EBV caused induction of a variety of Lewis-related glycoepitopes including sLeX (Table 5). These data suggest, that much of the model studies involving HSV-1 (**I, II, III**) and CMV [73] may be of value also for interpretation of results obtained from CMV- or EBV-infected cells from healthy blood donors, and possibly also from immunosuppressed patients infected with these viruses.

One problem associated with the current diagnostic monitoring of CMV and EBV is that high levels of virus DNA in the blood do not always correlate with clinical status [10]. One intriguing question is therefore whether herpesvirus-induced expression of sLeX or related glycoepitopes is of any pathogenic relevance, as proven for the HTLV-1 skin colonisation [85] or for the sLeX-

dependent metastatic behaviour of several tumours [78, 81, 155, 188, 189]. One could consider the possibility that sLeX or other related Lewis structures are major independent virulence factors of clinical significance (as suggested in the model of Fig 2), complementing high viral DNA levels for disease progression in immunosuppressed patients. If so, it could be expected that no direct correlation between CMV and EBV DNA levels on one hand and the number or intensity of cells expressing sLeX or LeY on the other hand should be found. The data of the present study, aimed at clarifying the status of sLeX and LeY in this context, is preliminary and carried out on total leukocyte fractions from immunosuppressed patients and therefore they do not permit any detailed conclusions (Table 6). However, three distinct features are discernible: (i) Some but not all patients with high levels of viral DNA express high levels of sLeX/LeY. (ii) There is no correlation between virus DNA levels and the degree of Lewis antigen expression. (iii) Absence of combination of concomitant sLeX and LeY expression is in accordance with the LeY/sLeX switch presented above. This last aspect is of interest since LeY is the natural ligand to thrombomodulin, an important modulator of inflammation, and expression of LeY may therefore be associated with a quite different biological behaviour than sLeX expression on CMV-infected leukocytes [190].

It is evident that further studies are needed to evaluate to what extent CMV- and EBV-induced selectin ligands and related structures contribute to the progress of virus-induced disease in immunocompromised patients. Should such studies indicate that sLeX/LeY are important factors for development of CMV or EBV disease in these patients, future results from the present study could be of relevance in two respects. Firstly, measurements of levels of leukocyte expression of selectin ligands may complement the current monitoring of CMV and EBV levels in blood for improved prediction of imminent CMV/EBV disease. Secondly, interference with selectin ligands is now under consideration as anti-metastatic therapeutics and a few inhibitors have reached clinical trials [191]. Should the selectin-dependent model, presented in Fig. 2, for spread of CMV- or EBV-infected circulating leukocytes into adjacent tissue be valid, then it is plausible that the same type of agents could be used as a complement to traditional antiviral treatment in immunosuppressed patients.

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