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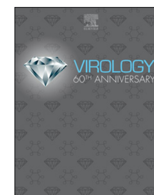

Interleukin-6 and type 1 interferons inhibit varicella zoster virus replication in human neurons

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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*.

1. 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, 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 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), anti-tumor necrosis factor alpha (TNFα) and anti-interferon alpha (IFNα, 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, TNFα and type 1 IFNs (IFNα and IFNβ) 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 TNFα did not inhibit VZV replication in human neurons *in vitro*.

2. Results

2.1. 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

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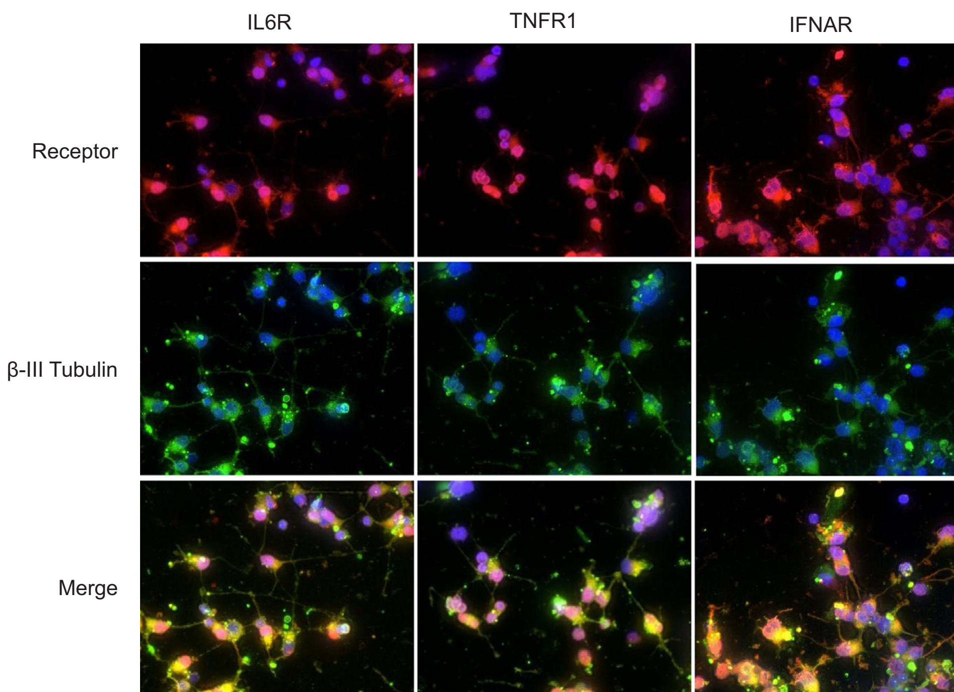

Fig. 1. Neurons express cytokine receptors *in vitro*. Uninfected iPSC-derived human neurons were permeabilized then co-immunostained for indicated cytokine receptor (top row) and β-III tubulin (middle row), followed by fluorescently-conjugated secondary antibodies. IL-6 receptor (IL-6R, left), tumor 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 = 60×; blue, DAPI staining of nuclei.

neurons were tested for the presence of receptors for IL-6 (IL-6R), TNFα (TNFR1 and TNFR2), and type 1 IFNs, IFNα and IFNβ (IFNAR). TNFα can bind to either TNFR1 or TNFR2 (Hohmann et al., 1989), whereas IFNAR is the receptor for both IFNα and IFNβ (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 down-regulate 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). Co-localization 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).

2.2. IL-6 and type 1 IFNs, but not TNFα, 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, TNFα, IFNα or IFNβ 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 ± 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 ± 0.28%; VZV + IL-6, 5.13 ± 1.30%; VZV + TNFα, 21.15 ± 19.55%; VZV + IFNα, 2.99 ± 1.30%; IFNβ, 6.52 ± 3.64%).

To test the hypothesis that IL-6, TNFα 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. TNFα 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

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