Robust gene expression changes in the ganglia following subclinical reactivation in rhesus macaques infected with simian varicella virus

Nicole Arnold¹,² · Christine Meyer³ · Flora Engelmann¹ · Ilhem Messaoudi¹,²,³

Abstract Varicella zoster virus (VZV) causes varicella during acute infection and establishes latency in the sensory ganglia. Reactivation of VZV results in herpes zoster, a debilitating and painful disease. It is believed that VZV reactivates due to a decline in cell-mediated immunity; however, the roles that CD4 versus CD8 T cells play in the prevention of herpes zoster remain poorly understood. To address this question, we used a well-characterized model of VZV infection where rhesus macaques are intrabronchially infected with the homologous simian varicella virus (SVV). Latently infected rhesus macaques were thymectomized and depleted of either CD4 or CD8 T cells to induce selective senescence of each T cell subset. After T cell depletion, the animals were transferred to a new housing room to induce stress. SVV reactivation (viremia in the absence of rash) was detected in three out of six CD8-depleted and two out of six CD4-depleted animals suggesting that both CD4 and CD8 T cells play a critical role in preventing SVV reactivation. Viral loads in multiple ganglia were higher in reactivated animals compared to non-reactivated animals. In addition, reactivation results in sustained transcriptional changes in the ganglia that enriched to gene ontology and diseases terms associated with neuronal function and inflammation indicative of potential damage as a result of viral reactivation. These studies support the critical role of cellular immunity in preventing varicella virus reactivation and indicate that reactivation results in long-lasting remodeling of the ganglia transcriptome.

Keywords Latency · Herpesvirus · Herpes zoster · Sensory ganglia · RNA-Seq

Introduction

Varicella zoster virus (VZV) is a human neurotropic alpha herpesvirus that causes varicella (chicken pox) during primary infection. VZV establishes latency in the sensory ganglia where it can reactivate to cause herpes zoster (HZ, shingles). The risk of getting HZ significantly increases with age, from an average of 3 cases per 1000 adults 40–50 years old to 10 cases of HZ per 1000 adults aged ≥80 years (Keating 2013). In addition, the incidence of HZ in individuals receiving immunosuppressive drugs such as those with autoimmune diseases, cancer and organ transplant recipients is higher (Arvin 2000; Rusthoven et al. 1988; Wilson et al. 1972; Yawn et al. 2011). VZV has also been shown to reactivate following stressful situations (Schmader et al. 1990), such as during and after spaceflight (Cohrs et al. 2008) and in medical students (Uehakim et al. 2011).

It is generally believed that VZV reactivation is due to the loss of VZV-specific cell-mediated immunity (Terada et al. 1994), since antibody titers remain relatively stable with increasing age (Levin et al. 2008; Oxman 2009; Weinberg et al. 2009). Similarly, VZV reactivation in hematopoietic stem cell transplants was associated with low CD4 T cell counts in the absence of significant changes in antibody titers (Vermont...
et al. 2014). Finally, T cell responses, but not antibody titers, negatively correlate with HZ disease severity (Onozawa et al. 2006; Park et al. 2004). However, the role of CD4 versus CD8 T cell immunity in preventing VZV reactivation is not well understood. Previous studies showed that the frequency of VZV-specific CD4 T cells declines more dramatically with age compared to VZV-specific CD8 T cells, suggesting a greater role for CD4 T cells in reactivation, but this hypothesis has yet to be formally tested (Asanuma et al. 2000; Weinberg et al. 2009).

In this study, we investigated the role of CD4 and CD8 T cell immunity in preventing herpes zoster using a non-human primate model of VZV infection. In this model, rhesus macaques are inoculated intrabronchially with simian varicella virus (SVV), a homologue of VZV. We previously showed that this model recapitulates the essential features of VZV infection in humans including varicella and establishment of latency in sensory ganglia (Messaoudi et al. 2009). Moreover, as described for VZV, SVV reactivates under conditions of immune suppression (Mahalingam et al. 2010; Mahalingam et al. 2007b; Ouwendijk et al. 2013; Traina-Dorge et al. 2015). To investigate the role that T cell senescence plays in SVV reactivation, rhesus macaques latently infected with SVV were first thymectomized and then depleted of either CD4 or CD8 T cells. This method leads to a profound loss of circulating T cells, which coupled with the absence of naïve T cell output (due to the thymectomy) results in robust homeostatic proliferation of the few T cells that have escaped depletion. This extensive proliferative burst accelerates T cell senescence in the depleted T cell population (Cho et al. 2000; Goldrath et al. 2000; Murali-Krishna and Ahmed 2000; Neujahr et al. 2006; Sheu et al. 2014). Finally, the animals were moved to a new room to induce stress.

SVV reactivation was detected in 42% of depleted animals (two CD4-depleted and three CD8-depleted animals). Moreover, average viral loads in the sensory ganglia from the animals that experienced a reactivation event were significantly higher. Finally, large gene expression changes were detected in ganglia collected from animals that underwent reactivation. Collectively, these data support the hypothesis that both CD4 and CD8 T cells play a critical role in preventing SVV reactivation and that SVV reactivation remodels the ganglia transcriptome.

Results

T cell depletion after thymectomy results in permanent loss of naïve T cells

Analysis of SVV replication kinetics and host responses in the 16 animals used in this study were previously published (Haberthur et al. 2014). All animals generated robust T and B cell responses as evidenced by the detection of antigen-specific T cells and antibodies (Haberthur et al. 2014). At 148 days post-infection (DPI), all 16 animals were thymectomized, and 29 days post-thymectomy (DPT), 12 animals were depleted of either CD4 or CD8 T cells (n = 6/group) while the remaining 4 animals served as thymectomized non-depleted controls. At 364 DPT, all animals were moved to a different room to induce environmental stress (Fig. 1a).

Thymectomy alone (control animals) had minimal impact on the frequency of circulating white blood cells (WBC) including lymphocytes and neutrophils (Fig. 1b–d). Similarly, thymectomy alone did not impact the frequency of circulating CD4 T, CD8 T, and CD20 B cells (Fig. 2a). Furthermore, very few changes were noted in the frequency of naïve and memory CD4 T, CD8 T, and B cell subsets (Fig. 2d, g, j). In contrast, CD4 depletion resulted in a significant decrease in the number of circulating lymphocytes (Fig. 1c) due to the loss of CD4 T cells (Fig. 2b) and, more specifically, naïve CD4 T cells (Fig. 2e), which remained significantly decreased for the remainder of the study. This change was accompanied by an increase in the frequency of terminally differentiated CD4 EM T cells (Fig. 2e). Frequency of CD8 T cells initially increased at 153 DPT in CD4-depleted animals but then decreased as CD4 numbers partially recovered (Fig. 2b). Interestingly, we also saw a significant increase in CD8 EM T cells and a decrease in naïve and TEM CD8 T cells in the CD4-depleted animals (Fig. 2h). No changes in B cell subsets were noted in the CD4-depleted animals (Fig. 2k).

As described for CD4 T cells, CD8 depletion resulted in a profound and sustained loss of CD8 T cells while the frequency of CD4 T cells and CD20 B cells remained relatively stable (Fig. 2e). This loss was also primarily driven by a significant reduction in the number of naïve CD8 T cells (Fig. 2i), which was accompanied by a transient increase in TEM CD8 T cells followed by an increase in the EM frequency (Fig. 2i). Intriguingly, the frequency of CD4 EM T cells increased while that of naïve and CM CD4 T cells decreased following CD8 depletion (Fig. 2f). The CD8-depleted animals had a significant increase in their switched memory B cell population after depletion (Fig. 2i).

T cells undergo robust homeostatic proliferation following depletion

The changes in frequency of naïve and memory T cells in the depleted animals prompted us to investigate the role of homeostatic proliferation. In the thymectomized non-depleted group, we observed a transient increase in proliferation in CD4 T cells and B cells, but not CD8 T cells 55–83 DPT (Fig. 3a–g). Following CD4 and CD8 T cell depletion, we observed robust proliferation in CD4 T, CD8 T, and B cells.
as early as 29 DPT (Fig. 3). In the CD4-depleted animals, the magnitude of CD4 T cell proliferation was much more robust than that of CD8 T cells and B cells (Fig. 3b–h). Interestingly, in the CD8-depleted animals, CD4 T cell proliferation occurred earlier than CD8 T cell proliferation and reached similar magnitude (Fig. 3c, f). Although proliferation of naïve T cells was observed in both CD4-depleted and CD8-depleted groups, the naïve T cell numbers remained significantly reduced due to their rapid conversion to memory phenotype cells in the absence of thymic output.

T cell depletion followed by environmental stress results in subclinical reactivations

SVV reactivation was monitored by measuring SVV viral loads in the whole blood using quantitative real-time PCR (qPCR) as well as visual inspection for zoster lesions. After almost a year with no signs of reactivation, all of the animals were moved to a different room in the facility to induce stress (Fig. 1a). No obvious rash was detected in the animals; however, SVV DNA was detected in whole blood (WB) from five animals (two CD4 depleted (one male and one female) and three CD8 depleted (two male and one female)) approximately 20–48 days after the move (383–412 DPT) (Fig. 4a). To characterize the immune response during SVV reactivation, we measured changes in SVV-specific IgG titers and frequency of antigen-specific T cells. We observed no significant increases in IgG titers or CD4 T cell responses in the animals that experienced reactivation (Fig. 4b–e). Increased CD8 T cell responses were detected in some of the depleted animals 412–454 DPT regardless of whether they experienced renewed viremia (Fig. 4f).

SVV reactivation results in increased viral loads and long-lasting transcriptional changes in the ganglia

No additional reactivation events were detected during the next several months. We therefore elected to euthanize the animals at 646 DPT and investigate changes in SVV viral loads and transcriptional profiles in the sensory ganglia following SVV reactivation. Significantly higher viral loads were observed in the trigeminal (TG) and dorsal root ganglia thoracic (DRG-T) of animals that showed signs of SVV reactivation compared to the animals that did not (Fig. 5a). No viral loads were detected in lumbar/sacral dorsal root ganglia (data not shown).

In order to determine the host transcriptome changes that occur following reactivation, we performed RNA-Seq on the DRG-T collected from three animals that experienced a reactivation event (two CD4 depleted, one CD8 depleted) and four animals that showed no signs of reactivation (two controls, one CD4 depleted and one CD8 depleted) (Table 1). A principle component analysis (PCA) showed a clear distinction between the reactivated and the control groups (Fig. 5b). Overall, we observed 1202 differentially expressed genes (DEGs) defined as having a fold change (FC) ≥2, a false discovery rate (FDR) of ≤0.05, and a median RPKM value of ≥5 (199 upregulated, 1003 downregulated) (Fig. 5c). To understand the biological implication of these transcriptional changes, we carried out a functional enrichment using Metacore™, which requires the use of human homologues. Of the 1202 DEGs, 641 had human homologues (120 upregulated and 521 downregulated) (Supplemental Dataset). Changes in expression of four DEGs were confirmed by RT-PCR (Fig. 6).
Genes upregulated in ganglia of animals that experienced SVV reactivation play a role in regulating cell cycle and gene expression.

Only 120 characterized DEGs were found to be upregulated in ganglia of animals that experienced SVV reactivation. Several of the 30 most upregulated genes regulate cell cycle progression (Fig. 7a) such as AMD1 (FC 4) which regulates Myc expression (Zhang et al. 2012), POC1B (FC 3) involved with centriole assembly (Keller et al. 2009), and the transcriptional regulator TAF1D (FC 4, confirmed by RT-PCR, Fig. 6a) (Wieczorek et al. 1998). Additional genes were involved with apoptosis such as HIF1A (FC 3) (Rapino et al. 2005) and MAP4K5 (FC 3) (Iacobas et al. 2003), and protein degradation...
such as UFD1L (FC 3) (Johnson et al. 1995) and CLPX (FC 3) (Flynn et al. 2003). Other highly upregulated genes play a role in neuronal development such as SNAPC3 (FC 3) (Wang et al. 2004), OLIG3 (FC 3) (Liu et al. 2008), and ACVR2A (FC 3) (O’Keeffe et al. 2016) (Fig. 7a). Two antiviral genes were also amongst the 30 most upregulated DEGs: TRIM23 (FC 3), which enhances antiviral innate responses through the RIG-I/MDA5-mediated pathway (Arimoto et al. 2010), and UFL1 (FC 4), which inhibits NF-kappaB signaling pathway (Kwon et al. 2010).

Functional enrichment showed that most of the upregulated DEGs mapped to “cellular metabolic process” (Table 2), some of which also enriched to GO term “response to glucose.” Examples of genes that mapped to these GO processes include those involved with double-stranded DNA damage repair (BAZ1A, FC 3) (Lan et al. 2010), transcriptional regulation (MIER1, FC 3) (Blackmore et al. 2008), mRNA degradation (CNOT7, FC 2) (Aslam et al. 2009), and regulation of circadian calcium rhythms in neurons (ARN7, FC 2) (Ikeda and Ikeda 2014). An additional 17 genes that mapped to “multicellular organismal reproductive process” played a role in microtubule stabilization (Emanuele and Stukenberg 2007) (CEP57, FC 3, confirmed by RT-PCR, Fig. 6b), anion transport (SLC26A8, FC 3 (Lohi et al. 2002)), and neuron development (RARB, FC 2 (Maden 2007)) (Fig. 7b).

**Fig. 3** Depletion results in robust homeostatic proliferation. Magnitude and kinetics of proliferation (means ± SEM) of CD4 T cell subsets were measured in a control, b CD4-depleted, and c CD8-depleted animals; CD8 T cell subsets in d control, e CD4-depleted, and f CD8-depleted animals; and B cell subsets in g control, h CD4-depleted, and i CD8-depleted animals were determined by measuring Ki67 expression within specific lymphocyte subsets by flow cytometry (one plus sign P < 0.05, two plus signs P < 0.01, three plus signs P < 0.001 for CM; one ampersand P < 0.05, two ampersands P < 0.01, three ampersands P < 0.001 for naïve T cells; one dollar sign P < 0.05, two dollar signs P < 0.01, three dollar signs P < 0.001 for switched memory; two ampersands P < 0.01, three ampersands P < 0.001 for IgM memory; one dollar sign P < 0.05, two dollar signs P < 0.01, three dollar signs P < 0.001 for exhausted memory; one number sign P < 0.05, two number signs P < 0.01, three number signs P < 0.001 for naïve B cells)

Genes downregulated in ganglia of animals that experienced SVV reactivation may contribute to neuronal damage.

Several of the downregulated genes play a role in neuronal development. The most downregulated gene in our data set was GPR137 (FC 400, confirmed by RT-PCR, Fig. 6c), a G protein-coupled receptor essential for controlling pain transmission in the trigeminal ganglia (Manteniotis et al. 2013) (Fig. 8a). Several of the downregulated DEGs were involved with axon regeneration such as NDEL1 (FC 123) (Toth et al. 2008), AMIGO3 (FC 200) (Kuja-Panula et al. 2003), THY-1 (FC 110) (confirmed by RT-PCR, Fig. 6d) (Rege and Hagood 2006), and INA (FC 89) (Baum and Garriga 1997). Additional
downregulated genes were involved in neuron differentiation and stabilization such as **FERD3L** (FC 104) (Ono et al. 2010), **BASP1** (FC 200) (Goodfellow et al. 2011), and **HSP A12B** (FC 119) (Ono et al. 2010). Finally, other highly downregulated genes played a role in cell proliferation (**MZF1**, FC 124) (Gaboli et al. 2001), DNA damage repair (**XRCC3**, FC 121) (Kurumizaka et al. 2001), and protein transport (**SSR4**, FC 183) (Nagasawa et al. 2007) (Fig. 8a).

Functional enrichment showed that the most statistically significant GO process to which downregulated DEGs mapped is **axo-dendritic-transport** which includes genes encoding neurofilaments **NEFL** (FC 7) and **NEFM** (FC 4), and the neurodevelopment protein **NDEL1** (FC 123) (Table 2). Several of the 215 downregulated genes that mapped to “cellular component organization” (Table 2) regulate neuronal gene expression including several histone genes such as **ATF5** (FC 158) (Angelastrø et al. 2005) and **BAIAP2** (FC 155) (Oda et al. 1999). In addition, several highly downregulated genes mapped to “metabolic process” (Table 2) such as **PGAM2** (FC 149, glycolysis) (Kondoh et al. 2005), **CBR3** (FC 130, prostaglandin metabolism) (Miura et al. 2008), and **HEXIM2** (FC 54, transcription regulation) (Yik et al. 2005). Of the 177 genes that mapped to the GO process “response to stress,” 43 directly interact with each other (Fig. 8b) including several that interact with the transcription factor **APP** (FC 7), which regulates neurite outgrowth (Bekris et al. 2011), and histone deacetylase class II (**HDAC**, FC 5).

Additional bioinformatics analysis of the downregulated DEGs revealed enrichment to process networks associated with the cell cycle (Table 2) including DNA repair such as **RAD9** (FC 44) (Lieberman et al. 1996); microtubule assembly, e.g., **TUBA1B** (FC 7) (Kumar et al. 2010); and cell cycle progression, e.g., **ANAPC13** (FC 9) (Yamaguchi et al. 2015). Several downregulated genes also enriched to the process network “immune response-antigen presentation” (Table 2) such as **ICAM** (FC 37), **NFKBIB** (FC 12), and several heat shock proteins (**HSP A1L**, FC 95), **HSP A8** (FC 8), **HSPCA** (FC 5), and **HSP90** (FC 2) (Fig. 8c). Finally, analysis of the downregulated genes using a diseases biomarker database revealed enrichment to several diseases related to the neuronal damage and mental health diseases (Table 2). The DEGs that enriched to “basal ganglia diseases” played a role in axon regeneration such as **NGF** (FC10) (Lindsay 1988) and **SNCG** (FC 59) (Buckingham et al. 2008); neuron differentiation, e.g., **SUZ12** (FC 6) (Mazzoni et al. 2013); and CNS inflammation such as **CCR7** (FC 8) (Buonamici et al. 2009) (Fig. 8d).
SVV reactivation leads to greater changes in gene expression relative to latent SVV infection

We also compared changes in gene expression in ganglia collected from animals that experienced a reactivation event and those that did not experience reactivation (Arnold et al. 2016). Overall, we detected 967 DEGs (403 upregulated, 325 downregulated) in ganglia from animals that did not have a reactivation and 1200 DEGs (641 upregulated, 559 downregulated) in the ganglia from animals that did. A comparison of these two data sets showed 728 genes were differentially expressed in ganglia obtained from either reactivated or non-reactivated animals relative to naïve ganglia, while 239 DEGs were only detected in ganglia of non-reactivated animals and 427 DEGs in ganglia from reactivated animals (Fig. 9a).

Functional enrichment was then performed on all three sets of DEGs to better understand the biological implications of these gene expression changes (Table 3). Genes that were differentially expressed in both sets of ganglia relative to naïve enriched to biological and disease processes related to metabolism, cell death, neurogenesis, and infections (Table 3). Most of the common genes mapped to “metabolic process” directly interacted with one another (Fig. 9a) and were regulated by the transcription factor NRF2, which regulates oxidative stress (Li et al. 2005) (Fig. 9b). In addition, all of the common genes that mapped to the disease process “infection” had the same directional change; however, DEGs detected in ganglia from animals that experienced a reactivation had a

Table 1  DRG-T viral loads of the samples used for RNA-Seq analysis

| Sample | Reactivated | Viral load |
|--------|-------------|------------|
| CD4 depleted #3 | Y | 555 |
| CD4 depleted #5 | Y | 189 |
| CD8 depleted #6 | Y | 2091 |
| Control #2 | N | 64 |
| Control #3 | N | 236 |
| CD4 depleted #6 | N | 152 |
| CD8 depleted #5 | N | 21 |
higher FC (Fig. 9c). The upregulated immune genes included antiviral genes such as *IFI16* and *DDX58*, T cell genes such as *IL-7* and *CD274*, and costimulatory molecules such as *CD80* and *CD46*. Downregulated immune genes included phagocytic markers *CD68* and *CD63*, as well as regulatory T cell transcription factor *FOXP3* (Fig. 9c). DEGs

**Fig. 6** Gene validation. Taqman assays were done on a TAF1D (upregulated), b GPR137 (downregulated), c THY1 (downregulated), and d CEP57 (upregulated). \( n = 3 \) for reactivated (R) and \( n = 4 \) for non-reactivation (NR). One asterisk \( P < 0.05 \), two asterisks \( P < 0.01 \) compared to NR.

**Fig. 7** Gene enrichment analysis of the upregulated genes in reactivated animals. Heat map analysis of the a 30 most upregulated and b upregulated genes mapping to "multicellular organismal reproductive process." NR non-reactivated, R reactivated.
detected only in ganglia from non-reactivated animals also enriched to biological and disease processes related to metabolism, cell adhesion, central nervous diseases, and viral infections. Genes that enriched to “central nervous system diseases” and “viral infection” were primarily upregulated and played a role in neuron growth such as NGF (FC 7, regulates neuron growth) (Indo et al. 1996), DSC1 (FC 95, neural circuit regulation (Zhang et al. 2013)), and CYR61 (FC 6, dendrite growth (Malik et al. 2013)) (Fig. 9d). Interestingly, DEGs detected only in reactivated animals enriched to biological and disease processes related to cell cycle and neurodegenerative diseases. Genes that mapped to “neurodegenerative diseases” played a role in neuro-transmission such as intermediate filaments (e.g., NEFL, FC 11 and GFAP, FC 293), neurotransmitters (e.g., TAC1, FC 14) and synaptic vesicle exocytosis (e.g., CLPX2, FC 80) (Fig. 9e).

### Discussion

Age and immune status are the primary risk factors for getting herpes zoster. The goal of this study was to investigate the role of CD4 and CD8 T cell immunity in preventing herpes zoster. Using the rhesus macaque animal model of SVV infection, we were able to artificially age either the CD4 or CD8 T cell population in latently infected animals by performing a thymectomy followed by CD4 or CD8 T cell depletion. The profound loss of T cells coupled with the absence of naïve T cell output from the thymus results in robust...
homeostatic proliferation in order to repopulate the T cell compartment, which in turn leads to accelerated T cell senescence (Cho et al. 2000; Goldrath et al. 2000; Murali-Krishna and Ahmed 2000; Neujahr et al. 2006; Sheu et al. 2014). Our data showed that T cell numbers of the depleted subset do not recover to baseline levels no matter how robust the homeostatic proliferation. Similar findings have been reported in mice depleted of their lymphocytes (Neujahr et al. 2006). Interestingly, we observed increased proliferation in the non-depleted T cell subset and B cells, suggesting all lymphocytes are able to sense and respond to the available space created by the depletion.

Thymectomy and accelerated aging of the T cell compartment alone were not sufficient to cause reactivation. This observation explains the relatively low rates of reactivation in older individuals ≥80 years of age where the reactivation rate is 10 per 1000 persons/year despite the uniform presence of immune senescence. Similarly, the rate of VZV reactivation in HIV+ subjects is 9.3 per 1000 persons/year (Blank et al. 2012; Pergam et al. 2011). We therefore induced stress by moving the animals to a different room in the facility. Although we were not able to measure cortisol levels, previous studies in non-human primates have shown that room changes increase cortisol levels in the feces and hair in rhesus macaques for up to 1 year (Davenport et al. 2008). Also, fecal cortisol increases have also been observed in chimpanzees moved to a new facility (Reimers et al. 2007). In addition, temporary moves of 2 days or less have also been shown to increase cortisol levels in rhesus, bonnet, and cynomolgus macaques as well (Novak et al. 2013).

SVV reactivation was observed in two (33%) CD4-depleted and three (50%) CD8-depleted animals as evidenced by the presence of SVV DNA in blood and increased viral loads in the ganglia. This is in line with the incidence rate of HZ in bone marrow transplant patients, which had been reported to reach 43% (Chen et al. 2014; Koc et al. 2000; Novak et al. 2013). However, our reactivation rates are lower than those observed in macaques that have undergone whole body irradiation coupled with immune-suppressive treatments (Mahalingam et al. 2010), in line with the reduced severity of immune suppression due to accelerated senescence of only one T cell compartment. In this study, we did not detect a zoster rash. VZV reactivation in the absence of rash is referred to as zoster sine herpete (Blumenthal et al. 2011) and has been reported in HIV-positive patients, bone marrow transplant recipients, patients with leukemia, astronauts, and cynomolgus macaques subjected to stress from travel and isolation (Birlea et al. 2011; Ljungman et al. 1986; Mahalingam et al. 2007a).

SVV reactivation was observed in both CD4- and CD8-depleted animals, which suggests a role for both CD4 and CD8 T cells in the prevention of reactivation. Viral loads in the blood seemed to be slightly higher in the CD4-depleted animals; however, since only two CD4-depleted animals experienced reactivations, we could not determine whether this increase was significant. These observations are in line with clinical studies that have indicated a critical role for CD4 T cells in prevention of VZV reactivation. Specifically, HZ is more prevalent in HIV+ patients (Onunu and Uhunmwangho 2004). Furthermore, rapid recovery of CD4 T cells, but not CD8 T cells, after stem cell transplants is associated with reduced rates of cytomegalovirus reactivation (Drylewicz et al. 2016). Previous studies from our lab have also shown a greater role for CD4 T cells in the resolution of acute SVV infection where CD4-depleted animals had higher viral loads and prolonged disease and are unable to establish latency in the ganglia (Haberthur et al. 2011; Meyer et al. 2013). On the other hand, CD8+ cells in the trigeminal ganglia play a critical role in the prevention of HSV-1 reactivation in mice (St Leger and Hendricks 2011), which indicates that immunological control of reactivation may differ between these two closely related viruses.

In contrast to what has been previously reported for VZV, no significant changes in antibody titers were detected during SVV reactivation. Similarly, no increase in SVV-specific CD4 T cell responses was detected in animals that experienced a reactivation event. On the other hand, CD8 T cell responses were increased in several depleted animals regardless of renewed SVV viremia, but in none of the non-depleted controls. This observation suggests that depleted animals were more susceptible to the stress induced by the room change, but only some of the animals experienced a reactivation. Our data differs from previous reports where increased T cell responses have been observed in transplant recipients following subclinical reactivations (Ljungman et al. 1986). Although we were unable to detect a robust immune response in the peripheral blood, SVV reactivations could have triggered a local immune response within the ganglia. Unfortunately, we were unable to address this question since the ganglia tissue was collected nearly 1 year after viremia was detected.

Viral loads were higher in the ganglia collected from animals that experienced a reactivation compared to those collected from animals that did not. The increased SVV viral loads could have been due to viral replication during reactivation as previously reported for VZV and SVV (Ouwendijk et al. 2013; Reichelt et al. 2008) and/or additional seeding of the ganglia after renewed SVV viremia. However, it is also possible that the viral loads in the ganglia of the reactivated animals were higher to begin with, which made them more susceptible to reactivation. In addition to increased viral loads,
RNA-Seq analysis revealed distinct transcriptional profiles between the DRG-T of animals that experienced a reactivation event and those that did not despite the fact that our analysis was carried out 1 year after SVV viremia was detected. This suggests that SVV reactivations can cause long-lasting gene expression changes in the ganglia. Genes that were downregulated after reactivation play a critical role in neuron differentiation and axon regeneration as well as DNA repair indicative of significant damage to the ganglia, while genes that were upregulated after SVV reactivation play a role in cell proliferation and neuronal development, suggesting tissue repair. Although we were not able to examine the ganglia tissue at the time of reactivation, our gene expression data are supportive of previous studies that reported severe necrosis and inflammation of the ganglia recovered from patients with HZ at the time of death (Gowrishankar et al. 2010; Lungu et al. 1995).

Recent studies from our lab have shown a downregulation of neuronal genes during acute infection (Arnold et al. 2016); therefore, we compared the DEGs detected after acute SVV infection (100 dpi) and those detected after SVV reactivation. Only 48 common DEGs were identified, most of which were downregulated after both acute infection and reactivation. Several of these DEGs play a role in synaptic plasticity (THRA, KIRREL, and IGF58) (Martin et al. 2015; Ray and Treloar 2012; Vallortigara et al. 2009), neuronal growth (MBP, STMN2, PRPH, and RHOG) (Eriksson et al. 2008; Franke et al. 2012; Westerlund et al. 2011), and cell cycle such as ARF1, HDLBP, and BOP1 (Molyneux et al. 2014; Strezoska et al. 2000; Yorimitsu et al. 2014). Although a limited number of DEGs were shared between these studies, both revealed a robust number of downregulated genes that were involved in neuronal function, which suggests that both viral entry and viral reactivation cause neuronal damage but may affect different neuronal functions.

We also compared the transcriptome of ganglia from animals that experienced a reactivation and those that did not to ganglia from naïve animals. This analysis showed larger transcriptional changes in ganglia from animals that experienced a reactivation, further supporting the hypothesis that reactivation leads to additional remodeling of the ganglia transcriptome compared to latent SVV infection. A significant number of common DEGs were detected in ganglia from animals that experienced or did not experience a reactivation event, indicating that SVV infection exerts the largest transcriptional impact. In addition, several common genes enriched to infectious diseases; however, the reactivated ganglia showed larger gene expression changes in line with renewed viral replication such as that seen during reactivation. Additionally, we detected additional DEGs that were unique to ganglia from animals that experienced a reactivation. Genes that were differentially expressed only in ganglia from animals that experienced a reactivation event enriched to neurodegenerative diseases in line with the known complications of neuralgia and ganglia necrosis often observed after VZV reactivation (Gabutti et al. 2016).

In summary, data described in this study provides additional evidence that supports a critical role of T cells in the prevention of varicella virus reactivation. This study is also the first to characterize the gene expression changes that occur after SVV reactivation. Remarkably, significant changes in expression of genes important for neuronal function were altered nearly a year after reactivation, suggesting sustained remodeling of the ganglia transcriptome. These results provide additional insight into the development of post-herpetic neuralgia caused by neuronal damage that can last for years (Schmader 1998).

Methods

Animals and sample collection

Sixteen colony-bred Rhesus macaques (Macaca mulatta, RM) 3–4 years of age and of Indian origin were used in these studies. All the animals were housed and handled in accordance with the Oregon National Primate Research Center Institutional Animal Care and Use Committee (protocol #0779). The 16 RM were intrabrachinally inoculated with $4 \times 10^5$ PFU SVV as previously described (Haberthur et al. 2014). At 148 days post-infection, all the animals were thymectomized. At 29, 32, and 37 DPT, six animals (two male and four female) were depleted of CD4 T cells using the humanized monoclonal antibody OKT4-HulG at a dose of 50 mg/kg. Another six animals (two male and four female) were depleted of CD8 T cells using a mouse-human chimeric monoclonal antibody cM-T807 at a dose of 5 mg/kg on days 29, 32, and 37 post-thymectomy. The remaining four animals (all males) served as controls. All the animals were moved to a different room 364 DPT and were then euthanized ~646 DPT. All procedures were done under ketamine anesthesia to minimize pain. Necropsy was carried out in accordance with the recommendation of the American
Veterinary Association guidelines for euthanasia. Sensory ganglia tissue (trigeminal, dorsal root ganglia (DRG)-cervical, DRG-thoracic, and DRG-lumbar sacral) was collected at necropsy. Tissues were then flash frozen or stored in trizol at $-80^\circ$. Peripheral blood mononuclear cells (PBMC) were isolated over a density gradient cell separation medium (Corning, Manassas, VA) and resuspended in RPMI with 10% FBS and PSG.

| GO process                                             | # genes | FDR          |
|--------------------------------------------------------|---------|--------------|
| **DEGs detected in naïve verus ganglia from reactivated and non-reactivated animals**                      |         |              |
| Metabolic process                                      | 432     | 1.221E−09    |
| Positive regulation of biological process              | 255     | 5.265E−06    |
| Regulation of cell death                               | 111     | 2.139E−05    |
| Regulation of cellular component organization           | 107     | 8.362E−05    |
| **Process networks**                                   |         |              |
| Muscle contraction                                     | 19      | 2.758E−02    |
| Development neurogenesis axon guidance                  | 21      | 7.962E−02    |
| **Diseases**                                           |         |              |
| Infection                                              | 61      | 3.331E−04    |
| Bacterial infections and mycoses                       | 67      | 8.560E−04    |
| Connective tissue diseases                             | 123     | 1.65E−03     |
| RNA virus infections                                   | 53      | 1.684E−03    |
| **DEGs detected only in naïve verus ganglia from non-reactivated animals**                               |         |              |
| Cell-cell adhesion                                     | 34      | 1.671E−03    |
| Wnt signaling pathway, planar cell polarity pathway    | 9       | 6.722E−03    |
| Regulation of type I interferon production             | 9       | 8.779E−03    |
| Metabolic process                                      | 159     | 1.21E−02     |
| Viral process                                           | 29      | 1.169E−02    |
| **Process networks**                                   |         |              |
| Cytoskeleton_regulation of cytoskeleton rearrangement   | 12      | 1.80E−03     |
| Cell adhesion_cell junctions                           | 10      | 6.359E−03    |
| Protein folding nucleus                                | 6       | 6.913E−03    |
| **Diseases**                                           |         |              |
| Central nervous system diseases                        | 53      | 3.314E−04    |
| Virus diseases                                          | 38      | 4.470E−04    |
| DNA virus infections                                   | 24      | 1.011E−03    |
| Herpesviridae infections                               | 15      | 2.827E−03    |
| **DEGs detected only in naïve verus ganglia from reactivated animals**                                   |         |              |
| Metabolic process                                      | 323     | 7.163E−08    |
| Cell cycle                                             | 79      | 2.076E−07    |
| Organelle organization                                 | 146     | 3.141E−07    |
| Regulation of synaptic plasticity                      | 21      | 2.93E−05     |
| **Process networks**                                   |         |              |
| Signal transduction_ESR1-nuclear pathway               | 13      | 1.579E−02    |
| Protein folding_folding in normal condition            | 10      | 6.954E−02    |
| Neurophysiological process_long-term potentiation      | 8       | 6.954E−02    |
| **Diseases**                                           |         |              |
| Basal ganglia diseases                                 | 39      | 3.423E−08    |
| Gliosis                                                | 5       | 3.603E−08    |
| Cerebral arterial diseases                             | 5       | 1.193E−07    |
| Neurodegenerative diseases                             | 77      | 5.964E−04    |
| Trigeminal neuralgia                                   | 5       | 3.881E−06    |
DNA was extracted from whole blood using the Qiagen genomic DNA extraction kit (Qiagen, Valencia, CA). Ganglia tissue was digested in proteinase K solution (20 mg/ml) overnight, and DNA was extracted using the Qiagen genomic DNA extraction kit (Qiagen, Valencia, CA). Viral loads were determined as previously described by real-time PCR using primers and probes specific for ORF21 and measured on the ABI StepOne instrument (Applied Biosystems, Foster City, CA).

**Identification of immune cell populations**

PBMC cells were stained with antibodies against CD8β (Beckman Coulter), CD4 (eBioscience, San Diego, CA), CD28 (BioLegend), CD95 (BioLegend), and CCR7 (BD Pharmingen), which allowed the delineation of central memory (CM; CD28+ CD95+ CCR7+), transitional effector memory (TEM; CD28+ CD95+ CCR7−), and effector memory (EM; CD28+ CD95+ CCR7) CD4 and CD8 T cells. PBMC cells were also surface stained with antibodies against CD20, IgD (Southern Biotech, Birmingham, AL) and CD27 (Biolegend) to delineate IgM (IgD+ CD27+), class-switched memory (IgD− CD27+), exhausted memory (IgD− CD27−), and naïve (IgD= CD27−) B cell subsets. Cells were fixed and permeabilized according to manufacturer recommendations (BioLegend) before the addition of a Ki67-specific antibody (BD Pharmingen). The samples were analyzed using the LSRII instrument (Becton, Dickinson and Company, San Jose, CA) and FlowJo software (TreeStar, Ashland, OR).

**Intracellular cytokine staining**

PBMC were stimulated ex vivo with SVV lysate (1 μg) in the presence of brefeldin A (Sigma, St. Louis, MO) for 14 h. Cells were then stained with antibodies against CD4 and CD8β. Cells were then fixed, permeabilized (BioLegend), and stained for IFN-γ (eBioscience) and TNF-α (eBioscience). Samples were then acquired on the LSRII instrument and data was analyzed using FlowJo software.

**RNA-sequencing analysis**

RNA was extracted from ganglia tissue homogenized in trizol using a bead beater and zirconia/silica beads followed by extraction using the Ambion Purelink RNA Mini Kit (Life Technologies, Carlsbad, CA). RNA library preparation was done using the NEXTflex® Rapid Directional RNA-Seq kit (BIOO Scientific, Austin, TX). DNA libraries were then multiplexed and sequenced on the Illumina HiSeq2500 (Illumina, San Diego, CA) platform. All data analysis steps were performed with the RNA-Seq workflow module of the systemPiperR package available on Bioconductor (Girke 2015; Huber et al. 2015). Next-generation sequencing (NGS) quality reports were generated with the seeFastq function defined by the same package. RNA-Seq reads were mapped with the splice junction aware short read alignment suite Bowtie2/Tophat2 (Kim et al. 2013; Langmead and Salzberg 2012) against the Macaca mulatta genome sequence downloaded from Ensembl (Cunningham et al. 2015). The default parameters of Tophat2 optimized for mammalian genomes were used for the alignments. Raw expression values in the form of gene-level read counts were generated with the summarizeOverlaps function (Lawrence et al. 2013). Only reads overlapping the exonic regions of genes were counted, while reads mapping to ambiguous regions of exons from overlapping genes were discarded. Analysis of differentially expressed genes (DEGs) was performed with the GLM method from the edgeR package ( Anders et al. 2013; Robinson et al. 2010) (SRP097695). For comparison to naïve ganglia, we leveraged RNA-Seq data from our recent publication (SRP072525 (Arnold et al. 2016)). In all analyses, differentially expressed genes (DEGs) were defined as those with a fold change ≥2, a false discovery rate (FDR) ≤0.05, and a median RPKM ≥5. Enrichment analysis was performed using MetaCore software (GeneGo, Philadelphia, PA).

**Gene expression change validation**

RNA was reverse transcribed using random hexamers and SuperScript ® IV RT using the SuperScript ® IV First-Strand Synthesis System (Invitrogen, Lithuania) to generate cDNA. Taqman gene expression assays (Thermo Fisher, Waltham, MA) of selected genes and housekeeping gene (RPL32) were carried out using 100 ng of cDNA in duplicate on the ABI StepOne instrument (Applied Biosystems). mRNA expression levels were calculated relative to our housekeeping gene (RPL32) using 2−ΔΔCt calculations.

**Statistical analysis**

Graphing was performed with GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). One-way repeated-measures analysis of variance (ANOVA) with Dunnett’s multiple comparison post-test was used to explore differences relative to and pre-thymectomy (−6 DPT) values. Unpaired t test was used to determine significance of ganglia viral loads and gene validation.

**Acknowledgements** We would like to thank Vanessa Rico for her help with blood processing and some of the flow cytometry assays. This work was funded by the National Institute of Health (NIH) (R01AG037042-06).

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.
varicella virus gene expression in sensory ganglia. J Virol 87:8294–8306
Miura T, Nishinaka T, Terada T (2008) Different functions between hu-
man monomeric carbonyl reductase 3 and carbonyl reductase 1. Mol
Cell Biochem 315:113–121
Molyneux SD, Waterhouse PD, Shelton D, Shao YW, Watling CM, Tang
QL, Harris IS, Dickson BC, Pharmapalan P, Sandve GK, Zhang X,
Bailey SD, Berman H, Wunder JS, Ivzvak Z, Lupien M, Mak TW,
Khokha R (2014) Human somatic cell mutagenesis creates geneti-
cally tractable sarcomas. Nat Genet 46:964–972
Murali-Krishna K, Ahmed R (2000) Cutting edge: naive T cells
masquerading as memory cells. J Immunol 165:1733–1737
Nagashara K, Higashi T, Hosokawa N, Kaufman RJ, Nagata K (2007)
Simultaneous induction of the four subunits of the TRAP complex
by ER stress accelerates ER degradation. EMBO Rep 8:483–489
NeuJahr DC, Chen C, Huang X, Markmann JF, Cobbold S, Waldmann H,
Sayed M, Hancock WW, Turka LA (2006) Accelerated memory cell
homeostasis during T cell depletion and approaches to overcome it.
J Immunol 176:4632–4639
Novak MA, Hamef AB, Kelly BJ, Detmmer AM, Meyer JS (2013) Stress,
the HPA axis, and nonhuman primate well-being: a review. Appl
Anim Behav Sci 143:135–149
Oda K, Shiratsuchi T, Nishinaka T, Inazawa J, Yoshikawa H, Taketani Y,
Nakamura Y, Tokino T (1999) Identification of BAIAP2 (BAI-as-
associated protein 2), a novel human homologue of hamster IRSp53,
whose SH3 domain interacts with the cytoplasmic domain of BAII1.
Cyto genetic Cell Genet 47:75–82
O’Keefe GW, Gutierrez R, Howard L, Laurie CW, Osorio C, Gavalda N,
Wyatt SL, Davies AM (2016) Region-specific role of growth differ-
centation factor-5 in the establishment of sympathetic innervation.
Neural Dev 11:1
Ono Y, Nakatani T, Minaki Y, Kumai M (2010) The basic helix-loop-
helix transcription factor Nato3 controls neural activity in mes-
encephalic floor plate cells. Development 137:1987–1906
Onozawa M, Hashino S, Sakahata M, Fujisawa K, Nakamura T,
Nakagawa M, Kahta Ka Kondo T, Ota S, Tanaka J, Issumara M,
Asaka M (2006) Relationship between preexisting anti-varicella-
zoster virus (VZV) antibody and clinical VZV reactivation in hema-
topoietic stem cell transplantation recipients. J Clin Microbiol 44:
4441–4443
Onu Y, Uhmwoangwo A (2004) Clinical spectrum of herpes zoster in
HIv-infected versus non-HIV infected patients in Benin City,
Nigeria. West Afr J Med 23:300–304
Ouwendijk WJ, Abendroth A, Traina-Dorge V, Getu S, Stein M, Walshill M,
Andrew GC, Osterhaus AD, Gilden D, Verjans GM, Mahalingam R (2013)
T-cell infiltration correlates with CXCL10 expression in ganglia of
cynomolgus macaques with reactivated varicella-zoster virus neuropathogenesis in human dorsal root ganglia. J
Virol 82: 3971–3983.
Rege TA, Hagoed JS (2006) Thy-1 as a regulator of cell-cell and cell-
matrix interactions in axon regeneration, apoptosis, adhesion, mig-
tation, cancer, and fibrosis. FASEB J 20:1045–1054
Reichert M, Zerboni L, Arvin AM (2008). Mechanisms of varicella-
zoster virus neuropathogenesis in human dorsal root ganglia. J
Virol 82: 3971–3983.
Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor
package for differential expression analysis of digital gene expres-
sion data. Bioinformatics 26:139–140
Russofen JJ, Ahlgren P, Elhamik P, Pinfold P, Reid J, Stewart L, Feld R
(1988) Varicella-zoster infection in adult cancer patients. A popu-
lation study. Arch Intern Med 148:1561–1566
Schmader K (1998) Postherpetic neuralgia in immunocompetent elderly
people. Vaccine 16:1768–1770
Schmader K, Studenski S, MacMillen J, Gruffenberg S, Cohen HJ (1990)
Are stressful life events risk factors for herpes zoster? J Am Geriatr
Soc 38:1188–1194
Sheu TT, Chiang BL, Yen JH, Lin WC (2014) Premature CD4+ T cell
aging and its contribution to lymphopenia-induced proliferation of
memory cells in autoimmune-prone non-obese diabetic mice. PLoS
One 9:e89379
St Leger AJ, Hendricks RL (2011) CD8+ T cells patrol HSV-1-infected
griggial ganglia and prevent viral reactivation. J Neurovirol 17:
528–534
Strezoska Z, Pestov DG, Lau LF (2000) Bop1 is a mouse WD40 repeat
nuclear protein involved in 28S and 5. 8S RRNA processing and
60S ribosome biogenesis. Mol Cell Biol 20:5516–5528
Terada K, Kawano S, Yoshihito K, Morita T (1994) Varicella-zoster virus (VZV)
reactivation is related to the low response of VZV-specific immunity
after chickenpox in infancy. J Infect Dis 169:650–652
Toth C, Shim SY, Wang J, Yang J, Neumayer G, Belzil C, Liu WQ,
Martinez J, Zocheodine D, Nguyen MD (2008) Ndel1 promotes axon
regeneration via intermediate filaments. PLoS One 3:e2014
Traina-Dorge V, Doyle-Meyers LA, Sanford R, Manfred J, Blackmon
A, Wellish M, James S, Alvarez X, Midkiff C, Palmer BE, Dehara
E, Gilden D, Mahalingam R (2015) Simian varicella virus is present
in macrophages, dendritic cells, and T cells in lymph nodes of rhesus
macaques after experimental reactivation. J Virol 89:9817–9824
Uchakina PN, Parish DC, Dane FC, Uchakina ON, Scheetz AP, Agarwal
NK, Smith BE (2011) Fatigue in medical residents leads to reacti-
vation of herpes virus latency. Interdiscip Perspect Infect Dis 2011:
41
Vallortigara J, Chassande O, Higeret P, Onderlin V (2009) Thyroid hor-
mone receptor alpha plays an essential role in the normalisation of
adult-onset hyperthyroidism-related hypertension of synaptic
dynamic plasticity target genes in striatum. J Neuroendocrin 21:49–56
Vermont CL, Jol-van der Zijde EC, Hissink Muller P, Ball LM, Bredius
RG, Vossen AC, Lankester AC (2014) Varicella zoster reactivation after
hematopoietic stem cell transplant in children is strongly cor-
related with leukemia treatment and suppression of host T-
lymphocyte immunity. Transpl Infect Dis 16:188–194
Wang CC, Kadota M, Nishigaki R, Kazuki Y, Shirayoshi Y, Rogers MS,
Gojobori T, Ikeo K, Oshimura M (2004) Molecular hierarchy in
neural plasticity target genes in striatum. J Neuroendocrinol 21:49–56
Weinberg A, Zhang JH, Oxman MN, Johnson LR, Hayward AR,
Caulfield MJ, Irwin MR, Clair I, Smith JG, Stanley H, Marchese
RD, Harbecke R, Williams HM, Chan IS, Arheiten RD, Gershon AA,
Schoedel F, Morrison VA, Kaufman CA, Kraus SE, Schmader KE,
Davis LE, Levin MJ (2009) Varicella-zoster virus-specific immune
responses to herpes zoster in elderly participants in a trial of a clini-
cally effective zoster vaccine. J Infect Dis 200:1068–1077

© Springer J. Neurovirol. (2017) 23:520–538

357
Westerlund N, Zdrojewska J, Padzik A, Komulainen E, Bjorkblom B, Rannikko E, Tataruk T, Garcia-Frigola C, Sandholm J, Nguyen L, Kallunki T, Courtney MJ, Coffey ET (2011) Phosphorylation of SCG10/stathmin-2 determines multipolar stage exit and neuronal migration rate. Nat Neurosci 14:305–313

Wieczorek E, Brand M, Jacq X, Tora L (1998) Function of TAF(II)-containing complex without TBP in transcription by RNA polymerase II. Nature 393:187–191

Wilson JF, Marsa GW, Johnson RE (1972) Herpes zoster in Hodgkin’s disease. Clinical, histologic, and immunologic correlations. Cancer 29:461–465

Yamaguchi M, Yu S, Qiao R, Weissmann F, Miller DJ, VanderLinden R, Brown NG, Frye JJ, Peters JM, Schulman BA (2015) Structure of an APC3-APC16 complex: insights into assembly of the anaphase-promoting complex/cyclosome. J Mol Biol 427:1748–1764

Yawn BP, Wollan PC, Kurland MJ, St Sauver JL, Saddier P (2011) Herpes zoster recurrences more frequent than previously reported. Mayo Clin Proc 86:88–93

Yik JH, Chen R, Pezda AC, Zhou Q (2005) Compensatory contributions of HEXIM1 and HEXIM2 in maintaining the balance of active and inactive positive transcription elongation factor b complexes for control of transcription. J Biol Chem 280:16368–16376

Yorimitsu T, Sato K, Takeuchi M (2014) Molecular mechanisms of Sar/Arf GTPases in vesicular trafficking in yeast and plants. Front Plant Sci 5:411

Zhang D, Zhao T, Ang HS, Chong P, Saiki R, Igarashi K, Yang H, Vardy LA (2012) AMD1 is essential for ESC self-renewal and is translationally down-regulated on differentiation to neural precursor cells. Genes Dev 26:461–473

Zhang T, Wang Z, Wang L, Luo N, Jiang L, Liu Z, Wu CF, Dong K (2013) Role of the DSC1 channel in regulating neuronal excitability in Drosophila melanogaster: extending nervous system stability under stress. PLoS Genet 9:e1003327