Control of Herpes Simplex Virus Type 1 Latency in Human Trigeminal Ganglia

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Persistence is half the way.

Fortune cookie

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1 Abstract

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus that infects humans and, after a primary lytic infection, establishes lifelong latency in the sensory neurons of the trigeminal ganglia (TG). HSV-1 latency is accompanied by a chronic immune cell infiltration of the TG, the infiltrate being mainly composed of $CD8^+$ T cells. These T cells are believed to control viral latency, but cellular and viral factors like viral microRNAs are also considered to play a crucial role in the establishment and maintenance of viral latency.

In the present work, it was investigated whether the tissue-infiltrating T cells are clonally expanded, which would indicate that these T cells are activated by antigen. By applying complementarity determining region 3 (CDR3) spectratyping and immunohistochemistry, several clonal expansions were identified in the TG-resident T cells. In addition, several T cells were present that seemed to be unspecific bystander T cells. Strikingly, some expanded T-cell clones were present in the right and left TG of the same individual. This strongly suggests that similar antigens are present in both TG and that the infiltration of immune cells to the TG is driven by antigen.

The morphology of the TG was investigated by immunohistochemistry and *in situ* hybridization. Analysis of the distribution of T cells throughout the TG provided puzzling results: unexpectedly, most neurons surrounded by T cells did not harbour the only known prominent transcript during latency, the latency associated transcript (LAT). Whether these neurons do actually harbour latent virus was addressed by a combination of LAT *in situ* hybridisation, T-cell immunohistochemistry, and single cell analysis of laser microdissected sensory neurons by PCR. This analysis revealed that only LAT^+ neurons were harbouring HSV-1 DNA and viral microRNAs. Also, mRNA for a viral gene product was only detected in LAT^+ neurons. All analysed LAT^- neurons were devoid of viral microRNAs and DNA of HSV-1. DNA of HSV-2 or varicella-zoster virus (VZV) was not detected in any of the excised neurons. Altogether this indicates that in the vast majority of infected human neurons, HSV-1 latency is not directly controlled by T cells, but rather by cellular or viral factors like the miRNAs. Our data suggest that $CD8^+$ T cells only come into action if these mechanisms are overrun.

2 Introduction

2.1 Immune system

The body has to constantly protect itself from a variety of pathogens like viruses, bacteria, fungi or parasites, as well as from mutated autologous cells. It is the role of the immune system to protect the organism against such threats. Two systems have evolved to accomplish this. The first line of defence is the innate immune response. Immediately after crossing the surface barriers of skin or mucosa, pathogens are identified as non-self by invariant receptors recognising common features of pathogens. This then triggers an inflammatory process. The innate immune system consists of white blood cells like macrophages, dendritic cells, granulocytes, mast cells, and natural killer cells, as well as the soluble factors of the complement system. If pathogens overcome these first control mechanisms, the innate immune system paves the way for the adaptive immune response and prevents accelerated pathogen reproduction whilst the adaptive response is generated. In contrast to the innate immune system, the effectors of the adaptive immune response recognise specific antigens and are able to memorise them. These effectors of the adaptive immune response are the lymphocytes, divided into T cells and B cells. T cells play the central role in cell-mediated immunity whereas B cells, by generating specific antibodies, represent the humoral branch of the adaptive immune response. The downside of such a highly specific immune response is the rejection of transplants or autoimmune disorders of the immune system.

2.1.1 T cells

T cells originate from lymphoid progenitor cells in the bone marrow but their maturation mostly takes place in the thymus (Chaplin, 2010). The rearrangement of the T-cell receptor (TCR), as well as selection of T cells with a functional, non-autoreactive TCR, takes place there. After maturation, T cells are released from the thymus and migrate between the bloodstream and lymphatic organs as mature naïve T cells, until they encounter their respective antigen. Activation of T cells depends on presentation of antigens by antigen-presenting cells (APC) on major histocompatibility complex (MHC), and a further co-stimulatory signal. Upon antigen recognition T cells proliferate, giving rise to a clonal expansion, and finally differentiate into effector T cells. Most effector T cells die eventually, but some persist after

antigen clearance and differentiate into memory cells, which can be reactivated quickly upon a second encounter with the respective antigen.

There are two main subsets of T cells (Chaplin, 2010), each carrying one of two different surface receptors, CD4 or CD8. $CD4^+$ T cells recognise antigens presented on MHC class II, which is present on immune cells only. The main function of $CD4^+$ T cells is to modulate the actions of other immune cells by the secretion of cytokines or direct interaction. There are a variety of regulatory T cells of both subsets, for example the most commonly known $CD4^+$ $FoxP3^+$ regulatory T cells (Sakaguchi et al., 2009). Regulatory T cells are able to suppress other immune cells and play a crucial role in the induction of immune tolerance. $CD8^+$ T cells are stimulated by antigen presented on MHC class I, which is present on most cells of the body. The major task of $CD8^+$ T cells is to detect and destroy cells that are infected by intracellular pathogens or altered by mutagenesis. As this work focuses on herpesvirus infection, both $CD8^+$ T cells, key players in anti-viral immunity, as well as MHC class I molecules, are explained in detail in the following sections.

2.1.1.1 $CD8^+$ T cells

$CD8^+$ T cells, also called cytotoxic T cells, are capable of inducing death in somatic cells infected by a virus or other intracellular pathogens, as well as in tumour cells (Russell and Ley, 2002). The characteristic feature of these T cells is the surface molecule CD8, which binds to MHC class I molecules and stabilises the interaction of the TCR with the MHC molecule. Upon recognition of a specific peptide:MHC class I complex by the TCR, and a co-stimulatory signal provided by the APC, naïve $CD8^+$ T cells differentiate into cytotoxic T cells. Without the co-stimulatory signal, anergy or apoptosis is induced in T cells reactive to peptide:MHC complexes, preventing immune responses to self antigens. If a fully differentiated $CD8^+$ T cell encounters its respective antigen, the TCRs and the associated co-receptors cluster at the site of cell-cell contact, forming an immunological synapse. This also leads to a reorientation of the cytoskeleton, in order to focus the release of effector molecules. There are two classes of effector molecules produced by effector $CD8^+$ T cells: cytokines and cytotoxins. Cytokines are small soluble proteins that can modulate the actions of cells. The major cytokine released by $CD8^+$ T cells is interferon-γ (IFN-γ), which induces a non-cytotoxic antiviral response, but cytokines of the tumour necrosis factor (TNF) family are also secreted by $CD8^+$ T cells. The second set of effector molecules are the cytotoxins, which are synthesised and stored in specialised cytotoxic granules during the activation of a naïve $CD8^+$ T cell (Russell and Ley, 2002). Perforin, one of the cytotoxic proteins, delivers the other contents of

the cytotoxic granules to the membrane of the target cell. Another component of the granules is a family of serine proteases, the granzymes, which trigger apoptosis in the target cell. Granzyme B cleaves and activates caspase-3, which in turn triggers the caspase cascade, leading to the degradation of DNA and apoptosis. The last component of the cytotoxic granules in humans, granulysin, has antimicrobial properties. $CD8^+$ T cells can also induce apoptosis in other cells by the interaction of Fas with Fas ligand. As both transmembrane proteins are expressed on activated lymphocytes, this mechanism is primarily employed to control lymphocyte numbers. The mechanism by which CD8+ T cells induce apoptosis in target cells is highly precise, as only infected target cells are killed while neighbouring healthy cells are spared.

2.1.1.2 T-cell receptor

T cells recognise their respective antigen, presented on MHC, via the TCR expressed on the surface of all T cells. The TCR is a heterodimer, which in the majority of T cells is composed of one α- and one β-chain (Rudolph et al., 2006). Each of the two TCR chains has a C-terminal transmembrane region with a short cytoplasmic tail. The extracellular N-terminal portion consists of a constant (C) and a variable (V) region. Peptide:MHC complexes are recognised via the V region. The whole TCR complex consists of one α and β TCR chain together with the CD3 complex (Clevers et al., 1988). The CD3 complex, which is composed of one γ- and one δ-chain, as well as two ε- and ζ-chains, all with long cytoplasmic tails, is involved in signal transduction. The structure of the TCR is depicted in **Fig. 1**. Each TCR complex is associated with the co-receptor CD4 or CD8 which binds to MHC class I or II, respectively.

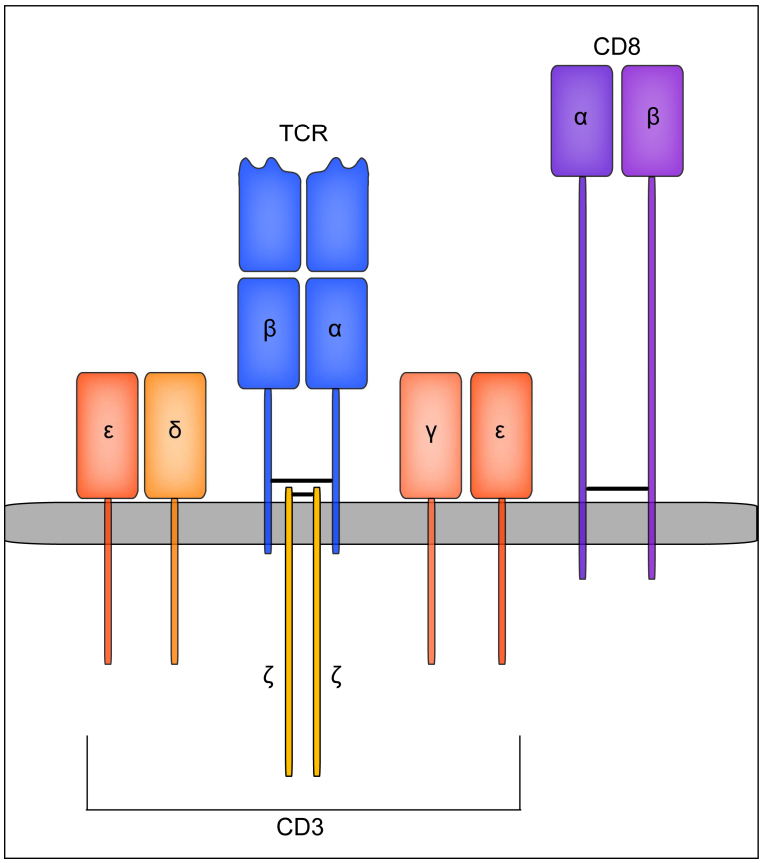

Fig. 1: Schematic model of the T-cell receptor complex.
Depicted is an α:β TCR in blue, which recognises the peptide:MHC class I complex on somatic cells. Signalling occurs via the CD3 complex (in orange), consisting of CD3ε:CD3δ and CD3ε:CD3γ heterodimers and one ζ:ζ homodimer. Further, the α:β heterodimeric CD8 co-receptor is depicted here in purple. The CD8 molecule binds to MHC class I present on most somatic cells. (Scheme according to (Murphy et al., 2008))

The variability of the TCR, and therefore the ability to recognise a broad spectrum of antigens, is generated by somatic recombination – a more or less random joining of gene segments – which takes place during T-cell development in the thymus. The β-chain of the TCR is composed of a variable (V), a diversity (D), a joining (J), and a constant (C) gene segment, whereas the α-chain lacks the D segment (**Fig. 2**). The α-chain locus on chromosome 14 consists of 70-80 Vα, 61 Jα, and 1 Cα gene segments. The β-chain locus, on the other hand, is located on chromosome 7 and encodes for 52 Vβ, 2 Dβ, 13 Jβ, and 2 Cβ gene segments (Arden et al., 1995) (Lefranc and Lafranc, 2001). The rearrangement of the different loci is mediated by two enzymes, the recombination activation genes (RAG) 1 and 2. The sequence of gene rearrangement in α:β T cells starts with the rearrangement of the β-chain locus in CD4 and CD8 double-negative thymocytes. First, Dβ segments rearrange to Jβ segments and subsequently the Vβ segment rearranges to DJβ segments (Krangel, 2009). The

successful rearrangement of one β-chain leads to the expression of this β-chain, together with a surrogate α-chain on the surface of the thymocyte, and gene rearrangement is halted. This ensures the allelic exclusion of the β-chain, followed by a rapid cell proliferation and expression of both TCR co-receptors CD4 and CD8. When the proliferation phase ends, rearrangement of Vα with Jα is induced.

During TCR rearrangement, random nucleotides are added or deleted at the Vβ-Dβ and Dβ-Jβ junctions, as well as at the Vα-Jα junctions, thus generating an extremely variable region, the complementarity determining region 3 (CDR3). This hypervariable region forms the antigen-binding site of the TCR (Rudolph et al., 2006). The deletion or insertion of nucleotides leads to variation in length of approximately 6 to 8 amino acids between different T-cell clones. The CDR1 and 2, which mainly mediate contact to the MHC, are germline encoded within the V gene segments. The combination of different gene segments, the pairing of two TCR chains and the insertion or deletion of random nucleotides leads to a possible total diversity ranging from 10^{15} to 10^{20} TCR molecules (Miles et al., 2011).

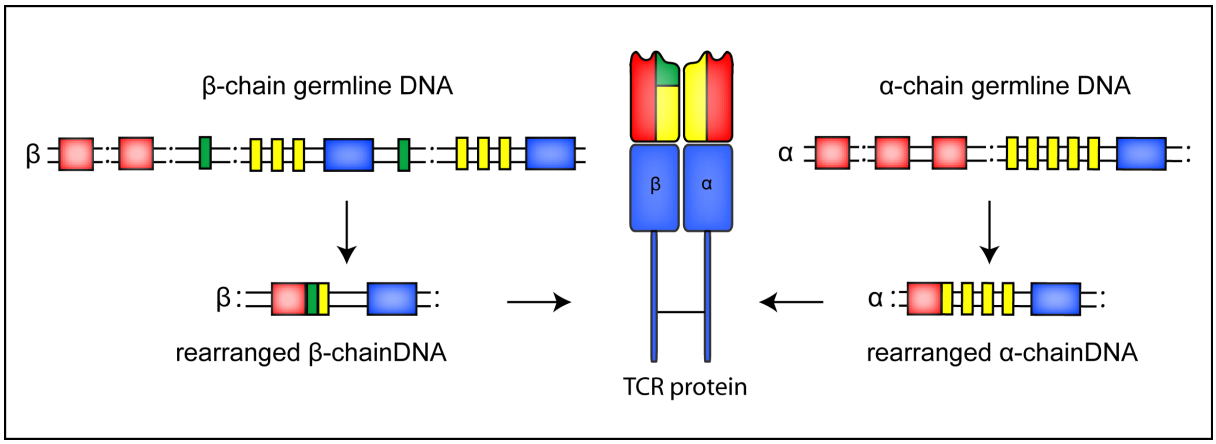

Fig. 2: Somatic recombination of β-chain and α-chain V(D)J segments.
TCR α and β genes are rearranged during T-cell development in the thymus. One of each of the V(D)J gene segments is chosen randomly, and gene segments are subsequently linked by somatic recombination. In the final TCR-molecule, the CDR3 region, composed of the Vβ-Dβ-Jβ and Vα-Jα junctions, forms the antigen-binding cleft. (Scheme according to (Murphy et al., 2008))

Once a functional α:β TCR can be expressed on the cell surface, double-positive thymocytes are selected by their recognition of MHC loaded with self-antigen. Thymocytes reacting too strongly or not at all to self-peptide:MHC complexes are deleted in the thymus. CD8 and CD4 double-positive thymocytes that react with low affinity to MHC class I or MHC class II become CD4+ or CD8+ T cells, respectively, and are released into the bloodstream (Chaplin, 2010).

Recognition of a specific peptide:MHC class I complex by the TCR of a T cell leads to clustering of TCR components, with subsequent phosphorylation by the TCR-associated tyrosine kinases Lck and Fyn. This leads to recruitment of ZAP-70, which in turn induces a signal transduction cascade leading to an increase of the intracellular calcium concentration and finally to the activation of several transcription factors (Lin and Weiss, 2001).

As many viruses have evolved strategies to downregulate MHC class I expression, $CD8^+$ T cells need to be able to react to very low levels of antigens to evoke an effective immune response. As a matter of fact, it was shown that some $CD8^+$ T cells may even become activated by such low numbers as one to three peptide:MHC class I complexes (Sykulev et al., 1996) (Purbhoo et al., 2004).

2.1.2 Major Histocompatibility Complex

The major histocompatibility complex (MHC) is a large cluster of genes, encoding membrane glycoproteins that bind pathogen-derived peptides and display them on the cell surface to T cells. In humans, these genes are encoded on chromosome 6 and are called human leukocyte antigen (HLA). To defend the organism against a vast variety of pathogens, the MHC has to be able to bind a wide range of pathogen-derived peptides. Therefore, the MHC locus is polygenic and both MHC alleles are expressed co-dominantly. Further, the MHC genes are highly polymorphic, meaning there are various different alleles present in the population.

There are two main classes of MHC molecules. MHC class I, which is expressed on almost all somatic cells except the erythrocytes, presents peptides derived from the cytosol to $CD8^+$ T cells. On some somatic cells such as neurons, expression of MHC class I molecules occurs only after induction by an inflammatory milieu (Neumann et al., 1995). $CD4^+$ T cells on the other hand, recognise antigen only in the context of MHC class II, which is expressed solely on immune cells like macrophages, dendritic cells or B cells. Peptides derived from proteins in intracellular vesicles, taken up by endocytosis, are presented on MHC class II.

The MHC further encodes many other proteins involved in antigen processing and the production of peptide:MHC complexes.

2.1.2.1 MHC class I

The MHC class I molecule is a heterodimer consisting of a membrane anchored α-chain and a non-covalently associated β2-microglobulin (Germain, 1994). The α-chain, encoded on chromosome 6 within the MHC locus, forms three domains, the α1, α2, and α3 domains (**Fig. 3**). In humans, genes encoding for the MHC class I α-chain are called HLA-A, -B, and-C. The highest allelic variation occurs within the α-1 and α-2 domains, which form the peptide-binding cleft. The β2-microglobulin, in contrast, is not polymorphic and not encoded within the MHC locus, but on chromosome 15. Binding of the TCR co-receptor CD8 is mediated by the α3 domain, which also binds the β2-microglobulin. The peptide-binding cleft of MHC class I molecules binds peptides typically 8 to 10 amino acids in length via hydrogen bonds and ionic interactions (Rudolph et al., 2006).

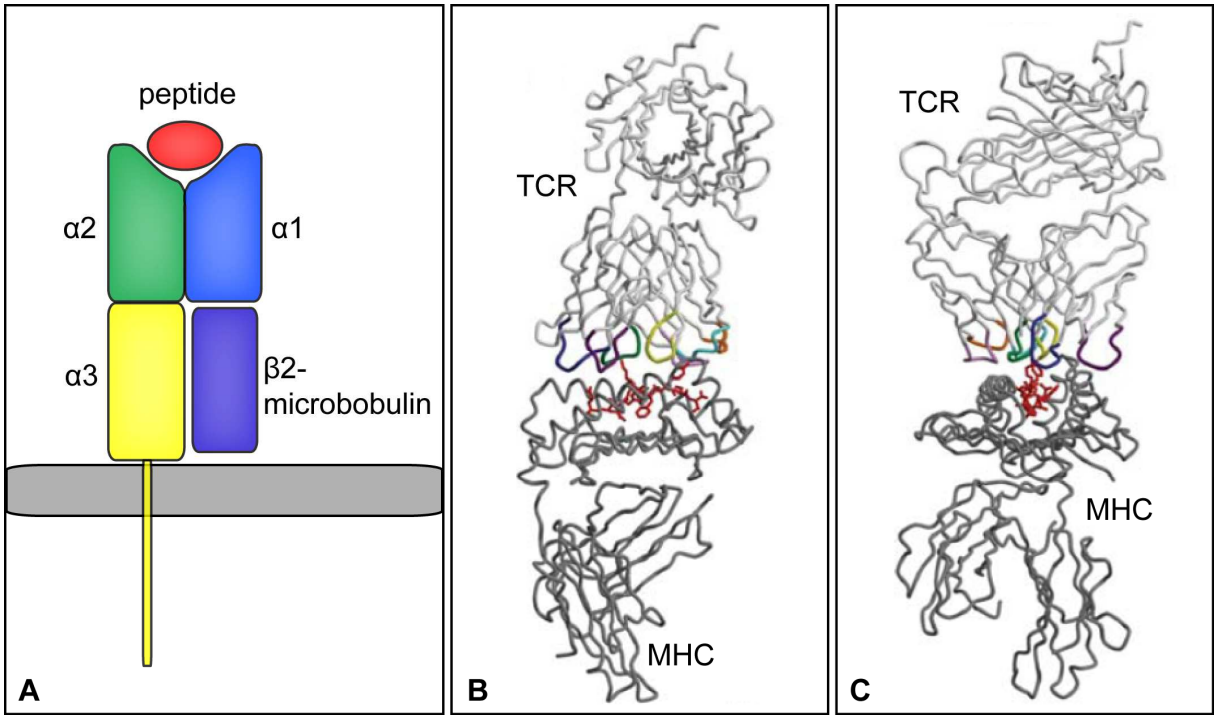

Fig. 3: Structure of the MHC class I molecule and binding of the TCR.
A: The MHC class I heterodimer composed of one α-chain, which folds into three domains (α1, α2, and α3) and the β2-microglobulin. (Scheme according to (Murphy et al., 2008)) B and C: Recognition of the peptide:MHC complex by the TCR. The CDRs of the TCR are binding the MHC molecule as well as the peptide (Rudolph et al., 2006).

Viruses and some other pathogens replicate inside their host cells. Some pathogen-derived proteins are degraded by the proteasome complex and subsequently transported into the lumen of the endoplasmic reticulum (ER) where the MHC class I molecules are synthesised. Transport of cytosolic peptides into the ER lumen is mediated by two heterodimer-forming proteins in the ER membrane, the transporters associated with antigen processing-1 and -2 (TAP1 and TAP2). In the ER lumen, the MHC class I α-chain is associated with chaperones until it finally binds β2-microglobulin and an adequate peptide. The final peptide:MHC class I complex is transported to the cell membrane via the Golgi apparatus (Germain, 1994). Many components of the antigen-presenting pathway are upregulated by IFN-γ, a cytokine produced upon viral infection.

MHC class I molecules can also present exogenous peptides by a mechanism called cross-presentation. This mechanism ensures that $CD8^+$ T cells can respond to pathogens that do not infect APCs. The main types of APCs able to present extracellular proteins on MHC class I are dendritic cells, which are then able to activate naïve $CD8^+$ T cells (Heath and Carbone, 2001).

2.2 Herpes simplex virus type 1

Herpes simplex virus type 1 (HSV-1) is a herpesvirus which frequently infects humans, causing a variety of diseases ranging from relatively harmless cold sores to ocular herpes, the leading cause of blindness caused by infection in industrialised countries. After primary infection, usually occurring via the mucosa of the mouth, HSV-1 establishes latency in sensory neurons, a state that lasts for the life of the host. Viral latency is characterised by retention of a functional viral genome and very limited gene expression without production of infectious virus particles. Due to certain triggers, such as stress, UV light, or immune suppression, the virus can reactivate from the latent state and cause recurrent infections at the original site of infection. As well as cell culture systems, several animal models of infection, including mice and rabbits, are used to study the life cycle of HSV-1.

2.2.1 Taxonomy and Structure

HSV-1 belongs to the family Herpesviridae, a group of large double-stranded (ds) DNA viruses infecting a broad range of animals from molluscs to higher vertebrates (Modrow et al., 2010). Even though infections by different herpesviruses lead to different symptoms, particle morphology and molecular properties are very similar within this virus family. The name herpes is derived from the Greek word “herpein” meaning “to creep” which refers to the creeping spread of HSV-1 skin lesions (Whitley and Roizman, 2001). The characteristic feature of all herpesviruses is the ability to switch between lytic infection and latency.

HSV-1 virions consist of an icosahedral nucleocapsid composed of 162 capsomers that contains the dsDNA genome, a lipid envelope with up to 12 embedded glycoproteins, and the protein-filled tegument in between (**Fig. 4**). The viral glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, gN) play a role in adsorption to and penetration of the cell, as well as in eliciting protective antibodies. Some of the tegument proteins, which enter the host cell along with the virus, play important regulatory functions in inducing viral replication, such as VP16 and vhs (virus host shutoff) (Modrow et al., 2010).

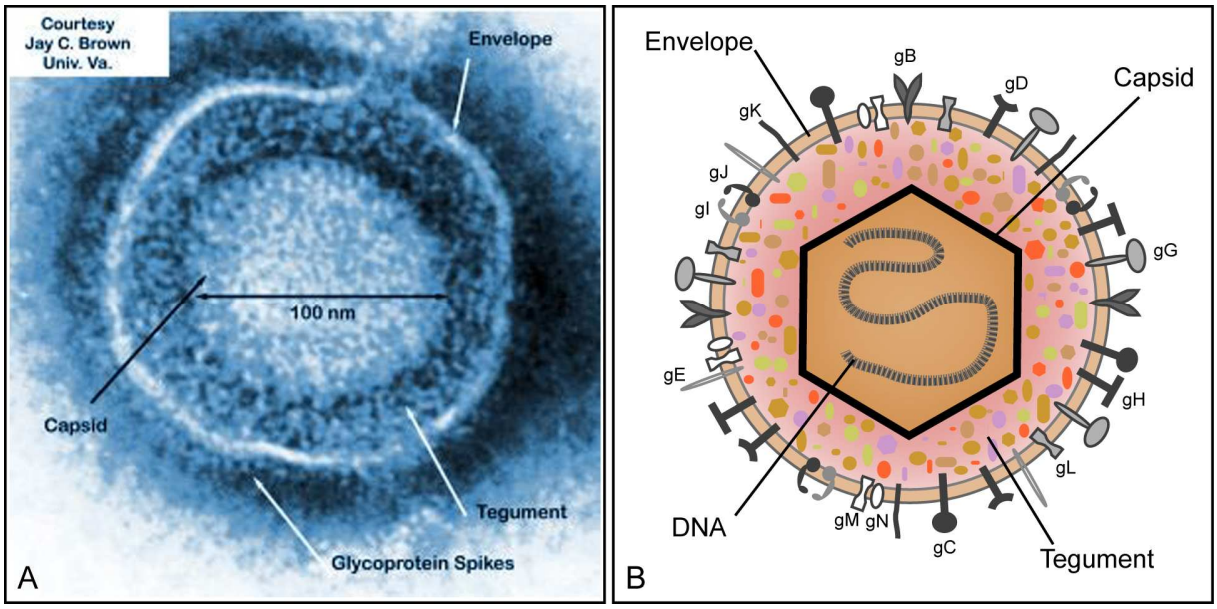

Fig. 4: Structure of the HSV-1 virion.

A: Electron microscopy micrograph of a HSV-1 virion (courtesy of Jay Brown, University of Virginia). B: Schematic structure of the HSV-1 virion, showing the lipid envelope containing glycoproteins, the nucleocapsid and the tegument.

The dsDNA genome of HSV-1 – about 152,000 base pairs in length – is divided into a long and a short segment. The virus genome encodes for about 84 proteins with multiple functions (Whitley and Roizman, 2001), and in total has approximately 89 open reading frames (ORF), some of them partially overlapping (Rajcani et al., 2004). Six distinct regions can be found on the HSV-1 genome (**Fig. 5**). The unique long (U_L) and short (U_S) regions are flanked by two pairs of inverted repeats, the long repeats (R_L / b) and the short repeats (R_S / c). Additionally, at both ends of the linear molecule, there are the relatively short a sequences. They function in circularisation of the DNA upon infection. There are three origins of replication (ori), where the replication of the genome starts. One resides in the middle of the U_L region (ori_L) and the other two are in the R_S (ori_S) (Modrow et al., 2010). Apart from a few exceptions, most HSV-1 transcripts are not spliced (Rajcani et al., 2004). Upon infection the genome circularises and exists as an episome in the nucleus of the host cell.

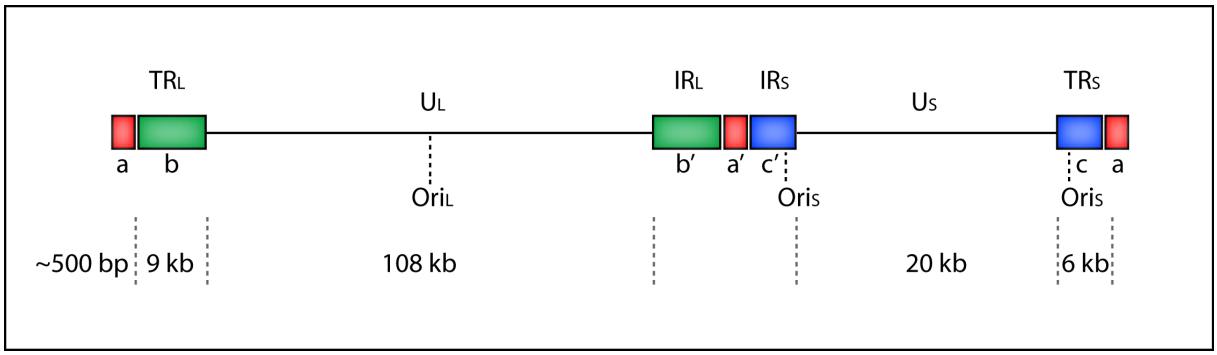

Fig. 5: Scheme of the HSV-1 genome.

The HSV-1 genome consists of one unique long (U_L) and one unique short (U_S) sequence being separated by inverted repeats at the termini (TR_L and TR_S) as well as in the middle (IR_L and IR_S).

Members of the Herpesviridae family can be grouped by their host cell range and their replicative properties (Wagner and Bloom, 1997). Alphaherpesviruses can infect a relatively broad spectrum of host cells, have a short replication cycle, and establish lifelong latency in neurons. Betaherpesviruses, on the other hand, have a narrow host cell range, a slow replication cycle, and establish latency in leukocytes. The third group of herpesviruses, the Gammaherpesviruses, have a narrow host cell range, establish latency in B lymphocytes, and vary in length of the replication cycle. The Gammaherpesviruses can cause cancer by inducing proliferation in the infected cells. **Table 1** lists the eight herpesviruses infecting humans, grouped by subfamily.

Table 1: Human herpesvirus classification of the Herpesviridae Family
(According to the International Committee on Taxonomy of Viruses)

Subfamily	Genus	Species
Alphaherpesvirinae	Simplexvirus	Herpes simplex virus type 1
		Herpes simplex virus type 2
	Varicellovirus	Varicella-zoster virus
Betaherpesvirinae	Cytomegalovirus	Human cytomegalovirus
	Roseolovirus	Human herpesvirus 6
		Human herpesvirus 7
Gammaherpesvirinae	Lymphocryptovirus	Epstein-Barr virus
	Rhadinovirus	Kaposi's-sarcoma-associated herpesvirus

2.2.2 Lytic Infection

HSV-1 infection of epithelial or mucosal cells results in lytic infection of the host cell followed by production of infectious virus. During HSV-1 lytic infection, viral genes are expressed in a highly regulated cascade. Lytic infection usually results in the release of infectious virus particles and death of the host cell.

2.2.2.1 Entry into the host cell

Infection of a host cell begins with the attachment of HSV-1 to host cell receptors via glycoproteins in its lipid envelope (Whitley and Roizman, 2001). Attachment is mediated by binding of gC to heparan sulphate proteoglycan on the host cell, followed by binding of gD to a modified heparan sulphate or HVEM (herpesvirus entry mediator). Fusion of the host cell membrane with the virus membrane requires formation of a complex of gB with gH/gL (Eisenberg et al., 2011), after which the capsid, along with tegument proteins, gets released into the host cytoplasm. HSV-1 can also enter host cells via receptor-mediated endocytosis or cell to cell transmission (Modrow et al., 2010). The capsid and some tegument proteins are then transported via microtubules to nuclear pores, where viral DNA is released into the nucleus. Other tegument proteins, such as vhs, appear to remain in the cytoplasm. Vhs causes disaggregation of polyribosomes and degradation of cellular RNA thereby priming the cell to virus production (Wagner and Bloom, 1997). Upon entry into the nucleus, viral DNA is associated with histones and therefore silenced (Roizman, 2011).

2.2.2.2 Viral Replication

Viral genes are transcribed in a sequential manner. First the immediate early or α-genes are transcribed, which in turn activates transcription of early or β-genes. Finally, after viral DNA synthesis, the late or γ-genes are transcribed. The replication cycle of HSV-1 is very short, as mature virions are formed within 8 hours after infection in some cell culture systems (Wagner and Bloom, 1997). A scheme of the lytic cycle of HSV-1 infection is depicted in **Fig. 6**.

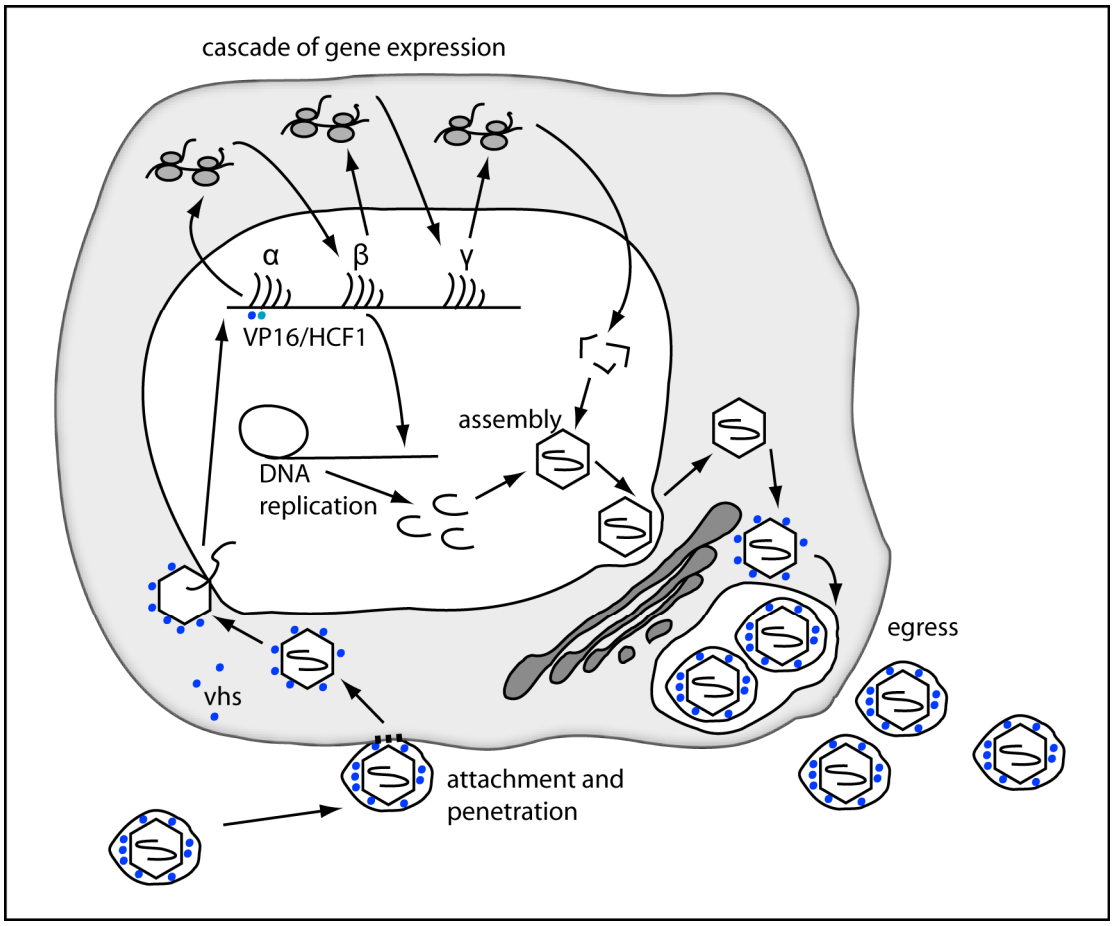

Fig. 6: Lytic life cycle of HSV-1.

Primary lytic infection of epithelial or mucosal cells starts with attachment and penetration of HSV-1. The capsid is then transported to the nuclear membrane and the linear dsDNA is injected into the nucleus. Subsequently, the genome circularises and the cascade of viral gene expression is induced by the binding of VP16 together with HCF1 to α-gene promoters. Assembled virions bud through the nuclear membranes, are enveloped at the trans-Golgi network and released from the infected cell.

Transcription of α-genes

Binding of the α-gene promoter by the tegument protein VP16, together with two cellular factors Oct1 (octamer binding protein 1) and HCF1 (host cell factor 1), leads to recruitment of a histone demethylase, which demethylates histones present on viral DNA and thereby initiates transcription of α-genes (Roizman, 2011). The five α-genes named infected cell protein (ICP) ICP0, ICP4, ICP22, ICP27, and ICP 47 are transcribed first (Wagner and Bloom, 1997). The capped and polyadenylated α-mRNAs are transported into the cytoplasm, where they are translated into proteins that are in turn re-imported into the nucleus, where they promote transcription of early genes. ICP0, ICP4, and ICP27 are potent transactivators of viral early and late gene transcription, whereas ICP22 promotes transcription of viral late genes (Wagner and Bloom, 1997). ICP47 functions in export of viral mRNA from the nucleus (Modrow et al.,

2010). Apart from activating RNA polymerase II, ICP4 can also downregulate expression of the latency-associated transcript (LAT) and all α-genes, including itself (Deluca, 2011). ICP0 promotes viral early transcription by binding to a repressor complex (HDAC/CoREST/LSD1/REST) which then gets detached from the viral genome (Roizman, 2011). Another function of ICP0 is mediated by its E3 ubiquitin ligase activity which marks proteins for proteasomal degradation. ICP0 was found to induce degradation of ND10 nuclear bodies, which are dynamic structures in the nucleus consisting of a large number of proteins with functions in stress response, transcriptional control and innate immune response (Everett, 2011).

Transcription of β-genes

Presence of viral α-proteins in the nucleus leads to expression of β-genes, which all encode for non-structural proteins involved in the replication of viral DNA. Seven β-proteins need to be present in the nucleus to ensure viral DNA replication: the viral DNA polymerase (UL30), the helicase/primase complex (UL5, UL52, UL8), an ori binding protein (UL9) and two DNA binding proteins (UL42 and UL29) (Ward and Weller, 2011). Another important β-protein is the viral thymidine kinase, which catalyses the phosphorylation of thymidine. After accumulation of sufficient levels of β-proteins in the nucleus, synthesis of multiple copies of viral DNA by the viral DNA polymerase is initiated. This also leads to a shutdown of IE and E gene expression (Wagner and Bloom, 1997). DNA replication takes place in replication compartments in the nucleus, forming at previous ND10 sites which were dissociated by the E3 ligase activity of ICP0 (Ward and Weller, 2011). Replication of viral DNA starts at the oris and occurs by the rolling circle principle, leading to viral DNA containing multiple units of the viral genome in concatemers.

Transcription of γ-genes

The HSV-1 γ-genes, which are mainly transcribed after DNA replication, encode for 30 structural or core proteins. These genes are subdivided into γ1-genes, expressed in small amounts before viral DNA replication, and γ2-genes that are absolutely dependent on DNA replication for expression (Wagner and Bloom, 1997). After transcription, the mRNAs are exported form the nucleus to the cytoplasm where they are translated into protein. These proteins are then transported back into the nucleus, where assembly of the virus capsid and subsequent packaging of viral DNA takes place.

2.2.2.3 Nuclear egress and envelopment

During lytic infection, the nuclear membrane is modified by the insertion of herpes glycoproteins. HSV-1 capsids, enclosing the viral DNA, bud through the inner lamella of the nuclear membrane, thus acquiring the initial envelope. This envelope then fuses with the outer nuclear membrane, and the bare capsids produced are then released into the cytoplasm (Baines and Roberts, 2011). At the trans-Golgi network, viral capsids associate with tegument proteins including VP16 and vhs, and are finally released at the plasma membrane, with an envelope formed by trans-Golgi vesicles. Nucleocapsids associated with tegument can also spread to other cells by syncytium formation (Modrow et al., 2010).

2.2.2.4 Lytic cycle proteins and inhibition of host-defence

Several viral proteins expressed during lytic infection have functions in inhibiting host-defence. Of the HSV-1 mechanisms known to inhibit anti-viral response, the functions of ICP47 are best known. This protein has been demonstrated to bind to the cytoplasmic domain of TAP (Hill et al., 1995), which is positioned within the ER membrane. Binding of ICP47 stops TAP from transporting peptides to the endoplasmic reticulum, where they would be associated with MHC class I and presented to $CD8^+$ T cells on the cell surface.

ICP0 has also been shown to have an effect on host-defence, via its degradation of ND10 nuclear bodies. These distinct nuclear structures are built up of several proteins, with functions in chromatin modification, DNA damage responses, stress responses, regulation of gene expression, and innate immune responses that are upregulated by IFN-γ (Everett, 2011). Therefore, HSV-1 ICP0 renders the cell unsusceptible to IFN-γ. The role of ICP0 as an IFN-γ antagonist is further emphasised by its ability to work against STAT (Signal Transducer and Activator of Transcription) or IFN-γ receptor-dependent repression of the virus (Halford et al., 2006).

An intrinsic mechanism to protect cells against viral infection is the detection of dsRNA by host protein kinase R (PKR). Activated PKR can in turn induce the IFN-dependent immune response, apoptosis, and activation of the central translation initiation factor eIF-2α, which subsequently blocks all protein synthesis. Viral ICP34.5 can prevent this translational block (Whitley and Roizman, 2001). Another HSV-1 protein counteracting PKR is Us11. It binds to dsRNA, which therefore cannot be recognised by PKR (Modrow et al., 2010). The cellular intrinsic defence mechanisms are further impaired by the HSV-1 vhs-induced destruction of

cellular mRNA, inhibition of DNA transcription, and blocking of mRNA splicing (Whitley and Roizman, 2001).

Immune evasion mechanisms are also mediated by some of the viral glycoproteins (Modrow et al., 2010). For example, gC can bind to the complement component C3b. Furthermore, gE and gI bind to the Fc region of antibodies, thereby preventing the binding of the Fc-domain of antibodies by immune effector cells. These two glycoproteins also mediate cell-to-cell spread of HSV-1 without release of infectious virus, whereby the virus can avoid encounters with neutralising antibodies.

Eliminating infected cells by apoptosis is a crucial mechanism in limiting viral spread. HSV-1 has evolved several mechanisms to block programmed cell death. For instance, the α-genes ICP4, ICP22, and ICP27 are anti-apoptotic proteins (Cotter and Blaho, 2011). Furthermore, U_S3, U_S11, U_L14, gJ, and gD are believed to inhibit apoptosis caused by cell injury (Cotter and Blaho, 2011).

2.2.3 Latency

A primary infection with HSV-1, which usually occurs at the oral mucosa, results in lytic infection with lysis of the epithelial or mucosal cells, and subsequent release of infectious virus. Free HSV-1, released from productively infected cells, can then enter sensory nerve fibres innervating the site of inoculation. The virus then travels via retrograde transport to the cell body of the neurons, which are located in the trigeminal ganglia (TG). There, HSV-1 establishes lifelong latency (Baringer and Swoveland, 1973), which is characterised by the existence of a functional viral genome without the production of infectious virus (**Fig. 7**). During latency only minimal expression of viral genes occurs. The only transcript abundantly expressed is the latency-associated transcript (LAT), but recently minimal expression of other viral transcripts has also been described in latently infected human (Derfuss et al., 2007) and mouse TG (Kramer and Coen, 1995) (Chen et al., 1997) (Feldman et al., 2002) (Chen et al., 2002a). Latency is divided into three steps. First, during establishment of latency, viral DNA circularises upon entry into the nucleus and is silenced by association with histones. Second, throughout the maintenance of latency only minimal expression of viral genes occurs. The third step is spontaneous reactivation from latency, when new infectious virions are formed (**Fig. 8**).

2.2.3.1 Establishment of latency

In human HSV-1 infection, events taking place after primary infection cannot be studied. However, studies in the mouse model reveal that entry of the virus into the TG is followed by a short phase of increasing virus titres, during which infectious virus can be recovered from the TG (Liu et al., 1996). It is not yet resolved whether HSV-1 is transferred from neuron to neuron, or if multiple neurons are infected at the periphery. As no local sensory loss induced by neuronal death can be observed, it could be speculated that neurons either do not die upon viral replication or that viral replication does not take place. Despite this, an infiltration of immune cells into the primary infected TG occurs. The initial immune infiltration in the TG is composed of cells of the innate immune system (Shimeld et al., 1995) (Liu et al., 1996), which later changes, from day seven post infection onwards, to mostly $CD8^+$ T cells of the adaptive immune response (Liu et al., 1996). The appearance of $CD8^+$ T cells is concurrent with elimination of viral replication (Simmons and Tscharke, 1992). Establishment of viral latency is not only promoted by the immune system, however, as HSV-1 was found to establish latency in the TG of mice deficient in an innate and adaptive immune system (Ellison et

al., 2000). Upon entering the nucleus of its host cell, viral DNA immediately circularises and is associated with histones (Efstathiou et al., 1986) (Deshmane and Fraser, 1989). HCF1, which is needed for induction of viral α-gene expression by VP16, is present in the cytoplasm of unstressed neurons, unlike in non-neuronal cells where it is located in the nucleus. Without the VP16/HCF1/Oct1 complex, the viral genome remains silenced, as associated histones as well as the HDAC/CoREST/LSD1/REST repressor complex, are not removed (Roizman, 2011) (**Fig.7**). Active regulation by the virus itself does not seem to be a prerequisite for establishing latency, as no viral gene product was shown to be absolutely required (Wagner and Bloom, 1997). Thus, latent infection seems to be the consequence of a failure to enter the lytic cascade.

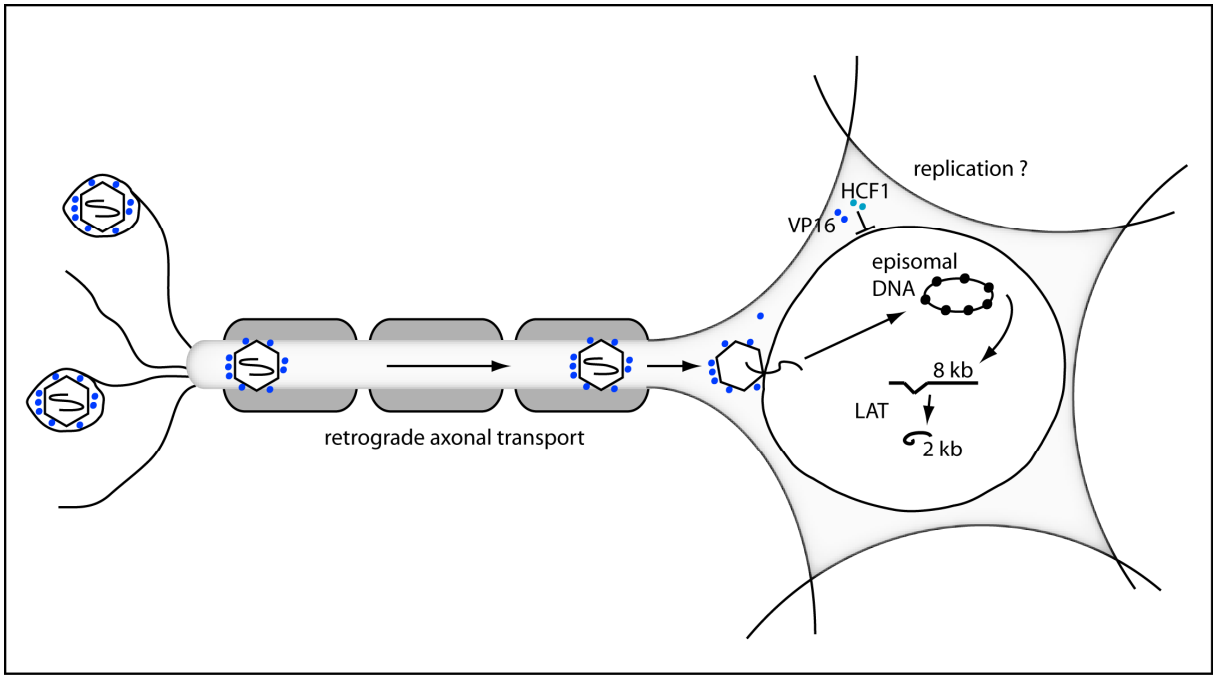

Fig. 7: Establishment of HSV-1 latency.

HSV-1 enters axonal termini at the site of the primary infection. Viral capsids are transported in a retrograde manner to the nerve cell body. Upon injection of viral DNA into the nucleus, the DNA circularises, and associates with chromatin. During latency the viral genome persists as an episome. As HCF1 is not present in the nucleus of unstressed neurons, the VP16/HCF1 complex cannot initiate α-gene expression and thereby viral lytic gene expression is silenced. During latency only LAT is expressed.

2.2.3.2 Maintenance of latency

In latently HSV-1-infected sensory neurons, the only abundantly expressed viral gene products are the LATs (Stevens et al., 1987) (Croen et al., 1987). The primary 8.5-kb polyadenylated transcript is spliced into a 2-kb LAT stable intron, which accumulates in neuronal nuclei (Wagner and Bloom, 1997). It is still a matter of debate whether functional peptides or proteins are derived from this transcript (Henderson et al., 2009). As LAT is the only easily detectable transcript in latently infected neurons, many functions in establishment and maintenance of latency, as well as in reactivation, have been attributed to it. Studies with LAT$^-$ mutants in animal models of infection suggest a minor role of LAT in latency. It was shown that LAT is not essential, because most LAT$^-$ mutants do establish latency and can reactivate (Perng et al., 1994) (Chen et al., 1997). Furthermore, in mice, not all sensory neurons harbouring HSV-1 DNA express LAT to detectable levels (Mehta et al., 1995) (Chen et al., 2002b). Infection of mice with LAT$^-$ mutants did however result in decreased numbers of latently infected TG neurons (Thompson and Sawtell, 1997) and a decreased reactivation rate in explant cultures of mouse TG (Carr et al., 1998). The importance of LAT in promoting spontaneous reactivation seems to vary between species, as the effect of LAT$^-$ mutants is more prominent in rabbits (Perng et al., 1994) (Hill et al., 1990) than in mice (Margolis et al., 2007). Spontaneous reactivation of HSV-1 from latency also seems to vary between species as it is a much more common event in humans and rabbits than in mice. Therefore, the effect of LAT on the latency-reactivation cycle might be underestimated in small animal models with a shorter life expectancy, and therefore a shorter latency period, than humans.

One mechanism by which LAT could be maintaining latency might be via the transcriptional control of ICP0 (**Fig. 8**), as LAT expression is associated with an increase in unspliced ICP0 transcripts (Chen et al., 2002a) (Maillet et al., 2006). ICP0 has to be present to ensure complete reactivation from latency, with production of infectious virus (Halford and Schaffer, 2001) (Thompson and Sawtell, 2006). For some of the recently described 16 HSV-1 microRNAs, an antagonistic effect on viral α-gene products has also been suggested. microRNAs are short RNA molecules of about 22 nucleotides in length, capable of hampering protein production by binding to complementary mRNA. Most of the HSV-1 microRNAs are encoded within the primary LAT transcript, or in genomic regions close to LAT (Cui et al., 2006) (Umbach et al., 2008) (Umbach et al., 2009) (Jurak et al., 2010), and are differentially expressed in productive versus latent infection in mice and humans (Umbach et al., 2008) (Umbach et al., 2009) (Jurak et al., 2010). Umbach *et al*, 2008 showed that microRNA H2-3p and microRNA H6 are able to reduce ICP0 and ICP4 expression, respectively, *in vitro*.

Conversely, no significant effect on establishment and maintenance of latent infections was observed in mice infected with HSV-1 mutants deficient in the LAT-derived microRNAs and microRNA H6 (Kramer et al., 2011). It therefore remains to be resolved, whether viral microRNAs and LAT might be more important in humans. With sRNA1 and sRNA2, two more small RNAs have been mapped to the LAT region. Both were shown to function in inhibiting productive infection and apoptosis in mice (Perng and Jones, 2010).

During latency HSV-1 DNA is in a heterochromatic state with histone markers typical of a repressed chromatin structure (Knipe and Cliffe, 2008). This enhanced assembly of heterochromatin, especially in promoters of lytic genes, was shown to correlate with the presence of LAT (Wang et al., 2005b).

LAT was further shown to inhibit apoptosis, thereby promoting survival of latently infected neurons (Perng et al., 2000) (Hamza et al., 2007). The anti-apoptotic effects of LAT are mediated by the inhibition of caspase 8- and caspase 9-induced apoptosis (Henderson et al., 2002) as well as inhibition of caspase 3 activation by Granzyme B released from $CD8^+$ T cells (Jiang et al., 2011).

2.2.3.3 Immunological control of latency

Immunosuppression is commonly accepted as a trigger for reactivation and severe HSV infection in humans (Montgomerie et al., 1969) (Naraqi et al., 1977), thereby implying a role of the immune system in control of HSV-1. In fact, a persisting immune cell infiltration, together with anti-viral cytokines and chemokines like IFN-γ and TNF-α, were described in latently HSV-1-infected sensory ganglia in mice (Shimeld et al., 1995) (Cantin et al., 1995) (Halford et al., 1996) and also in humans (Theil et al., 2003a). Immune infiltrates in latently HSV-1-infected human TG have been identified to consist primarily of $CD3^+$ T cells, as well as some $CD68^+$ macrophages. The majority of infiltrating immune cells are effector memory $CD8^+$ T cells, showing markers of recent activation by antigens (Derfuss et al., 2007) (Verjans et al., 2007). Very few $CD4^+$ T cells can be found in the human TG at all, usually spread only among the axons (Theil et al., 2003a). In HSV-1-uninfected TG, only scattered $CD3^+$ T cells are present. A strong correlation between infiltrating T cells and latent infection of the TG by HSV-1, but not varicella-zoster virus (VZV) or human herpesvirus 6 (HHV-6), was demonstrated (Theil et al., 2003a) (Hüfner et al., 2006) (Hüfner et al., 2007).

In the mouse model of latent HSV-1 infection, expression of Granzyme B is considered as a marker for HSV-1-specificity, along with ongoing activation by chronic stimulation (van Lint et al., 2005). In humans, some of the $CD8^+$ T cells present in the TG also express

Granzyme B. However, a substantial number of TG infiltrating $CD8^+$ T cells that show markers of activation do not co-express Granzyme B (Derfuss et al., 2007) (Verjans et al., 2007). Therefore, some T cells might represent unspecific bystander T cells, which were attracted by the inflammatory milieu and entered the TG only because of their activation status. Entry of activated but unspecific T cells into the TG has also been described in mice (van Lint et al., 2005).

The T cells present in human TG were shown to have clonally expanded TCR β-chains (Derfuss et al., 2007) and TCRγ-loci (Verjans et al., 2007), pointing towards an antigen-driven proliferation of these T cells. Moreover, cultured T-cell lines derived from HSV-1 latently infected TG, were reactive against HSV-1 proteins (Verjans et al., 2007).

The TG infiltrating T cells in humans are mainly found to be clustered around neuronal cell bodies (Derfuss et al., 2007). A peculiarity of these T cells is that most of them do not directly surround LAT^+ neurons. This was also found in mice, where spontaneously reactivated neurons expressing viral proteins were surrounded by T cells, but most LAT^+ neurons were found to be free of associated inflammatory cells (Feldman et al., 2002).

The presence of activated $CD8^+$ T cells in the neuronal tissue raises the question of whether MHC class I is expressed by infected neurons. Neurons themselves could present antigen on MHC class I to T cells, as Neumann *et al*., 1995 showed inducible expression of MHC class I on neurons by IFN. On the other hand, neuron supporting satellite cells could also play a role in antigen presentation (van Velzen et al., 2009). Furthermore, HSV-1 lytic infection in mouse TG was described as inducing MHC class I expression on neurons and satellite cells (Pereira et al., 1994).

The functions of these T cells in HSV-1 latency have mostly been studied in the mouse model of infection. TG infiltrating $CD8^+$ T cells were shown to prevent HSV-1 from reactivating in mouse TG *ex vivo* cultures in a dose-dependent, antigen-specific and MHC-restricted manner (Liu et al., 2000) (Khanna et al., 2003). It was shown that this is mediated by IFN-γ (Liu et al., 2000) and Granzyme B (Knickelbein et al., 2008). The vast majority of TG infiltrating $CD8^+$ T cells in latently infected mice with a C57BL/6 genetic background were specific for HSV-1. About 50% of the local $CD8^+$ T cells recognise the immuno-dominant epitope $gB_{(498\text{-}505)}$ (Khanna et al., 2003). The other T cells mostly recognise subdominant epitopes belonging to early or late gene products expressed before viral DNA synthesis (Sheridan et al., 2009) (St Leger et al., 2011). It still has to be resolved which antigen triggers the $CD8^+$ T-cell infiltration in latently HSV-1-infected human TG.

As destruction of neurons is seen very rarely in mice (Decman et al., 2005) (Esaki et al., 2010) and never in humans (Theil et al., 2003a), infiltrating $CD8^+$ T cells apparently do not release their full cytotoxic capacity. Secretion of anti-viral cytokines, like IFN-γ or TNF-α, appears to be the major mode of action of local $CD8^+$ T cells. IFN-γ was demonstrated to suppress viral replication immediately after reactivation (Cantin et al., 1999) as well as at late stages (Decman et al., 2005). This was partly mediated by the inhibition of ICP0 expression (Decman et al., 2005). The release of cytolytic granules by $CD8^+$ T cells present in latently infected TG was, however, also observed. Granzyme B, which normally initiates apoptosis by cleavage of caspase 3, was shown to degrade ICP4, a viral α-protein required for efficient viral gene expression (Knickelbein et al., 2008) (**Fig. 8**).

2.2.3.4 Reactivation from latency

The most common trigger for reactivation of latent HSV-1 is stress. It has been shown that psychological stress, either by disruption of the social hierarchy within mouse colonies (Padgett et al., 1998), or by restraint (Bonneau, 1996) (Freeman et al., 2007), induced reactivation in mice by reducing the number and functionality of HSV-1-specific $CD8^+$ T cells. In humans, immunosuppression caused by stress also led to increased reactivation of HSV (Sainz et al., 2001).

Glucocorticoids not only reduce T-cell numbers in the TG of mice latently infected with HSV-1 (Himmelein et al., 2011), but can also regulate gene expression and cause changes in the chromatin status (Adcock, 2000). This could lead to activation of viral gene expression. An influence of the neuronal excitation status on HSV-1 viral replication has also been demonstrated (Zhang et al., 2005). Increased neuronal excitability inhibited viral replication, whereas decreased activity of neurons enhanced viral replication. In addition, inhibition of histone deacetylases, leading to an increase in transcription, was shown to enhance reactivation of HSV-1 from latency (Roizman, 2011). These observations suggest that reactivation of HSV-1 might be triggered by signals leading to an increase in transcriptional activity of the host neuron (Wagner and Bloom, 1997). The molecular mechanism might be that in stressed neurons, HCF1 is translocated into the nucleus (**Fig. 8**). If transcriptional activity is also increased within this neuron, VP16 *de novo* synthesis might occur (Thompson et al., 2009), leading to activation of α-promoters. Subsequently, α-proteins like ICP0 or ICP4 can act as transactivators of viral gene expression, leading to complete reactivation. At this stage LAT-derived microRNAs or the infiltrating $CD8^+$ T cells could still repress viral gene expression by acting on the transactivating α-transcripts or -proteins. It has been proposed that gene

expression during reactivation, at least in explanted mouse TG, is not as ordered as in lytic infection (Tal-Singer et al., 1997) (Du et al., 2011). Presumably, as no sensory loss is associated with repeated reactivation, no neuronal death occurs during the release of virus. Infectious virions are transported in an anterograde manner to the initial site of infection, where HSV-1 infects epithelial or mucosal cells and enters the lytic phase. Apparent lesions are less frequent than short asymptomatic shedding of the virus (Mark et al., 2008).

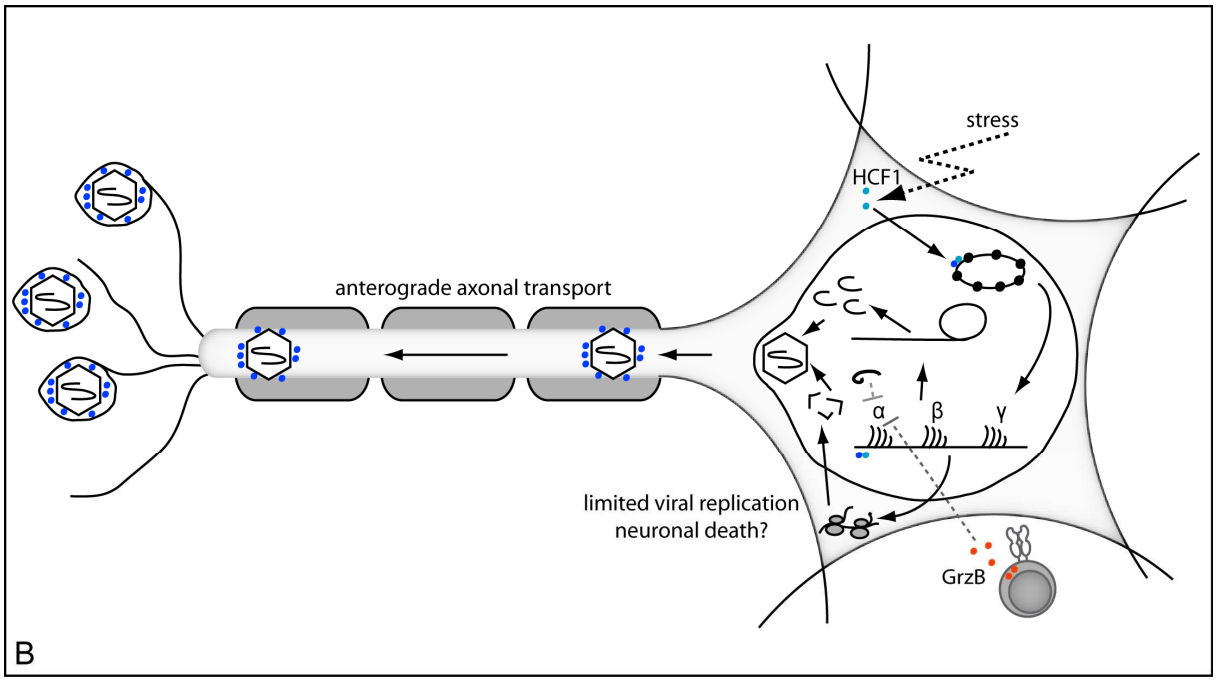

Fig. 8: Control of HSV-1 latency and reactivation.

Control of viral latency is believed to be mediated by LAT, infiltrating $CD8^+$ T cells, and host cell factors. In stressed neurons HCF1 is translocated into the nucleus and, together with *de novo* synthesised VP16, can activate α-gene expression. LAT and $CD8^+$ T cells act to repress further viral gene expression, but if these control mechanisms are overrun, limited viral replication takes place. Resulting viral particles are subsequently transported by anterograde axonal transport to the neuron termini. This process probably does not result in lysis of the nerve cell.

2.3 Objectives

The basic question underlying this study is that of the molecular mechanisms that trigger the immune response to latent HSV-1 in human TG, an infection with limited viral protein production in a site with minimal immune control. This will be addressed from the T cell and the neuronal perspective in HSV-1's natural site of latency, the human TG. The long term aim of our work is to understand how HSV-1 latency is maintained and which molecular triggers lead to reactivation.

The first aim of this study is to examine the impact of lifelong chronic HSV-1 infection on the TCR repertoire of TG-infiltrating T cells. Previous studies have demonstrated that the TG infiltrating $CD8^+$ T cells show markers of recent activation by antigens and are clonally expanded. To detect expanded T cell clones, CDR3 spectratyping was applied to TG of latently HSV-1-infected individuals. Detection of pervasive T-cell clones with structurally homologous or even identical T cell receptors within the TG infiltrating T cells would indicate that these T cells share reactivity to similar antigenic epitopes.

The second aim is to investigate what mechanisms lead to the puzzling observation made in HSV-1 latency that most neurons expressing LAT – the only prominent transcript during latency – are free of directly associated T cells, and that most T cells are clustered around LAT^- neurons. It has been described in mice that some neurons contained viral DNA but do not accumulate LAT to detectable levels. A recent study also showed that LAT encodes several viral microRNAs, some of which were shown to downregulate expression of the viral α-proteins ICP0 and ICP4 *in vitro*. These α-proteins are required for efficient reactivation with *de novo* production of virions. Therefore we hypothesised that neurons devoid of LAT contain viral DNA, and that due to the resulting lack of post-transcriptional control by viral microRNAs, HSV-1 α-proteins might be expressed. This potential expression of viral proteins would explain why the infiltrating T cells are clustered around some neurons and not others. This will be addressed in the current study by combining LAT *in situ* hybridisation and immunohistochemistry for T cells with subsequent single cell analysis of laser microdissected neurons for the presence of viral DNA, microRNA, and mRNA.

3 Results

This cumulative thesis consists of two accepted publications. In the following results section, these publications are included and the contribution of the author to the respective publications is indicated. All publications of the author, including those which are not included in this thesis, are listed separately (list of publications; chapter 7).

3.1 Clonal expansions of $CD8^+$ T cells in latently HSV-1-infected human trigeminal ganglia

Held K, Eiglmeier I, Himmelein S, Sinicina I, Brandt T, Theil D, Dornmair K, Derfuss T (2011) Clonal expansions of CD8+ T cells in latently HSV-1-infected human trigeminal ganglia. J Neurovirol., DOI 10.1007/s13365-011-0067-9

The author of this doctoral thesis contributed to Held *et al*. (2011b) by performing CDR3 spectratyping as well as all immunohistochemical stainings, by carrying out data analysis and by writing major parts of the manuscript.

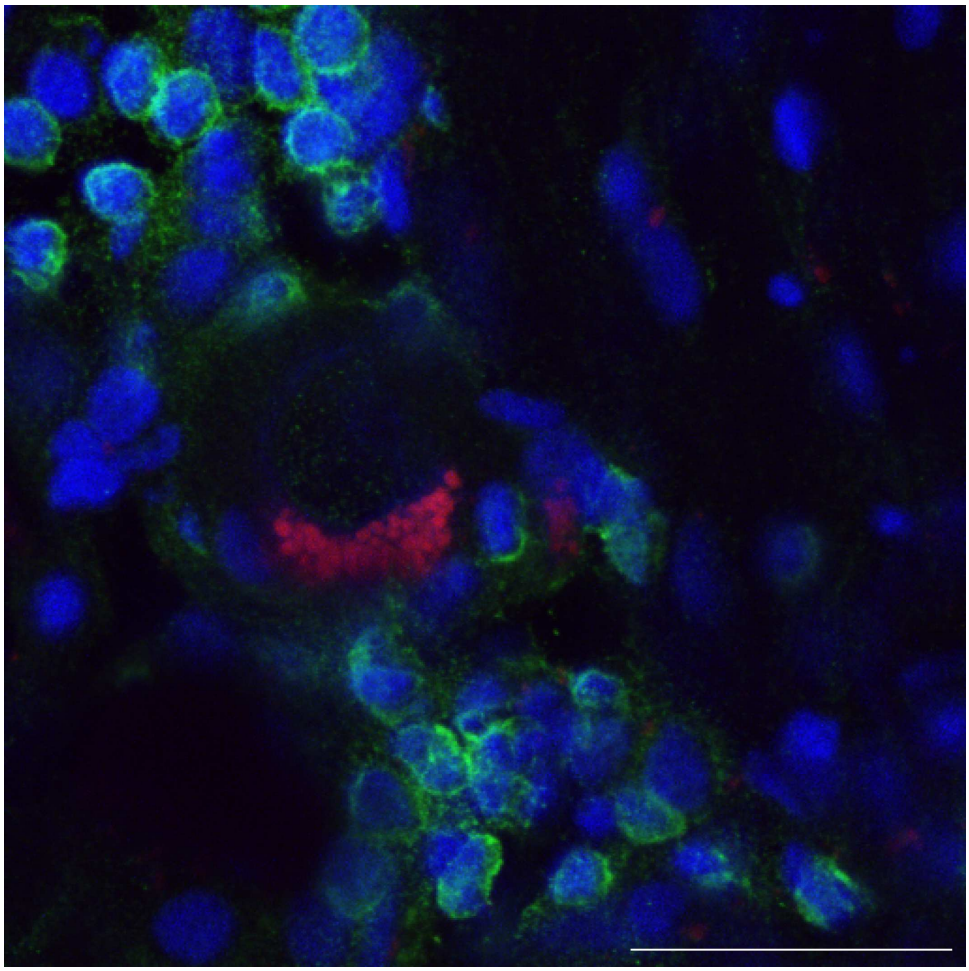

Fig. 9: T cells surrounding a neuron.
Anti-CD3 immunohistochemistry (green) on a 10 µm frozen TG section. The red signal originates from lipofuscin. Scale bar, 50µm.

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DOI 10.1007/s13365-011-0067-9

SHORT COMMUNICATION

Clonal expansions of CD8$^+$ T cells in latently HSV-1-infected human trigeminal ganglia

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Abstract Herpes simplex virus type 1 latency in trigeminal ganglia (TG) is accompanied by a chronic immune cell infiltration. The aim of this study was to analyse the T-cell receptor β-chain repertoire in latently HSV-1 infected human TG. Using complementarity-determining region 3 spectratyping, 74 expanded β-chain sequences were identified in five TG. No clone appeared in more than one subject. Similar clones were present in the right and the left TG of two subjects. This indicates that these T cells are primed in the periphery and recognise the same antigen in the TG of both sides.

Keywords HSV-1 · Latency · CD8$^+$ T cells · Human · Trigeminal ganglia

Introduction

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus, which, after productive infection of the mucosa, enters the local nerve endings and establishes lifelong latency in the sensory neurons of the trigeminal ganglia (TG) (Baringer and Swoveland 1973). The only prominent viral transcript during latency is the latency-associated transcript (LAT) (Stevens et al. 1987). Other viral transcripts are only expressed on a very low level during latency in human TG (Derfuss et al. 2007). Nevertheless, HSV-1 latency in human TG is accompanied by a prominent immune response (Theil et al. 2003). Most of the infiltrating immune cells are CD8$^+$ T cells which are key players in the control of viral infections.

Electronic supplementary material The online version of this article (doi:10.1007/s13365-011-0067-9) contains supplementary material, which is available to authorized users.

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These $CD8^+$ T cells are believed to control the latency state of HSV-1 in an antigen-specific, T-cell receptor (TCR)-mediated and non-cytolytic manner as shown in the mouse model (Knickelbein et al. 2008). In mice, most TG infiltrating $CD8^+$ T cells are specific for HSV-1 with about 50% recognising a specific epitope on HSV-1 glycoprotein B (Khanna et al. 2003; St Leger et al. 2011). The specificity of T cells infiltrating human TG remains to be elucidated.

The TCR is a heterodimeric cell surface protein, consisting of one α and one β chain. Each of these chains is composed of a variable (V), a joining (J) and a constant (C) region. The β chain further contains a diversity (D) region in between the V and the J region. During rearrangement of the TCR α and β chains, random nucleotides are inserted or deleted by the nucleotide transferase at the V-(D)-J junctions, thereby generating a hypervariable region, termed complementarity-determining region 3 (CDR3). Recognition of antigenic peptides bound to major histocompatibility complex (MHC) molecules is mediated by three CDRs. Two CDRs are germline-encoded. The main contribution to recognition of the antigenic peptide, however, comes from the hypervariable, non-germline-encoded CDR3 loop. Because random nucleotides are inserted at the CDR3, CDR3 lengths of different T-cell clones may vary from zero to more than ten amino acids. CDR3 lengths of polyclonal T-cell populations follow a Gaussian distribution, whereas the outgrowth of particular clones is indicated by a preferred CDR3 length. CDR3 spectratyping is a PCR-based method that measures the length distributions of the β chains. It allows the analysis of TCR repertoire diversity and the identification of prominent clones (Pannetier et al. 1995). Here, we applied CDR3 spectratyping of TCR β chains to T-cell infiltrates in several human TG to analyse the local T-cell repertoire and to search for public T-cell clones, shared between individuals.

Results

Prominent T-cell infiltrates in latently HSV-1 infected human TG

To verify the correlation between HSV-1 latency and the infiltration of T cells into the TG, ganglia sections were stained for CD3 and the HSV-1 or VZV infection state was determined by nested PCR. Immunohistochemical staining of the human TG sections revealed higher numbers of infiltrating T cells in TG positive for HSV-1 by nested LAT RT-PCR. The two LAT negative cases showed significantly lower numbers of T cells in immunohistochemistry (Fig. 1a, b). On average, the number of $CD3^+$ T cells in latently HSV-1 infected TG was 39.54 cells per 0.1 mm^2, whereas uninfected TG only contained 11.64 cells per 0.1 mm^2 (Fig. 1c and Table 1). There was no statistically significant difference in the T-cell counts between VZV infected vs. uninfected TG ($p>0.05$ Mann–Whitney U test) (Fig. 1a–c).

Clonally expanded T-cell populations in latently infected human TG

TG of four subjects were analysed by CDR3 spectratyping (subjects 01 to 04). For two individuals, TG of both sides were assessed (subjects 01 and 03). These TG had well-preserved RNA, were positive for LAT and showed T-cell infiltrates. An HSV-1 non-infected TG from subject 04 was used as a control sample.

CDR3 spectratyping was carried out for each TG. We used 26 Vβ-specific primers combined with 13 Jβ-specific primers, which resulted in 338 PCR products per TG. PCR products were separated on a polyacrylamide gel to obtain the overall distribution of CDR3 lengths. Mono- or oligoclonal expansions are indicated by single peaks over a polyclonal Gaussian background (Fig. S1 in the Electronic supplementary material). CDR3 spectratyping revealed 22 single peaks from the right TG and 26 peaks from the left TG of subject 01. In the one TG analysed from subject 02, 20 peaks were found. In subject 03, 22 peaks from the right TG and 18 peaks from the left TG were identified. From the negative control, subject 04, 14 peaks were obtained. PCR products from all obtained peaks were sequenced. Only if readable sequences were acquired, clones were considered as expanded.

In all TG, several clonally expanded TCR β chains were detected. In total, we identified 74 β-chain sequences of clonal expansions in the latently infected ganglia and 10 expanded β chains in the negative control (Table S1 in the Electronic supplementary material). In subject 01, 34 out of 338 possible Vβ/Jβ combinations showed clonal expansions. For the other subjects 02 and 03, 15 and 25 out of 338 possible Vβ/Jβ combinations were clonally expanded. Table 2 shows identical or homologous amino acid sequences of TCR β chains that were found in the left and the right TG of subjects 01 and 03. The clones with homologous CDR3 sequences which were identified in both ganglia belonged to identical Vβ families. The CDR 1 and 2 regions are germline-encoded within the V gene segments of the β chain and mainly mediate contact to the MHC. TCRs featuring the same Vβ segment already share two of three CDRs and are therefore more homologous than TCRs with different Vβ segments. The CDR3, which is most relevant for peptide binding, is either identical or homologous in the clonally expanded TCRs listed in Table 2. The amino acids at positions 3 to 5 after the conserved cystein are considered to play a major role in recognition of the

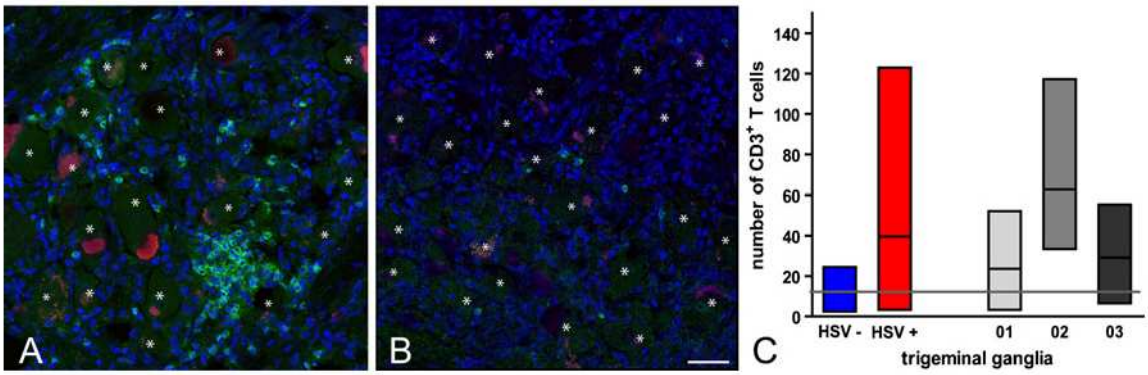

Fig. 1 Prominent T-cell infiltrates in latently HSV-1 infected human TG. **a**, **b** Representative micrographs of latently HSV-1 infected (**a**) and uninfected (**b**) human TG sections stained for CD3 (*green*). *Scale bar* 50 μm. The *red signal* is autofluorescence of lipofuscin. Neurons are indicated by *asterisks*. **c** Numbers of $CD3^+$ T cells per 0.1 mm^2 in non-infected (*blue*; *n*=2) vs. HSV-1 infected (*red*; *n*=5) TG. *Grey bars* show T-cell counts for those TG used for CDR3 spectratyping (01 to 03). *Bars* depict the range and mean of the T-cell numbers. The *grey line* indicates the mean T-cell count of HSV-1 negative TG

antigen presented by MHC class I (Rudolph et al. 2006). Therefore, TCRs with common motifs in these amino acids recognise similar antigens. Interestingly, in subject 03 for each of the two identical clones occurring in both TG, homologous clones sharing the same Vβ segment and amino acids with similar properties in their CDR3 were identified.

An inter-individual comparison of the TCR repertoires obtained from TG showed that no clone appeared in more than one subject. Hence, all clones were private to each subject. This difference between individuals reflects the heterogeneous HLA background, although subjects shared some HLA class I alleles (Table 1). However, in each of the two individuals where both TG were analysed, two matching clonally expanded TCR β chains were found on each side. These TCRs not only displayed identical amino acid sequences, but also identical nucleotide sequences. The absence of clones shared by other analysed subjects excludes the possibility that the identical clones in both TG were detected due to contaminations.

Localisation of clonally expanded TCRs in human TG

CDR3 spectratyping of RNA isolated from TG analyses all TCR molecules with no differentiation of $CD8^+$ or $CD4^+$ T cells. However, in latently HSV-1 infected TG $CD8^+$ T cells dominate the immune cell infiltrate (Theil et al. 2003). To morphologically identify expanded T-cell clones, sections of TG showing clonal expansions of T cells within the Vβ1 family were double-stained for Vβ1 and CD8. Clones featuring the Vβ1 chain were present quite frequently in the TG of subjects 02 and 03. Among the $CD8^+$ T-cell infiltrates surrounding neurons, several cells expressing the Vβ1 chain could be identified, whilst not all T cells surrounding one neuron expressed the same Vβ chain. Figure 2 shows representative micrographs of subjects 02 and 03 with clusters of $CD8^+$ T cells. Some of these T cells also express the Vβ1 chain. The vast majority of cells that stained positive for Vβ1 also stained positive for CD8. Moreover, all identified $V\beta1^+$ T cells were located adjacent to neurons.

Table 1 Overview of tissue samples used in the present study

Subject	Gender	Age	HSV-1	VZV	$CD3^+$T cells	HLA-A	HLA-B	HLA-C
01	m	56	+	–	23.6	0101	0801	0304
							1501	0701
02	m	41	+	+	62.7	0101	0801	0602
						0301	3701	0701
03	m	78	+	+	29.2	2402	0801	0701
						3001	4006	1502
04	m	62	–	+	12.5	nd.	nd.	nd.
05	m	36	–	+	10.7	nd.	nd.	nd.
06	f	17	+	+	76.9	nd.	nd.	nd.
07	f	61	+	–	24.0	nd.	nd.	nd.

The HSV-1 and VZV infection state plus the average T-cell count per 0.1 mm^2 are listed. The HLA class I alleles expressed by the three HSV-1 infected subjects used for CDR3 spectratyping are also stated

nd not done, *m* male, *f* female

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Table 2 Similar and identical β chains in the right and left TG of subjects 01 and 03

Subject	side	Vβ	NDN	Jβ	FU
01	right	1	C A S T L T G G A G Y N E Q F F G P G	2.1	198
01	right	1	C A S S V A V N T D T Q Y F G P G	2.3	261
01	left	1	C A S S V G G P N Q P Q H F G D G	1.5	185
01	left	11	C A S S L S R T G V N Y G Y T F G S G	1.2	723
01	right	11	C A S S L S R T G V N Y G Y T F G S G	1.2	540
01	right	13.2	C A S S P S Q G G H Q P Q H F G D G	1.5	239
01	left	13.2	C A S T W S G R S Y G Y T F G S G	1.2	250
01	right	23	C A S S L R Q S Y E Q Y F G P G	2.7	303
01	left	23	C A S S L R Q S Y E Q Y F G P G	2.7	953
03	left	11	C A S S E W V S G S E Q Y F G P G	2.7	798
03	right	11	C A S S E W V S G S E Q Y F G P G	2.7	1138
03	left	11	C A S S E Y W G T G T G E L F F G E G	2.2	191
03	right	17	C A S S P D R A G G Y T F G S G	1.2	326
03	left	17	C A S S P D R A G G Y T F G S G	1.2	466
03	right	17	C A S S P G H L Y E Q Y F G P G	2.7	744

The amino acid sequence of the CDR3 is listed. Peak height is given in fluorescent units (FUs). CDR3 sharing amino acids with similar properties in positions 3 to 5 after the conserved cysteine are surrounded by *dashed lines* (i.e. subject 01 Vβ1 position 3: T and S are both amino acids with polar, neutral side chains, sharing similar hydropathy and L and V in position 2 both possess hydrophobic non-polar, neutral side chains). TCRs identical in their amino acid structure are surrounded by *double-lined boxes*

Discussion

We show here, that latently HSV-1 infected human TG show increased numbers of infiltrating T cells compared to HSV-1 uninfected TG. CDR3 spectratyping of the TCR β chain revealed several clonally expanded T-cell clones with certain β chains. These expansions were private to the assessed individuals, and no public T-cell responses could be identified, even though some HLA alleles were shared between individuals. However, it might be possible that particular TCR chains from expanded clones that were detected in one patient were present in other patients, but escaped detection because they were hidden in the polyclonal background. For several infections or autoimmune diseases as well as tumours (Dong et al. 2010; Junker et al. 2007; Schwab et al. 2009; Skulina et al. 2004; Pellkofer et al. 2009; Puisieux et al. 1994; Miles et al. 2011) biases in the T-cell receptor repertoire were identified. Some of these studies showed several T-cell clones appearing in different anatomical sites. A former study examining the TCR usage in HSV-2-specific $CD8^+$ T cells derived from blood showed a strong bias in the TCR repertoire with public TCR usage (Dong et al. 2010). Such pervasive clonal expansions are usually interpreted as being driven by sustained activation through a persisting antigen. One could speculate that lifelong exposure to HSV-1 antigens may shape the T-cell receptor repertoire in human TG. Infiltrating T cells in latently HSV-1-infected TG do not seem to exhibit public TCR usage. However, clones that had homologous or even identical amino acid sequences could be identified in both TG of two individuals. T cells with homologous TCRs are likely to respond to similar antigens. In subject 01 as well as subject 03, two clones with identical amino acid sequences were detected in both TG. These were even identical in their nucleotide sequence, which may suggest that these clones originated from the same T cell. This T cell probably encountered its respective antigen in the periphery, proliferated and afterwards migrated into both TG. HSV-1-specific T cells present

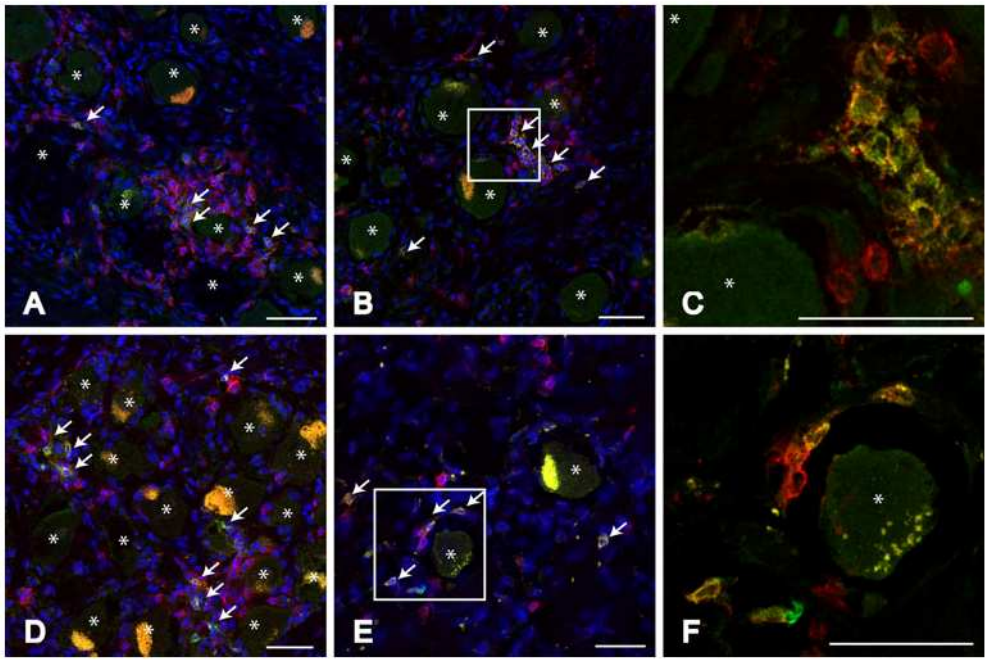

Fig. 2 Clusters of expanded T cells in human TG. Representative micrographs of human TG sections stained for CD8 (*red*) and Vβ1 (*green*) from S02 (**a**, **b**, **c**) and S03 (**d**, **e**, **f**). C and F are enlargements of **b** and **e**, respectively. $CD8^+$ $V\beta1^+$ T cells are indicated by *white arrows* in micrographs **a**, **b**, **d** and **e**. *Scale bars* 50 μm. The *yellow signal* within the neurons originates from lipofuscin. Neurons are indicated by *asterisks*

in human TG could be produced in the periphery during primary infection, may be also a reactivation event, and subsequently migrated to the latently infected tissue.

Staining for expanded Vβ chains revealed that the expanded T cells mostly belong to the $CD8^+$ subset, and that only some T cells in one cluster surrounding a neuron share the same Vβ chain. The presence of different clones in T-cell clusters around neurons could indicate that not all T cells react to the same antigen. Therefore, the TCR repertoire present in human TG seems to be more heterogeneous than in mice. This further suggests that some of the infiltrating T cells might represent unspecific bystander T cells, which were attracted by the inflammatory milieu, like already proposed by Verjans et al. in 2007. This phenomenon has also been seen in the mouse model (van Lint et al. 2005) where both specific and non-specific T cells persist in ganglia harbouring latent HSV-1.

Taken together, our data show that the TCR repertoire of infiltrating T cells in human TG is complex and differs between individuals. However, single identical T-cell clones can be found in both TG of the same individual, indicating presence of the same antigen in both TG. The fact that neuron surrounding T cells are clonally expanded adds further evidence to the significance of these immune cells in latency and reactivation.

Material and methods

The Ethics Committee of the Medical Faculty of the Ludwig Maximilian University of Munich approved the use of human TG autopsy samples. TG of both sides were removed from seven subjects 6 to 24 h after death. None of the subjects had any history of neurological disorders or an active orolabial herpes infection. Table 1 lists gender, age, HSV-1 infection state, T-cell counts and HLA-type of the TG used in this study. Ganglia were embedded directly after removal in Jung Tissue freezing medium (Leica Microsystems, Nussloch, Germany) and were stored at −80°C. Frozen sections of 10 μm were cut for immunohistochemistry and mounted on positively charged slides (Superfrost Plus, Menzel, Braunschweig, Germany). Slides were subsequently stored at −20°C. RNA and DNA were isolated from ten 30-μm sections.

RNA extraction and HSV-1/VZV PCRs

RNA was extracted using Qiazol (Qiagen, Hilden, Germany) and the miRNeasy Mini Kit (Qiagen). The quality of the isolated RNA was analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) combined with the Agilent RNA 6000p Kit (Agilent Technologies). Subsequently, DNA was extracted from the organic phase. After addition of back extraction buffer and phase separation by centrifugation, DNA present in the aqueous phase was precipitated by adding isopropanol. The DNA pellet was then washed three times with 75% ethanol and solubilised with water.

To assess the HSV-1 infection state of the TG, a nested LAT RT-PCR was done as described before (Derfuss et al. 2007). Furthermore, the VZV infection state was specified by nested PCR for the ORF 63 (Outer: 5′-CGCACTGGAATGTGACGTAT, 3′-TCCCCGTCTCGATAACAATC; inner: 5′-TGAAGACGATAGCGACGATG, 3′-CCCGTCTGGTTCACAAGAAT).

Immunohistochemistry

Immunohistochemistry was performed for CD3, CD8 and Vβ1. CD3 was detected using the polyclonal rabbit anti-human CD3 antibody (1:500; Dako Cytomation, Glostrup, Denmark) and visualised with a biotinylated goat anti-rabbit immunoglobulin antibody (1:100; Dako Cytomation), followed by Cy2-labelled streptavidin (1:100; Dianova, Hamburg, Germany) or HRP-conjugated streptavidin (1:100; Dako Cytomation) and incubation in DAB (Dako Cytomation). A dual staining for CD8 and Vβ1 was conducted using the LT8 mouse anti-human CD8α antibody (1:50; AbD Serotec, Düsseldorf, Germany) labelled with Cy3 (FluoroLinkTM MAb Cy3 labelling kit, Amersham Biosciences, Buckinghamshire, England) and the FITC-labelled Vβ1 BL37-2 antibody (1:25; Immunotech, Marseille, France). The Vβ1 signal was enhanced with an anti-FITC Alexa Fluor 488 labelled secondary antibody (1:100; Invitrogen, Karlsruhe, Germany). Antibodies to other Vβ families prominently expanded in the assessed TG were either not available or did not give reliable staining results. Stained sections were analysed by confocal imaging.

To obtain numbers of infiltrating $CD3^+$ T cells, five randomly selected fields of view (0.123 mm^2) were counted with an objective of ×20. Data were statistically analysed using Microsoft Excel 2003 and GraphPad Prism 5.

CDR3 spectratyping

The CDR3 lengths of TCR β chains were analysed by CDR3 spectratyping as described before (Junker et al. 2007; Schwab et al. 2009). In brief, cDNA was reverse-transcribed using a Cβ-specific primer and SuperScript II reverse transcriptase (Seitz et al. 2006). For increased sensitivity, a semi-nested TCR Vβ gene family-specific PCR with 25 Vβ-specific (Monteiro et al. 1996) and two different Cβ-specific primers (SpTy-β-out (Junker et al. 2007) and Cβ-reverse (Monteiro et al. 1996)) was carried out followed by a subsequent runoff reaction for every product using fluorescence-labelled Jβ-specific primers (Puisieux et al. 1994). The obtained PCR products were separated by electrophoresis according to their CDR3 length on an ABI377 DNA sequencer (Applied Biosystems, Darmstadt, Germany). Mono- or oligoclonal expansions appear as peaks of certain CDR3 length over a Gaussian background of polyclonal T cells (Fig. S1 in the Electronic supplementary material). Peak height is measured in fluorescent units (FU), the relative signal intensity. Higher numbers indicate more prominent clonal expansions. Peaks over 180 FU were considered as an actual aberration from the polyclonal background. Finally, Vβ-Jβ amplification products of expanded clones were reamplified and directly sequenced. Only clones for which we obtained a readable sequence were considered as clonally expanded.

The Vβ nomenclature described in Arden et al. (1995) is used throughout this manuscript. The subjects chosen for spectratyping were HLA typed by the Labor für Immungenetik und Molekulare Diagnostik, Munich, Germany.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Arden B, Clark SP, Kabelitz D, Mak TW (1995) Human T-cell receptor variable gene segment families. Immunogenetics 42:455–500

Baringer JR, Swoveland P (1973) Recovery of herpes-simplex virus from human trigeminal ganglions. N Engl J Med 288:648–650

Derfuss T, Segerer S, Herberger S, Sinicina I, Hufner K, Ebelt K, Knaus HG, Steiner I, Meinl E, Dornmair K, Arbusow V, Strupp M, Brandt T, Theil D (2007) Presence of HSV-1 immediate early genes and clonally expanded T-cells with a memory effector phenotype in human trigeminal ganglia. Brain Pathol 17:389–398

Dong L, Li P, Oenema T, McClurkan CL, Koelle DM (2010) Public TCR use by herpes simplex virus-2-specific human CD8 CTLs. J Immunol 184:3063–3071

Junker A, Ivanidze J, Malotka J, Eiglmeier I, Lassmann H, Wekerle H, Meinl E, Hohlfeld R, Dornmair K (2007) Multiple sclerosis: T-cell receptor expression in distinct brain regions. Brain 130:2789–2799

Khanna KM, Bonneau RH, Kinchington PR, Hendricks RL (2003) Herpes simplex virus-specific memory CD8+ T cells are selectively activated and retained in latently infected sensory ganglia. Immunity 18:593–603

Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL (2008) Noncytotoxic lytic granule-mediated CD8 + T cell inhibition of HSV-1 reactivation from neuronal latency. Science 322:268–271

Miles JJ, Douek DC, Price DA (2011) Bias in the alphabeta T-cell repertoire: implications for disease pathogenesis and vaccination. Immunol Cell Biol 89:375–387

Monteiro J, Hingorani R, Peroglizzi R, Apatoff B, Gregersen PK (1996) Oligoclonality of CD8+ T cells in multiple sclerosis. Autoimmunity 23:127–138

Pannetier C, Even J, Kourilsky P (1995) T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today 16:176–181

Pellkofer HL, Voltz R, Goebels N, Hohlfeld R, Dornmair K (2009) Cross-reactive T-cell receptors in tumor and paraneoplastic target tissue. Arch Neurol 66:655–658

Puisieux I, Even J, Pannetier C, Jotereau F, Favrot M, Kourilsky P (1994) Oligoclonality of tumor-infiltrating lymphocytes from human melanomas. J Immunol 153:2807–2818

Rudolph MG, Stanfield RL, Wilson IA (2006) How TCRs bind MHCs, peptides, and coreceptors. Annu Rev Immunol 24:419–466

Schwab N, Bien ChG, Waschbisch A, Becker A, Vince GH, Dornmair K, Wiendl H (2009) CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. Brain 132:1236–1246

Seitz S, Schneider CK, Malotka J, Nong X, Engel AG, Wekerle H, Hohlfeld R, Dornmair K (2006) Reconstitution of paired T cell

receptor alpha- and beta-chains from microdissected single cells of human inflammatory tissues. Proc Natl Acad Sci U S A 103:12057–12062

Skulina C, Schmidt S, Dornmair K, Babbe H, Roers A, Rajewsky K, Wekerle H, Hohlfeld R, Goebels N (2004) Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. Proc Natl Acad Sci U S A 101:2428–2433

St Leger AJ, Peters B, Sidney J, Sette A, Hendricks RL (2011) Defining the herpes simplex virus-specific CD8+ T cell repertoire in C57BL/6 mice. J Immunol 186:3927–3933

Stevens JG, Wagner EK, vi-Rao GB, Cook ML, Feldman LT (1987) RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science 235:1056–1059

Theil D, Derfuss T, Paripovic I, Herberger S, Meinl E, Schueler O, Strupp M, Arbusow V, Brandt T (2003) Latent herpesvirus infection in human trigeminal ganglia causes chronic immune response. Am J Pathol 163:2179–2184

van Lint AL, Kleinert L, Clarke SR, Stock A, Heath WR, Carbone FR (2005) Latent infection with herpes simplex virus is associated with ongoing CD8+ T-cell stimulation by parenchymal cells within sensory ganglia. J Virol 79:14843–14851

Verjans GM, Hintzen RQ, van Dun JM, Poot A, Milikan JC, Laman JD, Langerak AW, Kinchington PR, Osterhaus AD (2007) Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. Proc Natl Acad Sci U S A 104:3496–3501

Supplementary material

Table S1 All expanded β-chains obtained from the TG used in this study.

Amino acid sequences of CDR3 are listed. Peak height is given in fluorescent units (FU). Subjects 01 to 03 tested positive for HSV-1. Subject 04 testing negative for HSV-1 served as negative control.

Subject	side	Vβ	NDN	Jβ	FU
AC01	R	1 C A S	T L T G G A G	Y N E Q F F G P G	2.1	198
AC01	R	1 C A S S V	A V N	T D T Q Y F G P G	2.3	261
AC01	L	1 C A S S V	G G P	N Q P Q H F G D G	1.5	185
AC01	L	2 C S A R	A P G R H	E Q Y F G P G	2.7	184
AC01	L	3 C A S	R A G T G D	N E K L F F G S G	1.4	195
AC01	R	3 C A S S	F P G T S	S T D T Q Y F G P G	2.3	1801
AC01	L	5.1 C A S S L	G E G G	S Y E Q Y F G P G	2.7	544
AC01	L	5.2 C A S S	D R G Q	S P L H F G N G	1.6	368
AC01	L	5.2 C A S S	P Q R G	E K L F F G S G	1.4	310
AC01	L	5.2 C A S S L	A R G L R A S	T D T Q Y F G P G	2.3	745
AC01	L	5.2 C A S S L	G D	S N Q P Q H F G D G	1.5	542
AC01	L	11 C A S	R T A G	N T G E L F F G E G	2.2	220
AC01	L	11 C A S S	L S R T G V	N Y G Y T F G S G	1.2	723
AC01	R	11 C A S S	L S R T G V	N Y G Y T F G S G	1.2	540
AC01	L	11 C A S S E	Y P	Y E Q Y F G P G	2.7	568
AC01	R	13.1 C A S	F P P G G V	S Y N E Q F G X G	2.1	230
AC01	R	13.1 C A S	R N G G	N Q P Q H F G D G	1.5	213
AC01	L	13.1 C A S	S L G E G	N E Q F F G P G	2.1	266
AC01	R	13.2 C A S	S P S Q G G H	Q P Q H F G D G	1.5	239
AC01	L	13.2 C A S	T W S G R S	Y G Y T F G S G	1.2	250
AC01	L	14 C A S	R P L G Q G S	Q P Q H F G D G	1.5	210
AC01	L	14 C A S S	I S G G L D	Y E Q Y F G P G	2.7	485
AC01	R	14 C A S S	L L Q G	N Y G Y T F G S G	1.2	1046
AC01	R	17 C A S S	A P G Q S	Y E Q Y F G P G	2.7	1469
AC01	L	17 C A S S	S T G G D P	S Y E Q Y F G P G	2.7	675
AC01	L	18 C A S S	P T G D G	E Q Y F G P G	2.7	450
AC01	R	20 C A W S	G P V S V G T V	T D T Q Y F G P G	2.3	312
AC01	R	21 C A S S	L G T G D	T D T Q Y F G P G	2.3	1888
AC01	L	22 C A S S	D M G P	G Y T F G S G	1.2	520
AC01	L	22 C A S S	G P S E I	E Q F F G P G	2.1	350
AC01	R	23 C A S S	L R Q	S Y E Q Y F G P G	2.7	303
AC01	L	23 C A S S	L R Q	S Y E Q Y F G P G	2.7	953
AC01	R	23 C A S S	L W T E D	T E A F F G Q G	1.1	420
AC01	R	23 C A S S	N P L A G V	T D T Q Y F G P G	2.3	453
AC02	R	1 C A S S V	G G G	E Q Y F G P G	2.7	650
AC02	R	2 C S A	I P I T G P H	E Q Y F G P G	2.7	560
AC02	R	2 C S A	P Q G A	E Q F F G X G	2.1	540

AC02	R	3	C A S	T Y L D R P	Y E Q Y F G P G	2.7	800
AC02	R	3	C A S S	T Y P L A E V P T T	Y N E Q F F G P G	2.1	260
AC02	R	5.1	C A S S	F R A G T S R S T	N E Q F F G P G	2.1	330
AC02	R	5.1	C A S S L	S P P	N T E A F F G Q G	1.1	160
AC02	R	5.2	C A S S L	V Q G D	T G E L F F G E G	2.2	240
AC02	R	11	C A S S E	S T G S L A	P Q H F G D G	1.5	425
AC02	R	13.1	C A	S G T G Y	Y G Y T F G S G	1.2	500
AC02	R	13.1	C A S S Y	D G	S T D T Q Y F G P G	2.3	630
AC02	R	14	C A S S	D R T G S	E Q F F G P G	2.1	260
AC02	R	14	C A S S	S S G T A	Y G Y T F G S G	1.2	210
AC02	R	22	C A S S E	M T A	Y E Q Y F G P G	2.7	880
AC02	R	22	C A S S S	Y I E P T G	N S P L H F G N G	1.6	2930
AC03	L	1	C A S S	A Q G T G K P R	E Q Y F G P G	2.7	587
AC03	L	1	C A S S	P P G Q G T L G A	E A F F G Q G	1.1	250
AC03	R	3	C A S S	H R G G	I E Q Y F G P G	2.7	286
AC03	R	5.1	C A S S	R D	F Y N E Q F F G X G	2.1	256
AC03	L	8	C A S	R N D R E	E Q Y F G P G	2.7	1582
AC03	L	11	C A S S E	W V S G S	E Q Y F G P G	2.7	798
AC03	R	11	C A S S E	W V S G S	E Q Y F G P G	2.7	1138
AC03	L	11	C A S S E	Y W G T G	T G E L F F G E G	2.2	191
AC03	L	13.2	C A S S	N R A A G E Y	T Q Y F G P G	2.3	941
AC03	R	13.2	C A S S	P P P G	D T Q Y F G P G	2.3	1673
AC03	L	13.2	C A S S	R T R Q F A	N T G E L F F G E G	2.2	323
AC03	R	14	C A S S	I P	N T G E L F F G E G	2.2	304
AC03	L	14	C A S S	Q G D S S	N E Q F F G P G	2.1	843
AC03	R	14	C A S S	S T G F Y	Y E Q Y F G P G	2.7	729
AC03	R	17	C A S S	P D R A G	G Y T F G S G	1.2	326
AC03	L	17	C A S S	P D R A G	G Y T F G S G	1.2	466
AC03	R	17	C A S S	P G H L	Y E Q Y F G P G	2.7	744
AC03	L	18	C A S S P	W E R T G	S Y E Q Y F G P G	2.7	558
AC03	R	20	C A	S T L Q P	Y N E Q F F G P G	2.1	207
AC03	R	20	C A W	N P G I G	Q P Q H F G D G	1.5	220
AC03	L	20	C A W S	P G W T S G	T Q Y F G P G	2.3	1359
AC03	R	20	C A W S	P N R A D	Y G Y T F G S G	1.2	371
AC03	L	20	C A W S V	Q	N Y G Y T F G S G	1.2	594
AC03	L	21	C A S S L	G Q G A S	S Y N E Q F F G P G	2.1	271
AC03	L	22	C A S S E	S G K R I S	G Y T F G S G	1.2	270
AC04	L	1	C A S	V R A T	S T D T Q Y F G P G	2.3	630
AC04	L	5.1	C A S S	P G Q G G	N Q P Q H F G D G	1.5	320
AC04	L	8	C A S S	I K T G R G	G K L F F G S G	1.4	240
AC04	L	8	C A S S	P P S A G S L G V	E A F F G Q G	1.1	180
AC04	L	11	C A S S	G Q G W G	T E A F F G Q G	1.1	560
AC04	L	13.1	C A S S	V S S G M N	T D T Q Y F G P G	2.3	460
AC04	L	14	C A S S	F G T G E	G Y T F G S G	1.2	600
AC04	L	14	C A S S	G G T G G	N Q P Q H F G D G	1.5	480
AC04	L	20	C A W	T W S S G L	Y N E Q F F G P G	2.1	180
AC04	L	23	C A S S L	R R G Q	P Q H F G X G	1.5	280

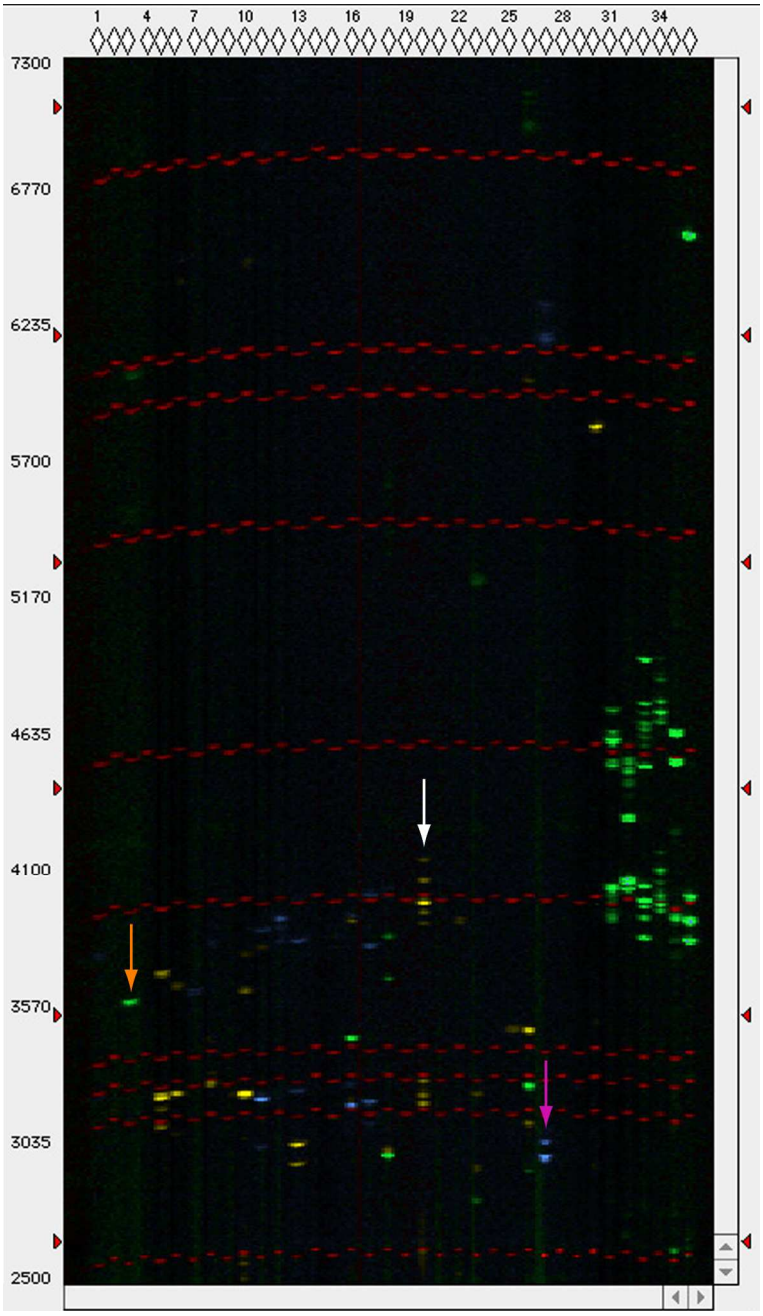

Fig. S1 Micrograph showing a representative DNA sequencer gel.

PCR products separated on this gel stem from subject 04, the negative control, in which mono- (orange arrow) or oligoclonal expansions (pink arrow) appear as peaks of certain CDR3 length over a Gaussian background of polyclonal T cells (white arrow). PCR products from Vβ-Jβ amplification were applied to lanes 1 to 30, whereas in lanes 31 to 36 Vβ-Cβ PCR products were separated.

3.2 Expression of herpes simplex virus 1-encoded microRNAs in human trigeminal ganglia and their relation to local T-cell infiltrates

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The author of this doctoral thesis contributed to Held *et al*. (2011a) by conducting most experiments except microRNA quantitative polymerase chain reactions (PCR) and the immunohistochemical staining for Fig. 1A. Furthermore, the author analysed all data and wrote parts of the manuscript.

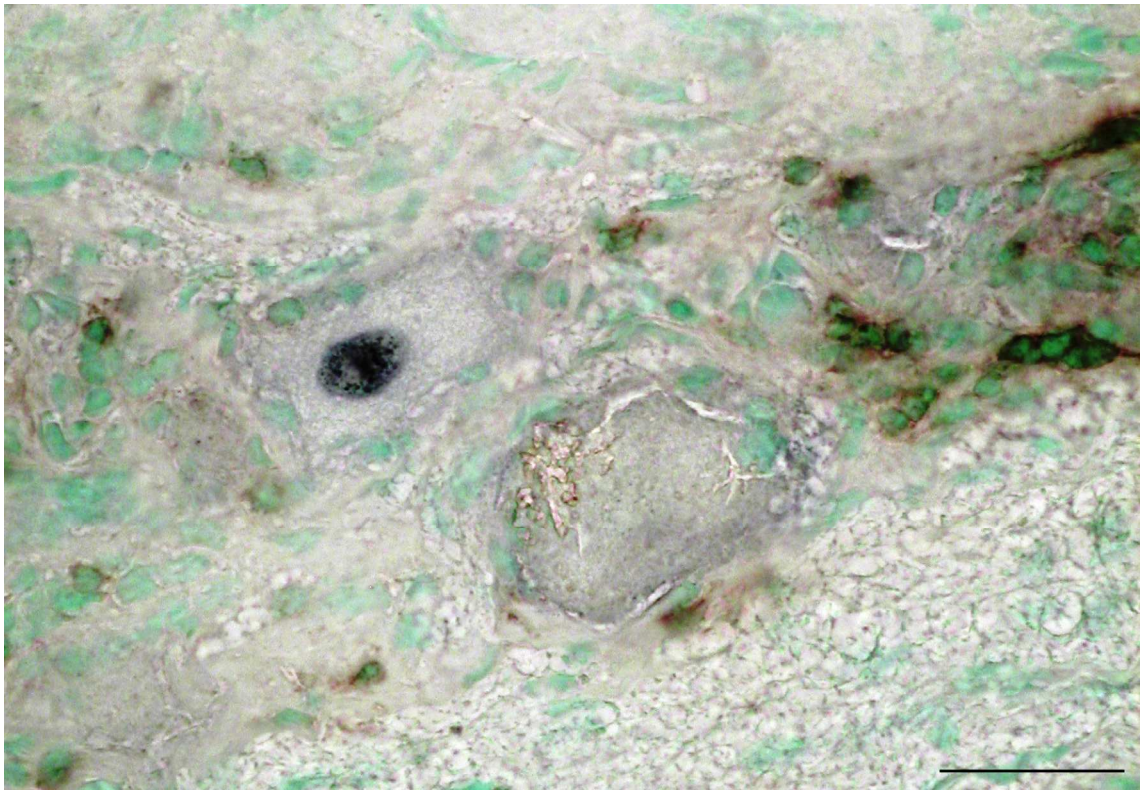

Fig. 10: Latently HSV-1-infected neuron and TG infiltrating T cells.
LAT *in situ* hybridisation (dark nuclear staining) and anti-CD3 immunohistochemistry (brown surface staining) on a 10 µm frozen TG section, counterstained with methyl green. Scale bar, 50µm.

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Expression of Herpes Simplex Virus 1-Encoded MicroRNAs in Human Trigeminal Ganglia and Their Relation to Local T-Cell Infiltrates▽

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Herpes simplex type 1 (HSV-1) is a neurotropic virus which establishes lifelong latency in human trigeminal ganglia (TG). Currently, two nonexclusive control mechanisms of HSV-1 latency are discussed: antiviral CD8$^+$ T cells and viral microRNAs (miRNAs) encoded by the latency associated transcript (LAT). We investigate here to what extent these mechanisms may contribute to the maintenance of HSV-1 latency. We show that only a small proportion of LAT$^+$ neurons is surrounded by T cells in human TG. This indicates that viral latency in human TG might be controlled by other mechanisms such as viral miRNAs. Therefore, we assessed TG sections for the presence of HSV-1 miRNA, DNA, and mRNA by combining LAT *in situ* hybridization, T-cell immunohistochemistry, and single cell analysis of laser-microdissected sensory neurons. Quantitative reverse transcription-PCR (RT-PCR) revealed that LAT$^+$ neurons with or without surrounding T cells were always positive for HSV-1 miRNAs and DNA. Furthermore, ICP0 mRNA could rarely be detected only in LAT$^+$ neurons, as analyzed by single-cell RT-PCR. In contrast, in LAT$^-$ neurons that were surrounded by T cells, neither miRNAs nor the DNA of HSV-1, HSV-2, or varicella-zoster virus could be detected. These data indicate that the majority of LAT$^+$ neurons is not directly controlled by T cells. However, miRNA expression in every latently infected neuron would provide an additional checkpoint before viral replication is initiated.

Herpes simplex virus type 1 (HSV-1) is a human herpesvirus with a specific tropism for sensory neurons. After a primary infection of the oral cavity, the virus migrates along the axons of the trigeminal nerve to reach the cell bodies of sensory neurons within the trigeminal ganglion (TG), where it establishes life-long latency (1, 20). This latent state is characterized by the expression of only one prominent viral transcript, called latency-associated transcript (LAT). It accumulates in the nuclei of the infected neurons, where it can easily be visualized by applying *in situ* hybridization (21). In addition, there is evidence from animal models (3, 4, 7, 13, 17) and also from human postmortem studies (6) that viral immediate-early genes are expressed at very low levels during viral latency.

Latent HSV-1 infection in human TG is accompanied by immune cell infiltrates that are mainly composed of CD8$^+$ T cells (23). This infiltration has previously been seen in TG of latently infected mice (16), where it has been demonstrated that most of the local CD8$^+$ T cells are specific for HSV-1 and can block the virus from reactivation (11, 15, 19). This protective effect of T cells could be mediated partly by gamma interferon (5, 14). Moreover, granzyme B produced by local CD8$^+$ T cells was shown to degrade the HSV-1 immediate-early protein ICP-4 *in vitro* (12). Since this viral protein is important for viral replication, lack of ICP-4 might stop reactivation of HSV-1 at a very early stage. Interestingly, the local granzyme B production was not accompanied by neuronal apoptosis. The CD8$^+$ T-cell effector functions were demonstrated in *ex vivo* cultures of mouse TG (12, 15). It was also found in humans, the natural host of HSV-1, that CD8$^+$ T cells isolated from HSV-1 latently infected human TG proliferate in reaction to protein derived from HSV-1 (29). Furthermore, HSV-1-positive human ganglia show an increased expression of inflammatory cytokines (6, 23). The infiltrating CD8$^+$ T cells also express chemokine receptors and show features of clonal expansion, indicating an antigenic stimulus as the cause of the local T-cell proliferation (6).

LAT is believed to play a role in the establishment and maintenance of latency (3, 25) and reactivation (9, 18), but thus far no LAT-derived protein has been found. Recently, it has been shown that HSV-1 encodes several microRNAs (miRNAs), some of them originating from the LAT region (10, 26, 27). The discovery that HSV-1-encoded miRNAs have a regulatory capacity for viral genes helps to show how the virus itself might control its latency state. Two of these miRNAs were shown to reduce expression of the immediate-early proteins ICP0 and ICP4 *in vitro* (26), thereby potentially inhibiting viral reactivation from latency since these two immediate-early proteins are known to be major transactivators for viral gene expression. Further, ICP34.5, a neurovirulence factor (2), is a potential target for other viral miRNAs. In this way, LAT-encoded miRNAs may facilitate the establishment and main-

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TABLE 1. Tissue sample overview used in the present study with alphaherpesvirus infection state[a]

Subject	Gender	Age (yr)[b]	Tissue	Presence (+) or absence (–) of:	
				HSV-1 latency	VZV latency
1	F	52	TG	+	+
2	M	12	TG	+	–
3	F	85	TG	+	ND
4	F	71	TG	+	+
5	M	39	TG	+	–
6		5 wks	FG	–	–
7		5 wks	FG	–	–
8	M	56	TG	+	+
9	M	77	TG	–	+
10	F	80	TG	+	+
11	M	62	TG	–	+
12	F	61	TG	+	–
13	M	41	TG	+	–
14	M	78	TG	+	+
15	M	36	TG	–	–
16	F	32	TG	–	+
17	M	35	TG	–	+
18	M	34	TG	+	+
19	F	17	TG	+	–
20	M	29	TG	–	–

[a] F, female; M, male; TG, trigeminal ganglia; FG, facial ganglia; ND, not done.
[b] Expressed in years except as noted.

tenance of latency. The presence of the HSV-1-encoded miRNAs has been described in whole RNA from lytic infected cells and also latently infected mouse and human TG (27).

We hypothesized that by downregulating viral proteins, which may be potential viral candidate antigens for the infiltrating T cells, the viral miRNAs may reduce recruitment of antiviral $CD8^+$ T cells to infected neurons. Therefore, we assessed single laser-microdissected neurons for the presence of viral miRNA in relation to LAT expression and immune cell infiltrates using a combination of *in situ* hybridization, immunohistochemistry, and quantitative reverse transcription-PCR (qRT-PCR). This concomitant analysis of viral miRNAs, LAT, viral DNA, viral mRNA, and T cells on the single-cell level helps to clarify to which proportion the viral miRNAs and host T cells may contribute to the maintenance of HSV-1 latency in human TG.

MATERIALS AND METHODS

The Ethics Committee of the Medical Faculty of the Ludwig Maximilian University of Munich approved the use of autopsy samples for the present study. TG of both sides were removed from 16 subjects 6 to 24 h after death. None of them had lesions suggestive of an active orolabial herpes infection or a history of cranial nerve disorders. Gender, age, and alphaherpesvirus infection state are listed in Table 1. Ganglia were embedded immediately in Jung tissue freezing medium (Leica Microsystems, Nussloch, Germany) and subsequently stored at −80°C. Frozen sections (10 μm) were cut and mounted on positively charged slides (Superfrost Plus; Menzel, Braunschweig, Germany) for immunohistochemistry or on membrane-covered PET slides (Zeiss, Jena, Germany) for laser catapulting microscopy (LCM). Slides were subsequently stored at −20°C or at −80°C, respectively. Further, 10 30-μm tissue sections were collected for RNA extraction. Paraffin sections (4 μm) on positively charged slides were prepared from several TG after fixation in 4% buffered paraformaldehyde for 24 h and paraffin embedding.

***In situ* hybridization and immunohistochemistry.** The 25-bp oligonucleotide probe (Dig-LAT probe [5′-CAT AGA GAG CCA GGC ACA AAA ACA C-3′]) used for the detection of the LAT was synthesized and labeled with digoxigenin (Eurofins MWG Synthesis GmbH, Ebersberg, Germany). An *in situ* hybridization for LAT, followed by CD3 immunohistochemistry, was carried out on TG sections as described previously (6, 22, 24). The polyclonal rabbit anti-human CD3 antibody (Dako Cytomation, Glostrup, Denmark) was detected by using a biotinylated goat anti-rabbit immunoglobulin antibody (Dako Cytomation), followed by Cy2-labeled streptavidin (Dianova, Hamburg, Germany) or horseradish peroxidase (HRP)-conjugated streptavidin (Dako Cytomation) and incubation in diaminobenzidine (DAB; Dako Cytomation).

The frequency of different T-cell neuron interaction patterns was assessed in paraffin sections and cryosections of 19 TG. These sections were concomitantly stained for LAT and CD3. The frequencies of the different T-cell neuron interaction patterns were analyzed by counting all of the neurons of one TG section under the microscope. To confirm the consistency of the pattern, five consecutive sections were stained for LAT, as well as CD3, and analyzed.

Varicella-zoster virus (VZV) latency was determined by staining TG sections for the VZV protein 62 as described in a previous study (23). In brief, frozen TG sections were incubated with the anti-VZV immediate-early gene 62 antibody (Chemicon [Millipore], Billerica, MA). Subsequently, the antibody was detected with a biotinylated rabbit anti-mouse immunoglobulin antibody (Dako Cytomation), followed by HRP-conjugated streptavidin (Dako Cytomation) and incubation in DAB (Dako Cytomation).

HSV-1 miRNA expression in human TG. Total RNA was isolated from human ganglia with Qiazol (Qiagen, Hilden, Germany) and subsequently purified using an miRNeasy minikit (Qiagen).

For LCM, the tissue was covered with *n*-propanol to prevent drying and to inhibit RNase activity, and immediately transferred to a PALM Microbeam-Z microscope. Neurons meeting our criteria (positive or negative *in situ* signal for LAT; positive or negative for local CD3 T-cell clusters) were marked electronically. After evaporation of the *n*-propanol, five marked neurons were microdissected and laser pressure catapulted into single reaction tubes, which were immediately stored on dry ice. After microdissection of the neurons, it was visually verified that all five neurons were actually captured.

RNA from five pooled LCM cells per sample was prepared with a combination of the miRNeasy minikit and an RNeasy microkit (Qiagen). miRNAs were transcribed using a TaqMan miRNA reverse transcription kit (Applied Biosystems, Darmstadt, Germany) and miRNA specific stem-looped primers (26). These stem-looped primers bind to the miRNAs and generate a longer cDNA transcript in the RT reaction, which can easily be amplified in a normal qPCR.

qPCR was performed on the ABI 7900 (Applied Biosystems) using the qPCR core kit and uracyl *N*-glycosylase (both from Eurogentec, Cologne, Germany). Primers and probes for qPCR were designed as described recently (26). The total amount of transcribed RNA equivalent used per PCR was 0.8 ng. The miRNA let7a was used for normalization with the ΔC_T method (26).

Viral DNA in single neurons. DNA was extracted from five pooled microdissected neurons or T-cell infiltrates as described before (31). The copy numbers for HSV-1, HSV-2, or VZV DNA in single neurons were determined on the ABI 7900 cycler (Applied Biosystems) using an Artus HSV-1/2 PCR kit or an Artus VZV PCR kit (both from Qiagen), respectively. The mean efficiencies of the PCRs ranged between 96 and 105%. Titration of viral DNA revealed that 10 copies could be detected with a 100% recovery.

HSV-1 ICP0 mRNA expression in single neurons. First, cDNA of whole TG was analyzed for the expression of ICP0 by nested PCR as described previously (6). Only positive TG were used for microdissection.

To preserve mRNA, membrane-covered PET slides (Zeiss, Jena, Germany) were stained for 5 min with a Cy3-labeled anti-CD8 antibody (clone LT8 [AbD Serotec, Dusseldorf, Germany] and the Cy3 MAb labeling kit [GE Healthcare, Chalfont St. Giles, United Kingdom]). Neurons surrounded by $CD8^+$ T cells were microdissected and immediately stored on dry ice. To obtain sufficient amounts of mRNA, five neurons were pooled. For each of the pools, a multiplex nested LAT and ICP0 PCR was performed as previously described (6). The first round of amplification was carried out in one reaction tube for both products, whereas in the second round of PCR LAT and ICP0 were amplified separately. cDNA from HSV-1-infected Vero cells was used as a positive control. PCR products were separated by using a 2% agarose gel. The identity of the PCR products was verified by sequencing.

Statistics. Statistical analysis was performed by using GraphPad Prism 5 and Microsoft Office Excel 2003. When Gaussian distributions of results were given, the two-sided Student *t* test was used. The results without Gaussian distributions were analyzed by the Mann-Whitney U test. Differences with a P value of 0.05 were considered statistically significant.

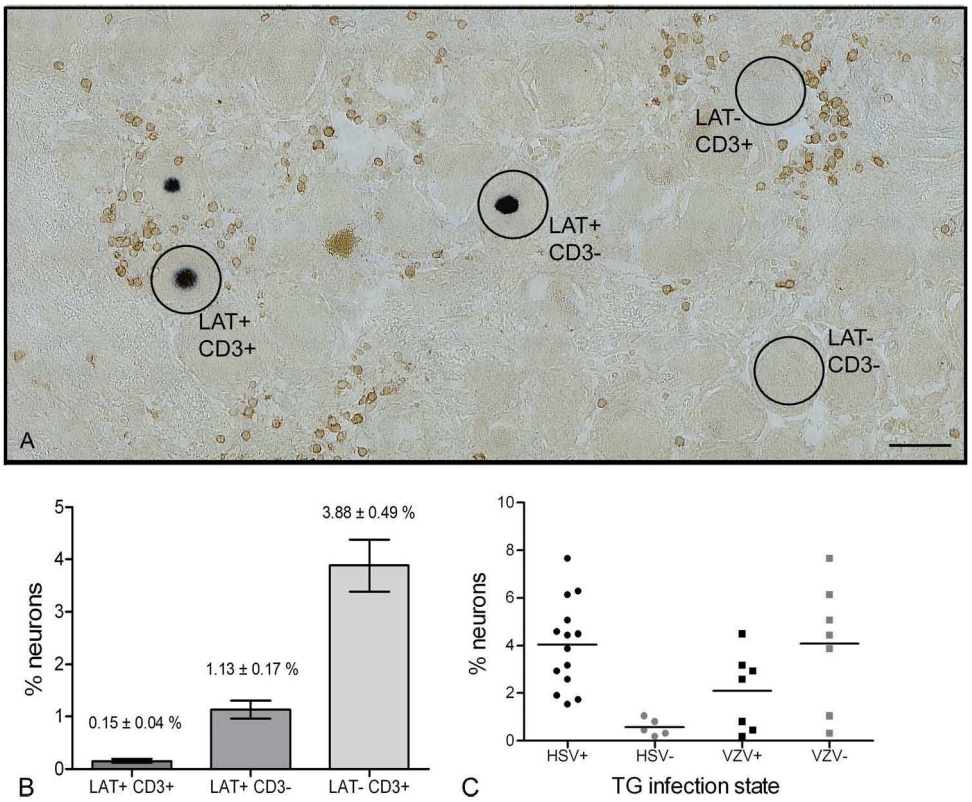

FIG. 1. (A) LAT *in situ* hybridization (dark nuclear staining) and anti-CD3 immunohistochemistry (brown surface staining) on a 4-μm paraffin section of human TG. All four different T-cell neuron interactions are depicted in this micrograph. Scale bar, 50 μm. (B) Percentage of neuronal patterns in latently HSV-1 infected human TG with range. The values are given as means ± the SEM of the total neurons of one TG section. All bars exhibit highly significant differences (Student *t* test: $P < 0.005$). (C) Percentage of neurons surrounded by T cells in latently HSV-1- or VZV-infected TG versus noninfected human TG. The difference in the percentage of neurons surrounded by T cells in latently HSV-1-infected ganglia versus non-HSV-1-infected ganglia is significant (Student *t* test, $P = 0.001$), whereas this is not the case with VZV-infected versus noninfected ganglia (Student *t* test, $P = 0.472$).

RESULTS

Frequency of different T-cell neuron interaction patterns. Four different T cell neuron interactions can be envisioned: (i) LAT-positive neurons that are surrounded by T cells, (ii) LAT-positive neurons that are not surrounded by T cells, (iii) LAT-negative neurons that are surrounded by T cells, and (iv) LAT-negative neurons that are not surrounded by T cells (Fig. 1A). To quantify the different T-cell neuron interaction patterns, all neurons in one TG section of 19 different ganglia were counted. Only 0.15% ± 0.04% (mean ± the standard error of the mean [SEM]) of neurons showed a LAT hybridization signal and were surrounded by T cells. The majority of the LAT^+ neurons, 1.13% ± 0.17% of the total neurons, were not surrounded by T cells. This means that only 13.16% of all LAT^+ neurons were surrounded by T cells. Furthermore, 3.88% ± 0.49% of the neurons of one TG section showed surrounding T-cell infiltrates, although they were negative for LAT by *in situ* hybridization (Fig. 1B). Examination of consecutive sections showed the same frequencies of the different T-cell neuron interaction patterns. It is widely accepted that HSV-1 is the trigger for the infiltration of immune cells into the TG, but VZV is also known to establish latency in the sensory neurons of the human TG. To test the hypothesis that the T cells infiltrate the TG due to HSV-1 and not VZV, the occurrence of neurons surrounded by T cells was compared between latently HSV-1-infected and noninfected TG and latently VZV-infected and noninfected TG. HSV-1-infected TG show significantly higher amounts of neurons surrounded by T cells than HSV-1-negative TG (Student *t* test: $P = 0.001$). This is not the case for VZV (Fig. 1C). The percentage of LAT^+ neurons overall also correlated with the percentage of neurons surrounded by T cells, meaning that TG with a high percentage of LAT^+ neurons usually also exhibited more T-cell infiltrates ($P = 0.0045$, $r^2 = 0.4095$). In TG negative for latent HSV-1, only scattered T cells were present.

HSV-1 miRNA expression in human TG. In the present study we examined 10 human TG from five subjects for the expression of HSV-1 miRNAs. Three subjects were latently HSV-1 infected, and two subjects were HSV-1 negative as determined by LAT *in situ* hybridization. In addition, two facial ganglia (FG) of two different HSV-1-negative cases were also assessed in the present study. The 10 TG and two FG were screened by qPCR for the expression of five HSV-1-encoded miRNAs (H2-3p, H3, H4-3p, H4-5p, H5, and H6). The results were normalized against miRNA let7a levels. All assessed miRNAs were present in the latently infected TG, except for

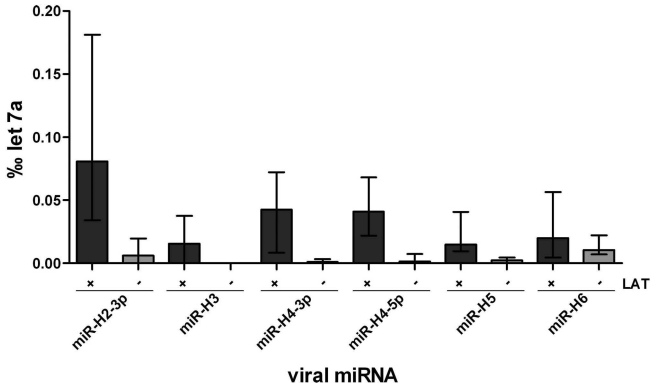

FIG. 2. Expression of HSV-1 miRNAs in latently HSV-1-infected versus noninfected human TG. The expression of the viral miRNAs is shown in ‰ let7a (median with interquartile range), a human housekeeping miRNA. All LAT^+ TG show expression of viral miRNAs, whereas hardly any expression is seen in LAT^- TG. The difference in the viral miRNA expression of latently HSV-1-infected versus noninfected TG is statistically significant for the miRNAs H2-3p, H4-3p, H4-5p, and H5 ($P < 0.05$ [Mann-Whitney U test]).

miRNA H3 in one subject (Fig. 2). The two FG from HSV-1-negative cases showed results comparable to those for the HSV-1-negative TG.

HSV-1 miRNA expression in single neurons. On the cellular level of the TG it becomes apparent that only a minority of latently infected neurons are surrounded by T cells. Differential expression of viral miRNAs might contribute to this finding. Higher levels of viral miRNAs may lower the amount of viral antigen presented to T cells due to their ability to block transcription of immediate-early genes (26).

Therefore, we analyzed the expression of viral miRNAs in single neurons using a combination of *in situ* hybridization, immunohistochemistry, and LCM, followed by qPCR. Thus, the expression of viral miRNAs could be analyzed on the single-cell level in relation to LAT expression and T-cell infiltration.

Frozen TG sections were stained for LAT and $CD3^+$ T cells, and neurons were then extracted by laser microdissection (Fig. 3A to F). Five neurons from each group (LAT^+ neurons with surrounding T cells, LAT^+ neurons without surrounding T cells, LAT^- neurons with surrounding T cells, and LAT^- neurons without surrounding T cells) were pooled and analyzed by qRT-PCR for HSV-1 miRNAs. HSV-1 miRNAs were only expressed in LAT^+ neurons and not in LAT^- neurons (Fig. 3G). The expression level of viral miRNAs did not differ in LAT^+ neurons surrounded or not surrounded by infiltrating T cells (Fig. 3H). In contrast to whole-tissue RNA (Fig. 2), on the single-cell level miRNA H6 showed an obvious difference in expression in LAT-positive versus LAT-negative neurons (Fig. 3G and H).

No viral DNA in single LAT^- neurons surrounded by T cells. Since 95.51% of the neurons that were surrounded by T cells were negative for LAT and did not show any expression of viral miRNAs, these neurons were screened for the presence of viral DNA in order to determine the potential trigger for the immune cell infiltration. LAT^+ and LAT^- neurons, surrounded or not surrounded by T cells, were microdissected and assessed for the occurrence of HSV-1, HSV-2, and VZV DNA by qPCR. Further T-cell infiltrates that surrounded neurons were microdissected and analyzed. HSV-1 DNA was only detected in LAT^+ neurons and not in LAT^- neurons (Fig. 3I). All of the 12 LAT^+ neuron pools were positive for HSV-1 DNA and all of the 16 LAT^- neuron pools were negative for HSV-1 DNA. There was no obvious difference in the HSV-1 DNA copy number of LAT^+ neurons surrounded or not surrounded by T cells ($P > 0.05$ [Mann-Whitney U test]). A broad distribution in the amount of HSV-1 DNA copies per neuron was observed (Fig. 3I). The HSV-1 DNA copy numbers ranged from 1 to 558 copies per neuron pool.

Neither HSV-2 nor VZV DNA was present in any of the neurons (data not shown). Further, no herpesviral DNA could be found in the T-cell infiltrates, which is in accordance with the neurotropism of the *Alphaherpesviridae* (data not shown).

HSV-1 ICP0 mRNA expression in single neurons. To identify a potential antigenic trigger for the immune cell infiltration, neurons that were surrounded by $CD8^+$ T cells were analyzed for the presence of the immediate-early gene ICP0 mRNA. ICP0 was chosen because it is a potent activator of gene expression with functions in reactivation, and it has also been reported to be present at low levels in latently infected human TG (6). Therefore, it may be a possible antigenic trigger to infiltrating T cells. Expression of ICP0 was found in whole RNA from two of six analyzed TG. Frozen sections from these two positive TG were used for microdissection of neurons surrounded by $CD8^+$ T cells. A total of 16 pools of five neurons each from two different subjects were assessed for the presence of LAT and ICP0 mRNA. LAT transcripts were amplified from one of the neuron pools. This pool also showed an ICP0 amplification signal. Sequencing confirmed the identity of the transcripts.

DISCUSSION

The present study aimed at deciphering to what extent $CD8^+$ T cells and viral miRNA are involved in the HSV-1 latency process in human sensory neurons. It has been assumed that $CD8^+$ T cells and miRNAs are both implicated in the control of viral latency (15, 26). Different interaction patterns of these two mechanisms can be proposed: (i) latently infected neurons need both control mechanisms to keep the latent state, and (ii) latent infection is controlled in some neurons by miRNA in the others by T cells. To distinguish which interaction pattern occurs during HSV-1 latency in humans, TG were assessed by immunohistochemistry for the presence of T cells and by *in situ* hybridization for the presence of LAT, followed by laser microdissection of single neurons on the same histological section. Our investigation demonstrated that only a minority of the LAT^+ neurons were surrounded by T cells. This is in line with previous findings from the mouse model and human TG (7, 23, 29). It can be assumed that these neurons surrounded by T cells represent foci of viral gene expression beyond the expression of LAT. This is supported by our demonstration of ICP0 and LAT in single neurons that were surrounded by T cells.

Surprisingly, many LAT^- neurons were surrounded by T cells. To exclude that this finding is due to an artifact (e.g., neurons were not cut at the nuclei level, where LAT accumulates during latency), T cells and LAT were simultaneously

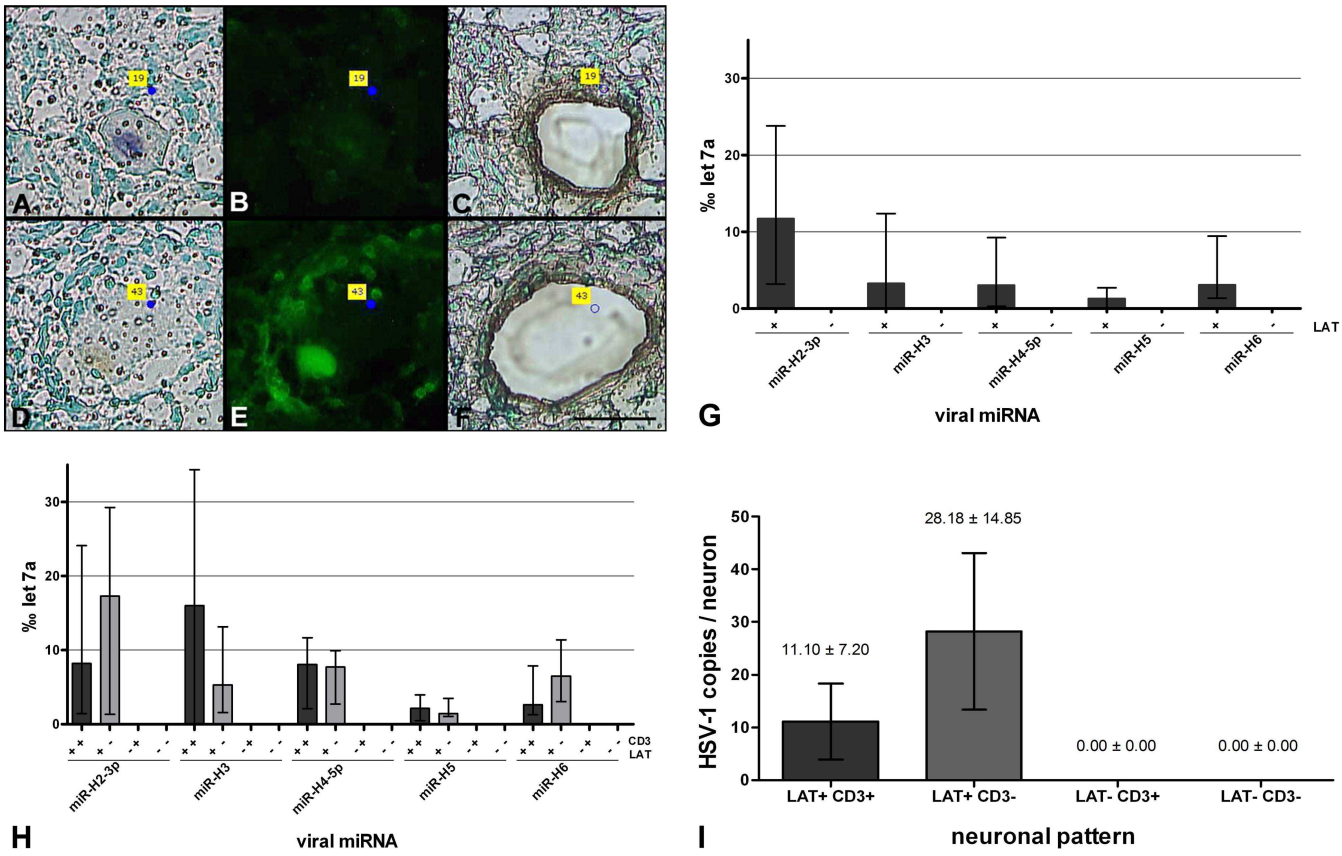

FIG. 3. Analysis of single neurons. (A to F) Representative micrographs of TG sections on membrane-covered PET slides used for LCM. Panels A and D show human TG sections stained for LAT by *in situ* hybridization. The micrographs show a LAT^+ ("19") and a LAT^- ("43") neuron. Panels B and E show CD3 staining of the same TG sections showing neurons surrounded ("43") or not surrounded ("19") by T cells. Panels C and F show neurons that were microdissected. Magnification, ×400. Scale bar, 50 μm. (G and H) HSV-1 miRNA expression in single neurons. The results are given in ‰ let7a (median with interquartile range). In panel G, the expression of viral miRNAs in LAT^+ versus LAT^- neurons is shown. Viral miRNAs are only present in LAT^+ neurons. The difference in the expression of viral miRNAs in LAT^+ versus LAT^- neurons is highly significant for all miRNAs ($P < 0.001$ [Mann-Whitney U test]). In panel H, HSV-1 miRNA expression in LAT^+ and LAT^- neurons with respect to T cells is shown. An abundant expression of viral miRNAs was detected in LAT^+ neurons but not in LAT^- neurons. There is no obvious difference in miRNA expression with regard to the T-cell infiltration. (I) Viral DNA in single microdissected neurons. The values are given as means ± the SEM of the HSV-1 DNA copies per neuron. HSV-1 DNA was only detected in LAT^+ neurons. There is no significant difference between neurons surrounded or not surrounded by T cells. No HSV-2 or VZV DNA was detected in any of the neurons (data not shown).

stained on consecutive sections. These findings confirmed that T cells are frequently found around LAT-free neurons (data not shown). Furthermore, no HSV-1, HSV-2, or VZV DNA was present in any of the LAT^- neurons, whereas all LAT^+ neurons contained HSV-1 DNA. This is in contrast to earlier studies in humans (31) and mice (reviewed in reference 30) wherein LAT could be detected by PCR also in neurons that were found to be negative by *in situ* hybridization. These contradictory results could be explained by the use of frozen samples in the present study, a different probe for the *in situ* detection of LAT, and possibly a more specific PCR protocol.

It still remains unresolved why T cells surround LAT^- neurons. One explanation could be that these or neighboring neurons were the origin of former herpesviral reactivations. This would be in line with reports demonstrating that memory T cells remain resident in the skin long after a resolved HSV-1 infection (8). The local cytokine milieu produced by antiviral T cells following repeated reactivations of HSV-1 might also keep other T cells, the so-called bystander T cells, in the ganglion (28). Finally, it cannot be definitely excluded that these neurons host other viruses or minuscule amounts of viral antigen which are not detectable with the techniques applied in the present study. However, it was shown in animal studies with LAT-deficient mutants that neurons with low DNA copy numbers are less likely to reactivate (18). Therefore, a prominent immune response around neurons with only little viral DNA would not be expected.

Besides immune cells, HSV-1-encoded miRNAs were considered as an additional control mechanism for viral latency. Using RNA extracted from whole TG, we observed the expression of miRNAs H2-3p, H3, H4-3p, H4-5p, H5, and H6 in latently infected TG. This is in accordance with the results of Umbach et al. (27). On the single-cell level, we could detect HSV-1 miRNA exclusively in LAT^+ neurons. However, there was no significant difference in the expression of viral miRNAs in neurons that were or were not surrounded by T cells.

Based on these findings, we propose the following model for the control of HSV-1 latency in humans: in the majority of

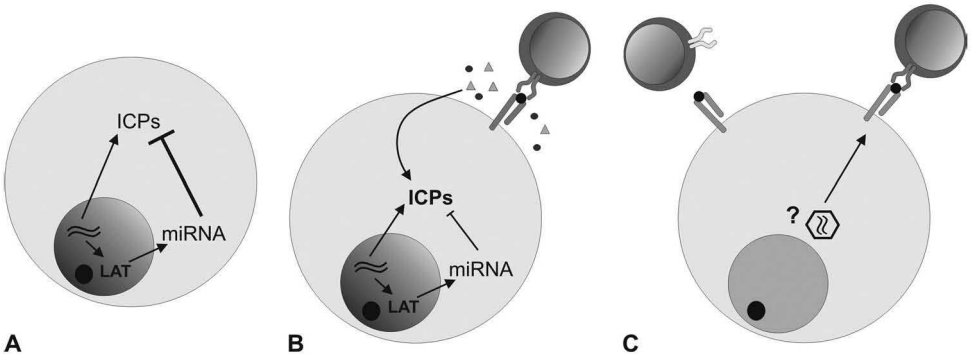

FIG. 4. Proposed model of latent HSV-1 infection in human TG. (A) In the majority of latently HSV-1-infected neurons miRNA levels and cellular mechanisms are sufficient to block the virus from reactivation. (B) In a small percentage of latently infected neurons, the equilibrium is shifted toward viral gene production, and immediate-early proteins get translated. The $CD8^+$ T cells infiltrate and keep the virus in the latent state. (C) The T cells surrounding LAT^- neurons may be attracted by as-yet-unknown factors (former HSV-1 reactivations, a different virus, or nonspecific by the inflammatory milieu).

latently HSV-1-infected neurons, cellular and viral control mechanisms, such as the viral miRNAs, are sufficient to block the virus from reactivation. In a small percentage of latently infected neurons, the virus escapes these control mechanisms, and viral gene expression is initiated. This leads to the presentation of viral antigens on MHC. As a consequence, $CD8^+$ T cells are attracted as a backup to keep the virus in the latent state by a noncytolytic mechanism (Fig. 4).

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REFERENCES

1. **Baringer, J. R., and P. Swoveland.** 1973. Recovery of herpes simplex virus from human trigeminal ganglions. N. Engl. J. Med. **288:**648–650.
2. **Brown, S. M., A. R. MacLean, J. D. Aitken, and J. Harland.** 1994. ICP34.5 influences herpes simplex virus type 1 maturation and egress from infected cells in vitro. J. Gen. Virol. **75:**3679–3686.
3. **Chen, S. H., M. F. Kramer, P. A. Schaffer, and D. M. Coen.** 1997. A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. J. Virol. **71:**5878–5884.
4. **Chen, S. H., et al.** 2002. Neither LAT nor open reading frame P mutations increase expression of spliced or intron-containing ICP0 transcripts in mouse ganglia latently infected with herpes simplex virus. J. Virol. **76:**4764–4772.
5. **Decman, V., P. R. Kinchington, S. A. Harvey, and R. L. Hendricks.** 2005. Gamma interferon can block herpes simplex virus type 1 reactivation from latency, even in the presence of late gene expression. J. Virol. **79:**10339–10347.
6. **Derfuss, T., et al.** 2007. Presence of HSV-1 immediate-early genes and clonally expanded T cells with a memory effector phenotype in human trigeminal ganglia. Brain Pathol. **17:**389–398.
7. **Feldman, L. T., et al.** 2002. Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. Proc. Natl. Acad. Sci. U. S. A. **99:**978–983.
8. **Gebhardt, T., et al.** 2009. Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat. Immunol. **10:**524–530.
9. **Hill, J. M., F. Sedarati, R. T. Javier, E. K. Wagner, and J. G. Stevens.** 1990. Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology **174:**117–125.
10. **Jurak, I., et al.** 2010. Numerous conserved and divergent microRNAs expressed by herpes simplex viruses 1 and 2. J. Virol. **84:**4659–4672.
11. **Khanna, K. M., R. H. Bonneau, P. R. Kinchington, and R. L. Hendricks.** 2003. Herpes simplex virus-specific memory $CD8^+$ T cells are selectively activated and retained in latently infected sensory ganglia. Immunity **18:**593–603.
12. **Knickelbein, J. E., et al.** 2008. Noncytotoxic lytic granule-mediated $CD8^+$ T cell inhibition of HSV-1 reactivation from neuronal latency. Science **322:**268–271.
13. **Kramer, M. F., and D. M. Coen.** 1995. Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. J. Virol. **69:**1389–1399.
14. **Liu, T., K. M. Khanna, B. N. Carriere, and R. L. Hendricks.** 2001. Gamma interferon can prevent herpes simplex virus type 1 reactivation from latency in sensory neurons. J. Virol. **75:**11178–11184.
15. **Liu, T., K. M. Khanna, X. Chen, D. J. Fink, and R. L. Hendricks.** 2000. $CD8^+$ T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. J. Exp. Med. **191:**1459–1466.
16. **Liu, T., Q. Tang, and R. L. Hendricks.** 1996. Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection. J. Virol. **70:**264–271.
17. **Margolis, T. P., et al.** 2007. Spontaneous reactivation of herpes simplex virus type 1 in latently infected murine sensory ganglia. J. Virol. **81:**11069–11074.
18. **Perng, G. C., et al.** 1994. The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency. J. Virol. **68:**8045–8055.
19. **Sheridan, B. S., T. L. Cherpes, J. Urban, P. Kalinski, and R. L. Hendricks.** 2009. Reevaluating the CD8 T-cell response to herpes simplex virus type 1: involvement of CD8 T cells reactive to subdominant epitopes. J. Virol. **83:**2237–2245.
20. **Stevens, J. G., and M. L. Cook.** 1971. Latent herpes simplex virus in spinal ganglia of mice. Science **173:**843–845.
21. **Stevens, J. G., E. K. Wagner, G. B. vi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.
22. **Theil, D., et al.** 2001. Prevalence of HSV-1 LAT in human trigeminal, geniculate, and vestibular ganglia and its implication for cranial nerve syndromes. Brain Pathol. **11:**408–413.
23. **Theil, D., et al.** 2003. Latent herpesvirus infection in human trigeminal ganglia causes chronic immune response. Am. J. Pathol. **163:**2179–2184.
24. **Theil, D., et al.** 2003. Dually infected (HSV-1/VZV) single neurons in human trigeminal ganglia. Ann. Neurol. **54:**678–682.
25. **Thompson, R. L., and N. M. Sawtell.** 1997. The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. J. Virol. **71:**5432–5440.
26. **Umbach, J. L., et al.** 2008. MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature **454:**780–783.
27. **Umbach, J. L., M. A. Nagel, R. J. Cohrs, D. H. Gilden, and B. R. Cullen.** 2009. Analysis of human {alpha}-herpesvirus microRNA expression in latently infected human trigeminal ganglia. J. Virol. **83:**10677–10683.
28. **van Lint, A. L., et al.** 2005. Latent infection with herpes simplex virus is associated with ongoing $CD8^+$ T-cell stimulation by parenchymal cells within sensory ganglia. J. Virol. **79:**14843–14851.
29. **Verjans, G. M., et al.** 2007. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. Proc. Natl. Acad. Sci. U. S. A. **104:**3496–3501.
30. **Wagner, E. K., and D. C. Bloom.** 1997. Experimental investigation of herpes simplex virus latency. Clin. Microbiol. Rev. **10:**419–443.
31. **Wang, K., T. Y. Lau, M. Morales, E. K. Mont, and S. E. Straus.** 2005. Laser-capture microdissection: refining estimates of the quantity and distribution of latent herpes simplex virus 1 and varicella-zoster virus DNA in human trigeminal ganglia at the single-cell level. J. Virol. **79:**14079–14087.

4 Discussion

To study the TCR repertoire of the T-cell infiltrates present in human TG, CDR3 spectratyping was applied. Several clonal expansions were found within all assessed TG. One finding of particular note is that identical TCR chains were found in the left and right ganglia from the same individual. This provides direct evidence for antigen-driven T-cell responses. These expansions were not shared between individuals, which can be expected due to the different HLA haplotypes of the subjects.

Analysis of the distribution of the immune cell infiltrates amongst neuronal cell bodies revealed that most LAT^+ neurons are not closely associated with T cells. Quite a few neurons negative for LAT by *in situ* hybridisation were, however, surrounded by T cells. This raises the question of whether these LAT^- neurons surrounded by T cells harbour latent HSV-1 genomes. In mouse TG it has been established that numerous LAT– neurons containing HSV-1 DNA are present (Chen et al., 2002a). Our present examination of single laser microdissected neurons from human TG showed that only LAT+ neurons actually harbour HSV-1, indicating a more fundamental role of LAT in human HSV-1 latency.

Both studies add further knowledge of the role of the TG infiltrating CD8+ T cells and viral transcripts like LAT and microRNAs in the maintenance of HSV-1 latency in its natural host, the human. Altogether, the results indicate that latency is tightly controlled by viral and cellular control mechanisms. The host immune system, in this case the virus-specific $CD8^+$ T cells, only come into action if these mechanisms are overrun.

4.1 The infiltrating T cells are clonally expanded

To investigate how latent viral infections may shape the TCR repertoire of the TG infiltrating T cells, the sequences of hypervariable TCR CDR3-regions were analysed (chapter 3.1). Given that HSV-1 establishes lifelong latency in the sensory neurons and repeatedly reactivates from there, it can be assumed that the lifelong exposure to viral antigen has shaped the TCR usage of the local T cells. Further, as herpesviruses are estimated to be 400 million years old, and HSV-1 and humans have therefore co-existed for a very long time, some TCRs universally reactive to this virus might have evolved. Such strong biases in the blood TCR repertoire have been described for herpes simplex virus type 2 (HSV-2) (Dong et al., 2010) as well as the human herpesviruses Epstein-Barr virus (EBV) and cytomegalovirus (HCMV) (Venturi et al., 2008). In the present study, using CDR3 spectratyping, clonally expanded TCR β-chains were detected in all analysed TG. Even though the analysed subjects shared some HLA alleles, however, no public T-cell clones occurring in more than one subject were identified. Studies analysing the TCR repertoire present in the brain of multiple sclerosis patients showed that distinct T-cell clones appeared in different anatomical sites (Skulina et al., 2004) (Junker et al., 2007). Here, the right and the left TG from two subjects were screened independently for clonal expansions. Some homologous clones were found to occur in both TG. Homologous clones share Vβ-segments and further exhibit amino acids with similar properties in their CDR3. Amino acids in positions three to five after the conserved cysteine have been shown to be especially important for antigen binding (Rudolph et al., 2006). The presence of homologous clones indicates that these T cells proliferated to similar antigenic epitopes. Remarkably, each of the subjects from which both TG were analysed, revealed two identical clones in both TG. These clones present in the right and the left TG even shared identical nucleotide sequences, suggesting that they originate from the same T-cell clone, which was probably primed in the periphery and subsequently infiltrated both latently infected TG.

Staining for expanded Vβ-chains in the present study revealed that not all of the $CD8^+$ T cells clustered around a neuronal cell body belong to the same T-cell clone, indicating different reactivities in these T-cell clusters. This also hints towards an infiltration of the TG by activated but unspecific bystander T cells. Verjans, *et al* 2007 have also proposed this on the basis that most T cells present in TG express markers of activation but only a few co-express Granzyme B, which is considered as a marker for specificity in mice (van Lint et al., 2005). Van Lint *et al*, 2005 further found that initial infiltration of activated T cells into latently

infected mouse TG was independent of T-cell specificity. Infiltration of activated but unspecific T cells to sites with a chronic inflammatory milieu has also been described in rheumatoid arthritis patients (Scotet et al., 1999). $CD8^+$ T cells reactive against viral proteins derived from EBV and HCMV were found to be enriched within inflamed joints. In the present study homologous and even identical clones were detected in the right and the left TG, and thus it seems likely that these clones were attracted by specific antigens expressed in both TG.

On the other hand, in mice latently infected with HSV-1, it was shown that almost all local T cells are reactive against HSV-1-derived antigenic epitopes (St Leger et al., 2011), with $gB_{(498\text{-}505)}$ being the immuno-dominant epitope in C57BL/6 mice. In mice the overall immunological control of HSV-1 therefore appears to be more thorough than in humans. There are three obvious reasons for this: first, HSV-1 ICP47 restricts MHC class I antigen presentation less efficiently in mice than in humans (Orr et al., 2007). Second, rare expression of numerous viral proteins was found to occur in mouse TG (Feldman et al., 2002), but not in humans (Theil et al., 2003a). Third, laboratory mice are only infected by HSV-1 and do not show an age-related accumulation of $CD8^+$ T cells reactive to other viruses like HCMV, as seen in elderly humans (Karrer et al., 2009).

Given that the epitopes responsible for triggering the immune infiltration into HSV-1-infected TG in humans have not yet been identified, the candidate antigens of the clonally expanded TG infiltrating T cells can only be speculated. HSV-1 can be considered as the initial trigger, since a positive correlation between these TG infiltrating immune cells and the presence of latent HSV-1, but not VZV or HHV-6, could be established (Theil et al., 2003a) (Hüfner et al., 2006) (Hüfner et al., 2007) (Arbusow et al., 2010). Also, the two studies presented here show significantly more T cells in latently HSV-1-infected TG than in TG negative for HSV-1. Both the number of $CD3^+$ T cells (chapter 3.1), as well as the number of neurons surrounded by $CD3^+$ T cells (chapter 3.2), are elevated in latently HSV-1-infected human TG. This was not observed when VZV-infected TG were compared to VZV-uninfected TG. Most convincingly, Verjans *et al*, 2007 showed that some T-cell lines isolated from human TG were reactive to B cells presenting HSV-1, but not VZV, antigens.

In humans, it is more feasible to study the reactivity of blood-derived T cells than TG derived ones. It was shown that human $CD4^+$ and $CD8^+$ blood T cells differ in antigen-specificity (Mikloska and Cunningham, 1998). $CD4^+$ T cells, which usually encounter free virus, mostly recognised structural viral proteins produced late in the lytic cycle. On the other hand, $CD8^+$ T cells mainly reacted to viral proteins produced early in the lytic cycle, therefore being able to kill virus infected cells before viral replication starts. In searching for candidate T-cell

antigens in human latency, however, it has to be considered that HSV-1 remains almost transcriptionally silent during this period. So far, no viral antigens were found to be expressed in human TG during latency (Theil et al., 2003a), and whether LAT, the only readily detectable transcript during latency, encodes for a protein is still a matter of debate (Henderson et al., 2009). The α-proteins ICP0 and ICP4 might be candidate antigens, though, as low level expression of these genes was detected in some latently HSV-1-infected human TG by Derfuss et al, 2007, as well as in the present study.

Future work might identify the antigenic epitopes for $CD8^+$ T cells in latently HSV-1-infected human TG. This could be done with human TG, obtained very shortly after death, and therefore containing viable T cells. Stable T-cells lines could be generated from these TG and used for an exact epitope mapping. Alternatively, tissue-infiltrating T cells in close contact with neurons could be excised from TG sections by laser microdissection with subsequent reconstitution of the TCR as described in (Seitz et al., 2006). The reconstituted TCR could then be used to screen for the antigen (Siewert et al., 2011). Identification of the TCR repertoire present in the TG could also clarify whether the reactivity of tissue-residing T cells differs from blood T cells. Further studies might also address changes in the human TCR repertoire occurring with ageing, and therefore prolonged exposure to latent HSV-1. It further remains to be resolved whether a single immuno-dominant epitope can be identified in humans or if – as is more likely – the epitopes vary, especially among subjects with different HLA-alleles.

4.2 Colocalisation of infiltrating T cells and latent HSV-1 in human TG

Since it was shown that several herpesviruses can establish latency in human TG (Liedtke et al., 1993) (Pevenstein et al., 1999) (Theil et al., 2003b) (Hüfner et al., 2007), it is important to determine which virus triggers the immune infiltration into the TG. So far, a strong correlation between HSV-1 latency and infiltrating T cells has been demonstrated for whole ganglia, and no co-occurrence of immune cells with VZV or HHV-6 was found (Theil et al., 2003a) (Hüfner et al., 2006) (Hüfner et al., 2007). It was further shown that T-cell infiltrates are localised in the same division of human TG as HSV-1 latently infected neurons (Hüfner et al., 2009). In humans, usually infected via the mouth, the mandibular and maxillary division were found to mostly harbour latent HSV-1 as well as T cells. In accordance with this, in mice infected with HSV-1 via the eye, a more prominent T-cell infiltration to the ophthalmic division was observed (Khanna et al., 2003). Reactivity of the $CD8^+$ T cells from human TG was demonstrated to HSV-1 but not VZV (Verjans et al., 2007), which may be the most convincing argument.

Surprisingly, it was noted that most T cells did not surround neurons that showed a positive staining signal in LAT *in situ* hybridisation (Theil et al., 2003a) (Derfuss et al., 2007). In the present study (chapter 3.2), a detailed quantification of this peculiarity was carried out by counting T-cell-neuron interaction patterns on TG sections co-stained for HSV-1 LAT and $CD3^+$ T cells. It was shown that 4.2% of all TG neurons were closely surrounded by T cells, with 95.5% of them negative for LAT by *in situ* hybridisation. 1.3% of all TG neurons were positive for LAT, with only 13.3% of LAT^+ neurons being surrounded by T cells. However, overall numbers of LAT^+ neurons still correlated with the overall number of neurons surrounded by T cells in the TG. Staining of consecutive sections confirmed the consistency of these results. The numbers of LAT^+ neurons in human TG are in line with the report of Wang *et al*, 2005a, who detected LAT expression in 0.2 to 1.5 % of human TG neurons.

There are several potential explanations for the three different T-cell-neuron interaction patterns observed. (I) In mice it has been established that several neurons harbour HSV-1 DNA, but do not accumulate LAT to detectable levels (Chen et al., 2002b). This was confirmed in a study using human TG (Wang et al., 2005a). We therefore hypothesised that the neurons surrounded by T cells but devoid of LAT, do actually contain HSV-1 DNA. (II) To attract $CD8^+$ T cells, viral proteins need to be expressed and presented on MHC class I. As mentioned above, viral LAT-derived microRNAs were shown to facilitate downregulation of ICP0 and ICP4 translation *in vitro* (Umbach et al., 2008). This led to the hypothesis that

differential expression of viral microRNAs might cause the different T-cell-neuron interaction patterns. In neurons with no detectable LAT expression, microRNA levels would be low, and therefore viral proteins might be minimally expressed, leading to the attraction of T cells. (III) In LAT expressing neurons not surrounded by T cells, microRNA levels seem to be sufficient to block viral protein synthesis.

To study why the infiltrating T cells are clustered around some neurons and not around others, TG sections were stained for LAT and CD3, and subsequently single neurons were excised using laser microdissection. Microdissected neurons were then analysed by PCR for the presence of viral DNA, HSV-1 microRNAs, and mRNA for the α-gene ICP0. Signs of the virus were only detected in neurons positive for LAT by *in situ* hybridisation, and neither the amount of viral DNA nor the amount of viral microRNAs correlated with the T-cell infiltrates. Furthermore, the T cells were also not attracted by HSV-2 or VZV, as neurons surrounded by T cells tested negative for these viruses by quantitative PCR.

The present study is the first to show that, in humans, all latently infected neurons do express LAT. This indicates that LAT plays a more important role in HSV-1 latency in humans than in small animal models of infection. The contradictive results to an earlier study in human TG (Wang et al., 2005a), which analysed the presence of HSV-1 and VZV DNA in human TG and found HSV-1 DNA also in LAT^- neurons and non-neuronal cells, might be explained by differences in the experimental procedures. Firstly, in the present study frozen TG were used, instead of paraffin fixed TG. Further, a different *in situ* hybridisation protocol and probe, as well as different quantitative PCR protocols, were used. The present use of a PCR kit approved for diagnostics should rule out unspecific positive results. Also, Wang *et al*, 2005a used a laser capture microdissection PixCell II microscope (Arcturus Engineering, Inc., USA) to excise single neurons, where a membrane is melted onto the neurons in question. This might lead to contaminations by nearby HSV-1 positive neurons. Here, laser catapulting microdissection (Zeiss, Germany) was used, thereby avoiding such contaminations. This might explain why Wang *et al*, 2005a found HSV-1 DNA also in non-neuronal cells. However, average HSV-1 copy numbers, as well as the observation that the majority of neurons do not harbour more than 20 HSV-1 DNA copies, are in accordance with the results of the work presented in this thesis. To ensure sensitivity of our quantitative PCR protocol, titration experiments were carried out, resulting in a recovery rate of 50% for one HSV-1 copy, diluted in DNA from HSV-1 negative TG.

Our observation that LAT is expressed in all latently HSV-1-infected TG neurons was also confirmed by F. Catez and P. Lomonte (personal communication). A double-fluorescent *in*

situ hybridisation for HSV-1 DNA and LAT on human TG sections did not show DNA^+ neurons that were negative for LAT.

One major question still remains. What actually attracts T cells to neurons devoid of LAT and viral DNA? It can be speculated that these neurons used to be latently infected by HSV-1 but the virus reactivated and was subsequently cleared by the immune system. As it is not known whether neurons die upon viral reactivation, or whether some viral genomes do remain latent in neurons after a reactivation event, this cannot be excluded. Moreover, it has been shown for HSV-2 that T cells remain in the skin long after infectious virus has been cleared (Gebhardt et al., 2009). The T cells surrounding LAT^- neurons could also be virus-unspecific bystander T cells that have been attracted by the inflammatory milieu. It also cannot be excluded that these neurons harbour very little viral DNA, which was below the detection limit of the PCR protocol applied here. $CD8^+$ T cells are able to react to such small numbers of antigen:MHC class I complexes as 1 to 3 on one cell (Sykulev et al., 1996) (Purbhoo et al., 2004). A study analysing the differences in the transcriptome of neurons surrounded by T cells versus neurons free of T cells might give further insight into this paradox. This might also resolve the question of whether MHC class I expression is upregulated in these neurons.

4.3 Model of latent HSV-1 infection in humans

The overall conclusion of this study is that the T cell population observed throughout human TG is triggered by latent HSV-1 infection. It was demonstrated that these T cells possess clonally expanded TCR β-chains. These clones were private to the assessed individuals as no T-cell clone occurred in different individuals. However, homologous and identical clones present in both TG of one individual were found. This indicates presence of the same T-cell antigen in both TG. The fact that T-cell clones identical even in their nucleotide sequence were found in both TG implies that these clones originate from the same T cell. This T cell was most probably primed in the periphery during primary HSV-1 infection. Upon arrival of the virus in the TG, some of these activated T-cell clones, along with other activated T cells, infiltrated into the tissue. Repeated recognition of their respective antigen resulted in proliferation and secretion of cytokines and chemokines, creating an inflammatory milieu that attracted more T cells, some of which could be unspecific (**Fig. 11**).

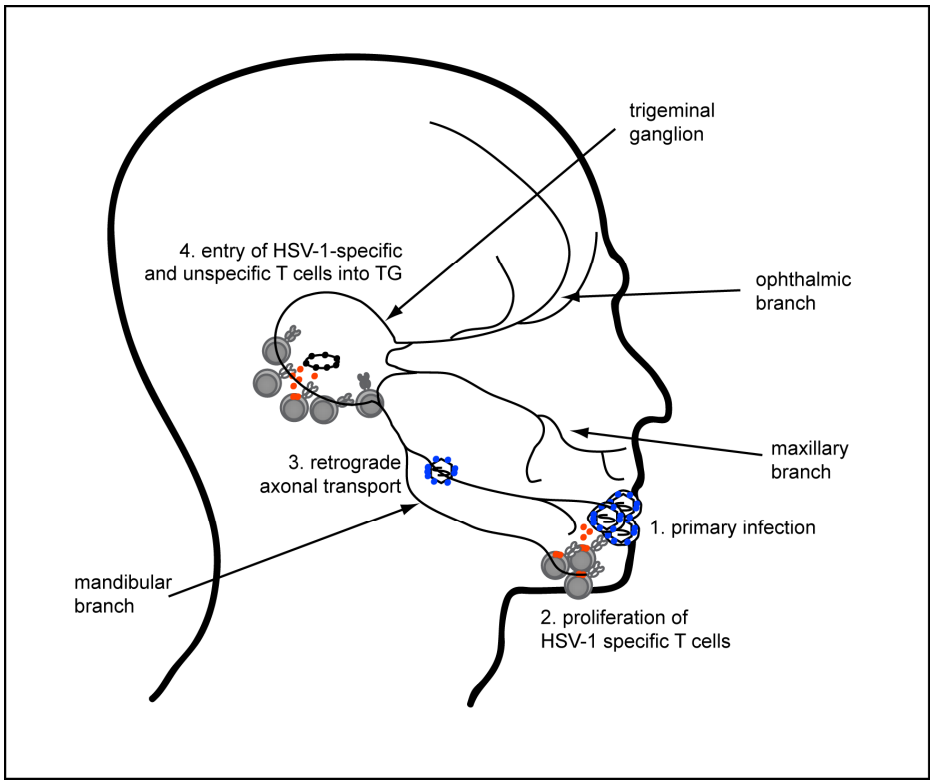

Fig. 11: Schematic model of the events after HSV-1 primary infection with special focus on T cells. Upon primary infection, HSV-1 proliferates in the mucosal and epithelial cells. HSV-1 specific $CD8^+$ T cells are activated and proliferate. After HSV-1 is transported via retrograde axonal transport to the TG, also activated virus-specific $CD8^+$ T cells enter the TG. Recognition of their respective antigens leads to secretion of cytokines and chemokines, resulting in the attraction of other activated T cells, some probably also unspecific.

An important finding of the current study is that LAT, as the only prominent transcript during latency, is expressed in all neurons containing HSV-1 DNA. LAT therefore seems to play a more crucial role in the maintenance of latency in humans than in small animal models of infection. An intra-species variation in the effect of LAT on spontaneous reactivation has already been shown (Hill et al., 1990) (Perng et al., 1994) (Margolis et al., 2007). This correlates with the ability of HSV-1 to reactivate spontaneously, which is a hallmark of human infection. Spontaneous reactivation is also more common in rabbits than in mice (Wagner and Bloom, 1997). Additionally, in the present study the LAT-derived microRNAs, as well as microRNA H6, were found to be present in all latently infected neurons. It was shown that microRNA H2-3p and microRNA H6 were downregulating ICP0 or ICP4 translation, respectively, *in vitro* (Umbach et al., 2008), thus ensuring that an interruption in viral gene repression does not lead to spontaneous reactivation.

Overall, the following model for the maintenance of HSV-1 latency can be envisioned: in most latently infected neurons, restriction of viral transcription by packaging of the viral genome into heterochromatin is sufficient to maintain latency. However, if transient instabilities in the heterochromatin occur, viral microRNAs encoded by the LAT locus are present to hamper transcription of viral α-proteins. The induction of full blown viral replication is thereby prevented (**Fig. 12A**). Triggers like stress might, however, induce changes in the chromatin structure of viral genomes that overrun these control mechanisms. Then the tissue-infiltrating $CD8^+$ T cells might step in and, via the secretion of IFN-γ and Granzyme B, hinder full blown reactivation of the virus (**Fig. 12B**).

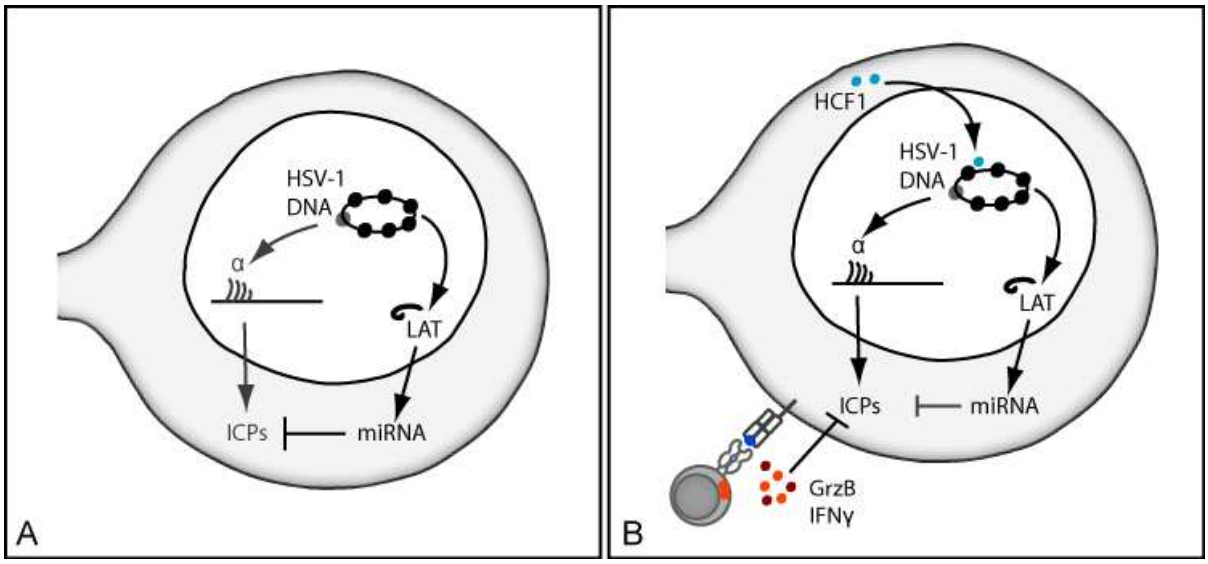

Fig. 12: Model of the maintenance of viral latency in neurons.

A: In most neurons latency is maintained by the combined actions of chromatin control and LAT.

B: Stress might lead to increased transcriptional activity within the neuron as well as translocation of HCF1 into the nucleus. This might overrun the capacity of microRNAs, and the $CD8^+$ T cells serve as a second line of defence, stopping viral replication in a non-cytolytic manner.

5 Bibliography

Adcock, IM (2000). Molecular mechanisms of glucocorticosteroid actions. Pulm. Pharmacol. Ther., 13, 115-126.

Arbusow, V, Derfuss, T, Held, K, Himmelein, S, Strupp, M, Gurkov, R, Brandt, T, Theil, D (2010). Latency of herpes simplex virus type-1 in human geniculate and vestibular ganglia is associated with infiltration of CD8+ T cells. J. Med. Virol., 82, 1917-1920.

Arden, B, Clark, SP, Kabelitz, D, Mak, TW (1995). Human T-cell receptor variable gene segment families. Immunogenetics, 42, 455-500.

Baines, JD, Roberts, KL (2011). Nuclear egress and envelopment of HSV. In Alphaherpesviruses: Molecular Virology. Weller, SK (ed). Caister Academic Press: Norfolk, UK, pp. 195-206.

Baringer, JR, Swoveland, P (1973). Recovery of herpes-simplex virus from human trigeminal ganglions. N. Engl. J. Med., 288, 648-650.

Bonneau, RH (1996). Stress-induced effects on integral immune components involved in herpes simplex virus (HSV)-specific memory cytotoxic T lymphocyte activation. Brain Behav. Immun., 10, 139-163.

Cantin, E, Tanamachi, B, Openshaw, H (1999). Role for gamma interferon in control of herpes simplex virus type 1 reactivation. J Virol., 73, 3418-3423.

Cantin, EM, Hinton, DR, Chen, J, Openshaw, H (1995). Gamma interferon expression during acute and latent nervous system infection by herpes simplex virus type 1. J Virol., 69, 4898-4905.

Carr, DJ, Halford, WP, Veress, LA, Noisakran, S, Perng, GC, Wechsler, SL (1998). The persistent elevated cytokine mRNA levels in trigeminal ganglia of mice latently infected with HSV-1 are not due to the presence of latency associated transcript (LAT) RNAs. Virus Res., 54, 1-8.

Chaplin, DD (2010). Overview of the immune response. J. Allergy Clin. Immunol., 125, S3-S23.

Chen, SH, Kramer, MF, Schaffer, PA, Coen, DM (1997). A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. J Virol., 71, 5878-5884.

Chen, SH, Lee, LY, Garber, DA, Schaffer, PA, Knipe, DM, Coen, DM (2002a). Neither LAT nor open reading frame P mutations increase expression of spliced or intron-containing ICP0 transcripts in mouse ganglia latently infected with herpes simplex virus. J. Virol., 76, 4764-4772.

Chen, XP, Mata, M, Kelley, M, Glorioso, JC, Fink, DJ (2002b). The relationship of herpes simplex virus latency associated transcript expression to genome copy number: a quantitative study using laser capture microdissection. J. Neurovirol., 8, 204-210.

Clevers, H, Alarcon, B, Wileman, T, Terhorst, C (1988). The T cell receptor/CD3 complex: a dynamic protein ensemble. Annu. Rev. Immunol., 6, 629-662.

Cotter, CR, Blaho, JA (2011). Apoptosis modulation during Herpes Simplex Virus Replication. In Alphaherpesviruses: Molecular Virology. Weller, SK (ed). Caister Academic Press: Norfolk, UK, pp. 207-218.

Croen, KD, Ostrove, JM, Dragovic, LJ, Smialek, JE, Straus, SE (1987). Latent herpes simplex virus in human trigeminal ganglia. Detection of an immediate early gene "anti-sense" transcript by in situ hybridization. N. Engl. J Med., 317, 1427-1432.

Cui, C, Griffiths, A, Li, G, Silva, LM, Kramer, MF, Gaasterland, T, Wang, XJ, Coen, DM (2006). Prediction and identification of herpes simplex virus 1-encoded microRNAs. J Virol., 80, 5499-5508.

Decman, V, Kinchington, PR, Harvey, SA, Hendricks, RL (2005). Gamma interferon can block herpes simplex virus type 1 reactivation from latency, even in the presence of late gene expression. J Virol., 79, 10339-10347.

Deluca, NA (2011). Functions and mechanism of action of the herpes simplex virus regulatory protein, ICP4. In Alphaherpesviruses: Molecular Virology. Weller, SK (ed). Caister Academic Press: Norfolk, UK, pp. 17-38.

Derfuss, T, Segerer, S, Herberger, S, Sinicina, I, Hufner, K, Ebelt, K, Knaus, HG, Steiner, I, Meinl, E, Dornmair, K, Arbusow, V, Strupp, M, Brandt, T, Theil, D (2007). Presence of HSV-1 immediate early genes and clonally expanded T-cells with a memory effector phenotype in human trigeminal ganglia. Brain Pathol, 17, 389-398.

Deshmane, SL, Fraser, NW (1989). During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J. Virol., 63, 943-947.

Dong, L, Li, P, Oenema, T, McClurkan, CL, Koelle, DM (2010). Public TCR use by herpes simplex virus-2-specific human CD8 CTLs. J. Immunol., 184, 3063-3071.

Du, T, Zhou, G, Roizman, B (2011). HSV-1 gene expression from reactivated ganglia is disordered and concurrent with suppression of latency-associated transcript and miRNAs. Proc. Natl. Acad. Sci. U. S. A, 108, 18820-18824.

Efstathiou, S, Minson, AC, Field, HJ, Anderson, JR, Wildy, P (1986). Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J. Virol., 57, 446-455.

Eisenberg, RJ, Heldwein, EE, Cohen, GH, Krummenacher, C (2011). Recent progress in understanding herpes simplex virus entry: relationship of structure to function entry. In Alphaherpesviruses: Molecular Virology. Weller, SK (ed). Caister Academic Press: Norfolk, UK, pp. 131-152.

Ellison, AR, Yang, L, Voytek, C, Margolis, TP (2000). Establishment of latent herpes simplex virus type 1 infection in resistant, sensitive, and immunodeficient mouse strains. Virology, 268, 17-28.

Esaki, S, Goshima, F, Katsumi, S, Watanabe, D, Ozaki, N, Murakami, S, Nishiyama, Y (2010). Apoptosis induction after herpes simplex virus infection differs according to cell type in vivo. Arch Virol, 155, 1235-1245.

Everett, RD (2011). The role of ICP0 in counteracting intrinsic cellular resistance to virus infection. In Alphaherpesviruses: Molecular Virology. Weller, SK (ed). Caister Academic Press: Norfolk, UK, pp. 51-71.

Feldman, LT, Ellison, AR, Voytek, CC, Yang, L, Krause, P, Margolis, TP (2002). Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. Proc. Natl. Acad. Sci. U. S. A, 99, 978-983.

Freeman, ML, Sheridan, BS, Bonneau, RH, Hendricks, RL (2007). Psychological stress compromises CD8+ T cell control of latent herpes simplex virus type 1 infections. J Immunol, 179, 322-328.

Gebhardt, T, Wakim, LM, Eidsmo, L, Reading, PC, Heath, WR, Carbone, FR (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. Nat. Immunol, 10, 524-530.

Germain, RN (1994). MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell, 76, 287-299.

Halford, WP, Gebhardt, BM, Carr, DJ (1996). Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. J Immunol, 157, 3542-3549.

Halford, WP, Schaffer, PA (2001). ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. J Virol., 75, 3240-3249.

Halford, WP, Weisend, C, Grace, J, Soboleski, M, Carr, DJ, Balliet, JW, Imai, Y, Margolis, TP, Gebhardt, BM (2006). ICP0 antagonizes Stat 1-dependent repression of herpes simplex virus: implications for the regulation of viral latency. Virol. J, 3, 44-67.

Hamza, M. A., Higgins, D. M., Feldman, L. T., and Ruyechan, W. T. (2007) The latency-associated transcript of herpes simplex virus type 1 promotes survival and stimulates axonal regeneration in sympathetic and trigeminal neurons. J.Neurovirol., 13, 56-66

Heath, WR, Carbone, FR (2001). Cross-presentation in viral immunity and self-tolerance. Nat. Rev. Immunol., 1, 126-134.

Henderson, G, Jaber, T, Carpenter, D, Wechsler, SL, Jones, C (2009). Identification of herpes simplex virus type 1 proteins encoded within the first 1.5 kb of the latency-associated transcript. J. Neurovirol., 15, 439-448.

Henderson, G, Peng, W, Jin, L, Perng, GC, Nesburn, AB, Wechsler, SL, Jones, C (2002). Regulation of caspase 8- and caspase 9-induced apoptosis by the herpes simplex virus type 1 latency-associated transcript. J Neurovirol., 8 Suppl 2, 103-111.

Hill, A, Jugovic, P, York, I, Russ, G, Bennink, J, Yewdell, J, Ploegh, H, Johnson, D (1995). Herpes simplex virus turns off the TAP to evade host immunity. Nature, 375, 411-415.

Hill, JM, Sedarati, F, Javier, RT, Wagner, EK, Stevens, JG (1990). Herpes simplex virus latent phase transcription facilitates in vivo reactivation. Virology, 174, 117-125.

Himmelein, S, St Leger, AJ, Knickelbein, JE, Rowe, A, Freeman, ML, Hendricks, RL (2011). Circulating herpes simplex type 1 (HSV-1)-specific CD8+ T cells do not access HSV-1 latently infected trigeminal ganglia. Herpesviridae., 2, 5-15.

Hüfner, K, Arbusow, V, Himmelein, S, Derfuss, T, Sinicina, I, Strupp, M, Brandt, T, Theil, D (2007). The prevalence of human herpesvirus 6 in human sensory ganglia and its co-occurrence with alpha-herpesviruses. J Neurovirol., 13, 462-467.

Hüfner, K, Derfuss, T, Herberger, S, Sunami, K, Russell, S, Sinicina, I, Arbusow, V, Strupp, M, Brandt, T, Theil, D (2006). Latency of alpha-herpes viruses is accompanied by a chronic inflammation in human trigeminal ganglia but not in dorsal root ganglia. J Neuropathol. Exp. Neurol., 65, 1022-1030.

Hüfner, K, Horn, A, Derfuss, T, Glon, C, Sinicina, I, Arbusow, V, Strupp, M, Brandt, T, Theil, D (2009). Fewer latent HSV-1 and cytotoxic T cells occur in the ophthalmic division than in the maxillary and mandibular divisions of the human trigeminal ganglion and nerve. J Virol., 83, 3696-3703.

Jiang, X, Chentoufi, AA, Hsiang, C, Carpenter, D, Osorio, N, Benmohamed, L, Fraser, NW, Jones, C, Wechsler, SL (2011). The herpes simplex virus type 1 latency-associated transcript can protect neuron-derived C1300 and Neuro2A cells from Granzyme B-induced apoptosis and CD8 T-cell killing. J. Virol., 85, 2325-2332.

Junker, A, Ivanidze, J, Malotka, J, Eiglmeier, I, Lassmann, H, Wekerle, H, Meinl, E, Hohlfeld, R, Dornmair, K (2007). Multiple sclerosis: T-cell receptor expression in distinct brain regions. Brain, 130, 2789-2799.

Jurak, I, Kramer, MF, Mellor, JC, van Lint, AL, Roth, FP, Knipe, DM, Coen, DM (2010). Numerous conserved and divergent microRNAs expressed by herpes simplex viruses 1 and 2. J Virol., 84, 4659-4672.

Karrer, U, Mekker, A, Wanke, K, Tchang, V, Haeberli, L (2009). Cytomegalovirus and immune senescence: culprit or innocent bystander? Exp. Gerontol., 44, 689-694.

Khanna, KM, Bonneau, RH, Kinchington, PR, Hendricks, RL (2003). Herpes simplex virus-specific memory CD8+ T cells are selectively activated and retained in latently infected sensory ganglia. Immunity., 18, 593-603.

Knickelbein, JE, Khanna, KM, Yee, MB, Baty, CJ, Kinchington, PR, Hendricks, RL (2008). Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. Science, 322, 268-271.

Knipe, DM, Cliffe, A (2008). Chromatin control of herpes simplex virus lytic and latent infection. Nat. Rev. Microbiol., 6, 211-221.

Kramer, MF, Coen, DM (1995). Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. J Virol., 69, 1389-1399.

Kramer, MF, Jurak, I, Pesola, JM, Boissel, S, Knipe, DM, Coen, DM (2011). Herpes simplex virus 1 microRNAs expressed abundantly during latent infection are not essential for latency in mouse trigeminal ganglia. Virology, 417, 239-247.

Krangel, MS (2009). Mechanics of T cell receptor gene rearrangement. Curr. Opin. Immunol., 21, 133-139.

Lefranc, M-P, Lafranc, G (2001). The T-cell receptor FactsBook. Academic Press: London.

Liedtke, W, Opalka, B, Zimmermann, CW, Lignitz, E (1993). Age distribution of latent herpes simplex virus 1 and varicella-zoster virus genome in human nervous tissue. J. Neurol. Sci., 116, 6-11.

Lin, J, Weiss, A (2001). T cell receptor signalling. J. Cell Sci., 114, 243-244.

Liu, T, Khanna, KM, Chen, X, Fink, DJ, Hendricks, RL (2000). CD8(+) T cells can block herpes simplex virus type 1 (HSV-1) reactivation from latency in sensory neurons. J Exp. Med., 191, 1459-1466.

Liu, T, Tang, Q, Hendricks, RL (1996). Inflammatory infiltration of the trigeminal ganglion after herpes simplex virus type 1 corneal infection. J Virol., 70, 264-271.

Maillet, S, Naas, T, Crepin, S, Roque-Afonso, AM, Lafay, F, Efstathiou, S, Labetoulle, M (2006). Herpes simplex virus type 1 latently infected neurons differentially express latency-associated and ICP0 transcripts. J Virol., 80, 9310-9321.

Margolis, TP, Elfman, FL, Leib, D, Pakpour, N, Apakupakul, K, Imai, Y, Voytek, C (2007). Spontaneous reactivation of herpes simplex virus type 1 in latently infected murine sensory ganglia. J Virol., 81, 11069-11074.

Mark, KE, Wald, A, Magaret, AS, Selke, S, Olin, L, Huang, ML, Corey, L (2008). Rapidly cleared episodes of herpes simplex virus reactivation in immunocompetent adults. J Infect. Dis., 198, 1141-1149.

Mehta, A, Maggioncalda, J, Bagasra, O, Thikkavarapu, S, Saikumari, P, Valyi-Nagy, T, Fraser, NW, Block, TM (1995). In situ DNA PCR and RNA hybridization detection of herpes simplex virus sequences in trigeminal ganglia of latently infected mice. Virology, 206, 633-640.

Mikloska, Z, Cunningham, AL (1998). Herpes simplex virus type 1 glycoproteins gB, gC and gD are major targets for CD4 T-lymphocyte cytotoxicity in HLA-DR expressing human epidermal keratinocytes. J. Gen. Virol., 79 (Pt 2), 353-361.

Miles, JJ, Douek, DC, Price, DA (2011). Bias in the alphabeta T-cell repertoire: implications for disease pathogenesis and vaccination. Immunol. Cell Biol., 89, 375-387.

Modrow, S, Falke, D, Truyen, U (2010). Molekulare Virologie. Spektrum: Heidelberg.

Montgomerie, JZ, Becroft, DM, Croxson, MC, Doak, PB, North, JD (1969). Herpes-simplex-virus infection after renal transplantation. Lancet, 2, 867-871.

Murphy, K, Travers, P, Walport, M (2008). Janeway's Immunobiology. Garland Science Publishing.

Naraqi, S, Jackson, GG, Jonasson, O, Yamashiroya, HM (1977). Prospective study of prevalence, incidence, and source of herpesvirus infections in patients with renal allografts. J. Infect. Dis., 136, 531-540.

Neumann, H, Cavalie, A, Jenne, DE, Wekerle, H (1995). Induction of MHC class I genes in neurons. Science, 269, 549-552.

Orr, MT, Mathis, MA, Lagunoff, M, Sacks, JA, Wilson, CB (2007). CD8 T cell control of HSV reactivation from latency is abrogated by viral inhibition of MHC class I. Cell Host. Microbe, 2, 172-180.

Padgett, DA, Sheridan, JF, Dorne, J, Berntson, GG, Candelora, J, Glaser, R (1998). Social stress and the reactivation of latent herpes simplex virus type 1. Proc. Natl. Acad. Sci. U. S. A, 95, 7231-7235.

Pereira, RA, Tscharke, DC, Simmons, A (1994). Upregulation of class I major histocompatibility complex gene expression in primary sensory neurons, satellite cells, and Schwann cells of mice in response to acute but not latent herpes simplex virus infection in vivo. J Exp. Med., 180, 841-850.

Perng, GC, Dunkel, EC, Geary, PA, Slanina, SM, Ghiasi, H, Kaiwar, R, Nesburn, AB, Wechsler, SL (1994). The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency. J. Virol., 68, 8045-8055.

Perng, GC, Jones, C (2010). Towards an understanding of the herpes simplex virus type 1 latency-reactivation cycle. Interdiscip. Perspect. Infect. Dis., 2010, 262415.

Perng, GC, Jones, C, Ciacci-Zanella, J, Stone, M, Henderson, G, Yukht, A, Slanina, SM, Hofman, FM, Ghiasi, H, Nesburn, AB, Wechsler, SL (2000). Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. Science, 287, 1500-1503.

Pevenstein, SR, Williams, RK, McChesney, D, Mont, EK, Smialek, JE, Straus, SE (1999). Quantitation of latent varicella-zoster virus and herpes simplex virus genomes in human trigeminal ganglia. J. Virol., 73, 10514-10518.

Purbhoo, MA, Irvine, DJ, Huppa, JB, Davis, MM (2004). T cell killing does not require the formation of a stable mature immunological synapse. Nat. Immunol., 5, 524-530.

Rajcani, J, Andrea, V, Ingeborg, R (2004). Peculiarities of herpes simplex virus (HSV) transcription: an overview. Virus Genes, 28, 293-310.

Roizman, B (2011). The checkpoints of viral gene expression in productive and latent infection: the role of the HDAC/CoREST/LSD1/REST repressor complex. J. Virol., 85, 7474-7482.

Rudolph, MG, Stanfield, RL, Wilson, IA (2006). How TCRs bind MHCs, peptides, and coreceptors. Annu. Rev. Immunol, 24, 419-466.

Russell, JH, Ley, TJ (2002). Lymphocyte-mediated cytotoxicity. Annu. Rev. Immunol., 20, 323-370.

Sainz, B, Loutsch, JM, Marquart, ME, Hill, JM (2001). Stress-associated immunomodulation and herpes simplex virus infections. Med. Hypotheses, 56, 348-356.

Sakaguchi, S, Wing, K, Onishi, Y, Prieto-Martin, P, Yamaguchi, T (2009). Regulatory T cells: how do they suppress immune responses? Int. Immunol., 21, 1105-1111.

Scotet, E, Peyrat, MA, Saulquin, X, Retiere, C, Couedel, C, Davodeau, F, Dulphy, N, Toubert, A, Bignon, JD, Lim, A, Vie, H, Hallet, MM, Liblau, R, Weber, M, Berthelot, JM, Houssaint, E, Bonneville, M (1999). Frequent enrichment for CD8 T cells reactive against common herpes viruses in chronic inflammatory lesions: towards a reassessment of the physiopathological significance of T cell clonal expansions found in autoimmune inflammatory processes. Eur. J. Immunol., 29, 973-985.

Seitz, S, Schneider, CK, Malotka, J, Nong, X, Engel, AG, Wekerle, H, Hohlfeld, R, Dornmair, K (2006). Reconstitution of paired T cell receptor alpha- and beta-chains from microdissected single cells of human inflammatory tissues. Proc. Natl. Acad. Sci. U. S. A, 103, 12057-12062.

Sheridan, BS, Cherpes, TL, Urban, J, Kalinski, P, Hendricks, RL (2009). Reevaluating the CD8 T-cell response to herpes simplex virus type 1: involvement of CD8 T cells reactive to subdominant epitopes. J Virol., 83, 2237-2245.

Shimeld, C, Whiteland, JL, Nicholls, SM, Grinfeld, E, Easty, DL, Gao, H, Hill, TJ (1995). Immune cell infiltration and persistence in the mouse trigeminal ganglion after infection of the cornea with herpes simplex virus type 1. J Neuroimmunol., 61, 7-16.

Siewert, K, Malotka, J, Kawakami, N, Wekerle, H, Hohlfeld, R, Dornmair, K (2011). Unbiased identification of target antigens of CD8+ T cells with combinatorial libraries coding for short peptides. Nat. Med., in print.

Simmons, A, Tscharke, DC (1992). Anti-CD8 impairs clearance of herpes simplex virus from the nervous system: implications for the fate of virally infected neurons. J. Exp. Med., 175, 1337-1344.

Skulina, C, Schmidt, S, Dornmair, K, Babbe, H, Roers, A, Rajewsky, K, Wekerle, H, Hohlfeld, R, Goebels, N (2004). Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. Proc. Natl. Acad. Sci. U. S. A, 101, 2428-2433.

St Leger, AJ, Peters, B, Sidney, J, Sette, A, Hendricks, RL (2011). Defining the herpes simplex virus-specific CD8+ T cell repertoire in C57BL/6 mice. J. Immunol., 186, 3927-3933.

Stevens, JG, Wagner, EK, vi-Rao, GB, Cook, ML, Feldman, LT (1987). RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science, 235, 1056-1059.

Sykulev, Y, Joo, M, Vturina, I, Tsomides, TJ, Eisen, HN (1996). Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. Immunity., 4, 565-571.

Tal-Singer, R, Lasner, TM, Podrzucki, W, Skokotas, A, Leary, JJ, Berger, SL, Fraser, NW (1997). Gene expression during reactivation of herpes simplex virus type 1 from latency in the peripheral nervous system is different from that during lytic infection of tissue cultures. J. Virol., 71, 5268-5276.

Theil, D, Derfuss, T, Paripovic, I, Herberger, S, Meinl, E, Schueler, O, Strupp, M, Arbusow, V, Brandt, T (2003a). Latent herpesvirus infection in human trigeminal ganglia causes chronic immune response. Am J Pathol, 163, 2179-2184.

Theil, D, Paripovic, I, Derfuss, T, Herberger, S, Strupp, M, Arbusow, V, Brandt, T (2003b). Dually infected (HSV-1/VZV) single neurons in human trigeminal ganglia. Ann. Neurol., 54, 678-682.

Thompson, RL, Preston, CM, Sawtell, NM (2009). De novo synthesis of VP16 coordinates the exit from HSV latency in vivo. PLoS. Pathog., 5, e1000352.

Thompson, RL, Sawtell, NM (1997). The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. J. Virol., 71, 5432-5440.

Thompson, RL, Sawtell, NM (2006). Evidence that the herpes simplex virus type 1 ICP0 protein does not initiate reactivation from latency in vivo. J Virol., 80, 10919-10930.

Umbach, JL, Kramer, MF, Jurak, I, Karnowski, HW, Coen, DM, Cullen, BR (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. Nature, 454, 780-783.

Umbach, JL, Nagel, MA, Cohrs, RJ, Gilden, DH, Cullen, BR (2009). Analysis of human {alpha}-herpesvirus microRNA expression in latently infected human trigeminal ganglia. J Virol., 83, 10677-10683.

van Lint, AL, Kleinert, L, Clarke, SR, Stock, A, Heath, WR, Carbone, FR (2005). Latent infection with herpes simplex virus is associated with ongoing CD8+ T-cell stimulation by parenchymal cells within sensory ganglia. J Virol., 79, 14843-14851.

van Velzen, M, Laman, JD, Kleinjan, A, Poot, A, Osterhaus, AD, Verjans, GM (2009). Neuron-interacting satellite glial cells in human trigeminal ganglia have an APC phenotype. J Immunol, 183, 2456-2461.

Venturi, V, Chin, HY, Asher, TE, Ladell, K, Scheinberg, P, Bornstein, E, van Bockel, D, Kelleher, AD, Douek, DC, Price, DA, Davenport, MP (2008). TCR beta-chain sharing in human CD8+ T cell responses to cytomegalovirus and EBV. J. Immunol., 181, 7853-7862.

Verjans, GM, Hintzen, RQ, van Dun, JM, Poot, A, Milikan, JC, Laman, JD, Langerak, AW, Kinchington, PR, Osterhaus, AD (2007). Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. Proc. Natl. Acad. Sci. U. S. A, 104, 3496-3501.

Wagner, EK, Bloom, DC (1997). Experimental investigation of herpes simplex virus latency. Clin. Microbiol. Rev., 10, 419-443.

Wang, K, Lau, TY, Morales, M, Mont, EK, Straus, SE (2005a). Laser-capture microdissection: refining estimates of the quantity and distribution of latent herpes simplex virus 1 and varicella-zoster virus DNA in human trigeminal ganglia at the single-cell level. J Virol., 79, 14079-14087.

Wang, QY, Zhou, C, Johnson, KE, Colgrove, RC, Coen, DM, Knipe, DM (2005b). Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proc. Natl. Acad. Sci. U. S. A, 102, 16055-16059.

Ward, SA, Weller, SK (2011). HSV-1 DNA replication. In Alphaherpesviruses: Molecular Virology. Weller, SK (ed). Caister Academic Press: Norfolk, UK, pp. 89-112.

Whitley, RJ, Roizman, B (2001). Herpes simplex virus infections. Lancet, 357, 1513-1518.

Zhang, CX, Ofiyai, H, He, M, Bu, X, Wen, Y, Jia, W (2005). Neuronal activity regulates viral replication of herpes simplex virus type 1 in the nervous system. J. Neurovirol., 11, 256-264.

6 Abbreviations

APC	antigen-presenting cell
CDR	complementarity determining region
ds	double-stranded
EBV	Epstein-Barr virus
ER	endoplasmic reticulum
g	glycoproteins
HCF1	host cell factor 1
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICP	infected cell protein
IFN	interferon
kb	kilo base pairs = 1,000 base pairs
LAT	latency associated transcript
MHC	major histocompatibility complex
n	number
Oct1	octamer binding protein 1
ORF	open reading frame
ori	origin of replication
SEM	standard error of the mean
TAP	transporter associated with antigen processing
TCR	T-cell receptor
TG	trigeminal ganglia
TNF	tumour necrosis factor
vhs	virus host shutoff
VZV	varicella-zoster virus

7 List of publications

Included in this thesis:

Held K, Junker A, Dornmair K, Meinl E, Sinicina I, Brandt T, Theil D, Derfuss T (2011) Expression of herpes simplex virus 1-encoded microRNAs in human trigeminal ganglia and their relation to local T-cell infiltrates. J Virol., 85, 9680-9685

Held K, Eiglmeier I, Himmelein S, Sinicina I, Brandt T, Theil D, Dornmair K, Derfuss T (2012) Clonal expansions of CD8+ T-cells in latently HSV-1-infected human trigeminal ganglia. J Neurovirol., 18, 62-68

Not included in this thesis:

Held K, Thiel S, Loos M, Petry F (2008) Increased susceptibility of complement factor B/C2 double knockout mice and mannan-binding lectin knowout mice to systemic infection with Candida albicans. Mol Immunol., 45, 3934-3941

Arbusow V, Derfuss T, **Held K**, Himmelein S, Strupp M, Gurkov R, Brandt T, Theil D (2010) Latency of herpes simplex virus type-1 in human geniculate and vestibular ganglia is associated with infiltration of CD8+ T cells. J Med Virol., 82, 1917-1920

Held K, Derfuss T. (2011) Control of HSV-1 latency in human Trigeminal Ganglia - current overview. J Neurovirol., 17, 518-27 (Review)

Kim SM, Bhonsle L, Besgen P, Nickel J, Backes A, **Held K**, Vollmer S, Dornmair K, Prinz JC (2012) Analysis of the paired TCR α- and β-chains of single human T cells. In preparation

8 Zusammenfassung

Das Herpes-Simplex-Virus Typ 1 (HSV-1) ist ein doppel-strängiges DNA Virus, welches Menschen infiziert und nach einer primären lytischen Infektion eine lebenslange Latenz in den sensorischen Neuronen der Trigeminus-Ganglien (TG) etabliert. Die HSV-1 Latenz wird von einer chronischen Immunzellinfiltration begleitet, welche hauptsächlich aus $CD8^+$ T-Zellen besteht. Es wird angenommen, dass diese T-Zellen die virale Latenz kontrollieren. Jedoch scheinen auch zelluläre und virale Faktoren, wie zum Beispiel virale microRNAs eine zentrale Rolle in der Etablierung und dem Erhalt der Latenz zu spielen.

In der vorliegenden Arbeit wurde untersucht, ob die in die TG eingewanderten T-Zellen klonal expandiert sind. Dies würde bedeuten, dass sie durch ein Antigen aktiviert wurden. Unter der Anwendung von CDR3-Spektratypisierung, einer Methode welche die Längenverteilung der hypervariablen Region 3 des T-Zell-Rezeptors analysiert, sowie Immunhistochemie, konnten klonale Expansionen bei den T-Zellen in den TG nachgewiesen werden, wovon einige unspezifische „bystander" T-Zellen zu sein schienen. Bemerkenswerterweise wurden einige identische T-Zell-Klone im rechten und linken TG eines Individuums gefunden, was auf die Anwesenheit gleicher Antigene in beiden TG schließen lässt. Außerdem kann daher angenommen werden, dass eine Immunzellinfiltration in die TG durch Antigene ausgelöst wird.

Die Morphologie der TG wurde mittels Immunhistochemie und In-situ-Hybridisierung untersucht. Die Analyse der Verteilung der T-Zellen im TG führte zu unerwarteten Ergebnissen. Die meisten der von T-Zellen umgebenen Neurone enthielten kein Latenz-assoziiertes Transkript (LAT), welches das einzige bekannte häufig transkribierte Genprodukt der viralen Latenz darstellt. Eine Kombination aus LAT In-situ-Hybridisierung, T-Zell Immunhistochemie und PCR-Analyse von einzelnen, durch Lasermikrodissektion gewonnenen Neurone sollte Aufschluss darüber geben, ob diese Neurone tatsächlich Viren enthalten. Diese Analyse zeigte, dass nur LAT^+ Neurone HSV-1 DNA oder virale microRNAs enthielten. So konnte auch mRNA für ein virales Genprodukt nur in LAT^+ Neuronen nachgewiesen werden. In keinem der hier analysierten LAT^- Neurone konnten virale microRNAs sowie HSV-1 DNA nachgewiesen werden. DNA für HSV-2 oder Varizella-Zoster-Virus (VZV) war in keinem der untersuchten einzelnen Neurone vorhanden. Zusammengefasst lässt sich für den Menschen sagen, dass in der Mehrheit der infizierten Neurone die HSV-1 Latenz nicht direkt durch T-Zellen kontrolliert wird, sondern eher durch zelluläre oder virale Faktoren wie zum Beispiel die viralen microRNAs. Unsere Daten lassen darauf schließen, dass $CD8^+$ T-Zellen nur dann in Aktion zu treten scheinen, wenn diese Faktoren nicht mehr ausreichen.

9 Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 25.04.2012.....Kathrin Held.....
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Erklärung

Hiermit erkläre ich, *

☒ dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

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*) Nichtzutreffendes streichen

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11 Curriculum vitae

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Publikationen

Held K, Thiel S, Loos M, Petry F (2008) Increased susceptibility of complement factor B/C2 double knockout mice and mannan-binding lectin knowout mice to systemic infection with Candida albicans. Mol Immunol., 45, 3934-3941

Arbusow V, Derfuss T, **Held K**, Himmelein S, Strupp M, Gurkov R, Brandt T, Theil D (2010) Latency of herpes simplex virus type-1 in human geniculate and vestibular ganglia is associated with infiltration of CD8+ T cells. J Med Virol., 82, 1917-1920

Held K, Junker A, Dornmair K, Meinl E, Sinicina I, Brandt T, Theil D, Derfuss T (2011) Expression of herpes simplex virus 1-encoded microRNAs in human trigeminal ganglia and their relation to local T-cell infiltrates. J Virol., 85, 9680-9685

Held K, Derfuss T. (2011) Control of HSV-1 latency in human Trigeminal Ganglia - current overview. J Neurovirol., 17, 518-27 (Review)

Held K, Eiglmeier I, Himmelein S, Sinicina I, Brandt T, Theil D, Dornmair K, Derfuss T (2012) Clonal expansions of CD8+ T-cells in latently HSV-1-infected human trigeminal ganglia. J Neurovirol., 18, 62-68

Kim SM, Bhonsle L, Besgen P, Nickel J, Backes A, **Held K**, Vollmer S, Dornmair K, Prinz JC (2012) Analysis of the paired TCR α- and β-chains of single human T cells. In preparation

Kongresse

09/2007	37. Jahrestagung der Deutschen Gesellschaft für Immunologie, Heidelberg Poster: “Susceptibility of complement deficient mouse strains to systemic infection with Candida albicans” (Posterpreis)
08/2010	35th Annual International Herpesvirus Workshop, Salt Lake City Poster und Vortrag: “HSV-1 microRNA expression at the single cell level in human trigeminal ganglia” (Reisestipendium)
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