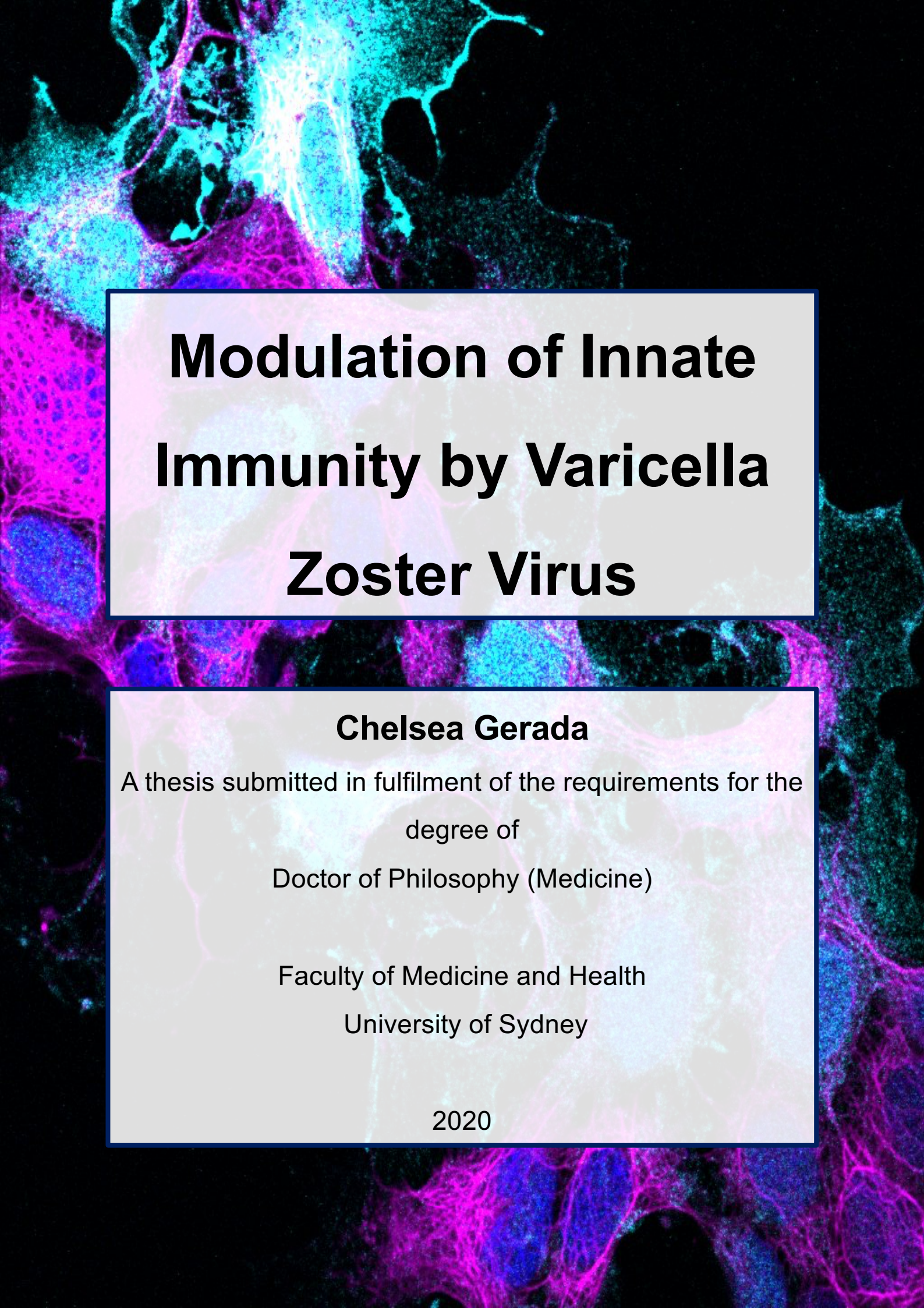

Modulation of Innate Immunity by Varicella Zoster Virus

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A thesis submitted in fulfilment of the requirements for the degree of

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Declaration

The work presented in this thesis was completed in the Faculty of Medicine and Health at the University of Sydney under the supervision of Prof. Allison Abendroth, Dr. Megan Steain, and A/Prof. Barry Slobedman.

This declaration is to certify that to the best of my knowledge the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Chelsea Gerada

Acknowledgments

“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”
— J.R.R. Tolkien, The Hobbit or There and Back Again

I started my PhD hoping that I would gain some skills that would make me employable and that I would make some sort of contribution to science. Looking back on everything now, I realise I have gained so much more than those initial aims. I have gained a belief in myself, a feeling that I know what I am doing and that I can make difficult decisions. I have grown my passion for science and now have something that I know will always be a part of my life, because I really can’t imagine doing anything else. I also have gained multiple mentors and friendships that have helped me grow. This personal and professional growth wouldn’t have been possible without a multitude of people who I want to thank.

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when I was struggling which I am super grateful for. Carolyn, your compassion and guidance always made me feel I had someone to go to when things weren't working out so well. Your determination and love of science is very inspiring, and I know you will do great things in your PhD. Max you are such a champion, I loved our conservations on the bachelor franchise and that I had another comrade to back me up against Felix and his questionable taste in multimedia. Your sense of humour helped me get through tough lab days and I am now officially passing on the baton of being one of the only VZV researchers in the southern hemisphere.

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Abstract

Varicella zoster virus (VZV) is a ubiquitous human alphaherpesvirus, which causes varicella (chicken pox) during primary infection. During primary infection, the virus infects the skin and gains access to sensory nerve termini. Through retrograde transport, VZV infects neurons in the dorsal root ganglia (DRG) where the virus establishes lifelong latency. VZV can reactivate to cause herpes zoster (shingles), which encompasses a broad variety of complications such as post herpetic neuralgia (PHN), which is defined as neuropathic pain that persists at least 3 months beyond the appearance of the herpes zoster rash. The mechanisms which govern the ability of VZV to establish and maintain lifelong latency, as well as cause PHN are still unclear and could provide therapeutic targets to help prevent and treat PHN. VZV has been shown to regulate various aspects of innate immunity and this has been linked with the ability of the virus to maintain latency. This study aimed to uncover novel mechanisms of VZV innate immunomodulation to increase understanding of contributing factors to both latency maintenance and the establishment of PHN.

The ability of VZV to modulate apoptosis, a non-inflammatory form of programmed cell death, has been linked with the ability of the virus to maintain lifelong latency in neuronal cells. VZV open reading frame (ORF) 63 had been associated with the ability of VZV to protect neuronal cells from apoptosis, however this had never been confirmed in human cells. This study (Chapter 2) utilised VZV ORF63 expressing neuronal (SH-SY5Ys) and keratinocyte (HaCaTs) human cell lines, to further characterise the ability of VZV ORF63 to inhibit apoptosis in a cell type specific manner. It was confirmed that VZV ORF63 expressed in isolation could protect SH-SY5Ys from apoptosis induction. Interestingly, VZV ORF63 also protected HaCaTs from apoptosis induction and this seemed to be linked to its relocalisation to the cytoplasm during apoptosis. This suggests that VZV ORF63 may be able to protect multiple cell types from apoptosis.

Whilst viruses such as VZV can inhibit apoptosis, other aspects of immunity can compensate for viral inhibitory mechanisms. In HSV-1 infection, cytotoxic T cells (CTL) can prevent HSV-1 reactivation and this has been attributed to the granzyme B cleavage of HSV ICP4. In other viral infections, granzyme B cleavage sites in viral

gene products can perturb granzyme B cleavage of apoptotic substrates. Interestingly, no apoptosis is evident in the context of the CTL control of HSV-1 reactivation. Natural killer (NK) cells have also been shown to play critical role in the control of alphaherpesviruses infections and utilise granzyme B to induce apoptosis in target cells. Thus, the aim of Chapter 3 was to identify novel VZV and HSV-1 proteins with granzyme B cleavage sites and to assess whether these proteins could modulate NK cell mediated cytotoxicity. VZV ORF4, VZV ORF62 and HSV ICP27 were found to be cleaved by granzyme B, however in an NK cell cytotoxicity assay, only VZV ORF4 was able to inhibit NK cell apoptosis induction in target cells. The granzyme B cleavage site in VZV ORF4 was identified via site directed mutagenesis. The mutation of the granzyme B cleavage site in VZV ORF4 did not alter its ability to protect against NK cell mediated cytotoxicity, suggesting a novel mechanism for NK cell cytotoxicity inhibition by VZV ORF4. Together this data broadens our understanding of VZV immunoevasive mechanisms in the context of innate immunity and highlights a potential role for granzyme B in the modulation of VZV lytic infection and reactivation.

The ability of VZV to modulate innate immunity could contribute to the development of PHN, as aspects of innate immunity can alter sensory neuronal functioning. For example, the skin microenvironment can alter peripheral sensory neuron functioning via the release of nociceptive substances and cytokines. VZV can infect and modulate key skin cell types such as keratinocytes which lie in close proximity to sensory neurons. Therefore, this study aimed to characterise VZV modulation of keratinocyte inflammatory transcriptional and secretory profile to identify potential factors which could alter neuronal functioning (Chapter 4). Of the transcripts examined, inflammatory cytokine transcripts were the most regulated by VZV infection, therefore supernatant was analysed for inflammatory cytokines, chemokines and growth factors. VZV infection altered the secretion of various cytokines, chemokines and growth factors in HaCaTs. Interestingly, VZV infection increased interleukin 1 receptor antagonist (IL1-RA) secretion from HaCaTs at all time points examined post infection. Together this suggests that VZV can modulate the inflammatory profile of keratinocytes which could contribute to PHN development.

To examine whether strain specific differences in VZV could contribute to PHN development four clinical isolates (2 PHN and 2 non-PHN) were examined in regard

to their growth, ability to induce cell death and modulation of ARPE-19 inflammatory cytokine/chemokine expression (Chapter 4). The clinical isolates examined did not exhibit any differences in growth or ability to induce cell death in ARPE-19s. Supernatant from infected cells was analysed to determine whether VZV clinical isolates could regulate inflammatory responses. No differences were noted between isolates; however, it was clear that VZV infection decreased IL-6 secretion and increased IL-1RA secretion. It was also shown that all isolates could downregulate IL-1α secretion from ARPE-19s suggesting that in the context of the skin, VZV can downregulate inflammatory factors and upregulate anti-inflammatory factors. This alteration of inflammatory factors could contribute to VZV pathogenesis in the skin.

Together this work identifies novel VZV immunomodulatory functions which would contribute to the ability of VZV to maintain lifelong latency and alter PHN development.

Publications of this study

Gerada C, Steain M, McSharry BP, Slobedman B, Abendroth A. 2018. Varicella-Zoster Virus ORF63 Protects Human Neuronal and Keratinocyte Cell Lines from Apoptosis and Changes Its Localization upon Apoptosis Induction. J Virol 92 (12): e00338-18.

Gerada C, Steain M, Campbell TM, McSharry BP, Slobedman B, Abendroth A. 2019. Granzyme B Cleaves Multiple Herpes Simplex Virus 1 and Varicella-Zoster Virus (VZV) Gene Products, and VZV ORF4 Inhibits Natural Killer Cell Cytotoxicity. J Virol 93 (22): e01140-19

Presentations of this study

International Meetings

2016 **16th International Congress of Immunology** (Melbourne, VIC, Australia) *'VZV ORF63 in the Inhibition of Apoptosis'* - Poster

2018 **43rd International Herpesvirus Workshop** (Vancouver, Canada*)* *'VZV ORF63 Inhibits Apoptosis in both Neuronal and Keratinocyte Cell Lines and Changes its Localisation upon Apoptosis Induction'* - **Oral** and Poster

2019 **14th World Congress on Inflammation** (Sydney, NSW, Australia) 'Modulation of Keratinocyte Inflammatory Profile by Varicella Zoster Virus' - Poster

National Meetings

2015 **8th Australasian Virology Society Meeting** (Hunter Valley, NSW, Australia) *'VZV ORF63 in the Inhibition of Apoptosis'* – Poster

2017 **9th Australasian Virology Society Meeting** (Glenelg, ADL, Australia*)*

'VZV ORF63 Inhibits Apoptosis in both Neuronal and Keratinocyte Cell Lines and Changes its Localisation upon Apoptosis Induction' - Poster

2019 **10th Australasian Virology Society Meeting** (Queenstown, New Zealand) *'Granzyme B cleaves multiple VZV and HSV-1 proteins and VZV ORF4 inhibits NK cell dependent cytotoxicity'* -**Oral** and Poster

Local Meetings

2017 **Charles Perkins Centre 3rd Early Mid-Career Research Symposium** (Sydney, NSW, Australia) *'The Chickenpox Virus and the Philosopher's Stone'* – **Oral** for lay audience

2017 **Australian Society of Immunology NSW branch meeting** (Bowral, NSW, Australia) *'VZV ORF63 Inhibits Apoptosis in both Neuronal and Keratinocyte Cell Lines Induction'* – **Oral**

2018 **Charles Perkins Centre 4th Early Mid-Career Research Symposium** (Sydney, NSW, Australia) '*The Chickenpox virus and the Holy Grail'*- Poster for lay audience

2019 **Australian Society of Immunology NSW branch meeting** (Kiama, NSW, Australia) *'Granzyme B cleaves multiple VZV and HSV-1 proteins and VZV ORF4 inhibits NK cell dependent cytotoxicity'*- **Oral**

2019 **Charles Perkins Centre 5th Early Mid-Career Research Symposium** (Sydney, NSW, Australia) *'Alien vs Predator: An epic battle between viral infection and NK cells'*- **Oral** for lay audience

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List of figures in this study

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List of tables in this study

Abbreviations

18SRNA	18 subunit ribonucleic acid
3-OH HS	Heparan sulfate 3-O sulfotransferase
ADCC	antibody dependent cell mediated cytotoxicity
AIF	apoptosis inducting factor
ANOVA	analysis of variance
ARPE-19	retinal pigment epithelial cell
ASC	Apoptosis speck-like protein with CARD domain
ATTC	American Type Culture Collection
Bcl-XL	B-cell lymphoma-extra large
BH	Bcl-2 homology
BSA	bovine serum albumin
CAD	caspase activated DNase
CC3	cleaved caspase 3
cFLIP	cellular FLICE inhibitory protein
CNS	central nervous system
COX2	cyclooxygenase-2
CPE	cytopathic effect
CRLR	calcitonin receptor like receptor
CTL	cytotoxic lymphocyte
CTV	cell trace violet
DAPI	4,6 diamidino-2-phenylinodle
DC	dendritic cell
DED	death effector domain

DISC	death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DRG	dorsal root ganglia
DW	distilled water
E	Early
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular-signal-regulated kinases
FACS	fluorescence-activated cell sorting
FADD	Fas-Associated protein with Death Domain
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FLICE	FADD-like IL-1β-converting enzyme
G-CSF	granulocyte-colony stimulating factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage-colony stimulating factor
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HRP	horse radish peroxidase
HSV-1	herpes simplex virus 1

HVEM	herpes virus entry mediator
ICAM	intercellular adhesion molecules
ICP	intracellular protein
IDE	insulin degrading enzyme
IE	immediate early
IFN	interferon
IL	interleukin
IP-10	interferon induced protein 10
IRF3	interferon regulatory factor 3
IRF7	interferon regulatory factor 7
IRL	intrinsic repeat long
IRS	intrinsic repeat short
IU	international units
JAK	janus-kinase signal transducer
KLD	kinase, ligase DpnI
KSHV	Kaposi's sarcoma-associated herpes virus
L	Late
LAK	lymphokine activated killer
LAT	latency associated transcript
MACS	magnetic-activated cell sorting
MCP-1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MIP	monocyte inflammatory protein
MLKL	mixed lineage kinase domain like protein

MMRV measles mumps rubella vaccine

MOMP mitochondrial outer membrane potential

MRC Medical Research Council

ND-10 Nuclear domain 10

NEB New England Bioscience

NGF nerve growth factor

NIR near infrared

NK natural killer cell

NKT natural killer T cell

NLR nod like receptor

ORF open reading frame

P2XR4 P2X purinoreceptor 4

PBS phosphate buffered saline

PDGF platelet derived growth factor

PE phycoerythrin

PFA paraformaldehyde

PGE2 prostaglandin E2

PHN post herpetic neuralgia

PI3K phosphoinositide 3-kinases

PRRs pattern recognition receptors

PUMA p53 upregulated modulator of apoptosis

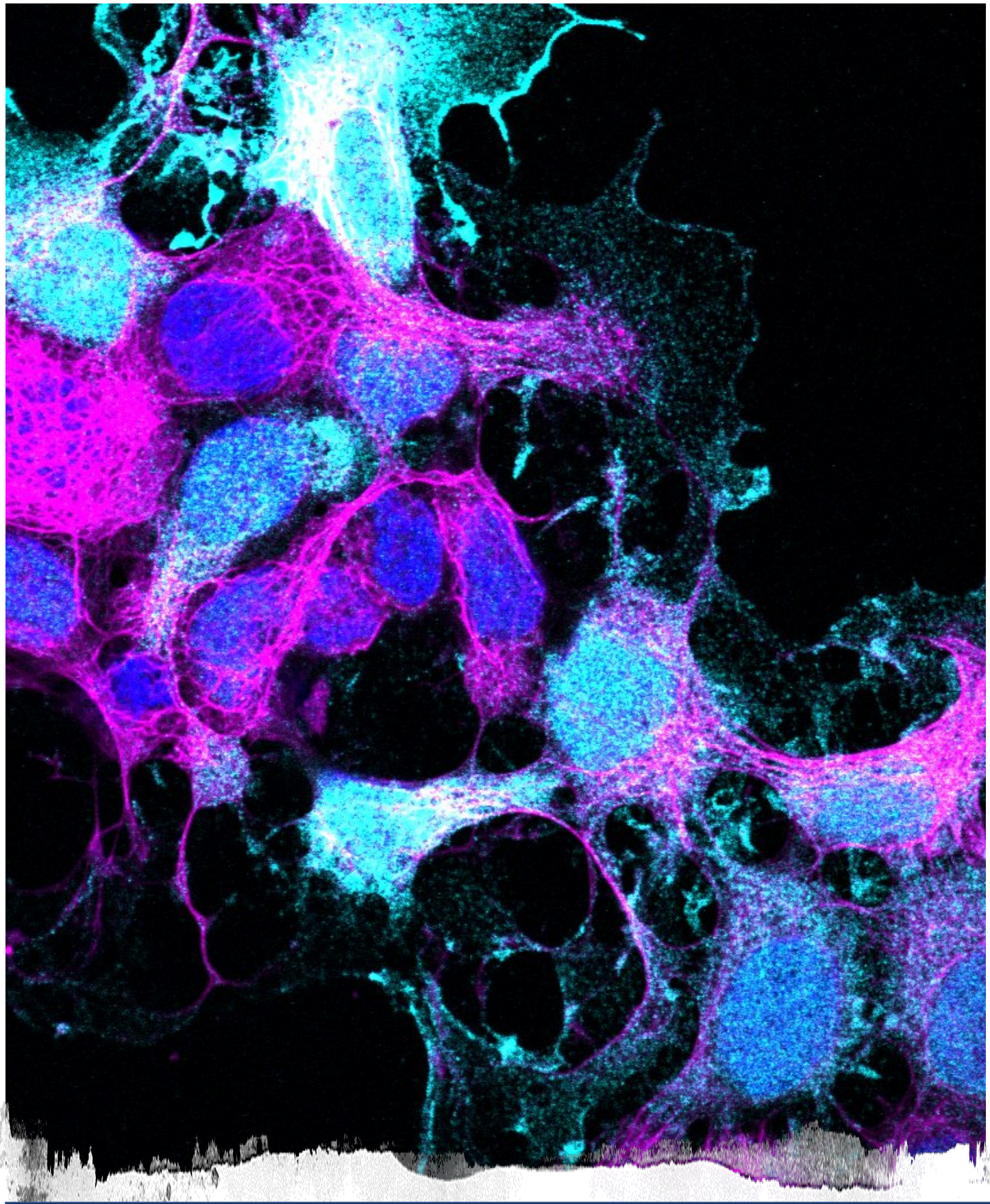
PVDF polyvinylidene difluoride

RANTES regulated on activation, normal T expressed and secreted

RIP receptor-interacting serine/ threonine-protein

RNA	ribonucleic acid
RPM	rotations per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SA	streptavidin
SCID	severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
SVV	simian varicella virus
TAP	transporter associated with antigen processing
TCR	T cell receptor
TG	trigeminal ganglia
Th1	T helper 1
TLR	toll like receptor
TNF	tumour necrosis factor
TRADD	tumour necrosis factor receptor type 1-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL R1/ R2	TNF-related apoptosis-inducing ligand receptor 1/2
TRL	terminal repeat long
TRPV1	transient receptor cation channel subfamily V member 1
TRS	terminal repeat short
UK	United Kingdom
UL	unique long

US	unique short
USA	United States of America
VEGF	vascular endothelial growth factor
VLT	VZV latency associated transcript
VZV	varicella zoster virus
XIAP	X chromosome-linked inhibitor of apoptosis protein



Chapter 1: Introduction

CHAPTER 1

1 The Herpesviridae family

The *Herpesviridae* family encompasses approximately 130 viruses, which can cause disease in a range of human and animal hosts (Pellett and Roizman, 2013). However, only 9 of the viruses in this family are tropic for the human host. Despite causing clinically distinct disease, these viruses have similar physical and biological properties. The human herpesviruses include herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesviruses 6A (HHV-6A), 6B (HHV-6B), 7 (HHV-7) and Kaposi's sarcoma associated herpesvirus (KSHV) (Pellett and Roizman, 2013).

Herpesviruses have a similar virion architecture, comprising of a double stranded (ds) deoxyribonucleic acid (DNA) genome (Gibson, 1996), an icosahedral capsid, tegument and envelope. The genome resides within the icosahedral capsid, which is composed of 162 capsomeres. In between the capsid and envelope there is an amorphous protein layer known as the tegument that contains essential and non-essential viral proteins. These structures are enclosed by a lipid bilayer known as the envelope, which also contains viral glycoproteins (Pellett and Roizman, 2013).

As well as having a similar virion structure, herpesviruses also share similar biological traits. These include the ability to establish both latent and lytic infection, nuclear DNA transcription and capsid assembly as well as a range of virally encoded enzymes. However, these viruses also possess distinguishing properties such as duration of replication, cell tropism and species-specificity, allowing them to be categorised into 3 subfamilies (Table 1.1). These subfamilies include the alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae (Pellett and Roizman, 2013).

Previously, the classification of herpesviruses was dependent upon biologically distinct features such as the site of latent infection. Due to developments in sequencing technology, the herpesvirus family is now defined by similarity in genome homology (Pellett and Roizman, 2013). The herpesvirus subfamilies, their associated human herpesviruses, the diseases they cause and defining characteristics are detailed in Table 1.1 (Pellett and Roizman, 2013).

Table 1.1 Classification of human herpesviruses, defining properties and associated human disease.

Subfamily	Defining Characteristics	Human herpesvirus and associated disease
Alpha (α)	• Rapid spread in cell culture • Variable host range • Short replicative cycle • Latency established in sensory ganglia	• Herpes simplex virus type 1 (HSV-1): Cold sores and genital herpes • Herpes simplex virus type 2 (HSV-2): Cold sores and genital herpes • Varicella zoster virus (VZV): Chickenpox and shingles
Beta (β)	• Slow spread in cell culture • Limited host range • Long replicative cycle • Latency established in lymphoreticular cells, secretory cells, myeloid progenitor cells kidney cells	• Human cytomegalovirus (HCMV): Congenital and immunocompromised infection • Human herpesvirus 6 A and B (HHV-6A and HHV-6B): Roseola • Human herpesvirus 7 (HHV-7): Roseola
Gamma (γ)	• *In vitro* replication in lymphoblastoid cells • Limited host range • T or B cell tropism • Latency established in lymphoid tissue	• Epstein-Barr virus (EBV): Glandular fever • Kaposi's sarcoma associated herpesvirus (KSHV): Kaposi's sarcoma

2 Alphaherpesvirus virion and lifecycle

2.1 Virion

Both HSV-1 and VZV share a conserved virion structure which consists of 4 structural components; the core, nucleocapsid, tegument and envelope (Straus et al., 1988, Laine et al., 2015) (Figure 1.1A). Virus particles can range from 155-240 nm in diameter with an icosahedral capsid which is typically 125 nm in diameter (Laine et al., 2015). Within the core, one copy of the linear ds DNA viral genome is arranged as a torus and is surrounded by the nucleocapsid (Furlong et al., 1972). Hexamers make up the facets of the icosahedral nucleocapsid, whilst pentamers form the vertices (Fong et al., 1973). 162 capsomeres make up the nucleocapsid and these capsomeres have a 5:3:2 axial symmetry (Almeida et al., 1962). The tegument lies between the nucleocapsid and envelope and is composed of proteins which are later enveloped with components of host cellular membranes (Cook and Stevens, 1970), causing the tegument to appear as a heterogeneous organised layer (Morgan et al., 1968, Owen et al., 2015). The envelope is spherical in shape and incorporates viral glycoproteins. HSV-1 and VZV encode 11 known homologous glycoproteins, with HSV-1 having 3 unique glycoproteins (Roizman and Campadelli-Fiume, 2007).

2.2 Genome

Similar to other members of the *Herpesviridae* family, both HSV-1 and VZV are genetically stable, a property which is demonstrated by little nucleotide variation between isolates (Norberg et al., 2004). The VZV genome is the smallest of the alphaherpesviruses at 129 kilobase pairs (kbp) (Pellett and Roizman, 2013), whilst the HSV-1 genome is larger at approximately 152 kbp (Wagner et al., 1974). The HSV-1 genome is composed of at least 80 open reading frames (ORFs) (Roizman and Campadelli-Fiume, 2007), whilst the VZV genome is composed of 71 unique ORFs (Davison and Scott, 1986). Both HSV-1 and VZV viral genomes consists of two distinct regions known as the unique long region (UL) and the unique short region (US), which are separated by an internal repeat region and are flanked by terminal repeats (Figure 1.1B). Both genomes are linear, but due to an unpaired cytosine at the 5’ end and guanine at the 3’ end, the genome can circularise during latency (Kinchington et al., 1985, Jackson and DeLuca, 2003).

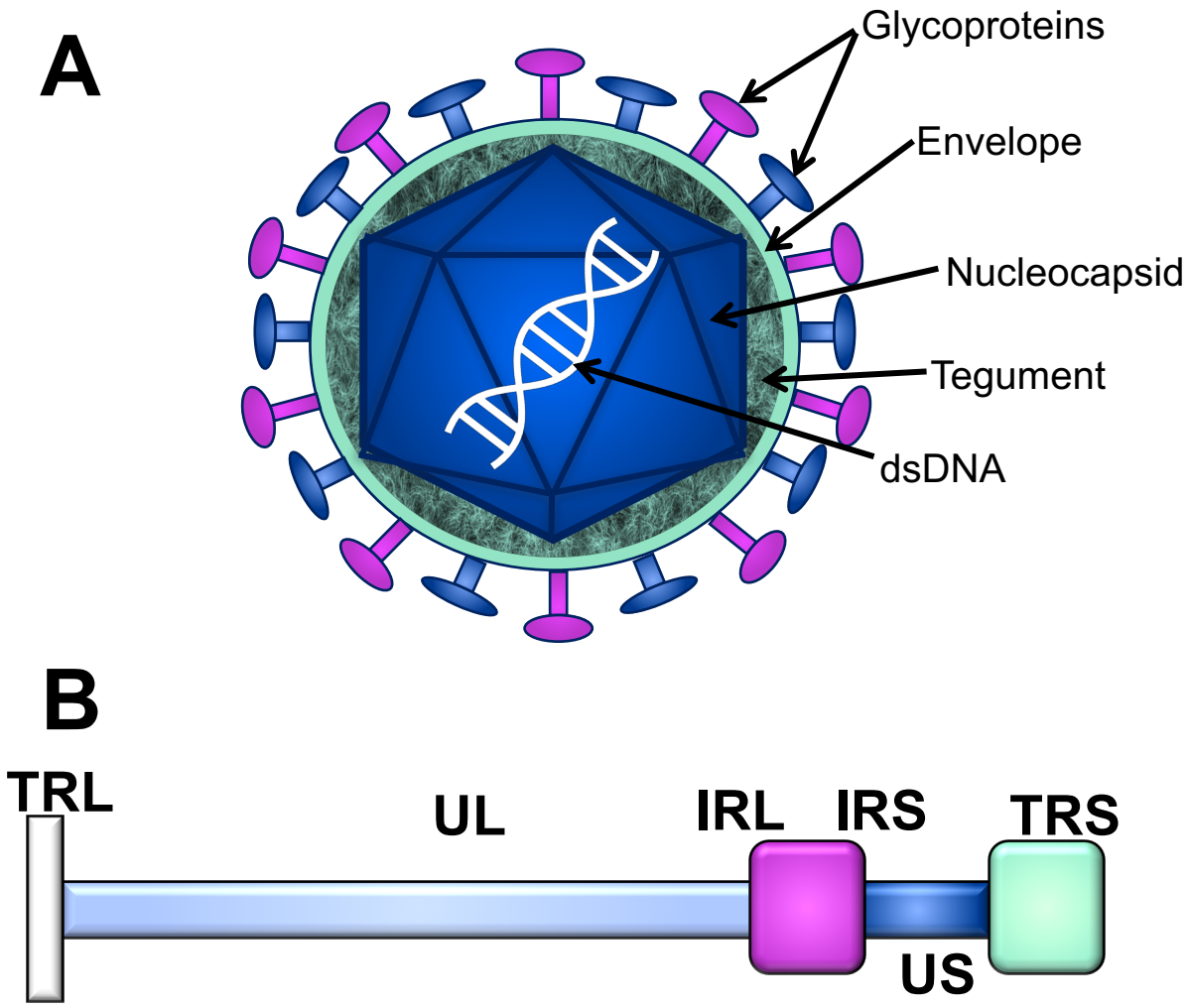


Figure 1.1 Alphaherpesvirus virion structure and genome.
Alphaherpesviruses contain glycoproteins that are crucial for attachment and entry into the host cell. These glycoproteins are embedded in the viral envelope. The VZV double stranded (ds) DNA genome is enclosed in an icosahedral nucleocapsid. The tegument protein layer lies between the nucleocapsid and envelope (A). Alphaherpesvirus genomes consist of a unique long (UL) and a unique short (US) region that are separated by an internal region which is flanked by internal repeats (internal repeat long IRL, and internal repeat short IRS). Each end of the genome consists of a tandem repeat, tandem repeat long (TRL) and tandem repeat short (TRS) (B).

2.3 Life cycle

Generally, herpesviruses have a consistent pattern of cell entry, temporal gene expression, replication and egress from the host cell (Figure 1.2). Alphaherpesvirus productive infection is initiated by virus entry and replication within the host cell and results in the cellular exit of newly synthesised infectious progeny (Honess and Roizman, 1974, Pellett and Roizman, 2013). Alphaherpesviruses, like all other herpesviruses, also have the ability to establish lifelong latent infection, in which the viral genome is maintained in an infected cell for an extensive period (Kennedy et al., 2015). In human ganglia, the site of alphaherpesvirus latency, viral genomes are highly associated with chromatin which can restrict viral gene and protein expression, disabling the ability of the virus to produce infectious progeny (Gary et al., 2006, Azarkh et al., 2010, Kristie, 2015). Reactivation from latency is identified via an increase in viral gene expression and the production of virions (Pellett and Roizman, 2013).

VZV can be distinguished from other members of the alphaherpesvirus family as it exhibits a highly restricted host specificity to human and simian cells (Weller et al., 1958, Myers and Connelly, 1992, Cohen et al., 1999). During primary infection both HSV-1 and VZV are tropic for a variety of cell types which contributes to viral spread and pathogenesis. In contrast to other alphaherpesviruses, VZV is mainly cell associated *in vitro* and therefore infection does not culminate in the release of infection virions in cell culture (Weller, 1953).

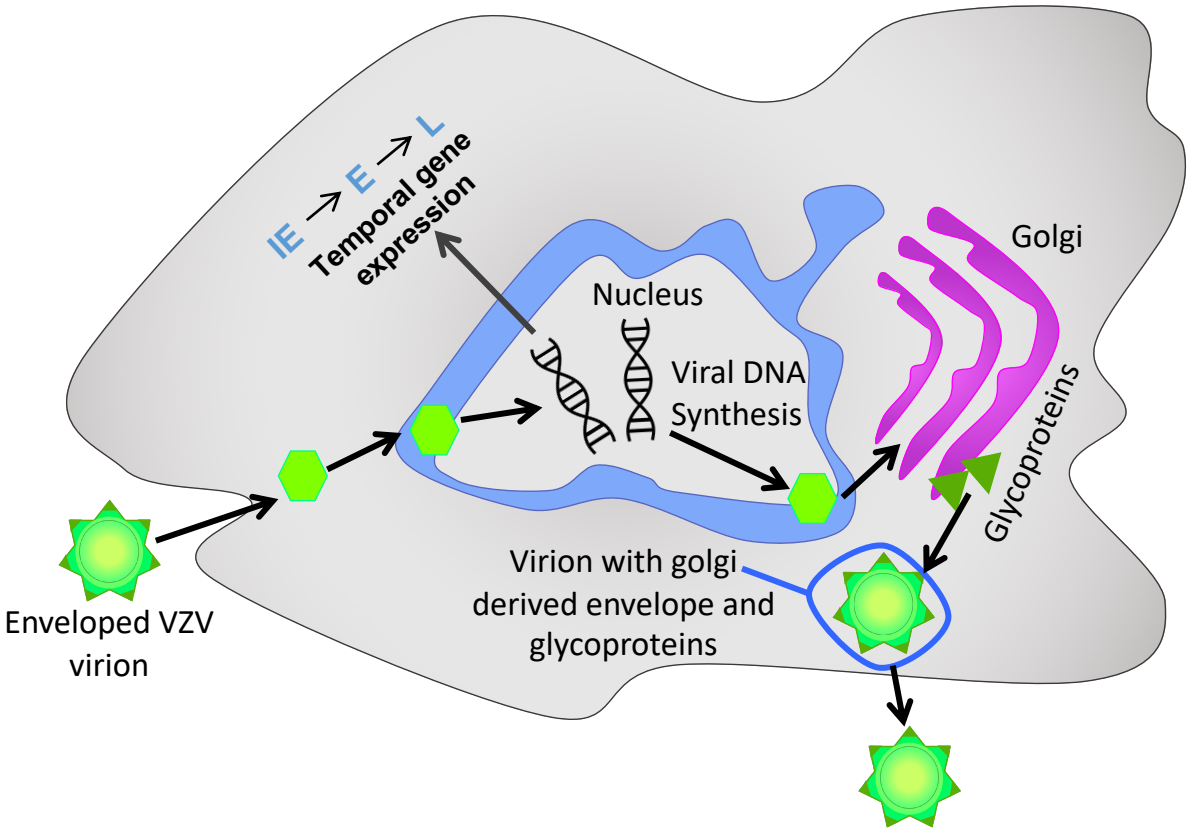


Figure 1.2 Alphaherpesvirus temporal gene expression during lytic infection.
For alphaherpesvirus entry, enveloped virions fuse with the host cell membrane. Capsids are then transported into the nucleus where temporal gene expression is highly regulated. Immediate early (IE) genes are first expressed, followed by early (E) genes and then late (L) genes. Once viral DNA is synthesized capsids are assembled and released into the cytoplasm. From there the virus can obtain an envelope and glycoproteins from the golgi apparatus. Newly synthesized virions then exit the cell.

2.3.1 Entry

Herpes virions use glycoproteins on the viral envelope to interact with cellular receptors to enter host cells (Heldwein and Krummenacher, 2008). Both HSV-1 and VZV tether to the host cell via viral glycoproteins binding to cell-surface heparan sulfate proteoglycans (Zhu et al., 1995a, Laquerre et al., 1998, Sathiyamoorthy et al., 2017). HSV-1 then requires glycoprotein D (gD) to bind either herpesvirus entry mediator (HVEM), nectin-1 or 3-O-sulfated heparan sulfate proteoglycan (3-OS HS), to allow the virus to anchor to the cellular plasma membrane and initiate fusion (Agelidis and Shukla, 2015). VZV does not encode a gD homolog and the precise nature of VZV glycoprotein interactions and the mechanisms mediating attachment are yet to be elucidated (Sathiyamoorthy et al., 2017). The mediators of VZV cell tropism are unknown, however, it has been shown that electrostatic interactions between VZV glycoproteins gB and gE and cell surface glycosaminoglycans, such as heparin sulphate, mediate the attachment of VZV virions to the cell membrane (Zhu et al., 1995a, Zhu et al., 1995b). The mannose 6-phosphate receptor has also been suggested to facilitate VZV entry into the host cell via interaction with gE (Zhu et al., 1995b, Chen et al., 2004).

Following attachment, VZV gB, gH and gL regulate the fusion of the viral envelope and cell membrane (Duus and Grose, 1996, Oliver et al., 2009, Vleck et al., 2011). Interestingly, gB interacts with myelin-associated glycoprotein to promote cell-cell fusion in neural tissue (Suenaga et al., 2010). Additionally, the sialic acids on gB are essential for this interaction (Suenaga et al., 2015). gE and gI form heterodimers and these heterodimers are postulated to bind to insulin degrading enzyme (IDE) to promote VZV cell entry (Olson and Grose, 1998, Li et al., 2006). However, in the dorsal root ganglia (DRG) this interaction is dispensable for infection, but crucial for neurovirulence (Zerboni and Arvin, 2011, Zerboni et al., 2011). Analysis of gE has also indicated that IDE binds to a truncated precursor gE protein in the cytoplasm rather than a receptor-ligand binding interaction (Carpenter et al., 2010). gH and gB contain endocytosis motifs that allow fusion of VZV virions with the cell membrane (Pasieka et al., 2004, Maresova et al., 2005). The αV intergrin subunit has also been shown to mediate VZV gB/ gH-gL fusion (Yang et al., 2016). Both HSV-1 and VZV enter the cell

via fusion or an endocytic pathway which allows the virus to release its capsid into the cytoplasm to initiate the replication process (Heldwein and Krummenacher, 2008).

2.3.2 Replication and gene expression

After viral entry, tegument proteins and the nucleocapsid are transported into the nucleus. Currently for VZV, the viral proteins and host cytoskeletal elements involved in this process are unknown, however this has been extensively characterised for HSV-1 (Sodeik et al., 1997, Arvin and Gilden, 2013, Döhner et al., 2018). Characteristic of the herpesvirus family, both HSV-1 and VZV gene expression follows in a highly regulated temporal cascade (Inchauspe et al., 1989, Perera et al., 1992). The temporal classification of genes for HSV-1 has been extensively characterised, however is yet to be fully elucidated for VZV. This is due to limited production of cell free VZV *in vitro* and subsequent difficulty in producing a synchronous *in vitro* VZV infection (Pellett and Roizman, 2013).

The first genes expressed after viral entry are immediate early (IE) genes. IE gene products are present in the tegument and are expressed independently of *de novo* protein synthesis, 3-4 hours post infection (p.i.) (Honess and Roizman, 1974). Through their ability to transactivate, these gene products enable the transcription and translation of other viral genes. Specifically for VZV, ORFs 4, 10 and 62 have been identified as initiators of viral protein synthesis (Kinchington et al., 1995, Zerboni et al., 2014). Cellular ribonucleic acid (RNA) polymerase from TATA box homologous sites within viral promoters allows for transcription of early (E) gene products to occur so that viral replication can be initiated (Wagner et al., 1995). Through the use of single cell confocal microscopy and electron microscopy it has been shown that newly synthesised ORF62, the major VZV transactivator, can appear within 1 hour p.i. and can establish prereplication sites within 4 hours (Reichelt et al., 2009). Interestingly, in addition to having critical transactivation capacities many IE genes encode for intrinsic/innate immune evasion. This is identifiable in VZV ORF62, which, in addition to its transactivation capacities, is an inhibitor of type 1 interferon (IFN) production through targeting interferon response factor 3 (IRF3) (Inchauspe et al., 1989, Sen et al., 2010).

Another IE gene product, VZV ORF63 is incorporated into the tegument and has a duplicate copy at ORF70 (Jackers et al., 1992, Kinchington et al., 1995). VZV ORF63 has crucial trans-regulatory activity and is linked with the establishment of latency (Jackers et al., 1992, Cohen et al., 2004) due to the repression of the expression of VZV genes including ORF4, 28 and 62 (Jackers et al., 1992, Bontems et al., 2002, Hoover et al., 2006). Additionally, ORF63 interferes with the type 1 IFN response through interferon regulatory factor 7 (IRF7) and eukaryotic translation initiation factor 2 alpha (eIF2α) (Ambagala and Cohen, 2007, Verweij et al., 2015). Intriguingly, ORF63 has also been shown to protect neuronal cells from apoptosis (Hood et al., 2006). Therefore, the intrinsic modulatory functions conferred by IE gene expression allow viral replication to occur and through transactivation capabilities, facilitate the synthesis of E gene products.

HSV IE genes include infected cell protein (ICP) 27, ICP4, ICP47, ICP22 and ICP0 which are viral transactivators and aid in E gene expression (Kennedy et al., 2015). HSV ICP4, ICP27 and ICP0 are homologous to VZV ORF62, ORF4 and ORF61 respectively (Kennedy et al., 2015). HSV ICP22 is genetically homologous to VZV ORF63 and encodes a similar transactivating activity, however its ability to protect against apoptosis remains unclear (Hood et al., 2006). HSV ICP47 is an IE gene that does not have a VZV homolog and can bind to transporter associated with antigen processing (TAP) and interferes with major histocompatibility complex (MHC) I synthesis and cell surface expression (Tomazin et al., 1996).

E genes encode for the machinery necessary for viral replication. For VZV these proteins include a DNA polymerase encoded by ORF28 (Abele et al., 1988) and a DNA binding protein encoded by ORF29 (Roberts et al., 1985, Kinchington et al., 1988). Other VZV proteins which have DNA binding properties include ORF51 protein, the VZV origin binding protein (Chen and Olivo, 1994, Webster et al., 1995) and a nucleocapsid protein ORF40 (Vafai et al., 1990). E genes also encode for a range of viral kinases including a thymidine kinase encoded by ORF36 (Sawyer et al., 1986). ORF47 and ORF66 proteins have serine-threonine phosphorylating activity and are incorporated in the capsid and tegument (Sato et al., 2003). ORF66 has similar substrates to protein kinase A and phosphorylates matrin 3 to modulate RNA

processing (Erazo et al., 2011). ORF66 also interacts with histone deacetylases to promote transcription (Walters et al., 2009). HSV-1 also contains many E ORFs with the ability to bind DNA and facilitate viral replication, such as ICP8 and UL52 (Boehmer and Lehman, 1993, Biswas and Weller, 1999, Arvin et al., 2007).

DNA replication occurs after E gene expression. Within the nucleus, DNA becomes circularised via complimentary unpaired nucleotides and rolling circle replication creating head-tail concatemers (Jacob et al., 1979, Ecker and Hyman, 1982, Kinchington et al., 1985). Isomerization can occur via homologous recombination (Kinchington et al., 1985). Viral nucleases cleave concatemers so they can be packaged into new virions (Jacob et al., 1979). Late (L) gene products are produced after E gene products have initiated replication. L gene products are usually structural proteins, such as glycoproteins and nucleocapsid proteins, essential for the production of infectious viral progeny (Gruffat et al., 2016). Through fluorescent and nanogold labelling of cells it has been shown that expression of structural proteins can also occur earlier in infection (Reichelt et al., 2009). VZV glycoproteins play a crucial role in mediating VZV entry and, similarly to IE and E genes, have a role in promoting VZV pathogenesis. For example, gE (ORF68) and gI (ORF67) heterodimer formation acts as a receptor for the Fc domain of immunoglobulin (Ig) G and can therefore block effector functions of IgG which facilitate the immune response, such as opsonisation and complement activation (Litwin et al., 1990, Yao et al., 1993). HSV-1 glycoproteins, as described previously, also enable viral entry and encode immunomodulatory functions to facilitate viral pathogenesis; for example, gC can modulate complement activation (Friedman et al., 1984).

2.3.3 Virion assembly and egress

In cell culture VZV is highly cell associated, however *in vivo* VZV virions are effectively released from cells (Caunt and Taylor-Robinson, 1964, Grose et al., 1979). This is in contrast to HSV-1 in which cell free virus is readily released *in vitro* (Baines and Roizman, 1992). Thus, as VZV is highly cell associated *in vitro*, it is difficult to study virion assembly and egress, and more is known in the context of HSV-1. In the current model for alphaherpesvirus egress, nucleocapsids pass through the inner and outer nuclear membranes to be delivered into the cytoplasm: involving envelopment, fusion and de-envelopment (Grose and Ng, 1992). Envelopment of naked nucleocapsids

occurs in the cisternae of the golgi apparatus (Wang et al., 1998). To aid the attachment of the naked nucleocapsids to the cisternae, tegument proteins adhere to the cytosolic surface (Hambleton et al., 2004). In an alternate model, infectious virions are enveloped upon nuclear release (Harson and Grose, 1995). Glycoproteins such as gE are involved in VZV egress and thus are crucial for VZV spread (Hambleton et al., 2004). It has also been demonstrated that the phosphorylation of ORF9 by ORF47 is crucial for the egress of VZV and that the acidic cluster of ORF9 is key to this role (Riva et al., 2015, Lebrun et al., 2018).

3 VZV and HSV-1 epidemiology and pathogenesis

3.1 Epidemiology

3.1.1 Epidemiology of VZV infection

VZV is a ubiquitous human pathogen that causes significant morbidity despite a readily available vaccine (Kawai et al., 2014, Drolet, 2017). VZV causes two clinically distinct diseases; chicken pox (varicella) during primary infection and shingles (herpes zoster) after reactivation from latency (Weller and Witton, 1958) (Figure 1.3). In contrast to a number of other medically significant viral pathogens, host factors determine the risk of severe morbidity and mortality in VZV infection (Arvin and Gilden, 2013). Historically, before vaccination was introduced, VZV was widespread in infants/children and caused devastating secondary complications such as encephalitis and pneumonia (Preblud, 1986, Jackson et al., 1992). It was reported that in the United States of America (USA) the incidence of varicella was equal to the birth rate and 11,000 of these cases resulted in hospitalisation (Marin et al., 2011). This in turn meant that almost all children became latently infected with VZV. Surprisingly, despite a vaccine being introduced, latent infection of children with VZV is postulated to still occur today, as the vaccine cannot protect against latent infection (Arvin and Gilden, 2013, Sadaoka et al., 2016). In Australia, it has been estimated that there are approximately 240,000 cases of varicella each year (Gidding et al., 2003). Additionally, 83% of children aged 10-14 are seropositive for VZV IgG (Gidding et al., 2003).

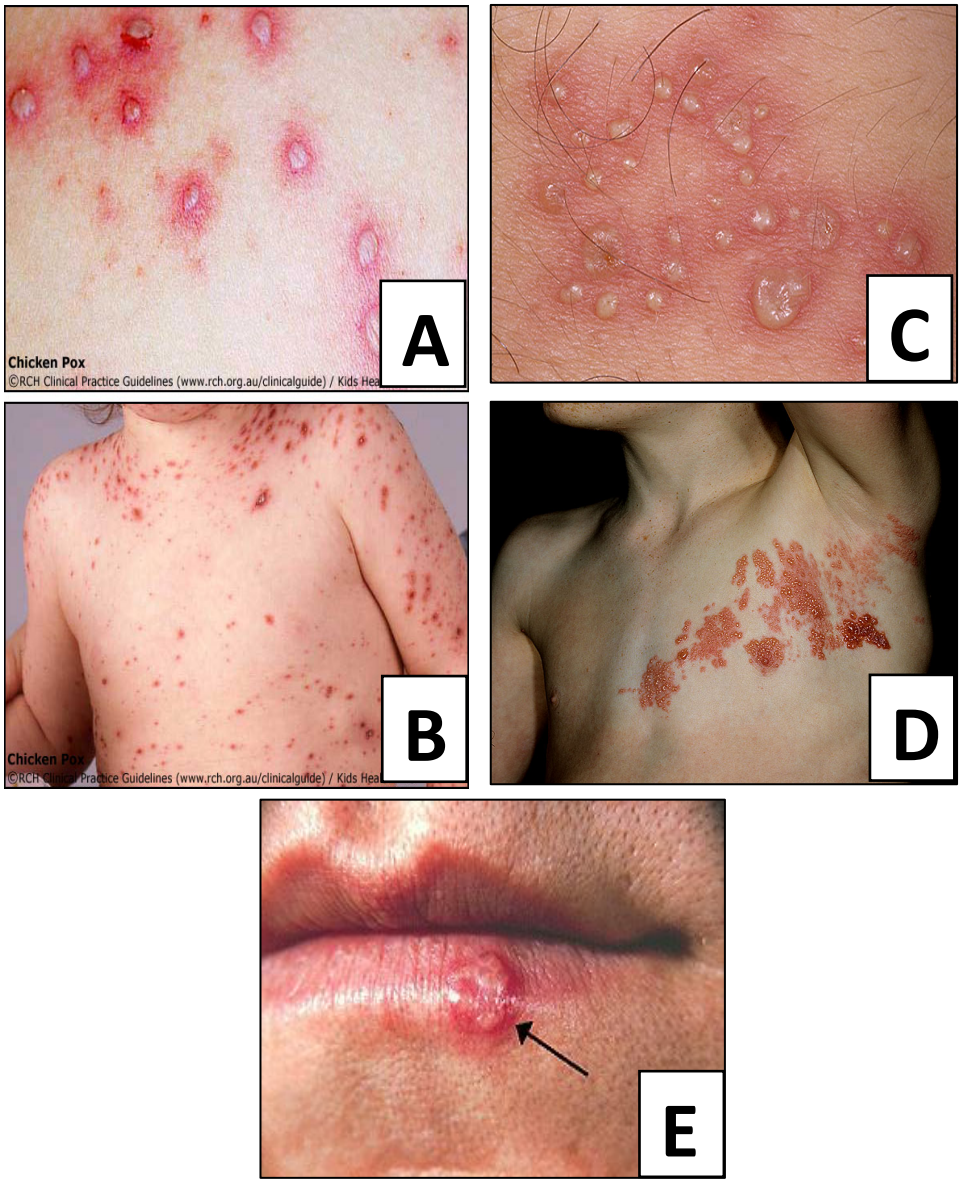


Figure 1.3 Clinical appearances of varicella, herpes zoster and oral herpes lesions.
Varicella causes a vesiculopustular rash (A). This rash is usually disseminated and may cover the entire body (B). Herpes zoster also causes a vesiculopustular rash to form on the epidermis (C). The herpes zoster rash develops unilaterally, typically across one dermatome (D). HSV-1 causes oral herpes which is more localised, usually resulting in a vesiculopustular lesion or cold sore (E). Images sourced from http://www.rch.org.au/kidsinfo/fact_sheets/Chickenpox_Varicella/, http://hardinmd.lib.uiowa.edu/dermnet/shingles45.html and https://en.wikipedia.org/wiki/Herpes_simplex

This is problematic as the age adjusted incidence of herpes zoster is increasing and the cause of this is unknown (Donahue et al., 1995, Stein et al., 2009). Herpes zoster causes significant morbidity and reduces sufferers' quality of life (Weinberg, 2007). It has been calculated that in the USA there is a 30% lifetime risk of experiencing herpes zoster and that the mean age of onset is 59.4 years (Yawn and Gilden, 2013). In Australia, the herpes zoster incidence rate between 1998-2006 was 10/1000 persons (Stein et al., 2009). Concurrently, the risk of post herpetic neuralgia (PHN) also increases with age (Choo et al., 1997, Drolet, 2017). PHN is one of many severe complications that can result from herpes zoster and is defined as a severe neuropathic pain that lasts for months to years after the resolution of the herpes zoster rash (Watson et al., 1988). From data collected between 1998-2006, the PHN incidence rate was estimated at 1.45/1000 persons in the Australian population (Stein et al., 2009).

3.1.2 Epidemiology of HSV-1 infection

Similar to VZV, HSV-1 is ubiquitous in the human population, however unlike VZV there is no commercially available vaccine for HSV-1. HSV-1 infection can cause oral herpes and genital herpes, and similar to VZV becomes latent and can reactivate. However reactivation events for HSV-1 are more frequent than for VZV, and do not cause a clinically distinct disease from primary HSV-1 infection (Pellett and Roizman, 2013) (Figure 1.3). HSV-1 is widely prevalent, with the seroprevalence in Australia being approximately 76% and world-wide approximately 90% (Cunningham et al., 2006, Arvin et al., 2007, McQuillan et al., 2018). Using mathematical modelling, HSV-1 seroprevalence is predicted to decline to 54.8% in the USA in 2018 and to 48.5% in 2050 due to decreased exposure during childhood (Ayoub et al., 2019). Compared with the other serotype HSV-2, HSV-1 infections are more frequent particularly in developed countries (Cunningham et al., 2006).

3.2 Pathogenesis of VZV infection

3.2.1 Varicella

VZV initially causes varicella within the human host and was one of the most common childhood infectious diseases (White et al., 1991). VZV can be transmitted via the inhalation of respiratory droplets or ingestion of vesicle fluid (Leclair et al., 1980, Gustafson et al., 1982) (Figure 1.4). Characteristic symptoms of varicella include an itchy, vesicular disseminated rash, malaise and low grade fever (Arvin and Gilden, 2013). As mentioned previously, varicella can cause a variety of serious complications such as encephalitis and vasculopathy, however the incidence of these has decreased with the introduction of vaccination (Appelbaum et al., 1953, Liese et al., 2008, Nagel et al., 2008, Elena et al., 2011). In spite of this, immunocompromised individuals are still at risk of these complications (Wiegering et al., 2011). Interestingly, if varicella is contracted during adulthood, symptoms are generally more severe and there is an increased risk of complications, however the reasons for this phenomenon are unknown (Gershon, 2008, Marin et al., 2008).

Initially, VZV replicates in the upper respiratory mucosa, where it is believed to be transported to lymph nodes such as the tonsils via dendritic cells (DCs) (Bogger-Goren et al., 1984, Abendroth et al., 2001b, Sen et al., 2014). It is postulated that the virus comes into contact with and infects T cells in these secondary lymphoid tissues (Abendroth et al., 2001b, Morrow et al., 2003). T cell tropism is crucial for the development of varicella and furthermore VZV has been shown modulate T cell function (Moffat et al., 1995, Ku et al., 2002, Sen and Arvin, 2016, Jones et al., 2019). Primary viremia occurs during an incubation period of 10-21 days in the lymph nodes and mononuclear cells, which is consistent with the clinical symptoms of varicella such as fever and malaise (Arvin and Gilden, 2013). Secondary viremia occurs after the virus has replicated in the liver and spleen (Grose, 1981, Arvin and Gilden, 2013). It has been demonstrated that during viremia VZV infected T cells upregulate skin homing markers required to transport VZV to the dermis, resulting in the infection of epidermal cells (Ku et al., 2004).

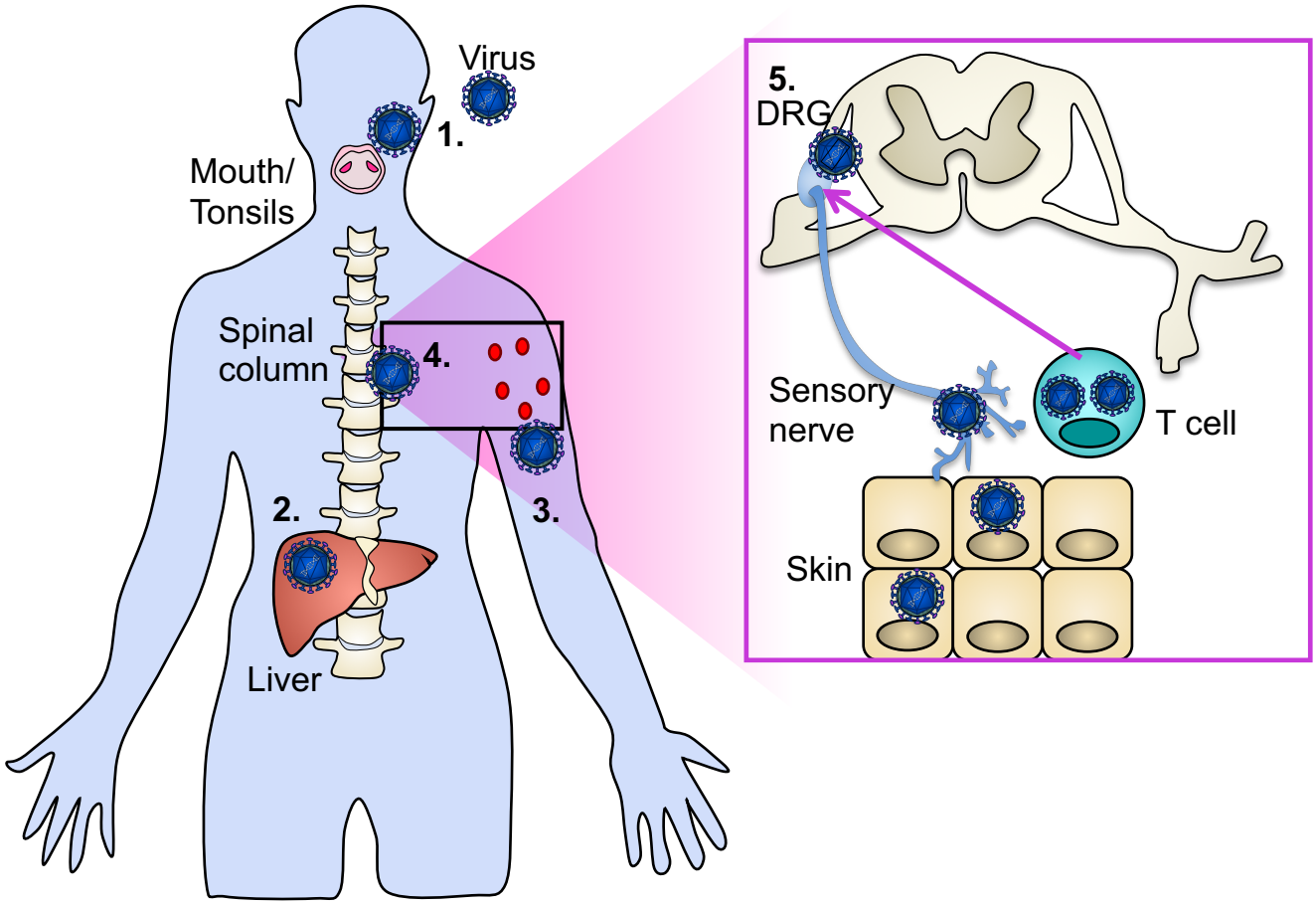


Figure 1.4 Pathogenesis of Varicella Zoster Virus.
VZV virions are inhaled (1) causing the respiratory mucosa to become infected. Within the respiratory mucosa immature dendritic cells (DCs) are proposed to become infected. VZV infected DCs drain in tonsillar lymph nodes, which are postulated to transmit virus to T cells, resulting in primary viremia. Secondary viremia occurs in the liver and spleen causing the virus to disseminate to the reticuloendothelial system (2). Infected T cells upregulate skin homing markers and travel to skin, causing the characteristic varicella rash (3). During varicella VZV infects epidermal sensory nerve termini and via retrograde axonal transport infects the sensory ganglia (4 & 5). Alternatively it has been proposed that VZV is trafficked to the sensory ganglia by infected T cells (5). Within the sensory ganglia, VZV establishes life long latency in neurons and has the potential to reactivate to cause herpes zoster.

VZV productively infects a wide variety of cell types in the skin such as Langerhans cells (LCs), keratinocytes and fibroblasts (Nikkels et al., 1995, Annunziato et al., 2000, Cunningham et al., 2010, Huch et al., 2010). VZV modulates the signalling pathways of these cell types to enhance viral spread and dissemination (Jones and Arvin, 2003, Black et al., 2009, Huch et al., 2010). For example, VZV decreases the secretion of IFNα by plasmacytoid DCs *in vitro* (Huch et al., 2010) and alters the capacity of keratinocytes to stimulate T cell responses (Black et al., 2009). The virus produces a vesicular, disseminated rash, which is initially maculopapular in nature in the skin (Arvin and Gilden, 2013). Lesions develop into fluid filled vesicles which contains infectious virus (Ross et al., 1962). The cutaneous rash is usually resolved within 1-2 weeks in which the lesions crust over and slough off (Mueller et al., 2008). Through skin cells in cutaneous VZV lesions or via T cell viremia, the virus is transported in a retrograde fashion to the sensory ganglia, where it can establish a lifelong latent infection (Hope-Simpson, 1965, Zerboni et al., 2005b, Mueller et al., 2008).

VZV latency is characterised by the presence of viral DNA in ganglionic neurons, limited viral gene transcription and the potential for reactivation (Gilden et al., 1983, Croen et al., 1988, Grinfeld and Kennedy, 2004). Recently, the transcriptional activity of VZV during latency has been more extensively characterised using ultra-deep virus-enriched RNA sequencing of latently infected trigeminal ganglia (TG) (Depledge et al., 2018). A spliced VZV mRNA antisense to VZV ORF61, termed VZV latency associated transcript (VLT), was the most abundant transcript produced during latency. Interestingly, this transcript encodes for a protein which has late kinetics both *in vitro* and in shingles skin lesions, however the function of the transcript during latency is yet to be elucidated (Depledge et al., 2018). The only other VZV transcript detected during latency was the VZV ORF63 transcript which has been previously been detected during latency in both rodent and human ganglia (Cohen et al., 2004, Depledge et al., 2018).

3.2.2 Herpes zoster

VZV can later reactivate from lifelong latency to cause herpes zoster, which characteristically involves dermatomal pain, malaise, fever, headache and a skin rash, typically across a single dermatome (Guess et al., 1985, Donahue et al., 1995,

Schmader and Dworkin, 2008). The chance of experiencing herpes zoster increases with age and a decline in VZV specific T cell immunity (Hayward and Herberger, 1987, Buchbinder et al., 1992, Yawn and Gilden, 2013), however the molecular mechanisms which govern reactivation are yet to be elucidated. IE genes ORF61 and ORF63 have been linked to latency and reactivation. It is proposed that ORF61 protein is required for reactivation as it causes the nuclear import of the major transregulatory protein ORF63. (Walters et al., 2008, Gershon et al., 2010). Additionally, ORF61 protein dismantles host nuclear domain (ND)-10 structures which usually work to silence viral gene expression and are important in the outcome of HSV-1 infection (Everett et al., 2010). ORF63 inhibits neuronal apoptosis (Hood et al., 2006), which would be crucial for the maintenance of latency and successful reactivation. In *in vitro* models of VZV latency the inhibition of phosphoinositide 3-kinases (PI3K) and the removal of neurotropic factors such as nerve growth factor (NGF) can cause VZV reactivation, highlighting the potential importance of PI3K and NGF in maintaining VZV latency (Baird et al., 2019).

Immune surveillance and T cell dependent proteolysis of HSV viral proteins is critical for regulating HSV reactivation, however analysis of human trigeminal ganglia demonstrates few to no T cells surrounding neurons containing latent VZV (Hüfner et al., 2006, Verjans et al., 2007, Knickelbein et al., 2008). VZV regulation of MHC presentation has been suggested to account for this lack of T cell presence (Eshleman et al., 2011). Additionally, it has been suggested that VZV proteins could be sequestered within cytoplasmic vesicles in neurons, thus making them unavailable for antigen presentation (Verjans et al., 2007). However, it remains unclear as to why there is a lack of resident cytotoxic T cells in VZV latently infected ganglia. Therefore, more research is required to understand the complexity of the maintenance of VZV latency and the initiation of reactivation.

During VZV reactivation, all classes of VZV genes are expressed and infectious virions are produced (Arvin and Gilden, 2013). In DRG xenografts implanted in severe combined immunodeficiency (SCID) hu mice, VZV replicates in both neurons and satellite cells, causing fusion and polykaryocyte formation (Reichelt et al., 2008). Neuron-satellite cell fusion has also been observed in ganglia from patients with herpes zoster at the time of death and is suggested to amplify the spread VZV to

neuronal cell bodies in the DRG (Esiri and Tomlinson, 1972) . VZV reactivation is often associated with inflammation, haemorrhagic necrosis and degeneration of related motor and sensory roots (Gilden et al., 2000, Leinweber et al., 2006, Oaklander, 2008).

Reactivation involves anterograde transport of the virus from the reactivating ganglia to the innervated dermatome (Arvin and Gilden, 2013). Transport has been suggested to occur along myelinated nerves which terminate in the epidermis near hair follicles (Muraki et al., 1996, Markus et al., 2011, Baird et al., 2013). The mechanisms involved in VZV transport from the DRG to the skin have not been comprehensively investigated. Stem cell derived neurons have been used to develop an *in vitro* model of VZV latency and reactivation to address this under investigated area (Markus et al., 2015). The transport of virus to the epidermis causes the infection of epithelial cells and the generation of the characteristic herpes zoster rash, which occurs across a single dermatome (Abendroth et al., 2001b). The appearance of the rash can be preceded by a unilateral dermatomal pain, otherwise known as zoster associated pain (ZAP) (Haanpää et al., 1999). Additionally, herpes zoster decreases cutaneous innervation at the site of the rash (Rowbotham and Fields, 1996). On occasion, VZV reactivation results in pain with a dermatomal distribution without the appearance of a rash; this is known as zoster sine herpete (Gilden et al., 1994).

Herpes zoster causes a variety of debilitating complications especially for immunocompromised hosts. Reactivation in the trigeminal ganglia can cause ophthalmic diseases such as conjunctivitis, iridocyclitis or Ramsay Hunt syndrome (Kawai et al., 2014). Immunocompromised individuals who suffer from herpes zoster are predisposed to conditions such as Gullian-Barre syndrome, vasculopathy and ventriculitis (Kawai et al., 2014). The most prolific complication of herpes zoster is post herpetic neuralgia (PHN), which can severely impact the sufferer's quality of life (Gupta and Smith, 2012).

3.2.3 Post herpetic neuralgia (PHN)

PHN can be defined as the presence of pain more than one month after the resolution of the herpes zoster rash (Kost and Straus, 1996, Gupta and Smith, 2012). The types of pain experienced by patients can be described as a deep aching or burning pain, electric shock-like pains, hyperesthesia (exaggerated responses to stimuli) and

dysesthesia (increased painful sensitivity to touch). Additionally, some patients report allodynia (pain response to innocuous stimuli) and an extreme itching sensation (Kost and Straus, 1996, Gupta and Smith, 2012). The chronic neuropathic pain experienced during PHN can cause mental illness and sleep disturbance (Drolet, 2017). There are a variety of factors which could predispose patients to developing PHN. These include patient factors such as age, sex and immune status (Forbes et al., 2016) as well as viral factors. Viral factors which could affect the likelihood of developing PHN include novel variants of VZV with altered pathogenesis, as well as VZV induced changes in the ganglia and skin.

During herpes zoster, it has been shown that the skin is inflamed, and signs of haemorrhagic necrosis and neuronal loss may develop (Kleinschmidt-DeMasters and Gilden, 2001). Inflammation in the peripheral nerves can cause demyelination, Wallerian degeneration and sclerosis (Kost and Straus, 1996). Additionally, changes are noted in the central nervous system during herpes zoster which could influence the development of neuropathic pain; these include degeneration of the dorsal horn of the spinal cord, unilateral segmented myelitis and letpomeningitis (Kost and Straus, 1996). Pathologically, it has been identified that patients with PHN have increased atrophy of the dorsal horn in the spinal column (Watson et al., 1991). Therefore, it is clear changes in both the skin and the ganglia can lead to pathological sequelae during herpes zoster which could influence the development of PHN.

The effect of VZV reactivation in ganglia has been studied in regard to the immune cell infiltrate and through use of rat models (see 3.2.3.1). However, the involvement of the skin microenvironment in PHN development has remained largely unstudied. This is in spite of the fact that transient receptor potential cation channel subfamily V member 1 (TRPV1) expression in epidermal keratinocytes is increased in patients with PHN, highlighting a potential role of the skin in PHN development (Han et al., 2016). Altogether, it is unclear why particular patients develop PHN. It is known that VZV can infect and modulate cells present in both the skin in the periphery and in the ganglia, which could contribute to the development of PHN. Additionally, a variety of patient factors have been found to correlate with PHN development such as age, gender, diabetes mellitus and recent trauma (Forbes et al., 2016). The contribution of VZV modulation of the ganglia and skin to PHN development will be discussed below.

3.2.3.1 Contribution of ganglia to PHN development

The DRG is a network of sensory and central neurons which allows sensory information to be transmitted from peripheral sites such as the skin to the central nervous system (CNS), where it can be processed by the brain (Krames, 2015). Therefore, the DRG plays a critical role in nociceptive signalling. As mentioned previously, the DRG is the site of VZV latency and reactivation, however there are limited studies which have explored the impact of reactivation on human ganglia function (Zerboni et al., 2014).

Recently, animal models of VZV induced pain have been developed, including a rat model in which rats are inoculated with VZV in the foot pad (Dalziel et al., 2004, Guedon et al., 2015). The rats displayed signs of allodynia for up to 35 days p.i., however the virus underwent an abortive infection, suggesting that VZV does not need to replicate to cause pain in this model (Kinchington and Goins, 2011). VZV DNA was found in the DRG of the rats and this was associated with differential host gene expression patterns that were related to nociception (Guedon et al., 2015). A human DRG xenograft mouse model has also been utilised and has identified VZV infection of satellite cells and polykaryon formation between neurons and satellite cells, which could affect neuronal function and therefore contribute to PHN development (Reichelt et al., 2008). Using this model it has also been shown that VZV infection in the DRG induced pro-inflammatory responses, which could influence the development of neuropathic pain (Zerboni and Arvin, 2015). Simian varicella virus (SVV) causes a similar pathogenesis to VZV in monkeys and has also been used to investigate VZV induced changes in ganglia (Mahalingam et al., 2010, Sorel and Powers, 2018). SVV reactivation in rhesus macaques caused a systemic pro-inflammatory response (Traina-Dorge et al., 2014). Additionally, T cell infiltration was detected in the ganglia of cynomolgus macaques during SVV reactivation (Ouwendijk et al., 2013). This T cell infiltration was linked to an increase in the chemokine CXCL10 (Ouwendijk et al., 2013). The effect of this immune cell infiltration on the functionality of sensory neurons in the DRG of SVV infected macaques is yet to be investigated and could provide some fascinating insights into PHN development.

There has been limited work on the effect of VZV reactivation in human DRG due to limited availability of human ganglionic samples. Ganglia isolated from individuals who

had experienced herpes zoster between 1 to 4.5 months before death have been utilised to characterised immune cell infiltrate into the ganglia following herpes zoster (Gowrishankar et al., 2010). This infiltrate consisted of non-cytolytic CD8+ T cells with smaller proportions of CD4+ T cells, natural killer (NK) cells and macrophages (Gowrishankar et al., 2010). The effects of natural VZV reactivation on the ganglia has been further characterised by the study of ganglia isolated from individuals who had active herpes zoster at the time of death (Steain et al., 2014). Ganglia from the site of reactivation demonstrated pathology such as necrosis, secondary to vasculitis or haemorrhage, which could contribute to PHN development (Steain et al., 2014). It was observed that cells of the immune system such as CD8+ T cells localise to the ganglia. T cells were closely associated with neurons in the ganglia, however there was little evidence of neuronal apoptosis (Steain et al., 2014). Whilst T cells were not causing neuronal apoptosis, it is possible that they, along with other infiltrating immune cells, could secrete substances that would affect neuronal function. VZV infects satellite cells in the human DRG xenograft mouse model, and it would be of interest to determine how this affects neuronal function, due to their role in controlling neuronal functional homeostasis (Zerboni and Arvin, 2015). More recently studies of ganglia from a patient with PHN have revealed that B cells, CD4+ and CD8+ T cells infiltrate the ganglia (Sutherland et al., 2019). Interestingly, ganglia from the PHN patient had similar levels of CD4+ and CD8+ T cells whereas ganglia from herpes zoster patients had a predominance of CD4+ T cells (Sutherland et al., 2019).

3.2.3.2 Contribution of skin to PHN development

The skin is a complex microenvironment comprised of sensory neuron termini, immune cells and a variety of skin cells such as fibroblasts, keratinocytes and LCs. VZV has been found to infect these cell types (Hayward et al., 1986, Sexton et al., 1992, Huch et al., 2010) and thus could modulate this microenvironment to affect peripheral neuronal signalling. Keratinocytes are of particular interest as they have been found to have many functions in the skin, such as barrier formation, regulation of the immune microenvironment and more recently, regulation of neuronal cells (Pasparakis et al., 2014, Ritter-Jones et al., 2016, Hesselink et al., 2017). Keratinocytes have close functional contact with sensory afferent nerves and stimulation of keratinocytes alone can cause nociception-related responses (Baumbauer et al., 2015). A broad variety of receptors, neuropeptides and

neurotransmitters are expressed by keratinocytes and have been found to directly affect neuronal nociceptive function (Hesselink et al., 2017). Several studies have highlighted that these cells have a key role in the development of neuropathic pain through communication with a variety of cell types such as neurons and immune cells (Radtke et al., 2010, Hou et al., 2011, Pang et al., 2015, Hesselink et al., 2017).

VZV has been shown to infect keratinocytes *in vitro* and *in vivo* in both varicella and herpes zoster skin lesions (Nikkels et al., 1995, Han et al., 2016) and furthermore can regulate keratinocyte-immune cell interactions (Black et al., 2009) (more detail will be provided in Section 5.4.2.1). Despite evidence of VZV regulating keratinocyte differentiation (Jones et al., 2014) and immune function (Black et al., 2009) there has been little research on the ability of VZV to modulate keratinocyte nociceptive capacity. VZV modulation of this cell type during herpes zoster may contribute toward peripheral sensitisation in PHN. One study analysing herpes zoster skin lesions demonstrated that TRPV1 mRNA and protein was increased in epidermal keratinocytes and this correlated with the degree of pain experienced by the patients (Han et al., 2016). As TRPV1 had been implicated in the development of neuropathic pain, this suggests that VZV may increase the nociceptive properties of keratinocytes. However, it has not been investigated whether other properties are influenced by VZV infection, such as the release of nociceptive molecules by keratinocytes. This information is critical as it could provide novel therapeutic targets for reducing or eliminating the burden of PHN on the human population. One clinical trial used topical therapy with cannabinoid agonists on patients with facial PHN and found that 5/8 patients experienced a mean pain reduction of 87.8% (Phan et al., 2010). Interestingly, cannabinoid receptors are present on both neurons and keratinocytes in the skin (Hesselink et al., 2017), therefore it would be interesting to determine *in vitro* whether cannabinoid agonists affect VZV infection and pathogenesis of both neurons and keratinocytes. With the recent development of a rat model of VZV induced pain (Guedon et al., 2014) it may also be possible to elucidate the molecular and behavioural effects of cannabinoid receptor agonists *in vivo*.

3.3 Pathogenesis of HSV-1

Similarly to VZV, HSV-1 is usually contracted in childhood to young adulthood, however reactivation events can occur throughout life (Thellman and Triezenberg, 2017). Pathogenesis of HSV-1 begins when mucosal surfaces or abraded skin are inoculated via direct contact with infectious virus (Kumar et al., 2016). Previously, initial HSV-1 infection was most prevalent in oropharyngeal mucosa, however HSV-1 is now also being detected in the genital tract (Ryder et al., 2009). HSV-1 can cause both ocular and cutaneous infections (Toma et al., 2008, Rolinski and Hus, 2014). During primary HSV-1 infection the virus gains access to sensory nerve termini and is transported in a retrograde fashion to the TG, where the virus establishes lifelong latency (Antinone and Smith, 2010, Kumar et al., 2016). During latency, a latency associated transcript (LAT) is produced in neurons, which inhibits apoptosis and is critical for the development of spontaneous reactivation (Perng et al., 1994, Carpenter et al., 2015). Many triggers have been found to stimulate HSV-1 reactivation such as facial trauma, sun exposure and stress (Grinde, 2013). Once the virus has reactivated, HSV-1 travels in an anterograde fashion along the sensory nerves to the skin, where asymptomatic shedding or the formation of lesions can occur (Wisner et al., 2011). As stated previously, HSV-1 reactivation is recurrent, typically occurring multiple times within the host's life (Kumar et al., 2016). Interestingly, HSV-1 does not commonly cause neuropathic pain even though it is closely related to VZV and shares many features of pathogenesis (Dalziel et al., 2004). The reason for this is yet to be elucidated, however some factors may include; location of reactivation, differential immune control of reactivation events and age that reactivation events occur (Choo et al., 1997, Gupta and Smith, 2012).

HSV-1 can cause more severe disease in immunocompromised hosts, similarly to VZV (Herget et al., 2005). This is due to the importance of the immune response to control viral spread and pathogenesis (Arvin et al., 2007). HSV-1 is the most common cause of infectious encephalitis which can be fatal or result in severe neurological sequelae (Tyler, 2018). Newborns are susceptible to developing neurological problems from HSV-1 infection, due to their immunonaive status and immature/developing immune system (Kimberlin, 2007).

3.4 Treatment and vaccination

3.4.1 Treatment and vaccination for VZV

Due to potential complications arising from varicella and herpes zoster, pharmaceutical agents are often used when an individual is diagnosed with either condition. Acyclovir is most commonly used, along with the derivative nucleoside analogues famciclovir and valacyclovir (Elion et al., 1977, Bean et al., 1982, Kim et al., 2014). By using antivirals, the period of viral shedding decreases and there is a decrease in lesion formation and subsequent pain (Balfour Jr et al., 1983, Balfour et al., 1992, Sackel et al., 2014). Some novel agents such as FV-100, a bicyclic nucleoside analog, have been developed to expand the options for treating VZV (McGuigan et al., 2007, Pentikis et al., 2011). Even though there are safe antiviral pharmaceuticals available, these drugs do not stop the virus establishing latency and/or reactivating. Therefore, there has been an emphasis in current research on prevention via the use of vaccines (Hambleton and Gershon, 2005).

The vOka vaccine (Varivax) is a live attenuated VZV strain, which was derived from the clinical Oka strain after 30 passages in guinea pig and human fibroblasts (Takahashi et al., 1975, Asano et al., 1985, Ndumbe et al., 1985). Attenuation has been suggested to be due to a decreased ability to replicate in skin cells (Zerboni et al., 2005a). However, vOka has no impairment in infecting T cells and can still downregulate MHC class I expression (Moffat et al., 1998, Abendroth et al., 2001a). vOka infected DCs upregulate the host T helper (Th) 1 response to infection by the upregulation of IFN-γ and interleukin (IL) -12, when compared to other virulent VZV strains (Gutzeit et al., 2010).

Routine vaccination in Australia was introduced in 2005 and recently the development of the measles, mumps and varicella (MMRV) vaccine has aided in simplifying administration and compliance (Nolan et al., 2002, Kuter et al., 2006). A major problem with this vaccine is the ability of vOka to establish latency and potentially reactivate to causes herpes zoster; this reactivation is rare but has been well documented (Sharrar et al., 2000, Wise et al., 2000, Gershon, 2001, Uebe et al., 2002, Sauerbrei et al., 2003). Viral mutations which could stop the virus entering and becoming latent in neuronal cells should be investigated to decrease the ability of the virus to reactivate

and cause herpes zoster. Some candidate genes have been identified, such as ORF63 as it was shown to be critical for the establishment of latency in a cotton rat latency model (Cohen et al., 2004). ORF66, gI, ORF10 and ORF7 have also been suggested as candidate genes which are important for VZV neurovirulence (Zerboni and Arvin, 2011).

To address the incidence and severity of herpes zoster in the ageing population, a vaccine was designed for the prevention of herpes zoster. This vaccine consists of the same strain used for the varicella vaccination but is 14 times more concentrated (Oxman et al., 2005). It has resulted in a reduction of herpes zoster by 49-51%, a decrease in disease severity and a decreased incidence of PHN, in randomised placebo controlled trials (Oxman et al., 2005, Gelb, 2008, Levin et al., 2008). Despite this, a two-dose vaccination trial did not improve antibody responses within individuals over 70 years of age (Vesikari et al., 2013). Suggested improvements to this vaccine include administering multiple doses of higher potency vaccine and designing attenuated vaccines which overexpress immunogenic VZV epitopes (Arvin, 2008). A more recent development, is the use of an adjuvanted recombinant gE subunit vaccine (Leroux-Roels et al., 2012). The vaccine has shown higher immunogenicity than the current vOka vaccine as well as having a good safety profile (Leroux-Roels et al., 2012, Lal et al., 2015, Cunningham et al., 2016, Sacks, 2019).

Despite recent progress in the development of novel treatment and vaccination strategies for herpes zoster, around 20-35% of individuals will experience herpes zoster and, of these, around 10-30% will develop PHN (Drolet, 2017). Studies have shown PHN can negatively affect measures such as productivity, mental health and life enjoyment (Drolet, 2017). Therefore, more research is needed to determine how VZV interacts with and modulates factors essential to viral pathogenesis such as the immune response, so that more effective prevention and treatment strategies can be developed.

3.4.2 Treatment and vaccination for HSV-1

Currently, there is no commercially available vaccine for HSV-1 or HSV-2, however there are preventative and therapeutic vaccines in development. Acyclovir is a nucleoside drug which is used to treat oral and genital herpes, other analogues such

as penciclovir are also used (Arduino and Porter, 2006). These pharmaceutical agents selectively inhibit the viral DNA polymerase and as such cause minimal cytotoxicity to the host (Kim et al., 2014). Systemic use of acyclovir was found to reduce the duration of symptoms of recurrent HSV-1 infection (Arduino and Porter, 2006). Therefore, pharmaceuticals can be used to manage the symptoms and duration of HSV-1 infection. To prevent HSV-1 disease, several vaccines are being developed. Herpevac is a subunit vaccine which contains a truncated gD2 in HSV-2 and showed 58% vaccine efficacy for the prevention of genital HSV-1 disease and 32% efficacy for prevention of primary infection in seronegative women (Johnston et al., 2016). However, this vaccine did not protect against HSV-2 disease. There are also several live attenuated vaccines in the pre-clinical phase with varying levels of success (Johnston et al., 2016, Carr and Gmyrek, 2019, Royer et al., 2019).

4 The innate immune response in controlling viral pathogenesis

The innate immune response is critical in the early detection and control of viral disease, as well as the development of effective adaptive immunity, to limit viral spread and pathogenesis. Once the virus has entered a host cell, subsequent host cell damage or detection of viral components can trigger various forms of programmed cell death to stop viral spread to neighbouring cells (Upton and Chan, 2014) (Figure 1.5). Pattern recognition receptors (PRRs) allow cells to sense viral components such as dsDNA and cause the production of type I IFNs and inflammatory cytokines in a cell type specific manner (Takeuchi and Akira, 2009). These critical cytokines cause neighbouring cells to enter into an antiviral state which can limit the spread of viral infection (Hoffmann et al., 2015).

Through the production of cytokines and chemokines, innate immune cells such as monocytes, NK cells and DCs can migrate to the infected area and become activated to help control the virus locally and begin a cascade of adaptive immunity (Takeuchi and Akira, 2009). All of these components work synergistically and have been found to be critical to limiting viral spread.

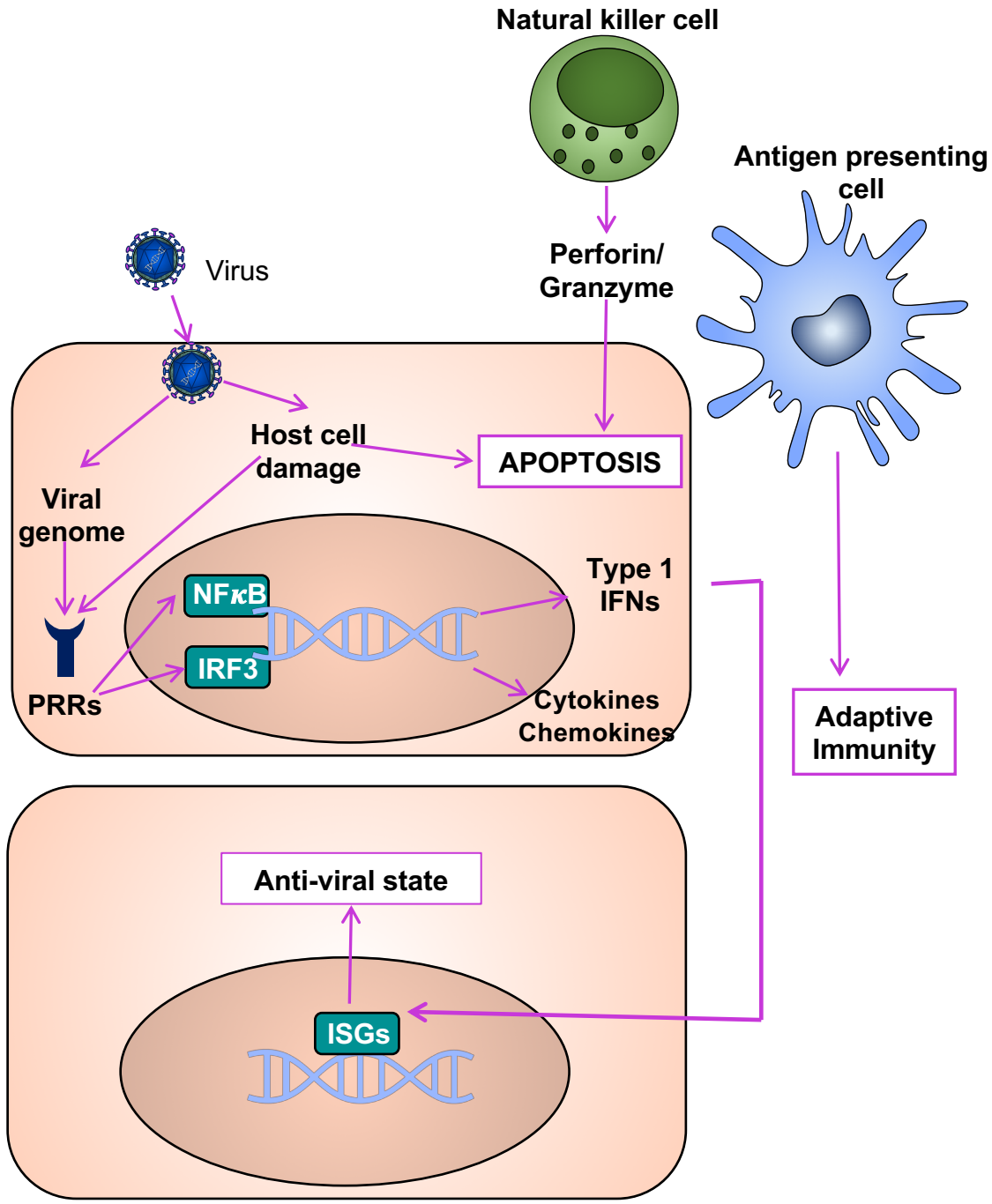


Figure 1.5 Innate immunity against viral infection.
When viruses enter a cell the viral genome and subsequent host cell damage can be sensed by pattern recognition receptors (PRRs). PRRs lead to the activation of transcription factors such as NFκB and interferon regulatory factor 3 (IRF3). Activation of NFκB results in the production of pro-inflammatory cytokines and chemokines, which can attract and activate immune cells at the site of infection. Activation of IRF3 results in the production of type I interferons (IFNs), which can cause the activation of interferon stimulated genes (ISGs) in neighbouring cells, resulting in an anti-viral state. Host cell damage and detection by PRRs can also lead to the induction of programmed cell death such as apoptosis. NK cells can recognise virally infected cells and kill them through various means, the most targeted being the delivery of perforin and granzyme to the target cell, resulting in apoptosis induction. Antigen presenting cells (APCs) can take up and process dying cells, and present antigenic peptides on MHC to T cells, leading to the induction of adaptive immunity.

4.1 Programmed cell death

The main forms of programmed cell death initiated by viral infection include apoptosis, necroptosis and pyroptosis (Upton and Chan, 2014). Apoptosis is a non-inflammatory form of programmed cell death which can be distinguished by the cleavage of caspase 3 and has been considered to be the main cell death mechanism (Jänicke et al., 1998). Necroptosis is an inflammatory form of cell death which shares some of the apoptosis biochemical pathway. If particular components of the apoptosis pathway are inhibited, necroptosis can be initiated, eventually causing the phosphorylation of mixed lineage kinase domain like pseudokinase (MLKL) and the formation of pores at the cell membrane (Kang and Tang, 2016). Pyroptosis is mediated by the inflammasome which contains the pattern recognition receptor (PRR) NOD-like receptor (NLR), the adaptor protein apoptosis-associated speck-like protein containing CARD (ASC) and caspase 1. Inflammasome activation causes cell membrane disruption and is therefore also an inflammatory form of programmed cell death (Kang and Tang, 2016). The dynamics and classifications of the types of programmed cell death are constantly evolving; for the purposes of this literature review apoptosis will be the major focus.

4.1.1 Apoptosis

Apoptosis is utilised by both the innate and adaptive immune response to kill virally infected cells and limit viral spread. When a cell senses it has been infected via pathogen associated molecular patters or damage associated molecular patterns, type 1 IFNs are produced that act to induce an anti-viral state in neighbouring cells (Samuel, 1991). Simultaneously, programmed cell death pathways such as apoptosis are initiated to stop the virus spreading (Benedict et al., 2002). Apoptosis is a programmed form of cell death which is non-inflammatory and contains distinct biochemical pathways (Figure 1.6), which are highly complex and involve an energy dependent cascade of molecular events (Samali et al., 1999, Fink and Cookson, 2005). Three apoptosis pathways have been identified: the extrinsic, intrinsic and perforin/granzyme pathways. All of these pathways converge in the cleavage of caspase 3, the major hallmark of apoptosis induction. This causes DNA fragmentation, nuclear and cytoskeletal protein degradation, formation of apoptotic bodies and engulfment by phagocytes (Elmore, 2007).

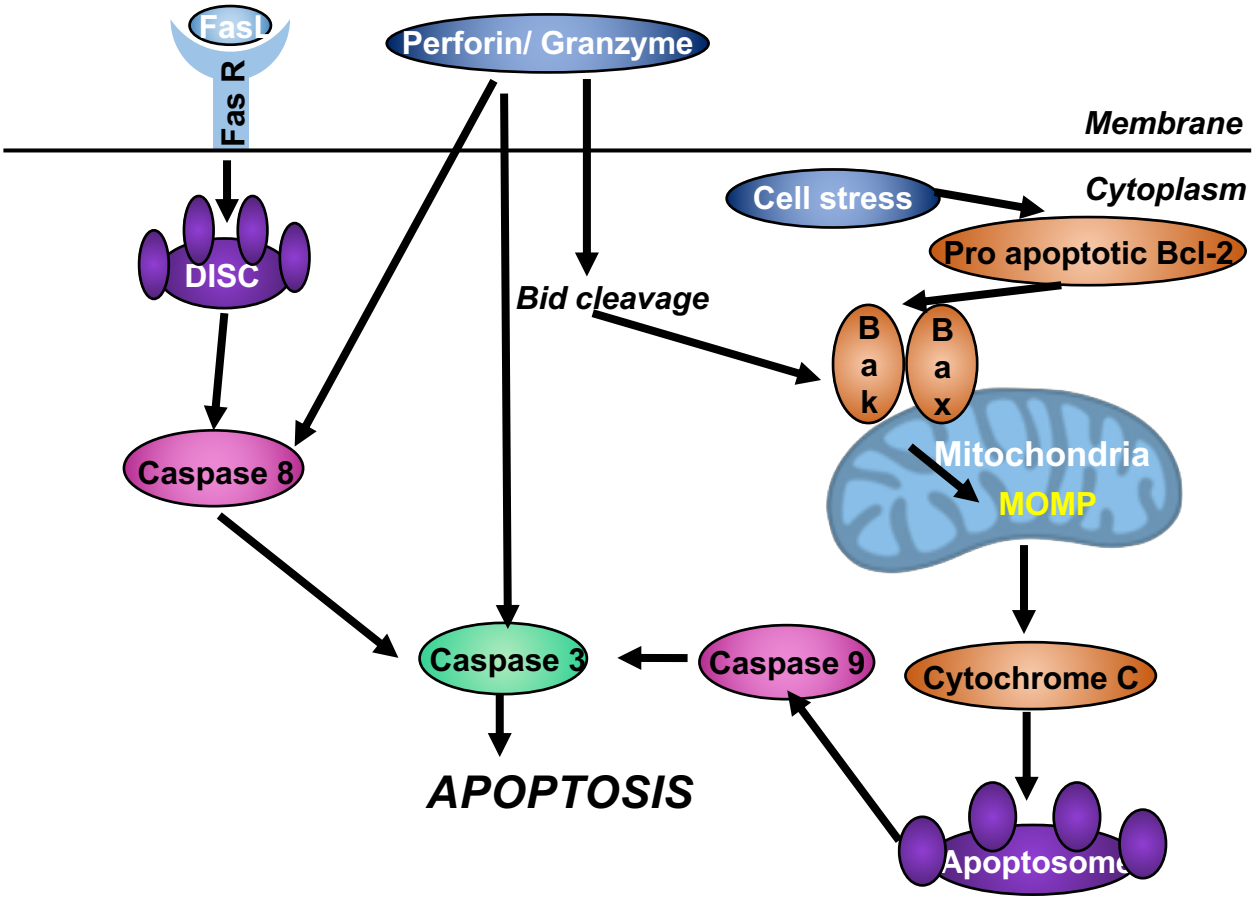


Figure 1.6 Summary of the extrinsic, granzyme B and intrinsic apoptotic pathways.
The extrinsic apoptosis pathway can be stimulated by ligands such as Fas ligand, binding to their corresponding receptors. These receptors contain a death effector domain (DED) that allows them to associate with cytoplasmic adapter proteins. These adaptor proteins associate with caspase 8 to form a DISC and this causes the cleavage of pro-caspase 8 to form caspase 8. Caspase 8 can then cleave pro-caspase 3 for the execution phase of apoptosis to be induced. Caspase 8 can also cleave Bid to form truncated Bid (tBid), which is involved in the activation of the intrinsic apoptotic pathway. In the intrinsic apoptotic pathway, cellular stress activates damage response molecules, which activate pro-apoptotic Bcl-2 family members. These Bcl-2 family members activate pro-apoptotic molecules Bax and Bak, which cause permeabilization of the outer membrane of the mitochondria and the release of cytochrome C. Cytochrome C associates with other pro-apoptotic molecules to form the apoptosome. This causes the cleavage of pro-caspase 9 and release of active caspase 9 which can then cleave pro-caspase 3 to induce apoptosis. Perforin and granzyme B are delivered to a cell via a CTL or NK cell, perforin forms a pore in the membrane of the cell to allow targeted release of granzyme B. Granzyme B can cleave a variety of apoptotic molecules such as caspase 8, Bid and caspase 3 to cause the induction of apoptosis.

4.1.1.1 Extrinsic apoptotic pathway

The extrinsic signalling pathway is dependent on transmembrane receptor mediated interactions. This involves death receptors that are members of the tumour necrosis factor (TNF) receptor gene family such as Fas receptor, tumor necrosis factor receptor 1 (TNFR1) and TNF-related apoptosis-inducing ligand receptor I/II (TRAIL RI/II) (Locksley et al., 2001) (Figure 1.6). These receptors interact with their respective ligands Fas, TNF-α and TRAIL, and cause the recruitment of cytoplasmic adapter proteins such as Fas-associated protein with death domain (FADD), tumor necrosis factor receptor type 1-associated death domain protein (TRADD) and receptor-interacting serine/threonine-protein (RIP) (Grimm et al., 1996, Hsu et al., 1996). After this has occurred a death-inducing signalling complex (DISC) is formed which results in the autocatalytic activation of pro-caspase 8 (Kischkel et al., 1995). When caspase 8 has been activated, the execution phase of apoptosis can be triggered via the cleavage and activation of caspase 3 (Stennicke et al., 1998). This pathway is negatively regulated via molecules such as cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (cFLIP) to ensure that apoptosis does not occur unnecessarily (Kataoka et al., 1998). cFLIP has been found to limit caspase 8 activation via interference with DISC formation (Scaffidi et al., 1999, Djerbi et al., 2001). In some cells, the activation of the extrinsic pathway can cause the activation of the intrinsic pathway. Caspase 8 can cause the cleavage and activation of BH3 interacting-domain death agonist (Bid) (Li et al., 1998, Luo et al., 1998). Bid can then interact with Bax or and B-cell lymphoma 2 (Bcl-2) homologous antagonist killer (Bak) to induce mitochondrial outer membrane permeabilization (MOMP), causing an intrinsic amplification loop (Kluck et al., 1999, Shelton et al., 2009).

4.1.1.2 Intrinsic apoptotic pathway

The intrinsic signalling pathways that induce apoptosis are activated by a wide variety of non-receptor mediated stimuli and produce intracellular signals that are mitochondrial-initiated events (Elmore, 2007). Signals that induce this intrinsic apoptotic pathway may act in a negative or positive manner. Negative signals include absence of growth factors, hormones and cytokines, whilst positive signals include radiation, toxins, hypoxia, viral infections and free radicals (Elmore, 2007). This pathway is initiated by the activation of BH3-only pro-apoptotic proteins such as Bid, Bim and p53 upregulated modulator of apoptosis (PUMA), which promote the

oligomerisation of the pro-apoptotic proteins Bax and Bak in the mitochondrial outer membrane, thus instigating MOMP (Zong et al., 2003, Kim et al., 2009). In contrast other BH domain proteins such as Bcl-2 and B-cell lymphoma-extra-large (Bcl-XL) inhibit MOMP via neutralizing pro-apoptotic family members (Emily et al., 2001). MOMP causes the release of cytochrome C into the cytosol (Desagher et al., 1999) where it can induce the formation of the apoptosome via interaction with apoptotic protease activating factor 1 (Apaf-1) and pro-caspase 9 (Zou et al., 1999). The process results in the activation of caspase 9 (Zou et al., 1999) which then activates effector caspases such as caspase 3 (Li et al., 1997). Simultaneously, Smac/DIABLO is released from the mitochondria allowing it to interact with the anti-apoptotic protein X chromosome-linked inhibitor of apoptosis protein (XIAP) (Du et al., 2000). Usually XIAP binds to caspase 3, 7 and 9 in order to prevent inappropriate caspase activation (Takahashi et al., 1998, Srinivasula et al., 2001). When Smac/DIABLO interacts with XIAP, this function is antagonized so apoptosis can occur (Du et al., 2000). A second group of pro-apoptotic proteins are released from the mitochondria when the cell has been committed to die. These proteins include apoptosis inducing factor (AIF), endonuclease G and caspase activated deoxyribonuclease (CAD). AIF causes DNA fragmentation in the nucleus (Candé et al., 2002) whilst endonuclease G causes the cleavage of nuclear chromatin (Li et al., 2001). CAD is cleaved by caspase 3 and causes oligonucleosomal DNA fragmentation and advanced chromatin condensation (Sakahira et al., 1998).

4.1.1.3 Granzyme/Perforin mediated apoptosis

Both NK cells and cytotoxic T lymphocytes (CTLs) can kill infected cells and induce apoptosis via two pathways. They can upregulate their expression of FasL to induce extrinsic apoptosis (Suda et al., 1995, Zamai et al., 1998) and can also induce apoptosis in virally infected cells via the perforin and granzyme pathway (Lowin et al., 1995, Shresta et al., 1995). Perforin is a transmembrane pore-forming molecule, which enables the delivery of granules containing granzymes through pores into the target cell (Liu et al., 1995). Granzymes are serine proteases which, like caspases, recognise specific tetrapeptide sequence motifs (P4-P3-P2-P1) and cleave proteins after the aspartate residue at P1 (Wee et al., 2011).

Granzyme B can cleave caspases (Smyth and Trapani, 1995) such as pro-caspase 10 and pro-caspase 3, as well as pro-apoptotic Bcl-2 family members such as Bid, to activate the intrinsic pathway and directly induce apoptosis (Andrade et al., 1998, Barry et al., 2000). Granzyme A activates caspase independent pathways via inducing DNA nicks to induce apoptosis (Beresford et al., 1999, Fan et al., 2003). When mice are deficient in granzyme A and B, a delayed form of cell death occurs where there is a lack of phosphatidyl serine and a subsequent reduction in phagocytosis by antigen presenting cells (APCs) (Hoves et al., 2011). This delayed and less immunogenic cell death is hypothesised to be due to granzyme M and granzyme H (Voskoboinik et al., 2015). Granzyme M induces caspase independent apoptosis through the cleavage of survivin, leading to degradation of the anti-apoptotic survivin-XIAP complex (Hu et al., 2010). Granzyme H has also been shown to induce apoptosis through cleavage of Bid and CAD (Hou et al., 2008). Interestingly, many granzymes such as granzyme B have also been found to cleave proteins which do not regulate apoptotic cell death such as nuclear and cytoskeletal components as well as membrane receptors and viral proteins (Wee et al., 2011).

4.2 Innate immune cells

The production of cytokines and chemokines at the site of viral infection can attract innate immune cells such as monocytes, macrophages, NK cells and DCs (Takeuchi and Akira, 2009). Both macrophages and DCs are APCs which can uptake and process viral components to display on MHC class II to activate and enhance adaptive immunity (Mercer and Greber, 2013). Both cell types can also produce a variety of cytokines to help limit viral spread (Mercer and Greber, 2013). NK cells are cytotoxic lymphocytes which are able to recognise cells lacking expression of surface MHC class I (Stern et al., 1980, Lanier, 2008). Many viruses have evolved strategies to downregulate the expression of MHC class I and II to avoid detection by the adaptive immune system. Thus, NK cells are an important line of defence against such viruses (Lanier, 2008). The activation of NK cells is complex involving both activating and inhibitory ligands as well as the absence of MHC class I molecules (Martinet and Smyth, 2015). Once an NK cell has recognised a target cell, it can form an immunological synapse through which perforin is delivered and forms a pore in the target cell to allow granzymes to access the target cell, which initiates apoptosis

(Afonina et al., 2010). NK cells can also express FasL to induce extrinsic apoptosis and cytokines such as IFNγ, which can limit viral spread (Björkström et al., 2016).

5 VZV and HSV immune system interactions

The way in which both HSV-1 and VZV interact with the various cell types they encounter during infection determines disease outcome. Therefore, it is important to understand the mechanisms these viruses use to further viral replication and spread, so that we can develop targeted strategies to eliminate the occurrence of VZV and HSV-1 induced disease and complications. The innate and adaptive immune response is critical in the control of alphaherpesvirus infection and disease (Zerboni et al., 2014). This is best demonstrated by the fact that individuals who have genetic or acquired immunodeficiencies have persistent and often fatal alphaherpesvirus infections (Nikkels et al., 1997, Etzioni et al., 2005, Dropulic and Cohen, 2011). As such, it is not surprising that alphaherpesviruses modulate and infect various cells of the innate and adaptive immune system to further viral propagation (Zerboni et al., 2014, Yang et al., 2019). The following sections will outline the immune response that develops toward VZV and HSV-1 infections and the ways in which these alphaherpesviruses interact with these cells, particularly focusing on the intrinsic and innate immune response.

5.1 Immune responses to HSV-1

Similar to other herpesviruses, the immune response to HSV-1 requires both the innate and adaptive arms to effectively control primary infection and limit viral reactivation from latency. As mentioned previously, HSV-1 reactivation does not result in neuropathic pain as often as VZV and this could be due to a more dynamic immune control during reactivation. The immune response to HSV-1, as opposed to VZV, has been more extensively characterised due to the availability and use of murine models (Kollias et al., 2015).

5.1.1 Immune response to primary HSV-1 infection

When HSV-1 accesses the skin during initial infection, skin cells release a cascade of inflammatory cytokines and chemokines which recruit and activate a variety of immune cells (Chew et al., 2009). In particular, type I IFNs are critical, which is demonstrated by the dissemination of the virus in mice lacking the IFN-α/β receptor (Leib et al., 1999). Both IFN-γ and TNF are critical in limiting viral spread and enhancing both

innate and adaptive immune responses (Minami et al., 2002). NK cells are a major source of these cytokines and have been implicated in controlling lytic HSV-1 infection (Habu et al., 1984, Nandakumar et al., 2008). This can occur through enhancing DC-T cell interactions and presenting antigen to T cells (Kim et al., 2015). DCs migrate to draining lymph nodes to initiate HSV-specific T cell immunity (Chew et al., 2009). $CD4^+$ T helper cells secrete cytokines to control infection and can help prime $CD8^+$ CTLs (Chew et al., 2009). CTL function is required to clear replicating virus in the skin and limit spread to the nervous system (van Lint et al., 2004). In comparison to T cell mediated immunity, the humoral immune response to HSV-1 is not as dominant in controlling infection (Chew et al., 2009).

5.1.2 Immune response to HSV-1 latency and reactivation

HSV-1 reactivation from latency occurs more frequently than VZV and this can perhaps be attributed to different immune control (Figure 1.7). CTLs have been found to inhibit HSV-1 reactivation via the secretion of cytokines and the release of granzyme B (Knickelbein et al., 2008), and this will be discussed in more detail in Section 5.2. HSV-specific T cells are observed in latently infected ganglia in both human and murine models (Khanna et al., 2003, Verjans et al., 2007, Chew et al., 2009). Their constant presence may be due to low level lytic viral protein expression (Arbusow et al., 2010, Russell and Tscharke, 2016).

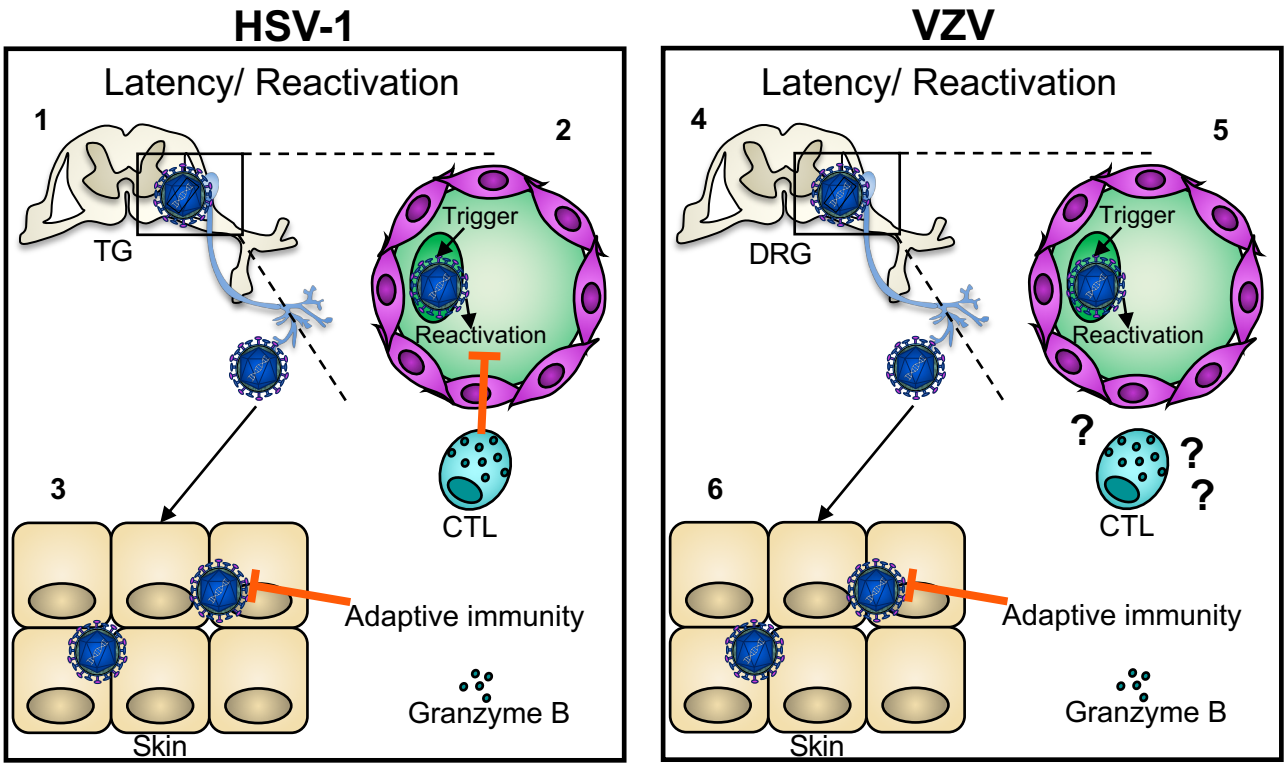


Figure 1.7 Immune response during alphaherpesvirus latency and reactivation.
HSV-1 becomes latent in the trigeminal ganglia (TG) and reactivates multiple times throughout life (1). During latency, CTLs are found surrounding latently infected neurons without neuronal apoptosis being evident (2). A trigger causes activation of viral replicative machinery which can activate CTLs to release granzyme B. Granzyme B can cleave viral components to inhibit reactivation without causing neuronal apoptosis (2). If the immune system is compromised, HSV-1 can travel down the sensory nerves in a retrograde fashion to reach the skin, where immune memory and the adaptive immune response eliminates the virus (3). In the case of VZV, the virus becomes latent in the dorsal root ganglia (DRG) and reactivates less frequently throughout life than HSV-1, with most reactivation events occurring later in life (4). It is unclear during VZV latency, what immune cells are present in the ganglia, however during reactivation CTLs infiltrate and lie in close proximity to sensory nerves, similarly to HSV-1 (5). Whether these cells can release granzyme B to control the reactivation of the virus remains to be seen, but neuronal apoptosis is not observed. In hosts with a compromised immune system, the virus travels to the skin of the innervating dermatome and similarly to HSV-1 the memory and adaptive immune response act to control the virus (6).

5.2 HSV-1 encoded immune evasion

There has been extensive research on the ability of HSV-1 to interfere with both the innate and adaptive immune responses. In regard to innate immunity, HSV-1 inhibits various forms of cell death such as apoptosis and necroptosis to further viral propagation (Yu and He, 2016). Some of the HSV-1 gene products that inhibit apoptosis include Us3 which inhibits granzyme B cleavage of Bid (Cartier et al., 2003), LAT which protects Neuro2A cells from granzyme B induced apoptosis (Jiang et al., 2011) and glycoprotein J which inhibits FasL and granzyme B induced caspase activation (Jerome et al., 2001). ICP22 has been linked to both apoptosis induction and apoptosis inhibition (Aubert et al., 1999, Hagglund et al., 2002). This could be due to an alternatively spliced gene product Us1.5 which may induce apoptosis, whilst the full length ICP22 protects from apoptosis (You et al., 2017). Other innate immune responses that HSV-1 curtails include TLR recognition of the virus, the type I IFN response, MHC processing and ND10 bodies which allows the virus to spread and replicate (Orr et al., 2005, Daubeuf et al., 2009, Tavalai and Stamminger, 2009, Su et al., 2016, Yuan et al., 2018).

HSV-1 also interferes with various aspects of the adaptive immune response such as MHC recognition, T cell function, DC viability and function, natural killer T (NKT) cell function and antibody neutralisation (Kruse et al., 2000, Friedman, 2003, Chew et al., 2009, Leib et al., 2009, Lubinski et al., 2011, Rao et al., 2011, Yang et al., 2015). During both primary infection and reactivation these mechanisms allow the virus to spread effectively. As mentioned previously, in a murine model of HSV-1 infection, $CD8_+$ T cells were shown to prevent HSV-1 reactivation (Liu et al., 2000). In *in vivo* and *ex vivo* murine ganglia cultures, $CD8_+$ T cells were found to directly release granzyme B in lytic granules to neurons without caspase activation (Knickelbein et al., 2008). It is not clear whether this is due to neurons being innately resistant to cell death or whether HSV-1 gene products may function to inhibit CTL induced cytotoxicity. Knickelbein and colleagues (2008) demonstrated that granzyme B was required for the maintenance of HSV-1 neuronal latency and this was attributed to the cleavage of ICP4 which contained a granzyme B cleavage site. HSV-1 ICP4 is a potent transactivator (Smith et al., 1993) and seems to be crucial in the reactivation of HSV-1 as it can repress LAT promoters (Rivera-Gonzalez et al., 1994).

Interestingly, it is yet to be elucidated whether any other HSV-1 genes contain granzyme B cut sites. It would also be advantageous to determine whether this phenomenon occurs in human cells as previous work has been done in a murine context. Previous studies identified that CTLs play an important role in the control of HSV-1 reactivation, however there are other immune cells such as NKT and NK cells which can also release granzymes into target cells, thus it would be of interest to explore the role of these cell types in the control of HSV-1 reactivation.

5.3 Immune responses to VZV

5.3.1 Immune response to primary VZV infection

In primary VZV infection both the innate and adaptive arms of the immune system are initiated. Similarly to HSV-1, early in infection the innate and intrinsic immune system is responsible for limiting viral spread and replication. Infected cells undergo cell death and the type 1 IFN response is activated to limit viral spread (Zerboni et al., 2014). This is demonstrated by the successful use of human leukocyte derived IFN for the treatment of varicella in children being treated for cancer (Arvin et al., 1982). NK cells are crucial for the control of VZV infection (Tilden et al., 1986, Vossen et al., 2005, Malavige et al., 2010) as NK cell deficiency and dysfunction causes fatal primary varicella infection (Etzioni et al., 2005). NK cells are able to lyse VZV infected fibroblasts *in vitro* (Ihara et al., 1984) and cause apoptosis of target cells through the perforin granulysin pathway (Hata et al., 2001).

The humoral immune response against VZV involves both neutralising antibodies and antibodies specific for viral proteins. These antibodies can lyse infected cells through activation of the complement pathway or with NK cells via antibody-dependent cellular cytotoxicity (ADCC) (Abendroth and Arvin, 2000, Arvin, 2008). Antibodies may assist in limiting viral spread within the first 72 hours p.i., however humoral immunity plays a limited role in controlling VZV infection (Arvin, 2008). This is identifiable in cases of children with agammaglobulinemia, where VZV infection is no more severe in comparison to healthy children (Laing et al., 2018).

In contrast, VZV-specific cell-mediated immunity is critical in resolving primary infection (Arvin, 1992). This can be seen in patients who are immunosuppressed or immunocompromised, as they are at higher risk of persistent viremia and life

threatening dissemination (Dolin et al., 1978, Groth et al., 1978). Both $CD4^+$ and $CD8^+$ T cells are required for the cell-mediated response against VZV (Abendroth and Arvin, 1999). This is accompanied by the secretion of Th1 like cytokines such as IL-2, IL-10, IL-12 and IFN-γ, which direct the production of different antibody isotypes and clonal expansion of VZV specific lymphocytes (Arvin et al., 1986, Zhang et al., 1994, Zhang et al., 1995, Jenkins et al., 1998). In VZV infection, both $CD8^+$ and $CD4^+$ CTLs recognise and lyse infected cells, and this is in contrast to the classic model of cell-mediated immunity (Hickling et al., 1987, Diaz et al., 1989, Huang et al., 1992).

5.3.2 Immune response to VZV latency and reactivation

As mentioned previously, knowledge concerning the immune response during VZV latency is lacking due to limited model systems. Even though the adaptive immune response is critical for the control of primary infection, it cannot prevent the establishment of lifelong latency (Abendroth and Arvin, 2000). However, it may play a role in the prevention of reactivation of the virus. This is demonstrated by the correlation between the increased incidence of herpes zoster and the decline of T cell activity (Miller, 1980, Park et al., 2004). During herpes zoster, an influx of immune cells are observed in the DRG due to the release of CXCL10 from uninfected surrounding neurons (Steain et al., 2011). Analysis of DRG from patients with active herpes zoster demonstrated that T cell infiltration predominates, however infiltrating NK cells have also been observed in DRG from individuals with herpes zoster 1-4.5 months preceding death (Gowrishankar et al., 2010, Steain et al., 2014). Similarly to HSV-1, CTLs have been found in close proximity to neurons in the DRG during natural VZV reactivation without significant apoptosis induction (Steain et al., 2014), however it is unknown whether granzyme B can prevent reactivation in a non-apoptotic manner. It is also unknown whether the VZV homolog of HSV ICP4, VZV ORF62, or any other VZV gene products contain granzyme B cleavage sites.

Months after herpes zoster, $CD8^+$ T cells infiltrate into the ganglia, however the cytotoxic potential of the $CD8^+$ T cells is diminished as shown by a lack of granzyme B staining (Gowrishankar et al., 2010). Once the virus has reactivated to cause herpes zoster, the immune response in the skin is very similar to primary infection (Steain et

al., 2012). This drives prolonged VZV-specific immunity and explains why the occurrence of a second episode of herpes zoster is rare (Vossen et al., 2004).

5.4 VZV modulation of the immune response

Similar to HSV-1, VZV targets various components of the intrinsic, innate and adaptive immune response to ensure successful viral replication and spread. During primary infection it is crucial for the virus to reach the sensory nerve termini in the skin, so that lifelong latency can be established. It is also critical that neuronal survival is maintained so that the latent pool of virus is not diminished. Therefore, it is critical to elucidate the mechanisms by which VZV can evade the immune response to potentially develop novel therapeutic targets. Due to the importance of the immune response in regulating the homeostasis of inflammation, a better understanding of this modulation by VZV may also help increase our understanding of the development of PHN.

5.4.1 VZV modulation of the adaptive immune response

VZV targets many components of the adaptive immune response to allow viral spread (Zerboni et al., 2014). VZV appears to evade host recognition during the initial incubation period following VZV inoculation, suggesting that the virus encodes proteins which allow T lymphocyte evasion (Arvin, 1996). *In vitro*, VZV blocks the IFNγ induced upregulation of MHC class II and downregulates cell-surface expression of MHC class I (Cohen, 1998, Abendroth et al., 2000, Abendroth et al., 2001a, Eisfeld et al., 2007). MHC class I molecules are retained in the Golgi apparatus of infected cells, while in contrast, MHC class II surface expression is blocked through VZV interference with the JAK/STAT pathway (Abendroth et al., 2000, Zerboni et al., 2014). This modulation may help the virus evade and delay T cell responses (Abendroth et al., 2000). Additionally, VZV downregulates the expression of intracellular adhesion molecule 1 (ICAM-1) on the cell-surface of keratinocytes (Nikkels et al., 2004). ICAM-1 aids in the recruitment of inflammatory cells and as such may reduce the antigen presenting capacity of keratinocytes (Lebedeva et al., 2005). Furthermore, VZV infection was shown to reduce the capacity of IFNγ treated keratinocytes to stimulate antigen specific T cells, as well as decreasing their ability to upregulate MHC class I in response to IFNα, TNF and TLR3 ligand (Black et al., 2009). Collectively, this

suggests that VZV manipulation of skin cells such as keratinocytes can impair T cell responses.

Interestingly, VZV can also infect T cells directly, through contact with VZV infected immature DCs, which aids in viral dissemination during varicella (Abendroth et al., 2001b, Ku et al., 2002). In the SCID-hu mouse model, memory $CD4_+$ T cells could mediate the transfer of infectious virus to the skin (Ku et al., 2004). Recently, single cell mass cytometry has been utilised to investigate VZV infection of human tonsil T cells. It was shown that VZV infection changed T cell surface proteins to resemble a skin trafficking profile in a TCR independent process (Sen et al., 2014). Therefore, it is clear that VZV directly modulates T cell markers for trafficking purposes.

5.4.2 VZV modulation of innate immune responses

VZV has also been shown to modulate different aspects of the innate immune response to ensure viral spread and the production of infectious viral progeny (Zerboni et al., 2014). VZV modulates cytokine responses such as the type 1 IFN response (Ambagala and Cohen, 2007, Zerboni et al., 2014, Verweij et al., 2015). ORF63 blocks the effects of IFNα by degrading IRF9 and interfering with the phosphorylation of signal transducer and activator of transcription (STAT) 2, causing the prevention of ISG induction (Ambagala and Cohen, 2007, Verweij et al., 2015). It has been suggested that IFNα may be critical in the establishment and maintenance of latency, as it limits VZV replication within human skin xenographs in the SCID-hu mouse model (Ku et al., 2004). ORF62 inhibits the function of IRF3 and thus the production of type 1 IFN (Sen et al., 2010). VZV has also been shown to interfere with the NFκB pathway via ORF61, which is another crucial component of inducing an antiviral state (Sloan et al., 2012).

In addition to impairing the ability of cells to induce an antiviral state, VZV also affects different cells of the innate immune system. VZV differentially modulates the expression of NKG2D ligands, which are inducible-self proteins that can be expressed during cell stress and bind to NKG2D receptors on NK cells (Raulet, 2003, Campbell et al., 2015). Differential expression of NKG2D ligands during VZV infection limits NK cell activity (Campbell et al., 2015). As well as modulating the capacity of target cells

to activate NK cells, VZV productively infects and modulates NK cell function *in vitro* (Campbell et al., 2019). Specifically, VZV infection of NK cells inhibits NK cell degranulation as well as IFNγ and TNF production (Campbell et al., 2019). As mentioned previously, NK cells have been found in patient DRG samples after herpes zoster (Gowrishankar et al., 2010). It would be interesting to elucidate whether they with CTLs could control VZV reactivation in a similar manner to HSV-1 and whether VZV could block target cell death upon granzyme B release. Recently, VZV has also been shown to productively infect human monocytes and impair their ability to endocytose and differentiate into macrophages (Kennedy et al., 2019).

VZV also has the capacity to extensively modulate DC function. VZV infection of mature monocyte derived dendritic cells (MDDCs) causes the downregulation of functionally important cell-surface molecules, namely CD80, CD83 and CD86. Additionally, VZV infection of plasmacytoid dendritic cells (pDCs) causes the abrogation of IFNα production (Morrow et al., 2003, Huch et al., 2010). The low cell surface expression of costimulatory molecules corresponds to an impaired ability to stimulate T cell proliferation (Abendroth et al., 2001b). As DCs are an important class of APC, VZV infection may delay immunosurveillance, and this could be due to modulation of the NFκB pathway by VZV ORF66 (Sloan et al., 2012). As mentioned previously, VZV can also manipulate the ability of skin cells such as keratinocytes to stimulate both innate and adaptive immune responses; this will be described in the following Section (5.4.2.1).

5.4.2.1 VZV modulation of keratinocytes

VZV has been found to infect keratinocytes *in vitro* and *in vivo* in both varicella and herpes zoster skin lesions (Nikkels et al., 1995, Han et al., 2016). Through the utilisation of RNAseq, VZV infection was shown to affect the differentiation of keratinocytes by reducing cytokeratins and desmosomal proteins which could promote blistering and desquamation of the epidermis for efficient VZV spread (Jones et al., 2014).

Whilst VZV affects keratinocyte differentiation, it does not induce significant cell death in immortalised human keratinocytes for up to 10 days p.i. (Black et al., 2009). This

suggests that VZV infection could be delaying apoptosis induction in keratinocytes and thus warrants further investigation. A detailed analysis of the ability of VZV to cause different forms of cell death in keratinocytes has not been performed and would be interesting considering the role of keratinocytes in regulating the microenvironment of the skin. It has been established that VZV does not interfere with autophagy induction in keratinocytes from VZV skin lesion samples (Carpenter et al., 2011). This is in contrast to a mouse model of HSV-1 infection, where autophagy was not induced in keratinocytes as the type 1 IFN response was sufficient to protect against HSV-1 infection (Yordy et al., 2012).

As well as regulating keratinocyte differentiation and cell death induction, VZV also regulates the communication between immune cells and keratinocytes. VZV infection of keratinocytes inhibits the upregulation of MHC class I and class II following IFNγ treatment and consequently VZV infected keratinocytes stimulated antigen-specific T cells to a lesser degree (Black et al., 2009). Additionally, in this system VZV was able to inhibit MHC class I upregulation by IFNα, TNF and TLR3 (Black et al., 2009). This finding conflicts with a previous report suggesting that in herpes zoster skin lesions, VZV infected cells only downregulated MHC class II (Nikkels et al., 2004). This disparity could be due to differences between an *in vitro* system and using *ex vivo* clinical samples. Nikkels *et al.* (2004) also identified that VZV infection caused a decrease in the expression of ICAM-1 in human keratinocytes and suggested this could cause a reduction in their capacity to present antigen to lymphocyte function-associated antigen 1 (LFA-1) ligand expressing T cells. Increased levels of Th1 inflammatory cytokines such as IFNγ, TNF and IL-6 were observed in herpes zoster skin (Nikkels et al., 2004).

The ability of VZV infection to cause inflammatory cytokine release from keratinocytes has been demonstrated in HaCaT cells, where VZV was shown to induce the expression of SOCS3 (Choi et al., 2015). This is not surprising considering dsRNA causes activation of TLR3 in keratinocytes, leading to the production in type 1 IFN (Tohyama et al., 2005). It is clear that VZV can regulate keratinocyte communication with the immune system, however it would be of interest to explore the impact of VZV

infection on keratinocytes in relation to communication with other components of the skin microenvironment, such as neurons.

5.4.2.2 VZV modulation of apoptosis

As previously mentioned, VZV delays apoptosis induction in keratinocytes and circumvents apoptosis induction in neurons. VZV, like many of the Herpesviridae family, has dedicated a large proportion of its genome to evading the host immune response (Arvin and Gilden, 2013). The first challenge the virus must overcome is the intrinsic response initiated within the cells it infects. Part of this intrinsic response is the activation of cell death pathways such as apoptosis. VZV like other herpesviruses, encodes genes which inhibit apoptosis to enable viral replication and spread (Galluzzi et al., 2008, You et al., 2017). The inhibition of apoptosis by VZV has been suggested to play an important role in VZV latency, reactivation and the development of PHN.

Interestingly, VZV modulates apoptosis in a cell type specific manner. VZV infection induces apoptosis in skin cells such as human fibroblasts (HFs) and MeWos, as well as immune cells (Hood et al., 2003, Koenig and Wolff, 2003, Brazeau et al., 2010). In contrast, VZV infection did not induce apoptosis in primary human DRG neurons (Hood et al., 2003). The lack of apoptosis induction in human neurons has also been observed in the context of the SCID hu mouse model and human neural derived stem cells (Baiker et al., 2004, Pugazhenthi et al., 2011, Yu et al., 2013). It has been observed that VZV infection of DCs results in the down-regulation of Fas from the cell-surface, however this has not been linked to whether these cells are protected from extrinsic apoptosis or a particular VZV ORF (Hu and Cohen, 2005). Several VZV gene products inhibit apoptosis, however only VZV ORF63 has been linked with the inhibition of apoptosis in neurons (Hood et al., 2006).

VZV ORF63 plays a role in the inhibition of neuronal apoptosis and has been suggested to be key to the maintenance of latency as its transcript is abundantly produced during latency (Cohen et al., 2004, Cohen et al., 2005, Hood et al., 2006). VZV ORF63 encodes for an IE protein and is present in duplicate in the VZV genome as VZV ORF70 (Debrus et al., 1995). Our lab has shown that a VZV ORF63 or VZV ORF70 knockout virus could not protect dissociated human sensory neurons from

apoptosis induction (Hood et al., 2006). The protective effect of VZV ORF63 was further established by transfection of rat sensory neurons with a VZV ORF63 expressing plasmid, where VZV ORF63 protected neurons from nerve growth factor (NGF) withdrawal induced apoptosis. However, it has not been established whether VZV ORF63 expressed in isolation can protect human neuronal cells from apoptosis induction. Furthermore, it is unclear whether VZV ORF63 can inhibit apoptosis in a cell type specific manner and the mechanism of action is yet to be identified. This information is crucial due to the association of VZV ORF63 with VZV latency and pathogenesis.

Other anti-apoptotic genes include VZV ORF66 which encodes a serine/threonine kinase and plays a role in the modulation of T cell apoptosis (Heineman et al., 1996, Schaap et al., 2005, Schaap-Nutt et al., 2006). When ORF66 protein expression is impaired, T cells undergo apoptosis more readily, suggesting that ORF66 may be preventing apoptosis induction in T cells (Schaap et al., 2005). The effect of ORF66 in other cell types is yet to be characterised, as is the mechanism of action. ORF12 interacts with the extracellular-signal regulated kinases (ERK) signalling pathway in MeWos and fibroblasts, to optimize the capacity for viral replication, avoid the antiviral response and inhibit apoptosis (Liu et al., 2012, Liu and Cohen, 2013). This interaction stimulates cell cycle progression and the inhibition of Bim, a key apoptotic protein (Liu and Cohen, 2013, Liu and Cohen, 2014). Currently, it is unclear whether ORF66 and ORF12 have anti-apoptotic effects in other cell types relevant to VZV pathogenesis such as neurons and additional immune cells such as NK cells, monocytes and DCs.

5.4.3 Relevance of VZV modulation of the immune response for pathogenesis and PHN development

It is clear that VZV manipulates many facets of the immune response for viral propagation in the human host. As mentioned above the development of PHN during herpes zoster can be devastating for patients, however how this condition develops is poorly understood (Gupta and Smith, 2012). It may be caused by intrinsic properties of the virus manipulating pathways which contribute to neuropathic pain development, or by various host factors such as age, sex and immune status (Forbes et al., 2016). The immune response has been implicated in the development of neuropathic pain in

various conditions. For example, in response to nerve injury many changes can occur in the DRG such as satellite cell proliferation and the recruitment of lymphocytes and polymorphonuclear cells (Calvo et al., 2012). These cells as well as the neurons of the DRG increase the release of pro-inflammatory cytokines and these mediators can increase DRG neuronal excitability in the development of neuropathic pain (Calvo et al., 2012). Therefore, it is crucial to understand the ways in which VZV can modulate the effector functions of immune cells such as NK cells and CTLs.

A variety of cytokines produced during an immune response have been linked to the development of neuropathic pain. Pro-inflammatory cytokines such as IL-1β and TNF attenuate neuron hyperexcitability in rodent models (DeLeo et al., 2000, Wolf et al., 2006, Clark et al., 2013). Additionally, chemokines such as monocyte chemoattractant protein 1 (MCP-1) have been proposed to increase nociceptive transmission after peripheral injury potentially through the activation of microglia (Thacker et al., 2009). As mentioned previously, VZV modulates cytokine and chemokine secretion from multiple cell types and this may be pertinent for determining therapeutic targets for the development of PHN (Zerboni et al., 2014).

VZV modulates various aspects of the skin microenvironment, such as keratinocyte function and pDC functioning (Black et al., 2009, Huch et al., 2010, Jones et al., 2014). However, it is unclear how VZV modulation of these cell types could relate to aberrant neuron functioning in the skin during PHN development. Understanding how VZV infection affects the inflammatory and nociceptive capacity of skin cell types is critical for broadening our understanding of the contribution of the skin to PHN development and yielding novel therapeutic targets for the treatment of PHN.

6. Aims

Alphaherpesviruses can exploit various aspects of the immune system and are therefore able to establish lifelong latency and cause significant morbidity, particularly in immunocompromised hosts. In particular, VZV reactivation from latency can cause serious complications such as PHN which have negative effects on both individual sufferers and the healthcare system. The innate immune response is critical in limiting viral access to sites of latency and responding to reactivation events. Therefore, by dissecting VZV immunoevasive mechanisms and modulation of key innate immune processes, novel therapeutic targets may be identified to alleviate the burden of herpes zoster and PHN. As mentioned, apoptosis is inextricably linked to the ability of VZV to establish and maintain a lifelong latent infection. In particular VZV ORF63 has been linked with the protection of neuronal cells from apoptosis, however this is yet to be confirmed in human neuronal cells. Additionally, it is unclear whether ORF63 can protect non-neuronal cells from apoptosis and the mechanism ORF63 utilises to prevent apoptosis. By further exploring the role of ORF63 in the prevention of apoptosis a greater understanding of VZV latency in neurons may be achieved.

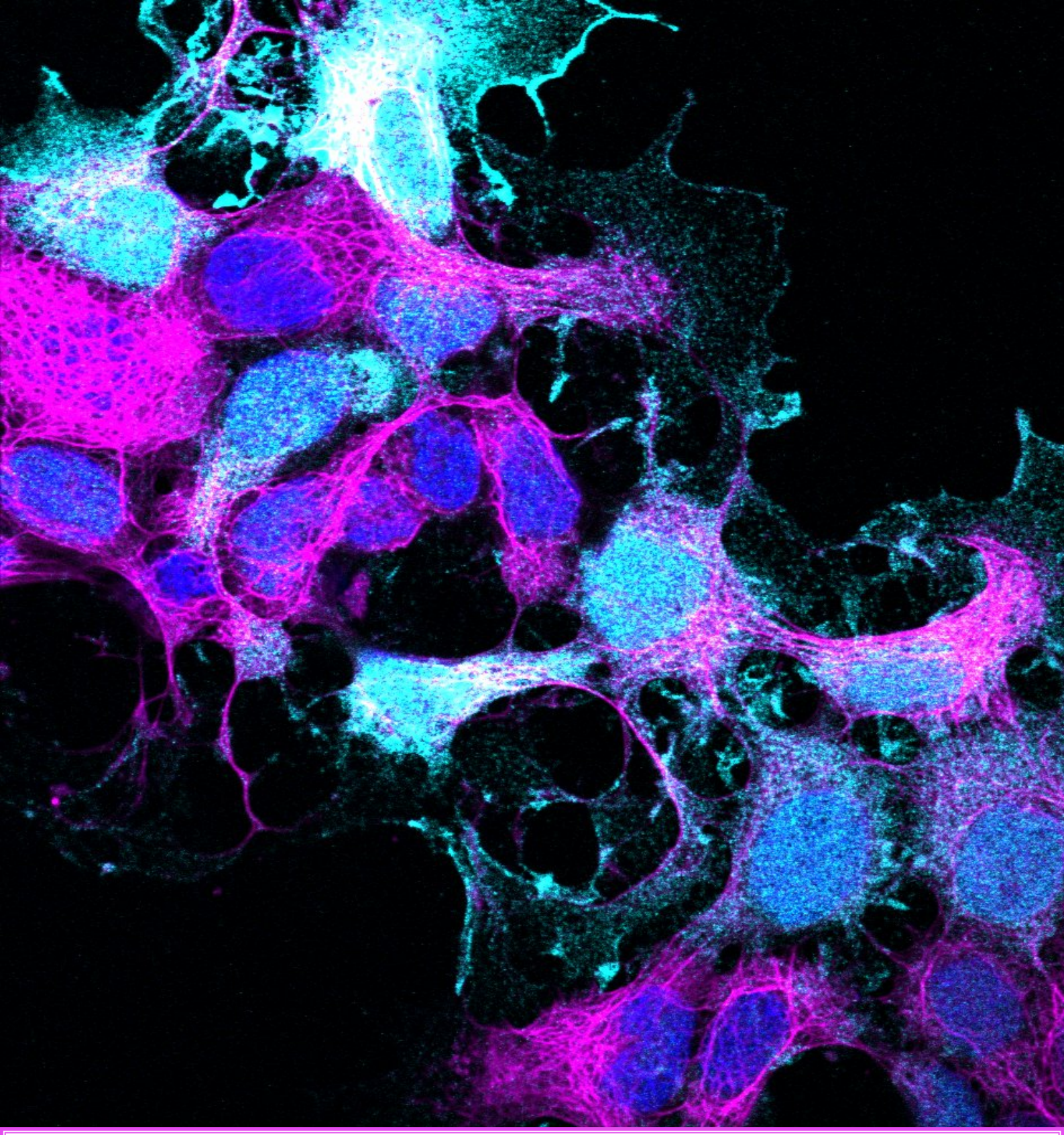
Immune cells such as NK cells and CTLs have the ability to induce apoptosis in virally infected target cells. This has been extensively studied in the context of HSV-1 where CTLs can prevent HSV-1 reactivation through the granzyme B cleavage of HSV ICP4, without apoptosis induction being evident. It is unclear whether the binding and cleavage of HSV ICP4 by granzyme B could out compete granzyme B binding to apoptotic substrates, causing an inhibition of immune cell cytotoxicity. CTLs and NK cells infiltrate ganglia during herpes zoster, and both may play a role in limiting the extent of VZV reactivation. It is unknown whether granzyme B can cleave VZV proteins as in HSV-1 reactivation, and whether this can affect immune cell cytotoxicity to target cells. By further characterising the link between cytotoxic immune cells, granzyme B and alphaherpesviruses, an increased understanding concerning viral immunoevasive strategies and immune cell control of viral reactivation will be achieved.

The ability of VZV to successfully reactive to cause herpes zoster leads to the development of PHN in some individuals. It is unclear as to why some patients develop

PHN and others do not. It is likely that a combination of viral and patient factors could influence the development of PHN, however this has not been extensively explored in the context of causational studies. VZV is closely related to HSV-1, however only VZV reactivation commonly leads to neuropathic pain development. This suggests there are VZV specific factors which can cause PHN development. VZV can modulate a variety of skin cell types, however as keratinocytes can directly modulate neuronal nociceptive activity, the ability of VZV to modulate keratinocyte inflammatory and nociceptive profiles should be further investigated. In general, there has been minimal investigation in the contribution of peripheral sites such as the skin to PHN development, despite some success in clinical trials using topical cannabinoids to treat PHN. It is also clear that mutations in VZV strains can cause functional consequences in regard to the pathogenesis of those strains. It is yet to be investigated whether there are genetic similarities in clinical isolates from PHN patients vs non PHN patients which could alter viral pathogenesis and therefore the likelihood of developing PHN. Exploration of potential causative factors of PHN could lead to the discovery of therapeutic targets for the treatment of PHN.

This doctoral research was achieved through the following aims:

1 To explore the ability of VZV ORF63 to protect against both neuronal and non-neuronal apoptosis in a human setting (Chapter 2)
2 To identify HSV-1 and VZV proteins with novel granzyme B cleavage sites and determine how this affects NK cell cytotoxicity (Chapter 3)
3 To determine the effect of VZV infection on keratinocyte inflammatory and nociceptive capacity (Chapter 4)
4 To determine whether strain specific differences in VZV clinical PHN and non PHN isolates can alter viral characteristics *in vitro* (Chapter 4)



Chapter 2: VZV ORF63 Protects Human Neuronal and Keratinocyte Cell Lines from Apoptosis

CHAPTER 2

Introductory statement

VZV has previously been shown to modulate apoptosis in a cell-type specific manner. VZV inhibits apoptosis in neurons, the site of latency establishment and induces apoptosis in cell types involved in viral dissemination. It is unclear whether this is due to different cell death vulnerabilities in neurons or whether this is directly controlled by the virus. VZV ORF63 has been implicated in the protection of neurons from apoptosis, however, has not been examined in isolation in a human setting. Furthermore, it is unclear whether VZV ORF63 expressed in isolation only protects neuronal cells from apoptosis. The findings of chapter 2 address the cell type specificity of VZV ORF63 apoptotic protection in human cell lines and begins to elucidate the mechanism of action of VZV ORF63.

The findings of Chapter 2 are presented as a published article, with author contributions outlined below.

Authorship attribution statement

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The convention for author placement is the listing of authors in order of contribution to the paper, with the senior author listed in last position.

This work was conceived and designed by CG, MS, BPM, BS and AA. At least 90% of the total work was completed by CG including performing the experiments, experimental validation, data analysis and data interpretation. CG prepared the manuscript and figures, with all other authors critically reviewing the manuscript. MS and AA provided supervision to CG; and AA were responsible for funding acquisition and project administration.

I have obtained permission to include the published material from the corresponding author, Allison Abendroth.

Signed: .....

Chelsea Gerada

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Signed: .....

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VIRUS-CELL INTERACTIONS

Varicella-Zoster Virus ORF63 Protects Human Neuronal and Keratinocyte Cell Lines from Apoptosis and Changes Its Localization upon Apoptosis Induction

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ABSTRACT There are many facets of varicella-zoster virus (VZV) pathogenesis that are not fully understood, such as the mechanisms involved in the establishment of lifelong latency, reactivation, and development of serious conditions like postherpetic neuralgia (PHN). Virus-encoded modulation of apoptosis has been suggested to play an important role in these processes. VZV open reading frame 63 (ORF63) has been shown to modulate apoptosis in a cell-type-specific manner, but the impact of ORF63 on cell death pathways has not been examined in isolation in the context of human cells. We sought to elucidate the effect of VZV ORF63 on apoptosis induction in human neuron and keratinocyte cell lines. VZV ORF63 was shown to protect differentiated SH-SY5Y neuronal cells against staurosporine-induced apoptosis. In addition, VZV infection did not induce high levels of apoptosis in the HaCaT human keratinocyte line, highlighting a delay in apoptosis induction. VZV ORF63 was shown to protect HaCaT cells against both staurosporine- and Fas ligand-induced apoptosis. Confocal microscopy was utilized to examine VZV ORF63 localization during apoptosis induction. In VZV infection and ORF63 expression alone, VZV ORF63 became more cytoplasmic, with aggregate formation during apoptosis induction. Taken together, this suggests that VZV ORF63 protects both differentiated SH-SY5Y cells and HaCaT cells from apoptosis induction and may mediate this effect through its localization change during apoptosis. VZV ORF63 is a prominent VZV gene product in both productive and latent infection and thus may play a critical role in VZV pathogenesis by aiding neuron and keratinocyte survival.

IMPORTANCE VZV, a human-specific alphaherpesvirus, causes chicken pox during primary infection and establishes lifelong latency in the dorsal root ganglia (DRG). Reactivation of VZV causes shingles, which is often followed by a prolonged pain syndrome called postherpetic neuralgia. It has been suggested that the ability of the virus to modulate cell death pathways is linked to its ability to establish latency and reactivate. The significance of our research lies in investigating the ability of ORF63, a VZV gene product, to inhibit apoptosis in novel cell types crucial for VZV pathogenesis. This will allow an increased understanding of critical enigmatic components of VZV pathogenesis.

KEYWORDS apoptosis, human herpesviruses, varicella zoster virus

Varicella-zoster virus (VZV) is a human alphaherpesvirus that can cause both varicella (chicken pox) and herpes zoster (shingles). Varicella results from initial VZV infection, where a variety of skin cells, such as keratinocytes, are infected to produce the characteristic varicella rash (1). The nerve endings of sensory neurons dock in the keratinocyte layer of the dermis, allowing the virus to infect sensory neurons and establish lifelong latency in the dorsal root ganglia (DRG) (1). VZV often reactivates later in life to cause herpes zoster, which encompasses a variety of debilitating complica-

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tions, one of the most significant being postherpetic neuralgia (PHN). PHN involves severe neuropathic pain that can last for months or years following the resolution of the herpes zoster rash (2). The mechanisms responsible for VZV's ability to establish latency, reactivate, and cause PHN have not been fully elucidated; however, it has been suggested that the ability of VZV to modulate apoptosis could play a critical role in these processes (3).

Apoptosis is a programmed, noninflammatory form of cell death that is utilized by the intrinsic, innate, and adaptive immune responses to eliminate potentially harmful cells (4). Biochemically, apoptosis can be triggered by two distinct signaling pathways, the extrinsic and intrinsic pathways. The intrinsic pathway is initiated by a diverse range of intracellular stimuli, such as DNA damage and endoplasmic reticulum (ER) stress. These stimuli activate proapoptotic Bcl-2 family members, which causes the oligomerization of Bax with Bak at the mitochondrial membrane (4–7). This results in disruption of the mitochondrial membrane potential and activation of initiator caspases (8). Anti-apoptotic Bcl-2 proteins can bind to proapoptotic Bcl-2 proteins to inhibit this process (9). Thus, the balance of these anti- and proapoptotic Bcl-2 family members is critical in the induction of apoptosis (4, 10). Initiator caspases can cleave effector caspases, such as caspase 3, resulting in morphological apoptotic changes (4, 11). In contrast, the extrinsic pathway is initiated by ligand-induced oligomerization of cell surface receptors, such as Fas, which causes the cleavage of initiator caspases like caspase 8 (12). Caspase 8 can cleave caspase 3, resulting in apoptosis; however, it can also cleave Bid, which can signal through the mitochondria to induce apoptosis (4, 13). Apoptosis is an important host defense mechanism utilized during viral infection to limit viral dissemination. As such, many viruses have evolved to modulate apoptotic pathways (14).

Members of the herpesvirus family have evolved a variety of different mechanisms to manipulate the apoptotic pathway, and this may facilitate viral spread, the maintenance of latency, and successful reactivation (15). Gammaherpesviruses, including Epstein-Barr virus (EBV), encode Bcl-2 homologs to disrupt apoptosis (16). Betaherpesviruses, such as human and murine cytomegalovirus (CMV), interfere with Bax and Bak to inhibit apoptosis, among other mechanisms (17–19). Alphaherpesviruses, such as herpes simplex virus 1 (HSV-1), have been found to inhibit proapoptotic Bcl-2 family members (20) and to regulate prosurvival signaling (21). Interestingly, VZV has been found to modulate apoptosis in a cell-type-specific manner (22). It has been reported that VZV induces apoptosis in skin cell types, such as human fibroblasts (HFs) (22) and MeWo cells (23), and in immune cells, such as T cells, B cells, monocytes (24, 25), and Vero cells (26). In contrast, VZV has been found to protect against apoptosis in human neurons (22, 27). This is thought to be critical to allow VZV to establish lifelong latency in the DRG. Interestingly, the ability of VZV to induce apoptosis in keratinocytes, a critical cell type in VZV pathogenesis, has not been fully characterized.

Several VZV gene products have been associated with the inhibition of apoptosis. Open reading frame 66 (ORF66) has been suggested to inhibit apoptosis in T cells (23, 28), and ORF12 has been shown to trigger extracellular signal-regulated kinase (ERK) phosphorylation to inhibit apoptosis in MeWo cells and human embryonic kidney (HEK293T) cells (29–31). The ORF63 gene is essential for VZV replication and is duplicated within the VZV genome (as ORF70); it is also abundantly transcribed in neuronal latency (32–34). Our laboratory has previously utilized an ORF63 single-knockout virus to determine that ORF63 can inhibit apoptosis in neuronal cells, but not in HFs (27). However, the ability of VZV ORF63 alone to protect against apoptosis has not been examined in the context of human neuronal cells. Furthermore, it is not clear whether this phenotype extends to other clinically relevant cell types, such as keratinocytes. Thus, it is important to elucidate the role of ORF63 in apoptosis in the context of both neuronal and nonneuronal cells due to its importance in latency and reactivation.

We constructed novel VZV ORF63-expressing SH-SY5Y and HaCaT cell lines via a lentivirus expression system to characterize the ability of VZV ORF63 to protect against apoptosis. SH-SY5Y cells are able to undergo intrinsic apoptosis but not extrinsic

apoptosis due to a lack of caspase 8 (35). In this report, we show that VZV ORF63 expression alone can inhibit apoptosis in differentiated human SH-SY5Y neuronal cells, extending our previous work (22, 27). We also establish that this phenotype is not exclusive to neurons, as VZV ORF63 expression alone can also protect a human keratinocyte line (HaCaT cells) from both extrinsic and intrinsic apoptosis. Furthermore, we begin to elucidate how ORF63 mediates its protective effect by showing that VZV ORF63 relocalizes during apoptosis induction in HaCaT cells, implying a potential modulatory function of ORF63 in the cytoplasm of infected cells.

RESULTS

Validation of VZV ORF63-expressing differentiated SH-SY5Y cells. Our laboratory has previously reported that VZV can protect neurons from apoptosis and determined that VZV ORF63 expression alone could protect rat neurons from nerve growth factor (NGF) withdrawal-induced apoptosis (22, 27). However, it is still not known whether VZV ORF63 alone can protect human neuronal cells from apoptosis. We have also previously demonstrated VZV productive infection of the differentiated SH-SY5Y neuroblastoma cell line (36); thus, these cells provide a suitable neuronal model to examine the effects of VZV ORF63 on apoptosis. To investigate this, a hemagglutinin (HA)-tagged VZV ORF63-expressing SH-SY5Y cell line was generated via the construction of a VZV ORF63 pseudovirus and subsequent transduction and selection (Fig. 1A to E). In parallel, an empty control pseudovirus was generated to create control transduced (CT) SH-SY5Y cells. The SH-SY5Y cell line can be differentiated to morphologically and biochemically resemble primary human neurons (37). Previously, we demonstrated that an all-*trans*-retinoic acid (ATRA) and brain-derived neurotrophic factor (BDNF) differentiation protocol causes SH-SY5Y cells to develop extensive neuronal processes and to upregulate markers that are present in primary human neurons, such as synaptophysin and neural cell adhesion molecule (NCAM) (36).

In three biological replicates, SH-SY5Y cells were differentiated using ATRA and BDNF. Both light microscopy images (Fig. 1A) and staining for NCAM by immunofluorescence assay (IFA) (Fig. 1B) showed the development of extensive neuronal processes in the differentiated VZV ORF63, CT, and untransduced (UT) SH-SY5Y cells that were absent from undifferentiated SH-SY5Y cells. Additionally, staining for synaptophysin demonstrated increased synaptophysin expression and localization to neurites when the VZV ORF63, CT, and UT SH-SY5Y cells were differentiated, in contrast to the undifferentiated cells, which demonstrated minimal staining (Fig. 1C). Altogether, this demonstrates that differentiating VZV ORF63, CT, and UT SH-SY5Y cells using an ATRA and BDNF treatment regime yields mature neuronal-like cells that are both morphologically and biochemically similar to primary human neurons. Immunostaining for HA and ORF63 showed that differentiated VZV ORF63 SH-SY5Y cells expressed ORF63 in both the nucleus and cytoplasm, which is consistent with previous findings (Fig. 1D) (38). Flow cytometry staining for intracellular HA revealed that on average 30% of cells were positive after selection and differentiation (Fig. 1E). As expected, no HA-specific staining was observed in control transduced or untransduced differentiated SH-SY5Y cells by either method (Fig. 1B to E).

VZV ORF63 inhibits staurosporine-induced apoptosis in differentiated SH-SY5Y cells. The intrinsic apoptotic pathway was examined in the differentiated SH-SY5Y cells via the use of staurosporine, an inhibitor of protein kinase C (PKC) (39). VZV ORF63, CT, and UT SH-SY5Y cells were differentiated on coverslips using ATRA and BDNF, as previously described (36), and treated with 0.5 μM staurosporine for 4 h. The cells were immunostained for the markers of apoptosis; DNA fragmentation via terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining and cleaved caspase 3 (CC3), to determine the extent of apoptosis induction (Fig. 2A to C). TUNEL and CC3 staining was readily observed within CT and UT SH-SY5Y cells; however, fewer TUNEL- and CC3-positive cells were seen in the ORF63 SH-SY5Y cells (Fig. 2A to C). To quantitate this difference, 10 independent fields of view for each cell type were enumerated for CC3- and TUNEL-positive cells over three independent

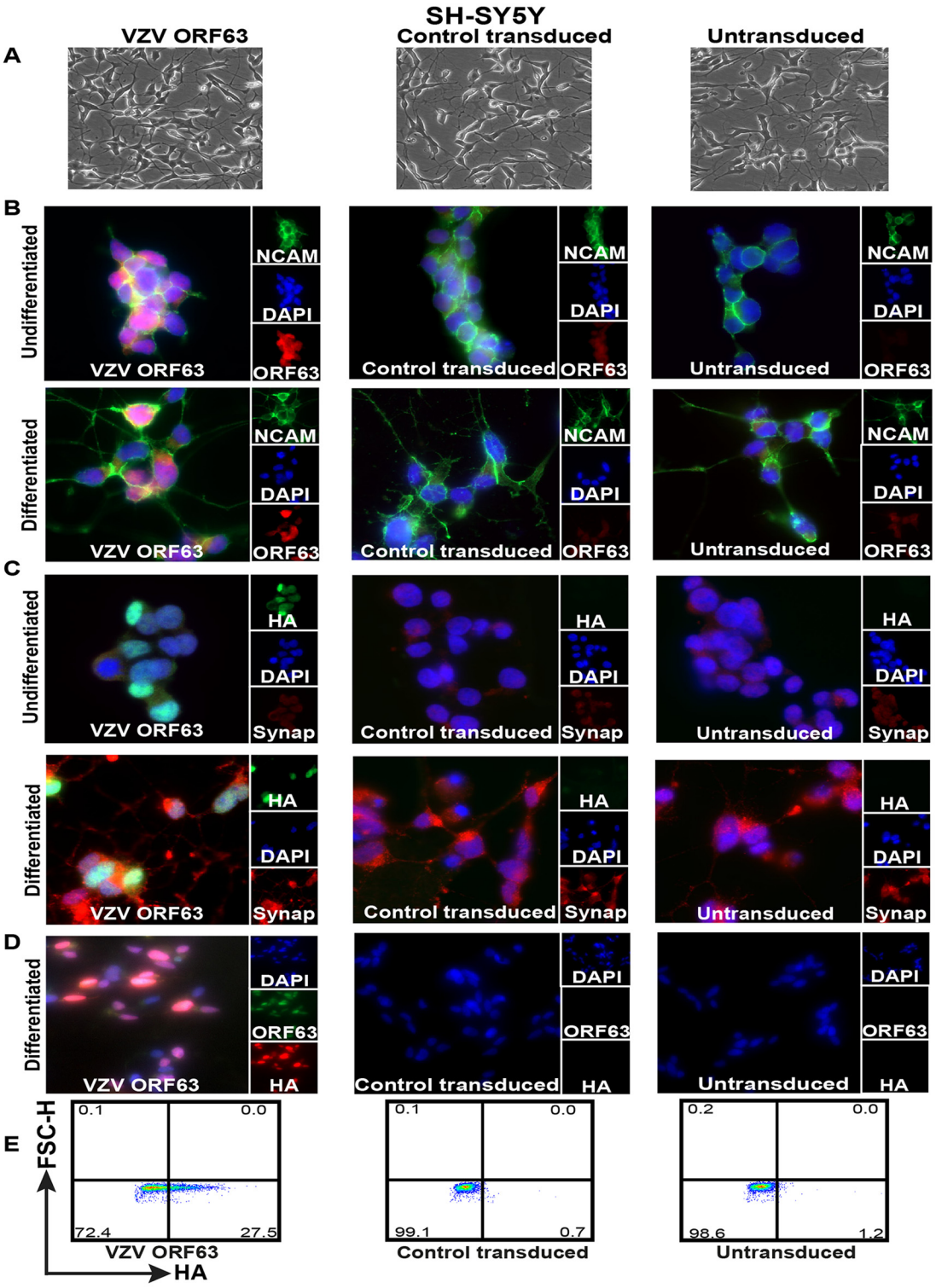

FIG 1 Validation of HA-tagged VZV ORF63-expressing differentiated SH-SY5Y cells. (A) SH-SY5Y cells (1.5×10^6) were transduced with VZV ORF63 or CT pseudoviruses and selected with 0.4 mg/ml G418 for 10 days to create VZV ORF63, CT, and untransduced SH-SY5Y cells. These cells were differentiated using 10 μM ATRA for 5 days and 50 ng/ml BDNF for 4 days. The cells were imaged by light microscopy at the end of the differentiation protocol; the images are shown at $\times$20 magnification. (B to D) VZV ORF63, control transduced, and untransduced SH-SY5Y cells (1×10^5) were differentiated on Matrigel-coated coverslips (13 mm; Knittel glass), fixed with 4% PFA, and stained for VZV ORF63 and NCAM (B), HA and synaptophysin (C), or VZV ORF63 and HA (D). The cells were counterstained with nuclear DAPI (blue) and were visualized by fluorescence microscopy. The images are shown at $\times$20 (D) or $\times$63 (B and C) magnification. (E) Additionally, 5×10^5 differentiated VZV ORF63, CT, and UT SH-SY5Y cells were fixed, permeabilized, stained for HA, and analyzed via flow cytometry. All the data presented are representative of three biological replicates, except for IFA staining (B and C), which is representative of two biological replicates.

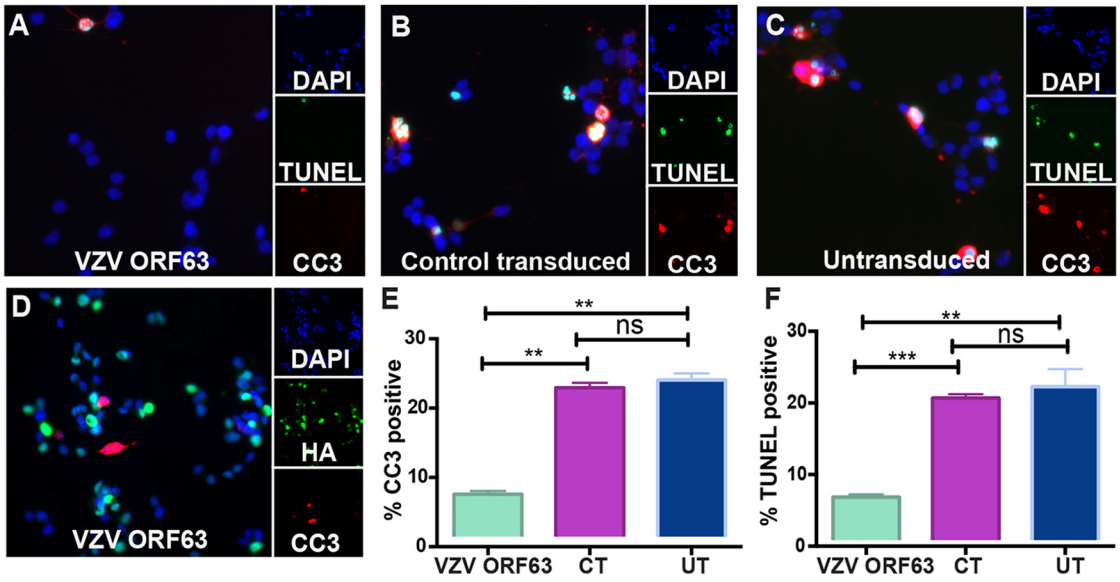

FIG 2 VZV ORF63 inhibits staurosporine-induced apoptosis in differentiated SH-SY5Y cells. (A to D) VZV ORF63 (A and D), CT (B), and UT (C) SH-SY5Y cells (1×10^5) were treated with ATRA for 5 days and differentiated on Matrigel-coated coverslips (13 mm; Knittel glass) via BDNF treatment. The cells were then treated with 0.5 μM staurosporine for 4 h to induce apoptosis and fixed with 4% paraformaldehyde. The cells were permeabilized and stained for CC3 (red) and TUNEL (green) (A to C) or HA (green) and CC3 (red) (D). (E) Cells were counterstained with nuclear DAPI (blue) and visualized by fluorescence microscopy. The images are shown at ×20 magnification and are representative of three biological replicates. (F) For cell counts, 10 different fields of view were imaged to calculate the percentage of CC3- or TUNEL-positive cells under each condition. (E and F) The error bars show standard errors of the mean (SEM), and statistical significance was established by Student's paired *t* test ns, not significant [$P \geq 0.05$]; **, $P < 0.01$; ***, $P < 0.005$.

experiments (Fig. 2E and F). We found that VZV ORF63 SH-SY5Y cells were significantly less TUNEL and CC3 positive than CT and UT cells (Fig. 2E and F). Additionally, no significant differences were found between CT and UT SH-SY5Y cells, suggesting transduction alone does not affect the ability of the cells to undergo apoptosis. To ascertain whether the HA-ORF63-expressing cells were protected from apoptosis, the cells were dually immunostained for HA and CC3 and assessed by fluorescence microscopy (Fig. 2D). No HA-ORF6-expressing cells were found to be CC3 positive. These data show that expression of VZV ORF63 alone is sufficient to inhibit staurosporine-induced apoptosis in differentiated human SH-SY5Y neuronal cells.

VZV rOka induces only a small degree of apoptosis in HaCaT cells. We have shown that VZV ORF63 can protect human neurons from apoptosis induction; however, it is unclear whether this phenotype can be observed in other clinically relevant cell types. Previously, our laboratory has shown that VZV-infected HFs are susceptible to apoptosis induction (22); however, other VZV genes, such as ORF12, have been shown to be protective in skin cells, such as MeWo cells (29). Keratinocytes have been shown to be infected in patient samples (40) and *in vitro* (41, 42). Interestingly, the ability of VZV to cause cell death in this cell type has not been fully characterized. We sought to characterize the ability of VZV strain rOka to induce apoptosis in HaCaT cells, a spontaneously immortalized human keratinocyte cell line (43). The HaCaT cell line has previously been shown to be infected with VZV (44) and thus was chosen as a suitable model for studying VZV proteins in keratinocytes.

VZV rOka-infected HaCaT cells or mock-infected HaCaT cells were stained with cell trace violet (CTV) and used to infect monolayers of HaCaT cells in a cell-associated manner at a 1:5 inoculum-to-cell ratio. CTV staining of the inoculating cells allowed the exclusion of the cells in subsequent flow cytometry analysis. VZV is highly cell associated *in vitro*, and therefore, cell-associated infections are standard practice to propagate infection; however, this results in asynchronous infection (45). Mock- and VZV-infected cells were collected at days 2, 3, and 5 postinfection (p.i.) for flow cytometry

detection of CC3 and LIVE/DEAD staining with Zombie NIR (Biolegend) (Fig. 3A). Both adherent and suspended cells were collected for flow cytometry analysis to ensure all dead cells were analyzed. VZV rOka-infected HaCaT cells were identified by VZV gEgI antigen (a late gene product) expression, and the cells were shown to be on average 20%, 28%, and 28% gEgI positive (gEgI$^+$) on days 2, 3, and 5, respectively, over three biological replicates (Fig. 4). Cells were classified as being live if they were Zombie NIR$^-$ and CC3$^-$, as undergoing other cell death if they were Zombie NIR$^+$ and CC3$^-$, as undergoing late apoptosis if they were Zombie NIR$^+$ and CC3$^+$, and as undergoing early apoptosis if they were CC3$^+$ and Zombie NIR$^-$ (Fig. 3A to E). Even by 5 days p.i., the VZV gEgI-positive cells had a percentage of live cells (measured by Zombie NIR staining) similar to those of both mock-infected and bystander gEgI-negative cells. In addition, results from 3 biological replicates showed that VZV gEgI-positive cells underwent significantly less other cell death at days 3 and 5 p.i. but slightly more early apoptosis at day 5 p.i. than both mock-infected and bystander gEgI-negative cells (Fig. 3B and E). However, for all other time points and types of cell death, VZV antigen-positive cells were comparable to mock-infected and gEgI-negative cells (Fig. 3B to E). To confirm that VZV rOka was not able to induce a large amount of apoptosis in the HaCaT cells, IFA analysis was conducted on rOka- and mock-infected HaCaT cells that were infected using the cell-associated method described above. Cells were collected each day for 3 days p.i. and stained for CC3, TUNEL, and VZV ORF40 (an early gene product) (Fig. 5). At all time points, there were similar levels of apoptotic cells among mock-infected and VZV rOka-infected cells, and the results were consistent in 3 biological repeats. Together, these results indicate that VZV rOka does not induce a large amount of apoptosis in HaCaT cells and suggest VZV gene products could delay virus-induced apoptosis.

Validation of VZV ORF63-expressing HaCaT cells. Our laboratory has previously suggested that the protective effect of ORF63 is neuron specific, as the loss of one of two copies of ORF63 from the virus resulted in an increase in apoptosis in neurons, but not in HFs (27). However, the ability of ORF63 alone to protect nonneuronal cell types from apoptosis has not been investigated. To study the protective effect of VZV ORF63 in a nonneuronal cell type, an HA-tagged VZV ORF63-expressing HaCaT cell line was generated via transduction with the VZV ORF63 pseudovirus (Fig. 6A and B). In parallel, a control pseudovirus was generated to create CT HaCaT cells. Immunostaining and microscopy revealed that the VZV ORF63 HaCaT cells expressed HA-tagged VZV ORF63 in both the nucleus and cytoplasm, which is consistent with the ORF63-expressing SH-SY5Y cells and previous reports (38) (Fig. 6A). On average, over three biological replicates, 50% of cells were determined to be HA positive after selection via flow cytometry (Fig. 6B). No specific HA staining was observed in control transduced or untransduced HaCaT cells by either method. Taking the data together, these HA-ORF63-expressing cell lines can be used to investigate the roles of ORF63 in a number of apoptosis pathways.

VZV ORF63 inhibits staurosporine- and FasL-induced apoptosis in HaCaT cells. Since VZV rOka induced minimal apoptosis in HaCaT cells, we sought to determine whether VZV ORF63 could protect against apoptosis in this cell type. Staurosporine and FasL were utilized to induce intrinsic and extrinsic apoptosis, respectively, to ascertain whether VZV ORF63 protection is specific to a particular type of apoptotic stimulus. VZV ORF63-expressing, CT, or UT HaCaT cells were treated with 0.5 μM staurosporine for 5 h and then stained for CC3 and TUNEL as described previously and visualized by fluorescence microscopy (Fig. 7A to C). Fewer CC3- and TUNEL-positive cells were observed in the VZV ORF63 HaCaT cells than in the CT and UT cells (Fig. 7A to C). To quantitate this difference, 10 independent fields of view for each cell type were enumerated for CC3- and TUNEL-positive cells (Fig. 7E and F). Significantly fewer VZV ORF63 HaCaT cells than CT and UT cells were CC3 positive and TUNEL positive (Fig. 7E and F). CT cells were not significantly different from UT cells, indicating that transduction alone does not affect the ability of the cell type to undergo apoptosis. Cells were

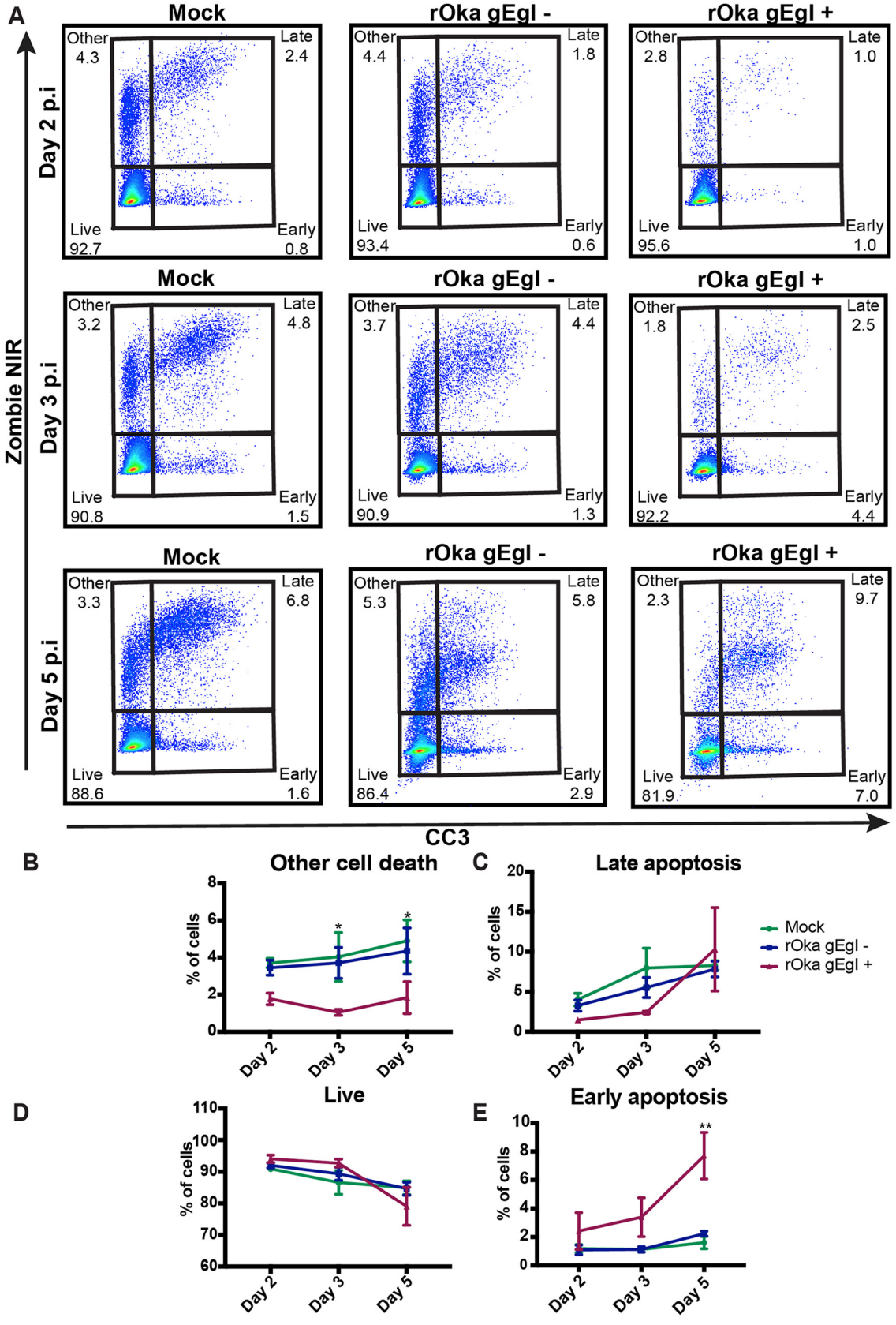

FIG 3 VZV rOka induces only a small degree of apoptosis in HaCaT cells. (A) HaCaT cells (5×10^5) were infected with either CTV-labeled VZV rOka inoculum or CTV-labeled mock inoculum at a ratio of 1:5 in a 6-well plate (Costar). Cells were collected at days 2, 3, and 5 p.i.; stained for VZV gEgI and CC3 and LIVE/DEAD stained to identify apoptotic cells; and analyzed by flow cytometry. The flow cytometry

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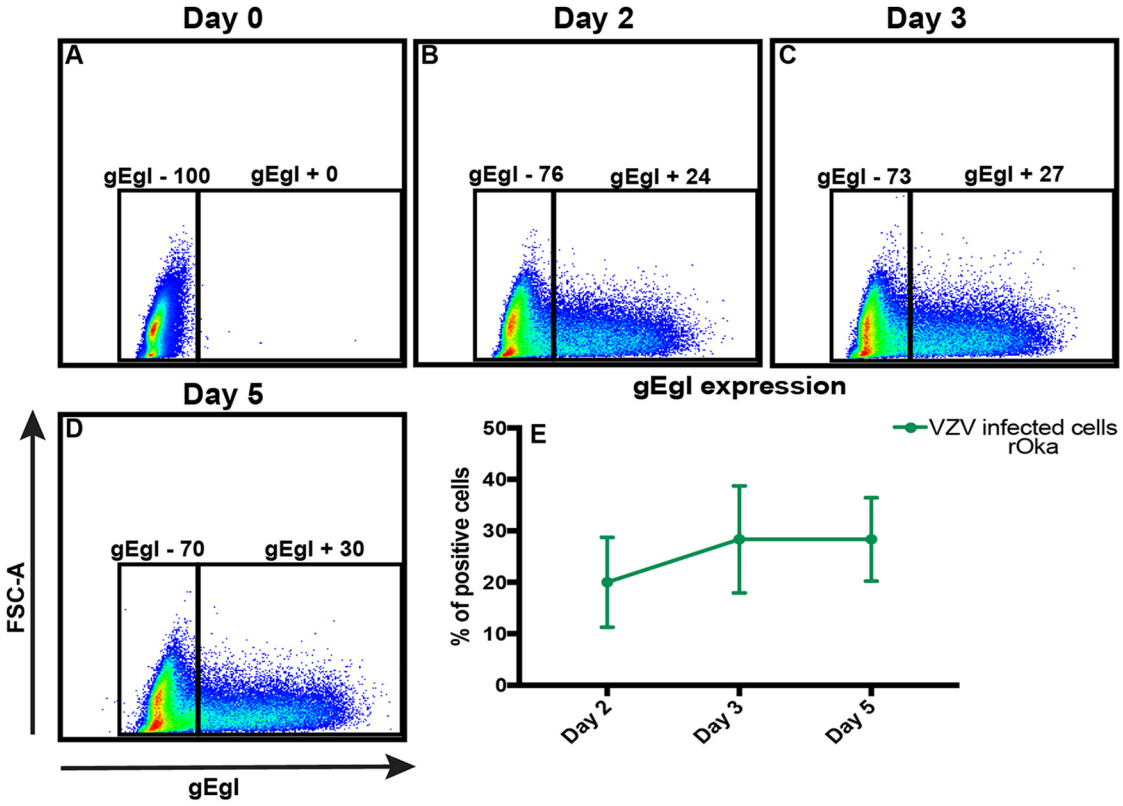

FIG 4 gEgI positivity of VZV rOka-infected HaCaT cells shown in Fig. 3. (A to D) HaCaT cells (5×10^5) were infected with either CTV-labeled VZV rOka inoculum or CTV-labeled mock inoculum at a ratio of 1:5 in a 6-well plate (Costar). Cells were collected at days 0, 2, 3, and 5 p.i.; stained for VZV gEgI and CC3 and LIVE/DEAD stained to identify apoptotic cells; and analyzed by flow cytometry. The flow cytometry plots are representative of three biological replicates. (E) Percentages of gEgI-positive HaCaT cells. The graphs are representative of the collation of three biological replicates. The error bars show SEM.

also dually immunostained for HA and CC3 (Fig. 7D), and the HA-ORF63-expressing cells were not CC3 positive.

For FasL-induced apoptosis, cells were treated with 100 ng/ml FasL for 4 h and immunostained for CC3 and TUNEL by fluorescence microscopy as previously described (Fig. 7G to L). The VZV ORF63 HaCaT cell population had significantly fewer CC3-positive cells than CT and UT HaCaT cells. There was minimal TUNEL staining in all three cell types (Fig. 7G to I). These differences were enumerated as described above, and it was clear that VZV ORF63-expressing cells were significantly less CC3 positive than CT and UT cells (Fig. 7K); however, this pattern was not discerned for TUNEL positivity, due to minimal TUNEL staining being observed (Fig. 7L). There were no significant differences between CT and UT cells. FasL-treated cells were also dually immunostained for CC3 and HA expression, and it was observed that VZV ORF63-expressing cells were not CC3 positive (Fig. 7J). Together, these data demonstrate that VZV ORF63 protects HaCaT cells from staurosporine- and FasL-induced apoptosis.

VZV ORF63 localization changes upon intrinsic apoptosis induction. It has become apparent that VZV ORF63 can protect both human HaCaT keratinocytes and differentiated human SH-SY5Y neuronal cells from apoptosis; however, it is still unclear how the protein modulates this effect. To begin to elucidate this, we sought to determine whether ORF63 localization changes during apoptosis induction. VZV ORF63

FIG 3 Legend (Continued)
plots are representative of three biological replicates. (B to E) Percentages of cells undergoing other cell death (B), late apoptosis (C), or early apoptosis (E) or that were alive (D) over the time course after infection. The graphs are representative of the collation of three biological replicates. The error bars show SEM. Statistical significance was established by a 2-way ANOVA using Tukey's multiple-comparison test. *, $P < 0.05$; **, $P < 0.01$.

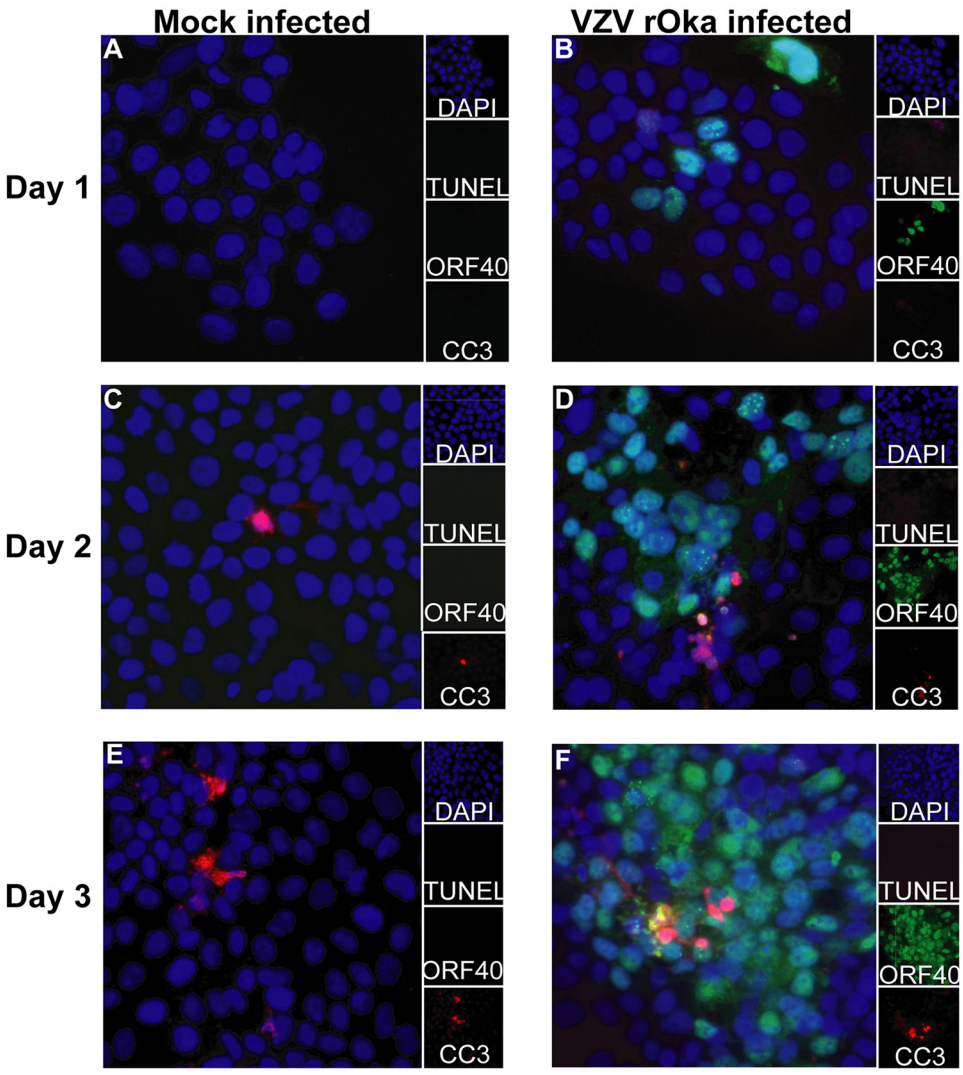

FIG 5 VZV rOka induces only a small degree of apoptosis over a 3-day time course measured by IFA. (A to F) HaCaT cells (1×10^5) were seeded onto coverslips (13 mm; Knittel glass) and infected with either VZV rOka inoculum or mock inoculum at a ratio of 1:5. Cells were collected at days 1, 2, and 3 p.i. and fixed with 4% paraformaldehyde. The cells were permeabilized and stained for CC3 (red) and VZV ORF40 (green) and TUNEL stained (magenta). The cells were counterstained with nuclear DAPI (blue) and were visualized by fluorescence microscopy. The images are shown at ×20 magnification and are representative of three biological replicates.

HaCaT cells treated with 0.5 μM staurosporine for 3 h (Fig. 8A) or untreated (Fig. 8B) were stained with MitoTracker deep red FM (Life Technologies), fixed, permeabilized, and immunostained for HA. Confocal microscopy revealed that VZV ORF63 expression became more cytoplasmic; additionally, there appeared to be formation of HA-positive aggregates.

To determine whether this change in localization was consistent with ORF63 protein expression during VZV infection, at day 3 p.i., VZV rOka-infected HaCaT cells were treated with 0.5 μM staurosporine (Fig. 8C and E) for 3 h or were left untreated (Fig. 8D and F). The cells were stained with MitoTracker deep red FM, fixed, permeabilized, and immunostained for VZV ORF63 or VZV ORF40. During VZV infection VZV ORF63 became markedly more cytoplasmic during apoptosis induction, and again, aggregate formation was identifiable (Fig. 8C and D). In contrast to this, ORF40 localization during apoptosis induction remained largely nuclear (Fig. 8E and F), suggesting that the phenotype observed for VZV ORF63 is a specific relocalization and not merely a consequence arising from cell stress associated with apoptosis. These data suggest that

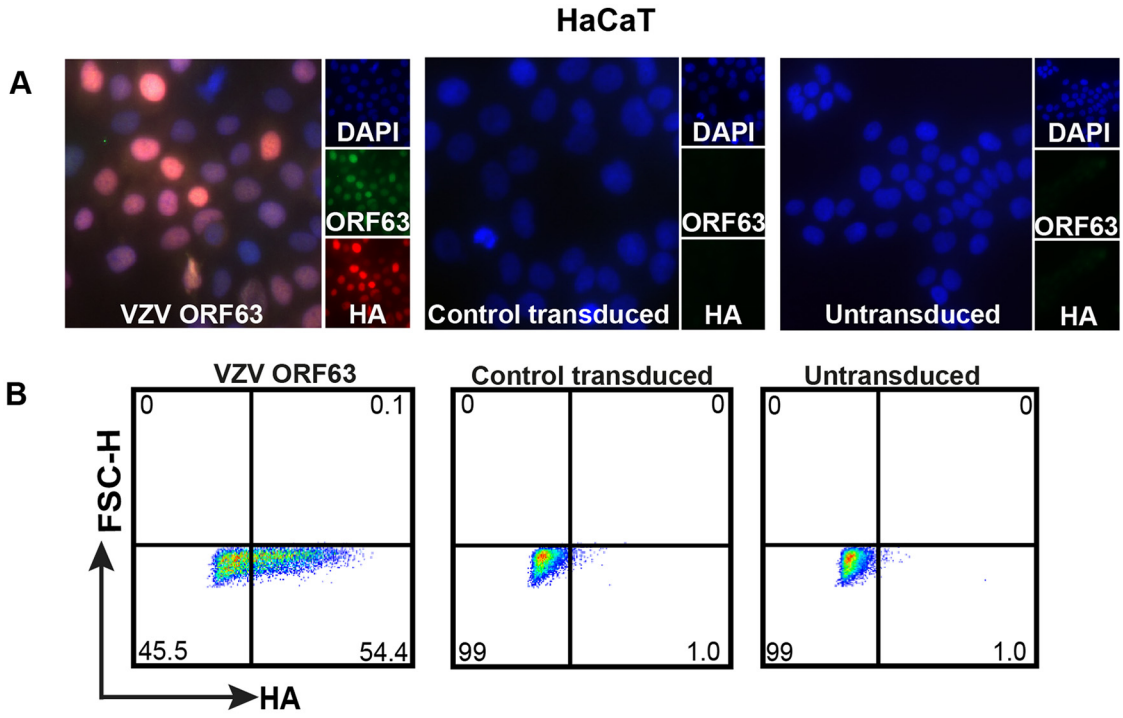

FIG 6 Validation of HA-tagged VZV ORF63-expressing HaCaT cells. (A) HaCaT cells (9×10^5) were transduced with VZV ORF63 or CT pseudoviruses and were selected with 0.5 mg/ml G418 for 10 days to create VZV ORF63, CT, and untransduced HaCaT cells. VZV ORF63, control transduced, and untransduced HaCaT cells (1×10^5) were seeded on coverslips (13 mm; Knittel glass), fixed with 4% PFA, and stained for HA (red) and VZV ORF63 (green). The cells were counterstained with nuclear DAPI (blue) and visualized by fluorescence microscopy. The images are shown at ×20 magnification. (B) Additionally, 5×10^5 VZV ORF63, CT, and UT HaCaT cells were fixed, permeabilized, stained for HA, and analyzed via flow cytometry. All the data presented are representative of three biological replicates.

VZV ORF63 relocalizes during apoptosis induction in human keratinocytes, implying a potential function for ORF63 in the cytoplasm.

DISCUSSION

The current study demonstrates that VZV ORF63 expressed in isolation in human neuronal cell and keratinocyte lines can protect against apoptosis induction. We also characterized the ability of VZV rOka to induce cell death in the human HaCaT keratinocyte line, observing that the virus delayed apoptosis induction over a 5-day time course. We began to elucidate the mechanism behind VZV ORF63 protection by demonstrating that VZV ORF63 protein changes its localization during apoptosis induction, suggesting interaction with proteins in the apoptotic pathway. These findings suggest that, due to its ability to modulate apoptosis in the skin and neuronal environments, VZV ORF63 may be a potential candidate for mutation to generate new attenuated VZV strains for use in vaccination (46).

VZV is a neurotropic virus that establishes lifelong latency in the DRG. As neurons are senescent, it is not surprising that the virus modulates apoptosis to ensure latency is maintained and that reactivation is successful. We have previously demonstrated that VZV ORF63 is associated with neuronal protection from apoptosis; however, there has been no direct evidence that VZV ORF63 expression alone can protect human neuronal cells from apoptosis. Through the construction of novel VZV ORF63-expressing SH-SY5Y cells, we have been able to dissect the protective phenotype of ORF63. We demonstrated that VZV ORF63 expression alone is enough to protect differentiated human SH-SY5Y neuronal cells from staurosporine-induced apoptosis, consolidating our previous work. It is important to note that differentiated SH-SY5Y cells have more properties of central neurons than of peripheral neurons (47, 48), and thus, it would be beneficial to repeat these experiments in primary human peripheral neurons. As VZV ORF63 is one of the most prominent transcripts produced in latency (32–34, 49), it is

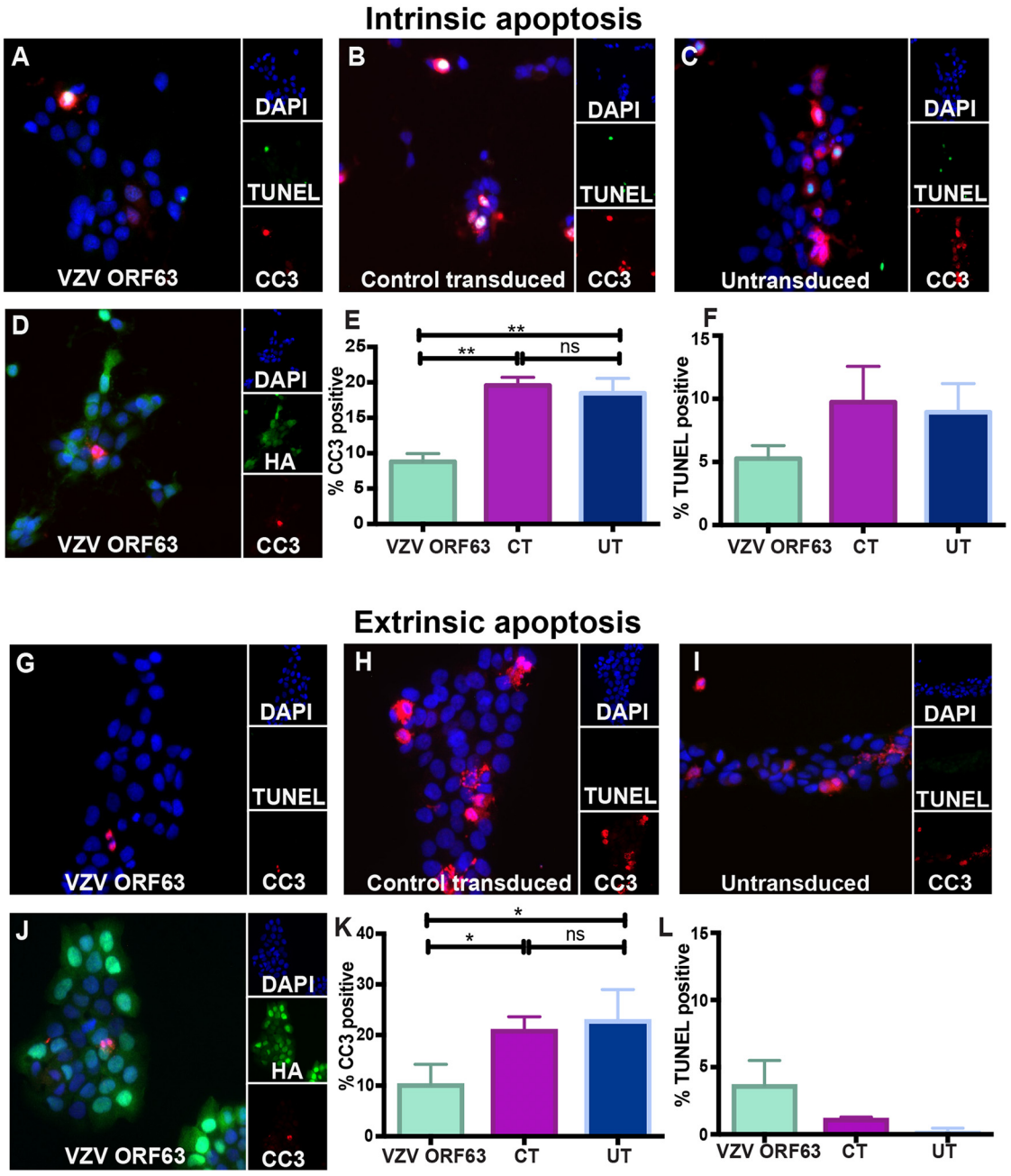

FIG 7 VZV ORF63 inhibits staurosporine- and FasL-induced apoptosis in HaCaT cells. VZV ORF63 (A, D, G, and J), CT (B and H), and UT (C and I) HaCaT cells (1×10^5) were treated with 0.5 μM staurosporine (A to F) or 100 ng FasL (G to L) for 5 h and fixed with 4% paraformaldehyde on coverslips (13 mm; Knittel glass). The cells were permeabilized and stained for CC3 (red) and TUNEL stained (green) (A to C and G to I) or stained for HA (green) and CC3 (red) (D and J). The cells were counterstained with nuclear DAPI (blue) and visualized by fluorescence microscopy. The images are shown at ×20 magnification and are representative of three biological replicates. For the cell counts, 10 different fields of view were imaged to calculate the percentage of CC3-positive (E and K) or TUNEL-positive (F and L) cells under each condition. The error bars show SEM, and statistical significance was established by Student's paired *t* test (ns, not significant [$P \geq 0.05$]; *, $P < 0.05$; **, $P < 0.01$).

conceivable that VZV ORF63's ability to inhibit apoptosis also enables the virus to establish latency, maintain latency, and reactivate.

Various studies have demonstrated that VZV induces apoptosis in cell types such as HFs (22), MeWo cells (23), and immune cells (e.g., T cells, B cells, and monocytes) (24, 25). The ability of VZV to induce or protect against cell death has not been fully characterized in keratinocytes. Sensory neurons of the DRG dock in the keratinocyte

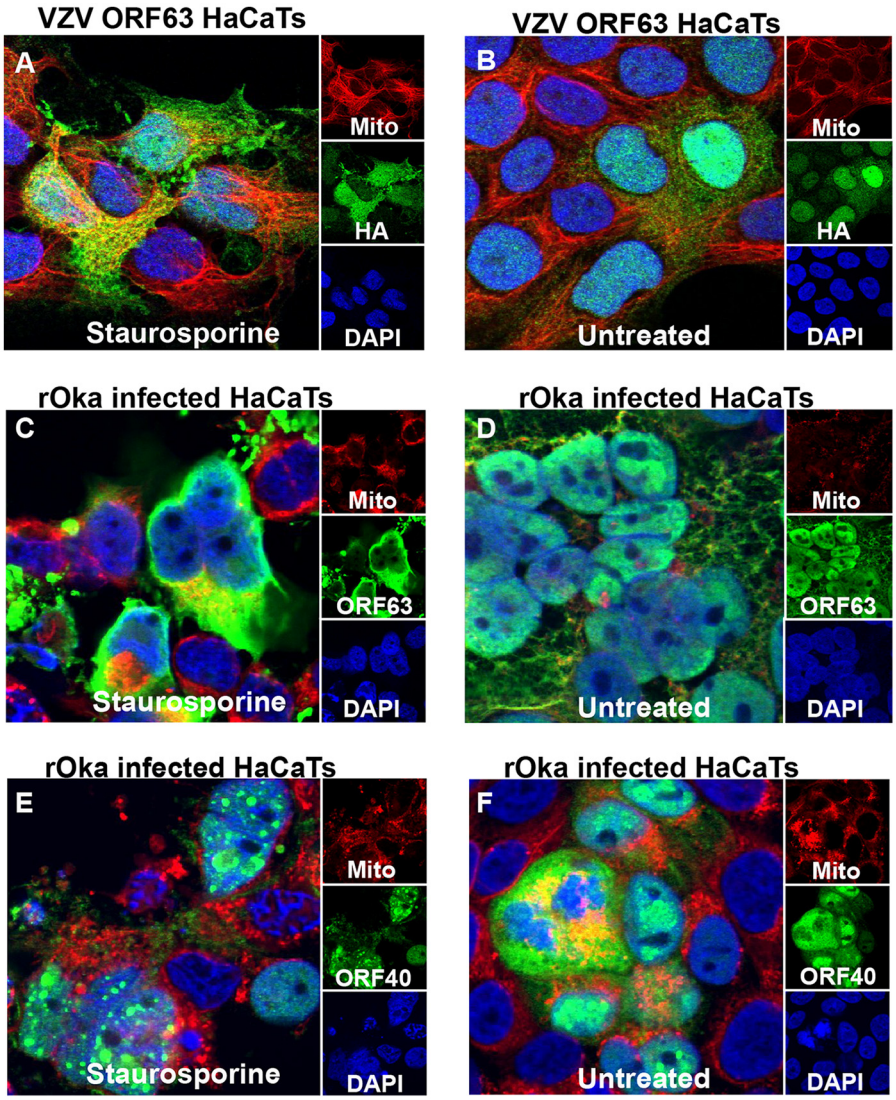

FIG 8 VZV ORF63 protein localization changes with staurosporine treatment. VZV ORF63-expressing HaCaT cells (A and B) and rOka-infected HaCaT cells (C to F) were treated with 0.5 μM staurosporine for 3 h (A, C, and E) or were left untreated (B, D, and F). The cells were stained with MitoTracker deep red (red); fixed with 4% paraformaldehyde; permeabilized; and stained for HA, VZV ORF40, or VZV ORF63 (green). The cells were counterstained with nuclear DAPI (blue) and visualized by fluorescence microscopy. The images are shown at $\times$63 magnification and are representative of three biological replicates.

layer of the epidermis (50), and therefore, modulation of cell death in keratinocytes could promote VZV infection of sensory neurons of the DRG. It is critical to explore the types of cell death induced by the virus due to their different inflammatory capacities, with apoptosis being noninflammatory and other forms of cell death, such as pyroptosis, necroptosis, and necrosis, being inflammatory (51). In the current study, we were able to distinguish between cells that were alive, in early apoptosis, in late apoptosis, or undergoing another form of cell death. Some reports in the literature suggest that HaCaT cells are capable of undergoing necroptosis (52, 53); however, in our and other's hands (E. Mocarski, Emory University, personal communication), using tumor necrosis factor (TNF) in combination with a second mitochondrion-derived activator of caspases (Smac) mimetic and pan-caspase inhibitor, HaCaT cells were not susceptible to necroptosis (Fig. 9). In the current study, we found that only a small percentage of human keratinocytes underwent early apoptosis up to 5 days p.i. with VZV rOka, indicating that the virus may delay apoptosis induction in these cells. Similar results were obtained by

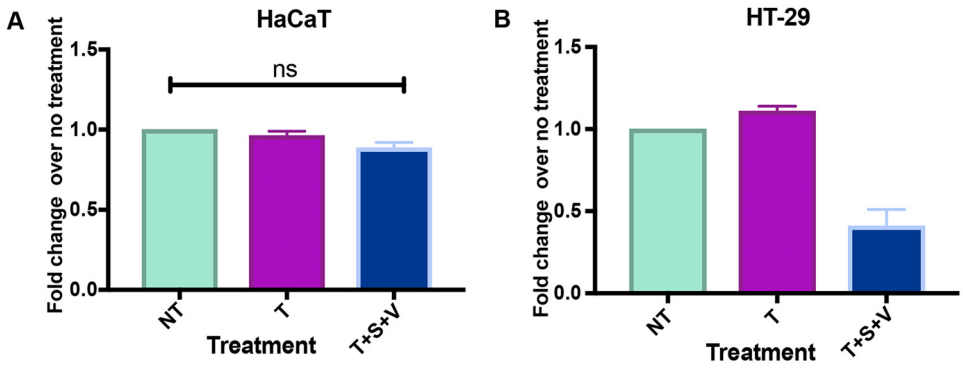

FIG 9 HaCaT cells cannot undergo necroptosis. HaCaT cells (3×10^4) (A) and HT-29 cells (2×10^4) (B) were seeded in triplicate in individual wells of a 96-well plate (Costar). To induce necroptosis (T+S+V), HT-29 cells and HaCaT cells were treated with 30 ng/ml TNF (T), 1 μM BV-6 (S), and 12.5 μM z-Vad (V) for 16 h. For the no-treatment control (NT), cells were treated with DMSO, and for the cell survival control (T), cells were treated with TNF alone. Cell viability was measured by CellTitre-Glo 2.0 assay using a Tecan plate reader. For HaCaT cells, three biological replicates were conducted; for HT-29 cells, two biological replicates were conducted. The error bars show SEM, and statistical significance was established by Student's paired *t* test (ns = not significant [$P \geq 0.05$]).

Black et al. (41), where VZV-infected human papillomavirus (HPV)-immortalized keratinocytes were negative for annexin V staining at 10 days p.i. Interestingly, VZV antigen-positive cells seemed to undergo less non-caspase 3-dependent cell death than mock-infected cells; therefore, it is prudent to explore the roles of different forms of cell death in the skin microenvironment.

Based on the observation that VZV delayed apoptosis induction in keratinocytes, we thought it would be interesting to explore the role of VZV ORF63 in that cell type. As a result, we constructed a novel VZV ORF63-expressing HaCaT cell line. VZV ORF63 was found to protect HaCaT cells from both staurosporine- and FasL-induced apoptosis, demonstrating that the protective effect provided by ORF63 is not exclusive to neurons or apoptotic stimuli. This is the first time that ORF63 has been shown to modulate FasL-induced extrinsic apoptosis. Confocal microscopy was utilized to examine VZV ORF63 protein localization during apoptosis induction, as it has been demonstrated that during infection, VZV ORF63 can localize to the mitochondria in human lung cells (54), which could be where VZV ORF63 modulates apoptosis. In both VZV-infected cells and VZV ORF63-expressing HaCaT cells, VZV ORF63 became more distinctly cytoplasmic upon apoptosis induction; however, localization to the mitochondria was not clear due to a diffuse ORF63 staining pattern. Interestingly, aggregate formation was also observed, indicating a potential protein-protein interaction. This change was shown not to be a by-product of cell death, as the same pattern was not observed with VZV ORF40, a protein with no known antiapoptotic functions.

It is important to elucidate the mechanism by which VZV gene products are able to protect against apoptosis, due to their importance in pathogenesis. VZV ORF63 is homologous to HSV ICP22, and both have been found to be antiapoptotic; however, there are key differences in their functions (55). HSV ICP22 produces a full-length ICP22 protein and an N-terminally truncated form called Us1.5 (56). Us1.5 acts to activate caspase 3 and thus induce apoptosis (57), while ICP22 has been suggested to antagonize p53 (58); however, it is not clear if this is responsible for ICP22 apoptosis inhibition (55). VZV ORF63 produces only a full-length protein, and unlike HSV ICP22, transcript and ORF63 protein are present during latency (34, 49). HSV encodes a latency-associated transcript known as LAT that has been shown to inhibit apoptosis by affecting apoptotic proteins, and also through the production of microRNAs that target apoptotic genes (55). VZV ORF63 is not known to encode any microRNAs or to transcriptionally regulate apoptotic proteins. Cell stress could activate the translation of the protein to inhibit apoptosis in latently infected neurons.

In summary, our results provide the first evidence that VZV ORF63 alone is enough

to protect differentiated human SH-SY5Y neuronal cells from apoptosis. Additionally, this is the first demonstration that VZV ORF63's protective effect is not neuron specific and can protect human HaCaT keratinocyte cells from both intrinsic and extrinsic apoptosis. This implicates VZV ORF63 as being crucial in VZV pathogenesis in the skin. We have begun to elucidate the mechanism of action of VZV ORF63 by showing that protein localization changes with apoptosis induction, suggesting protein-protein interactions with the apoptosis machinery. Altogether, our work helps to uncover the potential role of VZV ORF63 apoptosis modulation in VZV latency, reactivation, and ultimately pathogenesis.

MATERIALS AND METHODS

Cell lines. HEK293T cells (ATCC), HaCaT cells (Creative Bioarray), and HT-29 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 IU/ml penicillin and streptomycin.

SH-SY5Y neuroblastoma cells (ATCC) (59) were maintained in DMEM-F12 supplemented with 10% FBS and 50 IU/ml penicillin and streptomycin. To differentiate SH-SY5Y cells, the cells were treated with 10 μM ATRA for 5 days. Cells were then seeded onto Matrigel (Corning)-covered plates or flasks and treated with 50 ng/ml BDNF in serum-free medium for 4 days.

Viruses and infection of cell lines. VZV rOka (kindly provided by Ann Arvin, Stanford University) (60)-infected HaCaT cells were cocultivated with uninfected HaCaT cells at a 1:5 ratio.

HaCaT cells and undifferentiated SH-SY5Y cells were transduced with control or VZV ORF63 pseudoviruses, using 8 μg/ml of Polybrene. SH-SY5Y cells and HaCaT cells were selected with 0.4 mg/ml and 0.75 mg/ml G418, respectively, for 10 days. This generated VZV ORF63-expressing HaCaT cells, CT HaCaT cells, VZV ORF63-expressing SH-SY5Y cells, and CT SH-SY5Y cells. Undifferentiated ORF63-expressing SH-SY5Y and CT SH-SY5Y cells were differentiated before use in experiments.

Apoptosis and necroptosis treatments and cell viability measurement. To induce apoptosis, differentiated SH-SY5Y cells were treated with 0.5 μM staurosporine for 4 h and HaCaT cells were treated with 0.5 μM staurosporine for 5 h or with 100 ng/μl Fas ligand for 5 h. To induce necroptosis, HT-29 cells and HaCaT cells were treated with 30 ng/ml TNF, 1 μM BV-6 (Smac mimetic), and 12.5 μM z-Vad (pan caspase inhibitor) for 16 h, and cell viability was measured by CellTitre-Glo 2.0 assay (Promega, USA). For the no-treatment control, cells were treated with dimethyl sulfoxide (DMSO), and for the cell survival control, cells were treated with TNF alone.

Lentivirus construction, production, and infection. Primers (IE63_FHA_Eco, GGCGCAATAGAATTC TACCATGTACCCATACGATGTTCCAGATTACGCTTTTTGCACCTCACCGGC, and IE63_R_Bam, GGCCGAAGGAT CCCTACACGCCATGGGGGG) were utilized to amplify VZV ORF63 from the VZV pOka genome (Ann Arvin, Stanford University), to attach an HA tag to the N-terminal region of the protein, and to insert appropriate restriction enzyme (RE) cut sites. PCR products were purified (GE Healthcare; Illustra GFX PCR DNA and gel band kit) and digested with EcoRI-HF and BamHI-HF REs. The backbone plasmid (pCDH1-CMV-MCS-EF1-Neo cDNA cloning and expression vector [pCDH]; System Biosciences, USA) was also digested with EcoRI-HF and BamHI-HF and ligated with the VZV ORF63 PCR product using T4 DNA ligase (NEB); the resulting plasmid was called pCDH63. pCDH63 was transformed into *Escherichia coli* Stbl2 competent cells (NEB) and collected using a plasmid DNA purification kit (Nucleobond Xtra Midi plasmid DNA purification kit; Macherey-Nagel). pCDH63 was transfected into 293T cells with the packaging plasmids psPAX2 (Addgene) and pMD2G (Addgene) using Fugene HD (Promega) to create an HA-VZV ORF63-expressing pseudovirus. pCDH was transfected into 293T cells with the packaging plasmids psPAX2 and pMD2G using Fugene HD to create the corresponding control pseudovirus.

Flow cytometry. Mock-infected or VZV rOka-infected HaCaT cells were stained with 1 μl/ml CTV (Thermo Fisher Scientific) for 20 min at 37°C. The cells were then quenched in medium and seeded onto uninfected HaCaT cells at a ratio of 1:5. At days 2, 3, and 5 p.i., cells were collected for flow cytometry. The cells were stained with Zombie NIR and rabbit anti-VZV gEgI (Meridian Bioscience Inc.) that was conjugated to Dylight 488 (Serotec) at room temperature (RT) for 30 min protected from light and were then fixed and permeabilized (BD Fix Perm) at 4°C. The cells were stained with rabbit anti-CC3-phycoerythrin (PE) (BD Biosciences) overnight at 4°C. All samples were acquired on an LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FloJo software (Tree Star, Ashland, OR). CTV-labeled inoculum cells were excluded from the analysis.

TUNEL assay and MitoTracker staining. Cells were grown on coverslips and treated as indicated. The cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 15 min at RT. After washing with PBS, the cells were permeabilized with 0.1% Triton X (Sigma-Aldrich) for 10 min and blocked using 20% normal donkey serum (NDS) (Sigma-Aldrich). The cells were TUNEL stained (In Situ Cell Death Detection kit, fluorescein; Roche) for 1 h at 37°C.

For MitoTracker deep red FM staining, cells were stained with 0.3 μM MitoTracker deep red FM for 30 min at 37°C and washed with PBS. The cells were fixed with 4% PFA for 15 min at RT.

IFA. Cells were grown on coverslips and treated/infected as indicated. The cells were washed with PBS and fixed with 4% PFA for 15 min at RT. After washing with PBS, the cells were permeabilized with 0.1% Triton X (Sigma-Aldrich) for 10 min and blocked using 20% NDS (Sigma-Aldrich). The cells were incubated with primary antibodies or corresponding isotype controls for 1 h at RT. The cells were washed with PBS, incubated with secondary antibodies at RT for 30 min, and washed with PBS, and coverslips were mounted on glass slides using Prolong Gold Anti-Fade reagent with DAPI (4′,6-diamidino-2-

phenylindole) (Life Technologies). Staining was visualized on a Zeiss Axio Imager microscope or Zeiss LSM 510 Meta Spectral confocal microscope, and images were taken using Zen software (Zeiss) or LSM 510 (Zeiss), respectively. Images were pseudocolored using Fiji (Image J). For apoptosis quantification, 10 independent images of each coverslip were taken, and CC3- and TUNEL-positive cells were counted manually to calculate an average percentage of positive cells.

Antibodies. For IFA, cells were stained with the following primary antibodies as indicated: mouse anti-VZV ORF40 (NCP-1; 1:500; Meridian Bioscience Inc.), mouse anti-HA tag (6E2; 1:100; Cell Signaling Technology), rabbit anti-CC3 (D3E9; 1:150; Cell Signaling Technology), mouse anti-NCAM (123C3; 1:20; Cell Signaling Technology), rabbit anti-synaptophysin (Z66; 1:10; Invitrogen), and rabbit anti-VZV ORF63 (1:300; a gift from Paul Kinchington, University of Pittsburgh).

For IFA, cells were stained with the following secondary antibodies (1:250; Invitrogen) as indicated: donkey anti-rabbit IgG 488, 594, and 647 and donkey anti-mouse IgG 488, 546, 594, and 647.

Statistical analysis. *P* values were determined for IFA analysis using a paired 2-tailed Student *t* test. *P* values were determined for flow cytometry analysis via a 2-way analysis of variance (ANOVA) using Tukey's multiple-comparison test.

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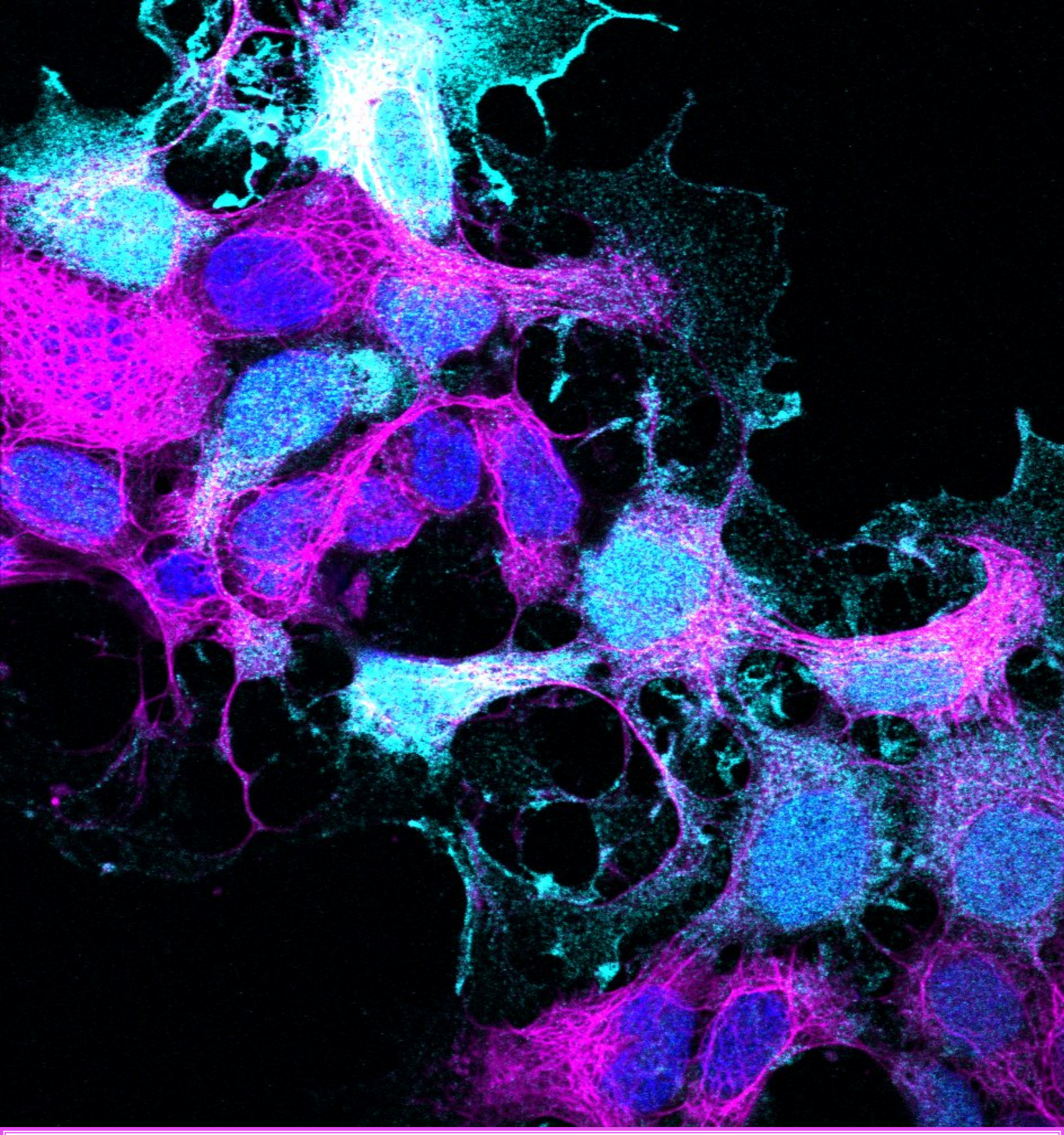

Chapter 3: Granzyme B cleaves multiple VZV and HSV-1 proteins and VZV ORF4 inhibits NK cell cytotoxicity

CHAPTER 3

Introductory statement

It is clear that cell death pathways such as apoptosis are critical in the pathogenesis of herpesvirus infection. As well as being an intrinsic defense mechanism, both the innate and adaptive immune response can utilize apoptosis, through the perforin/granzyme pathway, to kill virally infected target cells. Both cytotoxic T cells and NK cells have been shown as critical cell types in the control of alphaherpesvirus infection and have a role in both productive infection and the maintenance of latency. CTLs have been shown to prevent the reactivation of HSV-1 through granzyme B cleavage of HSV ICP4. However, the CTLs do not induce neuronal apoptosis in this context. It is unclear whether the possession of granzyme B cleavage sites in either HSV-1 or VZV can prevent cell death induced by cytotoxic immune cells as in the case of other viruses. The findings in Chapter 3 explore HSV-1 and VZV proteins with granzyme B cleavage sites and link this to their ability to inhibit NK cell mediated cytotoxicity.

The findings of Chapter 3 are presented as a published article, with author contributions outlined below.

Authorship attribution statement

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The convention for author placement is the listing of authors in order of contribution to the paper, with the senior author listed in last position.

This work was conceived and designed by CG, MS, TMC, BPM, BS and AA. At least 90% of the total work was completed by CG including performing the experiments, experimental validation, data analysis and data interpretation. CG prepared the manuscript and figures, with all other authors critically reviewing the manuscript. MS and AA provided supervision to CG; and AA were responsible for funding acquisition and project administration.

I have obtained permission to include the published material from the corresponding author, Allison Abendroth.

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Chelsea Gerada

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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VIRUS-CELL INTERACTIONS

Granzyme B Cleaves Multiple Herpes Simplex Virus 1 and Varicella-Zoster Virus (VZV) Gene Products, and VZV ORF4 Inhibits Natural Killer Cell Cytotoxicity

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ABSTRACT Immune regulation of alphaherpesvirus latency and reactivation is critical for the control of virus pathogenesis. This is evident for herpes simplex virus 1 (HSV-1), where cytotoxic T lymphocytes (CTLs) prevent viral reactivation independent of apoptosis induction. This inhibition of HSV-1 reactivation has been attributed to granzyme B cleavage of HSV infected cell protein 4 (ICP4); however, it is unknown whether granzyme B cleavage of ICP4 can directly protect cells from CTL cytotoxicity. Varicella zoster virus (VZV) is closely related to HSV-1; however, it is unknown whether VZV proteins contain granzyme B cleavage sites. Natural killer (NK) cells play a central role in VZV and HSV-1 pathogenesis and, like CTLs, utilize granzyme B to kill virally infected target cells. However, whether alphaherpesvirus granzyme B cleavage sites could modulate NK cell-mediated cytotoxicity has yet to be established. This study aimed to identify novel HSV-1 and VZV gene products with granzyme B cleavage sites and assess whether they could protect cells from NK cell-mediated cytotoxicity. We have demonstrated that HSV ICP27, VZV open reading frame 62 (ORF62), and VZV ORF4 are cleaved by granzyme B. However, in an NK cell cytotoxicity assay, only VZV ORF4 conferred protection from NK cell-mediated cytotoxicity. The granzyme B cleavage site in ORF4 was identified via site-directed mutagenesis and, surprisingly, the mutation of this cleavage site did not alter the ability of ORF4 to modulate NK cell cytotoxicity, suggesting that ORF4 has a novel immunoevasive function that is independent from the granzyme B cleavage site.

IMPORTANCE HSV-1 causes oral and genital herpes and establishes life-long latency in sensory ganglia. HSV-1 reactivates multiple times in a person's life and can cause life-threatening disease in immunocompromised patients. VZV is closely related to HSV-1, causes chickenpox during primary infection, and establishes life-long latency in ganglia, from where it can reactivate to cause herpes zoster (shingles). Unlike HSV-1, VZV only infects humans, and there are limited model systems; thus, little is known concerning how VZV maintains latency and why VZV reactivates. Through studying the link between immune cell cytotoxic functions, granzyme B, and viral gene products, an increased understanding of viral pathogenesis will be achieved.

KEYWORDS *Varicella zoster virus* (VZV), *Herpes simplex virus* (HSV), granzyme B, natural killer (NK) cells

Human alphaherpesviruses such as herpes simplex virus 1 (HSV-1) and varicella zoster virus (VZV) are characterized by their ability to establish life-long latency in sensory nerves during primary infection (1). Primary infection with HSV-1 can result in oral or genital herpes, whereas primary infection with VZV results in chickenpox (2). During primary infection, these viruses establish life-long latency in either the dorsal root (DRG) or trigeminal ganglia (TG) (2). For both VZV and HSV-1, reactivation and clinical severity is heightened in immunocompromised hosts, highlighting the impor-

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tance of the immune system in controlling alphaherpesvirus pathogenesis (3). Understanding how these viruses maintain life-long latency and reactivate is key to developing therapeutic strategies to prevent the potentially severe consequences of alphaherpesvirus reactivation.

HSV-1 latency has been studied in mouse models, in which cytotoxic T lymphocytes (CTLs) lie in close proximity to latently infected neurons (4). These CTLs have been shown to inhibit HSV-1 reactivation through the delivery of granzyme B and the subsequent cleavage of HSV infected cell protein 4 (ICP4) (5). Typically, granzyme B would induce apoptosis in target cells; however, this is not observed in HSV-1-infected neurons. Viral inhibition of granzyme B-induced apoptosis has been explored in the context of adenovirus, where the viral protein L4-100K has been shown to inhibit both granzyme B activity and CTL cytotoxicity (6). This function was linked to a granzyme B consensus motif in L4-100K. To date, it is unknown whether there are HSV-1 gene products other than HSV ICP4 that can be cleaved by granzyme B. As HSV ICP4 has a granzyme B consensus motif, it is pertinent to investigate whether HSV ICP4 can inhibit granzyme B function and CTL cytotoxicity, as this could explain the lack of CTL-induced apoptosis in the context of HSV-1 latency.

HSV-1 literature has focused on the role of CTLs in the prevention of HSV-1 reactivation; however, both CTLs and NK cells can utilize granzyme B to kill target cells. Typically, when CTLs or NK cells recognize a virally infected target cell, they create an immunological synapse with the target cell and directly secrete granules containing perforin and granzyme B along with other constituents. Perforin forms a pore in the target cell, allowing for the delivery of granzyme B. Granzyme B cleaves multiple apoptotic pathway components that converge on the cleavage of caspase 3, the executioner caspase. This ultimately results in the induction of apoptosis in the target cell.

VZV is genetically similar to HSV-1 (7); however, less is known about VZV latency and reactivation. In examination of postmortem latently infected TG samples, resident CTLs were shown to be directed against HSV-1 rather than against VZV (8). However, in postmortem DRG samples from individuals with active herpes zoster at the time of death, CTLs that are granzyme B positive were in close proximity to VZV-infected DRG neurons (9). This may highlight that, while ganglia resident CTLs are not directed toward VZV during latency, CTLs infiltrate into the ganglia in response to VZV reactivation. Interestingly, no neuronal cell death is observed with CTL infiltration during VZV reactivation, and the role of granzyme B remains unclear. NK cells have been found to infiltrate the DRG in samples that were collected 1 to 4.5 months after herpes zoster reactivation, and therefore it would be relevant to examine NK cells in the context of the relationship between VZV and granzyme B (10).

Therefore, this study sought to identify whether VZV encodes proteins that can be cleaved by granzyme B, as well as to identify additional granzyme B cleavage sites in HSV-1 proteins. In this study, HSV gene products and VZV gene products were selected for investigation on the basis of having predicted granzyme B cleavage sites. These gene products were expressed in human cells *in vitro* to examine the relationship between granzyme B, NK cells, and human alphaherpesviruses. In this report, we show that HSV ICP27, VZV open reading frame 4 (ORF4), and VZV ORF62 can be cleaved by granzyme B. We addressed the biological relevance of granzyme B cleavage of HSV-1 and VZV proteins in the context of NK cell cytotoxicity. We demonstrate that VZV ORF4 alone was able to inhibit NK cell-mediated cytotoxicity, indicating that possession of a granzyme B cleavage site is not sufficient to limit NK cell-mediated cytotoxicity. Surprisingly, mutation of the granzyme B cleavage site in VZV ORF4 did not alter the ability of ORF4 to inhibit NK cell-mediated cytotoxicity, demonstrating that this function is independent of the granzyme B cleavage site. This study increases our knowledge concerning HSV-1 and VZV proteins that may be targeted by granzyme B to limit viral spread, as well as characterizing a novel immunoevasive function for VZV ORF4 in the context of NK cell-mediated cytotoxicity. Overall, this broadens our knowledge

concerning the control of HSV-1 and VZV latency and VZV immunoevasive mechanisms during lytic infection and reactivation.

RESULTS

Granzyme B cleaves VZV ORF4, VZV ORF62, and HSV ICP27. The ability of CTLs to inhibit HSV-1 reactivation has been attributed to the cleavage of ICP4 by granzyme B (5). However, it is unclear whether other HSV-1 gene products also contain granzyme B cleavage sites and thus may be targeted by the immune response to inhibit viral reactivation. Furthermore, it is yet to be determined whether the closely related herpesvirus VZV encodes gene products that contain granzyme B cleavage sites. To determine whether there were novel granzyme B cleavage sites in VZV and HSV gene products, HSV-1 and VZV gene products were examined for potential granzyme B cleavage sites via the program GraBCas (11).

VZV ORF4, VZV ORF62, and HSV ICP27 all contained predicted multiple granzyme B cleavage sites. HSV ICP27 is an immediate-early gene that regulates mRNA synthesis and processing (12, 13). VZV ORF4 is the homolog of HSV ICP27 and an immediate-early gene that is essential for viral replication and the establishment of latency (14). VZV ORF62 is also an immediate-early protein and is a transactivator and has immunomodulatory roles (15, 16). Of note, all of these viral gene products have important roles in viral replication.

To test whether these viral proteins could be cleaved by granzyme B, plasmids were designed to express these viral proteins tagged to green fluorescent protein (GFP). Lysates from 293Ts transfected with the viral protein-GFP plasmids, as well as the parental plasmid (pGFP-C1), were generated (Fig. S1). These lysates were incubated with or without granzyme B (50 μM for VZV ORF4, VZV ORF62, HSV ICP4, and 100 μM for HSV ICP27; Millipore) and were analyzed for cleavage products via Western blot analysis. Granzyme B was able to effectively cleave VZV ORF4 (Fig. 1A) and VZV ORF62 (Fig. 1B), producing cleavage products of 65 kDa and 45 kDa, respectively. HSV ICP27 (Fig. 1C) was also cleaved, yielding a cleavage product of 40 kDa; however, the concentration of granzyme B required to demonstrate this phenotype was double that of the other gene products. No cleavage was observed when GFP was expressed alone (Fig. 1D). As expected from previous reports, HSV-1 ICP4 was cleaved by granzyme B, yielding a cleavage product of 40 kDa (Fig. 1E). These results were observed in at least 3 biological replicates. Using cleavage product sizes for HSV ICP27, VZV ORF4, and VZV ORF62, it was possible to further narrow down the predicted granzyme B cleavage site (Fig. 1F). These cleavage sites differed from previously predicted cleavage sites in HSV ICP4 (5) and adenovirus L4-100K (6).

To further demonstrate that granzyme B was able to specifically cleave VZV ORF4, VZV ORF62, and HSV ICP27 proteins, increasing concentrations of granzyme B were added to lysates to determine whether there was a dose-dependent response. Such a response was evident for VZV ORF4 (Fig. 1G) and VZV ORF62 (Fig. 1H), with the full-sized proteins diminishing and cleavage products increasing with increasing concentrations of granzyme B. This pattern was also observed for HSV ICP27 (Fig. 1I); however, double the amount of granzyme B was required to see this effect. As HSV ICP27 required double the amount of granzyme B to observe a cleavage product, it is possible that its granzyme B cleavage site may not be as efficiently recognized by granzyme B in comparison to the other gene products studied. These results were observed in 3 biological replicates. These experiments demonstrate for the first time that VZV ORF62, VZV ORF4, and HSV ICP27 are cleaved by granzyme B.

VZV ORF4 can restrict cell death by NK cell-mediated cytotoxicity at a low ratio. L4-100K in adenovirus can inhibit granzyme B function and CTL-induced cell death due to its granzyme B consensus motif (6). HSV ICP4 also contains a granzyme B consensus motif (5); however, this has not been linked to inhibition of immune cell-mediated cytotoxicity. NK cells also utilize granzyme B to kill virally infected target cells and are critical in the control of HSV-1 and VZV infection. Therefore, we sought to determine

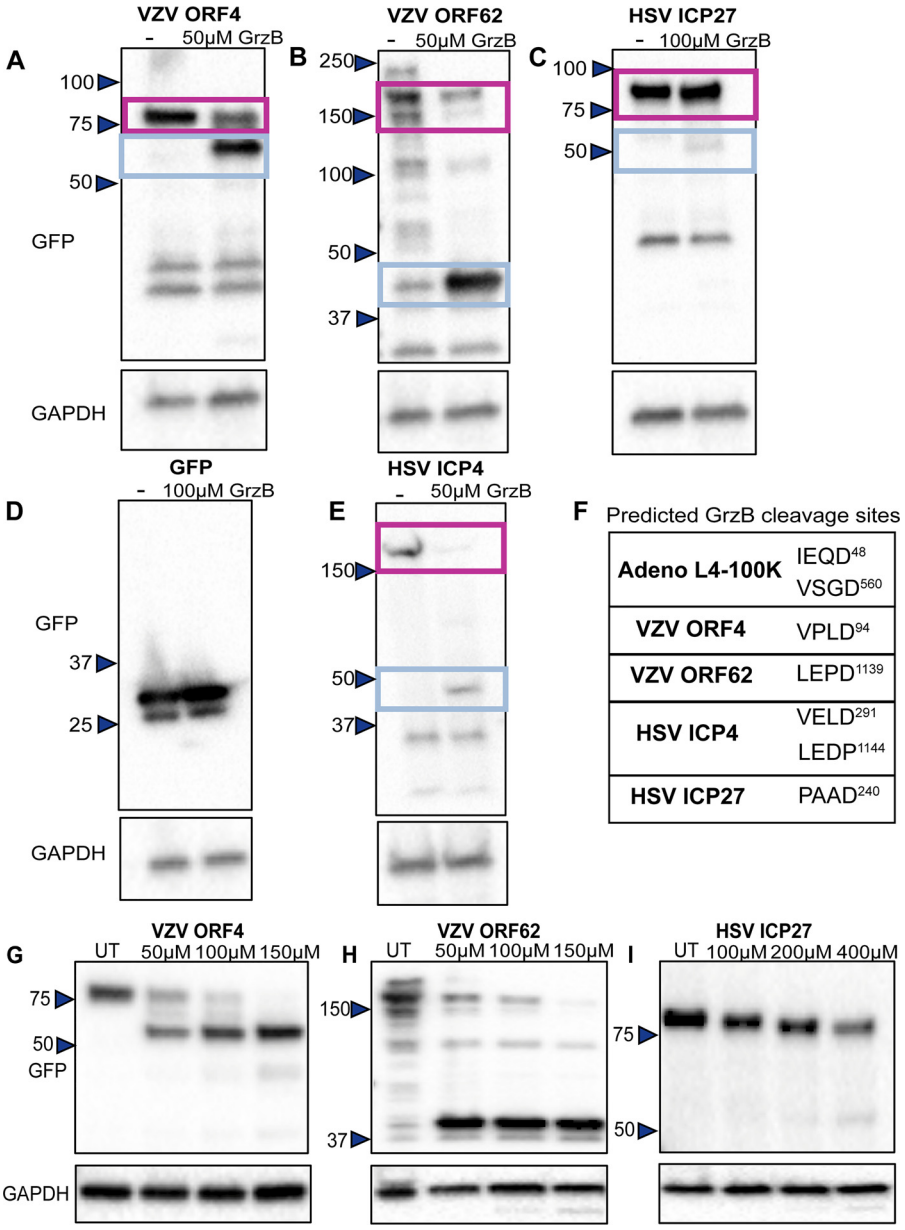

FIG 1 VZV ORF4, VZV ORF62, HSV ICP27, and HSV ICP4 are cleaved by granzyme B. 293Ts were transfected with VZV or HSV ORF-containing plasmids. Day 2 posttransfection cells were harvested and protein lysates generated for immunoblotting. ORF4 (A), ORF62 (B), and ICP4 (E) lysates were treated with 50 μM (1×) granzyme B (Merck), ICP27 (C) and green fluorescent protein (GFP) (D) lysates were treated with 100 μM (2×) granzyme B. Blots were probed for GFP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are representative of 3 biological replicates. Purple and blue boxes indicate the full-sized protein and the protein cleavage product, respectively. −, untreated; GrzB, granzyme B treated. Previously predicted granzyme B cleavage sites of HSV ICP4 and adenovirus L4-100K are displayed in conjunction with predicted granzyme B cleavage sites for VZV ORF4, VZVORF62, and HSV ICP27, based on the cleavage product size (F). VZV ORF4 (G), VZV ORF62 (H), and HSV ICP27 (I) containing lysates were either untreated (UT) or treated with increasing concentrations of granzyme B, as indicated. Blots were probed for GFP and GAPDH and are representative of 3 biological replicates. Solid arrows indicate protein size ladder.

whether the HSV-1 and VZV gene products that we identified to be cleaved by granzyme B would affect cell death by NK cell-mediated cytotoxicity.

To assess this in a human cell setting, human NK cells were isolated by CD56$^+$ magnetically activated cell sorting (MACS) selection and incubated overnight with interleukin-2 (IL-2) (Fig. 2A). We then assessed NK cell activation (CD69) and cytotoxicity against 293Ts that had been transfected with pGFP, pORF4, pORF62, pICP27, or pICP4,

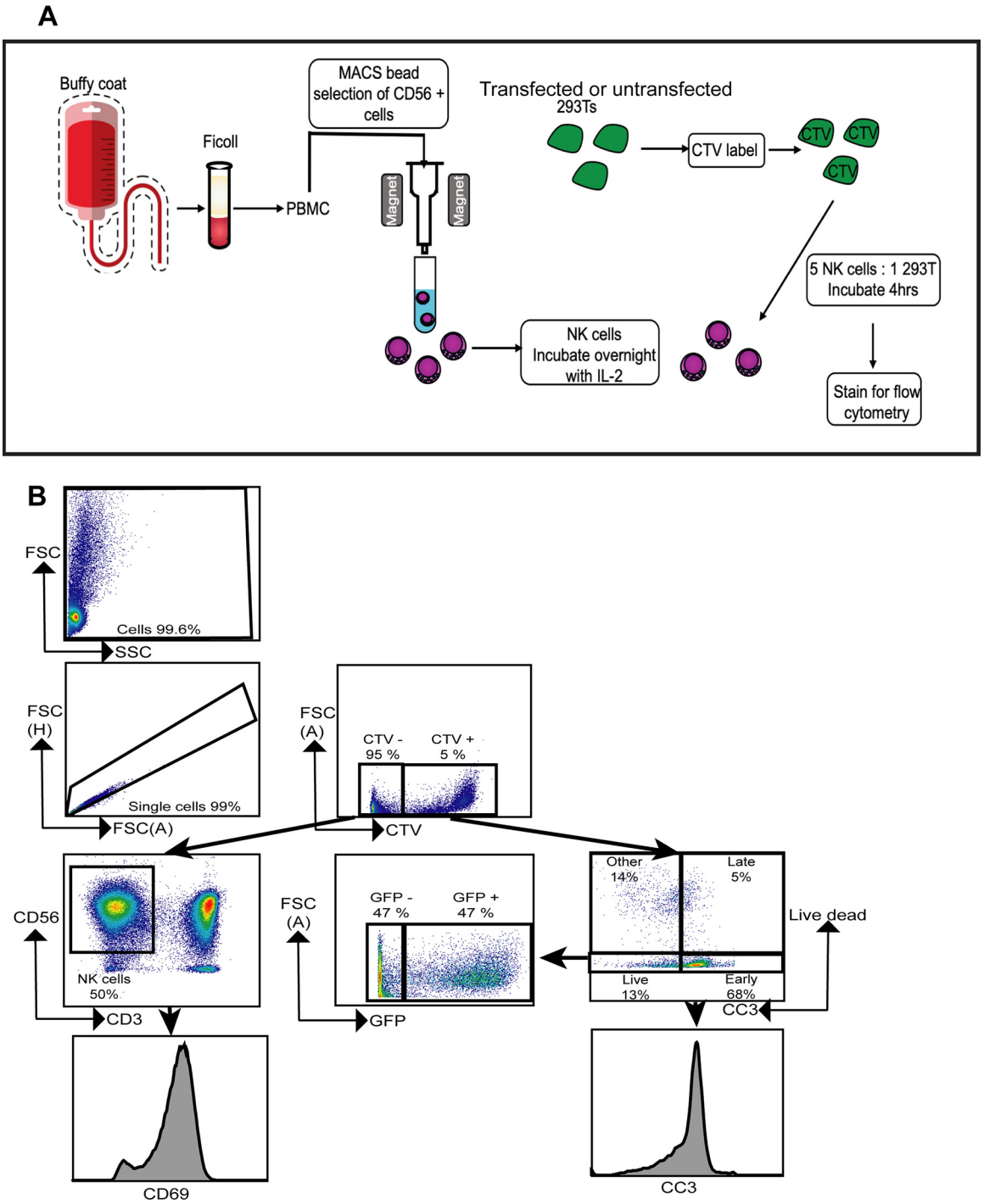

FIG 2 NK cell cytotoxicity assay setup and gating strategy. Peripheral blood mononuclear cells (PBMCs) were extracted from buffy coats using a Ficoll gradient and cryopreserved (A). NK cells were isolated from resuscitated PBMCs via MACS bead selection of $CD56^+$ cells and were treated overnight with IL-2. NK cells were cocultured with cell trace violent (CTV)-labeled 293Ts expressing the viral protein of interest or with GFP alone or were untransfected. Cells were incubated for 4 h and subsequently collected and stained for flow cytometry analysis. Cells were first gated on size and granularity (FSC-A versus FSC-H) (B). Single cells were gated for CTV. CTV^+ cells were classified as live ($CC3^-$, live/dead negative), early apoptotic ($CC3^+$, live/dead negative), late apoptotic ($CC3^+$, live/dead positive), or other cell death ($CC3^-$, live/dead positive) based on CC3 and live/dead staining. Within the live cell gate, GFP^+ cells were identified using GFP versus FSC-A. Median fluorescence intensity (MFI) of CC3 for CTV^+ cells was determined. NK cells were identified in CTV^- population by CD3 versus CD56 staining ($CD3^-$, $CD56^+$). In the NK cell population, CD69 MFI was measured.

or were left untransfected. 293Ts have previously been shown to efficiently activate NK cells (17). 293Ts were cell trace violet (CTV) labeled to allow for identification in subsequent flow cytometry analysis (Fig. 2B). NK cells were incubated with 293Ts at a 5:1 (high) or 1.5:1 (low) ratio for 4 h, and cells were analyzed by flow cytometry. 293Ts incubated without NK cells were used to determine whether any of the viral gene products affected 293T viability.

To assess the cytotoxicity of the NK cells, cell death in 293Ts was measured via CC3 and Zombie live/dead staining, as we have previously characterized (18). Live cells were defined as $CC3^-$ and live/dead negative, early apoptotic cells as $CC3^+$ and live/dead negative, late apoptotic as $CC3^+$ and live/dead positive, and nonapoptotic cell death was defined as $CC3^-$ and live/dead positive. Transfection of different viral gene products into cells can affect cell viability. To account for this, NK cell cytotoxicity was measured as the fold change in percentage of live cells after NK cell incubation relative to untreated transfected cells for each viral gene product (Fig. 3A and B). At a high NK cell ratio, we found no significant difference with NK cell coculture between 293Ts expressing any of the viral proteins compared to GFP-expressing or untransfected 293Ts (Fig. 3B). Furthermore, when cultured with NK cells, there was no significant difference in CC3 MFI between 293Ts expressing viral proteins, GFP-expressing 293Ts, or untransfected 293Ts (Fig. 3C and D). Together, these data demonstrate that at a 5:1 ratio, expression of VZV or HSV-1 viral proteins that are cleaved by granzyme B does not alter target cell apoptosis mediated by NK cell-mediated cytotoxicity.

In our assay, 293T cells were not 100% GFP positive. Therefore, the frequency of GFP^+ cells in the live gate was examined (Fig. 3E), with the expectation that GFP^+ cells would be enriched if the viral gene product was protective (Fig. 3E). To account for the differences in transfection efficiencies of the viral gene products, GFP changes are displayed as fold change of GFP^+ cells that have been incubated with NK cells relative to untreated transfected cells for each viral gene product.

Analyzing fold change of GFP^+ cells cultured with NK cells over GFP^+ cells cultured alone, we found no significant differences between 293Ts expressing ORF4, ORF62, ICP27, or ICP4 compared to 293Ts expressing GFP (Fig. 3F). However, there was a trend toward reduced live GFP^+ ICP4-expressing 293Ts, suggesting that cells expressing ICP4 were more vulnerable to NK cell-mediated cytotoxicity. Together, these data suggest that at a high NK cell to target ratio the HSV and VZV proteins examined could not protect cells against NK cell-mediated cytotoxicity.

It is possible that at high NK cell to target ratios, the NK cell response could overwhelm any potential protective effects of the viral gene products. Therefore, a lower NK cell to target cell ratio (1.5:1) was examined (Fig. 4). Strikingly, at a lower NK cell ratio, VZV ORF4-expressing 293Ts trended toward a higher frequency of live cells compared to those of 293Ts expressing other viral proteins and to controls cells, which was significant compared to that of ORF62-expressing 293Ts (Fig. 4A and B). VZV ORF4-expressing cells had, on average, 10% more live cells in the NK cell cytotoxicity assay than did control cells and cells expressing other viral genes. VZV ORF4-expressing cells also had a significant reduction in CC3 MFI in comparison to VZV ORF62-expressing cells and trended toward having a lower MFI in comparison to all other cells (Fig. 4C and D). This suggests that VZV ORF4 was able to protect against NK cell-mediated cytotoxicity at a low NK cell to target cell ratio. There were no significant differences in fold change of GFP^+ cells when cultured with NK cells for ORF4, ORF62, ICP27, or ICP4 in comparison to control cells (Fig. 4E and F). Overall, these data suggest that VZV ORF4 may be able to protect cells from NK cell-mediated cytotoxicity. This may not be due to the granzyme B cleavage site, however, as other granzyme B cleavage site-containing viral proteins did not affect NK cell-mediated cytotoxicity.

Herpesviruses encode proteins that can alter NK cell activation through interfering with target cell activating and inhibitory ligands (19). To ensure that the ability of VZV ORF4 to protect cells from NK cell-mediated cytotoxicity was not due to an inhibition of NK cell activation, CD69 expression was assessed via flow cytometry (Fig. 5B). NK cells were identified as $CD3^-$ and $CD56^+$ (Fig. 5A). Expression of viral proteins or of GFP

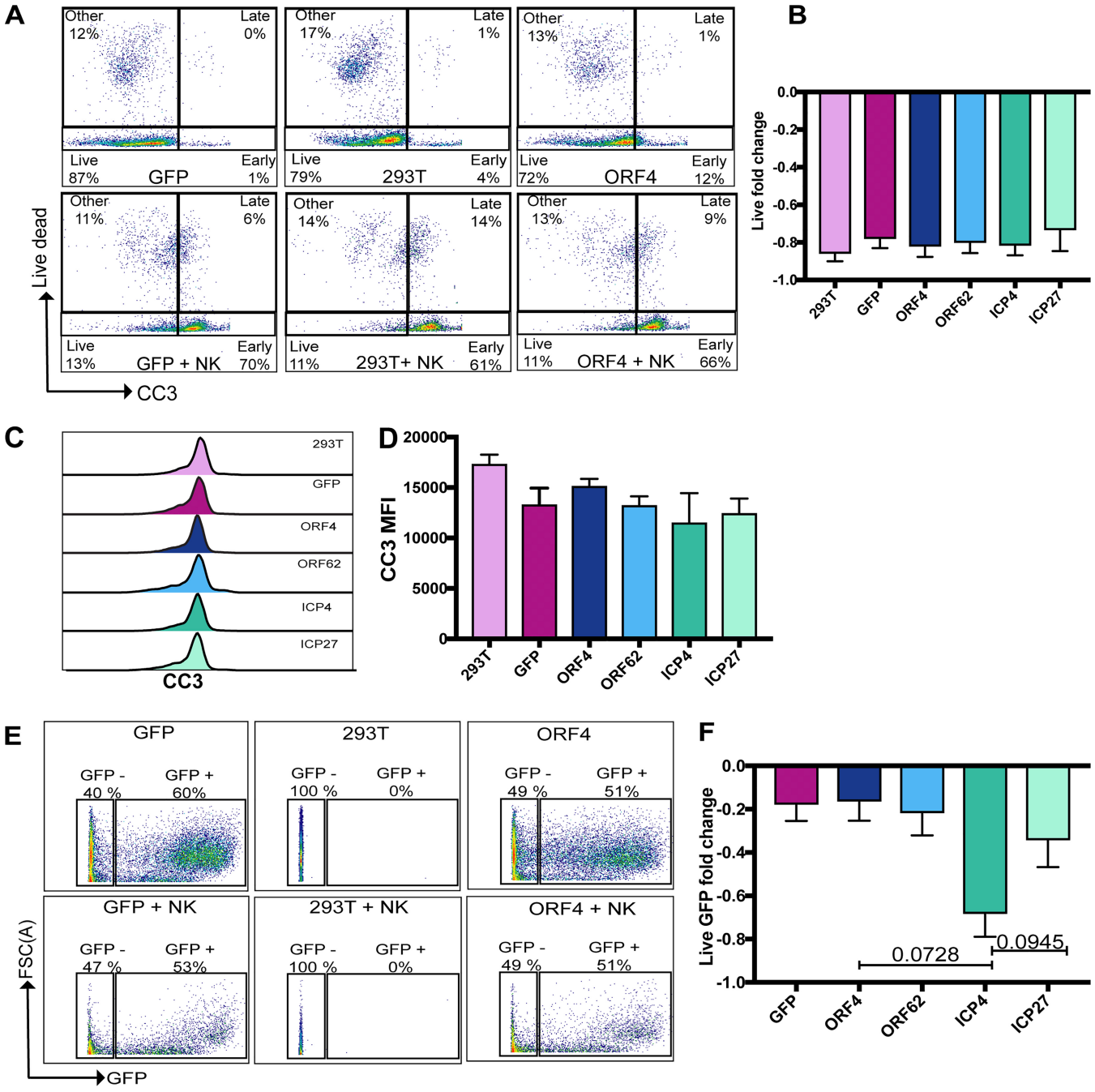

FIG 3 HSV and VZV gene products which can be cleaved by granzyme B cannot protect 293Ts against NK cell cytotoxicity at a high NK cell to target ratio. 293Ts were untransfected or were transfected to express GFP alone, HSV ICP27-GFP, HSV ICP4-YFP, VZV ORF4-GFP, or VZV ORF62-GFP. Two days posttransfection, 293Ts were incubated with interleukin 2 (IL-2)-treated NK cells isolated from PBMCs in an NK cell to target cell ratio of 5:1 or were cultured without NK cells. Cells were collected and analyzed via flow cytometry. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (A). Bar graph depicting fold change in live cells ± standard error of the mean (SEM) is representative of 3 biological replicates (B). Representative histograms of cleaved caspase 3 (CC3) MFI are displayed (C) and collated in a bar graph ($n = 3$, ± SEM) (D). GFP expression was measured in CTV$^+$ live cells. Representative flow plots for both untreated and NK cell-incubated 293Ts are displayed. GFP expression was measured in CTV$^+$ live cells. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (E). Bar graph depicting fold change in GFP$^+$ cells ± SEM is representative of 3 biological replicates (F). Statistical significance was established by a repeated measure one-way analysis of variance (ANOVA) using Tukey's multiple-comparison test.

alone did not significantly alter CD69 expression at either the high (Fig. 5B and C) or low ratio (Fig. 5D and E). This demonstrates that any differences in cell death in 293Ts was not due to differential NK cell activation.

Identification of the VZV ORF4 granzyme B cleavage site. As ORF4 appeared to be able to protect 293Ts against NK cell-mediated cytotoxicity, we wanted to identify

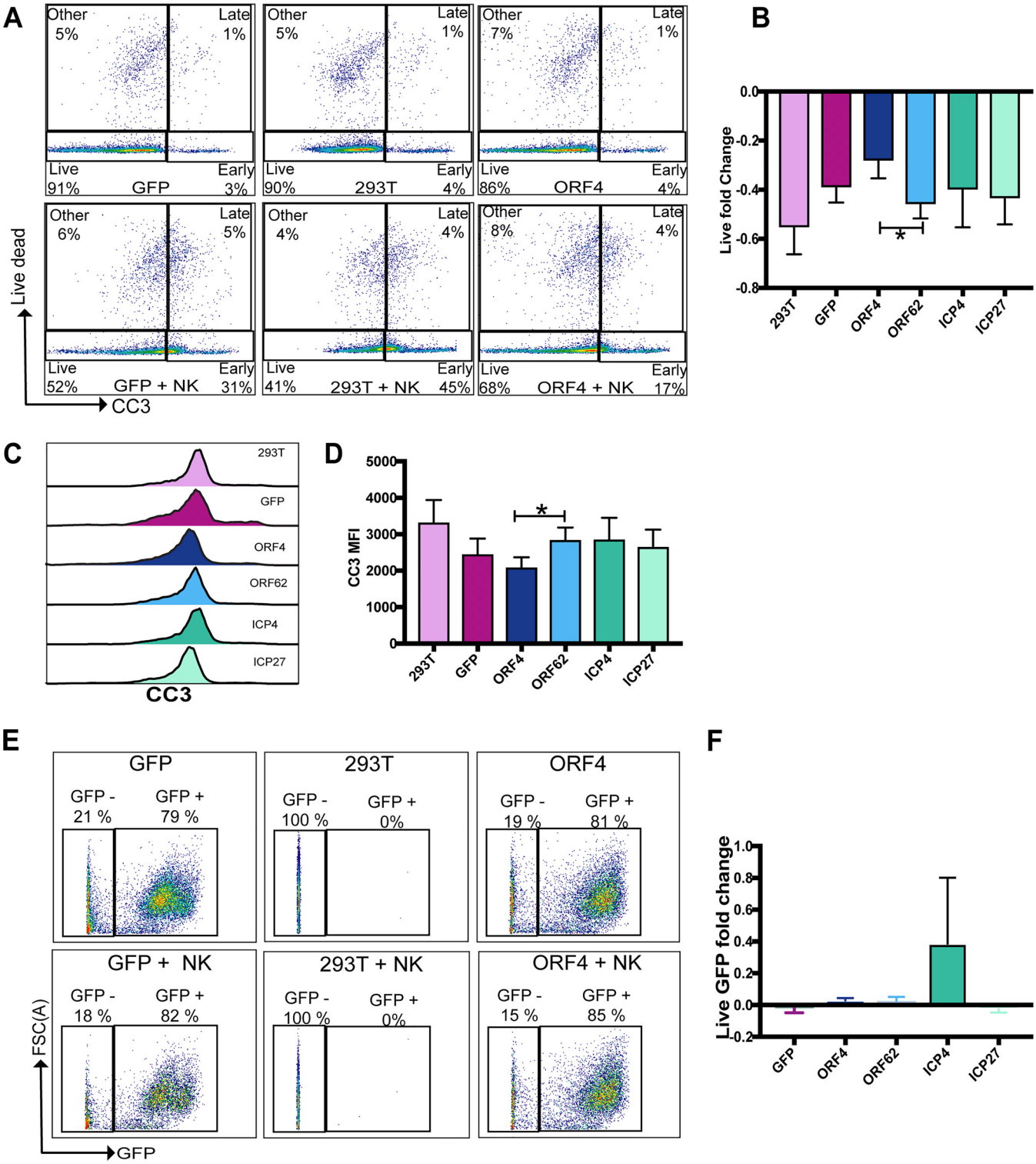

FIG 4 VZV ORF4 can protect 293Ts against NK cell cytotoxicity at a low NK cell to target ratio. 293Ts were transfected to express GFP alone, HSV ICP27-GFP, HSV ICP4-YFP, VZV ORF4-GFP, VZV ORF62-GFP, or were untransfected. Two days posttransfection, 293Ts were incubated with IL-2-treated NK cells at an NK cell to target cell ratio of 1.5:1 or were cultured without NK cells. Cells were collected and analyzed via flow cytometry. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (A). Bar graph depicting fold change in live cells ± SEM is representative of 3 biological replicates (B). Representative histograms of CC3 MFI are displayed (C) and collated in a bar graph ($n = 3$, ± SEM) (D). GFP expression was measured in CTV$^+$ live cells. Representative flow plots for both UT and NK cell-incubated 293Ts are displayed. GFP expression was measured in CTV$^+$ live cells. Representative flow plots are displayed for untransfected, GFP, and ORF4 293Ts (E). Bar graph depicting fold change in GFP$^+$ cells ± SEM is representative of 3 biological replicates (F). Statistical significance was established by a repeated measure one-way ANOVA using Tukey's multiple-comparison test. *, $P \leq 0.05$.

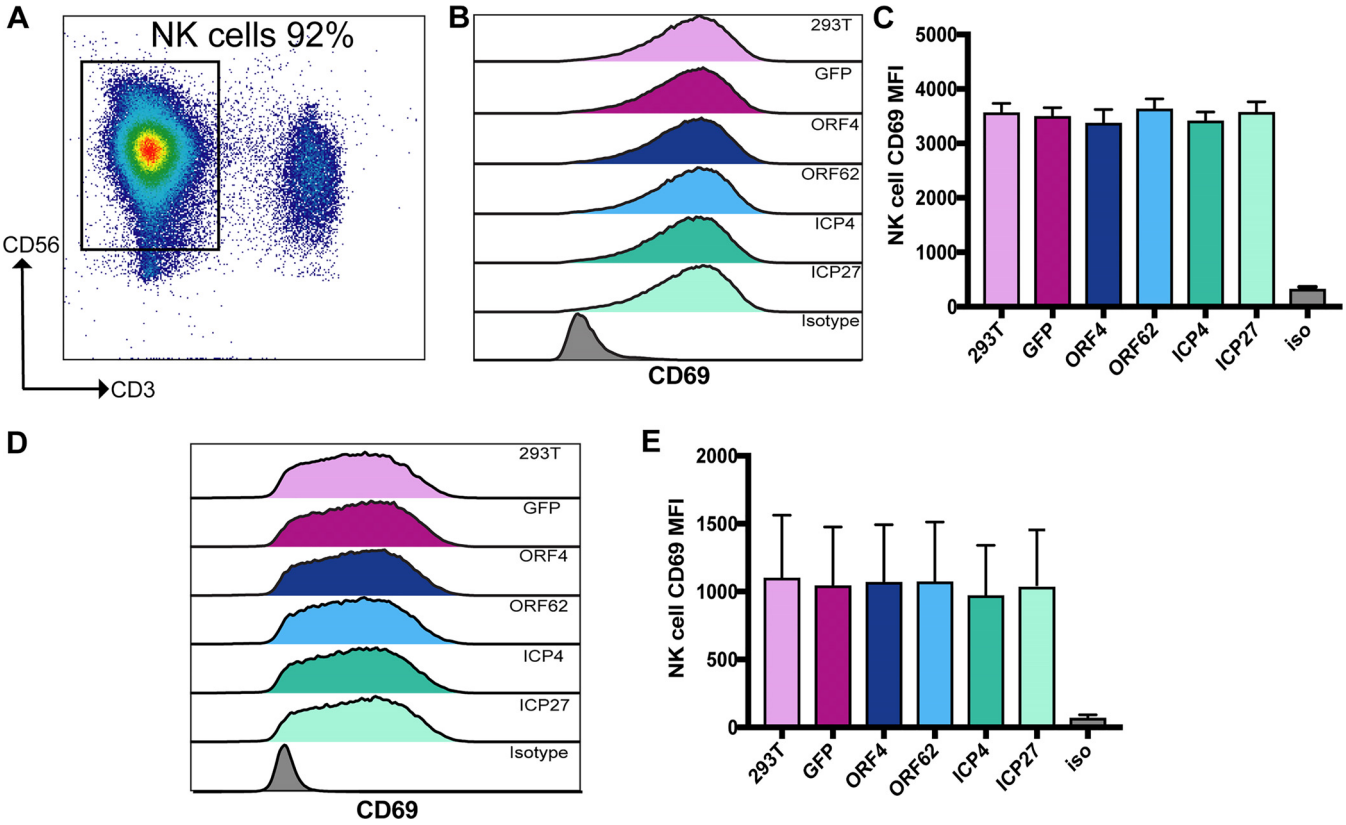

FIG 5 NK cell activation is not affected by transfected 293Ts at a high or low NK to target ratio. 293Ts were untransfected or were transfected to express GFP alone, VZV ORF4-GFP, VZV ORF62-GFP, ICP4-YFP, or HSV ICP27-GFP. Two days posttransfection, 293Ts were incubated with IL-2-treated NK cells isolated from PBMCs in an NK cell to target cell ratio of 5:1 (A to C) or 1.5:1 ratio (D and E) or were cultured without NK cells. Cells were collected and analyzed via flow cytometry. NK cells were identified as $CD56^+$ CD3−, as seen in the representative flow plot (A). Isotype control staining for CD69 was performed on NK cells cultured alone. CD69 expression was evaluated by histogram (B and D) and MFI collated in a bar graph (C and E) ($n = 3$, ± SEM).

the granzyme B cleavage site and mutate it to determine whether the granzyme B cleavage site was responsible for this effect. Using the initial GraBCas predictions, as well as cleavage product size, we predicted the granzyme B cleavage site (Fig. 6A). Previous studies have shown that by converting the aspartic acid (D) residue in the cleavage site to a glutamic acid, the ability of granzyme B to cleave these sites is abolished (20, 21). The granzyme B cleavage site in Bid was identified in both reports via mutation of D75 to glutamic acid (20, 21). Site-directed mutagenesis was performed on pORF4 to mutate the aspartic acid residue via a single base pair substitution (Fig. 6A). Successful single base pair substitution was confirmed by sequencing, and the resulting plasmid was termed pORF4M. pORF4 and pORF4M were transfected into 293Ts, and lysates were collected for digest with granzyme B. pORF4M without granzyme B digest produced the same 75-kDa band as pORF4-GFP. When incubated with granzyme B, the 65-kDa cleavage product was absent from ORF4M-expressing cells, suggesting that the mutant abolished the ability of granzyme B to cleave this gene product (Fig. 6B). This was observed in 3 independent biological replicates, indicating that the granzyme B cleavage site was successfully identified in VZV ORF4.

The ability of VZV ORF4 to inhibit NK cell cytotoxicity is independent from the granzyme B cleavage site. To determine whether the granzyme B cleavage site in ORF4 was responsible for its ability to limit cell death mediated by NK cell-mediated cytotoxicity, the low-ratio cytotoxicity assay was performed with 293Ts expressing GFP, ORF4, or ORF4M and then compared to untransfected 293Ts. Similarly to results shown in Fig. 3 and 4, the fold frequency of live cells when cocultured with NK cells over cells alone was calculated (Fig. 7A and B). As demonstrated in Fig. 5B, following culture with NK cells, a higher frequency of live cells was noted in 293Ts expressing ORF4 compared

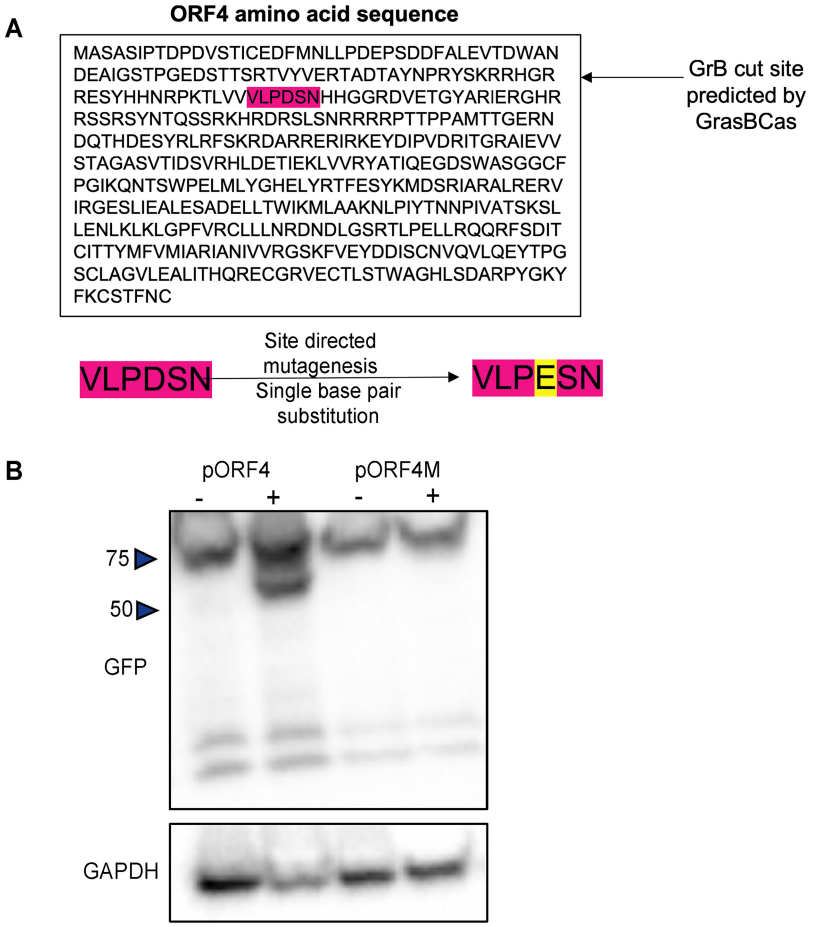

FIG 6 Identification of the ORF4 granzyme B cleavage site using site-directed mutagenesis. Potential sites of granzyme B cleavage were identified via GraBCas (11). The aspartate residue in the cleavage site was changed to a glutamic acid via a single base pair substitution using PCR (A). 293Ts were transfected with 1 μg of ORF4 or ORF4M plasmid. On day 2 posttransfection, cells were harvested and protein lysates generated for immunoblotting. Lysates were treated with 133 ng granzyme B (BioVision, USA). Blots were probed for GFP and GAPDH and are representative of 3 biological replicates (B). ± indicates untreated (−) and granzyme B treated (+). Solid arrows indicate protein size ladder.

to GFP-expressing or untransfected 293T control cells (Fig. 7A and B). Similarly to the previous assay, VZV ORF4-expressing cells showed on average 10% more live cells than did control cells and cells expressing other viral genes. Unexpectedly, ORF4M-expressing cells had a significantly higher fold change frequency of live cells compared to GFP-expressing cells (Fig. 7A and B). VZV ORF4M-expressing cells had on average 15% more live cells in the NK cell cytotoxicity assay than did control cells and cells expressing other viral genes. This trend is also observed in the CC3 MFI (Fig. 7C and D). When we examined live cells after NK cell coculture, significantly more GFP-expressing cells were present when 293Ts expressed ORF4M or ORF4 than in GFP-expressing 293Ts (Fig. 7E and F). Together, these data indicate that ORF4 has a previously unknown function that protects cells from NK cell-mediated cytotoxicity; however, this is not due to the presence of a granzyme B cleavage site.

VZV ORF4 does not protect 293Ts against staurosporine- or FasL-induced apoptosis. To determine whether the ability of VZV ORF4 to restrict cell death mediated by NK cell cytotoxicity was due to a broad antiapoptotic effect, ORF4-, ORF4M-, or GFP-expressing and untransfected 293Ts were treated with two independent apoptotic stimuli. As described previously, staurosporine induces the intrinsic apoptotic pathway, while FasL induces the extrinsic apoptotic pathway (18). Thus, treatment with these drugs would identify whether an antiapoptotic effect was specific

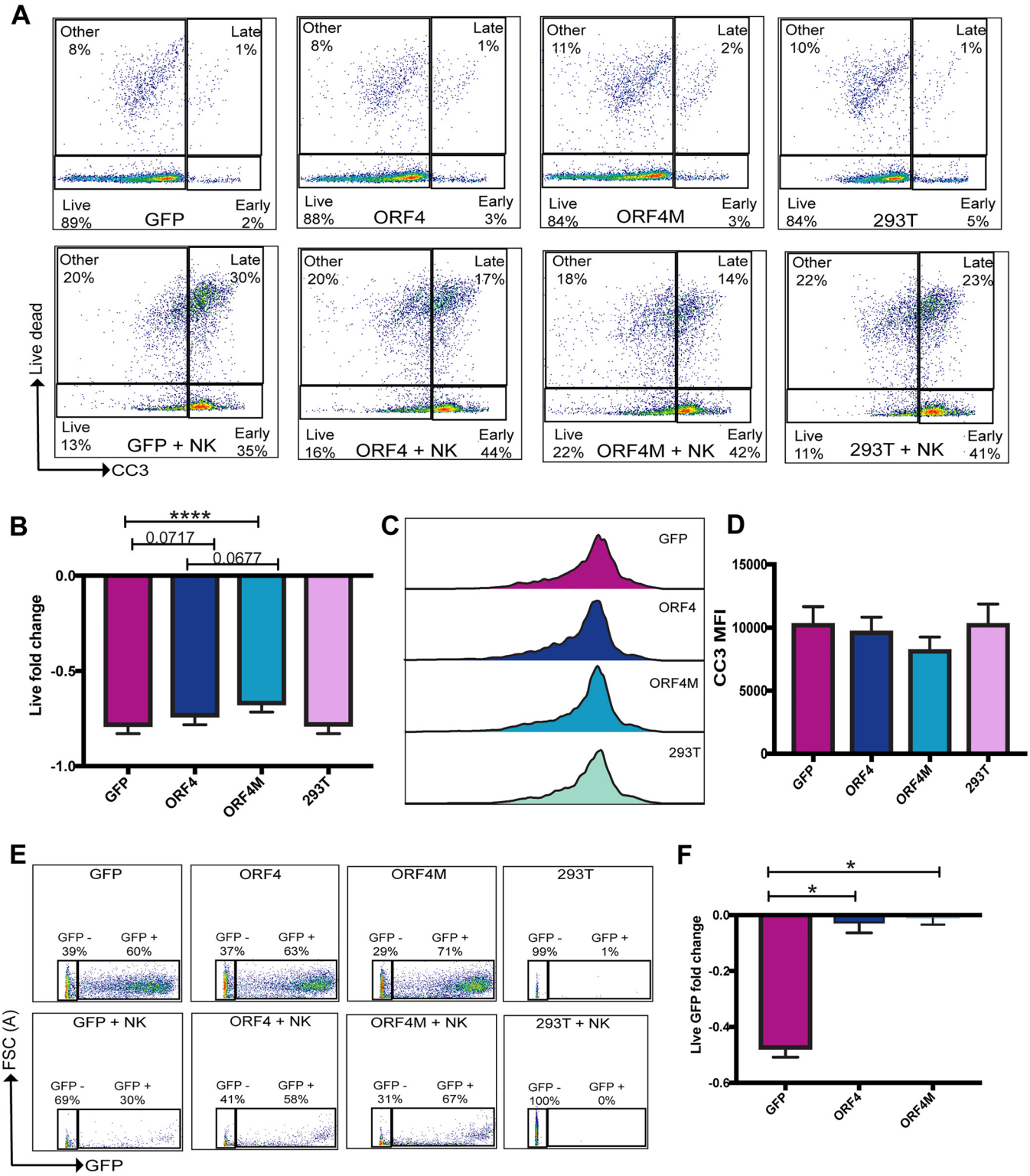

FIG 7 Both ORF4 and ORF4M can protect 293Ts from NK cell cytotoxicity at a low NK cell-to-target ratio. 293Ts were untransfected or were transfected to express GFP alone, VZV ORF4-GFP, or VZV ORF4M-GFP. Two days posttransfection, 293Ts were incubated with IL-2-treated NK cells isolated from PBMCs in an NK cell to target ratio of 1.5:1 or cultured without NK cells. Cells were collected and analyzed via flow cytometry. Representative flow plots are displayed (A). Bar graph depicting fold change in live cells ± SEM is representative of 3 biological replicates (B). Representative histograms of CC3 MFI are displayed (C) and collated in a bar graph ($n = 3$, ± SEM) (D). GFP expression was measured in CTV$^+$ live cells. Representative flow plots for both untreated and NK cell-incubated 293Ts are displayed. GFP expression was measured in CTV$^+$ live cells. Representative flow plots are displayed for untransfected, GFP, ORF4, and ORF4M 293Ts (E). Bar graph depicting fold change in GFP$^+$ cells ± SEM is representative of 3 biological replicates (F). Statistical significance was established by a repeated measure one-way ANOVA using Tukey's multiple-comparison test. *, $P \leq 0.05$; ****, $P \leq 0.001$.

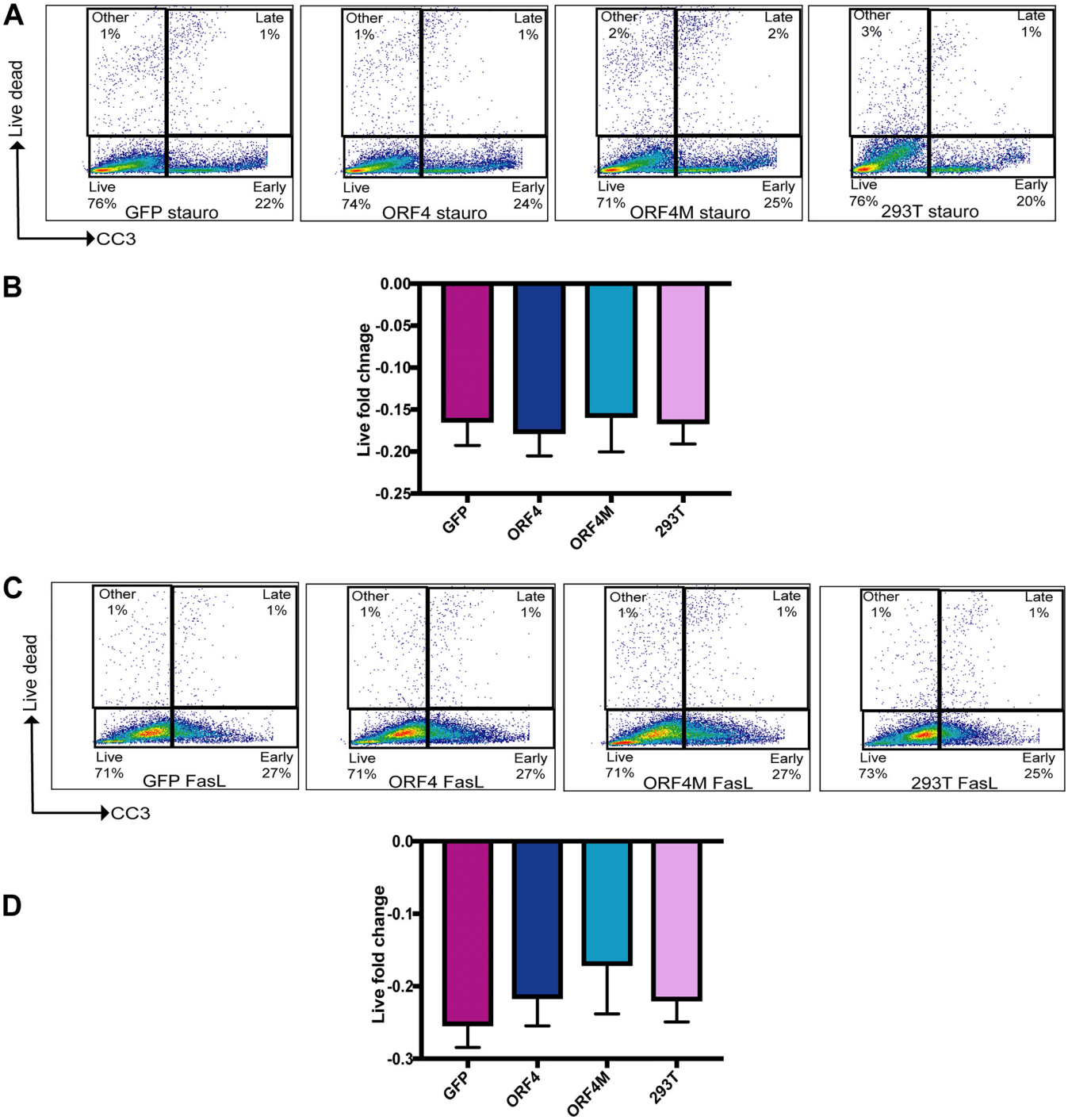

FIG 8 VZV ORF4 does not protect 293Ts against staurosporine- or FasL-induced apoptosis. 293Ts were untransfected or transfected with pGFP-C1, pORF4, or pORF4M. Two days posttransfection, 293Ts were treated with 1 μM staurosporine (A and B) or 200 ng/μl FasL (C and D) or were left untreated for 5 h. Cells were stained with Zombie NIR, permeabilized, fixed, stained for CC3, and analyzed via flow cytometry. Flow plots displayed are representative of 3 biological replicates (A and C). Bar graph depicting fold change in live cells ± SEM is representative of 3 biological replicates (B and D). Statistical significance was established by a repeated measure one-way ANOVA using Tukey's multiple-comparison test.

to a particular pathway. However, there were no significant differences in the fold change in live cell frequencies between 293Ts expressing ORF4 or ORF4M compared to GFP-expressing or untransfected 293Ts for either staurosporine (Fig. 8A and B) or FasL treatment (Fig. 8C and D). This suggests that the ability of ORF4 to limit cell death through NK cell cytotoxicity is not mediated by inhibition of extrinsic or intrinsic apoptosis induction.

DISCUSSION

Granzyme B has been shown to be critical to the regulation of HSV-1 reactivation by the immune system; however, it is unknown why granzyme B does not induce apoptosis in this context. VZV is closely related to HSV-1, but much less is known regarding control of VZV reactivation and lytic infection by the immune system. Overall, this study aimed to identify HSV-1 and VZV proteins that could be cleaved by granzyme B and assess whether these proteins could inhibit NK cell-mediated cytotoxicity. This study demonstrates that there are multiple VZV and HSV-1 proteins that are susceptible to cleavage by granzyme B; however, this does not necessarily confer resistance to NK cell-mediated cytotoxicity. A novel function of VZV ORF4 was identified in protecting 293Ts against NK cell-mediated cytotoxicity at a low effector to target ratio. Surprisingly, mutation of the granzyme B cleavage site in ORF4 did not change the ability of ORF4 to inhibit NK cell-mediated cytotoxicity, suggesting that this function is independent of the cleavage site. The ability of VZV ORF4 to modulate NK cell cytotoxicity did not seem to be due to an ability to inhibit extrinsic or intrinsic apoptotic pathways. Altogether, this work highlights a novel immunomodulatory function of VZV ORF4 and a more extensive role for granzyme B in limiting alphaherpesvirus infection.

The role of granzyme B in viral infections has been previously addressed in the context of adenovirus. It has been identified that adenovirus L4-100K can inhibit granzyme B function and lymphokine-activated killer (LAK) cell-mediated cytotoxicity through the possession of its granzyme B cleavage site (6). In the cases of HSV-1 and VZV, it appears that in the context of NK cell-mediated cytotoxicity a granzyme B cleavage site is not sufficient to inhibit NK cell cytolytic function. This difference could be due to the NK cells in our system also being able to kill through the FasL pathway, while the 293Ts were Fas deficient in the adenovirus study (6). It is important to examine the role of Fas and other extrinsic apoptotic inducers in this context, as they could induce apoptosis in target cells when granzyme B is inhibited. Both VZV and HSV contain proteins that can inhibit both intrinsic and extrinsic apoptotic pathways (22, 23), and thus granzyme B cleavage of critical viral proteins may be a fail-safe mechanism to ensure inhibition of viral spread even when cell death pathways are inhibited.

NK cell/CTL granules contain other granzymes that could compensate for the inhibition of granzyme B. In the case of adenovirus, granzyme H was found to target adenovirus proteins that were crucial for viral DNA replication to inhibit viral replication (24). Therefore, it would be interesting to examine the role of other granzymes in the context of alphaherpesvirus infection. Interestingly, the viral proteins we identified as being cleaved by granzyme B (HSV ICP27, VZV ORF4, and VZV ORF62) all have critical roles in viral replication and/or host evasion (12, 14, 16, 25, 26). This suggests that in the context of VZV infection, granzyme B could limit viral replication and spread when cell death has been inhibited, and this warrants further research.

In our NK cell cytotoxicity assays, the other components of cytolytic granules could be masking the ability of viral proteins to inhibit granzyme B function. It would be of interest to determine if the expression of HSV and VZV viral proteins could inhibit granzyme B-mediated cytotoxicity in an isolated system. In the process of this study, several attempts were made to deliver recombinant granzyme B to target cells by the bioPORTER protein delivery system; however, this method of delivery was ineffective at inducing granzyme B-mediated apoptosis at the cell confluence necessary to perform these experiments (Fig. S2). Therefore, investigating different methods of protein delivery/generating recombinant granzyme B may be necessary to directly assess the ability of HSV/VZV proteins to inhibit granzyme B function *in vitro*.

A novel mechanism of VZV ORF4 to inhibit NK cell-mediated cytotoxicity was identified in this study; however, the mutation of the granzyme B cleavage site revealed this was not due to the cleavage site. We investigated the possibility that VZV ORF4 could inhibit apoptosis; however, it did not inhibit either FasL- or staurosporine-induced apoptosis. This finding suggests that ORF4 limits NK cell-mediated cell death through an alternative mechanism, such as regulating NK cell detection of the target.

VZV ORF4 is the homolog of HSV ICP27; however, in our NK cell assay, only ORF4 could protect against NK cell cytotoxicity. This suggests that the ORF4 mechanism of immunoevasion is distinct from potential overlapping functions with HSV ICP27. Our previous work has shown that VZV and HSV-1 differentially modulate the expression of ligands to the activating NKG2D receptor expressed on NK cells (27). Even though coculture with ORF4-expressing 293Ts did not affect CD69 expression on IL-2-treated NK cells, it could have affected expression of NK cell ligands such as NKG2D ligands. Future studies could measure expression of NK cell ligands such as NKG2D ligands on ORF4-expressing cells and relate this back to a broader assessment of NK cell function.

In HSV-1 infection in mice, it was found that CTLs could inhibit viral reactivation without cell death induction, and this was attributed to granzyme B cleavage of ICP4 (5). In VZV infection, both NK cells and CTLs have been identified in ganglia of patients who have experienced herpes zoster (9, 10). The CTLs in this context were granzyme B positive, and there was no indication of neuronal apoptosis (9). It would be interesting to investigate the role of VZV ORF62 and VZV ORF4 in protecting neurons from granzyme B-induced apoptosis. Additionally, using cell lines such as the SH-SY5Y cell line or neurons derived from induced pluripotent stem cells (iPSCs), it may be possible to determine whether granzyme B treatment of VZV infected neurons could inhibit virus spread and drive the virus into a latent-like state. This direction would likely be impeded by the difficulty in achieving effective delivery of granzyme B, as described above, but would be of interest to examine in highlighting a potential role for granzyme B in controlling VZV reactivation.

In summary, we have found novel HSV-1 and VZV proteins that can be cleaved by granzyme B, including HSV-1 ICP27, VZV ORF4, and VZV ORF62. In contrast to adenovirus infection, the possession of a granzyme B cleavage site in VZV and HSV-1 proteins was not sufficient to protect against NK cell-mediated cytotoxicity. For the first time, we report that VZV ORF4 has a novel function in limiting NK cell-mediated cytotoxicity through a granzyme B cleavage site-independent mechanism. VZV ORF4 alone was not able to protect cells against FasL- or staurosporine-induced apoptosis, indicating that its ability to inhibit NK cell apoptosis is not due to a broad inhibition of apoptotic pathways. Altogether, this study broadens our knowledge concerning both viral mechanisms of immune modulation and the effects of granzyme B on alphaherpesvirus pathogenesis.

MATERIALS AND METHODS

Cell lines. HEK293T cells (ATCC) and ARPE-19 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50 IU/ml penicillin and streptomycin.

Plasmid construction and validation. PCR was performed to amplify HSV ICP27, VZV ORF62, and VZV ORF4 from HSV strain F or the VZV pOka genome, respectively, while incorporating restriction enzyme cleavage sites. PCR products were purified (Illustra GFX PCR DNA and gel band kit; GE Healthcare) and digested with SgrAI and NheI (VZV ORF62) or AgeI and NheI (HSV ICP27 and VZV ORF4). The backbone plasmid (pGFP-C1; Clontech) was also digested with AgeI and NheI and ligated with the VZV and HSV gene PCR products using T4 DNA ligase (NEB), and the resulting plasmids were termed pORF4, pORF62, and pICP27. In the resulting plasmids, GFP was tagged to the 3′ end of the viral ORFs. Plasmids were transformed into *Escherichia coli* 5-alpha competent cells (NEB) and isolated using a plasmid DNA purification kit (NucleoBond Xtra Midi plasmid DNA purification kit; Macherey-Nagel). pICP4-YFP was generously gifted by Roger Everett from the MRC Centre, University of Glasgow (Glasgow, Scotland). Sequencing was performed to ensure that the gene products were inserted into the backbone correctly.

Plasmid DNA transfections. On the day prior to transfection, 6×10^5 293T cells were seeded into a well of a 6-well plate (Corning). On the day of transfection, 1 μg of plasmid DNA was added to serum-free DMEM and FuGene HD (Promega) and was incubated for 15 min at room temperature (RT). The transfection mixtures were then added to previously seeded 293T cells. Two days posttransfection, GFP was imaged at $\times$10 magnification via a fluorescence microscope (Zeiss Axio).

Generation of protein lysates and granzyme B digest. Plasmids were transfected into 293Ts as described above. Medium was removed from cells at day 2 posttransfection, and cells were washed with cold phosphate-buffered saline (PBS). Cells were resuspended in cell lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 1% NP-40, and 1% Triton X-100) for lysis before supernatant was collected and stored at −20°C.

Protein lysates were treated with various concentrations of recombinant granzyme B (initial cleavage assay, Millipore; remaining assays, Enzo Life Sciences) as indicated or were left untreated and were incubated at 37°C for 1 h. Samples were denatured by heating in reducing sample buffer (Bio-Rad) and resolved by SDS-PAGE on precast 4 to 20% polyacrylamide gels (Bio-Rad). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and probed with primary antibodies. This was followed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Abcam) and incubation with enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific). The blot was imaged using a ChemiDoc imaging system (Bio-Rad). The following primary antibodies were used: mouse anti-GFP (B-2; Santa Cruz Biotechnology) and rabbit anti-human GADPH (FL-335; Santa Cruz Biotechnology).

Site-directed mutagenesis. PCRs were designed to substitute an amino acid to convert an aspartic acid to a glutamic acid in the VZV ORF4 plasmid using the Q5 site-directed mutagenesis kit (NEB). The forward primer was TATTACCCGAATCAAACCATC and the reverse primer was CAACAACCAAAGTTTTCG. PCR products were ligated using the kinase, ligase, and DpnI (KLD) enzyme mix (NEB) and were transformed into chemically competent *Escherichia coli*. Colonies were selected, and the plasmid was isolated via miniprep kit (NucleoSpin; Macherey-Nagel) and sequenced to determine whether they contained the mutated plasmid. Plasmids with the correct sequence were transformed into *Escherichia coli* 5-alpha competent cells (NEB) and isolated using a plasmid DNA purification kit (NucleoBond Xtra Midi plasmid DNA purification kit; Macherey-Nagel). The resulting plasmid was termed pORF4M.

Peripheral blood mononuclear cell isolation and NK cell separation. Buffy coats collected from healthy human donors were sourced from the Australian Red Cross Blood Service, with ethics approval from the University of Sydney (Sydney, Australia). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare). Cells were resuspended in PBS and cryopreserved. $CD56^+$ NK cells were isolated from cryopreserved PBMCs via magnetic bead separation using CD56 microbeads (Miltenyi Biotec, Germany) as in (28). Cells were resuspended in RPMI supplemented with 10% FBS (Lonza) and 1% (vol/vol) penicillin/streptomycin (Life Technologies) and enumerated with a hemocytometer. NK cells were left untreated or were incubated overnight with 200 U/ml IL-2 Improved Sequence (IS) (Miltenyi Biotec) for use in NK cell cytotoxicity assays.

NK cell cytotoxicity assay. 293Ts were either transfected with GFP constructs or were left untransfected. Cells were trypsinized and washed twice with PBS. Cells were labeled with cell trace violet (CTV) (Thermo Fisher Scientific) for 1 h at 37°C so they were distinguishable from NK cells during subsequent flow cytometry analysis. Labeled cells were quenched with supplemented DMEM for 5 min at 37°C, washed once with supplemented DMEM, and seeded into a 12 well plate. Previously isolated NK cells were counted and added to 293Ts at a 5:1 or 1.5:1 ratio, as indicated, and incubated together for 4 h at 37°C on a rocking platform to prevent cells from becoming adherent. Cells were subsequently harvested for flow cytometry analysis.

Apoptosis induction assay. 293Ts were transfected with pGFP-C1, pORF4, and pORF4M, as described, and at day 2 posttransfection, cells were left untreated, treated with 1 μM staurosporine (Sigma-Aldrich), or treated with 200 ng/μl FasL (Enzo Life Sciences) for 5 h and analyzed via flow cytometry as previously described (18).

Flow cytometry. Cells were collected and stained with Zombie NIR (BioLegend) per the manufacturer's instructions. For NK cell cytotoxicity assays, cells were surface stained with mouse anti-human CD56 BV605 (clone NCAM16.2; BD Biosciences), mouse anti-human CD3 PerCP-Cy5.5 (clone UCHT1; BD Biosciences), and mouse anti-human CD69 BV711 (clone FN50; BD Biosciences) at RT for 30 min. Cells were fixed and permeabilized with fix/perm buffer (BD) for 20 min at RT. Cells were stained with rabbit anti-human cleaved caspase 3 (CC3) phycoerythrin (PE) (clone C92-605; BD Biosciences) overnight at 4°C. Samples were acquired using an LSRFortessa flow cytometer. Data were analyzed with FlowJo software v10.0.6 (FlowJo, LLC, USA).

Immunofluorescence. Cells were grown on cover slips and transfected to express GFP alone or were left untransfected. At day 2 posttransfection, granzyme B (Enzo Life Sciences) was delivered to cells using the bioPORTER protein delivery system (Sigma-Aldrich) per the manufacturer's instructions. At 4 h posttreatment, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at RT. After washing with PBS, the cells were permeabilized with Triton X-100 (Sigma-Aldrich) for 10 min and blocked using 20% normal donkey serum (Sigma-Aldrich). The cells were incubated with rabbit anti-CC3 (D3E9, 1:150; Cell Signaling Technology) for 1 h at RT. Cells were washed with PBS, incubated with donkey anti-rabbit IgG 594 (1:250; Invitrogen) for 30 min, and washed with PBS, and coverslips were mounted on glass slides using Prolong Gold anti-fade reagent with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Staining was visualized on a Nikon E widefield microscope, and images were taken using Nikon software. Images were pseudocolored using Fiji (Image J). For cell quantification, thresholds for individual channels were determined by using the Huang method to create a binary image. The watershed method was used to separate cell staining, and automatic counting was performed using Fiji (Image J).

Statistical analysis. *P* values were determined for flow cytometry analysis via a repeated measure one-way analysis of variance (ANOVA) using Tukey's multiple-comparison test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.01140-19.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

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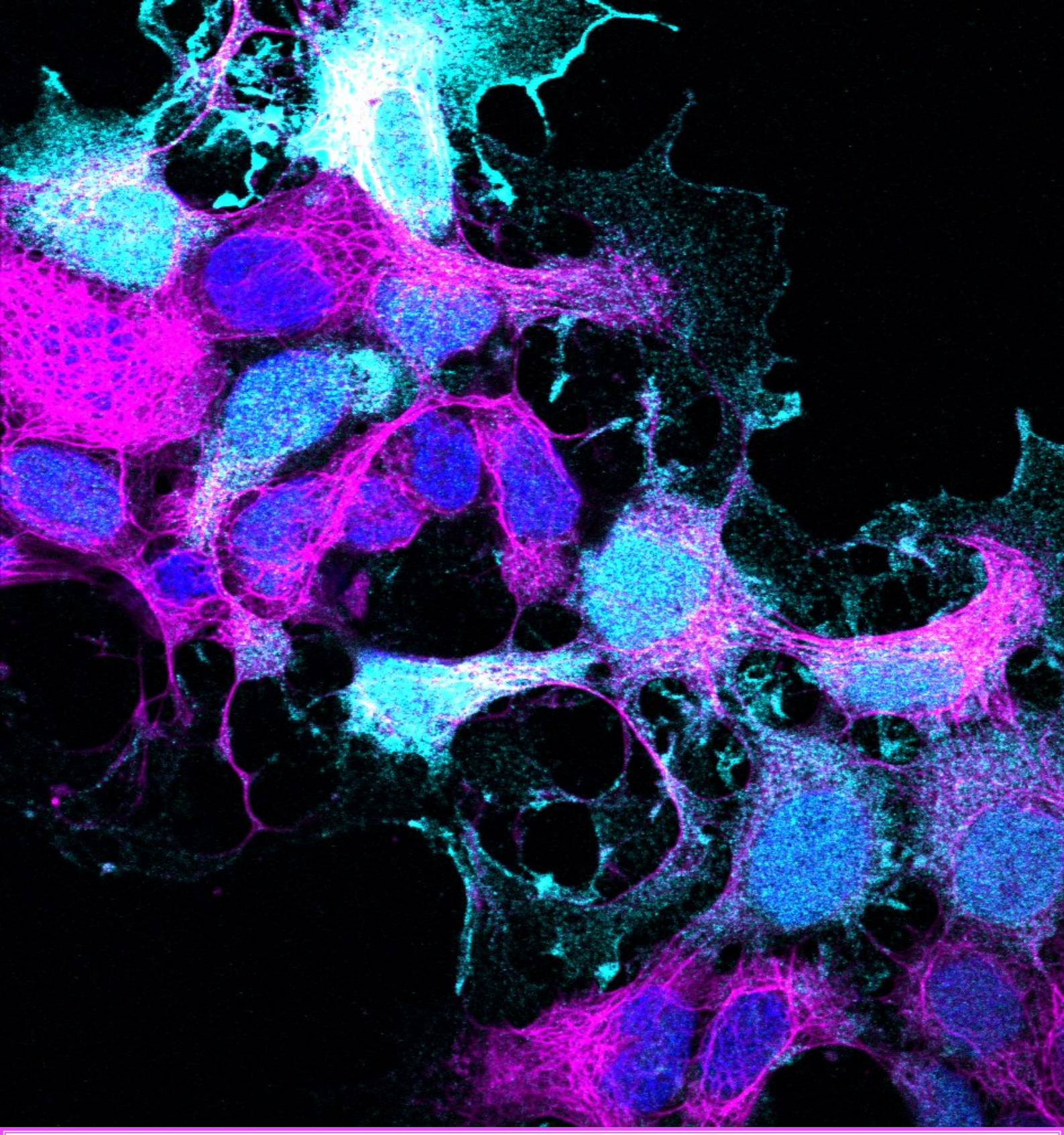


Chapter 4: VZV modulation of the skin microenvironment and clinical isolate variation in the context of PHN

CHAPTER 4

Introduction

The reactivation of VZV from latency causes herpes zoster, which may be associated with a broad variety of complications (Kawai et al., 2014). Post herpetic neuralgia (PHN) affects approximately 10-30% of patients who experience herpes zoster and can be defined as a neuropathic pain which lasts 3 months after the resolution of the herpes zoster rash and can persist for years (Drolet, 2017). PHN severely affects patient morbidity and there is a lack of effective treatments (Forbes et al., 2016, Drolet, 2017). Additionally, there is no way to predict whether a patient who experiences herpes zoster will develop PHN. Previous work presented as part of this thesis has demonstrated that VZV can modulate the induction of apoptosis (Gerada et al., 2018), as well as the ability of immune cells to kill virally infected target cells (Gerada et al., 2019). This modulation is thought to be critical for the maintenance of latency and the establishment of reactivation during herpes zoster and thus could contribute to the ability of VZV to cause PHN (Zerboni et al., 2014). However, HSV-1 encodes similar immunoevasive mechanisms to VZV and does not cause neuropathic pain after reactivation (Retamal-Díaz et al., 2016). This suggests that there are specific features of VZV pathogenesis that cause the development of neuropathic pain during herpes zoster. Through identifying VZV immunoevasive mechanisms and factors related to the ability of VZV to cause pathogenesis, new therapeutic targets may be identified for predicting the likelihood of developing PHN as well as its treatment.

Whilst it is unclear what specific features of VZV pathogenesis lead to the development of neuropathic pain, there has been extensive research into factors which can lead to neuropathic pain in non-viral settings (Calvo et al., 2012, McKelvey et al., 2015). It is clear that factors in both the peripheral nervous system and central nervous system are critical for neuropathic pain development through peripheral and central sensitization (Calvo et al., 2012). VZV can infect and modulate cells of both the DRG and peripheral nervous system in the skin and it is conceivable that modulation of these cell types could contribute to PHN development (Zerboni et al., 2014). There has been investigation into the effect of VZV infection in the DRG via the use of human DRG samples and animal models.

In a rat model of VZV induced pain, VZV DNA in the DRG was associated with differential host gene expression patterns that were related to nociception (Guedon et al., 2015). Additionally, it has been shown that sex steroids play a role in the pain response of rats following VZV infection (Stinson et al., 2017). This demonstrates that there may be some sex specific differences in PHN development. There has been some suggestion that PHN is experienced more often in females, however a recent metanalysis could not correlate gender with likelihood of developing PHN (Forbes et al., 2016). Post-mortem human DRG samples have revealed ganglia involved in the reactivation event, demonstrated pathology such as necrosis, secondary to vasculitis or hemorrhage, which could contribute to PHN development (Steain et al., 2014). Additionally, the immune cell infiltration in a ganglion from a patient with PHN has been investigated (Sutherland et al., 2019). Whilst there wasn't evidence of neuronal necrosis or cell death in this patient, there was an elevated proportion of $CD8^+$ T cells in comparison to a postmortem ganglia from a patient who suffered herpes zoster (Sutherland et al., 2019). Thus, VZV modulation of transcription, inflammation and immune cell infiltration in the DRG could contribute to sensitization of the nociceptive pathway during PHN.

Peripheral sensitization can be influenced by the microenvironment of the skin, as skin cells and immune cells can interact with neurons to alter their excitability (Calvo et al., 2012, Hesselink et al., 2017). Whilst there has been research on the ability of VZV to modulate various cell types in the skin (Zerboni et al., 2014), this has not been linked to the development of PHN. This is an area of interest as clinical trials using topical cannabinoids have been shown to be effective in PHN pain alleviation (Phan et al., 2010), suggesting an important role of the skin microenvironment in PHN development. Additionally, transient receptor cation channel subfamily V member 1 (TRPV1) levels in epidermal keratinocytes has been associated with pain development during herpes zoster (Han et al., 2016). Keratinocytes are an interesting cell type to consider in regards to neuropathic pain development as they lie in close proximity to sensory neurons and can secrete modulatory nociceptive substances (Baumbauer et al., 2015, Ritter-Jones et al., 2016, Hesselink et al., 2017).

VZV has been shown to infect and modulate keratinocyte function and thus this interaction could contribute to PHN development. VZV has been shown to regulate

keratinocyte development (Jones et al., 2014) as well as interfere with the ability of T cells to recognize infected keratinocytes (Nikkels et al., 2004), however there has been minimal research on the ability of VZV to modulate inflammation and the secretion of nociceptive substances from keratinocytes. The ability of VZV infection to cause inflammatory cytokine release from keratinocytes has been demonstrated in HaCaT cells, despite an increase in SOCS3 transcript expression *in vitro* (Choi et al., 2015). One of the issues faced by studying the effects of VZV on keratinocyte function *in vitro*, is that the cell associated nature of VZV (Grose et al., 1979) results in asynchronous infection and it is difficult to achieve a high percentage of infected cells. Therefore, it is unclear whether the VZV infected cells are directly responsible for inflammatory changes or whether VZV exposed bystander cells could be playing a role. It is critical to dissect whether VZV infection can directly modulate keratinocyte function to further understand possible contributing factors towards PHN development, and why topical therapy is effective in some PHN patients.

It is unknown whether patient factors or viral factors are more predictive of neuropathic pain development in herpes zoster. Novel variants of VZV have been shown to have altered pathogenesis, for example VZV gE mutants which exhibit faster cell-to-cell spread (Santos et al., 2000). It is therefore conceivable that mutations in VZV which alter pathogenesis could also alter the likelihood of developing PHN. As mentioned previously, it is unknown why some patients of a similar age and immunocompetence develop PHN and others do not. Therefore, assessing the ability of different wild type variants of VZV to differentially modulate cell types in the skin and ganglia is particularly pertinent.

In this study VZV modulation of HaCaT transcriptional and secretory profile was examined to identify novel viral modulation of factors in keratinocytes, which could influence PHN development. A bead separation approach was utilized to distinguish between the effect of VZV on transcription in bystander and productively infected HaCaT cells. Trends were observed in VZV modulation of HaCaT inflammatory cytokine transcripts and this modulation was also reflected in supernatants of VZV infected HaCaTs. Clinical VZV isolates from patients with or without PHN were characterized in regard to their growth kinetics, ability to cause cell death and modulation of cytokine, chemokine and growth factor secretion to determine whether

different features of these isolates could be linked with the potential to cause PHN. Together this work highlights some intriguing features of VZV modulation of skin cell types, which could contribute to PHN development.

Materials and methods

Materials

Table 4.1 Solutions and reagents

Product	Source	Storage	Use
Trypsin 0.5 % trypsin-EDTA (10x) containing 2 g/L sodium EDTA and 8.5 g/L sodium chloride	Gibco, Australia	-20 °C	Cell culture Supplied as 10X and diluted to 1X in 1X PBS (500 mL) (Lonza, Australia)
PE microbeads	Miltenyi Biotec, Germany	4 °C	Isolation of VZV gEgI+ cells
Ultracomp Ebeads	Thermo Fisher scientific, USA	4 °C	Compensation flow cytometry
Phosphate buffered saline (PBS)	Lonza, Australia	RT	Cell culture, washing flow cytometry
Brilliant II SYBR Green QPCR Master Mix	Agilent, USA	-20 °C	qRT-PCR

Table 4.2 Buffers

Product	Source	Storage	Use
Fix/perm buffer	BioLegend, USA	-20 °C	Used for fixation in flow cytometry
Fixation buffer	BioLegend, USA	-20 °C	Used for fixation in flow cytometry
FACS buffer: PBS, pH 7.2, 1% FCS and 2 mM EDTA	n/a	4°C	Washing and staining flow cytometry
MACS buffer: PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA	n/a	4°C	Washing in MACS bead separation
Fix perm wash buffer	BioLegend, USA	-20 °C	Immunostaining for flow cytometry

Table 4.3 Media and supplements

Product	Source	Additions	Storage	Use
Dulbecco's Modified Eagle Medium (DMEM) 10 % FCS with 4.5 g/L glucose, non-essential amino acids, inorganic salts and vitamins, and without sodium pyruvate	Lonza, Australia	Supplemented with 10 % (v/v) FCS (Serana, Australia) and 50 IU/mL penicillin/streptomycin (Gibco, USA)	4 °C	Warmed to 37 °C and used in cell culture

Table 4.4 Cell lines

Cell	Origin	Obtained from	Culture conditions	Split ratio
HaCaT	Human keratinocytes, spontaneously immortalized	Creative Bio Array, USA	Cultured in DMEM supplemented with 10 % FCS and 50 IU/ mL P/S. Cells were passaged up to 40 times at 37 °C with 5 % CO_2.	1:5, 1:10
ARPE-19	Human retinal pigment epithelial cell	ATTC, USA	Cultured in DMEM supplemented with 10 % FCS and 50 IU/ mL P/S. Cells were passaged up to 40 times at 37 °C with 5 % CO_2.	1:4, 1:5

Table 4.5 Kits

Kit	Manufacturer	Use	Items
Isolate II RNA mini kit	Bioline, USA	Used in the isolation of RNA	Kit includes: ISOLATE II filters, ISOLATE II RNA mini columns and collection tubes, collection tubes, lysis buffer RLY, wash buffer RW1, wash buffer RW2, membrane desalting buffer MEM, reaction buffer for DNase I RDN, DNase RNase-free, RNase-free water
Affinity script qPCR cDNA synthesis kit	Agilent, USA	Used in the conversion of RNA to cDNA	Kit includes: 2x cDNA synthesis master mix, AffinityScript RT/ RNase block enzyme mixture, Oglio(dT) primer, random primers, RNase-free H_2O
LegendPlex Human Anti-viral 13 plex: IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-α, IFN-β, IFN-λ1, IL-29, IFN-λ2/3, IL-28, IFN-γ, TNF, IP-10, GM-CSF	BioLegend, USA	Used in measuring the levels of cytokines/ chemokines in supernatants	Kit includes: setup beads 1: FITC beads, setup beads 2: PE beads. setup beads 3: raw beads, capture beads, detection antibodies, standard cocktail, lyophilized (1 vial), SA-PE, matrix B lyophilized (1 vial), assay buffer, wash Buffer, plate sealers, data analysis software dongle, V-bottom plate
BioPlex pro human cytokine 27 plex: FGF basic, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL1-RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-	Bio-Rad, Australia	Used in measuring the levels of cytokines/ chemokines in supernatants	Kit includes: coupled magnetic beads, detection antibodies, standards, control, assay buffer, wash buffer, sample diluent, standard diluent, detection antibody diluent, streptavidin-PE, a flat bottom plate, and sealing tape.

17A, IP-10, MCP-1, MIP-1 α, MIP-1β, PDGF-BB, RANTED, TNF, VEGF			
Human IL-1α ELISA Kit	Elisakit.com, Australia	Used in measuring the concentration of IL-1α in supernatants	Kit includes: wash buffer (20x), standard, assay diluent SB, anti-human IL-1α biotin labelled detection antibody, streptavidin-HRP conjugate, assay diluent 1B, strip wells, TMB substrate, stop solution

Table 4.6 Viruses

Virus	Source	Isolate
vOka	VARIVAX (varicella virus vaccine Oka/Merck Strain) (MSD)	Vaccine VZV strain, GenBank: AB097932.1
VZV-S	Professor Ann Arvin, Stanford University, USA	Clinical VZV strain, not sequenced
SUK57 (non PHN)	Professor Judith Breuer, University College London, UK (Ashrafi et al., 2010)	Clinical isolate, not sequenced
SUK66 (PHN)	Professor Judith Breuer, University College London, UK (Ashrafi et al., 2010)	Clinical isolate, not sequenced
SUK29 (non PHN)	Professor Judith Breuer, University College London, UK (Ashrafi et al., 2010)	Clinical isolate, not sequenced
Z226 (PHN)	Professor Judith Breuer, University College London, UK (Ashrafi et al., 2010)	Clinical isolate, not sequenced

Table 4.7 Flow cytometry antibodies and dyes

Antibody/ dye	Company	Primary/ Secondary	Concentration used	Storage
Cell trace violet (CTV)	Invitrogen, USA	Dye	1:1000	-20 °C
Zombie NIR	BioLegend, USA	Dye	1:1000	-20 °C
Rabbit anti-cleaved caspase 3 (CC3) PE clone C92-605	BD Biosciences, USA	Primary	1:20	4°C
Mouse anti-VZV gEgI conjugated to Dylight 488 (Serotec)	Meridian Bioscience Inc, USA	Primary	1:500	4°C

Table 4.8 Primers used for RT-PCR

Target	Direction	Sequence (5'→3')	Final concentration (μM)
TRPV1	TRPV1_F	GCCTGTCTTCATCATCCTGCTGCT	3.2
	TRPV1_R	GTTCTTGCTCTCCTGTGCGATCTTGT	
COX2	COX2_F	TTCAAATGAGATTGTGGGAAAT	3.2
	COX2_R	AGATCATCTGCCTGAGTATCTT	
18S RNA	18SRNA_F	GTAACCCGTTGAACCCCATT	3.2
	18SRNA_R	CCATCCAATCGGTAGTAGCG	
IL-8	IL-8_F	CTTGGCAGCCTTCCTGATTT	3.2
	IL-8R	TTTCCTTGGGGTCCAGACAGA	
IL-1ß	IL-1ß_F	GCTGAGGAAGATGCTGGTTC	3.2
	IL-1ß_R	GTGATCGTACAGGTGCATCG	
NGF	NGF_F	AGCGTAATGTCCATGTTGTTCTAC	3.2
	NGF_R	TGCTATCTGTGTACGGTTCTGC	
CRLR	CRLR_F	AGTGGCCAACAACCAGGCCTTAG	3.2
	CRLR_R	CTGCAAACACGGCCACCACAA	
ASC	ASC_F	ATCCAGGCCCCTCCTCAGT	3.2

	ASC_R	GTTTGTGAACCCTCGCGATAAG	
P2XR4	P2XR4_F P2XR4_R	GCTCATCCGCAGCCGCAAAG GTTGGTCACAGCCACGCCCTT	3.2
TNF	TNF_F TNF_R	CCTGCTGCACTTTGGAGTGA GATGAGGTACAGGCCCTCTG	3.2

Methods

Propagation of VZV

VZV infected HaCaTs were harvested when a cytopathic effect (CPE) of 2+ was reached. CPE ranges from 1-3 where a CPE of 1 is defined as 10% infection of the monolayer and 3+ as 80-90% infection of the monolayer. VZV infected cells were split onto a 30-40% confluent monolayer of uninfected HaCaTs at an appropriate ratio, mostly 1:4. Cells were maintained in supplemented DMEM at 37 °C, 5% CO_2 in tissue culture incubators.

Bead separation of gEgI+ and gEgI- VZV infected HaCaTs

HaCaTs were infected or mock infected in a cell-associated manner with VZV-S, VZV vOka or uninfected HaCaTs at a 1:3 ratio. On days 1, 3 and 5 post exposure cells were trypsanised and washed in FACS buffer. Additionally, supernatant was collected from all samples. Supernatant was centrifuged at 259 xg for 5 minutes and the supernatant aspirated and stored in 500 uL aliquots at -80°C. Cells were stained with mouse anti gEgI-PE for 30 minutes at 4 °C and washed twice in MACS buffer. Some cells from each condition were fixed for flow cytometry analysis using fixation buffer at RT for 15 mins. MACS anti-PE beads were added to VZV infected samples and were incubated for 15 mins at 4 °C. The MACS column apparatus was assembled, and the column prewashed with 3 mL MACS buffer. All samples were resuspended in 500 uL and applied to individual columns. Columns were washed three times with 3 mL of buffer and flow through VZV gEgI- cells were collected. The gEgI+ cells were extracted from the column using 5 mL MACS buffer. Cells were washed twice in PBS and some cells were fixed using fixation buffer at RT for 15 mins for subsequent flow cytometry analysis. RNA was collected from the remaining cells using RNA lysis buffer and stored at -80 °C.

RNA extraction

RNA extraction was performed using the Bioline Isolate II RNA mini kit. Cells were washed in PBS and 350 µL lysis buffer was added. Lysates were stored at -80 °C until use. On the day of RNA extraction, lysates were thawed and loaded onto an ISOLATE II filter. Samples were centrifuged at 11,000 xg for 1 minute. 350 µL 70% ethanol was added to filtered samples and mixed well before applying to the ISOLATE II RNA Mini column. Samples were centrifuged at 11,000 xg for 30 seconds. 350 µL membrane desalting buffer was applied to the membrane and samples were centrifuged at 11,000 xg for 1 minute. For each sample, 10 µL reconstituted DNase I was added to 90 µL Reaction buffer for DNaseI. 95 µL of this mixture was applied to the membrane and incubated for 10 minutes at RT. 200 µL buffer RW1 was added to the column and centrifuged at 11,000 xg for 30 seconds. 600 µL buffer RW2 was added to the column and centrifuged at 11,000 xg for 30 seconds. 250 µL buffer RW2 was added to the column and centrifuged at 11,000 xg for 2 mins. RNA was eluted by adding 20-30 µL RNase free H_2O and centrifuging at 11,000 xg for 1 min. The concentration and purity of the samples was assessed using the nanodrop reader (Thermo Fisher Scientific).

cDNA synthesis

The affinity script qPCR cDNA synthesis kit (Agilent, USA) was used to convert RNA into cDNA. The following components were added in order to a microcentrifuge tube: RNase-free H_2O to make reaction volume up to 20 µL, 10 µL first strand mastermix, 3 µL 0.1 µg/ µL random primers, 1 µL AffinityScript reverse transcription (RT)/ RNase Block enzyme mixture and the same concentration of RNA. No-RT controls were made by making the same reaction as above omitting the AffinityScript RT/ RNase Block. Samples were incubated at 25 °C for 5 mins for annealing, 42 °C for 15 minutes for cDNA synthesis and 95 °C for 5 mins to terminate the cDNA synthesis reaction. Some of the cDNA was pooled to make standards and the rest was diluted 1:10 with ultrapure H_2O.

qRT-PCR

PCR mastermixes were made for each primer set. The mastermix was composed per sample as follows 3.22 µL ultrapure H_2O, 6.5 µL Brilliant II Sybr Green (Agilent, Australia), 0.14 µL of forward and reverse 20 µM primer. 3 µL of template was added to 10 µL of mastermix. On each plate samples, standards, RT controls and H_2O

controls were run in duplicate. Samples were processed using a LightCycler 480 qRT-PCR machine (Roche Life Science). qRT-PCR was performed with a single 2-minute denaturation step at 50 °C followed by incubation at 95 °C for 2 minutes. 40 cycles were performed with denaturation at 95 ° C for 5 seconds followed by primer specific annealing and extension durations and temperatures. During these cycles fluorescence levels were acquired. After cycling, a step-wise increase in temperature from 60-90 °C was performed to create a melt curve.

Analysis of PCR

The average number of transcripts was normalized to the housekeeping gene 18S RNA, then infected cultures were normalized to their corresponding mock control sample. Statistical analysis was performed using a matched one-way ANOVA. Comparisons were considered to be significant if the p value was less than 0.05.

Legend plex assay to detect inflammatory cytokines in VZV infected HaCaT supernatant

Supernatants collected from mock, VZV-S and vOka infected HaCaTs at days 1, 3 and 5 post VZV exposure were assessed for 13 antiviral analytes using the LegendPlex Human Anti-Virus Response Panel (Table 4.5). Before commencing the assay, the 20x wash buffer was brought to RT and diluted with DW to make a 1x solution. The standard cocktail was reconstituted with 250 μL assay buffer and diluted appropriately to create standards. C0 standard consisted of assay buffer alone and C7 standard consisted of undiluted standard cocktail. Before use the assay components were warmed to RT and 25 μL assay buffer was added to all wells of a 96 well v bottom plate as well as 25 μL standard or sample. Samples and standards were run in duplicate. Pre-mixed beads were sonicated for 1 minute in a sonicator water bath and 25 μL were added to each well with intermittent vortexing to ensure the beads were suspended. The plate was sealed and shaken at RT and 500 RPM, protected from light for 2 hrs. Plate centrifuged at 250 xg for 5 minutes and supernatant was discarded. 200 μL washing buffer was added to each well and the plate was centrifuged again to remove the supernatant. 25 μL detection antibody was added to each well and the plate was sealed and shaken at RT and 500 RPM, protected from light for 1 hr. 25 μL streptavidin (SA)-PE was then added to each well and the plate

was sealed and shaken at RT and 500 RPM, protected from light for 30 minutes. Plate centrifuged at 250 xg for 5 minutes and supernatant was discarded. 200 μL washing buffer was added to each well and the plate was centrifuged again to remove the supernatant. Beads were resuspended in 150 μL wash buffer and were run on the LSR Fortessa (BD Biosciences). Results were analysed using the LegendPlex software (BioLegend). Concentration for each analyte was calculated by LegendPlex software and 2 technical replicates were averaged for each biological replicate (n=3). Fold changes in concentration were calculated via normalizing the corresponding mock. Statistical analysis was performed using a matched one-way ANOVA. Comparisons were considered to be significant if the p value was less than 0.05.

VZV clinical isolates

Clinical VZV isolates in MeWo cells from patients with and without PHN were kindly gifted from Professor Judith Breuer, University College London, UK (Table 4.6). These isolates were resuscitated onto MeWos and were monitored for growth and abnormal CPE. These isolates were split onto both HaCaTs and ARPE-19s at a 1:10 ratio of infection and were passaged until there were no remaining inoculum as determined by light microscopy. Stocks were then frozen down and resuscitated for experimental use.

VZV clinical isolate spread and cell death induction assessment by microscopy and flow cytometry

ARPE-19s or HaCaTs were seeded into 6 well plates and 6 hours later, clinical isolates and mock ARPE-19s or HaCaTs were cell trace violet (CTV) labelled for 30 mins in PBS at 37°C and were washed in media. CTV labelling is used to identify inoculum in a VZV cell associated infection (Kennedy et al., 2019). CTV labelled cells were used to infect previously seeded ARPE-19s at a 1:10 ratio. For HaCaT experiments CTV labelled cells were used to inoculate uninfected HaCaTs at a 1:5 ratio. Mock and viral inoculum were stained for gEgI and were fixed for analysis by flow cytometry. At days 1, 3 and 5 post exposure, light microscopy images were taken to observe the CPE of infected cells. Supernatant was collected at days 1, 3 and 5 post exposure, spun down and frozen at -80°C for subsequent analysis. Cells were trypsinized, washed once in PBS and stained with Zombie NIR at RT for 30 mins. For a live dead compensation

control cells were heated at 70°C for 5 mins. Cells were washed once in FACS buffer and were stained for gEgI at RT for 30 mins. Cells were washed once in FACS and were permeabilized and fixed with BD fix perm buffer at RT for 20 mins. Cells were washed twice in perm buffer and incubated with rabbit anti CC3-PE overnight at 4°C. Cells were washed once with perm buffer and once with FACS buffer before running on the LSR Fortessa.

BioPlex assay to detect cytokines, chemokines and growth factors in clinical isolate infected ARPE-19s and vOka infected HaCaTs

Supernatants collected from mock, SUK57, SUK66, SUK29 and Z226 infected ARPE-19s at days 1, 3 and 5 post exposure as well as supernatants from mock and vOka infected HaCaTs at days 1, 3 and 5 post exposure were assessed for 27 analytes using the Bio-Plex pro human cytokine 27 plex assay. The 27 analytes, minimal detectable concentration, maximal detectable concentration and range detected in both HaCaTs and ARPE-19s are detailed in Table 4.9.

Table 4.9 Range of analytes detected in BioPlex assay in HaCaTs and ARPE-19s.

Analyte	Min DC (pg/mL)	Max DC (pg/mL)	Range detected in ARPE-19 (pg/mL)	Range detected in HaCaTs (pg/mL)	Displayed
MIP1β	0.027	3153	6-11	0.9-2	Yes
IL-6	0.044	12138	24-600	10-100	Yes
IFNγ	0.25	39427	20-100	40-120	Yes
IL-1RA	0.61	176929	8-600	800-13000	Yes
IL-5	0.52	89976	0.52-6	0.52-6	No
GM-CSF	0.06	6128	0.6-2	0.6-23	Only HaCaTs
TNF	0.32	38113	2-7	9-25	Only HaCaTs
RANTES	0.5	32505	0.67-5.5	5.5-60	Only HacaTs
IL-2	0.2	42672	1-4	1-4	No
IL-1β	0.02	6429	0.37-1	0.37-1	No
Eotaxin	0.0051	2415	0.13-12	0.48-2	Only ARPE-19
Basic FGF	2.02	14215	10-150	5-30	Yes
VEGF	2.98	204405	7-900	10-3000	Yes
PDGF-BB	5.2	69945	70-200	13-1200	Yes
IP-10	1.77	38620	30-90	30-90	Yes
IL-13	0.047	17341	0.31-0.68	0.31-0.68	No
IL-4	0.087	5003	0.63-2.11	0.63-2.11	No
MCP-1	0.066	4910	150-2400	13-1800	Yes
IL-8	0.068	32464	5000-21000	171-2000	Yes
MIP1α	0.043	1465	0.16-0.34	0.16-0.34	No
IL-10	0.16	11109	0.4-1	0.4-1	No
G-CSF	3.09	110859	ND	ND	No
IL-15	7.37	160632	ND-30	ND-30	Yes
IL-7	0.13	63428	5-90	5-90	Yes
IL-12p70	0.091	52165	0.5-1	0.5-1	No
IL-17	0.37	105188	1.5-2.5	1.5-2.5	No
IL-9	0.33	61607	5-50	5-10	Yes

The luminex MAGPIX (Thermo Fisher Scientific, USA) underwent verification and calibration as per manufacturer's instructions before the assay was commenced. Kit components were brought to RT before use and supernatants were defrosted in the fridge. Wash buffer was diluted to 1x using DW before use. 50 μLs of beads were added to all wells of the 96 well plate and were washed twice in wash buffer using the Bio-Plex pro wash station. Standards and the control were reconstituted with DMEM (10% FCS + p/s) and incubated on ice for 30 minutes. The standard was diluted 1:4 in a series dilution to create 10 standards. 50 μLs of sample, standard, control or blank (DMEM) was added to the beads and was incubated on a plate shaker for 30 mins and 850 RPM at RT. The detection antibody was diluted in antibody diluent as per manufactures instructions. The plate was washed 3 times in 100 μL wash buffer using the BioPlex pro wash station and 25 μL of detection antibody was added to each well. The plate was incubated for 30 mins at 850 RPM and RT and washed 3 times in 100 μL wash buffer using the Bio-Plex pro wash station. Streptavidin-PE was diluted in assay buffer as per manufactures instructions and 50 μL was added to each well. The plate was incubated for 10 mins at 850 RPM and RT and then washed 3 times in 100 μL wash buffer using the bio-plex pro wash station. Beads were resuspended in 125 μL assay buffer by shaking at 850 RPM for 1 minute. Data was acquired on the luminex MAGPIX. Milliplex analyst software (MerckMillapore) was used for data analysis to generate standard curves and concentrations of each analyte. Fold changes in analyte concentration were calculated via normalizing the corresponding mock to generate a heat map using MORPHEUS software (Broad Institute). Statistical analysis was performed using a matched one-way ANOVA for ARPE-19 samples and a two tailed paired students T test for HaCaT samples. Comparisons were considered to be significant if the p value was less than 0.05.

IL-1α ELISA

Mock and vOka infected HaCaT supernatant and mock, SUK57, SUK66, SUK29 and Z226 ARPE-19 supernatant was analysed for IL-1α concentration using the ELISAkit.com human IL-1α ELISA. Kit components and supernatants were brought to RT before use and kit components were prepared as per manufacturer's instructions. HaCaT supernatants were diluted 1:2 in supplemented DMEM before use and ARPE-19 supernatants were run neat. The standard was reconstituted in assay diluent SB

and incubated at RT for 15 minutes with regular inversion. Supplemented DMEM was used to perform the serial dilutions of the standard. 50 µL of assay diluent SB to each well and 50 µL of standards, samples and zero standard controls (supplemented DMEM) was added and incubated at RT for 2 hours. All samples, standards and controls were run in duplicate. The plate was washed 4 times with wash buffer and 100 µL of biotin labelled detection antibody was added to each well. The plate was incubated at RT for 2 hours and then washed 4 times with wash buffer. 100 µL of freshly diluted streptavidin-HRP conjugate was added to each well and the plate was incubated for 45 mins at RT. The plate was washed 5 times with wash buffer and 100 µL TMB substrate was added to each well and incubated for 10 mins at RT protected from light. The reaction was stopped with 50 µL stop solution and the optical density (OD) was determined using the TECAN Infinite M1000 pro plate reader. The mean absorbance for each standard, sample and control was determined and the mean of the zero standard controls was subtracted. A 7-point standard curve from 2000 pg/mL to 31.25 pg/mL was generated and used to calculate the concentration of IL-1α in each sample.

Statistics

All statistical analyses were performed as stated using GraphPad Prism versions 6 and 7 (GraphPad Inc). Data was analyzed using either a two-tailed paired students T test or one-way repeated measure ANOVA as indicated.

Results

VZV-S and vOka cell-associated infection levels in HaCaT cells and MACS bead selection of gEgI+ HaCaT cells

To explore the effects of VZV on keratinocyte inflammatory and nociceptive profiles HaCaTs were utilized as an *in vitro* model, due to their ability to be easily propagated and infected by VZV (Choi et al., 2015, Gerada et al., 2018). As explained previously, VZV is highly cell associated *in vitro* (Grose et al., 1979) and using a cell-associated infection method HaCaTs were infected with either VZV-S, vOka or mock infected HaCaTs at a 1:3 ratio. The cell associated infection method is commonly utilized to inoculate uninfected cells and can result in an asynchronous infection (Shiraki et al., 1992). To determine whether the vaccine strain (vOka) was attenuated in regard to modulating keratinocyte inflammatory and nociceptive responses, the effects of VZV-S (a clinical isolate) and vOka on HaCaTs were compared. Cells were harvested at days 1, 3 and 5 post exposure and stained with zombie live dead to exclude dead cells in subsequent analysis. Through analysis of VZV gEgI staining it was clear that by day 3 on average 10% of VZV-S exposed HaCaTs were gEgI+ and 20% of vOka exposed HaCaTs were gEgI+ (Figure 4.1A). As the percentage of VZV gEgI positive cells varied between the three independent experiments with VZV-S and vOka on day 1 ,3 and 5 post exposure (Figure 4.1B & 4.1C), it was determined that infected cells needed to be separated from bystander cells for subsequent mRNA transcript analysis.

MACS bead separation was utilized to positively select VZV gEgI+ cells (Figure 4.2). Flow cytometry analysis of gEgI staining was used to assess the purity of MACS sorted populations. The positively selected fraction was on average 80% pure (gEgI+) for both VZV-S and vOka infected cells on days 1, 3 and 5 post exposure (Figure 4.3A-C & Figure 4.4A-C) and the remaining bystander cells were on average 90% pure (gEgI-), from 3 independent experiments. This purity of cell populations would allow for the effect of VZV infection in HaCaTs to be distinguished from the effect of exposure to VZV infected cells when examining inflammatory and nociceptive changes.

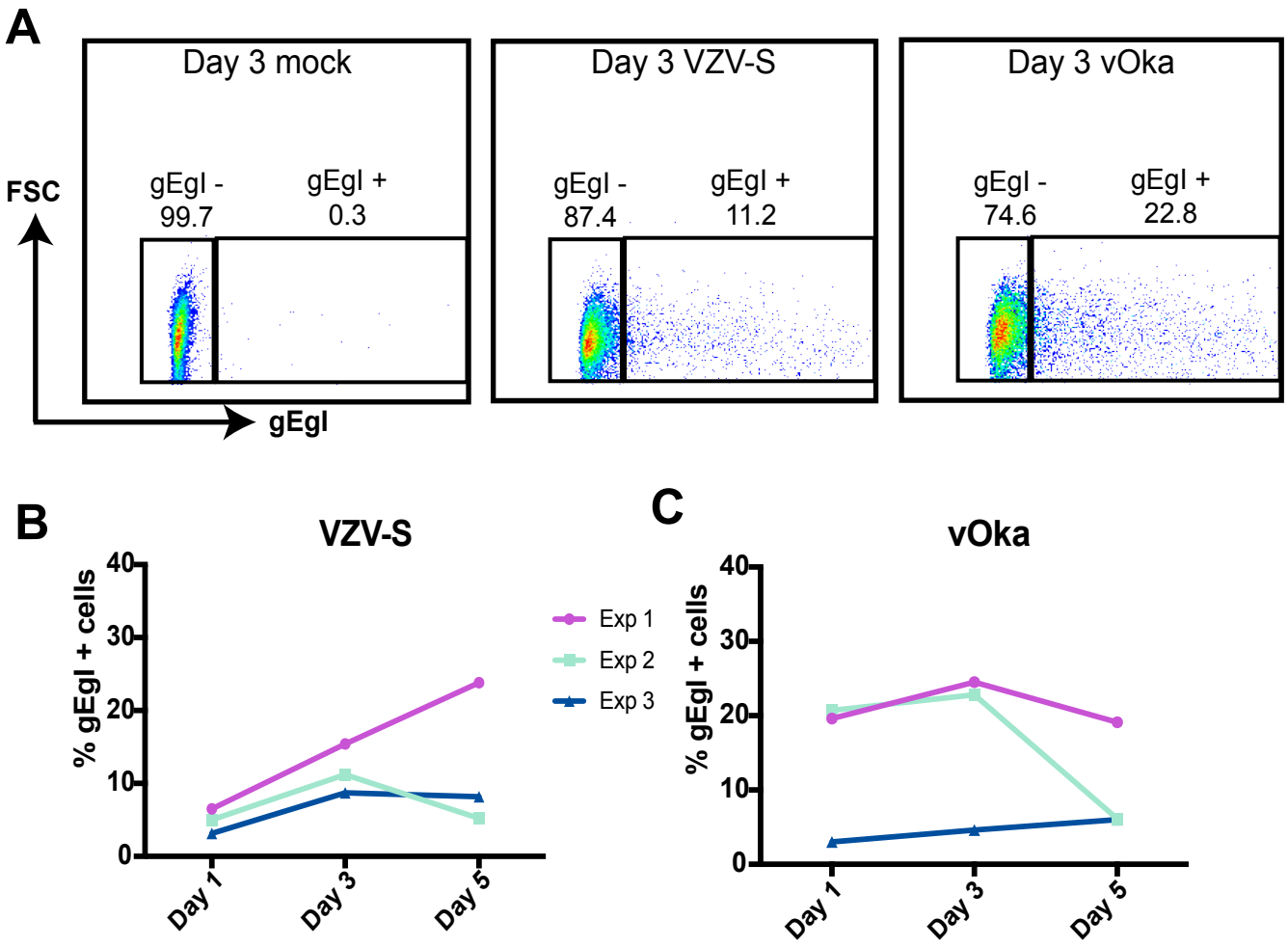


Figure 4.1 Kinetics of VZV gEgI during a 5-day cell associated infection detected by flow cytometry.
HaCaT cells were either mock, VZV-S or vOka infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post exposue, cells were collected and stained for gEgI and analysed by flow cytometry. Representative flow plots of day 3 for mock, VZV-S and vOka gEgI expression are displayed (A). Line graphs of gEgI expression for VZV-S (B) and vOka (C) at day 1,3 and 5 post exposure are plotted for each independent experiment (Exp) (n=3).

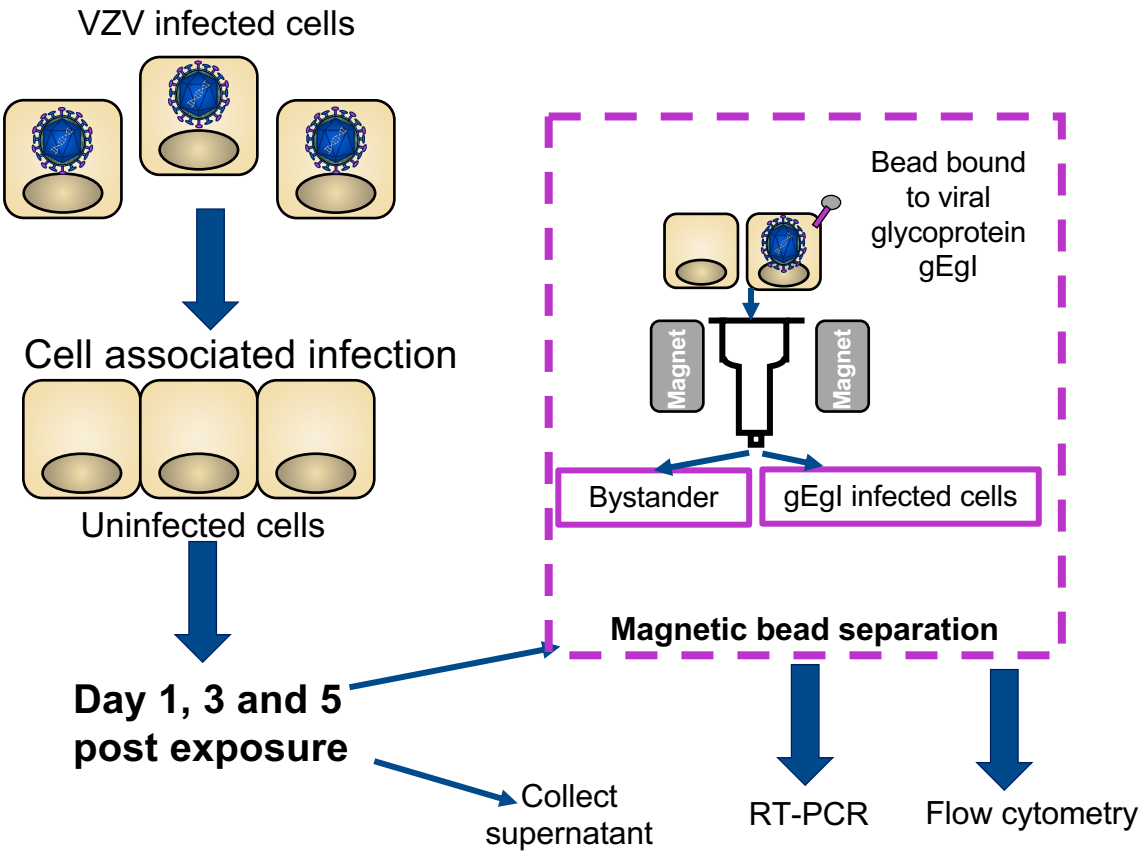


Figure 4.2 Schematic of magnetic bead separation of VZV infected cells and subsequent downstream analysis.
HaCaTs were either mock or VZV infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post exposure, supernatant was collected and magnetic bead separation was used to sort bystander ($gEgI^-$) and VZV infected ($gEgI^+$) cells. To do this cells at various days post VZV exposure were stained with mouse anti-gEgI PE and incubated with magnetic beads which bind to PE. Cells were then run through a magnetic column to separate bystander and infected cells. Success of sorting was assessed via flow cytometry and RNA was harvested from sorted cells and stored at -80°C for subsequent analysis.

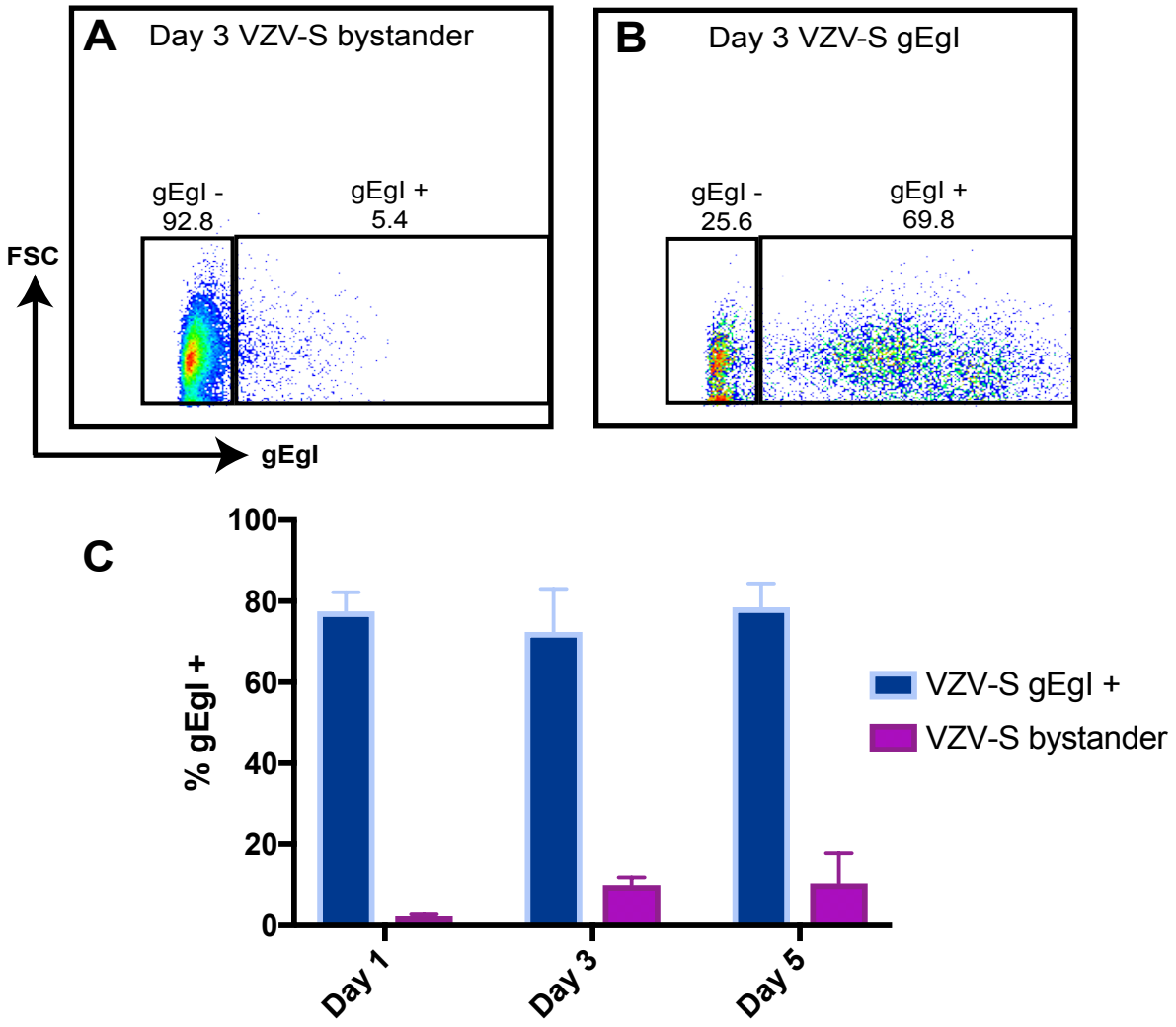


Figure 4.3 MACS bead separation of VZV gEgI positive VZV-S infected cells. HaCaTs were either mock or VZV-S infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post exposure gEgI^{+} (A) cells were separated from gEgI^{-} cells (bystanders) (B) using MACS bead separation. Cells were fixed and analysed via flow cytometry. Representative flow plots for gEgI positivity post-separation for bystander and gEgI^{+} cells at day 3 post exposure are displayed (A & B). gEgI^{+} cells and bystander cells (gEgI^{-}) are collated in a bar graph (n=3) (C).Error bars represent the SEM.

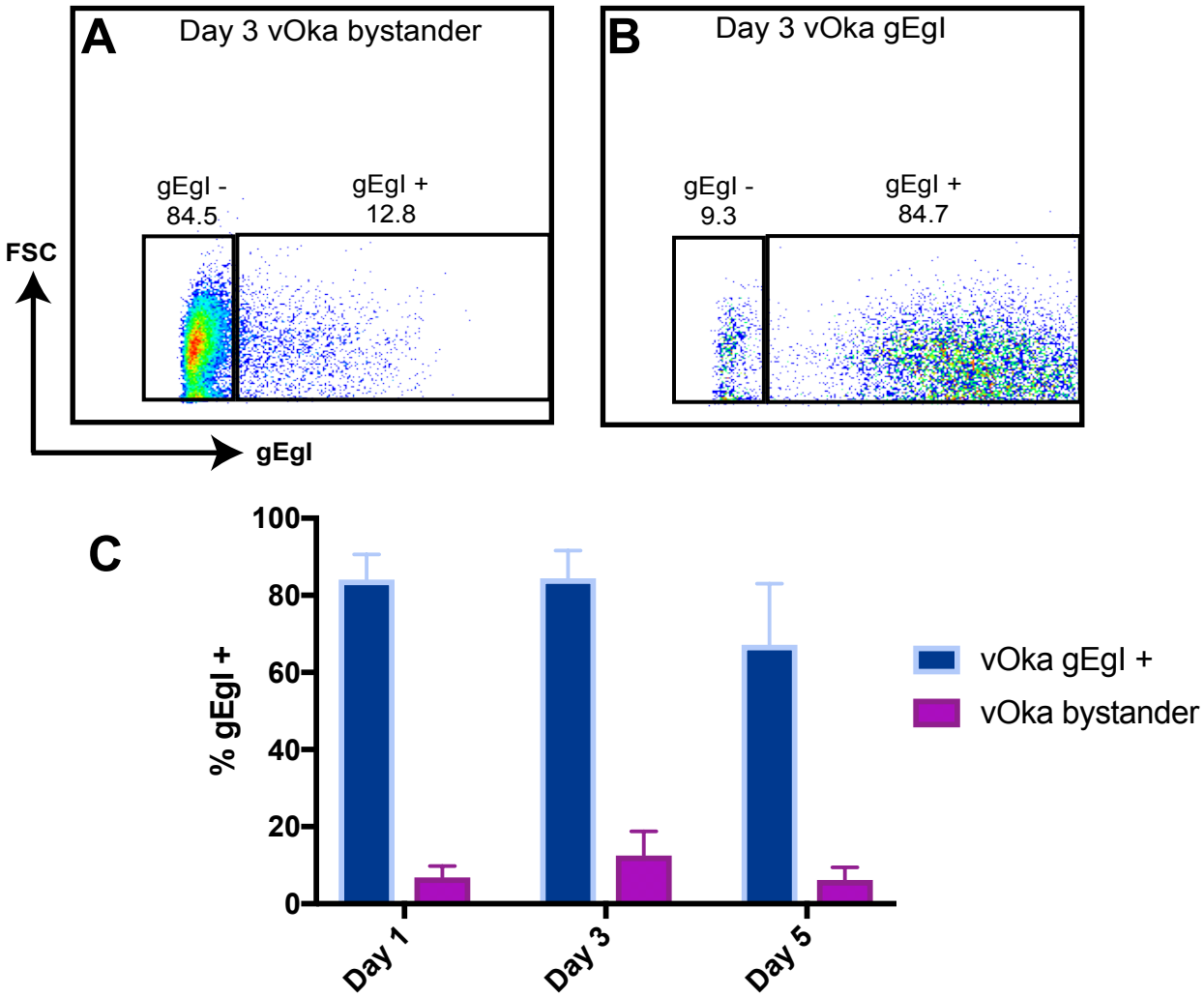


Figure 4.4 MACS bead separation of VZV gEgI positive vOka infected cells. HaCaTs were either mock or vOka infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post exposure gEgI^{+} cells (A) were separated from gEgI^{-} cells (bystanders) (B) using MACS bead separation. Cells were fixed and analysed via flow cytometry. Representative flow plots for gEgI positivity post-separation for bystander and gEgI^{+} cells at day 3 post exposure are displayed (A & B). gEgI^{+} cells and bystander cells (gEgI^{-}) are collated in a bar graph (n=3) (C). Error bars represent the SEM.

VZV-S and vOka modulation of pain and inflammatory associated transcripts in HaCaTs

As described previously, HaCaTs were mock, VZV-S or vOka infected and VZV gEgI positive HaCaTs were separated from antigen negative cells using MACS bead separation. mRNA was extracted from cells and converted to cDNA for RT-PCR analysis. A variety of transcripts related to pain, ion channels and receptors, inflammasome formation and inflammatory cytokines were examined in these cells to determine whether VZV infection could modulate the HaCaT nociceptive potential and inflammatory profile. All of the transcripts chosen were detectable in mock infected HaCaTs at a basal level and had a Ct value ≥ 25. Transcriptional changes in infected and bystander cells were normalized to the mock control. Pain associated transcripts (Figure 4.5) examined included cyclooxygenase 2 (COX-2), an enzyme critical for the production of prostaglandin E2 (PGE2) (Ma and Quirion, 2008). Injured nerve derived COX-2/PGE2 has been shown to contribute to the maintenance of neuropathic pain in aged rats, where PGE2 receptors where increased in DRG neurons (Ma et al., 2010). Additionally, nerve growth factor (NGF) a neuropeptide (Khan and Smith, 2015), which plays an important role in the sensitization of nociceptive neurons was examined. COX-2 transcripts were not significantly regulated by VZV-S or vOka in both bystander and gEgI positive cells in comparison to mock infected cells. NGF transcripts were also not significantly regulated.

Keratinocytes have been shown to upregulate pain associated ion channels and receptors in the context of neuropathic pain (Hesselink et al., 2017). Transient receptor potential cation channel subfamily V member 1 (TRPV1) (Christoph et al., 2006), P2X purinoceptor 4 (P2XR4) (Tsuda, 2016) and calcitonin receptor-like receptor (CRLR) (Hou et al., 2011) are receptors (Figure 4.6) which have been identified on human keratinocytes and have been shown to play a role in the generation of neuropathic pain. VZV-S or vOka did not significantly regulate the transcript of either TRPV1, P2XR4 or CRLR in comparison to mock infected cells. However, there was a trend for a TRPV1 transcript increase in both gEgI$_+$ cells and bystander cells for both VZV-S and vOka at all time points.

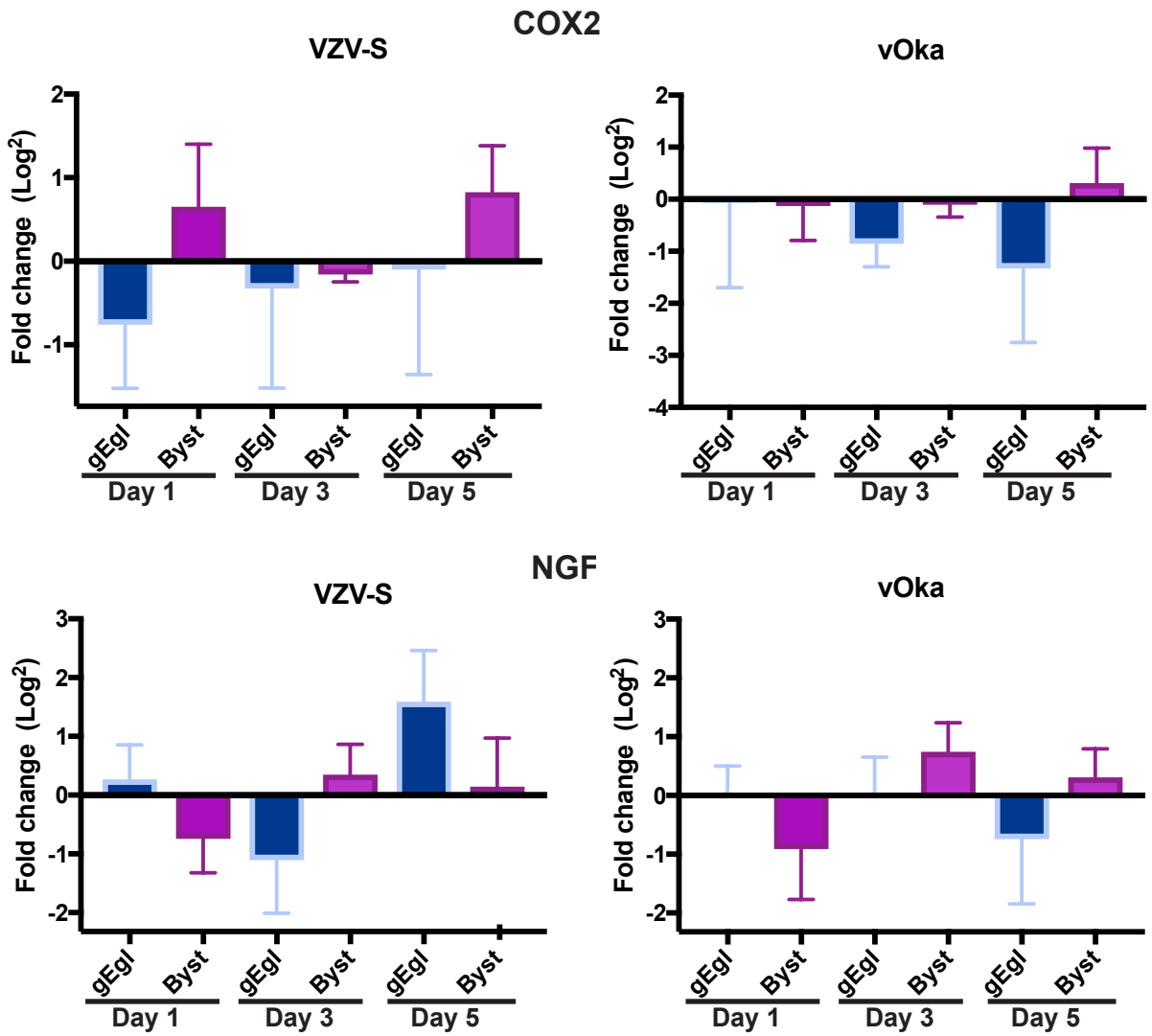


Figure 4.5 qRT-PCR fold change in pain associated transcripts in VZV-S and vOka exposed HaCaTs.

HaCaT cells were either mock, VZV-S or vOka infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post VZV exposure, cells were MACS bead separated based on gEgI positivity. Total cellular RNA was extracted from mock, bystander ($gEgI^-$) and virus infected ($gEgI^+$) cells at days 1, 3 and 5 post exposure and mRNA expression of human COX-2 and NGF was measured using qRT-PCR. Expression levels of NGF and COX-2 were normalized to the house keeping gene 18S RNA. VZV-S and vOka bystander and virus infected cell transcripts are depicted as fold change relative to mock. Statistics were generated using a one-way ANOVA test, and Tukey's multiple comparison post hoc test for each time point. Error bars represent the SEM for 3 independent experiments (n=3).

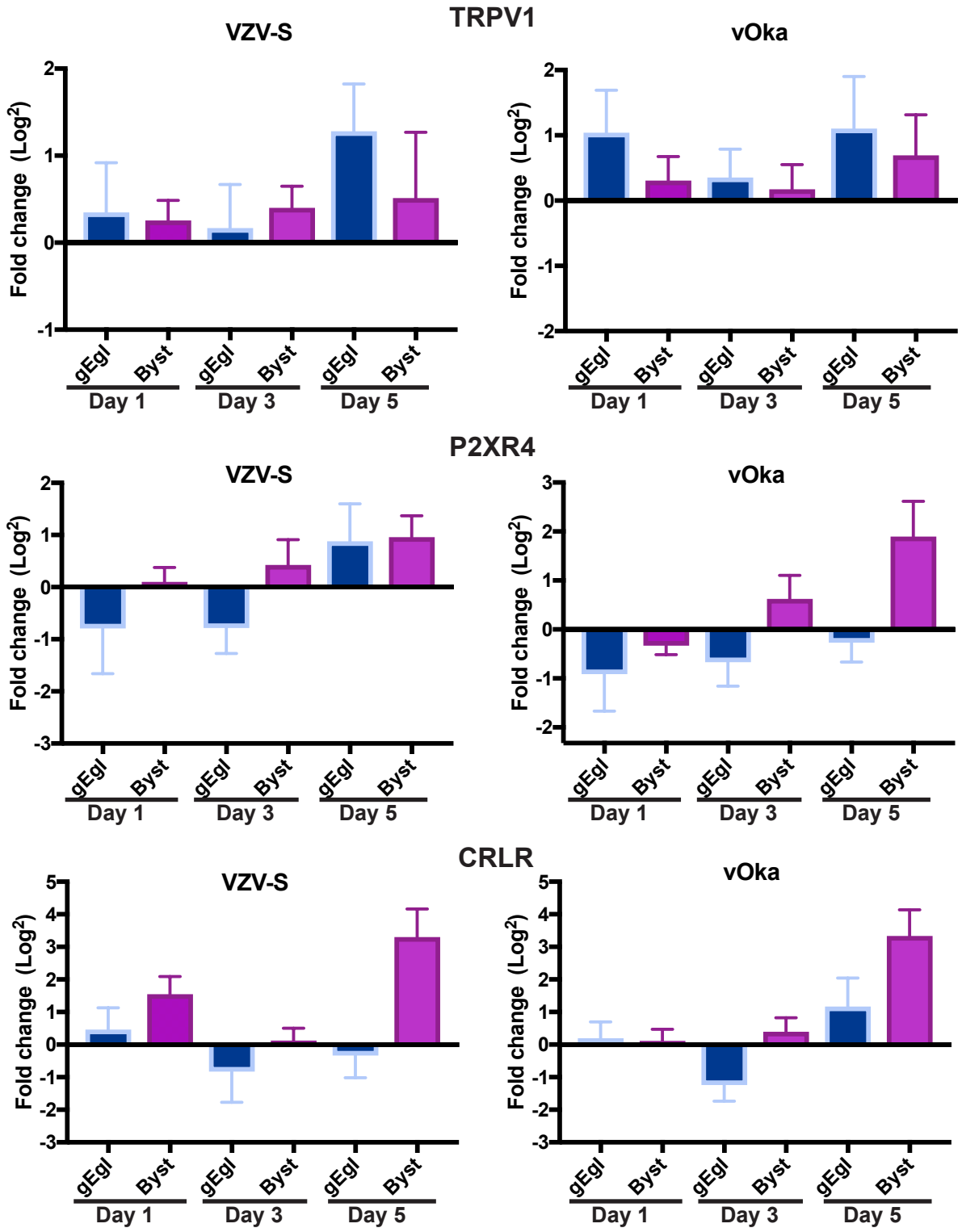


Figure 4.6 qRT-PCR fold change in ion channel and receptor transcripts in VZV-S and vOka exposed HaCaTs.

HaCaT cells were either mock, VZV-S or vOka infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post VZV exposure, cells were MACS bead separated based on gEgI positivity. Total cellular RNA was extracted from mock, bystander ($gEgI^-$) and virus infected ($gEgI^+$) cells at days 1, 3 and 5 post exposure and mRNA expression of human TRPV1, P2XR4 and CRLR was measured using quantitative qRT-PCR. Expression levels of TRPV1, P2XR4 and CRLR were normalized to the house keeping gene 18S RNA. VZV-S and vOka bystander and virus infected cell transcripts are depicted as fold change relative to mock. Statistics were generated using a one-way ANOVA test, and Tukey's multiple comparison post hoc test for each time point. Error bars represent the SEM for 3 independent experiments (n=3).

As there were no obvious changes in pain associated transcripts, inflammatory transcripts were examined. A variety of inflammatory cytokines have been shown to contribute to the development of neuropathic pain (Clark et al., 2013). Additionally VZV infection, has been shown to alter inflammatory cytokines and chemokines in skin cells (Choi et al., 2015, Graybill et al., 2017). There has been some evidence to suggest that VZV infection can cause inflammasome activation which results in the production of IL-1β (Nour et al., 2011). Inflammasome activation triggered by viral infection can potentiate anti-viral immune responses to control viral spread, however aberrant activation can cause neuronal tissue injury via IL-1β. Inflammasome related transcripts (Figure 4.7) examined included Inflammasome Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) a critical adaptor protein, and IL-1β, a cytokine implicated in neuropathic pain development (Grace et al., 2016). For both VZV-S and vOka there were no significant differences observed in bystander or gEgI+ cells in regard to ASC transcript, however there was a trend towards an increase in ASC transcript in bystander and gEgI+ cells across several timepoints. Similarly, for IL-1β transcripts, there were no significant differences observed in bystander or gEgI+ cells for VZV-S or vOka in comparison to mock infected cells. Towards later timepoints, IL-1β transcripts trended toward an increase in both bystander and infected cells.

VZV has been shown to inhibit NFκB function and thus could affect the transcription of multiple NFκB driven inflammatory cytokine transcripts (Sloan et al., 2012). Both IL-8 and tumour necrosis factor (TNF) are inflammatory cytokines (Figure 4.8) which have been associated with neuropathic pain (Clark et al., 2013). For both VZV-S and vOka there were no significant differences observed in bystander or gEgI+ cells in regard to the IL-8 transcript. TNF transcript was significantly decreased day 1 post exposure in both bystander and gEgI+ cells for VZV-S in comparison to mock infected cells. On days 3 and 5 post exposure the TNF transcript trended towards an increase in gEgI+ cells for VZV-S and vOka, however this was not statistically significant. Current analysis failed to find transcripts that were consistently and significantly regulated by VZV infection in HaCaTs. Assessment of protein abundance and function is critical to determine whether VZV can alter the nociceptive capacity of keratinocytes and influence PHN development.

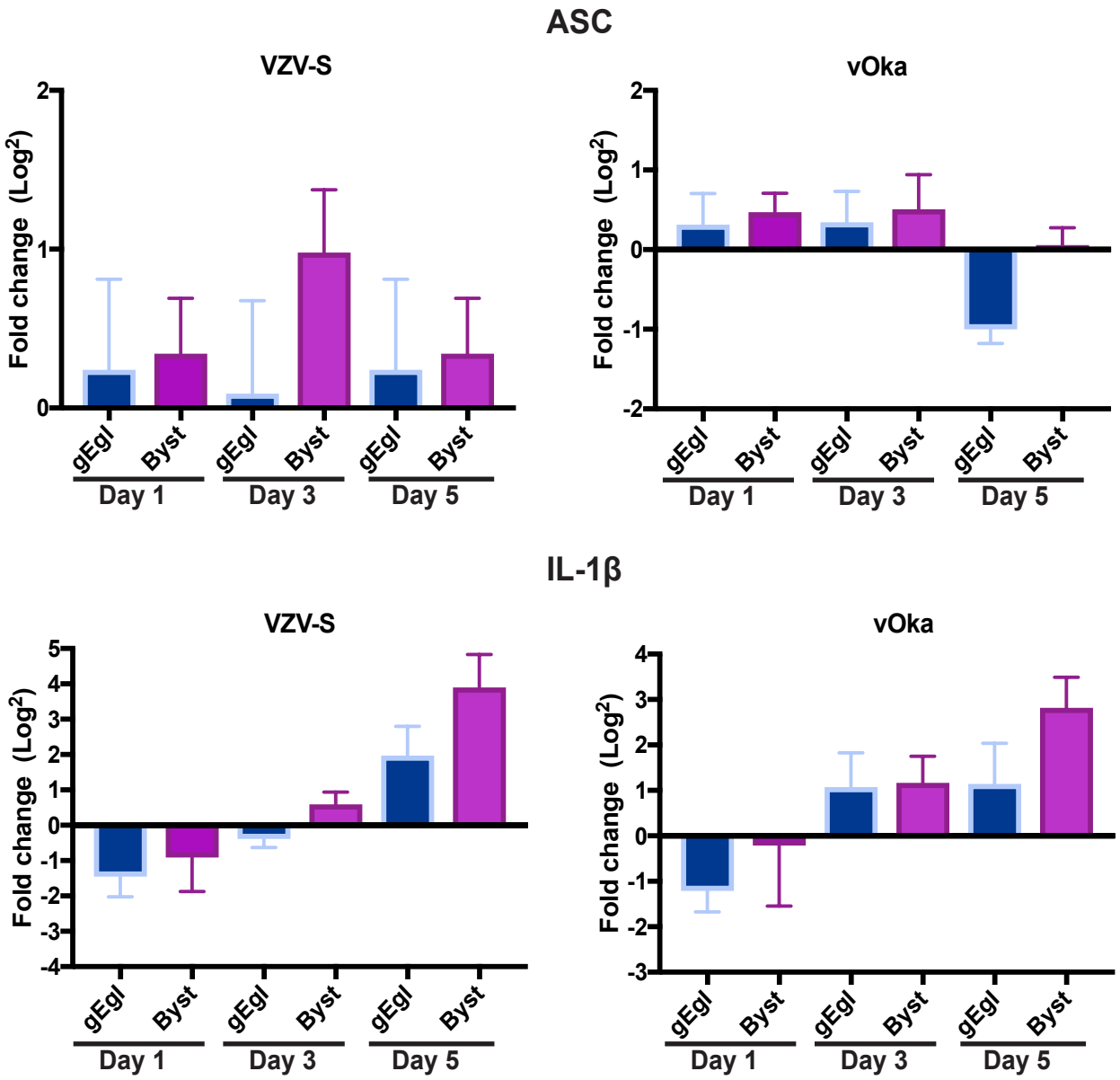


Figure 4.7 qRT-PCR fold change in inflammasome related transcripts in VZV-S and vOka exposed HaCaTs.

HaCaT cells were either mock, VZV-S or vOka infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post VZV exposure, cells were MACS bead separated based on gEgI positivity. Total cellular RNA was extracted from mock, bystander ($gEgI^-$) and virus infected ($gEgI^+$) cells at days 1, 3 and 5 post exposure and mRNA expression of human ASC and IL-1β was measured using qRT-PCR. Expression levels of ASC and IL-1β were normalized to the house keeping gene 18S RNA. VZV-S and vOka bystander and virus infected cell transcripts are depicted as fold change relative to mock. Statistics were generated using a one-way ANOVA test, and Tukey's multiple comparison post hoc test for each time point. Error bars represent the SEM for 3 independent experiments (n=3).

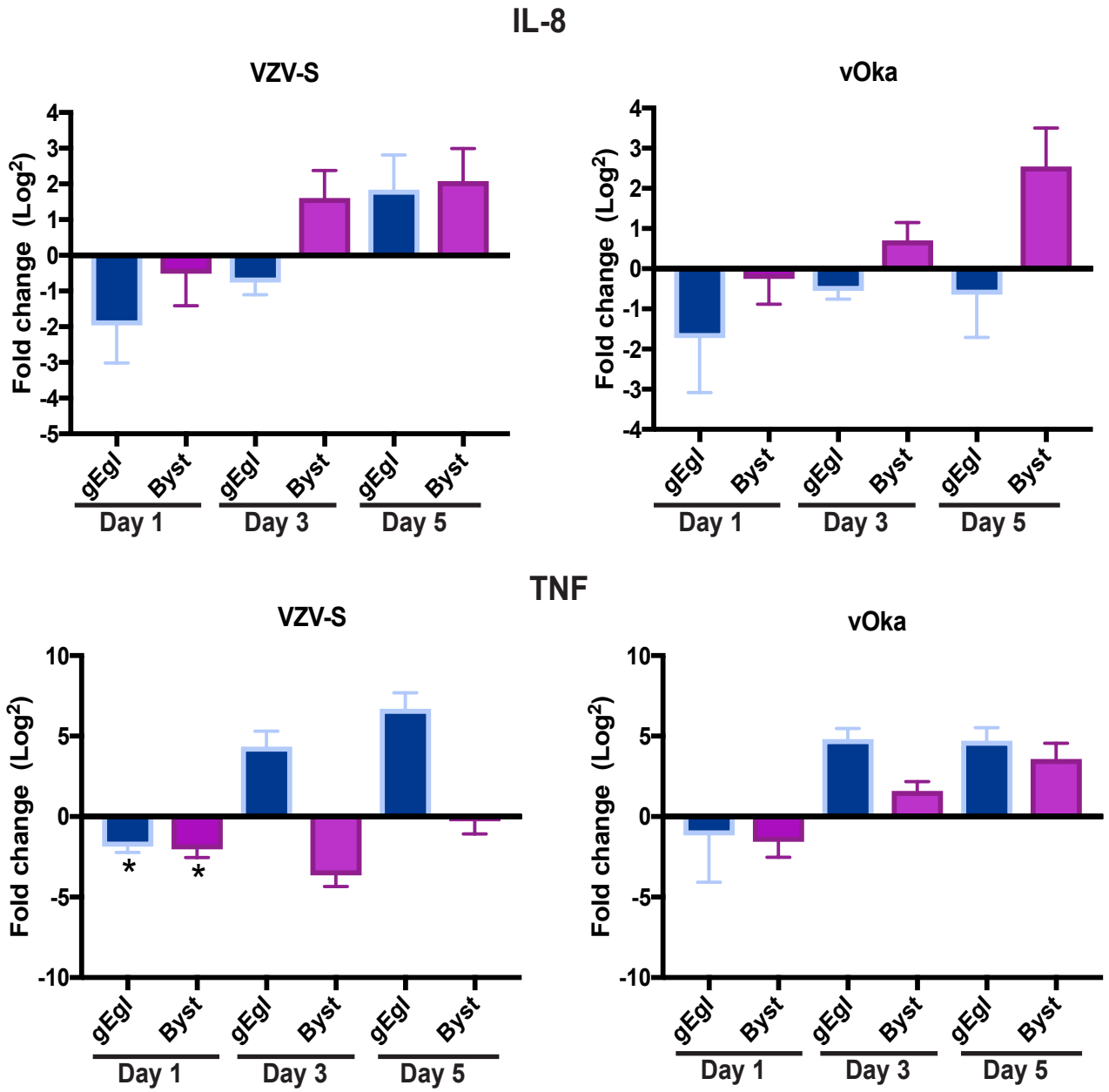


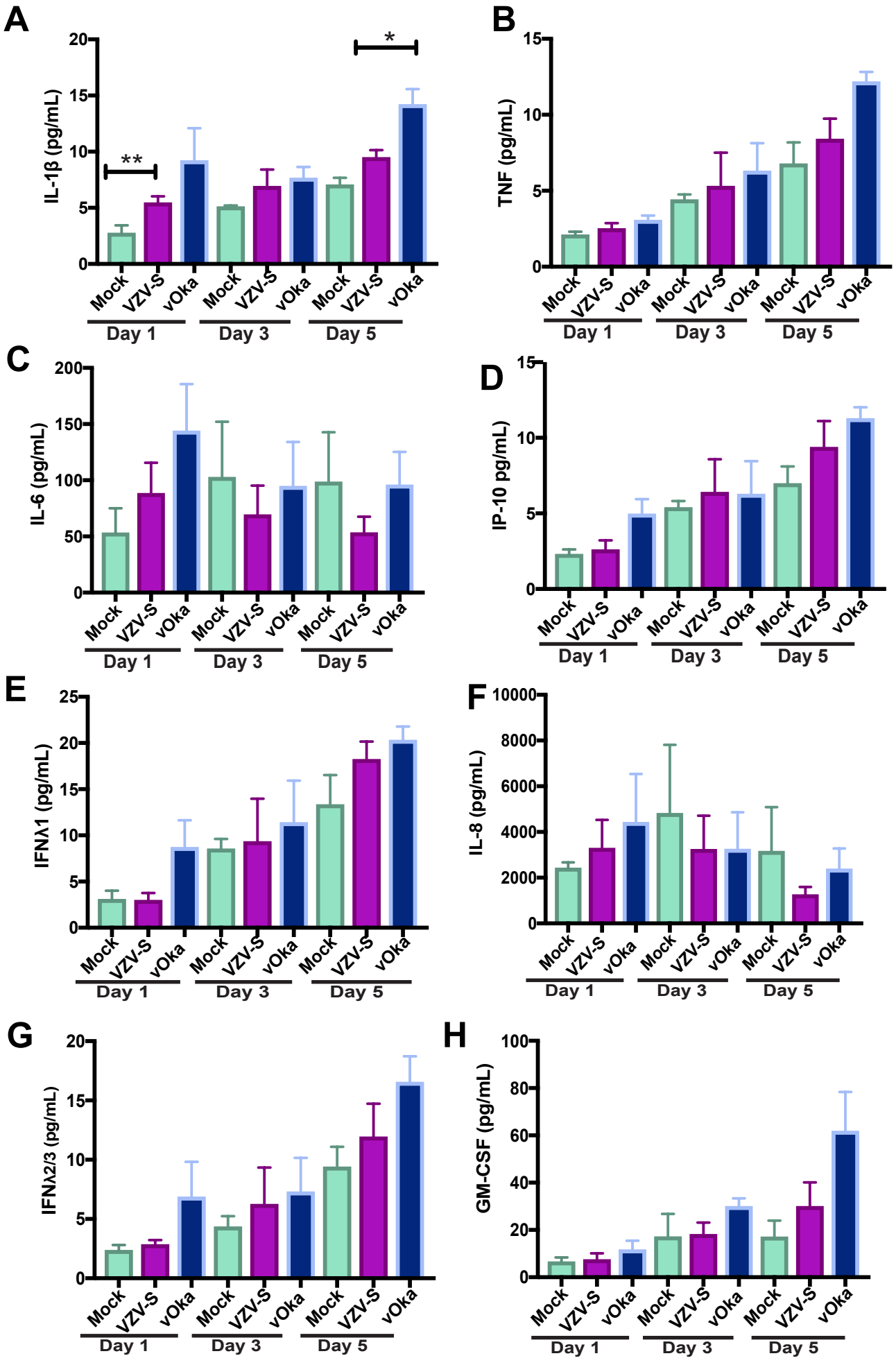
Figure 4.8 qRT-PCR fold change ininflammatory cytokine transcripts in VZV-S and vOka exposed HaCaTs.

HaCaT cells were either mock, VZV-S or vOka infected in a cell associated manner at a 1:3 ratio. On days 1, 3 and 5 post VZV exposure, cells were MACS bead separated based on gEgI positivity. Total cellular RNA was extracted from mock, bystander ($gEgI^-$) and virus infected ($gEgI^+$) cells at days 1, 3 and 5 post exposure and mRNA expression of human ASC and IL-1β was measured using quantitative qRT-PCR. Expression levels of ASC and IL-1β were normalized to the house keeping gene 18S RNA. VZV-S and vOka bystander and virus infected cell transcripts are depicted as fold change relative to mock. Statistics were generated using a one-way ANOVA test, and Tukey's multiple comparison post hoc test for each time point. Error bars represent the SEM for 3 independent experiments (n=3).

VZV-S and vOka modulation of inflammatory cytokines/chemokines in HaCaTs

Whilst transcripts were not significantly modulated with VZV infection alone, it was possible that infection could modulate the production and release of inflammatory cytokines/ chemokines. A variety of cytokines and chemokines have been shown to play a role in the development of neuropathic pain in the context of the skin microenvironment (Clark et al., 2013). Thus, to determine whether VZV infection could affect the cytokine and chemokine secretion from keratinocytes, supernatant was collected from mock, VZV-S or vOka infected HaCaTs at days 1,3 and 5 post VZV exposure and was analyzed for 13 analytes with the human antiviral LegendPlex assay. Analytes included IL-1β, TNF, IL-6, interferon gamma induced protein 10 (IP-10), interferon (IFN) λ1, IL-8, IFNλ2/3, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ (Figure 4.9A-I). IL-12 p70, IL-10, IFNα2 and IFNβ. IFNα2, IFNβ, IL-10 and IL-12 were also all tested, however were below the limit of detection at all time points in mock, VZV-S and vOka (data not shown). It is important to note that each day was analyzed separately, and therefore results were not compared between days.

At day 1, 3 and 5 post exposure VZV-S and vOka had a higher concentration of IL-1β, with VZV-S on day 1 and vOka on day 5 being statistically significant. For all other analytes there were no statistically significant differences in concentration between mock, VZV-S and vOka, however some interesting trends were identified. This is best visualized in the heat map presented in Figure 4.9J which displays cytokine/chemokine expression as fold change in relation to mock cells. It was seen that at all time-points both VZV-S and vOka increased the expression of IL-1β, TNF, IP-10, IFNλ1, IFNλ2/3, GM-CSF and IFN-γ. IL-6 and IL-8 were increased at day 1 post VZV-S and vOka exposure and were subsequently decreased at days 3 and 5 post exposure. Together this data suggests that VZV infection and/or exposure to VZV infected cells, modulates the secretion of cytokines and chemokines in HaCaT cells.


A
IL-1β (pg/mL)
20
15
10
5
0
**
*
Mock
VZV-S
vOka
Day 1
Day 3
Day 5
B
TNF (pg/mL)
15
10
5
0
C
IL-6 (pg/mL)
200
150
100
50
0
D
IP-10 pg/mL)
E
IFNλ1 (pg/mL)
25
F
IL-8 (pg/mL)
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G
IFNλ2/3 (pg/mL)
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GM-CSF (pg/mL)
100
80
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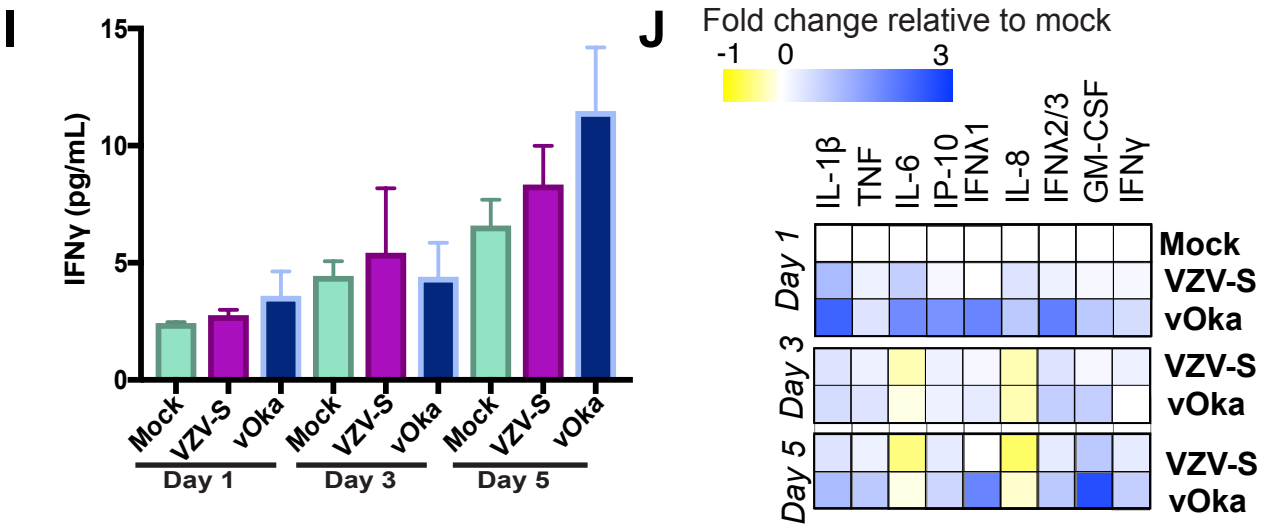


Figure 4.9 Analysis of mock, VZV-S and vOka infected HaCaT supernatant using the LegendPlex human anti-viral multiplex bead array.
Supernatants from days 1, 3 and 5 post VZV-S, vOka or mock exposed HaCaTs were collected and analysed via the LegendPlex human anti-viral response panel. Samples were run in duplicate and beads were run on the LSR Fortessa for analysis as per manufacturer's instructions. LegendPlex software was used to generate standard curves and calculate concentrations of each analyte per sample. Analytes displayed include IL-1β (A), TNF (B), IL-6 (C), IP-10 (D), IFNλ1 (E), IL-8 (F), IFNλ2/3 (G), GM-CSF (H), IFN-γ (I). Bar graphs are representative of 3 biological replicates and error bars indicate the SEM (A-I). Statistical significance was determined using a one-way ANOVA test, and Tukey's multiple comparison post hoc test for each time point * $P<0.05$ ** $P<0.01$. Heat map displays fold change relative to mock, averaged over 3 replicates (J).

Assessment of different characteristics of VZV clinical PHN and non PHN isolates

From previous data it appears that VZV infection of HaCaTs modifies inflammatory transcripts and some inflammatory cytokine secretion, which could be relevant for PHN development. However, it is unclear whether strain specific differences in VZV could contribute to the development of PHN. To address this gap in knowledge, clinical isolates from patients with or without PHN were kindly gifted from Professor Judith Breuer, UCL, UK. These isolates were analyzed based on their growth, cytopathic effect (CPE) induction, cell death induction and the secretion of inflammatory cytokines in infected cell cultures. It is critical to assess the growth and CPE induction of these viruses as any differences could contribute to the isolates ability to induce PHN. As previously mentioned, cell death induction by VZV plays a critical role in VZV pathogenesis (Zerboni et al., 2014) and may contribute to severity of symptoms and PHN.

Analysis of SUK57 and SUK66 CPE induction, virus spread and cell death induction

To determine whether there were any differences in the ability of SUK57 and SUK66 to induce CPE or spread in culture, CTV labelled mock, SUK66 (PHN) or SUK57 (non PHN) infected ARPE-19s were used to infect a fresh layer of ARPE-19s at a 1:10 ratio. At days 1, 3 and 5 post exposure VZV infected cells were imaged using light microscopy, supernatant was collected, and cells were stained and analyzed by flow cytometry. Light microscopy was utilized to determine whether plaque formation was comparable between the clinical isolates over the 5-day time-course (Figure 4.10). Multiple fields of view were examined, and images displayed are representative of 3 biological replicates. SUK57 and SUK66 caused similar CPE in regard to their ability to establish plaques in ARPE-19s at the time points examined.

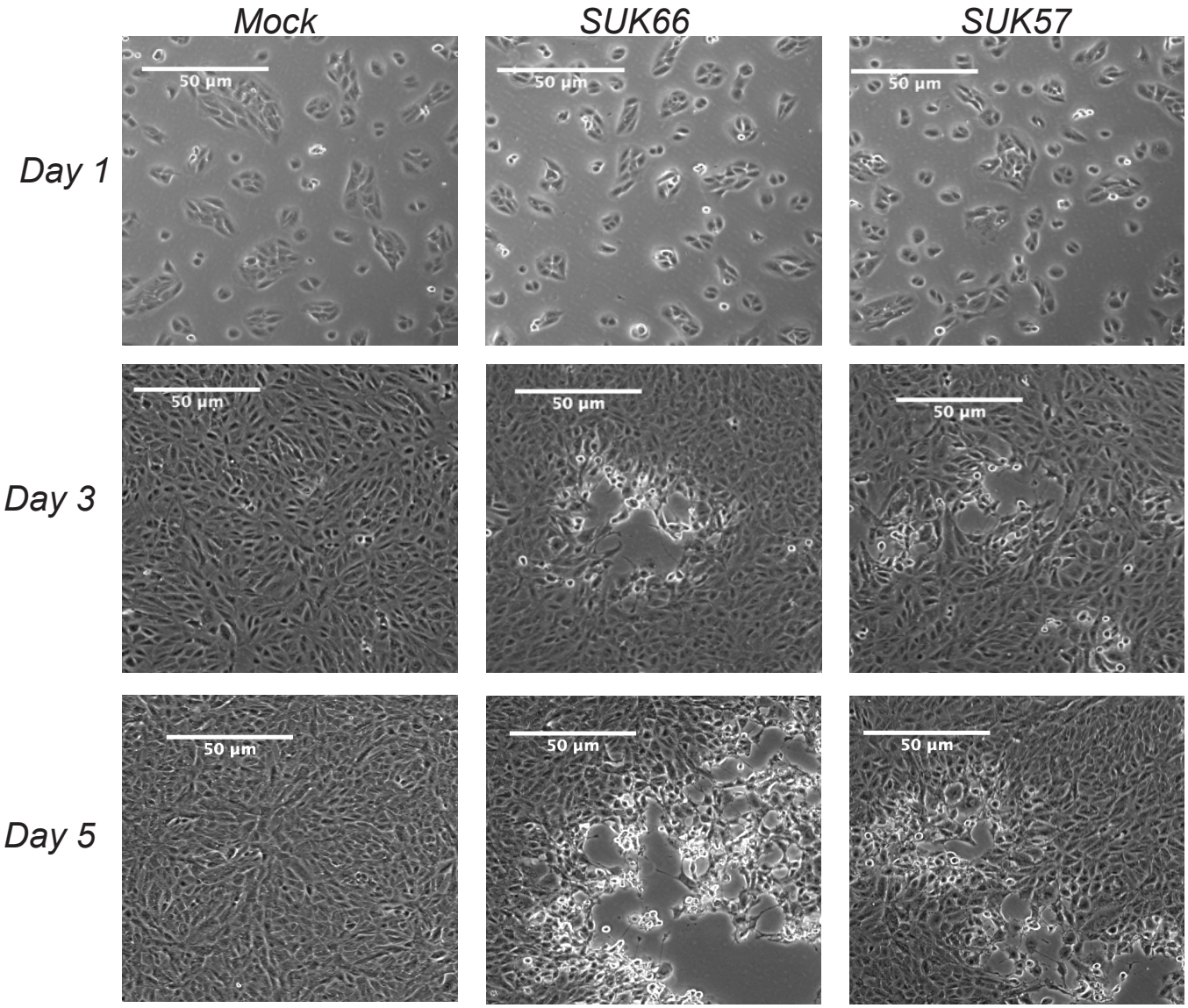


Figure 4.10 Light microscopy analysis of mock, VZV clinical isolates SUK66 and SUK57 infected ARPE-19s at days 1, 3 and 5 post exposure.
ARPE-19s were infected with mock, VZV clinical isolate SUK66 or SUK57 infected ARPE-19s at a 1:10 ratio. On days 1, 3 and 5 post exposure cells were imaged via light microscopy. Images are representative of 3 biological replicates and are at 10x magnification.

To quantitatively measure VZV spread, gEgI was measured using flow cytometry in CTV- cells to ensure the inoculum was not included in the analysis. The gating strategy for flow cytometry experiments is shown in figure 4.11. Figure 4.12A indicates representative flow plots for day 3 post inoculation with mock, SUK66 or SUK57 inoculum. At this timepoint it is clear there were no differences between the viral isolates in regard to the percentage of gEgI positive, CTV- cells. The percentage of gEgI positive cells in the inoculum was also measured to account for differences which could affect viral growth as is depicted in Figure 4.12B. As indicated, there was some variation in infection levels in SUK66 and SUK57 inoculum across the three replicates. Despite differences in gEgI positivity of inoculum, there weren't any significant differences identified in the percentage of gEgI positive cells at any day post exposure, indicating that SUK66 and SUK57 spread similarly in ARPE-19s (Figure 4.12C). This was observed in 3 biological replicates with individual growth curves being displayed for SUK66 and SUK57 in figure 4.12D and 4.12E respectively.

The amount of cell death induced by VZV isolates was also analyzed using CC3 and zombie live dead staining as previously described (Gerada et al., 2018). At all-time points examined, minimal cell death induction was observed in SUK66 and SUK57 infected ARPE-19s which were comparable to mock infected ARPE-19s. This is shown in the representative flow cytometry plots of 3 biological replicates seen in figure 4.13. Together this indicates in ARPE-19s SUK66 and SUK57 virus isolates spread similarly and don't induce significant levels of cell death at the time-points examined in this study.

As described previously, keratinocytes are an interesting cell type to examine in regard to VZV induction of PHN. Therefore, SUK66 and SUK57 infected HaCaT inoculum was generated to determine whether these viral isolates spread differently and induced cell death in HaCaTs. CTV labelled mock, SUK66 or SUK57 infected HaCaTs were used to infect uninfected HaCaTs at a 1:5 ratio. At days 1, 3 and 5 post exposure infected cells were imaged using light microscopy, supernatant was collected, and cells were stained and analyzed by flow cytometry. As identifiable by the light microscopy images taken SUK66 and SUK57 didn't establish plaques as effectively in HaCaTs in comparison to the ARPE-19s and produced minimal CPE (Figure 4.14).

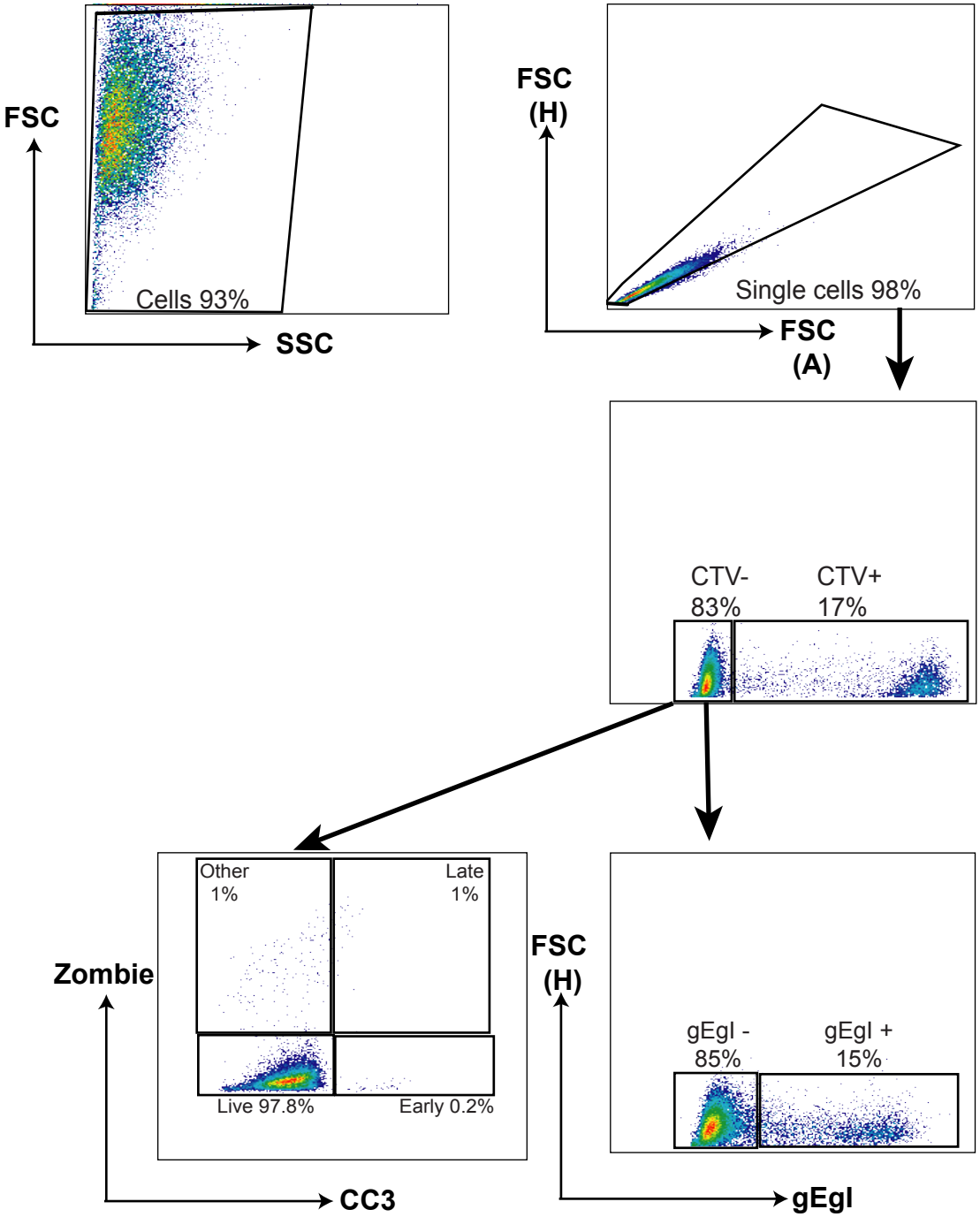


Figure 4.11 Gating strategy for clinical isolate assessment by flow cytometry. Cells were gated on forward scatter (FSC) and side scatter (SSC) and single ARPE-19 cells were identified by forwards scatter area (FSC-A) and forward scatter height (FSC-H). Single cells were then gated on cell trace violet (CTV) and FSC-H to identify CTV^+ and CTV^- cells, CTV^+ cells were excluded from further analysis. CTV^- cells were gated on gEgI and FSC-H to identify $gEgI^+$ cells and $gEgI^-$ cells. CTV^- cells were also gated on CC3 and zombie NIR to identify live ($CC3^-$ $Zombie^-$) early apoptotic ($CC3^+$ $zombie^-$), late apoptotic ($CC3^+$ $zombie^-$) and other cell death ($CC3^-$ $zombie^+$).

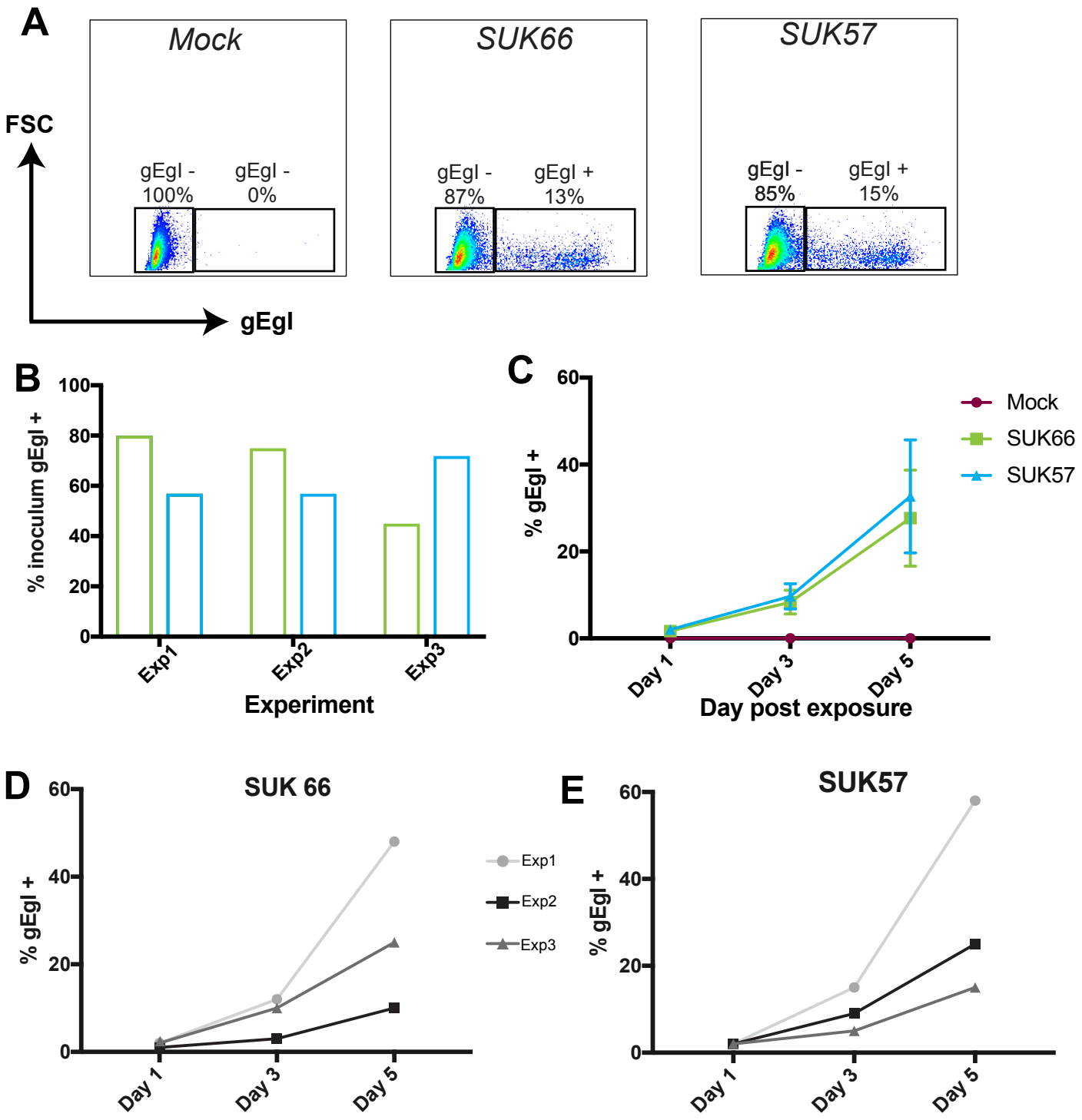


Figure 4.12 Flow cytometry assessment of gEgI kinetics in mock, SUK66 and SUK57 infected ARPE-19s.

ARPE-19s were infected with mock, VZV clinical isolate SUK66 or SUK57 infected ARPE-19s at a 1:10 ratio. Inoculum was CTV labelled for exclusion in subsequent analysis. At days 1, 3 and 5 post exposure cells were stained for gEgI and analysed via flow cytometry. Representative flow cytometry plots are indicative of 3 biological replicates at day 3 post exposure (A). Inoculum was stained for gEgI to determine the percentage of infected cells (B). Line graph error bars represent the SEM (C). Line graphs for individual replicates are displayed for SUK66 (D) and SUK57 (E).

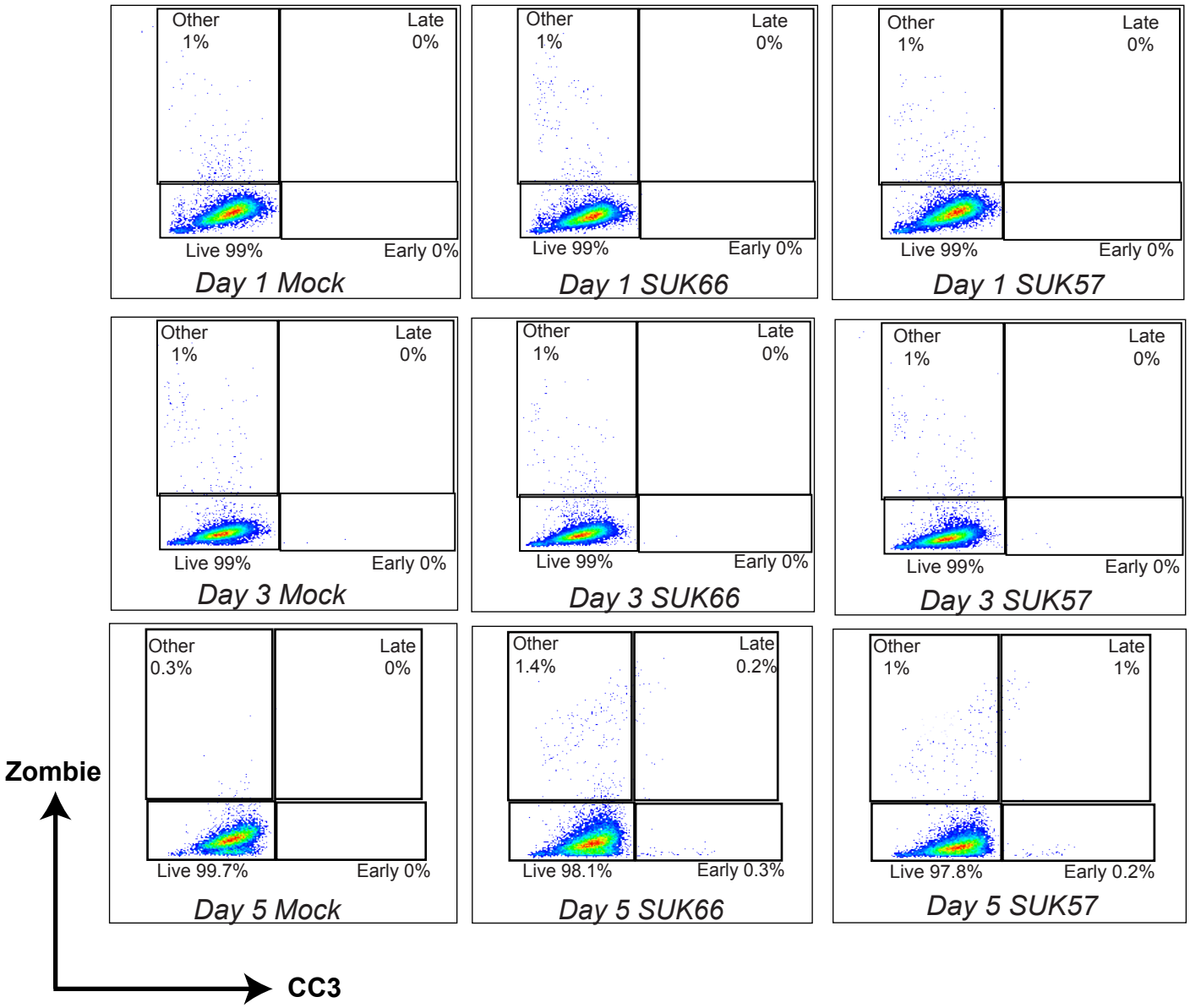


Figure 4.13 Flow cytometry assessment of cell death induction in mock, SUK66 or SUK57 exposed ARPE-19s at days 1, 3 and 5 post exposure.
ARPE-19s were infected with mock, VZV clinical isolate SUK66 or SUK57 infected ARPE-19s at a 1:10 ratio. Inoculum was CTV labelled for exclusion in subsequent analysis. At days 1, 3 and 5 post exposure cells were stained for cleaved caspase 3 (CC3) and zombie NIR, and was analysed via flow cytometry. Representative flow cytometry plots are displayed for 3 biological replicates.

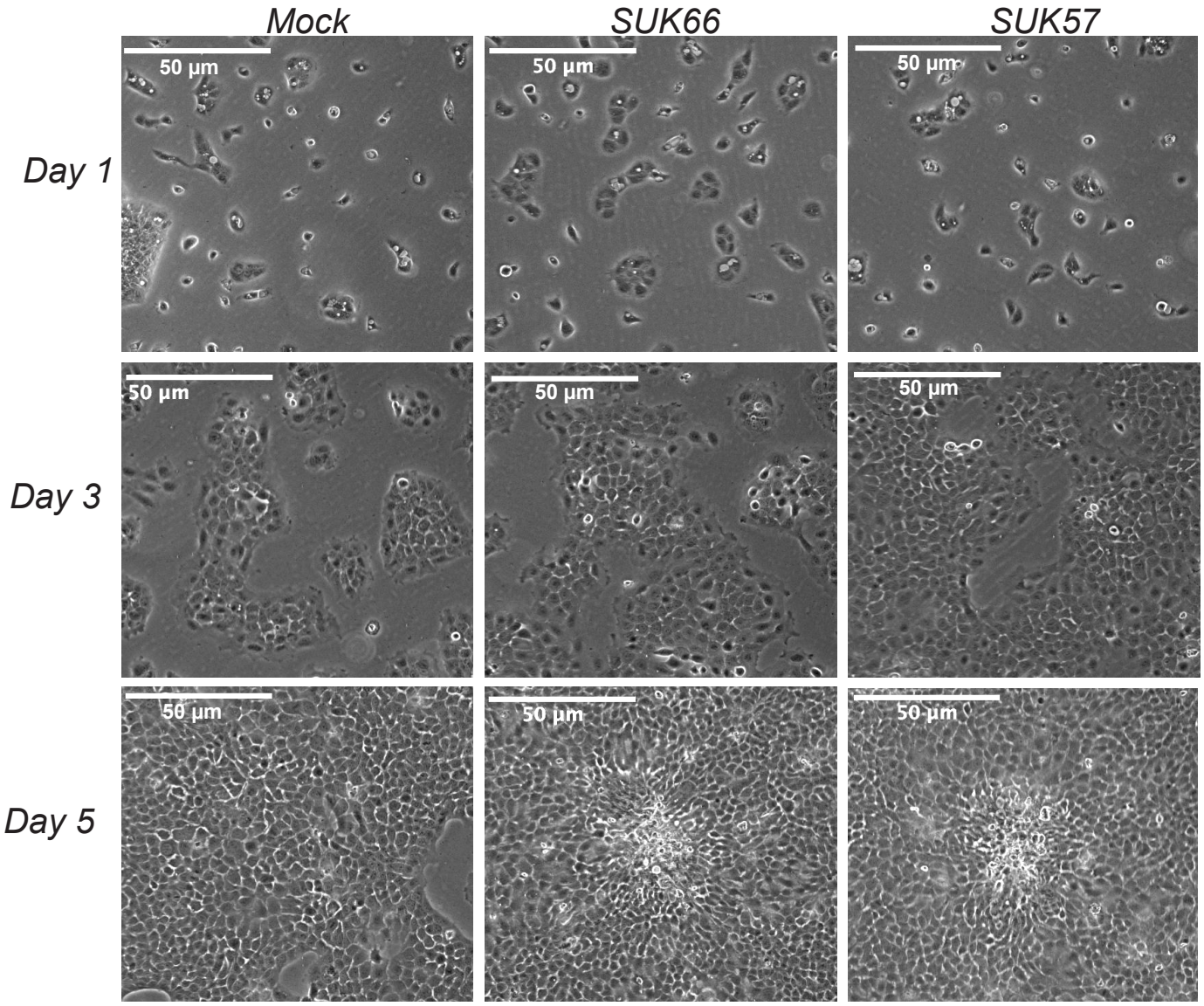


Figure 4.14 Light microscopy analysis of mock, SUK66 and SUK57 infected HaCaTs at days 1, 3 and 5 post exposure.
HaCaTs were infected with mock, VZV clinical isolate SUK66 or SUK57 infected HaCaTs at a 1:5 ratio. On days 1, 3 and 5 post exposure cells were imaged via light microscopy. Images are representative of 2 biological replicates and are at 10x magnification.

The observation of limited virus infection was also observed in gEgI quantification by flow cytometry, as only a small percentage of gEgI+ cells was achieved in the HaCaTs at all time points tested with both isolates (Figure 4.15A-E). This is despite 20-40% of the virus inoculum being gEgI positive (Figure 4.15B). At all time points examined, minimal cell death induction was observed in SUK66 and SUK57 exposed HaCaTs which were comparable to mock exposed HaCaTs (Figure 4.16). Due to the difficulty in achieving reasonable infection levels in the HaCaTs, despite optimizing inoculum ratios and serum concentrations in the media, they were mitigated from subsequent analysis.

Analysis of SUK29 and Z226 CPE induction, spread and cell death induction

To further examine whether PHN and non PHN isolates could have different CPE induction and spread in culture, SUK29 (non PHN) and Z226 (PHN) were examined in ARPE-19s. CTV labelled mock, SUK29 or Z226 infected ARPE-19s were used to infect a fresh layer of ARPE-19s at a 1:10 ratio. At days 1, 3 and 5 post exposure infected cells were imaged using light microscopy, supernatant was collected, and cells were stained and analyzed by flow cytometry. Light microscopy of SUK29 and Z226 infected cells in comparison to mock cells indicates that both viruses caused similar CPE to the other isolates examined (Figure 4.17). These images are representative of three biological replicates.

The spread of SUK29 and Z226 was assessed via flow cytometry measurement of gEgI+ cells. Figure 4.18A indicates representative flow plots for day 3 post inoculation with mock, SUK66 or SUK57 inoculum. At this timepoint there was no differences between the viral isolates in regard to gEgI positivity of CTV- cells. gEgI positivity of the inoculum was measured to account for differences which could affect viral growth (Figure 4.18B). As indicated, there was some variation in infection levels in SUK29 and Z226 inoculum across the three replicates. Despite differences in inoculum gEgI positivity, there weren't any significance differences identified in gEgI positivity at any day post exposure, indicating that SUK29 and Z226 spread similarly in ARPE-19s (Figure 4.18C). This was observed in 3 biological replicates with individual growth curves being displayed for SUK66 and SUK57 in figure 4.18D and 4.18E respectively.

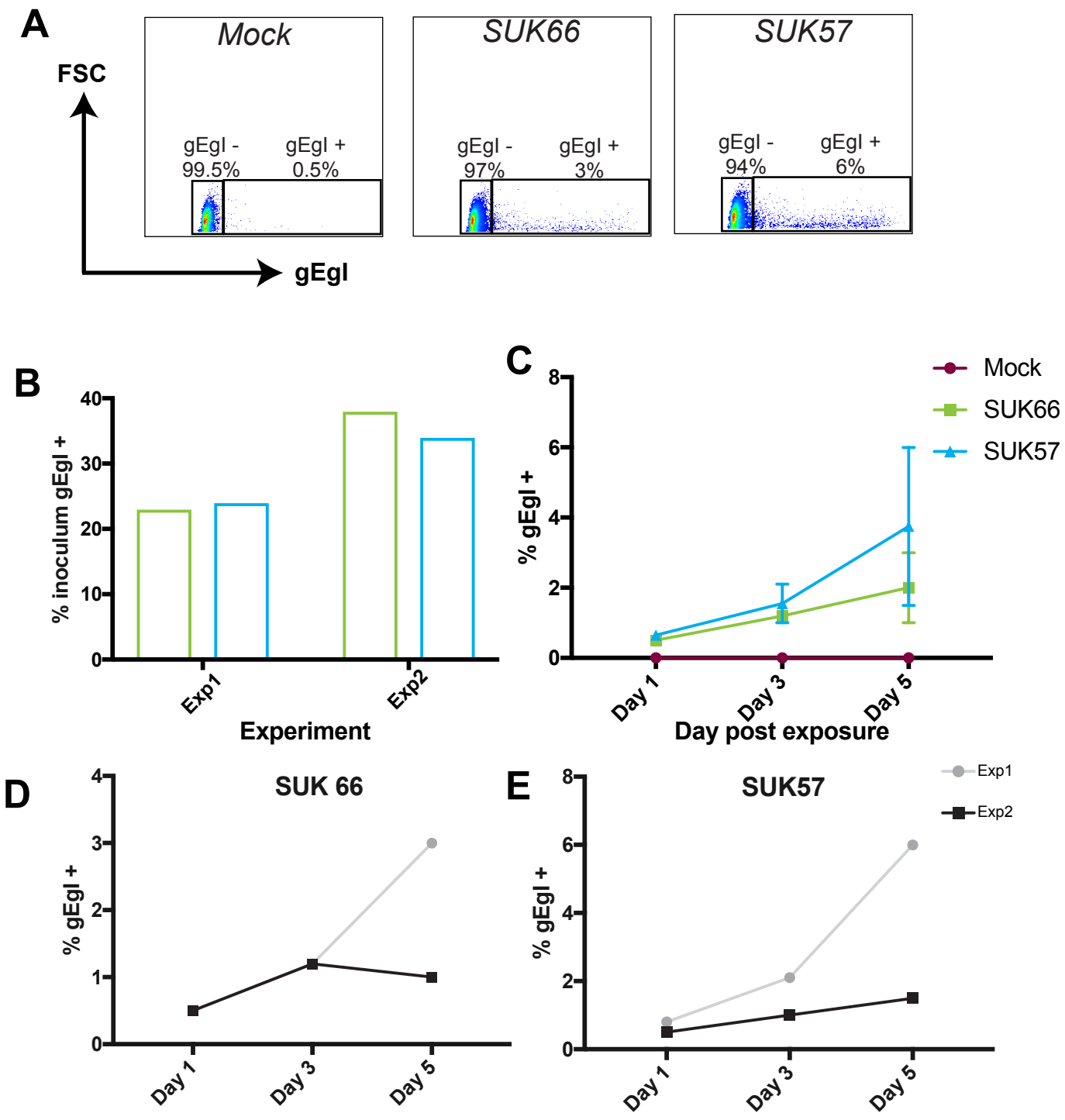


Figure 4.15 Flow cytometry assessment of gEgI kinetics in mock, SUK66 and SUK57 infected HaCaTs.

HaCaTs were infected with mock, VZV clinical isolate SUK66 or SUK57 infected HaCaTs at a 1:10 ratio. Inoculum was CTV labelled for exclusion in subsequent analysis. At days 1, 3 and 5 post exposure cells were stained for gEgI and analysed via flow cytometry. Representative flow cytometry plots are indicative of 2 biological replicates at day 3 post exposure (A). Inoculum was stained for gEgI to determine the percentage of infected cells (B). Line graph error bars represent the SEM (C). Line graphs for individual replicates are displayed for SUK66 (D) and SUK57 (E).

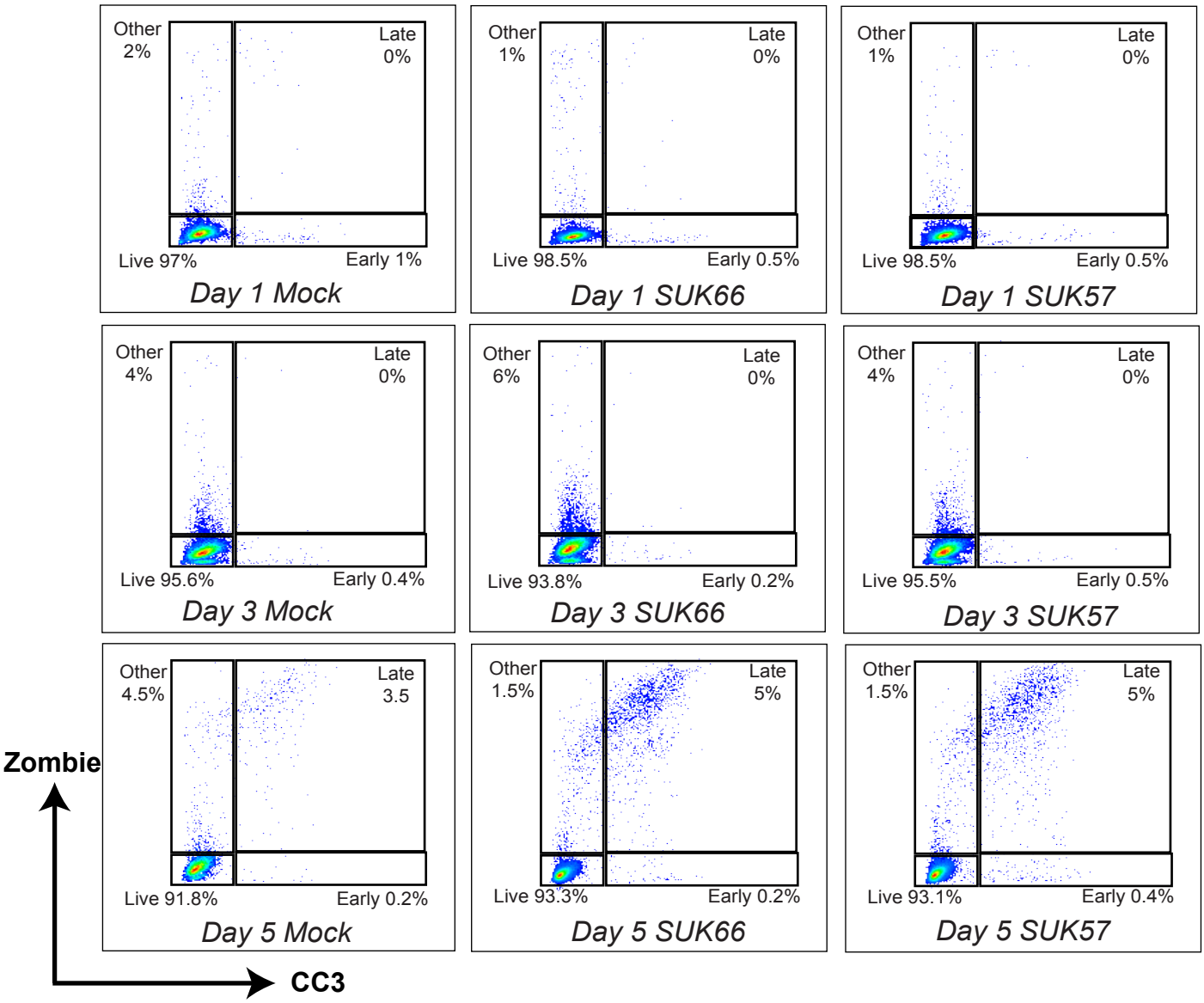


Figure 4.16 Flow cytometry assessment of cell death induction in mock, SUK66 or SUK57 exposed HaCaTs at days 1, 3 and 5 post exposure.
HaCaTs were infected with mock, VZV clinical isolate SUK66 or SUK57 infected ARPE-19s at a 1:10 ratio. Inoculum was CTV labelled for exclusion in subsequent analysis. At days 1, 3 and 5 post exposure cells were stained for cleaved caspase 3 (CC3), zombie NIR and analysed via flow cytometry. Representative flow cytometry plots are displayed for 3 biological replicates.

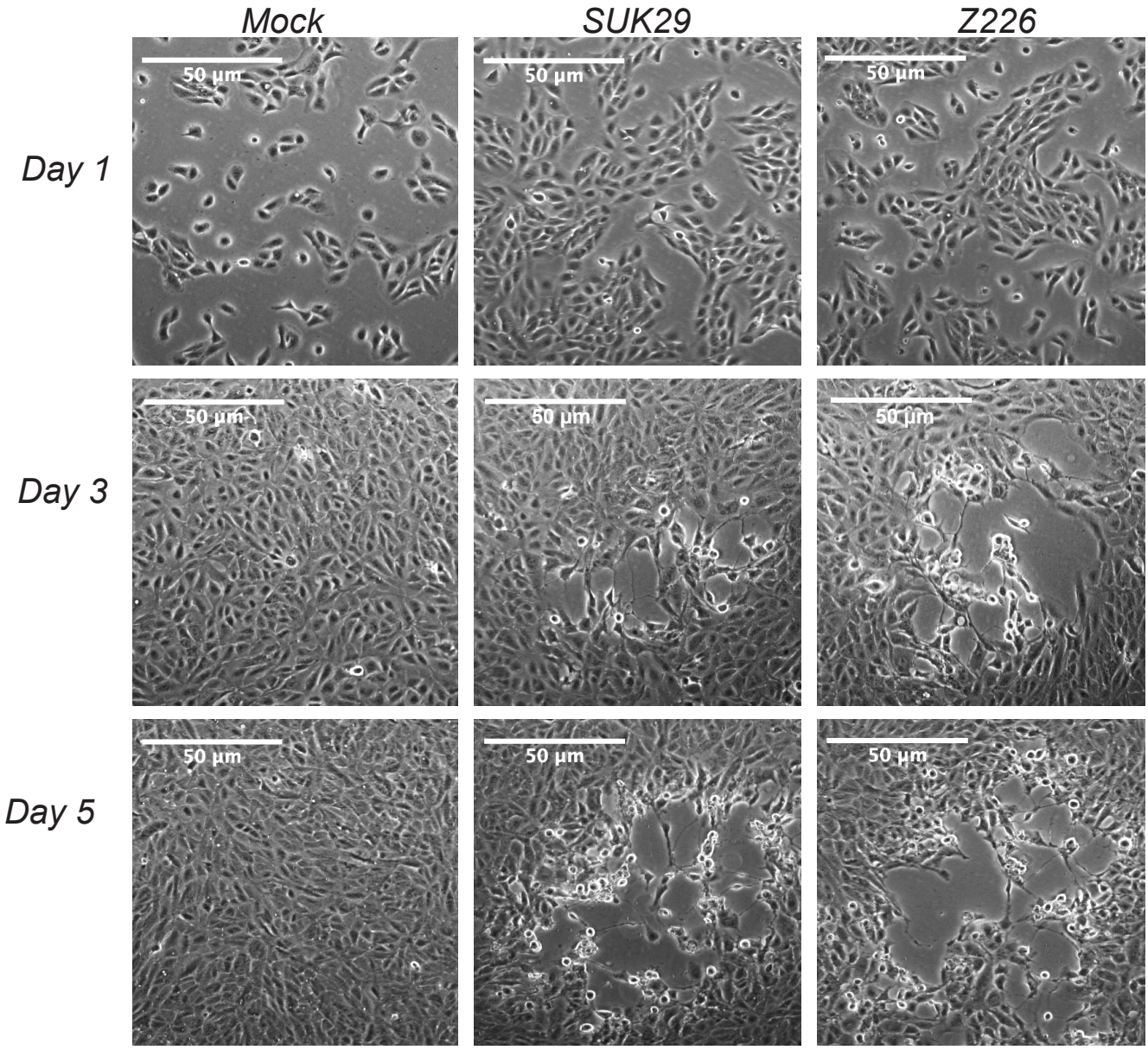


Figure 4.17 Light microscopy analysis of mock, SUK29 and Z226 infected ARPE-19s at days 1,3 and 5 post exposure.

ARPE-19s were infected with mock, VZV clinical isolate SUK29 or Z226 infected HaCaTs at a 1:5 ratio. On days 1, 3 and 5 post exposure cells were imaged via light microscopy. Images are representative of 3 biological replicates and are at 10x magnification.

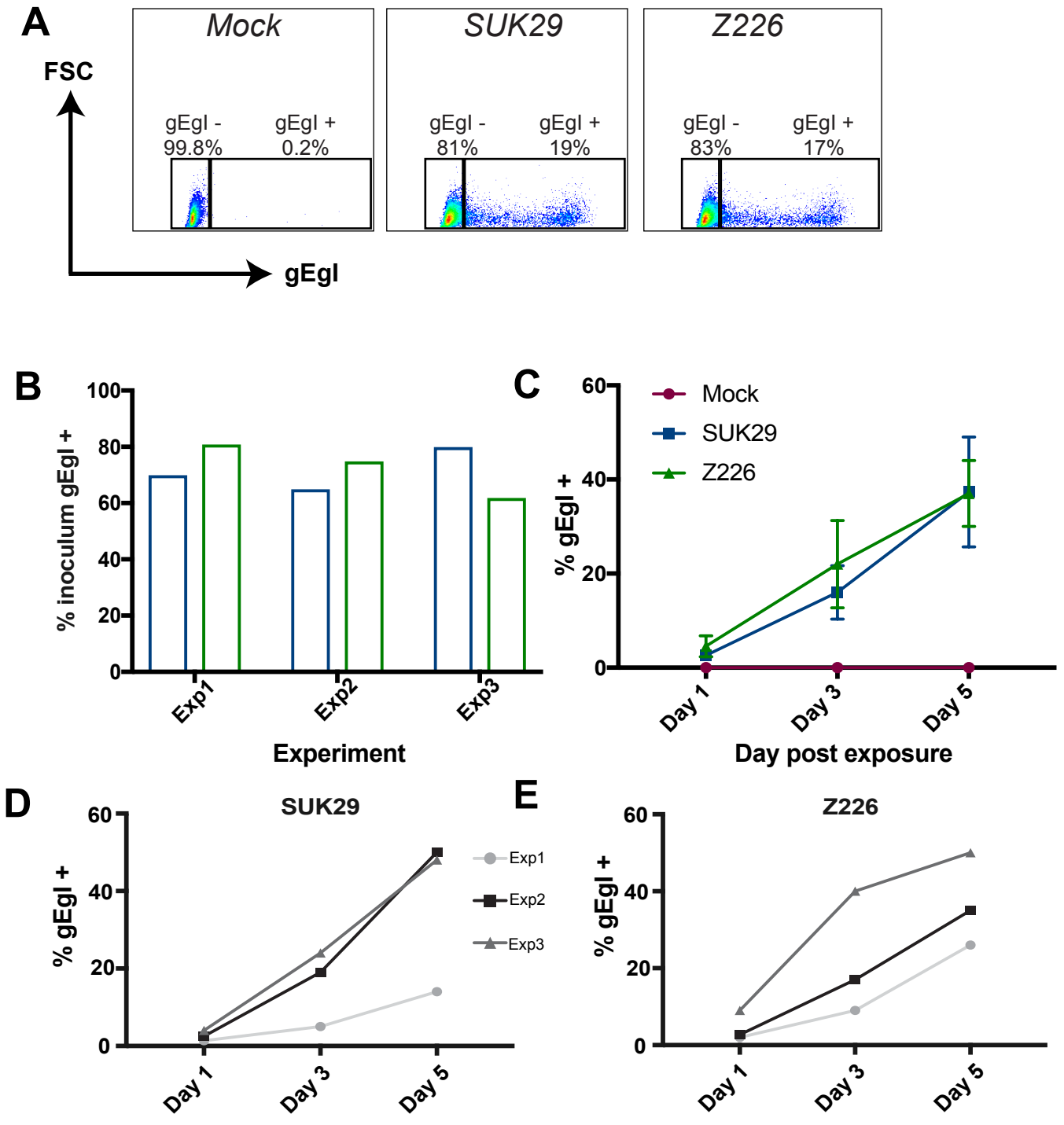


Figure 4.18 Flow cytometry assessment of gEgI growth kinetics in mock, SUK29 and Z226 infected ARPE-19s.

ARPE-19s were infected with mock, VZV clinical isolate SUK29 or Z226 infected ARPE-19s at a 1:10 ratio. Inoculum was CTV labelled for exclusion in subsequent analysis. At days 1, 3 and 5 post exposure cells were stained for gEgI and analysed via flow cytometry. Representative flow cytometry plots are indicative of 3 biological replicates at day 3 post exposure (A). Inoculum was stained for gEgI to determine the percentage of infected cells (B). Line graph error bars represent the SEM (C). Line graphs for individual replicates are displayed for SUK29 (D) and Z226 (E).

Cell death was also assed in mock, SUK29 and Z226 exposed ARPE-19 cultures (Figure 4.19). As can be seen in representative flow plots, at day 5 post exposure both SUK29 and Z226 began to induce more other cell death than mock infected cells (Figure 4.19A). The percentage of live cells was collated for each time point across 3 biological replicates (Figure 4.19B). At days 1 and 3 post exposure SUK29 or Z226 didn't induce cell death and was comparable to mock. However, at day 5 post exposure both viruses seemed to induce higher levels of other cell death, with Z226 induction of cell death approaching significance in comparison to mock cells. Through the linear regression plot it clear that cell death induction is related to percentage of gEgI positive cells (Figure 4.19C). Together this indicates that the clinical isolates did not differ in their ability to induce CPE, spread and induce cell death in ARPE-19s. Furthermore, this indicates that in the context of the skin, PHN and non PHN isolates may not have different pathogenic features.

Analysis of chemokine, cytokine and growth factor concentrations in clinical isolate and vOka HaCaT supernatant

Whilst VZV PHN and non PHN isolates did not appear to differentially spread and cause CPE in ARPE-19s, it is conceivable that their ability to cause inflammation in the context of the skin may differ. Previous work in this chapter identified that VZV could modulate the HaCaT secretory profile. Therefore, we wanted to determine whether the ability of VZV to modulate secretion of inflammation related substances was consistent across VZV clinical isolates. As explained previously, there was difficulty in achieving high infection levels in HaCaT cells using the clinical isolates, therefore ARPE-19s were utilized to assess the ability of VZV clinical isolates to modulate cytokine and chemokine secretion.

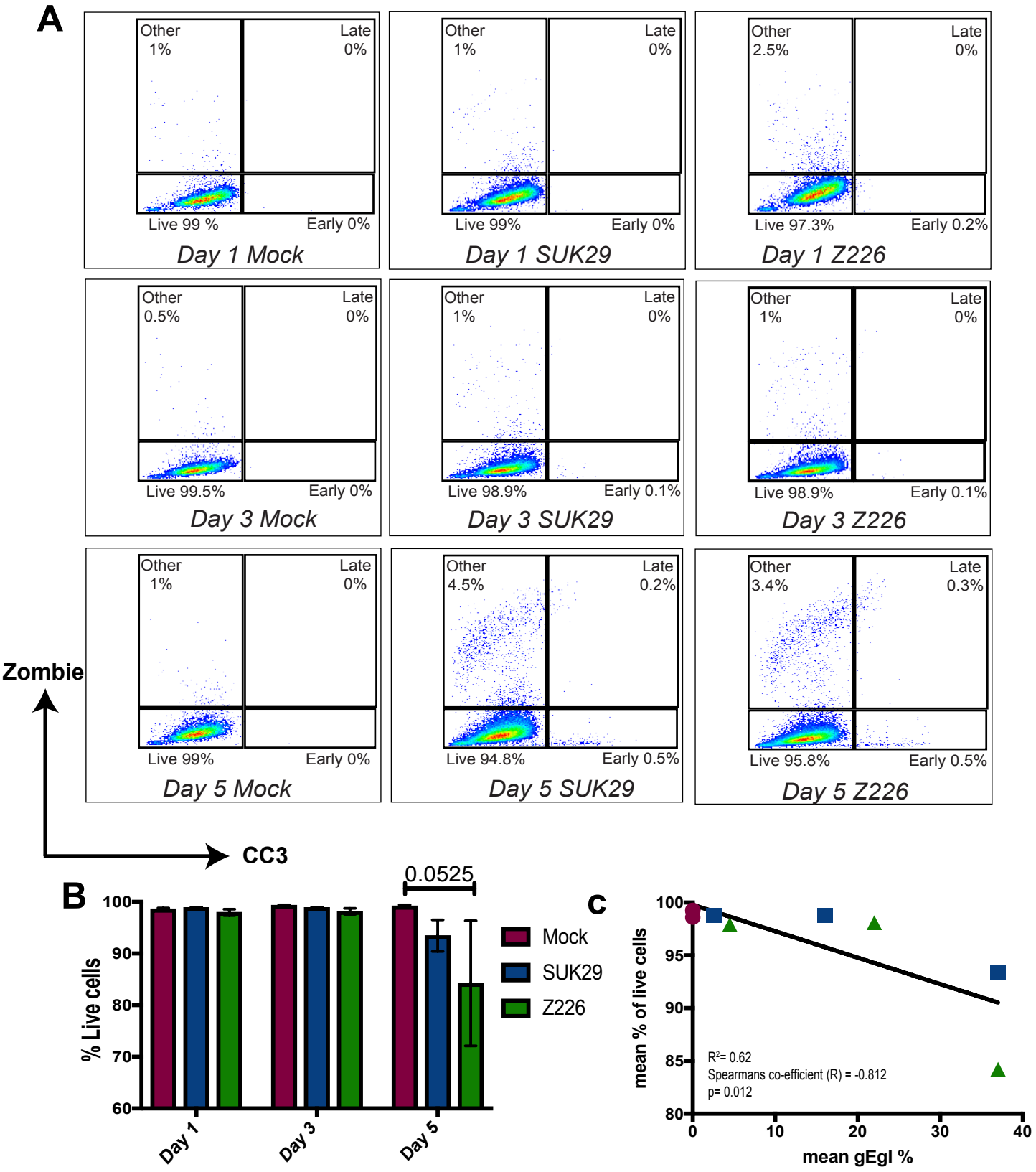


Figure 4.19 Flow cytometry assessment of cell death induction in mock, SUK29 or Z226 exposed ARPE-19s at days 1, 3 and 5 post exposure.
ARPE-19s were infected with mock, VZV clinical isolate SUK29 or Z226 infected ARPE-19s at a 1:10 ratio. Inoculum was CTV labelled for exclusion in subsequent analysis. At days 1, 3 and 5 post exposure cells were stained for cleaved caspase 3 (CC3), zombie NIR and analysed via flow cytometry. Representative flow cytometry plots are displayed for 3 biological replicates (A). Bar graphs are representative of 3 biological replicates and error bars indicate the SEM (B). Statistical significance was determined using a two-way ANOVA test, and Tukey's multiple comparison post hoc test.

Previous literature has shown that VZV can modulate CXCL8 and CCL26 chemokine expression in ARPE-19s (Graybill et al., 2017), therefore we wanted to expand the number of analytes in our analysis of VZV infected supernatants and utilized the BioPlex pro human cytokine 27 plex assay. To compare this to VZV modulation of HaCaT secretory profile mock and vOka infected HaCaT supernatants were also analysed using the BioPlex pro assay. Analytes examined and the ranges of concentrations detected in both HaCaTs and ARPE-19s is displayed in Table 4.9. IL-5, IL-2, IL-1β, IL-13, IL-4, MIP1α, IL-10, G-CSF, IL-12p70 and IL-17 were either not detected or detected below 5 pg/mL in both the HaCaTs and ARPE-19 supernatant and as such were excluded from subsequent analysis (Table 4.9). GM-CSF, TNF and RANTES were only secreted by HaCaT cells with vOka infection decreasing the expression of RANTES and TNF and increasing GM-CSF expression, however the changes observed were not statistically significant (Figure 4.22). Eotaxin was only secreted by ARPE-19s, however the concentration was small ranging from 0.13-12 pg/mL (Figure 4.21). None of the VZV clinical isolates tested regulated the secretion of Eotaxin from the ARPE-19s. MIP1β was secreted at low levels in ARPE-19s alone and infection with any VZV isolate did not significantly alter MIP1β secretion at the time points tested (Figure 4.21). IL-6, IFNγ, IL1ra, IL-8, IL-15, IL-7, IL-9 IP-10, MCP-1, Basic FGF, VEGF and PDGF-BB were secreted by both HaCaTs and ARPE-19s.

Interesting trends were observed in the ability of VZV to modulate cytokine secretion in both the ARPE-19s and HaCaTs. In the ARPE-19s, all VZV clinical isolates downregulated IL-6 at day 5 post VZV exposure (Figure 4.20). This was statistically significant for SUK66 in comparison to mock at day 5 post exposure. Interestingly, vOka infection in HaCaTs did not decrease the secretion of IL-6 (Figure 4.22). All isolates resulted in increased secretion of IL1-RA at day 5 post exposure in ARPE-19s, with this increase being significant for SUK57 infected ARPE-19s in comparison to mock (Figure 4.20). SUK57 also significantly increased IL1-RA in comparison to mock at day 1 post exposure. vOka infected HaCaTs had increased secretion of IL1-RA at days 1, 3 and 5 post infection, with the increase at day 5 trending towards statistical significance (Figure 4.22).

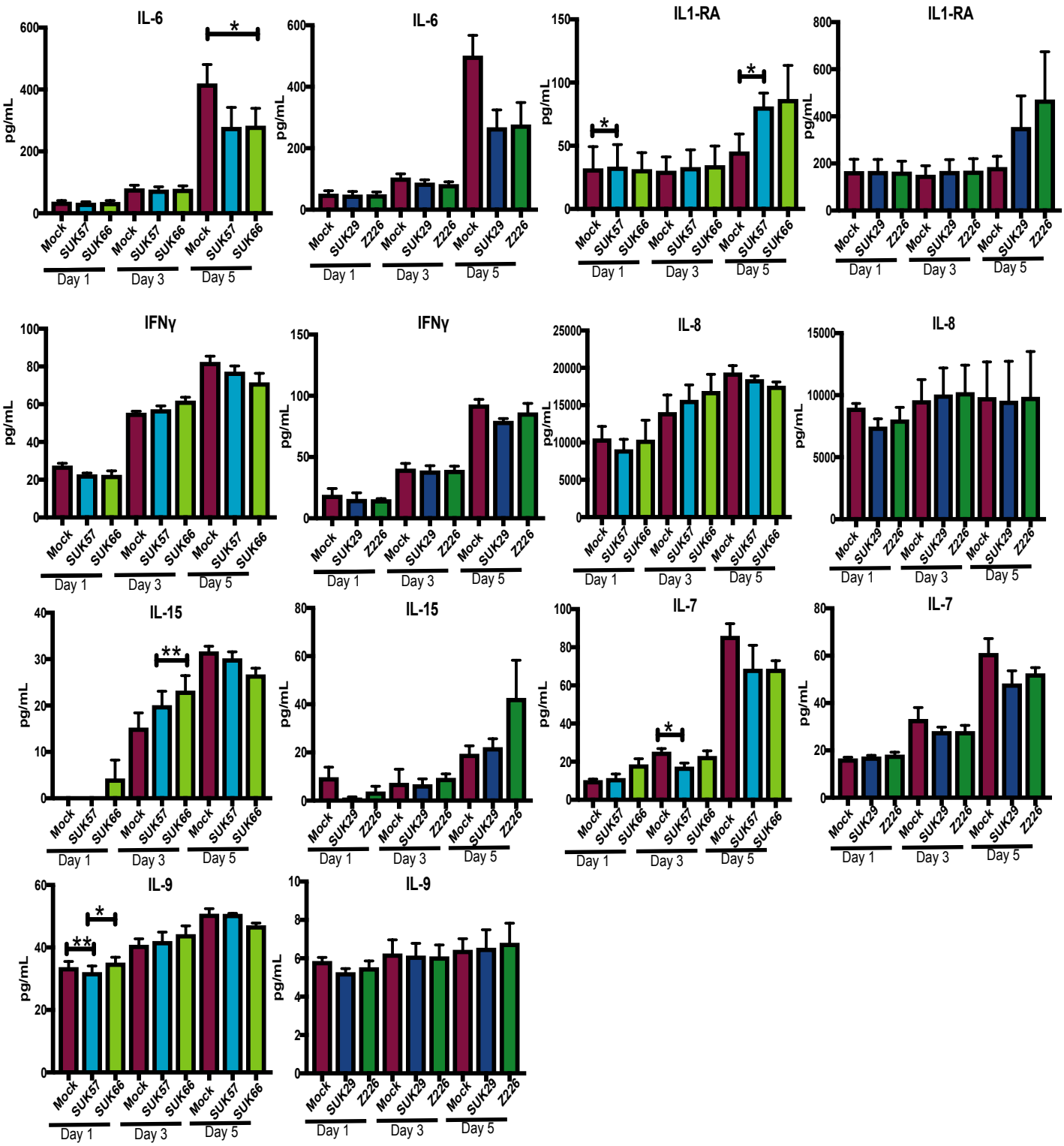


Figure 4.20 Modulation of ARPE-19 cytokine secretion by VZV clinical isolates SUK57, SUK66, SUK29 and Z226.

Supernatants from mock, SUK57, SUK66, SUK29 and Z226 infected ARPE-19s were collected and analysed via the BioPlex pro human cytokine 27 plex assay. Samples were run in duplicate and beads were run on the Luminex MAGPIX for analysis as per manufacturers instructions. MILLIPLEX software was used to generate standard curves and calculate concentrations of each analyte per sample. Cytokine analytes displayed include IL-6, IL-1RA, IFN-γ, IL-8, IL-15, IL-7 and IL-9. Bar graphs are representative of 3 biological replicates and error bars indicate the SEM. Statistical significance was determined using a one-way ANOVA test, and Tukey's multiple comparison post hoc test for each time point * $P<0.05$ ** $P<0.01$.

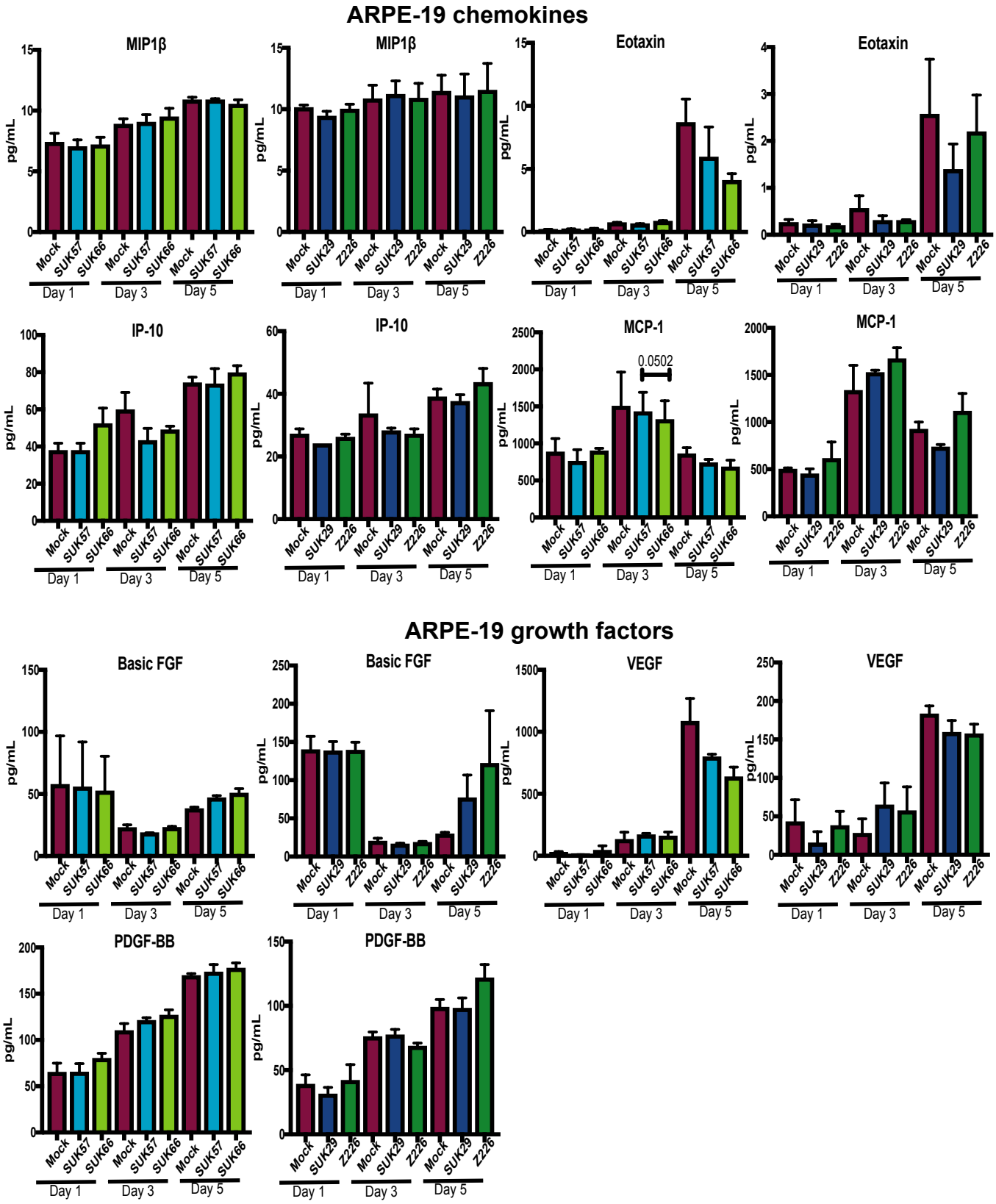
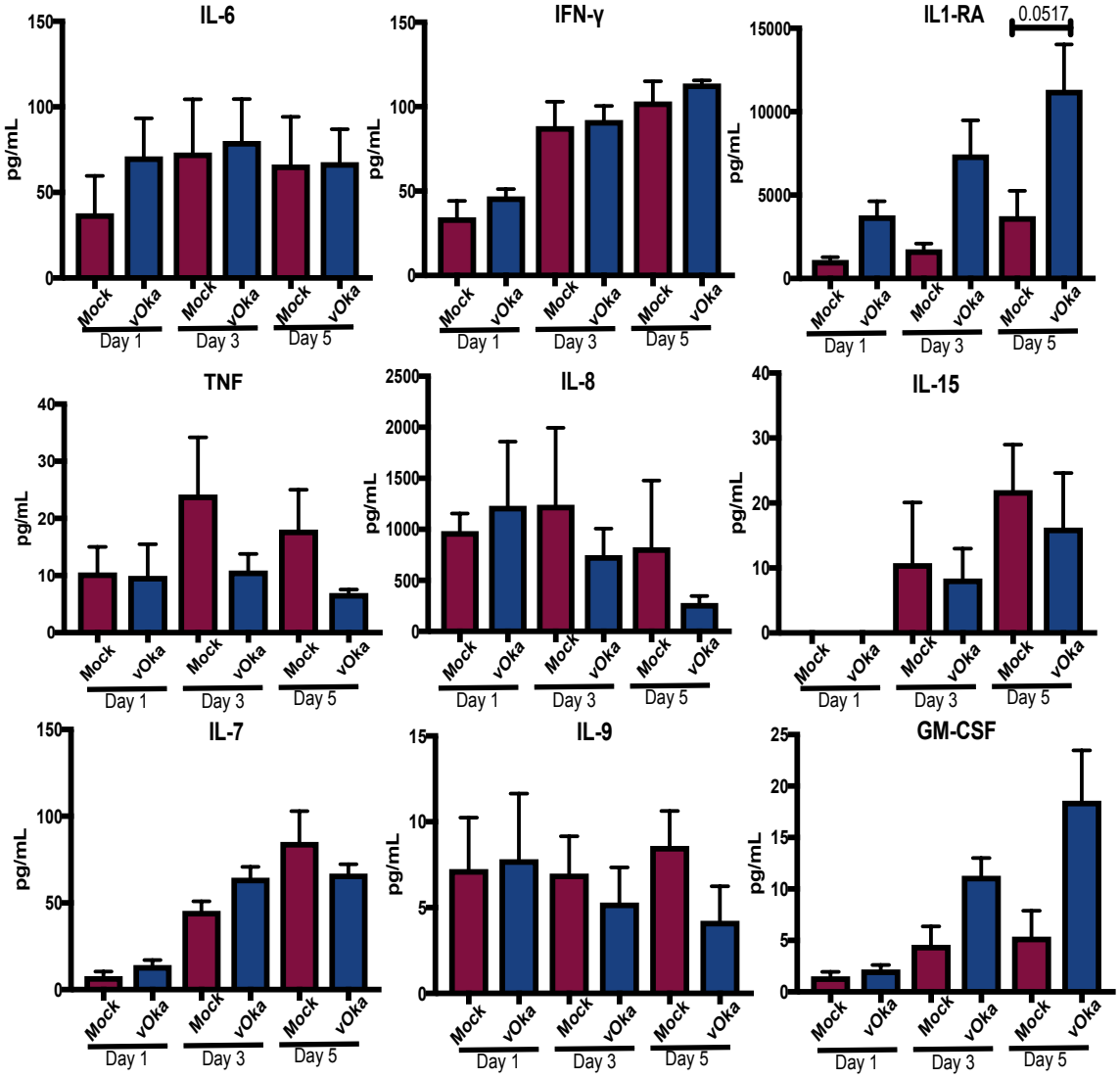


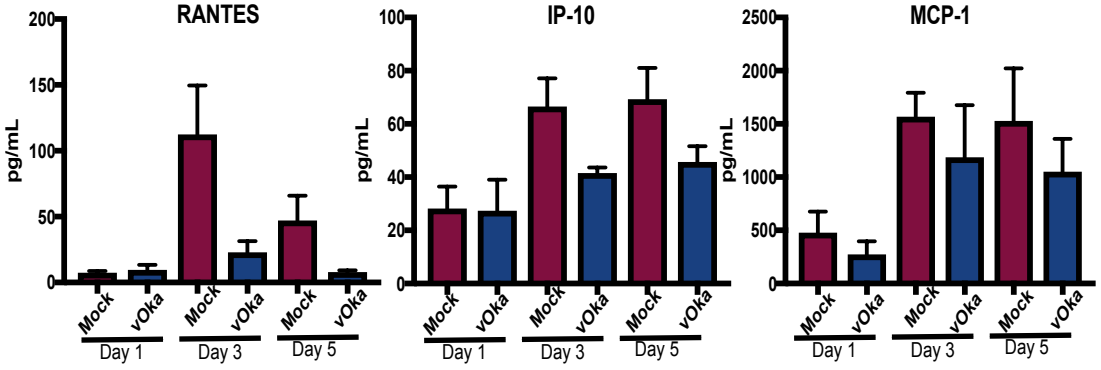
Figure 4.21 Modulation of ARPE-19 chemokine and growth factor secretion by VZV clinical isolates SUK57, SUK66, SUK29 and Z226.

Supernatants from mock, SUK57, SUK66, SUK29 and Z226 infected ARPE-19s at day 1, 3 and 5 post exposure were collected and analysed via the BioPlex pro human cytokine 27 plex assay. Samples were run in duplicate and beads were run on the Luminex MAGPIX for analysis as per manufacturer's instructions. MILLIPLEX software was used to generate standard curves and calculate concentrations of each analyte per sample. Analytes displayed include MIP1β, Eotaxin, IP-10, MCP-1, Basic FGF, VEGF and PDGF-BB. Bar graphs are representative of 3 biological replicates and error bars indicate the SEM. Statistical significance was determined using a one-way ANOVA test, and Tukey's multiple comparison post hoc test for each time point.

HaCaT cytokines



HaCaT chemokines



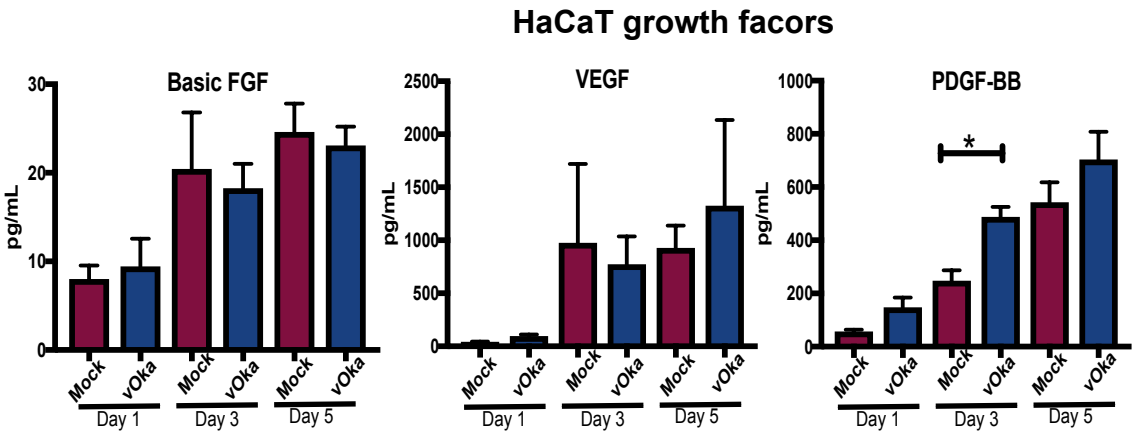


Figure 4.22 Modulation of HaCaT secretory profile by vOka.
Supernatants from mock and vOka infected HaCaTs at days 1, 3 and 5 post exposure were collected and analysed via the Bioplex pro human cytokine 27 plex assay. Samples were run in duplicate and beads were run on the Luminex MAGPIX for analysis as per manufacturer's instructions. MILLIPLEX software was used to generate standard curves and calculate concentrations of each analyte per sample. Analytes displayed include IL-6, IFN-γ, IL1-RA, TNF, IL-8, IL-15, IL-7, IL-9, GM-CSF, RANTES, IP-10, MCP-1, Basic FGF, VEGF and PDGF-BB. Bar graphs are representative of 3 biological replicates and error bars indicate the SEM. Statistical significance was determined using a paired two tailed students T test for each time point * $P<0.05$.

VZV infection of ARPE-19s or HaCaTs did not significantly alter the levels of IFNγ or IL-8 at the time points examined. SUK57 and SUK66 increased the secretion of IL-15 in ARPE-19s at day 3 post exposure, with this increase being significantly higher in SUK66 infected ARPE-19s (Figure 4.20). SUK29 and Z226 increased the secretion of IL-15 at day 5 post exposure, however this increase was not statistically significant. In contrast, vOka did not alter IL-15 secretion by HaCaTs (Figure 4.22). IL-7 secretion was decreased by all viral isolates tested at day 3 and 5 post exposure in ARPE-19s, with the decrease at day 3 in SUK57 infected ARPE-19s being statistically significant (Figure 4.20). vOka did not significantly alter IL-7 secretion in HaCaTs however a small decrease was observed at day 5 post exposure (Figure 4.22). The concentration of IL-9 was low in both HaCaT and ARPE-19 supernatants, however SUK57 significantly downregulated IL-9 secretion in comparison to mock and SUK66 at day 1 post exposure. (Figure 4.20 & 4.22).

VZV infection seemed to regulate chemokine and growth factor secretion to a greater extent in the HaCaT cells (Figure 4.22). None of the VZV isolates examined modulated the secretion of IP-10 to a significant extent in the ARPE-19s (Figure 4.21). In the HaCaTs vOka seemed to decrease the secretion of IP-10 at days 3 and 5 post exposure, however this decrease did not reach statistical significance. There were no obvious trends observed in the ability of the clinical isolates to modulate MCP-1 secretion in ARPE-19s. vOka decreased the secretion of MCP-1 in HaCaTs, however this decrease was not statistically significant. The clinical VZV isolates appeared to increase basic FGF secretion at day 5 post exposure, however this increase was not statistically significant. In comparison there was less basic FGF secretion in the HaCaTs and vOka did not appear to alter the concentration of basic FGF in the supernatant. vOka also did not alter VEGF secretion in the HaCaTs, similarly there were no obvious trends in the ability of the clinical isolates to modulate VEGF secretion in the ARPE-19s. PDGF-BB was not significantly altered by any of the clinical isolates at the time points tested in ARPE-19s, however vOka increased PDGF-BB expression at all timepoints examined in the HaCaTs and this increase was statistically significant at day 3 post exposure.

To further examine the results, fold changes for the analytes examined were calculated in comparison to mock supernatants, for each clinical isolate and vOka to generate a heat map (Figure 4.23). It is identifiable that there are no obvious differences in the ability of VZV PHN or non PHN clinical isolates to alter the secretory profile of ARPE-19s at the time points examined. Whilst some small differences were found, these were likely due to differences in infection level. Interestingly, VZV infection seemed to differentially modulate IL-6, FGF, PDGF-BB, IL-8 secretion in HaCaTs and ARPE-19s, however IL1-RA was consistently upregulated in both cell types by VZV infection. Together this data indicates that VZV infection can differentially modulate the secretory profile of ARPE-19s and HaCaTs, however this is not altered by whether the VZV strain was PHN or non PHN derived. Furthermore, clinical isolates did not differentially modulate cytokine, chemokine or growth factor secretion in the ARPE-19s suggesting that the ability of the isolates to cause neuropathic pain may not be due to differential modulation of skin cell secretory profiles.

Quantifying IL-1α release in vOka infected HaCaTs and clinical isolate infected ARPE-19s

One of the most consistent trends identified in the analysis of VZV modulation of ARPE-19 and HaCaT secretory profile, was the increase in IL1-RA seen in infected cell supernatants. IL1-RA binds to the IL-1 receptor to prevent IL-1 cytokines such as IL-1β and IL-1α binding and signaling through the IL-1 receptor (Ohlsson et al., 1990, Jensen, 2010). As such, the production of IL1-RA is critical in maintaining the balance of inflammation in the context of the skin (Jensen, 2010). As minimal IL-1β was detected (Table 4.9), it is possible that IL1-RA production by VZV infected HaCaTs and ARPE-19s could be in response to increased IL-1α levels. IL-1α is constitutively expressed by skin cells such as keratinocytes and epithelial cells to aid in skin barrier function, additionally viral infection can trigger its production (Jensen, 2010). To assess whether this was the case, an ELISA was performed to determine the concentration of IL-1α in the supernatants previously analyzed in the BioPlex. In the HaCaTs vOka infection increased IL-1α in the supernatant at days 1, 3 and 5 post exposure in comparison to mock infected HaCaTs, however this increase was not statistically significant (Figure 4.24A). Interestingly a different trend was observed in

the ARPE-19s infected with VZV clinical isolates. Small decreases in IL-1α in the supernatant was observed in SUK57 and SUK66 supernatant at days 3 and 5 post exposure in comparison to mock infected ARPE-19s, however this decrease was not statistically significant (Figure 4.24B). Larger decreases were seen with SUK29 and Z226 infection, with Z226 decreasing IL-1α secretion to a significant extent at day 5 post exposure (Figure 4.24C). The differences in infection levels between the HaCaTs and ARPE-19s may account for the different trends that were observed between isolates and cell types. Together this data shows that VZV may modulate IL-1α secretion and this could contribute to VZV pathogenesis in the skin.

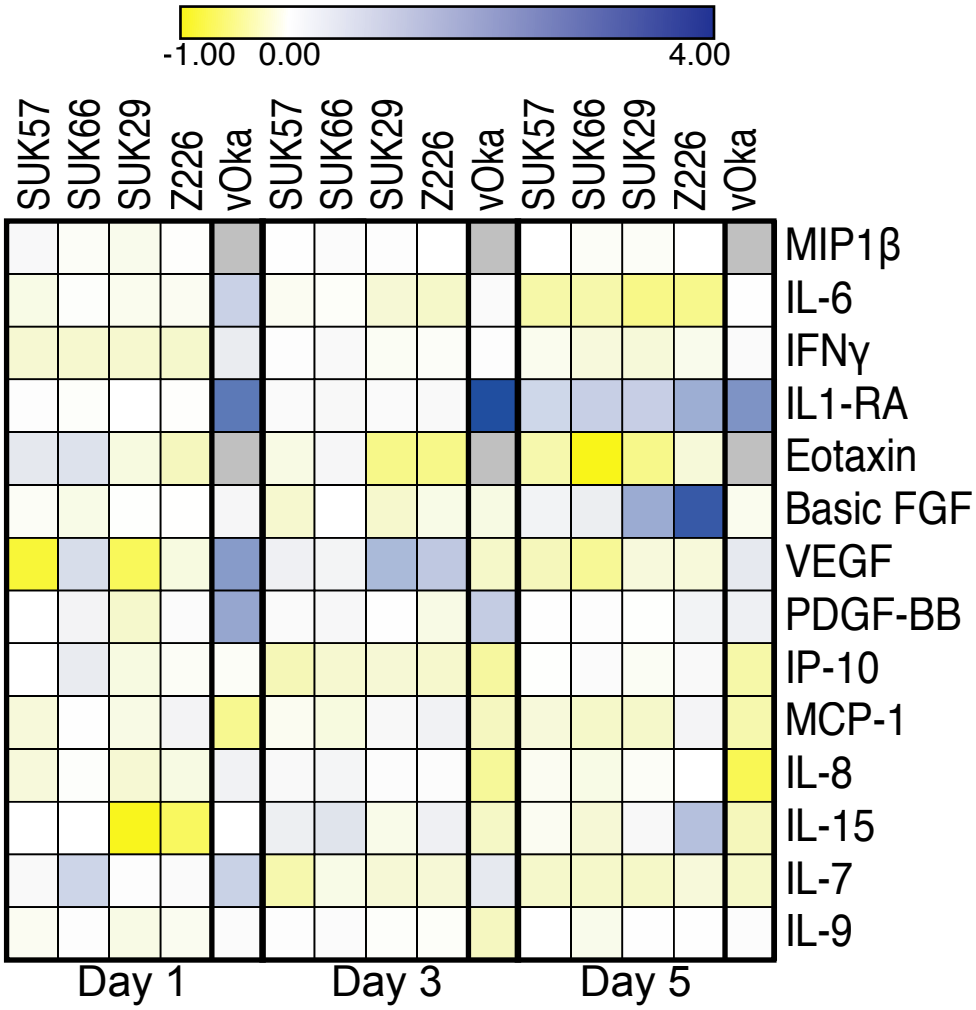


Figure 4.23 Heat map of fold change in cytokines, chemokines and growth factors induced by clinical isolates in ARPE-19s and vOka in HaCaTs.
Fold changes in cytokines, chemokines and growth factors induced by clinical isolates in ARPE-19s and vOka in HaCaTs were calculated and Morpheus (Broad Institute, https://software.broadinstitute.org/morpheus) was used to generate a heat map. Fold changes are relative to mock infected HaCaTs. SUK57 (non PHN) SUK66 (PHN) SUK29 (non PHN) Z226 (PHN).

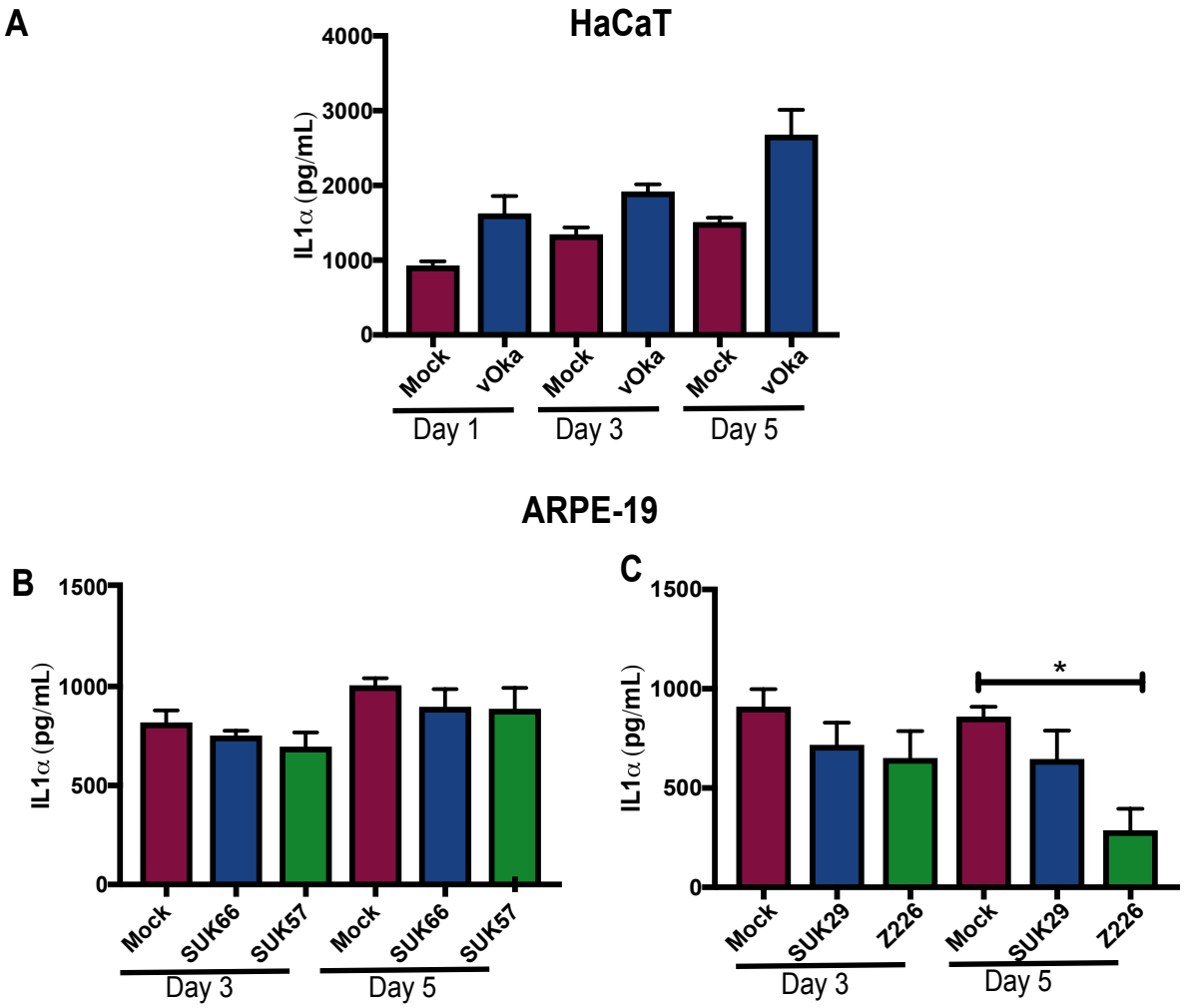


Figure 4.24 Modulation of IL-1α secretion by VZV in HaCaTs and ARPE-19s. Supernatants from vOka and mock infected HaCaTs at days 1, 3 and 5 post exposure (A) and from SUK57, SUK66, SUK29, Z226 and mock infected ARPE-19s (B & C) at days 3 and 5 post exposure were analysed for IL-1α secretion using the ELISAkit.com human IL-1α ELISA. Bar graphs are representative of 3 biological replicates and error bars indicate the SEM. Statistical significance was determined for the HaCaT data using a paired two tailed students T test for each time point and statistical significance was determined for ARPE-19 data using a two-way ANOVA test, and Tukey's multiple comparison post hoc test * $P<0.05$.

Discussion

Whilst keratinocytes have been identified as being modulated by VZV infection (Nikkels et al., 2004, Black et al., 2009, Jones et al., 2014, Choi et al., 2015) and as being critical in the development of neuropathic pain (Baumbauer et al., 2015, Hesselink et al., 2017), there has been little investigation into VZV modulation of keratinocyte inflammatory and nociceptive profile, in relation to PHN. Additionally, studies examining the effect of cell associated VZV infection on keratinocytes (Choi et al., 2015) have not examined the differences between infected cells and bystander cells. Choi *et al* (2015) inoculated HaCaT cells with VZV infected HFFs at a 1:1 ratio and examined various inflammation related transcripts at 4, 8, 24 and 48 hours. At earlier time points inoculation with VZV infected HFFs caused an increase in IFNα and IFNβ transcript. Additionally, suppressor of cytokine signaling (SOCS)1 & 3 was increased at all time points. However as inoculating cells were not excluded in analysis, it is possible the changes observed may be due to the inoculating HFFs rather than changes in the HaCaTs. In this chapter, vOka and VZV-S infected HaCaTs were used to inoculate uninfected HaCaTs to rule out effects seen in different inoculating cell types. Additionally, a technique using magnetic beads to separate VZV gEgI$^+$ and gEgI$^-$ bystander cells was optimized to allow for the assessment of VZV induced transcriptional changes in HaCaT cells.

VZV-S significantly down regulated TNF transcripts in both bystander and gEgI positive cells at day 1 post exposure, however increased at days 3 and 5 post exposure in gEgI$^+$ cells. TNF can cause the induction of cell death, can interact with neuroinflammatory signaling cascades and has been linked to peripheral and central sensitization in animal models (Leung and Cahill, 2010) and therefore could contribute to induction of neuropathic pain during PHN. All other transcripts examined such as pain associated and receptor/ion channel transcripts did not show any significant difference in gEgI$^+$ or bystander cells in comparison to mock cells, however some interesting trends were observed. It is possible that with further replicates, statistical significance may be observed.

TRPV1 transcript increased for both VZV-S and vOka in both gEgI$^+$ and bystander cells. This observation is intriguing as TRVP1 has been shown to increase in

epidermal keratinocytes in herpes zoster lesion biopsies in humans and is correlated with experiencing neuropathic pain (Han et al., 2016). Future investigation could determine whether VZV infection increases the protein expression of TRPV1 on the cell surface of HaCaTs. Additionally, as the transcript increase was evident in infected and bystander cells, it would be pertinent to determine whether a soluble factor could cause this increased TRPV1 transcript expression. The difficulty in preselecting transcripts for investigation in this study means that novel transcriptional regulation by VZV may be missed. Jones *et al* (2015) determined that VZV infection of undifferentiated keratinocytes effected the differentiation of these keratinocytes and promoted genes associated with blistering. More broadly, it may be useful for future experiments to utilize a single cell transcriptomic approach as in Jones *et al* (2015) to fully characterize the ability of VZV to induce nociceptive transcriptional changes in keratinocytes. By comparing changes in mock infected, bystander and VZV infected HaCaTs using the single cell approach novel factors regulated by VZV infection may be identified. Additionally, as HaCaTs can be undifferentiated (Lehen'kyi et al., 2007) it may be interesting to examine nociceptive/ inflammatory transcriptional changes induced by VZV in both undifferentiated and differentiated HaCaTs and primary human keratinocytes.

As mentioned, it is also pertinent to examine VZV modulation of the keratinocyte secretory profile as this could affect the skin microenvironment in the context of PHN. There have been some studies investigating VZV modulation of HaCaT proinflammatory cytokine secretion (Choi et al., 2015), however as mentioned these studies have not used VZV infected keratinocytes to infect other keratinocytes, meaning that results seen may be due to inoculating cells rather than the keratinocytes themselves. In this chapter VZV modulation of the keratinocyte secretome was examined using keratinocytes as the inoculating cell type and a human anti-viral LegendPlex kit to examine 13 analytes. IL-1β was significantly increased by VZV-S and vOka at days 1 and 5 respectively. VZV has been shown to induce NLRP3 inflammasome formation in THP-1s, primary lung fibroblasts and melanoma cells but has not been examined in keratinocytes (Nour et al., 2011). It is possible that as there was a low amount of IL-1β detected, it could have been released as the biologically inactive pro-form due to cell death rather than inflammasome formation (Hogquist et al., 1991). Additionally, IL-1β was not detected in our broader screen of vOka infected

HaCaTs supernatants using the Bio-Plex pro human cytokine 27 plex assay, suggesting that whilst significance was shown with the LegendPlex assay, IL-1β was not produced to a biologically relevant concentration. It may be interesting to examine whether VZV can cause IL-1β release in primary human keratinocytes, as IL-1β has been shown to influence neuron activity in the context of neuropathic pain (Clark et al., 2013). All other analytes identified were not significantly altered by VZV infection, however this could be attributed to variation in infection levels in the HaCaTs. Optimizing the cell associated infection of HaCaTs to yield more consistent infection levels and the use of another keratinocyte cell line or primary human keratinocytes may aid in determining whether the trends observed are biologically significant.

Some interesting trends in the HaCaT secretome were observed; in comparison to mock, both VZV-S and vOka increased TNF, IP-10, IFNλ1-3, GM-CSF and IFNγ whereas both IL-6 and IL-8 where downregulated. In the Bio-Plex assay VZV was shown to only downregulate IL-8 in HaCaTs, whereas IL-6 was downregulated in ARPE-19s. It is not clear whether this is due to differential VZV modulation of cytokine/ chemokine secretion in VZV infected cells or due to bystander cells responding to infected cells. Both IL-8 and IL-6 production is usually increased during viral infection, except in cases where viruses inhibit their production/ expression (Mogensen and Paludan, 2001, Tanaka et al., 2014). Therefore, it is possible that VZV is specifically modulating the expression of IL-8 and IL-6 in ARPE-19s and HaCaTs. VZV infection of fetal skin organ cultures caused an increase in IL-6 transcript and secretion into the media (Jarosinski et al., 2018). In this model multiple different skin cell types are present, and it is possible that both VZV infected and bystander cells could be responsible for IL-6 production. The organoid model is more reflective of VZV infection of the skin in comparison to using individual cell lines, it would be interesting to determine which cell type in the organoid model is responsible for IL-6 production and whether infected or bystander cells produce IL-6.

VZV has been shown to alter NFκB function in human dendritic cells and fibroblasts (Jones and Arvin, 2006, Sloan et al., 2012), therefore VZV modulation of transcription factors in keratinocytes may be an interesting area of future research in regards to differential cytokine expression. In the future, HaCaTs could be separated into gEgI positive and bystander cells and stimulated to further characterize the effect of VZV

infection on the ability of keratinocytes to secrete pro-inflammatory cytokines/ chemokines.

It is clear that VZV infection can induce changes to the HaCaT transcriptional and secretory profile, however it remains to be determined whether this can affect sensory neuron nociceptive capacity. Our lab has previously characterized the use of the SH-SY5Y neuronal cell line to study the effects of VZV infection on neuronal cells (Christensen et al., 2011). Using a transwell system it would be possible to coculture HaCaTs and SH-SY5Ys to determine whether VZV induced secretory changes in HaCaTs could affect neuronal nociceptive transcriptional changes and secretion of nociceptive factors. However as SH-SY5Ys are not biochemically similar to sensory neurons (Xie et al., 2010, Korecka et al., 2013) it may also be helpful to utilize induced pluripotent stem cells (iPSC) to differentiate into a sensory neuronal phenotype, to explore the keratinocyte neuronal interaction (Markus et al., 2015).

Investigation into VZV modulation of critical cell types in the skin and the way in which this could affect neuronal functioning, could increase our knowledge of contributing factors to PHN development. Another contributing factor to PHN development which has not been explored, is differences within VZV strains which could cause altered virus characteristics. Through assessment of clinical isolates from patients with or without PHN this chapter aimed to examine differences between VZV PHN or non PHN isolates in their ability to spread, to induce cell death and to modulate pro inflammatory cytokine/ chemokine secretion. All of the clinical isolates assessed in this study did not spread or cause CPE differently in the ARPE-19s. For future study, genetic analysis of the VZV clinical isolates via deep sequencing of virus RNA, could reveal genetic differences between isolates and give insight into potential altered viral characteristics. It is critical to determine whether differences seen between sequenced and unsequenced VZV strains is due to genome modifications which could affect the expression and functioning of VZV ORFs. The isolates grown in the HaCaT cells did not seem to spread as successfully as in ARPE-19s. It would be interesting to determine whether this is due to a specific VZV restrictive feature of the HaCaT cell line. The use of primary human keratinocytes to assess the clinical isolate infection and spread may be advantageous in future experiments.

In regard to cell death induction SUK57, SUK66 and SUK29 did not induce significant levels of cell death throughout the 5-day time-course in ARPE-19s. Z226 began to induce higher levels of cell death at day 5 in the time-course which approached statistical significance. This may be due to variation in the number of cells infected rather than a specific induction of cell death by Z226 in comparison to the other VZV strains. It would be interesting to examine what pathway VZV is inducing cell death in this context as the role of pyroptosis and necroptosis in VZV pathogenesis has yet to be examined.

It has previously been demonstrated that VZV can modulate ARPE-19 secretion of cytokines and chemokines to influence lymphocyte migration (Graybill et al., 2017). As previously mentioned, many cytokines and chemokines produced in the microenvironment of the skin have been linked with the development of neuropathic pain (Clark et al., 2013). In this chapter we examined the ability of different clinical VZV isolates to modulate ARPE-19 cytokine and chemokine secretion using the BioPlex pro 27 plex assay. vOka infected HaCaT supernatants were also analysed to compare VZV modulation of cytokines and chemokines in different cell types. In our study, all of the clinical isolates tested caused decreases in IL-6 and IL-7 in the ARPE-19s, whereas IL1-RA, and IL-15 were increased with VZV infection in the ARPE-19s. This decrease in IL-6 has been previously observed in VZV infected ARPE-19s and as IL-6 has been shown to contribute to resistance to other viral infections such as HSV-1 (Paludan, 2001), it would be interesting to determine the mechanistic basis of IL-6 downregulation during VZV infection (Graybill et al., 2017). Both IL-7 and IL-15 aid in the proliferation of NK cells (Lum et al., 2004) and NK cells have been shown to be critical in the control of VZV as demonstrated by patients with NK cell deficiencies who suffer severe disseminated varicella (Etzioni et al., 2005). Therefore, it may be interesting to treat NK cells with supernatant from VZV infected ARPE-19s and determine if NK cell proliferation is affected. Previous studies have reported that VZV infection causes decreased secretion of IFNγ and TNF in ARPE-19s (Graybill et al., 2017), however our study found no significant changes in these proinflammatory cytokines. This may be due to variability in infection levels or the use of different time courses.

Graybill *et al* (2017) demonstrated that VZV infection increased ARPE-19 secretion of CCL26 and IL-8, and decreased secretion of CCL11, MCP-1 and CCL13 at day 8 post infection. This resulted in an increased number of migrating $CD4_+$ and $CD8_+$ T cells. In our study, VZV infection did not significantly alter the secretion of chemokines, however this could be due to the utilization of a shorter time course. Additionally, the panel utilized was more focused towards cytokines, therefore it may be useful to utilize a panel directed towards measuring chemokines in VZV infected ARPE-19 supernatant. As mentioned, there were no differences in the ability of differing VZV isolates to modulate ARPE-19 secretion of chemokines and cytokines, suggesting that this is a relatively conserved feature.

The HaCaTs seemed to express higher levels of chemokines in comparison to the ARPE-19s. Particularly, T cell chemokines RANTES and IP-10 were secreted in a higher concentration, suggesting the HaCaTs may be a better tool for dissecting the effect of VZV infection on chemokine secretion. vOka infection of HaCaTs decreased secretion of both RANTES and IP-10 at days 3 and 5 post exposure. Seeing as RANTES is critical for T cell migration (Schall et al., 1990) it would be interesting to determine whether treatment with supernatant from vOka infected HaCaTs could alter T cell migration. IP-10 has previously been shown to increase in the context of VZV infected human DRG explants and was mainly produced by bystander neurons (Steain et al., 2011). Additionally in simian varicella virus (SVV), IP-10 expression correlated with an increase of T cell infiltration (Ouwendijk et al., 2013). In our current study, vOka was able to decrease the basal level of IP-10 secretion, this is not surprising considering IP-10 transcription is activated by the transcription factor NFκB and VZV has been shown to inhibit NFκB function (Sloan et al., 2012). However, in the SVV infection model and human ganglia explant model there are multiple cells producing proinflammatory cytokines in response to VZV infection which could cause the increase in IP-10. To further explore the mechanism of VZV induced downregulation of T cell chemokines VZV $gEgI_+$ and $gEgI_-$ HaCaTs could be sorted and stimulated to produce these chemokines with TNF and IFNγ. It is important to investigate the modulation of these chemokines in the ganglia and skin. Blocking antibodies to IP-10 and RANTES could be utilized in the SVV model to determine how removal of these T cell chemokines alters SVV progression.

Interestingly, in HaCaTs vOka significantly increased PDGF-BB secretion at day 3 post exposure. PDGF has been shown to play a role in fibroblast proliferation and the acceleration of wound healing (Beer et al., 1997). PDGF-BB has also been shown to activate nociceptive neurons and contribute to inflammatory pain (Barkai et al., 2019). Therefore, determining whether supernatant from vOka infected HaCaTs could affect neuron activation is important.

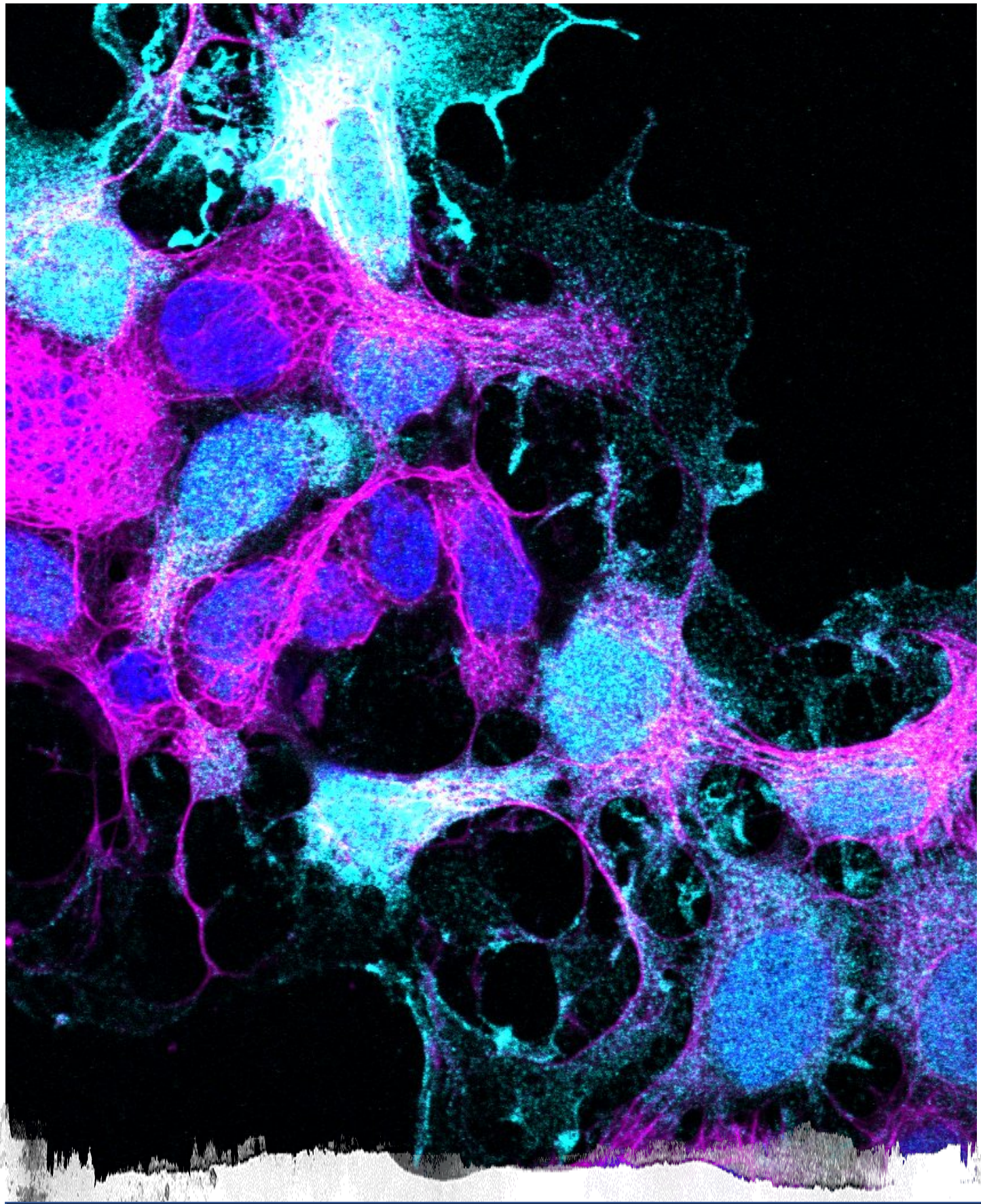
IL1-RA secretion was upregulated by all VZV strains tested in HaCaTs and ARPE-19s. IL1-RA inhibits both IL-1α and IL-1β from signaling through the IL-1 receptor and thus inhibits inflammation (Jensen, 2010). The IL-1α and IL-1β genes have been shown to participate in the modulation of IL1-RA production *in vivo* (Hurme and Santtila, 1998). Additionally, TLR stimulation has been shown to cause IL1-RA production (Rehani et al., 2009). In our study, we wanted to determine whether the increase in IL1-RA observed during VZV infection was due to an increase in IL-1α secretion. HSV-1 infection has been shown to cause the release of IL-1α in human keratinocytes to act as an alarmin (Milora et al., 2014). This release of IL-1α is critical in the control of HSV-1 infection as the *in vivo* recruitment of leukocytes in the epidermis is dependent on IL-1 signaling. Mice who were not able to signal via IL-1α had an increased mortality rate and greater viral dissemination, displaying the importance of IL-1α (Milora et al., 2014).

In our hands VZV infection did cause an increase in IL-1α secretion from the HaCaTs but not ARPE-19s. As the ARPE-19s had higher infection levels and IL-1α secretion was decreased, this suggests VZV infection may be able to inhibit IL-1α secretion. In the HaCaTs there was a lower infection level suggesting that IL-1α production was occurring in bystander cells and potentially functioning as an alarmin. This could be further investigated by performing flow cytometry on VZV infected HaCaTs and ARPE-19s to distinguish between IL-1α levels in infected and bystander cells. Overall, this suggests VZV may have the potential to skew the skin microenvironment towards being anti-inflammatory with decreased IL-1α secretion and increased IL-RA secretion. It would be interesting to examine the balance of IL-1α and IL1-RA during VZV infection *in vivo* by utilizing SCID hu human skin explant mice, as VZV targeting

of IL-1 signaling may represent a significant pathogenic mechanism for VZV replication and spread in the skin.

Conclusion

Whilst PHN remains an enigmatic feature of VZV pathogenesis there has been minimal investigation into VZV modulation of the skin microenvironment as a factor contributing to PHN development. In our study, VZV infection was shown to change the transcriptional and secretory profile of a keratinocyte cell line (HaCaT) and has highlighted targets such as TRPV1 and PDGF-BB which could be further investigated in the context of VZV induced neuropathic pain. From our study, it is clear that VZV can modulate the secretory profile of both ARPE-19s and HaCaTs. In particular IL-6 and IL1-RA seem to be strongly regulated and warrant further investigation in regard to VZV modulation of inflammation in the skin. Additionally, the ability of different clinical isolates to cause CPE and modulate the secretory profile of ARPE-19s was characterized. It was revealed that spread, CPE induction and the ability to modulate the secretory profile of ARPE-19s was fairly conserved between the VZV clinical isolates. This highlights that clinical isolate variation alone may not play a role in the development of PHN. Alternatively, it is possible that host factors such as immune status, age and sex may be a better predictor of PHN development. Interestingly, children and young adults rarely develop PHN (Petursson et al., 1998), suggesting that age is an important factor in PHN development.



Chapter 5: Discussion

CHAPTER 5

Introductory statement

During lytic infection within the skin, VZV gains access to sensory nerve termini, where it travels in an anterograde fashion to the dorsal root ganglia (DRG) and establishes lifelong latency (Valyi-Nagy et al., 2007, Eshleman et al., 2011). There is evidence to suggest that VZV sub-clinically reactivates throughout life, however reactivation resulting in clinically relevant disease such as herpes zoster usually occurs once and typically correlates with a decline in VZV-specific immunity (Levin et al., 2003, Freer and Pistello, 2018). This highlights the importance of the immune system in controlling VZV pathogenesis and is further emphasised by studies that show immunocompromised patients frequently suffer severe or fatal disseminated varicella (Etzioni et al., 2005). Therefore, we may gain insight into VZV latency and reactivation by investigating VZV immunoevasive mechanisms.

In contrast to HSV-1, little is known concerning how VZV establishes and maintains lifelong latency and what causes clinically relevant VZV reactivation (Valyi-Nagy et al., 2007). This is due to limited *in vivo* model systems to study VZV latency due to its host specificity as well as limited production of cell free virus *in vitro* (Myers and Connelly, 1992, Eshleman et al., 2011). There are a variety of *in vitro* neuronal models to study VZV but only recently have these been applied to the study of VZV latency (Markus et al., 2015, Sadaoka et al., 2016). The ability of VZV to inhibit apoptosis has been linked to the maintenance of lifelong latency and thus is a critical topic of research to investigate in regard to VZV pathogenesis during both lytic and latent infection (James et al., 2012). Herpes zoster can cause severe complications such as PHN, an intense neuropathic pain (Gupta and Smith, 2012). Interestingly, only a proportion of herpes zoster patients develop PHN, however it is unclear as to the factors which can predict PHN development. As previously mentioned, age is an important factor as children rarely develop PHN (Petursson et al., 1998). In the current study, we investigated VZV immunoevasive mechanisms and modulation of clinically relevant cell types *in vitro* to better understand contributing factors to the maintenance of latency, the establishment of reactivation and the development of PHN. As such this discussion will examine the implications of our findings within the context of current literature,

referencing VZV pathogenic mechanisms in the context of latency, reactivation and PHN.

In the first results chapter (Chapter 2) the role of VZV ORF63 in the cell specific inhibition of apoptosis was examined. This work confirmed that VZV ORF63 when expressed alone in SH-SY5Ys neuronal cells could protect these cells from apoptosis. Additionally, we determined that the anti-apoptotic effect of VZV ORF63 was not neuron-specific, as a keratinocyte cell line (HaCaTs) was also protected from apoptosis. This highlighted the possibility that VZV ORF63 could be critical for the establishment of latency in neuronal cells as well as VZV spread during lytic infection. The mechanism of VZV ORF63 inhibition of apoptosis was investigated and it was shown that the VZV ORF63 protein re-localised to the cytoplasm and formed aggregates in response to apoptotic stimuli. This suggests that VZV ORF63 could potentially be interacting with apoptotic protein structures such as the apoptosome within the cytoplasm to inhibit apoptosis.

In addition to investigation of VZV modulation of apoptosis, we explored the ability of alphaherpesviruses to modulate NK cell mediated cytotoxicity (Chapter 3). This was examined in the context of determining whether viral proteins with granzyme B cleavage sites could block granzyme B induced apoptosis. It was found that multiple HSV-1 and VZV proteins contained granzyme B cleavage sites, and as a result could be cleaved by granzyme B. As most of these proteins are essential for viral replication, this highlights a potential role for granzyme B in restricting HSV-1 and VZV lytic infection as well as reactivation. Despite these HSV-1 and VZV proteins containing granzyme B cleavage sites, only VZV ORF4 could protect 293Ts against NK cell mediated cytotoxicity. VZV ORF4 was identified as having a novel immunoevasive function in the inhibition of NK cell mediated cytotoxicity, highlighting another mechanism which could aid VZV spread and reactivation.

The third results chapter (Chapter 4) highlighted several factors which could contribute to the development of PHN. This included VZV modulation of keratinocyte RNA and secretory profile, in which VZV infection upregulated inflammatory cytokines, decreased chemokine expression and increased nociceptive molecules such as PDGF-BB. Additionally, we assessed the ability of clinical VZV isolates to spread,

induce cell death and modulate the APRE-19 secretome to determine whether strain-specific differences in VZV could affect the development of PHN. There were no differences in the ability of the VZV clinical isolates tested in this thesis, to spread or cause CPE in ARPE-19s. Additionally, it was shown that irrespective of VZV isolate, VZV infection upregulated the secretion of IL-1RA and decreased the secretion of IL-1α in ARPE-19s. This suggests that isolate variability may not play a major role in PHN development and that VZV may alter the balance of IL-1 cytokines in the skin to further viral pathogenesis. Overall, these results give us a broader understanding of how VZV immunoevasive mechanisms and modulation of key cell types could contribute to the maintenance of latency and the development of PHN.

VZV modulation of apoptosis

Induction of apoptosis by VZV

Many viruses induce apoptosis as a method of increasing viral dissemination and spread. VZV has been shown to induce apoptosis in multiple skin cell types such as MeWos (Brazeau et al., 2010) and human fibroblasts (Hood et al., 2003), as well immune cells such as T cells, B cells and monocytes (Koenig and Wolff, 2003, König et al., 2003, Kennedy et al., 2019). Interestingly, these are all critical cell types involved in VZV dissemination and spread. VZV-infected T cells have been shown to be capable of trafficking the virus to the skin in the SCID mouse skin xenograft model (Arvin et al., 2010). It would be interesting to investigate whether the induction of apoptosis in VZV specific CTLs by VZV would increase VZV dissemination to skin cells and neuronal cells. It may be possible to examine this *in vitro* by inoculating cells with VZV- infected T cells in the presence or absence of a caspase inhibitor. More recently, it has been demonstrated that NK cells (Campbell et al., 2018) and monocytes (Kennedy et al., 2019) are infected by VZV and pass on infection to epithelial cells *in vitro*. VZV caused apoptotic induction in monocytes at 48 hours p.i. (Kennedy et al., 2019). It would be interesting to further characterise the induction of apoptosis by VZV in these cell types and assess whether this increases the dissemination of VZV.

The induction of apoptosis by VZV and simian varicella virus (SVV) has been linked to a down-regulation of Bcl-2 in MeWos and monkey kidney cells respectively

(Pugazhenthi et al., 2009, Brazeau et al., 2010). It would be of interest to determine whether this occurs in all cell types in which VZV infection causes apoptosis or whether there are cell type specific mechanisms of apoptosis. Identifying whether the downregulation of Bcl-2 is a result of the host response to VZV infection or whether a VZV gene product could target Bcl-2 expression would be intriguing. In this thesis, the ability of VZV to induce apoptosis in the HaCaT keratinocyte cell line was assessed and, in this system, VZV did not induce significant amounts of apoptosis. This finding has been previously reported in VZV-infected human papillomavirus (HPV)-immortalized keratinocytes (Black et al., 2009). It would be useful to further examine VZV apoptosis induction in the context of primary human keratinocytes, as cell lines can have deficiencies in the apoptotic pathway, which makes them less sensitive to apoptosis induction (Lehman et al., 1993, Lombet et al., 2001). Through using cell lines which are either vulnerable or resistant to VZV induced apoptosis, it may be possible to determine which apoptotic pathway elements are involved in the induction of apoptosis by VZV.

Cell-type specific apoptosis inhibition by VZV: intentional or consequential?

In contrast to some skin cell types and immune cells, VZV does not induce apoptosis in neurons. This was first identified in the context of primary human sensory ganglionic neurons, a neuronal culture model which our lab has established (Hood et al., 2003). This phenomenon was subsequently demonstrated in various other neuronal models such as the non-obese diabetic SCID mouse human neural cell model, where neural cells from VZV infected human neural cell xenographs underwent minimal apoptosis (Baiker et al., 2004). Furthermore, VZV does not induce apoptosis in neurons derived from human neural stem cells (Pugazhenthi et al., 2011, Yu et al., 2013). The ability of VZV to protect neurons from apoptosis was attributed to ORF63, using a recombinant virus unable to express one copy of the diploid ORF63 gene (Hood et al., 2006). However, as ORF63 is a potent viral transactivator (Jackers et al., 1992), it was unclear whether this was due to an effect on another VZV ORF. Several VZV ORFs have been shown to have anti-apoptotic activity such as ORF66 in T cells and ORF12 in MeWos (Schaap et al., 2005, Liu et al., 2012) (Figure 5.1). In the current study, we were able to examine the effect of ORF63 in isolation by using a lentiviral expression system in human neuronal SH-SY5Y and HaCaT keratinocyte cell lines.

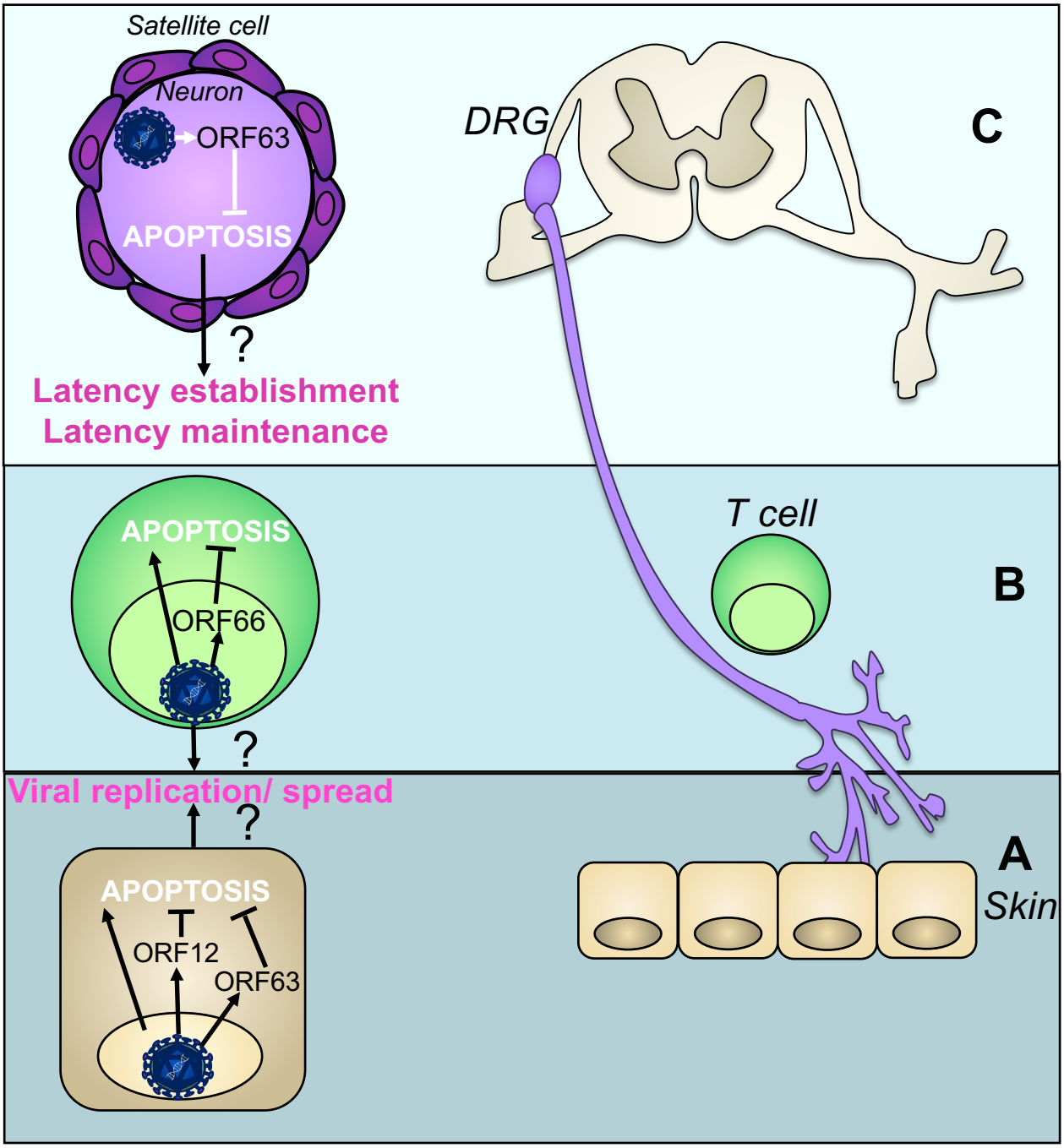


Figure 5.1 VZV modulation of apoptosis during lytic infection and the establishment of latency.
During lytic infection, VZV infects skin cells (A) such as keratinocytes, fibroblasts and epithelial cells. VZV induces apoptosis in skin cell types, despite the production of anti-apoptotic gene products such as VZV ORF12 and VZV ORF63, which may act to delay apoptosis to ensure efficient viral replication and spread. T cells (B) are are also infected by VZV during lytic infection and act as a conduit to transport VZV to the skin and dorsal root ganglia (DRG). VZV induces apoptosis in T cells as well as other immune cells. VZV ORF66 may act to delay T cell apoptosis to promote viral dissemination. VZV establishes life-long latency in neurons of the DRG (C). VZV ORF63 can inhibit apoptosis in these neurons which may aid in the establishment and maintenance of latency.

In this system, VZV ORF63 was able to protect both differentiated SH-SY5Ys and the HaCaT cell line from intrinsic and extrinsic apoptosis induction. This suggests that the ability of VZV ORF63 to protect against apoptosis induction is not cell type specific, unlike the induction of apoptosis by VZV (Figure 5.1). It is unclear as to why certain cell types are protected from apoptosis during VZV infection and others are not, however there is evidence to suggest that VZV alters the transcriptional profile of apoptotic genes differentially in neuronal cells and HFs (Markus et al., 2014). It is possible that anti-apoptotic viral genes, like VZV ORF63, delay apoptosis in VZV infected immune cells and skin cells, to allow the virus to replicate and the transport of the virus to neuronal cells, even though the cellular response ultimately compensates, and apoptosis is induced. This could be examined by expressing known VZV anti-apoptotic proteins in isolation in different skin and immune cells and assessing whether they can protect against apoptosis induction. If the regions of these anti-apoptotic proteins responsible for the inhibition of apoptosis are identified, it may be possible to mutate these regions in VZV and assess whether apoptosis is induced more rapidly.

VZV modulation of apoptosis: relevance to latency

VZV modulation of apoptosis in a cell specific manner is a crucial component of VZV research due to its link to VZV pathogenesis. As VZV establishes lifelong latency in neurons of the DRG, the inhibition of apoptosis in neurons is critical for viral maintenance of latency and the induction of reactivation (James et al., 2012). In contrast, within productive infection in the skin, the induction of apoptosis may aid in viral dissemination. It is critical to determine the mechanism of ORF63 apoptosis inhibition to begin to assess its role in viral pathogenesis. The ORF63 transcript is also one of the most abundant transcripts produced during VZV latency, so may play a role in apoptosis protection in this context (Depledge et al., 2018). LAT in HSV-1 infection has been shown to inhibit a variety of apoptotic stimuli (Jiang et al., 2011, Carpenter et al., 2015, You et al., 2017), and it would be interesting to assess if the ORF63 transcript was also capable of inhibiting apoptosis. In this study (Chapter 2) it was identified that ORF63 localisation changed to being predominantly cytoplasmic upon apoptosis induction. It is pertinent to assess whether VZV ORF63 has any apoptotic protein binding partners in the cytoplasm to begin to unravel its mechanism of action. In our study, potential apoptotic protein binding partners of VZV ORF63 were

evaluated using co-immunoprecipitation and mass spectrometry, with no obvious candidates being identified (data not shown). As the VZV ORF63 aggregates observed when apoptosis was induced were large in size, it may be pertinent to examine the insoluble fraction of protein lysates for protein binding partners in future studies.

With evolving models of VZV latency it may be possible to investigate the anti-apoptotic properties of VZV ORF63 transcript and protein in this context. Through the use of the VZV ORF63 expressing cell lines developed in this study, a complete VZV ORF63 / VZV ORF70 deficient virus could be made to assess the importance of VZV ORF63 for the establishment of latency. This could be assessed *in vitro* through the use of the human embryonic stem cell (hESC)-neuron fluorescent VZV system, where neuronal cultures are infected in the presence or absence of acyclovir (Markus et al., 2015). Latently infected cells test positive for the presence of the VZV genome, but are GFP negative indicating no protein expression. It has been demonstrated using this system that after a long period of GFP absence, disruption of NGF signalling can cause viral replication and subsequent GFP expression (Markus et al., 2015). However, using acyclovir to induce latency can be problematic due to damage to the viral genome which decreases the potential for reactivation (Laemmle et al., 2019). Another group has suggested that a low MOI, cell-free VZV infection of neurons, derived from hESC- derived neural stem cells can result in cultures which are positive for VZV DNA without lytic viral growth (Sadaoka et al., 2016). Additonally, as with the previous system, with NGF depletion, lytic infection was seen in previously latenly infected cultures. Via utilising a GFP expressing VZV deficient in both VZV ORF63 and ORF70 in this system, it would be possible to assess the importance of VZV ORF63 in the establishment and maintenance of latency. Additionally by using plasmids or an adenovirus transient transfection model, these hESC derived neurons could be engineered to overexpress VZV ORF63 to determine whether VZV ORF63 is protective against cell death induction in this system.

VZV modulation of apoptosis: relevance to the development of PHN

It is clear that the cell type specific induction of apoptosis by VZV, and the inhibiton of apoptosis by VZV gene products such as VZV ORF63 may play a critical role in both the lytic and latent stages of VZV infection. It is also perceivable that the modulation of cell death by VZV could contribute to the development of PHN. In post mortem ganglia samples from patients with herpes zoster at the time of death, neurons were not identified as being apoptotic, however surrounding satellite cells did display apoptotic markers (Steain et al., 2014). Satellite cells are critical for regulating neuronal function and have been shown to play a key role in the development of hypersensitivity during neuropathic pain (Ohara et al., 2009). Therefore, if VZV induces death of satellite cells this may cause sensory neuronal dysfunction in the DRG and could be a contributing factor to the development of PHN. This could be investigated via the infection of mixed satellite cell neuron cultures isolated from fetal DRG and assessment of cell death induction and neuronal function (Hood et al., 2006). In the SCID-hu mouse human DRG xenograph *in vivo* model, VZV caused an abortive infection and selective loss of mechanoreceptive neurons (Zerboni and Arvin, 2015). This was postulated to be due to either abrogated satellite cell functioning or the loss of ORF63 expression as a result of the abortive infection (Zerboni and Arvin, 2015). Using this model, it would be interesting to quantify the amount of cell death induced in satellite cells and determine if this could factor into the loss of mechanoreceptive neurons.

Interplay of granzyme B and viral immunoevasive mechanisms during alphaherpesvirus latency

The role of CTL and NK cell cytotoxicity in HSV-1 latency and reactivation

The modulation of apoptosis by alphaherpesviruses may contribute to their ability to maintain lifelong latency and cause clinically relevant reactivation. Apoptosis is utilised by cells in our innate and adaptive immune system to effectively eliminate virally infected cells in a targeted manner. CTLs and NK cells utilise cytotoxic granules containing perforin and granzymes to induce apoptosis in target cells via the formation of an immunological synapse. In a mouse model of HSV-1 infection, CTLs have been shown to inhibit HSV-1 reactivation from latency (Knickelbein et al., 2008). This was shown to be due to the cleavage of HSV-1 ICP4 by granzyme B. However, in this

system neuronal apoptosis was not evident, even though granzyme B was being delivered to these cells. This raised the question of whether this was due to HSV inhibition of apoptosis or whether neurons are intrinsically resistant to granzyme B induced apoptosis.

Adenovirus L4-100K has been shown to inhibit granzyme B function and therefore CTL cytotoxicity, through containing a granzyme B cleavage site (Andrade et al., 2001). In this current work (Chapter 3), the ability of HSV ICP4 to inhibit NK cell cytotoxicity was addressed in a human *in vitro* setting. HSV ICP4 when expressed in isolation in 293T cells could not inhibit NK cell induced cytotoxicity. This suggests that the possession of a granzyme B cleavage site does not automatically confer resistance to NK cell killing. It would be useful to investigate whether the same observation can be made in the context of human CTLs to identify whether this is specific to NK cell mediated death. Additionally, NK granules can contain other cytotoxic compounds such as granulysin and granzyme A which could mask the ability of HSV ICP4 to block granzyme B induced apoptosis in the context of NK cell cytotoxicity (Krzewski and Coligan, 2012). To address this, recombinant granzyme B could be delivered to HSV ICP4 expressing cells, to determine whether HSV ICP4 can inhibit granzyme B induced apoptosis. In this study (Chapter 3) recombinant granzyme B was found to induce low levels of apoptosis when delivered *in vitro* using the BioPorter protein delivery system. Therefore, future work could focus on optimising the delivery of biologically active recombinant granzyme B to target cells.

There are additional HSV-1 proteins with known anti-apoptotic functions which could contribute to the inhibition of apoptosis during latency and viral reactivation. LAT, glycoprotein J and Us3 have all been shown to directly inhibit granzyme B induced apoptosis (Jerome et al., 2001, Cartier et al., 2003, Jiang et al., 2011). Additionally, LAT and Us3 have been shown to inhibit CTL induced cytotoxicity (Cartier et al., 2003, Jiang et al., 2011). It would be interesting to determine the importance of these proteins in controlling HSV-1 reactivation *in vivo* by abrogating the expression of anti-apoptotic gene products and observing the effect this has on the ability of the virus to reactivate. To date, the role of these gene products in preventing NK cell mediated cytotoxicity has not been addressed.

NK cells have primarily been linked to the control of HSV-1 lytic infection (Egan et al., 2013) whilst their role in the control of HSV-1 reactivation has been understudied. NK cells have been reported to infiltrate the trigeminal ganglia in a mouse latency model (Liu et al., 1996). In the context of the closely related alphaherpesvirus VZV, NK cells were observed as part of the immune infiltrate in DRG samples from patients who had suffered from herpes zoster 1-4.5 months before death (Gowrishankar et al., 2010). This suggests that NK cells could play a critical role in the response to HSV-1 reactivation. It would be of interest to examine the role of NK cells in preventing/ controlling HSV-1 reactivation in a mouse latency model potentially by depleting CTLs or NK cells and observing the effect on HSV-1 reactivation. Additionally, it would be possible to assess the capacity of NK cells to prevent HSV-1 reactivation *in vitro* via utilising latently infected stem cell derived neurons (Pourchet et al., 2017, D'Aiuto et al., 2019).

The role of CTLs and NK cells in VZV latency and reactivation

In contrast to HSV-1, much less is known regarding the control of VZV reactivation and the maintenance of latency. This is due to the host specificity of VZV and limited models of VZV latency. There have been several small animal models where VZV infection *in vivo* has been reported to establish latency, however the virus does not reactivate, perhaps due to an abortive infection (Laemmle et al., 2019). SVV infection of rhesus macaques has been used as a *in vivo* model to explore the role of the immune system in latency and reactivation (Sorel and Powers, 2018). In this model depletion of CTLs or T helper cells caused an increase in subclinical reactivation, suggesting that T cells are important in controlling SVV reactivation (Arnold et al., 2017). Reactivation *in vivo* caused T cell infiltration into the ganglia with neurons being surrounded by CTLs (Ouwendijk et al., 2013). This parallels findings from the study of DRG samples from patients with herpes zoster at the time of death, where CTLs were found closely associated with neurons (Steain et al., 2014). Furthermore, herpes zoster is known to be associated with waning cell-mediated immunity (Miller, 1980, Levin et al., 2003), further highlighting the importance of T cells in controlling reactivation.

As stated previously, NK cells have also been found to infiltrate human ganglia in post-mortem human ganglia samples (Gowrishankar et al., 2010), but they have not been

examined in the context of SVV latency and reactivation. It would be possible to explore this via the depletion of NK cells in the SVV latency model to assess whether subclinical reactivation occurs as with T cell depletion. Interestingly, VZV-infected neurons in close proximity to granzyme B+ CTLs in DRG samples do not display signs of apoptosis (Steain et al., 2014). This is a similar observation to neurons resisting CTL cytotoxicity in the HSV-1 latency mouse model (Knickelbein et al., 2008). Rhesus macaques have been shown to contain CTLs and NK cells which are granzyme B+ and can cause target cells to undergo immune cell mediated cytotoxicity (Webster and Johnson, 2005, Morrow et al., 2010). Therefore, determining whether NK cells and T cells also could release granzyme B and prevent SVV reactivation would also be of interest. In the current study, several VZV proteins were identified as being cleaved by granzyme B *in vitro* so it would be of value to identify whether SVV proteins can by cleaved by granzyme B and assess what effect this has on lytic and latent SVV infection *in vivo*.

Role of granzyme B during lytic and latent HSV-1 and VZV infection

Whilst the ability of granzyme B to inhibit HSV-1 reactivation has been assessed, it is unclear whether granzyme B can also inhibit HSV-1 spread in a lytic infection setting. Utilising the *in vivo* mouse model, it would be possible to identify NK cell/ CTL infiltration during cutaneous HSV-1 infection and determine whether HSV ICP4 is cleaved. In the current study (Chapter 3), HSV ICP27 was identified as being cleaved by granzyme B, confirmation of this *in vivo* in HSV-1 infection of the skin would be of interest. Cleavage of HSV-1 proteins during infection and assessment of viral replication and spread could be performed *in vitro* via the delivery of recombinant granzyme B. Similarly to HSV-1, multiple VZV proteins including VZV ORF4 and VZV ORF62 were identified as being cleaved by granzyme B in the current study (Chapter 3) (Figure 5.2). Interestingly, all of these proteins have roles in viral replication and/or host immune evasion, therefore determining the effect of granzyme B on VZV replication and spread *in vitro* should be a component of future studies. Overall, the role of granzyme B in controlling HSV-1 and VZV lytic infection needs to be further characterised.

As granzyme B has been shown to prevent HSV-1 replication *in vivo*, it may be able to help drive the virus into a latent-like state *in vitro*. This could be assessed via HSV-

1 infection of hESC derived neurons, delivery of recombinant granzyme B and measurement of viral transcription and protein expression at various time-points post granzyme B treatment. Additonally, as multiple VZV proteins are cleaved by granzyme B, it may be pertinent to investigate the ability of granzyme B to drive VZV into a latent like-state in hESC derived neurons.

Role of VZV immunoevasive functions in relation to PHN

From our study, it is clear that VZV has immunoevasive mechanisms to inhibit NK cell cytotoxicity against virally infected target cells. Other work from our lab has also shown that VZV can infect NK cells and inhibit their cytolytic capacity and ability to secrete effector cytokines (Campbell et al., 2019). NK cells have also been shown to play a role in the development of neuropathic pain in animal models. In a nerve crush injury model in mice, NK cells were shown to target injured neurons and induce apoptosis (Davies et al., 2019). When NK cells were depleted, mice displayed high levels of mechanical sensitivity 30 days after the original injury (Davies et al., 2019). In this model, injured neurons upregulated the NK cell activating ligand RAE-1. To date, there have been no studies exploring whether human DRG neurons can upregulate NK cell activating ligands in response to injury or viral infection. It would be interesting to determine whether viral infection could cause the upregulation of NKG2D ligands on isolated human DRG neurons and whether NK cells could selectively target these neurons. If this is the case, the functional paralysis of NK cells by VZV and inhibition of cytotoxicity could cause damaged neurons to persist after VZV reactivation and contribute to the development of hypersensitivity in PHN. Overall, this highlights that VZV modulation of NK cell cytotoxicity through both the manipulation of target cells and the NK cells themselves, could be an interesting avenue of exploration in the development of PHN.

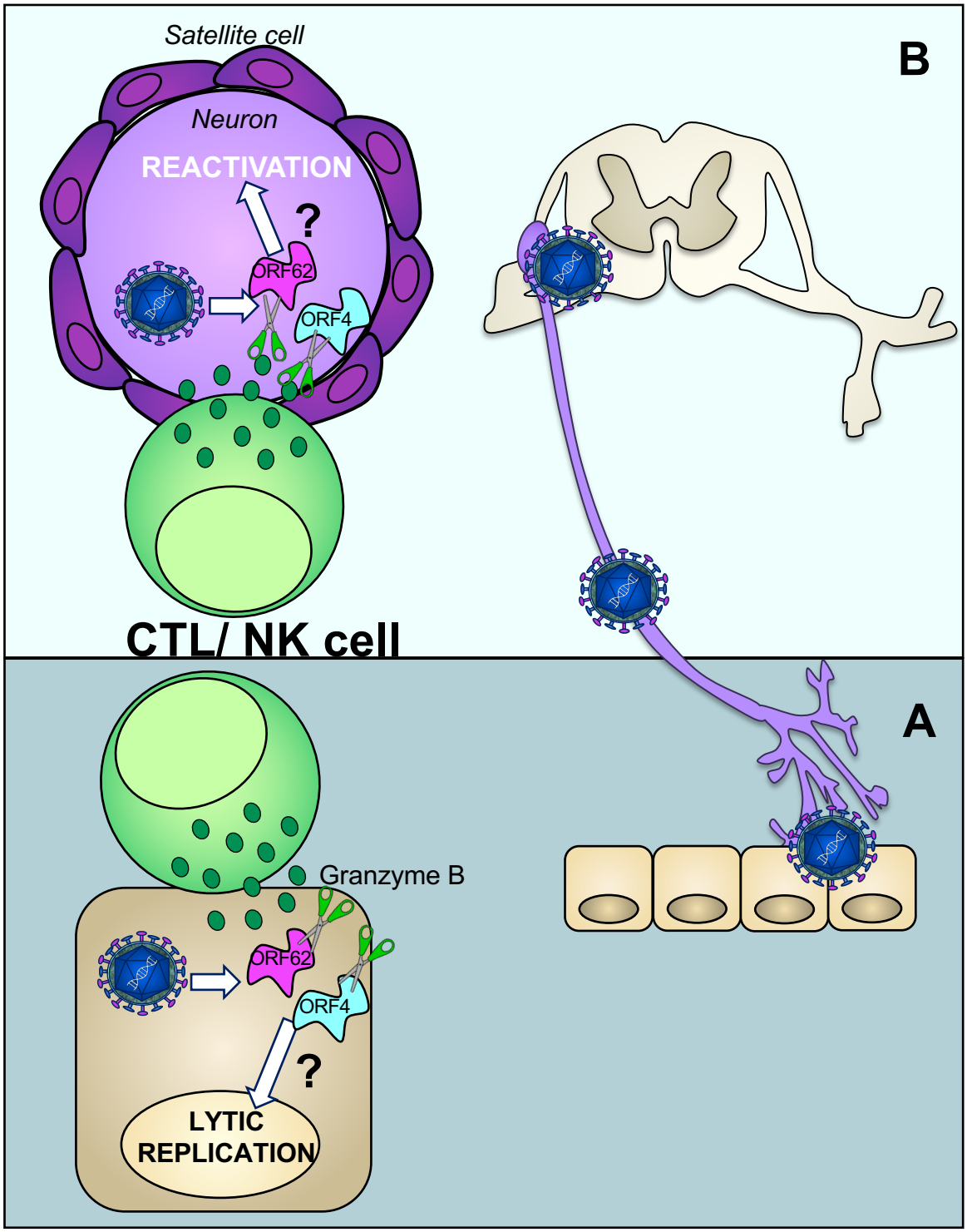


Figure 5.2 Granzyme B modulation of VZV pathogenesis.
During lytic infection, VZV produces proteins such as VZV ORF4 and VZV ORF62 which are essential for viral replication and spread (A). Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells can recognize virally infected target cells and deliver granzyme B through cytotoxic granules. In our study we have shown that granzyme B can cleave VZV ORF4 and VZV ORF62, however it is unclear whether this can effect lytic VZV replication. CTLs and NK cells can also infiltrate the ganglia post VZV reactivation (B). It is unclear in this setting whether delivery of granzyme B can prevent VZV reactivation through cleavage of VZV ORF4 and VZV ORF62.

Factors which contribute to the development of PHN

The development of PHN during herpes zoster is a fascinating facet of VZV pathogenesis that has been difficult to address due to limited model systems. Our study aimed to identify and characterise some of the factors which may contribute to the development of PHN such as modulation of key cell types and VZV clinical isolate variation. With the development of *in vivo* models of VZV-induced neuropathic pain it may be possible to explore these factors in more depth.

VZV modulation of the skin microenvironment

Skin cells such as keratinocytes have been shown to play a critical role in the development of neuropathic pain in animal models (Baumbauer et al., 2015). In punch biopsies of herpes zoster skin lesions, TRVP1 has been shown to be significantly upregulated in epidermal keratinocytes and this upregulation is positively correlated with the degree of pain (Han et al., 2016). Additionally, some patients with PHN respond to topical therapy with cannabinoids highlighting the skin microenvironment as a critical component in PHN (Phan et al., 2010).

Our study found that VZV infection could modulate the secretory profile of HaCaT cells (Figure 5.3). In general, VZV infection modulated factors critical for NK cell proliferation and caused a downregulation of chemokines critical for T cell migration. It would be interesting to determine whether these changes were capable of changing immune cell migration and function within the skin. It may be possible to examine this *in vitro* by utilisation of T cell migration assays and treatment of PBMCs with supernatant from VZV-infected HaCaTs (Graybill et al., 2017). It would also be pertinent to examine whether these changes occurred *in vivo* through the use of SCID hu skin explant mice or SVV infected rhesus macaques. Immune cells such as NK cells have been found to play a role in maintaining neuronal homeostasis. This is evident in a murine model where damaged neurons upregulated NKG2D ligands and were targeted by NK cells to aid in Wallerian degeneration (Davies et al., 2019). It would be interesting to determine whether VZV-infected human neurons also could be cleared by NK cells and whether VZV modulation of keratinocyte cytokine and chemokine secretion could affect NK cell cytotoxic potential. If damaged neurons during VZV reactivation were not eliminated by immune cells, this could contribute to the development of PHN.

Growth factors known to cause nociceptive neuron activation such as PDGF were increased by VZV infection of HaCaTs. It remains to be established whether supernatant from VZV-infected HaCaTs could affect neuronal functioning. This could be assessed *in vitro* by the generation of iPSC derived neurons and patch clamping, to determine whether treatment with VZV infected HaCaT supernatant affects the electrical potential and firing of neurons (Kiskinis et al., 2018). If interesting targets in VZV-infected HaCaT supernatant are identified, their contributions to PHN development could be assessed *in vivo* via the VZV-induced neuropathic pain rat model (Dalziel et al., 2004). For example, blocking antibodies to PDGF could be utilised in this model to determine whether targeting PDGF could have therapeutic benefits. HaCaTs can also be undifferentiated to biochemically resemble basal keratinocytes (Lehen'kyi et al., 2007). It would be interesting to determine whether the changes to the keratinocyte secretory profile induced by VZV are similar in undifferentiated HaCaTs, as basal keratinocytes are situated close to sensory nerve termini in the skin (Figure 5.3) Additionally, it may be of value to determine whether cannabinoids could affect keratinocyte and neuron functioning in this model to elevate neuropathic pain development. Overall a clearer understanding of VZV modulation of the skin microenvironment could yield some novel therapeutic targets for the treatment of PHN.

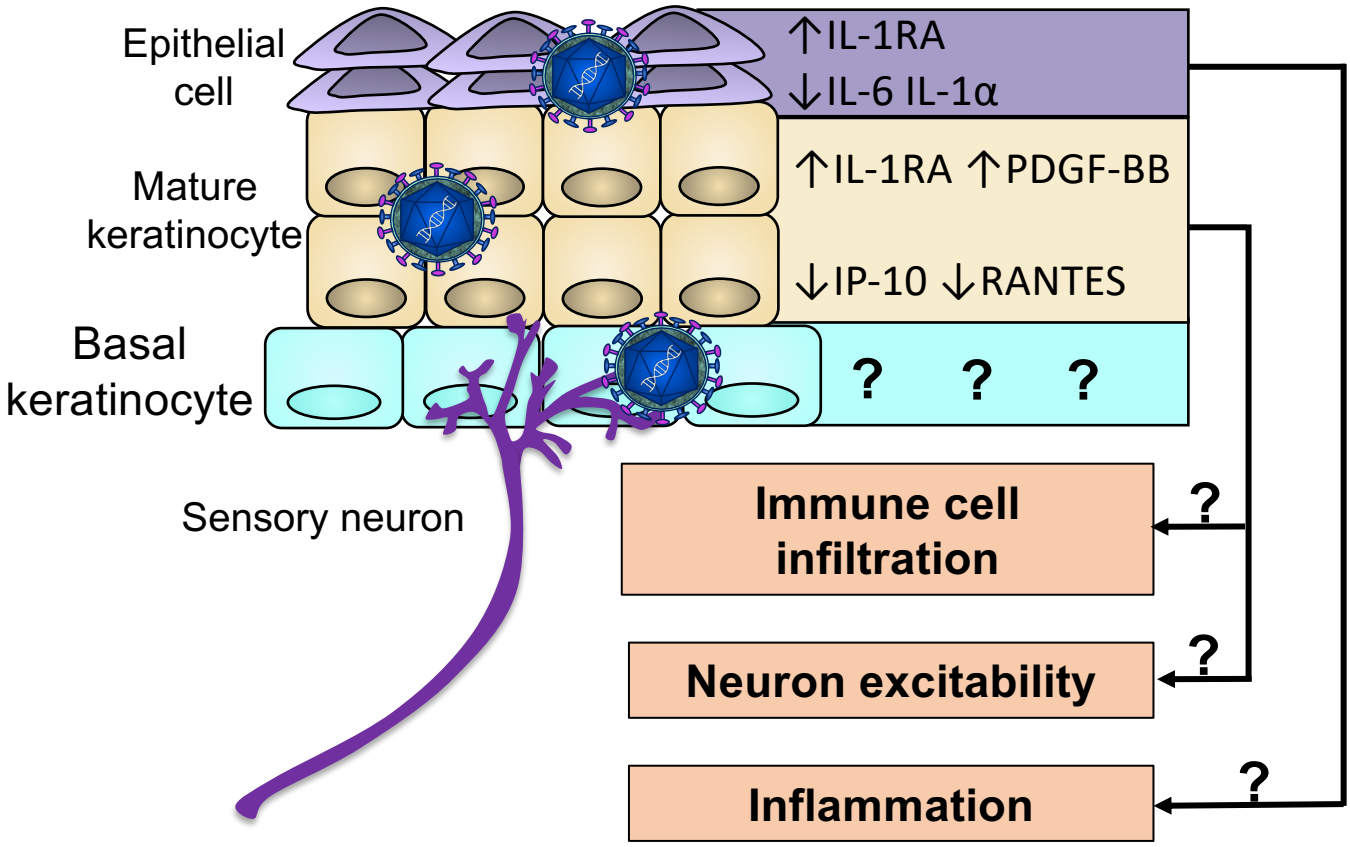


Figure 5.3 VZV modulation of the secretory profile of skin cells in the context of post herpetic neuralgia.
In our study we have shown that VZV can alter the secretory profile of epithelial cells and mature keratinocytes, this is yet to be examined in the context of basal undifferentiated keratinocytes. In epithelial cells, VZV infection alters the IL-1 axis by decreasing IL-1α and increasing IL-1RA secretion. Additionally, the secretion of IL-6 is downregulated with VZV infection suggesting a potential dampening of the inflammatory response. In keratinocytes, VZV was shown to decrease T cell chemokines such as IP-10 and RANTES potentially affecting T cell migration in the skin. Additionally, PDGF-BB secretion was increased by VZV infection which could alter sensory neuron excitability. VZV modulation of inflammation, immune cell infiltration and neuron excitability in the context of the skin could contribute to the development of peripheral sensitization and ultimately PHN.

Our study also analysed the effects of VZV infection on the secretory profile of epithelial cells (Figure 5.3). In contrast to previous studies, we did not observe significant changes in the chemokine profiles of VZV-infected ARPE-19s, however this may have been observed if a broader variety of chemokines were examined (Graybill et al., 2017). The major change we observed was alteration of the IL-1 axis in which VZV infection increased IL-1RA secretion and decreased IL-1α secretion. The IL-1 axis is critical in maintaining the barrier functions and inflammation of the skin (Jensen, 2010). It has previously been reported that HSV-1 infection of keratinocytes results in IL-1α secretion which acts as an alarmin in the skin (Milora et al., 2014). It would be interesting to determine whether VZV can inhibit IL-1α secretion to prevent its alarmin function or whether epithelial cells respond differently to keratinocytes in the context of alphaherpesvirus infection. Together VZV modulation of cells in the skin such as epithelial cells and keratinocytes could alter inflammation, immune cell migration and function, and neuronal excitability which could contribute to the development of PHN (Figure 5.3).

Viral isolate variation

As mentioned previously, VZV reactivation causes neuropathic pain to a greater extent than HSV-1 reactivation (Kinchington and Goins, 2011). This raises the question of whether there are unique pathogenic mechanisms utilised by VZV which causes neuropathic pain development. However, not all patients that experience VZV reactivation develop PHN (Kinchington and Goins, 2011). These facts lead us to raise the question of whether different VZV strains have different features which would influence their capacity to cause neuropathic pain. We received clinical VZV isolates from patients with and without PHN from Professor Judith Breuer, UCL, UK to address this question.

In this study, four isolates (2 non PHN and 2 PHN) were analysed to determine whether they induced CPE, spread and induced cell death similarly. In ARPE-19s all isolates were able to induce CPE, spread and induce cell death to a similar extent. This agrees with previous work in which PHN and non PHN isolates were found to grow to a similar extent in MeWos and have a similar viral gene expression (Ashrafi et al., 2010). Additionally, in regard to their ability to modulate ARPE-19 secretion of cytokines, chemokines and growth factors no differences were noted in the isolates

tested. Whilst we did not determine any differences in the context of skin cells, it may be more pertinent to test the ability of the isolates to grow and modulate neuronal cell function. It would be possible to assess this in the context of the SH-SY5Y cell line initially and if differences are found utilise iPSC derived neurons. The effects of PHN and non PHN VZV clinical isolates on neuron functioning has been examined in the context of the ND7/23-Nav1.8 rat DRG x mouse neuroblastoma hybrid cell line, where PHN-associated VZV was found to increase the sodium current amplitude in comparison to the non PHN VZV isolates (Kennedy et al., 2013). This suggests that PHN-associated isolates may alter neuron excitability and warrants further investigation (Kennedy et al., 2013). As VZV infection in rat/mouse neurons is abortive, it would be advantageous to use iPSC induced sensory neurons to further examine the effect of PHN VZV isolates on neuron excitability.

Additionally, testing further clinical isolates that are available will provide additional insights into whether strain specific differences can alter VZV pathogenicity in the skin and in neurons. Furthermore, it would be interesting to utilise these isolates in the rat model *in vivo* to determine if they cause different extents of neuropathic pain development.

Patient factors

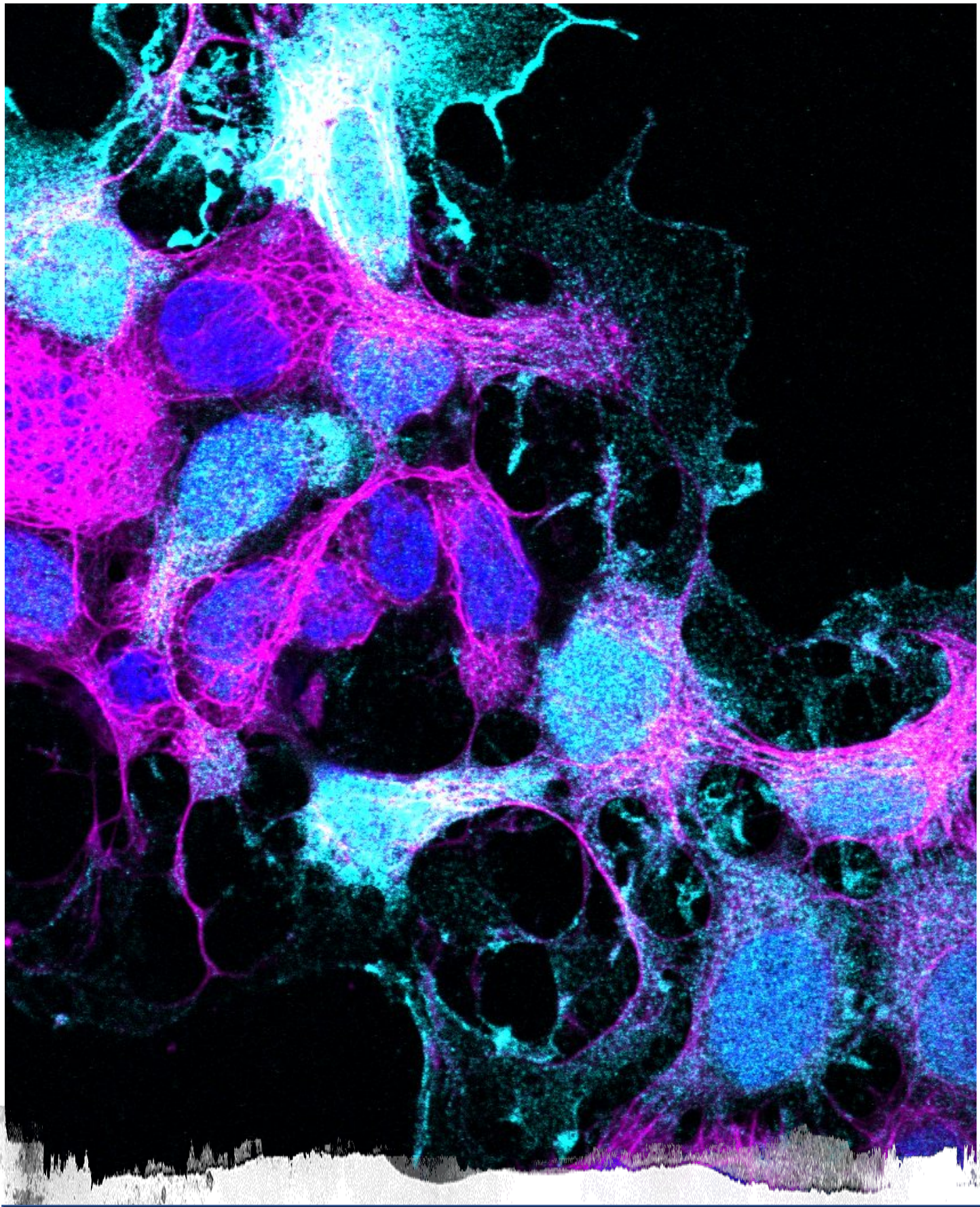
As discussed, there have been a variety of patient factors that have been linked to the development of neuropathic pain. In a recent systematic review, it was determined that older age was significantly associated with PHN development, whilst gender had conflicting reports in regard to predicting PHN development (Forbes et al., 2016). Additional factors such as immunosuppression, recent trauma and personality disorder syndromes have also been linked with an increased chance of PHN development (Forbes et al., 2016). With the rat model of VZV induced pain it is now possible to determine the potential biological basis behind these risk factors as most studies looking at PHN risk factors have mostly been correlational.

Whilst correlational studies have found conflicting reports in regard to the impact of gender on the risk of developing PHN, in the rat model of VZV induced orofacial pain female rats showed a longer affective response than male rats (Stinson et al., 2017). The phase of the female estrous cycle also influenced pain development which

suggests sex hormones may play a role in influencing PHN development (Stinson et al., 2017). Seeing as PHN mainly occurs in older patients, it would be interesting to utilise this model to determine whether any differences are observed between elderly rats and young rats in regard to VZV induced pain development.. Mature rats have shown differential microglial activation markers and neuropathic behaviours in response to L5 spinal nerve ligation in comparison to juvenile rats (Zeinali et al., 2016). Furthermore, it has been suggested that neuropathic pain development is supressed in younger rats by an anti-inflammatory response to nerve injury, therefore it would be interesting to identify if this anti-inflammatory response is present in young rats infected with VZV (McKelvey et al., 2015). Additionally, the effects of immunosuppression on VZV induced pain in rats could be studied. In this respect, it may be interesting to deplete individual immune subsets to determine if a particular subset is linked to neuropathic pain development.

Concluding statement

VZV is a ubiquitous human pathogen, whose ability to establish lifelong latency in the DRG can lead to devastating consequences in both immunocompromised and ageing populations (Zerboni et al., 2014). This is due to reactivation of VZV to cause herpes zoster which encompasses a broad variety of complications such as PHN (Gupta and Smith, 2012). Our understanding of how the virus maintains lifelong latency, reactivates and causes PHN is limited. The innate immune response is the first to respond to viral infection and reactivation, and VZV encodes many gene products with the ability to inhibit various aspects of innate immune response (Zerboni et al., 2014). Through studying the interplay between the host response and VZV immunoevasive mechanisms, novel therapeutic targets could be identified for the treatment of herpes zoster and PHN. In this study, different aspects of VZV evasion of the innate immune response were characterised, and the potential contribution of VZV modulation of keratinocytes and strain specific differences to the development of PHN were addressed. The role of VZV ORF63 in the inhibition of apoptosis was expanded to show that VZV ORF63 could inhibit apoptosis in both SH-SY5Ys and HaCaTs. This suggests that VZV ORF63 could play a crucial role in the establishment and maintenance of latency as well as viral replication and spread in the context of the skin. Additionally, a novel immunoevasive function for VZV ORF4 was uncovered in the context of NK cell cytotoxicity. Novel targets of granzyme B in both HSV-1 and VZV were identified, expanding the potential role of granzyme B for restricting viral replication in both lytic infection and reactivation. In regard to contributing factors to PHN development, VZV was shown to modulate keratinocyte secretion of cytokines and chemokines which could impact immune cell trafficking and function in the skin. Additionally, VZV was found to alter the IL-1 axis in both HaCaTs and ARPE-19s which could represent an immunoevasive mechanism utilised by VZV for successful spread in the skin. Clinical PHN and non-PHN isolates of VZV were compared to assess whether specific isolates had different characteristics *in vitro*, which could be linked to PHN development. No notable differences were observed in the isolates tested, suggesting PHN may be better predicted by patient factors. Together this work has increased our understanding of alphaherpesvirus pathogenesis, VZV immunomodulation and more broadly the ability of viruses to subvert human host defences.



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