J. Neurovirol.
DOI 10.1007/s13365-016-0468-x

MEETING REPORT

2016 Colorado alphaherpesvirus latency society symposium

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Received: 29 June 2016 / Accepted: 30 June 2016
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Introduction

The 6th annual symposium of the Colorado Alphaherpesvirus Latency Society (CALS) convened 68 investigators who authored over 2,866 PubMed-listed articles focused on herpesvirology and traveled over 130,000 miles to attend the 2-day conference last May 19–20, 2016. Clinical scientists and basic researchers from Australia, Israel, Germany, Belgium, England, Scotland, and from universities throughout the USA discussed new information about herpes simplex virus types 1 and 2, varicella zoster virus, bovine herpesvirus, and pseudorabies virus latency (Fig. 1). In addition to 29 oral presentations by established investigators, 10 posters together with oral summaries were presented by graduate students and new investigators. Dr. James Eberwine from the University of Pennsylvania, School of Medicine presented the plenary talk describing transcriptome variability in adult human and mouse neuronal cells with the insights it has provided into cell identity and function. This featured presentation by an expert non-virologist neuroscientist helped attendees view alphaherpesvirus latency from a different perspective. Another highlight of 2016 CALS was the Friday night fireside chat focused on topics of interest not formally covered during the presentations. The relaxed setting of The Christiania Lodge nestled in the heart of the Colorado Rocky Mountains provided an ideal location to establish new collaborations, strengthen existing partnerships and train the next generation of clinical/basic research scientists devoted to the ultimate elimination of disease caused by alphaherpesvirus reactivation. A brief summary of the presentations follows.

Martine Aubert, *Fred Hutchinson Cancer Research Center*, described a new therapy approach for HSV infection that targets virus in latent reservoirs. This approach involves the introduction of DNA double-strand breaks in latent HSV genomes by rare-cutting homing endonucleases (HE), leading to mutagenesis of essential viral genes and thereby disabling virus. Proof-of-concept studies were done first in vitro in sensory neurons from trigeminal ganglia (TG), cells in which HSV becomes latent following ocular infection, and then in vivo in a mouse ocular model of latent HSV infection. In both systems, adenovirus-associated virus vectors were used to deliver HSV-specific HEs. While AAV8 was most efficient and specific in vitro, AAV1 was optimal in vivo and able to reach over 25 % of neurons covering all three divisions of the TG following intradermal whisker pad injection. In cultured neurons derived from dissociated TG collected from mice at various times post-ocular HSV infection, i.e., 7 (acute), 14 (acute/late), and 32 (latent) days, exposure to HSV-specific HEs led to mutation in 2–8 % of viral genomes. Thus, the viral genome was susceptible to endonuclease-mediated mutagenesis regardless of the time of treatment postinfection, suggesting that both HSV lytic and latent forms were targetable. Mutagenesis frequency after endonuclease exposure was increased nearly 2-fold by treatment with a histone deacetylase inhibitor. Use of the ocular mouse model of latent HSV infection showed that a targeted endonuclease delivered to viral latency sites via whisker pad injection of AAV vectors induced mutation in 2–4 % of latent HSV genomes in vivo. These low levels of mutation in HSV latent genomes in HE-

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Published online: 12 July 2016

Fig. 1 Participants of the 6th Annual Symposium of the Colorado Alphaherpesvirus Latency Society. *Left to right*, *row 1*—Richard Thompson, Randall Cohrs, and Nicholas Baird; *row 2*—Thomas Goodwin, David Tscharke, Jeffrey Cohen, Robert Hendricks, Ann Morrison, Maria Nagel, Katherine Lee, and Padma Srikanth; *row 3*—Andrea Bertke, Angus Wilson, Shannan D. Washington, Don Gilden, Satish Mehta, Leigh Zerboni, Moriah Szpara, Tamera Goldstein, Paul Kinchington, and Philip Krause; *row 4*—Dan Carr, Chandra Menendez, Peter Kennedy, Martine Aubert, David Bloom, Brian Wigdahl, Mark Challberg, Carol Pierson, and Vicki Traina-Dorge; *row 5*—Todd Margolis, Seth Frietze, Ken Jones, Jennifer Lee, Deepak Shukla, Joel Rovnak, David Knipe, Susanne Himmelein, Donna Neumann, Lora McClain, Patrick Stuart, Ravi Mahalingam, Duane Pierson, Dallas Jones, and Bridget Sanford; *row 6*—Ron Goldstein, Kevin Egan, and Charles Grose; *row 7*—Benedikt Kaufer, Leonardo D'Aiuto, Greg Smith, Julian Scherer, Matthew Taylor, Jim Goodrich, Clinton Jones, James Eberwine, David Davido, Marielle Lebrun, Scott Schmid, Victor Hsia, Aaron Sanford, Catherine Sadzot, Homayon Ghiasi, and Edouard Cantin; *not included in the picture*—Laura Benjamin, Judy Breuer, Elisabeth Cohen, Robert Figliozz, and Elena Moraitis

treated mice led to a delay in viral reactivation compared to control animals in an ex vivo TG explant reactivation assay. Specifically, at day 1 post-explant, reactivation from TG was seen in 90 % of control animals, while in HE-treated animals, only 43 % of the TG showed reactivation. While the difference was not statistically significant, the data provide the first proof-of-principle for the use of a targeted endonuclease as an agent to treat latent viral infection in vivo.

Laura Benjamin, *University College London and University of Liverpool*, described the use of imaging to explore the association of VZV reactivation and surrogate markers of stroke and stroke subtype produced by VZV. To date, knowledge about the subtype of stroke and use of surrogate stroke predictors in the context of VZV is limited, thus impeding our understanding of this complication of VZV infection. We aim to determine: (1) the association of VZV reactivation and surrogate markers of ischemic stroke, (2) to develop a robust classification of stroke subtypes, and (3) to explore the association with VZV reactivation. This cross-sectional study will recruit adults with a first ever ischemic stroke. Surrogate stroke outcome will be defined by remodeling index and continuous measure of carotid wall thickness, using CT angiography. MRI stroke subtype will be determined by MRI imaging. The subtype of stroke classification will be developed as a consensus among experts. VZV IgG titers will be measured and any associations will be determined. VZV stroke will be defined by intrathecal VZV IgG or DNA. A sample size of 500 ischemic stroke cases, recruited over 1.5 years, will provide over 80 % power with a significance level of 0.05 to detect a 1/3 standard deviation difference using a continuous measure of carotid wall thickness, assuming a VZV IgG prevalence rate of 80 %. The study is 2 months in recruitment; 19/50 patients had an MRI head, of these, 13/19 (68 %) were large–medium-sized vessel, 10/19 (53 %) were single lesions, and 11/19 (58 %) were middle cerebral artery in origin. We have demonstrated feasibility of this study. How these stroke subtypes (and surrogate markers of stroke) relate to VZV stroke and high VZV antibody titer will be determined on completion.

Andrea Bertke, *Virginia Tech*, showed that sympathetic neuronal pathways differentially regulate HSV-1 and HSV-2 genital infection. After genital infection, HSV-2 reactivates more frequently than does HSV-1. While both HSV-1 and HSV-2 establish latency in sensory and autonomic ganglia, the autonomic nervous system innervates the face and genitalia differently, and autonomic neurons are exquisitely sensitive to stress hormones associated with recurrent disease. Thus, autonomic pathways may provide an alternative latent

reservoir for reactivating virus that contributes to different recurrence frequencies of HSV-1 and HSV-2. The contribution of sympathetic pathways to acute genital disease and recurrence frequency was tested in guinea pigs treated with 6-hydroxydopamine (6-OHDA) to ablate sympathetic neuronal axons before infection, making them unavailable for infection. Loss of sympathetic pathways significantly reduced HSV-1 acute severity ($p = 0.03$) and neurological signs ($p = 0.002$), but did not affect HSV-2 lesion severity, while HSV-1 and HSV-2 recurrences were reduced by 84 and 39 %, respectively. Furthermore, stress hormones differentially regulated HSV-1 and HSV-2 infection in sympathetic neurons. Thus, sympathetic pathways play a significant role in HSV-1 and HSV-2 acute and recurrent genital disease, with a substantially greater impact on HSV-1 due to selective modulation in autonomic neurons.

David C. Bloom, *University of Florida College of Medicine*, evaluated a differentiated human iPSC neuron model of HSV-1 latency. Although most knowledge about HSV-1 latency has come from animal models, in vitro-infected primary neuron culture models have recently gained increasing acceptance as a tool to study aspects of HSV-1 latency. Nonetheless, these models are limited by the fact that HSV-1 is a human virus and mechanisms of latency evolved through virus–human neuron interactions. Here, we evaluated human-induced pluripotent stem cells (hiPSCs) as a potential model to study HSV-1 infection. hiPSCs were generated from fibroblasts obtained through a skin biopsy of an adult and subsequently induced to differentiate into neurons. To establish latency, hiPSC-derived neurons were infected with an HSV-1 recombinant virus expressing EGFP and RFP under the control of ICP0 and GC promoters, respectively, at a multiplicity of infection of 0.3 in the presence of antivirals (E)-5-(2-bromovinyl)-2′-deoxyuridine along with interferon-α. Cultures were harvested at 8 h and at 7 days postinfection. Analysis of the transcriptional status of the neurons, as assessed by expression of the EGFP and RFP reporters as well as RT-qPCR analysis, revealed restricted expression of lytic genes at 7 days, with only the latency-association transcript abundantly detected. Chromatin immunoprecipitation assay to evaluate histone methylation status of H3K4 and H3K27 at the ICP0, IPC4 GC, and LAT promoters revealed a 50- to 100-fold increase in H3K27me3 from 8 h to 7 days and a corresponding loss of the H3K4me3 marker. Finally, treatment of the 7-day cultures with trichostatin or sodium butyrate resulted in reactivation of lytic gene transcription and production of infectious virus. These data suggest the promise of hiPSCs as a valuable in vitro model to study certain aspects of HSV-1 latency, especially those involving the effects of miRNAs and long noncoding RNA, which may be human-specific.

Judith Breuer, *University College London*, showed that host diversity provides insight into VZV pathogenesis. Whole-genome sequencing analysis of VZV genetic diversity in 44 samples obtained from vesicle fluid, trigeminal ganglia, CSF, and other body compartments, including multiple samples from different sites sampled from eight patients, showed that sequences from the same patient were identical at the consensus level and genetic signatures were not associated with neurotropism. Sample heterogeneity was lowest for vesicle fluid and ganglionic material and greatest in CSF. Results from two CSF samples were consistent with reactivation of viruses from more than one clade and provide evidence for recombination.

Edouard Cantin, *Beckman Research Institute of City of Hope*, demonstrated HSV-induced neurocognitive impairment (NCI) in latently infected mice. Despite antiviral therapy, NCI is common in individuals who survive HSV encephalitis (HSE), and recent reports have also documented NCI in seropositive healthy individuals without encephalitis. Analysis to test whether HSV-induced inflammation underlies NCI was carried out in mice infected with HSV 17+ and treated with PBS, acyclovir (ACV) or ACV + intravenous pooled human IgGs (IVIG) beginning day 4 postinfection (PI); a 7-day course of ACV with single dose of IVIG was given. Prior studies showed that IVIG exerts potent anti-inflammatory effects, including induction of regulatory T cells secreting IL-10 that prevent HSE. Infected mice treated with PBS or ACV had very high levels of anxiety compared to ACV + IVIG-treated mice that was indistinguishable from uninfected mice. Notably, IVIG reduced CNS inflammation and influx of Ly6Chi inflammatory monocytes (IMs) at day 7 PI, consistent with recent reports implicating IMs in stress-induced behavioral deficits. Unexpectedly, ACV protected against development of learning and memory deficits which were evident only in female mice, and IVIG antagonized the effects of ACV. Serum proteomic analysis revealed significant changes in numerous proteins at days 2, 5, and 7 PI, consistent with antagonistic effects for ACV and IVIG on specific proteins. Comparative analysis of proteomic profiles from male and female mice might provide clues to how ACV and IVIG exert antagonistic effects on learning and memory.

Dan Carr, *University of Oklahoma Health Sciences Center*, compared resistance to HSV-1 challenge in mice vaccinated with a mutant HSV-1 deficient in the nuclear location signal of ICP0 (0ΔNLS) or with HSV-1 gD-2 subunit or left unvaccinated. Based on findings indicating that 0ΔNLS-vaccinated CD-1 outbred mice are highly resistant to ocular HSV-1 challenge, resistance to infection was evaluated in immunocompetent wild-type (WT) C57BL/6J mice or mice deficient in IFNAR1 (CD118−/−) or B cells (μMT) vaccinated with the 0ΔNLS mutant virus or a gD-2 subunit vaccine. Like outbred mice, 0ΔNLS-vaccinated animals were resistant to infection compared to the gD-2 subunit vaccinated group. Efficacy was

preserved in CD118−/− but not in μMT-vaccinated mice. At day 30, there was a 2-log reduction in LAT expression in the TG of 0ΔNLS-vaccinated WT mice compared to the gD-2 subunit vaccinated group that correlated with a 2- to 3-log reduction in genome copy number in TG of latent 0ΔNLS-vaccinated mice compared to naïve or gD-2 subunit-vaccinated mice. There was also a significant loss of mechano-sensory function of the cornea over the course of infection that did not recover during latency. Changes in corneal neovascularization found in infected non-vaccinated mice were not evident in 0ΔNLS-vaccinated mice at day 30 postinfection (latency). Neutralizing antibody titers were significantly higher in 0ΔNLS-vaccinated WT mice than in gD-2 subunit-vaccinated animals, suggesting that humoral immunity is the major correlate of protection.

Jeffrey I. Cohen, *National Institutes of Health*, described humoral and cellular immune deficiencies associated with zoster. While most persons with herpes zoster have self-limited disease, patients older than age 50 and those with impaired cellular immunity are at higher risk of developing complications including postherpetic neuralgia and neurologic disease. Repeated episodes of herpes zoster, especially in young persons, are extremely rare. Interferon (IFN)-α blocks the spread of VZV in skin, and the treatment of zoster patients with IFN-α reduces virus dissemination and visceral disease. In collaboration with Lindsey Rosen and Steven Holland at the National Institutes of Health and with Don Gilden at the University of Colorado School of Medicine, we found that a patient with VZV vasculopathy that had a thalamic stroke and spinal cord lesions had neutralizing antibodies to IFN-α. Antibodies to IFN-γ have been previously associated with disseminated zoster in some adults from Thailand and Taiwan. In the United Kingdom, a study of people with herpes zoster who developed postherpetic neuralgia found that 5 % had neutralizing antibodies to IFN-α, IFN-γ, IL-6, or GM-CSF. Type 1 IFN signaling involves activation of STAT1 and 3. Two girls with gain-of-function mutations in STAT1 have been described with recurrent zoster. Young patients with autosomal dominant loss-of-function mutations in STAT3 were reported to have 6- to 20-fold higher rates of zoster than the general population and higher rates of recurrent zoster. Overall, these findings indicate that certain patients with complications of zoster or recurrent zoster have inherited or acquired immunodeficiencies that affect IFN signaling.

Leonardo D'Aiuto, *University of Pittsburgh*, described a new human neuronal progenitor cell (NPC)-based drug screening platform for CNS HSV-1 infections. The increasing prevalence of genital HSV-1 infections raises concerns about fetal infections, particularly CNS infections that could affect neurological development in light of the high susceptibility of NPCs to the virus. Acyclovir treatment does not increase the rate of neonatal survivors with normal neuronal development, underscoring the need to identify novel drugs exerting efficacious anti-herpetic activity at different stages of neuronal differentiation. While conventional antiviral drug screens use 2D monolayer cell cultures, increasing evidence suggests that 3D cultures have greater predictive value for in vivo testing because they mimic tissue architecture. Comparison of the anti-HSV-1 activity of proven antiviral drugs and 72 small molecules including nostodiones, quinazolinones, and epigenetic inhibitors in 2D and 3D cultures of human NPCs and neurons generated from induced pluripotent stem cells identified novel anti-HSV-1 compounds with high efficacy in both NPCs and neurons. Some compounds displayed drastic differences in infected 2D and 3D NPC cultures; the latter proved to be better predictors of antiviral drug activity in neurons compared to 2D cultures.

David J. Davido, *University of Kansas*, showed that mutations in HSV-1 UL39 impair pathogenesis in mice and constitute a potential vaccine against HSV-1. Efforts to generate HSV-1 mutants in the infected cell protein 0 (ICP0) gene identified a mutant, KOS-NA, that was severely impaired for acute replication in the eyes and TG of mice. Sequencing of the KOS-NA genome to identify the mutation(s) responsible for these impaired phenotypes revealed two non-synonymous mutations in the *UL39* gene, resulting in 2 amino acid changes in the encoded ICP6 protein. Repair of the *UL39* gene in the KOS-NA genome restored its acute replication phenotypes in mice. When the *UL39* mutations were inserted into the HSV-1 genome, virus replicated in the cornea and TG of mice at levels comparable to those of KOS-NA. Analysis to test whether KOS-NA might serve as a prophylactic vaccine showed that KOS-NA almost completely protected against corneal disease (keratitis) and greatly reduced latent infection by challenge virus. Together, the data indicate the important role of one or both amino acid substitutions in ICP6 in supporting acute replication and establishing latency, as well as the ability of KOS-NA to protect mice against HSV-1-induced corneal disease.

Kevin P. Egan, *Drexel University College of Medicine*, described HSV-1 replication kinetics and immune response in the lip scarification model of infection. HSV-1 is a human pathogen that replicates in epithelial cells of mucosal surfaces before becoming latent in the trigeminal ganglion. Disease can include erythematous pustular lesions in the oral mucosa. There is an established ocular infection model in the laboratory mouse which reproduces primary infection and latency seen in humans. However, most primary human infections occur within the lip and latency is established within a different branch of the trigeminal ganglion. To examine the kinetics of HSV-1 replication in the lip scarification model, the lower lip of the 3-month-old mice were scarified and inoculated with HSV-1; tissue was collected at 7 time points up to day 60 postinfection (PI) for detection of infectious virus and responding immune cells. Virus titers in the lip were high at early time points that resolved after 8 days. Lip pathology

peaked at 5 days PI and resolved by 15 days PI. CD8+ T cells were retained in the trigeminal ganglion at 30 and 60 days PI. These results show that the lip scarification model can reproduce human infection with characteristic pathology, resolution and retention of CD8+ T cells in the trigeminal ganglion.

Robert William Figliozzi, *University of Maryland Eastern Shore*, described zebrafish as models of human alphaherpesvirus infection and latency. Currently, mouse and rabbit species are the most commonly reported host for HSV models, each with various advantages and disadvantages. These models have improved our knowledge of HSV latency, but neither of them completely mimics every characteristic of infection and latency in the natural human host. Unlike HSV, VZV has no true non-primate animal model that can establish latency. Compared to zebrafish, these models represent a significantly higher cost and harbor greater humanitarian concerns. Analysis of individual zebrafish inoculated intraperitoneally with HSV-1 or VZV revealed genetic evidence of viral infection for 30 days post-injection and the ability to express viral RNA after stress. The ability of these fish to harbor human viral DNA for 30 days is indicative of viral infection and possibly latency. Further studies are needed to detect and isolate viral protein and infectious viral progeny from infected zebrafish, as well as to compare the effect of several potentially reactivating stress events.

Homayon Ghiasi, *Cedars-Sinai Medical Center*, described the relationship of primary HSV-1 replication in the eye, viral DNA levels in TG during latency and time to reactivation in TG of latently infected mice. Analysis of mice infected with virulent (McKrae) or avirulent (KOS, RE) strains of HSV-1 for virus titers in the eyes on days 3 and 5 postinfection (PI), level of viral gB DNA in TG on day 28 PI, and virus reactivation on day 28 PI as measured by explant reactivation following ocular HSV-1 infection showed less avirulent virus in the eye, even after corneal scarification, reduced latency and a longer time to reactivate than did the HSV-1 virulent strain. The time to explant reactivation of avirulent strains of HSV-1 was similar to that of the virulent LAT- McKrae-derived mutant. The viral dose with the HSV-1 McKrae strain affected levels of viral DNA and time to explant reactivation. Overall, the results suggest no absolute correlation between primary virus titer in the eye, viral DNA levels in latently infected TG and time to reactivation.

Don Gilden, *University of Colorado School of Medicine*, described successful antiviral treatment of a patient after 6 years of chronic progressive VZV brain infection. In 2010, 6 months after sacral 1–2 distribution zoster, a 63-year-old man developed transient global amnesia. Brain MRI showed a focal non-enhancing, left centrum semiovale T2 hyperintense lesion with ill-defined margins. Brain biopsy showed hypercellular white matter, but no neoplasm, inflammation, vasculitis, or necrosis. The patient was followed without intervention, but continued to have progressive cognitive problems for the next 4 years. In 2014, the patient heard a case presentation of someone with focal VZV encephalitis without rash who was treated successfully with oral valacyclovir. Thinking he might also have VZV brain infection, particularly since he had zoster 6 months before the onset of neurological disease, he arranged for analysis of the 2010 brain biopsy for VZV infection. Using immunofluorescence, a laboratory devoted to scientific research on VZV detected VZV antigen in the brain biopsy. In 2015, the brain biopsy was examined using immunohistochemistry by another VZV laboratory with a different anti-VZV antibody; again, VZV antigen was detected. Neurological examination in 2015 revealed significant cognitive impairment. After 2 weeks of treatment with intravenous acyclovir, his mental status was normal and he has been asymptomatic for >5 months. Unlike most neurotropic viruses which produce acute disease, VZV produces both acute and chronic neurological disease. To our knowledge, our patient represents the most extended case of VZV infection of the brain without inflammation, i.e., 6 years' duration, who responded rapidly and completely to treatment with intravenous acyclovir. Overall, VZV can cause chronic progressive focal or generalized neurologic disease, with or without a recent history of zoster. Despite a lengthy duration of infection, proper antiviral treatment can result in a favorable outcome.

Don Gilden, *University of Colorado School of Medicine*, presented evidence that a single virus causes granulomatous vasculitis with three different phenotypes. Granulomatous vasculitis is a necrotizing vasculitis that primarily affects arteries of the central nervous system. The characteristic pathological features of vessel wall damage and inflammation with multinucleated giant cells and/or epithelioid macrophages are seen in three serious human vasculitides: intracerebral VZV vasculopathy, giant cell arteritis (GCA), and granulomatous aortitis, suggesting a common etiology. Historically, virological analysis of intracerebral cerebral arteries exhibiting the pathology of granulomatous vasculitis has revealed productive VZV infection (Cowdry A inclusion bodies, VZV DNA, VZV antigen, and herpes virions). Similarly, virological analysis of temporal arteries (TAs) pathologically-verified to be GCA revealed VZV antigen, VZV DNA, and herpes virions. Overall, immunostaining no less than 50 sections of 104 TAs with antibody to VZV revealed the presence of VZV antigen in 70 % of TAs exhibiting the pathology of GCA (GCA-positive). Importantly, although many TA biopsies from patients with clinical and laboratory features of GCA did not exhibit the pathology of GCA (GCA-negative), 58 % of 100 GCA-negative TAs were also found to contain VZV antigen. Compared to normal TAs from age- and gender-matched control subjects over the age of 50, the detection of VZV was highly statistically significant. Because large arteries emanating from the heart (aorta, subclavian, and others) are frequently affected in GCA, we searched for VZV in aortas

pathologically verified to be granulomatous aortitis; VZV antigen was detected in all of 11 such aortas. The combined studies revealing VZV antigen in arteries from patients with intracerebral VZV vasculopathy, GCA and aortas from patients with granulomatous aortitis strongly indicated that these three forms of granulomatous vasculitis are all caused by VZV.

Ronald S. Goldstein, Bar-Ilan University, Israel, presented a scalable and easily manipulated experimental model for study of VZV latency. Study of herpesvirus latency is complicated by the difficulty in obtaining, growing and genetically modifying human neurons in culture. To facilitate the study of VZV latency, the use of the widely-used human embryonic kidney 293 cell line was used. Exposure of 293 cells to "high MOI" cell-free VZV resulted in viral entry to 10–15 % cells, as determined by two methods. A smaller percentage of 293 kidney cells exposed to VZV containing GFP fusions to ORF66 (early) and ORF23 (late) proteins exhibited fluorescence at 2–3 days postinfection (PI), but not at 4 days PI. By contrast, ORF62 (IE) was observed in 293 kidney cells for at least 8 days. VZV genomes were detected in 293 kidney cells by PCR and in situ hybridization for at least 10 days PI. Importantly, treating 293 kidney cells infected with VZV a week earlier with agents that elicit in vitro reactivation of VZV in stem cell-derived neurons increased VZV RNA and protein synthesis. Thus, this human culture cell model recapitulates key aspects of VZV latency. The ease of expansion and genetic modification of 293 kidney cells may make them a useful complementary system to study molecular aspects of VZV latency requiring large numbers of genetically manipulated cells.

James Goodrich, *University of Colorado Boulder*, described the effect of HSV-1 lytic infection on transcription of host cell and viral genes by RNA polymerase II (Pol II). Analysis using chromatin immunoprecipitation sequencing (ChIP-seq) to determine how levels of genome-bound Pol II change after HSV-1 infection revealed a near complete loss of Pol II occupancy throughout host cell mRNA genes, both in the gene body and downstream of the promoter region; no increases in Pol II occupancy consistent with robust transcriptional activation was detected. Concomitant with the loss of host genome Pol II occupancy, Pol II was observed covering the HSV-1 genome, reflecting a high level of viral gene transcription. Pol II ChIP-seq performed before and after heat shock (a cellular stress that causes changes in transcription), showed that the major transcriptional response to heat shock was repression; however, HSV-1 infection induced a more potent and widespread repression of Pol II occupancy than did heat shock. These studies provide new insights into how HSV-1 produced changes in the cellular program of gene expression and how virus co-opts host Pol II for its own use.

Charles Grose, *University of Iowa*, described how autophagy facilitates transport of VZV particles without degradation after VZV reactivation. The Grose laboratory previously described a proviral autophagy role during VZV infection in a 23-year-old man with focal encephalitis after VZV reactivation without rash. Examination of his brain biopsy by both confocal and electron microscopy revealed abundant astrogliosis, with autophagic vacuoles seen in infected astrocytes, but not xenophagy. Other infected cells also showed autophagy without xenophagy. Further analyses of the virion fraction from infected cells to test whether autophagy contributes to virus assembly and/or transport but not degradation during both primary infection and herpes zoster revealed microtubule-associated protein 1 light chain (LC3) and Ras-like GTPase 11 (Rab11) in immunoblotting assays; the same two proteins were detected by immuno-TEM on viral particles. Together, the data suggest that viral particles accumulate in single-membraned vesicular compartments decorated with components from both the endocytic pathway (Rab11) and the autophagy pathway (LC3-II) before egress. These VZV results also relate to the role of the ICP34.5 neurovirulence protein in herpes simplex virion degradation, since ICP34.5 alone does not prevent xenophagy.

Robert L. Hendricks, *University of Pittsburgh*, described the critical role of sympathetic corneal nerves in primary and reactivated herpes stromal keratitis (HSK). Primary HSK in mice is a neurologically controlled immunopathological disease that progresses in HSV-1-infected corneas long after latency is established in sensory ganglia. Recent findings demonstrate that severe HSK is triggered by loss of corneal sensory nerves and of the corneal blink reflex, leading to exposure-induced inflammation that can be prevented or reversed by tarsorrhaphy (surgically closing the eyelids). Analysis showed that HSK persistence requires hyper-innervation of the sensory nerve-depleted cornea by sympathetic nerves emanating from the superior cervical ganglion (SCG), and that both sympathetic hyper-innervation of the infected corneal stroma and persistent HSK can be prevented or ameliorated by depleting $CD4^+$ T cells, macrophages or by SCG excision. Moreover, the severe, diffuse UV-B light-induced recurrent HSK in NIH mice was associated with nearly complete loss of sensory nerves and hyper-innervation of the corneal stroma with sympathetic nerves, resembling primary HSK in C57BL/6 and BALB/c mice. In contrast, milder and more focal recurrent HSK in C57BL/6 mice is associated with sectoral loss of sensory nerves and hyper-innervation with sympathetic nerves. These data suggest that both primary and recurrent HSK in mice are regulated by loss of corneal sensory nerves and hyper-innervaton of the corneal stroma by sympathetic nerves.

Susanne Himmelein, *Ludwig-Maximilian-University Munich*, described anatomical preparation and characterization of human vestibular ganglia in the context of vestibular

neuritis. HSV-1 primarily infects the trigeminal ganglion and could reach the geniculate ganglion via the lingual nerve. From there, smaller numbers of cells in the superior vestibular ganglion (VG) could be infected via facio-vestibular anastomoses. After establishing latency in sensory neurons, reactivation can result in different diseases including vestibular neuritis, in which the superior portion of the vestibular nerve is involved more that the inferior part. An improved method was established for obtaining human VG from the temporal bone, involving rapid and gentle processing of post-mortem specimens that preserves the structural integrity of the tissue and allows rigorous anatomical evaluation. Various micro- and macro-anatomical studies of 35 VG samples obtained revealed neurons only in the stem (9/35), in the two branches (7/35), in one branch only (14/35), and both in the stem and branches (5/35), contrary to earlier publications. Single-cell RT-qPCR analysis to examine the frequency of latently infected neurons in various branches of the 35 VG revealed very few latently infected VGs, as analyzed by nested PCR and subsequent sequencing.

Victor Hsia, *University of Maryland Eastern Shore*, showed that a volatile organic compound (VOC), gamma-butyrolactone (GBL), was induced after acute HSV-1 infection and may affect virus latency. Initial analyses to test whether a VOC is produced during HSV-1 infection of epithelial cells that might influence subsequent neuronal infection to facilitate latency involved the use of two-dimensional gas chromatography/mass spectrometry to measure emission of VOCs in HSV-1-infected Vero cells; concentrations of GBL in particular increased significantly after 24 h. Analysis of the effects of GBL on HSV-1 replication using differentiated LNCaP cells as a proxy for human neurons showed that GBL triggered depolarization of differentiated LNCaP and decreased HSV-1 infection by repressing viral gene expression and replication. These results may provide useful clues in understanding the complex signaling pathways involved in primary HSV-1 infection and ultimate establishment of latency.

Clinton Jones, *Oklahoma State University Center for Veterinary Health Sciences*, presented data concerning activation of the canonical Wnt/β-catenin signaling pathway in sensory neurons of calves latently infected with bovine herpesvirus 1 (BoHV-1). Like most α-herpesviridae subfamily members, BoHV-1 becomes latent in sensory neurons. Recent studies indicated that the canonical Wnt/β-catenin signaling pathway is active in sensory neurons of calves latently infected with BoHV-1. β-catenin is a cellular transcription factor activated by the canonical Wnt signaling pathway but targeted for proteolysis in the absence of Wnt. A co-activator of β-catenin, HMGA1 (high mobility group AT-1 hook), was expressed at higher levels in TG of latently infected calves than in uninfected calves, whereas a Wnt antagonist, frizzled homolog 8 protein (FZD8), was expressed at higher levels in TG of uninfected calves. During dexamethasone-induced reactivation from latency, mRNA expression levels of two additional Wnt antagonists, dickkopf1 (DKK1), and secreted frizzled protein 2 (SFRP2), were induced in TG in correlation with reduced β-catenin protein expression in TG neurons 6 h after dexamethasone treatment. A viral protein expressed in latently infected sensory neurons (ORF2) was found to cooperate with HMGA1 to stimulate β-catenin-dependent transcription in mouse neuroblastoma cells. These findings may be relevant to the latency-reactivation cycle since the canonical Wnt/β-catenin signaling pathway interferes with neuro-degeneration but promotes neuronal differentiation. Conversely, chronic stress or increased corticosteroids induced DKK-1, which leads to neuronal damage in the hippocampus and ischemic neuronal death. SFRP2 may also inhibit neuronal survival because it induces cell death in the developing hindbrain. Overall, these results suggest that β-catenin expression in latently infected neurons promotes maintenance of BoHV-1 latency.

Dallas Jones, *University of Colorado School of Medicine*, described VZV-mediated regulation of the PD-1:PD-L1 pathway in human T cells. During primary infection, VZV infection of T cells promotes hematogenous spread of virus, the development of varicella and subsequent establishment of latency. Although the mechanism(s) by which VZV-infected T cells evade immune clearance is unknown, binding of the programmed cell death receptor-1 (PD-1) expressed on T cells with the programmed cell death-ligand 1 (PD-L1) expressed on target cells promoted T cell exhaustion and apoptosis. Flow cytometry analysis to test whether VZV-infected T cells upregulate PD-L1 during viremia to evade immune clearance showed that PD-L1 expression in VZV-infected CD4+ and CD8+ T cells was increased by 55 ($p = 0.008$) and 65 % ($p = 0.008$), respectively, along with a 60 ($p = 0.008$) and 70 % ($p = 0.008$) increase in PD-1 expression, respectively, as compared to mock-infected CD4+ and CD8+ T cells isolated from human peripheral blood mononuclear cells. Similarly, compared to mock-infected cells, VZV-specific CD8+ T cells that recognize the ORF18 or ORF34 peptide of VZV showed a 320 ($p = 0.0004$) and 300 % ($p < 0.0001$) increase in PD-L1 expression, respectively, and a 275 ($p = 0.0003$) and 200 % ($p = 0.0002$) increase in PD-1 expression, respectively. Compared to isotype controls, VZV-infected human brain adventitial fibroblasts co-cultivated with VZV-specific CD8+ T cells and treated with an anti-PD-L1 antibody showed a 40 % ($p < 0.05$) increase in interferon-γ secretion 24 h after co-cultivation, demonstrating that VZV-mediated induction of PD-L1 and successive induction of T cell exhaustion and apoptosis is reversible. Overall, these studies identify a novel mechanism of VZV-mediated PD-L1 upregulation in VZV-infected T cells that may contribute to T cell exhaustion and immune evasion, potentiating viremia, and the establishment of latency along the entire neuraxis.

Paul R. Kinchington, *University of Pittsburgh*, described the influence of HSV-1 expression on CD8 + T cell immunodominance, priming and ganglionic retention during infection of C57Bl6 mice. HSV-1 latency is associated with a long-term ganglionic T cell population (CD8+ cells) that influences virus reactivation. In C57BL/6 mice, half of HSV-1-specific CD8+ cells recognize a single epitope ($gB_{498–505}$), while the remainder recognize 18 epitopes on 11 viral proteins. Interestingly, ganglionic gB-CD8+ cells at latency are functional, while non-gB-CD8+ cells are impaired. Analysis of ganglionic CD8 populations developing in response to HSV-1 lacking $gB_{498–505}$ revealed no priming of gB-CD8+ cells but induction of a compensated ganglionic CD8 response with increased frequency and functionality of non-gB-CD8 populations. Similar analysis showed that HSV-1 expressing the $gB_{498–505}$ epitope from promoters of gB, CMV IE, ICP0, VP16, and gC restored the gB-CD8 compartment, but not to levels above the 50 % seen for wild-type virus, whereas $gB_{498–505}$ expression from certain late promoters failed to prime $gB_{498–505}$ T cell responses, despite efficient expression in lytic infections. Use of a dual infection model with concurrent normal HSV-1 T cell priming showed that efficient ganglionic retention of T cells at latency requires antigen presentation. These studies define aspects of how various HSV-1 expression kinetics shape the $CD8^+$ T cell repertoire in latently infected ganglia and provide clues about molding CD8+ cells to better inhibit HSV-1 reactivation.

Philip R. Krause, *US Food and Drug Administration*, *Center for Biologics Evaluation and Research*, *Office of Vaccines Research and Review*, described novel functions of HSV ICP27 in regulating host pre-mRNA processing. ICP27 is thought to play an important role in establishing HSV-2 latency. Based on recent studies indicating that ICP27 inhibits splicing in a gene- and intron-specific manner, high-throughput RNA sequencing was used to comprehensively investigate its role in cellular pre-mRNA processing. ICP27 selectively caused intron retention, promoted use of alternative 5′ splice sites, or induced expression of pre-mRNAs cleaved and polyadenylated from cryptic polyadenylation signals in introns, resulting in expression of previously unreported variants of >200 cellular genes. In each case, the use of a specific 5′ splice site was reduced. Mutation of nearby motifs that are conserved in ICP27-targeted sites eliminated ICP27-mediated splicing inhibition. ICP27-mediated effects were frequently coupled with changes in coding sequences, untranslated region sequences, and expression level of targeted genes. Through ICP27, HSV coordinates expression of these modified cellular genes with expression of its own genes, resulting not only in reduced expression of targeted genes, but also in expanding genomic material available to the virus to encode additional novel genes as infection and reactivation progresses.

Marielle Lebrun, *University of Liege*, described the role of VZV ORF9p and cellular adaptin protein-1 (AP-1) in late stages of virus maturation. Analysis of VZV ORF9p (HSV-1 VP22 homolog), an essential virus tegument protein, showed that it is phosphorylated by the viral kinase ORF47p, that phosphorylation of ORF9p is important for envelopment of cytoplasmic capsids (secondary envelopment); and that ORF9p interacts with the clathrin adaptor protein complex 1 (AP-1), the host protein complex that directs clathrin-mediated transport of membrane proteins between the trans-Golgi network and endosomes. Mutational analysis of ORF9p identified a motif that is important for AP-1 binding and VZV secondary envelopment. The amino-terminal region of OR9p contains a cysteine residue predicted to be palmitoylated and involved in secondary envelopment. Co-immunoprecipitation studies identified viral glycoproteins associated with AP-1. These results raise the possibility that ORF9p connects viral glycoproteins to the AP-1 complex, and that this bridge is dependent on palmitoylation of ORF9p. An understanding of the contribution of ORF9p to the biology of VZV awaits studies in infected dendritic and T cells to determine whether ORF9p is required for virus to enter neurons, become latent and reactivate.

Jennifer S. Lee, *Harvard Medical School*, described how HSV-1 ICP0 regulates the structure of latent viral chromatin. Latent infection involves minimizing viral protein expression so that the host immune system cannot recognize and eliminate infected cells. HSV-1 is thought to express non-coding RNAs such as the latency-associated transcripts (LATs) and miRNAs as the only abundant viral gene products during latent infection. Our analysis of HSV-1 mutant viruses provided strong genetic evidence that HSV-1 ICP0 is expressed during the establishment and/or maintenance of latent infection in murine sensory neurons in vivo. Studies of an ICP0 nonsense mutant virus showed that ICP0 promotes heterochromatin and both lytic and latent transcription, suggesting that ICP0 is expressed and functional, leading to the hypothesis that ICP0 promotes transcription of LAT in neurons, just as it promotes lytic gene transcription in epithelial cells. This study raises the new concept that a lytic viral protein can be expressed during latent infection and can play a dual role by regulating viral chromatin to optimize latent infection in addition to its epigenetic regulation during lytic infection. The results also raise the possibility that ICP0 might serve as a target for antiviral therapy in both lytic and latent infections.

Lora McClain, *University of Pittsburgh*, described the mechanism of action of the antiherpetic alkaloid trans-dihydrolycoricidine (R430). HSV-1 infection results in oral lesions, keratitis, and rarely encephalitis. After primary infection, HSV-1 establishes latency in sensory neurons for the lifetime of the host, and virus can be reactivated. Furthermore, HSV-1 may develop resistance to acyclovir (ACV), underscoring the need for compounds with a novel

mechanism of action. We previously reported the ability of R430 to significantly inhibit HSV-1 and VZV infection. The mechanism of action of R430 was examined by total RNA sequencing of human induced pluripotent stem cell-derived neurons grown in the presence or absence of R430 for 12 h. Differentially expressed genes were determined using unpaired t-tests and false-discovery rate correction with $p < 0.05$ and a fold-change of 1.5 as thresholds. Ingenuity Pathway Analysis indicated that EIF2 signaling, EIF4 and p70S6K regulation, mTOR signaling and TWEAK signaling were significantly dysregulated, identifying R430 as a member of a novel class of molecules that exert antiherpetic activity by inhibiting translation and by targeting the mTOR signaling pathway.

Chandra M. Menendez, *University of Oklahoma Health Sciences Center*, showed that HSV-1 activity persists in a unique region of the brain during latency and is associated with loss of resident T cell function. Early antiviral intervention is crucial to the survival of patients with herpes simplex encephalitis (HSE); however, many survivors suffer from long-term neurological deficits. The tissue residence of latent virus, other than in sensory neurons, and the potential pathogenic consequences of latency remain unclear. Characterization of HSV-1 lytic and latent infection in the CNS compared to that in trigeminal ganglia after ocular infection of mice unexpectedly revealed HSV-1 lytic cycle genes usually identified during acute infection that were expressed in CNS ependyma (EP) cells at 60 days postinfection (DPI). HSV-1-specific gB T cells and resident memory T cells persisted in the EP as opposed to TG, yet EP T cells were less capable of controlling HSV-1 infection ex vivo and secreted less IFN-□ by 60 DPI. These findings raise the possibility that persistent viral lytic gene expression after HSE elicits chronic inflammation in the CNS EP and may contribute to chronic neurologic sequelae in surviving HSE patients.

Elena Moraitis, *University College London and University of Liverpool*, studied mechanisms of VZV-related cerebral arteriopathy. The group determined whether VZV infection of human brain vascular adventitial fibroblasts (HBVAF) induces their differentiation into myofibroblasts, with changes in their proliferating and migratory capacity that potentially contribute to vascular remodeling. HBVAF transformation, proliferation, and migration were assessed by flow cytometry using staining with α-smooth muscle actin (α-SMA) antibodies, Click-iT Plus EdU assay kit and scratch assay, respectively, at 6 days postinfection. The fold-increase of percentage of α-SMA+ cells differed significantly between VZV- and mock-infected conditions in relation to resting baseline HBVAF levels [19.53-fold (SEM 3.38-fold) for VZV-infected cells compared with an 8.24-fold (SEM 1.37-fold) in mock-infected cells ($p = 0.03$)]. There was a significant increase in the percentage of EdU+ cells observed in VZV-infected cultures compared to mock-infected expressed as fold-change in relation to resting quiescent HBVAF [25.38-fold (SEM 3.37-fold) in VZV-infected cultures compared with 3.31-fold (SEM 0.09-fold) in mock-infected cultures ($p = 0.01$)]. Scratch assay showed a greater number of cells in the scratch area in the VZV-infected cultures. Overall, in HBVAF, VZV can trigger proliferation, differentiation to myofibroblasts and migratory capacity.

Maria A. Nagel, *University of Colorado School of Medicine*, showed that burning mouth syndrome (BMS) is associated with VZV. BMS is defined as chronic, orofacial burning pain for which no dental or medical cause is found. Pain persists and is often intractable despite treatment with antidepressants, analgesics, hormones, α-lipoic acid and anticonvulsants. We recently described a case of BMS in a 65-year-old woman that was due to HSV-1, virologically verified by the presence of HSV-1 DNA in her saliva and based on the disappearance of pain and viral DNA after treatment with oral valacyclovir. Since then, we have encountered two cases of BMS in a 61-year-old woman of 8 months' duration and in a 63-year-old woman of 2 years' duration, respectively, both of which were associated with increased serum levels of anti-VZV IgM antibodies and with pain that improved with antiviral treatment. Together, the findings point to the value of testing BMS patients not only for VZV or HSV-1 DNA in the saliva, but also for serum anti-VZV and anti-HSV-1 IgM antibodies. Both infections are treatable with oral antiviral agents.

Julian Scherer, *Princeton University*, showed that two-color herpesvirus discriminate inoculum from progeny virus particles. The establishment of a latent phase during the infectious cycle of alphaherpesviruses is closely linked to virion interactions with the axonal transport machinery. Axonal transport represents a highly dynamic process that connects distal entry sites with the site of replication/latency in the nucleus. Tracking of virion components undergoing axonal transport traditionally relies on imaging and labeling techniques with single fluorescent protein fusions. However, these techniques fail to distinguish virus inoculum from progeny. To separately analyze particle motility before and after replication, a two-color pseudorabies virus (PRV) variant, PRV180G, produced by growing PRV180 (RFP-tagged small capsid protein mRFP-VP26) in a cell line stably expressing mNeonGreen-VP26 was developed. During viral replication in this cell line, both green- and red-tagged VP26 are packaged, producing two-colored infectious virions. However, after PRV180G replication in neuronal cells, all progeny viral particles were only red. This approace was validated in modified Campenot neuronal chambers, which separate axons from the soma. Particles were observed to enter up to 18 h postinfection, which might have obscured previous analyses

of virion egress. Overall, PRV180G is a reliable marker of capsid destination and allows a more detailed analysis of axonal transport.

Greg Smith, *Northwestern University*, working in collaboration with Osefame Ewaleifoh, Bastian Zimmer, Shen-Ying Zhang, Luigi Notarangelo, Lorenz Studer, and Jean-Laurent Casanova, showed that TLR3 promotes HSV-1 latency by rapidly protecting CNS neurons from infection. HSV-1 is a highly neuroinvasive virus that is typically restricted to the human peripheral nervous system (PNS) where it establishes relatively benign latent infections. Rarely is this evolved homeostasis broken by spread to the brain in association with life-threatening herpes simplex encephalitis (HSE). A subset of HSE patients encode inborn errors in the TLR3 innate sensor pathway, but it is unclear how TLR3 functions in HSV-1 infection. Using human iPSC-derived PNS and CNS neurons from healthy controls and HSE patients, interferon was shown to establish anti-HSV states in these cells. However, control CNS neurons were uniquely resistant to HSV-1 in the absence of a pre-conditioned antiviral response. This inherent resistance resulted from an unexpected TLR3-triggered loss of susceptibility that was STAT1-independent. TLR3 was found to sense HSV-1 by an indirect mechanism that can be circumvented in vitro, which in turn led to the demonstration that TLR3 invokes a near-instantaneous resistance to HSV-1 in CNS neurons. These findings help explain the PNS-restricted neuroinvasion exhibited by alphaherpesviruses in their natural hosts.

Padma Srikanth, *Sri Ramachandra University*, described the anti-VZV antibody response in patients with fever and maculopapular rash (MPR) along with the presence of VZV in CSF of patients with meningitis/encephalitis. Most (50 %) patients demonstrated an IgG response suggesting past infection, 23.5 % showed both IgM and IgG (reactivation/reinfection) and 11.7 %, only IgM indicative of primary infection. No IgM/IgG response was detected in 14.7 %. Correlation of antibody with age showed an increase in IgG with age ($r = 0.53$) and a decline in IgM. Among IgM-positive patients was a 30-year-old woman with fever, rigor, and conjunctival congestion who was VZV PCR-positive (targeting ORF 8). Since there is an increasing pool of immunocompromised individuals in whom VZV may cause severe illness, it is important to screen patients with fever and MPR for VZV. VZV was detected in the CSF of 6 patients (5 pediatric, one adult) by qualitative real-time PCR and subsequently by qPCR. Two pediatric patients were neonates who responded well to intravenous acyclovir (mothers did not provide history of chickenpox during pregnancy/delivery). Three older children presented with aseptic meningitis that responded to intravenous acyclovir with resolution of CNS disease, although one subsequently developed low-grade spikes of persistent fever that abated upon anti-tuberculosis therapy. One child developed neurological sequelae at the time of detection and did not recover. One adult with breast cancer had a very high viral load of VZV. Since chickenpox is considered an innocuous self-limiting disease, it is important for clinicians to investigate atypical manifestations of VZV to initiate early therapy.

Patrick M. Stuart, *Saint Louis University*, described the critical role of sympathetic corneal nerves in primary and reactivated herpes stromal keratitis. Following corneal infection, HSV-1 becomes latent in trigeminal ganglionic (TG) neurons. Previous studies have shown that latency is maintained, at least in part, by HSV-specific CD8+ T cells that infiltrate the TG. In wild-type mice, this maintenance of latency effectively prevents reactivation to the level where virus can be detected at the cornea 1 week postinfection (PI). In contrast to wild-type mice, 10–20 % of mice unable to express CD28 shed virus 4–6 weeks PI, raising the possibility that CD28 costimulation plays a role in the establishment or maintenance of HSV-1 latency. Comparison of B6 and B6-CD28KO mice for viral load and extent of cells with latent virus in TG revealed significantly greater numbers of viral genomes in infected TG of B6-CD28KO mice before day 23 PI, whereas the number of latently infected cells in B6 mice greatly exceeded that in B6CD28KO mice until 21–40 days PI. These differences were not observed after this early time period. Analysis of the CD8+ T cell response in B6 and B6-CD28KO mice showed that the total number CD8+ T cells and the percentage of these cells specific for the gB peptide were much higher in B6 mice, a difference seen for the first 5 weeks PI but not later. Interestingly, functional analysis of these gB-responsive CD8+ T cells indicated no differences in the percentage of cells expressing IFNγ, TNFα, and CD107α. Likewise, the percentage of these cells expressing the T cell exhaustion markers PD-1 and TIM3 did not differ. These results indicate that mice with CD28 deficiency display reduced control of TG infection as evidenced by increased HSV-1 genome load and fewer neurons expressing LAT transcripts at early times after infection. Similarly, the numbers of HSV-1 gB-specific T cells were reduced during these early times. gB-specific T cells from CD28KO mice were functional and did not display signs of increased T cell exhaustion.

Moriah Szpara, *Pennsylvania State University*, showed that a homogeneous population of mature human neurons derived from differentiated human SH-SY5Y neuroblastoma cells can serve as a useful neuronal model for HSV-1 infection. Fully differentiated SH-SY5Y cells have a neuronal morphology and express higher levels of neuron-specific markers than their undifferentiated, epithelial-like progenitors. Neuronal SH-SY5Y cells are able to establish productive HSV-1 infection, with viral replication similar to that observed in undifferentiated SH-SY5Y cells or in the parental epithelial SK-N-SH cell line. Neuronal SH-SY5Y cells have distinct responses to infection as compared to their epithelial-like progenitors, as demonstrated by levels of early growth response gene 1 (Egr-1) and phosphorylated AKT/protein kinase B (p-

AKT). The differentiated SH-SY5Y neurons also express antiviral immune proteins. Thus, these cells provide an accessible and reproducible model for studying HSV interactions with human neurons and for investigating intrinsic immune responses in human neurons.

Matthew Taylor, *Montana State University*, discussed findings on the role of superinfection exclusion during spread of neuroinvasive alphaherpesvirus. Superinfection exclusion is a widely observed phenomenon initiated by a primary viral infection to prevent further infection of the same cell. The capacity for alphaherpesviruses to infect the same cell affects rates of interviral recombination and disease severity. Earlier research in vitro with neuronal cultures identified a co-infection restriction after anterograde directed (axon-to-cell) viral spread for both HSV-1 and pseudorabies virus (PRV). Thus, superinfection exclusion may influence anterograde spread restrictions. Analysis of recombinant viruses expressing fluorescent protein as markers of infection showed that most HSV-1 and PRV-infected cells exclude a secondary viral inoculum within 2 h postinfection. In contrast to previously published work, recombinant PRV with deletions in the viral glycoprotein D gene established superinfection exclusion of secondary viral inoculum. Further work identified a mouse embryonic fibroblast (MEF) cell line expressing a mutated TATA-box-binding protein (TBP) deleted in an N-terminal domain that did not support superinfection exclusion during HSV-1 or PRV infection. In neuronal cultures, MEFs with a deletion in the N-terminus of TBP were co-infected at a much higher rate than in wild-type MEFs, indicating that the absence of superinfection exclusion alleviates anterograde spread restriction. Overall, early gD-independent superinfection exclusion limits co-infection by HSV-1 and PRV after anterograde spread. Additional studies using a mixture of 3 fluorescently-labeled recombinant PRVs were carried out to test viral co-infection during transneural spread from the site of primary intravitreal infection in mice. All 3 viruses infected retinal cells, whereas subsequent anterograde virus spread from the optic nerve resulted in superinfection exclusion and only one virus was recovered, identical to results obtained in vitro. Interestingly, retrograde-directed spread (cell-to-axon) from the oculomotor nerve maintained high levels of co-infection, suggesting that co-infection restrictions during neuroinvasive spread are influenced by direction through neuronal circuits.

Richard Thompson, *University of Cincinnati College of Medicine*, showed that entry of HSV via axonal transport into sensory neurons represents a pivotal point where largely unknown mechanisms lead to latent or lytic infection in neurons. Regulation at this pivot point is critical for efficient widespread seeding of the nervous system and host survival. Our combined genetic and in vivo studies support a model in which regulated de novo VP16 expression in neurons mediates entry into the lytic cycle during the earliest stages of virus infection in vivo, based on three lines of evidence: (1) activation of the latent transcriptional program in trigeminal ganglion neurons infected from the body surface preceded activation of the lytic program by 12–14 h, while in individual neurons, the lytic program began as a transition out of this default latent program; (2) forced de novo expression of wild-type VP16, but not a transactivation-defective mutant, from the LAT promoter dramatically shifted the balance toward productive viral replication; and (3) identification of a region downstream of the VP16 promoter TATA box that regulates the transition from acute infection to latency.

David Tscharke, *Australian National University*, explored the effect of increased presentation of antigen to CD8+ T cells on HSV-1 pathogenesis. $CD8^+$ T cells have been implicated in the control of acute HSV infection as well as latency. Further, HSV-1 targets the $CD8^+$ T cell response with the immunomodulator ICP47, which may be expressed during latency. We explored the consequence of making virus-infected cells more visible to $CD8^+$ T cells in vivo using HSV-1 that expresses an extra copy of the immunodominant $CD8^+$ T cell epitope, $gB_{498–505}$, one of which targets the peptide directly to the endoplasmic reticulum (ER), thus circumventing ICP47. Growth of these viruses in the skin and ganglia of acutely infected mice was similar to that of a control HSV-1, as were the $CD8^+$ T cell responses elicited; however, mice infected with both viruses developed significantly larger skin lesions than those infected with control virus. Overall, expression of the extra $gB_{498–505}$ did not significantly alter the extent or stability of latency, irrespective of ER-targeting. While this study shows no effect of increasing antigen presentation on latent HSV-1 infection, it raises caution that enhanced immunopathology may be an unintended consequence of therapies aimed at augmenting $CD8^+$ T cell recognition of HSV.

Shannan D. Washington, *Louisiana State University Health Sciences Center*, showed that deletion of the HSV-1 Ctcf binding motif Ctrl2 enhances acute infection at the expense of latency in the mouse. During latency, the protein CTCF occupies 7 conserved CTCF binding domains in the HSV-1 genome. The CTRL2 site, downstream from the LAT enhancer, was enriched in the protein CTCF during latency and CTCF was evicted at early times post-reactivation in mice latently infected with 17*Syn*+. Luciferase assays using reporter constructs containing the CTRL2-binding domain showed that this motif was a functional insulator with both enhancer-blocking and -silencing capabilities, further suggesting a mechanistic role for this CTCF-binding domain in regulating latent HSV-1 gene expression. CTCF insulators can facilitate the formation of chromatin loops to regulate gene expression through spatial interactions. To test whether CTCF self-dimerization might facilitate the formation of chromatin loops in the latent HSV-1 genome, we recently combined conventional chromosome conformation capture assays with next-generation sequencing (3C-seq) using latently infected mouse TG. These experiments identified 3 potential loops, one of

which involved the CTRL2 domain of HSV-1 and a site near the VP16 promoter. Use of a recombinant virus with a deletion spanning only the CTRL2 domain revealed significant differences between the mutant and wild-type virus with respect to acute replication, mortality rates, latent viral genome loads and LAT expression in vivo. These data provide evidence that the latent HSV-1 genome may be maintained by spatial interactions via chromatin loops and that disruption of spatial interactions impedes the establishment of latency and/or reactivation in vivo.

Angus C. Wilson, *New York University School of Medicine*, described the control of host microRNA (miRNA) expression during HSV-1 infection. Little is known about the impact of HSV-1 on host regulatory RNAs, including miRNAs, small 19- to 22-nucleotide single-stranded RNA molecules that bind target mRNAs and suppress translation. Wilson and colleagues found that an evolutionarily conserved cluster of three cellular miRNAs (miR-183, miR-96, and miR-182) is strongly and selectively upregulated early in the HSV-1 replication cycle in primary neurons and fibroblasts. This increase was transcriptional and dependent on viral protein expression. The viral E3 ubiquitin ligase ICP0 was both necessary and sufficient for induction of the miR-183 cluster and was abolished by mutations that blocked either E3 ligase function or nuclear localization. Induction was mimicked by depletion of the host transcription factors ZEB1 (δEF1) and ZEB2 (SIP1), newly identified substrates for ICP0-mediated degradation. Depletion of ZEB1 in fibroblasts using siRNA led to increased HSV-1 replication. Manipulation of miRNA expression by targeting host transcription factors identifies a new function for ICP0 and provides an efficient mechanism for HSV-1 to broadly modify the host cell environment. The miR-183 cluster and ZEB proteins are master regulators of the epithelial–mesenchymal transition and may facilitate viral infection of mucosal epithelial cells during natural infection.

Acknowledgments Funding for this conference was provided, in part, by Public Health Service grant AG04651 (R.C.) from the National Institute on Aging, National Institutes of Health, and generous donations from Affymetrix eBioscience (a part of Thermo Fisher Scientific), Agilent Technologies, Inc., Bully Ranch, Cellular Dynamics International (a Fuijiflim Company), Colorado Mountain Express, FedEx (store #2004), Genewiz, Integrated DNA Technologies, Molly's Spirits, Research Products International, Rocky Mountain SIMS, Thermo Fisher Scientific, Tivoli Distributing Company, Tivoli Lodge, Walmart (store no. 4288), a private donation from The VZV Foundation, and a personal contribution from Don Gilden. We especially thank the owners of The Christiania Lodge at Vail. We thank Marina Hoffman for the editorial assistance.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.