Herpes Simplex Virus Type 1 Infection:

Features of immune surveillance and antiviral resistance

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Aspecten van immuun controle en antivirale resistantie

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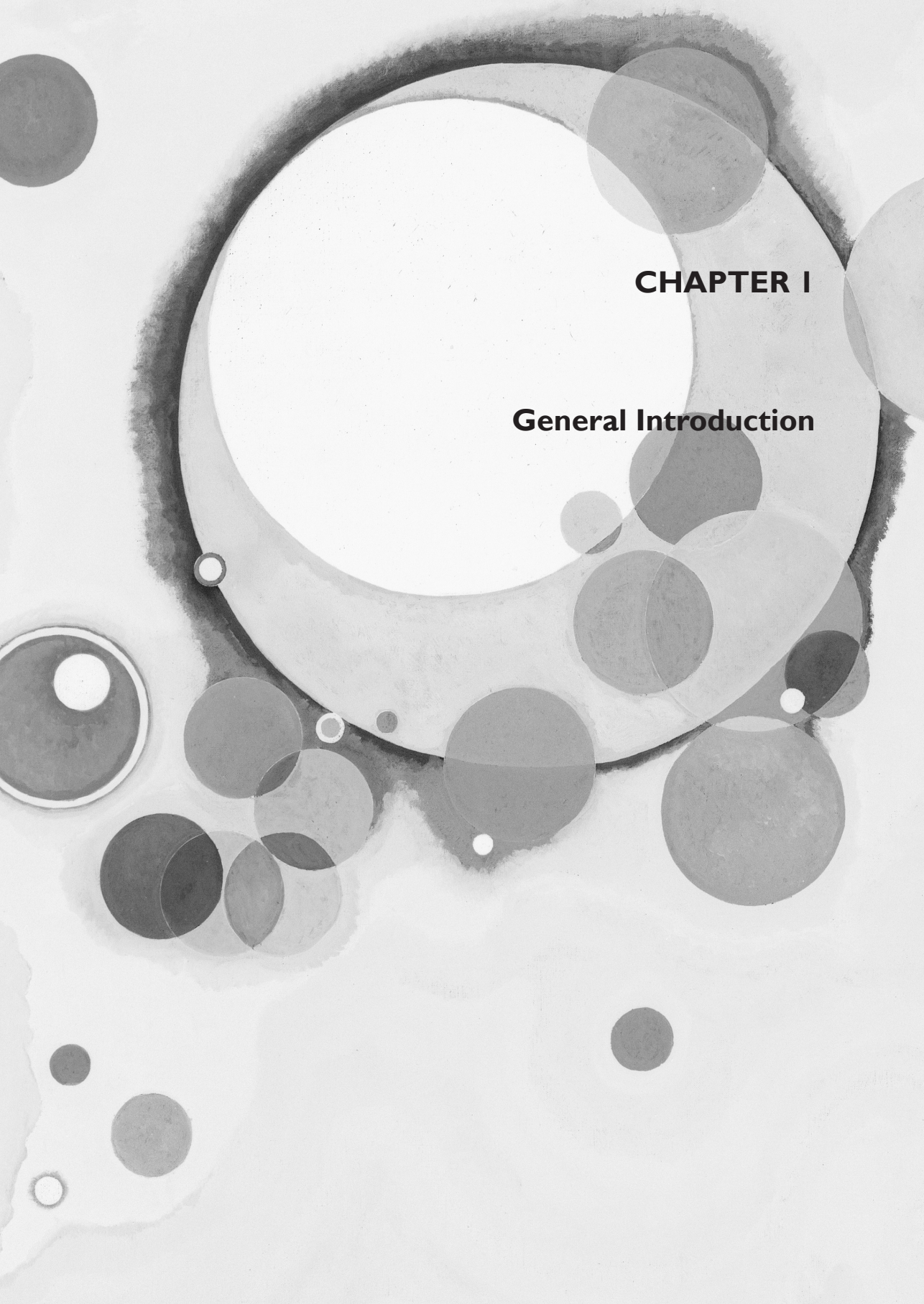

CHAPTER 1

General Introduction

Human herpesviruses

The family of Herpesviridae is highly disseminated in the animal world, with a wide range of host species. Because most animal species have at least one herpesvirus, the number of herpesviruses in nature is likely to exceed by far the 138 currently identified members [1-2]. To date, eight human herpesviruses have been identified: human herpesvirus 1 to 8 (HHV1-HHV8). They are classified on basis of their cell tropism and genome organization into subfamilies of Alpha-, Beta-, and Gammaherpesvirinae (Table 1) [2]. In addition to causing productive infections, a key feature of herpesviruses is their ability to establish latency. This non-productive phase lasts for the life time of the host, is characterized by a silent viral infection with intermittent reactivations, commonly clinical unapparent, resulting in intermittent shedding of infectious virus and spread throughout the population [2].

Alphaherpesvirinae members such as herpes simplex virus type 1 (HSV-1) have a relatively short replication cycle, rapidly destruct infected cells, and establish latency in sensory neurons. Members of the Betaherpesvirinae subfamily replicate more slowly and are largely cell-associated. Cytomegalovirus (CMV) establishes latent infections in epithelial cells of the secretory glands and kidneys. Gammaherpesvirinae members predominantly infect lymphocytes [2]. Moreover, gammaherpesviruses are known for their oncogenic potential, and are associated with malignancies such as Burkitt's lymphoma (for EBV), and Kaposi sarcoma (for KSHV) [3-4].

Table 1. *Subfamilies of human herpesviruses (HHV).*

Designation	Synonym	Subfamily	Infected cell types	
			Lytic	Latent
HHV-1	Herpes simplex virus type 1 (HSV-1)	α	Mucosa Epithelium	Neurons
HHV-2	Herpes simplex virus type 2 (HSV-2)	α	Mucosa Epithelium	Neurons
HHV-3	Varicella-zoster virus (VZV)	α	Mucosa Epithelium	Neurons
HHV-4	Epstein-Barr virus (EBV)	γ	B-cells Epithelium	B-cells
HHV-5	Cytomegalovirus (CMV)	β	Leukocytes Epithelium	Leukocytes Epithelium
HHV-6	Roseolovirus	γ	Leukocytes Epithelium	T-cells
HHV-7	Roseolovirus	γ	Leukocytes Epithelium	T-cells
HHV-8	Kaposi's sarcoma associated herpesvirus (KSHV)	β	Lymphocytes	B-cells

HSV-1: architecture, genomic organization and viral life cycle

Herpes simplex virus (HSV) is considered the prototypic herpesvirus. From the inside out, the HSV-1 virion consists of four main elements: 1) a core containing viral double-stranded DNA; 2) an icosahedral nucleocapsid; 3) a proteinaceous layer called the tegu-

ment; and 4) a lipid bilayered envelope containing viral glycoproteins (Figure 1). The virus particle is about 120 nm in diameter. The genome of HSV-1 is ~152,000 base pairs in size and encodes for 80 proteins. The double-stranded DNA genome consists of covalently linked components, designated as unique short (US) and unique long (UL) sequences [2]. The nucleocapsid is formed from at least eight viral proteins [5-6]. The surrounding tegument layer contains over 20 viral proteins. Nine glycoproteins are present in the viral envelope. Thirty-one HSV proteins are essential for viral replication [7].

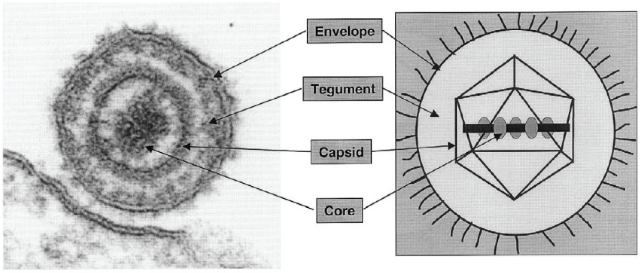

Figure 1. *Electron microscopy (left) and schematic representation (right) depicting the morphology of the herpes virion. Herpesvirus particles consist of four main elements. The inner core, containing the double-stranded DNA genome, is surrounded by a nucleocapsid shell. The proteinaceous tegument surrounds the nucleocapsid, which is in turn enclosed in a lipid envelope containing viral glycoproteins. Figure adapted from [8], reprinted with permission.*

Herpesvirus infection is launched with the attachment of viral glycoproteins to host cell surface receptors. Several HSV glycoproteins are involved in the entry of HSV-1, and mutant viruses that lack these glycoproteins cannot infect cells [9]. Cellular receptors facilitating HSV-1 entry include herpes virus entry mediator (HVEM), nectin-1 and paired immunoglobulin-like type 2 receptor alpha (PILRα). Binding of HSV-1 glycoprotein D (gD) to HVEM and nectin-1 results in attachment of the virus particle to the host cell membrane. These interactions bring the membranes in close proximity, allowing other viral glycoproteins to interact with cell surface molecules. Subsequent binding of viral gB to PILRα creates an entry pore for the viral nucleocapsid [7, 10-11]. The capsid is transported to the nuclear membrane, where viral DNA is released into the nucleus. Consequently, viral DNA is transcribed in a highly regulated fashion by the host polymerase II, although viral factors participate at all stages of infection. Within 2-4 hours after infection, immediate early (IE) genes are expressed. These IE proteins are primarily transcription factors that induce the expression of early (E) genes. The expression of E proteins peaks between 4-8 hours after infection, and E proteins are involved in viral DNA and nucleotide metabolism. E proteins promote DNA replication and expression of late (L) genes is then stimulated. The L genes primarily encode structural proteins which are involved in assembly of the newly formed capsids in the nucleus and virion formation. Progeny virus is released from infected cells via exocytosis (Figure 2) [7].

The two key stages of HSV-1 infections: lytic and latent

Viral particles are released from HSV-1–infected epithelial cells during lytic infection. In addition to the spread to neighboring epithelial cells, HSV-1 particles can enter sensory nerve endings innervating the infected target organ. Nucleocapsids are transported in a retrograde fashion within the axon to the cell body of the sensory neuron (Figure 3A)

HSV-1 replicates briefly and then establishes a latent infection persisting for the life time of the host. The viral genome is retained within the nucleus of the cell and no infectious virions or detectable viral proteins are produced. Viral gene expression is largely repressed except for latency-associated transcripts (LATs). These transcripts do not code for a protein, but are abundantly present and can be used to identify latently infected neurons [12].

Interruption of latency results in reactivation. Transcriptional activation of viral genes can result in the production of virions, which are transported in an anterograde fashion to the nerve endings. A recurrent productive infection can be established commonly at the anatomical site of the primary infection (Figure 3B). Reactivation may be asymptomatic or lead to recurrent lesions [7]. HSV-1 infections are generally acquired via the orofacial route resulting in a latent infection of the neuronal cell bodies of the innervating sensory ganglion, referred to as the trigeminal ganglion (TG). Triggers for reactivation are intermittent and can be of 'ganglionic' origin, such as peripheral nerve damage [13], or of 'skin' origin, including ultraviolet light or peripheral trauma [7, 14-15].

The trigeminal ganglion: site of HSV-1 latency

The TG is a collection of neuronal bodies converging from three major sensory nerves that innervate the facial region: the ophthalmic, maxillary and mandibulary nerve. Two TG are located on both sides of the face at the base of the skull innervating the face bilaterally. On each side, the ophthalmic branch diverges from the TG to the eye and nose, the maxillary nerve sprouts to the nose and upper lip, and the mandibular nerve carries motor and sensory information to the mouth and chin (Figure 4A). From the TG, one motor root and one sensory root project to the brainstem [16].

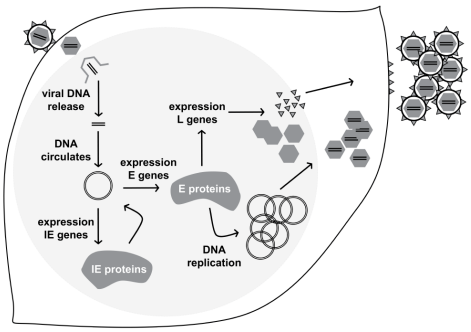

Figure 2. *Schematic representation of the HSV-1 lytic replication cycle in cells. Virus attachment to the target cell membrane results in the release of the nucleocapsid into the cell. The capsid is transported to the target cell nucleus, where viral DNA is released. Viral DNA is transcribed in a highly regulated temporal fashion. First, immediate early (IE) genes are expressed, and the encoded proteins regulate the expression of early (E) genes. The early proteins are involved in DNA replication and expression of late (L) genes. The late proteins are primarily structural proteins involved in virion assembly. Progeny virus is released by exocytosis.*

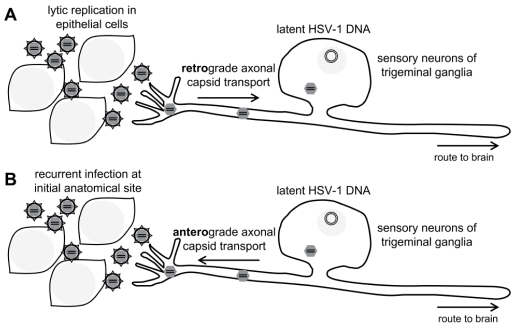

Figure 3. *Schematic representation of the two key stages of HSV-1 infections. (A) Lytic replication in epithelial cells results in release of infectious viral particles. Subsequently, these particles enter sensory nerve endings innervating the infected target site. Nucleocapsids are transported in a retrograde fashion within the axon to the neuronal cell body. HSV-1 establishes a latent infection inside sensory neuronal cell bodies. (B) Reactivation of latent HSV-1 from sensory neurons is associated with anterograde transport of HSV-1 nucleocapsids. Release of virus from sensory nerve endings allows for a recurrent lytic infection at the initial anatomical site.*

In the TG, sensory neuronal cell bodies are surrounded by a layer of satellite glial cells (SGC) (Figure 4B). Each neuron has its own sheath of 3-10 SGC, which appears as a continuous structure [17-18]. The neuron-SGC boundary is complex and involves multiple membrane interdigitations, resulting in a large surface contact area and control of migrating substances to and from the neuron. A complete glial sheath, like the one formed by SGC around sensory neurons, is unique and is not found in the central nervous system [18]. SGC are usually identified by their morphology and perineuronal location, and their role is to provide mechanical and nutritional support of interacting neuronal cell bodies [17]. In addition, SGC control the neuronal microenvironment by responding to chemical signals [17].

HSV-1 epidemiology and disease manifestations

Humans are the only natural host for HSV. However, in contrast to other HHV, HSV-1 and HSV-2 are able to infect various animal species in experimental infection settings. In humans, primary infections are usually acquired during early childhood upon close personal contact and are often asymptomatic. In the adult population, HSV-1 infection rates are estimated to reach numbers of 40% at 15 years of age, increasing with age up to 80% in the adult population >40 years of age [19-20]. In asymptomatic individuals, the presence of HSV-1 infection is assessed by testing the presence of HSV-1–specific antibodies in serum, based on gG antibodies to differentiate HSV subtypes, whereas symptomatic HSV-1 infections are validated by virus culture or PCR on vesicular fluids [7, 21]. A scraping of skin vesicles or vesicular fluid can be inoculated onto cell cultures that are susceptible to the cytopathic effects characteristic of HSV-1 replication. The cytopathic effect usually develops within several days after inoculation with infectious virus. Alternatively, real-time PCR can be performed directly on nucleic acids isolated from

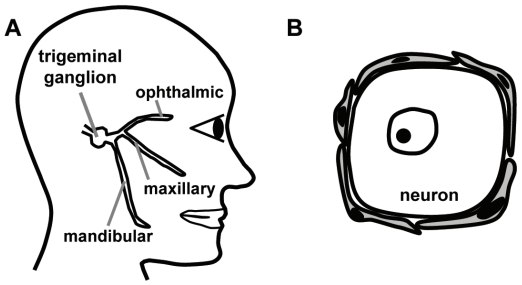

Figure 4. *The trigeminal ganglion. (A) Anatomic location of the trigeminal ganglion and its nerve branches conveying the sensory information of the face. (B) A sensory neuronal cell body in the trigeminal ganglion is surrounded by a sheath of satellite glial cells (filled grey).*

swabs. It offers a sensitive and quantitative result that is faster than culture methods [21]. The severity of clinical HSV-1 disease depends on a variety of factors, such as viral load, viral replication rate, as well as age and immune status of the host [22]. Symptomatic disease manifestations range from mild labial blisters (herpes labialis) in the immunocompetent population to severe systemic, neurological, or lethal disease in neonates and immunocompromised individuals [7]. Herpes labialis is the most common manifestation of HSV infections and outbreaks are estimated to affect 20-40% of HSV-1 seropositive adults [23]. Skin lesions are often preceded by a prodromal phase with local symptoms of tingling, burning, pain or itching. These symptoms are thought to result from early viral replication at sensory nerve endings and in the epidermis [24]. Vesicles form within a day after skin infection and persist in most persons for only 48 hours. Subsequently, lesions progress to an ulcerative and crusting stage and healing is usually complete within 10 days [7, 25].

A more rare HSV-1 disease manifestation is herpes whitlow, which is a self-limiting, cutaneous infection of the distal phalanx of the hand often affecting healthcare workers, children with oral herpes, and adults with genital HSV [7, 22, 26]. Herpes whitlow is caused by direct inoculation of the involved digit, for children by finger or thumb sucking or for healthcare workers by exposure of the digit of the dominant hand to patient's lesions. Clinical symptoms include swelling, erythema, vesicles, and severe local pain but infections heal within 4 weeks [26]. HSV-1 is increasingly associated with genital infections [27]. Symptoms are highly equivalent to those in the orofacial area, but recurrences are more infrequent and occur at a lower rate than for genital HSV-2 infections [28]. Hepatitis is a rare but life-threatening complication of HSV disease, often leading to acute liver failure and consequently death [29-30]. Diagnosis is often complicated by the absence of specific clinical symptoms, but quantification of HSV-1 DNA levels in plasma is known to correlate with liver function and disease severity in HSV-1 hepatitis patients [29]. Herpes simplex encephalitis is a severe infection of the central nervous system that is usually localized to the frontal and temporal lobes in adults. Symptoms are headache, fever, seizures, cognitive impairment and other neurological signs. It is uncommon, but has a high morbidity and mortality if not treated promptly [31]. Ocular HSV-1 infections affect the eye lid, conjunctiva, the cornea, uvea and retina [32]. The predominant form of recurrent ocular disease is herpetic keratitis which is a leading cause of corneal blind-

ness worldwide [33]. The pathogenesis of and immunity to ocular HSV infections are described later in this section.

HSV shedding is instrumental to virus transmission throughout the population [14, 34, 35]. Virus shedding is usually asymptomatic and transmission to susceptible hosts is dependent on intimate personal contact [14, 34]. Asymptomatic shedding is defined as the detection of HSV in the absence of lesions. Calculation on the frequency of HSV shedding estimated that seropositive subjects shed HSV DNA in tears or saliva at least once every 30 days [34]. Oral shedding occurs in episodes which are rapidly cleared by the immunocompetent host [36].

HSV-1 infections of the eye

HSV-1 infection is a major cause of ocular disease and can infect almost all parts of the eye. The human eye is a complex structure of transparent layers and photoreceptors. The anterior part of the eye consists of the cornea, the anterior chamber, the iris and the lens. The spherical space behind the lens contains vitreous fluid. The back of the eye consists of the retina, where photoreceptors are located (Figure 5). The eye has acquired an immunoprivileged status through evolution. The eye lacks lymphatic drainage and tissue-resident cells express immunosuppressive factors [37].

The most common HSV-1 infections of the eye affect the outer surface, the eye lids, and the conjunctiva lining the eye. If HSV-1 lesions affect the inner parts of the eye, they are primarily confined to the cornea. Ocular HSV infection may lead to unilateral or bilateral disease. The majority of corneal HSV-1 infections are superficial, where virus replication is confined to the corneal epithelium. Infectious epithelial keratitis (IEK) is caused by a cytopathic effect of the virus and is usually relatively mild. IEK lesions commonly heal without permanent loss of vision. Recurrent corneal infections are generally confined to the epithelial layer. However, in 20% of individuals with recurrent corneal HSV-1 disease, the infection spreads to the deeper stromal layers of the cornea [38]. It will cause stromal disease, necrotizing or non-necrotizing, referred to as herpetic stromal keratitis (HSK), which leads to irreversible corneal scarring and edema leading to progressive loss of vision with each subsequent recurrence. In contrast to IEK, which is solely a result of viral replication, corneal scarring in HSK is mainly due to the inflicting immunopathological processes in the cornea [37, 39]. For necrotizing HSK, which is less common than non-

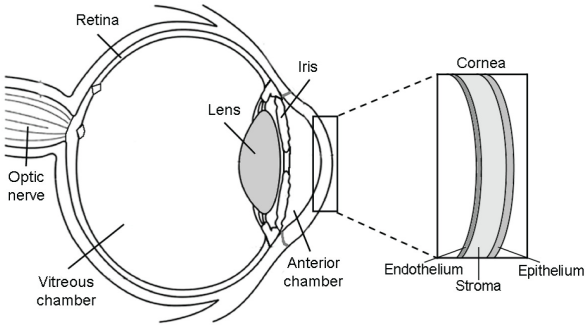

Figure 5. *Cross-sectional diagram of the human eye. The enlargement on the right shows the anatomical structure of the cornea where three distinct layers can be identified.*

necrotizing HSK, viral replication contributes to lesion formation [40]. Non-necrotizing HSK, also known as immune stromal keratitis, does not involve epithelial viral replication, and is usually accompanied by anterior chamber inflammation [40]. Studies on the pathogenesis of HSK have benefited from the experimental mouse infection model, where the cornea is scarified after which HSV-1 is inoculated. The susceptible mouse develops corneal inflammation that resembles the characteristics of HSK in humans. CD4 T-cells play an important role in corneal destruction [41-42]. In line with the keratogenic role of HSV-specific CD4 T-cells in the experimental HSK mouse model, CD4 T-cells isolated from corneal buttons from HSK patients are directed to various HSV-1 tegument proteins, and in this way are thought to contribute to the immunopathogenesis of HSV-1 ocular disease in humans [39, 43-45]. Not the direct cytopathic effect of the T-cells, but the complex interactions between infiltrating T-cells and cornea-resident cells are pivotal to the development of HSK, which are beyond the scope of this thesis.

HSV-1 can also infect the more posterior layers of the eye, such as the iris, ciliary body, choroid, retina and optic nerve. The concomitant inflammation is called uveitis. These infections have a more chronic nature. HSV-1 uveitis can be preceded by keratitis or present simultaneously, referred to as keratouveitis [46]. More commonly, it associates with anterior inflammation, involving the iris and the ciliary body, but in rare cases posterior uveitis can develop. The necrotizing retinitis, in its most severe form known as acute retinal necrosis (ARN), usually begins with vasculitis but can develop into retinal necrosis within 2-3 weeks [38, 46]. ARN is associated with a fulminant inflammation of the anterior chamber and/or vitreous chamber where inflammatory cells infiltrate the eye. It frequently results in retinal detachment, which is a major complication following HSV-1 posterior uveitis [46]. HSV-1 uveitis is due to both the cytopathic effect of the virus as well as the local T-cell mediated inflammatory response directed to the inciting virus.

HSV infections in immunocompromised individuals

The failure to limit viral replication in a timely fashion is a feature of HSV infections in the immunocompromised host. These patients suffer from atypical lesions which can persist for long periods of time. The recurrence of mucosal lesions is also more common in the immunocompromised host and extensive infections can develop rapidly. In addition, HSV may spread to other organs and cause pneumonia, hepatitis, encephalitis, and disseminated infection when it is not contained in time [7, 47-49]. HSV infections also increase the risk of HIV acquisition, in part due to HIV target cell influx in response to HSV replication in the (genital) mucosa [50-51]. Therapeutic or prophylactic treatment with antiviral agents is therefore especially warranted in immunocompromised patients to prevent serious clinical disease. In line with the observations that immunity is important to control HSV reactivation and peripheral shedding, immunocompromised patients have more frequent oral viral shedding which are cleared surprisingly rapid [14, 52-53].

Immunity to HSV infections

Upon mucoepithelial HSV infection, the host immune system responds by attempting to clear the virus from the infected tissue. First, the innate immune system is activated. This

inborn system is not specific for HSV, and starts rapidly after infection. It is mediated by neutrophils, macrophages and dendritic cells (DC), which are attracted to the site of infection. Macrophages and DC are antigen-presenting cells (APC) expressing a variety of pattern recognition receptors that can recognize proteins and nucleic acids which are shared among viruses [54]. Detection of these pathogen ligands triggers phagocytosis of the pathogens. Intracellular signaling pathways are activated resulting in the production of cytokines and shaping of the subsequent adaptive immune response. The innate immune response limits dissemination of the virus and initiates the adaptive arm of the immune system.

The adaptive immune system is mediated by T-cells and B-cells that, in contrast to the innate immune system, establish immunological memory. B-cells are activated by APC in lymph nodes draining infected sites. They can produce virus-specific antibodies, referred to as neutralizing antibodies, which inhibit virus attachment to new target cells. Virus surface glycoproteins are important targets of HSV-specific neutralizing antibodies. The second component of the adaptive immune response is composed of T-cells. Antiviral T-cells express either CD4 or CD8 proteins at their cell surface and are classified accordingly. T-cells use membrane-exposed T-cell receptors (TCR) to recognize antigen-derived peptides bound to human leukocyte antigen (HLA) molecules expressed at the APC surface. HLA molecules are polymorphic proteins expressed on virtually all nucleated cells and are classified in class I and class II HLA molecules. In general, HLA class I molecules present peptide antigens of intracellular origin to CD8 T-cells, whereas HLA class II molecules present antigens of extracellular origin to CD4 T-cells. However, phagocytosed antigens can also be presented by DC in HLA class I molecules, a process known as cross-presentation [55]. HLA molecules bind a variety of antigenic peptides, which are dictated by the structure of the peptide-binding groove on the HLA molecule. The genes encoding the HLA complex are highly polymorphic. Three genetic loci (A, B, and C) encode the heavy chain of the HLA class I molecule, allowing a maximum of six allelic variants per individual. The HLA class I molecule, independent of its allelic variant, dimerizes with beta-2 microglobulin to form stable membrane-associated HLA class I proteins. The genes for HLA class II encode the alpha and beta chains of three different HLA class II molecules, which are referred to as HLA-DP, HLA-DQ and HLA-DR. The alpha chain of HLA-DR is monomorphic, whereas there are four different genes encoding for the HLA-DR beta chain. These extensive polymorphisms ensure that most individuals inherit different variants of each gene, referred to as alleles, and helps coping with the structural diversity of pathogens and their antigens [56].

Virus-specific CD8 T-cells mainly recognize their cognate peptide bound to HLA-A or HLA-B molecules. The majority of peptides bound by HLA class I molecules are 8 to 10 amino acids in length and are bound by pockets in the HLA molecule. The antigenic requirements for HLA class I binding are strict due to the constraining binding pockets and the embedding conformation of the HLA-peptide interaction. These conditions allow computer algorithms to predict potential antigenic HLA class I allele-binding peptides from protein sequences. In HLA class II molecules, the two ends of the antigenic peptide are not pinned down into pockets. They can extend out at each end of the groove and therefore HLA class II allele-binding peptides are longer (10-30 amino acids) and more variable in amino acid sequence than HLA class I allele-binding peptides [56]. The

breadth of antigenic peptides presented by HLA molecules is not only restricted by their length or motif of amino acid residues. The timing and amount of antigen expression, e.g. during virus replication viral proteins are temporally expressed, will have an impact on the exposure of antigenic peptides to virus-specific T-cell repertoire. Moreover, peptides are derived from protein structures that are degraded for subsequent presentation in different HLA alleles expressed by the host. In case of HLA class II molecules, these peptides are generated in the intracellular vesicles of phagocytosed antigens. HLA class I peptides are cleaved from tagged intracellular proteins by the proteasome and loaded onto HLA molecules. These processes can be modified by pathogens and contribute to the availability of antigen peptides for HLA molecules and subsequent T-cell recognition [56-58].

In general, the TCR interacts with a unique HLA/peptide-complex on the APC or target cell membrane and each T-cell clone expresses a single TCR. The TCR is a heterodimer composed of an alpha and a beta chain. The diversity of TCR is gained through rearrangement of the encoding TCR genes [56]. When a T-cell interacts via its TCR with the HLA/peptide-complex on a target cell, the two cell membranes localize close to each other, allowing other protein-protein interactions to occur. The interaction of these co-receptors, such as the CD4 and CD8 proteins, are essential for effective T-cell stimulation. Activation of T-cells by APC triggers a wide range of intracellular signaling pathways leading to T-cell proliferation, differentiation, and a variety of effector functions. The latter can be direct cytotoxicity and, in the case of virus-specific T-cells, production of antiviral cytokines such as interferon (IFN) gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) that will inhibit viral replication [56].

CD8 T-cells exploit several mechanisms to control or eliminate virus-infected cells. They can induce lysis or apoptosis of the infected target cell, which is mediated by the release of perforin and granzymes. These proteins form pores in the target cell and cleave apoptosis-inducing cellular caspases, respectively. Apoptosis of infected target cells can also be induced by direct cell surface molecule interactions. Activated cytotoxic T-cells express Fas ligand, which binds to Fas molecules on the target cell. This interaction directly signals to the target cell to undergo apoptosis. Antigen-specific activation of CD4 T-cells results in the production of cytokines to shape the adaptive immune response, but CD4 T-cells can also exert cytotoxic effector functions [45].

In addition to the generation of effector immune cells, T-cell activation also results in the formation of immunological memory. Memory T-cells can circulate in the body for years, or remain with previously infected tissues and are efficiently triggered in the case of antigen-specific challenge [59-60]. This mechanism allows fast recruitment of antigen-specific T-cells to the site of an infection and contributes to rapid and specific clearance of pathogens. The recurrent nature of HSV skin infections is effectively controlled by HSV-specific CD8 and CD4 T-cells, indicating that these memory T-cells monitor infection and reactivation locally [61-62].

Effect of HSV infection on host immunity

The chronic and recurrent nature of HSV infections in humans has a profound effect on host immunity. It has been shown that HSV-1 infection inhibits DC maturation, the-

reby modulating the initiation of an adaptive immune response [63-64]. One of the key features of HSV-1 is its ability to evade immune recognition through interference with innate and adaptive immunity. HSV-1 proteins can block type I IFN production and inhibit antigen presentation on infected cells [65-68]. The HSV-1 protein infected cell protein (ICP)-47 downregulates HLA class I expression enabling the virus to evade CD8 T-cell recognition [57-58].

Long-term infection of tissues, albeit in latent state, has profound effects on tissue resident immunity. The retention of memory T-cells at the site of previous infection, for example in the TG, allows rapid mounting of a virus-specific immune response upon secondary antigenic stimulation [61-62, 67]. The stimulation of tissue resident T-cells depends on local APC, allowing T-cell expansion and activation at the site where their cognate antigen is present [67]. However, chronic antigenic stimulation of local T-cells can also affect their capacity to respond to those antigens. A state of T-cell exhaustion is characterized by poor effector functions and expression of inhibitory receptors and can result from increased or prolonged antigenic stimulation [68]. Indeed, T-cells retained in HSV-1–infected TG express markers of exhaustion, such as PD-1 and CD94/NKG2a [69, 70]. This T-cell functional impairment may have important consequences for the local T-cell antigen recognition and control of virus replication.

HSV-specific T-cells in mice and humans

The first studies on HSV-specific T-cells in humans focused on T-cells recovered from HSV-induced lesions in the eye or genital skin. It was shown that HSV-specific T-cells in genital lesions recognized diverse viral proteins [71-72] and CD4 T-cells isolated from HSV-1 infected human eyes were shown to be specific for various viral tegument proteins [39, 43-45]. In peripheral blood, gD and gB have been shown as antigenic targets of HSV-specific T-cells [72-74]. These studies focused on individual HSV proteins as antigenic targets. Recently, the breadth of HSV-1 specific blood T-cells was studied in great detail in HSV-1 seropositive immunocompetent individuals. An HSV-1 proteome-wide approach, covering all 80 HSV-1 encoded proteins, identified viral proteins UL39 and UL46 to be dominant T-cell targets in peripheral blood [75]. Infected humans recognized a mean of 17 and 23 HSV-1 antigens as CD8 and CD4 antigens, respectively, covered by multiple HLA alleles [75].

Knowledge on the HSV-1 specificity of T-cells is also gained from mouse studies. T-cells of the CD4 and CD8 subtype are induced upon experimental mouse HSV-1 infection. It is known that HSV-1 specific T-cells peak in quantity around 8 days post infection [76]. The inbred mouse strains that were used for these studies express a limited range of mouse major histocompatibility complex subtypes (referred to as H-2 alleles). The restricted H-2 variability leads to a strong bias in the repertoire of induced virus-specific T-cells. Consequently, the majority of the HSV-specific CD8 T-cells are directed to a single epitope in gB [77-78]. T-cells infiltrate the TG and exert local functions that are described later in this section.

Immune control of HSV-1 latency

HSV-1 latency in the TG is characterized by the absence of infectious viral particles. The HSV-1 genome persists in an episomal state in neuronal nuclei [79-80]. Most of the HSV-

1 infected neurons express LAT, which seem to be responsible for neuronal survival by preventing apoptosis [12, 81-84]. LAT sequences are complementary to the HSV-1 IE transcript of ICP0 [12]. Although there are some reports on open reading frames within the LAT region [85-86], it is generally believed that the role of LAT is not to code for a protein, but rather as a post-transcriptionally inhibiting RNA with anti-apoptotic and T-cell modulating functions [82, 87-92].

The detection of viral transcripts during latency in human TG is anecdotal [93]. However, T-cells have been found juxtaposed to HSV-1 infected neurons. These T-cells, conglomerates of CD4 and CD8 T-cells, do not seem to destruct latently HSV-1 infected neurons. The exact mechanisms by which T-cells recognize and control HSV-1 latency in neurons are topics of current research [94-97].

Most of the information on T-cells and HSV-1 latency is obtained through the use of the experimental C57BL/6 mouse model [98-99]. The T-cell response in HSV-1 infected mice is dominated by CD8 T-cells and can be separated in an acute (up to 10 days post infection) and a latent phase (>21 days post infection). T-cells retained within the TG of latently infected mice secrete IFN-γ and granzyme B and thereby inhibit viral reactivation [100-101]. The majority of the CD8 T-cells in TG of infected mice are specific for HSV-1 epitopes, in particular directed to a single epitope in the late protein gB [98]. By using TCR-binding tetramers, it was demonstrated that these T-cells are in close proximity to latently infected neurons and it was speculated that sensory neurons could directly regulate the effector function of these CD8 T-cells in mice [98, 102-103]. In addition, restraining mice experiencing physical stress compromised the CD8 T-cell mediated control of HSV-1 latency [104]. A non-cytotoxic role of CD8 T-cell secreted granzyme B was demonstrated that could degrade the HSV-1 IE protein ICP4, thereby inhibiting viral gene expression and thus blocking viral reactivation [105]. Although the majority of mouse CD8 T-cells are specific for gB, other HSV-1 antigens are also recognized by TG-infiltrating T-cells in C57BL/6 mice [106-107]. No IE gene products are targeted, whereas the majority of non-gB reactive CD8 T-cells are directed to early proteins such as UL29 and UL39 [107]. The mechanism of this specific targeting is unknown, but it appears favorable for the host to inhibit viral replication as early as possible. If TG-resident T-cells block viral reactivation at an early stage, it is expected that they are specific for HSV-1 proteins expressed with IE and E kinetics.

Despite the extensive knowledge that is gained by studying HSV-1 specific T-cells in mice, humans are the only natural host and reservoir of HSV-1. This is of significance when studying pathogen-specific immunity since virus and host co-evolved in time. Studies on T-cells in HSV-1 latently infected human TG have demonstrated the presence of a chronic immune response, which is composed of CD8 and CD4 T-cells, with an effector memory phenotype that localize to HSV-1 latently infected neurons [108-110]. In addition, limited IE genes were expressed in sensory neurons and T-cells were shown to be clonally expanded, suggesting that HSV-1 antigens are recognized in latently infected human TG [93, 110-112].

Therapeutic interventions for HSV infections

Besides vaccination, prevention of infection can be accomplished by avoiding contact with infectious secretions from shedding individuals, in particular those individuals with symptomatic herpetic lesions. Moreover, education, condom use and other hygienic measures can prevent or limit viral shedding [7]. In case of symptomatic disease, HSV infections can be treated with antiviral drugs to contain infection and limit viral transmission.

HSV vaccines

Vaccination remains the most cost-effective method to prevent or limit viral infections. However, prevention of HSV infections introduces unique problems because the virus establishes latency and reactivations occur in the presence of antiviral humoral and cell-mediated immunity. Clearance of latent virus in a non-replicating state has proven hitherto impossible. An HSV vaccine will therefore not lead to sterile immunity. Because it is known that the latent HSV load is related to the frequency of viral shedding, current vaccination strategies aim at limiting the latent HSV-1 burden, and reducing (a)symptomatic shedding.

Vaccination can be accomplished by live-attenuated virus vaccines, inactivated or killed virus vaccines, or by subunit vaccines. Live-attenuated HSV vaccines have been used as early as the 1920s, but were unsuccessful due to adverse events or lack of immunogenicity [113-114]. Subsequent vaccine strategies aimed at replication-defective mutants. A single cycle infectious HSV-2 vaccine entered clinical trials but had limited clinical efficacy in immunized persons [115-118]. More recently, gears shifted towards the use of subunit vaccines. In particular, the glycoproteins of HSV have received most of the attention. Glycoproteins gB and gD are the most used immunogens since these are the dominant targets for neutralizing antibodies and cell-mediated immunity in HSV-infected individuals [70-71]. An earlier vaccination trial with adjuvanted recombinant gD showed transient and partial protection only in HSV seronegative women [118]. A more recent HSV-2 gD vaccination trial was inefficacious, although some protection against HSV-1 genital disease was observed [119]. It is expected that vaccination with HSV-1 antigens targeted by T-cells involved in controlling latency and clearing virus from infected mucosa will enhance protection against HSV-1 infections or recurrences [75].

VZV vaccines

Although no effective HSV vaccine is on the market, a live attenuated vaccine for varicella-zoster virus (VZV), an HSV-related alpha-herpesvirus, was implemented in the United States in 1995. VZV is an Alphaherpesvirinae family member causing chickenpox during primary infection. Like HSV, VZV establishes latency in sensory ganglia of the host [120]. In contrast to HSV, VZV reactivation, known as herpes zoster, is a vesicular rash confined to regions of the skin served by a single dermatome, and is associated with waning cell-mediated immunity [120]. In 1974, Takahashi and colleagues empirically introduced a live-attenuated VZV vaccine that protected children from varicella infection [121]. The vaccine is still effective in preventing varicella in children, although the effect on preventing zoster has not been established and is currently questioned [122]. The VZV vaccine can establish latency [123], reactivate causing herpes zoster [124-126], and can be transmitted to susceptible individuals [127]. Moreover, mild breakthrough disease

has been reported, suggesting that the vaccine may be too attenuated to be effective in some vaccinees [128-129].

VZV vaccination of adults over 60 years of age is performed to boost immunity and reduce the occurrence of herpes zoster [122]. Vaccine efficacy may be improved by adapting vaccine constituents [130], but waning cellular immunity in the elderly will inevitably reduce vaccine immunogenicity and efficacy [131].

Treatment of HSV-1 infections

Treatment of herpes labialis in immunocompetent hosts is rarely warranted. However, incidental frequent and complicated HSV-1 infections can effectively be treated with antiviral agents. The first generation of antiviral drugs was based on direct disruption of (viral) DNA synthesis. Newer antiviral agents are prodrugs specifically activated by viral proteins, thereby enhancing its specificity and limiting toxicity.

Two classes of antiherpesvirus drugs have been developed. The first class of antivirals are the nucleoside analogues, which are prodrugs that require phosphorylation by viral thymidine kinase (TK). The excellent specificity and low toxicity profile of these drugs are accomplished by the high affinity of these drugs for viral TK instead of host cell kinases [132]. Therefore, these drugs are primarily converted to their active compounds in infected cells, hardly causing any side effects. Viral TK phosphorylated nucleoside analogues are subsequently phosphorylated by cellular kinases and triphosphate forms of the drugs compete with cellular deoxynucleoside triphosphates for incorporation into elongating DNA strands. The incorporation of these nucleoside analogues results in termination of chain elongation and block of viral replication (Figure 6) [47, 133].

The earliest antiviral drug in this class was idoxuridine, which proved to be effective in shortening the course of herpes labialis [134-135]. Its toxicity is short term and idoxuridine has poor water solubility. The use of idoxuridine to treat HSV infections was outshined by the discovery of acyclovir (ACV), which also has an excellent safety profile due to the selective phosphorylation by viral TK. The discovery of ACV was preceded by observations that adenine arabinoside (ara-A) was an excellent non-toxic antiviral with clinical value [136-138]. However, ara-A is rapidly inactivated in the host cell and attempts to identify inhibitors of this inactivation led to the discovery of ACV as a strong antiviral agent by itself [132, 139-140]. Professor Elion was awarded the 1988 Nobel Prize in Medicine, partly for the development of ACV. Subsequently, other nucleoside analogues have been approved for the treatment of HSV infections, such as penciclovir (PCV) and ganciclovir (GCV) [141-142]. A valine ester conjugated prodrug of ACV, valacyclovir (valACV), was developed which increased the oral bioavailability of ACV [143-145]. ValACV is converted to ACV by intestinal and hepatic first-pass metabolism, increasing the bioavailability by 3- to 5-fold [132]. GCV was initially developed to treat CMV infections, but it can also be used to treat HSV infections [47, 133, 146-149]. It can be given orally, but has limited bioavailability [150-151]. Therefore, valganciclovir has been developed. GCV can be used to treat ocular HSV infections, in particular because of its aqueous solubility which is better tolerated for topical application [152]. The second class of antiherpesvirus drugs includes direct HSV DNA polymerase inhibitors, such as cidofovir and foscarnet (FOS) (Figure 6). A major side effect of FOS is nephrotoxicity,

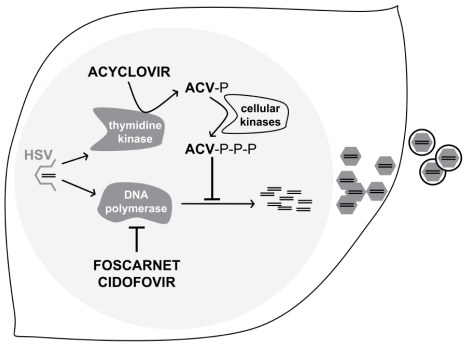

Figure 6. *The target of nucleoside analogues, such as acyclovir (ACV), and the mechanism of action of DNA polymerase inhibitors are schematically depicted. Selective phosphorylation (P) of ACV by HSV-encoded thymidine kinase and subsequent phosphorylation by cellular kinases is required for incorporation of these nucleoside analogues by viral DNA polymerase. ACV triphosphate acts as a terminator of viral DNA chain elongation. Foscarnet and cidofovir are direct DNA polymerase inhibitors.*

which may be reduced by dosage adjustment and adequate hydration [153].

Treatment for primary oral or genital HSV infections can be applied topically or systemically and is usually given for 7 to 10 days, whereas treatment for recurrent infections is episodic for 1 to 5 days to decrease symptoms. In addition, therapy can be given in a prophylactic fashion to prevent recurrences [154-156]. Suppressive therapy also decreases HSV asymptomatic shedding and risk of transmission [154, 157-158]. Treatment of severe disease, such as encephalitis and ARN, should include intravenous ACV or alternatively FOS [159]. Due to its intervention in viral DNA replication, current HSV-1 treatment is only active during lytic infections, but is ineffective against latent virus.

Anti-herpesvirus drugs have been of major value in the field of ophthalmology. In IEK, the topical application of ACV accelerates the elimination of replicating virus from the cornea [160]. At this stage of the disease, the local virus-specific immune response limits the spread of the virus, and concomitant use of steroids to dampen ocular inflammation may promote the spread of the virus into the stroma [161-162]. Antiviral treatment of HSK is directed at the presumed presence of infectious virus and aimed at dampening the accompanying intra-ocular inflammatory response. Oral ACV does not appear to contribute to resolution of established HSK [163], since most pathology is due to the local immune response [39, 164].

Oral ACV prophylaxis significantly reduced the rate of recurrent genital and orofacial HSV infections in immunocompetent individuals [165-166]. Subsequently, the Herpetic Eye Disease Study (HEDS) group conducted a series of trials to investigate the effect and impact of ACV treatment on ocular HSV disease. For HSV epithelial ocular disease, no apparent benefit of a 3-week course of ACV in preventing HSK was seen in the subsequent year [167], but a 12-month prophylactic ACV treatment significantly reduced the recurrence rate of IEK in patients irrespective of their disease history [168]. A prospective trial demonstrated that a 12-month course of ACV prophylaxis reduced the rate of

recurrent ocular and orofacial HSV-1 disease [166]. The greatest clinical benefit is obtained for HSK patients, as prolonged ACV therapy will prevent recurrences and reduces the risk of corneal scarring and loss of vision [156]. The benefit of the treatment did not sustain after stop of prophylaxis, demonstrating that prolonged ACV use is needed to prevent recurrence-associated morbidity [156].

Because HSV infections of immunocompromised hosts are usually more complicated, antiviral treatment is especially warranted. ACV therapy, in particular intravenous administration, is associated with shorter durations of viral shedding and more rapid healing of lesions [157]. Prophylaxis therapy is also of clinical value in immunocompromised patients, especially those at risk for infection such as transplantation patients [170].

HSV-1 antiviral resistance

Resistance of HSV-1 to antiviral treatment has become an important problem, especially among immunocompromised patients exposed to long-term antiviral therapy [171]. In immunocompetent individuals, however, the incidence of antiviral resistance, in particular against ACV, has remained below 1% [47, 172-173]. In most individuals, antiviral drug resistance is not associated with adverse clinical outcomes. This can be explained by the contribution of immunity to viral clearance [133]. Inherently, the prevalence of ACV resistance (ACV^R) in immunocompromised patients is higher [172, 174-177]. ACV-resistant HSV infections in immunocompromised hosts are associated with significant morbidity and even mortality due to the uncontrolled cytopathic effects of the virus [178-179].

In line with its mechanism of action, viral mutations conferring ACV^R have been found in the TK and rarely in the DNA polymerase gene [180-181]. HSV requires a functional DNA polymerase to replicate, whereas viral TK is dispensable for productive infection. Consequently, more ACV^R HSV-1 with a mutated and inactive TK protein rather than mutated DNA polymerase protein is found are detected. In fact, 95% of all ACV^R HSV-1 is due to mutations in the TK gene [182-184]. The HSV-1 TK gene is polymorphic by nature, exemplified by the observations that ACV^R-associated TK mutations were detected before the introduction of ACV in clinical practice [185]. Specific TK mutations conferring ACV^R have been studied in great detail. Three different phenotypes of ACV^R TK mutants have been described: 1) TK-negative mutants that lack TK activity, 2) TK mutants with reduced level of enzymatic activity, and 3) TK mutations with altered substrate specificity. The latter class of mutants constitutes a minority of all described TK mutations, and can phosphorylate endogenous thymidine, but does not bind ACV or other nucleoside analogues [186]. It is expected that ACV^R TK mutants display resistance towards other TK-dependent antivirals [133, 187-188]. However, TK mutants with reduced activity or altered specificity can display limited cross-resistance [189-191].

The HSV TK protein has several important sites involved in enzymatic activity: the ATP-binding site (amino acids 51-63), the nucleoside-binding site (amino acids 168-176), and the cysteine residue (amino acid 336), the latter which retains the three-dimensional structure of the active protein [192]. In addition, there are six highly conserved regions

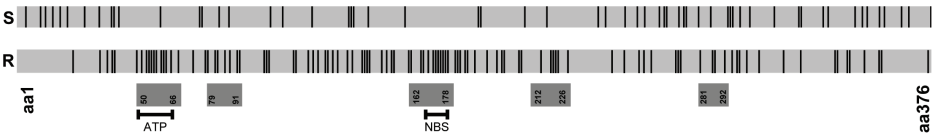

Figure 7. *The polymorphic viral thymidine kinase protein (376 amino acids [aa]) harbours mutations that do not affect acyclovir sensitivity of the respective HSV-1 strain (S), locations indicated on the upper bar. In contrast, aa mutations resulting in acyclovir resistance (R) are indicated on the lower bar. Conserved regions and ATP- and nucleoside binding sites (ATP and NBS, respectively) of the thymidine kinase protein are indicated by the darker grey boxes and the respective aa locations.*

within herpesvirus TK proteins, which appear important for the TK function. Numerous natural polymorphisms have been described for the TK gene, which are usually located outside the active and conserved sites of the enzyme, and merely represent the polymorphic nature of the gene [133, 193-194]. The genetic variation of the TK gene can even be exploited to identify and genotype individual HSV-1 strains [194-195]. Half of the mutations that confer ACV^R are due to nucleotide insertions or deletions, resulting in open reading frameshift and synthesis of a truncated, non-functional protein [182, 184]. The polymorphisms in the TK protein have been characterized in great detail and are summarized in Figure 7 [47, 133, 182, 187, 193-194].

HSV-1 variants with ACV^R TK-negative mutants are known to have limited pathogenicity and are impaired in their ability to reactivate from latency in experimental animal models [197-198]. In humans, ACV^R HSV-1 has been detected in sensory ganglia [196, 199]. Moreover, reactivation of ACV^R HSV-1 has been reported in clinical isolates, suggesting that ACV^R HSV-1 can reactivate from the innervating ganglion [189, 200-202]. The prevalence of ACV^R HSV-1 in herpetic keratitis patients is relatively high (6.4%) and can be reisolated from the same cornea during a subsequent recurrence [189, 202]. Unfortunately, alternative treatment options for ACV^R ocular HSV-1 disease are limited, mainly due to toxicity of other antiviral therapies [203].

Aims and outline of this thesis

HSV-1 infections can range from mild herpes labialis to sight-threatening ocular disease and life-threatening hepatitis and encephalitis in susceptible hosts. In the immunocompetent host, viral replication is effectively controlled at peripheral epithelial sites. However, HSV-1 is never cleared from the body, as it establishes latency in sensory neurons for the life time of the host. Viral replication is monitored locally and in part controlled by a virus-specific memory T-cell response. In other words: "herpes is for life". Nevertheless, intermittent reactivation from latency does occur, and may cause symptoms that require antiviral treatment. Antiviral treatment can also be given as prophylaxis, preventing recurrences but posing a risk for the development of antiviral resistance and refractory HSV-1 disease.
The research described in this thesis was aimed to study the control of HSV-1 infections,

by host immunity and by antiviral treatment. During latency, HSV-1 persists in sensory neurons of the TG. Latently infected neurons are surrounded by clusters of T-cells, and their role in the non-cytolytic control of HSV-1 replication appears essential. The HSV-1 antigens recognized by TG-resident T-cells are rational candidates for HSV-1 subunit vaccines. The research described in *Chapter 2* aimed to identify and characterize the HSV-1 target antigens of TG-resident T-cells in humans. Local antigen presentation is crucial for activation, maintenance and modulation of a tissue-resident antiviral T-cell response. Given their neuron-interacting localization, satellite glial cells could be key players in the neuron/T-cell cross-talk controlling HSV-1 latency in sensory ganglia. SGC are known to provide mechanical and nutritional support to the neuronal cell bodies. *Chapter 3* describes the study on the phenotype and function of TG-resident SGC in the context of antigen presentation [69]. When viral control in the TG fails, HSV-1 reactivates and infectious virus may be shed at the periphery. Shedding often occurs without symptoms, but can cause recurrent disease, especially in immunocompromised patients. It is known that HIV-infected individuals experience more frequent HSV shedding than healthy persons. The TG is also home to VZV, a family member of HSV, and shedding of both viruses may be interrelated. *Chapter* 4 describes the research on the kinetics and quantity of HSV-1 and VZV shedding in saliva of HIV-infected patients. Therapeutic or prophylactic treatment of HSV-1 infections is especially warranted when it involves ocular disease or infection of immunocompromised individuals. Two classes of drugs have been developed. The nucleoside analogues, such as ACV, are prodrugs requiring phosphorylation by viral thymidine kinase. The second class of antiherpesvirus drugs includes direct HSV DNA polymerase inhibitors. The specificity and low toxicity profile of the first class of drugs have led to its widespread use in treatment and prophylaxis of epithelial and ocular HSV-1 disease. However, long-term treatment of HSV-1 infections with ACV poses the risk of inducing antiviral resistance. Moreover, establishment of latency by ACV-resistant HSV-1 could foster recurrent ACV refractory disease. *Chapter 5* describes investigations into the presence of ACV-resistant HSV-1 in latently infected TG of immunocompetent individuals [196]. The prevalence of ACV-resistant HSV-1 is relatively high in HSV-1 keratitis patients and can reactivate to cause recrudescent ACV-resistant HSV-1 keratitis. The research described in *Chapter 6* expands on these studies and reports on the prevalence of ACV resistance in HSV-1 induced uveitis. Finally, the study described in *Chapter 7* aimed to identify risk factors for the development of ACV refractory herpetic keratitis in patients with severe recrudescent HSV-1 keratitis with stromal involvement.

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CHAPTER 2

Local CD4 and CD8 T-cell reactivity to HSV-1 antigens documents broad viral protein expression and immune competence in human ganglia

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Abstract

Herpes simplex virus type 1 (HSV-1) infection results in lifelong latency in trigeminal ganglion (TG) neurons with periodic reactivation leading to recrudescent herpetic disease. HSV-1 proteins are expressed in a temporally coordinated fashion during lytic infection, but their expression pattern in latency is unknown. Selective retention of HSV-1 reactive T-cells in human TG suggests their role in controlling latency by recognizing locally expressed HSV-1 proteins. We characterized the HSV-1 proteins recognized by virus-specific CD4 and CD8 T-cells recovered from human HSV-1 latently infected TG. T-cell clusters, consisting of both CD4 and CD8 T-cells, surrounded neurons and expressed mRNAs and proteins consistent with in situ antigen recognition and antiviral function. HSV-1 proteome-wide scans revealed that intra-TG T-cell responses included both CD4 and CD8 T-cells directed to 1-3 HSV-1 proteins per person. HSV-1 protein ICP6 was targeted by CD8 T-cells in 4 of 8 HLA-discordant donors. Tetramer staining in situ demonstrated clustering of HSV-1-specific CD8 T-cells around TG neurons. Intra-TG retention of virus-specific CD4 T-cells, validated to the HSV-1 peptide level, suggests trafficking of viral proteins from neurons to HLA class II-expressing non-neuronal cells for antigen presentation. The diversity of viral proteins targeted by TG T-cells across all kinetic and functional classes of viral proteins suggests broad HSV-1 protein expression in latently infected human TG. Collectively, the human TG represents an immunocompetent environment for both CD4 and CD8 T-cell recognition of HSV-1 during latency. The HSV-1 proteins recognized by TG-resident T-cells, particularly ICP6 and VP16, are potential HSV-1 vaccine candidates.

Introduction

The neurotropic human alphaherpesvirus herpes simplex virus type 1 (HSV-1) is endemic worldwide. It is acquired during early childhood via the orofacial route resulting in a lifelong latent infection of neurons in the bilateral trigeminal ganglion (TG). During latency no infectious virus is produced, virus transcription is directed to latency-associated transcripts (LATs) and microRNAs, and HSV-1 proteins are undetectable using standard methods [1, 2]. Latent HSV-1 periodically reactivates, producing infectious virus that may lead to symptomatic lesions. Both primary and recurrent disease may result in clinical disorders of variable severity or even death, emphasizing the unmet need for preventive and therapeutic vaccines. The candidate HSV subunit vaccines, based on the HSV glycoproteins B (gB) and gD tested in human phase III trials were not consistently effective [3-5]. Vaccines induced antigen-specific antibodies and CD4 T-cells, but not CD8 T-cells, arguing for novel vaccine formulations that include specific HSV-1 antigens targeted by both antibodies and CD4 and CD8 T-cells.

Studies in humans and HSV-1 mouse models suggest a pivotal role for virus-specific CD8 T-cells in control of HSV-1 latency. Virus-specific CD8 T-cells, expressing an activated effector memory T-cell phenotype, are selectively retained in HSV-1 latently infected ganglia [6-8]. In C57BL/6 mice, the HSV-specific intra-TG CD8 T-cells inhibit HSV-1 reactivation by secreting interferon-γ (IFN-γ) and granzyme B (grB), and are mainly directed against an immunodominant HSV-1 gB epitope [9-11]. In nature, however, HSV-1 only infects humans. Because HSV-1 infections in mice mimic, but are not equivalent to human disease it is important that findings from mouse models be confirmed and extended to humans. Moreover, the HSV-1 antigens recognized by human TG-infiltrating T-cells are rational candidates for HSV-1 subunit vaccines.

HSV-1 encodes at least 77 proteins that during lytic infection are sequentially expressed in a coordinated fashion as immediate early (α), early (β), leaky late (γ1) and true late proteins (γ2) [12]. Expression of γ2 proteins depends on viral DNA replication. While infectious virions eventually assemble in distal axonal structures after reactivation, the temporal expression and trafficking of HSV-1 proteins in human neurons during latency is unknown. We previously showed reactivity of human TG-derived CD4 and CD8 T-cells to whole HSV-1 [8], but not which proteins were susceptible to local immune recognition. The detection of transcripts encoding the HSV-1 α proteins ICP0 and ICP4 in latent human TG [13] suggests that this kinetic class of proteins is expressed during latency or early after reactivation. However, their accessibility to antigen processing and presentation within TG-resident cells for local T-cell surveillance is unclear. The aims of this study were to identify the HSV-1 proteins recognized by and the spatial orientation of virus-specific T-cells in HSV-1 latently infected human TG.

Materials and Methods

Clinical specimens from TG donors

Heparinized peripheral blood and paired TG were obtained from 35 subjects (median

age 70 yrs, range 51–98 yrs) at autopsy with a median post-mortem interval of 6.4 hrs (range 2.5–11.5 hrs). Causes of death were not related to herpesvirus infections. All subjects provided written informed consent for brain autopsy and use of clinical specimens. The study was performed according to the principles expressed in the Helsinki declaration. Blood was used to generate EBV-transformed B-lymphoblastic cell lines (BLCL) and for HLA typing as described [8]. Plasma HSV-1 IgG levels were determined by ELISA (Focus Diagnostics).

Generation and HSV-1-specificity testing of TG-TCL

TG-TCL were generated by phytohemagglutinin (PHA) stimulation of TG cell suspensions, and CD137-enriched T-cells, in the presence of γ-irradiated allogeneic PBMC and human IL-2 as described [8]. Antigen-specificity of TG-TCL was determined by IFN-γ ICC as described [16], using autologous or partially HLA class I-matched BLCL infected overnight with HSV-1 with a virus/cell-ratio of 10 or pulsed with 2 μM of HSV-1 peptides. Mock-infected BLCL were negative controls. Cells were stained for CD4, CD8, CD3 and IFN-γ (all from Becton Dickinson; BD) and analyzed by flow cytometry with Diva software (BD) as described [8].

Isolation of nucleic acids and PCR analysis

One-fifth of the dispersed TG cell suspension was used for RNA and DNA isolation [8]. RNA was reverse transcribed using oligo-dT and used for quantitative real-time PCR (qPCR) on an ABI Prism 7700 with Taqman Universal Master Mix and commercial intron-spanning primer/probe- pairs specific for human perforin, grB, CD8α, TNF-α, IFN-γ and β-actin (Applied Biosystems) per manufacturer. The relative transcript levels were determined by the formula 1,000x2(-delCt) where delCt equals Ct [(target gene) - Ct (β-actin)]. Intra-TG HSV-1 DNA load was determined by qPCR [14].

CD137-based enrichment of virus-reactive CD8 T-cells from TG-TCL

To enrich HSV-1 reactive CD8 T-cells, HLA-matched BLCL were infected overnight with HSV-1 with a virus/cell ratio of 10. TG-TCL were added at a ratio of 1:1 for the next 24 hrs. Cells were harvested, stained for CD3 (BD), CD8 (BD) and CD137 (Miltenyi). Cells expressing CD3, CD8, and CD137 were selected with a BD FACS Aria cell sorter, expanded by PHA stimulation and used in HSV-1 ORFeome screens as described [16].

HSV-1 ORFeome screen

The generation and validation of the HSV-1 ORFeome, covering a total of 74 HSV-1 ORFs, for functional T-cell assays has been detailed [16]. In short, each HSV-1 ORF was amplified and cloned into a custom-made vector fused to eGFP. Donor-matched HLA-I cDNA (in pcDNA3) and HSV-1 ORFs were expressed in Cos-7 cells (ATCC CRL-1651) by transfection [16]. All HSV-1 ORFs were transfected in duplicate and appropriate mock- or HSV-1 infection controls were included. After 48 hrs, ORF expression was confirmed by eGFP fluorescence and TG-TCL ($5x10^4$/well) were added to 104 transfected Cos-7 cells/well. After 24h, supernatants were collected for IFN-γ ELISA [16].

Whole HSV-1 ORFeome screens for CD4 targets were performed in duplicate as described [16, 27]. Gamma-irradiated HLA-DQ/DR-matched allogeneic PBMC were pulsed overnight with predefined dilutions of protein lysates of HSV-1 ORF-transfected Cos-7

cells [27] or HSV-1 proteins made with bacterial lysates [16], or peptides at 2 μM, and as controls UV-treated mock- and HSV-1-infected Vero cell lysates [16]. After 48h, [^{3}H]-thymidine was added and cells harvested to measure [^{3}H]-thymidine incorporation [16].

In situ analyses of human TG

In situ immunofluorescence was performed using allophycocyanin-labeled CD4 (clone RPA-T4; BD) and FITC-labeled CD8 (1A5; Monosan) monocloncal antibodies (mAbs). The APC signal was enhanced by the FASER system per manufacturer (Miltenyi). Sections were post-fixed with 4% formaldehyde, counterstained for DNA with DAPI (Invitrogen) and mounted with ProLong Gold Antifade Reagent (Invitrogen). For immunohistochemistry, paraffin sections and cryosections of human TG were stained as described [23]. The mAbs used were directed to CD8 (1A5; Monosan), grB (GrB-7; Dako), TIA-1 (2G9; Immunotech), CD3 (UCHT1; Dako) and CD137 (4B4-1; BD). Sections were counterstained with hematoxylin and mounted with glycerol gelatin.

In situ tetramer stainings were performed as described previously [19]. In brief, TG cryosections (8 μm) were fixed with 4% formaldehyde and incubated with 2-4 μg of the respective APC-conjugated HSV-1 peptide / HLA-I tetramers at 4°C for 20 hrs. Next, slides were washed and post-fixed in 4% formaldehyde. Slides were (counter-) stained with anti-CD8 (3B5; Invitrogen) and DAPI (Invitrogen), and mounted with ProLong Gold Antifade Reagent (Invitrogen). Fluorescent images were acquired on a Zeiss LSM700 confocal laser scanning microscope.

Statistical analysis

Statistical differences between were determined by Spearman correlation and Wilcoxon matched-pairs signed-rank test. $P<0.05$ were considered significant.

Results

Transcripts levels of T-cell cytolytic effector molecules and cytokines correlate with HSV-1 DNA load and CD8α transcript levels in human TG

To gain insight into the functional properties of human TG-residing T-cells we determined the transcript levels of the T-cell cytolytic effector molecules perforin and grB, and the cytokines IFN-γ and tumor necrosis factor-α (TNF-α) in 26 TG by real-time RT-PCR [14]. HSV-1 DNA was detectable in 17 of 26 (65%) TG. Transcription of the CD8 T-cell-specific gene CD8α, and perforin and grB correlated significantly with the intra-TG HSV-1 DNA load (Figure 1A). Additionally, the perforin, grB, IFN-γ and TNF-α mRNA levels correlated significantly with CD8α mRNA levels (Figure 1B). The data suggest that the TG-resident CD8 T-cell pool is not only increased with a higher intra-TG HSV-1 burden, but is also transcriptionally active to orchestrate an anti-viral function in situ. Finally, the HSV-1 DNA load and mRNA levels of CD8α, grB and IFN-γ correlated significantly between left and right TG indicating that both intra-TG latent HSV-1 burden and T-cell responses are symmetric intra-individually (Figure 1C).

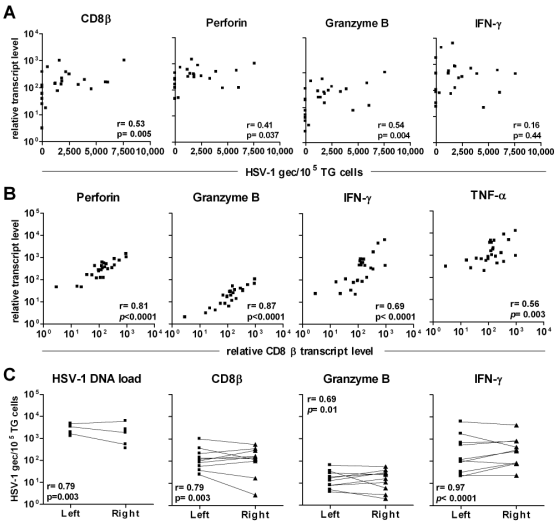

Figure 1. *Comparison of T-cell cytolytic granule and cytokine transcripts to HSV-1 DNA load and CD8α transcript levels in human TG (N=26). (A) Comparison of HSV-1 DNA load with relative transcript levels of CD8α, perforin, grB and IFN-γ. geq, genome equivalent copies. (B) Comparison between relative CD8α transcript levels and perforin, grB, IFN-γ and TNF-α. (C) Comparison of the HSV-1 DNA load and transcript levels of CD8α, grB and IFN-γ between paired left and right TG of individual donors. Spearman correlation (A and B) and Wilcoxon matched-pairs signed-rank test (C) were used for statistical analysis.*

Neuron-interacting T-cells in human TG express markers characteristic for T-cells recognizing antigen in situ

Latently infected human ganglia are infiltrated with T-cells that cluster near LAT+ neurons suggesting their role in controlling HSV-1 latency [7, 8]. To corroborate their potential protective role we performed in situ analyses on HSV-1 latently infected human TG. Neuron-interacting T-cell clusters consisted of CD4 and CD8 T-cells (Figure 2A). CD8 T-cells expressed both grB and the T-cell intercellular antigen-1 (TIA-1) consistent with their cytotoxic potential (Figure 2B). We have recently shown that CD137, a TNF receptor family member [15], is induced on HSV-1 reactive human CD4 and CD8 T-cells shortly after recognition of HSV in vitro [16]. Here, we demonstrated that TG-residing T-

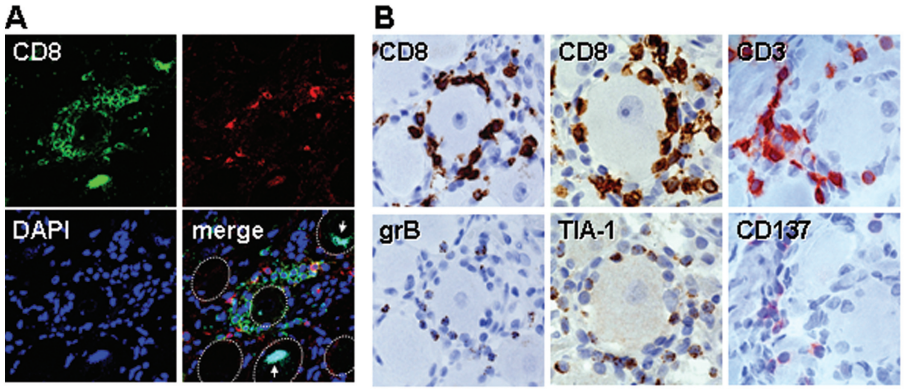

Figure 2. *Phenotype of T-cells in latently infected human TG. (A) Double immunofluoresence staining for CD4 and CD8 combined with DNA counterstaining (DAPI; blue nuclei). (B) Consecutive TG tissue sections stained for CD8 and granzyme B, CD8 and TIA-1, and CD3 and CD137. Sections were developed with diaminobenzidine (brown color) or 3-amino-9-ethylcarbazole (red color) and subsequently counterstained with hematoxylin (blue nuclei). Magnifications were: (A) X400 and (B) X1000. Representative images from 10 TG donors analyzed.*

Table 1. *Phenotype and HLA-restriction of HSV-1 reactive T-cells recovered from human TG.*

	Patient's HLA class I genotype		Percentage HSV-1 reactive T-cells			
Patient ID	**HLA-A allele 1; 2**	**HLA-B allele 1; 2**	**CD4 T-cells**	**CD8 T-cells**	**HLA-A allele 1; 2**	**HLA-B allele 1; 2**
TG1	A*0201;A*1101	B*0702; B*4402	nd	5	4; 0	1; 0
TG2	A*0201	B*1501; B*4402	5	18	5	13; 0
TG3	A*0101	B*0801	0.3	11	11	0
TG4	A*0201;A*0301	B*3501; B*4402	0	2	2; 0	0; 0
TG5	A*0301;A*3004	B*3501; B*4001	0	7	0; nd	0; 7
TG6	A*0301;A*2902	B*0702; B*4403	10	22	2; 1	19; 0
TG7	A*0301;A*3101	B*4001; B*5101	0	37	10; 9	18; 0
TG8	A*0101;A*02	B*07; B*0801	0	10	2; 3	5; nd
TG9	A*0201;A*6802	B*1402; B*5701	nd	5	1; 1	3; 0
TG10	A*0201;A*0301	B*3501	0	24	2; 22	0
TG11	A*0101;A*2902	B*0801; B*4403	nd	11.5	3; 2	3; 3.5
TG12	A*1101;A*3101	B*4001	4	10	3; 0	7

TG-derived T-cell lines were incubated with mock– and HSV-1–infected autologous BLCL and assayed by flow cytometry for intra-cellular IFN-γ expression. Patient HLA-I allele restricted HSV-1 reactive CD8 T-cell responses were defined using partially HLA–I matched BLCL. The values represent mean net Percentages of live/CD3-gated IFN-γ+ T-cells (HSV-1 minus mock) of at least 2 separate experiments. nd, not done.

cells express CD137 in situ, implicating that they have encountered their cognate antigen locally (Figure 2B).

Human TG-derived HSV-1-specific CD8 T-cells are directed to a restricted set of viral proteins

To identify the viral proteins recognized by human TG residing T-cells, T-cell lines (TCL) were generated by mitogenic stimulation of TG-derived T-cells from HSV-1 IgG seropositive donors. HSV-1–specific T-cells were phenotyped and enumerated by a flow cytometric intra-cellular IFN-γ (IFN-γ ICC) assay using mock– and HSV-1–infected autologous B-cell lines (BLCL) as antigen-presenting cells (APC). The median percentage of HSV-1–specific CD8 T-cells in 12 TG-TCL was 10% (range 2 to 37%) and four TG-TCL also contained HSV-1-specific CD4 T-cells (Table 1).

The HSV-1 proteins recognized by human TG-derived CD8 T-cells were determined using transfected Cos-7 cells as artificial APC, which expressed one of the donor's HLA-A and –B alleles in combination with 74 separate HSV-1 open reading frames (ORFs) [16]. First, we used a set of partially HLA–A or –B matched HSV-1-infected BLCL as APC to uncover both the diversity and identity of HSV-1 peptide-presenting HLA class I (HLA-I) alleles used by the CD8 T-cells. The data demonstrated that the virus-specific intra-TG CD8 T-cell response is mediated by 1 to 4 different HLA–A and –B alleles per person (Table 1). Next, we used the implicated HLA cDNAs to determine HSV-1 CD8 T-cell antigens using an HSV-1 ORFeome-wide screen [16]. We observed definitive hits in the HSV-1 ORFeome screen only when the net proportion of CD8 T-cells reactive with

Table 2. *HSV-1 antigens and epitopes recognized by CD8 T-cells recovered from human TG.*

		Kinetic class of the HSV-1 proteins recognized		
Patient ID	**HLA allele**	**Immediate early**	**Early**	**Late**
TG1	A*0201	-	-	UL6 protein
	A*0201			gB
TG2	A*0201	ICP0 aa642-651	ICP8 aa1096-1105	-
	B*1501	-	ICP6	-
TG3	A*0101	VP16 aa090-099	-	gL aa066-074
	A*0101	VP16 aa479-488		gK aa201-209
TG4	A*0201	-	-	UL25 protein
TG5	B*4001	-	ICP6	-
TG6	A*2902	-	-	VP13/14 aa508-516
	B*0702	-	Thymidine kinase	VP11/12 aa386-394
TG7	A*0301	ICP4 aa1096-1105	-	-
	A*3101	-	ICP6	-
	B*4001	VP16 aa163-175	ICP6	-
TG12	B*4001	-	ICP6	-

HLA allele by which the indicated proteins and peptides are recognized by CD8 T-cells. HSV-1 gene and protein names, and expression kinetic class, are from [1] and Genbank NC_001806. Not all gene products have separate names. The amino acid (aa) location of CD8 T-cell epitopes identified are in parentheses.

HLA-matched HSV-1-infected BLCL was >4%. TG-TCL with <4% net reactivity were incubated with HSV-1-infected HLA-matched BLCL and CD137 positive T-cells were selected at 18 hours as described to enrich HSV-1-specific responder T-cells [16], which were then non-specifically expanded and used for genome-wide screens.
In total, specific HSV-1 CD8 T-cell antigens were discovered for 8 of 12 HSV-1 reactive TG-TCL (Table 2). Figure 3 summarizes results of the HSV-1 ORFeome-wide screen of donor TG3 TCL, demonstrating 3 HSV-1 proteins (i.e., gK, gL, and VP16) targeted by HLA-A*0101–restricted HSV-1–specific CD8 T-cells. Among the 8 TG-TCL assayed, 13 different HSV-1 CD8 T-cell viral targets were identified with 1 to 3 viral proteins per TG-TCL (Table 2; Figure 3). VP16 and particularly ICP6 were recognized by multiple TG-TCL in the context of diverse HLA-A and –B alleles. In case of ICP6, 4 of 8 TG-TCL were positive and the protein was recognized via HLA-A*3101 (donor TG7), –B*1501 (donor TG2) and in 3 different TG donors via HLA-B*4001 (TG5, TG7 and TG12) (Table 2). Finally, candidate CD8 T-cell epitopes of several HSV-1 target proteins were predicted by in silico algorithms [17] and the epitopes were subsequently validated by IFN-γ ICC using corresponding synthetic peptides and HLA-matched BLCL as APC (Table 2 and Figure 3).

We recently studied antigenic targets of blood-derived HSV-1 specific CD8 T-cells in HSV-1 IgG seropositive healthy subjects using related methodologies [16]. Systemic HLA–A and –B restricted CD8 responses were directed to 14 HSV-1 ORFs on average per person and 45 HLA–A and –B allele restricted HSV-1 epitopes were identified [16]. To discern potential similarities between systemic and intra-TG HSV-1 peptide specific CD8 responses in HLA-I concordant individuals we tested TG-TCL of seven HLA-A/B allele matched TG donors for responses to HLA-appropriate HSV-1 peptides from our

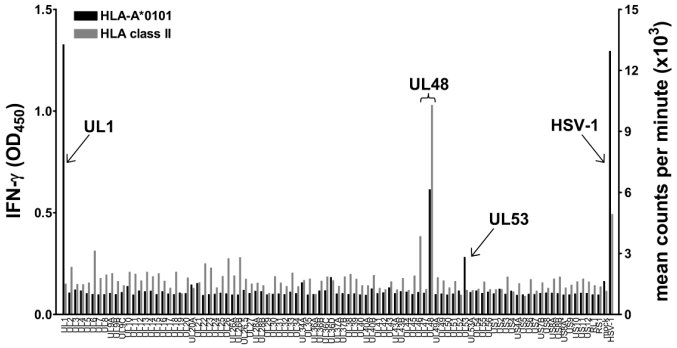

Figure 3. *HSV-1 antigens recognized by human TG-derived CD4 and CD8 T-cells. Representative data from the TG-TCL of donor TG3 for CD8 (black) and CD4 (grey) T-cell reactivity to proteins encoded by individual HSV-1 open reading frames (ORFs). Mean IFN-γ secretion levels, shown as arbitrary OD_{450} values, by TG-TCL exposed in duplicate to Cos-7 cells that co-express TG3-specific HLA-A*0101 and the individual HSV-1 ORFs arrayed in nominal genomic order on the x-axis. The CD4 T-cell reactivity was assayed by a proliferation assay presented as mean [^{3}H]-thymidine incorporation after TG-TCL co-culture with protein lysates from mock- and HSV-1 infected Cos-7 cells, and lysates from Cos-7 cells transfected with individual HSV-1 ORFs, in duplicate using γ-irradiated HLA-DQ/DR-matched allogeneic PBMC. The names of HSV-1 ORFs and corresponding proteins specifically recognized are indicated. VP16, virus protein 16; gK, glycoprotein K and gL, glycoprotein L.*

previous PBMC work [16]. Peptide-specific CD8 T-cell responses were detected in two TG-TCL. The TG-TCL of donor TG3 recognized four HLA-A*0101–restricted peptides: $gL_{66\text{-}74}$, $gK_{201\text{-}209}$, and two VP16 peptides $VP16_{90\text{-}99}$ and $VP16_{479\text{-}488}$. The HLA-A*2902–restricted VP13/14508-516 peptide was recognized by the TG-TCL of donor TG6 (Table 2; Table 3) [16].

Collectively, the data demonstrated that human intra-TG HSV-1–specific CD8 T-cell responses were directed to a relatively restricted number of viral proteins per person. However, even within the small population studied, we detected CD8 T-cell responses to HSV-1 proteins in diverse kinetic and structural classes (Table 2; Table 4). Notably, the HSV-1 β protein ICP6 was a prominent CD8 T-cell target in TG-TCL of 4 of 8 HLA-diverse TG donors involving 3 different HLA-I alleles.

HSV-1-specific human TG-derived CD4 T-cells recognize immediate early and late viral proteins

The intra-TG CD4 responses were analyzed in detail for donors TG2 and TG3 (Table 1). CD4 T-cells of donor TG2 responded to the HSV-1 α protein ICP47 and subsequent assays using whole ICP47-spanning peptides defined the antigenic region at residues 57-75 (Figure 4A-B). In case of donor TG3, CD4 T-cell reactivity was directed to the HSV-1 γ1 protein VP16 (Figure 4C). Application of truncated recombinant VP16 proteins and subsequently overlapping peptides identified two distinct antigenic regions located between residues 187-203 and 215-238 (Figure 4D). Besides being a structural viral protein, VP16 has also been implicated as a master initiator protein for HSV neuronal reactivation in murine studies [18].

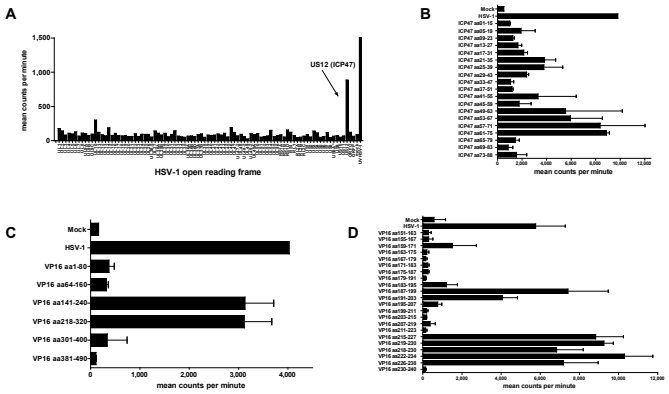

Figure 4. *Identification of the antigenic region of HSV-1 proteins recognized by CD4 T-cells in the TG-TCL of donors TG2 and TG3. (A) The CD4 T-cell reactivity was assayed by a proliferation assay presented as [^{3}H]-thymidine incorporation after co-culture of the TG-TCL of donor TG2 with lysates generated from mock- and HSV-1 infected Cos-7 cells and lysates from Cos-7 cells transfected with the individual HSV-1 ORF antigen using γ-irradiated HLA-DQ/DR donor TG2 matched allogeneic PBMC as APC. The names of the HSV-1 ORFs and corresponding proteins driving the positive responses are indicated. ICP47, infected-cell polypeptide 47. (B) Proliferation assay data of the TG-TCL of donor TG2 with γ-irradiated HLA-DQ/DR TG2-matched allogeneic PBMC pulsed with whole HSV-1 ICP47 protein spanning synthetic peptides (15-meric peptides with 10 amino acid (aa) overlap) as APC. (C) Proliferation assay data of the TG-TCL of donor TG3 with γ-irradiated HLA-DQ/DR donor TG3 matched allogeneic PBMC pulsed with the indicated recombinant HSV-1 VP16 protein fragments as APC. VP16, virus protein 16. (D) Proliferation assay data of the TG-TCL of donor TG3 with γ-irradiated HLA-DQ/DR donor TG3 matched allogeneic PBMC pulsed with HSV-1 VP16 protein fragment (aa151-240) spanning synthetic peptides (13-meric peptides with 8 aa overlap) as APC. Data are presented as mean counts per minute of duplicate experiments.*

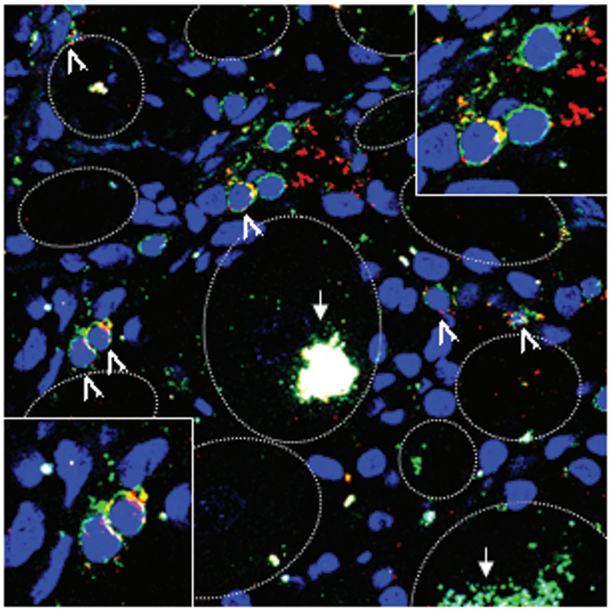

Figure 5. *HSV-1 epitope-specific CD8 T-cells are localized close to neuronal cell bodies in human TG. Representative image of the TG tissue of donor TG2 stained with DAPI (blue), anti-CD8 (green) and tetramers (red) that consisted of both the synthetic HSV-1 peptides ICP0$_{642-651}$ and ICP8$_{1096-1105}$ conjugated to HLA-A*0201. Inserts, lower left and upper right corner, are enlargements of areas containing tetramer positive CD8 T-cells. The white arrows and arrow heads signify autofluorescent granules and tetramer-positive CD8 T-cells. respectively. Neuron outlines are marked with a white dashed line. Magnification was X400.*

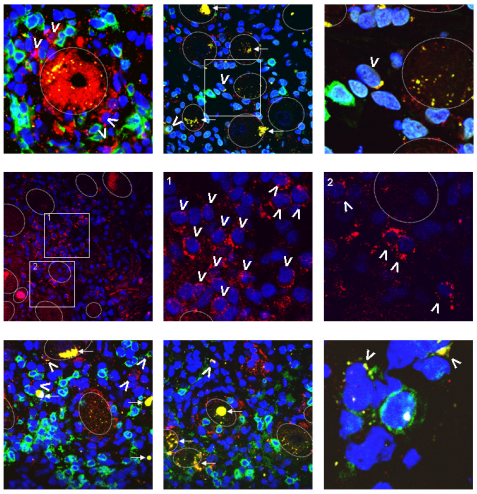

Figure 6. *HSV-1 epitope-specific CD8 T-cells are localized in the vicinity to neuronal cell bodies in human TG tissue. Representative optical section images of snap-frozen TG tissue of donor TG3 stained with DAPI (blue), anti-CD8 (green) and tetramers (red) that consisted of the synthetic HSV-1 peptides gL_{66-74} (upper panel), $VP16_{90-99}$ (middle panel) and $gK_{201-209}$ (lower panel) bound to HLA-A*0101. The white arrows and arrow heads signify autofluorescent granules and tetramer-positive cells, respectively. The boxed areas in the upper and middle panels are enlarged in the corresponding images to the right. Note that for the HLA-A*0201 /$VP16_{90-99}$ tetramer staining anti-CD8 was omitted. Neuron outlines are marked with a white dashed line. Magnifications were: X400 and in the enlargements X800.*

HSV-1 epitope specific CD8 T-cells localize in close proximity to neurons in human TG

The symmetry of the virus and T-cell parameters between paired TG facilitated studies on the spatial orientation of HSV-1 reactive CD8 T-cells in the contralateral snap-frozen TG specimen of the same donor by in situ tetramer staining [19]. HSV-1 CD8 T-cell epitopes and corresponding snap-frozen contralateral TG specimens were available for donors TG2 and TG3. HLA-A*0201 tetramers conjugated with $ICP0_{642-651}$ and $ICP8_{1096-1105}$, and HLA-A*0101 reagents with gL_{66-74}, $gK_{201-209}$, $VP16_{90-99}$ and $VP16_{479-488,}$ were validated on the corresponding TG-TCL (data not shown). HSV-1 tetramer+ CD8 T-cells preferentially clustered near neuronal cell bodies in TG of the respective donor (Figure 5, Figure 6).

Discussion

The host-pathogen standoff in human latent HSV-1 infection permits periodic epithelial shedding of infectious virus, and potential transmission, without overt host damage. In the present study, we demonstrated that the human TG is an immunocompetent organ capable of presenting viral antigens to both CD4 and CD8 T-cells, presumably over long periods of time, to maintain local enrichment of HSV-1–specific T-cells. The data suggest that the HSV-1 proteins expressed in latently infected human TG is unbiased to a specific class of kinetic or structural viral proteins and that the viral antigens identified herein are rational candidates for HSV-1 subunit vaccines.

In contrast to viral DNA and transcripts, viral proteins have not been detected in HSV-1 latently infected human ganglia [1, 13]. Viral protein synthesis may be shutdown or occur at low levels. T-cells are activated with only a few MHC/peptide-complexes making them highly sensitive and specific biosensors to detect extremely low-level expression of their cognate antigens [20]. The recognition of diverse HSV-1 proteins by human TG infiltrating T-cells implies their cognate antigen expression in situ. Moreover, the HSV-1 targets identified did not group to a specific kinetic or functional class of viral proteins

suggesting that HSV-1 protein synthesis is unbiased during latency in human TG. Alternatively, the T-cell response reported herein may be directed to local reactivating HSV-1. In contrast to humans, HSV-1 mouse models are either fatal or have tight neuronal latency in which spontaneous reactivation does not lead to peripheral release of infectious virus [21]. Nevertheless, latently infected TG of C57BL/6 mice contain neuron-interacting CD8 T-cells, directed to non-α HSV-1 proteins like gB and ICP6 [6, 11], implicating that full HSV-1 reactivation is not a prerequisite to retain infiltrating virus-specific T-cells in ganglia with diverse viral protein reactivity. The combined human and mouse data argue that this process involves recognition of the T-cells' cognate viral antigens produced locally in HSV-1 latently infected ganglia.

Activation of HSV-1-specific CD8 T-cells in latently infected murine ganglia is dependent on local CD4 T-cells, MHC class II and recruited blood-derived APC [22]. We have recently shown that satellite glial cells (SGC), which tightly envelop neuronal cell bodies in ganglia, are most likely not of neuroectodermal but of myeloid origin [23]. Human TG-resident SGC are related to macrophages and myeloid dendritic cells with regards to their phagocytic capacity and expression of CD45, co-stimulatory and HLA class II molecules [23]. The present study documents inclusion of CD4 T-cells in neuron-surrounding T-cell clusters and proves peptide-level recognition of HSV-1 by TG-resident CD4 T-cells in the natural host. Although local human APC driving the HSV-1-specific CD4 T-cell responses could be blood-derived or ganglion-resident [22, 24], it is unlikely that HLA class II negative neurons are directly involved. The data imply trafficking of HSV-1 proteins or remnants thereof from neurons to secondary APC. Given their localization and phenotype, SGC are candidate APC to create an immunocompetent but not overly inflammatory environment to support HSV-specific CD4 and CD8 T-cell responses within latently infected human TG.

An important and still unanswered question is the functional role of the HSV-specific T-cells documented in this report. In the absence of tools to selectively interrupt or bolster T-cells at specific anatomic sites in humans, this question is difficult to address. Surrogate data can be obtained from examining the phenotype and activation status of human TG-resident CD8 T-cells. Integrating human TG ex vivo flow cytometry and in situ data, it is evident that TG-resident CD8 express CD137 and grB, and low levels of CD27 and CD28, indicative of recent antigen encounter locally [8, 16]. mRNA expression for antiviral T-cell cytokines and lytic molecules was directly correlated with HSV-1 DNA levels (Figure 1), and lytic granule molecules were also detected at the protein level in neuron-surrounding CD8 T-cells (Figure 2). The fact that human TG-resident HSV-1-specific CD8 T-cells can massively expand in vitro and then display brisk virus-specific IFN-γ responses argues against an exhausted phenotype [16, 25]. Together with the remarkable localization of HSV-1-specific CD8 T-cells around neurons (Figure 5 and Figure 6), the data hitherto argue for a functional role for these cells in non-lytic control of neuronal HSV-1 latency in human TG in cooperation with local CD4 T-cells.

If this interpretation is correct, elicitation of T-cells capable of activity in the TG is a rational goal for preventative and therapeutic vaccines. Our findings have several implications for HSV-1 vaccine design. First, ICP6 was recognized by 4 of 8 TG donors in diverse HLA-I contexts. ICP6 is a ribonucleotide reductase subunit expressed prior to viral

DNA replication [1]. Because ICP6 was also a dominant target for the systemic CD8 response in HSV-1 seropositive subjects [16], this protein is an attractive vaccine candidate. Second, HSV-1 proteins of diverse kinetic and structural classes were recognized by TG CD8 T-cells (Table 2). These range from nonstructural α (ICP0 and ICP4) and β proteins (ICP6 and thymidine kinase) to late structural tegument (VP11/12 and VP13/14) and envelope glycoproteins (gB, gK and gL). Tegument protein VP16, recognized by both CD4 and CD8 TG-resident T-cells, is possibly a chameleon with both a hyper-early role in neuronal reactivation and a structural role in tegument assembly [18]. The cell biology implication of this finding is that diverse HSV-1 proteins are diverted from viral assembly and access the HLA class I pathway in neurons, or possibly surrounding APC after handover. Third, the apparent diversity of recognized HSV-1 antigens is lower in TG than in blood, where we detected a mean of 14 reactive HSV-1 ORFs per person using similar technology [16]. The restricted clonality of the human intra-TG T-cell response has been confirmed by T-cell receptor spectratyping [8, 26]. Surveys of more participants, ideally with parallel PBMC studies, will be required to determine if the breadth or fine specificity of the paired TG and systemic HSV-1 T-cell responses differ and to pick the best antigens for possible subunit approaches targeting ganglia as a locus of control. Manipulation of T-cell priming or boosting to imprint a ganglia-homing program via vaccination, without imparting an overly aggressive phenotype, is an equally important and challenging task that must be overcome to target the TG as an immunocompetent site for the purpose of HSV-1 latency control.

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Table 3. *HSV-1 peptide responses in TG-derived TCL of HLA class I concordant donors.*

HSV-1 peptide ID			Percentage peptide reactive CD8 T-cells						
HLA allele	**ORF**	**aa location**	**TG1**	**TG2**	**TG3**	**TG4**	**TG6**	**TG8**	**TG9**
A*0101	UL1	066-074	na	na	10	na	na	0	na
A*0101	UL39	512-520	na	na	nd	na	na	0	na
A*0101	UL41	259-268	na	na	nd	na	na	0	na
A*0101	UL46	354-362	na	na	nd	na	na	0	na
A*0101	UL47	360-368	na	na	nd	na	na	0	na
A*0101	UL47	566-574	na	na	nd	na	na	0	na
A*0101	UL48	090-099	na	na	1.5	na	na	0	na
A*0101	UL48	479-488	na	na	4	na	na	0	na
A*0101	UL53	201-209	na	na	3.5	na	na	0	na
A*0201	UL13	389-397	0	0	na	0	na	0	0
A*0201	UL25	367-375	0	0	na	0	na	0	0
A*0201	UL27	280-288	0	0	na	0	na	0	0
A*0201	UL27	448-456	0	0	na	0	na	0	0
A*0201	UL39	425-433	0	0	na	0	na	0	0
A*0201	UL40	184-192	0	0	na	0	na	0	0
A*0201	UL47	286-294	0	0	na	0	na	0	0
A*0201	UL47	374-382	0	0	na	0	na	0	0
A*0201	UL47	545-553	0	0	na	0	na	0	0
A*2902	UL25	170-179	na	na	na	na	0	na	na
A*2902	UL25	235-243	na	na	na	na	0	na	na
A*2902	UL26	022-030	na	na	na	na	0	na	na
A*2902	UL26	326-334	na	na	na	na	nd	na	na
A*2902	UL27	295-303	na	na	na	na	0	na	na
A*2902	UL27	641-649	na	na	na	na	nd	na	na
A*2902	UL29	460-468	na	na	na	na	0	na	na
A*2902	UL29	895-903	na	na	na	na	0	na	na
A*2902	UL46	093-101	na	na	na	na	0	na	na
A*2902	UL46	126-134	na	na	na	na	0	na	na
A*2902	UL46	224-232	na	na	na	na	0	na	na
A*2902	UL46	333-341	na	na	na	na	0	na	na
A*2902	UL47	508-516	na	na	na	na	1.5	na	na
B*0702	ICP0	698-706	na	na	na	na	0	0	na
B*0702	UL21	382-390	na	na	na	na	0	0	na
B*0702	UL49	281-290	na	na	na	na	0	0	na
B*0702	US1	070-078	na	na	na	na	0	0	na
B*0702	US7	022-030	na	na	na	na	nd	nd	na
B*0702	US7	097-105	na	na	na	na	0	0	na
B*0702	US7	195-203	na	na	na	na	0	0	na
B*0702	US7	230-238	na	na	na	na	0	0	na

The viral protein, amino acid location and the human leukocyte antigen (HLA) presenting the indicated peptide are indicated. ORF, open reading frame and aa, amino acid. TG-derived T-cell lines (TG-TCL) from the indicated TG donors were incubated with HLA-matched B-cell lines pulsed with the indicated peptides and assayed by flow cytometry for intra-cellular IFN-γ expression. The values represent the mean net percentages of live/CD3-gated IFN-γ (i.e., peptide minus mock B-cells used as antigen presenting cells) of at least 2 separate experiments. HSV-1 peptides scored positive are boxed. na, not applicable and nd, not done.

Table 4. *Characteristics of HSV-1 proteins recognized by human TG-derived CD4 and CD8 T-cells.*

Gene	Protein	Kinetics	Status	Virion	Function
RS1	ICP4	α	E	Yes	Repressor/transactivator
RL2	ICP0	α	nonE	Yes	Multiple functions: e.g., virus β and γ gene regulator and IFN type I evasion
UL1	Glycoprotein L	γ1	E	Yes	Complexed with glycoprotein H, virus entry
UL6	Not defined	γ1	E	Yes	Cleavage-packaging viral DNA
UL23	Thymidine kinase	β	nonE	Yes	Neurovirulence and target of acyclovir
UL25	Not defined	γ2	E	Yes	Virus penetration and capsid assembly
UL27	Glycoprotein B	γ1	E	Yes	Virus entry and syncytium formation
UL29	ICP8	β	E	No	ssDNA binding
UL39	ICP6	β	nonE	No	Large subunit of ribonucleotide reductase
UL46	VP11/12	γ1	nonE	Yes	Tegument phosphoprotein
UL47	VP13/14	γ1	nonE	Yes	Tegument phosphoprotein, modulates VP16 activity
UL48	VP16	γ1	E	Yes	Tegument, pre-formed transactivator
UL53	Glycoprotein K	γ2	E	No	Virion exocytosis and syncytium formation

Gene and protein names from [1] and Genbank NC_001806. Not all gene products have separate names. Kinetics: expression kinetics classification designated as α (immediate early), β (early), γ1 (late) and γ2 (late late). Expression of γ2 requires ongoing viral DNA synthesis. Status: essential (E) or non-essential (nonE) for virus growth in cell culture. Virion: presence or absence in highly purified virions [1].

CHAPTER 3

Neuron-interacting satellite glial cells in human trigeminal ganglia have an antigen presenting cell phenotype

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Abstract

Satellite glial cells (SGC) in sensory ganglia tightly envelop the neuronal cell body to form discrete anatomical units. This type of glial cell is considered neuroectoderm-derived and provides physical support to neuron somata. There are scattered hints in the literature suggesting that SGC have an immune-related function within sensory ganglia. Here, we addressed the hypothesis that SGC are tissue-resident APC. The immune phenotype and function of a large series (n=40) of human trigeminal ganglia (TG) were assessed by detailed flow cytometry, in situ analyses, and functional in vitro assays. Human TG-resident SGC (TG-SGC) uniformly expressed the common leukocyte marker CD45, albeit at lower levels compared to infiltrating T-cells, and the macrophage markers CD14, CD68 and CD11b. In addition, TG-SGC expressed the myeloid dendritic cell (mDC) marker CD11c, the T-cell co-stimulatory molecules CD40, CD54, CD80, and CD86 and MHC class II. However, the mature DC marker CD83 was absent on TG-SGC. Functionally, TG-SGC phagocytosed fluorescent bacteria, but were unable to induce an allogeneic mixed leukocyte reaction. Finally, TG-infiltrating T-cells expressed the T-cell inhibitory molecules CD94/NKG2A and PD-1, and the interacting TG-SGC expressed the cognate ligands HLA-E and PD-L1, respectively. In conclusion, the data demonstrate that human TG-SGC have a unique leukocyte phenotype, with features of both macrophages and immature mDC, indicating that they have a role as TG-resident APC with potential T-cell modulatory properties.

Introduction

Sensory ganglia are part of the peripheral nervous system (PNS). They contain cell bodies of sensory neurons establishing the connection between the periphery and CNS. Sensory ganglia lack a blood-nerve barrier and enclose high numbers of satellite glial cells (SGC) [1-3]. SGC are considered to be neuroectoderm-derived and involved in the maintenance of sensory neuron homeostasis by regulating extracellular ion and nutrient levels within sensory ganglia [2]. In contrast to CNS-resident glial cells, like astrocytes and microglia, SGC have a distinct interaction with neurons [2-3]. They directly associate with the neuronal soma, so that each neuronal cell body is completely surrounded by a sheet of several SGC providing physical support and a protective barrier [3]. The numerous fine invaginations between the neuron and SGC sheath illustrate their intimate association [2-3]. Upon mechanical injury to sensory neurons, SGC undergo morphological changes, proliferate, and up-regulate a variety of growth factors, cytokines and the glial marker glial fibrillary acidic protein [2, 4-5].

Human alpha-herpesviruses, like HSV, are a common threat to human sensory ganglia. HSV establishes a lifelong latent infection in neurons within sensory ganglia, predominantly the trigeminal ganglion (TG), and reactivates intermittently [6]. Recent studies in mice and humans emphasized the importance of infiltrating T-cells to control latent HSV infections in sensory ganglia [7-9]. Virus-specific T-cells are directly juxtaposed to latently infected neurons, produce cytokines and cytolytic effector molecules, but do not induce neuronal damage [7-8, 10-12]. Current data suggest that the neurons themselves or hitherto unrecognized resident cells in latently infected sensory ganglia induce and coordinate this non-pathogenic chronic T-cell response [8, 10-12].
Here, we addressed the hypothesis that SGC are tissue-resident APC. The availability of a series of fresh post-mortem human TG specimens enabled us to combine ex vivo and in situ analyses for the phenotypic and functional characterization of human TG-resident SGC (TG-SGC).

Materials and Methods

Clinical specimens

Heparinized peripheral blood (PB) and TG specimens, i.e. left and right TG, were obtained from 40 subjects (median age 79 yr, range 41-94 yr) at autopsy with a mean post-mortem interval of 6 hr (range 2.5-15.5 hr). The TG tissue panel consisted of 34 donors with a CNS disease (mainly Alzheimer's disease and Parkinson's disease) and 6 donors without evidence of CNS disease. The cause of death was not related to alpha-herpesvirus infections. No significant differences in the immunological parameters analyzed were detected between donors with or without a history of CNS disease (data not shown). Specimens were either snap-frozen (n=23) or transferred to tubes containing culture medium consisting of RPMI-1640 (Lonza) supplemented with heat-inactivated 10% FBS (Greiner) and antibiotics (n=17). Written informed consent from the donor or next of kin was obtained. The local ethical committees approved the study, which was conducted according to the tenets of the Declaration of Helsinki.

Generation of TG single cell suspensions

Generation of single cell suspensions from human TG was performed essentially as described previously [12]. In brief, the TG were fragmented and subsequently treated with Liberase Blendzyme 3 (0.2 units/mL, Roche) at 37°C for 1 hr. Dispersed cells were filtered through a 70 µm pore-size cell strainer (BD Biosciences), and the flow-through was collected in PBS containing 1% FBS. From the same donor, PBMC were isolated from heparinized PB (~4 ml/donor) by density gradient centrifugation on Ficoll-Hypaque [12]. Donor PBMC and TG single cell suspensions were directly used for phenotypic and functional analyses.

Flow cytometry

Donor-matched PBMC and TG cells were subjected to multi-color flow cytometric analyses using the following fluorochrome-conjugated mAbs: CD3-allophycocyanin (UCHT1; Dako), CD11b-PE (Bear-1; Beckman Coulter), CD11c-allophycocyanin (S-HCL3; BD Biosciences), CD14-FITC (TÜK4; Dako), CD40-FITC (5C3; BD Biosciences), CD45-PerCP (2D1; BD Biosciences), CD54-FITC (6.5B5; Dako), CD68-PE (Y1/82A; BD Biosciences), CD80-FITC (MAB104; Beckman Coulter), CD83-allophycocyanin (HB15e; BD Biosciences), CD86-PE (FUN-1; BD Biosciences), HLA-DR-PerCP (L243; BD Biosciences), CD94-FITC (DX22; eBioscience), NKG2A-allophycocyanin (131411; eBioscience), PD-1-PE (MIH4; eBioscience), and PD-L1-PE (MIH1; eBioscience). Cells were labeled according to the manufacturers' instructions and appropriate isotype- and fluorochrome-matched unrelated mAbs were included as negative controls. Cells and data were analyzed on a BD FACSCalibur flow cytometer and BD CellQuest Pro software (BD Biosciences).

In situ analyses

Snap-frozen TG were embedded in Tissue Tek OCT compound (Sakura) and cut into 6 µm sections on a Leica CM 3050S cryostat. Sections were fixed in acetone for 10 min and incubated with the following unconjugated mAbs according to the manufacturers' instructions: CD11b (ICRF44; BD Biosciences), CD11c (B-ly6; BD Biosciences), CD14 (TÜK4; Dako), CD16 (3G8; BD Biosciences), CD40 (5D12; Pangenetics), CD45 (2B11+PD7/26; Dako), CD54 (LB-2; BD Biosciences), CD64 (32.2; Dako), CD68 (KP1; Dako), CD80 (M24; Innogenetics), CD83 (Hb15a; Beckman Coulter), CD86 (1G10; Pangenetics), CD94 (HP-3B1; Immunotech), HLA-E (4D12; gift from D.E. Geraghty, Seattle, WA), PD-1 (MIH4; eBioscience), and PD-L1 (MIH1; eBioscience). Primary mAb were visualized using the avidin-biotin system (Dako) and 3-amino-9-ethylcarbazole (AEC; Sigma) as substrate, sections were counterstained with hematoxylin (Sigma), examined under a Zeiss Axioskop and photos were taken using a Nikon DC-U1 camera. For each donor and each marker, three sections and three fields per section were analyzed. Human tonsil sections were used as positive control tissue and appropriate isotype and conjugate negative control stainings were included.

For double stainings, sections were fixed in acetone and endogenous peroxidase activity and endogenous biotin were blocked before incubation with the first primary antibody CD14 (TÜK4) or CD45 (2B11+PD7/26). The first mAb was detected using an avidin-biotin-HRP system (Biogenex). Before substrate incubation, sections were incubated with normal mouse serum (10%) and a CD11c-PE mAb (B-ly6), which was visualized using an anti-PE secondary Ab (AbD Serotec) and an alkaline phosphatase-conjugated

tertiary Ab (Sigma-Aldrich). Slides were first developed with fast blue substrate, followed by incubation with AEC substrate solution.

Enrichment of PB- and TG-derived cell populations
Monocytes and TG-SGC were isolated using anti-CD14 microbeads and a MACS magnetic separator (Miltenyi Biotec) according to the manufacturer's instructions. T-cells were isolated from PBMC of healthy blood donors using anti-CD3 microbeads (Miltenyi Biotec). Flow cytometry confirmed that the enriched cell fractions contained >85% CD14+ cells and >95% CD3+ cells, respectively (data not shown).

Phagocytosis assay
TG single cell suspensions were incubated with fluorescein-labeled Escherichia coli (E. coli) K-12 strain bioparticles (Invitrogen) in a cell-to-particle ratio of 1:100 according to the manufacturer's instructions. After incubation at 37°C for 2 hr, cells were washed extensively and subjected to flow cytometry or used for immunocytological analyses. For the latter procedure, E. coli treated TG-SGC were enriched using anti-CD14 beads, spun down onto glass slides, fixed with 4% paraformaldehyde and stained with Alexa Fluor610-PE-conjugated anti-CD68 mAb (KP1; Dako). Cytospins were mounted in ProLong Gold anti-fade reagent with DAPI (Invitrogen) and analyzed on a confocal laser-scanning microscope (Zeiss LSM510 Meta). Pictures were made using multi-track recording with a 405 nm Diode, 488 nm Argon and 561 nm Diode laser to detect DAPI, fluorescein and Alexa Fluor610-PE, respectively.

AlloMLR assay
CD14-enriched TG-SGC, PB-derived monocytes and mature DC were used as stimulator cells in alloMLR assays. Due to the low number of monocytes recovered from the donors' PBMC, mature DC were generated from PB samples of healthy blood donors (n=2). To obtain mature DC, CD14-enriched PB-derived monocytes were cultured with IL-4 and GM-CSF for 6 days to generate immature monocytes-derived DC, and subsequently matured with a cytokine cocktail as described previously [13-14]. The mature DC phenotype, characterized by high CD80, CD83 and CD86 expression [15], was confirmed by flow cytometric analyses (data not shown). The effector cells, i.e. allogeneic PB T-cells, were labeled with CFSE (Invitrogen) at a final concentration of 0.5 μM. The stimulator cells were co-cultured with effector cells at a ratio of 1:10 at 37°C. At day 7, cells were harvested for flow cytometric analyses. Cells were stained with CD3-allophycocyanin (UCHT1; Dako) to discriminate between T-cells and stimulator cells.

Results

Human TG-SGC express typical macrophage markers
We have previously shown that TG-SGC uniformly express MHC class II suggesting that they have a role as APC [12]. Tissue-resident APC, including macrophages and dendritic cells (DC), express the common leukocyte marker CD45 enabling their distinction from stromal cells like fibroblasts. Paired TG-derived cells and PBMC samples were assayed for CD45 expression. In contrast to PBMC, the TG-derived CD45+ cell pool included 2 dis-

tinct cell populations: CD45high and CD45low cells (Figure 1A). Whereas the CD45high cells consisted mainly of T-cells (data not shown), all CD45low cells expressed the monocyte/macrophage marker CD14 (Figure 1B; Table 1). In situ analyses showed that CD14 was expressed by TG-SGC (Figure 1B). As hinted upon by a previous report [8], the macrophage-specific marker CD68 was expressed intracellularly but not on the surface of TG-SGC (Figure 1C; Table 1 and data not shown). Additionally, TG-SGC selectively expressed antigen uptake receptors like CD11b and CD11c (Figure 1D-E; Table 1), as well as CD16 and CD64 (data not shown). In situ double stainings confirmed the flow cytometry data and demonstrated co-expression of CD14, CD45 and CD11c on TG-SGC (Figure 2).

Table 1. *Marker expression on CD45low human TG-SGC.*

Marker	Percentage of Positive TG-SGC ± SD	No. of Donors
CD14	95.9 ± 4.7	9
CD68	97.3 ± 1.1	2
CD11b	92.3 ± 6.6	2
CD11c	88.5 ± 8.3	8
CD80	82.3 ± 18.5	5
CD86	94.8 ± 5.6	6
CD83	6.3 ± 4.1	3
CD40	91.9 ± 7.2	4

Data represent the average of TG-SGC that express the indicated marker determined by flow cytometry.

Human TG-SGC have an immature myeloid DC phenotype

The complement receptor CD11c is commonly used as a marker to discriminate between myeloid (mDC; CD11cpos) and plasmacytoid (CD11cneg) DC [16]. Maturation of mDC is characterized by upregulation or induction of surface markers like MHC class II and the co-stimulatory molecules CD80, CD83 and CD86 essential for T-cell interaction and stimulation [15]. Surface expression of CD83 is considered characteristic for functionally mature DC [17].

The expression of CD11c and MHC class II on TG-SGC prompted us to determine the expression of additional DC markers. Whereas the TG-SGC expressed both CD80 and CD86 (Figure 1F-G; Table 1), the mature DC marker CD83 could not be detected (Figure 1H; Table 1). Furthermore, TG-SGC co-expressed MHC class II and the T-cell adhesion molecule CD54 (Figure 1I). All markers determined were expressed uniformly on all TG-SGC, irrespective of the presence of interacting T-cells or latent alpha herpesvirus (data not shown). However, flow cytometry demonstrated CD40 expression on TG-SGC of several donors, whereas by in situ analyses some TG donors showed weak but differential CD40 expression on TG-SGC associated with T-cell clusters (Figure 1J; Table 1). Although not all donors and not all T-cell-cluster-associated SGC showed this CD40 staining pattern, we ascribe this discrepancy with the flow cytometry data to inter-donor variation, fixatives, or different clones of CD40 mAbs that were used in the separate assays. Table 2 presents a comparative overview of the phenotype of human TG-SGC.

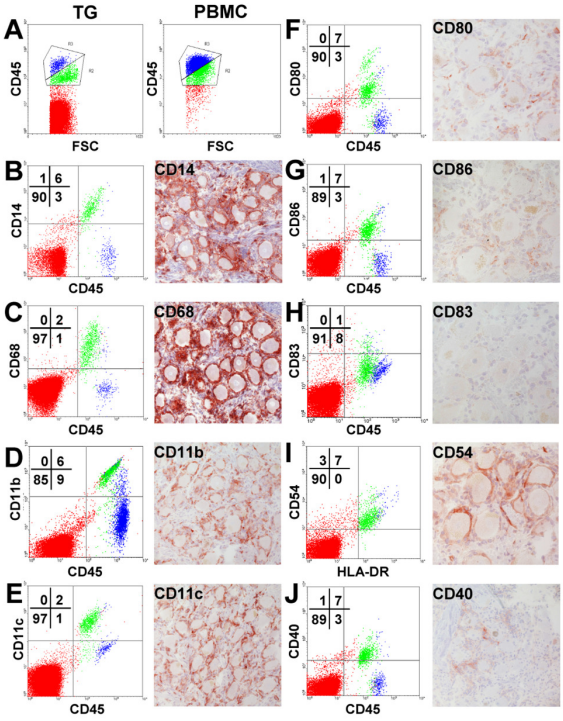

Figure 1. *Human TG-resident SGC express macrophage- and DC-specific markers. (A) Dot plots of paired TG cells (left) and PBMC (right) stained for CD45 to demonstrate that human TG harbor a unique cell population expressing CD45 at low levels (gate R2). CD45low and CD45high cells are arbitrarily green and blue in all dot plots, respectively. Subsequent panels show representative ex vivo flow cytometric analysis (left) (n = 14 donors) and in situ analysis (right) (n = 6 donors) of the expression of CD14 (B), CD68 (C), CD11b (D), CD11c (E), CD80 (F), CD86 (G), CD83 (H), CD54 (I), and CD40 (J) detected. The number for each quadrant in dot plot represents the percentage of cells expressing the indicated marker defined on matched isotype control mAb stainings. Sections were developed with AEC (bright red precipitate) and counterstained with hematoxylin (blue nuclei). Original magnifications are ×200 (B–E, and J) and ×400 (F–I).*

Human TG-SGC phagocytose bacterial particles

A critical role of macrophages is to phagocytose cellular debris and pathogens. Because the TG-SGC have a macrophage phenotype, we determined their capability to phagocytose bacterial particles. Whole TG cell suspensions were incubated with fluorescein-conjugated E. coli after which the phagocytic cell type was identified by flow cytometry. Bacteria were predominantly associated with the CD45low TG cells, identified herein as TG-SGC (Figure 3A). Because this assay does not discriminate between membrane bound and internalized bacteria, the E. coli treated TG-SGC were isolated using anti-CD14 magnetic beads and subsequently subjected to immunocytology. Confocal laser-scanning microscopy revealed that the bacteria co-localized intracellularly with the late endosome marker CD68 (Figure 3A). Conclusively, these data demonstrate that the TG-SGC have actively phagocytosed the bacteria.

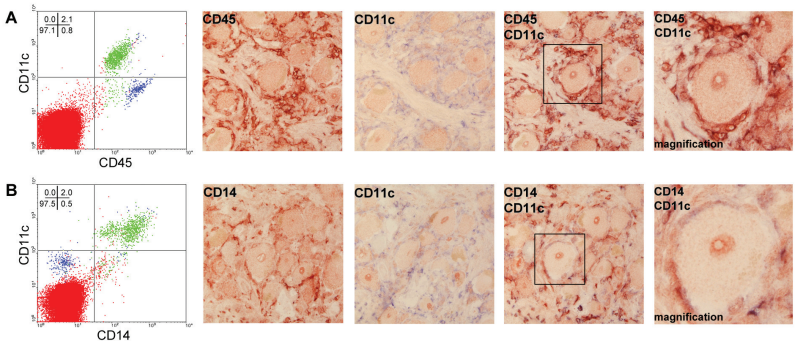

Figure 2. *Human TG-resident SGC express APC markers. Human TG single cell suspensions, and frozen TG biopsy specimens, were analyzed for the markers CD45 and CD11c (A), and CD14 and CD11c (B) in cytometry analysis (n = 14 donors) and double-color in situ analysis (n = 6 donors) on consecutive sections, respectively. CD45low and CD45high cells are arbitrarily green and blue in all dot plots, respectively. The number for each quadrant in the dot plot represents cells expressing the indicated marker. Slides were developed with AEC and Fast blue resulting in red and blue staining patterns, respectively. A double positive cell, stained purple, is enlarged for experiment (far right). Original magnification is ×200.*

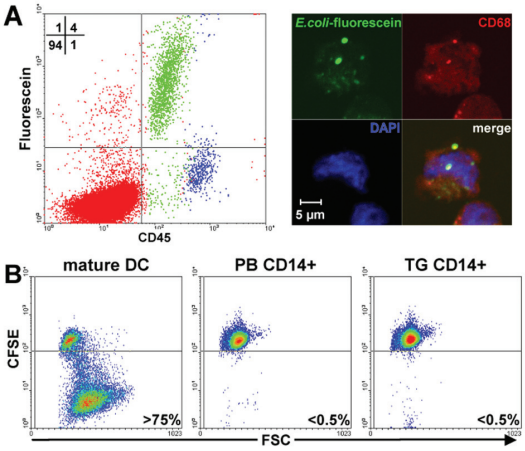

Figure 3. *Human TG-resident SGC share functional characteristics with macrophages and immature myeloid DC. (A) Human TG-SGC were incubated with fluorescein-conjugated bacteria to determine their phagocytic function by flow cytometry (left) and confocal laser scanning microscopy (right). CD45low and CD45high cells are arbitrarily green and blue in the dot plot, respectively (left). Cytospins of CD14-enriched TG-SGC treated with fluorescein-conjugated bacteria (bacteria in green) were stained for CD68 (late endosomes in red) and DAPI (cellular nuclei in blue) and examined by confocal laser scanning microscopy (right). (B) Dot plots of a representative allogeneic MLR using mature monocyte-derived DC generated from peripheral blood-derived monocytes of a healthy blood donor (DC, left), and CD14-enriched peripheral blood monocytes (PB CD14+, middle), and CD14-enriched TG-SGC (TG CD14+, right) recovered from the same TG donor, hereby used as stimulator cells in combination with CFSE-labeled allogeneic T cells. The percentage indicates the frequency of T cells that proliferated upon incubation at 37°C for 7 days. Results are representative of two experiments performed on two TG donors.*

Human TG-SGC are unable to induce an alloMLR

While immature mDC primarily function as phagocytes, DC maturation is associated with upregulation of co-stimulatory and MHC molecules, secretion of cytokines, down-regulation of phagocytic capacity and increased ability to induce T-cell responses [15]. It is well established that mature DC are potent stimulators of an alloMLR, a characteristic that distinguishes them from other APC [18]. Because TG-SGC expressed a myeloid DC phenotype, they were used as stimulator cells in alloMLR assays. From the same donor, PB-derived CD14+ monocytes and CD14+ TG-SGC were co-cultured with CFSE-labeled allogeneic T-cells. In contrast to mature monocyte-derived DC, both monocytes and TG-SGC were unable to induce T-cell proliferation (Figure 3B), indicating that human TG-SGC resemble immature mDC both phenotypically and functionally.

Human TG infiltrating T-cells express T-cell inhibitory molecules and TG-SGC the respective ligands

Although neuron-interacting CD8+ T-cells express cytolytic molecules, like perforin and granzyme B, neuronal damage is not observed in HSV-1 latently infected TG, suggesting that the cytolytic activity of the CD8+ T-cells is inhibited [7-12, 19]. Recently, Suvas et al. have shown that the NK inhibitory molecule complex CD94/NKG2A prevents CD8+ T-cell mediated TG neuron destruction in mice [19]. Whereas the majority of the TG-infiltrating HSV-specific CD8+ T-cells expressed CD94/NKG2A, both neurons and CD11b+ cells expressed the cognate ligand Qa-1b [19]. Analogous to the mouse, human TG-infiltrating T-cells co-expressed CD94 and NKG2A (Figure 4A). Moreover, the frequency of CD94/NKG2A+ T-cells in TG (mean frequency 13±4%) was higher compared to PB (3±1%), suggesting selective infiltration or differentiation of T-cells to express CD94/NKG2A locally. The cognate receptor HLA-E [20] was expressed throughout the TG tissue, including TG-SGC, and CD94 expression co-localized with CD3 within neuron-interacting T-cell clusters (Figure 4A).

In addition to NK inhibitory molecules, several studies have indicated that the molecule PD-1 and its ligand PD-L1 negatively regulate T-cell effector functions [21-25]. Both CD4+ and CD8+ TG-infiltrating T-cells expressed PD-1, but percentages and expression levels did not differ between donor-matched TG-derived T-cells (mean 29±7%) and PB T-cells (35±12%; Figure 4B). However, in situ analyses revealed that neuron-interacting T-cell clusters tended to have a higher PD-1 expression, compared to scattered T-cells (Figure 4B). Notably, PD-L1 expression was confined to TG-SGC and appeared to be higher on TG-SGC in proximity to the T-cell clusters (Figure 4C).

Discussion

For decades, SGC have been regarded as nursing cells providing physical support to neuron somata in sensory ganglia. The current study demonstrates that human TG-SGC have phenotypic and functional APC properties. Two main findings are reported. First, human TG-SGC have a unique leukocyte phenotype, with features of both macrophages and immature mDC. Second, TG-infiltrating T-cells expressed the T-cell inhibitory molecules CD94/NKG2A and PD-1, and the interacting TG-SGC expressed the cognate ligands HLA-E and PD-L1, respectively.

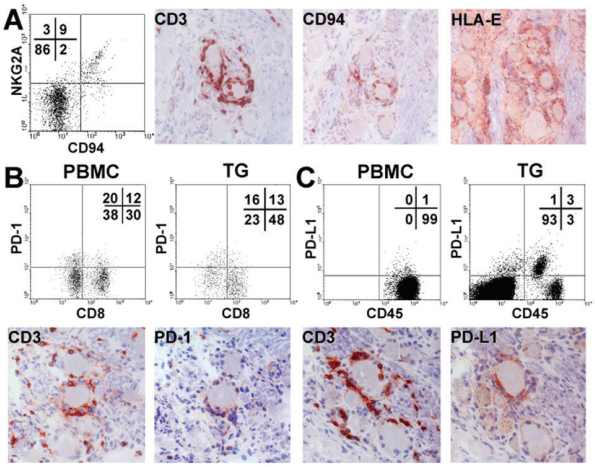

Figure 4. *Human TG-infiltrating T cells express inhibitory molecules. (A) Dot plot of ex vivo flow cytometry (left) on CD94 and NKG2A expression on gated T cells, and in situ analyses of CD3, CD94, and HLA-E on consecutive sections. (B and C) Dot plots of ex vivo flow cytometric (top) and in situ analyses (bottom) of CD3 and PD-1 (B), and CD3 and PD-L1 (C) on consecutive sections. The number for each quadrant in dot plot represents the percentage of cells expressing the indicated marker defined on matched isotype control mAb stainings. Dot plots in A and B are gated on CD3+ cells. Representative data from six TG donors are shown. Sections were developed with AEC (bright red precipitate) and counterstained with hematoxylin (blue nuclei). Original magnification is ×200.*

Current knowledge on CNS-resident glial cells advocate their role as critical participants in the healthy and diseased brain by maintaining axonal integrity and myelination, providing nutrients, controlling synapse formation and function, and immune regulation [26-30]. Whereas macroglia, like astrocytes and oligodendrocytes, are derived from the neuroectoderm [31], microglia express several leukocyte cell markers implicating their origin from myeloid progenitor cells [26, 32]. Microglia are the main CNS-resident APC which constantly sense and sample the brain environment and coordinate immune responses in response to danger signals [26-28]. They resemble macrophages and immature mDC and have been implicated in neurodegenerative disorders like multiple sclerosis [27, 29-30]. Both human and rodent microglia express low levels of the membrane molecule CD45, a marker commonly used to distinguish microglia (CD45low) from stromal cells and macroglia (both CD45neg) and infiltrating lymphocytes (CD45high) [33-34].

In contrast to CNS glial cells, the immune function of PNS-resident SGC is poorly defined. The data presented here demonstrate that human TG-SGC closely resemble CNS microglia both phenotypically and functionally (Table 2). Microglia and TG-SGC are CD45low, and express similar macrophage- and DC-associated markers, and T-cell co-stimulatory molecules (Figure 1 and 2; and Table 2) [26]. Furthermore, both cell types actively phagocytose bioparticles and are unable to induce primary T-cell responses (Figure 3) [35-36]. Hitherto, PNS-resident SGC have been considered to be neuroectoderm-derived [2-3, 37]. The current study challenges this concept, suggesting that human TG-SGC arise from myeloid progenitors analogous to microglia [26, 32].

Table 2. *Comparison of phenotype and functional characteristics of TG-SGC to other human APC[a].*

	Macrophage[b]	Immature DC[b]	Mature DC[b]	CNS Microglia[b]	TG-SGC
Phenotype					
CD14 and CD68	+	-	-	+[c]	+
CD16 and CD64	+	+	-	+	+
CD11b and CD11c	+	+	+	+	+
MHC class II	+	+	++	+	+
CD45	high	high	high	low	low
CD40 and CD54	+	+	++	+	+
CD80 and CD86	+	+	++	+	+
CD83	-	-	+	+[c]	-
Function					
Phagocytosis	+	+	-	+	+
Allogeneic MLR	-	-	+	+[c]	-

a, Results indicate the presence (+), intensity (++; high and low), or absence (-) of the markers or functional characteristics indicated.
b, Data previously described [14, 24, 28].
c, Upon stimulation with LPS, microglia express CD14 and CD83, and are able to induce an allogeneic MLR [24].

Recent data obtained by the Carbone's group support this hypothesis. The authors studied the local effector cells involved in maintaining virus-specific CD8+ T-cell responses that control HSV-1 latency in sensory ganglia of experimentally infected mice [38]. It was shown that CD8+ T-cell homeostasis was depending on a tripartite interaction that includes infiltrating CD4+ T-cells and recruited DC. The effector DC originated from circulating monocytes and expressed high levels of CD11b, CD11c, MHC class II, and F4/80. In situ analyses showed that the CD11c+ DC were occasionally found in close proximity to CD8+ T-cells, but more strikingly they appeared to surround the neuronal somata [38]. The comparable phenotype and anatomic localization of murine sensory ganglia-resident DC and human TG-SGC suggest that they represent the same cell type. This local APC may present the cognate HSV-1 antigens to infiltrating virus-specific CD8+ T-cells. Studies in mice support this notion [39-40]. Alternatively, HSV-1-specific CD8+ T-cells may penetrate the SGC-sheet to interact directly with the latently infected neurons [7]. Because neurons do not express MHC class II, infiltrating virus-specific CD4+ T-cells most likely interact with TG-SGC.

In contrast to a previous study on human TG, the majority of the investigated markers analyzed in this study were uniformly expressed by TG-SGC of the TG donors studied. This was irrespective of the donor's HSV and varicella zoster virus (VZV) serostatus, and the presence of infiltrated T-cells (data not shown). A major difference between the preceding and present study is the median age of the TG donors analyzed: 29 yrs versus 79 yrs, respectively [8]. Animal studies have demonstrated that aging induces the transition of naïve microglia into an activated state, characterized by upregulation of MHC class II

and CD68 [41-42]. Consequently, the discrepancy between both studies may in part be attributed to the relatively high age of the TG donors analyzed here.

It is generally established that TG-infiltrating CD8+ T-cells inhibit HSV-1 reactivation by means of interferon-gamma (IFN-γ) and cytolytic effector molecules [7-8, 12, 43-44]. Nevertheless, the latently infected neurons encountered are not damaged, suggesting that cytolytic T-cell effector functions are inhibited [8, 12, 43]. The expression of CD94/NKG2A on human TG-infiltrating T-cells is consistent with a previous study on mouse TG, demonstrating that blocking the CD94-NKG2A/Qa-1b interaction in ex vivo TG cultures resulted in neuronal cell lysis [19]. CD94 expression in human TG was selectively expressed by T-cells interacting with neuronal somata, suggesting an analogous role of the CD94-NKG2A/HLA-E interaction in human latently infected TG. Notably, Qa-1b was expressed by neurons, but also CD11b+ cells in mouse TG [19]. The CD11b+Qa-1b+ cells may represent the effector DC that are functionally involved in controlling local T-cell responses in HSV-1 latently infected mouse sensory ganglia [38].

In addition to CD94/NKG2A, the data on human TG suggest the involvement of the T-cell inhibitory molecule PD-1. Human TG-infiltrating T-cells and TG-SGC expressed PD-1 and PD-L1, respectively. Notably, the expression of both markers appeared to be higher within neuron-interacting T-cell clusters. Interferon stimulation upregulates PD-1 and PD-L1 expression on receptive cells [22, 45]. Consequently, the differential PD-1 and PD-L1 expression observed may be attributed to IFN-γ secreted by activated T-cells recognizing the latent virus. Functional studies are mandatory to investigate the role of both the HLA-E/CD94-NKG2A and PD-1/PD-L1 pathway to inhibit cytolytic T-cell effector function in human HSV-1 latently infected TG. Moreover, elucidation of the T-cell inhibitory mechanisms employed in the PNS may provide tools for the development of future therapeutic intervention strategies to counteract undue cell damage associated with T-cell-mediated chronic diseases.

In conclusion, the data presented in this study show that human TG-resident SGC have a unique leukocyte phenotype, sharing properties with macrophages and immature mDC. We hypothesize that TG-SGC are tissue-resident APC involved in sensing the local environment and the control of local T-cell responses to protect the irreplaceable neuronal somata in TG.

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CHAPTER 4

Longitudinal study on oral shedding of herpes simplex virus 1 and varicella-zoster virus in individuals infected with HIV

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Abstract

Primary herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) infection leads to a life-long latent infection of ganglia innervating the oral mucosa. HSV-1 and VZV reactivation is more common in immunocompromised individuals and may result in viral shedding in saliva. We determined the kinetics and quantity of oral HSV-1 and VZV shedding in HSV-1 and VZV seropositive individuals infected with HIV and to assess whether HSV-1 shedding involves reactivation of the same strain intra-individually. HSV-1 and VZV shedding was determined by real-time PCR of sequential daily oral swabs (n=715) collected for a median period of 31 days from 22 individuals infected with HIV. HSV-1 was genotyped by sequencing the viral thymidine kinase gene. Herpesvirus shedding was detected in 18 of 22 participants. Shedding of HSV-1 occurred frequently, on 14.3% of days, whereas solely VZV shedding was very rare. Two participants shed VZV. The median HSV-1 load was higher compared to VZV. HSV-1 DNA positive swabs clustered into 34 shedding episodes with a median duration of 2 days. The prevalence, duration and viral load of herpesvirus shedding did not correlate with CD4 counts and HIV load. The genotypes of the HSV-1 viruses shed were identical between and within shedding episodes of the same person, but were different between individuals. One-third of the individuals shed an HSV-1 strain potentially refractory to acyclovir therapy. Compared to HSV-1, oral VZV shedding is rare in individuals infected with HIV. Recurrent oral HSV-1 shedding is likely due to reactivation of the same latent HSV-1 strain.

Introduction

Herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) are closely related neurotropic human alpha-herpesviruses (αHHV) that are endemic worldwide. A hallmark of both viruses is the ability to establish a lifelong latent infection of sensory ganglia with intermittent reactivation and neuronal spread of the virus to innervating tissues [1]. αHHV shedding in bodily secretions and fluids, particularly saliva, contributes to virus transmission throughout the population [2-5]. Virus shedding is commonly asymptomatic, but may lead to recurrent herpetic lesions most commonly as cold sores for HSV-1 and as shingles for VZV. Recurrent HSV infections of the same anatomical location may be due to reactivation of the latent virus strain or superinfection with an exogenous strain. Whereas most sequential HSV-1 isolates from the same anatomical location of an individual are identical, HSV-1 isolates with different genome profiles have been described in patients with oral, genital and corneal herpesvirus infections [6-9]. Alternatively, multiple virus strains may have established latency in the same ganglion. Indeed, different HSV-1 strains have been detected in the same latently infected human ganglion, including the oral mucosa innervating trigeminal ganglion, indicating that recurrent oral HSV-1 shedding may be due to reactivation of genetically different latent HSV-1 strains [10, 11]. The host immune system is pivotal to limit reactivation from its ganglionic stronghold [12-14]. As such, individuals infected with HIV experience more severe and persistent herpetic lesions and may be at risk for central nervous system disease [12, 15-17]

Previous studies have described high oral HSV shedding frequencies in individuals infected with HIV compared to healthy persons seropositive for the respective herpesviruses [15, 18, 19]. Oral lesions present in individuals infected with HIV have been associated with shedding of herpesviruses in the oral cavity [20, 21]. Moreover, oral detection of herpesviruses is decreased in individuals treated with anti-retroviral or anti-herpesvirus drugs [22-24]. Limited number of studies have reported on VZV shedding in saliva or oral swabs of herpes zoster patients [25, 26], healthy individuals [26] and in one study on individuals infected with HIV [27]. These studies were mainly restricted to the detection of VZV only and limited to the analysis of one or a few consecutive saliva samples per individual. The trigeminal ganglion, which innervates the oral mucosa and eye, commonly contains both latent HSV-1 and VZV in co-infected individuals. Hence, the aim of this study was to determine the prevalence and kinetics of oral HSV-1 and VZV shedding in HSV-1 and VZV seropositive individuals infected with HIV and to assess whether HSV-1 shedding involves reactivation of the same strain intra-individually.

Materials and Methods

Study population

Individuals infected with HIV-1 were recruited between 1995 and 2007 at the University of Washington Virology Research Clinic (Seattle, WA) from a pool of unrelated research study participants known to comply with an intensive study protocol and asked to collect oral swab specimens at home daily for at least 30 days (Table 1). The median duration of HIV infection was 8 years (range 3 months to 18 years). Participants were instructed

to rub a Dacron swab across the buccal mucosa and tongue in the morning prior to showering or brushing their teeth, to place the oral swab in 1 mL of PCR transport medium and to store the sample at -20°C until laboratory processing [18]. Participants were eligible if they were HSV-1 and VZV seropositive, and agreed not to use anti-herpesvirus drugs, such as acyclovir (ACV) during the study. The use of anti-herpesvirus drugs was only monitored if prescribed by the University of Washington Virology Research Clinic (Seattle, WA). Participants #11 and #16 used famciclovir bidaily for 60 days in the year prior to collection of the oral specimens, but treatment was stopped two weeks before start of the study. At baseline, plasma HIV RNA load and blood CD4 T-cell counts were determined as described [18]. A log book recording symptomatic (herpetic) oral lesions was filed. Participants had routine clinic visits at the start and end of the study and irregularly during the study. During these visits brief visual oral exams were performed and a history of suspected herpetic oral lesions since the last visit was reviewed by the clinician and noted in the participant's chart. Except for participant #6, no evident abnormalities of the mouth (e.g. bleeding gum) or the neck were recorded and reported by the participants themselves during the sampling period. Written informed consent was given by the participants and the protocol was approved by the Institutional Review Board at the University of Washington (Seattle, WA). The study was performed according to the tenets of the Declaration of Helsinki.

Quantitative αHHV PCR analyses and HSV-1 thymidine kinase sequencing from oral swabs

DNA was extracted from swab medium as described [18]. Quantitative PCR (qPCR) assay for HSV-1 and VZV DNA was performed using an ABI prism 7500 and Taqman Universal Master Mix (both from Applied Biosystems, Foster City, CA) as reported [28]. The HSV-1 and VZV qPCR used published virus-specific primers and probes [29]. For standardization of HSV-1 and VZV Taqman assays, electron microscopy counted high-titer virus preparations and commercially available quantified DNA control panels (Advanced Biotechnologies) were used [29]. The lower limit of detection of both qPCR assays was 50 genome equivalent copies (geq)/mL. Cycle threshold values outside the linear range of the qPCR assay were considered as positive results, but could not be reliably quantified.

From a selected number of HSV-1 positive swabs (n=39), the entire HSV-1 thymidine kinase (TK) gene was amplified and sequenced as described [30]. The TK sequences were aligned to the consensus TK sequence of reference HSV-1 strain H129 (GenBank: GU_734772). The obtained HSV-1 TK sequences were deposited in the GenBank database under accession numbers JQ895543-JQ895556. Phylogenetic analysis was performed by estimating a maximum-likelihood unrooted tree of HSV-1 TK nucleotide sequences under the Kimura 2-parameter model and 1,000 bootstrap replications (MEGA 5.0 software).

Statistical analysis

Herpesvirus shedding episodes were defined as one virus DNA positive swab or a series of DNA positive swabs that were collected before and after at least two negative swabs. Any shedding episode could include one missing or one negative swab within the episode [18]. Statistical analyses were done using GraphPad Prism 4. Spearman's correlation

tests were used to determine correlations between herpesvirus shedding frequency, HIV viral load, CD4 T-cell counts or highly active anti-retroviral therapy (HAART). Mann-Whitney tests were used to compare shedding rates and median HSV-1 viral loads in HAART versus non-HAART persons and among shedding episodes of variable length. Differences were considered significant if $P<0.05$.

Results

Oral HSV-1 and VZV shedding in individuals infected with HIV

Twenty-two HSV-1 and VZV seropositive individuals infected with HIV were enrolled in the study. The median age was 42 years (range 22-61 years) and 20 were male. Fifteen participants were HSV-2 seropositive, and 6 persons (27%) were taking HAART (i.e. participants #1 to #6) (Table 1 and Figure 1). Participants had a median CD4 T-cell count of 268 cells/mL with an interquartile range (IQR) of 202-476 cells/mL and a median HIV RNA load of 37,900 copies/mL (IQR: 15,656-109,316 copies/mL) (Table 1). Whereas the CD4 T-cell counts were not different (Mann-Whitney test; P=0.45), the HIV RNA load was significantly lower in persons taking HAART compared to those not receiving HAART (Mann-Whitney test; P=0.01), respectively. A total of 715 oral swabs were obtained and analyzed for the presence and amount of HSV-1 and VZV DNA by qPCR. Samples were collected for a median of 31 days (IQR: 28-33 days), with 19 participants collecting for ≥30 days (Figure 1). Except for individual #6, none of the participants reported symptomatic herpetic oral lesions during the study period.

Four of the 22 (18%) persons shed neither HSV-1 nor VZV DNA during the study. From the 18 remaining persons, HSV-1 DNA was detected on 102 out of 715 sample days (14.3%) (Table 2). The HSV-1 DNA load was quantified in 97 swabs, with a median DNA load of 5,603 geq/mL (IQR: 1,073-56,050 geq/mL). Very low VZV DNA levels were detected in 7 swabs from two persons, and could be quantified in 1 sample (participant #4; 58 geq/mL). The median number of episodes of HSV-1 shedding was 2 and 1.5 episodes per 30 days among participants receiving HAART and those who were not receiving HAART (Mann-Whitney test; P=0.48), respectively. The frequency of HSV-1 and VZV shedding, and the maximum detected HSV-1 load, did not correlate with the participants' CD4 T-cell counts or HIV RNA load (Figure 1 and data not shown). All VZV DNA positive swabs were HSV-1 negative, with the exception of one swab from participant #17 in which both HSV-1 and VZV were detected (Figure 1).

The 102 HSV-1 positive swabs clustered into 34 distinct shedding episodes, with a median duration of 2 days (range 1-20 days) (Table 2). During the study period, 14 episodes (41%) of 1 day duration were detected with a median HSV-1 DNA load of 256 geq/mL (IQR: 124-3,555 geq/mL). Eleven episodes (32%) lasted ≥ 4 days, and 7 episodes where of unknown duration because swabs were positive at the beginning or end of the study (Figure 1 and Table 2). The median HSV-1 load of 3-day episodes (909,842 geq/mL) or ≥ 4-day episodes (106,000 geq/mL) was significantly higher compared to one-day episodes (Mann-Whitney test; P=0.005 and P=0.0001, respectively) (Figure 2). One of the participants (#6) reported symptomatic herpetic oral lesions at days 15 to 20 of the study.

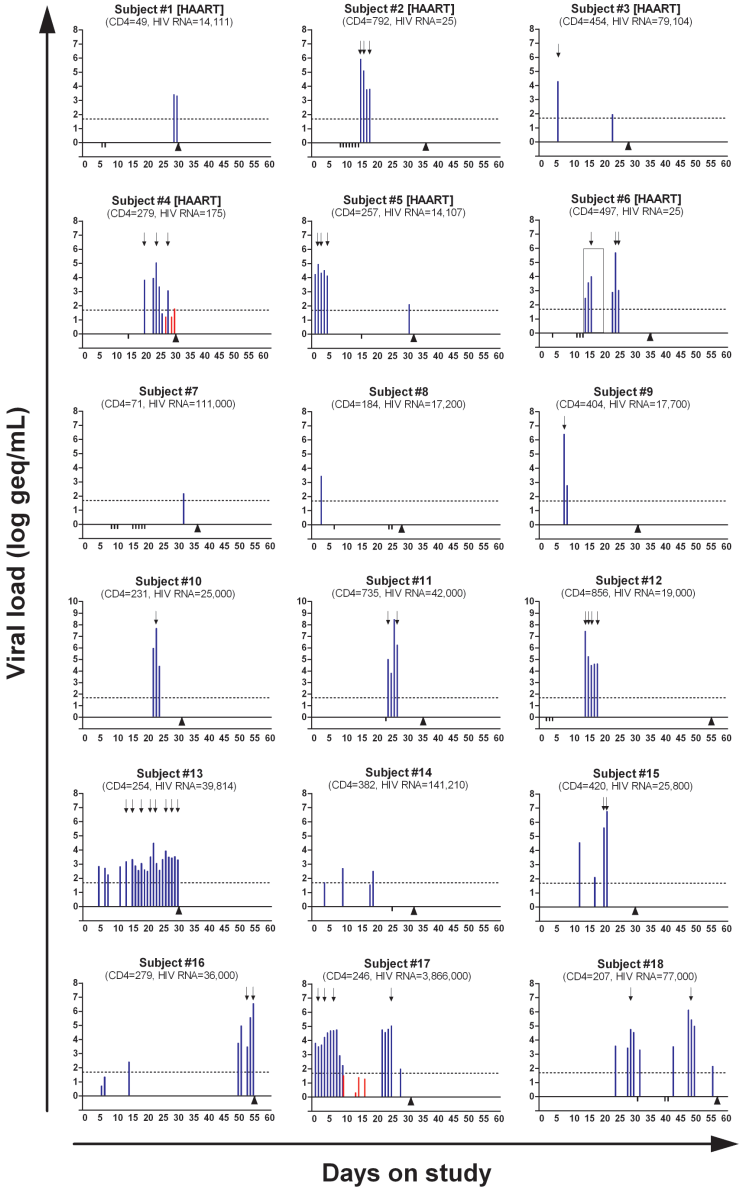

Figure 1. *Oral herpes simplex virus 1 (HSV-1) and varicella-zoster virus (VZV) shedding patterns in individuals infected with HIV. Viral loads [genome equivalent copies (geq) per mL] are plotted on the y-axis and the days on study on the x-axis. For each patient, CD4 T-cell counts (cells/μL) and HIV RNA loads (geq/mL) are specified. Individuals #1 to #6 were taking anti-retroviral therapy (HAART). The dotted line represents the lower limit of detection of the qPCR. Blue bars indicate HSV-1 shedding, red bars indicate VZV, and black bars below the x-axis indicate missing swabs. The solid box (graph of patient #6) indicates the presence of symptomatic herpetic oral lesions. Arrowheads denote the end of the swabbing period per individual and arrows indicate swabs that were used for HSV-1 thymidine kinase-based genotyping (see Table 3).*

Table 1. *Demographic and clinical characteristics of study subjects.*

Baseline characteristics	n = 22 subjects
Median age (range) in yrs	42 (22-61)
Male, n (%)	20 (91)
HSV serostatus, n (%)	
HSV-1 only	7 (32)
HSV-1 and HSV-2	15 (68)
Race/ethnicity, n (%)	
White	18 (82)
Black	2 (9)
Other	2 (9)
Anti-retroviral use during study, n (%)	6 (27)
Median (IQR) CD4 count, cells/μL	268 (202-476)
HAART treatment: yes	367 (153-645)
HAART treatment: no	240 (202-412)
Median (IQR) HIV RNA, geq/mL	37,900 (15,656-109,316)
HAART treatment: yes	7,141 (25-46,608)
HAART treatment: no	59,500 (25,400-113,500)

HSV-1, herpes simplex virus type 1; VZV, varicella-zoster virus; HAART, highly active anti-retroviral therapy; IQR, interquartile range; geq, genome equivalent copies.

HSV-1 DNA was detected in mucosal swabs at the start of symptoms and was undetectable during the resolution phase. Notably, the participant's second HSV-1 shedding episode, with a 2-log higher HSV-1 DNA load, was asymptomatic (Figure 1).

Genotyping of Oral HSV-1 in Individuals Infected with HIV

To determine if oral HSV-1 shedding involves reactivation of the same latent strain within and between shedding episodes, the entire TK gene from a selected set of HSV-1 DNA positive oral swabs was sequenced (Figure 1). Besides the causative role of TK mutations in ACV resistance (ACV^R), the hypervariability of the TK gene provides insight into the genetic composition of a virus isolate [11, 30, 31]. The HSV-1 TK genotype was determined from 14 participants with a median of 2.5 (range 1-8) oral swabs analyzed per person. The analyzed sequential oral swabs were obtained during one (n=10 participants) or of two subsequent HSV-1 shedding episodes (n=4 participants) (Table 3). Alignment of the TK sequences obtained with the corresponding sequence of the HSV-1 reference strain H129 revealed numerous TK gene nucleotide substitutions, including those resulting in amino acid mutations in the encoding TK protein. Notably, HSV-1 TK sequences of sequential oral swabs from each individual, both within and between shedding episodes, were identical suggesting reactivation and subsequent oral shedding of the same endogenous HSV-1 strain (Table 3). Most of the viruses shed by each individual had a unique TK nucleotide sequence clustering into distinct participant-specific phylogenetic clades (Figure 3). However, HSV-1 shed by participants #2 and #18, and participants #6 and #13, could not be differentiated based on the TK gene genotypes (Table 3 and Figure 3). The TK sequence homology was not due to contamination, since the parti-

Table 2. *Proportion of days, time points, and study participants with HSV-1 or VZV detected in at least one oral swab.*

	n = 22 subjects
Days sample collected, n	715
VZV DNA positive participants, n (%)	2 (9)
Days VZV detected, n (%)	7 (1)
HSV-1 DNA positive participants, n (%)	18 (82)
Days HSV-1 detected, n (%)	102 (14)
Duration of HSV-1 DNA positive episodes	34
1 day (%)	14 (41)
2 days (%)	5 (15)
3 days (%)	4 (12)
≥4 days (%)	11 (32)

HSV-1, herpes simplex virus type 1; VZV, varicella-zoster virus.

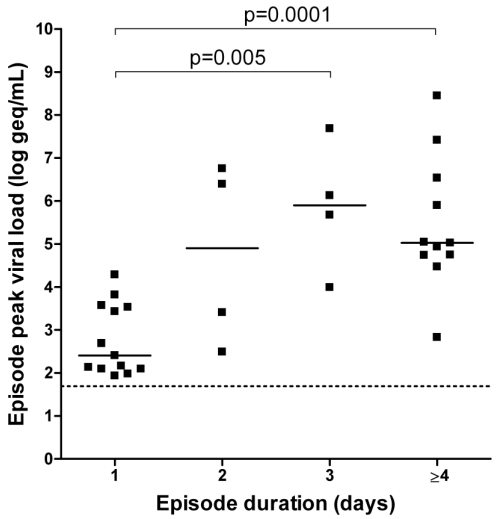

Figure 2. *Oral herpes simplex virus 1 (HSV-1) shedding characteristics of individuals infected with HIV. Episode duration (in days) is plotted against the peak HSV-1 viral load per individual (log-transformed geq/mL). Bars indicate the median viral load. The dotted line represents the lower limit of detection of the qPCR. The Mann-Whitney test was used to compare median viral loads and significant differences are indicated.*

cipants' samples were processed at different time points, all sequential swabs of each participant were identical (Table 3), and none of the aforementioned participants were family members or in any way related.

ACV^R HSV-1 is predominantly due to specific mutations in the drug-targeted TK protein leading to its defective or limited ability to convert ACV to ACV-monophosphate necessary to block HSV-1 replication [31]. Whereas the majority of the HSV-1 TK amino acid changes identified in the oral swabs were natural polymorphisms, 4 of 14 (29%) participants (#6, #10, #13 and #17) shed HSV-1 strains expressing ACV^R–associated TK protein variants suggesting that the respective viruses are unresponsive to ACV therapy (Table

Table 3. *Herpes simplex virus type 1 thymidine kinase variants detected in sequential oral swabs of HIV patients.*

Subject ID #	Sampling day	Thymidine kinase (TK) protein amino acid changes	GenBank accession no.
2	16, 17 & 19	I138V	JQ895543
3	6	S23N, E36K, Q89R, I138V, G240E & R281Q	JQ895544
4	21, 25 & 29	S23N, E36K, Q89R, I138V, G240E & R281Q	JQ895545
5	2, 3 & 5	L42P, Q89R, I138V, G251C, V267L, P268T, D286E & N376H	JQ895546
6	17, 25 & 26	C6G, R41H, Q89R, I138V, A192V, G251C, V267L, P268T, D286E & N376H	JQ895547
9	8	I138V & A316V	JQ895548
10	24	S23N, E36K, Q89R, I138V, G240E, R281Q & ***C336R***	JQ895549
11	25 & 28	C6G, L42P, Q89R, I138V, L267T, P268T & D286E	JQ895550
12	15, 16, 17 & 19	I138V & G240E	JQ895551
13	14, 16, 19, 22, 23, 27, 29 & 31	C6G, R41H, Q89R, I138V, A192V, G251C, V267L, P268T, D286E, N376H	JQ895552
15	21 & 22	C6G, I138V & G240E	JQ895553
16	54 & 56	I138V	JQ895554
17	2, 4, 7 & 26	C6G, ***del36E***, R41H, Q89R, I138V, A192V, G251C, V267L, P268T, D286E & N376H	JQ895555
18	30, 50	I138V	JQ895556

Sampling day refers to the oral HSV-1 shedding day of which the corresponding TK sequence of the indicated subject was determined. The analyzed sampling days are also depicted with arrows in Figure 1.

Amino acid changes are listed that are different from the HSV-1 TK reference sequence (GenBank No.: GU_734772). All sequential HSV-1 DNA positive swabs of the indicated days were identical within each subject. Underlined and bold/italic TK residue changes are unknown to affect acyclovir (ACV) sensitivity and published TK mutations leading to an ACV-resistant phenotype of the respective HSV-1 strain, respectively [11, 31-33]. All other residue changes are TK polymorphisms that are described not to affect ACV sensitivity. The mutation "del36E" refers to a deletion of "glutamic acid" at residue position 36 of the TK protein. HSV-1 DNA positive oral swabs of subjects #2 and #18, and #6 and #13, had identical HSV-1 TK nucleotide and protein sequences, respectively. In contrast, the TK sequences of the HSV-1 shed by subject #16 was different at the nucleotide level compared to the TK sequences of subjects #2 and #18.

The GenBank accession numbers of the HSV-1 TK sequences of the indicated oral swabs are provided.

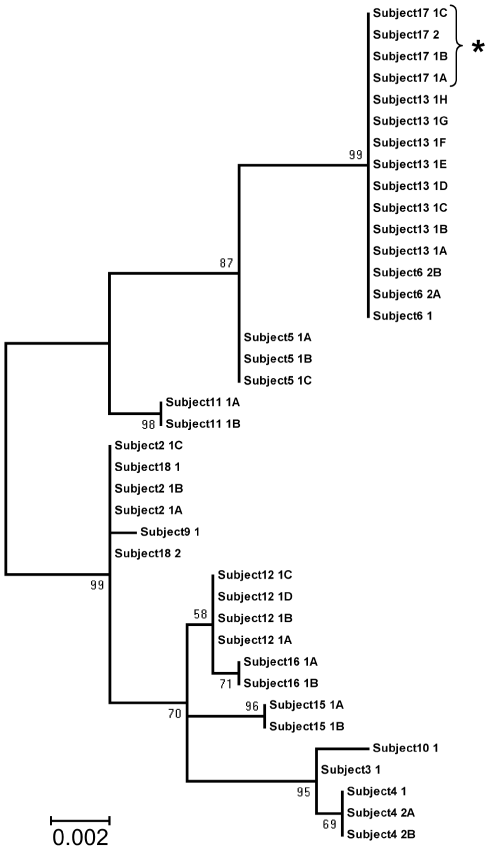

Figure 3. *Distinct oral herpes simplex virus 1 (HSV-1) thymidine kinase (TK) genotypes in individuals infected with HIV. Maximum likelihood unrooted phylogenetic tree of HSV-1 TK sequences was estimated under the Kimura 2-parameter model. The HSV-1 TK variants shown are coded by the participant's number, episode number (1 or 2), and swab within an episode (A to H). Selected bootstrap values are given. Scale bar represents number of nucleotide substitutions per site. *The TK variants from participant #17 are identical to those of individuals #1 and #6, except for a 3-nucleotide deletion (see Table 3).*

Discussion

The aim of this study was to examine the kinetics and quantity of oral HSV-1 and VZV shedding during a one-month daily sampling in individuals infected with HIV. It was found that shedding of HSV-1 occurs frequently, on 14.3% of days, whereas VZV shedding is very rare and at significantly lower genome copies. Based on the TK genotypes of sequential HSV-1 DNA positive oral swabs it was demonstrated that the participants shed genetically identical HSV-1 viruses, within and between HSV-1 shedding episodes, which were generally patient-unique. One-third of the participants shed a virus with an ACV^R TK genotype that potentially results in an HSV-1 strain refractory to ACV therapy.

Oral shedding of αHHV likely contributes to the epidemic spread within the human population [2-5]. Estimates on the frequency of HSV-1 shedding in immunocompetent individuals range from 0.5-76%. The sensitivity of virus detection techniques used, the number of persons and consecutive swabs sampled, and the time course of the studies may have attributed to the high variation reported [3, 34-37]. Studies that sampled saliva multiple times a day have revealed that 39% of oral HSV-2 reactivations are cleared within 12 hours [19]. A study in individuals infected with HIV demonstrated that HSV re-

activations are also of short duration and usually resolve before the onset of symptoms [18]. Consistent with previous reports, the current study detected HSV-1 shedding in 82% of the individuals infected with HIV at a frequency comparable to immunocompetent individuals [3, 18, 34-37]. The results corroborate with earlier data describing that a large proportion of the oral shedding episodes were cleared within 2 days and maximal viral loads per episode were significantly higher in episodes of prolonged duration [18, 19]. In this study, one participant (#6) reported oral lesions during one shedding episode that coincided with the detection of HSV-1 DNA in the mucosal swabs. Notably, the concurrent shedding episode of this participant, which was asymptomatic, had 2-log higher HSV-1 DNA copy numbers suggesting that the viral DNA load was not related to symptomatic herpetic oral lesions [21].

Subclinical reactivation of VZV has been less well studied and is largely evident in the elderly, in immunocompromised individuals and in herpes zoster patients [25, 26, 38, 39]. One study evaluated the prevalence of VZV in saliva of individuals infected with HIV and demonstrated low copy numbers in 3 of 59 (5.1%) participants [27]. A similarly low incidence and low copy numbers of oral VZV shedding was reported in the current study. The low prevalence of VZV shedding did not allow investigation of the potential interrelatedness of oral HSV-1 and VZV reactivation and shedding in individuals infected with HIV. Deprived VZV-specific T-cell immunity, as seen in immunocompromised individuals and the elderly, is a risk factor for VZV reactivation [14]. Future studies on more severe immunocompromised individuals, e.g. stem cell transplant patients, are warranted to study a potential interrelation between oral HSV-1 and VZV shedding [12, 27, 38].

HSV-1 and VZV are closely related human herpesviruses that establish a lifelong latent infection of sensory ganglia, yet HSV-1 shedding is much more frequent compared to VZV shedding. The different patterns in virus shedding resemble the differences observed in recurrent symptomatic HSV-1 and VZV infections. Recurrent HSV-1 lesions occur frequently, whereas individuals typically develop herpes zoster only once in a life time [40]. In contrast to HSV-1, VZV establishes latency in ganglia along the entire neuraxis, hence VZV reactivation from ganglia other than the trigeminal ganglia may be undetectable in saliva. However, previous studies have shown that VZV DNA and infectious virus can be detected during asymptomatic reactivation in astronauts and in zoster patients, irrespective of the affected dermatome [2, 25, 26]. Likewise, elderly vaccinated with the live-attenuated VZV vaccine shed viral DNA in saliva, suggesting that VZV reaches the saliva by viremic spread upon vaccination or viral reactivation [41].

It was previously shown that HSV-1/VZV co-infection correlates with the detection of both viruses in human trigeminal ganglia [42, 43]. Both viruses can be detected in the same trigeminal ganglion and even HSV-1 and VZV double-infected neurons have been described [44]. The higher ganglionic HSV-1 DNA load compared to VZV DNA load [43-45] may account for a higher HSV-1 reactivation frequency. Alternatively, viral determinants of HSV-1 latency, including intra-neuronal expression of latency-associated transcripts and microRNAs [46-49], which are not shared by VZV, may contribute to the differential αHHV reactivation patterns. Finally, CD8 T-cells are considered pivotal to control HSV-1, but not VZV latency in human trigeminal ganglia suggesting that different immune mechanisms act on the control of latency of both αHHVs [42, 43].