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MEETING REPORT

2017 Colorado alphaherpesvirus latency society symposium

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Introduction

The 7th annual symposium of the Colorado Alphaherpesvirus Latency Society (CALS) convened 70 investigators who have authored over 2473 PubMed-listed publications involving herpesvirology and who traveled 110,408 miles from 3 continents, 6 countries, and 22 states. The 2-day symposium consisted of 26 oral presentations by investigators to discuss advances in the research of herpes simplex types 1 and 2, varicella zoster virus, bovine herpesvirus virus type 1, pseudorabies virus, and simian varicella virus latency. In addition, 18 promising graduate students and postdoctoral fellows presented posters, each preceded by a short oral summary, and a small group of exceptional undergraduates whose interest in herpesvirology is just beginning were also hosted. New this year was a special session led by volunteers with first-hand experience in NIH grant reviews to discuss the inner workings of an NIH study section.

This year marked the beginning of the Don Gilden Memorial Lectureship, a grassroots endeavor initiated and generously supported by fellow alphaherpesvirus experts who wish to continue Dr. Gilden's desire to underscore the value of neuroscientists outside the field of virology in broadening the knowledge base. The inaugural Don Gilden Memorial Lecture, presented by Dr. Paola Sandroni from the Mayo Clinic, provided an overview of disorders of the autonomic nervous system. While an early spring blizzard prevented the musical tribute that traditionally follows the presentation, all enjoyed the silent ambiance of the quaint mountain town covered with snow. The relaxed setting of The Christiania Lodge and The Tivoli 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 (Fig. 1). A brief summary of the presentations follows:

Andrea S. Bertke, Virginia Tech, explored the effects of various environmental triggers on HSV-1 and HSV-2 reactivation in different types of peripheral neurons. Previous studies have shown that the viruses preferentially replicate and establish latency in different types of sensory neurons, as well as in neurons of the autonomic nervous system. To determine in which types of neurons HSV-1 and HSV-2 reactivate in response to triggers commonly associated with recurrent disease, this laboratory infected primary adult murine sensory and autonomic neuronal cultures, and either treated them with stress hormones or deprived them of target-derived neurotrophic factors to simulate epithelial injury. Deprivation of neurotrophic factors neurturin and glial cell-derived neurotrophic factor induced reactivation of HSV-1 and HSV-2 in sensory neurons expressing glial family receptors, while nerve growth factor deprivation led only to HSV-1 reactivation in sympathetic neurons. Treatment with stress hormones epinephrine (EPI) and corticosterone (CORT) selectively induced HSV-1 or HSV-2 reactivation; HSV-1 only reactivated in sympathetic neurons in response to EPI or CORT, while HSV-2 reactivated in both sensory and autonomic neurons but only in response to CORT. Thus, HSV-1 and HSV-2 reactivate from different types of sensory and autonomic neurons in

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Fig. 1 Participants of the 7th annual symposium of the Colorado Alphaherpesvirus Latency Society. (*Row 1, from left*) Maria Nagel, Victoria Quintana, Jane Dantinne, Randall Cohrs, Julianna Pieknik, Yoshiki Kawamura, Richard Thompson, Tao Peng, (*row 2*) Anna Blackmon, Kamel Khalili, Charles Calisher, Katherine Lee, Satish Mehta, Peter Kennedy, Shannan Washington, Mike Gershon, Anne Gershon, Dan Carr, Addilynn Beach, Andrew Bubak, (*row 3*) Nicholas Baird, Paul R (Kip) Kinchington, Benjamin Warner, Andrea Bertke, Chiharu Graybill, Duane Pierson, Martine Aubert, Ravi Mahalingam, Rebecca Powell-Doherty, Vicki Traina-Dorge, Edouard Cantin, Nicholas Taylor, Luis Schang, (*row 4*) Marius Birlea, Paola Sandroni, Xiaomi Chen, Ian Hogue, Georges Verjans, Patrick Lomonte, (*row 5*) Todd Margolis, Qiaojuan Zhang, Christina Como, Susanne Himmelein, Aaron Prattis, Ken Jones, (*row 6*) Ron Goldstein, Seth Frietze, Homayon Ghiasi, Stacey Efstathiou, Charles Grose, Gang Li, Anna Cliffe, Werner Ouwendijk, (*row 7*) Dallas Jones, Patrick Stuart, Leonardo D'Aiuto, Klaus Osterrieder, Matthew Taylor, Julian Scherer, Clinton Jones, Scott Schmid, David Davido, Dane Phelan, Bill Jacobs, Mercedes Romero, Carol Kulesza, Victor Hsia, Moriah Szpara, Hua Zhu, (*not pictured*) Tom Goodwin, Ruth Itzhaki, Bridget Sanford, and Nancy Sawtell

response to different environmental triggers, which may explain their different patterns and frequencies of recurrent disease.

Marius Birlea, University of Colorado School of Medicine, presented data on alphaherpesvirus reactivation and headache, noting that headache is the most common reason for referral to a neurologist. Headaches are classified as "primary," i.e. migraine, with no clear cause, and "secondary," with multiple causes, including infections. Headaches can be chronic and disabling, and treatment is often unsatisfactory. Besides postherpetic neuralgia, alphaherpesvirus reactivation from sensory ganglia, with or without rash, has been only anecdotally associated with chronic headaches. Positive specific serum IgM antibody is considered a surrogate of spontaneous or triggered viral reactivation and can persist for months in low titers. In a retrospective chart review of 260 randomly selected chronic headache patients of a single academic headache clinic, positive HSV-1 and/or HSV-2 IgM was detected in ~35%; most common headache types were chronic migraine, trigeminal autonomic cephalalgias, new daily persistent headache, and cranial neuralgias. HSV1 DNA shed in the saliva, also indicative of viral reactivation, was found in 4% of consecutive patients of the same clinic (separate study, preliminary data). The role of alphaherpesviruses in head and face pain awaits further studies including appropriate controls, with the goal of identifying new treatment options.

Ann Blackmon, University of Colorado School of Medicine, showed that VZV causes redistribution of claudin-1 and aberrant expression of E-cadherin and N-cadherin in human perineurial cells. VZV vasculopathy occurs after virus reactivates from ganglia, spreads along nerve fibers to arteries, leading to pathological vascular remodeling and stroke. Interestingly, VZV antigen is present in cells surrounding nerve fibers in the outermost adventitial layer of temporal arteries from VZV vasculopathy patients. These VZV-infected cells express the tight junction protein claudin-1, identifying them as perineurial cells, which form a barrier between the peripheral nerve and surrounding tissue. To test the hypothesis that during VZV spread along nerve fibers, virus disrupts tight junctions in perineurial cells, thus

potentiating infection of surrounding vascular cells, mock- and VZV-infected primary human perineurial cells (HPNCs) were compared for expression and distribution of cell adhesion proteins (claudin-1, E-cadherin, and N-cadherin) at 3 days postinfection. While there was no VZV-induced change in claudin-1 transcripts compared to mock-infected HPNCs, claudin-1 redistributed from the membrane/cytoplasm to the nucleus. Analysis of HPNCs treated with anti-claudin-1 or isotype control antibodies prior to VZV infection revealed no difference in the level of virus infection. Furthermore, mock-infected cells expressed E-cadherin and not N-cadherin, while VZV-infected cells expressed only N-cadherin, supporting the novel possibility of a VZV-induced epithelial-mesenchymal cell transition. Addition of conditioned media from VZV-infected HPNCs to uninfected HPNCs induced the same changes seen in VZV-infected cells. Overall, these findings suggest that (1) claudin-1 is not necessary for VZV infection; and (2) VZV-induced redistribution of claudin-1, downregulation of E-cadherin, and upregulation of N-cadherin is mediated by a soluble factor and may lead to disruption of tight junctions in perineurial cells, allowing viral spread from nerve fibers to surrounding vascular cells in VZV vasculopathy.

Andrew N. Bubak, University of Colorado School of Medicine, described findings potentially relevant to the pathophysiological mechanisms of postherpetic neuralgia (PHN), a complication of zoster involving persistent dermatomal distribution pain >3 months after rash and the leading cause of suicide in elderly chronic pain patients. Treatments for PHN are generally ineffective, in part, because analyses of the causal mechanism(s) are limited since VZV is an exclusively human virus, restricting studies to the only established zoster model, SVV infection of rhesus macaques. General neuropathic pain is mediated by activation of peripheral nociceptive C- and Aδ-fibers, which in turn release glutamate and substance P on spinal cord neurons located in the dorsal horn to transmit pain sensation. By contrast, non-nociceptive Aβ sensory fibers, which detect mechano-sensory stimuli, do not produce substance P and are not typically involved in pain signaling. In rodent models, chronic pain can be caused by increased substance P release at the dorsal horn due to an inflammation-induced, chemical phenotypic shift in non-nociceptive Aβ sensory fibers, such that they produce substance P. Similar mechanisms of neuropathic pain are likely to occur during zoster when productive VZV infection leads to inflammation in ganglia, as well as in PHN where persistent ganglionitis is seen. Preliminary studies to test whether SVV reactivation causes increased substance P production from virus-infected C-fibers and from phenotypically altered Aβ fibers showed that latently infected dorsal root ganglia contained Aβ sensory fibers without substance P, whereas reactivated ganglia contained substance P-positive Aβ sensory fibers. Further studies involving more ganglia from uninfected, latently infected and reactivated monkeys, as well as examination of corresponding spinal cord sections for post-synaptic alterations, promise to provide insight into the mechanisms of neuropathic pain caused by zoster and clues as to potential treatment targets for PHN.

Edouard Cantin, City of Hope, investigated the role of *Bacteriodes fragillis* polysaccharide A (PSA) immunomodulation of host immunity in preventing HSV-1 encephalitis (HSE). HSE results primarily from reactivated HSV-1 in adults and primary infections in newborns. Despite antiviral treatment, mortality remains high (~20%) and most survivors (>60%) have debilitating neurological deficits. Although IVIG reportedly prevents HSE by exerting potent IL-10-dependent immunomodulatory effects, the significant cost, shortage, and infrequent serious adverse effects of IVIG limit its widespread clinical use. Evaluation of PSA as a prebiotic to protect against HSE using 129 naïve mice treated with PBS or PSA over 21 days, challenged at 10× LD_{50}, and given a 7-day course of acyclovir starting on day 4 post-infection showed that most of the PSA-treated mice survived, while all PBS-treated mice succumbed to HSE. PSA suppressed brain invasion by $Ly6C^{high}$ inflammatory monocytes and neutrophils and induced IL-10-secreting $ICOS^+$ $CD73^+$ CD4 T cells and $CD73^+$ CD8 T cells. Interestingly, IL-10 derived from both T and B cells was essential for PSA-mediated protection. The findings suggest that the microbiota play an important role in regulating immunity to HSV and that pre/probiotics in combination with antivirals might act synergistically to limit HSV-induced inflammatory diseases.

Xiaomi Chen, University of Colorado School of Medicine, presented evidence that VZV establishes latency in human enteric neurons. VZV, a ubiquitous human alphaherpesvirus that produces varicella (chickenpox) on primary infection and establishes latency in cranial nerve, dorsal root, and autonomic ganglia along the entire neuronal axis, can reactivate to cause zoster (shingles), often complicated by postherpetic neuralgia, headache, blindness, or stroke. Previous studies in guinea pigs, along with clinical and autopsy samples from young individuals, detected VZV DNA in the gut, suggesting that the virus can establish latency in the enteric nervous ganglia. Indeed, our quantitative PCR analysis of neurons isolated from five fresh surgically removed gut sections of adults with no recent history of varicella or cutaneous signs of herpesvirus infection revealed VZV DNA in the jejunum, colon, and ileum, with the lowest average CT of 28.73 in 100 ng of total DNA. In addition, CLARITY was used to demonstrate neurons in the jejunum, although their size and distribution suggest a technical challenge in isolating pure neuronal samples.

Anna Cliffe, University of Virginia, described stress-induced changes to HSV-1 chromatin during reactivation. During latent infection of peripheral neurons, HSV-1 lytic gene promoters are assembled into silent heterochromatin,

repressing reactivation. Various stimuli are known to overcome this repression and trigger reactivation, including axotomy, heat shock, and loss of neurotrophic support, although how these stimuli result in upregulation of viral lytic gene expression from silenced promoters is still unclear. Inhibition of P13K-activity by LY294002 was shown to result in HSV-1 reactivation dependent on DLK/JIP3-mediated activation of JNK. Interestingly, LY294002-mediated reactivation occurs independently of histone H3K9me demethylase activity. Instead, reactivation is accompanied by increased H3S10 phosphorylation on histones that still maintain K9 methylation, a process known as a histone phospho/methyl switch. Preliminary analyses to determine whether additional reactivation stimuli act through the same pathway, with particular focus on forskolin-mediated reactivation since forskolin also triggers histone S10 phosphorylation in neurons and HSV reactivation, indicate that forskolin-mediated reactivation does involve JNK-dependent early (Phase 1) reactivation. However, despite the similarities in LY294002- and forskolin-mediated reactivation, the two compounds can act synergistically to enhance lytic gene expression. A histone phospho/methyl switch is thought to permit gene expression because repressive proteins that interact with methylated histone residues are evicted when the neighboring residue becomes phosphorylated, although transcriptional activation is dependent on the specific histone readers present on DNA. Analyses to determine which histone readers are evicted during the early stages of reactivation show that binding of Suz12, a component of the PRC2 complex, decreases during Phase I reactivation. Thus, even in the absence of histone demethylase activity, the viral chromatin associated with PRC2 is remodeled early in reactivation stages and permits gene expression.

Leonardo D'Aiuto, University of Pittsburgh School of Medicine, described human three-dimensional (3D) neuronal platforms for drug screening. Infection of neurotropic viruses can cause structural and functional changes in the CNS, causing long-term neurological sequelae. Drug discovery for CNS infections has been hindered by lack of human neuronal cell platforms suitable for drug screening and by the difficulties in generating cell cultures that reproduce aspects of the 3D architecture of tissues. This laboratory generated scaffold-free 3D neuronal cultures in optical active 96-well plates and 96-Transwell plates starting from neural progenitor cells (NPCs) derived from induced pluripotent stem cells (iPSC). To test their suitability for drug screening, 3D neuronal cultures were infected with HSV-1 along with increasing amounts of acyclovir. The IC50 of acyclovir was determined using both flow cytometry (FC) and the CX7 High Content Screening (HCS) platform (ThermoFisher, Inc). FC analysis of both 3D neuronal culture systems showed >80% cell viability and no significant well-to-well variation in both cell density and the ratios of Tuj1+ or MAP2+ cells to GFAP+ cells. The IC50 for acyclovir determined by both FC and HCS was comparable (3.144 and 3.121 μM, respectively). This laboratory's 3D human neuronal cultures, along with a novel confocal-based HCS platform (CX7), provide an unprecedented opportunity for a rapid and robust drug screening for CNS infections.

David Davido, University of Kansas, showed that inhibition of viral DNA replication limited the efficacy of an HSV-1 neuro-attenuated vaccine in mice. This laboratory previously showed that an HSV-1 mutant, KOS-NA, which contains a mutation in the UL39 gene, is significantly neuro-attenuated for viral replication and is highly protective against ocular HSV-1 challenge (i.e., keratitis) as compared to a replication-impaired ($ICP0^{-}$) virus in mice. To test whether a replication-defective form of KOS-NA would retain the efficacy of KOS-NA while increasing vaccine safety, KOS-NA/Δ29, in which the essential viral DNA replication gene UL29 is deleted, was generated. Evaluation of KOS-NA, KOS-NA/Δ29, and Δ29 viruses as potential prophylactic vaccines against HSV-1 ocular infection in mice suggested that KOS-NA, in a dose-dependent manner, was more effective in limiting weight loss, acute viral replication, blepharitis, and severe keratitis than KOS-NA/Δ29 and Δ29 viruses after ocular challenge. These results suggest that blocking replication of KOS-NA diminishes aspects of its efficacy as a potential prophylactic vaccine to reduce HSV-1 corneal disease, and that replication competence should be considered when designing vaccines against HSV-1.

Seth Frietze, University of Vermont, described CTCF occupancy on HSV-1 and VZV DNA in human trigeminal ganglia (TG). HSV-1 and VZV are ubiquitous human neurotropic alphaherpesviruses that establish latent infection in TG after primary infection. During latency, much of the viral genome is transcriptionally silenced by posttranslational modification of core histone proteins. Current thought suggests that the epigenetic markings of the latent virus genome are segregated by chromatin insulators, especially the CTCF-binding factor. To develop a topological profile of CTCF chromatin binding along the alphaherpesvirus genome in multiple human TG, a set of tiled oligonucleotides was used to enrich virus sequences in chromatin immunoprecipitation assays followed by deep sequencing (ChIP-seq) targeting the CTCF protein. The results identified read clusters associated with CTCF consensus motifs enriched over input, indicating that CTCF binds to distinct chromatin sites on the VZV and HSV-1 genomes. This consensus portrait of CTCF occupancy on alphaherpesviruses in human trigeminal ganglia might provide clues as to the mechanism(s) controlling virus gene transcription during latency and reactivation.

Michael D. Gershon, Columbia University, described VZV in the enteric nervous system (ENS). The ENS is essential for life and its dysfunction is potentially lethal. VZV establishes latency in enteric neurons after natural varicella infection or

varicella vaccination. Reactivation of VZV in enteric neurons ("enteric zoster") may cause unexplained visceral pain, chronic intestinal pseudo-obstruction, inflammatory bowel disease, or perforated ulcer, even in the absence of rash. The co-existence of otherwise unexplained gastrointestinal (GI) symptoms with the presence of DNA encoding VZV gene products in saliva of individuals experiencing active VZV infection suggests VZV reactivation in the ENS. Detection of VZV DNA, RNA, and/or protein in GI mucosal biopsies and/or relief of GI symptoms in a therapeutic trial of valacyclovir support the diagnosis of "enteric zoster." Evidence suggests that viremia occurring during varicella or after vaccination delivers VZV to enteric neurons. While VZV-infected lymphocytes fail to release infectious cell-free VZV, they do release exosomes containing large concentrations of transcripts encoding stimulator-of-interferon genes (STING), which are not expressed in naïve enteric neurons, but are highly expressed in enteric neurons that harbor latent VZV. Thus, STING expression transferred via exosomes to neurons might inhibit VZV proliferation in neurons after their infection, contributing to establishment of latency. VZV can be reactivated from latency in guinea pigs to cause disseminated zoster when the animals are immunosuppressed (tacrolimus) and given coricotrophin-releasing hormone (to mimic stress).

Homayon Ghiasi, Cedars-Sinai Medical Center, explored the role of herpesvirus entry mediator (HVEM) ligands in HSV-1 latency/reactivation. Previously, this laboratory reported that the latency-associated transcript (LAT) of HSV-1 upregulated HVEM (TNFRSF14: CD270), a member of the T necrosis factor receptor superfamily (TNFRSF), but had no effect on other HSV-1 receptors during latency. In addition, latency/reactivation was significantly reduced in $HVEM^{-/-}$ mice, indicating a significant role for HVEM in HSV-1 latency/reactivation. HVEM also engages the immunoglobulin superfamily members CD160, LIGHT, B and T lymphocyte attenuator (BTLA), and LTα. To test whether CD160, LIGHT, and BTLA are involved in latency/reactivation in the trigeminal ganglia (TG) of latently infected mice, the effect of LAT on latency/reactivation was evaluated in $CD160^{-/-}$, $LIGHT^{-/-}$, and $BTLA^{-/-}$ mice ocularly infected with LAT(+) and LAT(−) viruses. Unlike HVEM, LAT did not upregulate CD160, LIGHT, or BTLA in TG of latently infected mice. HSV-1 latency was significantly reduced in $CD160^{-/-}$, $LIGHT^{-/-}$, and $BTLA^{-/-}$ mice, while the level of reactivation was unaffected. Thus, while the absence of HVEM affected both latency and reactivation, the absence of CD160, LIGHT, or BTLA affected only latency. The level of HVEM was reduced in $CD160^{-/-}$, $LIGHT^{-/-}$, and $BTLA^{-/-}$ mice, suggesting that lower HVEM expression in these mice contributes to reduced latency. However, since these mice still had intact level of HVEM expression, the level of reactivation was not affected. These results suggest that the presence of HVEM and its upregulation by LAT enhance HSV-1 latency/reactivation, identifying a novel mechanism that manipulates homeostatic pathways involved in HSV-1 latency/reactivation.

Ronald S. Goldstein, Bar-Ilan University, demonstrated that VZV expresses short non-coding RNAs, including a potential microRNA (miRNA). Many herpesviruses express miRNAs that are involved in regulation of lytic and/or latent infection. Two previous screens using next-generation sequencing (NGS) of RNA extracted from latently infected cadaver ganglia or using computational methods found no miRNAs encoded by VZV (HHV-3). However, this laboratory detected the presence of several 20- to 24-nt RNAs in VZV-infected human embryonic stem cell-derived (hESC) neurons and fibroblasts by two independent bioinformatics analyses after an NGS study of small RNA. One small non-coding RNA (sncRNA) common to both analyses maps to the repeat region of the VZV genome, which is preferentially expressed during latency. The sncRNA is also predicted to have a stem-loop precursor, consistent with its identity as a miRNA. This candidate was detected in each of three independent biological repetitions of NGS of RNA from fibroblasts and neurons productively infected with VZV, and in multiple cell types infected with VZV by Taqman qPCR. Importantly, a synthetic RNA oligonucleotide antagonistic to this sequence increased the number of infectious loci of ARPE (retinal pigment epithelial cells) cells infected with cell-associated VZV. These results suggest that VZV, like other human herpesviruses, expresses and uses miRNAs to regulate infection.

Chiharu Graybill, University of Colorado School of Medicine, presented evidence that cell-associated vOka and rOka strains of VZV inhibit autophagic flux. T cell-mediated immunity is required to maintain latency of VZV and prevent shingles, which develops after virus reactivation. Although an age-dependent decline in VZV-specific immunity is associated with the increased frequency and severity of shingles in the elderly, other age-associated cellular changes are likely to contribute to limiting virus reactivation. Macroautophagy (herein referred to as autophagy) is a catabolic process in which unnecessary or dysfunctional cellular materials are encapsulated into double-membraned vesicles, i.e., autophagosomes, and delivered to lysosomes for subsequent degradation. Studies suggest that VZV infection induces autophagy and alters viral replication. Furthermore, several reports demonstrate the age-related decline in autophagy. To examine how VZV infection affects this age-dependent decline in autophagy, this group conducted ratiometric flow cytometry and immunoblotting analyses of the effect of cell-associated wild-type rOka and attenuated vOka strains of VZV on the default state of autophagy (basal autophagy) or serum/amino acid starvation (induced autophagy) in infected MRC5 fibroblasts. Both assays indicated that turnover of LC3-II, an autophagosomal

marker, was reduced in infected cells compared to uninfected controls, suggesting that both strains of VZV inhibit lysosomal degradation at the late stage of the autophagy pathway. This inhibition was more pronounced when cells were infected with rOka compared to vOka. Additionally, autophagic flux, a process wherein autophagosomes fuse with lysosomes and encapsulated material is subsequently degraded, was strongly inhibited when cells underwent serum/amino acid starvation. The discrepancy between these findings and those of previous studies might rest in the nature of our assays, which specifically focus on completed autophagic flux. Overall, this study provides some understanding of the relationship between VZV infection, autophagy, and aging.

Charles Grose, University of Iowa, presented evidence that the cellular stress response to VZV infection includes elevated transcription of interleukin-6 (IL-6). Increased IL-6 has been detected in the skin of patients with herpes zoster and in the cerebrospinal fluid of patients with zoster encephalitis. To pursue additional experimental studies of IL-6 responses to VZV infection, this laboratory adapted a fetal human skin-organ culture (SOC) model to newborn foreskin SOC; VZV replication proceeded similarly in both models. Samples for RNA extraction and PCR were obtained from VZV-infected monolayers and VZV-infected SOC, along with uninfected monolayers, uninfected SOC, and Poly(I:C)-stimulated cells. Experiments carried out in a 96-well microarray plate showed that the patterns of upregulated transcripts differed between VZV-infected cells and VZV-infected skin explants. Despite some overlap in transcripts upregulated in infected and uninfected skin explants, examination of the results from the 84 wells with different innate immune transcripts revealed a 32-fold elevated IL-6 level in the infected skin explant that was not present in the infected monolayer culture. This transcript was elevated to the same high level in cells stimulated with Poly(I:C). The IL-6 results in the PCR assay were reproduced by qPCR testing with newly designed primers. Thus, the cellular stress response to VZV infection of SOC includes highly elevated levels of IL-6 transcription.

William Halford, Southern Illinois University School of Medicine, Daniel J.J. Carr, University of Oklahoma Health Sciences Center, in Bill's absence, Dan described a Phase I clinical trial of a live HSV-2 ICP0- virus as a therapeutic vaccine for genital herpes. In animal models, live HSV-2 *ICP0*$^-$ viruses elicit superior protection against genital herpes relative to glycoprotein D-2 subunit vaccines. Live HSV-2 *ICP0*$^-$ viruses are avirulent in mice and guinea pigs, but their safe use in humans is unknown. In the Phase I clinical trial conducted to assess the tolerability of the live HSV-2 *ICP0*$^-$ virus HSV-2 0ΔNLS in genital herpes sufferers, individuals who reported 4 to 24 genital herpes outbreaks per year were screened to verify HSV-seropositivity and flown to the Federation of St. Kitts & Nevis to receive a three-shot immunization series. On each visit, blood was drawn and participants received intradermal immunizations of 0.2 ml containing 150 million pfu HSV-2 0ΔNLS in the calf. Nineteen of 20 participants reported that the live HSV-2 0ΔNLS vaccine produced symptoms milder than the genital herpes symptoms they had endured for years before vaccination. The 17 participants who completed the vaccination series reported an average 3.1-fold reduction in the frequency of genital herpes symptomatic days in the first 4–6 months post-vaccination. Intriguingly, several participants had negligible HSV-2-specific antibody levels before vaccination (despite having been HSV-2-infected for >3 years), but exhibited a 2- to 6-fold increase in total HSV-2 antibody levels by day-30 post-vaccination. Importantly, such increases in HSV-2 antibody levels temporally correlated with reductions in HSV-2 genital herpes symptoms. Together, the data suggest that the live HSV-2 0ΔNLS vaccine is well-tolerated by herpes sufferers and merits consideration as a therapeutic vaccine that reduces genital herpes symptoms in patients.

Susanne Himmelein, Ludwig Maximilian University Munich, explored the role of Toll-like receptors (TLRs) during latent HSV-1 infection in human trigeminal ganglia (TG). The exact mechanisms of HSV-1 latency and the intermittent reactivation are still largely unknown, as is the possible involvement of TLRs. Although earlier studies purported that neurons cannot express TLRs, a recent study demonstrated expression of TLR2, TLR4, TLR5, and TLR9 on neurons in the adrenal cortex. While most research on TLRs thus far has been performed in in vitro and small animal models, this laboratory investigated the expression of TLRs and cytokines on LAT-positive versus LAT-negative human ganglia and neurons and its association with cytokine expression. RT-qPCR revealed significantly higher expression of TLR9 at a single neuron level in infected versus uninfected neurons (Mann-Whitney U test $p = 0.001$), whereas TLRs 2, 3, and 4 were not significantly expressed on either infected or uninfected neurons. In response to viral infection, TLRs initiate a signaling cascade which elicits an antiviral immune response, including induction of inflammatory cytokines, production of chemokines, and induction of innate immune response of the host. In that context, analysis of the expression of TNF-α, IL-6, IFN-γ, and IL-1β revealed only higher expression of IFN-γ at the single-cell level in infected versus uninfected (Mann-Whitney U test $p = 0.018$). Recognition of CpG-motifs by TLR9 initiates intracellular signal transduction, leading to activation of NF-KB, which acts within the nucleus to control transcription of inflammatory cytokines. In mice, the immune response against HSV-1 is dependent on TLR9 and TLR2 and on IFN-γ production in the TG. The results from this work, which are similar to those found in mice, suggest that TLRs play the same important role in humans.

Ian B. Hogue, Princeton University, showed that pseudorabies virus (PRV) egress and spread involves constitutive secretory mechanisms and does not depend on action

potential firing in neurons. Newly assembled herpesvirus particles exit from infected cells by exocytosis. Using a novel live-cell fluorescence microscopy assay of viral egress, this group recently showed that PRV particles exploit constitutive secretory mechanisms governed by Rab6, Rab8, and Rab11 for exocytosis in non-neuronal cells. By contrast, neurons act through highly specialized secretory mechanisms for regulated exocytosis of synaptic and neuropeptide vesicles, governed by Rab3 and Rab27. Alphaherpesviruses spread along circuits of synaptically connected neurons in vivo, and infection of neurons correlates with increased action potential firing in vivo and in vitro. To test whether PRV uses regulated secretory mechanisms dependent on action potential firing for egress and spread from neurons, this group used a compartmentalized neuron culture system to measure transneuronal spread of PRV, together with pharmacological and optogenetics approaches to modulate neuronal activity. Unexpectedly, transneuronal spread of PRV did not correlate with action potential firing. Further analysis of the molecular mechanisms of viral egress using a live-cell fluorescence microscopy assay revealed no association of PRV particle egress with the regulated secretory proteins Rab3 and Rab27, but instead, use of the constitutive secretory pathway, even in professional secretory cells that do possess regulated exocytosis mechanisms. Thus, while both neurotransmission and spread of alphaherpesviruses follow synaptic circuits, these processes appear to be mediated by distinct molecular and cellular pathways.

Victor Hsia, University of Maryland Eastern Shore, described the participation of thyroid hormone (TH) in regulating of VZV reactivation and replication. TH has been suggested to control herpesvirus gene expression and replication in neurons via epigenetics through its nuclear receptors. This group previously showed that hypothyroidism patients were predisposed to herpes zoster, suggesting that TH deficiency is a risk factor for VZV reactivation. To test whether TH plays a role in regulation of VZV replication and thus reactivation, this laboratory first performed a clinical observational study using a comprehensive medical database at Kaiser Permanente Southern California to determine whether patients taking TH medication have reduced herpes zoster occurrence; the fully adjusted hazard ratio analyses indicated that patients receiving medication for TH deficiency exhibited a lower risk of zoster (HR, 0.60; 95% CI, 0.51–0.71). Analysis using a human neuron-like cell model to investigate TH-mediated regulation of VZV replication showed that TH treatment decreased VZV replication in vitro. Further analyses revealed that TH repressed the ORF36 and ORF68 but increased the latency-associated ORF63. Bioinformatics suggested a pair of palindromic thyroid hormone-responsive elements located in the VZV ORF36 (thymidine kinase) promoter between the TATA box and transcription initiation site. Transfection studies indicated that TH negatively regulated ORF36. Chromatin immunoprecipitation analysis suggested that repressive chromatin was enriched at the ORF36 promoter in the presence of TH. Together, these findings supported the hypothesis that TH participates in the regulation of VZV latency and reactivation by reducing viral replication.

Ruth F. Itzhaki, University of Oxford, noted the urgent need for effective Alzheimer's disease (AD) treatments, since AD afflicts ~30 million people worldwide and the number of cases is predicted to increase greatly. Based on her lab's work, supported by others, implicating herpes simplex virus type 1 (HSV1) in the etiology of AD, one possible treatment is the use of antiviral agents to slow AD progression. Her lab found (1) HSV1 DNA in many elderly brains, (2) HSV1 in brain, together with the genetic factor, type 4 allele of apolipoprotein gene (APOE-ε4), confers a high risk of AD, accounting for about 60% of cases, (3) APOE-ε4 is a risk factor for recurrent herpes labialis, and (4) latent HSV-1 in the brain can reactivate, with harmful outcomes. They further linked HSV1 directly to AD biomarkers: HSV1 infection of neural and other cell types in culture, and of mouse brains, causes the deposition of beta-amyloid (Aβ) and AD-like tau (P-tau), which are the main components, respectively, of the characteristic abnormalities, amyloid plaques, and neurofibrillary tangles, of AD brain. Even more significantly, most of HSV1 DNA in AD brains is localized specifically within amyloid plaques. Also, acyclovir (ACV), as well as several other antivirals, greatly inhibits HSV1-induced formation of Aβ and P-tau in cell cultures. Based on these findings, supported by >70 recent studies from other labs, using epidemiological, genetic, cell biological, and virological approaches, her team suggests that in older age, as the immune system declines, HSV1 enters the brain, where the virus establishes a latent infection, but can be reactivated repeatedly after by events such as peripheral infection, stress, or immunosuppression. Reactivation could causes localized productive infection, resulting in direct and also inflammatory damage, which is greater in APOE-ε4 carriers. Recurrent reactivation could then cause cumulative damage, eventually leading to AD, possibly via formation of Aβ and its toxic products.

William R. Jacobs, Jr., Albert Einstein College of Medicine, studied the key role of antibodies mediating antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (ADCP) that protect mice and guinea pigs from HSV-1 and HSV-2. This laboratory previously showed that HSV-2 deleted in glycoprotein D (HSV-2 Δ*gD*) elicits high-titer HSV-specific antibodies (Abs) that (i) provide complete protection in mice against both HSV-1 and HSV-2 in vaginal or skin scarification challenge models; (ii) induce robust ADCC and ADCP; and (iii) have broadened antigen specificities. Extension of those studies to guinea pigs showed that the HSV-2 Δ*gD* vaccine also provides complete protection and completely prevents the establishment of latent virus following vaginal challenge with either a highly virulent clinical

isolate or laboratory strain of HSV-2. Development of an HSV-2 Δ*gD::rfp* reporter strain by this laboratory enabled quantitation of the ADCC activity of vaccine-elicited guinea pig antibodies and the demonstration that HSV-2-immune guinea pig serum mediates ADCC and ADCP activity, correlating with the protective immune response. Together, the data demonstrate that vaccine primes robust anti-HSV humoral immunity with Abs capable of mediating FcγR effector functions in multiple animal models. Further studies are needed to delineate the antibody characteristics mediating these effector functions in order to harness prophylactic and therapeutic strategies to provide protective immunity.

Clinton Jones, Oklahoma State University, described the regulation of the canonical Wnt/β-catenin signaling pathway during the bovine herpesvirus 1 (BoHV-1) latency/reactivation cycle. The latency-related (LR) RNA encoded by BoHV-1 is abundantly expressed in latently infected sensory neurons. Although the LR gene encodes several products, ORF2 appears to mediate important steps during the latency/reactivation cycle because a mutant virus containing stop codons at the amino-terminus of ORF2 does not reactivate from latency in calves. Recent studies showed that β-catenin and a β-catenin coactivator, high-mobility group AT-hook 1 protein are readily detected in trigeminal ganglia (TG) neurons of calves latently infected with BoHV-1, but not in TG neurons of mock-infected calves or during early stages of reactivation from latency. Here, RNA-Seq studies identified more than 50 genes that are regulated by the canonical Wnt/β-catenin signaling pathway and are differentially expressed during latency. In general, positive regulators of the Wnt pathway, including Akt3, were expressed at higher levels in TG of latently infected calves versus mock-infected calves or calves treated with the synthetic corticosteroid dexamethasone to stimulate reactivation from latency. During reactivation from latency, several Wnt antagonists were stimulated, confirming earlier microarray studies. Akt3, but not Akt1, steady-state levels were stabilized by ORF2 in transfected mouse Neuro-2A neuroblastoma cells, and ORF2 induced Akt3 activation. Since the Wnt/β-catenin signaling pathway promotes synapse formation and remodeling and inhibits neuro-degeneration, these cellular factors might regulate certain aspects of the latency/reactivation cycle.

Dallas Jones, University of Colorado School of Medicine, described a human ex vivo model for VZV vasculopathy using cadaveric cerebral, aortic, and pulmonary arteries. VZV, which is latent in >90% of the world and reactivates in 50% by 85 years of age to produce zoster (shingles), can also spread transaxonally upon reactivation to infect intracranial, extracranial, and systemic arteries. Such spread involves infiltration of immune cells, which secrete soluble factors that contribute to pathological vascular remodeling to promote stroke, giant cell arteritis, and aortitis (VZV vasculopathy). Mechanistic studies of VZV vasculopathy have been hindered since VZV is an exclusively human virus. This laboratory developed a human explant model using cerebral, aortic, and pulmonary arteries obtained <24 h postmortem and mock- or VZV-infected. At 9 days postinfection, immunohistochemical analyses revealed VZV infection in all vascular beds analyzed, predominantly in the adventitia, accompanied by a thickened intima. Conditioned supernatants revealed a 5- to 50-fold induction of IL-8 and/or IL-6 in all vascular beds, recapitulating the elevated IL-8 and IL-6 seen in cerebrospinal fluid from VZV vasculopathy patients and in VZV-infected primary human vascular cells in vitro. In a parallel experiment, immunofluorescence analysis of mock- and VZV-infected cadaveric cerebral arteries to which neutrophils were added revealed neutrophils infiltrating the VZV-infected, IL-8-producing arteries but not in the mock-infected arteries. These results indicate that VZV-infected cadaveric cerebral, aortic, and pulmonary arteries have increased IL-8 and/or IL-6, contributing to a proinflammatory arterial environment that can damage vascular integrity, and that VZV-infected cerebral arteries producing IL-8 promote neutrophil infiltration. This ex vivo functional model promises to enhance the understanding of the inflammatory response and vascular damage that produces VZV vasculopathy.

Yoshiki Kawamura, Fujita Health University, presented data indicating that mutations of HSV-2 LAT-associated miRNAs, LAT promoter, and the ICP4-binding site do not control HSV species phenotypes in guinea pigs. Despite the long-standing observation that HSV LAT promoter deletion viruses show impaired recurrence phenotypes in relevant animal models, the mechanism by which these sequences exert this phenotypic effect remains unknown. This group constructed and evaluated four HSV-2 viruses with targeted mutations in the LAT promoter and LAT-associated miRNAs affecting the LAT TATA, the LAT ICP4 binding site, miR-I and miR-II, which both target ICP34.5, and miR-III, which targets ICP0. While the LAT TATA mutant caused milder acute infections than wild-type (WT), there was no difference in recurrence phenotype between these viruses. LAT and miRNA expression during latency were not impaired by this mutation, suggesting that other promoter elements may be more important for HSV-2 LAT expression. Mutation of the LAT ICP4 binding site also did not cause an in vivo phenotypic difference between mutant and WT viruses. The miR-I/II mutant was impaired relative to WT virus for acute infection and reactivation in vivo, but did not differ phenotypically from its rescuant, suggesting that these differences were due to spurious non-miRNA-related mutations. The miR-III mutant exhibited WT phenotypes in acute and recurrent phases of infection. While not ruling out an effect of these elements in human latency or reactivation, these findings do not identify a specific role for LAT or LAT-associated miRNAs in the LAT promoter deletion phenotype in guinea pigs. Thus, other

sequences in this region may play a more important role in the LAT-associated phenotype in animals.

*Katherine S. Lee, University of Colorado School of Medicine, ex*plored VZV infection of human retinal pigmented epithelial ARPE-19 cells as an in vitro model of VZV-induced uveitis. Reactivation of VZV can cause herpes zoster ophthalmicus (HZO). Some HZO patients develop uveitis, which is characterized by a lymphocytic inflammatory response that can lead to blindness. Difficulties in obtaining and examining ocular fluid have limited the understanding of the pathogenesis of VZV-induced uveitis. This group evaluated VZV infection of the cell line ARPE-19 as an in vitro model of VZV-induced uveitis. Immunoblotting and microscopy to monitor infection of ARPE-19 cells with ROka revealed the same sequence appearance of viral proteins as in MRC-5 fibroblasts, but with prolonged cellular survival. Production of chemotactic and proinflammatory cytokines from mock- and VZV-infected ARPE-19 cells and from cells treated with heat-inactivated VZV was measured with multiplex assays using supernatants from these cultures. Migration of $CD4^+$ and $CD8^+$ T lymphocytes towards the culture supernatants was quantitated using Transwell inserts. This group discussed their findings to provide evidence that VZV infection of ARPE-19 cells serves as a useful model to study the immunopathology associated with VZV-induced uveitis.

Gang Li, Harvard Medical School, demonstrated that HSV-1 microRNA miR-H5-5p targets the viral protein kinase US3. HSV-1 latency is characterized by abundant expression of LATs and viral microRNAs (miRNAs) miR-H2-miRH8, and much lower expression of viral mRNAs that are expressed abundantly during productive infection. The role(s) of viral miRNAs during latency is poorly understood, and almost nothing is known about the role of HSV-1 miR-H5. Most previous work on miR-H5 has focused on the miRNA corresponding to the 3′ end of the pre-miRNA hairpin (3p). This studied showed that both the 5p and 3p species of miR-H5 are expressed in neuronally derived Neuro-2a cells in culture, albeit weakly, and are highly expressed during latency in mouse trigeminal ganglia. The seed sequence of miR-H5-5p, GGGGGGU, is highly unusual. Using photo-activatable ribonucleoside-enhanced crosslinking and immunoprecipitation analysis of HSV-1-infected cells overexpressing miR-H5, this group found that miR-H5-5p binds to the mRNA for US3, a viral protein kinase with multiple functions during HSV-1 infection, including promoting nuclear egress and viral protein synthesis, and countering apoptosis and innate immunity. Cotransfection assays showed that a miR-H5-5p mimic can decrease the levels of US3 mRNA and protein, and that the 3′ untranslated region of US3 mRNA is sufficient for repression by miR-H5-5p. Using an HSV-1 mutant deficient for miRH5 expression and a rescued derivative, this laboratory is investigating the effects of the mutation on various parameters of acute and latent infection of mice, and the possibility that miR-H5 promotes latency by repressing US3 transcript levels during latency.

Patrick Lomonte, CNRS, presented evidence that promyelocytic leukemia (PML) nuclear bodies (NBs) are essential for latent HSV-1 genome chromatinization through a PML/histone H3.3/H3.3 chaperone axis. PML-NBs are nuclear relays of the cell-associated intrinsic antiviral activity. HSV-1 latency establishment is tightly controlled by PML-NBs, although their real implication is still elusive. A hallmark of HSV-1 latency is the interaction between its genome and PML-NBs, leading to the formation of viral DNA-containing PML-NBs (vDCP-NBs). Using an infected human primary fibroblast model reproducing the formation of vDCP-NBs, combined with an immuno-FISH approach developed to detect latent HSV-1, this laboratory showed that vDCP-NBs contain not only the histone variant H3.3 Daxx/ATRX chaperone complex, but also H3.3 itself. H3.3 was also detected in vDCP-NBs present in trigeminal ganglia (TG) neurons from HSV-1-infected wild-type mice or infected cultured TG neurons from transgenic mice expressing a tagged H3.3 (eH3.3) in place of the endogenous H3.3. ChIP-qPCR of eH3.3- or eH3.1-expressing infected human primary fibroblasts showed that latent viral genomes are chromatinized exclusively with H3.3 and not the canonical histone H3.1. Consequently, Daxx and ATRX complexes were found interacting with viral loci. Inactivation of PML by short RNAs significantly impacted the chromatinization of the latent viral genomes with H3.3 without any overall replacement of H3.3 by H3.1, consistent with the absence of co-localization of the histone H3.1 chaperone CAF-1 with latent viral genomes in the vDCP-NBs in vivo and in cell cultures. The depletion of Daxx or ATRX significantly impacted the localization of HSV-1 genomes with vDCP-NB, but only slightly affected the association of viral genomes with H3.3, suggesting that another H3.3 nucleosome assembly mechanism compensates for the lack of the Daxx/ATRX chaperone activity, and that the H3.3-dependent chromatinization of the naked/non-nucleosomal HSV-1 genome entering the nucleus does not necessarily require the formation of vDCP-NBs. Together, the findings demonstrate a specific epigenetic regulation of latent HSV-1 through an H3.3-dependent HSV-1 chromatinization involving the H3.3 chaperone complex Daxx/ATRX and probably other H3.3-associated chromatin assembly mechanisms. Additionally, this study identifies PML-NBs as major actors in H3.3 chromatinization through a PML/histone H3.3/H3.3 chaperone axis.

Werner J.D. Ouwendijk, Freie Universitaet Berlin, discussed VZV infection of the enteric nervous system (ENS). VZV establishes latency in neurons of sensory and autonomic ganglia, including those of the ENS. Varicellavirus reactivation from the ENS may cause enteric zoster, but the underlying mechanisms remain poorly understood. Because simian varicella virus (SVV) infection of

nonhuman primates mimics VZV pathogenesis in humans, this group investigated whether the SVV primate model can be used to study varicella virus infection of the ENS. SVV DNA was detected in gastrointestinal (GI) tract specimens from 2 of 4 latently infected rhesus macaques, consistent with the detection of low amounts of VZV DNA, but not HSV-1 DNA, in 5 of 18 (27%) human small intestine biopsies. While no SVV antigens were detected by immunohistochemistry, in situ hybridization of adjacent tissue sections revealed selective expression of ORF63 transcripts in neurons of both the myenteric plexus and submucosal plexus in latently infected rhesus macaques. During primary SVV infection, high quantities of SVV DNA and antigen were detected in the GI tract draining mesenteric lymph nodes of infected African green monkeys (AGM), suggesting involvement of T cells in virus dissemination to the ENS. In vitro infection of human and AGM peripheral blood mononuclear cells showed that both VZV and SVV infect predominantly memory T cells. Moreover, a subset of VZV- and SVV-infected T cells expressed the gut-homing receptor $\alpha 4\beta 7$ integrin particularly activated CD4 T cells expressing a central memory phenotype. Virus-infected T cells were readily detected in the GI tract of acutely SVV-infected AGM at 9 days after infection. Together, the data demonstrate that SVV infects and establishes latency in neurons of the ENS during primary infection. Because SVV reactivation can be experimentally induced, this model holds great potential to study enteric zoster.

Tao Peng, Fred Hutchinson CRC, showed that keratinocytes produce IL-17c to protect the peripheral nervous system during human HSV-2 reactivation. Despite frequent HSV reactivation, peripheral nerve destruction and sensory anesthesia are rare. This group found that a novel interaction between HSV-infected keratinocytes and the peripheral nerve system via IL-17c promotes neuron survival and neurite growth during reactivations. Skin biopsies obtained during asymptomatic human HSV-2 reactivation exhibited a higher density of nerve fibers relative to biopsies during virological and clinical quiescence. Keratinocytes, the cells in which HSV initially replicates, produced IL-17c during HSV-2 reactivation. IL-17RE, a receptor subunit specific for IL-17c signaling, was expressed on nerve fibers in human skin and sensory neurons in dorsal root ganglia. In cultured human primary keratinocytes, HSV infection and TLR agonists had additive effects in inducing IL-17c expression. Exogenous human IL-17c provided directional guidance and promoted neurite growth and branching in microfluidic devices. Exogenous murine IL-17c pre-treatment reduced apoptosis in HSV-2-infected primary neurons. Together, these results suggest that IL-17c is a neurotrophic cytokine that protects the peripheral nervous system during HSV reactivation. This interaction might explain the lack of clinical nerve damage from recurrent HSV infection and might provide novel avenues to understanding and treating sensory peripheral neuropathies.

Dane M. Phelan, University of Florida, showed that in vivo knock-down of the HSV-1 LAT reduces reactivation from latency. HSV establishes a life-long latent infection in peripheral nerve ganglia, during which the latent viral genomes are maintained as circular episomes in the nucleus of the neuron. Periodically, the virus reactivates from individual neurons and the newly produced virions are transported via the nerve fibers to the original site of infection, typically the mucosal epithelium of the lip or mouth (HSV-1) or the genitalia (HSV-2). During latency, most virus genes are silenced with the exception of one region of the genome encoding the LAT. This non-coding RNA was originally described to play a role in enhancing HSV-1 reactivation, although subsequent evidence that it blocks apoptosis and promotes efficient establishment of latency suggested that its effects on reactivation were secondary to establishment. This laboratory used an AAV vector to deliver a LAT-targeting hammerhead ribozyme to the latently infected neurons of rabbits previously infected with HSV-1. Reactivation of the latent HSV was then induced by transcorneal iontophoresis of epinephrine. The results showed that reduction of LAT accumulation after latency is established reduces the ability of the virus to reactivate. Thus, the HSV-1 LAT has separate functional roles during both the establishment and reactivation phases of HSV infection.

Julianna R. Pieknik, USUHS, described how HSV-2 reactivates from autonomic neurons in vivo. Most HSV-2 infections are transmitted from asymptomatic individuals, contributing substantially to viral spread through the population. Reactivation from sensory cell bodies of the dorsal root ganglia has been considered the most likely source of asymptomatically shed virus, although latent HSV-2 infects and establishes latency in autonomic ganglia. To further characterize neuronal sites from which HSV-2 can reactivate, this laboratory constructed a VP26 capsid fusion mutant of HSV-2 that includes a monomeric 13-kD CreiLOV protein and studied it in the guinea pig model of genital HSV-2 infection. Use of this new virus, which has a wild-type recurrence phenotype, enabled identification of sites harboring viral capsids (corresponding to productive, as opposed to latent infection) in fluorescently labeled cryosections of dorsal root ganglia, major pelvic ganglia, and the ganglion impar. During the latent phase of infection, HSV-2 virions were detected in parasympathetic and sympathetic autonomic neuronal subtypes, suggesting that reactivation from autonomic ganglia can be a source of viral shedding.

Rebecca D. Powell-Doherty, Virginia Tech, described the amyloid-β and p-tau anti-threat response to HSV-1 infection in a murine model of primary adult hippocampal neurons. Recent studies have noted a potential link between HSV-1 infection and the development of Alzheimer's disease (AD), the sixth leading cause of death in the USA. HSV-1 DNA has been detected in AD amyloid plaques, and treatment with the antiviral acyclovir (ACV) was reported to block the

accumulation of AD-associated proteins beta-amyloid (Aβ42) and hyperphosphorylated-tau (p-tau) in Vero and glioblastoma cells. This laboratory sought was to determine whether the accumulation of AD-related proteins is attributable to acute and/or latent HSV-1 infection in mature hippocampal neurons, a region of the brain severely impacted by AD. Primary adult murine hippocampal neuronal cultures infected with HSV-1 (strain 17+), with or without ACV, were analyzed via FISH and immunofluorescent staining for LAT and amyloid-β and p-tau expression during 5 days postinfection. HSV-1-infected neurons treated with ACV showed a 3.5-fold increased expression of Aβ42, while uninfected cultures exhibited no signs of Aβ42 overexpression. Furthermore, Aβ42 co-localized with HSV-1 LAT expression. On the other hand, p-tau expression was transiently elevated in the presence of ACV alone, as well as in infected (with or without ACV) neurons. These studies suggest that amyloid-β plaque accumulation is an antiviral response to viral LAT expression in lieu of apoptosis in adult neurons, while p-tau acts as a danger-associated molecular pattern in response to any perceived threat. In hippocampal neurons, either mechanism may ultimately progress to disease pathology with persistent infection.

Nancy Sawtell, Cincinnati Children's Hospital Medical Center, described animal model studies that strengthen the link between HSV and Alzheimer's disease. The development of a tractable animal model is important in determining whether HSV latent infection in the CNS can cause or exacerbate neurological disease. This laboratory's early attempts in Swiss Webster mice showed no significant CNS pathology or cognitive changes after long-term latency, despite the presence of many viral genomes in the CNS. This suggested the importance of the host genetic background in CNS disease. Indeed, a link between the human APOE4 allele as well as the polymorphism in IL10 with the risk of developing Alzheimer's disease has been reported. In both huAPOE4-targeted replacement mice and in IL10-null mice, long-term HSV latency was found to be associated with the development of CNS pathology. This group noted that similar to findings in humans, uninfected aged mouse brains contained pathology as well, although distinct differences were observed in the context of HSV latent infection in some cases. To determine whether the observed CNS pathology in the infected mice resulted in measurable cognitive changes compared to mock-infected aged mice, this laboratory recently performed a NASA-funded behavioral study to gain insight into the biological significance of the CNS changes observed.

Julian Scherer, Princeton University, described how a three-member complex of virus envelope proteins mediates anterograde transport and spread. Alphaherpesvirus genomes establish a reactivateable, quiescent infection in peripheral nervous system neurons of their natural hosts. Upon reactivation, viral progeny move via the axon from the neuronal cell body back to the periphery, where they can spread to new hosts. For transport, newly formed capsids rely on the cellular architecture and molecular motor systems of the neuron. In particular, pseudorabies virus (PRV) progeny in transport vesicles are sorted into axons by the microtubule-based kinesin motor Kif1a. Three viral envelope proteins, Us7, Us8, and Us9, are also required for efficient axonal sorting and anterograde transport, although the molecular mechanism that enables these proteins to recruit Kif1a remains unknown. This laboratory's analyses using GST-tagged cytoplasmic truncations provided evidence that PRV Us9, the smallest and most critical transport factor, is able to undergo phosphorylation-dependent self-association. Similarly, phosphorylation of Us9 increases its interaction with Us8, whereas a direct Us7 interaction is undetectable. Adenovirus-based transduction of the individual PRV proteins in superior cervical ganglion (SCG) neurons was used to test for rescue of sorting and spread defects of PRV mutants by trans-complementation. Such analysis also showed that simultaneous transduction of the three envelope proteins results in their co-localization to fast-moving axonal structures. These findings provide insight into mechanisms of kinesin recruitment required for axonal sorting and anterograde transport of PRV.

Patrick Stuart, Saint Louis University, described the role of costimulatory molecules in herpetic eye disease. B6-CD28−/− and B6-CD137L−/− mice were infected ocularly with HSV-1 and compared to infections of wild-type B6 mice. In both primary and recurrent disease, mice lacking CD28 had little or no disease, whereas mice lacking CD137L displayed increased disease as compared to wild-type B6 mice. Interestingly, while the number of genomes was greater in CD28−/− mice than in B6 mice, the number of LAT+ neurons was greater in wild-type mice. Monitoring of T cells in infected trigeminal ganglia revealed fewer CD8+ cells in CD28−/− mice than in wild-type B6 mice, although the percentages of CD107a+, IFN-γ+, and TNF-α+ cells were similar. By contrast, analysis of CD137L+ mice indicated much lower percentages of these functional markers. Recent comparison of wild-type virus infection with that of CD137L-expressing McKrae HSV-1 viruses showed that disease seen with the CD137L virus was significantly less extensive than that with wild-type virus, despite the similar number of genomes. These results indicate that the role of the two costimulatory molecules CD28 and CD137L differs and suggest that the T cell subsets display differential responsiveness to these two pathways.

Moriah L. Szpara, Pennsylvania State University, showed that father-to-son transmission of HSV results in near-perfect preservation of viral genome identity and in vitro phenotypes. The advent of high-throughput sequencing has provided an unprecedented view of the circulating diversity of all classes of human herpesviruses. Recent data demonstrate that human cytomegalovirus and VZV display substantial variation both within and between hosts. For HSV, this laboratory has

previously demonstrated sequence diversity among hosts in different geographic regions. However, the extent of viral genomic variation associated with a transmission event or with long-term maintenance in a single host remains unknown. To address this issue, the laboratory fully characterized viruses isolated from a father-to-son transmission event that occurred 17 years before the strains were isolated, enabling a first view of the degree of virus conservation after transmission and adaptation to a new host. Characterization of the pathogenicity of these isolates in a mouse ocular model of infection and sequencing of their full viral genomes revealed nearly perfect preservation of both their phenotype and genotype during transmission and over two decades of clinical recurrences. Given the close genetic relationship of the two familial hosts, it remains to be seen whether this conservation of sequence will extend to non-familial transmission events. However, in the context of recent observations of extensive intra-host variation, these data provide an initial demonstration that HSV genome identity can be preserved over a human generation.

Matthew P. Taylor, Montana State University, studied the effect of type 1 interferon (IFN) signaling on alphaherpesvirus coinfection and neuronal spread. IFNs are cytokines that antagonize viral replication and spread. Type 1 IFNs, including IFN-α and IFN-β, are stimulated upon HSV-1 and pseudorabies virus (PRV) infection of cells and neurons. Mice lacking IFN signaling exhibit increased viral replication and greater dissemination of infection in brain tissue. To test whether type 1 IFN signaling might mediate previously observed restrictions on HSV-1 coinfection during neuronal spread, this laboratory analyzed viral coinfection in IFN-deficient cells and mice. Initial evaluation of HSV-1 superinfection exclusion by fluorescence microscopy and flow cytometry in primary fibroblasts from mice lacking either the IFN-α/β receptor (IFNAR−/−) or IFN-β and sequentially coinfected with two different fluorescent protein-expressing HSV-1 recombinants revealed enhanced superinfection exclusion in IFN-deficient compared to wild-type cells. Next, the effect of IFN signaling on coinfection during neuronal spread was tested in mice injected into the vitreous humor with recombinant viruses expressing different fluorescent proteins, directly infecting retinal cells and subsequently spreading into the brain. Fluorescent protein co-expression was quantified by microscopy at sites of primary infection and secondary neuronal spread. IFNAR−/− mice exhibited increased numbers of infected cells, but no alteration in coinfection, as compared to wild-type mice. Thus, while IFN signaling does impact alphaherpesvirus replication and spread, it does not appear to have a significant impact on the mechanisms that regulate coinfection of HSV-1 and PRV in cells and neurons. Further studies are needed to determine whether other cellular antiviral responses are involved and to identify them and their effect on neuronal coinfection.

Nicholas Taylor, University of Maryland Eastern Shore, investigated the lexicon of alphaherpesvirus latency, i.e., the words used by students, experts, and authors. Little to no consensus exists among the alphaherpesvirus latency research community regarding the lexicon and definition of latency. This group compiled a literature review to curate the various interpretations of latency that CALS attendees have recently published and reveal a consensus lexicon based on the frequency of terms used by CALS attendees to describe latency in their publications. Additionally, the results from latency lexicon polling of undergraduate science majors, health professions students, and CALS attendees underscore the importance of clearly describing latency in related publications. This group performed an exhaustive literature search for latency definitions published since 2014 by CALS attendees that resulted in only 10 publications. Polling of CALS attendees revealed that epigenetics and LAT silencing are frequently considered to be important characteristics of latency, yet these characteristics only appeared in under 20% of the searched definitions. The goal of this review was not to restrict the pursuit of latency research by defining it, but to strengthen the efforts by initiating and enhancing the latency lexicon discussion.

Richard Thompson, University of Cincinnati, showed that long-term HSV-1 latent infection in the CNS of targeted huApoE4 allele knock-in mice resulted in cognitive impairment. A highly important yet under investigated question is the potential effect of long-term HSV latent infection in the CNS. The large number of individuals latently infected, together with compelling evidence that reactivation-competent viral genomes reside in neurons in both the peripheral and central nervous systems, raise the potential that periodic inflammatory insults in CNS, while each of low significance, accrue over time to cause or exacerbate other etiologies of neurological disease. An increasing number of reports correlate HSV infection with the development of schizophrenia, and HSV infection confers additional risk for developing Alzheimer's disease, especially in individuals carrying the APOE 4 allele. In the CNS, ApoE is the major transporter of cholesterol to neurons and, for reasons still unclear, the E4 allele is the greatest genetic risk factor for late-onset sporadic Alzheimer's disease, with homozygous Caucasians and Japanese at 10- to 30-fold greater risk. However, a direct causal relationship between latent viral infection and cognitive impairment in a reproducible model system has not yet been reported. In preliminary studies, this laboratory identified host genetic factors that promote the development of histopathology in the CNS in association with long-term latent HSV-1 infection, although it remained unclear whether these changes were associated with measurable cognitive/behavioral changes. As part of a study to understand risks associated with latent HSV infection and heavy ion radiation exposure, a battery of well-established behavioral

assessment assays was performed on parental and huApoE4 targeted knock-in mice using identical housing, handling, and stressors for groups under comparison. In two independent experiments, cognitive/behavioral studies revealed that the human ApoE4 allele was associated with significant deficits in the long-term HSV-1-infected groups ($n = 16$) compared to mock-infected groups ($n = 16$). A similar effect was not seen in the parental strain of mice, directly implicating the ApoE4 allele in the phenomenon.

Vicki Traina-Dorge, Tulane University, described the reactivation of simian varicella virus (SVV) in rhesus macaques following CD4+ T lymphocyte depletion. SVV infection of primates is the counterpart of human VZV infection. Rhesus macaques intrabronchially inoculated with SVV develop primary infection with viremia and rash, which resolve with clearance of viremia followed by establishment of latency. To assess the role of CD4+ T lymphocyte immunity in reactivation, monkeys were treated with a single 50 mg/kg dose of a humanized monoclonal anti-CD4 antibody. Within 1 week after treatment, circulating CD4+ T cells were reduced from 40–60% to 5–30% and remained low for 2 months. In all treated monkeys, very low viremia was documented. Zoster rash erupted after 7 days in the monkey with the most extensive CD4+ T cell depletion (5%) and in all other treated monkeys, at 28–55 days post-treatment. One of the treated monkeys showed recurrent zoster rash. All monkeys were euthanized 9–35 days after the last episode of zoster. Detection of SVV DNA in dorsal root ganglia correlated with the dermatomal location of zoster. Immunofluorescence analysis of skin rash, lung, lymph node, and ganglia revealed SVV ORF 63 protein mostly in sweat glands in skin, in type II cells within lung alveoli, in macrophages and dendritic cells in lymph nodes, and in neuronal cytoplasm of ganglia. SVV spread to multiple tissues upon CD4+ T cell depletion and reactivation suggests a critical role for CD4+ T cell immunity in controlling varicella virus latency.

Benjamin E. Warner, University of Pittsburgh School of Medicine, described a growth conditional mutation of VZV for study in the rat pain model of postherpetic neuralgia (PHN). VZV causes chickenpox upon primary infection, herpes zoster (shingles) upon reactivation, and considerable pain after zoster that may become chronic and debilitating, i.e., PHN. Injection of VZV into rat footpads induces chronic pain that may model pain associated with PHN. However, VZV does not fully replicate in primary cells derived from rats, and viral gene expression is required for onset of pain. Previous studies detected low copy numbers of VZV DNA in the dorsal root ganglia of rats early in infection, but not at later times. To test the possibility that VZV does not need to fully replicate to induce pain and that even some VZV gene expression is sufficient to alter neurons to signal pain, this laboratory developed a conditionally replicating VZV in which essential tegument protein ORF9 was fused to a segment of *Escherichia coli* dihydrofolate reductase (DHFR), a degron that allows for conditional degradation. In the presence of the cell-permeable antibiotic trimethoprim, VZV with ORF9-DHFR replicated, while in the absence, ORF9-DHFR was degraded through the ubiquitin ligase pathway and VZV replication was halted. The findings confirm that ORF9 is critical for VZV replication and provide the means to examine the role of full VZV replication in the rat pain model.

Shannan Washington, LSU Health Sciences Center, described the effects of CTCF depletion in neurons, resulting in reactivation of HSV-1 in latently infected rabbits. The protein CTCF may control transcriptional repression of lytic regions during HSV-1 latency. CTCF occupies the seven conserved CTCF-binding sites that flank the LAT and immediate-early (IE) regions. This laboratory previously showed that three of the seven sites were functional enhancer-blocking insulators, providing evidence that CTCF binding to individual domains is differential and LAT and IE transcription-dependent, and that CTCF nucleates the formation of chromatin loops during HSV-1 latency in vivo. Further, CTCF is dynamically evicted from the binding domains involved in loop formation at early times post-reactivation in mice latently infected with HSV-1. To test whether CTCF eviction precedes and facilitates HSV-1 reactivation, the laboratory used neuronally targeted CTCF knock-down and the efficient AAV8-GFP ocular delivery method of siCTCF to rabbits latently infected with HSV-1. Ocular delivery of the AAV8-GFP/siCTCF resulted in global reactivation in rabbits (80%) and robust viral shedding from the cornea (50%), even in the absence of classic reactivation stimuli such as transcorneal iontophoresis of epinephrine. These findings indicate that CTCF eviction precedes and allows reactivation, and suggest that CTCF controls the transcriptional program of HSV-1 through its insulator function and is required for the maintenance of HSV-1 latency.

Qiaojuan Zhang, University of Maryland Eastern Shore, described regulation of T-type Ca^{2+} channel expression by HSV-1 infection in trigeminal ganglia sensory neuron-like ND7-23 cells. Infection of sensory neurons by HSV-1 disrupts electrical excitability, altering pain sensory transmission. Because of their low threshold for activation, functional T-type Ca^{2+} channels regulate various cell functions, including neuronal excitability and communication. This group tested the effect of HSV-1 infection on the functional expression of T-type Ca^{2+} channels in differentiated ND7-23 sensory-like neurons using whole-cell patch clamp recordings to measure voltage-gated Ca^{2+} currents. Differentiation of ND7-23 cells led to a significant increase in T-type Ca^{2+} current densities, which in turn promoted the morphological differentiation of ND7-23 cells and triggered a rebound depolarization. HSV-1 infection of differentiated ND7-23 cells caused a significant loss of T-type Ca^{2+} channels from the membrane. HSV-1-evoked reduction in the functional expression of T-type Ca^{2+}

channels was mediated by several factors, including decreased expression of Cav3.2 T-type Ca^{2+} channel subunits and disruption of endocytic transport. Decreased functional expression of T-type Ca^{2+} channels by HSV-1 infection required viral protein synthesis and replication, but occurred independently of Egr-1 expression. These findings suggest that infection of neuron-like cells by HSV-1 significantly disrupts expression of T-type Ca^{2+} channels, which can result in morphological changes and functional compromise of in electrical excitability.

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Compliance with ethical standards

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