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Interleukin-6 and type 1 interferons inhibit varicella zoster virus replication in human neurons

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Abstract

Varicella zoster virus (VZV) is a neurotropic alphaherpesvirus that, following primary infection (varicella), establishes latency in sensory, autonomic, sympathetic and parasympathetic neurons, where it remains until reactivation (zoster). VZV-specific cell-mediated immune responses maintain VZV latency; thus, immunosuppressed and elderly persons are at risk of reactivation and associated neurological diseases. However, the cytokines produced by the immune system that control VZV in neurons are largely unknown. Therefore, to better understand how the immune system may restrict VZV in neurons, we studied interleukin-6, tumor necrosis factor-alpha and type 1 interferons for their ability to inhibit VZV replication in human neurons *in vitro*. Our studies revealed that VZV transcription and viral spread were significantly reduced by interleukin-6 and type 1 interferons, and to a lesser extent by tumor necrosis factor-alpha. These findings will help in understanding how the innate immune system limits virus replication in neurons *in vivo*.

Keywords

VZV; IL-6; TNF; Type 1 interferon; antiviral; neuron

Introduction

Varicella zoster virus (VZV) is an exclusively human neurotropic virus that, upon primary infection, causes varicella (chickenpox). After primary infection with wild-type VZV or vaccination with the live-attenuated varicella vaccine (Varivax, Merck), virus establishes latency in sensory ganglia neurons (Gershon et al., 2012; Gilden et al., 1987; Gilden et al., 1983; Mahalingam et al., 1990). Maintenance of VZV latency requires cell-mediated immunity and production of cytokines. Upon loss or suppression of VZV-specific immunity due to aging, immunosuppressive therapy or HIV/AIDS, VZV can reactivate (Asanuma et

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al., 2000; Koenig et al., 2013; Levin et al., 2003; Saylor et al., 2015; Weinberg and Levin, 2010; Zhang et al., 1994) to cause zoster (shingles), postherpetic neuralgia, vasculopathies (e.g., stroke and giant cell arteritis) and diseases of the central nervous system (e.g., myelopathy and meningoencephalitis) (Gilden et al., 2016; Minassian et al., 2015; Yawn et al., 2016).

The specific mechanisms of how cell-mediated immunity within VZV-infected human ganglia restricts VZV replication remain largely unknown, due in part to the lack of a suitable animal model; no small-animal model fully recapitulates the human disease when virus reactivates. Human ganglia from patients who died soon after zoster contain neurons expressing VZV antigen as well as large infiltrates of CD8⁺ cells (Gowrishankar et al., 2010). These immune cells are postulated to help establish and maintain VZV latency via production of cytokines, but the identity of such cytokines remains unknown. Notably, treatment of lupus and rheumatoid arthritis with anti-interleukin-6 (IL-6), antitumor necrosis factor alpha (TNF α) and anti-interferon alpha (IFN α , a type 1 IFN) increases a patient's likelihood of developing zoster (Cacciapaglia et al., 2015; Furie et al., 2017; Garcia-Doval et al., 2010; Khamashta et al., 2016; Mourgues et al., 2016; Strangfeld et al., 2009), suggesting these cytokines are important in inhibiting VZV replication and maintaining latency. Thus, the aim of this study was to investigate the role of IL-6, TNF α and type 1 IFNs (IFN α and IFN β) in suppressing VZV in human neurons using our established *in vitro* model of VZV-infected human neurons (Grose et al., 2013; Yu et al., 2013). In this study, we found that IL-6 significantly inhibits VZV replication and virus production in human neurons, type 1 IFNs were also inhibitory to VZV replication, but less so than IL-6 and TNF α did not inhibit VZV replication in human neurons *in vitro*.

Results

VZV-infected human neurons express cytokine receptors *in vitro*.

To ensure human neurons derived from induced pluripotent stem cells (iPSCs) cultured *in vitro* expressed the appropriate cytokine receptors, and thus would be expected to respond to cytokines, uninfected neurons were tested for the presence of receptors for IL-6 (IL-6R), TNF α (TNFR1 and TNFR2), and type 1 IFNs, IFN α and IFN β (IFNAR). TNF α can bind to either TNFR1 or TNFR2 (Hohmann et al., 1989), whereas IFNAR is the receptor for both IFN α and IFN β (Merlin et al., 1985). TNFR1 is expressed on nearly all cells, but TNFR2 is expressed on a limited population of cells including endothelial cells, microglia, oligodendrocytes, hippocampal neurons, cardiac myocytes, thymocytes and mesenchymal stem cells (Arnett et al., 2001; Bocker et al., 2008; Dopp et al., 2002; Grell et al., 1998; Irwin et al., 1999; McCoy and Tansey, 2008; Tartaglia et al., 1991; Yang et al., 2002). Neurons were also stained for β -III tubulin, a neuronal marker, to show neuronal cell morphology. As expected, analysis of uninfected iPSC-derived human neurons using fluorescence microscopy revealed the expression of IL-6R, TNFR1 and IFNAR (Fig. 1, top row) as well as β -III tubulin (Fig. 1, middle row). TNFR2 staining was negative, which was in line with our unpublished RNAseq results that indicated TNFR2 is not expressed at the mRNA level. Uninfected neurons were also negative for the non-neuronal marker glial fibrillary acidic protein (GFAP; data not shown).

It was of concern that VZV-infection of iPSC neurons may downregulate cytokine receptors, thereby making the neurons resistant to cytokine treatment. To test this, neuron cultures were infected with VZV for 21 days, after which neurons were fixed and surface protein-stained (non-permeabilized) for the indicated receptors as well as the viral glycoprotein E (gE). At 21 days post-infection (dpi), VZV-infected neurons (Fig. 2, top row) retained surface expression of all cytokine receptors (Fig. 2, middle row). Colocalization of gE and cytokine receptor is visualized in the merge (Fig. 2, bottom row) as a yellow color. As a negative control, infected, non-permeabilized neurons were stained with normal rabbit serum and a mouse isotype Ig, followed by Alexa-conjugated secondary antibodies, as described in Materials and Methods (Fig. 2, bottom row).

IL-6 and type 1 IFNs, but not TNF α , inhibit viral replication *in vitro*.

To demonstrate that cytokine treatment over the course of the experiments would not be toxic to neurons, both uninfected and VZV-infected neuronal cultures were maintained without cytokine (untreated control, UT) or with either IL-6, TNF α , IFN α or IFN β for 21 days. After 21 days, cytotoxicity was measured by lactate dehydrogenase (LDH) release into the culture supernatants. In uninfected cultures, IL-6 induced an $8.17 \pm 4.01\%$ cytotoxicity relative to the maximum release control, and no other cytokine induced any cytotoxicity. Also, acyclovir (ACV) toxicity was measured since ACV was used as a positive control to inhibit VZV replication (used in virus replication experiments, below); this treatment induced no cytotoxicity in uninfected cultures. Similarly, after 21 days of infection, neurons cultured in the presence of cytokine did not release any additional LDH compared to infection alone, indicating cytokine treatment did not accelerate death of infected neurons (LDH release, relative to uninfected, untreated maximum release control: VZV only, $4.70 \pm 0.28\%$; VZV+IL-6, $5.13 \pm 1.30\%$; VZV+TNF α , $21.15 \pm 19.55\%$; VZV + IFN α , $2.99 \pm 1.30\%$; IFN β , $6.52 \pm 3.64\%$). To test the hypothesis that IL-6, TNF α or either type 1 IFN would inhibit VZV replication in human neurons, neuronal cultures were either pretreated with cytokine 24 h prior to VZV infection or with medium lacking any cytokine (UT control). Pretreatment was done to recapitulate what may occur *in vivo*, where neurons experience cytokine exposure prior to retrograde transport of alphaherpesviruses from peripheral tissue and deposition of viral DNA into the neuron nucleus (Rosato and Leib, 2015; Song et al., 2016). Following infection, neurons were continuously cultured in the presence or absence of cytokine as described above for the toxicity assay. At 21 dpi, UT and cytokine-treated cultures were harvested and mRNA levels measured. Treatment with IL-6 resulted in a significant reduction of all viral mRNAs measured, irrespective of gene class, relative to UT cultures (ORF62 and ORF63, immediate early; ORF29, early; ORF68, late; $p < 0.05$) (Fig. 3A). However, treatment of VZV-infected neurons with either type 1 IFN had no significant effect on levels of ORF63 transcript ($p > 0.05$), but did significantly reduce all other viral genes examined. TNF α followed the trend of inhibiting viral transcription relative to UT cells, but to a lesser extent than other cytokines studied and was not statistically significant. As a positive control for inhibition of viral replication, a parallel culture of VZV-infected neurons was maintained in the presence of ACV; at 21 dpi, VZV transcripts were either not detectable or not quantifiable ($C_t > 35$) (Fig. 3A). From the same cultures that were used to measure mRNA abundance, DNA was also extracted and abundance of viral genomes measured (Fig. 3B). None of the treatments, including

acyclovir, caused a significant decrease in viral DNA abundance compared to UT. Next, the ability of the cytokines to limit virus spread was measured. Neurons were pretreated or remained UT, infected, then maintained as described in the methods. The ability of IL-6, TNF α or either type 1 IFN to limit VZV spread within neuronal cultures was examined by measuring viral plaque area at 21 dpi. At 21 dpi, VZV-infected neuron cultures without cytokine treatment had viral antigen covering large areas ($0.317 \pm 0.121 \text{ mm}^2$). Conversely, VZV-infected neurons cultured in the presence of either IL-6, TNF α , IFN α or IFN β had much reduced spread (virus area: IL-6, $0.059 \pm 0.022 \text{ mm}^2$; TNF α , $0.092 \pm 0.032 \text{ mm}^2$; IFN α , $0.017 \pm 0.003 \text{ mm}^2$; IFN β , $0.008 \pm 0.01 \text{ mm}^2$). Compared to UT, cytokine-treated cultures contained plaques that were reduced by: IL-6, $83 \pm 8\%$; TNF α , $50 \pm 22\%$; IFN α , $93 \pm 2\%$; and IFN β , $96 \pm 3\%$ (Fig. 3C).

IL-6 inhibits production of VZV in human neurons *in vitro*.

To analyze the quantity of infectious virus produced, cultures were pretreated or remained UT, infected, and cultured as above. At 21 dpi, neuron cultures were harvested, dispersed into single cell suspension then co-cultured on a monolayer of uninfected fibroblasts and plaques were allowed to form. Compared to UT, only IL-6-treated VZV-infected neurons had a significantly reduced ($p < 0.05$) number of plaques form (reduced $61 \pm 15\%$; Fig. 4); all other treatments reduced the average number of plaques, compared to UT, but did not reach statistical significance.

Discussion

How cell-mediated immunity and/or cytokines restrict VZV replication in ganglia *in vivo* is unknown. To better understand this process, we have examined effects of IL-6, TNF α and type 1 IFNs on VZV replication in human neurons. IL-6 exhibited the most consistent and strongest antiviral characteristics of the studied cytokines, significantly limiting transcription of all viral kinetic classes (Fig. 3A), as well as virus spread (Fig. 3C), and production of infectious particles (Fig. 4), compared to UT controls. The strong anti-viral nature of IL-6 in human neurons *in vitro* is supported by the high levels of IL-6 present during VZV reactivation *in vivo* causing VZV vasculopathy (Jones et al., 2016) and giant cell arteritis (Gilden et al., 2016; Martinez-Taboada et al., 2008).

While IL-6 demonstrated the most consistent antiviral effect, type 1 IFNs also exhibited antiviral properties. Type 1 IFNs significantly reduced the levels of VZV transcripts compared to untreated, with the exception of VZV ORF63 transcript (Fig. 3A), suggesting there may be a differential block of virus transcription by type 1 IFNs compared to IL-6. This may be explained by the fact that type 1 IFNs primarily signal through signal transducer and activator of transcription 1 and 2 (STAT-1 and -2), whereas IL-6 primarily signals through STAT-3.

TNF α has been shown to be strongly antiviral in non-neuronal cells (Ito et al., 1991; Torigoe et al., 2000); however, these studies indicate TNF α may be less antiviral in neurons as it did not significantly inhibit VZV replication in any of our assays. Notably, TNF α signals through several different pathways following binding to either TNFR1 or TNFR2 surface receptors (Leong and Karsan, 2000); future studies will examine if differential downstream

pathways are used in neuronal versus non-neuronal cell types in the context of VZV-infection.

Zostavax has a high ratio of non-infectious DNA to plaque forming units (PFU) and VZV replicates slowly in neurons compared to non-neuronal cells; therefore, differentiation between newly replicated DNA and adhered inoculum is difficult. As such, viral DNA abundance does not increase compared to input by 14 dpi (Baird et al., 2014b). This is likely the reason that, after 21 days of infection, the abundance of viral DNA in ACV- or cytokine-treated neurons, although reduced, is statistically the same as UT neurons (Fig. 3B). It is likely that, if cultures were permitted to incubate longer, differences in DNA abundance would be observed. Despite the lack of statistical significance, IL-6- and type 1 IFN-treated, as well as ACV-treated, cultures had reduced average DNA abundance compared to UT cultures at 21 dpi.

Overall, IL-6 and type 1 IFNs had an inhibitory effect on VZV in human neurons *in vitro*, where TNF α did not. Whereas this study utilized Zostavax as inoculum, it is possible a wild-type strain of VZV may respond differently to cytokine treatment. Future studies will examine how each cytokine specifically modulates virus replication, as well as additive effects when cytokines are combined.

Materials and Methods

Cells and viruses

Human induced pluripotent stem cell (iPSC; Cellular Dynamics International, Madison, WI)-derived neurons were cultured and infected at a low multiplicity of infection (MOI; approx. 0.001 to 0.0001) with vaccine-strain VZV (Zostavax; Merck, Kenilworth, NJ) as previously described (Baird et al., 2014a; Baird et al., 2015; Baird et al., 2014b). After 3 h of incubation, neurons were washed twice with medium to remove inoculum and cultured for 21 days. Neurons received a 50% medium change with fresh medium twice weekly. Human fetal lung fibroblasts (HFL; ATCC, Manassas, VA) were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum. All cultures were incubated at 37°C and 5% CO₂.

Cytokine treatment

Cytokines were prepared in water per the manufacturer's instructions (IL-6 and TNF α , Millipore, Temecula, CA; IFN α , PBL Assay Science, Piscataway, NJ; IFN β , Peprotech, Rocky Hill, NJ). Cytokines were used at concentrations observed in the literature: IL-6 and TNF α were each used at 100 ng/ml while type 1 IFNs were used at 10 U/ml, acyclovir was used at 50 μ M. For cytokine treatment of VZV-infected cultures, cultures were pretreated for 24 h prior to infection or remained untreated without any cytokine. During the 3 h infection period, cytokine was removed, after which neurons were washed twice with cell medium and cytokine was reintroduced at concentration indicated above. Cytokine-treated cultures, whether infected and uninfected, received 50% medium changes twice weekly with fresh medium containing fresh cytokine at concentration indicated above.

Cytotoxicity

To measure cytotoxicity, parallel cultures of both infected and uninfected neurons were prepared and were either untreated or treated with indicated cytokine as detailed above. After 21 days, cytotoxicity was measured using the Pierce LDH cytotoxicity assay kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer's instructions. All percent cytotoxicity measurements were calculated relative to maximum release control of uninfected, untreated cultures.

Immunofluorescent microscopy

To determine expression of cytokine receptors, neurons were cultured on either standard tissue culture plastic (12-well plates) or poly-D/laminin-coated glass coverslips (Corning, Corning, NY). Medium was removed and cells were washed once with 1X PBS, fixed in 4% paraformaldehyde for 20 min at 4°C, then either permeabilized with 0.1% Triton-X for 20 min at room temperature (internal staining, Fig. 1) or remained non-permeabilized (surface staining, Fig. 2), and blocked with 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Cytokine receptors, β -III tubulin or VZV glycoprotein E (gE) were identified by overnight incubation at 4°C with a primary antibody [anti-IL6R (cat #: sc-13947), -TNFR1 (cat #: sc-7895) and -TNFR2 (cat #: sc-8041), -IFNAR (cat #: sc-704), and -VZV gE (cat #: sc-56995), Santa Cruz Biotechnology, Dallas, TX; β -III tubulin (cat#: 60052), Stem cell Technologies, Cambridge, MA], followed by Alexa Fluor 594- or 488-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h at 4°C. As negative controls, non-permeabilized, infected neurons were surface stained with normal rabbit serum and mouse isotype Ig antibody followed by Alexa Fluor 594- or 488-conjugated secondary antibodies, respectively (Fig. 2). Cell nuclei were stained with DAPI for 5 min at room temperature. Images were captured using an Olympus (Olympus, Center Valley, PA) IX73 inverted fluorescent microscope fitted with an XM10 cooled CCD camera and processed using Cellsense (Olympus) software.

Nucleic acid isolation and qPCR/RT-qPCR

VZV-infected human neurons harvested at 21 dpi were analyzed for abundance of viral DNA and RNA. DNA and RNA were isolated using ZR Duet DNA/RNA Kit (Zymo Research, Irvine, CA), after which mRNA was isolated by binding to oligo(dT) beads (uMACS; Miltenyi, Bergisch Gladbach, Germany) and reverse transcribed using the Superscript IV cDNA Synthesis Kit (LifeTechnologies). Quantitative TaqMan PCR (FastStart Universal MasterMix, Roche, Basel, Switzerland) was performed on an ABI 7500 FAST to quantify viral DNA using primers targeted to a locus within ORF68 (F: GTACATTTGGAACATGCGCG; R: TCCACATATGAAACTCAGCCC) and cDNA with primers targeted to four different viral loci: ORF62 (F: CCTTGGAACCACATGATCGT; R: AGCAGAAGCCTCCTCGACAA), ORF63 (F: TAGCGACGATGATGGGTCTA; R: GTGCTCTCCTCTGATTCTTCTTC), ORF29 (F: GGCGGAACCTTCGTAACCAA; R: CCCCATTAACAGGTCAACAAAA), and ORF68 (same as DNA primers, above).

Plaque area measurement

VZV-infected cultures were cultured in the presence or absence of indicated cytokine as detailed above, then fixed at 21 dpi and stained for viral gE. Images of infected neurons were captured as above and area of infection was measured by manually outlining VZV antigen-positive neurons using Cellsense software.

VZV titration

To determine production of infectious virus, a single well of a 12-well plate containing VZV-infected neurons (5×10^5 cells; with or without cytokine treatment) was harvested at 21 dpi by brief trypsinization and pipetted vigorously to disperse neurons into a single cell suspension. The entire neuron suspension was added to 40% confluent monolayers of HFL cells and co-cultured for 7 days, at which time cells were fixed and stained using 0.1% crystal violet in 70% ethanol/1X PBS and plaques counted.

Statistical analysis

Statistical significance of mean plaque size and DNA inhibition were determined using one-way analysis of variance (ANOVA) between untreated and treated groups. Statistical significance of mRNA transcription inhibition was determined using a 2-way ANOVA (treatment x ORF) to compare treatments between different ORFs. The subsequent significant effect of treatments was followed by Fisher's LSD tests to compare untreated groups with all subsequent treatments. All statistical tests were set at a 0.05- α level and conducted using Prism 7 (GraphPad software, La Jolla, CA).

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FIG 1.

Neurons express cytokine receptors *in vitro*. Uninfected iPSC-derived human neurons were permeabilized then co-immunostained for indicated cytokine receptor (top row) and β -III tubulin (middle row), followed by fluorescently-conjugated secondary antibodies. IL-6 receptor (IL-6R, left), tumor necrosis factor receptor 1 (TNFR1, middle) and interferon- α/β receptor (IFNAR, right) stainings were all positive, whereas TNFR2 and GFAP stainings were negative (not shown). Merged images (bottom row) indicate all cells are positive for receptor and β -III tubulin. Magnification = 60X; blue, DAPI staining of nuclei.

FIG 2.

Viral infection does not downregulate cytokine surface receptors. Human neurons were infected with VZV for 21 days, then surface-stained (non-permeabilized) for viral glycoprotein E (row 1) and cytokine receptor (row 2), followed by fluorescently-conjugated secondary antibodies. VZV-infected neurons (red) retain surface expression of cytokine receptor (green), seen as yellow in merge (row 3). Infected neurons (non-permeabilized) were immunostained using control rabbit serum (GFP) and mouse isotype antibody (RFP) (row 4), followed by fluorescently conjugated secondary antibodies. Magnification = 40X; blue, DAPI staining of nuclei.

FIG 3.

Inhibition of viral replication. VZV-infected neurons were either untreated (UT) or treated with the indicated cytokine (IL-6, TNF α , IFN α , IFN β) or acyclovir for 21 days, when abundance of either viral mRNA (A) or viral DNA (B) was assessed using qPCR, or virus spread was assessed by measuring viral plaque area (C). Viral transcripts corresponding to VZV ORFs 62, 63, 29 and 68 were assayed. Values are fold-difference of abundance in treated culture relative to UT culture, calculated using the $2^{-\Delta C_t}$ method ($2^{(C_{t,UT} - C_{t,treated})}$) (a and B). Data are mean $\pm$ SEM from 3 experiments; ND, not detected; * indicates a *p* value <0.05.

FIG 4.

Production of infectious virus. VZV-infected neurons were either untreated (UT) or treated with the indicated cytokine (IL-6, TNF α , IFN α or IFN β) for 21 days. Neurons were harvested and co-cultured on uninfected HFL cells in the absence of cytokine. Plaques in fibroblast monolayers were detected and counted following crystal violet staining and represented as fold difference relative to UT culture. Data are mean $\pm$ SEM from 3 experiments; * indicates a *p* value <0.05 .