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HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) PATHOGENESIS AND HSV GENE THERAPY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

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To my family

ABSTRACT

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Herpes simplex virus type 1 (HSV-1) pathogenesis and HSV gene therapy of experimental autoimmune encephalomyelitis.
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Annales Universitatis Turkuensis
Turku 2013

The aim of this thesis was to develop new herpes simplex virus (HSV) vectors for gene therapy of experimental autoimmune encephalomyelitis (EAE), the principal model of multiple sclerosis (MS), and to study the pathogenesis of wild-type HSV-1 and HSV-1 vectors *in vivo*. By introducing potential immunomodulatory factors into mice with EAE we strived to develop therapies and possibly find molecules improving recovery from EAE. We aimed at altering the immune response by inducing favorable Th2-type cytokines, thus shifting the immune response from a Th1- or a Th17-response. Our HSV vector expressing interleukin (IL)-5 modulated the cytokine responses, decreased inflammation and alleviated EAE.

The use of a novel method, bacterial artificial chromosome (BAC), for engineering recombinant HSV facilitated the construction of a new vector expressing leukemia inhibitory factor (LIF). LIF is a neurotropic cytokine with broad functions in the central nervous system (CNS). LIF promotes oligodendrocyte maturation and decreases demyelination and oligodendrocyte loss. The BAC-derived HSV-LIF vector alleviated the clinical symptoms, induced a higher number of oligodendrocytes and modulated T cell responses.

By administering HSV via different infection routes, e.g. peripherally via the nose or eye, or intracranially to the brain, the effect of the immune response on HSV spread at different points of the natural infection route was studied. The intranasal infection was an effective delivery route of HSV to the trigeminal ganglion and CNS, whereas corneal infection displayed limited spread. The corneal and intranasal infections induced different peripheral immune responses, which might explain the observed differences in viral spread.

Keywords: Herpes simplex virus type 1 (HSV-1), vector, gene therapy, experimental autoimmune encephalomyelitis (EAE), bacterial artificial chromosome (BAC), cytokine, interleukin (IL), toll like receptor (TLR).

TIIVISTELMÄ

Michaela Nygårdas
Herpes simplex virus tyyppi 1 (HSV-1) infektion patogeneesi ja HSV-välitteinen geeniterapia kokeellisessa autoimmuuni-enkefalomyeliitissä.
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Turku 2013

Väitöskirjatutkimuksen tarkoituksena oli kehittää uusia herpes simplex virus (HSV)-pohjaisia geenikuljettimia (vektoreita) kokeellisen autoimmuuni-enkefalomyeliitin (EAE) hoitoon, ja tutkia HSV-1 luonnonkannan ja vektoreiden aiheuttamia immuunivasteita *in vivo*. EAE toimii multippeliskleroosin (MS-taudin) ensisijaisena koemallina. Tavoitteemme oli kehittää uusia hoitomuotoja, jotka perustuivat virusvälitteiseen geeninsiirtoon. Pyrimme muokkaamaan aivoja kohtaan suuntautuneita immuunivasteita tuomalla hermostoon viruksen avulla suotuisia Th2-tyypin sytokiineja, ja siten muuttamaan immuunivasteen tasapainoa pois Th17-tyypin vasteesta. Interleukiinia (IL)-5 tuottava HSV-vektori lievensi EAE:ta, kykeni vähentämään tulehdusta ja muokkaamaan taudille tyypillistä immunologista tilaa.

Kehitin bacterial artificial chromosome (BAC)-teknologialla uuden HSV-vektorin, joka ilmentää LIF-sytokiinia (leukemia inhibitory factor). LIF on neurotrooppinen sytokiini, jolla on useita vaikutusmekanismeja. LIF edistää oligodendrosyyttien kypsymistä ja estää demyelinaatiota ja oligodendrosyyttien tuhoa. HSV-LIF-vektorilla pystyimme lieventämään EAE-oireita ja lisäämään oligodendrosyyttien määrää aivoissa. Myös tautiin liittyvissä T-soluvasteissa tapahtui myönteisiä muutoksia.

Havaitsin työssäni, että autoimmuunitaudin hoitotutkimuksissa yleisesti käytetyllä SJL/J-hiirikannalla saavutettiin HSV:n tehokas kulkeutuminen aivoihin annostelemalla virus nenän kautta, mikä edesauttaa geenihoidon toteutusmahdollisuuksia. Viruksen annostelu muita reittejä ei ollut yhtä tehokasta tällä hiirikannalla. Eroavaisuudet immuunivasteessa voivat selittää vaihtelun viruksen leviämisessä.

Avainsanat: Herpes simplex virus tyyppi 1 (HSV-1), vektori, geeniterapia, kokeellinen autoimmuuni enkefalomyeliitti (EAE), bacterial artificial chromosome (BAC), sytokiini, interleukiini (IL), Tollin kaltainen reseptori (TLR).

TABLE OF CONTENTS

ABBREVIATIONS

AAV	adeno-associated virus
Act1	actin 1
α-TIF	alpha trans-inducing factor,VP16
AP-1	activator protein 1
APC	antigen-presenting cell
BAC	bacterial artificial chromosome
BBB	blood-brain barrier
bp	base pair
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BSA	bovine serum albumin
cDNA	complementary DNA
CNS	central nervous system
ConA	Concanavalin A
CoREST	corepressor of repressor element 1 silencing transcription
DAI	DNA-dependent activator of interferon regulatory factors
DEPC	diethylpyrocarbonate
DMEM	Dulbecco´s modified eagle medium
ds	double-stranded
E	early
EAE	experimental autoimmune encephalomyelitis
EF-1α	elongation factor 1 alpha
EIA	enzyme immunoassay
eIF-2α	eukaryotic translation initiation factor 2 alpha
egr-1	early growth response protein 1
ET cloning	named after the RecE and RecT proteins
FCS	fetal calf serum
FGF	fibroblast growth factor
FRT	Flip-recognition target sites
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HCF-1	host cell factor C1
HDAC	histone deacetylase
HHV	human herpesvirus
HSE	herpes simplex encephalitis
HSV	herpes simplex virus
ICP	infected cell protein
IE	immediate early
IFN	interferon
IL	interleukin
IR	inverted repeat
IRF	interferon regulatory factor

ISG	interferon stimulated gene
ISRE	interferon-stimulated response elements
IVIS	*in vivo* imaging system
JAK	Janus kinase
kb	kilo base pair
L	late
LAT	latency-associated transcript
LAP	latency-associated promoter
LIF	leukemia inhibitory factor
Lox	locus of X-over P1 site
LSD1	lysine-specific demethylase
LUC	luciferase
MBP	myelin basic protein
MEM	minimum essential medium eagle
MHC	major histocompatibility complex
miRNA	microRNA
MOG	myelin oligodendrocyte glycoprotein
MOI	multiplicity of infection
MS	multiple sclerosis
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor κB
NGF	nerve growth factor
NK	natural killer cell
Oct-1	octamer binding protein
ORF	open reading frame
ori	origin of DNA replication
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cells
PFU	plaque-forming unit
PI3K	phosphatidylinositol 3-kinase
PKR	dsRNA-dependent protein kinase
PLP	proteolipid protein
PNS	peripheral nervous system
polyIC	polyinosinic-polycytidylic acid
PP1α	protein phosphatase 1α
PPMS	primary progressive multiple sclerosis
PRR	pattern recognition receptors
PSM	positive selection marker
PT	pertussis toxin
recA	recombinase A gene
RIG-I	retinoid acid inducible gene I
RLR	RIG-I-like receptor

RPMI	Roswell Park Memorial Institute
RRMS	relapsing-remitting multiple sclerosis
RT	reverse transcriptase
RNA	ribonucleic acid
sacB	gene encoding levansucrase
SFV	Semliki Forest virus
SIP-1	Spingosine-1-phosphate
SPMS	secondary progressive multiple sclerosis
STAT	signal transducers and activators of transcription
TAP	transporter associated with antigen presentation
TBK1	TANK-binding kinase 1
TG	trigeminal ganglion
TGF-β	transforming growth factor β
Th	T helper cell
TIR	Toll/IL-1R
tk	thymidine kinase
TLR	toll like receptor
TNF	tumor necrosis factor
TR	terminal repeat
Tregs	T regulatory cells
TRIF	TIR domain-containing adaptor protein inducing IFN-β
UL	unique long
US	unique short
vhs	virion host shutoff
VCAM	vascular cellular adhesion protein
VP	virion protein
Zeo	Zeocin

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-IV) and on supplementary data.

I Broberg E. K., **Nygårdas M**, Salmi A. A and Hukkanen V. 2003. *Low copy number detection of herpes simplex virus type 1 mRNA and mouse Th1 cytokine mRNAs by Light Cycler quantitative real-time PCR.* Journal of Virological Methods 112: 53-65.

II **Nygårdas M.**, Paavilainen H. and Hukkanen V. Cytokine responses in acute and latent herpes simplex virus infection established by three different routes of delivery in SJL mice. Manuscript, submitted.

III **Nygårdas M.,** Aspelin C., Paavilainen H., Röyttä M., Waris M. and Hukkanen V. 2011. *Treatment of experimental autoimmune encephalomyelitis in SJL/J mice with a replicative HSV-1 vector expressing IL-5.* Gene Therapy 18: 646-655.

IV **Nygårdas M**., Paavilainen H., Müther N., Nagel C-H., Sodeik B., Röyttä M., Hukkanen V. 2013. *A herpes simplex virus-derived replicative vector expressing LIF limits experimental demyelinating disease and modulates autoimmunity.* PLoS ONE 8(5): e64200. doi:10.1371/journal.pone.0064200

1 INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus causing a wide range of disorders, including herpes labialis, genitalis, keratitis and encephalitis. Approximately 52% of the population are seropositive for HSV-1 in Finland and the number varies from 30 to 90% worldwide.

HSV-1 was the first herpesvirus to be discovered and has been widely studied. The deep knowledge available on HSV molecular biology has made it possible to develop vectors based on the virus. HSV-1 has several features making them good gene therapy vectors, especially for treatment of central nervous system (CNS) disease. HSV-1 has a large genome, enabling deletion of nonessential genes to allow insertions of relative large transgenes. HSV-1 is a neurotropic virus and can maintain a life-long state of latency in neurons. HSV-1-based vectors also possess the ability to infect a wide range of cell and tissue types.

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the CNS characterized by demyelination of neurons. MS is the most common disabling disease diagnosed in young adults, especially women causing progressive paralysis. Common symptoms are vision loss, weakness of the limbs and sensory disturbances. The disease affects approximately 7000 persons in Finland and over 2 million people worldwide. No curative treatment is available, hence, new therapies are desperatelly needed.

Experimental autoimmune encephalomyelitis (EAE) is an inducible inflammatory demyelinating disease of the CNS in rodents and has served as the main experimental model for the study of MS. EAE studies have greatly improved the knowledge on MS disease pathogenesis, and has served as the platform for the development of new therapies. Gene therapy is treatment of disease by delivering genes to correct or modulate abnormal gene functions. Viral vectors can be used for the efficient delivery and expression of therapeutic genes in target cells or tissues.

This thesis strived to develop new HSV vectors for gene therapy of EAE and to improve the knowledge on wild-type HSV and HSV vector pathogenesis and immune responses *in vivo* in SJL/ J mice.

2 REVIEW OF THE LITERATURE

2.1 Herpesviruses

The virus family *herpesviridae* consists of more than 200 herpesviruses infecting a wide range of hosts, including mammals, birds and reptiles. Common to all herpesviruses is that they are large double-stranded DNA (dsDNA) viruses with a similar virion structure. Virus replication is followed by cell death in the lytic infection cycle. All herpesviruses can also establish latency in their host. The herpesviruses encode themselves for enzymes involved in their nucleic acid metabolism, DNA synthesis and protein processing, which they use in a regulated manner together with the host cell machinery. The virus replication occurs in the host cell nucleus (Pellett & Roizman, 2007).

Herpesviruses can be divided into the subfamilies *Alpha-, Beta-* and *Gammaherpesvirinae* based on their biological properties (Pellett & Roizman, 2007). Characteristic for the *Alphaherpesvirinae* is their ability to infect a variable range of hosts, have a relatively short reproductive cycle, spread rapidly in cell culture and establish latent infections mainly in sensory ganglia. *Alphaherpesvirinae* is also known as the neurotropic subfamily of the herpesviruses. The human alphaherpesviruses, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are mostly known to cause blisters in the oral and genital area. Varicella-zoster virus (VZV), also a human alphaherpesvirus, causes chickenpox and zoster (shingles). Members of the *betaherpesvirinae* are human cytomegalovirus (HCMV), human herpes virus 6A, 6B and human herpes virus 7 (HHV-7). Betaherpesviruses have a longer replication cycle, grow slowly in culture and are lymphotropic. HCMV infection usually goes unnoticed in the host, but the virus can cause severe disease in immunocompromised hosts. The human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi´s sarcoma-associated herpesvirus (KSHV), also known as HHV-8, especially infect T- and B-cells. Taken together, nine herpesviruses (Table 1) are known to infect humans and cause a wide range of diseases. Once acquired, herpesviruses remain in their hosts for life.

Systematic name	Common name	Abbreviation	Subfamily
Human herpesvirus 1	Herpes simplex virus type 1	HSV-1	α
Human herpesvirus 2	Herpes simplex virus type 2	HSV-2	α
Human herpesvirus 3	Varicella-zoster virus	VZV	α
Human herpesvirus 4	Epstein-Barr virus	EBV	γ
Human herpesvirus 5	Human Cytomegalovirus	HCMV	β
Human herpesvirus 6A	Human Herpesvirus 6A	HHV-6A	β
Human herpesvirus 6B	Human Herpesvirus 6B	HHV-6B	β
Human herpesvirus 7	Human Herpesvirus 7	HHV-7	β
Human herpesvirus 8	Kaposi's sarcomavirus	HHV-8, KSHV	γ

Table 1. List of the human herpesviruses (http://ictvonline.org).

2.2 Herpes simplex virus type 1 (HSV-1)

HSV-1 causes a wide range of disorders in humans. It is mainly associated with orofacial infections, such as herpes labialis of the lip (also known as cold sores) and causes gingivostomatitis of the oral cavity. The epidemiology of HSV-1 is slowly changing, as HSV-1 has become an important cause of herpes genitalis cases in many countries (Bernstein *et al.*, 2012; Löwhagen *et al.*, 2000; Nilsen & Myrmel, 2000; Roberts *et al.*, 2003; Tran *et al.*, 2004; Xu *et al.*, 2006), a disease previously associated mainly with HSV-2. Thus, HSV-1 is mainly transmitted through non-sexual contact and is usually acquired during childhood, but an increasing number of primary HSV-1 infections are also acquired later in life.

In addition to these two most common disorders, HSV-1 may also cause infections of the eye, the herpes keratitis. Herpetic keratitis is the most important cause of blindness due to a viral infection (Liesegang, 2001). Occasionally, albeit rarely, HSV-1 causes herpes simplex encephalitis (HSE), a severe inflammatory disease of the central nervous system (CNS), with a high mortality rate of up to 70% (Whitley, 1991). HSE can be a consequence of either latent HSV-1 reactivated from the TG or it can originate from a primary infection (Steiner, 2011). Neonates and newborns are at risk to contract neonatal encephalitis if the mother is having a primary infection or a disease outbreak during pregnancy or during delivery (Baker, 2007; Brown *et al.*, 2003).

There are large differences in the prevalence of HSV-1 and HSV-2 infections depending on geography, population subgroups, age and gender. Asia has the lowest HSV prevalence, whereas the highest prevalence is seen in some African countries (Smith & Robinson, 2002). The HSV-1 and HSV-2 seroprevalence ranges from 30 to 90% and 4 to 80%, respectively (Pebody *et al.*, 2004; Smith & Robinson, 2002). In Finland, approximately 50% of the population are seropositive for HSV-1 and 13% for HSV-2 (Pebody *et al.*, 2004). Clinical signs, such as fever, pain and blisters, may appear 4-20 days after primary infection, but up to 80% of HSV-1 infections are asymptomatic. The virus is transmitted by close personal contact of mucosal surfaces of abraded skin. The highest risk of contracting an HSV infection is when a person has active lesions or blisters, but the virus can also be transmitted from a person without symptoms. The severity and frequences of outbreaks a person experiences depend on the immunological status and the site of infection. Persons with a weaker immune response usually have more severe disease. The preferential site of infection is orofacial for HSV-1 and genital for HSV-2. HSV-1 usually causes milder genital disease than HSV-2 (Engelberg *et al.*, 2003; Lafferty *et al.*, 1987; Lafferty *et al.*, 2000). Once infected, the person carries the virus for life. Symptoms can be prevented or alleviated by acyclovir therapy (*Zovirax*). Acyclovir is an acyclic guanosine analog, which binds the viral DNA

polymerase and thus inhibits viral DNA polymerase activity. Acyclovir is converted to its active form acyclovir triphosphate by the viral enzyme thymidine kinase (TK) and human cellular kinases. Acyclovir triphosphate competes with 2-deoxyguanosine triphosphate (dGTP) for the viral DNA polymerase and when incorporated into the viral DNA it terminates the replication due to the lacking 3` hydroxyl group (Elion, 1982; Reardon & Spector, 1989). However, drug-resistant HSV strains carrying mutations in the TK gene exist and indicate the need for new anti-viral therapies against HSV. Serious efforts are now being made in many laboratories to develop vaccines for prevention of HSV-1 and HSV-2 infection. To date the subunit HSV-2 vaccines have failed to provide protection against both HSV-2 and HSV-1 infection in clinical trials (Belshe *et al.*, 2012).

2.2.1 Structure and genome

HSV-1 is a large double-stranded DNA (dsDNA) virus with a diameter of 250 nm and encoding more than 80 proteins. The HSV-1 virion consists of four elements: the *core*, *nucleocapsid*, *tegument* and the outermost layer known as the *envelope* (Figure 1). The *envelope* is a lipid layer bearing embedded at least 8 of the 11 HSV-glycoproteins glycoprotein B (gB), gC, gD, gE, gG, gH, gI, gL, gM, gJ and gN (Roizman *et al.*, 2007). Some of the glycoproteins are important for entry into the host cell, some have functions in immune evasion. The lipids in the envelope originate from host cells membranes and are acquired during viral replication (van Genderen *et al.*, 1994).

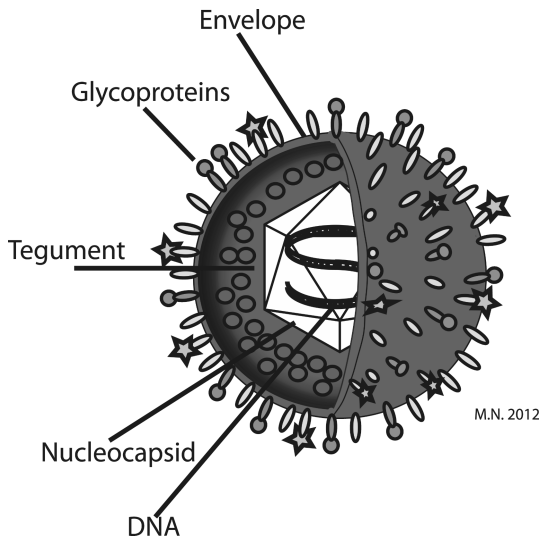

Figure 1. Schematic structure of the HSV-1 virion. The virion consists of four components: the core containing the double-stranded DNA genome, the capsid, the tegument and an outer lipid envelope containing glycoproteins.

The space between the envelope and the nucleocapsid is called the *tegument* (Roizman & Furlong, 1974). The tegument is composed of at least 20 viral proteins and is believed to be unstructured for most part (Grünewald *et al.*, 2003; Roizman *et al.*, 2007). Some of the tegument proteins are especially important for HSV replication: the viral protein (VP) 16, the virion host shutoff (vhs) protein (unique long (UL) 41) and VP1-2 (Roizman *et al.*, 2007). VP16, also known as α-trans-inducing factor (α-TIF), activates transcription of the first viral genes (Campbell *et al.*, 1984; Pellett *et al.*, 1985), whereas the vhs protein triggers shutoff of host cell protein synthesis (Kwong *et al.*, 1988; Read & Frenkel, 1983). VP1-2 (UL36) is the largest tegument protein and important for primary envelopment and binding of the capsid to the tegument (Cardone *et al.*, 2012; Reynolds *et al.*, 2001; Shiba *et al.*, 2000).

The icosahedral *capsid* consists of 162 capsomers (150 hexons and 12 pentons) of T=16 symmetry (Newcomb *et al.*, 1993; Schrag *et al.*, 1989). The outer shell of the capsid is composed of four viral proteins: VP5 (the major capsid protein), VP23, VP26 and VP19C (Zhou *et al.*, 1994). VP5 is the structural unit of the capsomers, while VP23 and VP19C make up the space between the capsomers (Newcomb *et al.*, 1993). VP26 is found at the distal tip of the capsid (Booy *et al.*, 1994; Zhou *et al.*, 1995). Other viral proteins; pUL25, pUL17 and pUL6, are also building blocks of the capsid. pUL6 is a portal protein located on the top of the capsid, forming a channel through which the viral DNA is packaged (Newcomb *et al.*, 2001; Trus *et al.*, 2004). pUL25 and pUL17 are involved in the packaging of the viral DNA into capsids (McNab *et al.*, 1998; Salmon *et al.*, 1998). Inside the capsid, the viral proteins VP22a and VP24 can be found. VP22a is a scaffold protein abundant in the pro-capsid (Rixon *et al.*, 1988). The viral protease VP24, a cleavage product of UL26, cleaves these scaffold proteins inside the pro-capsid when the viral DNA is packaged and the scaffold proteins are thereby removed (Baines, 2011). Scaffold proteins are therefore not found in the mature virion. Three types of capsids are found in infected cells; A (empty), B (intermediate) and C (full) capsids. Only the C capsids contain viral DNA and become mature virions (Gibson & Roizman, 1972).

Inside the capsid is the HSV-1 *genome*. The HSV-1 genome is a linear double-stranded DNA of 152 261 bp (strain 17^{+}; (McGeoch *et al.*, 1988a)). The genome has a G+C content of 68% (Kieff *et al.*, 1971). The genome is divided into a unique long (U_L) and a unique short (U_S) segment, which are flanked by inverted and terminal repeat sequences (Figure 2) (Wadsworth *et al.*, 1975). The repeats of the U_L and the U_S sequence are named the long repeats (IR_L, TR_L) and short repeats (IR_S, TR_S), respectively. At the ends are the terminal *a* sequences, a_L and a_S. These are important in circularization of the viral DNA and packaging of the genome into the virion (Deiss *et al.*, 1986; Mocarski & Roizman, 1981). A set of *a* sequences is also present at the L-S junction (*a*`). The *a* sequences can

vary in number of repeats with the exception of a_S, which only exist as one repeat (Hayward *et al.*, 1975; Roizman, 1979). The inverted *a`* sequence at the L-S junction enables genome recombination. The long and short unique sequences can exist inverted relative to each other and thus give rise to four different isomers (Delius & Clements, 1976; Roizman, 1979). These are present in equal amounts in wild-type virus (Hayward *et al.*, 1975)

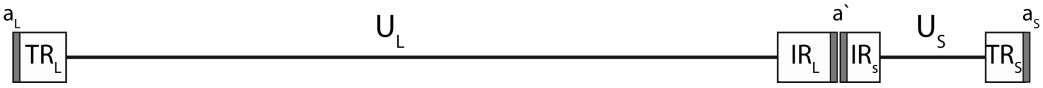

Figure 2. Schematic arrangement of the HSV-1 genome. The long unique (U_L) and short unique (U_S), the translated regions (TR_L, TR_S) and inverted repeats (IR_L, IR_S) are indicated. The *a* sequences are present in the terminal end and the L-S junction.

The HSV-1 genome encodes for at least 84 proteins and contains more than 100 genes. Approximately half of these proteins are essential for the HSV infection and the other half dispensable in cell culture. Gene overlaps are common and some of the transcripts are also found in opposite directions (Roizman *et al.*, 2007). The HSV genes are divided into three main kinetic classes: the α or immediate early (IE) genes which regulate the viral replication, the β or early genes that are important for viral DNA replication, and the γ or late genes which encode for structural virion proteins (Honess & Roizman, 1974).

2.2.2 HSV lifecycle

The HSV lifecycle can be divided into several phases*: entry, latency, genome transcription, DNA replication, late gene transcription, assembly* and *egress*. The whole process from attachment to the release of new virions takes approximately 18 hours in epithelial cells (Roizman *et al.*, 2007) (Figure 3).

Entry

HSV-1 can enter a cell via two mechanisms: 1) via direct fusion of its envelope with the plasma membrane, or 2) by endocytosis (Campadelli-Fiume *et al.*, 2012; Clement *et al.*, 2006; Gianni *et al.*, 2004; Milne *et al.*, 2005; Nicola *et al.*, 2005). Which of the two the virus chooses to use, is most likely cell type-dependent. Independent of the entry mechanism, the virus must first attach to the cell surface. The viral glycoproteins gB and gC mediate the binding of the virus to the cell surface receptor heparan sulphate, one type of glycosaminoglycan (GAG) chains at the cell surface (Shieh *et al.*, 1992; Spear *et al.*, 1992). This binding to heparan sulphate is not essential for HSV infection in cell culture, but enhances the infection (Campadelli-Fiume *et al.*, 2007). After binding to the cell surface, viral glycoprotein gD binds to entry receptors, such as herpesvirus entry mediator (HVEM), nectin-1/2, or 3-*O*-sulfotransferase

heparan sulphates (3-O-S HS) (Cocchi *et al.*, 2000; Montgomery *et al.*, 1996; Shukla *et al.*, 1999). Binding of gD to these receptors triggers fusion of the viral envelope with the cell membrane. Receptor-bound gD recruits the glycoproteins gB, and gH-gL, which then execute the fusion process (Campadelli-Fiume *et al.*, 2007; Spear & Longnecker, 2003). The nucleocapsid and tegument can thereafter enter the cytoplasm. Relatively recently, an additional role for gB in HSV entry was discovered. HSV-1 gB binds to paired immunoglobulin like-type 2 receptor (PILR)α on monocytes. This interaction was found to be equally important for HSV-1 infection as the gD-HVEM interactions in some cell types (Satoh *et al.*, 2008).

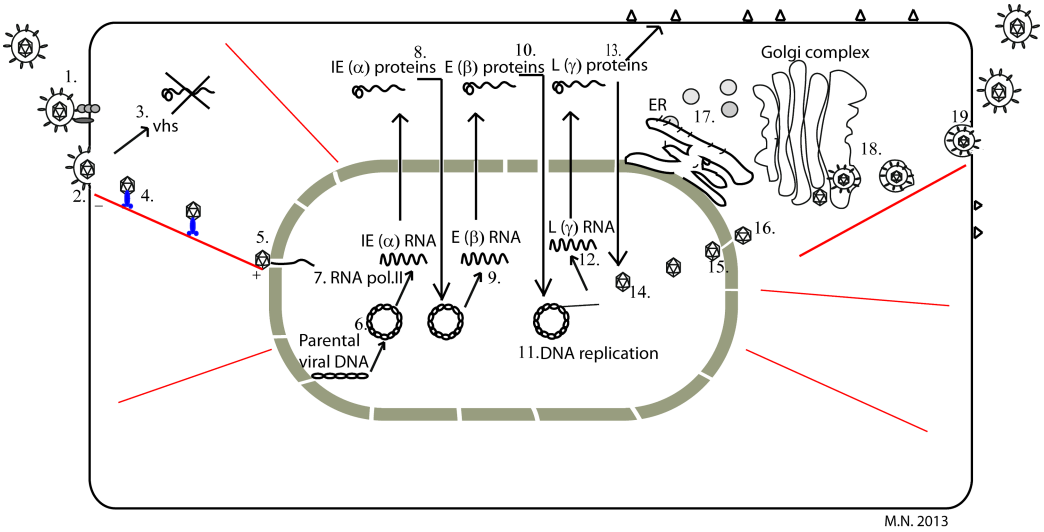

Figure 3. The HSV replication cycle. 1) HSV attaches to the cell by binding of gB and gC to GAG. Entry occurs by further binding of gD, gB, gH and gL to entry receptors. 2) The envelope fuses with the cell membrane. 3) The tegument protein vhs degrades mRNA. 4) The virion and some tegument proteins travel by retrograde transport along microtubules towards the nucleus. 5) At the nuclear pore viral DNA is released. 6) The viral DNA circularizes in the nucleus. 7) RNA polymerase II transcribes the viral DNA. The IE (α) genes are transcribed first. 8) The IE RNAs are translated in the cytoplasm. Some of these IE proteins enter the nucleus and activates the transcription of E (β) genes and L (γ) genes. 9) E (β) genes are then transcribed. 10) E (β) proteins are translated in the cytoplasm and enter the nucleus to activate DNA replication. 11) Replication of viral DNA occurs in the nucleus. 12) Transcription of L (γ) genes is activated by DNA replication and L (γ) proteins are translated in the cytoplasm. 13) Many of the L (γ) proteins are structural capsid proteins and some of these preassemble in the cytoplasm before entering the nucleus. 14) The capsid is assembled in the nucleus. 15) Capsids fuse with the nuclear membrane, and are pre-enveloped at the inner membrane and then de-enveloped at the outer nuclear membrane. 16) Naked capsids enter the cytosol. 17) Viral glycoproteins are synthetized in the endoplasmic reticulum (ER) and transported in vesicles. 18) Partially-tegumented capsids bud into the Golgi complex, where they receive their outer membranes and glycoproteins. 19) Virions are transported in vesicles to the outer cell membrane and are released from the cell by fusion. Modified from (Mettenleiter *et al.*, 2013; Roizman *et al.*, 2007).

Parts of the tegument and the nucleocapsid travel along cytoskeletal filaments, so called microtubules, in the cytoplasm towards the nucleus (Döhner *et al.*, 2002; Ojala *et al.*, 2000; Sodeik *et al.*, 1997; Topp *et al.*, 1994). Kinesin and dynein motor proteins drive this transport in a plus- and minus end-driven action. Kinesin is responsible for anterograde axonal transport (towards the periphery) and dynein is responsible for retrograde axonal transport (towards the nucleus) (Goldstein & Yang, 2000). Viral capsids bind to the motor proteins with the help of tegument proteins (Radtke *et al.*, 2010). The importance of this cellular transport machinery for HSV entry is showed by the fact that if the virus was to travel randomly in the cytoplasm towards the neuronal nucleus it would take it 230 years to move 1 cm (Sodeik, 2000). When HSV reaches the nucleus, the capsid is attached to the nuclear pore complex and the DNA is uncoated and released into the nucleus (Ojala *et al.*, 2000). The vhs protein degrades selectively host mRNA and shuts off cellular protein expression together with ICP27, and thereby induces a shift from cellular to viral replication (Feng *et al.*, 2005; Hardwicke & Sandri-Goldin, 1994; Taddeo *et al.*, 2013).

Genome transcription

In the nucleus the viral genome is transcribed. During the lytic infection HSV regulates a complex genetic program. Many cellular genes are suppressed and viral genes are expressed in a specific order and in a coordinated manner. Three main transcription classes exist; α (immediate-early (IE)), β (early (E)) and γ (late (L)) genes. The early and late genes can further be divided into β_1, β_2 and γ_1 (early late) and γ_2 (true late), giving rise to a total of five gene groups (Honess & Roizman, 1974; 1975; Swanstrom & Wagner, 1974).

As viral DNA enters the nucleus, it quickly circularizes and becomes coated by histones and other proteins (Garber *et al.*, 1993; Kent *et al.*, 2004; Poffenberger & Roizman, 1985). For viral transcription to start, the tegument protein VP16 is needed (McKnight *et al.*, 1987; Pellett *et al.*, 1985). VP16 and the host cell factor C1 (HCF-1) translocate to the nucleus and interact with the host cell octamer binding protein (Oct-1) at the promoters of the α genes (Kristie & Roizman, 1987). This VP16/Oct-1/HCF-1 complex further recruits lysine-specific demethylase (LSD1) and CLOCK histone acetyl transferase, which demethylate the histones (Kalamvoki & Roizman, 2011; Liang *et al.*, 2009). Hereby the transcription of the α genes is initiated, circa 2 to 4 hours post infection. LSD1 is normally part of a repressor complex consisting of the proteins HDAC1 or HDAC2, BRAF35 and the corepressor of repressor element 1 silencing transcription (CoREST). This complex is bound to REST at the response element RE1 on the DNA. It is likely that the recruitment of LSD1 also recruits the repressor complex to the viral gene promoters. Without the

VP16/Oct-1/HCF-1 complex the viral DNA remains bound to histones and is silenced (Roizman, 2011). This repressor system has therefore also been suggested an important role in the maintenance of latency (Du *et al.*, 2010).

The host RNA polymerase II transcribes the viral DNA with the help of several viral proteins (Alwine *et al.*, 1974; Costanzo *et al.*, 1977; McKnight *et al.*, 1987; Pellett *et al.*, 1985). Viral mRNA is exported to the cytoplasm where the viral proteins are translated. After translation, five of the six immediate early (IE) (α) proteins, the infected cell proteins 0 (ICP0), ICP4, ICP27, ICP22 and $U_S1.5$ are transported back to the nucleus to further stimulate the expression of the early (E) genes, the β genes (Roizman *et al.*, 2007). ICP0 is needed for the overall expression of β (E) and γ (L) genes; ICP0 binds to CoREST, displaces HDAC1 or -2, and as a consequence suppresses the silencing of the viral DNA via the repressor complex (Cliffe & Knipe, 2008; Gu *et al.*, 2005; Gu & Roizman, 2007). ICP4 is needed for the maximal expression of the β (E) and γ (L) genes (DeLuca *et al.*, 1985; Mavromara-Nazos *et al.*, 1986; Sandri-Goldin *et al.*, 1983) and interacts with RNA polymerase II transcription factors (Smith *et al.*, 1993). ICP27 has many important roles in virus replication, but the main function is the promotion of RNA export from the nucleus to the cytoplasm (Chen *et al.*, 2002; Sandri-Goldin, 1998). The β genes are expressed 4 to 8 hours post infection (Honess & Roizman, 1974).

DNA replication

Many proteins encoded by β genes are important for viral DNA replication and for the stimulation of late (L) or γ gene expression. The HSV DNA polymerase (UL30), its accessory protein (UL42), the helicase-primase complex (UL5, UL8, and UL52), the single-stranded DNA binding protein ICP8 (encoded by UL29) and the replication origin-binding protein (UL9) enter the nucleus and form the DNA replication complex (McGeoch *et al.*, 1988b; Wu *et al.*, 1988). Replication of viral DNA occurs at cellular nuclear domain 10 (ND10) sites (Maul, 1998; Maul *et al.*, 1996). The origin-binding protein (UL9) recognizes replication origin sites in the HSV genome (o*riS* and oriL) and the viral DNA replication is thereby initiated and occurs in a rolling circle mechanism (Muylaert *et al.*, 2011; Skaliter & Lehman, 1994). When viral DNA replication has started, transcription of γ genes peaks. Still, not all γ genes require viral DNA replication to occur for their sufficient expression. The γ genes mainly code for the structural proteins of the virion (e.g. VP16, gC, gB, gD), but also for the neurovirulence factor ICP34.5. Replication of viral DNA results in the formation of head-to-tail concatemers, which are cleaved at specific sites by the terminase enzyme complex (pUL15, pUL28 and pUL33) and thereby generating

monomeric HSV DNA (Adelman *et al.*, 2001; Baines, 2011; Vlazny *et al.*, 1982; Yang *et al.*, 2011).

Assembly

Assembly of the virion occurs in the nucleus in several steps and requires the proteins of the outer capsid shell (VP5, VP26, VP19C and VP23) and the scaffold protein VP22a. Capsid assembly probably begins with the formation of the portal ring by UL6 (Baines, 2011). Some of the capsid proteins, e.g. the major capsid protein VP5, and VP26 and VP23, cannot enter the nucleus alone and are therefore partially assembled in the cytoplasm before entering. VP5 must interact with the scaffolding protein, whereas VP23 can only enter the nucleus together with VP19C (Nicholson *et al.*, 1994; Rixon *et al.*, 1996). Empty pro-capsids are first assembled. At the approximate time of DNA packaging, the protease VP24 degrades the scaffolds proteins inside the shell (Trus *et al.*, 1996). One copy of the cleaved viral DNA is packed into each of these preassembled capsids (B-capsids). The filled, pre-assembled capsids mature into virions and bud through the nuclear membrane in a process called egress.

Egress

The egress of HSV-1 virions from the nucleus, through the cytoplasm and out of the host cell is still not fully understood, especially how the virus receives its tegument and envelope. First, the capsids acquire a primary envelope when they bud through the inner nuclear membrane to the perinuclear space (Mettenleiter, 2002; Mettenleiter *et al.*, 2013). Three theories exist on how HSV exits the nucleus. Initially it was proposed that the capsid acquires its envelope by budding through the nuclear envelope and then moves through the endoplasmic reticulum (ER) and the secretory pathway to the cell surface for release (the luminal or single-envelopment hypothesis) (Enquist *et al.*, 1998; Leuzinger *et al.*, 2005; Mettenleiter *et al.*, 2013). Another theory suggests that the nuclear capsids are transported directly through dilated nuclear pores into the cytoplasm (Leuzinger *et al.*, 2005; Mettenleiter *et al.*, 2013). Today the most accepted model is the “de-envelopment-re-envelopment” theory. According to this hypothesis the capsids acquire a primary envelope by budding through the inner nuclear membrane, but they are then de-enveloped when they fuse with the outer nuclear membrane at release to the cytoplasm. The cytoplasmic capsids are finally re-enveloped (final envelopment) by budding into vesicles of the cytoplasmic membranes such as the Golgi network, where also the glycoproteins are acquired (Enquist *et al.*, 1998; Mettenleiter *et al.*, 2013; Nagel *et al.*, 2008; Turcotte *et al.*, 2005). Many viral genes are involved in this process. The viral

genes UL31 and UL34 (VP1-2) are needed for the primary envelopment, since virus mutants lacking these genes are trapped in the nucleus (Chang *et al.*, 1997; Reynolds *et al.*, 2001; Roller *et al.*, 2000). The tegumentation occurs in the cytoplasm and involves complex protein-protein interactions (Mettenleiter, 2002; Miranda-Saksena *et al.*, 2002). It has been shown that most of the tegument and the glycoproteins can self-assemble in the cytoplasm (Szilágyi & Cunningham, 1991). The result of this secondary envelopment is a mature virus particle within a cellular vesicle, which is then transported to the plasma membrane. Finally, the virus is released from the infected cell by fusion of the vesicle and the plasma membrane (exocytosis) (Mettenleiter, 2002; Mettenleiter *et al.*, 2006).

2.2.3 Pathogenesis

HSV-1 can infect several different cell types. Primary infections most commonly occur in epithelial cells of the oral cavity or the genital area. The virus replicates in the cells, producing new infectious virions, the most important task of a virus. After a period of lytic infection in epithelial cells, the virus spreads to nerve endings where it enters dorsal root ganglion (DRG) sensory neurons (Goodpasture, 1929; Goodpasture & Teague, 1923; Stevens & Cook, 1971). The virus is transported along the axons in a retrograde manner to the neuronal cell body, where it establishes a latent infection in a non-integrated form for the entire lifetime of the human host (Rock & Fraser, 1983). The site of latency is usually determined by the site of primary infection. After infection of the orofacial area the virus establishes latency in the trigeminal ganglion (TG). In the case of genital herpes, the virus usually travels to the lumbosacral sensory nerves (Simmons, 2002). Stress, UV light, a compromised immune response, or additional infections can re-activate the virus from its latent state. The virus travels via anterograde transport to the peripheral tissue and causes either a recurrent infection or asymptomatic shedding. Recurrence of HSV-1 infection usually occurs at the location of the primary infection (Simmons, 2002).

Effects on the host cell

The HSV-1 infection affects the host cell in many ways. Since HSV-1 has evolved together with the human host, the virus has developed several mechanisms to control the host responses. As the virus enters the cell, physical changes occur, including structural changes of cellular membranes, modifications of the microtubule network, and fragmentation and enlargement of the nucleus (Simpson-Holley *et al.*, 2005; Ward *et al.*, 1998). The HSV infection inhibits host transcription, RNA splicing and transport, and protein

synthesis to make way for the viral gene expression on the cost of cellular gene expression. The vhs protein (UL41) is important for the shutdown of the host protein synthesis early in infection (Kwong *et al.*, 1988; Read & Frenkel, 1983). Vhs is a minor structural component of the tegument (Smibert *et al.*, 1992), which degrades host and viral mRNAs in the cytoplasm (Kwong & Frenkel, 1987). Several studies have indicated that vhs is an RNase or a subunit of an RNase (Everly *et al.*, 2002; Krikorian & Read, 1991; Zelus *et al.*, 1996). ICP27 (UL54) inhibits host cell RNA splicing and collaborates with vhs to reduce the amount of host mRNA during the infection, which leads to a decrease in host protein synthesis (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994). When the viral DNA enters the nucleus, the host attempts to assemble it into chromatin structures with histones (Cereghini & Yaniv, 1984). HSV-1 counteracts this host response with the help of VP16, which reduces the amount of histones on IE promoters, and thus promotes formation of the active form of chromatin (Herrera & Triezenberg, 2004; van Leeuwen *et al.*, 2003).

HSV-1 infection induces virus-induced cell death, a mechanism developed by the host to stop the virus from replicating and spreading to neighboring cells. Three to six hours into the infection, viral and cellular mediators are produced that have a role in the inhibition of apoptosis (Goodkin *et al.*, 2004). HSV-1 prevents this host cell death via the action of several viral genes: ICP4, ICP22, ICP27, the viral protein kinase US3, glycoproteins gJ and gD and the latency associated transcripts (LATs) (Aubert & Blaho, 1999; Aubert *et al.*, 1999; Jerome *et al.*, 1999; Leopardi & Roizman, 1996; Leopardi *et al.*, 1997; Peng *et al.*, 2003; Perng *et al.*, 2000; Zhou *et al.*, 2000). ICP4 and ICP27 are essential for blocking HSV induced apoptosis, either directly or by inducing the expression of the other anti-apoptotic genes. While ICP22 has a regulatory function, US3, gD and gJ have a role in the prevention of apoptosis (reviewed in (Goodkin *et al.*, 2004)).

Neurovirulence

The alphaherpesviruses are neurotropic viruses, with the ability to replicate in neurons of the CNS. In the HSV-1 genome the neurovirulence gene $\gamma_1$34.5 is located in the inverted repeats and exists thus in two copies (Chou *et al.*, 1990; Dolan *et al.*, 1992). Viruses deleted of this gene are much less virulent and mice survive even a 100 000 times higher dose given intracranially compared to wild-type HSV-1 infection (Chou *et al.*, 1990; McKie *et al.*, 1994). Deletion mutants do not spread very efficiently and they are less likely to successfully establish latency (Markovitz *et al.*, 1997; Whitley *et al.*, 1993). Even though $\gamma_1$34.5 is necessary for virus replication in the CNS, it is not essential for replication in *in vitro* cell cultures (Chou *et al.*, 1990).

Another important function of $\gamma_1$34.5 is to prevent too early shutoff of host protein synthesis (Figure 4). When cells are infected with wild-type HSV-1, the double-stranded RNA-dependent protein kinase (PKR) recognizes double-stranded RNA and is consequently phosphorylated and activated (Chou *et al.*, 1995; Proud, 1995). PKR in turn activates and phosphorylates the eukaryotic translation initiation factor 2α (eIF-2α), which leads to a shutoff of host and viral protein synthesis and thereby viral replication (Chou *et al.*, 1995). The C-terminal domain of the gene product of $\gamma_1$34.5, ICP34.5, inhibits this shutoff by binding to the cellular protein phosphatase 1α (PP1α), which leads to a dephosphorylation of eIF-2α (He *et al.*, 1997) (Figure 4). In cells infected with $\gamma_1$34.5-deletion mutants a premature shutoff of protein synthesis occurs when the viral DNA synthesis is initiated (Chou & Roizman, 1992).

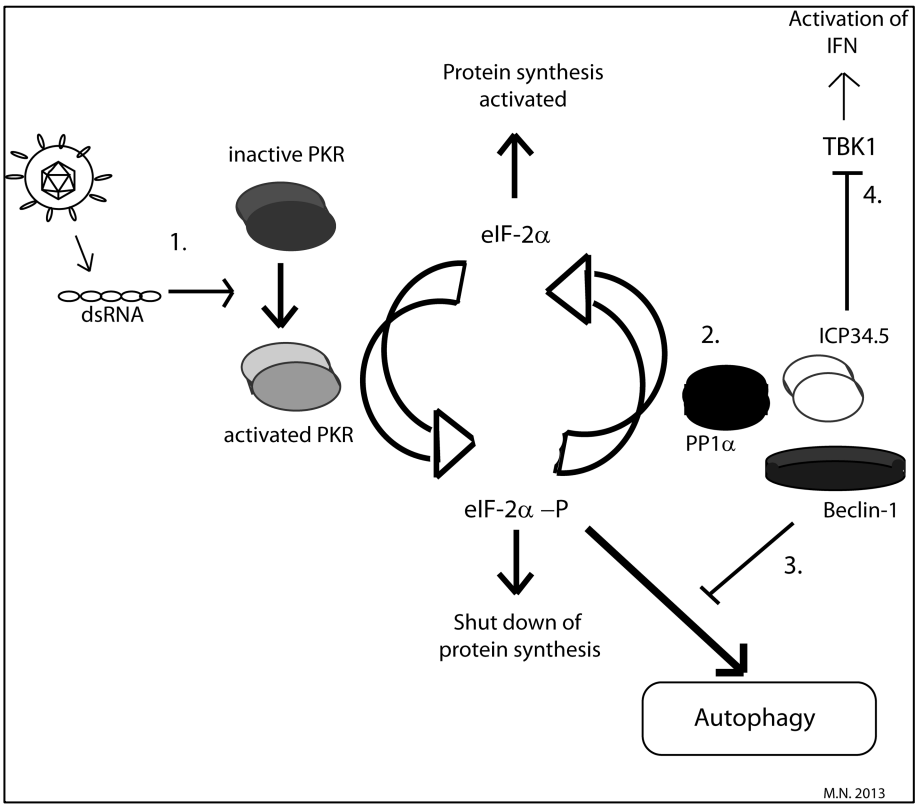

Figure 4. **ICP34.5 prevention of protein synthesis shutoff and autophagy.** 1) PKR recognizes viral dsRNA in the cell and induces shut-down of protein synthesis via phosphorylation of eIF-2α. 2) ICP34.5 binds to PP1α and thereby dephosporylates eIF-2α. Consequently, the protein synthesis is again activated. This binding to PP1α also inhibits PKR-induced autophagy. 3) ICP34.5 also inhibits autophagy by binding to Beclin 1. 4) ICP34.5 binds to TBK1 and thereby inhibits induction of interferons. Modified from (Hukkanen *et al.*, 2002).

The $\gamma_1$34.5 neurovirulence gene also has a function in autophagy (Figure 4). Autophagy is a mechanism used by cells to recycle cytoplasmic material to the lysosome for degradation, but it also functions as an antiviral mechanism to target virus for degradation (Levine & Klionsky, 2004; Tallóczy *et al.*, 2006). This microbial degradation pathway has also been termed "xenophagy" (Levine, 2005). The autophagic degradation of HSV-1 is activated by cellular PKR and phosphorylation by eIF-2α. ICP34.5 inhibits this degradation of HSV-1 (Tallóczy *et al.*, 2002; Tallóczy *et al.*, 2006). Another mechanism by ICP34.5 to block autophagy is by binding of the N-terminal domain of ICP34.5 to Beclin 1, a mammalian antiviral autophagy protein (Figure 4). This function is critical for HSV-1 neurovirulence (Orvedahl *et al.*, 2007). ICP34.5 also suppresses the induction of other cellular antiviral genes by directly targeting the TANK-binding kinase (TBK) 1, a major factor of the toll-like receptor (TLR)-dependent and independent nucleic acid sensing pathways, and thereby prevents induction of interferons (Verpooten *et al.*, 2009).

Latency

All herpes viruses have the capability to remain in a latent state, ensuring the survival of the virus throughout the lifetime of the host. After the lytic cycle, HSV-1 travels through retrograde transport to the cell body of neurons in the ganglion. The transport of the viral genome into the nucleus of a sensory neuron either leads to productive infection or establishment of latency. What determines this fate is not fully understood (Wilson & Mohr, 2012).

The latent state of infection is characterized by an intact circular viral genome in the nucleus without production of infectious virus. During latency viral gene expression is restricted (Steiner *et al.*, 1988), and the only gene products present are the latency-associated transcripts (LATs) (Croen *et al.*, 1987; Stevens *et al.*, 1987; Wagner *et al.*, 1988a) and a set of viral microRNAs (miRNAs). HSV is known to express 16 viral miRNAs and most of these are encoded by the primary LAT (Cui *et al.*, 2006; Jurak *et al.*, 2010; Umbach *et al.*, 2008).

LAT is an 8.5-kb primary transcript, yielding the stable nonlinear 2.0-kb and 1.5-kb introns localized to the nuclei of neurons (Farrell *et al.*, 1991; Rødahl & Haarr, 1997; Wagner *et al.*, 1988a; Wu *et al.*, 1996) (Figure 5). The LAT promoter (LAP1) is located approximately 700 bp upstream of the 2.0-kb LAT and is neuron specific (Hukkanen *et al.*, 1990; Zwaagstra *et al.*, 1990). In addition, another regulatory element, known as LAP2, is found between LAP1 and the 2.0-kb LAT (Goins *et al.*, 1994). LAT is important for the viral control of latency, but how LAT exerts this control is not fully known. LAT may also enhance the establishment of latency, since mice infected with LAT deletion viruses have fewer neurons containing viral DNA in the TG compared to wild-

type HSV-1 infection (Thompson & Sawtell, 1997). It has been proposed that LAT silences the expression of the lytic genes by heterochromatin formation in these genome regions (Kwiatkowski *et al.*, 2009; Wang *et al.*, 2005). LAT also inhibits apoptosis of infected neurons (Perng *et al.*, 2000; Thompson & Sawtell, 2001) and in this way promotes the survival of the infected neurons. Reactivation is also controlled by LAT, since LAT deletion mutants do not reactivate efficiently after infection (Bloom *et al.*, 1994; Bloom *et al.*, 1996; Hill *et al.*, 1990).

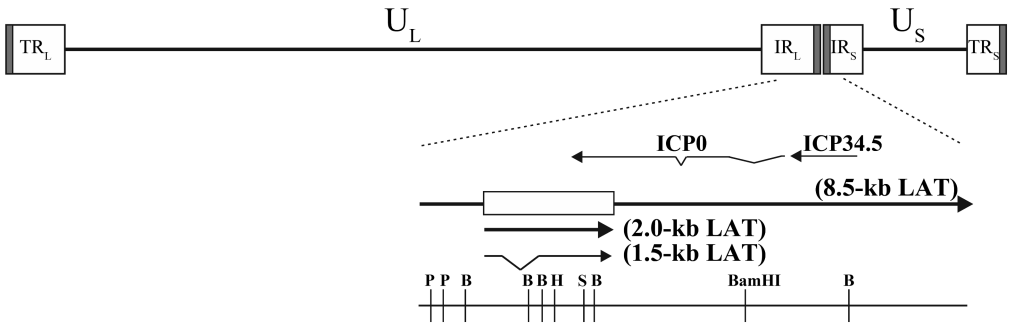

Figure 5. Map of the LAT locus in the prototypic HSV-1 (17^+) genome. The long unique (U_L) and short unique (U_S), the translated regions (TR_L, TR_S) and inverted repeats (IR_L, IR_S) are indicated. The 2.0-kb and 1.5-kb LATs within the 8.5-kb LAT are shown. ICP0 and ICP34.5 are transcribed in the opposite direction on the parallel nucleotide strand. Commonly used restriction endonuclease sites are marked with the abbreviations; P=PstI, B= BstEII, H=HpaI, S=SalI, BamHI. Modified from (Bloom *et al.*, 1996; Fareed & Spivack, 1994; Wagner *et al.*, 1988b).

Two LAT miRNAs, the miR-H6 and miR-H2-3p, inhibit the expression of ICP4 and ICP0, respectively (Umbach *et al.*, 2008). The authors suggested that this suppression of ICP4 and ICP0 might facilitate the establishment and maintenance of viral latency. ICP0 is transcribed on the antisense strand in the opposite direction of LAT and share the last 750 bp with the 2.0-kb LAT (Stevens *et al.*, 1987; Wagner *et al.*, 1988a) (Figure 5). LAT might inhibit the ICP0 expression by an antisense mechanism (Perng & Jones, 2010). Roizman et al have suggested that LAT and the miRNAs might have a role of surveillance and suppressing low level transcription of viral genes such as VP16 and ICP0 (Roizman *et al.*, 2011).

Besides the LATs and miRNAs, the latent state of infection is affected by other viral and host factors. In latently infected neurons, VP16 and HCF1 are retained in the cytoplasm and do not enter the nucleus (Kristie *et al.*, 1999). The HDAC-1 or -2/CoREST/LSD1/REST complex also has a role in silencing the viral DNA (Roizman, 2011). As a consequence transcription of the IE genes is not

initiated and the viral genes are silenced, which is important for the establishment of latency. The repressor complex has further been shown to be active only in the peripheral nervous system (PNS), not in CNS, indicating that HSV does not induce latency in the CNS (Roizman *et al.*, 2011). ICP27, a gene essential for productive infection in replicating cells, restricts productive infection in primary sensory neurons, as seen with deletion mutants growing to higher titers than wild-type HSV-1. Also ICP22 deletion mutants have shown a similar phenotype. Thus, ICP27 and ICP22 might also have an important role in the establishment of latency (Margolis *et al.*, 2012). Host factors important for the maintenance of latency include nerve growth factor (NGF) and the consequential phosphatidylinositol 3-kinase (PI3-K) signaling, thus, blocking viral reactivation (Camarena *et al.*, 2010; Wilcox *et al.*, 1990). One explanation could be that the neurons undergo apoptosis in the absence of NGF. As shown by Du et al, induction of apoptosis in the TG leads to de-repression of latently infected cells and to accelerated viral gene expression (Du *et al.*, 2012).

Periodically the virus reactivates from the latent state in the neurons, resulting in expression of the lytic genes and production of replicating virus. The mechanism behind the reactivation is still unknown, but is initiated by external stimuli (such as stress, UV light, trauma), that in turn stimulate HSV activation (Cushing, 1905). As mentioned earlier, for gene expression to occur, the VP16/Oct1/HCF1 complex needs to bind to the α gene promoters and thereby initiate protein synthesis. HCF1 is normally located in the cytoplasm in neurons and is only transported to the nucleus if they are stressed (Kristie *et al.*, 1999). VP16 is not expressed during latency either and needs to enter the nucleus in order to initiate transcription. One hypothesis is that VP16, as well, might be translocated to the nucleus after a stress signal (Thompson *et al.*, 2009), but other theories also exist. Since ICP0 can activate all classes of HSV genes, it is likely that some expression of ICP0 is needed for reactivation. Indeed, ICP0 mutants do reactivate inefficiently from latently infected TGs, also when there is the same amount of viral DNA present in the ganglion as in wild-type HSV infection (Halford *et al.*, 2001; Halford & Schaffer, 2001). Others have shown that ICP0 is not needed for the actual initiation of reactivation, but that it is needed for the production of infectious virus after reactivation (Thompson & Sawtell, 2006). The immune system also has a great impact on the control of latency. It is known that latently infected ganglia contain infiltrating immune cells (Liu *et al.*, 1996). $CD8^+$ T lymphocytes might inhibit reactivation, or more likely, inhibit the spread of reactivated virus (Halford *et al.*, 1996; Liu *et al.*, 2000; Shimeld *et al.*, 1997). The $CD8^+$ T cells produce interferon-γ (IFNγ), which plays an important role in the control of reactivation, especially to limit the replication of HSV-1 shortly after reactivation (Cantin *et al.*, 1999).

Most of the current data on HSV latency is received from animal models, especially the mouse model. These models often share a similar approach; animals are infected peripherally by corneal, intranasal, flank or footpad infection, the virus replicates in the local epithelia and is thereafter transported to the respective sensory ganglia. Approximately 14-28 days post infection, replicating virus is no longer detected and the virus is said to be latent (Bloom *et al.*, 2010; Hill *et al.*, 1975; Steiner & Kennedy, 1995). HSV does not sporadically reactivate *in vivo* in mice, but exposure to thermal stress (42°C for 10 min) has successfully been used to reactivate HSV in the mouse (Sawtell & Thompson, 1992). The latently infected TGs can be dissected from the mouse and HSV-1 is then reactivated by incubating the TG in tissue culture medium for several days, a procedure called "explantation-induced reactivation" (Stevens & Cook, 1971), or "explant culture" in short. The rabbit model resembles human infection in that sporadic reactivations occur. In addition, reactivation can be induced with adrenergic agents such as epinephrine or iontophoresis (Caudill *et al.*, 1986; Devi-Rao *et al.*, 1997). Besides animal models, cell culture models have been established. Cells used for the study of latency include primary neuronal cells lines, primary sympathetic neurons from rat superior cervical ganglia (SCG), and whole dorsal root ganglia cultures (Arthur *et al.*, 2001; Camarena *et al.*, 2010; Danaher *et al.*, 1999; Du *et al.*, 2012; Kobayashi *et al.*, 2012; Wilcox & Johnson, 1988). In these *in vitro* models, the lytic HSV infection is dampened to achieve long-term infections and reactivation is induced with different chemical compounds (reviewed in (Wilson & Mohr, 2012). Even though true latency cannot be established in vitro, cell culture models have proven a cost-effective and ethical alternative to *in vivo* models.

2.2.4 Immune responses

HSV-1 evokes an immune response in the host, involving components of both the innate and adaptive immune system. While the innate response is the first line of defense against a herpesvirus infection and determines the outcome of the infection, the adaptive immune response has an important role in the disease progression, virus spread and latency (Chew *et al.*, 2009). The primary HSV-1 infection induces inflammation at the site of infection. Leukocytes, lymphocytes, macrophages and natural killer (NK) cells infiltrate the infection site. Macrophages directly kill the infected cells, whereas other cells express antigens by the help of major histocompatibility complex (MHC) molecules. T cells will recognize these antigens and exert their effector responses, either directly or by the expression of cytokines (reviewed in (Broberg & Hukkanen, 2005)). The interferon (IFN) responses function to control the acute infection, whereas NK cells and lymphocytes are important in the control of persistent infections (Vollstedt *et al.*, 2004).

Innate immunity

The innate immune system has an important role in recognizing intruding pathogens and in limiting their replication and spread. It is well characterized that the innate response to HSV-1 involves the induction of type 1 interferons, including interferon (IFN)-α and IFN-β, and the functions of NK cells and macrophages (reviewed in (Chew *et al.*, 2009; Ellermann-Eriksen, 2005; Melchjorsen *et al.*, 2009)).

The first line of defense against an HSV-1 infection is the physicochemical barrier formed by the epithelial layer of the skin (reviewed in (Hukkanen *et al.*, 2010)). A healthy, intact skin protects the host from an HSV-1 infection. Saliva and other body fluids give further protection to the mucosal layer, containing chemicals that have been shown to have antiviral functions. These include lactoferrin, defensins and secretory leukocyte protease inhibitors (Galen *et al.*, 2007; Gu *et al.*, 1995; Hazrati *et al.*, 2006; Välimaa *et al.*, 2009; Välimaa *et al.*, 2002).

When a pathogen enters the cell, pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) (e.g. different nucleic acid structures) and signal to the host that an intruder is present (Figure 6). Toll-like receptors (TLRs) and retinoid acid inducible gene-I (RIG-I)-like receptors (RLRs) are PRRs, known to have a function in the HSV infection (Melchjorsen *et al.*, 2009; Rasmussen *et al.*, 2009). TLRs recognize microbes on the cell membrane (TLR1, 2, 4 and 6) or in the intracellular compartment, such as endosomes (TLR3, 7, 8 and 9), while RLRs detect viral nucleic acids in the cytoplasm (Akira, 2006; Melchjorsen *et al.*, 2009). The DNA-dependent activator of interferon regulatory factors (DAI) is a sensor for cytoplasmic DNA and is also indicated to have a role in HSV recognition (Takaoka *et al.*, 2007). Recently, another DNA sensor, the IFN-inducible receptor IFI16 has been found to recognize HSV in the cytoplasm and/or nucleus and to be responsible for the induction of antiviral IFN-β and inflammasome activation (Johnson *et al.*, 2013; Orzalli *et al.*, 2012; Unterholzner *et al.*, 2010).

Of the studied TLRs, TLR2, TLR3 and TLR9 are known to recognize HSV-1. TLR9 recognizes unmethylated CpG rich DNA, such as viral and bacterial DNA. It has been shown that TLR9 recognizes HSV DNA in plasmacytoid dendritic cells (pDCs) and that this TLR9 signaling is required for the IFN-α response to HSV in these cells (Krug *et al.*, 2004; Lund *et al.*, 2003; Rasmussen *et al.*, 2007). Other cells, such as macrophages and conventional DC are able to produce IFN-α in response to HSV-1 in a TLR9 independent manner (Hochrein *et al.*, 2004; Rasmussen *et al.*, 2007). TLR2 recognizes HSV through its glycoproteins (Leoni *et al.*, 2012) and has been shown to be responsible for inflammatory cytokine responses, both in microglia cell culture and in HSV-1

encephalitis (Aravalli *et al.*, 2005; Wang *et al.*, 2012). Mice lacking TLR2 are also less susceptible to HSV-1-induced encephalitis (Kurt-Jones *et al.*, 2004). TLR3 recognizes dsRNA and thereby viral nucleic acids (Cheng *et al.*, 2007). Deficiency in TLR3 has been reported to increase the risk of encephalitis in humans (Zhang *et al.*, 2007). Agonists of TLR3 and 9 have been beneficial in prevention of initial and recurrent herpes genitalis and encephalitis (Ashkar *et al.*, 2003; Boivin *et al.*, 2012; Boivin *et al.*, 2008; Gill *et al.*, 2006), indicating the importance of these TLRs in the immune response against HSV.

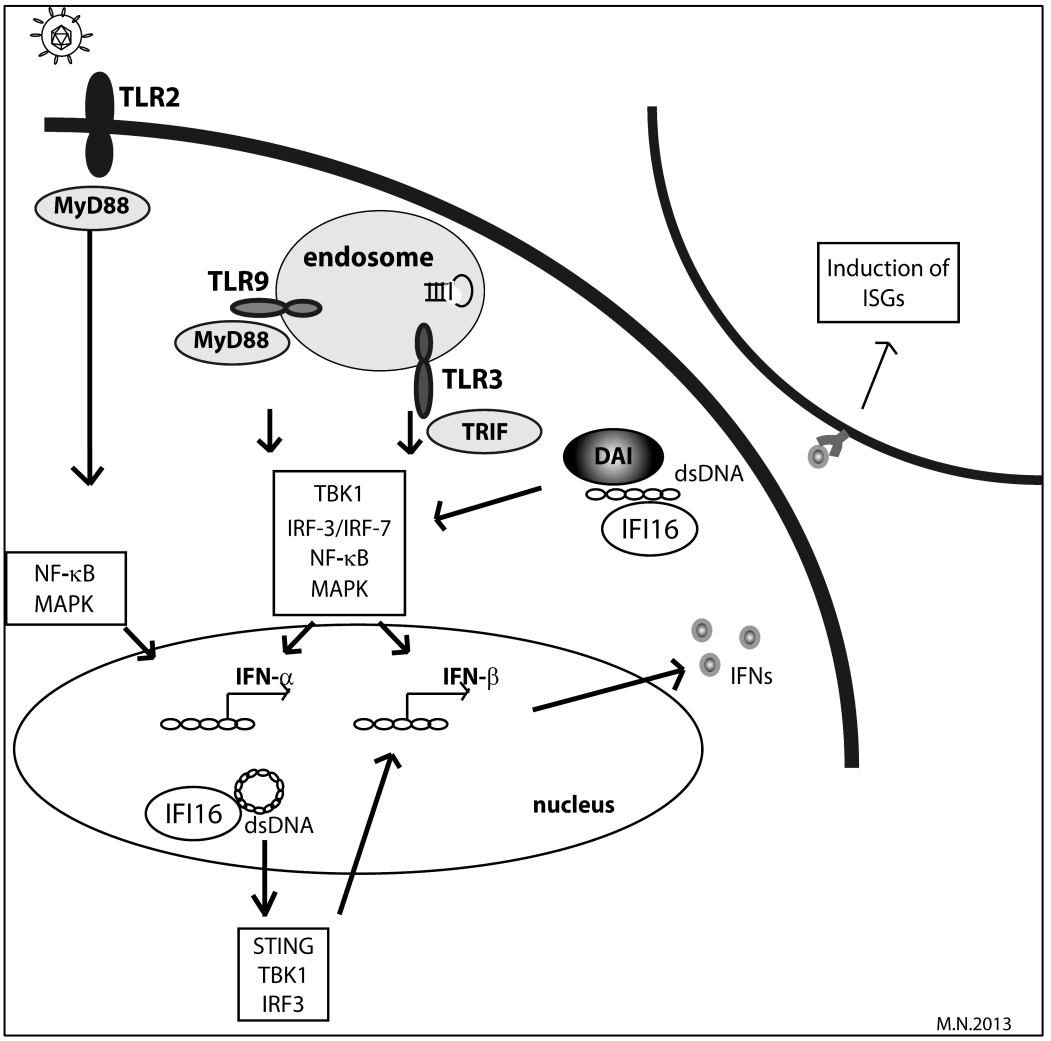

Figure 6. Induction of innate immune responses by PRRs in HSV infection. HSV is recognized by TLR2 at the cell surface and by TLR3 and TLR9 in endosomes. DAI senses viral cytoplasmic DNA. IFI16 recognizes both cytoplasmic and nucleic viral DNA. The recognition leads to activation of NK-κB, IRF-3 and 7, and MAP kinases. These molecules enter the nucleus and activate transcription of type I interferons (IFNs). The type I IFNs stimulate the expression of interferon stimulated genes (ISGs) and genes for other cytokines. Modified from (Hukkanen *et al.*, 2010; Melchjorsen *et al.*, 2009; Orzalli *et al.*, 2012; Paludan *et al.*, 2011).

The recognition of PAMPs by TLRs recruits adaptor molecules such as myeloid differentiation factor 88 (MyD88) or TIR domain-containing adaptor protein inducing IFN-β (TRIF). This further activates cellular transcription factors, such as NF-κB, activator protein 1 (AP-1) and interferon regulatory factor 3 (IRF-3), which leads to the consequential production of antiviral cytokines, such as type I IFNs, inflammatory cytokines and induction of maturation of dendritic cells (Kawai & Akira, 2006; Melchjorsen *et al.*, 2009; Mossman & Ashkar, 2005) (Figure 6). IFN-α and IFN-β are produced by many different cell types. IFN-β is mainly expressed by fibroblasts and epithelial cells, whereas IFN-α species are produced by macrophages, dendritic cells and leukocytes (Mossman & Ashkar, 2005). The type I IFNs exert multiple biological effects, including antiviral effects, stimulation of T cells, NK cells, macrophages and dendritic cells, and induction of apoptosis, to mention a few (Ellermann-Eriksen, 2005; Mossman & Ashkar, 2005). Secreted IFN-α and IFN-β bind to their receptor, leading to an activation of Janus kinase (Jak)-1 and Tyrosine kinase (Tyk)-2, which phosphorylate the signal transducer and activator of transcription (STAT) 1 and 2. These translocate to the nucleus where they bind to interferon-stimulated response elements (ISRE) in the promoter region of interferon stimulated genes (ISGs) (e.g. PKR) and initiate gene transcription (reviewed in (Mossman & Ashkar, 2005)) (Figure 6). Both IRF-3 and IRF-7 become activated after virus infection and lead to induction of many different ISGs. IRF-3 is essential for IFN-β expression and both IRF-3 and IRF-7 are meeded for IFN-α expression. Activated ISGs can then induce an antiviral state in the host cell (reviewed in (Kawai & Akira, 2006; Mossman & Ashkar, 2005)).

IFNs are essential for the protection against HSV-1 and are thus important in the control of the acute infection (Vollstedt *et al.*, 2004). The importance of type I IFNs in *in vivo* HSV-1 infection was first suggested, when differences in infectivity in mouse strains were linked to differences in the ability to quickly produce type I IFNs and in their level of NK cell function (C57BL/6 mice being resistant and BALB/c mice being susceptible) (Ashkar & Rosenthal, 2003; Halford *et al.*, 2004; Halford *et al.*, 1997; Lopez, 1981). It was then shown that IFN-α and –β are produced locally in peripheral HSV infection and can effectively limit the acute replication of HSV (Halford *et al.*, 1997; Hendricks *et al.*, 1991; Leib *et al.*, 1999). As mentioned, plasmacytoid dendritic cells (pDCs) are important producers of IFN-α in response to viral infection. These cells also function as antigen presenting cells, which together with cytokines stimulate T lymphocyte proliferation in the adaptive immune system (Liu, 2005). PDCs also produce interleukin (IL)-12 in response to HSV-1 infection (Krug *et al.*, 2004). In herpes stromal keratitis, pDCs have been found in the skin of herpetic lesions, where they express IFN-α, in addition to activating the adaptive immune response (Donaghy *et al.*, 2009). Type I IFNs activate NK cells, also known to be important for the control of HSV-1 infection. NK cells decrease the mortality

in encephalitis (Adler *et al.*, 1999) and depletion of NK cells leads to increased mortality in ocular HSV infection (Ghiasi *et al.*, 2000). NK cells are known to be an early source of IFN-γ, and thereby link the innate and the adaptive immune system (Mossman & Ashkar, 2005).

Macrophages are phagocytic cells, activated by the secreted type I IFNs. Macrophages are important in the antiviral response to HSV-1 during the first hours and days of infections (Ellermann-Eriksen, 2005; Liu *et al.*, 1996). Activated macrophages produce nitric oxide (NO), which can inhibit HSV infection (Croen, 1993). Macrophages are an important source of IL-12 and TNF-α (Kodukula *et al.*, 1999) and also express type I IFNs and IL-6 (Cheng *et al.*, 2000). Depletion of macrophages does not affect virus replication early in the infection, and they probably have a minor role in the limitation of the initial infection (Cheng *et al.*, 2000; Kodukula *et al.*, 1999). On the other hand, macrophages are important for the development of an adaptive immune response, since their depletion leads to much higher virus titers later in infection (day 8 post infection) in the cornea (Cheng *et al.*, 2000).

In addition to type I interferons, pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are expressed in the early host response to an HSV-1 infection. TNF-α is produced by many cell types during HSV-1 infection, including macrophages, microglia, astrocytes and T cells (Kodukula *et al.*, 1999; Lokensgard *et al.*, 2001; Shimeld *et al.*, 1997; Walev *et al.*, 1995). TNF-α expression is detected early in the infected TG and cornea, and has been shown to increase when the virus infection is cleared (He *et al.*, 1999; Hukkanen *et al.*, 2002; Shimeld *et al.*, 1997). TNF-α-knockout mice have a more severe corneal infection and a higher mortality rate (Minagawa *et al.*, 2004) and neutralization of TNF-α leads to increased HSV replication in the TG (Kodukula *et al.*, 1999). A strong expression of TNF-α has also been detected in brains of infected mice and was found to be important for the protection against HSV-1 encephalitis (Sergerie *et al.*, 2007). The same group also found that IL-1β is important for the protection against HSV-1 encephalitis. The lack of TNF-α and IL-1β leads to a suppressed innate immune response and thus, to an increased spread of the virus in the brains (Sergerie *et al.*, 2007). IL-6 is important in the defense against HSV-1. Epithelial cells show up-regulated IL-6 mRNA expression after infection with HSV-1 (Kanangat *et al.*, 1996a), and IL-6 is also induced in HSV-1 infected TG (Halford *et al.*, 1996; Shimeld *et al.*, 1997). IL-6 deficient mice have a higher mortality than wild-type mice after ocular and intranasal infection (LeBlanc *et al.*, 1999; Murphy *et al.*, 2008). The main producers of IL-6 are macrophages and monocytes, but also human and mouse leukocytes express IL-6 (Murphy *et al.*, 2008; Paludan, 2001).

Gamma-delta (γδ) T cells are distinct cells with properties typical for both the innate and adaptive immune system. These innate-like γδ T cells reside in tissues such as epidermis, lungs, intestine and uterus and respond rapidly and effectively to infectious agents (Sciammas & Bluestone, 1999; Vantourout & Hayday, 2013). They have much lower antigen receptor diversity than other T cell populations and are rarely antigen-specific. γδ T cells might have an important role in controlling HSV infection in epithelial cells in e.g. the genital tract, as they have been found to recognize glycoprotein I (gI) of HSV-1. This recognition do not require expression of MHC class molecules on the cells (Johnson *et al.*, 1992). γδ T cells have also been found to express cytokines in response to early HSV-1 infection, which will be discussed in more detail in the chapter "Adaptive immunity".

To evade the innate immune response, HSV has developed several strategies. Even though an HSV-1 infection induces a rapid activation of IRF-3, virus replication seems to limit the downstream activation of ISGs (reviewed in (Mossman & Ashkar, 2005)). The viral genes ICP4, ICP27 and ICP0 are important for escaping type I IFN signaling by inhibition of NF-κB, STAT1 and by IRF3 and IRF7 activation (Johnson *et al.*, 2008b; Lin *et al.*, 2004; Melchjorsen *et al.*, 2006; Melroe *et al.*, 2004). The US3 protein might also be involved in the control of IFN responses by inhibiting TLR3 responses (Peri *et al.*, 2008). ICP34.5 inhibits the TBK1 activated IFN responses (Verpooten *et al.*, 2009) and counteracts the type I IFN-induced activation of PKR (He *et al.*, 1997). HSV-1 has also mechanisms to avoid neutralization by complement. Glycoprotein C (gC) can bind to the C3b component of the complement system and thereby inhibit complement-mediated neutralization (Friedman *et al.*, 1984; Fries *et al.*, 1986; McNearney *et al.*, 1987). gC counteracts complement activation by blocking the binding of C5 and properdin to C3b (Hung *et al.*, 1994; Kostavasili *et al.*, 1997). *In vivo* studies with gC-deletion mutants have further shown the efficiency of gC to circumvent the complement activation (Lubinski *et al.*, 1999).

Adaptive immunity

The adaptive immune responses involve both humoral responses by B cells and effector mechanisms conducted by cytotoxic $CD8^+$ T cells and $CD4^+$ T helper cells. For the adaptive immune response to be activated, the pathogen needs to be ingested by dendritic cells, resident in most tissues. These dendritic cells travel to lymph nodes where they activate T and B lymphocytes. MHC class I and MHC class II molecules on antigen presenting cells (APC, e.g. dendritic cells) present viral antigens to $CD8^+$ T and $CD4^+$ T cells, respectively. Naïve $CD4^+$ T cells proliferate and differentiate into 4 different subtypes of effector T

cells, dependent on the antigen signal they receive together with a co-stimulatory signal and the cytokine profile present (polarizing cytokines) (Figure 7). Cytokines, such as IFNs and interleukins (IL) are small protein signaling molecules, important for the communication between immunological cells. Several cytokines have been reported to be up-regulated in response to an HSV-1 infection, both at the site of infection and in the ganglion and CNS.

Whereas the innate immune response is important for the control of the initial infection, T lymphocytes play a major role in the recovery from acute infection (Schmid & Rouse, 1992). $CD8^+$ T cells are cytotoxic cells with a main function in recognizing and destroying infected cells. These cells are essential for the clearance of HSV-1 infection from the TG (Simmons & Tscharke, 1992), eyes (Stuart *et al.*, 2004) and skin and nerves (van Lint *et al.*, 2004). Studies also indicate that the primary cytolytic effect of $CD8^+$ T cells is dependent on functional $CD4^+$ T cells (Jennings *et al.*, 1991; Smith *et al.*, 1994). The $CD8^+$ T cells secrete antiviral cytokines, such as TNF-α and IFN-γ in response to HSV infection (Cantin *et al.*, 1995; Liu *et al.*, 1996).

IFN-γ is a type II IFN, mainly produced by T lymphocytes and NK cells, but also by macrophages and APC. The production of IFN-γ by APC and NK cells is important for the early non-specific immune response (innate immunity) discussed earlier and function in linking the innate and the adaptive immune responses (Ellermann-Eriksen, 2005). Of the lymphocytes, $CD8^+$ T cells and $CD4^+$ T helper type 1 (Th1) cells are the main IFN-γ producers. The function of IFN-γ in the HSV infection has been extensively studied. Viral clearance is dependent upon IFN-γ activation, as shown in studies with knock-out mice and with neutralizing anti-IFN-γ antibodies (He *et al.*, 1999; Kodukula *et al.*, 1999; Smith *et al.*, 1994). The source of the IFN-γ expression in these experiments vary from γδ T cells of the innate immune system to $CD4^+$ and $CD8^+$ T cells of the adaptive immune response. IFN-γ, expressed by $CD8^+$ T cells, also has a role in the control of latency, as discussed previously (Cantin *et al.*, 1999; Lekstrom-Himes *et al.*, 2000; Liu *et al.*, 2001). Not surprisingly, IFN-γ expression has been detected both during the acute and latent infection in TG (Broberg *et al.*, 2002; Kodukula *et al.*, 1999), and also in the brain (Broberg *et al.*, 2002).

Also $CD4^+$ T cells are involved in the clearance of HSV-1 infection (Shimeld *et al.*, 1997; Smith *et al.*, 1994). In the absence of $CD8^+$ T cells, $CD4^+$ T cells are sufficient to clear the infection from both peripheral and neuronal sites (Johnson *et al.*, 2008a). On the other hand, $CD4^+$ T cells have been suggested to be responsible for the inflammatory response in HSV keratitis (Niemialtowski & Rouse, 1992). The $CD4^+$ T cells can be divided into four main populations: Th1, Th2, Th17 and T regulatory cells (Tregs) (Figure 7). Th1 cells are characterized

by the expression of IFN-γ, but they also express the pro-inflammatory cytokines IL-2 and TNF (Zhu *et al.*, 2010). The role of IFN-γ was discussed above. IL-12, expressed by dendritic cells, macrophages and monocytes of the innate immune response, induce proliferation of Th1 cells and expression of IFN-γ (reviewed in (Trinchieri *et al.*, 2003)). IL-12 has been shown to have a protective function in the HSV-1 infection when type I IFNs are absent (Vollstedt *et al.*, 2004). Both IL-12p35 and IL-12p40 are detected early in HSV infection (Kanangat *et al.*, 1996b). IL-23 and IL-27 are IL-12-related cytokines that are also involved in the induction of IFN-γ and Th1 development (Watford *et al.*, 2003). IL-23 has been shown to be up-regulated in the TG and brains during HSV-1 infection (Broberg *et al.*, 2002). The role of IL-27 in HSV-1 infection is not known.

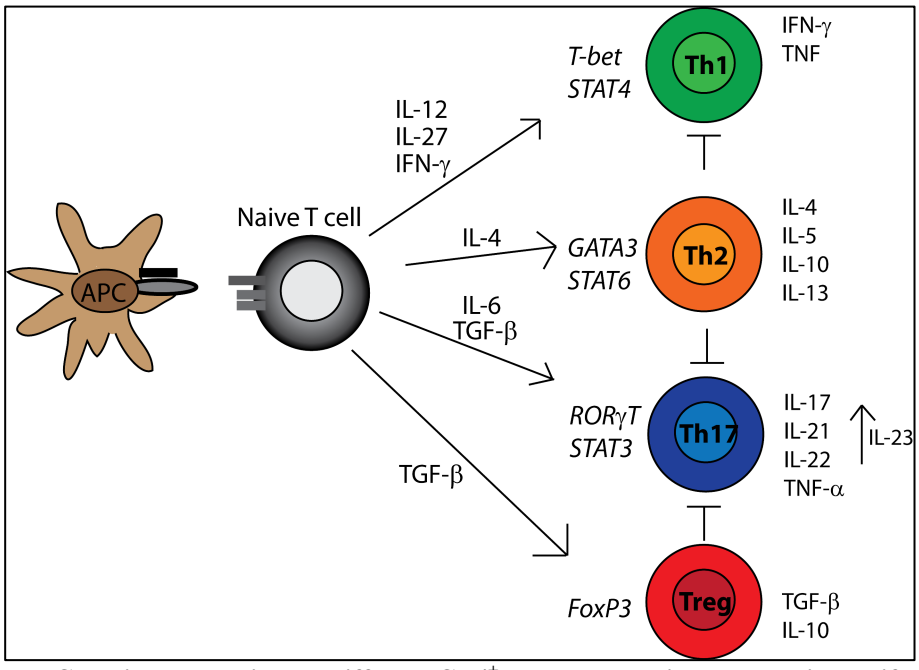

Figure 7. Cytokine expression by different CD4$^+$ T cell populations and their proliferation profile. Antigen presenting cells (APC) activate naïve T cells to differentiate into T helper (Th) cells type 1 (Th1), Th2, Th17 and T regulatory cells (Tregs) in the lymph nodes. Transcription factors are written in italics. Based on (Rouse & Sehrawat, 2010; Zhu *et al.*, 2010).

Th17 cells are another class of pro-inflammatory CD4$^+$ T cells, characterized by the expression of IL-17 (Park *et al.*, 2005), in addition to IL-21 and IL-22. These cells are activated by the expression of IL-6, TGF-β and IL-1β, whereas IL-23 is important for the maintenance of Th17 cells (reviewed in (van de Veerdonk *et al.*, 2009)). Besides Th17 cells, IL-17 is also produced by γδ T cells and NK cells of the innate immune response (Korn *et al.*, 2009). In herpetic stromal keratitis, IL-17 can be detected in early and late infection. The early IL-17

production is ascribed γδ T cells, whereas later in infection also Th17 cells can be found in the lesions (Suryawanshi *et al.*, 2011). Mice lacking the IL-17 receptor show milder keratitis, probably due to fewer infiltrating neutrophils (Molesworth-Kenyon *et al.*, 2008).

The Th2 cell lineage expresses the cytokines IL-4, IL-5, IL-10 and IL-13, and is involved in the activation of B cells (Rouse & Sehrawat, 2010). Both IL-4 and IL-10 have been detected in HSV-1 infection (Liu *et al.*, 1996; Shimeld *et al.*, 1999; Shimeld *et al.*, 1997), but some of these data are controversial, probably due to different mouse models and virus strains used (reviewed in (Broberg & Hukkanen, 2005; Hukkanen *et al.*, 2002)). Increased IL-4 levels have been correlated with increased HSV-1 replication in the eye (Ghiasi *et al.*, 1998). Later it has been suggested that IL-4 might function by down-regulating IL-2 in the HSV-1 infection, since STAT6 knockout mice show high IL-2 levels and lower HSV-1 titers. When IL-2 is depleted in these mice, also HSV-1 titers increase (Ghiasi *et al.*, 2002). IL-10 has anti-inflammatory functions in HSV infected cornea (Sarangi *et al.*, 2008; Yan *et al.*, 2001) and is a cytokine involved also in the functions of Tregs, discussed next.

Tregs´ main function is to limit the effector responses by other T cell populations and thereby minimize tissue damage (reviewed in (Belkaid & Tarbell, 2009)). Two subtypes exist, the naturally occurring Tregs and inducible Tregs. The transcription factor forkhead box P3 (FoxP3) is crucial for the development of naturally occurring Tregs. The signature cytokines of Tregs include IL-10 and transforming growth factor (TGF)-β, which contribute to the suppressive activity of Tregs (Miyara & Sakaguchi, 2007). The role of Tregs in HSV-1 infection includes the control of $CD8^+$ T cell proliferation and effector functions. Tregs have been shown to control eye immunopathology in both the mouse and the rabbit infection model (reviewed in (Belkaid & Tarbell, 2009)). In mice with depleted Tregs, HSV-1 infection has resulted in keratitis with increased severity (Sarangi *et al.*, 2008; Suvas *et al.*, 2004). The immune responses controlling the inflammation in HSV-1-induced keratitis involve both IL-10 and natural Tregs, which function independent of each other (Sarangi *et al.*, 2008). Another function of the Tregs in keratitis is minimizing the induction of infiltrating $CD4^+$ T cells (Suvas *et al.*, 2004). Tregs also seem to have a function in controlling the $CD4^+$ and $CD8^+$ T cell responses in HSV infection of neonates (Fernandez *et al.*, 2008).

HSV-1 infection induces production of neutralizing antibodies. Neutralizing antibodies have a protective role in genital herpes caused by HSV-2 (Seppanen *et al.*, 2006; Sherwood *et al.*, 1996; Zeitlin *et al.*, 1996), but the same protection is not as clear in HSV-1 infections. The higher susceptibility to encephalitis and keratitis in ocular HSV-1 infection in B cell deficient mice (Deshpande *et al.*,

2000), might rather be due to lower induction of T cell-mediated immunity (reviewed in (Chew *et al.*, 2009))

HSV-1 possesses mechanisms to circumvent the adaptive immune system. To inhibit the clearance of virus by cytotoxic $CD8^+$ T cells, HSV-1 inhibits the MHC class I antigen processing and presentation pathway. The HSV-1 protein ICP47 (the product of the US12 gene) binds to the transporter associated with antigen presentation (TAP), and thereby inhibits it and the MHC class I antigen complex to assemble (Früh *et al.*, 1995; Hill *et al.*, 1995). Consequently the infected cells are unable to present antigen and they avoid recognization and destruction by $CD8^+$ T cells. HSV-1 can also circumvent neutralizing antibodies by attaching its glycoprotein gE/gI complex to the Fc domain of IgG, and thereby blocks Fc-mediated antibody responses (Johnson *et al.*, 1988).

HSV vaccines

Successful development of effective vaccines against HSV needs to consider the viral immune evasion strategies. An ideal vaccine should prevent the individual from productive infections and latent infection, and it should induce a cellular immune response in addition to neutralizing antibodies. So far vaccines have mainly been developed against HSV-2. Vaccine approaches include inactivated virus, DNA-based vaccines, subunit vaccines, replication-incompetent virus and live-attenuated viruses (reviewed in (Johnston *et al.*, 2011)). Even though many of the developed vaccines have had promising results in animal models, so far satisfying results from clinical trials have not been reported. Vaccines tested in clinical trials include glycoprotein subunit vaccines, containing gD2 and/or gB2 (Corey *et al.*, 1999; Stanberry *et al.*, 2002). Glycoprotein gD2 is considered a good vaccine antigen candidate, as it elicits strong antibody responses in HSV-2 infection. Both the Chiron and the GlaxoSmithKline (GSK) subunit vaccines were reported to induce neutralizing antibody responses and $CD4^+$ T cell responses in vaccinated individuals (Bernstein *et al.*, 2005; Langenberg *et al.*, 1995). Even so, the Chiron subunit vaccine failed to prevent infection (Corey *et al.*, 1999). In a first study, the GSK subunit vaccine protected HSV-1- and HSV-2-double-seronegative women against disease, but not against the infection. Men and HSV-1-seropositive women were not protected (Stanberry *et al.*, 2002). In a further study (Herpevac trial), no protection against HSV-2 genital disease was seen. Instead a protection against HSV-1 genital disease was reported (Belshe *et al.*, 2012). The adjuvant formulation is of major importance in these subunit vaccines, inducing protective immune responses. For example, the GSK subunit vaccine adjuvant consists of alum and 2-O-deacylated-monophosphoryl lipid A (AS04), activating a stronger Th1 immune response than other adjuvants, such as MF59 used in the Chiron vaccine (Belshe *et al.*, 2012; Johnston *et al.*, 2011).

Other vaccines that have been promising in animal models include the subunit vaccine containing HSV-2 gG2 (Görander *et al.*, 2012) and several live-virus vaccines, such as HSV-2 vaccine constructs with deletions in the essential early genes UL5 and UL29 and UL41 (vhs) (dl5-29 and dl5-29-41L) (Hoshino *et al.*, 2009; Hoshino *et al.*, 2008) or deletions of ICP0 (Halford *et al.*, 2011). HSV-1 vaccine constructs with deletion in gE (Brittle *et al.*, 2008) or overexpressing gD in combination with a deletion of UL9 (origin of binding protein) (Brans & Yao, 2010) are also interesting vaccine candidates. Since herpesviruses have evolved so many mechanisms to evade host responses, it is difficult to foresee what antigen combination will be the winning concept. The use of subunit vaccines is challenging, as the use of a single antigen in the vaccine might not be enough to induce protecting immunity. The use of live attenuated vaccines might be a good alternative, as the only functional human herpesvirus vaccine available to this date is a live attenuated vaccine against varicella-zoster virus (VZV). Live attenuated vaccines against HSV can potentially induce stronger cell-mediated immune responses, and by also removing viral genes evolved in immune evasion the vaccine might become even more effective (Johnston *et al.*, 2011).

2.3 Viral vectors for gene therapy

Gene therapy is the treatment of inherited or acquired disease by delivering genetic material, such as DNA, to manipulate an individual's genes or correcting abnormal genes (American Society of Gene and Cell Therapy, http://www.asgct.org). Viral vectors can be utilized as vehicles for delivery of such therapeutic genes. Ideally, a vector for gene therapy should effectively express the therapeutic gene at the target site, but not have harmful effects on surrounding cells. Toxic or potentially harmful viral genes are most often deleted or inactivated to make the vector safe (Kay *et al.*, 2001).

The most frequently used viruses for construction of gene therapy vectors are retroviruses, adenoviruses, adeno-associated virus (AAV), lentivirus and HSV, but gene therapy vectors have also been constructed from many other viruses, including vaccinia virus, baculovirus and Semliki Forest viruses (SFV). Depending on the target disease, different vectors are suitable for different therapies. Tissue and cell tropism, transgene expression capacity and duration, and vector immunogenicity are import factors affecting the choice of a vector (Kay *et al.*, 2001; Thomas *et al.*, 2003). Some of the features of the main group of gene therapy vectors are described in Table 2.

Vector	Insert capacity	Vector genome forms	Tropism	Advances	Limitations
Adenovirus	*8 kb	Episomal	Broad	Efficient transduction of most tissues	Immunogenic Transient gene expression Pre-existing immunity
Adeno-associated virus (AAV)	Less than 5 kb	Episomal (less than 10 % integrated)	Broad	Non-inflammatory	Small packaging capacity Helper virus needed
Retrovirus	8 kb	Integrated	Dividing cells	Persistent gene transfer in dividing cells	Only transduce dividing cells. Low stability Risk of oncogenesis
Lentivirus	8 kb	Integrated	Broad	Persistent gene transfer in most tissues	Integration might induce oncogenesis
HSV-1	*40 kb	Episomal	Neurotropic	Large transgene capacity Strong neurotropism	Transient gene expression in non-neuronal cells

Table 2. The main group of viral vectors and some of their features. *Capacity is represented for replication-defective viruses. HSV-1 amplicons and helper-dependent adenoviruses can have even larger inserts. (Based on (Thomas *et al.*, 2003; Vannucci *et al.*, 2013)).

Viral vectors can be modified to specifically infect the target cell types in a process called vector targeting (reviewed in (Waehler *et al.*, 2007)). For example, transcriptional targeting is the use of specific promoters, functional only in the target tissue cell types, for the expression of the therapeutic gene. The use of microRNA target sequences can be used to detarget the expression of transgenes from certain types of cells. Thus, these vectors can infect many different cell types, but they express transgenes only in the target cells. Transductional targeting, on the contrary, is the modification of the virus to infect only target cells. This can be achieved e.g. by transferring viral attachment proteins from another virus strain to the new recombinant vector (a process called pseudotyping). Pseudotyping has been mostly used for the modification of retroviral, lentiviral and AAV vectors. Adaptor proteins, linking the viral attachment protein and the target cell receptors, represent another way of vector targeting. Ligands capable of redirecting the vector, and thus enabling recognition of target cells by single vector particles can be inserted into the vector capsid or envelope by genetic modification (Waehler *et al.*, 2007). The major hurdle the vectors encounter is the antiviral immune response they induce in the host. Adenovirus vectors are the most immunogenic vectors, inducing

cytotoxic T lymphocyte responses, promoting neutralizing antibody synthesis and cytokine-mediated inflammatory responses. Attempts to overcome such antiviral immunity have been undertaken by constructing helper virus-dependent vectors completely empty of viral genes (Thomas *et al.*, 2003; Vannucci *et al.*, 2013). Lentivirus and AAV vectors are less immunogenic. In many cases, the virus-induced immune response and other toxic side effects are dose-dependent. In clinical trials vectors may also elicit responses that differ in strength in different individuals (Thomas *et al.*, 2003).

By now, a total number of 1902 clinical gene therapy trials have been conducted or are ongoing in 31 different countries (Ginn *et al.*, 2013) (http://www.wiley.com//legacy/wileychi/genmed/clinical/). Although the huge potential for treatment of disease, the gene therapy-related risks are in many cases still considered too high for clinical use. Fortunately, research has improved the safety of many vectors, enabling their use also in clinical trials. Most of the clinical trials are conducted in the USA, where 1199 trials (63%) have been performed. Europe stands for 26% of the trials, with the United Kingdom (UK) performing the largest number of studies (198 trials, 10.4%). In Finland six clinical trials have been reported. Cancer is clearly the most common disease to be targeted in gene therapy trials (64.3%), followed by inherited monogenic diseases (mutations in single genes) and cardiovascular disease (8-9% each). Neurological diseases represent 1.9% of all clinical gene therapy trials. Adenoviruses and retroviruses are the most common gene therapy viral vectors, used in 23.2% and 19.4% of the trials, respectively. A retrovirus was the first virus used for successful clinical treatment of a genetic disorder called X-linked severe combined immunodeficienct (SCID-X1). Here, a Moloney leukemia virus-derived vector, expressing an IL-2 receptor gamma subunit, was used to transduce the patients´ hematopoietic stem cells *ex vivo* and then reinfused the cells into the patients. Unfortunately, 5 of the 20 patients treated developed leukemia some years later, and this severe side-effect could be directly coupled to the virus vector used. The use of integrating virus vectors has therefore been under debate (reviewed in (Biasco *et al.*, 2012)). Currently, two adenovirus vectors are in clinical use in China. These are replication-incompetent adenovirus vectors expressing a functional p53 gene (Gendicine and Oncorine). P53 is a naturally occurring tumor suppressor gene, which is often found to be mutated in different types of cancers. This vector has been used for the successful treatment of head and neck squamous cell carcinoma (reviewed in (Räty *et al.*, 2008)). HSV-1 has so far been used in 60 clinical trials (3.1%), mainly targeting gliomas (Ginn *et al.*, 2013) (http://www.wiley.com//legacy/wileychi/genmed/clinical/), but is gaining success in other therapy applications as well (discussed below).

2.3.1 Herpes simplex virus vectors

HSV-1-based vectors have several features making them good gene therapy vector candidates. They are neurotropic, but can be genetically modified so that they are not neurovirulent; they have a large transgene size capacity, and can maintain a life-long state of latency in neurons, which can be utilized for long-term transgene expression. HSV-1-based vectors possess the ability to infect a wide range of cell/tissue types, including both dividing and non-dividing cells, but they do not integrate into the host cell genome. HSV viral vectors are promising vehicles, especially for the transfer of genes into the CNS (Berges *et al.*, 2007). Three types of HSV vectors exist, amplicons, replication-defective HSV vectors and conditionally-replicating HSV vectors (Figure 8). The choice of vector is dependent of the goal of the therapeutic intervention.

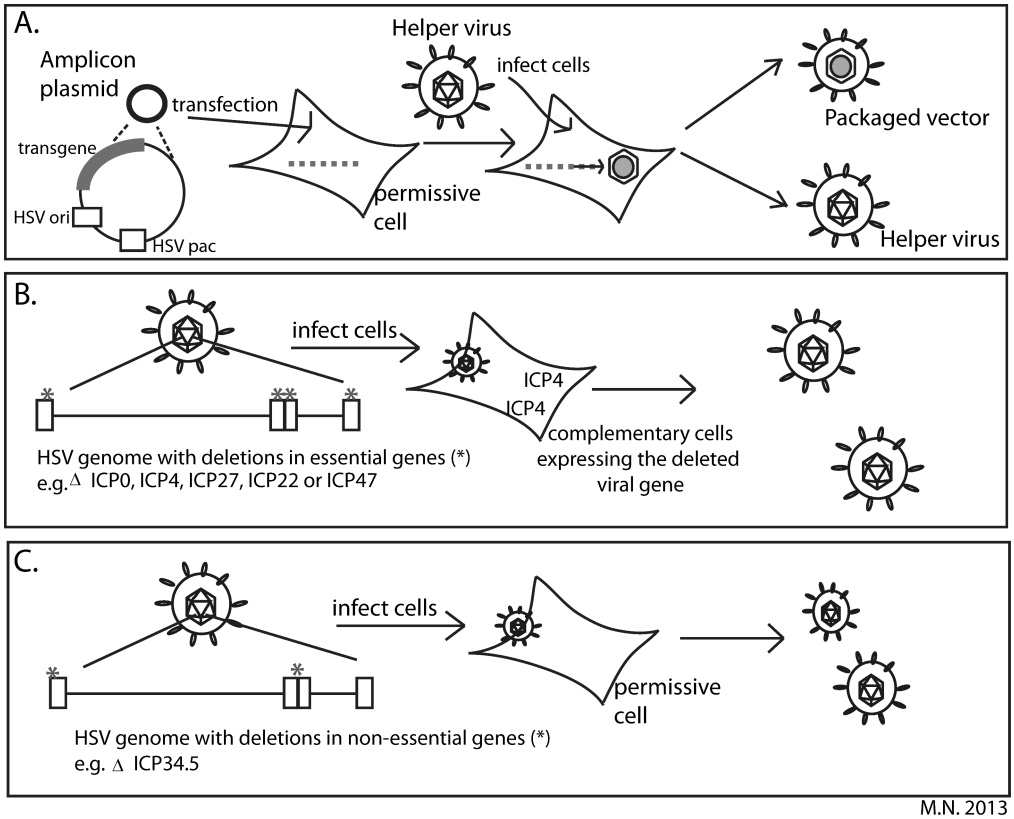

Figure 8. Different types of HSV vectors. A) HSV amplicons. A plasmid is constructed containing the transgene and an HSV *ori* for DNA replication (HSV ori) and an HSV packaging signal (HSV pac) for packaging the genomes into viral capsids. The plasmid is transfected into permissive cells and co-infection with a helper-virus provides all the enzymes and proteins needed for assembling the amplicon vector. B) Replication-defective HSV vectors have deletions in essential genes, which must be supplemented by the cell line in order for the virus to replicate in cell culture. C) Conditionally-replicating HSV vectors have deletions in non-essential genes, such as ICP34.5. These cells function normally in cell culture, but might be attenuated *in vivo*.

Amplicons are helper-dependent vectors that carry a minimal amount of viral genes (Spaete & Frenkel, 1982). This enables them to deliver very large foreign genomes (up to 150 kbp) to target cells and to be less toxic to the host (Tsitoura & Epstein, 2010). The name amplicon originates from the fact that multiple copies of a transgene can be amplified in a head-to-tail arrangement and be inserted into the virus particle. The amplicon genome originates from a plasmid carrying a packaging signal (*pac*) and an origin of replication (*ori*) from HSV-1. A helper virus is needed for the expression of proteins involved in amplification and packaging of the amplicon vector genome (Spaete & Frenkel, 1985). Since the HSV amplicon resembles wild-type HSV-1 in structure and other properties, these vectors can trigger a host response affecting the transgene expression. The main drawback with amplicons is indeed their short transgene expression and possible contamination by the helper virus (de Oliveira & Fraefel, 2010; de Silva & Bowers, 2009). By combining properties of other viruses with those of the HSV amplicon one can improve the vector efficiency and the stability of transgene expression. This can be achieved by introducing elements allowing the amplicon DNA to be integrated into the host genome, for example by the help of AAV or retrovirus components. Alternatively, the amplicon DNA can be maintained as a replicating episome by using the oriP and EBNA-1 gene elements from EBV. These types of vectors are called hybrid vectors (reviewed in (de Oliveira & Fraefel, 2010; de Silva & Bowers, 2009). HSV amplicons have been used in preclinical studies for a number of different CNS diseases, including Parkinson´s disease, Alzheimer´s disease, glioblastoma multiforme, ischemia, chronic lymphocytic leukemia and hereditary ataxia (reviewed in (de Silva & Bowers, 2011; Jerusalinsky *et al.*, 2012)).

Replication-defective or *non-replicative* vectors contain deletions in essential viral genes, such as ICP4, ICP0, ICP22, ICP27 and ICP47 (vhs), impairing the lytic replication and reactivation (reviewed in (Manservigi *et al.*, 2010)). Propagation of virus stock must therefore be prepared in complementing cell lines. Replication-defective HSV vectors expressing a wide range of therapeutic genes have been used in therapy of animal models of Alzheimer´s disease, Parkinson´s disease, multiple sclerosis (MS), chronic pain, diabetes, lysosomal disorders and epilepsy (Chattopadhyay *et al.*, 2003; Furlan *et al.*, 2007; Glorioso & Fink, 2009; Goss *et al.*, 2002; Hong *et al.*, 2006; Martino *et al.*, 2000; Martino *et al.*, 2005; Paradiso *et al.*, 2009; Puskovic *et al.*, 2006; Wolfe *et al.*, 2009; Yamada *et al.*, 1999) (reviewed in (Manservigi *et al.*, 2010)).

Conditionally-replicating HSV vectors have deletions in non-essential genes, such as the neurovirulence gene $\gamma_1$34.5, which render them to replicate only in certain cell types *in vivo*. HSV mutants deleted of the $\gamma_1$34.5 gene are non-neurovirulent (Chou *et al.*, 1990; Chou & Roizman, 1992; Whitley *et al.*, 1993), but have nevertheless the potential to spread to other cells of the CNS

(Markovitz *et al.*, 1997). HSV vectors lacking $\gamma_1$34.5 are able to replicate in rapidly dividing cells but not in post-mitotic neurons, making such oncolytic vectors promising candidates for therapy of CNS tumors (Campadelli-Fiume *et al.*, 2011; Martuza, 2000). Oncolytic vectors optimally replicate and cause lysis and death of infected tumor cells. The antitumor efficacy of HSV vectors deleted of $\gamma_1$34.5 has been further increased by expressing different immune-stimulating factors, such as IL-4, IL-10 (Andreansky *et al.*, 1998), IL-12 (Hellums *et al.*, 2005; Markert *et al.*, 2012; Parker *et al.*, 2000) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Liu *et al.*, 2003; Parker *et al.*, 2006). The Amgen Oncology´s HSV vector with deletions in $\gamma_1$34.5 and ICP47, and expressing the cytokine GM-CSF is currently in phase III clinical trials for treatment of melanoma (previously known as OncoVEX$^{GM\text{-}CSF}$, now named Talimogene laherparepvec) (Hu *et al.*, 2006; Senzer *et al.*, 2009). Other applications for conditionally-replicating HSV include gene therapy of ischemic brain injury, chronic pain, lysosomal disorders and multiple sclerosis (MS) (Antunes Bras *et al.*, 1998; Berges *et al.*, 2006; Broberg *et al.*, 2001; Laing *et al.*, 2010). Gene therapy models of MS will be further discussed in chapter 2.4.3. “Treatment of MS and EAE”.

Problems with the use of HSV vectors include the toxicity and immune responses to the HSV vectors, in addition to the relative short duration of transgene expression in many cell types. Toxicity can be decreased by removing certain viral genes, such as the immediate early genes. The immune response to HSV vectors depend on the purity of the vector stock, the route of delivery, the type of transgene and the number of injections needed (Manservigi *et al.*, 2010). HSV vectors deleted of $\gamma_1$34.5 have been reported to be toxic to ependymal cells (Kesari *et al.*, 1998; Lasner *et al.*, 1998). Changes in the cytokine expression after infection with $\gamma_1$34.5-deletion vectors have also been reported (Broberg *et al.*, 2004a). Since a large percentage of the population carries antibodies against HSV, pre-existing immunity could pose a problem. However, replicative HSV circumvents host immune responses effectively and pre-existing immunity has not been found to affect the vector induced immune responses (Brockman & Knipe, 2002; Chahlavi *et al.*, 1999). Vector administration in close proximity to the target tissue might also lower the risks of any vector inhibition by the immune response (Delman *et al.*, 2000). HSV vectors can be administered into the CNS either by direct intracranial injection, intrathecally, or through peripheral organs, such as the eye or nose (Frampton *et al.*, 2005). Intranasal administration of wild-type HSV-1 has proven effective in SJL/J mice (Broberg *et al.*, 2004a; Hudson *et al.*, 1991). Delivery of a TK-deletion vector intracranially have not reactivated latent wild-type HSV-1 (Wang *et al.*, 1997), further indicating that a previous infection with HSV would not necessarily be an obstacle for HSV gene therapy. Long-term gene expression could be

accomplished in neurons by delivering the transgene under the LAT promoter (Berges *et al.*, 2005).

HSV vectors have traditionally been constructed using a site-directed mutagenesis-protocol developed by Roizman and co-workers in 1981 (Post & Roizman, 1981). This "marker rescue method" relies on the thymidine kinase (TK) gene as a selection marker and requires several cloning steps. First, the initial TK needs to be removed. Then, the TK is reintroduced to a specific site in the viral genome via homologous recombination of a TK-plasmid and the viral genome. Thereafter the actual mutations or transgene insertions can be made in additional homologous recombination steps. Finally, the TK is usually also repaired (Post & Roizman, 1981). Another method for constructing recombinant HSV is the use of HSV cosmids. A set of cosmids is constructed to contain the whole genome collectively. The cosmids are co-transfected and overlapping fragments enables reconstitution of the viral genome (Cunningham & Davison, 1993). The "marker rescue method" is very time-consuming and several viral intermediates need to be constructed and purified before mutagenesis. The use of cosmids may instead lead to a higher risk of unwanted mutations (Parker *et al.*, 2011). Some years ago, the herpesvirus CMV was first cloned as a bacterial artificial chromosome (BAC), a system which has simplified the procedure significantly. The BAC methodology for constructing herpes simplex vectors will be discussed in more detail next.

2.3.2 Construction of HSV vectors as bacterial artificial chromosomes

Bacterial artificial chromosomes (BACs) are vectors able to carry very large inserts up to 300 kb (Shizuya *et al.*, 1992). BACs are mini F plasmids, 7 kb or longer in length (O'Connor *et al.*, 1989). They can quite easily accommodate the whole herpes viral genome and they have been utilized for construction of recombinant herpes viruses (Messerle et al 1997). This technique maintains the viral genome as a BAC in *Escherichia coli (E. coli)*. Viral progeny is produced after transfection of the recombinant BAC plasmid into eukaryotic cells. With this method any genetic modifications should be possible (Adler *et al.*, 2003).

The first herpes BAC was constructed for murine cytomegalovirus (Messerle *et al.*, 1997) and since then herpes-BACs have been created for all human herpesviruses, except for HHV-6B and HHV-7 (Borenstein & Frenkel, 2009; Borst *et al.*, 1999; Delecluse *et al.*, 1998; Horsburgh *et al.*, 1999; Meseda *et al.*, 2004; Nagaike *et al.*, 2004; Saeki *et al.*, 1998; Stavropoulos & Strathdee, 1998; Zhou *et al.*, 2002). A typical BAC vector contains an origin of replication, bacterial replication genes (*repE*) and genes to control the replication rate (e.g. *parA* and *parB*). In addition, an antibiotic resistance marker (e.g. chloramphenicol), a selection marker and 500-2000 bp flanking sequences

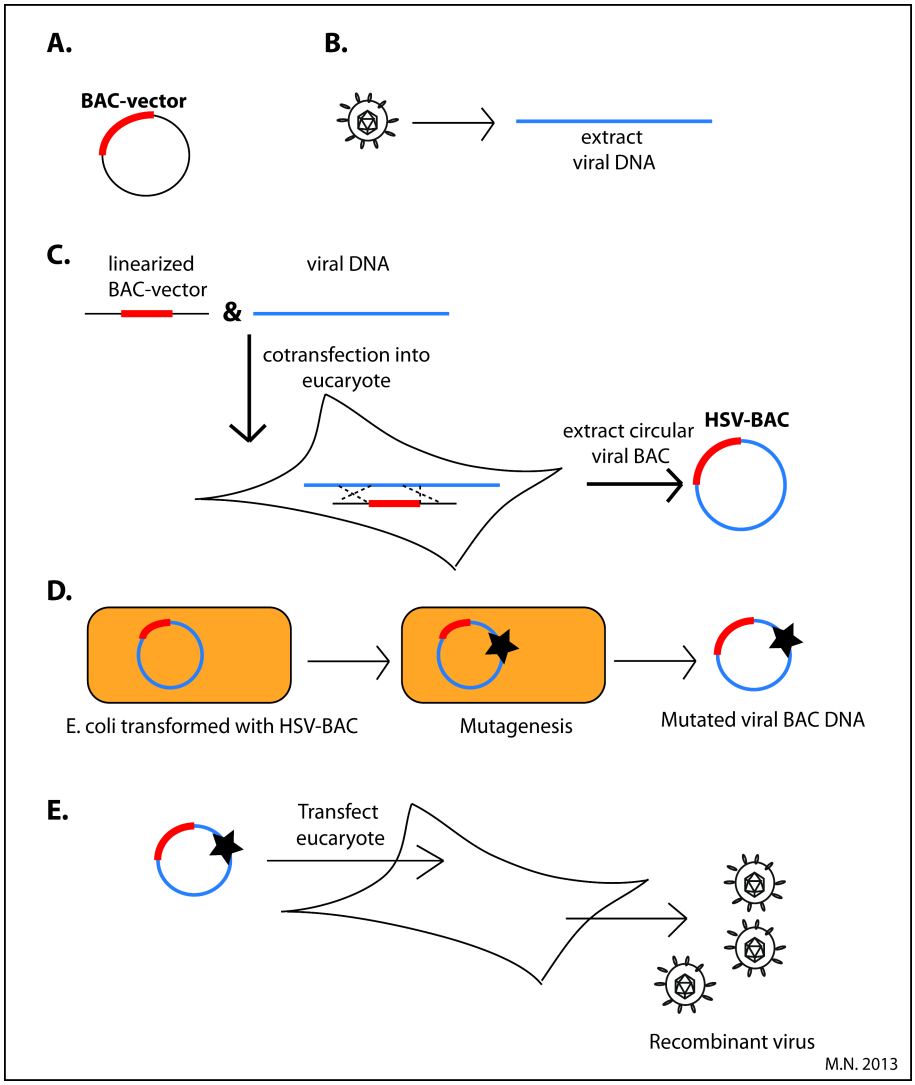

Figure 9. Construction of viral bacterial artificial chromosomes (BACs). A) A BAC vector containing required bacterial genes for replication and maintenance, and homologous flanking sequences (marked in red) is constructed. B) Eukaryotic cells are infected with HSV-1 and circular viral genome is recovered with Hirt extraction. C) The linearized BAC vector and viral DNA is cotransfected into eukaryotes and viral DNA containing BAC sequences is recovered. D) Viral-BAC DNA is transformed into *E. coli*. Mutagenesis is performed while viral-BAC is maintained in bacteria. Recombinant viral BAC DNA is extracted. E) Viral BAC DNA is transfected into eukaryotes and recombinant virus is recovered. (Modified from (Messerle *et al.*, 1997; Nagel, 2006; Wagner *et al.*, 2002)).

homologous to the target site in the viral genome are needed for selection of the correct clones and homologous recombination. The linearized BAC vector is co-transfected with the viral DNA into permissive cells (Figure 9). Homologous recombination will occur between the BAC vector and the viral genome and a BAC containing the viral sequence is produced. With the help of a selection

marker (such as β-galactosidase) BAC clones containing the viral genome is recognized. Circular viral DNA can be isolated and purified from the cells according to the method of Hirt (a method for extraction of extrachromosomal DNA) (Hirt, 1967), and transformed into *E.coli* (Figure 9). Bacterial cells containing the viral BAC are then selected based on the antibiotic selection marker and purified DNA is analyzed by restriction enzyme digestion and gel electrophoresis. (Reviewed in (Warden *et al.*, 2011)).

When a stable viral BAC has been isolated, different types of site-specific mutagenesis can be performed on it to construct viral mutants. When the specific alterations of the viral BAC have been confirmed, large amounts of DNA is produced and then transfected into mammalian cells. Since the insertion of the BAC sequences might attenuate the virus, it is desirable that the BAC vector is removed from the final viral genome. This is achieved by surrounding the BAC vector with loxP sites, enabling excision of the BAC vector from the viral BAC genome upon transfection into eukaryotic cells expressing Cre-recombinase (Adler *et al.*, 2001; Smith & Enquist, 2000; Wagner *et al.*, 1999).

The BAC technology has become important for the study of herpes pathogenesis, gene function and vaccine development. The location of the BAC sequences in the viral genome affects the attenuation of the mutant virus. Many of the first HSV BACs were replication-defective or non-infectious *in vivo* due to distortion of viral genes, such as the TK and viral packaging elements, by the BAC vector (Horsburgh *et al.*, 1999; Saeki *et al.*, 1998; Stavropoulos & Strathdee, 1998). Later, infectious HSV BACS have been constructed with the BAC element inserted either between two HSV genes or in the TK locus, but where TK is repaired (Nagel *et al.*, 2008; Tanaka *et al.*, 2003). Several studies have shown that the construction of HSV-1 BACs does not alter the viral characteristics drastically (Gierasch *et al.*, 2006), even though some alterations due to homologous recombination might occur. For example, modifications of the *a* sequences and loss of oriL have been reported (Nagel *et al.*, 2008). The BAC method is faster than the conventional methods (e.g. the marker rescue method or the use of cosmids), and viral BACs are more stable than other viral mutants (reviewed in (Warden *et al.*, 2011). Still, careful characterization of the constructs must be performed in order to detect any unwanted mutations.

Mutagenesis of viral BACs

Site-specific mutagenesis of the virus BAC genome can be accomplished via homologous recombination in special *E. coli* strains by using a positive selection marker. For recombination of linear DNA fragments in *E. coli*, the bacterial strains should have the functions of RecE and RecT or redα (exo), redβ (bet) and

redγ (gam). Redα (or RecE) is a 5´-3´exonuclease that creates single-stranded 3´overhangs and redβ (RecT) mediates the binding to complementary single-stranded DNA. These recombination proteins can be introduced into bacterial strains such as DH10B containing the viral BAC, by transformation of the plasmid pKD46 (Wagner & Koszinowski, 2004). Bacterial strains have also been developed for constitutive expression of the red-recombination functions. These include GS1783 and DY380 (Lee *et al.*, 2001; Tischer *et al.*, 2010). Several protocols exist for making mutations to the BAC viral genome, including ET recombination (named after the proteins RecE and RecT), a two-step replacement (shuttle mutagenesis) and *en passant*-mutagenesis (Borst *et al.*, 2004; Tischer *et al.*, 2006; Wagner & Koszinowski, 2004), depicted in Figure 10.

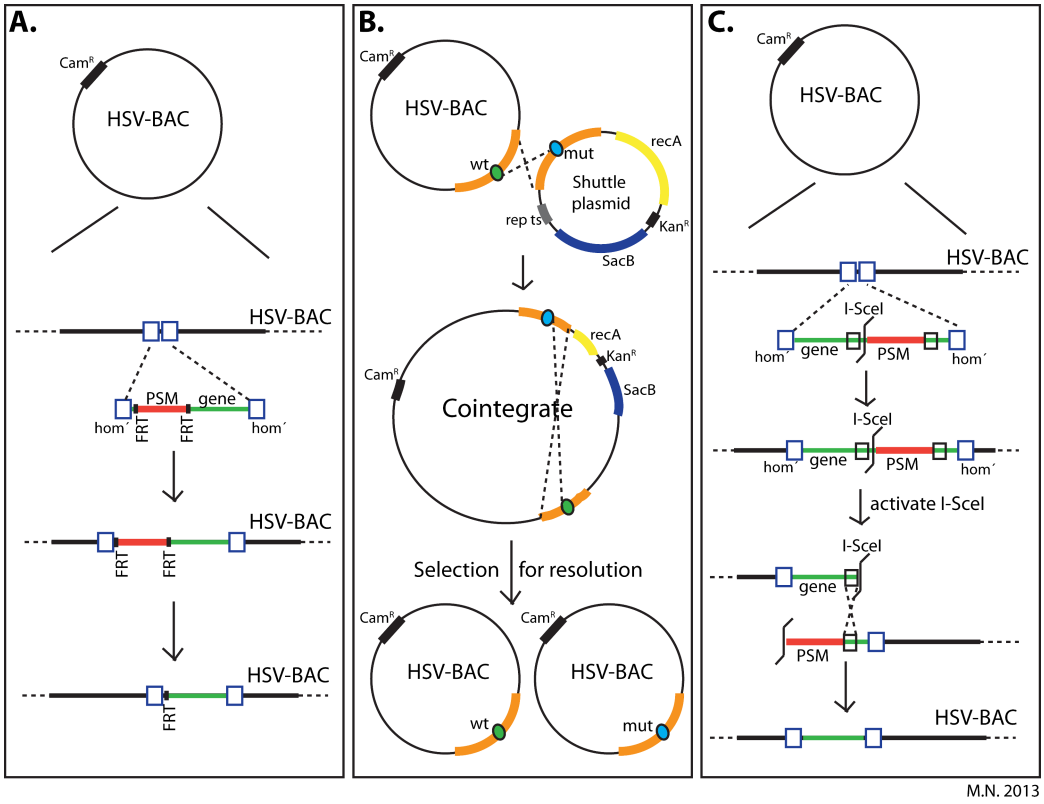

Figure 10. Mutagenesis of viral BACs by different methods. Insertion of a transgene into a viral BAC with A) ET cloning, B) two-step replacement method (shuttle mutagenesis) or C) *en passant*-mutagenesis. The methods are described in more detail in the text below.
Abbreviations: Cam^R, chloramphenicol resistance marker; FRT, Flip-recognition target site; hom´, homologous sequences; PSM, positive selection marker (e.g. kanamycin resistance); recA, rec A protein mediates recombination between plasmid and BAC; rep ts, provides temperature-sensitive replication: SacB, provides sucrose sensitivity; wt, wild-type.

ET cloning is a one-step procedure where a linear DNA fragment is inserted into the viral BAC by homologous recombination (Figure 10A) (Muyrers *et al.*, 1999; Wagner & Koszinowski, 2004). The DNA fragment to be inserted should contain a selection marker, for example kanamycin resistance, to enable selection of correct clones. The selection marker can be removed by flanking it with FLP recognition target sites (FRT) or loxP sequences (Wagner & Koszinowski, 2004). The enzymes FLP recombinase and Cre excise the DNA at the respective sites (Adler *et al.*, 2000; Cherepanov & Wackernagel, 1995).

The two-step replacement procedure entails the use of shuttle plasmids (Figure 10B). The shuttle plasmid contains the modified allele or insert, surrounded by flank sequences homologous to the integration site (Borst *et al.*, 2004). By homologous recombination the complete shuttle plasmid is inserted into the viral BAC genome, giving rise to a cointegrate. For this step to occur, the bacterial strain needs to express recA. The cointegrate can resolve via homologous recombination with the flank sequences. Only approximately 5% of the resolved clones contain the mutant allele, 5% the original allele, and 90% the cointegrate. To select against the cointegrate, a sacB gene can be inserted into the shuttle plasmid, enabling identification of the BAC clones with either of the alleles (Borst *et al.*, 2004).

The *en passant*-mutagenesis enables recombination without leaving any extra sequences in the final viral BAC (Figure 10C and Figure 14) (Tischer *et al.*, 2010; Tischer *et al.*, 2006). Here, a duplication sequence in addition to the selection marker is inserted in the linear DNA fragment. An I-SceI restriction site is also inserted for removal of the marker cassette in a second recombination step. I-SceI is a homing endonuclease, recognizing an 18 bp recognition sequence rarely present in genome sequences (Colleaux *et al.*, 1986). With this method, insertions, deletions and point mutations of the viral BAC genome can be accomplished.

2.4 Experimental autoimmune encephalomyelitis (EAE)

2.4.1 EAE - the primary model of multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS, where autoreactive immune cells attack the myelin sheath surrounding the nerve axons (reviewed in (Compston & Coles, 2008; Noseworthy *et al.*, 2000)). Inflammation, demyelination, axonal loss and gliosis are all features of the disease. The destruction of the myelin sheath leads to an impairment of the nerve signals, with subsequent clinical symptoms such as vision loss, ataxia, motor dysfunctions, and finally paralysis. MS can be divided into subtypes,

depending on the disease progress. Relapsing-remitting MS (RRMS) occurs in 80-85% of the patients, where symptoms occur for a period of time and then disappear. About 65% of the patients with RRMS will eventually develop secondary progressive MS (SPMS), where a gradual worsening of the disease occurs. Primary progressive MS (PPMS) is diagnosed in 15-20% of the patients, with clinical signs progressively worsening from onset of the disease (Compston & Coles, 2008).

The exact mechanisms behind the development of MS remain unknown. Several factors, including environmental factors, virus or bacterial infections and genetic susceptibility have been proposed (Ascherio & Munger, 2007a; b; Hafler *et al.*, 2007; Peltonen, 2007). There is a higher incident of disease in northern Europe and USA and in southern Australia, whereas MS is less common in countries at the equator (Compston & Coles, 2008). MS affects approximately two million people worldwide and 7000 persons in Finland, three of four patients being women (http://www.ms-liitto.fi/english) (Compston & Coles, 2008). To date, there is no cure for MS, but immunomodulative therapies, such as interferon-β, glatiramer acetate and natalizumab, may delay progression of the disease (Comi *et al.*, 2001; Jacobs *et al.*, 1996; Kappos *et al.*, 2009; Polman *et al.*, 2006; Rudick *et al.*, 2006). Much of our knowledge about MS pathophysiology and therapy targets is received from animal studies.

Experimental autoimmune encephalomyelitis (EAE) is a T-cell mediated inflammatory disease of the CNS and functions as the primary animal model for multiple sclerosis (MS) (Lando *et al.*, 1980; Rivers & Schwentker, 1935; Zamvil & Steinman, 1990). Symptoms include tail atonia, limb paralysis, weight loss and incontinence. EAE can be induced by immunization with myelin-derived antigens in adjuvants (Amor *et al.*, 1994; Linington *et al.*, 1993; Mendel *et al.*, 1995; Tuohy *et al.*, 1988; Zamvil & Steinman, 1990) or by adoptive transfer of myelin-activated T cells (Pettinelli & McFarlin, 1981). Depending on the animal strain and the induction protocol, disease pathogenesis differs in the different models (Owens, 2006). The most common models include induction of Biozzi AB/H mice, SJL mice and DA rats with myelin antigens such as myelin basic protein (MBP) and proteolipid protein (PLP), whereas C57BL/6 mice can be induced with the minor myelin protein myelin oligodendrocyte glycoprotein (MOG) (Amor *et al.*, 1994; Mendel *et al.*, 1995; Slavin *et al.*, 1998; Tuohy *et al.*, 1989), (reviewed in (Kipp *et al.*, 2012)). Freund´s adjuvant and pertussis toxin are used to further induce an autoimmune inflammatory response by affecting the BBB and T cells (Billiau & Matthys, 2001; Jee & Matsumoto, 2001; Linthicum *et al.*, 1982; Richard *et al.*, 2011). One explanation to the varying susceptibility and disease pathogenesis has been explained by differences in the Th1/Th2 biases and MHC haplotypes (Baker *et al.*, 1995). SJL/J and C5BL/6 mice are Th1 prone and susceptible, whereas BALB/c is Th2

prone and less susceptible (Butler *et al.*, 2002). The repertoire of disease models can be an advantage, broadening our understanding of the disease pathogenesis and might facilitate the translation from animal to human therapies.

2.4.2 Disease pathogenesis

The definite mechanisms contributing to the immune-mediated demyelination remain unknown. Autoreactive T cells recognizing myelin antigens are present in the blood circulation of both healthy persons and MS patients. These T cells have to be activated in order for an autoimmune reaction to be induced (Costantino *et al.*, 2008). "Molecular mimicry" and "bystander activation" have been suggested as inducers of the autoimmune response in MS. Molecular mimicry is the triggering of T cell activation by viral or other foreign antigens, with similarities to the host cell (Libbey *et al.*, 2007). "Bystander activation" proposes that an infection exposes self-antigens in the host, and that these self-antigens together with cytokines and chemokines further activate T cells to target myelin (Fujinami *et al.*, 2006; McCoy *et al.*, 2006). Independent of the underlying mechanism, studies of EAE indicate that the autoreactive T cells and antibodies activated in the periphery travel in the circulation to the brain, where vascular cellular adhesion molecules (VCAM)-1 and matrix metalloproteinases (MMP) facilitate the T cell trafficking across the blood-brain barrier (BBB) (Gold *et al.*, 2006; Noseworthy *et al.*, 2000) (Figure 11). Activated T cells expressing IFN-γ, TNF-α, and other inflammatory cytokines, further activate APC in the CNS, including astrocytes, microglia and macrophages. Recognition of myelin by MHC molecules on these APC activates T cells and induces expression of cytokines. Consequently macrophages and $CD8^+$ T cells are activated, which attack the myelin sheath (Noseworthy *et al.*, 2000) (Figure 11). Myelin-specific antibodies, in addition to the activated T cells and induced inflammatory cytokines, contribute to the demyelination process. When these antibodies have bound to myelin, they attack the myelin sheath and further recruit effector cells to the site (Gold *et al.*, 2006; Hukkanen *et al.*, 1983; Hukkanen *et al.*, 1982; Ryberg, 1978). In normal healthy mice these antibodies do not cause disease, since they do not reach the brain in sufficient amounts (Litzenburger *et al.*, 1998) (Figure 11).

Cytokines are an important part of the host's immune response against pathogens and pro-inflammatory cytokines are believed to be essential for the onset of autoimmune reaction in MS and EAE (Issazadeh *et al.*, 1995a; Liblau *et al.*, 1995). These cytokines function by sustaining the inflammation in the CNS, activating myelin-specific T cells and recruiting effector cells from the periphery to the CNS (Kieseier *et al.*, 1999) (Figure 11). Most of the earlier studies on T cell functions in EAE and MS have focused on the Th1/Th2

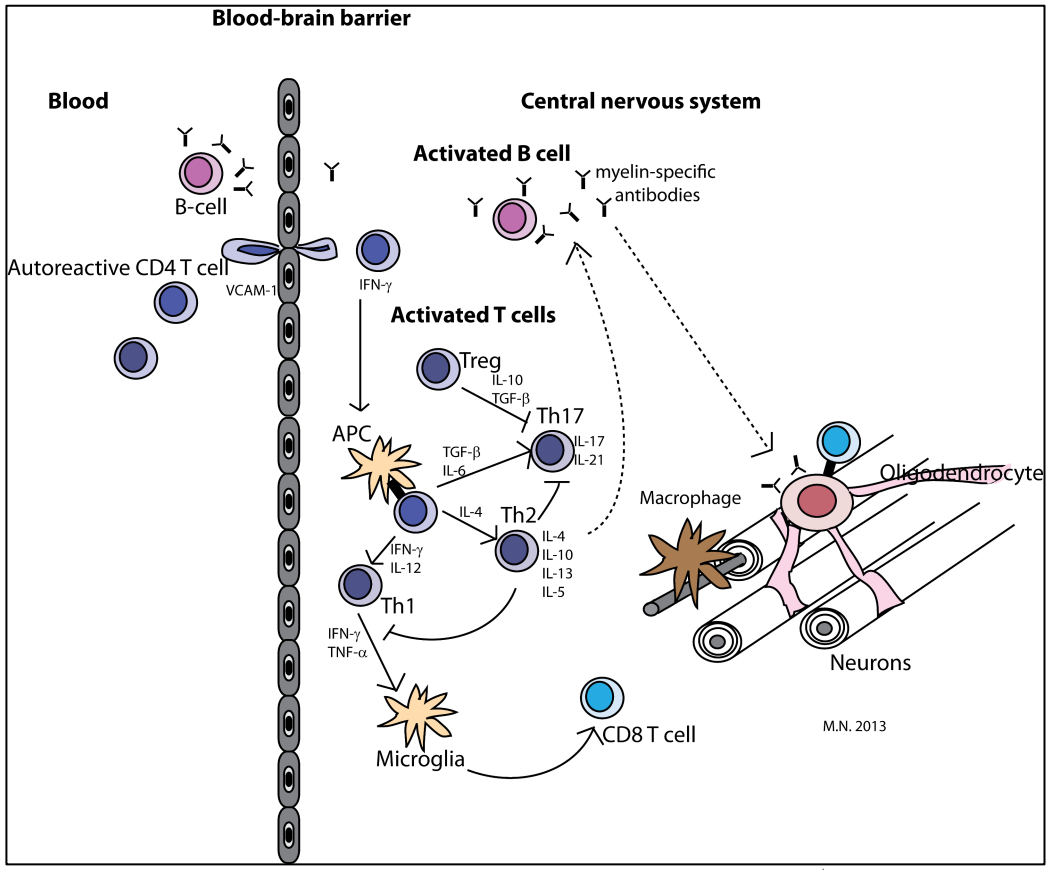

Figure 11. Immunopathological mechanisms of EAE. Autoreactive $CD4^+$ T cells and B cells are activated in the periphery and reach the blood-brain barrier (BBB) via the blood. VCAM-1 facilitates the trafficking over the BBB. Activated T cells express cytokines, which further activate antigen presenting cells (APC). The MHC molecules on these APCs recognize myelin peptides, and activate different T cell populations (Th1, Th2 and Th17). Cytokines produced by the activated T cells activate macrophages and $CD8^+$ T cells, which attack the myelin sheath. Autoreactive myelin-specific antibodies further recruit effector molecules to the site of inflammation. T regulatory cells (Tregs) suppress the effects of the self-reactive T cells. (Modified from (Hukkanen *et al.*, 2012; Noseworthy *et al.*, 2000)).

paradigm (Nicholson & Kuchroo, 1996), but is today known to also involve Th17 cells and Tregs (Goverman, 2009). The Th1 cells, induced by IL-12 and expressing IFN-γ, TNF and IL-2, were until some years ago believed to be the essential T cell population involved in the pathology and onset of the disease. This theory was partly based on the observations that several Th1 cytokines are up-regulated during disease (Issazadeh *et al.*, 1995a; Issazadeh *et al.*, 1995b; Olsson, 1995). Increased amounts of IFN-γ, IL-2, IL-12 and TNF-α have also been detected in MS patients (Balashov *et al.*, 1997; Gallo *et al.*, 1988; Hohnoki *et al.*, 1998; Lu *et al.*, 1993; Maimone *et al.*, 1991). The disease-promoting functions of these Th1 cytokines are further supported by studies showing that

TNF-α is directly toxic for oligodendrocytes (Selmaj & Raine, 1988), that treatment with antibodies against IL-12 ameliorates EAE (Ichikawa *et al.*, 2000; Leonard *et al.*, 1995) and that IFN-γ treatment exacerbates the disease (Panitch *et al.*, 1987). Still, some of these cytokines might also have protecting properties, since depletion of IFN-γ with antibodies exacerbates the disease (Billiau *et al.*, 1988).

The role of Th1 cells in the EAE pathogenesis was questioned, when induction of EAE in IL-12 and IL-23 knock-out mice showed that IL-23 and not IL-12 is the crucial cytokine in CNS inflammation (Becher *et al.*, 2002; 2003; Cua *et al.*, 2003). IL-23 was further reported to drive the expression of IL-17, expressed by Th17 cells (Langrish *et al.*, 2005), but is now believed to be important for the maintenance and longterm survival of the Th17 cell population (Stritesky *et al.*, 2008). Expression of TGF-β and IL-6 induces the proliferation of Th17 cells (McGeachy *et al.*, 2007). Th17 cells are now considered to have an important pathogenic role in the disease (Hofstetter *et al.*, 2005; Langrish *et al.*, 2005; Park *et al.*, 2005). It is likely that both Th1 and Th17 cells contribute to the pathogenesis, since both cell types can induce EAE, but might do so in different ways (Kroenke *et al.*, 2008; Luger *et al.*, 2008; Stromnes *et al.*, 2008) (reviewed in (El-behi *et al.*, 2010)).

Tregs are important in maintaining the balance between tolerance and immunity (Sakaguchi, 2004) and suppress pathogenic autoreactive T cells (Miyara & Sakaguchi, 2007; O'Connor & Anderton, 2008; Sakaguchi, 2005). They control neuroinflammation in EAE, as seen with elevated amounts of Tregs during disease recovery and increased EAE severity when depleted (McGeachy *et al.*, 2005; Montero *et al.*, 2004; Stephens *et al.*, 2005). Treg function is associated with the expression of TGF-β and IL-10 (Zhang *et al.*, 2004; Zhang *et al.*, 2006) (reviewed in (O'Connor & Anderton, 2008)). Already some years ago TGF-β1 was reported to have a regulatory role in EAE (Johns *et al.*, 1991; Kuruvilla *et al.*, 1991; Racke *et al.*, 1991). Thus, this reported role of TGF-β in EAE might be linked to the role of Tregs. Th2 cells have also been associated with the recovery phase of EAE and MS (Cua *et al.*, 1995). A disease limitating role has been suggested especially for IL-4 and IL-10 (Bettelli *et al.*, 1998; Kennedy *et al.*, 1992; Racke *et al.*, 1994). The function of Th2 cells on EAE and MS pathogenesis include down-regulation of Th1 responses and suppression of inflammation (Hill & Sarvetnick, 2002).

2.4.3 Treatment of EAE and MS

The treatments available today for therapy of MS include IFN-β1a, IFN-β1b, glatiramer acetate, natalizumab and mitoxantrone (reviewed in (Barten *et al.*, 2010)). The exact therapeutic mechanism of IFN-β and glatiramer acetate is still

unclear, but they have been proposed to work by interfering with T cell activation (Schmied *et al.*, 2003; Theofilopoulos *et al.*, 2005). Furthermore, IFN-β reduces MHC class II expression in the brains by modulating the APC and impairing $CD4^+$ T cell functions (Axtell & Steinman, 2008; Prinz *et al.*, 2008). Natalizumab (Tysabri, Elan Pharmaceuticals Inc., San Francisco, USA) is a monoclonal antibody against α4β1 integrin (VLA4) blocking the influx of leukocytes across the BBB (Rudick *et al.*, 2006). One severe side-effect of natalizumab treatment, is a higher risk of developing progressive multifocal leukoencephalopathy (PML) after polyoma virus JC infection (Berger & Koralnik, 2005). Mitoxantrone is used for treatment of RRMS and SPMS, but due to enhanced risk of severe side-effects, it is used only when other drugs are ineffective. Since the available treatments are only partially effective and not suitable for all patients, new therapies are needed. Drugs in Phase III trials include spingosine-1-phosphate (SIP-1) receptor antagonist (Fingolimod by Gilenia Biogen Idec, Cambridge, USA) and cladribine (Cohen *et al.*, 2010; Comi *et al.*, 2012; Giovannoni *et al.*, 2010; Kappos *et al.*, 2010). SIP-1 receptors are located on lymphocytes and are responsible for their movement from peripheral lymph nodes. Cladribine inhibits DNA synthesis and reduces the amount of $CD4^+$ and $CD8^+$ T cells, cytokines and chemokines. (reviewed in (Barten *et al.*, 2010)).

Systemic delivery of anti-inflammatory molecules has limitation in therapy since cytokines rarely cross the BBB (Saunders *et al.*, 1999). There are several therapeutic approaches for treatment of EAE, including systemic administration of cytokines, DNA vaccination, stem cell therapy and viral vectors (reviewed in (Baker & Hankey, 2003; Fissolo *et al.*, 2012; Karussis & Kassis, 2008; Reekmans *et al.*, 2012; Slavin *et al.*, 2002)). By using viral vectors for the delivery of cytokines directly to the CNS, higher levels and longer transgene expression can be achieved in addition to circumventing the peripheral immune responses (Martino *et al.*, 2001).

Viral therapy of EAE

Vectors based on retrovirus, adenoviruses, HSV, SFV, vaccinia virus and lentivirus have been used to express transgenes for gene therapy of EAE (reviewed in (Baker & Hankey, 2003)). Retroviruses have mainly been used as retrovirally transduced cell carriers to insert therapeutic genes into T cells, which are then reintroduced in the mice (Chen *et al.*, 1998). Lentivirus vectors have also been used to modify T cells by engineering therapeutic Tregs (Fransson *et al.*, 2012). Lentivirus vectors have further been constructed to express therapeutic agents such as cytokines, growth factors and short hairpin RNA against Actin 1 (Act1) targeted to the CNS, causing modulations of the

immune responses and protecting the CNS (Maiorino *et al.*, 2012; Slaets *et al.*, 2010; Yan *et al.*, 2012). Vaccinia virus has been used as vectors for treatment of EAE by expressing myelin peptides, but also for systemic delivery of cytokines (Genain *et al.*, 1997; Wang *et al.*, 1999; Willenborg *et al.*, 1995).

Th2 cytokines have been utilized in therapy of EAE, with the initial aim to shift the immune response from a Th1 dominance towards a Th2 profile. Administration of IL-4 by viral vectors has shown beneficial effects in EAE recovery. Replication-defective HSV vectors expressing IL-4 inhibited the progression of disease in both Biozzi AB/H mice and rhesus monkeys (Furlan *et al.*, 2001; Poliani *et al.*, 2001), and a conditionally replicating HSV vector with deletions in $\gamma_1$34.5 and expressing IL-4 ameliorated EAE in BALB/c mice (Broberg *et al.*, 2001). Helper-dependent adenovirus vectors expressing IL-4 also facilitated recovery from EAE (Butti *et al.*, 2008). The therapeutic function behind these IL-4 therapies included increase in IL-4 expression, reduced CNS inflammation and demyelination, and recruitment of anti-inflammatory Tregs (Broberg *et al.*, 2004b; Broberg *et al.*, 2001; Butti *et al.*, 2008; Furlan *et al.*, 2001; Poliani *et al.*, 2001). Another Th2 cytokine, IL-10, has also been utilized in gene therapy studies, but with controversial results. A lentivirus vector expressing IL-10 administered intranasally ameliorated EAE (Jerusalmi *et al.*, 2003), whereas a $\gamma_1$34.5-deleted HSV vector or an adenovirus vector did not have a significant effect on the disease course (Broberg *et al.*, 2001; Croxford *et al.*, 2001). IL-25 is a cytokine with anti-inflammatory functions, including Th17 regulation and induction of Th2 responses (Kleinschek *et al.*, 2007). By using a lentivirus expressing IL-25, neuroinflammation was partly inhibited and macrophages were modified to a more anti-inflammatory phenotype (Maiorino *et al.*, 2012). IL-1β is a proinflammatory cytokine known to be pathogenic in MS and EAE. A non-replicative HSV vector deleted of ICP4, ICP27 and ICP22, and expressing the IL-1 receptor antagonist gene (IL-1Ra) delayed the disease onset and severity in EAE in C57BL/6 mice. The therapeutic mechanism was associated with fewer infiltrating macrophages and decreased amount of pro-inflammatory cytokine response (Furlan *et al.*, 2007).

Therapies promoting remyelination or protection against demyelination would be of great of importance. Gene therapy targeting neurotropic growth factors, especially affecting oligodendrocytes has been attempted. One such factor is fibroblast growth factor (FGF)-II, which is known to promote migration and proliferation of oligodendrocyte precursor cells. A replication-deficient HSV vector expressing FGF-II was used to treat EAE in C56BL/6 mice (Ruffini *et al.*, 2001). The FGF-II viral therapy led to an increase in the number of oligodendrocytes in demyelinated areas and a reduction of myelin-reactive T cells and macrophages (Ruffini *et al.*, 2001). More studies regarding the action of FGF-II is needed, since it has been reported that FGF-II also inhibits myelin

production by oligodendrocytes (Goddard *et al.*, 2001). Transforming growth factor beta 1 (TGF-β1) is a cytokine affecting the myelinating capacity of oligodendrocytes and inhibiting differentiation of T cells (Diemel *et al.*, 2003; Gorelik *et al.*, 2002). A systemically administered Semliki Forest virus (SFV) vector expressing TGF-β1 reduced the disease severity in EAE, but the mechanism remained to be elucidated (Vähä-Koskela *et al.*, 2007). Another interesting gene candidate is leukemia inhibitory factor (LIF). Endogenous LIF has been shown to enhance oligodendroglial maturation and limit autoimmune demyelination and oligodendrocyte loss (Butzkueven *et al.*, 2006; Butzkueven *et al.*, 2002). LIF-producing lymphocytes and macrophages have also been detected in both active and chronic MS lesions (Vanderlocht *et al.*, 2006). Gene therapy with viral vectors expressing LIF has also been introduced, where a lentivirus vector expressing LIF significantly inhibited relapses in a model of EAE (Slaets *et al.*, 2010).

3 AIMS OF THE STUDY

The aim of this doctoral study was to gain better knowledge about HSV-1 pathogenesis and subsequent immune responses *in vivo* with the purpose of developing new HSV-1 vectors for gene therapy of EAE. This study focused on understanding the HSV-1 infection in the SJL/J mouse, since this animal strain is commonly used in studies of experimental autoimmune encephalitis. We wanted to investigate the cytokine profile in response to HSV-1 infection and to HSV-1-mediated cytokine gene therapy of EAE.

The specific aims of the study were:

1) To develop sensitive quantitative PCR-based detection of cytokine mRNA in tissue samples.

2) To study the spread and replication of HSV-1 in SJL/J mice upon infection via different administration routes and to investigate the consequent immune responses.

3) To develop novel HSV-1 vectors for gene therapy of EAE, utilizing the BAC methodology.

4) To study the effects of HSV vectors expressing immunomodulatory factors, especially Th2 cytokines and LIF, in EAE.

4 MATERIALS AND METHODS

4.1 PCR standards (I-IV)

External standards were constructed for each target of interest to be quantified with LightCycler real-time PCR (I) or Rotor-Gene real-time PCR (II-IV). The cytokine standards were cDNA from RNA, or in some cases cDNA plasmids. Plasmids were used for IFN-γ (a kind gift from Dr Jorma Määttä, Åbo Akademi University, Turku, Finland), IL-12p40, IL-12p35 (a kind gift of Professor Ueli Gubler, Hoffman-La Roche Inc., Nutley, NJ, USA), IL-23p19 (IL-23), β-actin, (described in (Broberg *et al.*, 2003)), IL-4 and IL-10. For amplification of the viral transcripts VP16 and HSV-1 gD, the plasmid pRB3717 (McKnight *et al.*, 1987) or purified HSV-1 viral DNA was used. Standards for all other cytokines were prepared from mouse tissue RNA. The RNA was converted to cDNA by reverse transcription (see 4.12) and finally a longer cDNA transcript was amplified with PCR, covering the target sequence for each cytokine. TLR9 was an exception, where the same primers were used for making the standard and as detection primers. Primers used for the PCR amplification of standards are listed in Table 3.

The PCR products were purified with either NucleoSpin Extract II-kit (Macherey Nagel, Düren, Germany), or by running the products on a Tris-Borate-EDTA (TBE)-gel or a Tris-Acetate-EDTA (TAE)-gel and cleaning the excised band with a QiaexII gel extraction kit (Qiagen, Hilden, Germany). The concentration was measured from the purified products or viral DNA and the corresponding copy numbers (per µl) were calculated with the following equation:

$$\frac{N_A \text{ [copies/mol]} \times \text{concentration [µg/ul]} \times \text{dilution factor } (10^{-6})}{\text{product length [bp]} \times 660 \text{ DA/bp [g/mol]}}$$

$$= \text{amount [copies per µl]}$$

(Where N_A= Avogadro´s constant, 6.022×10^{23} and DA=Daltons, where 660 g/mol is the molar mass for dsDNA. Modified from (I)).

The standard stock concentrations were made to contain 10^8 or 10^9 copies per 2 µl.

Table 3. Primer sequences for amplification of quantitative real-time PCR standards.

Target gene (studies used)	Primer name	Sequence (5` - 3`) (or reference)	Amplicon length (bp)	Genbank reference
IL-17 (III, IV)	IL17f	acg tca ccc tgg act ctc c	271 bp	NM_010552
	IL17r	cgc ggg tct ctg ttt agg		
mLIF (IV)	mLIF-238f	ggg agc cct ctt ccc atc ac	546 bp	NM_008501
	mLIF-783r	cta gaa ggc ctg gac cac c		
mTLR9 (II-III)	TLR9 sense	(Prinz *et al.*, 2006)	294 bp	NM_031178
	TLR9 antisense	(Prinz *et al.*, 2006)		
mTLR2 (II-III)	mTLR2f	cga gtc tgc ttt cct gct g	295 bp	NM_011905
	mTLR2r	cag ctc gct cac tac gtc tg		
mTLR3 (II, III)	TLR3sense	(Prinz *et al.*, 2006)	345 bp	NM_126166
	TLR3antisense	(Prinz *et al.*, 2006)		
MyD88 (II, III)	mMyD88f	aag cag cag aac cag gag tc	374 bp	NM_010851
	mMyD88r	agg ctg agt gca aac ttg g		
mIL-5 (II-IV)	IL-5f	agg atg ctt ctg cac ttg ag	319 bp	NM_010558
	IL-5r	gtc tct cct cgc cac act tc		
mIFNα6 (II-IV)	mIFNa6 f	gct ctg tgc ttt cct gat gg	336 bp	NM_206871
	mIFNa6 r	tgc tga tgg agg tca ttg c		
mIFNα4 (II-IV)	mIFNa4 f	tag gct ctg tgc ttt cct ca	464 bp	NM_010504
	mIFNa4 r	ggc tgt gtt tct tct ttc tca g		
mIFNβ (II-IV)	mIFNb f	cgt tcc tgc tgt gct tct c	309 bp	NM_010510
	mIFNb r	ctg tct gct ggt gga gtt ca		
mGAPDH (II-IV)	mGAPDHf	acc cag aag act gtg gat gg	341 bp	NM_008084
	mGAPDH r	gca tcg aag gtg gaa gag tg		
mFoxP3 (II, IV)	FoxP3 f	gtg gcc tca atg gac aag ag	304 bp	NM_054039
	FoxP3 r	cga aca tgc gag taa acc aa		
mGATA3 (II, IV)	GATA3 f	tac cac cta tcc gcc cta tg	347 bp	NM_008091
	GATA3 r	cat tag cgt tcc tcc tcc ag		
mTbet (II, IV)	mTbet f	tcc atg tac gca tct gtt ga	338 bp	NM_019507
	mTbet r	atc ttg ggc ggg tat tga g		
mRORγT (II, IV)	mRORgT f	gaa agc agg agc aat gga ag	299 bp	NM_011281
	mRORgT r	gat gat gga aag cca gtt cc		
mTGF-β (II, IV)	mTGFb f	acg gaa tac agg gct ttc g	369 bp	NM_011577
	mTGFb r	gtg tgt cca ggc tcc aaa ta		
mSTAT3 (IV)	mSTAT3 f	cag ggt gtc aga tca cat gg	372 bp	NM_011486
	mSTAT3 r	tgg tcg cat cca tga tct ta		
mIL-6 (II, IV)	mIL6 f	ctg gga aat cgt gga aat ga	345 bp	NM_031168
	mIL6 r	agg aga gca ttg gaa att gg		
mIL-27 (II)	mIL27 f	act ctg ctt cct cgc tac ca	339 bp	NM_145636
	mIL27 r	cga gac agg aca agc tcc a		

4.2 Primers for quantitative PCR (I-IV)

Primers for quantitative amplification of cytokines of mouse origin and viral gene transcripts were designed to amplify approximately 120-200 bp of the coding sequence. Suitable primers were designed with the program Primer 3 (http://primer3.wi.mit.edu/). Primers were ordered from Oligomer (Helsinki, Finland) or OliGold (Seraing, Belgium). The primer sequences for HSV-1 gD (Hukkanen *et al.*, 2000), VP16, IL-23, IL-12p40, IL-12-p35, IL-4, IFN-γ, β-actin (Broberg *et al.*, 2003; Broberg *et al.*, 2002), TLR9 (Prinz *et al.*, 2006), TLR2, TLR3, MyD88, IL-5, IL-10, IL-17, IFN-α4, IFN-α6, IFN-β (III), RORγT, FoxP3, T-bet, GATA3, STAT3, TGF-β, IL-6, mLIF, GAPDH (IV) and IL-27 (II) have been published.

4.3 Quantitative real-time PCR of HSV transcripts and cytokines (I-IV)

PCR amplifications were performed using the quantitative real-time LightCycler PCR System (I) (Roche Diagnostics GmbH, Mannheim, Germany) or Rotor-Gene (II-IV) (Corbett Research, Mortlake, Victoria, Australia) with the use of Sybr Green chemistry. Each reaction was performed in a total volume of 20 µl. For LightCycler, the mixture contained 2 µl cDNA sample, 3 mM $MgCl_2$, 20 pmol of each primer, 10% LightCycler – DNA Master Sybr Green I buffer (Roche) and 220 ng TaqStart antibody (Clontech Laboratories Inc. Palo Alto, CA, USA). For the Rotor-Gene reactions, 2 µl cDNA sample, 20 pmol primers and 10 µl Quantitect Sybr Green PCR enzyme buffer mixture (Qiagen) (III) or 10 µl Maxima Sybr Green qPCR Master Mix (Fermentas, part of ThermoScientific, Waltham, MA, USA) (II, IV) were added. The enzyme mixtures were prepared in a separate PCR clean room. For HSV-1 DNA PCR, 4 µl of DNA sample was added to the mixture and only 10 pmol of primers were added to the enzyme mixture.

The LightCycler reactions were performed under the following conditions: initial denaturation at 95ºC for 30 s, 50 cycles of 95ºC for 1 s / 55ºC or 60ºC for 5 s / 72ºC for 20 sec, followed by one cycle of melting curve from 55ºC to 98ºC, or from 55ºC to 94ºC at a transition rate of 0.1ºC / s. For Rotor-Gene, the reactions were carried out using the following conditions: initial denaturation at 95ºC for 15 min, 45 cycles at 95ºC for 15 s, 55ºC or 60ºC for 30 s, 72ºC for 45 s, followed by generation of melting curve from 72ºC to 95ºC. The annealing temperatures 55ºC or 60ºC were chosen based on the primer sequences. The annealing temperature was 55ºC for HSV-1 gD (DNA), IFN-γ, IL-4, IL-10, IL-5, IL-23, TLR9, TLR3, TLR2, IFN-β, RORγT, GATA3, STAT6, TGF-β, IL-6,

IL-27, and 60ºC for VP16, β-actin, GAPDH, IL-12p35, IL-12p40, IL-17, IFN-α6, IFN-α4, MyD88, FoxP3 and T-bet.

The melting temperature analysis was used to verify the specificity of the correct products. Copy numbers were normalized to β-actin (I, III) or to both β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (II, IV).

4.4 Mice (I-IV)

Six-week-old female BALB/c mice were obtained from the Central Animal Laboratory, University of Turku (Turku, Finland) (I). Pathogen free 4-6 week old female SJL/J mice were obtained from the Central Animal Laboratory, the University of Turku (III) or Harlan Laboratories (UK) (II, IV). The mice were maintained at the animal facility of the Microbiological Institute, University of Turku under permits LSLH-2002-5757/Ym-23 (I), ESLH-2005-3572/Ym-23 (III) and ESLH-2008-05-05175/Ym-23 (II, IV) from the National Animal Experiment Board in Finland. In studies III and IV, the recombinant viruses were used under the notification 19/M/07 of the National Board for Gene Technology, Finland.

4.5 Viruses (I-IV)

Viruses used in the studies (I-IV) are listed and described in Table 4.

Table 4. Viruses used in studies I-IV.

Virus	Description	Source, reference	Study
HSV-1 (F)	wild-type	ATCC, Bethesda, MD	I
HSV-1 (17^+)	wild-type	Everett R., Glasgow, UK	II
R3659	Δ TK Δ $\gamma_1$34.5, p α27-TK in $\gamma_1$34.5	Roizman B., (Lagunoff & Roizman, 1995)	III
R8308	Δ $\gamma_1$34.5, p egr1-IL-10 in $\gamma_1$34.5	Roizman B., (Andreansky *et al.*, 1998)	III
R8316	Δ $\gamma_1$34.5, p egr1-IL-5 in $\gamma_1$34.5	Roizman B., (Hellums *et al.*, 2005)	III
HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-Zeo	Δ $\gamma_1$34.5, ZeoR in 5´ $\gamma_1$34.5	Sodeik B & Messerle M MHH, Germany (IV)	IV
HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-LIF	Δ $\gamma_1$34.5, EF1α-LIF in 5´ $\gamma_1$34.5	This study (IV)	IV

4.6 Viral vectors (III-IV)

Herpes simplex virus type 1 (HSV-1), both wild-type and recombinant, were used for infection studies. Figure 12 depicts the structures of the recombinant viruses. The viral vectors R3659 (Lagunoff & Roizman, 1995), R8308 (Andreansky *et al.*, 1998) and R8316 (a kind gift from Dr. Bernard Roizman, University of Chicago, Chicago, IL, USA) are based on the HSV-1 strain F (III) and were constructed using conventional recombination techniques (Post & Roizman, 1981) (Figure 12A). The BAC-derived viral vectors HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-Zeo and HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-LIF (Figure 12B) were constructed from the HSV-1 (17^+) strain in collaboration with Dr. Beate Sodeik (Hannover Medical School, Hannover, Germany).

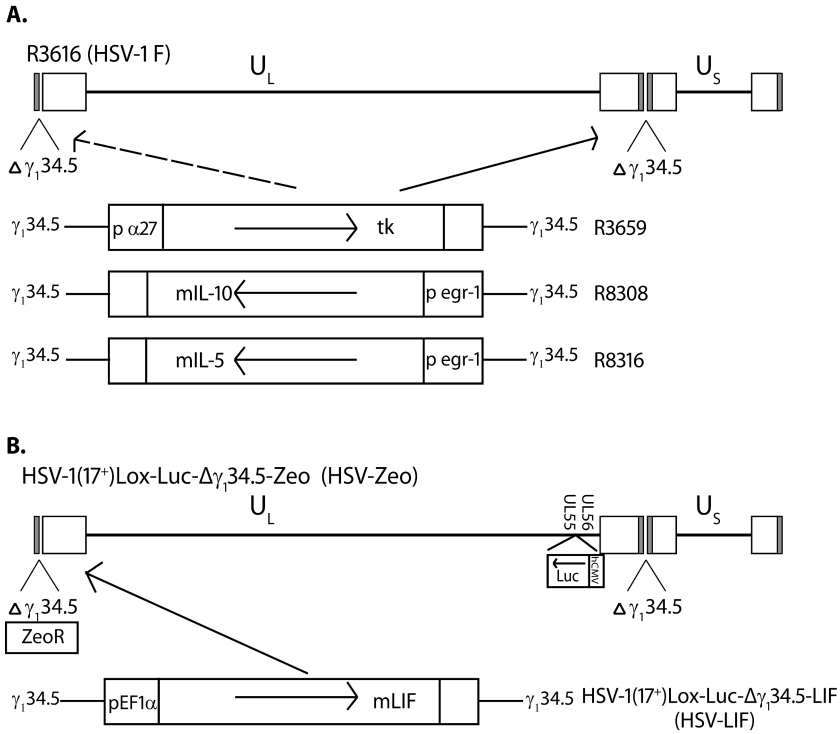

Figure 12. Schematic representation of the HSV-1 vectors used in the studies (III-IV). A) R3659, R8308 and R8316 were constructed based on the HSV-1 (F) vector R3616 (Andreansky *et al.*, 1998). The expression casettes were inserted into both $\gamma_1$34.5 loci. Dashed line indicates that the transgene is in reversed orientation in the left $\gamma_1$34.5 locus. B) HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-LIF (HSV-LIF) was constructed based on the BAC-derived HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-Zeo (HSV-Zeo) vector. The LIF expression cassette was inserted into the left $\gamma_1$34.5 locus. The arrows in the boxes indicate the gene orientation. Abbreviations: p egr-1: early growth response protein 1 promoter; pEF1α, elongation factor 1α promoter; ZeoR, zeocin resistance; mIL-10, murine interleukin-10; mIL-5, murine IL-5; mLIF, murine leukemia inhibitory factor.

Virus stocks were grown in Vero cells (American Type Culture Collection, ATCC, CCL-81). Cells were grown to 80-100% confluence in roller flasks and infected at a MOI of 0.01 – 0.1 in 100 ml of 2% FCS DMEM. The virus was left to grow until cytopathic effects were seen and all cells were infected. Supernatant and cells were collected, cells were centrifuged down, the supernatant was discarded and the cell pellet was dissolved in 2 ml 9% sterile milk (per roller). The dissolved cells were freeze-thawed three times and sonicated three times for 30 s. The titer of the resulting virus stock was determined by plaque titration.

The amount of transgene expressed from the vectors was analyzed from the supernatants of infected cell cultures. In study III, cytokine expression from the HSV vectors expressing IL-10 and IL-5 was tested from supernatants with enzyme immunoassay (EIA) (eBioscience, San Diego, CA, USA). CSM rat neuronal cells were infected with R8308 and R8316 and supernatants were collected at 24h post infection. The amounts of IL-10 and IL-5 were 850 pg/ml and 5.1 pg/ml for R8308 (IL-10) and R8316 (IL-5), respectively. In study IV, the LIF expression from the HSV-1(17^{+})Lox-Luc-$\Delta\gamma_1$34.5-LIF vector was measured using mouse LIF Quantikine ELISA kit (R&D Systems Europe, Oxon, UK). Infected T98G and Vero cells expressed 1 pg/µl and 2-3 pg/µl of recombinant LIF, respectively.

A transgene-specific PCR was set up for identification of transgene transcripts. The primers were designed to anneal to the promoter sequence and to the coding sequence of the transgene. The following primers were used; IL-5s: cagtggtgaaagagaccttga and HBVAva1: gagaagggtcgtccgcaggat (IL-5 transgene detection (III), egrplus1: agtcgcgagagatccca and IL10a: tgagggtcttcagcttctcac (IL-10 transgene detection (III) and EF1a–LIF f (p-LIF f): tggaatttgccctttttgag and EF1a–LIF r (p-LIF r): tgagctgtgccagttgattc (LIF transgene detection (IV).

The recombinant viruses were used under the notification 19/M/07 of the National Board for Gene Technology, Finland.

4.6.1 Construction of a BAC-derived HSV vector (IV)

Cloning of expression plasmid

An expression cassette for murine LIF was constructed by amplifying the LIF gene sequence from brain samples of healthy mice with primers mLIF ATG-L: agc tgg tac ccc cat aat gaa ggt ctt ggc cgc agg gat tgt gcc ctt act gct gct ggt tct gca ctg gaa aca cgg ggc a*gg gag ccc tct tcc cat cac* and mLIF R: agct agct agct agct aag ctt *cta gaa ggc ctg gac cac c* (annealing sequencing are in italics and the added restriction sites HindIII and KpnI are underlined). The amplified LIF

sequence spanned the complete coding sequence of murine LIF (nucleotides 218-835, GenBank NM_008501). The PCR product was digested with KpnI and HindIII restriction enzymes (Fermentas) and cloned into the plasmid pCDNA3.1(-)EF1α (a kind gift from Jay Nelson, Oregon Health & Sciences University, Portland, USA). The resulting plasmid pCDNA3.1-EF1αLIF expresses murine LIF from the elongation factor 1α (EF1α) promoter (Figure 13A).

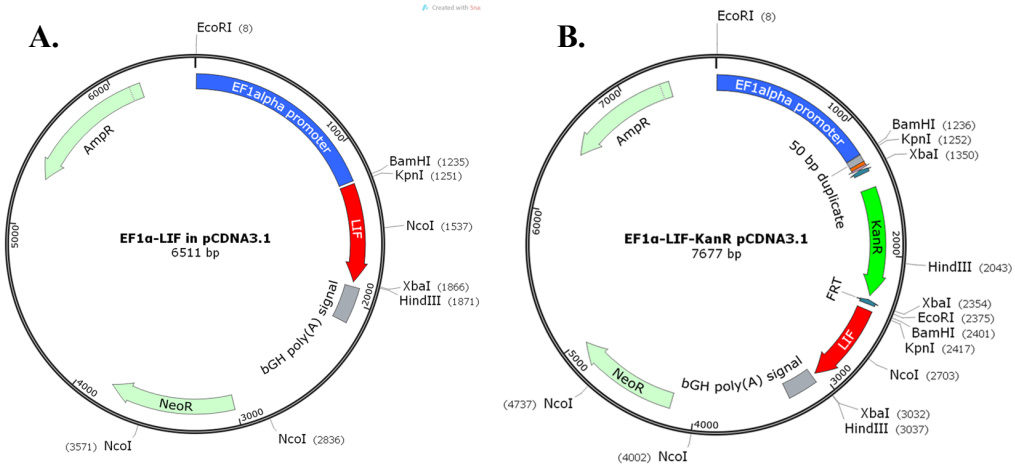

Figure 13. LIF plasmids for BAC mutagenesis. (A) The pcDNA3.1-EF1αLIF plasmid expresses murine LIF under an EF1α promoter. (B) The pcDNA3.1-EF1αLIF-Kan plasmid contains kanamycin resistance (KanR) and I-SceI sequences inserted into the LIF sequence for *en passant* mutagenesis. Restriction sites and their location are shown. Maps were drawn with SnapGene Viewer.

En passant-mutagenesis – insertion of LIF into the viral genome

Insertion of the LIF expression cassette into the HSV-1 (17^+) sequence is outlined in Figure 14. As a backbone virus, HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-Zeo or HSV-Zeo for short, was used (IV). This virus was deleted of both copies of the $\gamma_1$34.5 gene (690 bp was deleted of the 3´copy and 620 bp of the 5´copy). The 5´ copy of $\gamma_1$34.5 was replaced with a Zeo resistance marker and a luciferase expression cassette was inserted between UL55 and UL56 (Complete mutagenesis is described in IV). The TK gene was repaired and functional.

To construct an HSV-1 vector expressing LIF, the ZeoR gene in the 5' copy of $\gamma_1$34.5 in HSV-Zeo was replaced with LIF (under the EF1α promoter) using the *en passant* mutagenesis protocol (Tischer *et al.*, 2010; Tischer *et al.*, 2006). A kanamycin resistance cassette (KanR) together with 50 bp of homologous sequences of EF1α-LIF and the homing endonuclease I-SceI-site were amplified

with primers LIF-KanL2: tag cgg atc cga gct cgg tac ccc cat aat gaa ggt ctt ggc cgc agg gat tgt gcc ctt ata ggg ata aca ggg taa tcg att t*ag gac gac gac gac gac aag taa* and LIF-KanR: agc tag ctg gat cc*c agg aac act taa cgg ctg a* from the plasmid pGP704-Kan (Loewendorf *et al.*, 2004) using the Phusion High-Fidelity DNA polymerase (Fermentas) (annealing sequences are in italics). The PCR product and the pcDNA3.1-EF1αLIF plasmid were cut with BamHI, run on TAE-gel electrophoresis and correct bands were excised and cleaned with QiaexII gel extraction kit (Qiagen). They were then ligated with T4 ligase (Fermentas) yielding the plasmid pcDNA3.1-EF1αLIF-Kan (Figure 13B).

Thereafter, the EF1α, LIF, a homing endonuclease I-SceI-site, KanR and the homologous sequences were amplified with primers LIF-ICP34.5-L: ccc ggg ccc acg ggc gcc gtc cca acc gca cag tcc cag gta acc *aat tcg gag tgc ctc gtg ag* and LIF-ICP34.5-R: agg ccg cct cgg gtg taa cgt tag acc gag ttc gcc ggg ccg gct ccg cg *ctg gtt ctt tcc gcc tca g*, containing approximately 80 bp overhanging sequences complementary to sequences upstream and downstream of the insertion site (nucleotides 603-1259 of the $\gamma_1$34.5 gene in HSV-1(17^+)). The PCR product was cleaned with NucleoSpin Extract II-kit (Macherey-Nagel) and then digested with DpnI to remove any remaining template plasmid DNA (methylated during bacterial cloning). The DpnI digested PCR product was further cleaned by phenol-chloroform purification and ethanol precipitation. The finalized cleaned PCR product was then used to transform the electrocompetent *E. coli* GS1783 cells (Tischer *et al.*, 2010) containing the HSV-Zeo BAC. Transformed bacteria were cultivated on Luria Broth (Miller´s LB Broth Base, Invitrogen Life Technologies, Carlsbad, CA, USA) plates containing 30 µg/ml kanamycin and 17-34 µg/ml chloramphenicol at 32°C. Separated colonies were then cultivated at 32°C overnight in 10 ml LB supplemented with kanamycin and chloramphenicol and DNA was extracted with reagents from the NucleoBond BAC 100 kit (Macherey & Nagel), using a modified protocol. Briefly, overnight cultures were pelleted by centrifugation at 4000 rpm for 15 min. The pellet was resuspended in 200 µl of solution S1 (NucleoBond BAC 100 kit) and moved to eppendorf tube. Cells were lysed with 300 µl of solution S2 (NucleoBond BAC 100 kit) and incubated for 5 min at room temperature. 300 µl neutralization buffer S3 (NucleoBond BAC 100 kit) was added and the cells were incubated on ice for 10 min. The cell lysate was pelleted at 4°C for 15 min and the supernatant was transferred to new tubes. DNA was precipitated from the supernatant with isopropanol and 70% ethanol. DNA was dissolved in 30 µl of Tris-EDTA buffer. Clones containing the EF1α-LIF-Kan cassette were analyzed with restriction enzyme digests and TBE-gel electrophoresis.

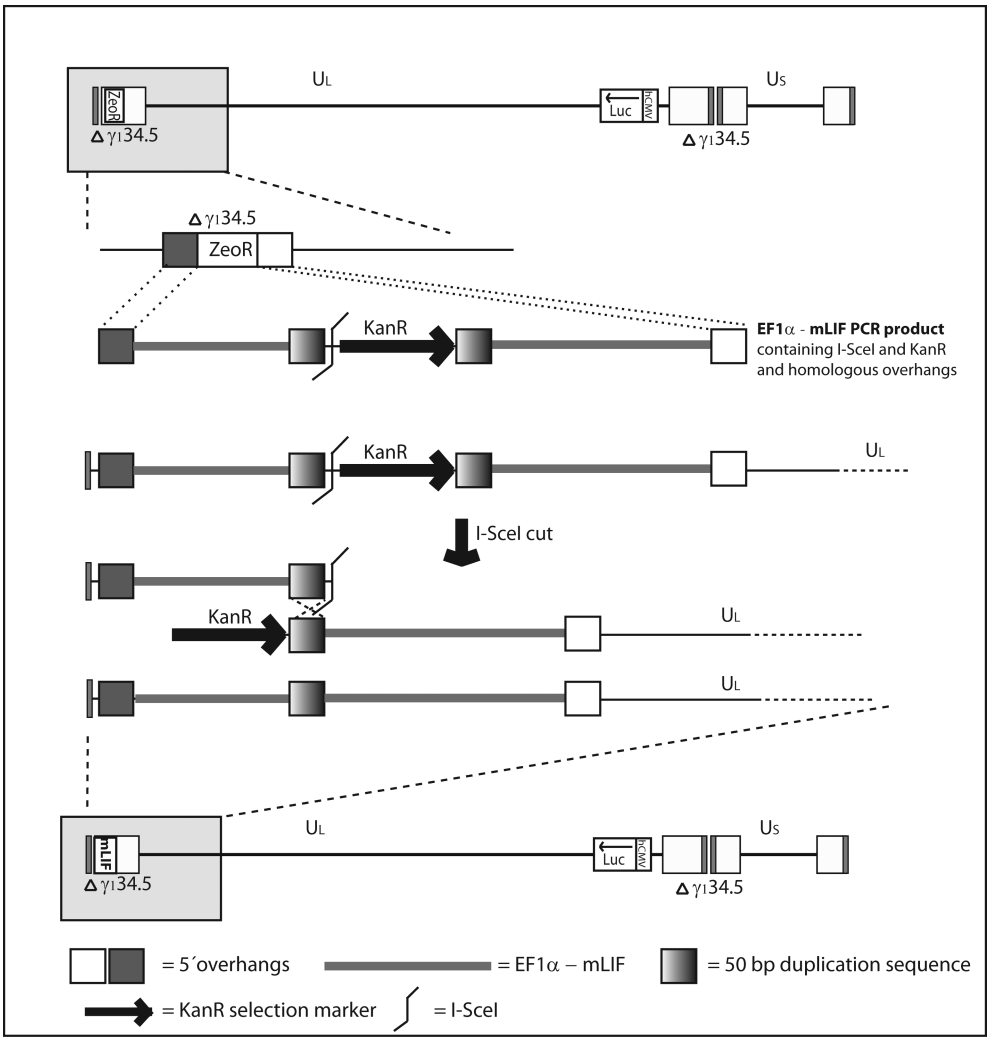

Figure 14. Insertion of EF1α-LIF into the left copy of $\gamma_1$34.5 of HSV-Zeo using the *en passant* mutagenesis protocol. The backbone vector HSV-Zeo contains a Zeocin resistance (ZeoR) marker in the left copy of $\gamma_1$34.5 in addition to a luciferase (Luc) gene under the human cytomegalovirus (hCMV) promoter inserted between UL55 and UL56. Both copies of $\gamma_1$34.5 are partially deleted. The *en passant* method entails the use of an antibiotic resistance marker, such as kanamycin (KanR), a 50 bp homologous sequence and the homing endonuclease I-SceI recognition site. These sequences are removed in the endpoint cloning steps by activating I-SceI. The final HSV-LIF BAC contains the inserted transgene murine LIF (mLIF) expression cassette.

To remove the kanamycin resistance cassette from the HSV-BAC genome, the homing endonuclease I-SceI was induced by L-Arabinose. First, a single BAC colony was incubated overnight in LB supplemented with chloramphenicol and kanamycin. Second, 100 µl of overnight culture was incubated with 2 ml of LB containing chloramphenicol (but no kanamycin), for 4 h at 32°C, followed by an addition of 2 ml of LB containing chloramphenicol and 0.1% (w/v) L(+)-

Arabinose (Sigma-Aldrich, Steinheim, Germany) and incubation for 1 h at 32°C. Red$\alpha\beta\gamma$ enzyme expression from the GS1783 bacteria was induced by incubation at 42°C for 30 minutes, followed by an additional 2 h incubation at 32°C. The culture was first plated on LB plates supplemented with chloramphenicol and 1% (w/v) L-arabinose (masterplate 1). The next day, colonies were grown on LB plates supplemented with chloramphenicol and kanamycin to see if kanamycin resistance had been removed (masterplate 2). Kanamycin-sensitive clones were selected for restriction digests and gel electrophoresis analysis. The resulting HSV-BAC was named HSV-1(17^+)Lox-Luc-$\Delta\gamma_1$34.5-LIF or for short, HSV-LIF.

Reconstitution of recombinant HSV-1(17^+)Lox-Luc-$\Delta\gamma_1$34.5-LIF

BAC-DNA was prepared with NucleoBond BAC 100 kit (Macherey & Nagel, Düren, Germany), as described before (Nagel *et al.*, 2008). Vero cells (ATCC, Manassas, VA, USA) were transfected with approximately 2 µg of BAC-DNA with MBS mammalian transfection kit (Stratagene, La Jolla, CA, USA) and cultured until viral plaques developed. High titer virus stocks were grown in Vero cell roller flasks until all cells showed cytopathic effects. Cells and supernatant were centrifuged and the cell pellet was dissolved in 2 ml of 9% sterile milk (per roller). Cells were freeze-thawed three times and sonicated three times for 30 s. The resulting cell-derived virus stock was titered and stored at -70°C.

Detection of transgene expression

To detect LIF expression from the transgene, T98G human glioblastoma cells (VTT Technical Research Centre, Turku, Finland) and Vero cells were grown in 24-well plates and infected with 0.5 or 5 multiplicity of infection (MOI) of HSV-LIF. As controls, cells were infected with HSV-Zeo or HSV-1 (17^+) wild-type, or were left uninfected. At 24 h and 48 h post infection, supernatants were collected and cells were lysed with a buffer containing 1.2 mM MOPS, pH 8.0, 3.5 mM EDTA, 0.1% NP-40 and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Cells were boiled for 5 min at 95°C, and the protein concentration was measured using the BCA protein assay kit (Pierce, ThermoScientific, Erembodegem, Belgium). 30 µg of total protein was separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Samples were transferred to Amersham Hybond-P membranes (GE Healthcare Europe GmbH, Munich, Germany). Blots were blocked overnight in phosphate buffered saline (PBS) containing 10% non-fat dry milk and 0.1% Tween-20. Membranes were incubated with the primary rabbit polyclonal antibody LIF M-179, (Santa Cruz biotechnology, Santa Cruz, CA, USA), washed with PBS containing 0.1%

Tween-20 and incubated with a horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark) and developed with enhanced chemiluminescence (ECL) (Pierce, ThermoScientific).

4.7 *In vivo* infections (II)

HSV-1 was administered to mice by intranasal, corneal or intracranial route. Before infections, mice were anaesthetized using Avertin (Tribromoethanol) (Sigma-Aldrich or Fluka, now part of Sigma-Aldrich, Schnelldorf, Germany). Mice infected via the corneal or intracranial route also received 100 µl (per 20 g bodyweight) of the analgesic drug Temgesic (buprenorfin 0.015 mg/ml, Reckitt Benckiser, UK) subcutaneously at the infection time point. The suitable viral dose was determined in pilot studies and was based on previous observation in the BALB/c model (Broberg *et al.*, 2004a). A dose of 10^5 PFU in 10 µl was administered to the nose or the cornea, and 10^3 PFU HSV-1 was administered intracranially.

4.8 EAE induction (III, IV)

The EAE induction protocol for SJL/J mice was set up in a series of preliminary experiments. In these experiments the dosage of pertussis toxin (PT) was optimized. PT was administered intravenously or intraperitoneally at different time points. The amount of proteolipid protein (PLP) and Freund´s complete adjuvant remained the same in all pilot experiments (see below). Pertussis toxin was administered intravenously at a dose of 1) 200 ng in 100 µl on days 0 and 3 or 2) 100 ng in 100 µl on days 0 and 3. Intraperitoneally pertussis toxin was given in the amounts of: 1) 200 ng in 200 µl on day 0 and day 1 or 2) 200 ng in 250 µl on day 0 and day 1. The intraperitoneal administration route was chosen for further experiments.

For therapy experiments, mice were induced for EAE by immunization with 50 µg myelin proteolipid protein 139-151 $(PLP)_{139\text{-}151}$ (PolyPeptide Group, previously NeoMPS, Strasbourg, France) in Freund´s incomplete adjuvant (Difco Laboratories), supplemented with 200 µg *Mycobacterium tuberculosis* (strain) and 12.5 µg *M. butyricum.* (*Mycobacteria* are added to incomplete Freund´s adjuvant to make up complete Freund´s adjuvant). The adjuvant was administered in both footpads, 50 µl each. 200 ng pertussis toxin (Alexis Biochemicals, Enzo Life Sciences, Plymouth Meeting, PA, USA) in 250 µl was administered intraperitoneally at day 0 and day 1 post EAE induction to boost the immune response.

4.9 Treatment of EAE (III, IV)

Mice induced for EAE were infected with HSV-1 vectors at day 6 post EAE induction. Mice were anesthetized with Avertin before infections. In study III, 10^7 PFU (in 10 µl) of the HSV-1 (F) vectors R8308 (IL-10) and R8316 (IL-5) were administered to the nostrils of anesthetised mice, 5 µl per nostril. The backbone virus R3659 and uninfected EAE mice were included as controls.
In study IV, 10^7 PFU (in 10 µl) of the HSV-1 (17^+) BAC-derived vectors HSV-LIF or HSV-Zeo were administered to anesthetized mice intracranially in the left parietal cerebral cortex (parenchymal), at the approximate anteroposterior level of bregma -0.1 cm and lateral level of -0.1 cm. Control groups consisted of untreated EAE mice and EAE mice infected with UV-irradiated vector (HSV-LIF inactivated with UV-light for 30 min, resulting in a 10^4-fold reduction of titer, with a residual titer of 5 x 10^3 PFU).

4.10 Clinical evaluation of EAE (III-IV)

Animals induced for EAE were observed daily for clinical symptoms. The clinical scores were 0, healthy; 1, fur ruffling; 2, tail atonia; 3, hind limb paralysis; 4, tetraplegia or moribund; 5, dead. Changes in weight were recorded weekly. The cumulative disease index was calculated by summing the daily clinical scores, and by dividing the sum by the number of animals in each group.

4.11 Dissection and processing of tissues (II-IV)

Mice were sacrificed under CO_2 anesthesia at different time points (in study II: on days 3, 7 and 31; in study III-IV: days 9/10, 14 and 21 after EAE induction). In study II, the uninfected control mice were sacrificed on day 7. Mice were perfused with sterile PBS via the left chamber of the heart. Samples were taken from the brains for virus culture and DNA/RNA preparation (II-IV). The rest of the brains was fixed in 10% formaldehyde solution, phosphate buffered, pH 7 (FF-Chemicals Ab, Haukipudas, Finland) for microscopy (III, IV). Samples from the trigeminal ganglia (TGs) were taken for virus culture, and for DNA and RNA extraction (II-IV). Samples from spinal cords were taken for DNA and RNA extraction and the rest was fixed in 10% formaldehyde solution (phosphate buffered pH 7), for microscopy (III-IV). From the paraffin blocks, 4 µm sections of brain and spinal cord samples were cut and transferred onto glass microscope slides (III-IV).

4.12 DNA extraction (II-IV)

DNA was extracted from brain and TG samples with EZNA tissue extraction kit (OMEGA, Bio-tek, Norcross, GA, USA) according to manufacturer´s manual in studies II-IV. Due to the limited amount of tissue sample available, DNA was extracted from TGs that had been processed for virus culture.

4.13 RNA extraction and reverse transcription (I-IV)

RNA was extracted with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) (I, III) or Tri Reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA) (II, IV) according to manufacturers' instructions. Shortly, 1 ml of Trizol or Tri Reagent was used for brain samples and 500 µl was used for TG samples (II-IV). Spinal cord samples were processed with 500 µl of Tri Reagent (IV). In study II, Qiagen All Prep-kit (Qiagen, Hilden, Germany) was used for RNA extractions, according to manufacturer's instructions. Cell culture samples were collected from 6-well plates in 1 ml of Trizol/well (I). RNA was diluted in 20 µl of water treated with diethylpyrocarbonate (DEPC). Samples were stored at -70ºC until cDNA transcription.

RNA was treated with DNase before cDNA transcription in studies II and IV. 8 µl RNA sample was mixed with 1 µl of 10 x DNase buffer, 1 µl DNase and 0.2 µl RNAse inhibitor (RiboLock RNase inhibitor), all from Fermentas (Fermentas). Samples were incubated at 37ºC for 30 min, whereafter 1 µl EDTA stop-solution was added and samples were incubated for 10 min at 65ºC.

RNA was transcribed into cDNA according to two protocols. In studies I and III, RNA was transcribed as follows: 0,023 U $pd(T)_{12-18}$ (0,7 µl of 33 U/ml) (Amersham Biosciences/GE Healthcare Bio Science, Pittsburgh, PA, USA) and 13,5 µl DEPC-water were mixed and 5 µl RNA sample was added. The sample mixture was incubated at 70ºC for 5 min and then put on ice. M-MLV RT 5 x Buffer, 5 mM dTNP, M-MLV Reverse transcriptase RNase H Minus enzyme (40 units) and RNase inhibitor (8 units), all from Promega (Madison, WI, USA) was then mixed in a PCR clean room and 5,7 µl mixture was added to the samples. The RT-reaction was carried out in a PerkinElmer PCR machine (Waltham, MA, USA) under the following conditions; 37ºC for 60 min followed by 90ºC for 5 min, whereafter the samples were cooled to 4ºC.

In studies II and IV, RNA was transcribed into cDNA as follows: 56 pmol $Oligo(dT)_{18}$ (Fermentas) and 27 µl DEPC-treated water were mixed and 10 µl DNase-treated RNA sample was added. The sample mixture was incubated at

70ºC for 5 min and put on ice. M-MLV RT 5 x Buffer, 5 mM dNTP, RevertAid H Minus M-MLV Reverse transcriptase (80 units) and RiboLock RNase inhibitor (8 units), (all from Fermentas) were then mixed in a PCR clean room and added to the samples. The RT-reaction was carried out in a PerkinElmer PCR machine (Waltham, MA, USA) under the following conditions; at 42ºC for 60 min followed by 90ºC for 5 min or 70ºC for 10 min, whereafter the samples were cooled to 4ºC. cDNA was stored at -70ºC and used for quantitative real-time RT-PCR.

4.14 Plaque titration (II-IV)

Vero cells (ATCC, CCL-81) were seeded in 6-well plates and were left to grow until confluent. Cells were washed once with 0.1% BSA in RPMI and overlaid with dilutions of the virus stock or other virus samples. Cells were incubated for 1-2 hours at room temperature, before changing the medium to minimum essential medium Eagle (MEM) containing 7% heat inactivated fetal calf serum (FCS) and 20 µg/ml human IgG (Sanquin, Amsterdam, Netherlands). Plates were incubated at 37°C $C0_2$ for 3-4 days until plaques were visible. Culture medium was removed and cells were fixed with cold methanol for five minutes. Plaques were visualized by staining cells with 0.1% crystal violet in 2% ethanol for 10 minutes. Excess stain was removed and cells were washed with tap water.

4.15 Virus culture of tissue samples (II-IV)

Brain and TG samples were homogenized by shaking the samples in tubes with glass beads (Assistant Ø 1 mm, VWR International Oy, Espoo, Finland) at 4°C for 2 x 45 s with Mikro-dismembrator II device (Medical Braun Ab, Espoo, Finland) (study III-IV) or with one stainless steel bead (5mm, Qiagen) at 4°C for 3 min 50 $s/_1$ with TissueLyser Single-bead dispenser (Qiagen) (II). The tissue samples were centrifuged at 4°C for 30 s. In study III, the TG samples were diluted 1:1 and the brain samples 1:10 in RPMI medium containing 0.1% BSA. In study II and IV the homogenized TG and brain samples were diluted 1:1 in RPMI medium containing 0.1% BSA. Vero cells in 6-well culture dishes were then overlaid with 0.5 ml virus dilution in duplicates. Cells were incubated for 1-2 hours at room temperature before changing the medium to minimum essential medium Eagle (MEM) containing heat inactivated 7% FCS and 20 µg/ml human IgG (Sanquin, Amsterdam, Netherlands). Cells were then incubated for four days at 37°C (CO_2-incubator), fixed in methanol for 5 minutes and stained with 0.1% crystal violet in 2% ethanol. Samples taken on day 21 post induction were incubated in culture medium for five days at 37°C (CO_2-incubator) before homogenization and plaque titration to reactivate the latent virus.

4.16 *In vivo* biophoton imaging (IV)

Virus vectors expressing luciferase can be visualized in living animals with biophoton imaging. The spread of the HSV vectors expressing luciferase in mice was analyzed using the IVIS Imaging System 50 Series apparatus (Xenogen, Caliper Life Sciences, Affligem, Belgium). Mice were injected intraperitoneally with 3 mg in 150 µl of beetle luciferin (Promega, Madison, WI, USA) and anesthetized under isoflurane flow. 5-10 minutes later the mice were moved to the dark chamber of the IVIS 50 apparatus and images were acquired for 1-5 minutes. Images were taken from day 1 to day 5 post infection.

4.17 Neuropathological examinations (III-IV)

Formaldehyde-fixed 4 µm paraffin brain sections were stained with hematoxylin and eosin (HE-staining). A professional neuropathologist scored the severity of inflammatory infiltrates in the CNS (HE-staining). The scoring was: 0, no infiltration; 1, perivascular infiltration; 2, perivascular inflammatory cuffs; 3, inflammation of the brain substance. Pictures were taken with a Carl Zeiss Axiovert 200M microscope and Zeiss AxioCam camera, using AxioVision Software (Carl Zeiss, Göttingen, Germany).

Fixed samples were deparaffinized and rehydrated by incubating slides overnight at 60°C and washed in Xylene (2 x 5min), 100% EtOH, 96% EtoH and 70% EtoH (2 x 2min each). Antigen unmasking was achieved by heat treatment in 10 mM citric acid buffer. Unspecific signal was blocked by incubating samples with 2-10% of normal serum of the same host species as the secondary antibody. Sections were incubated overnight at 4°C with the primary antibody.

In study III, immunohistochemistry was performed to analyze the cell composition of infiltrates in brains and spinal cords. Samples were stained for leukocytes (CD45, AbDSerotec, Oxford, UK, 1:2000), T lymphocytes (CD4, Abcam 1:1000; CD8, AbDSerotec, 1:100), and antigen presenting cells (F4/80, AbDSerotec 1:80). All antibodies were diluted in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA). Signals were detected using Vectastain ABC-kit (Vector Laboratories, Burlingame, CA, USA) according to manufacturer´s instruction and stained with diaminobenzidine (DAB).

In study IV, immunohistochemical stainings were done using the following antibodies: HSV-1 (1:1000, Biogenex, San Ramon, CA, USA), astrocytes (GFAP 1:2000, Abcam, Cambridge, UK), myelin basic protein (MBP 1:200, Abcam) and oligodendrocytes (CNPase 1:10000, Covance, Princeton, NJ, USA). MBP was visualized using Vectastain (Vector laboratories, Burlingame,

CA, USA), GFAP and HSV-1 with Brightvision (Immunologic, Duiven, Netherlands) and CNPase with mouse on mouse-kit (Biocare Medical, Concord, CA, USA). For all antibodies diaminobenzidine (DAB) staining was used. In study IV, oligodendrocytes (CNPase staining) were quantified from the rim of the white matter from corresponding frames of the brain sections of each animal. Four to seven frames were used for each brain section. Positive cells were calculated from photographs using Image J software (Abramoff *et al.*, 2004) and normalized to the size of the brain area.

4.18 Lymphocyte proliferation (III)

Spleens were collected from healthy female SJL/J mice and lymphocytes were isolated with Lympholyte-M density gradient (Cedarlane Laboratories, Hornby, Ontario, Canada) according to the instructions of the manufacturer. A total of 125 000 cells in 100 µl RPMI (Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% FCS, Hepes and glutamine were plated per well in 96-well round bottomed microtitre plates (Costar). The cells were incubated in triplicates as such or with 30 ng/ml of either of the cytokines IL-4, IL-5 or IL-10 (EIA-kit, eBioscience, San Diego, CA, USA) alone or in combination with 2 µg/ml Concanavalin A (ConA) (Sigma-Aldrich Corp. St.Louis, MO, USA) or 250 µg/ml polyinosinic-polycytidylic acid (PolyIC) (Sigma-Aldrich) for 24 h at 37°C, 5% CO_2. Cells and supernatants were collected and stored at –70°C. RNA and cDNA were extracted from the cells with 100 µl of Trizol reagent as described in chapter "4.13 RNA extraction and reverse transcription".

4.19 Statistical analysis (II-IV)

In study II, cytokine, TLR and signature transcription factor mRNA expression in HSV infected mice was compared to uninfected mice. The p values were calculated with the Mann Whitney U test using the Statview software (Abacus Concepts, Berkeley, CA, USA).

In study III and IV, clinical disease scores were compared for statistically significant differences between mouse groups on different days. Cytokine, TLR and signature transcription factor mRNA expression in HSV-treated EAE mice were compared to uninfected EAE mice. The cumulative index, calculated by summing the daily clinical scores and dividing by the number of animals in each group, was compared for statistically significant differences between mouse groups on different days. Statistical difference in inflammation scores was analyzed between treatment and control groups. Statview software

was used to perform the Mann Whitney U tests for all analyzes above. In study IV, the differences in oligodendrocyte numbers were calculated with the T-test (Statview software). A p value of ≤ 0.05 was considered statistically significant.

5 RESULTS

5.1 Real-time PCR for detection of HSV and cytokine mRNA (I-IV)

To evaluate the use of quantitative real-time PCR for detection of low copy cytokine and viral gene transcripts in cell culture and mouse tissue samples, two different detection systems were set up for LightCycler. In study I, PCR of HSV VP16 and the cytokines IL-12p35, IL-12p40 and IFN-γ was performed using either dsDNA binding dye Sybr Green I, or using target-specific hybridizing fluorescent probes. The probe system was ten times more sensitive than Sybr Green I. The detection range for cytokines was from one hundred to ten million copies and for HSV VP16 mRNA from one to ten million copies with the probe detection system. With Sybr Green I even 10 copies of VP16 mRNA could be detected (I).

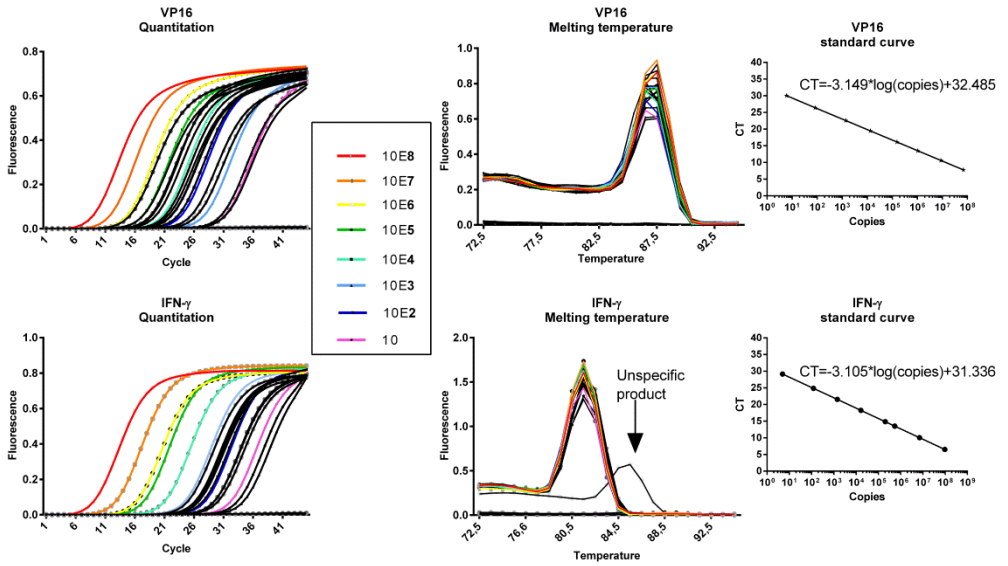

Figure 15. Quantitative real-time PCR with Rotor-Gene. Quantitative real-time PCR of VP16 and IFN-γ. For both VP16 and IFN-γ the quantitation data, the melting curve analysis and standard curve are shown. Standards are shown in color and samples in black.

In studies II-IV, the Rotor-Gene quantitative RT-PCR system was used. Rotor-Gene has a larger sample capacity and it is possible to analyze up to 72 or 100 samples compared to 32 samples with the LightCycler system. With the Rotor-Gene system we used dsDNA binding dye Sybr Green and melting temperature analysis to study the expression of cytokines and HSV gene transcripts (Figure 15). With this detection system we were able to detect below 10 copies of both HSV transcripts and cytokines.

5.2 Spread of HSV-1 in SJL/J mice (II-IV)

5.2.1 Spread of wild-type HSV-1 by three administration routes (II)

In order to study the spread of HSV-1, SJL/J mice were infected with wild-type HSV-1 by three different routes; intranasal, corneal (10^5 PFU HSV-1), or intracranial route (10^3 PFU HSV-1) (II). Samples were taken on days 3 and 7 post infection, representing the acute infection, and on day 31, representing the latent phase. By all three infection routes, viral DNA could be detected in the TG and in the brains of infected mice during the acute infection, indicating that HSV-1 spread from the peripheral sites to the TG and CNS. Replicating virus, studied with virus culture and VP16 quantitative real-time PCR, was only found in the TG and brains of mice infected intranasally or intracranially. No TG or brain samples from corneally infected mice were positive in virus culture, but in a few TGs low levels of VP16 expression could be detected on day 7 post infection. Eye swab samples taken daily from corneally infected mice were positive for replicating virus during the acute infection (day 1 to day 7 post infection) (II: Figure 2). The eye infection also led to strong clinical symptoms around the eyes, further indicating the presence of replicating virus in the eyes. HSV-1 established a latent infection in the TG of intranasally infected mice, and was able to reactivate from the TG after explant culture. TGs from mice infected by the corneal route did not reactivate in explant culture.
The intracranial infection led to a high amount of replicating virus both in the brains and TG. Mice were sacrificed by day 7 or earlier, if showing severe symptoms. Only one intranasally infected mouse died and all mice survived the corneal infection (II, Table 1). These results indicate that HSV-1 spread more efficiently to the TG and CNS after the intranasal infection compared to the corneal infection in SJL mice. The possible immunological explanations to the observed differences in virus spread are discussed later.

5.2.2 Spread and replication of $\gamma_1$34.5-deletion HSV vectors (III-IV)

The $\gamma_1$34.5-deletion vectors R3659, R8308 and R8316 were administered intranasally to SJL/J mice with EAE at a dose of 10^7 PFU (III). All three viruses spread to the TG in the majority of the infected mice, as studied with viral DNA PCR for HSV-1 gD. No virus was detected in the brains of infected mice, indicating that there was a limited spread of the vectors deleted of the neurovirulence gene.
In study IV, 10^7 PFU of the $\gamma_1$34.5-deletion vectors HSV-Zeo and HSV-LIF were administered by intracranial injection to the left hemisphere of the brain. Replicating virus could be detected in virus culture from brains and TG on day 3 post infection. Viral DNA could also be detected from brains, TGs and spinal cords of infected mice, indicating that the virus was able to spread from the brains to the TG and spinal cord.

Since these BAC-derived HSV vectors express luciferase under an hCMV promoter, the spread of the viruses could be studied *in vivo* using the *in vivo* imaging system (IVIS) luminometer. In a preliminary study, the intranasal and intracranial routes of administration were compared by infecting mice with the BAC-derived vectors HSV-Zeo and HSV-LIF. After intranasal administration the vectors did not reach the brain, whereas upon intracranial administration, virus could be detected in brains, TG and spinal cords (Figure 16). In study IV, both HSV-LIF and HSV-Zeo infected mice displayed high numbers of photons on day 1 post infection (IV: Figure 3A). Both vectors also spread to the spinal cord. By day 4 post infection, the luminescence signal declined (IV: Figure 3A).

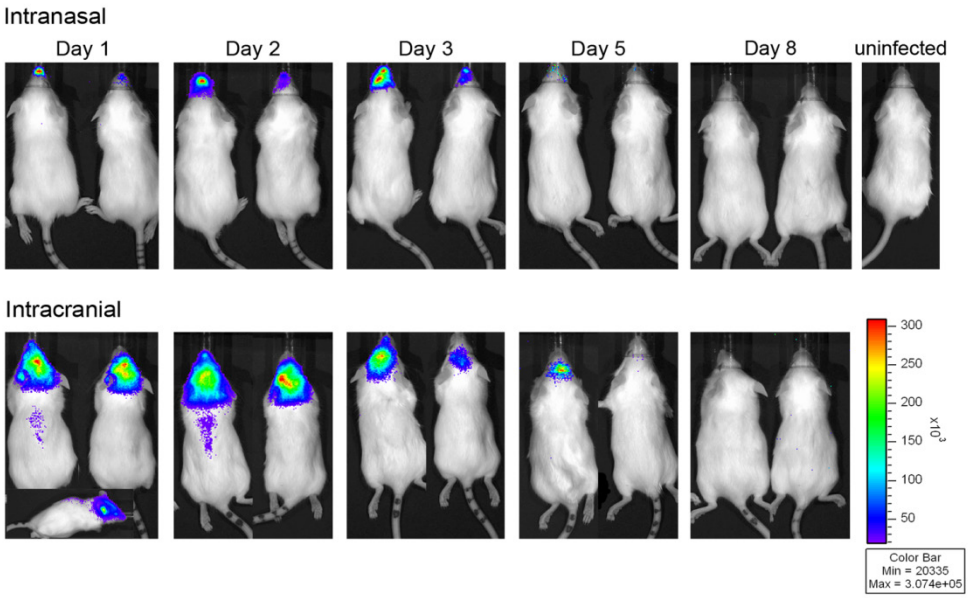

Figure 16. In vivo detection of luciferase expression in SJL/J mice infected intranasally or intracranially with $\gamma_1$34.5-deletion HSV vectors. The same two mice are shown for each day.

The distribution of HSV within the brain was studied by immunohistochemistry using an anti-HSV-1 antibody (IV: Figure 3B-E). The vectors could be detected in the brain parenchyma, especially near the third ventricle, the aqueduct and in the corpus callosum, and in ependyma of the ventricles of the brain.

5.3 Immune response to HSV-1 infection in SJL/J mice (II)

The use of HSV vectors in gene therapy requires good knowledge about the host responses evoked by the virus infection. The presence of markers of both the innate and adaptive immune responses was analyzed by quantitative real-time PCR from mice infected with wild-type HSV-1 intranasally, corneally or intracranially. Expression of TLR and type I interferons was chosen to represent

the innate immune responsen and cytokines and T cell population markers the adaptive T helper cell immune response.

5.3.1 Peripheral immune responses

The peripheral immune response was analyzed from nose and eye tissue of mice infected by the intranasal and corneal route, respectively. The intranasal infection evoked an increase in the mRNA expression in the nose of all three TLRs studied (TLR2, TLR3 and TLR9) during the acute infection (Figure 17 and II: Figure 3). An increase was also seen for MyD88, the adapter molecule of TLR2 and TLR9, on day 3 post infection. Type I IFNs were not significantly affected (Figure 17 and II: Figure 3). In the eye of mice infected by the corneal route, TLR2 was the only up-regulated TLR, and also MyD88 mRNA expression was increased in comparison to uninfected mice (Figure 15 and II: Figure 4). An increase in IFN-β mRNA expression was detected in early infection, whereas IFN-γ was up-regulated at all studied time points in comparison to uninfected mice (Figure 17 and II: Figure 4). These results indicate that the corneal infection induced a stronger IFN response at the peripheral tissue compared to the intranasal infection.

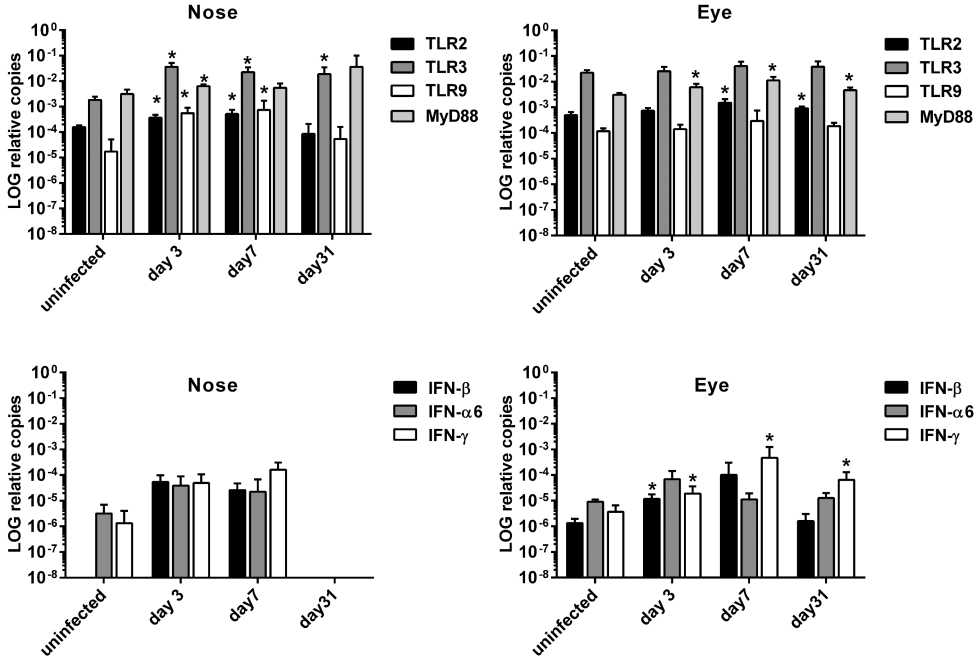

Figure 17. TLR and interferon responses in the peripheral tissue after corneal and intranasal HSV-1 infection of SJL mice. * $p < 0.05$ and ** $p < 0.01$ in comparison to uninfected mice.

The intranasal infection evoked a local Th1 response, as seen by an expression of IFN-γ and a significant increase in IL-23 and IL-27 mRNA expression in the nasal tissue (II: Figure 3). Surprisingly, the detected IFN-γ expression was not significantly elevated by the HSV-1 infection. In early infection IL-5 mRNA expression was up-regulated. Also the Th17 cell population might be involved in the host responses to HSV-1 infection, since RORγT, IL-17, IL-6 and TGF-β mRNA expression was elevated in the nose. The increase in IL-10 mRNA expression on day 7 post infection could have a role in regulating these inflammatory responses (II, Figure 3). The corneal infection induced a significant increase in T-bet and IL-12p40 mRNA expression during the acute infection. Expression of TGF-β, IL-10 and RORγT mRNAs were also increased in the eyes of infected mice, indicating that also Th17 and Treg cell populations might be involved in the immune response to corneal HSV-1 infection (II: Figure 4).

5.3.2 Immune responses in the trigeminal ganglia

The immune response in the trigeminal ganglia (TG) of mice upon infection with HSV-1 by three different infection administration routes was studied. Since both the intranasal and the intracranial infection led to a replicative virus infection of the TG (and the corneal infection did not), we mainly focused on the immune response in these two infection models. The intranasal infection route was also used to study the immune responses of the latent infection, since virus was able to reactivate from the TG in this infection group.

In the TG, TLR2 and TLR9 mRNA levels were increased during the acute infection in mice infected by all infection routes (II: Figure 5). TLR2 was up-regulated throughout the acute infection, whereas TLR9 was mainly increased on day 7 post infection. The intracranial infection also induced an up-regulation of MyD88. An induction of IFN-β was seen for all groups, but with a slightly slower response in the corneal group. During the latent phase, only TLR3 was up-regulated in the TG of intranasally infected mice (II: Figure 5).

In the TG, T-bet was up-regulated during the acute intranasal and intracranial infection (II: Figure 5). Of the Th1 cytokines, IL-27 mRNA was increased. The intranasal infection led to an increase in IL-5 and IL-10 mRNA levels in the TG, at day 3 and day 7, respectively. TGF-β was up-regulated in the TG of both intranasally and intracranially infected mice. In the TG of intracranially infected mice an up-regulation of IL-6 was also detected. These changes in addition to an increase in FoxP3 and IL-10, as well as a down-regulation of RORγT (II: Figure 5) indicate a role of Tregs during the acute intracranial infection. During the latent phase of intranasal infection, the mRNA expression of the studied

cytokines was quite low and significant changes were seen only for GATA3, a marker for Th2 cells, and for RORγT and TGF-β, where GATA3 and RORγT were down-regulated in the intranasal infection (II: Figure 5).

5.3.3 Immune response in the CNS

In brains of mice, the intracranial HSV-1 infection induced a strong IFN response, including type I IFNs and IFN-γ (II: Figure 6). Also the intranasal infection led to an increase in IFN-γ response, albeit with a slight delay. The virally induced IFN-γ response is in line with the later detection of replicating virus in the brains of intranasally infected mice. All three TLRs studied (TLR2, 3 and 9) and MyD88 were up-regulated in the brains after intracranial infection. Mice infected intranasally also had increased levels of TLR2, TLR9 and MyD88 on the later time point (day 7 post infection).

The intracranial HSV-1 infection led to an up-regulation of several cytokines in the brains (Figure 18). An increase was seen for T-bet and IL-12p40 on day 7, while IL-27 was elevated on both day 3 and day 7 (Figure 18 and II: Figure 6). The intracranial HSV-1 infection also induced IL-17 and IL-6 mRNA expressions, but down-regulated transcription of RORγT. Expression of FoxP3, TGF-β and IL-10 was up-regulated in the intracranial infection, again indicating a role for Tregs in controlling the infection. An increase in the Th2 responses (GATA3 and IL-5) was detected in the acute intracranial infection. The intranasal infection induced increased IL-27, IL-17, TGF-β and IL-10 levels during the acute infection. The expression of some cytokines remained high in the brains also at later time points, including GATA3, IL-5 and TGF-β.

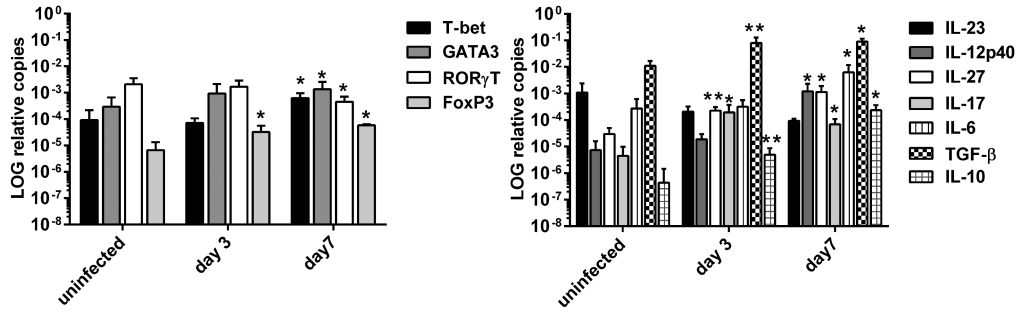

Figure 18. Cytokine immune response in brains of intracranially infected mice. * $p < 0.05$ and ** $p < 0.01$ in comparison to uninfected mice.

5.4 Construction of a LIF-expressing HSV vector using BAC mutagenesis (IV)

To construct an HSV vector expressing LIF for gene therapy of EAE, we utilized the BAC-methodology for efficient and safe mutagenesis of the HSV-1 genome. A pCDNA3.1 plasmid expressing murine LIF under an EF1α promoter was constructed by amplifying LIF from mouse tissues and inserting the PCR product into pCDNA3.1(-)EF1α (a kind gift from Jay Nelson, Oregon Health & Sciences University, Portland, USA).

The construction of the vector HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-LIF (HSV-LIF) is described in detail in the the "Material and methods" section. Shortly, an EF1α-LIF expression cassette was inserted into the left copy of $\gamma_1$34.5 in the genome of HSV-1(17^+)Lox-Luc-Δ$\gamma_1$34.5-Zeo-BAC (HSV-Zeo) using the *en passant* mutagenesis (Tischer *et al.*, 2010; Tischer *et al.*, 2006). EF1α, LIF, KanR and I-SceI sequences were amplified from the pcDNA3.1-EF1αLIF-Kan plasmid with primers containing recognition sequences homologous to the 5´and 3´ flanking the ZeoR sequence. Insertion of the EF1α-LIF-Kan cassette replaced the ZeoR sequence from the genome (Figure 19A and IV: Supplementary figure 2). The KanR selection cassette was removed by activation of I-SceI, removing the KanR sequence and the excess 50 bp duplication sequence. Correct clones were again analyzed with restriction digests and electrophoresis (Figure 19B-C and IV: Supplementary Figure 2).

Virus was reconstituted by transfecting DNA of a verified BAC clone into Vero cells. Four to seven days post transfection viral plaques were visible in cell cultures. Expression of mLIF was verified with immunoblot (IV: Figure 2) and ELISA. Infected cell culture samples expressed 1 pg/µl and 2-3 pg/µl of recombinant LIF in the neuronal cell line T98G and Vero cells, respectively, at 24 h post infection.

In mice infected intracranially with the HSV-LIF vector, transgene expression was studied with a LIF transgene-specific PCR (annealing to the promoter and LIF coding sequence). Recombinant-LIF was detected in all HSV-LIF infected mice on day 9 post induction (day 3 post induction), but in some mice also on day 14 and day 21. The total LIF mRNA levels were increased in brains, TG and spinal cords in mice infected with HSV-LIF at early time points.

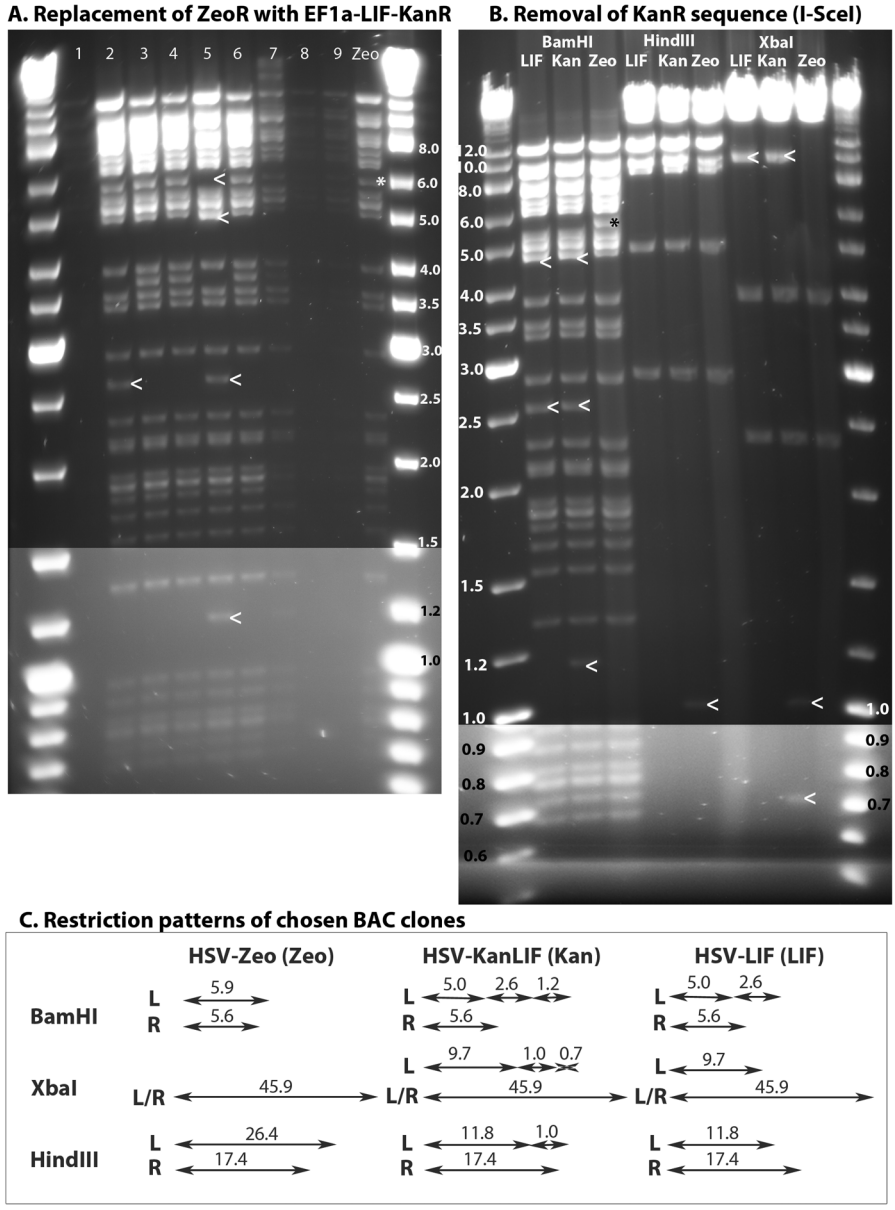

Figure 19. Restriction analysis of HSV-LIF BAC clones. A) BamHI restriction digest analysis of the replacement of ZeoR with EF1α-LIF-KanR in the left copy of $\gamma_1$34.5. Clone no 5 had all correct bands. B) BamHI, HindIII and XbaI restriction analysis of one HSV-1(17$^+$)Lox-Luc$\Delta\gamma_1$34.5-LIF (HSV-LIF) BAC clone compared to HSV-1(17$^+$)Lox-Luc$\Delta\gamma_1$34.5-KanLIF (HSV-KanLIF) and HSV-1(17$^+$)Lox-Luc$\Delta\gamma_1$34.5-Zeo (HSV-Zeo). C) Restriction patterns after digestion with BamHI, HindIII and XbaI in HSV-Zeo, HSV-KanLIF and HSV-LIF. L= band shifts covering the left $\gamma_1$34.5 region, R = band shifts covering the right $\gamma_1$34.5 region. New bands are marked with <.

5.5 Gene therapy of EAE with $\gamma_1$34.5-deletion HSV vectors (III-IV)

5.5.1 EAE induction in SJL/J mice

To set up an EAE model suitable for therapy studies, an induction protocol of EAE in SJL/J mice was determined in preliminary experiments. The PLP-adjuvant administered subcutaneously into the footpads was the same for all experiments, but the amount of pertussis toxin and route of administration varied in the pilot experiments. Pertussis toxin is known to facilitate the autoimmune reaction to the myelin proteins (PLP) and to increases the influx of inflammatory cells into the central nervous system (Hofstetter *et al.*, 2002; Jee & Matsumoto, 2001; Linthicum *et al.*, 1982; Silver *et al.*, 1999).

Intravenous administration of PLP (both 100 ng and 200 ng) led to severe EAE, with disease grades of 4 and with a survival rate less than 50%. After intraperitonal injection of pertussis toxin almost 100% of mice survived and the mice had disease grades ranging from 1.5 -4. Most mice had a maximum disease grade of 3. For gene therapy studies the induction protocol was chosen to entail intraperitonal administration of 200 ng pertussis toxin in 250 µl of PBS. SJL/J mice induced for EAE according to this protocol (III-IV), developed symptoms in 100% of mice at approximately 9-11 days post induction, with the peak of disease occurring at day 13-15.

5.5.2 Clinical disease

To study the therapeutic effect of different HSV vectors on EAE, mice were induced for EAE and treated with $\gamma_1$34.5-deletion HSV vectors expressing the Th2 cytokines IL-10 (R8308) or IL-5 (R8316) (III) or LIF (IV). Mice were infected with 1 x 10^7 PFU of the respective virus at day 6 post EAE induction. The Th2-expressing vectors were administered intranasally and the BAC-derived vectors by intracranial injection. The dose was determined in preliminary studies and the maximum non-toxic deliverable dose was chosen. EAE mice treated with the $\gamma_1$34.5-deletion HSV vectors were followed daily and clinical signs were recorded.

Study III included four groups, all induced for EAE: 1) no infection, 2) infection with the control virus R3659 (no cytokine transgene), 3) infection with R8308 (IL-10) and 4) infection with R8316 (IL-5). A significant decrease in clinical disease scores was detected after day 15 post induction in mice infected with R8316 (IL-5) (Figure 20A; III: Figure 1). In addition, the cumulative index was significantly lower for the mice treated with R8316 in comparison to untreated mice (III: Table 1). Treatment with R8308 (IL-10) caused no significant

alleviation of EAE in comparison to untreated mice, but a lower cumulative index was obtained (Figure 20A, III; Table 1). The control virus R3659 also alleviated the EAE severity during the recovery phase starting from day 18 (Figure 20A; III: Figure 1), indicating a beneficial effect of the backbone vector during the late phase of the EAE.

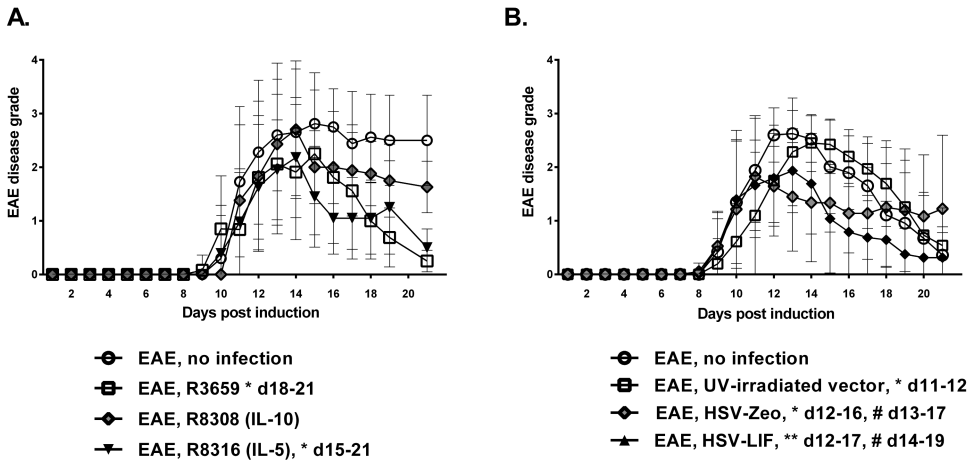

Figure 20. HSV vector therapy of EAE. (A) Treatment of EAE with HSV-1 F-based vectors expressing Th2 cytokines or control virus (III). (B) Treatment of EAE with BAC-derived HSV-1 (17^{+}) vectors, expressing LIF or control virus (IV). * $p< 0.05$ and ** $p<0.01$ when compared with untreated EAE mice. # $p<0.05$ when compared to UV-inactivated HSV-LIF.

In study IV, EAE mice were treated with BAC-derived HSV vectors. The study included four groups of SJL/J mice, all induced for EAE: 1) no treatment, 2) infection with UV-irradiated vector (HSV-LIF treated with UV resulting in titer reduction by 10,000-fold), 3) infection with HSV-Zeo, and 4) infection with HSV-LIF. Treatment with the HSV-LIF resulted in lower disease scores than in untreated or UV-irradiated vector treated EAE mice (Figure 20B; IV: Supplementary figure 5). The cumulative index was also significantly lower for the HSV-LIF treated mice starting at day 14 post induction, when compared to untreated EAE mice (IV: Figure 4). In addition, a significant difference was detected in the disease cumulative index for the HSV-LIF treated group in comparison to all other groups at later time points. The control virus HSV-Zeo also lowered the disease grades early in the treatment, but later the disease scores resumed (Figure 20B; III: Supplementary figure 5). The UV-irradiated vector did not have any alleviating effect on EAE disease. In this study, mice were followed up to 60 days post induction to study any possible effects on relapses, but the mice did not develop a relapsing-remitting disease (IV: Supplementary figure 5).

5.5.3 Inflammation

To study the amount of lymphocyte infiltrates and pathological changes typical for EAE, brain and spinal cord paraffin sections were stained with hematoxylin-eosin (HE). Cellular infiltrates were found in the brains and spinal cords of all groups of mice (III-IV). Mice treated with the HSV vector expressing IL-5 (R8316) and the control virus R3659 both had a significantly lower number of infiltrates in brains during recovery (III: Figure 2). These were shown to consist of leukocytes (CD45), including $CD4^+$ and $CD8^+$ T-lymphocytes (III: Figure 3 and 4). There was no significant difference in the composition of the infiltrates, but a trend of lower $CD4^+$ to $CD8^+$ ratio in HSV-treated mice was seen. There was also a trend towards fewer F4/80-positive cells in HSV-treated mice compared to untreated EAE mice. The HSV-LIF vector did not affect the number of infiltrating lymphocytes when compared to control groups (IV). Since LIF is known to have a role in oligodendrocyte proliferation and thereby might promote remyelination, the distribution of myelin basic protein (MBP) in brains and spinal cords was investigated. A typical demyelination did not occur in any of the groups studied, but inflammatory lesions disrupting the myelin could be detected (IV: Figure 5). When studying the number of oligodendrocytes, we found a significantly higher number of oligodendrocytes in the brains of HSV-LIF-treated EAE mice compared to controls (IV: Figure 6). We were not able to determine if this was due to a proliferation of oligodendrocytes or a decrease in apoptosis.

5.5.4 Modulation of the immune response in EAE by cytokine expressing vectors

Expression of TLRs and cytokines

Several TLRs have been shown to have a function in the pathogenesis of EAE. Since also HSV induces immune responses through TLRs we investigated the effect of TLR mRNA expression and subsequential type I interferon responses in brains and TGs after treatment with the HSV vectors expressing IL-5 (R8316) or IL-10 (R8308), or the control virus R3659. In addition, several inflammatory cytokines were studied in brain and TG tissue. The detected changes in TLR and cytokine expression in the TGs represented direct host responses to the intranasal vector administration, whereas changes in host responses in the brains represented modulations of EAE. The virus-induced responses in the TG may also have affected the outcome of EAE by an indirect mechanism related to the cytokine balance in the nervous system.

TLR and interferon responses

In the TG, only TLR3 of the studied TLRs was affected by the Th2 cytokine-expressing vectors. R8316 (IL-5) and R8308 (IL-10) significantly repressed the TLR3 mRNA expression on day 10 and day 14 post EAE induction (Figure 21). R3659, instead, induced the TLR9 mRNA expression early in the infection and MyD88 at later time points. R8316 (IL-5) and R8308 (IL-10) treated mice had significantly lower MyD88, TLR2, TLR3 and TLR9 mRNA levels than the control virus R3659, indicating that the Th2 cytokines lowered these responses induced by HSV. Whereas the control virus R3659 induced a type I IFN response early in the infection on days 9 and 14, the R8316 (IL-5) vector up-regulated the IFN-α4, IFN-α6 and IFN-β mRNA expression only on day 14 post induction in the TG (Figure 21). The IFN-β induction seen in R3816 (IL-5) vector treated mice was also significantly higher than in R3659 infected mice. R8308 (IL-10) treatment seemed to have the similar effect as IL-5-HSV treatment on type 1 interferons in the TG, but a significant decrease was only seen for IFN-α6 on day 21 post induction. On day 21, the IFN responses were down-regulated in the mice infected with the Th2-cytokine expressing HSV vectors.

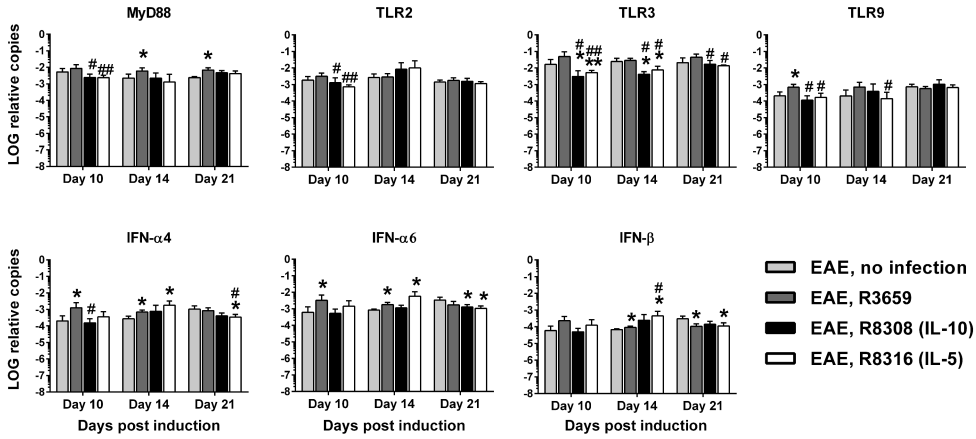

Figure 21. TLR and interferon expression in TG after intranasal HSV gene therapy of EAE mice. * and ** indicate significance where $p< 0.05$ or $p<0.01$, respectively, compared to uninfected EAE mice. # and ## indicate significance where $p< 0.05$ or $p<0.01$, when compared to the control virus R3659.

In brains, all three TLRs studied were significantly down-regulated in mice infected with the R8316 (IL-5) vector during the onset of the EAE disease and R8308 (IL-10) treatment induced similar responses (III: Figure 5). During the peak of disease (day 14), expression of TLR2, TLR9 and Myd88 mRNA was decreased in R8316 (IL-5) and R8308 (IL-10) vector-treated mice, R8316 (IL-5) significantly. A clear difference between the two cytokine vectors was seen in

the TLR3 responses, where the R8308 (IL-10) significantly down-regulated the TLR3 mRNA expression on day 14 post EAE induction and R8316 (IL-5) led to a small increase (III: Figure 5). The control virus R3659 up-regulated all studied TLRs and MyD88 in the brains at all studied time points, indicating that the Th2 cytokines expressed from the vectors was affecting the TLR responses.

The mRNA expression of the type 1 IFNs followed a common pattern in the brains. R8316 (IL-5) treatment significantly up-regulated IFN-α4, IFN-α6 and IFN-β mRNA expressions at day 10 and day 21 post EAE induction in brains (III: Figure 5). The R8308 (IL-10) vector significantly up-regulated the IFN mRNA levels during the later time points on both day 14 and 21. R3659 only induced a type I IFN response on day 14 post induction (day 8 post infection) (III: Figure 5).

Cytokines

The expression of several cytokines often associated with inflammatory disorders was studied from brain and trigeminal ganglion (TG) samples, including IL-4, IL-5, IL-10, IFN-γ, IL-17, IL-23, IL-12p35 and IL-12p40 mRNA. In the TG, infection with R8316 (IL-5) significantly increased the mRNA expression of IL-5, compared to both untreated EAE mice and mice infected with R3659 (III: Figure 7E). Mice infected with the IL-5 virus also showed significantly higher amounts of IL-10 in the TG at day 14, but the levels decreased in the recovery phase (III: Figure 7F). These results indicate that R8316 (IL-5) induced a Th2 type response in the TG. The induced Th2 response might have further up-regulated the IL-5 expression. The R8308 (IL-10) virus did not have a similar effect on the Th2 responses. The IL-17 mRNA expression was higher in the TG of both R8316 (IL-5) and R8308 (IL-10) infected mice, but only significantly so in the R8316 (IL-5) treatment (III: Figure 7A). Neither of the cytokine expressing viruses significantly affected the IL-12p40 or IFNγ mRNA expression in the TG, but the control virus R3659 did up-regulate the IL-12p40 expression (III: Figure 7C).

The HSV vectors expressing the Th2 cytokines IL-5 or IL-10 both induced an increase in IL-10 mRNA expression in brains compared to untreated EAE mice and R3659 infected EAE mice (III: Figure 6G). The R8308 (IL-10) vector down-regulated the pro-inflammatory cytokine IL-17 during onset of disease (day 10) and repressed IL-12p40 transcription during the acute EAE phase (day 14) (III: Figure 6A and C). Both R8308 and R8316 induced lower IL-12p40 mRNA levels than R3659, which stimulated IL-12p40 throughout the disease (III: Figure 6C). R8316 (IL-5) suppressed IL-12p35 during recovery (day 21) (III: Figure 6D). No significant changes were seen in IFN-γ mRNA expression

compared to untreated mice, though the Th2 cytokine expressing HSV vectors had lower IFN-γ levels than R3659 (III: Figure 6E).

Stimulation of spleen cells with Th2 cytokines

To elucidate the direct effect of Th2 cytokines on cytokine and TLR mRNA expression in our SJL/J mice, we isolated spleen cells of SJL/J mice and stimulated them with Concanavalin A (ConA, stimulates T cell proliferation) or polyinosinic-polycytidylic acid (PolyIC, a TLR3 agonist). We then treated them with recombinant IL-4, IL-10 or IL-5, either as such or in combination with the ConA/PolyIC-stimulation. We found that the IL-17 mRNA expression was lower when splenocytes were stimulated with IL-5 together with ConA, but increased by IL-5 as such and by IL-10 together with PolyIC (Figure 22A).

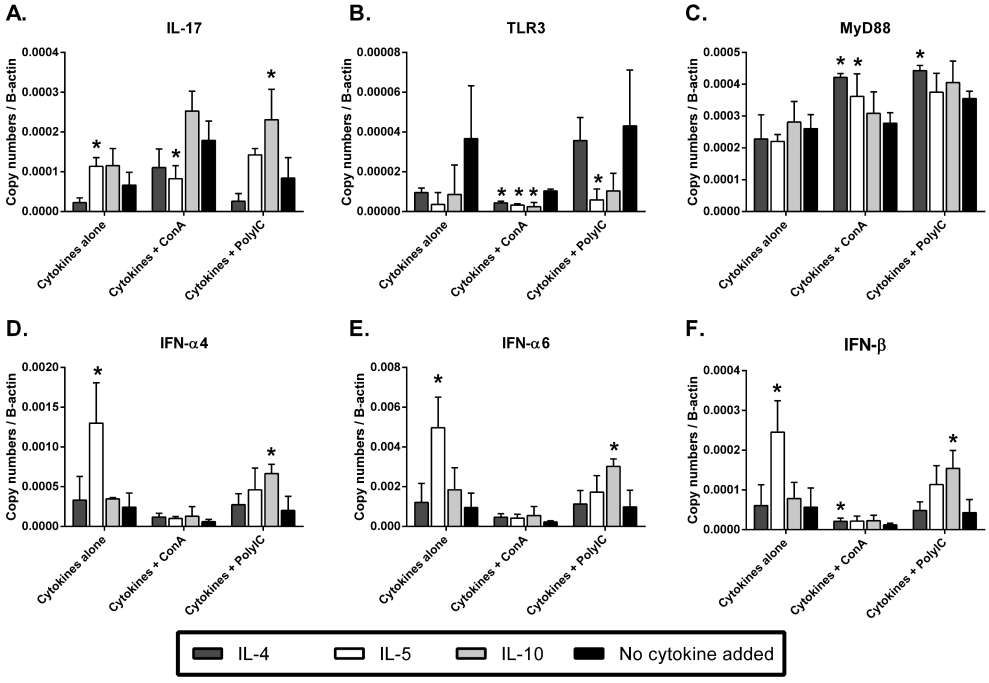

Figure 22. Stimulation of spleen cells from SJL/J mice with Th2 cytokines. Spleen cells were isolated and stimulated with with ConA or PolyIC, in combination with the Th2 cytokines IL-4, IL-5 or IL-10. * $p<0.05$ when compared to no cytokine-treatment.

All three cytokines inhibited TLR3 transcription in ConA-stimulated conditions. In addition, IL-5 down-regulated the TLR3 mRNA expression in PolyIC stimulation conditions (Figure 22B). The type 1 IFNs (IFN-α4, IFN-α6 and

IFN-β) showed an up-regulation upon stimulation with IL-5 alone (Figure 22D-F). Similarly, IL-10 in combination with PolyIC increased the mRNA levels of type 1 interferons. Both IL-4 and IL-5 up-regulated the MyD88 mRNA expression in combination with ConA (Figure 22C). This suggests that the interferon response in SJL/J spleen cells is partly MyD88-mediated.

5.5.5 Modulation of cytokine responses in EAE with HSV-LIF (IV)

While the Th2 cytokine-expressing HSV vectors could modulate the expression of type I IFNs, the same was not seen for the HSV-LIF vector. There were no significant changes in IFN-β mRNA levels in the brains, however a decrease in IFN-α6 mRNA levels was detected after HSV vector treatment (IV: Figure 7). In spinal cords the IFN-β mRNA expression was down-regulated after HSV-LIF treatment, but the backbone-virus stimulated IFN-α6 expression (IV: Figure 8). Also the HSV vector did not induce significant changes in IFN-γ mRNA levels in the brains of treated mice (IV: Figure 7). In spinal cords, both HSV-Zeo and HSV-LIF induced an increase in IFN-γ mRNA levels early in infection and HSV-LIF also on day 14 (day 8 post infection) (IV: Figure 8).

Since LIF has been shown to modulate T cell responses, we decided to analyze several autoimmunity and inflammation-related cytokines and T-cell population markers from brain and spinal cord samples by quantitative RT-PCR. Of the Th2 cytokines, IL-5 was induced in the HSV-LIF treated group on day 21 post induction in both the brain and spinal cord (IV: Figure 7 and 8). As typical modulators of Tregs, the levels of IL-10, TGF-β and FoxP3 were studied. IL-10 mRNA expression was elevated in both brains and spinal cords in the HSV-LIF and the HSV-Zeo treatment groups during onset of disease (IV: Figure 7 and 8). In HSV-LIF treated mice, an increase in TGF-β was detected in brains, whereas IL-6 tended to decrease, providing a net balance favoring Treg development (IV: Figure 7). For both the HSV-LIF and the control HSV-Zeo, there was a significant decrease in IL-6 mRNA levels in brains during recovery. The HSV-LIF treatment further induced a significant reduction of IL-17 expression during recovery in the brains (IV: Figure 7). A similar tendency was also seen in the spinal cords, but it was not significant. The cytokine IL-23 was not significantly altered in brains of HSV-treated mice. There were no significant changes in the T cell population markers FoxP3, RORγT or T-bet mRNA expression in the brains of HSV vector treated mice (IV: Supplementary figure 8).

In spinal cords, no clear differences in the immune response between HSV-LIF and HSV-Zeo vector-treated groups. TGF-β and IL-6 levels increased in both HSV vector treatment groups during onset of disease, but only HSV-LIF down-regulated the IL-6 mRNA expression during later timepoints (IV: Figure 8). IL-

23 mRNA expression was significantly decreased in the spinal cords of both HSV-treated groups during disease onset (IV: Figure 8). The Treg cell population marker FoxP3 was significantly elevated in both the HSV-LIF and HSV-Zeo treated mice on day 9 post induction (IV: Supplementary figure 8). This was also the case for T-bet mRNA expression, which could suggest a shift from Th17 towards Th1 and Treg dominance.

6 DISCUSSION

6.1 Quantitative RT-PCR for detection of cytokines and viral transcripts (I-IV)

In study (I) a quantitative RT-PCR method was established for the detection of low copy numbers of mouse cytokines and viral transcripts, applying the Roche LightCycler system (Wittwer *et al.*, 1997). In studies (II-IV) we analyzed cytokine and viral gene expression with Rotor-Gene. Cytokines are relatively short-lived mediators of the immune response and it can be difficult to detect sensitive changes in their expression with protein-based methods, such as immunoassays, flow cytometry and proteomics. Quantitative real-time PCR is a sensitive method, which can detect very low copy numbers of mRNA. Even though changes in mRNA levels do not always correlate to protein expression, detected mRNA levels can give a good indication of what might also be occurring on a protein level.

In study (I) detection of cytokines and HSV-1 VP16 with Sybr Green I dsDNA binding dye was compared to detection with probes. Sybr Green I binds to the minor groove of dsDNA and fluoresces upon binding to dsDNA during the annealing and elongation steps of PCR. The fluorescence signal is recorded at the end of the elongation phase, when the maximal signal is emitted. Since the Sybr Green system is an unspecific detection system, melting temperature analysis is needed for identification of correct products from unspecific product or primer-dimers. This is not required with the specific probe-detection system. There, two probes hybridize to the amplified DNA and the emitted signal from the acceptor dye is measured at the end of the annealing step. Depending on the initial target DNA concentration in the sample, the signal increase starts in different cycles.

Both the LightCycler and Rotor-Gene system were able to detect low copy numbers of mouse gene transcripts and viral genomes. With LightCycler we could detect copy numbers in the range of 10 and 100 copies per reaction with the probes and Sybr Green dye, respectively. The sensitivity of the probe detection was thus one log better (I). In study (II-IV), we were able to detect copy numbers at the range of 10 copies using the Sybr Green dye and Rotor-Gene. Even though no specific probe was included in these tests, unspecific products could be identified by differences in the products´ melting temperatures. As shown in study (I), detection of medium or high copy numbers was more reproducible than that of low copy numbers. Similar results have also been reported by others (Overbergh *et al.*, 1999; Yin *et al.*, 2001), where detection with probes was more sensitive for the reproduction of low copy numbers (Yin *et al.*, 2001). Copy numbers were calculated based on the dilution

curve of external standards of known concentration, either plasmid DNA and cDNA transcripts. Both types of standards gave good results in our testing settings, but in most tests cDNA standards were used. Due to supercoiling of plasmid DNA, cDNA standards might be more suitable for quantitative PCR (Hou *et al.*, 2010). All cytokine and viral copy numbers were normalized to β-actin or GAPDH. By doing so, we were able to measure a quantitative value, independent of the amount of starting material.

There are some differences between Rotor-Gene and LightCycler. Rotor-Gene has a capacity of up to 100 samples per run, in comparison to 32 with LightCycler. In Rotor-Gene the rotor is running at all times, enabling a more steady temperature for the samples. Another difference is the use of glass capillaries versus plastic tubes. Plastic tubes are more easily handled and stored, but glass capillaries might have an advantage in detection sensitivity. Both Rotor-Gene and LightCycler were suitable for the detection of mouse cytokines, but Rotor-Gene was chosen for further studies, due to larger sample capacity and availability. The probe detection system was not chosen for all tests, since good probes are not always easy to design and due to its higher costs.

6.2 Spread and replication of HSV-1 in SJL/J mice (II-IV)

6.2.1 Wild-type HSV-1

In study (II) we investigated the spread of and host responses to wild-type HSV-1 (17^+) in SJL/J mice, administered by three different routes: corneal, intranasal or intracranial. The SJL/J mouse is not frequently used in studies of HSV pathogenesis, but is a common model in experimental autoimmune encephalomyelitis (EAE). Since HSV vectors can be utilized for gene delivery in EAE, it is important to study the immune activation caused by the HSV-1 infection in these mice.

In the SJL/J mice, HSV-1 replicated in peripheral tissue during the acute infection in both intranasally and corneally infected mice. Viral DNA was amplified from TG and brain tissues of mice infected by all three routes, but replicating virus was only detected in these tissues after intranasal and intracranial administration. Intracranial HSV-1 infection caused severe encephalitis, as expected. The intranasal HSV-1 infection led to spread of replicating virus to the brains of SJL/J mice, as has been reported also by others (Hudson *et al.*, 1991). In intranasally infected mice, latent HSV-1 was also able to reactivate from the TG. We therefore propose that in the SJL/J mouse, the intranasal infection is more effective for HSV delivery to the TG and CNS than the corneal infection. To further study neuroinvasiveness differences, tracer

viruses expressing markers, such as lacZ, could be utilized to find what cells are infected after delivery by different routes (Rødahl & Haarr, 2000).

Corneal infection is a common animal model of HSV-1 infection, used for the study of keratitis and latency. These studies have mainly been conducted in rabbits, or in the C57BL/6 and BALB/c mice strains (Caudill *et al.*, 1986; Foster *et al.*, 1986; Metcalf & Michaelis, 1984; Norose *et al.*, 2002; Stuart & Keadle, 2012). The limited spread from the eyes in our study, might depend on differences in susceptibility between animal strains (Halford *et al.*, 2004; Kastrukoff *et al.*, 1986; Lopez, 1975) and differences in neurovirulence and neuroinvasiveness of different viral strains (Dix *et al.*, 1983; Lopez, 1975; Wang *et al.*, 2013). Another explanation might lie in the viral dose administered. In our study we administered a total dose of 10^5 PFU to the eyes. In many studies higher doses have been used (Leib *et al.*, 1989). Some differences in the peripheral immune responses of the intranasal infection and the corneal infection were also detected, which could explain the differences in virus pathogenesis after these routes. The immune response to these infections will be discussed further in "6.3. Immune response to wild-type HSV-1 infection".

6.2.2 $\gamma_1$34.5-deletion HSV vectors

HSV-1 deleted of the neurovirulence gene $\gamma_1$34.5 is non-neurovirulent, but is able to spread to some cell types in the CNS, including ependymal cells (Markovitz *et al.*, 1997; Whitley *et al.*, 1993). In study (III) and (IV), we studied the spread of different $\gamma_1$34.5-deletion vectors after intranasal and intracranial administration. The HSV-1 (F)-based vectors R3659, R8308 and R8306 were administered intranasally at a dose of 10^7 PFU. These vectors spread to the TG, but not to the brain, as analyzed with viral DNA PCR (III). In the BALB/c model, intranasal infection of $\gamma_1$34.5-deletion vectors has been effective in spread to the CNS (Broberg *et al.*, 2004a). When administering 10^7 PFU of the HSV-1 (17^+) BAC-derived $\gamma_1$34.5-deletion vectors HSV-Zeo and HSV-LIF intracranially, replicating virus was detectable in the brains for 3-5 days and also to some degree in the spinal cords during the acute infection. All infected mice had viral DNA in the TG during the acute infection and in some mice also at later time points. Thus, these vectors were able to spread to the spinal cord and the TG from the injection site in the parenchyma at the left parietal cerebral cortex. Since ependymal cells were positive for HSV-1 at the ventricles, the virus most likely spread along the ventricles or cerebrospinal canal (Martino *et al.*, 2001). Some $\gamma_1$34.5-deletion vectors have been reported to be cytotoxic in the brain (Kesari *et al.*, 1998; Lasner *et al.*, 1998). In our model, the ependymal cell lining remained intact and no enlargement of the ventricles was detected. The lower cytotoxicity could be explained by possible additional mutations in

the viral genome of the BAC-derived vectors, such as loss oriL and modifications of the *a* sequences (Nagel *et al.*, 2008).

If the experimental setting requires replicating virus or a higher amount of virus vector to be present in the CNS, a direct administration of the $\gamma_1$34.5 by intracranial route is more suitable than peripheral delivery. Nevertheless, intranasal vector administration has also been effective for modulation of immune responses in the TG and CNS (III).

6.3 Immune response to wild-type HSV-1 infection (II)

6.3.1 Innate immunity

The innate immune responses against HSV-1 infection include activation of the type I IFNs by PRRs, such as the TLRs, and induction of cytokine expression. In study (II), we infected SJL/J mice with wild-type HSV-1 by intranasal, corneal or intracranial administration. In intranasal HSV-1 infection, expression of TLR2, TLR3 and TLR9 were all up-regulated in nasal tissue, but did not lead to an activation of IFN-α or IFN-β. In infected eyes, only TLR2 was up-regulated, and an induction of IFN-β was also observed. This induction of IFN-β could have limited the spread from the cornea, as it is known that IFN-α and IFN-β can inhibit HSV replication (Halford *et al.*, 1997; Hendricks *et al.*, 1991; Leib *et al.*, 1999). IFN-γ was up-regulated at all studied time points in the eyes of corneally infected mice. The early expression might originate from cells of the innate immune system. Since IFN-γ is known to have strong antiviral activity in HSV-1 infection (He *et al.*, 1999; Kodukula *et al.*, 1999; Smith *et al.*, 1994), these responses might explain the reduced spread to the TG and CNS from the eyes.

The intracranial infection induced a strong up-regulation of TLR2 and TLR9 throughout the infection, and an increase in TLR3 was detected on day 7 post infection. Both IFN-β and IFN-α6 were also up-regulated in the brains of intracranially infected mice. These findings are in line with previous studies, where a role for TLR2 and TLR3 in HSV encephalitis has been suggested (Kurt-Jones *et al.*, 2004; Zhang *et al.*, 2007). Furthermore, TLR2 activation has been shown to induce inflammatory responses in the brain (Wang *et al.*, 2012). Thus, the strong immune responses to HSV in the brains of intracranially infected mice could be partly due to the innate immune responses.

Similar responses were also seen in the TG, where TLR2 mRNA expression was up-regulated throughout the acute infection after all three administration routes, and TLR9 levels were elevated at day 7 post infection. IFN-β levels were also

elevated in the TG during the acute infection of all three HSV-1 routes. Since only viral DNA and no replicating virus was detected in mice infected by the corneal route, these findings suggest that replicative virus is not needed for an induction of the innate immune responses in the TG.

6.3.2 Adaptive immune responses

Both $CD4^+$ and $CD8^+$ T cells are known to be involved in the HSV-1 induced immune responses. In our study we did not differentiate between different cell types, rather, the induced expression of cytokines and T cell transcription factors was studied with quantitative RT-PCR.

In study II, we found that the intranasal infection induced a strong peripheral Th17 response, with an increase in RORγT, IL-23, IL-17, IL-6 and TGF-β. Th1 responses included an up-regulation of IL-27 and IL-23, but surprisingly not IFN-γ. A role for Th17 cells in inducing inflammation in HSV keratitis (Sehrawat *et al.*, 2008; Suryawanshi *et al.*, 2011) has been reported, but its function in intranasal infection has not been reported previously. The function of IL-27 in HSV-1 infection also remains to be elucidated, but this pro- and anti-inflammatory cytokine is known to induce Th1 responses on one hand and suppress Th17 responses on the other (Batten *et al.*, 2006; Hunter, 2005; Stumhofer *et al.*, 2006). The observed up-regulation of IL-27 in HSV-1 infection of the nose, TG, and brain tissue indicates an important role for this cytokine both in the periphery and in the CNS.

The corneal HSV-1 infection induced expression of TGF-β and IL-10 in the eyes, in addition to the IFN responses mentioned earlier. Also TGF-β and IL-10 might limit the infection, as IL-10 and Tregs are known to be involved in the control of keratitis (Sarangi *et al.*, 2008). The intracranial infection led to a strong inflammatory response, but also induced a Treg and Th2 response in the brains. The up-regulation of FoxP3, TGF-β, IL-10 (Treg) and GATA3 and IL-5 (Th2) probably control the massive immune responses occurring in the CNS.

6.4 Gene therapy of EAE with HSV-1 $\gamma_1$34.5-deletion vectors (III-IV)

The Th1/Th2 paradigm has long been targeted in studies for therapeutic approaches of EAE, attempting to shift the immune response from a Th1 profile towards a Th2 milieu. Th2 cytokines, such as IL-4 and IL-10 have been administered systemically as recombinant proteins or delivered by gene therapy approaches to treat EAE. IL-5 is another Th2 cytokine, which has not been widely studied for its therapeutic potential. IL-5 is mainly associated with eosinophil functions (Sanderson, 1992), but has also been reported to be up-

regulated in glatiramer acetate-treated MS patients (Sanna *et al.*, 2006; Wiesemann *et al.*, 2001; Wiesemann *et al.*, 2003). There might therefore be a therapeutic potential for IL-5. Besides cytokines affecting T cell populations, immunological factors affecting the growth and functions of oligodendrocytes, the myelin-producing cells, are interesting therapeutic transgenes. LIF has several features making it a promising therapeutic candidate, due to its capability to promote oligodendrocyte differentiation and survival, as well as modulating T cell responses (Azari *et al.*, 2006; Cao *et al.*, 2011; Gao *et al.*, 2009; Kerr & Patterson, 2005).

In study III and IV, we infected SJL/J mice induced for EAE with HSV-1 vectors deleted of $\gamma_1$34.5 and expressing IL-5 (R8316) or IL-10 (R8308) intranasally, or LIF intracranially. Both R8316 (IL-5) and HSV-LIF significantly ameliorated EAE when administered at day 6 post EAE induction, whereas R8308 (IL-10) did not. Interestingly, also the backbone viruses R3659 and HSV-Zeo had a slight disease alleviating effect. These findings are in line with our previous reports where R3659 also alleviated EAE in the BALB/c mouse (Broberg *et al.*, 2001). The alleviated effect may be based on the ability of R3659 to induce systemic Th2 responses, as shown by Broberg (Broberg *et al.*, 2004a). The mice infected with R8316, R8308, R3659 and HSV-LIF all had significantly lower cumulative index at the end of the acute disease. The HSV-LIF treatment further resulted in significantly lower cumulative index scores in comparison to all control groups.

The EAE disease in both studies (III, IV) included pathological changes, including inflammatory infiltrates in the brain and spinal cord. The R8316 (IL-5) vector and R3659 significantly reduced the amount of infiltrating cells in the brains during recovery, and R8308 (IL-10) had a similar tendency. No significant differences in the infiltrate composition were detected. HSV-LIF and HSV-Zeo did not have a significant effect on the number of infiltrates. Demyelination was not detected, but we found that the infiltrates disrupted parts of the myelin, both in the brain and spinal cord. This is line with studies showing that demyelination occurs more often in relapses than in the acute disease (Amor *et al.*, 1994; Baker *et al.*, 1990; Raine *et al.*, 1978) (reviewed in (Baker *et al.*, 2011; Kipp *et al.*, 2012)). Therefore, to study demyelination and myelin repair in EAE, relapsing-remitting models should be used. The SJL/J PLP-model is considered a relapsing-remitting EAE model. Even though we followed the mice for a longer period of time, up to 60 days (study IV), we did not detect clear relapses. This might be explained by the young age of the animals in our model, as normally EAE is induced in mice older than 8 weeks.

The molecular mechanisms behind the therapeutic efficacy of the Th2 cytokine expressing vectors and HSV-LIF differ, as expected. The HSV vectors

expressing IL-5 or IL-10 induced an up-regulation of Th2 cytokines and also IFN-β mRNA expression in the CNS. R8316 (IL-5) treatment induced IFN-β at all studied time points in the brains, whereas IL-10 caused an increase during the later time points. R8316 (IL-5) also induced a strong increase in both IL-5 and IL-10 mRNA expression in the TG and of IL-10 expression in the brains, while R8308 (IL-10) did so to a lesser extent. The mechanism of IFN-β on EAE and MS is still not completely known, but studies indicate that it is important for the recovery from EAE. Mice without functional IFN-β or its receptor have more severe EAE (Guo *et al.*, 2008; Prinz *et al.*, 2008; Teige *et al.*, 2003). IFN-β also affects T cell function by suppressing up-regulation of MHC II molecules on macrophages and microglia, thus interfering with antigen presentation and activation of cytotoxic T cells (Axtell & Steinman, 2008). Type I IFNs can further decrease the production of Th1 cytokines such as IFN-γ and TNF-α (Noronha *et al.*, 1993), both known to have pro-inflammatory functions. This might be coupled to the reported increase in production of IL-4 and IL-10 by IFN-β (Kozovska *et al.*, 1999; Rudick *et al.*, 1998; Rudick *et al.*, 1996).

To study the mechanism behind the Th2 cytokines on the immune response, we stimulated spleen cells with ConA and PolyIC in the presence of Th2 cytokines. IL-5 stimulation alone up-regulated all type I IFNs and IL-10 and IL-4 were also capable of inducing IFN responses, in combination with PolyIC and ConA, respectively. These results further indicate an effect of the Th2 cytokines on the type I IFN responses. In addition, we found that all three Th2 cytokines: IL-5, IL-10 and IL-4, down-regulated the expression of TLR3 in combination with ConA-stimulation. These results supported our observations in R8316 (IL-5) and R8308 (IL-10) treated EAE mice, where a down-regulation of TLR3 was detected in the TG. A decrease in the mRNA expression of TLR2, TLR3 and TLR9 in brains was also found. Others have also reported an inhibition of TLR3 and TLR4 by Th2 cytokines (Mueller *et al.*, 2006). The up-regulation of IFN-β mRNA expression in R8316 (IL-5) and R8308 (IL-10) treated mice is therefore not likely to be induced by TLRs. Importantly, a role for TLRs in EAE has been proposed. MyD88, the signal-transducer for TLR2 and TLR9, is crucial for the induction of EAE (Prinz *et al.*, 2006). Thus, the decrease in MyD88 mRNA expression during disease onset in R8308 (IL-10) and R8316 (IL-5) treated mice might also explain the milder clinical disease. Therefore, the therapeutic mechanism was most likely a combination of several effects induced by IL-5. Since R8308 (IL-10) induced similar changes in the immune response as R8316 (IL-5), there might still be some unexplored mechanisms behind the function of IL-5 in EAE.

The therapeutic efficacy of LIF in EAE did not involve type I IFNs. Instead we detected modifications in cytokines of the Th17 and Treg cell populations. HSV-LIF treatment significantly down-regulated IL-17 mRNA expression

during recovery in brain tissue. In addition, TGF-β mRNA expression was up-regulated during disease onset, whereas a decrease was seen in IL-6 expression during recovery in brains and spinal cords of HSV-LIF treated EAE mice. Taken together, these results support the previous findings, that LIF regulates the Th17/Tregs axis (Gao *et al.*, 2009). Interestingly, HSV-LIF also significantly up-regulated IL-5 during recovery in EAE in both the brains and spinal cords, indicating a protective role for this cytokine (III). Furthermore, also the back-bone vector HSV-Zeo induced some beneficial modifications of the cytokine response. Both vectors induced an increase in the IL-10 mRNA expression in brains and spinal cords. TGF-β mRNA expression was also induced and IL-23 mRNA expression was down-regulated at early time points in the spinal cords after HSV vector treatment. IL-23 has a function in maintaining the Th17 cell population (Stritesky *et al.*, 2008) and the detected decrease in IL-23 could therefore have therapeutic relevance in EAE.

By studying neuropathological changes in response to the HSV vector treatments, we detected a higher number of oligodendrocytes in the brains of HSV-LIF treated mice. This is an important finding, since oligodendrocytes are the cells producing myelin and important for remyelinating events. We were not able to determine if the increase in oligodendrocyte number was due to anti-apoptotic effects on oligodendrocytes or a stimulation of oligodendrocyte proliferation. Both of these functions have been described LIF (Azari *et al.*, 2006; Kerr & Patterson, 2005; Slaets *et al.*, 2008). In mice with EAE, endogenous administration of LIF has prevented oligodendrocyte death (Butzkueven *et al.*, 2006; Butzkueven *et al.*, 2002), and gene therapy with a lentivirus expressing LIF was able to prevent relapses and reduce demyelination (Slaets *et al.*, 2010). As suggested by others, LIF might also have additional mechanisms for neuron protection, other than oligodendrocyte-related (Gresle *et al.*, 2012).

7 CONCLUSIONS

In this study, quantitative real-time PCR was established and applied for the sensitive detection of mouse cytokines and viral transcripts with LightCycler. Two technologies, one using Sybr Green I dsDNA dye-detection and the other specific probes, were compared. We found that the use of probes was more sensitive in the detection of mouse cytokines, whereas VP16 was detected equally well with both systems. Using Rotor-Gene PCR together with Sybr Green dye, we were able to detect also low copy numbers. Both PCR technology platforms (LightCycler and Rotor-Gene) worked well for detection of cytokine mRNAs and viral transcripts in cell culture and tissue samples, but careful analysis of melting temperatures was needed when using the Sybr Green dsDNA dye. Quantitative real-time PCR is a valuable technique, enabling many tests to be performed from a small sample size and detection of low mRNA levels.

In animal models of HSV-1 infection different outcomes are determined by the animal strain and route of administration. In this study HSV-1 was administered by intranasal, corneal or intracranial route in SJL/J mice, which are used for studies of EAE, the model disease of MS. We found that HSV-1 spread more efficiently to the TG and CNS after intranasal infection compared to corneal infection. The different host responses induced by the intranasal and corneal infection might explain the differences observed in viral spread. Interestingly, the intranasal infection induced local Th17 immune responses, whereas the corneal infection led to strong IFN-γ induction. In addition, an increase in IFN-β was observed. Since both IFN-β and IFN-γ are known to have antiviral functions in HSV infections, the expression of these cytokines might have limited the virus spread to the TG and CNS. The intracranial infection led to a severe infection of the brain, with spread also to the TG. The induced host response in the brain included Th1, Th2, Th17 and Treg cells of the adaptive immunity, but also strong TLR2 and IFN expression. The cytokine IL-27 was observed to be upregulated in many tissues, indicating a role of IL-27 in HSV-1 infection. The function of IL-27 should be studied further, since its function in HSV-1 infection is not well characterized and could also have implications for gene therapy with HSV vectors.

Two gene therapy studies were included in this thesis (III, IV). Both studies show that HSV vectors are good gene therapy vectors in treatment of autoinflammatory CNS disease. The $\gamma_1$34.5-deletion vectors induced disease-alleviating effects and induced beneficial cytokine responses. An HSV vector expressing IL-5 significantly ameliorated the disease, lowered inflammation and induced Th2 cytokine responses and an up-regulation of IFN-β. Another HSV vector expressing IL-10 also modulated the cytokine expression, but did not affect the clinical disease. The HSV-LIF vector, developed using the BAC-

technology, significantly reduced clinical EAE symptoms, induced a higher number of oligodendrocytes and modulated T cell responses in a favorable manner. Study IV also showed that BAC-derived vectors can be used as gene therapy vectors, as no adverse effects were detected. Further, we demonstrated that both the intranasal and the intracranial route of administration of HSV can be utilized in gene therapy studies. Finally, the safety of the vectors should be carefully studied and modifications elaborated in order to achieve longer transgene expression.

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