Virus Research 238 (2017) 226–235

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Simian varicella virus causes robust transcriptional changes in T cells that support viral replication

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ARTICLE INFO

Keywords:
Varicella zoster virus
T cell
RNA-Seq
Rhesus macaque

ABSTRACT

Varicella zoster virus (VZV) causes varicella (chickenpox) during acute infection. Several studies have shown that T cells are early and preferential targets of VZV infection that play a critical role in disseminating VZV in to the skin and ganglia. However, the transcriptional changes that occur in VZV-infected T cells remain unclear due to limited access to clinical samples and robust translational animal models. In this study, we used a nonhuman primate model of VZV infection where rhesus macaques are infected with the closely related Simian Varicella Virus (SVV) to provide novel insights into VZV-T cell interactions. RNA sequencing of bronchial alveolar lavage-resident T cells isolated from infected rhesus macaques show that SVV infection alters expression of genes important for regulation of gene expression, cell cycle progression, metabolism, and antiviral immunity. These data provide insight into cellular processes that may support viral replication, facilitate SVV dissemination, and evade host defense.

1. Introduction

Varicella Zoster Virus (VZV) is a highly contagious neurotropic alpha herpes virus that causes varicella (chickenpox) during acute infection. VZV is spread through the inhalation of virus particles or through direct contact with infectious fluid from skin lesions (Arvin, 2001; Grose, 1981). VZV establishes latency in sensory ganglia, from which it can reactivate causing herpes zoster (shingles), a painful disease most commonly affecting the elderly and immunocompromised individuals. Previous studies have shown that T cells are highly susceptible to VZV infection and may play a critical role in its dissemination to the skin and ganglia. Specifically, direct inoculation of fetal thymus/liver implanted under the kidney capsule of SCID-hu mice with VZV-infected fibroblasts results in the infection of CD4+ and CD8+ thymocytes (Moffat et al., 1995). In addition, *in vitro* experiments have shown that VZV has a high propensity to infect tonsillar T cells (Ku et al., 2002). Moreover, co-culture experiments showed that activated tonsillar CD4 T cells with skin homing markers were more likely to be infected with VZV (Ku et al., 2002). Importantly, human tonsillar CD4 T cells infected *in vitro* with VZV, but not fibroblasts, intravenously injected into SCID-hu mice were able to transport VZV to fetal human skin explant resulting in development of varicella rash (Ku et al., 2004) and fetal dorsal root ganglia xenografts (Zerboni et al., 2005).

Although these *in vitro* studies suggest that T cells play a critical role in VZV pathogenesis, none of these findings have been confirmed using T cells isolated from varicella patients. Moreover, the strict host tropism of VZV has precluded the development of animal models. An alternative is to use rhesus macaques intra-bronchially infected with the homologous simian varicella virus (SVV). This model mimics the key characteristics of VZV infection including the development of varicella, cellular and humoral immune responses, the establishment of latency in sensory ganglia, and reactivation (Messaoudi et al., 2009; Mahalingam et al., 2010; Mahalingam et al., 2007; Kolappaswamy et al., 2007). As described for VZV, we and others have demonstrated that SVV primarily infects T cells that traffic to the ganglia as early as 3 days post-infection (Ouwendijk et al., 2013; Arnold et al., 2016a). Moreover, we showed that T cells isolated from the broncho-alveolar lavage (BAL) during acute infection supported SVV replication (Arnold et al., 2016a). These data firmly establish the importance of T cells in SVV pathogenesis making this model ideal for investigating how VZV infection alters T cell behavior and function. In this study, we used this animal model to investigate the transcriptional changes that SVV infection induces within CD4 and CD8 T cells isolated from the BAL during acute infection. Our results show that SVV induces robust transcriptional changes involved with chromatin assembly, translation, cell cycle and cellular metabolism. In addition, several gene expression changes reveal possible mechanisms by which SVV may evade the host response.

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http://dx.doi.org/10.1016/j.virusres.2017.07.004
Received 28 January 2017; Received in revised form 24 May 2017; Accepted 4 July 2017
Available online 08 July 2017
0168-1702/

Table 1
Samples used for RNA-Seq Analysis.

Animal ID	DPI	Cell Type	Viral Load
28081	3	CD8	411,559
28081	3	CD4	320,688
17036	7	CD8	10,674
27180	10	CD8	404
27916	10	CD8	517
Control #1	UNIF	CD8	0
Control #2	UNIF	CD8	0
Control #3	UNIF	CD8	0
Control #4	UNIF	CD8	0
Control #1	UNIF	CD4	0
Control #2	UNIF	CD4	0
Control #3	UNIF	CD4	0
Control #4	UNIF	CD4	0

2. Methods and materials

2.1. Animals and samples

CD4 and CD8 T cells were isolated from bronchial alveolar lavage (BAL) samples collected from 8 colony-bred Indian origin Rhesus macaques (*Macaca mulatta,* RM) 3–5 years of age previously described in (Haberthur et al., 2013) using Magnetic Cell Sorting (MACs). Four of these animals were inoculated intrabronchially with 4×10^5 PFU wild-type SVV as previously described and were euthanized 3, 7 and 10 days post infection (DPI) (Table 1) (Haberthur et al., 2013). Briefly, BAL cells were first incubated with CD4 microbeads (Miltenyi Biotec, San Diego) for 20 mins at 4 °C. Cells were then washed and applied to the magnetic column to capture CD4 T cells. The negative fraction was then stained with anti-CD8 PE (Beckman Coulter) for 20 mins in the dark at 4 °C followed by a wash and incubation with anti-PE beads (Miltenyi Biotec) for 15 mins in the dark at 4 °C. Cells were then washed and the cell suspension was applied to a second magnetic column to isolate CD8 T cells. To determine purity, cells were stained with antibodies directed against surface markers CD4 (Tonbo Biosciences, San Diego, CA) and CD8β (Beckman Coulter, Brea, CA). Samples were acquired using an LSRII instrument (Becton, Dickinson and Company, San Jose, CA) and analyzed using Flowjo software (TreeStar, Ashland, OR). All samples used had a purity of > 90%.

2.2. RNASeq and bioinformatics

RNA was extracted from purified CD4 and CD8 T cells using the Ambion Purelink RNA Mini Kit extraction kit (Life Technologies, Carsbad, CA). RNA library preparation was done using the New England Biolab (NEB) Next Ultra Direction RNA Prep kit for Illumina (Ipswich, MA). We were unable to generate a library of the CD4 T cells isolated from the 7 DPI and 10 DPI animals. Therefore, we only have paired CD4/CD8 libraries from the animal euthanized 3 DPI and our control animals; and only a CD8 T cell library from the animals euthanized 7 DPI and 10 DPI (Table 1). Libraries were next multiplexed and sequenced on the Illumina NextSeq (Illumina, San Diego, CA) platform at single-ends 75bps. All data analysis steps were performed with the RNA-Seq workflow module of the *systemPiperR* package available on Bioconductor (Huber et al., 2015; Girke, 2015). Next generation sequencing quality reports were generated with the *seeFastq* function defined by the same package. Reads were mapped with the splice junction aware short read alignment suite Bowtie2/Tophat2 (Langmead and Salzberg, 2012; Kim et al., 2013) 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 that overlapped with exonic regions of genes were counted, while reads that mapped 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 (Robinson et al., 2010; Anders et al., 2013). Differentially expressed genes (DEGs) were defined as those with a fold change of ≥ 2, a false discovery rate (FDR) of ≤ 0.05 and an average reads per kilobase per million (RPKM) value ≥ 5. These FC and FDR cut off values are well-established statistical criteria for transcriptomic experiments (Conesa et al., 2016). Functional enrichment analysis was performed using MetaCore™ software (GeneGo, Philadelphia, PA) to identify significant gene ontology (GO) and Network Processes. When conducting the longitudinal analyses, an FDR cut-off could not be applied for the 7 DPI data set due to the fact that only 1 sample was analyzed. Heatmaps, volcano plot, and PCA were generated using gplots, ggplot2 and DESeq2 package in R.

2.3. Validation of gene expression changes

Remaining RNA from samples was reverse transcribed using random hexamers and SuperScript ® IV RT in the SuperScript ® IV First-Strand Synthesis System (Invitrogen, Lithuania) to generate cDNA. Taqman gene expression assays (Thermo Fisher, Waltham, MA) of candidate genes and housekeeping gene (RPL32) were used with 50 ng of cDNA and carried out in duplicate on the ABI StepOne instrument (Applied Biosystems). mRNA expression levels were calculated relative to the housekeeping gene (RPL32) using $2^{-}\Delta Ct$ calculations.

BAL cells collected 0 and 7 DPI during the course of another previous study (Arnold et al., 2016b) were stained with antibodies against CD8β (Beckman Coulter), CD4 (eBioscience, San Diego, CA), CD3 (Biolegend, San Diego, CA), CD2 (Biolegend), TLR4 (ThermoFisher, Waltham, MA), and IGFR2 (ThermoFisher). The samples were analyzed using the Attune NxT (ThermoFisher). Mean Fluorescence Intensity (MFI) was used to calculate FC of protein expression levels 7 DPI compared to 0 DPI.

3. Results

3.1. BAL-resident t cells isolated from SVV infected animals show robust transcriptional changes

We recently reported that SVV primarily infects both CD4 and CD8 T cells in the BAL, which can support viral replication (Arnold et al., 2016a). To determine SVV-induced transcriptional changes within T cells, we performed RNA-Seq on BAL-resident T cells shown to express viral transcripts (measured through AmpliSeq technology (Arnold et al., 2016a)) and uninfected BAL T cells (4 paired CD4/CD8 T cell samples isolated from 4 control animals) (Table 1). A principle component analysis (PCA) shows that infected and uninfected T cells have distinct transcriptional profiles (A).

We first identified global differences between the two groups of samples. Overall, a total of 1463 (739 upregulated and 724 downregulated) differentially expressed genes (DEGs) were detected, of which 1130 (586 upregulated and 544 downregulated) were characterized (Fig. 1B, Supplemental Dataset 1). Changes in expression of 4 DEGs were confirmed using qRT-PCR and that of an additional 5 DEGs were confirmed at the protein level using flow cytometry (Supplemental Fig. 1). Upregulated DEGs enriched to network processes related to inflammation and cell cycle (Fig. 1C) and gene ontology (GO) processes related to immunity, metabolism and gene expression (Fig. 1D). Downregulated DEGs enriched to process networks involved with phagocytosis and signaling (Fig. 1C) and GO processes involved with intercellular transport and metabolism (Fig. 1D).

3.2. SVV infection upregulates cell cycle processes and immune responses

Many of the upregulated genes, played a role in gene regulation,