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CHARACTERISATION OF THE IMMUNE RESPONSE FOLLOWING NATURAL HERPES ZOSTER REACTIVATION AND DURING POST-HERPETIC NEURALGIA

A THESIS SUBMITTED BY

JEREMY PHILIP SUTHERLAND

IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

2013

THE UNIVERSITY OF SYDNEY

SYDNEY MEDICAL SCHOOL
THE UNIVERSITY OF SYDNEY

CENTRE FOR VIRUS RESEARCH
WESTMEAD MILLENNIUM INSTITUTE
Declaration

The work presented in this thesis was completed by the author at the Department of Infectious Diseases and Immunology, University of Sydney and the Centre for Virus Research, Westmead Millennium Institute under the supervision of A/Prof Allison Abendroth, A/Prof Barry Slobedman and Dr Megan Steain. I certify that this thesis is my own work and all materials previously published or written by another author have been acknowledged and fully cited.

Jeremy Sutherland

August, 2013
Abstract

Varicella Zoster Virus (VZV) is responsible for two clinically distinct diseases; varicella (or chickenpox) during primary infection, and herpes zoster (or shingles) following reactivation. Herpes zoster involves active viral replication within the dorsal root ganglia (DRG), as well as in the innervated skin. Post-herpetic neuralgia (PHN) is the most common complication of herpes zoster. During PHN patients experience intense persisting pain generally in the same distribution as the original herpes zoster rash. There is very little known about the underlying pathology and pathogenesis of PHN.

One of the main reasons underlying this lack of understanding is the high species-specificity of VZV; animal and in vitro models are quite limited and study requires suitable human tissue. Previous studies involving limited histological examinations have revealed the presence of an immune infiltration and general cell loss within the DRG innervating the affected area. Our laboratory gained access to rare DRG samples that were surgically-excised from a PHN-affected patient in an attempt to control their pain. Additionally we have obtained DRG samples obtained at post-mortem examination from herpes zoster-affected patients and normal control patients. This has provided a unique opportunity to perform a detailed characterisation of the immune response to the virus within human sensory ganglia, as well as investigate viral gene expression and viral genome levels within the samples. An immune infiltration was detected in the PHN-affected patient material, and was composed of both CD4+ and CD8+ T cells with cytolytic potential, as well as B cells. Immunofluorescent investigation did not demonstrate any viral gene expression within the tissue, however molecular techniques showed that the VZV genome was present in the PHN-affected ganglia material at a level approximately seven-fold greater than that seen in a ganglion obtained from a patient with an active herpes zoster rash at the time of death.

In both primary infection and reactivation, VZV infection involves a similar exanthematous stage, and skin lesions are histopathologically indistinguishable from each other. However our understanding of the immune response in the skin during PHN is very limited. We obtained skin biopsy material from PHN-affected and control patients at least 6 months post-resolution of their herpes zoster-affected rash. This allowed the detailed examination of dendritic cell and T cell subsets present within the skin biopsy material of both patient types to identify any factors that may
predispose an individual to ongoing pain or resolution. The skin material from the PHN-affected patients contained a greater number of the immune cell subsets examined. Yet, there was no VZV antigen expression detected within these tissue samples.

We also gained access to an extensive tissue bank comprised of skin biopsies obtained from patients following diagnosis of herpes zoster. Each patient was followed for at least 6-12 months, and at each visit a biopsy was taken from the most painful area of skin and clinical details recorded. This tissue material was utilised in blinded immunofluorescent assays to identify different subsets of dendritic cells and T cells. There was no VZV antigen expression identified within this tissue material, however when biopsies were taken lesions were avoided. Different T cell and dendritic cell subsets were examined over time, and matched with the patients’ clinical histories in order to identify any factor that may correlate with reported pain quality and levels. Despite investigating multiple factors and cell types there was no marker identified that predisposed to ongoing pain or a prompt recovery.

Overall this thesis provides the first evidence of an ongoing chronic inflammatory process consisting predominantly of both CD4\(^+\) and CD8\(^+\) T cells within DRG from a PHN-affected patient many years post resolution of the herpes zoster rash. Within this chronically inflamed tissue, there is a large amount of VZV-specific DNA. In stark contrast to this, there does not seem to be any ongoing immunohistological process within the skin of patients post-herpes zoster. These findings have increased our knowledge on the immune reaction within human ganglia and skin following herpes zoster and during PHN and suggest that the best immune correlates of VZV diseases arising from VZV reactivation in ganglia are those that occur within the affected DRG rather than those that occur at cutaneous sites.
Acknowledgements

I would like to thank my supervisor A/Prof Allison Abendroth, for her guidance and support throughout the years. Thank you also to my co-supervisor A/Prof Barry Slobedman for your insight throughout the years. Thank you both for giving me this opportunity.

Thank you to my co-supervisor Dr Megan Steain. Your endless patience, enthusiasm, support and willingness to laugh with me at my mistakes helped me to keep going.

I would like to thank our collaborators in this research: A/Prof Michael Buckland of the Department of Pathology at the University of Sydney, Dr Michael Rodriguez of the Department of Forensic Medicine at the University of Sydney, Dr Karen Petersen of the University of California, San Francisco and Dr Frank Rice of Albany Medical College. Without your precious sample material this research would not have been possible.

I would like to thank the VZV laboratory members through the years: Jenna, Liz, Kavi, Jen, Rodney, JB and Kate. Thank you to the CMV laboratory members through the years: Brian, Selmir, Jo, John, Sam, Simone, Winnie and the Sarahs. Thanks for your assistance, advice, support and encouragement. I feel honoured for having the opportunity to complete my research with such a great group of people, and am thankful for your friendship.

Thanks to the Blackburn gang for the laughs and all: Jono, Chris, Sam, Sara, Claudio, Zainun and Rahma. Thanks to Rebecca and Catherine: you make the department happen. Thanks to Belinda and Becky, sushi was amazing. Thanks to everyone in IDI and Behavioural Sciences for putting up with me for so long.

I would like to thank the members of the Histopathology Laboratory and in particular Dr Jane Radford. You provided me with much needed advice and help, especially when things stopped working.

I would like to thank Dr Louise Cole of the Advanced Microscope Facility at the Bosch Institute. Thanks for all your training, troubleshooting and chats. Also thankyou for allowing me to use well beyond my allocated weekly time limit through the night when no one else was looking.
Thanks to everyone who provided tissue, cells, slides, antibodies, reagents, expertise and everything along the way. I can’t possibly acknowledge you all individually, but know that this project would not be complete without your help. I am eternally grateful.

Thanks to my wonderful thesis proof-readers: Megan, Brian, Michal, Caroline, Julian and Laura. Thanks for helping me out with the final stages, when I was starting to lose hope. Thanks to my aunt Dr Louise Sutherland. You went above and beyond with your reading and editing suggestions, and you were always available for a chat.

Thanks to my mates. You kept me sane.

Thanks goes to my family, for being there whenever I needed you and for what ever I needed you for. Thanks to Mum and Dad, for supporting me despite my decisions. Thanks to my brothers: James, Myles and Toby, for being around when I needed time off and being willing to sit through the occasional practice presentations.

And final thanks to my God, for providing me with such a great opportunity, and enabling me to complete it.
Publications and other material arising from work in this thesis

Publications


Presentations


Scholarships and Awards

• Australian Postgraduate Award 2009-2012
• Sydney Medical School Research Top-Up Scholarship 2009-2012
• Daphne Goulston Scholarship 2011
• Poster Award – 6th Australasian Virology Society Meeting, Kingscliff NSW, 2011
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Abbreviations

α  Alpha
ADCC  Antibody-dependent cellular cytotoxicity
AIDS  Acquired immunodeficiency syndrome
ARN  Acute retinal necrosis
β  Beta
β2m  Beta-2 microglobulin
BDNF  Brain derived neurotropic factor
bp  Base pairs
BSA  Bovine serum albumin
CCD  Charge coupled device
CD  Cluster of differentiation
cDNA  Complementary deoxyribonucleic acid
CNS  Central nervous systems
CPE  Cytopathic effect
CTL  Cytotoxic T lymphocyte
δ  Delta
DAB  Diaminobenzidene
DAPI  4’,6-diamidino-2-phenylindole
°C  Degrees centrigrade
DC  Dendritic cell
dDC  Dermal dendritic cell
dH2O  Distilled H2O
DMEM  Dulbecco’s modified essential medium
DNA  Deoxyribonucleic acid
DNase  Deoxyribonuclease
dATP  Deoxyadenosine triphosphate
dCTP  Deoxycytidine triphosphate
dGTP  Deoxyguanosine triphosphate
dH2O  Distilled water
dNTP  Deoxyribonucleotide
dTTP  Deoxythymidine triphosphate
DPX  DePeX mounting media
DRG  Dorsal root ganglia
ds  Double stranded
E  Early
EBV  Epstein-Barr virus
EDTA  Ethylene diamine tetra acetic acid
FAM  6-carboxyfluorecein
FBS  Fetal bovine serum
γ  Gamma
g  Grams
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
gB  Glycoprotein B
gC  Glycoprotein C
gE  Glycoprotein E
gH  Glycoprotein H
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>gI</td>
<td>Glycoprotein I</td>
</tr>
<tr>
<td>gK</td>
<td>Glycoprotein K</td>
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<tr>
<td>gL</td>
<td>Glycoprotein L</td>
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<tr>
<td>gM</td>
<td>Glycoprotein M</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<tr>
<td>HFF</td>
<td>Human foreskin fibroblasts</td>
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<tr>
<td>HHV</td>
<td>Human herpesvirus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HSV-1</td>
<td>Herpes simplex virus type 1</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes simplex virus type 2</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
</tr>
<tr>
<td>ICP</td>
<td>Infected cell protein</td>
</tr>
<tr>
<td>IDE</td>
<td>Insulin degrading enzyme</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescent assay</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>infHFF</td>
<td>VZV-infected human foreskin fibroblasts</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>IRl</td>
<td>Internal repeat long</td>
</tr>
<tr>
<td>IRs</td>
<td>Internal repeat short</td>
</tr>
<tr>
<td>ISH</td>
<td>In-situ hybridisation</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s Sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>L</td>
<td>Late/long/litres</td>
</tr>
<tr>
<td>LAT</td>
<td>Latency associated transcript</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser capture microdissection</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>m²</td>
<td>Square metres</td>
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<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetres</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitres</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MNC</td>
<td>Mononuclear cell</td>
</tr>
<tr>
<td>MPR&lt;sup&gt;ci&lt;/sup&gt;</td>
<td>Cation-independent mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
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<td>nm</td>
<td>Nanometres</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
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<td>PHN</td>
<td>Post-herpetic neuralgia</td>
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<tr>
<td>pmol</td>
<td>Picomole</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<td>pOka</td>
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<td>PORN</td>
<td>Progressive outer retinal necrosis</td>
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<tr>
<td>qRT-PCR</td>
<td>Qualitative reverse transcription polymerase chain reaction</td>
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<tr>
<td>rER</td>
<td>Rough endoplasmic reticulum</td>
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<td>RHS</td>
<td>Ramsay Hunt syndrome</td>
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S  Short
SCID  Severe combined immunodeficiency
sER  Smooth endoplasmic reticulum
SOC  Super optimal broth with catabolite repression
SOR  Site of reactivation
SVV  Simian varicella virus
T10  Thoracic level 10
T11  Thoracic level 11
T12  Thoracic level 12
TAE  Tris-acetate
TBS  Tris buffered saline
TdT  Terminal deoxynucleotidyl transferase
TG  Trigeminal ganglia
TIA-1  T cell intracytoplasmic antigen 1
tRNA  Transfer RNA
TRl  Terminal repeat long
TRs  Terminal repeat short
U  Units
UCSF  University of California, San Francisco
UL  Unique long
µm  Micrometres
µm²  Square micrometres
US  Unique short
uniHFF  Uninfected human foreskin fibroblasts
| V1       | Ophthalmic branch of trigeminal nerve |
| V2       | Maxillary branch of trigeminal nerve  |
| V3       | Mandibular branch of trigeminal nerve |
| VAS      | Visual analogue scale                |
| vOka     | VZV vaccine (Oka strain)             |
| v/v      | volume per volume                    |
| VZV      | Varicella zoster virus               |
| w/v      | weight per volume                    |
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Ethics details

Work in this thesis was performed in accordance with ethics guidelines of the University of Sydney and Western Sydney Area Health Service.

Ethics approval for research presented in this thesis was obtained from the University of Sydney Human Research Ethics committee. Committees operate under the National Statement on Ethical Conduct in Human Research (2007) issued by the National Health and Medical Research Council (NHMRC) in accordance with the NHMRC Act, 1992 (Cth).

Project title: Mechanisms of Immune Modulation by Varicella Zoster Virus during Cutaneous Infection

HREC Protocol #7906

Project title: Immunopathogenesis of Varicella Zoster Virus

Additional title: Effects of Varicella Zoster Virus Infection in Human Ganglia

HREC Protocol #9857
Chapter 1 - Literature review

1.1 The *Herpesviridae* family

The *Herpesviridae* family is a large ubiquitous group of viruses, with most animal species serving as possible hosts for at least one *Herpesviridae* family member (Pellett and Roizman, 2007). Viruses in this family rarely infect more than one species, but cause a broad range of diseases in their hosts. To date there have been more than 130 members of this family identified (Pellett and Roizman, 2007), with 8 known to infect humans. These are termed human herpesviruses (HHVs), and include Herpes Simplex virus 1 (HSV-1) or HHV-1, Herpes Simplex virus 2 (HSV-2) or HHV-2, Varicella Zoster virus (VZV) or HHV-3, Epstein-Barr virus (EBV) or HHV-4, Human Cytomegalovirus (HCMV) or HHV-5, HHV-6, HHV-7 and Kaposi's sarcoma-associated herpesvirus (KSHV) or HHV-8 (Pellett and Roizman, 2007).

1.1.1 Properties of *Herpesviridae*

All *Herpesviridae* family members share a common virion structure and a broad set of biological properties. Herpesviruses encode a wide range of viral enzymes involved in nucleic acid metabolism, DNA synthesis, and protein processing (Roizman and Baines, 1991). Viral DNA synthesis and capsid assembly occurs in the nucleus of infected cells, and virions are enveloped as they are released from the nucleus (Roizman and Baines, 1991, Pellett and Roizman, 2007). However it is not known whether there is an initial de-envelopment and re-envelopment as the virion moves through the cell, or if it remains enveloped throughout the process (Pellett and Roizman, 2007). Herpesviruses all have a lytic infection cycle, which includes the production of infectious progeny and the subsequent destruction of the infected cell. They also have the ability to establish a life-long latent infection in their natural hosts. Within latently infected cells the viral genome takes a closed circular structure, there is limited viral gene expression and the virus retains the capacity to reactivate (Pellett and Roizman, 2007).

Viruses are included in this family based on the architecture of their virions. A typical herpesvirus virion consists of a linear double stranded DNA (dsDNA) core, surrounded by an icosadeltahedral capsid consisting of 162 capsomeres and is approximately ~110 nm in diameter (Figure 1.1). Between the envelope and capsid there is an amorphous protein layer known as the tegument. The tegument has no
Figure 1.1 – Herpesvirus virion structure

The common virion structure shared between herpesvirus family members. This consists of a dsDNA genome contained within a nucleocapsid, surrounded by tegument proteins, enclosed within a viral envelope studded with various glycoproteins.

(Image courtesy of Gavin Morrow, University of Sydney)
distinctive features, but may appear fibrous in negative staining (Pellett and Roizman, 2007). The virus is enclosed in an envelope containing glycoprotein spikes, and viral particles are between 180-300 nm in diameter (Pellett and Roizman, 2007).

1.1.2 Classification of Herpesviridae

*Herpesviridae* family members are further classified into three subfamilies according to biological properties, replication efficiency, cytopathology and characteristics of latent infection (Roizman, 1979, Anonymous, 1973). These subfamilies are *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*.

1.1.2.1 *ALPHAHERPESVIRINAe*

Members of the *Alphaherpesvirinae* subfamily characteristically have a relatively short replication cycle, spread rapidly in cell culture, and efficiently destroy infected cells during lytic infection. These viruses have the ability to establish latency primarily in sensory ganglia (Croen et al., 1988, Roizman and Baines, 1991). Examples of human herpesviruses classified in this subfamily are the herpes simplex viruses and varicella zoster virus.

1.1.2.2 *BETAHERPESVIRINAe*

Viruses in the *Betaherpesvirinae* subfamily have a restricted host range, with a long replication cycle and slow spread in cell culture. Cells infected with these viruses frequently become enlarged due to cell-to-cell fusion (Pellett and Roizman, 2007). Latency is established in tissues such as secretory glands, kidneys, lymphoreticular cells and some myeloid progenitor cells (Bevan et al., 1993, Pellett and Roizman, 2007, Slobedman et al., 2010). Examples of human herpesviruses in this subfamily are HCMV and HHV-7.

1.1.2.3 *GAMMAHERPESVIRINAe*

Members of the *Gammaherpesvirinae* are classified according to a tropism for replication and latency in lymphoblastoid cells. Although some members have a lytic cycle in epithelial and fibroblastic cell types, viruses in this group are generally specific for either T or B lymphocytes. This allows further classification of this subfamily into gamma-1 (γ-1) and gamma-2 (γ-2) subfamilies, depending on tropism for T or B lymphocytes respectively. Included in this subfamily are EBV and KSHV; EBV has the ability to immortalise infected cells *in vitro*, and has been associated with Hodgkin’s lymphoma and Burkitt’s lymphoma, while KSHV commonly causes
Kaposi’s sarcoma in immunocompromised individuals (Landais et al., 2005, Rickinson and Moss, 1997, Speck and Ganem, 2010).

1.2 Varicella Zoster Virus

Varicella Zoster Virus – also called HHV-3, is a human herpesvirus that belongs to the alphaherpesvirinae subfamily. It is the only member of the Varicellovirus genus known to infect humans (Arvin and Gilden, 2007). Primary infection causes varicella (or chickenpox), and reactivation from latency results in herpes zoster (shingles).

1.2.1 The virion and viral genome

The VZV virion structure follows the general characteristics of all herpesviruses. It consists of four main components; a linear dsDNA genome core enclosed within three layers: a nucleocapsid, a tegument, and outermost an envelope with protruding viral glycoprotein spikes (Figure 1.1).

The VZV core consists of a linear dsDNA genome of around 125 000 base pairs (bp) in length (Arvin and Gilden, 2007). The complete sequence was published in 1986 by Davison and Scott, and was found to consist of two joined segments known as Long (L) and Short (S) (Figure 1.2) (Davison and Scott, 1986). The L segment is comprised of a unique long region (UL) (around 105 000 bp) which is flanked by two inverted repeat regions; terminal repeat long (TRL) and internal repeat long (IRL) (both 88.5 bp in length). The S segment is comprised of a unique short region (US) (around 5 232 bp) flanked by two internal repeat regions; terminal repeat short (TRS) and inverted repeat short (IRS) (each 7 319.5 bp in length) (Arvin and Gilden, 2007, Davison and Scott, 1986, Ecker and Hyman, 1982). The VZV genome contains at least 71 unique open reading frames (ORFs), three of which are present as duplicates in the IRS and TRS regions (Arvin and Gilden, 2007). The duplicated genes are ORF62, ORF63 and ORF64, which are also present as ORF71, ORF70 and ORF69 respectively (Arvin and Gilden, 2007).

The VZV nucleocapsid contains the viral genome, and is indistinguishable in appearance from that of other herpesviruses. Electron micrographs have shown a structure of 162 capsomeres with a 5:3:2 axial symmetry. In this icosohedron structure of 80-120 nm, pentameric proteins form the vertices and hexameric elements comprise its facets (Arvin and Gilden, 2007).
Figure 1.2 – Varicella zoster virus genome structure

The varicella zoster virus genome consists of a linear double-stranded DNA molecule containing two regions known as Long (L) and Short (S) segments. The L segment contains a unique long region (UL) flanked by two inverted repeat regions (TR_L and IR_L). The S segment contains a unique short region (US) flanked by two internal repeat regions (TR_S and IR_S).
The nucleocapsid is surrounded by the tegument; an amorphous material with no
distinctive features. It does contain several major transcriptional regulatory proteins,
including proteins encoded by ORFs 4, 47, 62 and 63 (Kinchington et al., 1995,
Kinchington et al., 1992, Stevenson et al., 1994).

Enveloped VZV virions are usually spherical in shape, and range from 180-200 nm in
diameter (Arvin and Gilden, 2007) (Figure 1.3). The viral envelope consists of
patches of host cell membranes modified to display virally encoded glycoproteins
(Arvin and Gilden, 2007). The virus passes through various cellular organelles during
maturation and consequently the exact origin of this envelope is unknown (Achong
 glycoproteins each have a homologous glycoprotein on the HSV-1 virion (Litwin et
al., 1992), and are the major antigenic determinants of the VZV virion in antibody
responses (Arvin and Gilden, 2007).

1.2.2 Replication of Varicella Zoster Virus

1.2.2.1 VIRION ATTACHMENT AND ENTRY

The VZV replication cycle begins with attachment of the virion to the host cell. Like
other herpesviruses this is thought to occur by fusion of the viral envelope with the
cytoplasmic membrane (Arvin and Gilden, 2007). Initial contact between the virion
and host cell occurs via non-specific electrostatic interactions between viral
glycoproteins present in the virion envelope and cellular glycosaminoglycans (such
as heparin sulphate) (Zhu et al., 1995a). VZV glycoprotein B (gB) has been
implicated in this stage of viral entry (Jacquet et al., 1998). Following these initial low-
affinity interactions, the VZV virion engages specific cell-surface receptors, two of
which have been potentially identified, to strengthen virion attachment to the host
cell. The cation-independent mannose-6-phosphate receptor (MPR\(^{ci}\)) has been found
to interact with phosphorylated N-linked complex oligosaccharides, which are present
on some viral glycoproteins, and works to facilitate infection of stable cell lines with
cell-free VZV. However this receptor does not mediate cell-to-cell spread of the virus

The second proposed VZV-specific cell-surface receptor is the insulin degrading
enzyme (IDE), which has been shown to facilitate both cell-associated and cell-free
VZV infection in a range of cell types (Li et al., 2006). This is thought to occur
through IDE interacting with glycoprotein E (gE), which has been found to be an
essential glycoprotein in VZV infection (Li et al., 2006). Furthermore gE residues 24-
Figure 1.3 – Electron micrographs of varicella zoster virions

Transmission electron micrographs of varicella zoster virions (arrows) in infected human explant dorsal root ganglia. (A) Viral particles are present within the cytoplasm (arrow head) and extracellular space between cells (black arrow). Microtubular structures (white arrow) are indicative of a neuronal cell. (B) Magnified region of inset in A, showing a fully assembled extracellular, enveloped virion.

(Picture courtesy of Kavitha Gowrishankar, University of Sydney from Gowrishankar et al., 2007)
71 were shown to be required for IDE binding, and the native secondary structure is also important (Li et al., 2007). However more recently it was found that IDE bound the precursor form of the gE protein rather than the mature membrane-associated form. Consequently the IDE:gE interaction is more likely an interaction that occurs within the cytosol of already infected cells rather than a receptor/ligand interaction that occurs during virion attachment (Carpenter et al., 2010). In a cell-free VZV model of infection virion entry occurs in a cholesterol-dependent manner, and may undergo receptor-mediated endocytosis (Hambleton et al., 2007).

### 1.2.2.2 Viral gene expression

Following VZV entry into the host cell, the virion uncoats and the nucleocapsid and tegument proteins are transported into the nucleus. This results in the release of the viral genome, and the initiation of VZV gene expression. This is presumed, like other herpesviruses, to occur in a highly regulated and ordered cascade. The 71 ORFs encoded in the VZV genome can be divided into three distinct groups based on their expression patterns and timing requirements (Honess and Roizman, 1974). These groups are the immediate early (IE or α), early (E or β) and late (L or γ) genes.

The first viral proteins to be expressed are the IE proteins. These act as major transcription regulators for both the early and late genes (Hambleton and Gershon, 2005). Three different IE proteins that are present in the viral tegument are encoded by ORF4, ORF62 and ORF63 (Debrus et al., 1995, Defechereux et al., 1997, Forghani et al., 1990b), and are homologous to the HSV-1 IE genes infected cell protein (ICP) 27, ICP4 and ICP22 respectively (Arvin and Gilden, 2007). A fourth, ORF61, is not a component of the viral tegument, but is homologous to the HSV-1 IE gene ICP0 (Arvin and Gilden, 2007), and newly synthesised IE61 protein can be detected along with IE62 as early as 1 hour post-infection (Reichelt et al., 2009).

The predominant IE protein is IE62, which is the major transcriptional regulatory protein. IE62 is derived from transcripts of both ORF62 and ORF71, which are located in the inverted repeat section of the US region (Davison and Scott, 1986). During infection of a permissive host cell, IE62 localises primarily within the nucleus. However co-expression with ORF66 protein results in cytoplasmic accumulation, which is observed late in the VZV infection cycle (Kinchington et al., 2000). IE62 has the capacity to transactivate many VZV genes from all three kinetic classes (Kinchington et al., 1992), and is required for both cell-to-cell spread in vivo, as shown in the human skin tissue xenografts in the severe combined
immunodeficiency (SCID) mouse model (or SCID-hu model), and for VZV replication in vitro (Sato et al., 2003a).

The exact function of IE63 is less clear although it has been shown to be important in maintaining viral virulence. ORF63 is required for growth in cell culture and ORF63 mutants were either non-virulent or had reduced virulence in the SCID-hu skin model, but mutations had no effect on replication in the SCID-hu T cell model (Baiker et al., 2004a, Sommer et al., 2001). IE63 has been shown to be part of the viral tegument (Kinchington et al., 1995). IE63 is located primarily within the nucleus during productive infection, however early in the infection it is found in both the nucleus and cytoplasm (Arvin and Gilden, 2007). IE63 has been shown to interact with IE62 to upregulate the transactivation of the VZV glycoprotein promoter, and may also interact with the cellular RNA polymerase (Lynch et al., 2002). ORF63 may also encode an anti-apoptotic factor and has been shown to protect neurons from apoptosis in vitro (Hood et al., 2006). Furthermore IE63 is the most prevalent and abundant transcript observed in latently infected human ganglia (Cohrs and Gilden, 2007).

ORF4 encodes another IE protein, which is predominantly located in the cytoplasm of infected cells, however on co-expression with IE62, it is primarily located within the nucleus (Defechereux et al., 1996). ORF4 protein has also been shown to be part of the viral tegument (Kinchington et al., 1995). ORF4 is an essential viral gene, as deletion has been shown to result in the loss of infectious virus (Sato et al., 2003b), and the protein product has also been shown to dimerise and transactivate other viral genes (Defechereux et al., 1997, Sato et al., 2003b).

Following the expression of functional IE proteins, E genes are expressed, and traditionally peak expression is thought to occur 5-7 hours post-infection (Honess and Roizman, 1974), however more recent work has shown it occurs much earlier (Reichelt et al., 2009). E genes primarily encode enzymes and transcription factors important for viral DNA replication and phosphorylation, and nucleotide metabolism (Kinchington and Cohen, 2000). The E gene ORF28 encodes the large subunit of the viral DNA polymerase, and ORF16 is believed to encode the small subunit (Arvin and Gilden, 2007, Davison and Scott, 1986). This enzyme complex is required for viral DNA replication (Arvin and Gilden, 2007). Other viral enzymes essential for replication are the single-stranded DNA binding protein (encoded by ORF29) and the origin binding protein (encoded by ORF51) (Kinchington et al., 1988, Stow et al.,
The protein encoded by ORF29 has a predominantly nuclear localisation within productively infected cells (Kinchington et al., 1988).

Other important E proteins are encoded by ORF36 (viral thymidine kinase); ORF18 and ORF19 (ribonucleotide reductase); and ORF47 and ORF66 (two different serine/threonine protein kinases) (Davison and Scott, 1986, Mahalingam et al., 1990, Smith and Smith, 1989).

The protein encoded by ORF29 has a predominantly nuclear localisation within productively infected cells (Kinchington et al., 1988).

The protein product of ORF47 has a cytoplasmic localisation in VZV-infected cells, and is a component of the virion capsid and tegument (Stevenson et al., 1994). ORF47 is essential for virus replication in human dendritic cells and human T cells, and for spread to other cells in vitro (Hu and Cohen, 2005, Sato et al., 2003c). ORF47 is also required for replication in human skin (Moffat et al., 1998b). Targeted ORF47 mutations have shown that a functional ORF47 protein kinase is required for replication and cell-to-cell spread in human skin in vivo, however this may involve some interaction between the ORF47 and IE62 proteins (Besser et al., 2003). More recently, ORF47 protein has been shown to interfere with innate immune responses by inhibiting the activation of the interferon regulatory factor 3, and functions to regulate many viral proteins throughout the infection cycle (Vandevenne et al., 2011, Kenyon and Grose, 2009).

Another component of the virion capsid and tegument is the ORF66 protein, which is also located within the cytoplasm of virally infected cells (Stevenson et al., 1994). The protein product of ORF66 can phosphorylate IE62, which inhibits its translocation to the nucleus (Kinchington et al., 2000). ORF66 protein has also been implicated in the downregulation of the major histocompatibility complex (MHC) class I expression in VZV-infected human fibroblasts (Abendroth et al., 2001a, Eisfeld et al., 2007), suggesting the gene product plays a central role in immune evasion strategies employed by the virus. ORF66 protein expression is required for phosphorylation of the essential nuclear matrix protein matrin 3 at a unique site, affecting its cellular localisation throughout the infection cycle (Erazo et al., 2011).

The final class of viral genes to be expressed in the VZV replication cycle are the L genes. L gene expression depends on prior protein expression from the IE and E genes (Honess and Roizman, 1974). L genes predominantly encode major structural proteins, including components of the tegument and nucleocapsid, as well as the viral glycoproteins. Examples of L genes include ORF33.5, ORF33, ORF40, ORF21 and ORF10, as well as the glycoproteins (Arvin and Gilden, 2007).
ORF33.5 encodes an assembly protein which is thought to act as a scaffolding protein for nucleocapsid assembly, and is located in the 3' region of ORF33 (McMillan et al., 1997). The major constituents of the nucleocapsid are encoded by the ORF21 and ORF40 genes (Mahalingam et al., 1998, Vafai et al., 1990). The ORF10 gene product forms part of the viral tegument (Kinchington et al., 1992), and is important for viral virulence (Moriuchi et al., 1993).

There have been 8 VZV glycoproteins identified to date (Storlie et al., 2008b). The most abundant is gE, which is encoded by ORF68 (Montalvo et al., 1985). gE is required for VZV replication, and has been shown to induce tight junction formation in epithelial cells, and hence increase cell-to-cell contact (Mo et al., 2002, Mo et al., 2000). As well as the monomeric and dimeric forms, gE is also found as a heterodimer with either glycoprotein I (gI) on infected cells (Kimura et al., 1997) or glycoprotein H (gH) on VZV virions (Maresova et al., 2005). The gE:gI complex is formed through a cysteine rich region, the loss of which has been shown to differentially affect cell-to-cell spread and viral entry (Berarducci et al., 2009).

Although generally considered a L gene product, one recent study found that gE accumulated in the Golgi apparatus as early as 4 hours post-infection (Reichelt et al., 2009). Unlike the HSV homologue, VZV gE appears to play an important role in regulating membrane fusion and syncytial formation (Reviewed in Cole and Grose, 2003, Pasieka et al., 2004). Interestingly there have been gE-mutant viruses isolated from clinical cases (Santos et al., 1998, Tipples et al., 2002), and these viruses show accelerated spread in both cell culture and the SCID-hu mouse model (Santos et al., 2000). The N-terminal region of gE has been shown to be important for infection in skin and T cell models (Berarducci et al., 2010).

While gE is indispensable for VZV replication in vitro, gI, encoded by ORF67, has been shown to be dispensable in some cell types (Mallory et al., 1997). However mutations in or deletions of gI resulted in disrupted syncytium formation, and altered conformation and distribution of gE in infected cells, which may indicate that gI assists in the cell-to-cell spread of the virus in vitro (Mallory et al., 1997). While gI is dispensable for viral infection of human melanoma cells (Cohen and Nguyen, 1997), it is essential for viral replication in human skin and T lymphocytes as shown in the SCID-hu mouse model (Moffat et al., 2002). Other investigations using the SCID-hu mouse model have shown a gI-deletion mutant virus failed to establish a persistent infection in human dorsal root ganglia (DRG) xenografts in the SCID-hu mouse.
model, suggesting that gI is required for the establishment of latency in vivo (Zerboni et al., 2007).

Glycoproteins C (gC) and K (gK), encoded by ORF14 and ORF5 respectively, have also been shown to be important in viral replication (Mo et al., 1999, Moffat et al., 1998a). gC is dispensable for growth in cell culture (Cohen and Seidel, 1994). However it is important for virus growth in skin implants in the SCID-hu mouse model (Moffat et al., 1998a). gC expression levels on VZV-infected cells varies between different viral strains (Kinchington et al., 1990). Interestingly limited gC expression occurs within the first 48 hours of infection, while other glycoproteins are easily detectable within 24-48 hours (Storlie et al., 2008a). Partial and full deletions in gK using the VZV cosmid system were shown to prevent viral replication in melanoma cell lines (Mo et al., 1999).

gB, encoded by ORF31, is the second most abundant viral glycoprotein (Arvin and Gilden, 2007), and an epitope within gB induces a neutralising antibody response (Forghani et al., 1990a). Recombinant gB has the ability to bind cellular heparin sulfate proteoglycans, and the recombinant protein delays the virion entry into cells (Jacquet et al., 1998). Consequently it is thought to be important in virion attachment and entry into host cells (Vafai et al., 1984).

gH is encoded by ORF37, and is the third most abundant glycoprotein (Arvin and Gilden, 2007). Neutralisation of gH via a monoclonal antibody obstructs VZV entry, cell-to-cell spread, and the transport of virions out of infected cells (Ito et al., 1993, Rodriguez et al., 1993). In addition, prophylaxis treatment with an anti-gH antibody in the SCID-hu mouse skin xenograft model results in significantly reduced VZV replication in skin (Vleck et al., 2010). Endocytosis induced by gH has been shown to play an important role in facilitating fusion of virally infected cells (Pasieka et al., 2004). Treatment of VZV-infected human embryonic lung cell cultures with an anti-gH neutralising antibody prevented the spread of virus within the cultures, and resulted in a latent-like state with restricted viral gene expression within infected cells (Shiraki et al., 2011). Specific domains of gH have been shown to play critical roles in viral fusion and skin tropism (Vleck et al., 2011).

When co-expressed with gH, glycoprotein L (gL), encoded by ORF60, has the ability to facilitate syncytia formation (Cole and Grose, 2003, Duus et al., 1995), and gL may act as a chaperone for gH allowing for complete maturation and expression on the cell surface (Duus and Grose, 1996).
Glycoprotein M (gM), encoded by ORF50, has only recently been characterised, and although it is dispensable for viral growth, it is important for efficient cell-to-cell spread in cell culture (Yamagishi et al., 2008, Sadaoka et al., 2010).

1.2.2.3 Virion Assembly and Release

The L genes are the final class to be expressed in the VZV infection cycle, and the replication cycle within a cell is completed within 9-12 hours (Reichelt et al., 2009). At this stage in the infection cycle, VZV DNA that has been previously replicated is packaged into capsids, transported out of the nucleus, and acquires an envelope. The origin of this envelope is unknown, as virions progress through various cellular organelles during maturation and egress from the cell (Achong and Meurisse, 1968, Wang et al., 2001, Zhu et al., 1995b). It appears that there is an initial envelopment at the nuclear membrane and subsequent de-envelopment in the cytoplasm, with the final envelopment occurring in the trans Golgi network (Gershon et al., 1994). The glycoproteins produced during L gene synthesis are incorporated into the virion envelope during maturation, and assembled virions are released from the cell (Cook and Stevens, 1970).

The assembly and release of virions has not been well characterised in vivo, however infectious progeny virions are released into aerosols and vesicular fluids, serving to facilitate infection of other susceptible hosts (Ozaki et al., 1996). In contrast, VZV infection in cultured cells is highly cell-associated (Taylor and Moffat, 2005). To date, there are only two cell types known to release infectious progeny in vitro when infected with VZV – explanted human fetal dorsal root ganglia (DRG) (Gowrishankar et al., 2007) and human embryonic stem cell-derived neuron cultures (Markus et al., 2011).

VZV infection of cells in vitro results in relatively low titres compared to other alphaherpesviruses such as the herpes simplex viruses. Early examinations of VZV-infection cell cultures suggested that VZV virions featured an envelope that broke down on virion exit from the nucleus (Cook and Stevens, 1968). More recent work has examined the egress of VZV virions within infected cells in culture (Carpenter et al., 2008), and found that there may be a large dissociation between capsid development and viral envelopment, which could result in these relatively low viral titres.
1.2.3 Clinical features of VZV infection

Primary VZV infection manifests as the generalised exanthematous disease of varicella (chickenpox) (Figure 1.4). Early symptoms which can precede the appearance of the rash by up to 48 hours include fever, malaise, headache and abdominal pain (Arvin and Gilden, 2007). The rash begins as maculopapular lesions that quickly become fluid-filled vesicles, and then become pustular and crust over within 2-3 days (LaGuardia and Gilden, 2001). Typically the rash first appears on the face and scalp, followed by the trunk and extremities (Cohen et al., 1999). The fluid within the vesicles contains infectious virus, which may serve as a source of viral transmission to other susceptible hosts who come in direct contact (Weller, 1953). After the appearance of the rash, common symptoms include fever, anorexia and listlessness. Varicella in the immunocompetent host is a mild disease, with lesion formation ceasing within 7 days (LaGuardia and Gilden, 2001). However complications can include secondary bacterial infections – particularly by Streptococcus pyogenes and Staphylococcus aureus, as well as pneumonia and central nervous system involvement, resulting in a more severe and potentially life-threatening condition (Fleisher et al., 1981, Heininger and Seward, 2006). Immunocompromised patients are also more susceptible to prolonged lesion formation and other more severe forms of secondary infections, including pneumonia, encephalitis and even death (Balfour, 1988, Jantsch et al., 2011).

Herpes zoster (shingles) is a clinically distinct disease caused by VZV. It is an exanthematous condition which results from the reactivation of endogenous latent VZV (Figure 1.5). Although the lesions are histopathologically indistinguishable from varicella (Oxman, 2000), the restricted distribution of the rash seen in herpes zoster distinguishes it from primary VZV infection. The herpes zoster rash is generally restricted to one dermatome, although it may spread up to three (one above and one below the affected area) in the immunocompetent host (Cohen et al., 1999). This reflects the intraneural spread of virus to the skin, rather than the general viremia that occurs in varicella (Oxman, 2000). The herpes zoster rash is usually preceded by and always accompanied with severe neuropathic pain within the same distribution (Cohen et al., 1999, Haanpaa et al., 1999), while other symptoms include fever, chills, malaise and gastrointestinal disturbances (Arvin and Gilden, 2007). Greater prodromal pain has been associated with a high viral load (Quinlivan et al., 2007). In some cases the severe pain persists for months to years following the resolution of the herpes zoster rash (Cohen et al., 1999, Steiner et al., 2007). This is called post-herpetic neuralgia (PHN), and is the most common and debilitating complication
Figure 1.4 – Varicella rash

Photograph of the back of a young male presenting with a serious presentation of the characteristic varicella rash.

Source:

(Accessed 15 August 2011)
Figure 1.5 – Herpes zoster rash

Photograph of a male with herpes zoster rash on the trunk.

Source:

http://missinglink.ucsf.edu/lm/DermatologyGlossary/dermatomal.html
(Accessed 15 August 2011)
associated with herpes zoster (Hope-Simpson, 1965, Hope-Simpson, 1975, Steiner et al., 2007). PHN will be discussed in more detail in section 1.5.

Other common conditions which may result from VZV reactivation include Ramsay Hunt Syndrome (RHS) and Bell’s palsy (or idiopathic peripheral facial palsy). RHS is a peripheral facial nerve palsy and is usually accompanied by a vesicular rash on the ear or in the mouth (Gilden et al., 2010), and has been associated in some patients with a rise in VZV-specific antibody titres and the presence of VZV DNA in skin, saliva and blood mononuclear cells (MNCs) (Hato et al., 2000, Murakami et al., 1998). Similarly it has been found that some cases of Bell’s palsy are also accompanied with seroconversion to VZV and the presence of VZV DNA in blood MNCs (Morgan and Nathwani, 1992, Terada et al., 1998).

There are other less common complications of VZV infection. For example VZV can productively infect the large and small cerebral arteries resulting in vasculopathy, where the virus is detectable within the affected arteries (Gilden et al., 1996, Salazar et al., 2011). Patients commonly present with headache, fever, and change in mental status, and the condition may ultimately result in stroke (Mueller et al., 2008).

VZV can also cause myelitis, with two distinct clinical presentations. The first is a self-limiting condition known as “postinfectious myelitis”. This usually occurs in immunocompetent individuals days to weeks after primary VZV infection or reactivation (Gilden et al., 1994a, Rosenfeld et al., 1993). The second is a progressive and sometimes fatal myelitis which is often seen in immunocompromised individuals, and is most commonly associated with acquired immunodeficiency syndrome (AIDS) (Chang et al., 2009, Lionnet et al., 1996, Manian et al., 1995).

VZV is also associated with two common clinical forms of necrotising retinitis: acute retinal necrosis (ARN) and progressive outer retinal necrosis (PORN). ARN is more likely to be HSV-related (Tan et al., 2001, Thompson et al., 1994, Sounshi et al., 1988), and can occur in both immunocompetent and immunocompromised individuals, while PORN is caused almost exclusively by VZV (Engstrom et al., 1994) and is most common in AIDS patients with very low CD4+ T cell counts (Guex-Crosier et al., 1997). VZV is also the main pathogen that is associated with many atypical necrotising retinopathies (Garweg and Bohnke, 1997).

Other complications include intractable itching, often complicated by the lack of other feeling within the affected area, termed post-herpetic itch (Oaklander, 2008, Oaklander et al., 2002, Oaklander et al., 2003, Semionov and Shvartzman, 2008).
Another complication is poliosis, or the depigmentation of hair, which has also been
described in other inflammatory conditions (Wu et al., 2006).

Other clinical diseases caused by VZV include varicella in pregnancy, disease in the
immunocompromised and newborn, congenital infections, varicella encephalitis and
varicella vasculitis (Arvin and Gilden, 2007).

1.2.4 Epidemiology

VZV is a ubiquitous human pathogen, and has a world-wide geographic distribution.
Restriction enzyme digests of VZV DNA isolated from unrelated clinical patients have
shown epidemiologically related and unrelated isolates (Quinlivan et al., 2002), with
comparative genome analysis showing polymorphisms within the glycoproteins that
differentiate between strains present in different regions of the world (Storlie et al.,
2008b). These different viral strains have very similar virulence and prevalence
(Takayama et al., 1989).

In most temperate climates more than 90% of the population are infected with VZV
before adolescence (Fairley and Miller, 1996), while in tropical climates only half of
those under 24 years old have been exposed (Mandal et al., 1998). This may be due
to climate, social conditions, or a combination of factors, but the varicella infection
rate is very high when susceptible individuals move from tropical to temperate zones
(Ooi et al., 1992). The annual incidence of varicella in the United States has been
estimated at 3.5 million cases per year (Arvin, 1996), however due to the introduction
of a widespread vaccination program the incidence in some areas has decreased by
up to 90% (Guris et al., 2008). In Australia, there are 0.19 cases of congenital
varicella per 100 000 live births per annum (Khandaker et al., 2011). Although
varicella is usually a benign disease, it has been estimated that in Australia, there
was an average of 7 deaths and 1100 hospitalisations due to varicella per year
(McIntyre et al., 2002). VZV causes annual epidemics of varicella, especially in
temperate regions during the late winter and spring. These probably begin with
sporadic cases in children exposed to reactivated VZV in adults. Years of higher
incidence of varicella are generally followed by periods where few cases occur (Choo
et al., 1995, Wharton, 1996).

There is an estimated 1 million cases of herpes zoster per year in the United States
(Weaver, 2007). In Australia the incidence of herpes zoster disease and
complications are comparable to that seen in the United States (Araujo et al., 2007).
Specifically, it is estimated to be up to 8.3 cases per 1000 people, with around
159 000 cases of herpes zoster in 1999 alone (MacIntyre et al., 2003). Susceptibility to herpes zoster is governed by a previous varicella infection. Thus given the high percentage of the population who have had varicella, most people are at risk of VZV reactivation and herpes zoster. Herpes zoster is very rare in childhood – occurring at a rate of 1.6 cases per 1000 people per year in children and adolescents up to 19 years old (Petursson et al., 1998). In Australia, neonatal herpes zoster occurs at a rate of 2.0 cases per 100 000 lives births per annum (Khandaker et al., 2011). One risk factor for the development of herpes zoster at an early age is a primary varicella infection during the first year of life or intrauterine infection with varicella (Baba et al., 1986, Guess et al., 1985, Takayama et al., 2000). Herpes zoster is more common in individuals older that 45 years, with the risk of virus reactivation increasing with the individual’s age (Brisson et al., 2001, Hope-Simpson, 1965, Ragozzino et al., 1982). It has been estimated that by age 70, the risk of developing herpes zoster is around five times that of middle age, and twelve times that of a child under 10 years old (Hope-Simpson, 1967). Rates of herpes zoster recurrence have been shown to be comparable to the incidence of initial herpes zoster reactivation, however recurrence is higher in women and anyone aged over 50 (Yawn et al., 2011). New cases of herpes zoster are diagnosed evenly throughout the year, with no seasonal variation at all (Hope-Simpson, 1965). The incidence of herpes zoster is increased in immunocompromised patients such as those with HIV, leukaemia or following bone marrow or renal transplants (Balfour, 1988). These clinical observations suggest that VZV reactivation and herpes zoster is associated with a decrease in cell-mediated immunity.

The most common neurological complication of herpes zoster is PHN (Kost and Straus, 1996), and is reported to be the third most common cause of neuropathic pain in the United States (Bennett, 1998). Despite regional differences, the epidemiology of PHN in Australia are comparable with those seen in the United States (Araujo et al., 2007). PHN lasting between 4 weeks to 10 years occurs in around 10% of all herpes zoster patients (Ragozzino et al., 1982). Pain lasting longer than 1 month accounts for up to 10% of all herpes zoster encounters with primary care physicians, with over 90% requiring prescribed treatment (Conway et al., 2006). Another more recent study found that 19.5% and 13.7% of patients experienced PHN to some degree at 1 and 3 months after diagnosis with herpes zoster (Gauthier et al., 2009). The age of onset of herpes zoster closely correlates with the incidence of PHN (Arvin, 1996). Studies have shown the risk of herpes zoster developing into PHN ranges from 43 to 47.5% in patients over 50 years (Brown, 1976, De Moragas
and Kierland, 1957), while for patients 30-49 years of age the risk is under 4% (Hope-Simpson, 1965). The incidence increases dramatically with age, and more than one third of the herpes zoster patients over 80 years will experience PHN (Hope-Simpson, 1975, Johnson et al., 2010). Children who experience herpes zoster are not at risk of PHN (Hope-Simpson, 1965, Petursson et al., 1998). In older patients, over 20% of PHN-affected patient experience symptoms for more than one year (Arvin and Gilden, 2007, Johnson et al., 2010).

1.3 The human nervous system

The human nervous system is divided into the central nervous system (CNS), consisting of the brain and spinal cord, and the peripheral nervous systems (PNS), consisting of sensory branches of cranial, spinal and peripheral nerves, and specialised nerve endings (Moore and Dalley, 1999). Collections of nerve cell bodies outside the CNS are called ganglia, for example a cranial ganglion or spinal ganglia (Ross and Pawlina, 2003).

1.3.1 Components of the human nervous system

The functional unit of the nervous system is the neuron. These are polarised cells which consist of cell bodies, axons, dendrites and terminals (Kandel et al., 2000). Neurons are arranged as an integrated communication network, and are able to receive stimuli from other cells, and in response conduct electrical impulses to other parts of the nervous system via their processes (Ross and Pawlina, 2003).

Another important component of nervous tissue are the supporting cells. These are non-conducting cells which surround individual neurons. These non-neuronal, non-excitable cells serve to physically support, electrically insulate, and provide nutrients for their associated neurons (Ross and Pawlina, 2003). In the CNS these cells are called neuroglia, or simply glia, while in the PNS they are called Schwann cells and satellite cells (Moore and Dalley, 1999).

1.3.1.1 THE PERIPHERAL NERVOUS SYSTEM

The PNS consists mainly of nerve fibres and their supporting Schwann cells (Ross and Pawlina, 2003), which are held together by three distinct types of connective tissue. From the inner-most outwards these connective tissue layers are the endoneurium, which surrounds each individual nerve fibre; the perineurium, which
surrounds each bundle of nerves, or fascicles; and the epineurium, which surrounds the entire peripheral nerve and fills the space between fascicles (Ross and Pawlina, 2003). These tissues serve to protect, insulate and isolate each peripheral nerve. Nerves are also encased within Schwann cells, which function to insulate and provide nutrients for each nerve fibre. The junctions between Schwann cells are termed the nodes of Ranvier. At these gaps the plasma membrane of the neuron is exposed to the extracellular environment, which enables efficient conduction of neural impulses along the axon (Kandel et al., 2000). Schwann cells also secrete essential nutrients and growth factors, and along with fibroblasts within the endoneurium and epineurium, help maintain the structure of the PNS (Moore and Dalley, 1999).

Each bundle of nerves, or axons, originate within the cell body from an area known as the axon hillock. This can be distinguished from the cell body as it usually lacks both Golgi apparatus and rough endoplasmic reticulum, as well as other large cytoplasmic vesicles (Ross and Pawlina, 2003). Each axon can either project locally, or extend from a cell body in one area of the nervous system to a nerve cell in a completely different section of the nervous system (Carpenter, 1983). The distal portion of each axon divides into extensive networks of nerve termini, which form synapses within the spinal cord (Carpenter, 1983).

1.3.1.2 THE DORSAL ROOT GANGLIA

In the PNS the cell bodies of sensory neurons are situated in the DRG or trigeminal ganglia (TG). These are bundles of neurons located immediately adjacent to the spinal cord (see Figure 1.6), and covered in a capsule of connective tissue which forms part of the epineurium and perineurium of the spinal nerves (Moore and Dalley, 1999). Unlike other neurons the DRG neurons do not possess dendrites, but instead have a unipolar axon consisting of peripheral and central branches. These axonal branches are the conducting units, and are utilised to convey impulses from receptors in the periphery into the CNS (Ross and Pawlina, 2003).

Neurons are post-mitotic cells and consequently do not undergo a replication cycle, however the internal structures do undergo regular turnover (Ross and Pawlina, 2003). The neuronal cell bodies correspond to the dilated section of the neuron. This section has a globular or pear-like shape with a diameter of between 60 to 120 µm (Kandel et al., 2000). The neuronal cell bodies contain a central or paracentral nucleus with a prominent nucleolus, surrounded by a perinuclear cytoplasm (Ross
Figure 1.6 – The dorsal root ganglia

The spinal nerves convey information from receptors in the skin and other organs to the spinal cord.

(A) Dorsal root ganglia are situated immediately adjacent to the spinal cord. These contain the cell bodies of sensory neurons, with projections to both the spinal cord and receptors in the periphery.

(B) A cross sectional view of an adult dorsal root ganglia and adjacent regions.

Source:
(A) http://faculty.washington.edu/chudler/gif/vert3.gif (accessed 15 August 2011)

(B) http://www.uoguelph.ca/zooloogy/devbio/miller/013654fig7-7.gif (accessed 15 August 2011)
Within the neuronal cytoplasm there is rough endoplasmic reticulum (called Nissl bodies when seen under light microscopy), smooth endoplasmic reticulum, ribosomes, mitochondria, Golgi apparatus, lysosomes, microtubules, neurofilaments and transport vesicles (Ross and Pawlina, 2003). Surrounding the neuron is a layer of satellite cells that tightly encapsulate the cell body (see Figure 1.7).

1.3.1.3 **THE TRIGEMINAL GANGLIA (TG)**

The TG is located in a cavity in the temporal bone, and is the sensory ganglion that generates most of the information from the orofacial complex in humans (Drake et al., 2009). There are three major divisions in the TG, each resulting in a branch which innervates a separate dermatome, and exits the cranium at a different site. The first division – the ophthalmic branch – (V1) innervates the area of skin above the eyes and forehead. The second division – the maxillary branch – (V2) innervates the region of skin below the eyes and above the mouth. The third division – the mandibular branch – (V3) innervates the skin over the jaw but sparing the angle of the mandible. The TG is made up of large and small unipolar neurons, their associated satellite cells and fibres (Drake et al., 2009).

1.4 **Pathogenesis of VZV infection**

The pathogenesis of VZV infection is comprised of three unique phases: inoculation with VZV leads to primary infection or varicella (chickenpox). During this phase a latent infection is established within sensory ganglia and maintained throughout the life of the host. The virus may reactivate from latency to cause a clinically distinct disease, herpes zoster (shingles) (Figure 1.8).

1.4.1 **Primary infection**

VZV infection occurs via the mucosa of the upper respiratory tract or conjunctiva when a susceptible individual comes into contact with aerosols containing infectious virus, although it can also occur from direct contact with infectious fluid from the vesicles of an infected individual (Grose, 1981, LaGuardia and Gilden, 2001) (Figure 1.6). Following inoculation there is an incubation period that lasts for 10-21 days, during which the infected host is asymptomatic and the virus moves from the infection site to the skin where viral replication occurs (Arvin and Gilden, 2007, Ku et
Figure 1.7 – Dorsal root ganglia (DRG) structure

An adult DRG sample stained via immunofluorescent assay using an anti-S100B antibody which labels satellite cells (green) and counterstained with DAPI to show cell nuclei (blue).

The full microscope image (A) shows both neuronal cell bodies (yellow arrow) and nerve bundles (pink arrow). In a higher power we can see the neuronal cell body (yellow) is encapsulated by satellite cells (red arrows)
Figure 1.8 – Pathogenesis of VZV infection

Primary VZV infection is thought to begin with inoculation of the mucosal membranes of the respiratory tract. After this there is an extended incubation period during which VZV (shown as green dots) spreads from the site of inoculation to skin and other organs (A). VZV then causes the characteristic varicella rash (red dots) (B). At some stage during primary infection VZV establishes lifelong latency in the dorsal root ganglia (purple dots) (C). Reactivation from latency later in life results in herpes zoster which is characterised by a rash usually restricted to a single dermatome (red ovals) (D). The most common complication of herpes zoster is post-herpetic neuralgia, where pain (orange oval) persists generally in a similar distribution of the herpes zoster-associated rash for months following resolution of the rash itself (E).

(Image courtesy of and adapted from Chantelle Hood, University of Sydney)
al., 2004). It has been proposed that dendritic cells (DCs) within the respiratory mucosa are the first cell type to be infected with VZV and may be responsible for the transport of VZV from the infection site to draining lymph nodes, and the subsequent infection of T lymphocytes (Abendroth et al., 2001b). This is thought to be followed by a primary viremia, during which the virus is transported to the liver and other reticuloendothelial cells (Arvin and Gilden, 2007). Initial symptoms develop around 14 to 16 days post infection, and during the final days of the incubation period a second viremia occurs. It has been hypothesised that the different phases of viremia during varicella are accompanied by the migration of VZV-infected T lymphocytes from the capillaries into the tissues (Moffat et al., 2007). Once here, VZV quickly invades cutaneous epithelial cells, resulting in the characteristic varicella rash (Asano et al., 1990). VZV has been shown to display a tropism for both CD4+ and CD8+ T lymphocytes in the SCID-hu mouse model (Moffat et al., 1995), and in particular for T lymphocytes that display skin homing markers (Ku et al., 2002).

The first varicella lesions become apparent 24 to 48 hours after initial symptoms (Moffat et al., 2007). Viral DNA, proteins and intact virions can be detected in cells of the dermis and epidermis of VZV lesions (Annunziato et al., 2000, Nikkels et al., 1993). As epidermal cells within lesions deteriorate, a fluid develops and fills the vesicle. This fluid contains cell-free virus, and can provide another transmission route on direct contact with a susceptible individual (Weller, 1953).

Varicella is normally resolved within 7 days, and requires the development of VZV-specific immunity. This is covered in more detail in subsequent section 1.6. Complications can arise during varicella, the most common of which is secondary bacterial infection, which can lead to a severe and life-threatening disease (Fleisher et al., 1981, Heininger and Seward, 2006).

1.4.2 Latency

At some stage during primary infection, VZV is transported to the sensory ganglia, primarily the DRG (Figure 1.7), where a latent infection is established and maintained throughout the life-time of the host (Gilden et al., 1983). Currently the method of virus transport to the ganglia, the exact cell-type infected during latency, and the underlying mechanisms by which the virus establishes and maintains latency are not fully understood. There have been two proposed models by which the virus gains access to sensory ganglia. The first is direct transmission through the bloodstream via infected T lymphocytes. This model is supported by recent work in the human
DRG xenograft SCID-hu mouse model, where VZV-infected T cells were shown to be capable of infecting DRG tissues (Zerboni et al., 2005). The second model, analogous to the establishment of HSV latency, involves retrograde axonal transport of virions from infected dermal and epidermal tissues (Abendroth et al., 2001a). This is favoured by immunohistochemical analysis which have shown the presence of VZV antigens in both Schwann cells and nerve termini (Silverstein and Straus, 2000).

The exact cell-type in which latent VZV infection occurs has been the source of some controversy. Many studies, utilising different techniques, have shown neurons to be the exclusive site of latency (Hyman et al., 1983, Gilden et al., 1987), while others have identified latent VZV infection of satellite cells (Croen et al., 1988, Meier et al., 1993). Other studies have shown that VZV establishes a latent infection predominantly in neurons, but also in some satellite cells (Dueland et al., 1995, Kennedy et al., 1998, Kennedy et al., 1999, Lungu et al., 1995). Another study utilised highly sensitive laser capture microdissection (LCM) and real time polymerase chain reaction (PCR) and found that latent VZV resides primarily, if not exclusively, within neurons (Wang et al., 2005). Other studies have utilised very specific nucleic acid separation techniques to show VZV latency almost exclusively within neurons (LaGuardia et al., 1999, Levin et al., 2003).

The VZV genome is thought to be maintained within latently infected cells in a non-integrated circular concatameric form, as PCR analysis has revealed that the genome termini are adjacent (Clarke et al., 1995). The exact amount of VZV DNA maintained within latently infected sensory ganglia is unknown, and studies have measured from between 6-31 copies of VZV DNA per $10^5$ ganglionic cells (Mahalingam et al., 1993) to up to 9046 copies per $10^5$ ganglionic cells (Cohrs et al., 2000). Wang and co-workers in the LCM study calculated that around 1.0-6.9% of neurons harbour latent VZV, with around 6.9 viral genomes per infected cell (Wang et al., 2005).

Although latency is characterised by the cessation of viral DNA synthesis, late gene expression, and production of infectious progeny (Silverstein and Straus, 2000), the virus retains the ability to reactivate to cause a productive infection. In HSV-1 latent infection of sensory neurons, viral gene expression is restricted to latency-associated transcripts (LATs) (Kent et al., 2003). VZV does not contain any known LAT homologues, however there is restricted VZV gene transcription in latent infection of sensory ganglia (Cohen, 2007). In contrast to HSV-1, VZV does not encode any viral microRNAs in latently infected cells (Umbach et al., 2009). Also there have been a
range of VZV ORFs detected in latently infected human sensory ganglia in various studies, with ORF4, ORF18, ORF21, ORF29, ORF62, ORF63 and ORF66 consistently detected as RNA transcripts (Cohrs et al., 1996, Cohrs et al., 2003, Kennedy et al., 2000, Kennedy et al., 1999, Lungu et al., 1998, Mahalingam et al., 1996, Theil et al., 2003, Inoue et al., 2010). These genes products are located within the cytoplasm of latently infected cells – contrasting with a predominantly nuclear localisation during productive infection of permissive cells (Lungu et al., 1998). It has been suggested that these proteins are sequestered from the nucleus to prevent their transcriptional function and preserve the virus in a latent state (Lungu et al., 1998).

One study demonstrated that ORF63 is the most prevalent transcript in latently infected human ganglia, suggesting it has an important role in maintaining viral latency (Cohrs and Gilden, 2007). However there is controversy about these latency associated proteins, with more recent papers detecting very low expression levels, and one in particular showing that IE63 protein expression was a very rare occurrence, and may actually indicate a pending or subclinical reactivation rather than a latent infection (Zerboni et al., 2010). This recent study also provided evidence that the previous reports of higher expression levels may in fact be due to inherent background present in the formalin-fixed and paraffin-embedded ganglionic tissue samples used.

More recently Nagel and co-workers used a highly sensitive multiplex reverse transcription PCR (RT-PCR) approach previously developed (Nagel et al., 2009) which was able to detect transcripts from all 68 unique VZV ORFs to study the viral transcriptome during latency in human ganglia (Nagel et al., 2011). This recent study was able to detect transcripts mapping to VZV ORF4, ORF40, ORF29, ORF62 and ORF63 which had been previously identified during VZV latency. Additionally, transcripts mapping to VZV ORF11, ORF41, ORF43, ORF57 and ORF68 were also detected – none of which had been previously reported (Nagel et al., 2011). ORF63 remained the most prevalent transcript detected (Nagel et al., 2011). Consequently the VZV transcripts that have been detected by multiplex RT-PCR during latency come from all kinetic classes of the virus life cycle – challenging the classical belief that early and late genes require a productive infection for transcription to occur.
1.4.3 VZV Reactivation

Reactivation from latency results in herpes zoster, which can occur months to years following the resolution of primary VZV infection. It has been suggested that like other herpesviruses there are periodic episodes of subclinical reactivations which may help maintain the VZV latent infection (Arvin, 1996). This is supported by the detection of asymptomatic VZV viremia in both bone marrow transplant recipients, elderly adults and healthy asymptomatic blood donors (Devlin et al., 1992, Quinlivan et al., 2007, Wilson et al., 1992).

Current information on the cellular and viral events responsible for VZV reactivation is quite limited. The underlying cause(s) of reactivation and reversion to productive infection is not yet known. The risk of herpes zoster increases with age (Hope-Simpson, 1965), and it has been suggested that the age-related reduction in cell-mediated immunity may allow VZV reactivation to occur (Schmader, 2007). This assertion is also supported by the higher incidence and greater severity of reactivation that occurs in immunocompromised and elderly patients (Alessi et al., 1988, Balfour, 1988). Interestingly it has been reported that VZV DNA and viral shedding occurs in astronauts following their return from spaceflight, and spaceflight is known to reduce cell-mediated immunity (Cohrs et al., 2008). Other noted triggers for viral reactivation include immunosuppression, trauma, x-irradiation, malignancy, and other infections (Kennedy, 2002, Strangfeld et al., 2009).

During VZV reactivation the full repertoire of viral genes is expressed, infectious virions are formed and are believed to be transported from the sensory ganglia to the nerve termini in the skin via anterograde axonal movement (Arvin and Gilden, 2007). This transport is believed to be along myelinated nerves which terminate within the epidermis near hair follicles and sebaceous glands (Muraki et al., 1996), and results in the infection of epithelial cells, causing the characteristic herpes zoster rash. Within the immunocompetent individual this takes the form of a unilateral vesicular rash usually localised to one dermatome (Abendroth et al., 2001a). This axonal movement model is supported by the detection of VZV proteins and inclusion bodies within small nerves in the dermis beneath the site of the vesicles (Annunziato et al., 2000). The appearance of the rash is sometimes preceded by a prodrome of unilateral dermatomal pain (Haanpaa et al., 1999). As accurate diagnosis of herpes zoster depends on the appearance of the characteristic rash, this prodrome is often mistaken for other conditions such as angina, HSV infection and lumbar radiculopathy (Dworkin et al., 2008a). Some episodes of VZV reactivation do not
produce a rash at all, but rather pain with a dermatomal distribution accompanied by a rise in VZV antibody titres (Gilden et al., 1994b). This condition is called zoster sine herperte (Murata et al., 2010).

VZV DNA has been detected in 20% of saliva samples taken from herpes zoster-affected patients 15 days post-rash onset (Mehta et al., 2008). The same study showed that a higher level of viral DNA detectable by PCR in the saliva correlated with a higher amount of pain reported by the patient (Mehta et al., 2008). VZV DNA has been reported to remain in the blood of 80% of patients with herpes zoster, and higher viral load seems to correlate with higher reported pain levels (Personal communication by J. Breuer noted in Gershon et al., 2010). Interestingly, VZV DNA has been shown to persist in the blood for up to 6 months following the resolution of the herpes zoster-associated rash (Quinlivan et al., 2010).

Herpes zoster has been shown to decrease the level of cutaneous innervation at the site of the cutaneous rash (Oaklander et al., 1998, Rowbotham et al., 1996). Interestingly, during the most common complication of herpes zoster, PHN, the level of cutaneous innervation at the site of the previous herpes zoster rash remains low, as well as a smaller reduction at an unaffected contralateral site (Oaklander et al., 1998, Rowbotham et al., 1996). PHN also causes significant morbidity in affected individuals, and is dealt with in more detail in the subsequent section.

### 1.5 Post-herpetic neuralgia

#### 1.5.1 Post-herpetic neuralgia

PHN is the most common complication following herpes zoster (Kost and Straus, 1996). It is defined as pain within the distribution of the herpes zoster rash that continues beyond a 4-6 week period following the resolution of the herpes zoster rash (Steiner et al., 2007), however the sensitive area often exceeds the area affected by the herpes zoster rash (Watson et al., 1988a). This pain substantially interferes with the affected individual's physical, emotional and social functioning (Coplan et al., 2004), and results in significantly increased health care costs (Davies et al., 1994, Dworkin et al., 2007b). One study found treatment for PHN-affected patients could cost up to $9000 US each year (Dworkin et al., 2008b). The prognosis for affected patients is poor, with some patients suffering for many years, sometimes right up until death (Watson, 1998). Although in many cases the pain naturally
resolves, some experience a gradual increase in the severity of the condition over time (Watson, 1998). There are a range of different treatments available, pharmacological, interventional and surgical, however the efficacy of these vary, and in many cases the pain is intractable (Volmink et al., 1996, Wu and Raja, 2008). However a new treatment method involving the application of a liquid nitrogen cloud in order to cool the affected area has shown potential in relieving the pain associated with PHN (Calandria, 2011).

1.5.1.1 CLINICAL FEATURES

Clinical findings from PHN-affected patients indicate there is altered nervous system function, including areas of sensory loss and sensory gain (Watson, 1998). Patients affected with PHN experience a combination of three different types of pain: the first is a steady burning; the second is sharp, often lancinating, shock-like episodes of pain; and the third is pain on non-painful stimuli of the affected area, also called allodynia (Schmader, 2007). Allodynia can result from a cold wind or piece of clothing brushing across the affected area. This condition is generally less severe in the morning, and progressively worsens throughout the day (Ragozzino et al., 1982). Patients may also have altered pain and thermal sensitivity which extends beyond the pain-affected area (Alvarez et al., 2007).

1.5.1.2 PATHOGENESIS

The cause and pathogenesis of PHN is not known. As a neuropathic pain (a neuralgia), one hypothesis is that PHN may result from damage to the sensorineural pain pathway (Steiner et al., 2007). The sensorineural pain pathway is involved in protecting against tissue damage, as pain triggers a behavioural response such as withdrawing from harm or resting to allow adequate time for healing (Oaklander, 2008). Characteristics of this type of pain include a discrepancy between the patient's complaint of pain and the lack of a corresponding tissue injury, as well as a progressive worsening of pain during the day (Oaklander, 2008). Neuralgias are closely linked to the malfunctioning of sensory neurons that transmit pain signals (Oaklander, 2008). Neurons transmit signals using an electrochemical gradient via a coordinated cycle of polarisation, depolarisation and repolarisation of the cell membranes. Damage to neuronal cells can result in incorrect initiation of this cycle, leading to pathologic signalling (Bennett, 1994).

In PHN, it is thought the structure of neurons within the reactivated ganglia may be altered during herpes zoster (Bennett, 1994). This may be due to different factors,
including direct cell death caused by virus infection and neuronal cell damage due to the inflammatory response (Bennett, 1994). In surviving nerve fibres, the population may shift from a balance between large inhibitory and small excitatory fibres towards a preponderance of smaller fibres (Watson, 1998). This may lead to pathologic signalling, either spontaneously or in response to the lightest of tactile stimulation.

An alternative explanation for the pathogenesis of PHN is the presence of a persistent viral infection and ongoing replication within the sensory neurons following the resolution of the herpes zoster rash (Steiner et al., 2007). During the productive phase of viral replication, viral antigens are produced. These viral antigen expressing neuronal cells may produce an immune reaction resulting in a general inflammation within the affected sensory ganglia. This inflammation would include virus-specific T lymphocytes that could recognise VZV antigens presented in association with MHC molecules. Infected cells may include neurons or the supporting satellite cells, resulting in a depolarisation of the neuronal cell membrane and the initiation of a pain signal (Bennett, 1994). Evidence that PHN may be produced by low-level ganglionitis due to VZV infection of ganglionic cells includes the detection of viral proteins within MNCs in the blood of PHN-affected patients for months to years following the resolution of the herpes zoster rash (Gilden et al., 2003), but only for a period of months in uncomplicated cases of herpes zoster or varicella (Gilden et al., 1989, Vafai et al., 1988). Alternatively it has been suggested that persistent viral infection within the sensory ganglia could lead to abnormal nerve impulses (Schon et al., 1987), and not be dependent on immune-cell mediated killing of VZV infected cells.

While at this point it is unclear what mechanisms are responsible for the clinical manifestations of PHN, it has been suggested that there are multiple mechanisms that contribute to the different aspects of the syndrome due to the different types of pain and the large variability in severity between patients (Pappagallo et al., 2000). One such mechanism, which has been suggested to be the cause of the allodynia related pain, is the cross-reactivity between antibodies for VZV IE62 and the cellular protein brain derived neurotropic factor (BDNF) (Hama et al., 2010) – an important neuronal protein in pain hypersensitivity. Alternatively it may be caused by aberrant immune cell function; in a rat model of another neuropathic pain – graded chronic constriction injury – it was found that adoptive transfer of splenocytes from high pain to low pain individuals also increases the likelihood and severity of allodynia occurring in the recipient (Grace et al., 2011). VZV has been shown to induce neuropathic changes in rat dorsal root ganglia during chronic infection, inducing
behavioural changes that may be due to pain, and is relieved through the use of analgesic drugs (Hasnie et al., 2007, Garry et al., 2005).

1.5.1.3 PATHOLOGY

There is very little known about the underlying pathology of PHN. Previous studies involving PHN and the assessment of DRG have been limited to general and brief histological examinations of human ganglia. One early post-mortem study of an individual who experienced PHN for several months prior to death found diffuse and focal infiltrations of inflammatory cells (Smith, 1978). Another study found a general loss of both axons and myelin within the affected area (Watson et al., 1988b). A follow-up study on post-mortem DRG samples confirmed these findings, and noted evidence of a general inflammatory process occurring in some PHN-affected individuals, but not all cases studied (Watson et al., 1991). This study also noted atrophy of the spinal dorsal horn, as well as cell, axon, and myelin loss only in patients with persistent PHN. Persistent inflammation and lymphocytic infiltrate within the affected areas in long-term herpes zoster survivors has been previously noted, but these cases had alternate causes of death and no clear pain at time of death, and as such this inflammation cannot be directly attributed to PHN (Head and Campbell, 1900). The major limitation in studying the pathogenesis of PHN is the dearth of appropriate human ganglia samples.

Other studies have focused on studying skin biopsies from both herpes zoster and PHN-affected patients. One study of skin samples from herpes zoster-affected patients revealed that there were significantly lower numbers of infiltrating cells present in the skin of patients who later went on to develop PHN compared to those that experienced no complications, despite the systemic cytokine and antibody responses showing no differences between the two groups (Zak-Prelich et al., 2003).

Studies comparing skin samples from PHN and non-PHN-affected patients found that PHN-affected patients featured large-scale loss of epidermal neurites at the site of pain (Oaklander et al., 1998, Rowbotham et al., 1996). Interestingly, one of these studies utilised multiple biopsies from different sites to show that while the level of innervation at a distal site was similar between PHN-affected and non-PHN-affected patients, subjects with PHN had lost about 80% of their epidermal neurites at the shingles-affected site and about 30% at the contralateral site (Oaklander et al., 1998). This generalised loss of neurites within the epidermis has also been seen in a patient with severe post-herpetic itch (Oaklander et al., 2002). Another study
compared the amount of sensory nerves present in the skin tissue of both the PHN-affected and contralateral sites of PHN-affected individuals, and found that substantially different pattern of innervation at the PHN-affected site (Petersen et al., 2002).

Clinical human tissue samples are relatively difficult to obtain, and consequently other studies have looked at the expression of viral DNA and proteins within the blood of PHN-affected individuals. VZV-specific proteins (gE and gB) were detected within MNCs of patients affected with PHN 1-4 years following acute herpes zoster, but not in cases of uncomplicated herpes zoster (Vafai et al., 1988). Viral DNA present in MNCs within the blood of patients following varicella persists for very limited periods following the resolution of the varicella rash (Gilden et al., 1989).

1.5.2 Epidemiology and risk factors

PHN is the third most common cause of neuropathic pain in the United States (Bennett, 1998). The incidence of PHN rapidly rises with age (Hope-Simpson, 1975). It is very rare in patients under the age of 30 (Hope-Simpson, 1975), and around half of all herpes zoster patients from age 60 on will experience PHN to some extent (De Moragas and Kierland, 1957). The incidence continues to increase with advancing age, as generally does the duration of PHN (De Moragas and Kierland, 1957, Hope-Simpson, 1975, Watson et al., 1988a).

Besides increased age, other risk factors for PHN include immunosuppression, female gender, the presence of a prodrome to herpes zoster, and the occurrence of a more severe episode of herpes zoster (Balfour, 1988, Jung et al., 2004, McKendrick et al., 2009). Although no one of these risk factors can accurately predict the occurrence of PHN, patients with multiple factors have a substantially increased risk of developing the condition. A Japanese study found three HLA alleles were significantly associated with PHN, but not herpes zoster, when compared to normal controls (Sumiyama et al., 2008). One study found that long-term pain was associated with certain characteristics that could be identified during acute illness such as moderate to severe acute pain, severity of rash and prodromal pain (McKendrick et al., 2009). On the other hand, PHN develops in around 5-10% of patients who exhibited none of these risk factors (Jung et al., 2004). Although in an early study there was no correlation between viral loads during herpes zoster and the development of PHN (Quinlivan et al., 2007), another more recent study from the same group performed over a longer time frame found the opposite (Quinlivan et al.,
2010). Thus the relevance of viral loads during herpes zoster and the incidence of PHN remains unclear. In most cases the pain resolves naturally and spontaneously (Volmink et al., 1996), however pain persists in many cases for longer than three months post-herpes zoster, and in around half of all cases for greater than a year (De Moragas and Kierland, 1957, Hope-Simpson, 1975, Ragozzino et al., 1982).

1.5.3 Treatment and Prevention

Treatment of PHN is quite challenging. It is made more difficult as the underlying cause and pathogenesis are unknown. Like many other types of neuropathic pain, PHN can be resistant to many therapies (Wu and Raja, 2008). There are many different therapies available, including pharmacological, interventional and surgical approaches. Although prevention strategies have been complicated by the lack of understanding of the events leading to PHN, a novel vaccine has the capacity to reduce the occurrence of PHN in herpes zoster patients by up to two thirds (Oxman et al., 2005). The implementation of the varicella zoster vaccine is discussed in more detail in section 1.8.

There are many different pharmacologic agents that are used in the treatment of PHN. These include various systemic agents such as tricyclic anti-depressants, anti-epileptics, analgesics and opioids, as well as topical therapies including local anaesthetics and capsaicin (Wu and Raja, 2008). The most common of these is tricyclic antidepressants, which are widely used for the symptomatic treatment of many neuropathic pains (Reviewed in Zin et al., 2008), however these therapies cause significant adverse effects particularly among the elderly (Collins et al., 2000, Sindrup and Jensen, 1999, Volmink et al., 1996). Anti-epileptics have also demonstrated some efficacy with side-effects less detrimental to a patient’s quality of life (Dworkin et al., 2003, Rice et al., 2001, Rowbotham et al., 1998, Sabatowski et al., 2004, Stacey et al., 2008), however the methodologies of some of these studies have been called into question (Gehling and Tryba, 2002, Bowsher, 2002, Low and Dotson, 1998). To compound this, many anti-epileptics can produce undesirable side effects such as delirium and dependence (Kruszewski et al., 2009).

The use of opioids in the treatment of neuropathic pains has also been quite controversial (Arner and Meyerson, 1988). One study has shown opioids provide rapid and stable pain relief during PHN (Fan and OxyContin Tablets Postmarketing Surveillance Study Group, 2008). Other studies have investigated local, non-
systemic agents such as capsaicin-impregnated patches or lidocaine-medicated plasters, both with promising results (Backonja et al., 2008, Liedgens et al., 2008).

However the aforementioned therapies generally provide unsatisfactory results when used as the only treatment strategy (Dworkin et al., 2007a). Consequently a combinatorial treatment regime which combines first, second, and third-line options of pharmacotherapies is the normal course of treatment (Dworkin et al., 2007a). These treatment regimes are decided upon on a case-by-case basis, and changes to therapy are common before a successful and stable treatment regime is reached (Hall et al., 2008).

A recent paper reported the use of a liquid nitrogen spray applied so as to cool the skin along the affected dermatome at weekly sessions for 30 seconds each treatment (Calandria, 2011). This approach resulted in a good or excellent improvement in 97% of the 47 PHN-affected patients before the sixth treatment. The author suggests it is a very efficient way of calming the pain associated with PHN, however does concede that the mechanism behind this treatment is currently unknown (Calandria, 2011).

Despite the large amount of pharmacologic agents available, there is no one established and effective treatment strategy available for PHN (Wu and Raja, 2008), and many patients experience little relief from pain despite considerable side effects of treatments (Watson, 1995).

There have been some reports of anti-viral treatment relieving the pain of PHN (Gilden et al., 2003). In one study a patient diagnosed with PHN had detectable levels of VZV DNA within blood MNCs, and she was treated with an anti-viral agent (famciclovir). This resulted in a complete relief of her pain within 1 month. The patient was followed over an 11 year period, and voluntarily ceased treatment on 5 separate occasions. Each time pain returned within a few days, accompanied by detectable levels of viral DNA within blood MNCs (Gilden et al., 2003).

In another small clinical study of anti-viral treatment of PHN 15 patients with PHN lasting for more than 3 months (an average of 12 months) were treated with intravenous acyclovir for 14 days, followed by oral valacyclovir for 1 month (Quan et al., 2006). At the end of the study 53% of the patients had experienced a clinically meaningful reduction in the pain. However more large-scale controlled studies of the use of anti-viral treatment in PHN have not shown the same dramatic success (Reviewed in Li et al., 2009, McKendrick et al., 2009), and consequently anti-virals
are not currently a recommended therapy for PHN following the disappearance of the herpes zoster rash.

Alternative therapies have also been trialled in the treatment of PHN. These include the effect of pulse radiofrequency (Kim et al., 2008), treatment using botulinum toxin A (Sotiriou et al., 2009), and acupuncture (Valaskatgis et al., 2008). These have mostly shown positive results, with successful reduction in the burden of pain. Another study found that PHN patients had significantly lower plasma concentrations of vitamin C when compared to healthy volunteers, and restoring the vitamin C level to normal brought about a decrease in episodes of pain (Chen et al., 2009). However this only affected spontaneous episodes of pain, and vitamin C treatment did not reduce episodes of burning or allodynia (Chen et al., 2009). Overall these alternate therapies have only been trialled in small scale studies or case reports, and each study requires larger clinical trials and more investigation into the underlying mechanisms of action.

A wide variety of interventional strategies have been examined as potential PHN treatments. These include the use of sympathetic nerve blocks, which can provide transient pain relief (Wu et al., 2000). Other approaches include epidural and intercostal nerve blocks. Overall the long-term effectiveness of these in PHN treatment is unknown (Wu and Raja, 2008). PHN greatly reduces the sufferer's quality of life, thus psychological interventions may be important for the emotional and mental health of the affected individual (Wu and Raja, 2008).

Surgical approaches to PHN treatment are quite limited. One option that has been used in a clinical trial on a PHN-affected patient involved the removal of the top layer of skin from the pain-affected area (Petersen et al., 2002). This resulted in a temporary relief of pain and a reduction in the use of medication, but on its return both the severity of pain and size of the affected area increased, and the required pharmacological intake also increased, and consequently is not recommended as a therapeutic strategy for PHN (Petersen and Rowbotham, 2007). Other possible approaches include the use of a pump to deliver a continuous supply of an analgesic to the area (Angel et al., 1998), or the complete removal of the affected sensory ganglia via a dorsal root ganglionectomy (Colli et al., 2008). Efficacy of these interventions has yet to be evaluated for use in the treatment of PHN.
1.6 Immune responses to Varicella Zoster Virus

Primary VZV infection initiates responses from both the innate and adaptive arms of the immune system. Early involvement of the innate immune system is likely to be responsible for restricting viral replication and spread of infection. This non-specific response involves both natural killer (NK) cells and interferon (IFN) production. NK cells are able to directly lyse VZV-infected fibroblasts *in vitro* (Ihara et al., 1984), and synthesise granulysin, a cytolytic protein which enhances target cell death (Hata et al., 2001), and have been shown to play some role during acute primary varicella infection (Malavige et al., 2010). In fact complete NK cell deficiency causes fatal primary varicella infection (Etzioni et al., 2005).

The humoral response against VZV consists of antibodies specific for viral proteins and neutralising antibodies. Antibodies lyse infected cells by either activating the complement pathway or in conjunction with NK cells through antibody-dependent cellular cytotoxicity (ADCC) (Abendroth and Arvin, 2000, Arvin and Abendroth, 2007). VZV specific antibody isotypes consist of IgM, IgG and IgA antibodies, which are detectable within the serum of a host during varicella (Arvin, 1996). These VZV specific antibodies recognise a variety of different epitopes, mostly viral glycoproteins, as well as other regulatory and structural proteins and viral enzymes (Arvin, 2001, Dubey et al., 1988, Giller et al., 1989, LaRussa et al., 1990). Although these antibodies may assist in limiting viral spread within the first 72 hours post infection, as shown by the effectiveness of passive immunoglobulin transfer directly after VZV exposure (Arvin and Gilden, 2007), humoral immunity appears to play a limited role in controlling VZV infection. This is evident in the similar severity of varicella in immunocompromised and healthy children, and the occurrence of uncomplicated varicella in children with congenital agammaglobulinemia (Arvin, 1987). Antibodies, along with cell-mediated immunity, are involved in preventing re-infection from exogenous VZV at mucosal sites (Bogger-Goren et al., 1982, Bogger-Goren et al., 1984). It has been suggested that humoral immunity may also play a role in the initial blocking of viral reactivation (Arvin, 1996), but is not the only barrier as declining antibody titres have been shown to have no effect on the rate of viral reactivation (Gershon and Steinberg, 1981).

In contrast to humoral responses, VZV-specific cell-mediated immunity is critical in containing and resolving primary infection (Arvin, 1992). This is supported by clinical observations of patients unable to acquire cellular immunity through immunosuppression. These individuals are at a higher risk of persistent viremia and
life-threatening dissemination during varicella (Arvin, 1987). The required T lymphocyte response against VZV includes both CD4+ and CD8+ T cells (Abendroth and Arvin, 1999). This response is accompanied by the secretion of T helper 1-like cytokines, including interleukin (IL)-2, IL-10, IL-12, and IFN-γ, which direct the clonal expansion of viral-specific lymphocytes and the production of different antibody isotypes (Arvin et al., 1986, Jenkins et al., 1998, Zhang et al., 1994, Zhang et al., 1995). In the classic model of cell-mediated immunity the cytotoxic T lymphocyte (CTL) response involves MHC class I restricted CD8+ T lymphocyte killing of virally infected cells (Abbas and Lichtman, 1991). However in VZV and other herpesvirus infections, both MHC class I-restricted CD8+ and MHC class II-restricted CD4+ CTLs are involved in the recognition and lysis of infected cells (Diaz et al., 1989, Hickling et al., 1987, Huang et al., 1992).

A recent genome-wide analysis of T cell responses utilising the simian varicella virus (SVV) model to specific VZV ORFs (Haberthur et al., 2013). This study demonstrated that during acute SVV infection both CD4+ and CD8+ T cells responded to both IE and E proteins, with a trend towards higher CD8+ responses. CD4+ T cell responses were predominantly towards the structural proteins during acute infection, while CD8+ T cells responded to L proteins during latency (Haberthur et al., 2013). While these findings generally correlated with previous work in human disease, a similar analysis is yet to be performed using human samples.

Although the adaptive immune system is central to the control of primary infection and prevention of re-infection, it is unable to prevent the establishment of latency or eradicate the latent virus from the host (Abendroth and Arvin, 2000). However it is predicted to play an important role in the prevention of viral reactivation. Clinical evidence for this includes a higher incidence of herpes zoster in the immunosuppressed and elderly, as increased age corresponds with a decline in T lymphocyte activity (Arvin, 1987). However declining cellular immunity may not be the only factor that leads to viral reactivation, as herpes zoster does not occur in all seropositive elderly individuals.

The immune response against herpes zoster is very similar to that seen during primary infection. The main difference is a more rapid response in herpes zoster, due to the pre-existing VZV-specific memory lymphocytes which were primed during primary infection (Vossen et al., 2004). This leads to prolonged VZV-specific immunity, and this subsequent exposure and resulting increase in immunity may help explain why the occurrence of a second episode of herpes zoster is rare.
1.6.1 Varicella Zoster Virus encoded immune evasion

VZV has evolved several different immune evasion strategies in order to persist in the human population. These are aimed towards avoiding immune detection and promoting viral spread (Arvin and Abendroth, 2007). The virus appears to evade host recognition during the initial incubation period of up to 21 days following VZV inoculation (Arvin et al., 1996), and so must encode some immunomodulatory proteins responsible for this transient evasion of T lymphocytes. VZV has been shown in vitro to specifically block the IFN-γ-induced upregulation of MHC class II (Abendroth et al., 2000), and to downregulate the cell-surface expression of MHC class I (Abendroth et al., 2001a). MHC class II surface expression is blocked through interference with the Jak/Stat signalling pathway, while the MHC class I molecules are retained within the Golgi apparatus of infected cells (Reviewed in Arvin and Abendroth, 2007). These effects may allow the virus to evade CD4+ T lymphocyte and CD8+ T lymphocyte responses (Abendroth et al., 2000, Arvin, 2001). VZV has also been shown to downregulate surface expression of the intercellular adhesion molecule 1 (ICAM-1) in infected cells (Nikkels et al., 2004). ICAM-1 is important in recruitment of inflammatory cells, and downregulation of this molecule may provide another means of virally encoded immune evasion.

VZV has the ability to infect T lymphocytes directly (Ku et al., 2002) and through transfer from VZV-infected immature DCs (Abendroth et al., 2001b), and is thought to utilise these cell types for the initial dissemination of virus during varicella. Experiments in the SCID-hu mouse model have shown that VZV-infected T cells, and predominantly memory CD4+ T cells, can mediate the transfer of infectious virus to skin (Ku et al., 2004). During VZV skin infection, expression of IFN-α is downregulated in infected cells, allowing the formation of vesicles on the skin surface (Ku et al., 2004).

VZV infection of human DCs results in the selective downregulation of functionally important cell-surface molecules, namely CD80, CD83 and CD86 (Morrow et al., 2003). Low cell-surface expression of these costimulatory molecules corresponds to an impaired ability to stimulate T cell proliferation (Abendroth et al., 2001b). This impact of VZV infection on DCs, an important class of antigen presenting cells, may also delay immunosurveillance (Arvin and Abendroth, 2007), and may be due to modulation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway by ORF66 (Sloan et al., 2012).
It has been shown that ORF63, the most prevalent VZV transcript in latently infected ganglia, blocks the effects of IFN-α (Ambagala and Cohen, 2007). IFN-α is known to limit VZV replication within human skin xenografts in the SCID-hu mouse model, and consequently the ability of ORF63 to block this cytokine may play a central role in the establishment and maintenance of a latent infection (Ku et al., 2004). Another more recent study found that IE62 blocks the function of the interferon regulatory factor 3 (IRF3) by interfering with its phosphorylation (Sen et al., 2010). VZV has been shown to interfere with NF-κB pathway, which is an integral part of the induction of the “anti-viral state” (Jones and Arvin, 2006), while ORF66 has been shown to be important in downregulating the IFN-γ signalling pathway (Schaap et al., 2005). Consequently VZV encodes many different immunomodulatory gene products that are likely to greatly inhibit the anti-viral immune response in infected cells.

1.7 Immune responses in chronic pain states

Historically inflammation and neuropathic pain syndrome have been considered distinct conditions, however there is more and more evidence that they may be related. Inflammation is a well-characterised and studied process involving a cascade of different immune cell types such as mast cells, neutrophils, macrophages and T cells. These cells have all been shown to release compounds that can both contribute to and ameliorate pain (Reviewed in Machelska, 2011). A common underlying mechanism of neuropathic pain is the presence of inflammation at the damaged or affected site (Vallejo et al., 2010) and evidence suggests that these immune cells contribute to neuropathic pain in the periphery (Reviewed in Thacker et al., 2007).

In the peripheral nervous system, tissue resident macrophages have been shown to migrate immediately following nerve injury to the site of injury, and display an active phenotype (Mueller et al., 2001). Further inflammation is promoted by the secretion of chemokines, matrix metalloproteases, and vasoactive mediators from activated macrophages, Schwann cells/satellite glial cells, and injured nerve axons to attract immune cells, increase blood flow to the area and cause swelling (Perrin et al., 2005, Shubayev et al., 2006, Zochodne et al., 1999). These changes result in a dense cellular infiltrate, mainly composed of macrophages, T lymphocytes and mast cells, which forms within 2 days of injury.
Immune cells have been shown to both ameliorate and contribute to pain. In a mouse model it was shown that CD4+ T lymphocytes were responsible for endogenous opioid-mediated analgesia in an antigen-specific manner (Boue et al., 2011). Peripheral immune cells have also been shown to actively contribute to nociceptor hypersensitivity and the resulting allodynia in a rat model of central nervous system chronic pain (Grace et al., 2011). Chemokines also have been implicated in the pathogenesis of pain states in the DRG. In addition, the upregulation of cytokine and/or chemokines within the nervous system, combined with tissue damage and infection has been proposed as a mechanism for hyperexcitability of neurons, causing pain (Miller et al., 2009, Abbadie et al., 2009). Characterisation of the host immune response within the ganglia and the skin during PHN has yet to be elucidated, and is the focus of this study.

1.8 Varicella zoster virus vaccines

The current varicella vaccine is a live attenuated virus using the Oka virus vaccine strain (vOka). The parental strain (pOka) was isolated from varicella skin lesions on a 3 year old boy (Takahashi et al., 2008), and then passaged 11 times in human embryonic lung fibroblasts at 34°C, and then 12 times in guinea pig embryo fibroblasts at 37°C (Takahashi et al., 2008, Takahashi et al., 1975). The exact genetic basis of attenuation has not been determined, however the vaccine and parental viral genomes have nucleotide differences which cause amino acids changes in all classes of viral proteins (Argaw et al., 2000, Arvin and Gilden, 2007, Gomi et al., 2002, Quinlivan et al., 2011). This vaccine strain has a lower efficiency of replication in human skin (Moffat et al., 1998a), however it remains unattenuated in both T cells and neural cells in vivo (Baiker et al., 2004b, Moffat et al., 1998a).

The current varicella vaccine was also expected to decrease the occurrence of herpes zoster. Although vOka still establishes a latent infection within sensory ganglia, it seems to have a lower rate of clinical reactivation (Edmunds and Brisson, 2002). This is likely due to the lower efficiency of vOka to replicate in human skin, leading to either lower initial access to neuronal cells following vaccination, or lower probability of causing a clinical reactivation (Baiker et al., 2004b). However general vaccination may lead to a higher occurrence of herpes zoster due to a loss of vaccine-induced immunity over time (Chaves et al., 2007). A recent study has found that the incidence of herpes zoster in the United States has increased over the years.
1993-2006 despite the introduction of the varicella vaccine, but there was no evidence to attribute this increase to the vaccine program (Leung et al., 2011). The Australian national varicella vaccination program has lead to a reduction in both congenital and neonatal varicella since its introduction in 2005 (Khandaker et al., 2011). Additionally, although rare, the vaccine strain can still reactivate and result in severe illness in both the immunocompromised and immunocompetent host (Han et al., 2011, Tseng et al., 2010, Iyer et al., 2009, Bryan et al., 2008).

Another vaccine formation has been developed for herpes zoster, and was shown to reduce the incidence of both herpes zoster and PHN in at-risk groups by up to a half and two thirds respectively (Oxman et al., 2005). This vaccine consists of the same viral strain at a fourteen times higher dose (Oxman et al., 2005). Viral DNA of the vaccine strain is detectable within the saliva of many recipients for over 28 days (Pierson et al., 2011). This vaccine provides a cost-effective and efficient preventative measure to prevent both herpes zoster and PHN, however does not always prevent either condition (Johnson, 2009, Najafzadeh et al., 2009). The duration of protective immunity provided, and the effects of vaccinating younger or at-risk groups is unknown.

1.9 The human skin

The skin is a highly complex organ, and at around 2 m$^2$ is the largest in the human body – comprising of around 6% of the total body weight (Tobin, 2006). It is constantly in a process of regeneration and has many important immunologic and metabolic functions.

1.9.1 Structure and function

There are two distinct layers within the human skin: the epidermis and the dermis.

1.9.1.1 The Epidermis

The epidermis is the outermost layer of the skin. It is typically around 50 – 100 µm thick (Rees, 2004), and consists of hardening epithelium that terminates at mucocutaneous junctions (such as the mouth), and is disrupted by pores of glands and hair fibres (Tobin, 2006). The primary cell type within the epidermis is keratinocytes, with smaller numbers of both Langerhans cells and melanocytes.
This layer is undergoing constant renewal, with cells gradually progressing from the innermost to outer levels, and it is estimated that the keratinocyte population within the epidermis is completely replaced around every 30 days (Farage et al., 2007). Within the epidermis there are four main “strata”, or layers, with different cell types restricted to the different layers (Nestle et al., 2009, Narayan, 2009) (Figure 1.9).

**1.9.1.2 THE DERMIS**

The dermis lies directly below the epidermis. Despite its greater volume when compared to the epidermis, the dermis contains far fewer cells (Ross and Pawlina, 2003). It can be roughly divided into two areas: a thin superficial area directly adjacent to the epidermis called the papillary region, and a thicker and deeper region known as the reticular region (Farage et al., 2007). The papillary region contains thin projections that interdigitate with the epidermis, strengthening the connection between the two (Farage et al., 2007). The reticular region consists mainly of a fibrous and amorphous extracellular matrix surrounding nerves, blood vessels, hair follicles, sebaceous and sweat glands, receptors and dermal cells (Tobin, 2006).

**1.9.2 Skin immune function and surveillance**

The epidermis is home to several important immune cell types, including macrophages and mast cells, as well as DCs and different subtypes of T cells (Nestle et al., 2009). The main resident immune cell in the epidermis is the Langerhans cell (Nestle et al., 2009). These are immature dendritic cells that are present at high levels in the epidermis, and perform an important immune surveillance function (Valladeau and Saeland, 2005). In addition, there are also T cells, mainly CD8⁺ lymphocytes, present in the lower layers of the epidermis (Krueger and Stingl, 1989).

In comparison to the rather simple histology of the epidermis, the dermis features a much greater cellular diversity with a more complicated anatomy. Cells present include macrophages, mast cells, fibroblasts and nerve endings, as well as many specialised immune cell types including dermal DCs, and both conventional (CD4⁺ T helper lymphocytes) and unconventional (γδ T cells and natural killer) T lymphocytes (Nestle et al., 2009). Another important immune cell, the plasmacytoid dendritic cell (pDC), is normally rare in healthy human skin however has an important role in inflammatory conditions (Cella et al., 1999, Colonna et al., 2004, Valladeau and Saeland, 2005), as well as in the acute phases of innate and adaptive immune
Figure 1.9 – Multi-layered structure of human skin

A haematoxylin and eosin stained section of human skin with the major structural features labelled. The epidermis is divided into 4 main layers: the stratum corneum, the stratum granulosum, the stratum spinosum and the stratum germinativum, and contains keratinocytes and other epidermal cells.

Underlying the epidermis is the dermis, which contains many different cells types, including fibroblasts.

Source
http://www.seas.ucla.edu/~pilon/Skin1.htm
(Accessed 15 August 2011)
responses, such as in herpesvirus infections (Donaghy et al., 2009, Huch et al., 2010).

1.9.3 VZV infection of human skin

VZV is thought to spread to the skin during primary infection using a tropism for human tonsillar CD4+ T lymphocytes which express skin homing markers (Ku et al., 2002, Ku et al., 2004). Once in the skin, VZV is able to replicate within all layers of the skin, causing typical lesions and resulting in the release of virions from infected cells with infectious virus present within the vesicular fluid and resulting scab (Annunziato et al., 1996, Annunziato et al., 2000, Nikkels et al., 1993, Nikkels et al., 1995a, Taylor and Moffat, 2005).

Previous studies have shown that there is a dramatic shift in the numbers of important immune cells within the skin during herpes zoster, with a reduction in tissue resident Langerhans cells within the epidermis, and an increase in pDCs – highly inflammatory dendritic cells (Colonna et al., 2004) – within herpes zoster-affected skin (Gutzeit et al., 2010, Huch et al., 2010). VZV antigen has been observed in skin biopsies during both varicella and herpes zoster, and has been shown to inhibit the ability of pDCs to express IFN-α, and consequently delay the immune response within the skin (Huch et al., 2010).

There have also been several studies comparing PHN-affected and normal skin material, which have been dealt with in more detail in section 1.4. Characterisation of human skin and specifically persistence of VZV antigen expression and immune cell composition in the skin of PHN-affected versus non-PHN-affected individuals has yet to be elucidated, and forms a large part of this study.
1.10 Aims and Hypotheses

Remarkably, despite the pivotal role of ganglionic infection to PHN, a survey of the literature over the past 100 years reveals few studies that have reported any examination of ganglia following herpes zoster and PHN, and many of those that have are restricted to basic histological observations of post-mortem material. This PhD project involves a detailed immunohistological assessment of VZV infection and immune cell infiltration in human ganglia and skin biopsy samples obtained following natural reactivation and subsequent development of PHN. There is a critical need for this work to further our understanding of the involvement of the cause and pathogenesis of PHN.

Currently it is not known whether there is a factor which predisposes some herpes zoster-affected individuals to developing PHN. There has yet to be a detailed study into any underlying immune cell involvement and persistence of VZV antigen expression during herpes zoster or PHN. It is also important to compare data from herpes zoster patients who developed PHN and those that experienced an uncomplicated recovery in an effort to find any distinguishing factor(s) between the two outcomes.

In order to further our understanding of the underlying cause and pathogenesis of PHN, this PhD study sought to focus on two critical sites of VZV infection during natural VZV reactivation – the ganglia and the skin. The specific aims were to:

1a Perform a detailed immunohistological analysis of human ganglionic material surgically excised from a PHN-affected individual years following resolution of the herpes zoster-associated rash, and compare and contrast with post mortem material taken from herpes zoster-affected patients and control patients in order to:

• Characterise any difference in local immune response and cellular immune infiltrate in the DRG and the spinal nerves.

• Identify and characterise VZV antigen expression within the ganglia material.

1b Perform a molecular analysis VZV-specific nucleic acid levels in order to assess the VZV nucleic acid levels within the material.
2a Perform an immunohistological analysis of skin biopsy samples obtained from patients who suffered from PHN-affected and control patients in order to:

- Characterise the cellular immune infiltrate in the skin of PHN-affected and control patients, at different sites within the same individuals
- Identify and characterise VZV antigen expression within the skin biopsy material.

2b Perform a molecular analysis of VZV-specific nucleic acid levels within the human skin biopsy material.

3 Perform an immunohistological analysis of skin biopsy samples obtained from patients suffering from herpes zoster over time in order to

- Identify VZV antigen expression within the skin biopsy material
- Compare and contrast immune cell phenotype over time in human skin biopsies from herpes zoster-affected patients who experienced uncomplicated herpes zoster and herpes zoster resulting in PHN.
- To identify any relation between the frequency or phenotype of immune cells, the presence or absence of VZV antigen expression, and the clinical level of pain reported by the patient.

So in regards to these aims, I hypothesise that:

- VZV reactivation in human ganglia and human skin during herpes zoster and PHN is defined by unique cellular infiltrates comprised of subsets of inflammatory cells.
- VZV gene expression in human ganglia and human skin during PHN results in productive viral antigen expression in a small subset of cells.
- The dynamics of the immune infiltration in the skin of herpes zoster patients will differ between those that develop PHN and those that experience an uncomplicated recovery.
- The presence of VZV antigen expression will correlate with greater clinically reported pain levels.
- The immune cell phenotype will differ between patients who report higher or lower clinical pain levels.
1.11 Research plan

One major difficulty in studying PHN is that the DRG samples used in many studies are taken from post-mortem examinations of patients, which can take place up to days after death. This raises the possibility that they may not be a true representation of the situation in vivo. Recently our lab came into possession of DRG samples that were surgically removed from a live patient suffering from PHN in order to relieve pain. These precious and rare samples will form the basis for some of the experiments in this project, and will be compared to a ganglia sample surgically excised from a patient with no evidence of PHN or herpes zoster, and post-mortem ganglia samples taken from both herpes zoster-affected and control patients.

While skin biopsy samples are relatively easier to obtain than DRG samples as the procedure is less intrusive, there has been relatively limited work into herpes zoster and PHN involving skin samples. Currently it is not known whether there is an ongoing factor which predisposes some herpes zoster-affected individuals to developing PHN. Nor has there been a study which correlates clinical data with any underlying immune involvement during herpes zoster or PHN.

Through a collaboration with the University of California, San Francisco (UCSF) we have access to skin biopsy samples taken over time from herpes zoster-affected patients who later went on to either develop PHN or had a complete recovery, along with clinical questionnaires which examine the patient’s experience of pain and other related details. This offers a unique opportunity to perform a detailed immunohistological assessment of VZV infection and immune infiltration in skin biopsy samples taken from single patients over time. By correlating the immunohistological findings with the clinical data, it may shed light on the potential mechanisms which cause one herpes zoster-affected patient to develop PHN, while another experiences no complications and makes a complete recovery.

All of the included ganglia samples have been formalin fixed, paraffin embedded, cut into consecutive sections and mounted on microscope slides. This allows the use of consecutive mirror image 5 µm sections to link together examination of basic histology, identification of the phenotype of infiltrating cells, and assessment of the extent of viral antigen expression via immunofluorescent assay (IFA) and immunohistochemistry (IHC) as described below.
The skin biopsy samples have been formalin fixed, snap frozen, and cut in consecutive 14 µm sections. These sections will also be used in IFAs to identify immune cells and viral antigen positive cells. Although these sections are also consecutive mirror images, due to the thicker nature of the sections it is more difficult to compare the same regions across different slides.

To characterise any immune infiltration in DRG and skin samples from patients suffering from herpes zoster and PHN, IFA and/or IHC will be performed on the paraffin-embedded and frozen sections utilising a variety of antibodies specific for different immune cells (such as different T cell subsets, B cell and dendritic cell specific cell markers). Assuming this is successful, their proximity to VZV-infected cells and other cells will be assessed by performing dual IFA stains for VZV antigens and immune cells within the same section, and comparing the corresponding locations of different markers examined over consecutive sections.

To assess viral gene expression during PHN, IFA will initially be performed on paraffin-embedded DRG and skin samples from patients suffering from PHN using antibodies specific for the VZV protein IE63, and the protein complex between gE and gl (a marker of productive infection). In addition to this, reverse transcriptase real time PCR analysis will be performed to check for any viral transcripts present in the tissue.

This study will further our understanding of the pathogenesis of VZV infection of human ganglia and skin in vivo, and has the potential to greatly enhance our limited understanding of PHN.
Chapter 2: Materials and Methods

2.1 Buffers and Solutions

2.1.1 General reagents

1% Agarose (low melting point) for paraffin-embedded cell pellets

0.5 g low gelling temperature agarose powder (Sigma, USA) was dissolved in 50 mL 1x phosphate buffered saline (PBS) by heating.

100% Ethanol

Undenatured ethanol 100% (Fronine Laboratory Supplies, Australia).

95% Ethanol

Undenatured ethanol 100% (Fronine Laboratory Supplies, Australia) was added to distilled H\textsubscript{2}O (dH\textsubscript{2}O) to give a final concentration of 95% v/v.

90% Ethanol

Undenatured ethanol 100% (Fronine Laboratory Supplies, Australia) was added to dH\textsubscript{2}O to give a final concentration of 90% v/v.

70% Ethanol

Undenatured absolute ethanol (Fronine Laboratory Supplies, Australia) was added to dH\textsubscript{2}O to give a final concentration of 70% v/v.

Histolene

Dipentene 100% (Fronine Laboratory Supplies, Australia).
10 M NaOH
Prepared by dissolving 80 g NaOH pellets (Sigma-Aldrich, Australia) in 200 mL ultra pure H$_2$O.

25x PBS
Prepared by dissolving 200 g NaCl (Sigma-Aldrich, Australia), 28.7 Na$_2$HPO$_4$ (Sigma-Aldrich, Australia), 5 g KCl (Sigma, USA) and 5 g KH$_2$PO$_4$ (Sigma, USA) in 300 mL ultrapure H$_2$O. The pH was adjusted to approximately 7.4 using concentrated hydrochloric acid solution (37%) (Sigma-Aldrich, Australia) before being made up to 1 L final volume with ultra pure H$_2$O (Sigma-Aldrich, Australia).

1x PBS
Prepared by diluting 80 mL 25x PBS stock with 1920 mL dH$_2$O to a final volume of 2 L.

Proteinase K
Prepared by resuspending Proteinase K powder (Amresco, USA) in ultra pure H$_2$O to a final concentration of 20 mg/mL. Solution was stored at -20°C in 1 mL aliquots.

Xylene
Dimethylbenzines (Fronine Laboratory Supplies, Australia).

2.1.2 Cell culture reagents

Dulbecco’s Modified Eagle’s Media (DMEM)
DMEM (Gibco, USA) contains 4.5 g/L D-glucose, L-glutamine and sodium pyruvate.
Heat inactivated foetal bovine serum (FBS)

FBS (JRH Biosciences, USA) was heat inactivated at 56°C for 30 minutes, and then stored at -20°C until needed.

MACS buffer

Prepared by diluting human AB serum (Invitrogen, USA) and 0.5 M EDTA (pH 8.0) (Gibco, USA) to final concentrations of 1% v/v and 5 mM respectively in 1x PBS.

Supplemented DMEM

DMEM was supplemented with 10% v/v heat inactivated FBS and 50 IU/mL penicillin streptomycin (Gibco, USA) and was stored at 4°C until needed.

Trypsin Versene

Concentrated 10x Trypsin (0.12% v/v) /versene (0.05% v/v) (Gibco, USA) was diluted 1:4 in 1x PBS. Solution was stored at -20°C until needed.

2.1.3 Molecular biology reagents

1% Agarose gel

2 g of UltraPure Agarose (Invitrogen, USA) was dissolved in 100 mL 1x TAE by heating. Volume was then made up to 200 mL. Gel mix was allowed to cool. When gel had almost set 1 μL ethidium bromide solution was added to a final concentration of 50 ng/mL, mixed well by swirling and gel was poured into gel tank (Bio-Rad Laboratories, Australia) with end formers in place, well comb was then set in place and gel allowed to set. Prior to loading of samples, end formers and well comb were removed and gel tank was filled with 1x TAE to cover the gel.

Ammonium Acetate solution

5 M ammonium acetate solution in dH₂O (Fluka, Australia)
**Ampicillin stock solution**

Prepared by dissolving 0.5 g ampicillin powder (Sigma, USA) in 10 mL dH₂O to a final concentration of 50 mg/mL. Stock solution was stored at -20°C in 1 mL aliquots.

**100x BSA**

1 mg/mL bovine serum albumin (New England Biolabs, USA)

**DNA ladder mixture**

GeneRuler DNA ladder mix (100-10000bp) (Fermentas, Canada).

**DNA loading buffer**

Blue/Orange 6x loading dye (Promega, USA).

**10 mM dNTP mix**

PCR Nucleotide Mix. Contains the sodium salts of dATP, dCTP, dGTP, dTTP, each at a concentration of 10 mM in water (Invitrogen, USA).

**25 mM dNTP mix**

PCR Nucleotide Mix. Contains the sodium salts of dATP, dCTP, dGTP, dTTP, each at a concentration of 25 mM in water (Promega, USA).

**Ethidium Bromide solution**

3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide, 10 mg/mL (Sigma, USA).

**GoTaq Flexi DNA Polymerase**

500 U, 5 U/µL with 5x green reaction buffer (Promega, USA).
Isopropanol

2-Propanol (Sigma, USA).

Kanamycin stock solution

Prepared by dissolving 0.5 g kanamycin sulfate (Sigma, USA) in 10 mL dH₂O to a final concentration of 50 mg/mL. Stock solution was stored at -20°C in 1 mL aliquots.

Luria Bertani (LB) agar

Prepared by dissolving 35 g LB agar powder (Sigma, USA) in 1 L of ultra pure H₂O and autoclaved for 15 minutes at 121°C. Agar was allowed to cool and then mixed with appropriate antibiotic (ampicillin or kanamycin, to 50 µg/mL) before being poured into plates (BD Biosciences, Australia) and allowed to set. Plates were stored at 4°C until required.

LB broth

Prepared by dissolving 20 g LB broth powder (Sigma, USA) in 1 L of ultra pure H₂O and autoclaved for 15 minutes at 121°C. Broth was stored at 4°C until required.

Magnesium Chloride Solution

25 mM MgCl₂ solution (Promega, USA).

10x NEBuffer 1

Solution containing 10 mM Bis-Tris-Propane-HCL, 10 mM MgCl₂, 1 mM Dithiothreitol (New England Biolabs, USA).

SacI

Restriction enzyme (20 000 units/mL) (New England Biolabs, USA)
SOC media
S.O.C. medium (Invitrogen, USA) consisting of 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate and 20 mM glucose.

25x TAE buffer
Prepared by dissolving 242 g Tris, 57.1 mL glacial acetic acid (Sigma-Aldrich, Australia) and 100 mL 0.5 M EDTA (pH 8.0) (Gibco, USA) in 1 L distilled H₂O. Distilled H₂O was added to make volume up to 2 L.

1x TAE buffer
Prepared by diluting 40 mL 25x TAE buffer in 960 mL dH₂O.

2.1.4 Immunohistochemistry reagents

Acid alcohol differentiator solution
Prepared by mixing 4 L 70% ethanol solution with 10 mL concentrated hydrochloric acid solution (37%) (Sigma-Aldrich, Australia).

Azure B solution
Prepared by dissolving azure B powder (Sigma-Aldrich, Australia) in ultrapure dH₂O to a final concentration of 1%.

Biotin blocking system
Endogenous biotin blocking system (Dako, Australia) consisting of avidin solution and biotin solution.
**Diaminobenzidine (DAB) working solution**
Liquid DAB+ Substrate Chromogen System (Dako, Australia). Prepared immediately prior to use by mixing DAB+ chromogen solution and DAB+ substrate buffer in the ratio of 1 drop to 1 mL respectively.

**DPX**
DePeX Mounting Media (Electron Microscopy Science, USA).

**Eosin counter-stain**
Alcoholic eosin (Fronine Laboratory Supplies, Australia).

**3% H₂O₂ solution**
Prepared by diluting 45 mL hydrogen peroxide solution (30%) (Sigma-Aldrich, Australia) in 405 mL dH₂O.

**Haematoxylin stain**
Haematoxylin solution (Fronine Laboratory Supplies, Australia).

**IHC antibody dilution buffer**
10% (v/v) rabbit or goat serum (depending on the primary antibody) (Sigma, USA) in 1x TBS.

**IHC blocking solution**
20% (v/v) rabbit or goat serum (depending on the primary antibody) (Sigma, USA) in 1x TBS.
*Scott’s blueing solution*

Prepared by dissolving 14 g NaHCO₃ (Sigma-Aldrich, Australia) in 2 L dH₂O, and 80 g MgSO₄ (Sigma-Aldrich, Australia) in 2 L dH₂O, and then mixing the two solutions to make a 4 L solution.

2.1.5 Immunofluorescent assay (IFA) reagents

10x (0.1 M) citrate buffer pH 6.0

Prepared by dissolving 42.02 g citric acid (anhydrous) (Univar, USA) in 1.8 L dH₂O, and the pH was adjusted to pH 6.0 using concentrated 10 M NaOH (Sigma-Aldrich, Australia). Distilled H₂O was added to produce a final volume of 2 L.

1x (0.01 M) citrate buffer pH 6.0

Prepared by diluting 10x (0.1 M) citrate buffer in dH₂O to a final concentration or 1x (0.01 M).

*Cryochrome freezing compound*

Shandon cryochrome compound sampler – containing red, yellow, green and blue-coloured cryo-preservation compound (Thermo Fisher Scientific, USA).

*EDTA unmasking solution*

Prepared by diluting 1 mL 0.5 M EDTA (pH 8.0) (Gibco, USA) in 500 mL dH₂O to a final concentration of 1 mM.

*Gelatin slide coating*

Prepared by dissolving 1% w/v gelatin (Sigma, USA) and 0.1% chromium (III) potassium sulfate (Sigma-Aldrich, Australia) in dH₂O
IFA antibody dilution buffer

10% v/v normal donkey serum (NDS) (Sigma, USA) in 1x tris buffered saline (TBS) or 1x PBS.

IFA blocking buffer

20% v/v NDS (Sigma, USA) in 1x TBS or 1x PBS.

IFA blocking and dilution buffer (cryopreserved sample material)

Prepared by dissolving 10 g bovine serum albumin (BSA) (Sigma, USA) and 2 mL Triton X-100 (Sigma-Aldrich, Australia) in 1 L 1x PBS to final concentrations of 1% and 0.2% respectively.

Neutral Buffered Formalin (NBF) (10%)

Neutral buffered solution containing 10% formalin (approximately 4% formaldehyde) (Sigma, USA).

Prolong Gold with 4’,6-diamidino-2-phenylindole (DAPI)

Prolong Gold antifade reagent with DAPI (Molecular Probes, USA).

10x Sudan black stock solution

Prepared by dissolving 6 g Sudan black B (Sigma-Aldrich, Australia) in 200 mL 70% ethanol. Solution was stored at room temperature and protected from light.

Sudan black autofluorescence eliminator

Prepared by diluting 10 mL 10x Sudan black stock solution in 90 mL 70% ethanol. Solution was then syringe filtered through a 0.22 µm filter (Millipore, USA) and protected from light.
10x TBS
Prepared by dissolving 121.14 g Tris (Sigma-Aldrich, Australia) and 175 g NaCl (Sigma-Aldrich, Australia) in 1.5 L ultra pure H₂O. The pH was adjusted to approximately 7.6 using concentrated hydrochloric acid solution (37%) (Sigma-Aldrich, Australia) before being made up to 2 L final volume with ultra pure H₂O.

1x TBS
Prepared by diluting 200 mL 10x TBS stock with 1800 mL dH₂O to a final volume of 2 L.

2.2 Antibodies
Primary antibodies used for single and dual immunofluorescent staining are listed in Table 2.1. Primary antibodies used for immunohistochemistry are listed in Table 2.2. Isotype control antibodies for staining experiments are listed in Table 2.3. Fluorochrome-conjugated secondary antibodies used for immunofluorescent staining are listed in Table 2.4. Secondary enzyme-labelled antibodies utilised in immunohistochemistry are listed in Table 2.5.

2.3 Primers
Primer sets and sequences for PCR are shown in Table 2.6. Primers specific for VZV amplify a small sequence in the untranslated region between VZV ORF8 and ORF9a. Primers specific for the UL-42 region of HSV-1 were taken from a previously published paper (Cassinotti and Siegl, 1998). Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from an in-house design kindly provided by Dr Megan Steain.

Primers used in the creation of plasmid constructs and to check the sensitivity of real time PCR reactions are also listed in Table 2.6. Primers specific for VZV ORF31 (also known as VZV glycoprotein B) amplify a 853 bp fragment including the region detected by the gB-specific real time primer set, and were designed using the online tool provided by the National Centre for Biotechnology Information (NCBI). Primers
specific for VZV ORF63 amplify a 544 bp fragment, including the region detected by the IE63-specific real time primer set, and were also designed using the online tool provided by the NCBI. Primers specific for VZV ORF68 (also known as VZV glycoprotein E) amplify a 721 bp fragment including the region detected by the gE-specific real time primer set, and were previously designed by Dr Chantelle Hood (Westmead Millennium Institute).

### Table 2.1: Primary antibodies used in IFA

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Optimal Dilution</th>
<th>Optimal concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-human CD3</td>
<td>Leica Microsystems Inc, Germany</td>
<td>1:10</td>
<td>20 µg/mL</td>
</tr>
<tr>
<td>Goat anti-human CD4</td>
<td>R&amp;D Systems, USA</td>
<td>1:10</td>
<td>10 µg/mL</td>
</tr>
<tr>
<td>Rabbit anti-human CD8</td>
<td>Abcam, USA</td>
<td>1:100</td>
<td>2 µg/mL</td>
</tr>
<tr>
<td>Mouse anti-human CD20</td>
<td>Leica Microsystems Inc, Germany</td>
<td>1:10</td>
<td>18 µg/mL</td>
</tr>
<tr>
<td>Mouse anti-human CD45RO</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>1:100</td>
<td>1 µg/mL</td>
</tr>
<tr>
<td>Mouse anti-human CD45RA</td>
<td>Santa Cruz Biotechnology, USA</td>
<td>1:100</td>
<td>1 µg/mL</td>
</tr>
<tr>
<td>Mouse anti-human TIA-1</td>
<td>Abcam, USA</td>
<td>1:50</td>
<td>2 µg/mL</td>
</tr>
<tr>
<td>Rabbit anti-cow S100</td>
<td>Dako, Denmark</td>
<td>Predilute</td>
<td>(unknown*)</td>
</tr>
<tr>
<td>Mouse anti-human S100</td>
<td>Millipore, USA</td>
<td>1:200</td>
<td>1:200</td>
</tr>
<tr>
<td>Mouse anti-human HLA-DR (BDCA-2)</td>
<td>Leica Microsystems Inc, Germany</td>
<td>1:50</td>
<td>(unknown*)</td>
</tr>
<tr>
<td>Goat anti-human DC-SIGN</td>
<td>R&amp;D Systems, USA</td>
<td>1:50</td>
<td>2 µg/mL</td>
</tr>
<tr>
<td>Rabbit anti-human Langerin</td>
<td>Leica Microsystems Inc, Germany</td>
<td>1:200</td>
<td>(unknown*)</td>
</tr>
<tr>
<td>Mouse anti-VZV glycoprotein E</td>
<td>Millipore, USA</td>
<td>1:10</td>
<td>100 µg/mL</td>
</tr>
<tr>
<td>Mouse anti-VZV glycoprotein E:glycoprotein I complex</td>
<td>Meridian Life Science, USA</td>
<td>1:250</td>
<td>4 µg/mL</td>
</tr>
<tr>
<td>Rabbit anti-VZV ORF63</td>
<td>Provided by Professor R. Mahalingam, University of Colorado School of Medicine</td>
<td>1:1000</td>
<td>(unknown**)</td>
</tr>
</tbody>
</table>

* - Concentration unknown as not provided by manufacturer. Isotype matched to antibody dilution
** - Concentration unknown as provided as serum. Isotype serum matched to antibody dilution
Table 2.2: Primary antibodies used in IHC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Optimal Dilution</th>
<th>Optimal concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-VZV glycoprotein E</td>
<td>Millipore, USA</td>
<td>1:10</td>
<td>100 ug/mL</td>
</tr>
<tr>
<td>Mouse anti-VZV glycoprotein E:glycoprotein I complex</td>
<td>Meridian Life Science, USA</td>
<td>1:250</td>
<td>4 ug/mL</td>
</tr>
<tr>
<td>Rabbit anti-VZV ORF63</td>
<td>Provided by Professor R. Mahalingam, University of Colorado School of Medicine</td>
<td>1:1000</td>
<td>(unknown*)</td>
</tr>
<tr>
<td>Mouse anti-VZV glycoprotein B</td>
<td>Dr C. Grose, University of Iowa</td>
<td>1:10</td>
<td>(unknown*)</td>
</tr>
<tr>
<td>Rabbit anti-VZV ORF29</td>
<td>Dr P.R. Kinchington, University of Pittsburgh</td>
<td>1:250</td>
<td>(unknown*)</td>
</tr>
<tr>
<td>Mouse anti-CD68</td>
<td>Leica Microsystems Inc, Germany</td>
<td>1:10</td>
<td>(unknown**)</td>
</tr>
</tbody>
</table>

* - Concentration unknown as provided as serum. Isotype serum matched to antibody dilution
** - Concentration unknown as not provided by manufacturer. Isotype matched to antibody dilution

Table 2.3: Isotype controls used in IFA and IHC

<table>
<thead>
<tr>
<th>Isotypes</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG 1</td>
<td>Invitrogen, USA</td>
<td>As required to match primary antibody concentration</td>
</tr>
<tr>
<td>Mouse IgG 2a</td>
<td>Invitrogen, USA</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG 2b</td>
<td>Invitrogen, USA</td>
<td></td>
</tr>
<tr>
<td>Normal Rabbit IgG</td>
<td>R&amp;D Systems, USA</td>
<td></td>
</tr>
<tr>
<td>Normal Rabbit Serum</td>
<td>Sigma, USA</td>
<td></td>
</tr>
<tr>
<td>Normal Goat IgG</td>
<td>R&amp;D Systems, USA</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: Secondary antibodies used for IFA

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Optimal Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 546 donkey anti-mouse IgG</td>
<td>Molecular Probes, USA</td>
<td>1:200</td>
</tr>
<tr>
<td>Alexa Fluor 594 donkey anti-mouse IgG</td>
<td>Molecular Probes, USA</td>
<td>1:200</td>
</tr>
<tr>
<td>Alexa Fluor 488 donkey anti-rabbit IgG</td>
<td>Molecular Probes, USA</td>
<td>1:200</td>
</tr>
<tr>
<td>Alexa Fluor 647 donkey anti-rabbit IgG</td>
<td>Molecular Probes, USA</td>
<td>1:200</td>
</tr>
<tr>
<td>Alexa Fluor 488 donkey anti-goat IgG</td>
<td>Molecular Probes, USA</td>
<td>1:200</td>
</tr>
</tbody>
</table>
Table 2.5: Secondary antibodies used for IHC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Optimal Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal rabbit anti-mouse immunoglobulin. Biotin. Affinity-isolated F(ab’)2</td>
<td>Dako, Australia</td>
<td>1:200</td>
</tr>
<tr>
<td>Polyclonal goat anti-rabbit immunoglobulin. Biotin. Affinity isolated</td>
<td>Dako, Australia</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2.6: PCR primer specificities and sequences

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VZV ORF9a</td>
<td>Forward</td>
<td>TCG GAT TCG GAA TAG TTT GGC TAC A</td>
<td>365</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACA CGG CAG ACC GGG TAT TAA AC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CGA GAT CCC TCC AAA ATC AA</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTC TTC TGG GTG GCA GTG AT</td>
<td></td>
</tr>
<tr>
<td>HSV-1 UL-42</td>
<td>Forward</td>
<td>GCT TCG TGG TGC TGG TT</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTC GTC CCT GAC GAC AC</td>
<td></td>
</tr>
<tr>
<td>VZV ORF31 fragment</td>
<td>Forward</td>
<td>GGA ACC TGC ACC GGG GAA CT</td>
<td>853</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTC CAC CGT CCC GGA CCC AT</td>
<td></td>
</tr>
<tr>
<td>VZV ORF63 fragment</td>
<td>Forward</td>
<td>GCG GCG CAT ATG CCT CGA CT</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGC TTC GTG TCG ACG TCG CTT</td>
<td></td>
</tr>
<tr>
<td>VZV ORF68 fragment</td>
<td>Forward</td>
<td>TTT CAA GAC ACC CGG TTC AAT CTC G</td>
<td>721</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAT ACA AAC TCC GTA TAT GAG CTC T</td>
<td></td>
</tr>
</tbody>
</table>

Primer sets and sequences for real time PCR reactions are shown in Table 2.7. The GAPDH-specific primer pair was previously designed and kindly provided by Dr Kavitha Gowrishankar (Westmead Millennium Institute). VZV-specific primers detecting VZV gB (ORF31), VZV IE63 and VZV gE (ORF68) were previously designed and kindly provided by Dr Joshua Bowles (Westmead Millennium Institute). The albumin-specific primer pair used in quantitative real time PCR reactions has been previously published (Douek et al., 2002). Information regarding the VZV ORF28-specific primer pair and probe was generously provided by Prof. Randall Cohrs of the University of Colorado, Denver.
Table 2.7: Real time PCR primer specificities and sequences

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>TCA CCA GGG CTG CTT TTA AC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAC AAG CTT CCC GTT CTC AG</td>
</tr>
<tr>
<td>VZV gB (ORF31)</td>
<td>Forward</td>
<td>CCA GAC CTA CCT TGC CAG AG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCG TCG GGA TCT GGT ATT TC</td>
</tr>
<tr>
<td>VZV ORF63</td>
<td>Forward</td>
<td>GAG GAG AGC ACC GAT TCT TG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTT TCG CGG CTG TAT ATT CC</td>
</tr>
<tr>
<td>VZV gE (ORF68)</td>
<td>Forward</td>
<td>CCG AGA CTT GGA GCT TTT TG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CGG CCA ACT GAT CCT CTT TA</td>
</tr>
<tr>
<td>Albumin</td>
<td>Forward</td>
<td>TGC ATG AGA AAA CCG CAG TAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTG GTG AAC A-G CCG ACC AT</td>
</tr>
<tr>
<td>VZV ORF28</td>
<td>Forward</td>
<td>CGA ACA CGT TCC CCA TCA A</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCC GGC TTT GTT AGT TTT GG</td>
</tr>
<tr>
<td>VZV probe (Iowa)</td>
<td>Forward</td>
<td>(FAM) -CCA GGT TTT AGT TGA TAC CA</td>
</tr>
</tbody>
</table>

Note: (FAM) signifies the fluorophor 6-carboxyfluorescin

Primers utilised in sequencing reactions are shown in Table 2.8. These primers were designed and kindly provided for use by Bin Wang (Westmead Millennium Institute), and are specific for regions either side of the insert site of the pGEM-T-Easy vector (Promega, USA).

Table 2.8: Primers used in plasmid sequencing

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T-Easy vector</td>
<td>pGEMTa</td>
<td>TCT CCC ATA TGG TCG ACC T</td>
</tr>
<tr>
<td></td>
<td>pGEMTb</td>
<td>TCA CGA CGT TGT AAA ACG AC</td>
</tr>
</tbody>
</table>

2.4 Commercial Kits

RecoverALL Total Nucleic Acid Isolation kit (Applied Biosystems, USA)

QIAquick PCR purification kit (Qiagen, Australia)

pGEM-T Easy Vector System (Promega, USA)

QIAprep Spin Miniprep Kit (Qiagen, Australia)

QIAfilter Plasmid Maxi (Qiagen, Australia)
2.5 Cells

2.5.1 Cell culture techniques

2.5.1.1 Resuscitating cells

Cells were removed from -80°C and then immediately thawed in water at room temperature, and subsequently transferred to a falcon tube (BD Biosciences, Australia) containing 9 mL of appropriate culture media. Cells were centrifuged at 524 x g for 5 minutes at room temperature and supernatant aspirated and discarded. Cells were rinsed once in 1x PBS, centrifuged at 524 x g for 5 minutes at room temperature and supernatant aspirated and discarded. Cells were resuspended in tissue culture media and transferred to a tissue culture flask of the appropriate area. Flasks were incubated at 37°C and 5% CO₂.

2.5.1.2 Harvesting cells

Cell media was aspirated and discarded to remove residual media. Cells were washed with 1x PBS, which was aspirated and discarded. To remove cells from flask 1.5 mL trypsin versene was added to each 150 mL tissue culture flask, and flasks were incubated at 37°C for 5 minutes. Media was then added and flasks were agitated to remove cells from the tissue culture flask surface. Cells were then used to create paraffin-embedded or frozen control cell blocks or for molecular analysis.

2.5.1.3 Splitting cells

Cells were harvested as previously described, and then seeded into new tissue culture flasks at a lower density. Flasks were incubated at 37°C and 5% CO₂.

2.5.1.4 Infecting cells

When a cytopathic effect (CPE) of 2+ to 3+ was achieved by the virus, where 1+ represents 10% of cells infected and 4+ represents 100% of cells infected as demonstrated by plaque formation, the cells were harvested as previously described. The VZV-infected cells were seeded onto uninfected cells to achieve a cell-associated infection at the desired ratio.

2.5.1.5 Creation of control cell blocks

To harvest cells for the creation of paraffin-embedded or frozen control cell blocks, cells were harvested from culture as previously described (Section 2.5.1.2) and the
cell suspension was transferred to a 15 mL Falcon tube (BD Biosciences, USA). Cells were pelleted by centrifugation at 524 x g for 5 minutes at room temperature and supernatant aspirated and discarded. Cell pellet was rinsed once in 1x PBS, and centrifuged at 524 x g for 5 minutes at room temperature and supernatant aspirated and discarded. Cell pellet was then mixed well with 10 mL neutral buffered formalin and fixed at 4°C overnight. Cell were centrifuged at 524 x g for 5 minutes at room temperature, and the cell pellet was rinsed twice with 1x PBS, centrifuged at 524 x g for 5 minutes at room temperature and supernatant aspirated and discarded.

2.5.1.5.1 Paraffin-embedded cell block creation

For the creation of paraffin-embedded cell blocks the cell pellet was resuspended in 0.8% agarose (low gelling temperature) and allowed to set. Agarose cell pellets were then transferred to a histopathology tissue cassette (ProSciTech, Australia) and transferred to the University of Sydney Histopathology laboratory for overnight processing and paraffin-embedding. Sectioning was performed on a microtome (Thermo Fisher Scientific, USA) and 5 µm sections were placed on Superfrost Plus microscope slides (Thermo Fisher Scientific, USA). Slides were stored at room temperature until required.

2.5.1.5.2 Frozen cell block creation

For the creation of frozen cell blocks the cell pellet was resuspended in cryochrome freezing compound, transferred to a plastic cryoembedding mould (Fisher Scientific, USA) containing a contrasting colour of cryochrome freezing compound and snap frozen in liquid nitrogen. Blocks were stored at -80°C until sectioning. Sectioning was performed on a Shandon cryostat (Thermo Fisher Scientific, USA), and sections were placed on gelatin-coated microscope slides. Slides were stored at -80°C until required.

2.5.2 Cell types

2.5.2.1 Human foreskin fibroblasts (HFFs)

Human foreskin fibroblasts (HFFs) were derived from surgically removed normal human foreskin tissue. In culture, HFFs were grown in DMEM supplemented with 10% FBS and 50 IU/mL penicillin streptomycin (Gibco, USA) at 37°C 5% CO₂. HFFs were passaged up to 25 times.
2.5.2.2 VZV-INFECTED HFFs

VZV rOka strain was propagated in HFF monolayers in supplemented DMEM and incubated at 37°C and 5% CO₂. When a CPE of 2+ to 3+ was achieved by the virus, the cells were harvested seeded onto uninfected HFFs (uniHFFs) to achieve a cell-associated infection at the desired ratio.

2.5.2.3 CD14⁺ IMMUNE CELL ISOLATION

CD14⁺ peripheral blood mononuclear cells (PBMCs) were kindly provided by Rodney Henriquez (VZV Research Laboratory, The University of Sydney). Unscreened human donor blood was provided under University of Sydney Ethics approval by the Australia Red Cross and PBMCs were isolated via density gradient sedimentation using endotoxin free Ficoll Hypaque (GE Healthcare, USA) at 430 x g with no brake at room temperature for 20 minutes. Buffy coats were collected and washed 3 times with 1x PBS at 270 x g at 4°C for 5 minutes. Buffy coats were washed once in ice cold MACS buffer at 270 x g at 4°C for 5 minutes. PBMCs were then incubated in 300 µL MACS buffer and 150 µL magnetically labelled anti-human CD14 microbeads (Miltenyi Biotec, USA) at 4°C for 30 minutes. PBMCs were then washed in MACS buffer at 270 x g at 4°C for 5 minutes then filtered through a MACS LS column attached to a Quadro MACS separator (Miltenyi Biotec, USA). The column was then washed twice with 5 mL MACS buffer and the positive fraction discarded. The remaining immune cells were used in the creation of paraffin-embedded and cryopreserved cell blocks.

2.6 Molecular biology techniques

2.6.1 Nucleic acid extraction

To recover DNA and RNA from paraffin-embedded tissue sections, the RecoverALL total nucleic acid isolation kit (Applied Biosystems, USA) was used as per the manufacturer's instructions. Paraffin-embedded sections were dissolved in xylene and physically scraped off the slides and placed in a 2 mL microcentrifuge tube. To remove all paraffin, 1 mL xylene was added to the samples, vortexed briefly and then heated to 50°C for 3 minutes. Samples were then centrifuged at 20 800 x g for 2 minutes to pellet the tissue. Supernatants were removed and discarded. Pellets were washed twice in 100% ethanol, vortexed to mix, then centrifuged at 20 800 x g for 2
minutes to pellet and supernatants discarded. Samples were then centrifuged for a further 2 minutes to remove any residual ethanol, and then allowed to air dry.

Digestion buffer and protease solutions were added to samples according to manufacturer instructions and samples were mixed gently. Samples were incubated at 50°C for 3 hours, and 400 µL was removed for RNA extraction. Remaining samples were incubated at 50°C for a total of 48 hours for DNA extraction. After incubation, isolation additive was added to each sample and vortexed briefly to mix. 100% ethanol was then added to each sample and pipetted gently to mix. Portions of samples were transferred to a filter cartridge within a clean collection tube and centrifuged at 10 600 x g for 1 minute, and flow through was discarded. This was repeated until the entire sample had passed through the filter. Wash 1 was then added to the filter cartridge and then centrifuged at 10 600 x g for 30 seconds, and flow through was discarded. Wash 2/3 was added to the filter cartridge and then centrifuged at 10 600 x g for 30 seconds, and flow through was discarded. Samples were centrifuged for an additional 30 seconds to remove residual fluid from the filters. DNase or RNase was added to filters as described in the manufacturer's instructions, depending on DNA or RNA isolation, and samples were incubated as required (30 minutes at room temperature for RNA isolation, 30 minutes at 37°C for DNA isolation). Wash 1 was added to filter cartridges, and samples were incubated for 60 seconds at room temperature and then centrifuged at 10 600 x g for 30 seconds. Flow through was discarded. Two washes of wash 2/3 were added to each filter cartridge and samples were centrifuged at 10 600 x g for 30 seconds and flow through discarded after each wash. Samples were centrifuged at 10 600 x g for an additional 60 seconds to remove any residual fluid from filters. Elution solution was preheated to 95°C. Filter cartridges were placed in clean collection tubes and 50 µL preheated elution solution was applied to the centre of the filter. Samples were allowed to sit at room temperature for 60 seconds, and then centrifuged at 20 800 x g for 60 seconds to collect eluant. The process was repeated with a second volume of preheated elution solution to ensure complete elution of isolated nucleic acid. Samples were quantitated on a Nanodrop spectrophotometer (Thermo-Scientific, USA) to check nucleic acid concentration and purity. DNA samples were stored at -20°C until required. RNA samples were stored at -80°C until required.

2.6.2 Polymerase chain reaction (PCR)

Primers used for PCR reactions are shown in Table 2.6.
2.6.2.1 **PCR REACTION CREATION**

Master mix was prepared, and reactions were set up for 30 µL total volume. Approximately 10 µL template DNA and 2.5 U GoTaq Flexi DNA polymerase were added to each reaction, with final concentrations of 1x Green GoTaq Flexi Buffer, 100 nM MgCl₂, 5 nM nucleotide mix, 20 pM of each primer, with reaction volume made up to 50 µL using ultra pure H₂O.

2.6.2.2 **PCR MACHINE CYCLING PARAMETERS**

Cycling parameters for all gene specific primers were as follows: initial denaturation step at 94°C for 4 minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute per kilobase of expected product. A final extension of 72°C for 10 minutes was included after cycling to ensure full elongation of the final product.

2.6.3 **DNA separation via agarose gel electrophoresis**

DNA samples were mixed with gel loading buffer in a 6:1 ratio. PCR reaction mixtures using Green GoTaq Flexi Buffer already contained gel loading buffer. DNA ladder was mixed with loading buffer in a 1:1 ratio. Samples were loaded onto a 1% TAE agarose gel and run at 100 volts for 60 minutes. Gel was imaged via EC3 imaging system (UVP Biomedicals, USA).

2.6.4 **Purification of PCR products**

Protocol involved the use of the QIAquick PCR purification kit (Qiagen, Australia) according to the manufacturer’s instructions. After checking PCR reactions were successful via agarose gel electrophoresis as described earlier, remaining PCR reaction mixtures were mixed with Buffer PBI in a ratio of 5 volumes buffer to 1 volume reaction mixture and mixed well. This was transferred to a QIAquick spin column in a 2 mL collection tube and centrifuged at 17 900 x g for 1 minute at room temperature, and flow-through discarded. QIAquick spin column was washed with 750 µL Buffer PE and centrifuged at 17 900 x g for 1 minute at room temperature, and flow-through discarded. QIAquick spin column was centrifuged at 17 900 x g for 1 minute at room temperature to remove any residual solution, and spin column was transferred to a new collection tube. DNA was eluted by adding 30 µL Buffer EB to the centre of the QIAquick membrane, incubating for 1 minute, and then centrifuged.
at 17900 x g for 1 minute at room temperature. Purified DNA was stored at -20°C until required.

2.6.5 DNA Ligation for plasmid creation

DNA ligation reactions were performed utilising the pGEM-T Easy vector system (Promega, USA). Briefly: 3 μL purified insert DNA were mixed with 2x Rapid Ligation Buffer, pGEM-T Easy Vector and T4 DNA ligase to final concentrations of 1x, 50 ng, and 3 Weiss units respectively. Optimal insert concentration was around 8 ng, however if unsuccessful reactions using different molar ratios were utilised. Reaction mixtures were incubated overnight at 4°C, and then utilised in bacterial transformations, as described in Section 2.6.10.1.

2.6.6 DNA sequencing

Primers used for sequencing reactions are shown in Table 2.8.

DNA sequencing reactions were created by mixing 600-1500 ng template DNA and 10 pmol primer in a total volume of 12 μL. Sequencing was performed by the Australian Genome Research Facility at the Westmead Millennium Institute.

2.6.7 DNase treatment and cDNA synthesis

Nucleic acid extracts were initially treated with DNase to ensure the complete removal of genomic DNA. This was performed by mixing 1-5 μg of total RNA with DNase I (amplification grade) (Ambion, USA) and 10x DNase I buffer (Ambion, USA) to final concentrations of 1 unit and 1x respectively. Solutions were incubated at 37°C for 15 minutes, and then 1 μL DNase I stop solution (Invitrogen, USA) was added and mixed well. DNase enzyme was then inactivated by incubating the solution at 65°C for 10 minutes, and transferred to ice.

DNase treated mixture was placed on ice, and reverse transcription was performed by mixing template RNA with dNTP mix (Invitrogen, USA) and random primers (Invitrogen, USA) and incubating at 65°C for 5 minutes. Reaction mix was then placed on ice and 5x RT buffer (Invitrogen, USA), DTT (Invitrogen, USA), RNaseOUT (Invitrogen, USA) and Superscript III Reverse Transcriptase were added. Final reaction mixture concentrations were 0.5 mM dNTP mix, 150 ng random primers, 1x RT buffer, 5 mM DTT, 40 Units RNaseOUT and 200 Units Superscript III Reverse Transcriptase. Mixture was mixed well via pipette, and then incubated at 25°C for 5 minutes, 50°C for 1 hour and then 70°C for 15 minutes. Reaction mix was
then transferred to 37°C, and treated with 2 Units RNase H (Invitrogen, USA) for 30 minutes to remove residual RNA. Resulting cDNA was stored at -20°C until required.

2.6.8 Real time PCR analysis of cDNA samples

Primers used for real time PCR reactions are shown in Table 2.7.

2.6.8.1 Real time PCR reaction creation

Real time PCR was performed using the SYBR green system (Invitrogen, USA). Master mix containing 0.54% (v/v) SYBR green, primers and RNase/DNase free water to a volume of 9.5 µL was added to each sample containing 3 µL cDNA. Positive control samples consisted of cDNA obtained from VZV-infected and uninfected cell cultures, and was kindly provided by Jenna Christensen (VZV Research Laboratory, The University of Sydney). Negative control samples consisted of master mix only with RNase/DNase free water. In addition, corresponding reverse transcriptase negative samples were included for all samples.

2.6.8.2 Real time PCR cycling parameters

All samples were processed using a Rotorgene 6000 qRT-PCR machine (Qiagen, Australia). Real time PCR cycling conditions were as follows: a single 2 minute denaturation step at 50°C followed by incubation at 95°C for 2 minutes. 50 cycles were then carried out with denaturation at 95°C, annealing and extension at 60°C for 30 seconds. During this annealing/extension step fluorescence levels were acquired. Following cycling, a step-wise increase in temperature from 60 – 90°C as performed to generate a melt curve. Data was analysed using Rotogene 6000 Software (Qiagen, Australia).

2.6.9 Quantitative real time PCR analysis of DNA levels

Primers and probes used in quantitative real time PCR reactions are shown in Table 2.9.

Plasmid constructs for use as standards had been previously created and were kindly provided for use by Dr Megan Steain. Plasmids consisted of pGEM-T Easy backbone with a small coding section for human albumin or VZV ORF28 inserted which contained the region detected by the corresponding real time primers.
2.6.9.1 STANDARD CURVE CREATION

Plasmid constructs were linearised to improve detection efficiency. Restriction digest was performed by mixing a known quantity or plasmid with ScaI, NEB buffer 1 and BSA to final concentrations of 50 units, 1x and 1x respectively. Reaction was incubated at 37°C for 2 hours, and then incubated at 65°C for 20 minutes to heat inactivate the enzyme. Digests were checked by running on a gel to check complete digestion.

A standard curve was created by calculating the copy number of plasmid included in the restriction digestion, and then performing tenfold serial dilutions of this reaction. Diluted and digested plasmid standards were then used in a real time PCR analysis to check that the standard curve was acceptable, before being used for quantitative real time PCR analysis of sample material.

2.6.9.2 QUANTITATIVE REAL TIME PCR ANALYSIS OF HUMAN ALBUMIN DNA

Quantitative real time PCR analysis of human albumin DNA levels was perform using the SYBR green system (Invitrogen, USA). Master mix containing 0.54% (v/v) SYBR green buffer, primers and RNase/DNase free water to a volume of 8.5 µL was added to each sample containing 4 µL DNA. A standard curve which had been previously created and tested as described earlier was included in each PCR run, and all samples were run in duplicate to ensure reliability. Negative control samples consisted of master mix mixed with RNase/DNase free water.

All samples were processed using a Rotorgene 6000 qRT-PCR machine (Qiagen, Australia). Real time PCR cycling conditions were as follows: a single 2 minute denaturation step at 50°C followed by incubation at 95°C for 2 minutes. 45 cycles were then carried out with denaturation at 95°C for 10 seconds, annealing at 62°C for 15 seconds and extension at 72°C for 20 seconds. During these annealing and extension steps fluorescence levels were acquired. Following cycling, a step-wise increase in temperature from 60 – 90°C as performed to generate a melt curve. Data was analysed using Rotorgene 6000 Software (Qiagen, Australia).

2.6.9.3 QUANTITATIVE REAL TIME PCR ANALYSIS OF VZV ORF28 DNA

Quantitative real time PCR analysis of VZV ORF28 DNA levels was perform using the Rotor-Gene Probe PCR Kit (Qiagen, USA). Master mix containing reaction buffer, primers, probe and RNase/DNase free water to a volume of 8.5 µL was added to
each sample containing 4 µL DNA. A standard curve which had been previously created and tested as described earlier was included in each PCR run, and all samples were run in duplicate to ensure reliability. Negative control samples consisted of master mix mixed with RNase/DNase free water.

All samples were processed using a Rotorgene 6000 qRT-PCR machine (Qiagen, Australia). Real time PCR cycling conditions were as follows: a single 3 minute denaturation step at 95°C. 50 cycles were then carried out with denaturation at 95°C for 3 seconds, annealing and extension at 60°C for 10 seconds. During this annealing/extension step fluorescence levels were acquired. Data was analysed using Rotogene 6000 Software (Qiagen, Australia).

2.6.10 Bacterial cultures

2.6.10.1 BACTERIAL TRANSFORMATION (HEAT SHOCK METHOD)

Plasmid or ligation mix was stored on ice. JM109 competent cells (Promega, USA) were defrosted on ice. Plasmid and competent cells were then mixed in a ratio of 1:10. Plasmids were mixed gently through competent cells by gentle stirring of pipette tip only. Mixtures were placed in a water bath at 42°C for 30 seconds, and then incubated on ice for 2 minutes. SOC media (Invitrogen, USA) was then mixed with bacterial mixture and tubes were incubated at 37°C and 225 rpm for 60 minutes. Cells were then transferred to agar plates as described below.

2.6.10.2 BACTERIAL GROWTH

2.6.10.2.1 Agar plate growth

Bacterial cells were transferred from previous culture to LB agar plates. If from LB broth culture, plates were allowed to dry on bench for 45 minutes. Plates were transferred to 37°C incubator for overnight growth.

2.6.10.2.2 Broth growth

Cultures were set up using large conical flasks. LB broth and appropriate antibiotic (ampicillin or kanamycin) solution were pre-warmed and mixed well, and then cultures were inoculated with bacterial cells from previous culture. Bacterial cultures were grown in shaking incubators at 37°C overnight.
2.6.10.3 BACTERIAL COLONY PCR

Bacterial colony PCR was utilised to check that the colony in question contained the desired plasmid construct. It was performed as described earlier, and bacterial colonies were scraped from plate growth and transferred to PCR tube directly before reactions were placed in PCR machine for cycling. PCR cycling conditions were performed as described earlier.

2.6.10.4 PLASMID MINIPREP

Plasmid minipreps were performed using the QIAprep Spin Miniprep Kit (Qiagen, Australia). Briefly bacterial cells from overnight culture were harvested by centrifugation at 6000 x g for 10 minutes at room temperature, and supernatant was discarded. Bacterial pellet was resuspended in 250 µL Buffer P1. 250 µL Buffer P2 was added to bacterial mixture and mixed well by inversion. 350 µL Buffer N3 was added to bacterial mixture and mixed immediately by inversion. Mixture was then centrifuged at 17 900 x g for 10 minutes to form a compact white pellet. Supernatants were then transferred to a QIAprep spin column and centrifuged at 17 900 x g for 1 minute, and flow through discarded. The QIAprep spin column was then washed by adding 0.5 mL Buffer PB and then centrifuged at 17 900 x g for 1 minute, and flow through discarded. QIAprep spin column was then washed with 0.75 mL Buffer PE and centrifuged at 17 900 x g for 1 minute, and flow through discarded. QIAprep spin column was centrifuged for an additional minute at 17 900 x g to remove any residual wash buffer. QIAprep spin column was then transferred to a clean microcentrifuge tube, and plasmid DNA eluted by adding 30 µL Buffer EB to the centre of the spin column, incubated at room temperature for 1 minute, and centrifuged at 17 900 x g for 1 minute. Plasmid DNA was then stored at -20°C until required.

2.6.10.5 PLASMID MAXIPREP

Plasmid maxipreps were performed using the QIAfilter Plasmid Maxi Kit (Qiagen, Australia). Briefly bacterial cells from overnight culture were harvested by centrifugation at 6000 x g for 15 minutes at 4°C, and supernatant was discarded. Bacterial pellet was resuspended in 10 mL Buffer P1 and mixed well. 10 mL Buffer P2 was added to mixture and mixed tubes inverted 6-8 times, and then incubated at room temperature for 5 minutes. 10 mL pre-chilled Buffer P3 was then added to tubes, mixed well by inverting, and the bacterial cell lysate was then transferred to a QIAfilter cartridge and incubated at room temperature for 10 minutes. During this
Incubation a QIAGEN-tip 500 was prepared by adding 10 mL Buffer QBT and allowing the column to empty by gravity flow. The bacterial cell lysate was then filtered through the QIAfilter Cartridge into the QIAGEN-tip and allowed to flow through the column by gravity flow. The QIAGEN-tip was then washed with two lots of 30 mL Buffer QC. Plasmid DNA was then eluted from QIAGEN-tip with 15 mL Buffer QF. DNA was precipitated by adding 10.5 mL isopropanol to the mixture, mixed well by inverting and centrifuged at 15,000 x g and 4°C for 30 minutes. Supernatant was carefully decanted, and pellets washed with room temperature 70% ethanol, and the centrifuged at 15,000 x g and 4°C for 30 minutes. Supernatant was carefully decanted and pellets air-dried. Pellet was resuspended in TE buffer. Plasmid DNA concentration and purity was checked using a Nanodrop spectrophotometer (Thermo-Fisher Scientific, USA) and plasmid was stored at -20°C until required.

2.6.10.6 Long term bacterial storage

To store bacterial cells for longer periods 200 µL of overnight cultures were mixed well with 800 µL of glycerol (Sigma, USA), and then tubes were stored in -80°C. Cells were checked for viability at least 1 month after storage by streaking cells on to LB agar plates and growing overnight.

2.7 Immunohistochemistry (IHC)

2.7.1 Haematoxylin and eosin stain

Slides were deparaffinised by washing in two changes of Xylene for 10 minutes each. Slides were re-hydrated by successively washing twice in 100% ethanol, twice in 95% ethanol, and once in 70% ethanol for 3 minutes each, and were then rinsed in running H₂O for 3 minutes. Slides were incubated in haematoxylin solution for 2 minutes and then rinsed quickly in running H₂O. Slides were dipped in acid alcohol solution three times to remove excess stain and then rinsed quickly in running H₂O. Slides were incubated in Scott’s Blueing solution for 30 seconds and then rinsed in running H₂O. Slides were examined under a microscope to ensure adequate staining levels. If extra staining was required slides were re-immersed in haematoxylin and subsequent steps were followed. If staining was adequate, slides were washed in running H₂O for 3 minutes. Slides were then washed in 70% ethanol for 30 seconds,
before being counterstained in eosin for 20 seconds. Slides were then dehydrated rapidly through graded ethanol (from 70% to 100%) and cleared in two washes in histolene of 2 minutes each. Slides were then coverslipped with DPX and allowed to dry overnight.

2.7.2 Immunohistochemistry (IHC)

Primary and secondary antibodies utilised in IHC procedures are listed in Table 2.2 and Table 2.5 respectively. Isotype control antibodies are shown in Table 2.3.

2.7.2.1 Single IHC Staining with High Temperature Antigen Unmasking

Slides were deparaffinised and rehydrated as in Section 2.7.1. Slides were then dipped in running H₂O and washed in 1x TBS for 5 minutes. Endogenous peroxidases were blocked by incubating sections in 3% H₂O₂ solution for 10 minutes, and then slides were rinsed in distilled H₂O. Sections were unmasked by incubating slides in 1x citrate buffer for 13 minutes at 93°C. Slides were left in 1x citrate buffer, taken off the heat and allowed to cool for 20 minutes. Slides were washed in 1x TBS for 5 minutes. Biotin blocking was then performed using the Biotin-blocking system (Dako, Australia). Avidin solution was applied to sections and slides were incubated for 10 minutes. Slides were then rinsed in 1x TBS. Biotin solution was applied to sections and slides were incubated for 10 minutes. Slides were then rinsed in 1x TBS for 2 minutes. Sections were circled using a Dako pen (Dako, Australia) and IHC blocking solution was applied to the sections, then incubated in a humidified chamber for 30 minutes at room temperature. Primary antibody diluted in IHC antibody dilution buffer was applied to the sections, then incubated in a humidified chamber for 2 hours at room temperature. Slides were washed in three changes of 1x TBS for 3 minutes each. IHC secondary antibody solution – containing biotinylated secondary antibody diluted in IHC antibody dilution buffer – was then applied to the sections, and incubated in a humidified chamber for 30 minutes at room temperature. Slides were then washed in three changes of 1x TBS for 3 minutes each. IHC enzyme solution – containing Streptavidin-HRP (Dako, Australia) diluted in IHC antibody dilution buffer – was then applied to the sections, which were incubated in a humidified chamber for 30 minutes at room temperature. Slides were then washed in three changes of 1x TBS for 3 minutes each. DAB working solution was mixed immediately prior to use, added to the sections and incubated for 5 minutes at room temperature. DAB working solution was removed from sections and the reaction was stopped by rinsing sections with dH₂O for 5 minutes. Slides were then transferred to
running H₂O for at least 10 minutes. Sections were then counterstained with azure blue or haematoxylin and mounted using DPX.

2.7.3 Azure Blue counterstain

Slides were counterstained in azure B solution for 4 minutes, before being washed in running H₂O for 5 mins. Slides were then dehydrated by washing subsequently in 70% ethanol for 30 seconds, 95% ethanol for 40 seconds, 95% ethanol for 50 seconds, and in two changes of 100% ethanol for 1 minute each. Slides were then washed in two changes of histolene for 2 minutes each before being coverslipped (ProSciTech, Australia) with DPX and allowed to dry overnight.

2.7.4 Haematoxylin counterstain

Slides to be counterstained in haematoxylin for 10 seconds, before being washed in running H₂O for at least 10 minutes. Slides were dehydrated by washing subsequently in 70% ethanol once, twice in 95% ethanol, and then twice in 100% ethanol for 3 minutes each. Slides were then washed in two changes of histolene for 2 minutes each before being coverslipped (ProSciTech, Australia) with DPX and allowed to dry overnight.

2.8 Immunofluorescent assay (IFA)

Primary and secondary antibodies utilised in immunofluorescent assays are shown in Table 2.1 and Table 2.4 respectively. Isotype control antibodies are shown in Table 2.3.

2.8.1 Immunofluorescence staining of paraffin embedded sections with high temperature antigen unmasking

Slides were deparaffinised and re-hydrated as per Section 2.7.1. Antigen retrieval was performed as per Section 2.7.2.1. Following this, slides were washed in 1x TBS for 5 minutes. Sections were circled using a Dako pen (Dako, Australia), and blocking solution (20% NDS in 1x TBS or 1x PBS) was applied to the sections which were then incubated in a humidified chamber for 30 minutes at room temperature. Blocking solution was discarded by tapping slides, and primary antibody solution (either single primary antibody for single stain or mix of primary antibodies for dual and triple stains) diluted in IFA antibody dilution buffer (10% NDS in 1x TBS) was
applied to the sections, which were then incubated in a humidified chamber for 2 hours at room temperature. Slides were washed in three changes of 1x TBS for 3 minutes each. Secondary antibody solution (either single or multiple antibody mix) diluted in IFA antibody dilution buffer was applied directly to the sections, which were then incubated in a humidified chamber for 60 minutes at room temperature. Samples were protected from light as much as possible for the remaining steps. Slides were washed in three changes of 1x TBS for 3 minutes each, and were coverslipped (ProSciTech, Australia) using Prolong Gold with DAPI, and then sealed with nail polish. Slides were stored at 4°C and protected from light.

2.8.2 Autofluorescence masking

This protocol was utilised to mask autofluorescence in sections during immunofluorescence assays where necessary.

Slides were stained according to immunofluorescence protocols. After primary antibody incubation and subsequent rinses 1x sudan black solution was applied to sections and slides were incubated at room temperature for 5 minutes. Excess solution was tipped off slides, slides were dipped briefly in 70% ethanol and then excess solution was allowed to drain off. Slides were then rinsed in 1x PBS, and normal staining protocol continued.

2.8.3 Gelatin coating of slides

Gelatin coating solution was made fresh and placed into a large container. Plain uncoated microscope slides (Mikro-Glass, Australia) to be used for collection of cryopreserved material were loaded into slide carriers, and then dipped briefly into warm gelatin solution. Slide carrier was gently shaken to ensure no air bubbles remained. Slide carriers were then removed, excess solution drained back into container, and then slide carrier was banged on the bench to ensure that no air bubbles or droplets of solutions remained on the slides. Slides were left in the carrier to dry overnight, and gelatin coating solution was stored in the fridge. Protocol was repeated on slides the following day.

Slides were then transferred to slide boxes on the third day, and stored in a cool, dark and dry place and were used within a month from coating.
2.8.4 Immunofluorescence staining of frozen samples with low temperature antigen unmasking

2.8.4.1 Single Immunofluorescence Staining

Unfixed tissue sections were fixed in 10% neutral buffered formalin for 4 hours prior to being used. Slides were rinsed twice in 1x PBS for 20 minutes each before being unmasked in 1 mM EDTA at 60°C for 3 hours. Slides were allowed to cool at room temperature for 30 minutes, and rinsed in 1x PBS for 30 minutes. Slides were then incubated in blocking buffer for 2 hours. Sections were circled using a Dako pen (Dako, Australia), primary antibody mix (in blocking buffer) was applied to sections and then incubated overnight in a sealed humidified chamber at room temperature. Slides were then washed three times in 1x PBS for 20 minutes each. Secondary antibody mix (in blocking buffer) was then applied and slides incubated for 3 hours in a humidified chamber at room temperature. Slides were protected from light for the remainder of the protocol. Slides were washed 4 times in 1x PBS for 25 minutes each. Slides were then coverslipped with 5 μL Prolong Gold with DAPI applied to each section. Slides were sealed with nail polish and stored at 4°C.

2.8.4.2 Dual Immunofluorescence Staining

Fixation (if required), unmasking and initial primary antibody were performed as with single immunofluorescence. Following initial primary antibody incubation slides were rinsed in 1x PBS for 6-8 hours. The second primary antibody mix (in blocking buffer) was then applied to sections and slides were incubated overnight in a sealed humidified chamber at room temperature. Slides were then washed three times in 1x PBS for 20 minutes each. Secondary antibodies specific for the relevant primary antibodies were applied as a mix (in blocking buffer) and slides incubated for 3 hours in a humidified chamber at room temperature. Slides were protected from light for the remainder of the protocol. Slides were washed 4 times in 1x PBS for 25 minutes each. Slides were then coverslipped with 5 μL Prolong Gold with DAPI applied to each section. Slides were sealed with nail polish and stored at 4°C.

2.8.4.3 Triple Immunofluorescence Staining

Fixation (if required), unmasking and initial and second primary antibodies were performed as with dual immunofluorescence. Following initial primary antibody incubation slides were rinsed in 1x PBS for 6-8 hours. The third primary antibody mix (in blocking buffer) was then applied to sections and slides were incubated overnight.
in a sealed humidified chamber at room temperature. Slides were washed three times in 1x PBS for 20 minutes each. Secondary antibodies specific for relevant primary antibodies were applied as a mix (in blocking buffer) and slides incubated for 3 hours in a humidified chamber at room temperature. Slides were protected from light for the remainder of the protocol. Slides were washed 4 times in 1x PBS for 25 minutes each. Slides were then coverslipped with 5 µL Prolong Gold with DAPI applied to each section. Slides were sealed with nail polish and stored at 4°C.

2.9 Microscopy and analysis

All microscopes, cameras and analysis software utilised were from the Advanced Microscopy Facility of the Bosch Institute (University of Sydney, Australia).

Confocal microscopy was performed utilising a Zeiss LSM 510 Meta Spectral confocal microscope (Carl Zeiss, Australia).

Bright field microscopy was performed utilising a Zeiss AxioPlan 2 upright microscope with an AxioCam HR digital monochrome CCD camera (Carl Zeiss, Australia) for images of immunofluorescent stains, or an AxioCam HR digital colour CCD camera (Carl Zeiss, Australia) for images of immunohistochemical stains.

Cell counts, tissue area and cell frequencies were calculated with the aid of the Zeiss AxioVision LE software (Carl Zeiss Pty Ltd, Australia).

Each results chapter contains specific information on the methods utilised to visualise stained tissue material and counting methods utilised.
Chapter 3 – Characterisation of immune cell phenotype in human dorsal root ganglia obtained from a patient with post-herpetic neuralgia, and comparison with other human ganglia samples obtained from patients at different stages post-herpes zoster.

3.1 Introduction

VZV is an ubiquitous human pathogen that causes two similar yet clinically distinct diseases – varicella and herpes zoster. Although the lesions are histopathologically indistinguishable in the two syndromes (Oxman, 2000), the general rash location differs considerably. The most common complication of herpes zoster is PHN - a severe neuropathic pain which can persist for months to years after the resolution of the herpes zoster-associated rash (Cohen et al., 1999, Steiner et al., 2007, Hope-Simpson, 1965, Hope-Simpson, 1975). To date there is very limited information about both the host immune response and the extent of viral replication within the ganglia during PHN.

This study aims to address this lack of knowledge through an immunofluorescent examination of rare human ganglia material obtained surgically from a PHN-affected patient, in comparison with other valuable post-mortem herpes zoster-affected and control ganglia material. Additionally nucleic acids were extracted from this material to assess the extent of viral infection within these samples.

The underlying pathology and pathogenesis of PHN is relatively unknown. Multiple theories have been offered as explanation. However research to date has been restricted due to the lack of a suitable animal model of PHN, and the very rare nature of clinical sample material. There have been very few studies into clinical PHN samples, and even fewer have utilised human ganglia material.

Previous studies involving PHN and the assessment of DRG have been limited to general and brief histological examinations of human ganglia. One histological study of ganglia obtained post-mortem from an individual who experienced PHN for several months prior to death found diffuse and focal infiltrations of inflammatory cells (Smith,
Another study found a general loss of both axons and myelin within the affected area of a single patient suffering from PHN (Watson et al., 1988b). A follow-up study on post-mortem DRG samples confirmed these findings, and noted evidence of a general inflammatory process occurring in some PHN-affected individuals (Watson et al., 1991). This study also noted atrophy of the spinal dorsal horn, and cell, axon, and myelin loss were only found in patients with persistent PHN. Persistent inflammation and lymphocytic infiltrate within the affected areas in long-term herpes zoster survivors had been previously noted, but these cases had other causes of death and no clear pain at time of death, and as such this inflammation cannot be directly attributed to PHN (Head and Campbell, 1900).

Overall these earlier histological based studies have been quite limited in their scope. This is most likely due to the experimental limitations at the time of publication, as well as the very limited number of samples available. Given the importance of the ganglia and the cellular and virological events that may be occurring during PHN, we sought to source ganglia from PHN-affected individuals. In this respect, we have obtained sample material surgically excised from a PHN-affected patient, as well as very valuable herpes zoster-affected and control material. This naturally infected ganglia material obtained post-herpes zoster and many months later during PHN is enough to allow a detailed examination of VZV DNA levels, the presence of active VZV gene transcription and antigen expression, and the constitution of infiltrating immune cells within this naturally infected material.

### 3.2 Patient material

#### 3.2.1 Human ganglionic material used in this study

A major limiting factor in advancing our understanding of the interaction of VZV with cells within human ganglia and the role the immune response may play during reactivation and PHN in vivo has been obtaining suitable human ganglia material. This study utilises precious PHN-affected ganglia material that was surgically excised from a PHN-affected patient. Also included are rare post-mortem material recovered from herpes zoster-affected patients and control material for comparison.

All tissue specimens were obtained in accordance with ethics guidelines of the University of Sydney, and informed consent of the donors was obtained where required.
3.2.1.1 PHN PATIENT MATERIAL

PHN-affected patient material was obtained from Dr Michael Buckland, originally of St Vincent's Hospital, and now of the Department of Pathology, University of Sydney. This patient was diagnosed with acute myeloid leukaemia in 2001, and consequently underwent a bone marrow transplant in 2002. During recovery from this procedure the patient experienced herpes zoster in the right thoracic region from T9 to T12. Following this the patient experienced prolonged PHN at levels T10 to T12.

After all other treatments options failed the patient was offered a ganglionectomy in an attempt to control the pain. This was performed in November 2006 – and material was removed from the patient’s right T11 region. PHN re-occurred, and in June 2007 a second ganglionectomy was performed – this time removing both the right T10 and T12 ganglia. The three ganglia samples obtained were cut into smaller parts, formaldehyde fixed and embedded in paraffin blocks. Figure 3.1 shows the locations of the removed ganglia.

The T11 ganglion obtained from the first surgery (PHN1) was made into three separate paraffin blocks containing tissue from different parts of the affected ganglion – one from the perikaryon region of the DRG (referred to as the neuronal area) (PHN1a) and two from the region of nerve bundles (PHN1b and PHN1c). Only the first block from this sample had been stained previously during my final year’s undergraduate honours work (Sutherland, 2008). However replicate stains, new IFA and IHC staining combinations, counts and frequency analyses were performed on all three paraffin embedded blocks during this study.

There were two ganglia obtained at the second surgery. The first (PHN2) from T10 level was made into two blocks – one each from the neuronal area (PHN2a) and nerve bundle region (PHN2b). The first block from this sample had mainly been stained previously during my honours work (Sutherland, 2008). Replicate stains, new IFA and IHC staining combinations, counts and frequency analyses were performed during this study. Sections from the second paraffin embedded block were immunostained for the first time in this study.

The second ganglion from this surgery (PHN3) from the T12 level and only one block was deemed suitable for this study and contained tissue located in the nerve bundle region (PHN3a). Another block was available, but following sectioning was found to contain very little tissue (PHN3b).
Figure 3.1 - Location of ganglia samples from the PHN-affected patient

PHN-affected ganglia material used in this study were obtained from the right thoracic region. PHN1 was the right T10 ganglion, PHN2 was the right T11 ganglion and PHN3 was the right T12 ganglion.

Ganglia diagram adapted from: http://www.backpain-guide.com/Chapter_Fig_folders/Ch06_Path_Folder/4Radiculopathy.html (accessed 29/2/12)
All herpes zoster patient material was generously provided by Dr Michael Buckland from the Department of Pathology, University of Sydney. Samples were supplied as formalin-fixed paraffin-embedded blocks, which were sectioned into consecutive 5 μm sections and mounted on Superfrost plus microscope slides (Thermo Fisher Scientific, USA) by Dr Jane Radford formerly of the Histopathology Laboratory of the University of Sydney. Off cuts from each block were also collected in clean eppendorf tubes to be utilised for nucleic acid extraction and subsequent molecular analysis.

### 3.2.1.2 HERPES-ZOSTER-AFFECTED PATIENT MATERIAL

Also included for comparison are rare post-mortem samples obtained from patients who had an active VZV reactivation event at or near the time of death. In this study ganglia from three different patients were included. Figure 3.2 shows the location of ganglia samples from the herpes zoster-affected patient material.

The first herpes zoster ganglion sample (HZ1) was obtained post-mortem. The patient was a 31 year old female who had been on immunosuppressive therapy (prednisone and mycophenolate) for five years following a renal transplant which was the result of preeclampsia which lead to end-stage renal failure. Past medical history also noted cardiomyopathy diabetes mellitus and hypertension. Six months prior to death the patient developed disseminated herpes zoster secondary to immunosuppression and EBV-associated post-transplant lymphoproliferative disorder (lymphomatoid granulomatosis grade 3). The patient also had complicating hepatitis and pneumonia. One month later she had recurrent herpes zoster complicated by thrombotic thrombocytopenic purpura and splenic vein thrombosis requiring splenectomy. Secondary transverse myelitis and demyelinating neuropathy was also diagnosed 4 months prior to death. One week prior to death the patient presented with headaches and confusion, and an MRI demonstrated multiple lesions on both cerebral hemispheres consistent with acute encephalitis. Multiple pulmonary emboli and deep vein thromboses were noted post-mortem. Cerebrospinal fluid taken at autopsy was PCR positive for VZV and EBV. The ganglion utilised in this study was a TG, which at autopsy tested PCR positive for VZV but negative for EBV and other infectious agents (Rodriguez, 2010). This ganglion sample contained tissue from the neuronal region of the TG. Sections from this ganglion were used in new IFA staining combinations, and all counts and frequency analyses were performed during this study.
Figure 3.2 - Location of ganglia samples obtained from HZ-affected patients

(A) The HZ1 ganglion sample was from the trigeminal region, however side was unknown.

(B) The HZ2 ganglion sample was from the upper left cervical region. Other ganglia included in the analysis were the HZ2 SOR (right trigeminal ganglia) and HZ2 contra (left trigeminal).

(C) The HZ3 ganglion sample was from the right lumbar region. The HZ3 SOR ganglion sample was from the tenth lumbar ganglion on the left side.

Ganglia diagrams adapted from: [http://www.uihealthcare.org/Adam/?/HIE%20Multimedia/2/18069](http://www.uihealthcare.org/Adam/?/HIE%20Multimedia/2/18069) (accessed 29/2/12)
The second herpes zoster ganglia sample (HZ2) was obtained post-mortem from a patient who at the time of death had active herpes zoster. The patient was a 62 year old male with acute myeloid leukaemia, which was diagnosed at autopsy. The patient had begun experiencing herpes zoster approximately 3 weeks prior to death in the right ophthalmic division of the TG. The rash was still evident at autopsy and there was some dissemination to the thoracic region (Rodriguez, 2010). Ganglionitis was observed in both TG and cervical DRG. The ganglion from this case utilised in this study was from the left cervical region (HZ2), and contained tissue from the nerve bundle region of the DRG. The site of reactivation (SOR) – the right TG (HZ2 SOR) and left TG (contralateral to SOR) (HZ2 contra) had been previously stained by Dr Megan Steain in a study which focused on the neuronal regions of the tissue (Steain et al., 2013) and these slides were utilised in this study to examine regions of nerve bundles within the ganglia material.

The third herpes zoster ganglion sample (HZ3) was obtained post-mortem from a patient with an active case of herpes zoster at the time of death. The patient was a 93 year old male with frontotemporal dementia who died due to aspiration pneumonia. Medical records indicated that this patient developed a herpes zoster rash approximately 17 days prior to death, which was treated with antibiotics (according to clinical notes) (Rodriguez, 2010). At autopsy there was a haemorrhagic vesicular and ulcerated rash in a left lumbar dermatome. The innervating ganglion was noted to be inflamed. The ganglion utilised in this study was a right lumbar DRG, not noted at autopsy to be inflamed, and contained tissue from the nerve bundle region of the DRG. As with HZ2, the SOR – the left lumbar ganglion (HZ3 SOR) had been previously stained by Dr Megan Steain in a study which had focused on the neuronal regions of the tissue (Steain et al., 2013), and these slides were utilised in this study to examine the regions of nerve bundles within the tissue.

All herpes zoster-affected patient material was generously provided by Dr Michael Rodriguez from the Department of Forensic Medicine, University of Sydney. Samples were supplied as formalin-fixed paraffin-embedded blocks, which were sectioned into consecutive 5 µm sections and mounted on Superfrost plus microscope slides (Thermo Fisher Scientific, USA) by Dr Jane Radford formerly of the Histopathology Laboratory of the University of Sydney. Off cuts from each tissue block collected as above for nucleic acid extraction and subsequent molecular analysis.
3.2.1.3 Control patient material

Post-mortem ganglia samples from two patients who did not present with herpes zoster were included as controls. The first control patient ganglia material (CON1) was obtained post-mortem from a 73 year old female who died from metastatic adenocarcinoma. This ganglion was from the lower thoracic area, and contained tissue from the neuronal area of the DRG. This sample had been stained previously during my honours work (Sutherland, 2008). New IFA and IHC staining combinations, replicate stains, all counts and frequency analyses were performed during this study.

The second control patient material (CON2) was obtained post-mortem from a 73 year old female who died from a head injury and previously suffered from cardiovascular disease. This sample contained tissue from the nerve bundle region of the DRG. This study is the first use of this sample material.

VZV sero-status of control patients is unknown.

All control patient material was generously provided by Dr Michael Rodriguez from the Department of Forensic Medicine, University of Sydney. Samples were supplied as paraffin-embedded blocks, consecutive 5 µm sections were collected onto Superfrost plus microscope slides (Thermo Fisher Scientific, USA) by Dr Jane Radford formerly of the Histopathology Laboratory of the University of Sydney. Off cuts from each tissue block collected as above for nucleic acid extraction and subsequent molecular analysis.

3.2.2 Control material

Normal tonsil samples had been previously provided by Dr. Tony Henwood (The Children’s Hospital at Westmead, Australia). These were supplied as formaldehyde-fixed tissue blocks embedded in paraffin as described earlier.

Control cell blocks were created from both uninfected and VZV-infected human foreskin fibroblasts, and CD14- depleted human PBMCs as described in Section 2.5.2.3. CD14- human PBMCs were generously donated by Rodney Henriquez (Varicella Zoster Virus Research Group, University of Sydney).
3.3 Results

3.3.1 Histology of patient ganglia samples

Previous reports have found extensive cellular infiltration in human ganglia obtained via autopsy from PHN-affected individuals (Head and Campbell, 1900, Watson et al., 1991). Thus, my initial examination of patient DRG material was a haematoxylin and eosin stain (H&E) of 5 μm paraffin-embedded ganglionic sections shown in Figures 3.3 and 3.4.

In all ganglionic sections there is the presence of lipofuscin, seen as a brown pigment within neurons. This a normal feature of human ganglionic material, and is made up of lipid containing residues of lysosomal degradation that builds up within neurons throughout the normal ageing process (Ross and Pawlina, 2003).

There were three separate tissue blocks obtained from PHN ganglia 1. The first (PHN1a) showed limited areas containing neurons, with an infiltration of immune cells concentrated within the outer capsule which surrounds the ganglia. The second (PHN1b) featured a cross-sectional view of the nerve bundles with an infiltration of immune cells concentrated around one nerve bundle and throughout the capsule. The third block (PHN1c) contained a longitudinal view of nerve fibres with a small infiltration of immune cells throughout the tissue. Representative images of H&E staining on ganglionic material from PHN1a, PHN1b and PHN1c are shown in Figures 3.3 A, B and C respectively.

There were 2 separate tissue blocks from PHN ganglia 2. The first (PHN2a) showed areas of neurons, along with an infiltration of immune-like cells throughout the entire ganglia with some focal points of immune infiltrations within the ganglia. The second (PHN2b) featured tissue containing a cross-sectional view of many smaller nerve bundles with limited immune-like cells. Representative images of H&E staining of ganglionic material from PHN2a and PHN2b are shown in Figures 3.3 D and E respectively.

There were 2 separate tissue blocks available from PHN ganglia 3. The first (PHN3a) consisted of a longitudinal view of nerve fibres without obvious infiltration of immune-like cells, and a representative image of H&E staining on this sample is shown in Figure 3.3 F. The second block (PHN3b) contained a very limited tissue region and was not utilised in subsequent experiments.
Haematoxylin and eosin staining of PHN ganglionic sections showing general histology of PHN-affected ganglionic material. Samples shown are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E) and PHN3a (F).

An example of a neuron and the encapsulating satellite cells are indicated by the white and blue arrows heads respectively. Examples of nerve bundles are indicated by the yellow arrows.

There are infiltrations of small round cells in both PHN1a, PHN1b and PHN2a (A, B and D) (indicated by black arrows) that bear the characteristics of immune infiltrations. An example of lipofuscin, which shows as a small brown granular pigment within neurons, is indicated by the green arrow. PHN1b, PHN1c and PHN2b (B, C and E) show a cross sectional view of nerve bundles, while PHN3a (F) is exclusively nerve bundles sectioned longitudinally along the fibres.
Figure 3.4 - Haematoxylin and eosin staining of herpes zoster-affected and control ganglia samples

Haematoxylin and eosin staining of HZ-affected and control ganglia samples showing general histology of HZ-affected and control ganglionic material. Samples shown are HZ1 (A), HZ2 (B), HZ3 (C), CON1 (D) and CON2 (E).
Interestingly, within PHN ganglia 2 there was also an area of altered morphology which was not observed in any of the other ganglia samples studied. This area also showed general loss of neuronal profiles via H&E staining (Fig. 3.5A). Neuronal cell death has been shown to lead to a proliferation of satellite cells in place of the adjacent neuron in a formation known as a Nodule of Nageotte (Ramon y Cajal, 1959). To identify if this PHN ganglia sample contain regions of proliferating satellite cells caused by neuronal cell death an IFA was performed using a primary antibody specific for S100B – a satellite cell and myelin marker. The resulting staining pattern is shown in Figure 3.5 B. S100B+ cells were readily detected around neurons, however there were no S100B+ cells observed within the region of altered morphology. Consequently the altered morphology observed in the PHN ganglia 2 sample does not appear to correlate with any detectable satellite cell proliferation and may be a region of fibrosis. Alternatively the altered morphology observed within PHN2 could simply be due to an infolding of the outer capsule of the DRG – thus giving rise to an abnormal appearance within an otherwise normal histology.

Overall the PHN-affected ganglionic material featured prominent infiltrations of immune-like cells, with PHN ganglia 2 containing the largest infiltration, along with a region of altered cellular morphology. Smaller infiltrations of small round cells that bear the characteristics of immune cells were also identified in PHN ganglia 1. There were no obvious immune-like infiltrations in PHN ganglia 3.

The histology of the herpes zoster-affected ganglia sample HZ1 showed normal neuronal regions of ganglia with no obvious immune infiltration. A representative image of H&E staining on this sample is shown in Figure 3.4 A.

The block available from HZ2 showed several distinct regions of tissue, each featuring cross-sectional views of large nerve fibre bundles with no obvious infiltrations of immune-like cells. A representative image of H&E staining on this sample is shown in Figure 3.4 B. Of the two other blocks previously stained by Dr Megan Steain the SOR and contralateral site both featured neuronal and nerve bundle regions with small areas of immune infiltration spread evenly throughout the ganglia samples (Steain et al., 2013).

The tissue sample utilised from HZ3 showed a small area of tissue which featured a cross-sectional view of several nerve fibre bundles with limited infiltrations of immune-like cells. A representative image of H&E staining on this sample is shown in Figure 3.4 C. The SOR – previously stained by Dr Megan Steain - featured both
Figure 3.5 – Assessment of morphology in the neuronal region of PHN ganglia sample 2.

(A) Haematoxylin and eosin staining and (B) IFA showing an area of altered morphology within PHN ganglia 2, indicated by the red arrows. The IFA was performed using the anti-S100B primary (green) which is a satellite cell and myelin marker, and is counterstained with DAPI in blue. The area of altered morphology is absent of (A) neuronal profiles, and contains (B) no nerve bundles or satellite cells. This region continued throughout the neuronal area of PHN ganglia 2. Neuronal cell bodies are marked with a yellow “N”.

neuronal and nerve bundle areas with a large and extensive immune infiltration within the ganglia sections (Steain et al., 2013).

The histology of the control ganglia sample (CON1) showed normal neuronal regions of ganglia with no immune infiltration, and the presence of large and small neurons each surrounded by satellite cells. A representative image of H&E staining on this sample is shown in Figure 3.4 D.

The second control tissue sample (CON2) featured two distinct areas of tissue that both had cross-sectional views of nerve fibre bundles with no obvious infiltrations of immune-like cells. A representative image of H&E staining on this sample is shown in Figure 3.4 E.

There were infiltrations of immune-like cells in both herpes zoster-affected tissue and PHN-affected tissue, but not in control material. The infiltrations within the PHN-affected material was present in both PHN ganglia 2 and to a lesser extent PHN ganglia 1. The infiltrations of small round cells observed in herpes zoster-affected tissue were mainly restricted to the ganglia innervating the rash area (Data presented in Steain et al., 2013). There was no infiltration of small round immune-like cells in control ganglia material.

### 3.3.2 Detection of VZV DNA in human ganglia following herpes zoster and PHN

In order to check for the presence of viable cellular DNA, and identify the presence of VZV DNA nucleic acid extraction and PCR amplification was performed. PCR amplification provides a convenient method for amplifying DNA fragments, and by using a specific primer pair this technique is able to detect sequences of DNA with high sensitivity and specificity. DNA extraction was performed on all ganglia samples except the ganglia region from PHN1 as there was not enough tissue available. Controls included DNA extracted from VZV-infected HFFs (infHFFs) in culture, as well from HSV-1 cell culture supernatant. A no template negative control was also included. Primers used in PCR to amplify GAPDH, VZV and HSV DNA regions are listed in Section 2.3.

As the material used in this study was paraffin-embedded, initially it was important to show the presence of amplifiable DNA within the sections. This was done using primers specific for the housekeeping gene GAPDH predicted to give a PCR product of 350 bp. The resulting PCR product were run on a 1% TAE agarose gel with a
suitable DNA ladder mix, shown in Figure 3.6 A. A band of the expected size was detected in PHN1a, PHN1b, PHN2a, PHN2b and PHN3a. There was no band detected in PHN3b. A band of the expected size was detected in HZ1 and CON1, but not in HZ2, HZ3 or CON2. This shows successful extraction of intact DNA from the PHN1a, PHN1b, PHN2a, PHN2b, PHN3a, HZ1 and CON1 ganglia samples. Although there was no GAPDH detection in PHN3b, HZ2, HZ3 and CON2 samples, corresponding with no viable DNA from each of these samples they were included in subsequent PCRs for consistency.

Using the same primer set, a product was detected in the infHFF culture DNA sample at the expected size (350 bp). There was no PCR product detected in the HSV-1 culture supernatant, but this is to be expected as it should contain HSV-1 virions only without any cellular DNA. There was no PCR product present in the no-template control, showing no contamination of the PCR.

To detect the presence of VZV DNA in each of the samples, a PCR was performed using primers specific for VZV ORF9a, which amplifies a product of 365 bp. The resulting PCR products were run on a 1% agarose gel, shown in Figure 3.6 B. The positive control infHFF featured a band at the expected fragment size. A band of the expected size was detected in PHN1a, PHN2a, PHN2b and PHN3a. No band was detected in PHN1b or PHN3b. A band of the expected size was also detected in HZ1. No bands were detected in HZ2, HZ3, CON1 or CON2. This shows successful PCR detection of VZV-specific DNA in the PHN1a, PHN2a, PHN2b, PHN3a and HZ1 ganglia samples, but not in PHN1b, PHN3b, HZ2, HZ3, CON1 or CON2.

HSV-1 is a highly related alphaherpesvirus, which unlike VZV has been shown to cause an immune infiltration in latently infected human ganglia (Verjans et al., 2007). In order to detect any HSV-1-specific DNA in the ganglia samples a PCR was performed using previously published HSV-1-specific primers (Cassinotti and Siegl, 1998) which produce a amplified PCR product of 208 bp. The resulting PCR products were run on a 1% agarose gel, shown in Figure 3.6 C. The positive control HSV-1 infected culture supernatant featured a band at the expected fragment size. A PCR product of the expected size was also observed in the HZ1 ganglia sample. No other ganglia sample showed detectable HSV-1 DNA. No PCR product was detected in the infHFF culture DNA samples, as expected. There was no PCR product present in the no-template controls, showing no contamination of the PCR mixture.

Consequently it was possible to isolate viable DNA from all PHN1 and PHN2 samples, and from PHN3, as well as from the HZ1 and CON1 samples. Significantly
Figure 3.6 - Detection of GAPDH and VZV DNA, but not HSV DNA in patient ganglia samples

DNA isolated from all PHN, HZ and Control ganglia samples was utilised in PCR reactions and then loaded onto 1% TAE agarose gels. PCR reactions used primers specific for the housekeeping gene GAPDH (A), VZV ORF9a (B) and a HSV-1-specific fragment (C), with band sizes shown above.

Each PCR reaction included control DNA extracted from infected HFF in cell culture, as well as the supernatant (containing HSV-1 virions) from an HSV-1 infected culture. Gels also included a DNA ladder (Fermentas GeneRuler DNA Ladder Mix) containing bands from 100 bp to 100 000 bp, with major band sizes labelled.
VZV DNA and not HSV-1 DNA was also detected by PCR in the PHN1a, all PHN2 and the PHN3a samples, demonstrating any immune cell infiltrate present in these ganglia was not due to an HSV-1 infection. VZV DNA was also detected in the HZ1 ganglia sample. HSV-1 DNA was only detected in the HZ1 ganglia sample.

3.3.3 Quantitation of VZV DNA in human ganglia samples

As VZV DNA was detected in several PHN-affected ganglia samples it was decided to investigate the relative amounts of VZV DNA present in each sample. This was carried out by performing two independent quantitative real time PCR analyses to detect human albumin and VZV ORF28. Human albumin is present as a single copy in each cell and is suitable as a standard in quantitative real time PCR (Douek et al., 2002), while VZV ORF28 contains a region unique to VZV – not present in any other related herpesviruses, allowing the specific detection of only VZV DNA. Reaction conditions, primer and probe sequences were kindly provided Prof Randall Cohrs of the University of Colorado, Denver.

To compare ganglia samples the number of copies of VZV ORF28 DNA was normalised against the number of copies of human albumin. Results are shown as “VZV DNA load per $10^5$ cells” to allow easy comparison between these results and previously published work (Eshleman et al., 2011). The relative results (from 2 replicates) are in shown in Figure 3.7. On average there was 6.9 times more VZV-specific DNA present in the ganglionic sample from a PHN-affected individual (PHN2a) than in the site of an active herpes zoster reactivation (HZ3 SOR).

Unfortunately there was insufficient DNA material to perform a full analysis on all ganglia samples, consequently only material from the neuronal region of PHN2 (PHN2a), HZ3 SOR and CON2 samples were completely analysed in two independent experiments.

This data suggests that there may be a higher viral DNA burden in PHN-affected ganglia than in ganglia following active herpes zoster reactivation.

3.3.4 RNA analysis of VZV-specific transcripts in human ganglia samples

As the viral DNA load in one of the PHN-affected ganglionic samples was found to be much higher than that detected in the ganglion at the site of reactivation, it investigated whether any active viral replication was present within the ganglia samples. This was assessed using reverse transcriptase real time PCR analysis of
VZV-specific DNA levels were measured via quantitative real time PCR utilising a primer specific for a unique VZV coding region. Results were normalised to human albumin (present in 1 copy per each cell), and then the number of copies per $10^5$ cells calculated.

DNA from the neuronal area of PHN2 (PHN2a), the site of reactivation from HZ3 (HZ3 SOR) and CON2 were fully analysed. Unfortunately analysis of other ganglia samples was not possible as there was insufficient DNA to perform all experiments.

On average there was 6.9x more copies of VZV present in the neuronal region of PHN2 than in the HZ3 site of reactivation.
VZV-specific mRNA transcripts of three viral genes: gB, gE and IE63. IE63 is an immediate early gene, and may also be expressed during latent infection however VZV gB and gE are both late genes, only expressed during in productively infected cells (Reichelt et al., 2009).

3.3.4.1 Plasmid Constructs

Initially it was important to measure the sensitivity of the primer pairs used. To this end, plasmid constructs were created containing the coding region detected by the real time PCR primer pairs. Plasmid construction was performed using the pGEM-T Easy vector system (Promega, USA). Regions of interest were amplified in PCR reactions (Section 2.6.2), confirmed via gel electrophoresis (Section 2.6.3), purified (Section 2.6.4) and utilised in ligation reactions (Section 2.6.5). Bacterial cells were transformed via heat shock (Section 2.6.10.1), and then amplified via bacterial cultures (Section 2.6.10.2). Constructs were screened for the region of interest via bacterial colony PCR (Section 2.6.10.3), before plasmid isolation (Section 2.6.10.4), and sending the resulting purified DNA for sequencing at the Australian Genome Research Facility (Section 2.6.6). The resulting plasmid constructs are called pGEM-gB-RT, pGEM-gE-RT and pGEM-IE63-RT and plasmid maps are shown in Figure 3.8 were created utilising PlasMapper (Dong et al., 2004).

3.3.4.2 Primer Sensitivity Testing

Utilising the plasmid constructs from above, plasmids were linearised by restriction enzyme digestion and serial dilutions of plasmid stocks were created and using the molecular weight of the plasmids the copy number of each dilution calculated. These serial dilutions were then utilised as templates in real time PCR reactions to measure the primer sensitivity of each primer pair. The IE63 and gB-specific primer pairs reliably and reproducibly amplified a product from 100 copies of their target sequences per reaction, while the gE-specific primer pair reliably and reproducibly amplified a product from 1000 copies of target sequence per reaction. Detection below this level was possible, however serial dilutions were less precise, and consequently it could not be reliably detected. Data is shown in Appendix 1.

3.3.4.3 Real Time PCR Analysis of VZV-Specific Gene Expression in Human Ganglia Samples

After checking sensitivity of the primers, RNA was extracted from human ganglia samples, and utilised in reverse transcription reaction to create cDNA. This cDNA
Figure 3.8 – Maps of plasmid constructs created for real time analysis

Plasmid constructs were created by PCR amplifying and purifying DNA fragments containing the region of interest, ligating into pGEM-T-Easy vector system (Promega, USA) and transfecting competent E.coli cells.

pGEM-gB-RT (A) contains a 853 bp region of VZV gB. pGEM-gE-RT (B) contains a 721 bp region of VZV gE. pGEM-IE63-RT (C) contains a 544 bp region of VZV IE63.

Maps shown include the PCR fragment in pink, ampicillin resistance gene in orange and unique restriction enzyme sites labelled in blue.
was utilised as templates in real time PCR analysis in order to detect VZV-specific transcripts, with real time analysis performed using primers for both a viral transcript (either gB or gE) and the house keeping gene GAPDH concurrently. Unfortunately analysis using an NanoDrop spectrophotometer (Thermo Scientific, USA) indicated that the RNA extracted from all ganglia samples was degraded (likely due to the heavy fixation process each ganglia was subjected to), and consequently the cDNA was not of sufficient quality and detection of both VZV and house keeping transcripts was not possible, despite the positive control (cDNA obtained from RNA infected cell culture material) working well. The IE63-specific primer pair was not able to be used as there was insufficient template material to continue. Therefore we were not able to use RNA to establish VZV-specific gene expression in human ganglia samples.

3.3.5 Presence and distribution of viral antigen expression in human ganglia following herpes zoster and during PHN

Although there is very little known about the underlying pathology and pathogenesis of PHN one of the main hypotheses offered is the presence of ongoing active viral infection within the ganglia (Steiner et al., 2007). VZV reactivation within the ganglia leads to herpes zoster, however PHN can be present months to years following the resolution of the zoster rash (Watson, 1998). Our laboratory has previously shown the presence of VZV antigen positive cells within post mortem ganglia samples taken from herpes zoster-affected patients (Gowrishankar, 2008, Gowrishankar et al., 2010), however a more recent study has shown that this may due to cross-reactivity between components of the antibody solution with human blood group proteins rather than viral antigen expression (Zerboni et al., 2012). More recently we have been able to demonstrate the presence of multiple VZV antigens in post mortem ganglia samples from another herpes zoster-affected patient, however at a much lower level and with a different cellular distribution than previously reported (Steain et al., 2013). In this study VZV IE63 antigen positive cells were observed within the ganglia of one herpes zoster-affected patient, and in another herpes zoster-affected patient anti-VZV gE and anti-VZV gE:gi complex antibody staining was observed within neuron-satellite cell units, along with some small infiltrating cells also staining positive for VZV gE. To date there has been no reported direct demonstration of productively infected cells or VZV antigen staining in PHN-affected ganglia material, despite detection of VZV-specific proteins within mononuclear cells in the blood of PHN-affected patients (Gilden et al., 2003).
VZV antigen detection within human ganglionic samples from a PHN-affected sample was attempted with the VZV-specific antibodies listed below in Table 3.1. Both immunofluorescent staining with sudan black autofluorescence masking and immunohistochemical staining with azure blue counterstain methods (see Sections 2.8.2 and 2.7.3) were attempted. In each experiment staining was successful on positive control infHFF cell blocks and VZV-infected fetal explant ganglia material. Control stains were performed in parallel by applying a matched antibody isotype at the same concentration to ganglia material. Negative controls included uniHFF and uninfected fetal explant ganglia material, as well as control human ganglia material.

There was no VZV antigen detection in the PHN patient material with any method attempted. There was no VZV antigen expression detected in the herpes zoster or control patient material. In total, 5 different markers were utilised a minimum of 3 times each. Some of these experiments were attempted simultaneously and in conjunction with those reported in Steain et al. (2013), which successfully demonstrated the presence of low level VZV antigen (IE63, gE and gE:gi) expression in both ganglia and infiltrating cells in post-mortem material from patients with active herpes zoster.

Therefore despite the higher viral DNA burden demonstrated in PHN-affected ganglia by quantitative real-time PCR we were not able to confirm the presence of any active viral replication by IFA staining.

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-glycoprotein E</td>
<td>(Millipore)</td>
</tr>
<tr>
<td>Mouse anti-glycoprotein E:glycoprotein I complex</td>
<td>(Meridian Life Sciences)</td>
</tr>
<tr>
<td>Mouse anti-glycoprotein B</td>
<td>(Dr C. Grose, University of Iowa)</td>
</tr>
<tr>
<td>Rabbit anti-ORF29</td>
<td>(Prof P. R. Kinchington, University of Pittsburgh)</td>
</tr>
<tr>
<td>Rabbit anti-IE63</td>
<td>(Dr R. Mahalingam, University of Colorado)</td>
</tr>
</tbody>
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3.3.6 Constitution and distribution of infiltrating immune cells in human ganglia in vivo

Previously during my undergraduate honours work I performed an analysis of the constitution and distribution of immune cells present in one ganglion sample each from PHN1 and PHN2, as well as in the HZ1 and CON1 samples. To do this ganglia sections were stained for a variety of immune markers including CD3, CD4, CD8 (for T cell subsets), CD20 (for B cells), CD68 (for macrophages and other large cells), CD57 (for NK cells), CD1a (for dendritic cells), CD45RA (for naive T cells), CD45RO (for memory T cells), TIA-1 (T cell intracytoplasmic antigen) and granzyme B (cytotoxic T cells and NK cell marker). All of these samples contained tissue from the neuronal region of the DRG.

Following on from this work we gained access to additional PHN patient ganglionic material which consisted mainly of regions of nerve bundles from the various ganglia. As there has not been to date an analysis of the infiltrate within the different regions of the ganglia, this project encompassed a detailed comparison of the immune cell infiltrate in PHN, herpes zoster and control material for both the neuronal and nerve bundle areas. There has also been no analysis of immune cell infiltration and viral antigen expression across the different phases of VZV infection of ganglia in vivo. To this end, ganglia and nerve bundle samples from individuals with active viral reactivation (HZ2 and HZ3), 6 months post-herpes zoster (HZ1) as well as years post-herpes zoster during PHN were all immunostained and examined by fluorescent microscopy.

All samples were IFA stained and analysed for CD3, CD4, CD8, CD20, CD68, CD45RA, CD45RO and TIA-1. Sensory neurons typically lack expression of MHC molecules, however it has been reported that HSV-1 infection of mouse sensory neurons causes an upregulation of these functionally important immune molecules (Pereira et al., 1994). All ganglia samples were also consequently used to examine the expression of HLA-DR to check for any upregulation of MHC class II on professional antigen-presenting cells.

To ensure accurate interpretation of IFA staining experiments a number of controls were included. The positive control material used was either human tonsil tissue or the CD14+ PBMC cell block. Negative control material was unHFF sections. Isotype controls were also performed to confirm the specificity of antibody staining, and included applying the appropriate isotype control antibody to matched ganglionic
sections. A representative panel of microscope images for each of the successful immune cell markers is shown for each IFA stain.

3.3.6.1 IMMUNE CELL ANALYSIS

Initial analysis during honours of the number of each cell type detected was performed by calculating the number of each cell marker detected as a percent of all small round cells (Sutherland, 2008). This was investigated to compare results with another study performed in our lab that was being performed at around the same time (Gowrishankar, 2008, Gowrishankar et al., 2010). However as the process was laborious and at times imprecise due to the subjective issue of what constitutes a “small round cell”, for this study cell marker numbers were analysed as frequency of positive cells per unit area – and more specifically number of positive cells per 100 000 µm². This allows for a more robust analysis that is more easily performed on new samples, and is more feasible to compare between samples stained and counted at different times.

Following IFA staining of sections, microscope images were captured using the Zeiss AxioPlan 2 upright microscope with an AxioCam HR digital monochrome CCD camera (Carl Zeiss, Australia) for images of immunofluorescent stains, or an AxioCam HR digital colour CCD camera (Carl Zeiss, Australia) for images of immunohistochemical stains. Microscope and cameras were part of the Bosch Advanced Microscopy Unit (University of Sydney, Australia). Cell counts and cell frequencies were calculated by counting the number of positive cells for each cell specific marker in 8-20 random images per section (limited only by the size and condition of the section). The total surface area examined in each micrograph was then calculated with the aid of the Zeiss AxioVision LE software (Carl Zeiss Pty Ltd, Australia). The frequency of positive cells was obtained by dividing the number of positive cells by the total area of the tissue which was then expressed as “positive cells per 100 000 µm²”. This analysis was performed for the cell markers CD3, CD4, CD8, CD20 and TIA-1. In addition, all IFA slides previously stained during honours were re-analysed using this method to allow a direct comparison.

An alternate method was utilised for HLA-DR and CD45RA/RO analysis as individual cell expression was difficult to identify, instead regions of expression were analysed. These markers were investigated in terms of relative expression: HLA-DR expression was compared across the various ganglia samples taken during active zoster, many months post herpes zoster and in a PHN-affected individual. This enabled the
comparison in HLA-DR expression levels between the difference disease states following natural VZV reactivation in vivo. CD45RA expression was compared to CD45RO expression within the same ganglia to identify the prominent immune cell type present – naive (CD45RA⁺) or memory (CD45RO⁺).

3.3.6.2 Detection of CD3⁺ T cells

T cells are an important effector cell type in the body’s immune system, especially in viral infections (Abbas and Lichtman, 1991), and during VZV infection T cell mediated immune responses have been shown to be crucial in the control of primary VZV infection (Arvin, 1992). To determine whether T cells were present within the PHN sample material and compare the relative cell numbers and distribution with that seen in herpes zoster and control ganglia samples, dual IFA was performed using the rabbit anti-S100B and mouse anti-CD3 primary antibodies specific for satellite cells/myelin and T cells respectively. The S100B marker allowed for further distinction between infiltrating cells and satellite cells. The secondary antibodies used to detect antibody binding were the Alexa Fluor 488 donkey anti-rabbit (green) and Alexa Fluor 594 donkey anti-mouse (red).

Representative images of CD3⁺ T cells staining (red) on each ganglia samples are shown in Figure 3.9. CD3 is a cell surface marker on T cells, and the staining for the anti-CD3 antibody shows a cell membrane-associated pattern. S100B is a calcium binding protein, and staining for S100B (green) is present within the cytoplasm and nucleus of satellite cells and myelin bundles. Specificity of the staining was confirmed by staining ganglia with isotype control antibodies (Fig 3.9 O).

The neuronal area of PHN1 (Fig. 3.9 A) had an infiltration of CD3⁺ T cells within the capsule region, and a smaller number of T cells in close proximity to neurons. The nerve areas of PHN1 (Fig. 3.9 B and C) showed a marked infiltration of CD3⁺ cells surrounding one nerve bundle, and a diffuse infiltration throughout the remaining tissue. The neuronal area of PHN2 (Fig. 3.9 D) had the largest infiltration of T cells, with cells spread throughout the ganglia within neuronal regions. The nerve bundles of PHN2 (Fig. 3.9 E) showed a very limited number of CD3⁺ cells. The nerve region of PHN3 (Fig. 3.9 F) showed a diffuse infiltration of CD3⁺ cells throughout the ganglia with two small areas of high infiltration.

HZ1 (Fig. 3.9 G) featured a CD3⁺ T cell infiltration mainly restricted to the capsule and outer edges of the neuronal regions. The nerve bundles of HZ2 (Fig. 3.9 H) showed limited CD3⁺ infiltrate spread throughout the tissue section. There was a
Figure 3.9 - CD3+ T cell detection in human ganglia

Human ganglia samples were stained by immunofluorescence utilizing antibodies specific for CD3 and S100B, detecting CD3 T cells (red) and satellite cells (green) respectively. The cell nuclei were counterstained with DAPI (blue). White arrows indicate CD3 T cell detection, and orange arrows indicate larger infiltrations of CD3 T cells. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E), PHN3a (F), HZ1 (G), HZ2 (H), HZ2 SOR (I), HZ2 contra (J), HZ3 (K), HZ3 SOR (L) and CON1 (M). Limited CD3 T cells were observed in CON2 (N). Positive control human tonsil material showed clear staining of both CD3 T cells and S100B follicular dendritic cells (not shown). Isotype staining on ganglia section (O) and background staining on uniHFF sections (not shown) were minimal.
diffuse infiltrate of CD3$^+$ T cells restricted to the nerve bundles of both HZ2 SOR and HZ2 contra, with some small foci (Fig. 3.9 I and J). The nerve bundles of HZ3 (Fig. 3.9 K) featured a diffuse infiltration of CD3$^+$ cells throughout the tissue section. The HZ3 SOR featured a large infiltration of CD3$^+$ T cells spread throughout the nerve cell bundles and to a lesser degree within the capsule region (Fig. 3.9 L).

CON1 (Fig. 3.9 M) and CON2 (Fig. 3.9 N) contained only a few sporadic CD3$^+$ T cells spread randomly within the section. CD3$^+$ T cell staining was clearly observed in the positive control human tonsil material (data not shown) while background and non-specific staining on the negative control (uniHFF) and isotype control stained sections was minimal (Fig. 3.9 O).

The frequency of CD3$^+$ T cells in human ganglia samples is shown in Figure 3.10. Each cell count is in “Positive cells per 100 000 $\mu m^2$”. The average value for the neuronal regions (Fig. 3.10 A) was 35.81 for PHN1, 14.33 for PHN2, 7.20 for HZ1 and 2.74 for CON1. The average value for the nerve bundle areas (Fig. 3.10 B) was 18.24 for PHN1, 1.51 for PHN2, 5.19 for PHN3, 0.71 for HZ2, 5.04 for HZ2 SOR, 7.04 for HZ2 contra, 12.74 for HZ3, 23.99 for HZ3 SOR and 1.62 for CON2.

The greatest infiltration of CD3$^+$ T cells was seen in the ganglia obtained from the site of reactivation of HZ3, (rash began 17 days prior to and was present at death). CD3$^+$ T cell frequencies in the PHN-affected ganglia were greater than those seen in HZ2 and HZ1 – reactivation occurring 3 weeks prior to death (and still present at death) and occurring 6 months prior to death. Significantly all the PHN-affected ganglia material showed the presence of infiltrating CD3$^+$ T cells greater than that observed in normal control ganglia.

### 3.3.6.3 Detection of CD4$^+$ T Helper Cells

Different subsets of T cells perform different functions within the human immune system. CD4$^+$ T helper cells are important in mediating humoral immune mechanisms (Abbas and Lichtman, 1991), and VZV-specific CD4$^+$ T cells are essential for the resolution of varicella (Abendroth and Arvin, 1999). In many herpesvirus infections (including VZV) CD4$^+$ T cells have been shown to perform cytolytic functions (Arvin et al., 1991). To detect the presence of CD4$^+$ T cells within the PHN sample material and compare the relative amounts and distribution with that seen in herpes zoster and control ganglia samples, immunofluorescence was performed using the goat anti-CD4 primary antibodies specific for cell markers on T helper cells. The secondary antibody used was Alexa Fluor 594 donkey anti-goat
Figure 3.10 - Frequency of CD3⁺ T cells in human ganglia samples

Human ganglia samples were stained by immunofluorescence, imaged and the number of positive cells per field of view counted. The total surface area of each field of view was calculated, cell counts averaged, and the frequency of positive cells per 100 000 um² for both the neuronal (A) and nerve bundle (B) regions of the ganglia samples are shown. For each sample stains were performed in duplicate, with 8-20 random fields of view examined per stain limited only by the size and condition of each tissue sample. Columns display the mean cell count for each sample, with the minimum and maximum shown by the square and triangle respectively.
Sections were also stained with rabbit anti-S100B and Alexa Fluor 488 donkey anti-rabbit (green) to allow better distinction between infiltrating cells and satellite cells.

Representative images of CD4⁺ T cell staining for each ganglia sample are shown in Figure 3.11. CD4 is a cell surface marker on T helper cells, and successful staining shows a membrane-associated pattern (red staining). CD4⁺ cells were found distributed throughout all human ganglia samples assessed. S100B is a calcium binding protein, and staining for S100B (green) is present within the cytoplasm and nucleus of satellite cells and myelin bundles. Specificity of the staining was confirmed by staining ganglia with isotype control antibodies (Fig 3.11 O).

The neuronal region of PHN1 (Fig. 3.11 A) featured a low frequency of CD4⁺ T cells within the ganglia as well as an infiltration in the capsule region. The nerve areas of PHN1 (Fig. 3.11 B and C) featured an infiltration of CD4⁺ cells very similar to that seen with the CD3 staining of these sections - a marked infiltration of CD4⁺ cells surrounding one nerve bundle, and a diffuse infiltration throughout the remaining tissue. The neuronal region of PHN2 (Fig. 3.11 D) featured infiltrations of CD4⁺ T cells found throughout the ganglia. The nerve bundles of PHN2 and PHN3 (Fig. 3.11 E and F) showed a very limited number of CD4⁺ cells.

The neuronal region in HZ1 (Fig. 3.11 G) showed CD4⁺ T cells generally restricted to the outer regions of the neuronal areas of the ganglia and capsule region. The nerve bundle regions in HZ2 (Fig. 3.11 H) featured a very limited number of CD4⁺ T cells. There was some diffuse CD4⁺ T cells spread throughout the nerve bundles of HZ2 SOR, with two separate foci in one bundle of nerves and the capsule region (Fig. 3.11 I). HZ2 contra featured a limited infiltration of CD4⁺ T cells throughout the nerve bundle regions (Fig. 3.11 J). The nerve bundle regions in HZ3 (Fig. 3.11 K) featured a diffuse infiltrate of CD4⁺ T cells spread throughout the tissue section. The HZ3 SOR featured a large infiltration of CD4⁺ T cells spread throughout the nerve cell bundles and to a lesser degree within the capsule region (Fig. 3.11 L).

The neuronal region of CON1 (Fig. 3.11 M) and CON2 (Fig. 3.11N) showed sporadic CD4⁺ T cells spread throughout the ganglia. Specific CD4⁺ staining was observed in the positive control human tonsil sections (tonsil not shown), while background and non-specific staining on the negative control (uniHFF) and isotype antibody control sections was minimal (Fig. 3.11 O).
Figure 3.11 - CD4⁺ T cell and S100B expression in human ganglia

Human ganglia samples were stained by immunofluorescence utilising antibodies specific for CD4 and S100B, detecting CD4 T cells (red) and satellite cells (green) respectively. HZ3 SOR (L) was stained for CD4 alone. The cell nuclei were counterstained with DAPI (blue). White arrows indicate CD4 T cell detection. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E), PHN3a (F), HZ1 (G), HZ2 (H), HZ2 SOR (I), HZ2 contra (J), HZ3 (K), HZ3 SOR (L) CON1 (M) and CON2 (N). Positive control human tonsil material showed clear staining of CD4 T cells and S100B expression on follicular dendritic cells (not shown). Isotype staining on ganglia section (O) and background staining on uniHFF sections (not shown) were minimal.
The frequency of infiltrating CD4\(^+\) T cells in human ganglia samples is shown in Figure 3.12. Each cell count is in “Positive cells per 100 000 \(\mu m^2\). The average count for the neuronal regions (Fig. 3.12 A) was 10.98 for PHN1, 1.96 for PHN2, 3.63 for PHN3 and 0.64 for CON1. The average count for the nerve bundle areas (Fig. 3.12 B) was 6.12 for PHN1, 1.29 for PHN2, 1.65 for PHN3, 0.48 for HZ2, 4.92 for HZ2 SOR, 1.31 for HZ2 contra, 3.13 for HZ3, 24.45 for HZ3 SOR and 1.84 for CON2.

The highest frequency of infiltrating CD4\(^+\) T cells was observed in the HZ3 SOR ganglia sample, while the PHN1 and HZ2 samples had similar levels. Higher levels of CD4\(^+\) T cells were observed in all PHN ganglia samples, above that observed in the controls. Control neuronal areas showed a very limited CD4\(^+\) T cell infiltration, however there was a similar frequency of CD4\(^+\) T cells in the control nerve bundle regions (CON2) compared to other VZV-affected ganglia samples.

\[\text{3.3.6.4 Detection of CD8}^+\ T\text{ Cytotoxic Cells}\]

Cytotoxic T cells are the principle cells involved in direct cell-mediated immune responses against viral infection, and are important for the control of primary VZV infection (Abbas and Lichtman, 1991, Abendroth and Arvin, 1999). CD8 is a characteristic marker of this subset of T cells (Abbas and Lichtman, 1991). To detect the presence of CD8\(^+\) cytotoxic T cells within the PHN sample material and compare the relative frequency and distribution of these cells with that seen in herpes zoster and control ganglia samples, immunofluorescence was performed using a rabbit anti-CD8 primary antibody specific for cell markers on cytotoxic T cells. The secondary antibody used to detect binding of the primary antibody was the Alexa Fluor 594 donkey anti-rabbit (red).

Representative images of CD8\(^+\) T cell staining on each ganglia sample are shown in Figure 3.13. CD8 is a cell surface marker on T cytotoxic cells, and successful staining showed a membrane-associated pattern. CD8\(^+\) T cells were readily detected and found distributed throughout all PHN ganglia samples, HZ1 and HZ3. Specificity of the staining was confirmed by staining of ganglionic sections with isotype control antibodies (Fig 3.13 O).

The neuronal area of PHN1 (Fig. 3.13 A) featured a relatively small number of CD8\(^+\) T cells within neuronal regions in the ganglia as well as a sizeable infiltration in the capsule region. The nerve bundle regions of PHN1 (Fig. 3.13 B and C) showed an infiltration of CD8\(^+\) cells very similar to that seen with CD3\(^+\) and CD4\(^+\) cells - a marked infiltration of CD8\(^+\) cells surrounding one nerve bundle, and a diffuse
Figure 3.12 - Frequency of CD4+ T cells in human ganglia samples

Human ganglia samples were stained by immunofluorescence, imaged and the number of positive cells per field of view counted. The total surface area of each field of view was calculated, cell counts averaged, and the frequency of positive cells per 100,000 um² for both the neuronal (A) and nerve bundle (B) regions of the ganglia samples are shown. For each sample stains were performed in duplicate, with 8-20 random fields of view examined per stain limited only by the size and condition of each tissue sample. Columns display the mean cell count for each sample, with the minimum and maximum shown by the square and triangle respectively.
Figure 3.13 - CD8⁺ T cell detection in human ganglia

Human ganglia samples were stained by immunofluorescence utilising an antibody specific for CD8 detecting CD8 T cells (red). The cell nuclei were counterstained with DAPI (blue). White arrows indicate CD8 T cell detection. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E), PHN3a (F), HZ1 (G), HZ2 SOR (I), HZ2 contra (J), HZ3 (K) and HZ3 SOR (L). There was limited CD8 T cell detection in HZ2 (H), CON1 (M) and CON2 (N). Positive control human tonsil material showed clear staining of CD8 T cells (not shown). Isotype staining on ganglia section (O) and background staining on unihFF sections (not shown) were minimal.
infiltration throughout the remaining tissue. The neuronal region of PHN2 (Fig. 3.13 D) featured relatively large infiltrations of CD8⁺ T cells found throughout the neuronal regions of the ganglia.

Limited numbers of CD8⁺ T cells were observed within the nerve bundle region of PHN2 (Fig. 3.13 E). The nerve bundle region of PHN3 (Fig. 3.13 F) showed a diffuse infiltrate spread throughout the tissue region.

The neuronal region of HZ1 (Fig. 3.13 G) showed CD8⁺ T cells generally restricted to the outer regions of the ganglia and capsule region. There were no CD8⁺ cells detected in the nerve bundle region of HZ2 (Fig 3.13 H). There was one focus of CD8⁺ cells within one nerve bundle in HZ2 SOR, with limited detection throughout the rest of the nerve tissue (Fig. 3.13 I), and limited detection within the nerve bundles of HZ2 contra (Fig. 3.13 J). There was a diffuse CD8⁺ infiltrate detected throughout the nerve bundles of HZ3 (Fig. 3.13 K), and a large infiltration of CD8⁺ cells within the nerve bundle regions of HZ3 SOR (Fig. 3.13 L).

No CD8⁺ T cells were observed in the neuronal regions CON1 (Fig. 3.13 M) or the nerve bundle regions of CON2 (Fig. 3.13 N). Specific staining was observed in the positive human tonsil material (data not shown), while background and non-specific staining on the negative control (uniHFF) and isotype control sections was minimal (Fig. 3.13 O).

The frequency of CD8⁺ T cells in human ganglia samples is shown in Figure 3.14. Each cell count is in “Positive cells per 100 000 μm²”. The average count for the neuronal regions (Fig. 3.14 A) was 8.44 for PHN1, 2.74 for PHN2, 0.21 for HZ1 and 0.00 for CON1. The average counts for the nerve bundle areas (Fig. 3.14 B) was 7.33 for PHN1, 0.77 for PHN2, 3.02 for PHN3, 0.41 for HZ2, 0.27 for HZ2 SOR, 0.55 for HZ2 contra, 4.32 for HZ3, 6.86 for HZ3 SOR and 0.10 for CON1.

CD8⁺ T cells were detected in comparable frequencies in all PHN1 samples and in the HZ3 SOR (17 days post reactivation with rash still present). However other than this sample, CD8⁺ T cells were generally present in greater abundance in the PHN-affected material compared to the other herpes zoster-affected and control ganglionic material.

3.3.6.5 Detection of TIA-1⁺ Cells

As CD8⁺ T cells were readily observed within the immune infiltration in herpes zoster and PHN-affected ganglia, I sought to determine the cytolytic nature of these cells. T-
Human ganglia samples were stained by immunofluorescence, imaged and the number of positive cells per field of view counted. The total surface area of each field of view was calculated, cell counts averaged, and the frequency of positive cells per 100,000 um² for both the neuronal (A) and nerve bundle (B) regions of the ganglia samples are shown. For each sample stains were performed in duplicate, with 8-20 random fields of view examined per stain limited only by the size and condition of each tissue sample. Columns display the mean cell count for each sample, with the minimum and maximum shown by the square and triangle respectively.
cell intracytoplasmic antigen 1 (TIA-1) is a marker found in cytolytic granules, and was detected using a mouse anti-TIA-1 antibody.

Representative images of TIA-1 immunofluorescent staining on each of the human ganglia samples are shown in Figure 3.15. TIA-1 is confined to cytoplasmic granules, and positive staining appears as small punctate areas (red staining). S100B is a calcium binding protein, and staining for S100B (green) is present within the cytoplasm and nucleus of satellite cells and myelin bundles. Specificity of the staining was confirmed by staining ganglia with isotype control antibodies (Fig 3.15 O).

There was staining of TIA-1+ cells observed within both the neuronal and nerve bundles regions of PHN 1 (Fig. 3.15 A, B and C) and PHN2 (Fig. 3.15 D and E), and more limited detection seen in the nerve bundles of PHN3 (Fig. 3.15 F).

TIA-1 expression was also clearly observed in the neuronal regions of HZ1 (Fig. 3.15 G), and the nerve bundles of HZ2 and HZ3 (Fig. 3.15 H and K respectively). No TIA-1 expressing cells were detected in the nerve bundles of HZ2 SOR and HZ2 contra (Fig. 3.15 I and J respectively), or HZ3 SOR (Fig. 3.15 L). Furthermore, no TIA-1 expressing cells were observed within the CON1 and CON2 samples (Fig. 3.15 M and N respectively).

The frequency of TIA-1+ cells in human ganglia samples is shown in Figure 3.16. Each cell count is in “Positive cells per 100 000 µm²”. The average count for the neuronal regions (Fig. 3.16 A) was 5.32 for PHN1, 2.57 for PHN2, 2.74 for HZ1 and 1.33 for CON1. The average count for the nerve bundle areas (Fig. 3.16 B) was 4.76 for PHN1, 3.23 for PHN2, 0.34 for PHN3, 0.08 for HZ2, 0.00 for HZ2 SOR, 0.00 for HZ2 contra, 0.80 for HZ3, 0.00 for HZ3 SOR and 0.00 for CON2.

These results demonstrate that within the PHN-affected sample material there were TIA-1+ cells spread throughout both neuronal and nerve bundle regions, and these were observed at a much greater frequency than that seen in the herpes zoster-affected and control material.

### 3.3.6.6 Detection of CD20+ B cells

B cells are an important effector cell type in the humoral immune system (Abbas and Lichtman, 1991). During VZV infection antibodies specific for viral proteins and neutralising antibodies are both important in activating the complement pathway or antibody dependent cellular cytotoxicity (Abendroth and Arvin, 2000, Arvin and Abendroth, 2007). B cells have also been previously detected in human ganglia.
Figure 3.15 - Cytolytic T cell detection in human ganglia

Human ganglia samples were stained by immunofluorescence utilising antibodies specific for TIA-1 and S100B, detecting cytolytic granules within cells (red) and satellite cells (green) respectively. HZ3 SOR (L) was stained for TIA-1 only. The cell nuclei were counterstained with DAPI (blue). White arrows indicate cytolytic T cell detection. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E), HZ1 (G), HZ2 SOR (I), HZ2 contra (J), HZ3 (K), HZ3 SOR (L) and CON1 (M). Limited cytolytic T cells were observed in PHN3a (F), HZ2 (H) and CON2 (N). Positive control human tonsil material showed clear staining of TIA-1 cytolytic immune cells and S100B follicular dendritic cells (not shown). Isotype staining on ganglia section (O) and background staining on uniHFF sections (not shown) were minimal.
Figure 3.16 - Frequency of TIA-1$^+$ cytolytic T cells in human ganglia samples

Human ganglia samples were stained by immunofluorescence, imaged and the number of positive cells per field of view counted. The total surface area of each field of view was calculated, cell counts averaged, and the frequency of positive cells per 100,000 $\mu m^2$ for both the neuronal (A) and nerve bundle (B) regions of the ganglia samples are shown. For each sample stains were performed in duplicate, with 8-20 random fields of view examined per stain limited only by the size and condition of each tissue sample. Columns display the mean cell count for each sample, with the minimum and maximum shown by the square and triangle respectively.
many weeks to months following herpes zoster (Gowrishankar, 2008, Gowrishankar et al., 2010). CD20 is a cell surface marker expressed exclusively on B cells. In order to determine whether B cells are present during PHN and compare the relative frequency and distribution of B cells with that seen in herpes zoster and control ganglia samples, IFA was performed using the mouse anti-CD20 primary antibodies followed by the Alexa Fluor 594 donkey anti-mouse secondary antibody (red). Sections were co-stained with rabbit anti-cow S100B to labelled satellite cells and nerves, detected with the Alexa Fluor 488 donkey anti-rabbit secondary antibody (green).

Representative images of CD20 staining from PHN-affected, herpes zoster-affected and control ganglia sample are shown in Figure 3.17. CD20 is a cell surface marker of B cells, and successful staining shows a membrane-associated pattern. S100B is a calcium binding protein, and staining for S100B (green) is present within the cytoplasm and nucleus of satellite cells and myelin bundles. Specific detection of CD20 was observed in human tonsil material (data not shown), while background and non-specific staining on the negative control (uniHFF) and isotype control sections was minimal (Fig. 3.17 O).

B cells were readily detected within the neuronal region of PHN1 (Fig. 3.17 A) and had a sizeable infiltration of B cells within the ganglia capsule, while the nerve bundle region of PHN1 (Fig. 3.17 B and C) showed a marked infiltrate centred around one nerve bundle with a diffuse infiltrate throughout the rest of the tissue section. However there was less CD20+ B cells detected in both the neuronal and nerve bundle regions of PHN2 (Fig. 3.17 D and E), and PHN3 (Fig. 3.17 F).

A limited number of CD20+ B cells were detected in the neuronal region of HZ1 (Fig. 3.17 G), and within the nerve bundles of the SORs for HZ2 and HZ3 (Fig 3.17 I and L respectively). Limited CD20+ B cells were detected in HZ2 contra and HZ3 (Fig. 3.17 J and K respectively) and the neuronal area of CON1 (Fig 3.17 M), while no positive staining cells were observed in HZ2 (Fig. 3.17 H) or in the nerve bundle region of CON2 (Fig. 3.17 N).

The frequency of CD20+ B cells in human ganglia samples is shown in Figure 3.18. Each cell count is in “Positive cells per 100 000 µm2”. The average count for the neuronal regions (Fig. 3.18 A) was 5.71 for PHN1, 0.75 for PHN2, 0.07 for HZ1 and 0.00 for CON1. The average count for the nerve bundle areas (Fig. 3.18 B) was 5.20
Figure 3.17 - CD20\(^+\) B cell detection in human ganglia

Human ganglia samples were stained by immunofluorescence utilising antibodies specific for CD20 and S100B detecting B cells (green) and satellite cells (red) respectively. Sections previously stained (I, J, L) and older sections (A, D, G, M) were labelled with CD20 only. The cell nuclei were counterstained with DAPI (blue). White arrows indicate CD20 B cell detection. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN3a (F), and HZ1 (G). There was limited CD20 B cell detection in PHN2b (E), HZ2 (H), HZ2 SOR (I), HZ2 contra (J), HZ3 (K), HZ3 SOR (L), CON1 (M) and CON2 (N). Positive control human tonsil material showed clear staining of CD20 B cells and S100B follicular dendritic cells (not shown). Isotype staining on ganglia section (O) and background staining on unHFF sections (not shown) were minimal.
Figure 3.18 - Frequency of CD20\(^+\) B cells in human ganglia samples

Human ganglia samples were stained by immunofluorescence, imaged and the number of positive cells per field of view counted. The total surface area of each field of view was calculated, cell counts averaged, and the frequency of positive cells per 100 000 um\(^2\) for both the neuronal (A) and nerve bundle (B) regions of the ganglia samples are shown. For each sample stains were performed in duplicate, with 8-20 random fields of view examined per stain limited only by the size and condition of each tissue sample. Columns display the mean cell count for each sample, with the minimum and maximum shown by the square and triangle respectively.
for PHN1, 0.00 for PHN2, 0.13 for PHN3, 0.00 for HZ2, 0.11 for HZ2 SOR, 0.00 for HZ2 contra, 0.00 for HZ3, 0.17 for HZ3 SOR and 0.00 for CON2.

These data demonstrates that CD20\(^+\) B cells are present in the greatest number in the PHN ganglia material when compared to all herpes zoster-affected and control ganglia material. In PHN sample 1 CD20\(^+\) B cells are present in a similar frequency throughout the different regions of the ganglion sample, in both neuronal and nerve bundle regions. No CD20\(^+\) B cells were detected in either of the control ganglia samples.

3.3.6.7 Detection of CD45RA and CD45RO Expressing Cells

Naive cells are immune cells that have not been exposed to antigen, while memory cells are immune cells that persist long after initial exposure to antigen (Abbas and Lichtman, 1991). Responses against herpes zoster have been shown to consist mainly of memory T cells (Vossen et al., 2004), and previous work on herpes zoster-affected ganglia material has confirmed the predominance of memory cells (Gowrishankar, 2008, Gowrishankar et al., 2010). The RA isoform of CD45 is a cell marker expressed on naive immune cells, included B cells, T cells and monocytes, while the RO isoform of CD45 is expressed on memory lymphocytes. To determine whether naive and memory cells are present within ganglia samples during PHN and to compare the relative frequency and distribution to that seen in herpes zoster, immunofluorescence was performed separately using mouse anti-CD45RA and mouse anti-CD45RO primary antibodies. The secondary antibody used to detect primary antibody binding was the Alexa Fluor 594 donkey anti-mouse (red).

Unfortunately due to the limited number of sections provided, staining for CD45RA and CD45RO was not performed on the HZ2 SOR and HZ2 contra samples. Representative images of CD45RA and CD45RO immunofluorescent staining on the various human ganglia samples are shown in Figure 3.19 and Figure 3.20 respectively. CD45RA and RO are cell surface markers of naive and memory immune cells respectively, and successful staining shows a membrane-associated pattern (red).

PHN1 showed CD45RA\(^+\) naive and CD45RO\(^+\) memory immune cells present within both the neuronal and nerve bundle areas of the ganglia as well as in an infiltration in the capsule region (Fig. 3.19 A, B and C, and Fig. 3.20 A, B and C respectively). PHN2 showed a small infiltrate of CD45RA\(^+\) naive and CD45RO\(^+\) memory immune cells spread within neuronal and nerve bundle areas (Fig. 3.19 D and E, and Fig.
Figure 3.19 - Naive immune cell detection in human ganglia

Human ganglia samples were stained by immunofluorescence utilising antibodies specific for CD45RA detecting naive immune cells (red). The cell nuclei were counterstained with DAPI (blue). White arrows indicate CD45RA immune cell detection. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E), PHN3a (F), HZ1 (G), HZ2 (H), HZ3 (I), HZ3 SOR(J) and CON1 (K) and CON2 (L). Positive control human tonsil material showed clear staining of CD45RA naive immune cells (not shown). Isotype staining on ganglia section (M) and background staining on unihFF sections (not shown) were minimal.
Figure 3.20 - Memory immune cell detection in human ganglia

Human ganglia samples were stained by immunofluorescence utilising antibodies specific for CD45RO detecting memory immune cells (red). The cell nuclei were counterstained with DAPI (blue). White arrows indicate CD45RO memory immune cell detection. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E), PHN3a (F), HZ1 (G), HZ2 (H), HZ3 (I), HZ3 SOR (J), CON1 (K) and CON2 (L). Positive control human tonsil material showed clear staining of CD45RO immune cells (not shown). Isotype staining on ganglia section (M) and background staining on uniHFF sections (not shown) were minimal.
3.20 D and E respectively). PHN3 showed a very limited infiltrate of CD45RA naive immune cells and a diffuse infiltration of CD45RO+ memory immune cells within the nerve bundle areas (Fig. 3.19 F and Fig. 3.20 F respectively).

HZ1 showed limited naive immune cell infiltration restricted to the outer edges of neuronal and capsule regions (Fig 3.19 G). There was a very limited infiltration of CD45RA+ naive immune cells detected in the neuronal areas of HZ2 and HZ3 (Fig. 3.19 H and I respectively), and the nerve bundle areas of HZ3 SOR (Fig. 3.19 J). There was not enough tissue available from HZ2 SOR and contra to perform this stain. HZ1 and HZ2 (Fig. 3.20 G and H respectively) showed CD45RO memory cell detection limited to the outer edges of the neuronal and nerve bundle areas, and the capsule region. There was a marked infiltration of CD45RO memory cells observed throughout the HZ3 and HZ3 SOR samples (Fig. 3.20 I and J respectively).

CON1 and CON2 both showed a low level of CD45RA+ naive immune cells and CD45RO+ memory immune cells spread throughout the ganglia (Fig. 3.19 K and L, and Fig 3.20 K and L respectively). Staining was successful on the positive human tonsil material (data not shown), while background and non-specific staining on the negative control (uniHFF) and isotype control sections was minimal (Fig. 3.19 M and Fig. 3.20 M).

PHN-affected sample material showed comparable levels of both CD45RA and CD45RO+ cells. However, all ganglia samples had more CD45RO+ cells present within the tissue samples.

### 3.3.6.8 DETECTION OF MHC CLASS II ISOFORM HLA-DR

Although normally sensory neurons do not express MHC class I or class II molecules, it has been previously shown that MHC-II (via human leukocyte antigen DR (HLA-DR)) is upregulated on satellite cells within ganglia during herpes zoster and in the recovery from a recent reactivation (Gowrishankar, 2008, Gowrishankar et al., 2010, Steain et al.). Consequently it was of interest to study whether this upregulation was maintained in human ganglionic material long after herpes zoster rash resolution and during PHN.

Representative images of HLA-DR (green) and CD4 (red) staining of each human ganglia sample are shown in Figure 3.21. In PHN1 there was a single region of HLA-DR upregulation in the small neuronal region of the tissue (Fig. 3.21 A). In the nerve bundle region of PHN1 there were areas of high HLA-DR expression generally
Figure 3.21 - CD4\(^+\) T cell and HLA-DR expression in human ganglia

Human ganglia samples were stained by immunofluorescence utilising antibodies specific for CD4 and HLA-DR, detecting CD4 T cells (red) and HLA-DR (green) respectively. The cell nuclei were counterstained with DAPI (blue). White arrows indicate areas of high HLA-DR expression. Samples are PHN1a (A), PHN1b (B), PHN1c (C), PHN2a (D), PHN2b (E), PHN3a (F), HZ1 (G), HZ2 (H), HZ2 SOR (I), HZ2 contra (J), HZ3 (K), HZ3 SOR (L) CON1 (M) and CON2 (N). Positive control human tonsil material showed clear staining of CD4 T cells and HLA-DR expression (not shown). Isotype staining on ganglia section (O) and background staining on uniHFF sections (not shown) were minimal.
accompanied by an infiltration of CD4⁺ T cells (Fig. 3.21 B and C). In PHN2 there was limited upregulation of HLA-DR in both the neuronal (Fig. 3.21 D) and nerve bundle regions (Fig. 3.21 E), which also occurred in conjunction with CD4⁺ T cell infiltration. In the nerve bundle regions of PHN3 there was some HLA-DR expression, which at times corresponded with CD4⁺ T cell infiltration (Fig. 3.21 F).

In the neuronal regions of HZ1 there was some HLA-DR expression, which occurred with CD4⁺ T cells in close proximity (Fig. 3.21 G). HLA-DR expression was low in the nerve bundle regions of HZ2 (Fig. 3.21 H). There was small areas of detectable HLA-DR expression in the nerve bundles of HZ2 SOR (Fig. 3.21 I) and HZ2 contra (Fig. 3.21 J), often accompanied by an infiltration of CD4⁺ T cells. In the nerve bundles of HZ3 there was some strong HLA-DR expression throughout the nerve bundles, often with CD4⁺ T cells in close proximity (Fig. 3.21 K). In the nerve bundles of HZ3 SOR there is diffuse HLA-DR expression accompanied by a large infiltrate of CD4⁺ T cells (Fig. 3.21 L).

In CON1 there was low level HLA-DR expression observed throughout the ganglion sample, although it is not associated with a CD4⁺ T cell infiltration (Fig. 3.21 M). In CON2 HLA-DR expression was limited to one area in the capsule of the nerve bundle regions, and accompanied by a CD4⁺ T cell infiltration (Fig. 3.21 N). Specific HLA-DR staining was observed in the positive control human tonsil and CD14⁺ cell block sections, while background and non-specific staining on the negative control (uniHFF) and isotype control sections was minimal (Fig. 3.21 O).

This data demonstrates there are areas of HLA-DR upregulation in many of the VZV-affected ganglia samples. The PHN-affected ganglia material showed large upregulation of HLA-DR in very small regions mainly restricted to the nerve bundles. There was no HLA-DR expression observed on neurons within the PHN-affected material. herpes zoster-affected material featured diffuse HLA-DR expression throughout the tissue. One of the control samples showed a small region of very high HLA-DR expression within the capsule of the ganglion sample.

### 3.4 Discussion

The focus of this study was to characterise the immune cell infiltration and viral gene expression in human ganglia samples that were surgically excised from an individual suffering from PHN, and to compare these samples to ganglia obtained post-mortem.
from patients who are suffering from or have recently suffered from herpes zoster and normal control ganglia samples. PHN is a very painful complication of herpes zoster, which results from VZV reactivation within human ganglia.

PHN is a relatively unstudied condition, and very little is known about the underlying pathology and pathogenesis. Due to the high species-specificity of VZV, studies must be done using cells and samples of human or simian origin (Arvin and Gilden, 2007). Animal models to study VZV disease are very limited, and in particular there is no current animal model for VZV reactivation and development of PHN. Consequently the very limited previous studies have utilised post-mortem material taken from herpes zoster-affected and PHN-affected individuals. These have reported both widespread cell loss and the presence of infiltrating immune-like cells (Head and Campbell, 1900, Watson et al., 1991, Watson et al., 1988b). However these studies were limited to basic histological examination, with no attempt to characterise the immune cell types involved in the infiltration or the extent and spread of active VZV infection.

Previously our lab has undertaken a detailed analysis of human ganglia obtained from individuals who presented with herpes zoster 1-5 months prior to death (Gowrishankar, 2008, Gowrishankar et al., 2010), with respect to characterising the different immune cells present as well as viral antigen expression in ganglionic cells. In these patients it was shown that the immune infiltration consisted predominantly of macrophages and non-cytolytic CD8+ T cells.

More recently, another study by our research group examined ganglia samples from two patients who had experienced herpes zoster (ie. zoster rash present) at the time of death (Steain et al., 2013). A details IFA analysis of ganglionic sections revealed a large inflammatory immune infiltrate present in both patient samples was comprised mainly of macrophages, natural killer cells, CD4+ T cells and cytolytic CD8+ T cells. Many of these T cells were closely associated with neurons within the reactivated ganglia. Both our laboratory’s previous studies mainly focused on the neuronal areas within the ganglia samples. This current PhD study has focused on neuronal areas and also examined in detail the nerve bundles, derived from these ganglia, but outside of the neuronal cell body areas. This is the first such study to characterise in detail the immune infiltration in the nerve bundles of patients at different stages of VZV reactivation. The HZ2 and HZ3 ganglia samples included in this study are the same samples utilised in Steain et al. (2013), however in the current study the nerve
bundle areas were examined, as the neuronal areas had previously been characterised.

The PHN ganglia samples used in this study were surgically excised from a PHN-affected patient from the lower thoracic level. This patient had suffered from herpes zoster, the rash had resolved and pain had continued for around 5 years. This offers an incredibly rare opportunity, and to the best of our knowledge there has been no previous study that has examined human ganglia material that has been surgically excised from a living patient suffering from PHN.

The herpes zoster-affected material utilised in this study was obtained from three different patients. HZ1 experienced a VZV reactivation 6 months prior to death but did not have any evidence of ongoing VZV replication at the time of death. HZ2 and HZ3 both had a VZV reactivation within weeks of death, and both had a visible herpes zoster rash at the time of death. This offers the opportunity to study different stages of VZV reactivation: active reactivation (HZ2 and HZ3), the recovery from VZV reactivation (HZ1), and long term pathology following herpes zoster in a PHN-affected individual (PHN samples).

Most of the patient material utilised in this study was obtained from patients who had undergone or were undergoing some type of immunosuppressive therapy, or were suffering from some type of immune-altering disorder. The PHN-affected patient was diagnosed with acute myeloid leukaemia, and consequently underwent a bone marrow transplant. The HZ1 patient was diagnosed with a EBV-associated lymphoproliferative disorder following a period of 5 years on an immunosuppressive treatment regime. The HZ2 patient was not currently nor had previously been on any immunosuppressive therapy, however at death was diagnosed with acute myeloid leukaemia. The control patient CON1 could be expected to be immunocompromised at the time of death due to metastatic adenocarcinoma. The herpes zoster patient HZ3, and control patient CON2 were not diagnosed with any direct immune-altering disease, or treated with any immunosuppressive treatment regime. However HZ3 was 93 years old at the time of death and therefore would be experiencing the normal decline in cell-mediated immune function which accompanies normal aging (Oxman, 2009). In an ideal case all patients would be immunocompetent and otherwise healthy, however realistically it is very rare to have access to the above post-mortem sample material and even more precious surgically-excised ganglia samples. VZV reactivation is also more likely to occur in patients who are
immunocompromised, or with age-related immune dysfunction (Hope-Simpson, 1965).

Post-mortem samples were obtained at time of autopsy. Fixation in formaldehyde would have commenced immediately on removal from the patient. Currently the time interval between time of death and commencement of autopsy is unknown, however generally is less than 1-2 days.

Basic histological examination of H&E stained PHN-affected sample material revealed the presence of a readily detectable immune infiltration in all the PHN ganglia samples. This was spread generally throughout neuronal and nerve bundle regions with distinct focal infiltrations in all PHN ganglia samples. This is consistent with previous studies on human ganglia from PHN-affected individuals which found evidence of an ongoing inflammatory process (Head and Campbell, 1900, Watson et al., 1991, Watson et al., 1988b). One early study of PHN-affected ganglia also found both diffuse and focal infiltrations of inflammatory cells, although examination was limited to the neuronal areas of the ganglia (Smith, 1978).

An extensive immune infiltration was also readily identified via basic histological examination of the neuronal regions of the sites of reactivation in active cases of VZV reactivation (HZ2 and HZ3), as identified in Steain et al. (2013). In this study on the neuronal areas from these same samples the immune infiltration was only present in the nerve bundles of HZ3 at the site of reactivation, and in a ganglion around the same region. Ganglia from HZ2 also featured a smaller but still significant immune infiltration in the neuronal regions.

H&E examination of samples from the 6 month post-zoster sample did feature a more limited immune infiltration, however this was restricted to the outer edges of the ganglia and capsule region. This result is in contrast to previous work in which there were varying amounts of infiltrating cells found in different herpes zoster-affected patients, however they were found spread generally throughout the ganglionic sections (Gowrishankar, 2008, Gowrishankar et al., 2010).

The PHN ganglionic samples used in this study were considerably smaller in size when compared to the herpes zoster and control samples. This may have been the result of the source of tissue. The PHN patient samples were removed from a live patient, and the priority of the surgeon is the well-being of the patient over removal of the intact ganglia samples, whereas the herpes zoster samples were removed during post-mortem examinations.
Interestingly one of the PHN-affected ganglia samples also featured altered morphology, with the loss of both neuronal profiles and no resulting proliferation of satellite cells. This is characteristic of fibrosis, and has been previously reported in studies of ganglia from PHN-affected patients (Smith, 1978, Watson et al., 1991, Watson et al., 1988b). One study also noted atrophy of the dorsal horn (Watson et al., 1991). The dorsal horn is directly innervated by sensory neurons which proceed from cell bodies contained within the DRG (Kandel et al., 2000). It is quite possible that the areas of fibrosis within the ganglia and the atrophy of the dorsal horn may be caused by the same pathological process. However on consultation with a neuropathologist the possibility was raised that this area of altered morphology may in fact be due to an infolding of the ganglion capsule itself (Professor M. Buckland, Personal interview, August 2011), and not any type of pathology. Unfortunately given the limited availability of the sample material we were not able to distinguish between these two scenarios.

In order to demonstrate the presence of DNA within the sections DNA was extracted from all available ganglia material. DNA extraction from the PHN1, PHN2 and PHN3a samples were successful, shown by the detection of a PCR product corresponding to the housekeeping gene GAPDH. DNA extraction was also successful for the HZ1 and CON1 samples. Unfortunately there was no GAPDH-specific product detected in the PHN3b, HZ2, HZ3 and CON2 samples, corresponding to no intact DNA extracted from these samples. This is likely due to degradation of cellular DNA due to the heavy fixation process. All samples used in this study were provided as formalin-fixed paraffin-embedded tissue blocks, and I had no choice in the fixation method.

In order to demonstrate the presence of VZV-specific DNA, PCR was performed using primers specific for VZV ORF9a. VZV specific DNA was detected in PHN1a, PHN2, and PHN3a. VZV specific DNA was also detected in the HZ1 sample. No VZV specific DNA was detected in PHN1b or CON1 despite successful DNA extraction. No VZV specific DNA was detected in PHN3b, HZ2, HZ3, or CON2, however DNA extraction was not successful on these samples.

Of the samples which showed successful extraction of DNA from the paraffin-embedded material, PCR using primers specific for the HSV-1 gene UL-42 showed no detectable HSV-1 within any PHN ganglia samples or the CON1 sample. Unfortunately the HSV-1 primers utilised in this study were highly specific to HSV-1, and do not cross-react with HSV-2 (Cassinotti and Siegl, 1998), and consequently we do not know the HSV-2 infection status of the patient samples utilised. However
given that the ganglia samples are not derived from the sacral region, it is less likely they would contain any HSV-2.

The HZ1 ganglia sample did show a detectable presence of HSV-1 DNA. As HSV-1 latency is almost ubiquitous within the human population (Smith and Robinson, 2002) and this sample was a trigeminal ganglia, it was expected to harbour a latent HSV-1 infection. HSV-1 latency has been previously shown to attract immune cells which surround latently infected cells within human ganglia. This process does not occur in response to VZV latency (Theil et al., 2003, Verjans et al., 2007). This ganglia sample displayed no foci for immune cells within any of the neuronal regions examined. The immune infiltration within this sample was limited to the outer regions of the ganglia. Thus it is unlikely that the main body of the infiltrating cells was in response to a latent HSV-1 infection.

Although PCR is useful in providing a qualitative result, and identifying the presence or absence of a specific DNA sequence, due to any potential differences in tissue fixation and processing, and the different lengths of time stored post-fixation it can not provide a reliable estimation on relative amounts of DNA. Consequently a quantitative real time PCR method was utilised to allow the calculation of the number of copies of VZV DNA per cell in each ganglia. Due to the very limited amount of DNA originally obtained from each ganglia sample, and the failure to isolate any viable DNA from some samples, complete analysis using this quantitative real time PCR approach was only possible on three ganglia samples: PHN2a, HZ3 SOR and CON2.

Interestingly the CON2 ganglia samples did not show any amplification of GAPDH, VZV or HSV-1 DNA utilising the conventional PCR approach. Conversely there was a detectable amount of β-actin within this sample via the quantitative real time PCR method. In both cases, similar amounts of input template were utilised, and consequently the real time PCR approach appears to be more sensitive.

It was also of great interest that this quantitative real-time PCR analysis showed that there was approximately seven-fold more copies of VZV DNA present in the PHN2a sample compared to that observed in the HZ3 SOR. Both of these samples feature a predominantly neuronal area, consisting mainly of the cell bodies of DRG neurons. The HZ3 patient experienced a reactivation of VZV within this ganglia just 17 days prior to death, with potential ongoing VZV replication occurring as shown by the presence of a rash at the time of death and the detection of VZV proteins within
several neurons (Steain et al., 2013). This patient was an immunocompetent patient suffering from dementia who died due to aspiration pneumonia. The PHN-affected patient did not show any clinical signs of VZV reactivation at the time of ganglionectomy. This could indicate an ongoing virological process within this PHN-affected patient which is confined to the DRG, giving rise to a greater VZV DNA load per cell than that seen in a herpes zoster-affected patient with a current and relatively extensive reactivation, and possibly contributing to the ongoing pathogenesis of PHN. Alternatively it may indicate that during herpes zoster a greater amount of viral replication occurred in this ganglia, increasing the latent virus burden, and may be a risk factor in the development of PHN.

Given the relatively high level of VZV DNA present in one of the PHN-affected, and one of the herpes zoster-affected samples, it was of interest to look for the expression of VZV-specific proteins from the different viral kinetic classes. Unfortunately all attempts to detect viral protein expression were unsuccessful despite the positive control material showing successful immunofluorescent detection of VZV antigens. In the same staining experiment HZ3 showed detectable VZV antigen expression using the same method, reported in Steain et al (Steain et al., 2013). This could be due to a low level of antigen expression – which seems unlikely due to the high viral load and presence of active replication in two of the herpes zoster-affected samples. Alternatively our current experimental approaches or antibodies may not be sensitive enough to detect viral protein expression in the human ganglia samples.

In fact to date, taking into account the many difficulties in performing immunohistochemistry on human formaldehyde-fixed paraffin-embedded material (Zerboni et al., 2012) there is only one recent reported detection of VZV-specific protein expression in human ganglia, and this was utilising the neuronal regions from the HZ3 SOR as reported in Steain et al. (2013). All attempts to detect VZV-expression in PHN-affected ganglia material performed during this current study were performed in tandem with the experiments reported in this paper. Thus despite VZV antigens being readily detected in the herpes zoster ganglia directed innervating active herpes zoster rash, no antigens were observed in the PHN-affected material.

It was also of interest to study any VZV-specific mRNA expression within the human ganglia samples. Previously during my honours work I was able to demonstrate the presence of viable mRNA specific to HLA-DRα (work not shown) via in-situ hybridisation (ISH) in satellite cells within the neuronal areas of PHN1, PHN2 and
HZ1. Unfortunately all attempts to detect a VZV-specific gene product via ISH were unsuccessful. In addition, RNA extraction from all ganglia samples was attempted. A real time PCR system was established, plasmid standards were created and the sensitivity of each primer pair was measured to ensure sensitivity was sufficient to ensure detection of low level mRNA copy numbers. Unfortunately there was little to no viable mRNA extracted from ganglia samples, and consequently VZV transcript analysis was not possible.

Significantly, the immunohistochemical analysis of PHN-affected ganglia samples revealed a large infiltration of immune cells, predominantly comprised of both CD4+ T cell and CD8+ T cells, as well as a large proportion of B cells and cytolytic cells. This immune cell infiltration appeared to be spread throughout the affected ganglia, in both the neuronal and nerve bundle areas. This immune cell infiltration was more comparable to that observed in the active herpes zoster samples rather than the samples isolated months post-herpes zoster.

There was considerable variability seen between the total number of CD3+ T cells, and the sum of CD4+ and CD8+ T cells. The counts for each stain were performed on random non-overlapping images of individual sections from different parts of the tissue block. For some of the larger tissue samples (the herpes zoster and control samples) the tissue area was considerably larger than the imaged regions. To control for this images areas were selected randomly, and multiple counts obtained and averaged to ensure a representative quantity was obtained overall. The difference in counts is most likely due to biological variability rather than the presence of rarer T cell types (NKT cells and γδ T cells).

The infiltration seen in the PHN-affected material which was obtained many years post occurrence of clinical VZV reactivation was much greater than that seen in herpes zoster-affected patient material just 6 months post-herpes zoster. The immune infiltration in the PHN-affected patient material was comparable to that seen in another immunosuppressed patient who experienced an acute VZV reactivation just 3 weeks prior to death with a rash still present at death. However the level of infiltration in the PHN-affected patient material was less than that seen in material obtained from an immunocompetent patient who experienced VZV reaction 17 days prior to death with rash still present at death.

Interestingly only the PHN-affected patient material (many years post VZV-reactivation) and the herpes zoster-affected material from the immunocompetent
patient (active VZV reactivation) showed the presence of B cells. IgG secreting B cells are known to be recruited to sites of chronic inflammation (Kunkel and Butcher, 2003). The lymphocytes (both B and T cells) in the PHN-affected material provides evidence of an ongoing inflammation in these samples, possibly a chronic inflammatory process.

This study included the first examination of the immune cell phenotype within the nerve bundles in the ganglia from a PHN-affected and herpes zoster-affected patients. The DRG is located in close proximity to multiple bundles of nerves. It is continuous with the dorsal root of the spinal cord, and in close proximity to the ventral root and the spinal nerve (previously identified in Figure 1.7). Unfortunately we cannot identify the exact origin of the nerve bundles that were examined during this study, nor can we ensure that the nerve bundles from the PHN-affected and herpes zoster-affected patients were from the same anatomical location.

Previous detailed immunohistochemical analysis of herpes zoster-affected ganglia samples have shown an immune infiltration consisting predominantly of macrophages, NK cells, CD4+ T cells and CD8+ T cells (Gowrishankar, 2008, Gowrishankar et al., 2010, Steain et al., 2013). Although staining for NK cells using antibodies specific for both CD56 and CD16 was attempted on patient samples in this study, neither was successful in providing clear identification of NK cells within the ganglia due to the expression of these markers by satellite cells, nerve sheaths and many other cell types. A similar problem was experienced when using an antibody specific for CD68 – a marker on macrophages, monocytes and other large cells which is also expressed on some satellite cells and nerve sheaths (van Velzen et al., 2009).

In all ganglia samples examined the immune cell infiltrate consisted mainly of a memory cell phenotype, shown through expression of the CD45RO antigen and lower expression of CD45RA. This predominance was to be expected due to the prior exposure to VZV antigens during primary infection and the subsequent development of memory cells. Only the PHN-affected patient samples also contained a significant proportion of CD45RA+ naive immune cells, and could be a result of some ongoing chronic inflammatory process.

There was also a significant proportion of TIA-1 expression in infiltrating immune cells. TIA-1 is a cytolytic marker present in T cells, as well as NK cells. TIA-1 is an mRNA binding protein which possesses nucleolytic activity against target cells when released from cytotoxic cells, resulting in apoptosis of the target cell (NCBI, 2013).
TIA-1 is expressed in cytotoxic cells irrespective of activation states. Staining for another cytolytic marker granzyme B was unsuccessful on this tissue. Granzyme B is a cytolytic marker that is upregulated on activated cytolytic cells, and would have both confirmed the cytolytic and activation state of the infiltrating cells. Cytotoxic CD4+ T cells have been described (Appay et al., 2002), and CD4+ T cells with cytolytic function have been shown to play an important role in the immune response to herpesvirus infections (Reviewed in Heller et al., 2006).

Steain et al. (2013) identified T cells in close proximity to neurons in post-mortem herpes zoster-affected ganglia material. These T cells appeared to have penetrated the satellite cell barrier surrounding the neurons. Despite CD4+ T cells being the predominant T cell subset detected in the herpes zoster reactivated ganglia samples, CD8+ T cells were far more likely to be interacting with the neuron-satellite cell barrier (Steain et al., 2013). These findings suggest that direct contact between neurons and T cells may occur in human ganglia during active herpes zoster. Although a significant amount of time was utilised examining the PHN-affected ganglia material, there were no observed instances where T cell or other immune cells appeared to have breached the satellite cell barrier and were in close proximity to neurons.

The infiltrating immune cells present within the PHN-affected ganglia material were distributed throughout the ganglia, with small foci of infiltration within both neuronal and nerve bundle regions. Previous work has shown that during active VZV reactivation infiltrating immune cells, predominantly CD8+ cytolytic T cells, are spread throughout the ganglia (Steain et al., 2013), and many months post herpes zoster infiltrating immune cells, predominantly non-cytolytic CD8+ T cells are spread throughout neuronal regions of the ganglia samples (Gowrishankar et al., 2010).

Sensory neurons do not normally express MHC molecules, however they can be expressed in response to viral infection (Redwine et al., 2001). Previous studies have shown an upregulation of both MHC I (via β2m expression) and MHC II (via HLA-DR expression) on satellite cells in human ganglia following VZV reactivation (Gowrishankar et al., 2010, Steain et al., 2013). In this current study HLA-DR expression was seen in areas throughout all patient samples, however expression appeared to be restricted to satellite cells. Satellite cells have been shown to have phagocytic capacity (van Velzen et al., 2009), and may play the role of professional antigen presenting cells within the ganglia (Verjans et al., 2007). HLA-DR expression varies significantly between patients, and comparison between patient samples is not
possible. β2m staining was attempted, but was unsuccessful on the PHN-affected patient material.

The PHN-affected patient material appears to be a site of chronic inflammation. Clinically the patient has been on an immunosuppressive treatment regime for many years. Despite this, there is a significant immune infiltrate into the dorsal root ganglia many years following the resolution of clinical VZV reactivation. This study has shown significant numbers of T cells and B cells, spread throughout both the neuronal and nerve bundle regions of the ganglia tissue. Due to the prolonged period of time from the resolution of the rash and the surgical extraction and subsequent fixation of tissue, for inflammatory cells to remain in the tissue suggests some ongoing chronic process that may contribute to the pathogenesis of PHN.

The immune infiltrate level seen in the PHN-affected material (from an immunosuppressed patient) was comparable to that seen in an immunocompetent herpes zoster-affected patient within 17 days of VZV reactivation who died with an extensive herpes zoster-associated rash at the time of death. The PHN-affected ganglia material also contained approximately 7 fold more VZV DNA than that seen in this currently herpes zoster-affected patient material. This provides some evidence that there may be some ongoing virological process present in the PHN-affected ganglia material that maybe important in maintaining a continued presence of infiltrating immune cells.

There are many other immune cell types that remain be investigated. Neutrophils, NK cells, mast cells, macrophages and other monocytes all play important roles in inflammation, and their role in PHN-related infiltration has yet to be elucidated. The activation state of the immune cells has yet to be demonstrated. Aberrant chemokine expression has also been shown to play some role in chronic pain states (Reviewed in White et al., 2007), but has yet to be examined during PHN. Unfortunately such studies are hampered by the limited availability of appropriate clinical samples, and could not be performed as part of this work.
Chapter 4 – Phenotype of immune cells in skin biopsy samples from post-herpetic neuralgia-affected and normal patients.

4.1 Introduction

VZV causes two clinically distinct diseases which both involve an exanthematous stage – the generalised rash seen in varicella and the more dermatomal restricted rash seen in herpes zoster. In both of these conditions the lesions are histopathologically indistinguishable from each other (Oxman, 2000). The most common complication of herpes zoster is PHN - a severe neuropathic pain which can persist for months to years after the resolution of the herpes zoster rash (Cohen et al., 1999, Steiner et al., 2007, Hope-Simpson, 1965, Hope-Simpson, 1975). To date there is limited information available about the host immune response in human skin during PHN, and whether there is any ongoing viral replication within the affected area.

This chapter aims to address this lack of knowledge through an immunofluorescent examination of rare human skin biopsies from both the active and contralateral sides of PHN-affected patients, as well as left and right sides from normal healthy control patients.

Previous studies have shown that there is a dramatic shift in the numbers of important immune cells within the skin during herpes zoster. In particular a reduction in tissue resident Langerhans cells within the epidermis, and an increase in plasmacytoid dendritic cells (pDCs), highly inflammatory DCs (Colonna et al., 2004) have been observed within herpes zoster-affected skin (Gutzeit et al., 2010, Huch et al., 2010).

Another study found significantly lower numbers of infiltrating lymphocytes present in the skin biopsies of patients who later develop PHN compared to those that experience no complications, despite the systemic cytokine and antibody responses being at comparable levels (Zak-Prelich et al., 2003). However this study was performed with single biopsies from each individual, which does not take into account the variation between individuals. It also did not explore the immune response of patients during PHN itself, but focused on herpes zoster.
As PHN is regarded as a neuropathic pain (Steiner et al., 2007), several studies have focused on the level of epidermal innervation. Early studies indicated there was extensive damage to both the peripheral and central nervous system in PHN-affected patients (Watson et al., 1991, Watson et al., 1988b), and clinical findings indicate altered nervous system function, showing both areas of sensory loss and sensory gain (Watson, 1998).

More detailed comparisons of skin biopsies obtained from both PHN and non-PHN-affected patients have shown that PHN-affected individuals feature a large-scale loss of epidermal neurites at the site of pain (Oaklander et al., 1998, Rowbotham et al., 1996). PHN patients show decreased innervation at both the affected and contralateral sites (Petersen et al., 2002, Oaklander et al., 1998). This is in contrast to patients who experience uncomplicated herpes zoster, who feature a decrease in the level of cutaneous innervation only at the site of the herpes zoster rash. The exact mechanism for this is unknown. However as VZV-infected neuronal cells are resistant to apoptosis (Hood et al., 2003), this difference in levels of innervation is unlikely to be due to direct virus killing of neuronal cells, but is more likely to be either through some effect of the host response or an indirect effect of the virus.

The underlying pathogenesis of PHN is still unknown. In some patients it has been shown that there may be ongoing viral replication during PHN. Viral proteins have been detected within circulating blood mononuclear cells in PHN-affected patients for months to years following the resolution of the herpes zoster rash (Gilden et al., 2003, Vafai et al., 1988). This prolonged period during which viral antigen expression can be detected does not occur in patients who experience an uncomplicated recovery from herpes zoster (Gilden et al., 1989). To date, there has not been an investigation of VZV antigen expression in skin samples from PHN-affected patients. Nor has a detailed immunofluorescent examination of the subsets of DCs and T cells present in PHN-affected skin been performed. The overall aim of this chapter is to address these important issues utilising valuable skin biopsies obtained from PHN-affected and control patients.

This research is unique in that as well as being able to compare skin biopsy samples taken from both PHN-affected and non-PHN-affected patients, skin biopsy samples taken from the contralateral side of each patient were also available. This was possible through a collaboration with Albany Medical College and the UCSF. Using both a biopsy from the affected and the contralateral side for each patient (or left and
right for control patients) we can account for any variation in natural immunity that may occur between individuals.

### 4.2 Patient and control material

Where applicable; all tissue specimens were obtained in accordance with ethics guidelines of both the University of Sydney and Western Sydney Area Health Service, and informed consent of the donors was obtained where required.

#### 4.2.1 Control cell block material

Initial protocol and antibody optimisations were performed on control cell blocks. Control cell blocks were generated using both uniHFFs and infHFFs, and CD14\(^-\) sorted human PBMCs. HFFs were cultured, infected with VZV and harvested as described in section 2.5. CD14\(^-\) human PBMCs were isolated as described in section 2.5, and were generously donated by Rodney Henriquez (VZV research laboratory, University of Sydney).

#### 4.2.2 Control patient skin material

Once antibody optimisation was successful on sections obtained from control cell blocks, antibodies were then trialled on human tissue material to ensure that the antibodies and staining technique were well established. Control human material consisted of cryopreserved human tonsil and skin tissue, kindly provided by Dr Heather Donaghy from the Westmead Millennium Institute in accordance with relevant ethics guidelines.

#### 4.2.3 Patient skin sample material

Patient skin biopsies were very generously provided by Professor Frank Rice of the Albany Medical College and Professor Karen Petersen of UCSF. Skin biopsy material included in the study was formaldehyde-fixed and cryopreserved (Petersen and Rowbotham, 2007, Petersen et al., 2002). Frozen material had been cryosectioned into 14 µm sections and mounted on gelatin-coated microscope slides. Multiple sections taken from different regions of each patient skin biopsy had been mounted on each slide. Where possible each slide contained a section from the beginning, middle and end of the tissue biopsy, as represented in Figure 4.1. This allowed multiple replicates to be represented on the one microscope slide.
Figure 4.1 - Processing of human skin punch biopsy material into slide mounted tissue

Biopsy samples were obtained from post-herpetic neuralgia affected and control patients through the use of a 3 mm biopsy punch (A), which provided a representative area of tissue from the relevant region (B). This biopsy sample was formaldehyde-fixed and cryopreserved, and then sectioned longitudinally, with multiple sections from different regions of the tissue sample mounted on the sample microscope slide (C).
In this initial pilot study skin biopsy material obtained from 5 PHN-affected patients and 5 normal control patients was utilised. Patient details including sample number, diagnosis, age, sex and biopsy location are shown in Table 4.1. This information was provided by our collaborators Professor Frank Rice (Albany Medical College) and Professor Karen Petersen (UCSF). The PHN-affected patients consisted of three females and two males, with an age range of 26 to 71 years old, and an average of 53.0 years. The normal control patients also consisted of three females and two males, with a similar age range of 22 to 68 years old, and an average of 52.4 years.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>Biopsy Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHN 1</td>
<td>PHN</td>
<td>66</td>
<td>F</td>
<td>T8 Dermatome</td>
</tr>
<tr>
<td>PHN 2</td>
<td>PHN</td>
<td>48</td>
<td>M</td>
<td>T6 Dermatome</td>
</tr>
<tr>
<td>PHN 3</td>
<td>PHN</td>
<td>54</td>
<td>F</td>
<td>L1 Dermatome</td>
</tr>
<tr>
<td>PHN 4</td>
<td>PHN</td>
<td>71</td>
<td>F</td>
<td>C5 Dermatome</td>
</tr>
<tr>
<td>PHN 5</td>
<td>PHN</td>
<td>26</td>
<td>M</td>
<td>Shoulder</td>
</tr>
<tr>
<td>Control 1</td>
<td>Normal control</td>
<td>61</td>
<td>F</td>
<td>T8 Dermatome</td>
</tr>
<tr>
<td>Control 2</td>
<td>Normal control</td>
<td>56</td>
<td>F</td>
<td>L1 Dermatome</td>
</tr>
<tr>
<td>Control 3</td>
<td>Normal control</td>
<td>55</td>
<td>M</td>
<td>T8 Dermatome</td>
</tr>
<tr>
<td>Control 4</td>
<td>Normal control</td>
<td>68</td>
<td>F</td>
<td>Deltoid</td>
</tr>
<tr>
<td>Control 5</td>
<td>Normal control</td>
<td>22</td>
<td>M</td>
<td>C4 Dermatome</td>
</tr>
</tbody>
</table>

Punch biopsy samples (3 mm) were taken from both the active (most painful area) and contralateral side (contra) for PHN patients or left and right sides for normal control patients. In total 3 slides containing 2-3 sections each from each biopsy sample were provided for use in this study.

4.3 Results

4.3.1 Immunofluorescent assay (IFA) development

Given the inherent difficulty in detecting antigen expression in formaldehyde-fixed and snap frozen biopsy material, particularly by IFA, an important initial step was to develop new IFA staining protocols. In addition, suitable positive control material matching the fixation and preparation of the test samples needed to be generated.

The patient sample material utilised in this chapter consisted of formaldehyde-fixed and snap frozen material – a type not previously used in our laboratory. Taking into
account the frozen nature of the samples, the previously established high temperature unmasking technique utilised in our laboratory with formaldehyde-fixed paraffin-embedded samples was unsuitable, and a new IFA protocol was required. To complicate this our laboratory had no available positive control material which adequately matched both the fixation and preservation methods of the test skin biopsy sample material with which to create and optimise the experimental procedure.

4.3.1.1 CONTROL MATERIAL CREATION

To overcome this initial hurdle a method of fixing and snap-freezing cell pellets was created. Control cell blocks were created using both uniHFFs and infHFFs, and CD14⁺ sorted PBMCs. Cells were fixed in 10% NBF for 4 hours at 4°C to match the fixation method used with the sample material (Petersen et al., 2002, Petersen and Rowbotham, 2007). Cells were centrifuged and rinsed in 1x PBS and resuspended in coloured cryochrome freezing compound. A freezing mould was then filled with coloured cryochrome freezing compound of a contrasting colour, and the cell suspension transferred to the centre of the freezing mould. The mould was immersed in liquid nitrogen to snap freeze the block of cryochrome freezing compound containing the cell suspension. The contrasting colours used allowed the differentiation between the supporting cryochrome compound and the suspended cells within the block, which enabled the cells to be easily cryosectioned and mounted on gelatin-coated slides.

Due to the very limited quantity of formaldehyde-fixed and snap-frozen skin biopsy sample material, it unsuitable for initial use in determining antibody suitability and optimising antibody concentrations. Instead, unfixed cryopreserved human tissue was obtained in order to trial antibodies on control human tissue after initial optimisation on cell blocks. Unfortunately we did not have access to any known VZV skin material, and consequently human tonsil tissue and normal skin tissue were used to trial all antibodies in conjunction with the infHFFs. This material was cryosectioned and mounted on gelatin-coated microscope slides. While different post-sectioning fixation methods were trialled, including methanol/acetone fixation and formaldehyde fixation, in order to more closely match the fixation of both the control cell blocks (10% NBF) to that of the precious patient derived sample material (4% formaldehyde), this control material was post-fixed immediately prior to use in 10% NBF for 30 min before continuing with the normal staining protocol.
4.3.1.2 *IFA Staining method development*

Initial protocol optimisation focused on different antigen unmasking techniques. As the patient biopsy material was formaldehyde-fixed, it is first necessary to perform an antigen retrieval step (or unmasking step) for successful detection of many antigens. Proteinase K digestion was found to be unsuitable due to its tendency to destroy cell surface markers (especially CD markers). This occurred at all proteinase K concentrations tested (from 10 to 25 µg/mL). Furthermore, the high temperature antigen unmasking technique commonly utilised in our laboratory for paraffin-embedded sections was found to be unsuitable as it melted the gelatin coating from the slide and destroyed the section at 95°C (data not shown). Instead of unmasking at high temperature, a range of lower temperatures were trialled (from 30°C to 80°C) for different lengths of time (from 20 min to 5 hours). Unmasking solutions of both citrate unmasking buffer and EDTA unmasking solution were tested. The optimal unmasking conditions were found to be EDTA unmasking solution at 55°C for 3 hours, with 30 min at room temperature to allow the slides to cool down. This was found to provide adequate unmasking of epitopes within the tissue with minimal damage to the tissue section.

Various blocking solutions were also trialled in the IFA staining method development. General blocking solution was 1x PBS containing between 1-20% serum (either NDS or BSA) with or without Tween 20 or Triton-X (0-0.5%). Blocking solution was applied for between 30 min to 3 hours. Optimal blocking conditions, which minimised both background staining levels and damage to the tissue material, were found to be 1% BSA and 0.2% Triton-X in 1x PBS for 2 hours. This solution was also used as an antibody dilution buffer.

Various wash solutions were also trialled, including both 1x TBS or 1x PBS alone, or wash solutions containing either Tween 20 or Triton-X (0-0.5%) with or without agitation. Optimal wash conditions, which minimised damage to the tissue sections were found with three changes of 1x PBS for 20 min each without agitation.

The final conditions employed were antigen unmasking at 55°C for 3 hours followed by a 30 min incubation at room temperature, blocking and antibody dilution buffer of 1% BSA and 0.2% Triton-X in 1x PBS for 2 hours, and optimal washing conditions of 3 changes of 1x PBS for 20 min each. This maintained suitable histology and enabled adequate detection of antigens within the tissue section.
4.3.2 Antibody optimisation

All antibodies were optimised for use with formaldehyde-fixed and cryopreserved tissue and IFA staining protocol. All successful primary antibodies which showed a robust level of specific staining and minimal background fluorescence and matching isotype controls are listed in Table 4.2.

Using the protocol outlined above, antibodies against specific cell surface markers were initially tested in single immunofluorescent stains on suitable control material (human skin, human tonsil tissue and CD14+ immune cells from peripheral human blood) and where appropriate on UCSF skin biopsy sample material. In parallel all sections were incubated with matched isotype control antibodies. Example images of IFA staining using antibodies specific for the pan-T cell marker CD3, the T helper cell marker CD4, the cytotoxic T cell marker CD8, the NK cell and other monocyte marker CD16, the B cell marker CD20 and the macrophage, monocyte and other large cell marker (cells of macrophage lineage) CD68 are shown in Figures 4.2 and 4.3. Specific staining with the antibodies against the cell surface markers CD3, CD4, CD8, CD16 and CD20 all showed the expected membrane-associated patterns. Staining with the antibody specific for CD68 showed the expected cytoplasmic staining pattern. No staining was observed in the sections incubated with isotype control antibodies (representative image shown in Figure 4.3E).

Example images of antibodies specific for the dermal dendritic cell marker DC-SIGN, the pDC marker DLEC (also called BDCA-2), and the Langerhans cell marker langerin are shown in Figure 4.4. The specific staining patterns observed above the isotype control stained sections showed the expected cellular distribution: DC-SIGN is cell-surface associated, langerin is a cytoplasmic marker and DLEC is a cell surface marker.

Example images of IFA staining of infHFFs with antibodies against VZV IE63, and the glycoprotein complex formed between gE and gI (gE:gI) are shown in Figure 4.5. Although these viral antigens normally display a cell membrane associated pattern in formaldehyde-fixed and paraffin-embedded material, in the formaldehyde-fixed and cryopreserved tissue they showed staining throughout infected cells. No specific staining was observed on unihHFFs incubated with VZV specific antibodies or sections incubated with isotype control antibodies.
Figure 4.2 - Optimisation of antibodies specific for different immune cells
Images of optimisation of antibodies for immunofluorescent staining on formaldehyde-fixed cryopreserved tissue. Staining in green is the specific antibody, while DAPI staining cell nuclei is shown in blue. Staining was performed on both CD14- immune cells isolated from peripheral blood (a) and on control human tissue (b) - either human skin (1-3) or human tonsil tissue (4-6). Antibodies used were anti-CD3 staining T cells (1), anti-CD4 staining T helper cells (2), anti-CD8 staining cytotoxic T cells (3), anti-CD16 staining NK cells (4), anti-CD20 staining B cells (5) and anti-CD68 staining macrophages (6). A representative isotype control image is shown in Figure 4.3.
Figure 4.3 - Optimisation of antibodies specific for different immune markers on human skin material

Images of trial immunofluorescent staining on patient skin sample material. Material consists of 3 mm bunch biopsy samples that have been formaldehyde-fixed and cryopreserved. Staining in green (on merged images) or greyscale (on cropped and single colour inset) is the specific antibody and DAPI labelling cell nuclei is shown in blue. Antibodies used were anti-CD3 staining T cells (A), anti-CD4 staining T helper cells (B), anti-CD8 staining T cytotoxic cells (C), and anti-CD68 staining macrophages (D). A representative isotype control image is also shown (E), and the epidermal and dermal regions of the biopsy sample have been labelled.
Figure 4.4 - Optimisation of antibodies specific for different dendritic cell markers

Images of trial immunofluorescent staining on skin biopsy material provided for optimisation. Material consists of 3 mm bunch biopsy samples (panels A-F) or human tonsil material (G-I) that had been formaldehyde-fixed and cryopreserved. Staining in green is the specific antibody and DAPI labelling cell nuclei is shown in blue. The first column shows merged images of antibody-specific staining (A, D, G), the second column shows single channel images of antibody-specific staining (B, E, H), while the third column shows merged images of isotype control staining (C, F, I). Antibodies used were anti-DC-SIGN staining dermal dendritic cells (A, B, C), anti-langerin staining Langerhan cells (D, E, F), and anti-DLEC staining plasmacytoid dendritic cells (G, H, I).
Figure 4.5 - Optimisation of antibodies specific for different VZV antigens
Images of trial immunofluorescent staining for VZV antigen on formaldehyde-fixed and cyopreserved cell pellets. Pellets consist of either VZV-infected (A, B, C) or uninfected human foreskin fibroblasts (D). Antigen-specific staining is shown in green, and counterstained cell nuclei with DAPI is in blue. Antibodies used were anti-VZV IE63 (A) and anti-VZV gEgl (B). A representative isotype control (C) and negative control of VZV-specific antibody applied to uninfected HFFs (D) is also shown.
Table 4.2 - Primary and isotype control antibodies optimised for IFA on formaldehyde-fixed cryopreserved skin biopsy material. Isotype control antibody dilutions were calculated to match the working concentration of the primary antibody solution.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Optimal dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-CD3</td>
<td>Leica Microsystems, Germany</td>
<td>1 in 10</td>
</tr>
<tr>
<td>Goat anti-CD4</td>
<td>R&amp;D systems, USA</td>
<td>1 in 10</td>
</tr>
<tr>
<td>Rabbit anti-CD8</td>
<td>Abcam, USA</td>
<td>1 in 100</td>
</tr>
<tr>
<td>Mouse anti-CD16</td>
<td>Leica Microsystems, Germany</td>
<td>1 in 40</td>
</tr>
<tr>
<td>Mouse anti-CD20</td>
<td>Leica Microsystems, Germany</td>
<td>1 in 10</td>
</tr>
<tr>
<td>Mouse anti-CD68</td>
<td>Leica Microsystems, Germany</td>
<td>1 in 10</td>
</tr>
<tr>
<td>Rabbit anti-DC-SIGN</td>
<td>Abcam, USA</td>
<td>1 in 500</td>
</tr>
<tr>
<td>Goat anti-DLEC (BDCA-2)</td>
<td>R&amp;D systems, USA</td>
<td>1 in 50</td>
</tr>
<tr>
<td>Mouse anti-Langerin</td>
<td>Leica Microsystems, Germany</td>
<td>1 in 200</td>
</tr>
<tr>
<td>Rabbit anti-VZV IE63</td>
<td>Prof R Mahalingam, University of Colorado Medical school</td>
<td>1 in 1000</td>
</tr>
<tr>
<td>Mouse anti-VZV gE:gI complex</td>
<td>Meridian Life Science, USA</td>
<td>1 in 250</td>
</tr>
<tr>
<td>Mouse IgG 2a</td>
<td>Invitrogen, USA</td>
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<tr>
<td>Mouse IgG 2b</td>
<td>Invitrogen, USA</td>
<td>(As required)</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>R&amp;D systems, USA</td>
<td>(As required)</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>R&amp;D systems, USA</td>
<td>(As required)</td>
</tr>
</tbody>
</table>

The patient material provided by collaborators was very limited in number, allowing for 2 repeats of 3 antibody combinations only. Consequently, to maximise the information obtained and gain information on the presence and localisation of more than one specific cell type in the skin biopsy sample, IFA with two or three specific antibodies was developed. Dual and triple IFAs allowing the co-detection of different antigens within the same sample, and provided valuable information about the relative positions of various immune cells and viral antigen expression. Thus antibody combinations were trialled on appropriate skin biopsy sample material from UCSF. The successful primary and secondary antibody combinations are described in Table 4.3.

Sample IFA images of the various antibody combinations used in immunofluorescent staining on control material are shown in Figures 4.6, 4.7 and 4.8. The first antibody combination (Figure 4.6) consisted of anti-CD4, anti-CD8 and anti-VZV gE:gI specific antibodies. Antibodies against CD4 and CD8 detect T helper and T cytotoxic lymphocytes respectively, while an anti-VZV gE:gI antibody detects the viral glycoprotein E-glycoprotein I complex in VZV-infected cells. All antibody staining patterns were similar to those observed in single stains.
Figure 4.6 - Immunofluorescent detection of CD4+ and CD8+ T cells and VZV antigen expression in human skin biopsy samples

Immunofluorescent staining for immune cells and VZV antigen expression. Antigen-specific staining in merged images is shown in green, red and purple and cell nuclei counterstained with DAPI is shown in blue. Antibodies used are anti-CD4 detecting T helper cells (green), anti-VZV gEgL detecting VZV-infected cells (red), and anti-CD8 detecting cytotoxic T cells (purple). Successful staining on UCSF sample material is shown as a merged colour image (A) along with the boxed section under a higher magnification (inset), along with the single monochrome images from each channel showing anti-CD4 (B), anti-VZV gEgL (C) and anti-CD8 (D). Controls shown include VZV-infected human foreskin fibroblasts (E), CD14+ immune cells isolation from peripheral human blood (F) and uninfected human foreskin fibroblasts (G).

A yellow arrow indicates CD4+ T cell detection and an orange arrow indicates CD8+ T cell detection. VZV-infected cells are not present in normal human skin, but were easily detected in the VZV infected HFF cell pellet (green arrows).
Figure 4.7 - Immunofluorescent detection of CD8⁺ T cells, Langerhan cells and plasmacytoid dendritic cells in human skin biopsy samples

Triple immunofluorescent of various immune cell markers. Antigen-specific staining is shown in green, red and purple and cell nuclei counterstained with DAPI is shown in blue. Antibodies used are anti-BDCA2 detecting plasmacytoid dendritic cells (green), anti-langerin detecting Langerhan cells (red), and anti-CD8 detecting T cytotoxic cells (purple). Successful staining on UCSF sample material is shown as a merged colour image and insets (A), along with the single monochrome images from each channel showing anti-langerin (B), anti-CD8 (C) and anti-BDCA2 (D). Controls shown include normal human skin (E), CD14⁺ immune cells isolated from peripheral human blood (F) and uninfected human foreskin fibroblasts (G).

An orange arrow indicates example CD8⁺ T cell detection and a green arrow indicates Langerhan cell detection. pDCs are not present in normal human skin, but were easily detected in the CD14⁺ immune cell pellet (white arrows).
**Figure 4.8 - Immunofluorescent detection of macrophages and VZV antigen expression in human skin biopsy samples**

Dual IFA detection of VZV antigen and immune cell marker CD68. Antigen-specific staining is shown in green and red, and cell nuclei counterstained with DAPI is shown in blue. Antibodies used are anti-VZV IE63 detecting VZV-infected cells (green) and anti-CD68 detecting macrophages (red). Successful staining on UCSF sample material is shown as a merged colour image (A), along with the single channel images from each channel showing DAPI (B), anti-IE63 (C) and anti-CD68 (D). Controls shown include infected human foreskin fibroblasts (E), CD14+ blood immune cells (F) and uninfected human foreskin fibroblasts (G).

A white arrow indicates example macrophage detection in normal human skin and the CD14+ immune cell pellet. VZV-infected cells are not present in normal human skin, but were easily detected in the VZV infected HFF cell pellet (orange arrows).
Table 4.3 - Combinations of primary antibodies, matching isotype controls and secondary antibodies utilised in IFAs on formaldehyde-fixed cryopreserved tissues

<table>
<thead>
<tr>
<th>Combination 1</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary antibodies</td>
<td>Anti-CD4 Goat IgG</td>
<td>Anti-VZV gE:gI Mouse IgG2a</td>
<td>Anti-CD8 Rabbit IgG</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 488 donkey anti-goat</td>
<td>Alexa Fluor 546 donkey anti-mouse</td>
<td>Alexa Fluor 647 donkey anti-rabbit</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Combination 2</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary antibodies</td>
<td>Anti-BDCA-2 Goat IgG</td>
<td>Anti-Langerin Mouse IgG2b</td>
<td>Anti-CD8 Rabbit IgG</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 488 donkey anti-goat</td>
<td>Alexa Fluor 546 donkey anti-mouse</td>
<td>Alexa Fluor 647 donkey anti-rabbit</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Combination 3</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secondary antibodies</td>
<td>Anti-CD68 Mouse IgG2a</td>
<td>Anti-VZV IE63 Matched NRS</td>
<td>(nil)</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 594 donkey anti-mouse</td>
<td>Alexa Fluor 488 donkey anti-rabbit</td>
<td></td>
</tr>
</tbody>
</table>

The second antibody combination (Figure 4.7) consisted of anti-BDCA-2, anti-CD8 and anti-langerin specific antibodies. The marker BDCA-2 is a marker of pDCs, langerin is a marker of tissue-resident dendritic cells Langerhans cells, and CD8 is a marker of CTLs. All antibody staining patterns were similar to those observed in single stains.

The third and final antibody combination (Figure 4.8) consisted of anti-CD68 and anti-VZV IE63 specific antibodies. CD68 is a marker of macrophages, monocytes and other large cells (of macrophage lineage), while anti-VZV IE63 detects the IE63 protein in VZV-infected cells. All antibody staining patterns were similar to those observed in single stains. No third antibody was used in this staining combination as there was none deemed suitable.

The above dual and triple IFA antibody staining combinations allowed the detailed analysis of the immune cell profile in formaldehyde-fixed and cryopreserved tissue.
4.3.3 Immunofluorescent analysis of cells present in human skin biopsies from PHN-affected and normal patients

To establish the immune cell profile in human skin biopsy samples from the active and contralateral sides of five PHN-affected patients, and the left and right sides of five control patients, IFAs were performed utilising our newly developed and optimised antibody staining combinations as described above.

The first antibody combination applied to human skin biopsy sections stained for CD4+ and CD8+ T cells in conjunction with VZV-infected cells by utilising the antibody specific for the viral proteins gE:gI. Representative images from two patients are shown in Figure 4.9. CD4+ and CD8+ T cells were detected in both PHN-affected and control tissue, as well as in control patients. Both subsets of T cells were detected throughout the dermis, generally within close proximity of the epidermis. Generally CD4+ T cells were detected at a greater frequency than CD8+ T cells in all skin biopsy samples. Positive staining for the VZV late gene marker gE:gI was not observed in either the PHN-affected patients or the control patients, despite the successful staining in the positive control infHFFs and low background staining levels on the isotype and negative controls.

The second antibody combination stained for pDCs, CD8+ T cells and Langerhans cells. Representative images from two patients are shown in Figure 4.10. pDCs were detected at a very low frequency in two patient samples only, and did not correlate with the presence of PHN. CD8+ T cells were detected in all samples, and were included to obtain a relative position in regards to the DC subsets identified in this combination. Langerhans cells were detected at varying frequencies in all patients, and the location and frequency were analysed (data shown below).

The third and final antibody combination stained for macrophages and VZV-infected cells by utilising an anti-VZV immediate-early protein IE63. The matched isotype staining for CD68 featured quite high background staining levels on the skin biopsy material, and no positive staining was observed above this. No IE63 staining was observed in any patient sample (data not shown). In these staining experiments, the positive controls showed clear positive staining, the isotype controls and negative controls showed low non-specific and background staining levels.

In each of the above stains matched isotype control antibodies were applied to skin biopsy material. Due to the very limited amount of patient skin samples provided,
Figure 4.9 - Immunofluorescent detection of CD4⁺ and CD8⁺ T cells and VZV antigen expression on sample human skin biopsy material

Representative images from immunofluorescent analysis of skin biopsy material. Sample material consists of 3 mm punch biopsy obtained from both PHN-affected and control patients. Material has been formaldehyde-fixed and cryopreserved. Antigen-specific staining is shown in green, red and purple, while DAPI is shown in blue. Samples shown are from patient PHN 2 - both the active (A) and contralateral sides (B), and from patient control 1 - both the left (C) and right sides (D). Antibodies used are anti-CD4 staining T helper cells (green), anti-CD8 staining T cytotoxic cells (purple), and anti-VZV gE/gI staining VZV-infected cells (red). Controls included CD14⁺ immune cells isolated from peripheral human blood (E) and VZV-infected human foreskin fibroblasts (F), as well as isotype control (G) and uninfected HFFs (H).

Orange arrows indicate example CD4⁺ T cell detection and white arrows indicate CD8⁺ T cell detection. VZV infected cells were not detected in human skin biopsy material, however were detected in VZV infected HFF cell pellets (green arrows).
Figure 4.10 - Immunofluorescent detection of CD8⁺ T cells, Langerhan cells and plasmacytoid dendritic cells in sample human skin biopsy material

Representative images from immunofluorescent analysis of skin biopsy material. Sample material consists of 3 mm punch biopsy obtained from both PHN-affected and control patients. Material has been formaldehyde-fixed and cryopreserved. Antigen-specific staining is shown in green, red and yellow, while DAPI is shown in blue. Samples shown are from patient PHN 2 – both the active (A) and contralateral sides (B), and from patient control 1 – both the left (C) and right sides (D). Antibodies used are anti-BDCA-2 staining plasmacytoid dendritic cells (green), anti-CD8 staining cytotoxic T cells (yellow), and anti-langerin staining Langerhan cells (red). Controls included control human skin (E) and CD14⁺ immune cells isolated from peripheral human blood (F), as well as isotype control (G) and uninfected HFFs (H).

White arrows indicate example CD8⁺ T cell detection and green arrows indicate Langerhan cells. Red arrows indicate example pDC detection.
slides from control 5 were used, and consequently experimental data from this patient is limited.

4.3.3.1 **Analysis of cell marker frequency and distribution in human skin biopsy samples from PHN-affected and control patients**

While IFA is useful to demonstrate the presence or absence of a specific expressed marker on cells, a quantitative analysis is required to better compare the frequency of a cell populations within the tissue. This requires enumeration of the number of positive IFA stained cells, and their relative position within the section. To allow proper analysis of the data obtained from the immunofluorescent assays performed on human biopsy samples from PHN-affected and control patients, two different quantitation methods were used.

**4.3.3.1.1 Analysis of the presence and distribution of immune cell subsets in human skin biopsy samples**

To analyse the presence of a rare cell marker, and the distribution of these cells within the section a semi-quantitative approach was utilised. It involved the examination of antibody staining for specific cell surface markers within a representative microscope images from each section, and assigning a score to each based on the presence or location of cells within the sample. Three independent sections were analysed to examine the location of Langerhans cells within the tissue, and the presence of pDCs. As biopsy samples were sectioned to provide multiple replicates of each biopsy on a single slide, this analysis was performed on up to three sections per biopsy sample for each IFA staining combination.

To analyse the location of Langerhans cells within the tissue samples, langerin staining was assessed according to presence of langerin$^+$ cells in the dermis, and their proximity to CD8$^+$ T cells. Results for langerin$^+$ cell scoring is shown in Table 4.4. Scoring was performed as follows: “norm” indicates tissue with langerin$^+$ cells present in the epidermis, “+” indicates langerin$^+$ cells within the dermis, while “+ TC” indicates langerin$^+$ cells present in the dermis in close proximity to CD8$^+$ T cells. This is illustrated in Figure 4.11.

Migration of Langerhans cells from the epidermis (as shown by langerin$^+$ cells present in the dermis) was seen more often in skin taken from PHN-affected patients (Langerhans cell migration present in 4/5 PHN-affected patients compared to 2/4 control patients). However migration was not restricted to the active side in PHN-
**Figure 4.11 - Analysis of Langerhan cell location in human skin biopsy material**

Representative images of immunofluorescent analysis of skin biopsy material showing scoring method for Langerhan cells. The epidermis and dermis layers are labelled. Antibodies used are anti-CD8 staining cytotoxic T cells (yellow), and anti-langerin staining Langerhan cells (red). Images were scored on the location of Langerhan cells: in the normal epidermal-restricted location (A), within the dermal region (B) and within the dermal region in close proximity to CD8 cytotoxic T cells (C).
affected patients (Langerhans cell migration in 3/5 PHN patients on active side, and 3/5 on contralateral side).

**Table 4.4 - Scoring of Langerhans cell presence in the dermis of patient skin biopsy samples.** Scoring is as follows: “norm” indicates normal epidermal distribution of langerin” cells, “+” indicates langerin” cells within the dermis, while “+ TC” indicates langerin” cells present in the dermis in close proximity to CD8⁺ T cells. The fraction in brackets is the number of sections in which the staining was observed followed by the total number of sections examined for each patient. Slides from patient Control 5 were used for isotype control (ND = staining not done).

<table>
<thead>
<tr>
<th>PHN-affected patients</th>
<th>Active</th>
<th>Contralateral</th>
<th>Control Patients</th>
<th>Left</th>
<th>Right</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHN 1</td>
<td>norm</td>
<td>+ (1/2)</td>
<td>Control 1</td>
<td>norm</td>
<td>+ TC (1/3)</td>
</tr>
<tr>
<td></td>
<td>+ TC (2/3)</td>
<td>+ (1/3)</td>
<td>Control 2</td>
<td>norm</td>
<td>+ TC (1/3)</td>
</tr>
<tr>
<td>PHN 2</td>
<td>(1/3)</td>
<td>norm</td>
<td>Control 3</td>
<td>norm</td>
<td>norm</td>
</tr>
<tr>
<td>PHN 3</td>
<td>norm</td>
<td>norm</td>
<td>Control 4</td>
<td>norm</td>
<td>norm</td>
</tr>
<tr>
<td>PHN 4</td>
<td>+ TC (2/3)</td>
<td>+ TC (1/3), + (2/3)</td>
<td>Control 5</td>
<td>(ND)</td>
<td></td>
</tr>
</tbody>
</table>

Langerhans cells were observed in close proximity to CD8⁺ T cells within the dermis in both PHN-affected and control material. There was no relationship between Langerhans cells in close proximity to T cells and the presence of PHN (Langerhans cells in close proximity to CD8⁺ T cells in 3/5 PHN-affected patients compared to 2/4 control patients), nor any link within PHN-affected patients (3/5 active PHN biopsies showed Langerhans cells and CD8⁺ T cells in close proximity, compared to 3/5 contralateral side).

As it was a rare event, BDCA-2 staining was assessed to be either positive or negative based on presence or absence of positive staining within the tissue section. Results are shown below in Table 4.5. BDCA-2⁺ pDCs were detected in two sections from the contralateral side of one PHN patient (PHN 2), and in one section from a control patient (Control 3). There was no relationship with the presence of pDCs and PHN when comparing opposite sides within PHN-affected patients, and when comparing PHN-affected and control patients.
Table 4.5 - Scoring of BDCA-2+ cells present in patient skin biopsy samples. Samples were scored either "+" (present) or "-" (absent). The fraction in brackets is the number of sections in which the staining was observed followed by the total number of sections examined for each patient. Slides from patient Control 5 were used for isotype control (ND = staining not done).

<table>
<thead>
<tr>
<th>PHN-affected patients</th>
<th>Active</th>
<th>Contralateral</th>
<th>Control Patients</th>
<th>Left</th>
<th>Right</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHN 1</td>
<td>-</td>
<td>-</td>
<td>Control 1</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PHN 2</td>
<td>-</td>
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</tr>
<tr>
<td>PHN 3</td>
<td>-</td>
<td>-</td>
<td>Control 3</td>
<td>-</td>
<td>+ (1/3)</td>
</tr>
<tr>
<td>PHN 4</td>
<td>-</td>
<td>-</td>
<td>Control 4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PHN 5</td>
<td>-</td>
<td>-</td>
<td>Control 5 (ND)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

4.3.3.1.2 Analysis of the frequency of immune cell subsets in human skin biopsy material

In order to gain a more complete understanding of the immune cell repertoire within the tissue samples, the frequency was calculated for the immune cell markers present in higher numbers: specifically Langerhans cells and T cell subsets. This involved counting the number of positive cells for each cell marker in up to three microscope images per section, for each section (limited only by the size and condition of the section). Three images per section were chosen as this covered a majority of the tissue area. The total surface area examined in each micrograph was then calculated with the aid of the Zeiss AxioVision LE software (Carl Zeiss Pty Ltd, Australia and New Zealand). The frequency of positive cells was then obtained by dividing the number of positive cells by the total area of the tissue. Fold changes were obtained by normalising patient values against the contralateral side (PHN-affected material) or the right site (control material). This analysis was performed for analysis of Langerhans cells in the epidermis, and CD4+ and CD8+ T cell subsets within the tissue. The dermis was not examined, as it was not possible to differentiate between migrating cells and cells present within vessel walls.

The frequency of Langerhans cells in the epidermis of patient skin biopsy samples is presented as fold change normalised to the contralateral or right side, shown in Figure 4.12. In 3/5 PHN-affected patients – patients PHN 1, PHN 4, PHN 5 – there was a reduction in the number of Langerhans cells in the epidermis of the PHN-affected side when compared to the contralateral side. The largest fold change was 1.4x seen in active side of patient PHN 2, and the smallest fold change was 0.6x seen in the active side of patient PHN 4. The average fold change in Langerhans cells in the epidermis of PHN-affected patient material was 0.9x.
Figure 4.12 - Relative frequency of Langerhan cells within the epidermis of human skin biopsy material

Fold change of Langerhan cells in the epidermis of patient skin biopsy samples. Following immunofluorescent detection of langerin, patient samples were imaged with up to 3 images per section per patient (constrained only by the presence and size of an epidermal region in the tissue). The number of langerin positive cells were counted, and the total surface area of the tissue calculated. Counts were converted to a frequency of positive cells per 100 000 μm². A fold change was calculated by dividing values by the contralateral (contra) or right side. Graphs show fold changes of Langerhan cells in PHN-affected patient biopsy material (A) and control patient biopsy material (B). The graph display the average values (columns), as well as the minimum and maximum values (triangles and crosses respectively). There is no data available for patient control 5 as sections were used as isotype controls.
In the control patient samples the frequency of Langerhans cells was considerably less varied between sides for each patient. The largest fold changes were 1.2x and 0.8x for patients Control 1 and Control 2 respectively. Patients Control 3 and Control 4 had similar levels of Langerhans cells staining in their left and right sides. The average fold change in Langerhans cells in the epidermis of all the control patient material was 1.0x.

The frequency of CD4$^+$ T cells present within the tissue of patient biopsy samples is presented as fold change normalised to the contralateral or right side, shown in Figure 4.13. CD4$^+$ T cells were detected primarily within the dermis of the PHN-affected skin biopsy samples, however there were rare CD4$^+$ cells detected within the epidermis. Three out of five PHN-affected patients showed an increase in frequency of CD4$^+$ T cells in their active side compared to the contralateral – patients PHN 1, PHN 2, PHN 4. The largest was an increase of 1.6x (PHN 2). The other two PHN-affected patients showed a lower frequency of CD4$^+$ T cells present in the active side, the lowest at 0.5x the contralateral side of patient PHN 3. Overall the average fold change in CD4$^+$ T helper cells in PHN-affected patient material was 1.1x.

In control patient samples there was overall less CD4$^+$ T cells detected in normal skin, and a lower range seen between different patient samples. The largest increase was 1.3x (patient Control 2), and the largest reduction was 0.5x (patient Control 4). On average there was a slight decrease (0.9x) in the frequency of CD4$^+$ T cells in skin biopsy material from the left compared to the right sides.

CD8$^+$ T cells were detected at a much lower frequency, with much greater variation within samples than CD4$^+$ T cells in all skin biopsy samples examined. The frequency of CD8$^+$ T cells within the tissue of patient biopsy samples is shown in Figure 4.14, and the variation within samples is shown through the greater range of minimum and maximum values as compared to previous results. Similar to CD4$^+$ T cells, CD8$^+$ T cells were primarily restricted to the dermis, with sporadic CD8$^+$ cell visible within the epidermis. Three PHN-affected patients – PHN 2, PHN 3, and PHN 4 – showed an increase in CD8$^+$ T cells on the active side, with the greatest increase – a 2.3 fold increase – seen in patient PHN 3. Patients PHN 1 and PHN 5 showed similar frequencies of CD8$^+$ T cells on the active and contralateral sides. On average, skin biopsy material from PHN-affected patients had a 1.5 fold increase in CD8$^+$ T cells on the pain side.

The frequency of CD8$^+$ T cells in the control patient material was also more varied than other immune cell types investigated. The greatest change was a 0.4 fold
Figure 4.13 - Relative frequency of CD4+ T cells in human skin biopsy material

Fold change of CD4+ T helper cells in patient skin biopsy samples. Following immunofluorescent detection of CD4, patient samples were imaged with up to 3 images per section per patient (constrained only by the size of the tissue). The number of CD4 positive cells were counted, and the total surface area of the tissue calculated. Counts were converted to a frequency of positive cells per 100 000 μm², and fold change calculated by dividing by the contralateral (contra) or right site. Graphs show fold change of T helper cells in PHN-affected patient biopsy material (A) and control patient biopsy material (B). The graphs display the average values (column), as well as the minimum and maximum values (triangles and crosses respectively). Data for control 5 is limited as sections were used for isotype control material.
Figure 4.14 - Relative frequency of CD8+ T cells in human skin biopsy samples
Frequency of CD8+ cytotoxic T cells patient skin biopsy samples. Following immunofluorescent detection of CD8, patient samples were imaged with up to 3 images per section per patient (constrained only by the size of the tissue). The number of CD8 positive cells were counted, and the total surface area of the tissue calculated. Counts were converted to a frequency of positive cells per 100,000 \( \mu m^2 \), and fold change calculated by dividing values by the contralateral or right side. Graphs show fold change of cytotoxic T cells in PHN-affected patient biopsy material (A) and control patient biopsy material (B). The graphs display the average values (columns), as well as the minimum and maximum values (triangles and crosses respectively). Data for patient control 5 is limited as sections from this sample were used for isotype controls.
decrease, seen in patient Control 1. On average the control patient material featured a limited increase of 1.1x more CD8$^+$ T cells on the left side.

Overall in all skin biopsy samples there was high variability in the frequency of Langerhans cells, and greater frequency of both CD4$^+$ and CD8$^+$ T cells in the skin biopsy samples from PHN-affected patients.

4.3.3.1.3 Analysis of frequency of immune cell subsets according to patient

While a comparison of the frequency of immune cells in each patient for the different cell markers is beneficial in showing a trend across patients, another useful analysis is the frequency of detection of each immune cell subset in each patient. This can be used to establish whether there is any difference in immune cell types in a specific patient. The fold changes of different immune cell markers are displayed for PHN-affected patient material and control patient material in Figures 4.15 and 4.16 respectively. While there are not any clear trends in the difference between cell marker frequencies, it is obvious that the frequencies of the cell markers investigated in this study are more variable in skin biopsies taken from PHN-affected patients compared to control patient biopsy material.

4.4 Discussion

This study has provided a detailed understanding of the numbers and type of dendritic cells and T cell subsets present in human skin during PHN. PHN is a very painful complication of herpes zoster, which results from VZV reactivation within human ganglia.

As VZV is a highly species-specific human herpesvirus, experimentation is generally restricted to the use of tissues of human origin. To date there is no experimental model that replicates the complete VZV infection cycle seen in vivo. The modelling of latency and reactivation in particular has been challenging, and experimental modelling of PHN has so far been beyond our capabilities.

Consequently studies into VZV reactivation and PHN in particular have required the use of clinical human tissue samples. This type of material has been difficult to acquire due to many different factors, and consequently studies performed previously have been quite limited in number and scope.
Figure 4.15 - Immune cell frequency in skin biopsy samples from PHN-affected patients

Frequency of different cell markers in each PHN-affected patient skin biopsy material. Following immunofluorescent detection of langerin, CD4 and CD8, patient samples were imaged with up to 3 images per section per patient (constrained only by the size of the tissue). The number of positive cells for each marker were counted, and the total surface area of the tissue calculated. Counts were converted to a frequency of positive cells per 100,000 μm², and fold change was calculated by dividing the values by the contralateral (contra). Graphs show the fold change of Langerhan cells (langerin positive), T helper cells (CD4 positive) and cytotoxic T cells (CD8 positive) for each patient sample. Samples are patients PHN 1 (A), PHN 2 (B), PHN 3 (C), PHN 4 (D) and PHN 5 (E).
Figure 4.16 - Immune cell frequency in skin biopsy samples from control patients

Frequency of different cell markers in each control patient skin biopsy material. Following immunofluorescent detection of langerin, CD4 and CD8, patient samples were imaged with up to 3 images per section per patient (constrained only by the size of the tissue). The number of positive cells for each marker were counted, and the total surface area of the tissue calculated. Counts were converted to a frequency of positive cells per 100 000 μm², and fold change was calculated by dividing the values by the right side. Graphs show the fold change of Langerhan cells (langerin positive), T helper cells (CD4 positive) and cytotoxic T cells (CD8 positive) for each control patient sample. Samples are patients control 1 (A), control 2 (B), control 3 (C) and control 4 (D).
One particular study performed by Zak-Prelch et. al. took skin biopsy samples from herpes zoster-affected patients, and then monitored the same patients for 6 months following herpes zoster for the development of PHN (Zak-Prelch et al., 2003). This study examined CD3, CD4, CD8 and CD56 expression in skin biopsies of herpes zoster lesions taken from patients presenting to hospital with herpes zoster. They found that there were significantly lower numbers of infiltrating lymphocytes present in skin biopsy samples taken during herpes zoster of patients who went on to develop PHN. However whether the immune infiltration persisted or was present in the skin following rash resolution and during PHN was unknown. Also this study does not take into account the possibility of individual variation in the amount of immune cell infiltration between patients, and utilised single punch biopsy samples from each patient involved (Zak-Prelch et al., 2003).

My study was made possible due to the provision of precious human biopsy material by collaborators at Albany Medical College and the UCSF. The skin material provided allows us to compare both between individuals – PHN-affected patient to control – and within individuals – PHN-affected side to contralateral side. This material allows us to account for any variation in immune response between individual patients.

The two initial patient cohorts – PHN-affected and control – represent similar ages and sex distribution. The skin biopsies were obtained at least six months after the resolution of the herpes zoster rash. Currently clinical data is limited to this information. It is not known whether control patients represent recovered herpes zoster patients at least 6 months post rash, or were normal patients without any VZV reactivation.

As with most clinical samples there was a very limited number available for use in this study. The work presented in this chapter was designed to form the foundation for a larger and more comprehensive study, using a larger patient cohort. This is the focus of Chapter 5 of this thesis.

Due to the limitation in the amount of human skin biopsy sample material available for this study a significant amount of time and effort was spent on method development. This method development and optimisation stage involved both the development of suitable control material, and the creation of an IFA staining protocol that enabled the examination of multiple markers within the same section. The development of a dual and triple IFA detection system that maintained the integrity of
the tissue and provided adequate antigen unmasking was critical for this work to progress,

Unfortunately there was no reproducible detection of VZV antigen positive cells within any of the PHN-affected skin patient material used in this study. Two different antibodies were utilised: one specific for a glycoprotein complex (gE:gI) and the other for an immediate early protein (IE63). These two viral antigens are normally present at relatively high levels during a productive infection (Reichelt et al., 2009). However at the start of my study both our laboratory and our colleagues did not have any fixation and preservation matched known VZV antigen positive tissue material. Thus we developed a suitable positive control for IFA staining, which was both fixation and preservation matched infHFF cell pellets, utilised as positive control material. Expression levels of VZV antigens in this productively infected material may be higher than that observed during clinical infection – especially if there is only a low level of persistent infection within the skin material, many months post-herpes zoster rash resolution. Consequently it is not known whether VZV antigen can be detected in patient sample material utilising our current protocol.

Ideally, if possible it would be best to attempt to obtain a biopsy sample of a herpes zoster lesion within days of its appearance and match the fixation and cryopreservation in order to assess the suitability of this method. None the less, we have previously used the anti-gE:gI antibody readily detected these VZV antigens by immunohistochemistry and immunofluorescence several days post-herpes zoster rash onset (Huch et al., 2010).

Another alternative explanation for the lack of VZV antigen detection in this tissue maybe that the VZV antigen expression is below the limit of the detection method. One of the VZV-specific antibodies utilised in this study (anti-VZV IE63) has been used by our laboratory to demonstrate the presence of very low level VZV infection in human ganglia samples (Steain et al., 2013). VZV antigen expression has also been demonstrated by previous work in our laboratory during active herpes zoster and many months following reactivation in both human skin biopsy and human ganglia samples (Gowrishankar et al., 2010, Huch et al., 2010).

Anti-viral treatment also has the potential to affect the presence of replicating VZV within the skin of patients both during herpes zoster and during the associated recovery period. It is possible it could affect VZV antigen expression during PHN to a point below detectable levels. Unfortunately it is unknown whether the patients
included in this study were treated with any anti-viral medication during the acute infection period.

Langerhans cells are the primary tissue resident dendritic cells present in the human epidermis (Ross and Pawlina, 2003). These cells are present in an immature phenotype in normal human skin and the upper respiratory tract. Upon exposure and capture of foreign antigen, Langerhans cells have the ability to migrate to draining lymph nodes and perform an important antigen presenting role through interaction with T lymphocytes (Valladeau and Saeland, 2005). Langerhans cells are not only restricted to the epithelial layer of the skin, but are also present in lymph nodes, hair follicles or other organs (Valladeau and Saeland, 2005).

In this study Langerhans cells were identified through the surface expression of the marker langerin. There is a population of langerin+ DCs that reside within the dermis that are a distinct population of cells, and perform a different function to Langerhans cells (Bursch et al., 2007, Ginhoux et al., 2007, Poulin et al., 2007). Although these cells are at a very low frequency (Romani et al., 2010), they are indistinguishable from migrating Langerhans cells with the staining method utilised, and consequently Langerhans cells were counted when confined to the epidermis only.

The frequency of Langerhans cells in human skin biopsy samples taken from PHN-affected patients was quite varied when compared to control patient biopsy material. In general, control patients had similar frequencies of Langerhans cells when comparing biopsies taken from left and right, while PHN-affected patients showed quite different levels when comparing active and contralateral biopsy material.

In 3 of the 5 PHN-affected patients, there was a greater frequency of Langerhans cells on the contralateral side compared to the active PHN-affected site. Whether this was an increase in the number of Langerhans cells on the contralateral side (proliferation of Langerhans cells), or a decrease in the number of Langerhans cells on the active side (migration of Langerhans cells out of the tissue) is unknown. In either case there is some factor that has caused a change in the dynamics of Langerhans cells from the normal steady state generally seen in the control patient biopsy material. The cause of this change from steady state dynamics observed in control patient material to the varied frequency seen in PHN-affected patient material is not known.

pDCs play an important role in the innate anti-viral immune response (Donaghy et al., 2009, Huch et al., 2010, Valladeau and Saeland, 2005). They are recruited to
sites of inflammation, and are inflammatory cells themselves, secreting many cytokines and chemokines that promote effector cell activation – in particular IFN-α (Cella et al., 1999, Fitzgerald-Bocarsly, 1993, Siegal et al., 1999). pDCs have been shown to play an important role in the control herpes simplex virus reactivation (Donaghy et al., 2009), as well as during both active varicella and herpes zoster in the skin (Huch et al., 2010).

pDCs were only detected in a single biopsy sample from two cases: the biopsy from contralateral side of PHN-affected patient PHN 2, and the biopsy from the right side of patient control 3. All samples from PHN-affected patients were obtained at least 6 months following the resolution of the herpes zoster-associated rash. Thus at this late time post-rash resolution, it is unlikely that pDCs would still be involved unless there was still an active and ongoing acute inflammatory process. Overall this infers that these cells play a limited role in the pathogenesis or control of PHN, and these cells seem to play a limited role months post herpes zoster, which is in stark contrast to what is observed in the skin during active herpes zoster (Huch et al., 2010). This suggests it is important to examine the dynamics of both Langerhans cells and pDCs over several time points following the appearance of the herpes zoster-rash in patients with and without PHN. This will be performed in the next chapter.

Dermal dendritic cells (dDCs) are another important subset of DCs present in human skin. These cells are present at high frequency throughout the human dermis. Cells expressing the dDC marker DC-SIGN have previously been shown to be present in similar frequencies in normal, varicella and herpes zoster-affected skin (Huch et al., 2010). Although we were able to successfully optimise an antibody against the dDC marker DC-SIGN, due to the limited number of slides it was not possible to study dDC frequency in the current study. In future it would be worthwhile examining the frequency of dDC in human skin samples from PHN-affected versus normal patients.

In the adaptive immune response again VZV infection, both CD4+ and CD8+ T cells are involved in control of viral replication through the destruction of infected cells (Abendroth and Arvin, 1999). Previous work done in our laboratory has shown that T cells are likely involved in the control of herpes zoster within the human ganglia (Gowrishankar et al., 2010, Steain et al., 2013), and despite the presence of T cells in close proximity with neurons in the reactivated ganglia there is no evidence of T cell induced neuronal apoptosis (Steain et al., 2013). Previous work done in this thesis (see Chapter 3) has shown that there is a large CD3+ T cell infiltrate within the ganglia of a PHN-affected patient many years post-resolution of the herpes zoster-
associated rash, however in contrast to that observed in ganglia during herpes zoster there were comparable levels of both CD4$^+$ and CD8$^+$ T cell subsets.

Based on the IFA staining of the skin biopsies, there was a greater number of T cells present in the skin biopsy tissue of PHN-affected patients, including both CD4$^+$ and CD8$^+$ T cells. As with the Langerhans cell frequency – there is no clear trend shown when comparing between the active and contralateral sides of PHN-affected individuals, or between the PHN-affected and control patient material. Previous work has found that lower numbers of infiltrating T cells within the skin may contribute to the development of PHN (Zak-Prelich et al., 2003). However that study examined biopsies of active herpes zoster lesions, while this current study avoided active lesions and concentrated on the painful area of skin only.

Initially the IFA examination of the macrophage marker CD68 was also planned. This was mainly due to the significant level of macrophages that have been shown within human ganglia during herpes zoster (Gowrishankar et al., 2010). The antibody was successfully optimised and used on trial material, and subsequently utilised in the examination of the PHN-affected and control skin samples. Unfortunately the IFA staining was not successful and resulted in very high background staining levels with no specific staining identifiable above the background level seen in the isotype control stained sections, and consequently the frequency of CD68 positive cells within the skin biopsy material remains unknown. In future, an antibody specific for another macrophage-specific cell surface marker could be utilised to study the frequency of this immune cell subsets in the sample material.

This study has utilised some very precious skin biopsy material comprising of matched biopsy samples taken from five PHN-affected and control patients. The biopsies were performed at least 6 months following the resolution of the herpes zoster rash. Biopsies were obtained from patients at a single time point only, providing a “snap shot” of immune cell subsets present during PHN. Although the two patient cohorts were generally matched in terms of median age and gender, it is unknown whether the control patients represent recovered herpes zoster patients matched to time post-rash, or normal patients who have not experienced herpes zoster. This could play a significant factor in the mechanics of immune infiltration and tissue resident immune cells.

This study was restricted in the different markers accessed mainly due to the very limited number of slides available. Although there were multiple sections present from each biopsy sample on each slide, it was not feasible to stain each section for
different markers due to the close proximity of the sections. Ideally multiple slides from each biopsy sample would be available, which would allow the examination of other related immune markers to study the presence of T cell subsets (CD45RA and CD45RO), B cells (CD20), NK cells (CD16), and cytotoxic markers (TIA-1 and Granzyme B). Other interesting immune markers include neutrophil (Ly-6G/-6C), mast cell (mast cell tryptase) and other innate and adaptive immune markers that have been associated with neuropathic pain (Machelska, 2011, Zhou et al., 2010, Vallejo et al., 2010, Labuz et al., 2010, Hama et al., 2010).

In future studies the kinetics of the different T cell and DC subsets (dDCs, Langerhans cells and pDCs) present within human skin could also be studied. This would require multiple biopsy samples taken from the one patient over time following diagnosis with herpes zoster, and is the focus of the next results chapter. While we have only looked at a single time point snapshot, the kinetics of immune cell infiltration over time would be interesting to examine. This is the focus of the study presented in the next chapter of this thesis.
Chapter 5 – Kinetics of immune cell infiltration over time in human skin biopsy samples obtained following herpes zoster.

5.1 Introduction

VZV is a common human infection that causes two clinical presentations – varicella and herpes zoster. PHN is the most common complication following herpes zoster (Cohen et al., 1999, Steiner et al., 2007, Hope-Simpson, 1965, Hope-Simpson, 1975). To date there has been no investigation into the dynamics of the immune infiltration or viral antigen expression in the skin over time and the level of pain experienced by the patient following the resolution of the herpes zoster-associated rash and the development of PHN.

This study aims to address this lack of knowledge through an immunofluorescent examination of rare sample material that includes biopsies obtained from multiple sites from patients during herpes zoster, and subsequent biopsies obtained from the same patients at intervals post-herpes zoster rash resolution along with matching detailed clinical data. This will enable a detailed examination of the subsets of immune cells present in the skin, the number and location of VZV antigen positive cells, and their relative location in regards to each other. The matching clinical data includes information on the size and severity of the painful and alloreactive areas of skin.

Previous studies have shown that there is a dramatic shift in the numbers of important immune cells within the skin during herpes zoster, with a reduction in tissue resident Langerhans cells within the epidermis, and an increase in pDCs – highly inflammatory dendritic cells (Colonna et al., 2004) – within herpes zoster-affected skin (Gutzeit et al., 2010, Huch et al., 2010). However whether this shift is maintained long-term post herpes zoster or in patients suffering PHN is unknown.

Another study found significantly lower numbers of infiltrating lymphocytes present during herpes zoster in the skin of patients who later developed PHN compared to those that experienced no complications, despite the systemic cytokine and antibody responses being at comparable levels (Zak-Prelich et al., 2003). It would be interesting to investigate whether difference in important immune mediators is
maintained following the resolution of the herpes zoster-associated rash, or if there are any other immune cell changes that may predispose a patient towards PHN instead of an uncomplicated recovery.

A common underlying mechanism of neuropathic pain is the presence of inflammation at the damaged or affected site (Vallejo et al., 2010) and evidence suggests that these immune cells contribute to neuropathic pain in the periphery (Reviewed in Thacker et al., 2007). In the peripheral nervous system, tissue resident macrophages have been shown to migrate immediately following nerve injury to the site of injury, and display an active phenotype (Mueller et al., 2001). Further inflammation is promoted by the secretion of chemokines, matrix metalloproteases, and vasoactive mediators from activated macrophages, Schwann cells/satellite glial cells, and injured nerve axons to attract immune cells, increase blood flow to the area and cause swelling (Perrin et al., 2005, Shubayev et al., 2006, Zochodne et al., 1999). These changes result in a dense cellular infiltrate, mainly composed of macrophages, T lymphocytes and mast cells, forming within 2 days of injury at the injury site (Zochodne et al., 1999).

While there have been studies into the location of VZV antigen expression in the skin during herpes zoster (Huch et al., 2010, Nikkels et al., 1995a, Nikkels et al., 1995b, Oxman, 2000), to date there has been no investigation of VZV antigen expression in human skin over time following herpes zoster. Furthermore, there have been no reported studies investigating the persistence of VZV antigen expression in human skin from patients with or without PHN following natural VZV reactivation. This study aims to address this important area by using two different viral markers, and investigate whether VZV antigen expression persists in the skin following herpes zoster, and whether there is any difference in dynamics of VZV antigen expression between patients who experience greater or lesser pain levels, or between patients who develop ongoing PHN compared to those who experience an uncomplicated recovery.

This study is novel as it involves the analysis of skin samples from multiple patients from the time they presented with herpes zoster, throughout the rash and following rash-resolution, along with monitoring the patients’ associated clinical pain levels. This study is possible through access to a tissue bank created by collaborators at the UCSF and the Albany Medical College. Punch biopsy samples were collected from multiple sites from patients who presented with a herpes zoster-associated rash, and from the same patients over subsequent visits. At each visit clinical data was
obtained, including several pain measurements. This offers a rare opportunity to study in detail any possible link between herpes zoster-associated pain levels and any ongoing virological or immunological processes in the skin.

5.2 Patient and control material

Where applicable; all tissue specimens were obtained in accordance with ethics guidelines of both the University of Sydney and Western Sydney Area Health Service.

5.2.1 Control cell block material

Initial protocol and antibody optimisation were performed on control cell blocks. Control cell blocks were generated using both uninfected and infHFFs, and CD14-sorted human PBMCs. HFFs were cultured, infected and harvested as described in section 2.5. CD14 human PBMCs were isolated as described in section 2.5, and were generously donated by Rodney Henriquez (VZV research laboratory, University of Sydney).

5.2.2 Control patient skin material

Once antibody optimisation was successful on control cell sections, antibodies were then trialled on human tissue material to ensure that the antibodies and staining technique were established. Control human material consisted of cryopreserved skin tissue, kindly provided by Dr Heather Donaghy from the Westmead Millennium Institute in accordance with relevant ethics guidelines.

5.2.3 Patient skin sample material

Patient skin biopsies were generously provided by Professor Karen Petersen of UCSF and Professor Frank Rice of Albany Medical College. The subjects were immunocompetent, in stable health, over the age of 50, and had cervical, thoracic, lumbar, or sacral outbreaks of herpes zoster. The subjects were required to have an average daily pain of at least 20 on the 0–100 mm pain visual analogue scale (VAS) over the last 48 hours at the time of the study inclusion visit. No subjects with facial or ophthalmic herpes zoster were included to avoid collection of skin biopsies from the face. All subjects provided written informed consent (Petersen et al., 2010).
The VAS is psychometric scale used to measure subjective information. For the UCSF tissue bank, patients were shown a numerical pain scale that corresponded to a measure of 0 to 100 mm (Petersen et al., 2010). Patients were asked to rate the severity of their pain on the scale, with 0 corresponding to no pain, and 100 corresponding to full pain. The VAS has been shown to be a reproducible measure of patient symptoms (Grant et al., 1999).

At the initial visit each patient’s areas of abnormal sensation and allodynia were mapped, and various sensory function tests were performed. Four sites were selected as follows: site 1 was placed in the most painful area in herpes zoster-affected skin, avoiding open lesions. Site 2 was placed in the second most painful area on the herpes zoster-affected skin. Site 3 was mirror-image to Site 1. Site 4 was a distant control site placed contralateral to and at least 2 dermatomes away from the level of the herpes zoster-affected dermatome; typically on the shoulder (Petersen et al., 2010). Sites studied are shown in Figure 5.1. Biopsies (3 mm punch biopsies) were obtained from all four sites on the first visit, and then from at least Site 1 and Site 3 on subsequent visits. Mostly full biopsy sets for each individual patient were provided for use in this study.

Each biopsy sample was assigned a code number from a random number list to maintain blinding during tissue microscopy and analysis. The specimens were immediately placed into a solution of ice-cold 4% paraformaldehyde in 0.1 M PBS at pH 7.4 for 4 h to fix, and then transferred through 3 one hour rinses of cold PBS before storage in PBS under refrigeration. After overnight incubation in 30% sucrose in PBS, the biopsies were rapidly frozen with CO₂ onto a chuck for sectioning by cryostat (Petersen et al., 2010).

Slides were archived frozen under glycerol mounted coverslips. Multiple sections taken from different regions of each biopsy sample had been mounted on each slide. This allowed multiple replicates to be represented on the one microscope slide. For each biopsy sample, 3 slides containing 2-3 sections each were provided for use in this study. In total samples from 27 patients have been completely examined, numbering 283 biopsy samples. Patient details are summarised in Table 5.1.

The average patient age was 67.8 years old, with a range of 46 to 85 years old. There were 13 male patients (average age 70.2, range 63 to 78 years old) and 14 female patients (average age 65.6, range 46 to 85 years old). Patients were followed for an average of 160 days (range of 130 to 210 days). There were at least 4 visits for each patient. All visits were recorded as days post herpes zoster rash onset. On
Figure 5.1 - Schematic of biopsy sites
Schematic of the four testing site, each 3 x 3 cm, marked for sensory examination and skin biopsies. Site 1 was placed in the most painful area in the herpes zoster affected skin, avoiding open lesions. Site 2 was placed in the second most painful area in the herpes zoster affected skin. Site 3 was mirror-image to Site 1. Site 4 was a standardised distant control site placed contralateral to and at least 2 dermatomes away from the level of the herpes zoster affected dermatome; typically 10 cm below and 10 cm lateral from the vertebra prominens.

Figure from Petersen et al., 2010.
Table 5.1 - Patient clinical details, including age, sex, and the approximate site of their herpes zoster reactivation. Also included is the day of the first and last visit, and the length of time they participated in the study. For each visit, the patient's pain level was recorded with the aid of a visual analogue scale (VAS). There were three measurements recorded; the average pain level over the last 24 hours (listed), as well as the minimum and maximum pain levels within the last 24 hours (shown in brackets).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/Sex</th>
<th>Site of HZ rash</th>
<th>Time (days post herpes zoster rash onset)</th>
<th>Pain score (VAS)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Visit 1</td>
<td>Visit 4</td>
</tr>
<tr>
<td>1</td>
<td>67M</td>
<td>T4 left</td>
<td>9</td>
<td>184</td>
</tr>
<tr>
<td>2</td>
<td>73M</td>
<td>T4 right</td>
<td>13</td>
<td>178</td>
</tr>
<tr>
<td>3</td>
<td>46F</td>
<td>C4 right</td>
<td>16</td>
<td>166</td>
</tr>
<tr>
<td>4</td>
<td>67M</td>
<td>T12 left</td>
<td>17</td>
<td>187</td>
</tr>
<tr>
<td>5</td>
<td>54F</td>
<td>T3 right</td>
<td>13</td>
<td>176</td>
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<td>C5 right</td>
<td>10</td>
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</tr>
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<td>7</td>
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<td>S1 left</td>
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<td>16</td>
<td>176</td>
</tr>
<tr>
<td>10</td>
<td>70M</td>
<td>T16 left</td>
<td>27</td>
<td>191</td>
</tr>
<tr>
<td>11</td>
<td>54F</td>
<td>T5 left</td>
<td>15</td>
<td>175</td>
</tr>
<tr>
<td>12</td>
<td>71F</td>
<td>L1 right</td>
<td>26</td>
<td>180</td>
</tr>
<tr>
<td>13</td>
<td>80F</td>
<td>T5 right</td>
<td>42</td>
<td>179</td>
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<td>14</td>
<td>67M</td>
<td>T1 right</td>
<td>19</td>
<td>180</td>
</tr>
<tr>
<td>15</td>
<td>77M</td>
<td>T4 right</td>
<td>11</td>
<td>169</td>
</tr>
<tr>
<td>16</td>
<td>61F</td>
<td>T6 right</td>
<td>44</td>
<td>185</td>
</tr>
<tr>
<td>17</td>
<td>85F</td>
<td>L3 right</td>
<td>28</td>
<td>174</td>
</tr>
<tr>
<td>18</td>
<td>64F</td>
<td>T5 right</td>
<td>9</td>
<td>219</td>
</tr>
<tr>
<td>19</td>
<td>69M</td>
<td>L4 left</td>
<td>14</td>
<td>182</td>
</tr>
<tr>
<td>20</td>
<td>67M</td>
<td>T5 right</td>
<td>16</td>
<td>207</td>
</tr>
<tr>
<td>21</td>
<td>63M</td>
<td>T8 left</td>
<td>14</td>
<td>147</td>
</tr>
<tr>
<td>22</td>
<td>73M</td>
<td>T4 left</td>
<td>44</td>
<td>187</td>
</tr>
<tr>
<td>23</td>
<td>82F</td>
<td>T7 right</td>
<td>34</td>
<td>185</td>
</tr>
<tr>
<td>24</td>
<td>66F</td>
<td>T3 right</td>
<td>36</td>
<td>166</td>
</tr>
<tr>
<td>25</td>
<td>67F</td>
<td>T3 right</td>
<td>19</td>
<td>183</td>
</tr>
<tr>
<td>26</td>
<td>78M</td>
<td>L1 left</td>
<td>15</td>
<td>184</td>
</tr>
<tr>
<td>27</td>
<td>69M</td>
<td>L5 left</td>
<td>49</td>
<td>200</td>
</tr>
</tbody>
</table>
average visit 1 was at 22 days (range 9 to 49 days), visit 2 was at 47 days (range 38 to 63 days), visit 3 was at 93 days (range 80 to 113 days) and visit 4 was at 181 days (range 147 to 219 days).

Information regarding co-morbidities and immune status was not available for each patient sample, however initial criteria for inclusion in the tissue bank required that subjects be immunocompetent, in stable health, over the age of 50, and have a cervical, thoracic, lumbar or sacral outbreak of herpes zoster (Petersen et al., 2010).

Pain levels for each patient were recorded at each visit. Pain was measured using a VAS, with pain regarded as clinically significant when measured at a score of 30 or above (Thyregod et al., 2007). All patients had a VAS of at least 20 to be included in the study, however only fifteen patients had an average VAS of 30 or above, with VAS at visit 1 missing for one patient. Out of the twenty-seven patients in the study, seventeen patients’ pain resolved during the study period. Ten patients experienced continuing pain beyond the study duration, however all VAS were below clinically significant levels during the study period. Of these ten patients, four last had VAS ≥ 30 at visit 1, five patients at visit 2, with one patient at visit 3. These patients are studied in further detail later in the chapter (see section 5.3.3.2).

Following IFA staining of the skin sections, the tissue was imaged using the Zeiss LSM 510 Meta confocal microscope. Three images per section were taken, and the number of positive cells for each cell marker counted. The total surface area examined in each micrograph was then calculated with the aid of the Zeiss AxioVision LE software (Carl Zeiss Pty Ltd, Australia and New Zealand). The frequency of positive cells was then obtained by dividing the number of positive cells by the total area of the tissue, and then multiplying to give “Positive cells per 100 000 $\mu$m$^2$”.

5.3 Results

5.3.1 Antibody optimisation

As with the previous chapter, the patient material provided by collaborators was very limited. Consequently, to maximise the information obtained and to gain information on the presence and localisation of more than one cell type in the skin biopsy sample, IFA using antibodies to two or three specific markers were examined concurrently on the sections provided. Dual and triple IFAs allowed the co-detection of different antigens within the same sample, and gave critical information about the
relative positions of various immune cells and viral antigen expression. Multiple sections on each slide allowed repeats to be performed within the one IFA experiment. Antibody combinations were established on appropriate control material as described in Chapter 4. The primary antibodies utilised are listed in Table 5.2. The successful primary and secondary antibody combinations are described below in Table 5.3.

Table 5.2 - Primary and isotype control antibodies utilised in immunofluorescent assays on formaldehyde-fixed cryopreserved skin biopsy material.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-CD3</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Goat anti-CD4</td>
<td>R&amp;D systems, USA</td>
</tr>
<tr>
<td>Rabbit anti-CD8</td>
<td>Abcam, USA</td>
</tr>
<tr>
<td>Mouse anti-langerin</td>
<td>Leica Microsystems, Germany</td>
</tr>
<tr>
<td>Goat anti-DLEC (BDCA-2)</td>
<td>R&amp;D systems, USA</td>
</tr>
<tr>
<td>Rabbit anti-DC-SIGN</td>
<td>Abcam, USA</td>
</tr>
<tr>
<td>Mouse anti-VZV gE:gI</td>
<td>Meridian Life Sciences, USA</td>
</tr>
<tr>
<td>Rabbit anti-VZV IE63</td>
<td>Prof R Mahalingam, University of Colorado Medical School</td>
</tr>
<tr>
<td>Mouse IgG2a</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td>Mouse IgG2b</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>R&amp;D systems, USA</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>R&amp;D systems, USA</td>
</tr>
</tbody>
</table>

Sample IFA images of the various antibody combinations used on control material are shown in Figures 5.2-5.4. The first antibody combination (Figure 5.2) consisted of anti-CD4, anti-CD8 and anti-VZV gE:gI. Antibodies against CD4 and CD8 detect T helper and cytotoxic T lymphocytes respectively, and anti-VZV gE:gI detects VZV-infected cells. All antibody staining patterns were similar to those observed in single stains, background staining on negative controls and non-specific staining on isotype control sections were minimal.

The second antibody combination (Figure 5.3) consisted of anti-CD3 and anti-VZV IE63. The marker CD3 is a pan T cell marker, and VZV IE63 is expressed early on in during the productive infection cycle in VZV-infected cells. All antibody staining patterns were similar to those observed in single stains, background staining on negative controls and non-specific staining on isotype control sections were minimal.
Figure 5.2 - Trial of staining combination 1 on human skin biopsy samples.

Normal human skin biopsy samples were stained by immunofluorescence utilising primary antibodies specific for the markers CD4, CD8 and VZV gEgL to detect CD4+ T helper cells (green), CD8+ cytotoxic T cells (purple) and VZV-infected cells (red) respectively. Sections were counterstained with DAPI to show the cell nuclei. Staining is shown on a patient skin sample utilised for trial antibody staining with a merged image (A) and single channels shown; anti-CD4 (B), anti-CD8 (C) and anti-VZV gEgL (D). Positive control staining on a CD14+ cell pellet showed clear detection of both CD4+ and CD8+ T cells (E), and positive control staining on VZV infected HFF cell pellet showed clear detection of VZV-infected cells (F). Isotype staining on normal human skin (G) and background staining on uninfected HFF sections (not shown) were minimal.

White arrows indicate CD4+ T cell detection and orange arrows indicate CD8+ T cell detection. VZV gEgL was not present in normal human skin, but was easily detected in the VZV infected HFF cell pellet (green arrows).
Figure 5.3 - Trial of staining combination 2 on human skin biopsy samples.

Normal human skin biopsy samples were stained by immunofluorescence utilising primary antibodies specific for the markers CD3 and VZV IE63 to detect T cells (red) and VZV-infected cells (green) respectively. Sections were counterstained with DAPI to show the cell nuclei. Staining is shown on a patient skin sample utilised for trial antibody staining with a merged image (A) and single channel images; anti-CD3 (B), anti-VZV IE63 (E) and DAPI (D). Positive control staining on VZV infected HFF cell pellet showed clear detection of VZV-infected cells (E), and on CD14+ cell pellet showed clear detection of CD3+ T cells (F). Isotype staining on normal human skin (G) and background staining on uninfected HFF sections (not shown) were minimal.

White arrows indicate CD3+ T cell detection. VZV-infected cells are not present in normal human skin, but were easily detected in the VZV infected HFF cell pellet (green arrows).
Figure 5.4 - Trial of staining combination 3 on human skin biopsy samples.

Normal human skin biopsy samples were stained by immunofluorescence utilising primary antibodies specific for the markers langerin, BDCA-2 and DC-SIGN to detect Langerhan cells (red), plasmacytoid dendritic cells (pDCs) (green) and dermal dendritic cells (purple) respectively. Sections were counterstained with DAPI to show the cell nuclei. Staining is shown on a patient skin sample utilised for trial antibody staining with a merged image (A) and single channel images; anti-BDCA-2 (B), anti-langerin (C) and anti-DC-SIGN (D). Positive control staining on a CD14⁺ cell pellet showed clear staining of pDCs (E). Isotype staining on normal human skin (F) and background staining on uninfected HFF sections (G) were minimal.

A white arrow indicate dermal dendritic cell detection and orange arrows indicate Langerhan cell detection. pDCs were not observed in normal skin, however were easily detected in the CD14⁺ cell block (green arrows).
The third antibody combination (Figure 5.4) consisted of anti-BDCA-2, anti-DC-SIGN and anti-langerin specific antibodies. The marker BDCA-2 is a marker of pDCs, langerin is a marker of tissue-resident dendritic cells Langerhans cells, and DC-SIGN is a marker of dermal dendritic cells. All antibody staining patterns were similar to those observed in single stains, background staining on negative controls and non-specific staining on isotype control sections were minimal.

Table 5.3 – Proposed combinations of primary antibodies, matching isotype controls and secondary antibodies optimised for use in immunofluorescent assays on formaldehyde-fixed cryopreserved tissues.

<table>
<thead>
<tr>
<th>Proposed combination 1</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-CD4</td>
<td>Anti-VZV gE:gI</td>
<td>Anti-CD8</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Mouse IgG2α</td>
<td>Rabbit IgG</td>
<td></td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td>Alexa Fluor 488 donkey anti-goat</td>
<td>Alexa Fluor 546 donkey anti-mouse</td>
<td>Alexa Fluor 647 donkey anti-rabbit</td>
</tr>
<tr>
<td>Proposed combination 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-CD3</td>
<td>Anti-VZV IE63 (nil)</td>
<td></td>
</tr>
<tr>
<td>Mouse IgG2α</td>
<td>Matched NRS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td>Alexa Fluor 594 donkey anti-mouse</td>
<td>Alexa Fluor 488 donkey anti-rabbit</td>
<td></td>
</tr>
<tr>
<td>Proposed combination 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-BDCA-2</td>
<td>Anti-Langerin</td>
<td>Anti-DC-SIGN</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Mouse IgG2b</td>
<td>Rabbit IgG</td>
<td></td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td>Alexa Fluor 488 donkey anti-goat</td>
<td>Alexa Fluor 546 donkey anti-mouse</td>
<td>Alexa Fluor 647 donkey anti-rabbit</td>
</tr>
</tbody>
</table>

The antibody staining combinations shown in Table 5.3 were then applied to the formaldehyde-fixed and cryopreserved tissue to enable the detailed analysis of the immune cell profiles present in the skin biopsy tissue taken at various times post-herpes zoster rash.

5.3.2 Immunofluorescent analysis present in human biopsy samples

Although initial antibody optimisation and staining trials on normal control material were successful, the combinations did not all transfer directly to the UCSF sample material. Of the optimised antibody combinations (seen above in Table 5.2), only
combination 3 (anti-BDCA-2/anti-langerin/anti-DC-SIGN primary antibodies) were successful. In combination 1 the anti-VZV gE:gl was unsuccessful, with significant non-specific background staining seen on the paraformaldehyde-fixed, cryopreserved and gelatin-mounted UCSF sample material. The staining for the anti-CD4 and anti-CD8 combination was still deemed successful as positive staining above low non-specific background staining was observed and all control sections were successful. Combination 2 (anti-CD3 and anti-VZV IE63 primary antibodies) was entirely successful. The final staining combinations utilised on the UCSF sample material are shown in Table 5.4.

Table 5.4 - Combinations of primary antibodies, matching isotype controls and secondary antibodies utilised in immunofluorescent assays on formaldehyde-fixed cryopreserved skin biopsy material.

<table>
<thead>
<tr>
<th>Order of antibody application to sections</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Combination 1</strong></td>
<td>Anti-CD4</td>
<td>Anti-CD8</td>
<td>(nil)</td>
</tr>
<tr>
<td>Goat IgG</td>
<td></td>
<td>Rabbit IgG</td>
<td></td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td>Alexa Fluor 488</td>
<td>Alexa Fluor 647</td>
<td>donkey anti-rabbit</td>
</tr>
<tr>
<td></td>
<td>donkey anti-goat</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Combination 2</strong></td>
<td>Anti-CD3</td>
<td>Anti-VZV IE63</td>
<td>(nil)</td>
</tr>
<tr>
<td>Mouse IgG2a</td>
<td></td>
<td>Matched NRS</td>
<td></td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td>Alexa Fluor 594</td>
<td>Alexa Fluor 488</td>
<td>donkey anti-rabbit</td>
</tr>
<tr>
<td></td>
<td>donkey anti-mouse</td>
<td>donkey anti-rabbit</td>
<td></td>
</tr>
<tr>
<td><strong>Combination 3</strong></td>
<td>Anti-BDCA-2</td>
<td>Anti-Langerin</td>
<td>Anti-DC-SIGN</td>
</tr>
<tr>
<td>Goat IgG</td>
<td></td>
<td>Mouse IgG2b</td>
<td>Rabbit IgG</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td>Alexa Fluor 488</td>
<td>Alexa Fluor 546</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td></td>
<td>donkey anti-goat</td>
<td>donkey anti-mouse</td>
<td>donkey anti-rabbit</td>
</tr>
</tbody>
</table>

5.3.2.1 IMMUNOFLUORESCENT ANALYSIS OF THE IMMUNE CELL SUBSETS IN HUMAN SKIN BIOPSY MATERIAL

To characterise the immune cell profile in multiple skin biopsy samples from the same patients over time from the most painful sites in patients post-herpes zoster,
along with a contralateral site and a contralateral and distal control site, IFAs were performed utilising the antibody staining combinations as described above.

In each of the IFAs, matched isotype control antibodies were applied to UCSF skin biopsy material. A biopsy sample was selected at random, and was utilised for application of isotype control antibodies for all staining experiments.

5.3.2.1.1 Immunofluorescent assay: staining combination 1

The first antibody combination applied to human skin biopsy sections stained for CD4\(^+\) and CD8\(^+\) T cells. Representative images from a single patient are shown in Figure 5.5. CD4\(^+\) and CD8\(^+\) T cells were detected in all biopsy samples examined.

5.3.2.1.2 Anti-CD3 T cell and anti-VZV IE63 staining

Immunofluorescent staining utilising the second combination – anti-CD3 and anti-VZV IE63 – was attempted on the first patients to be analysed (consisting of approximately 80 slides and 240 individual sections). After microscopic analysis of these sections there was no identified VZV IE63 expression within the skin biopsy sample material (data not shown). Staining was successful on positive control material, and there was minimal non-specific background staining. Both the UCSF skin biopsy material and the anti-VZV IE63 primary antibody had been kindly provided to us by collaborators, and both were extremely valuable in their own right. The anti-CD3 primary antibody was present on all T cells, however both CD4\(^+\) and CD8\(^+\) T cells were successfully identified in the previous IFA staining combination. Consequently this immunofluorescent assay was not performed on the remaining patient material. The remaining patient skin biopsy sample material was conserved for use in the future for detection of another VZV antigen.

5.3.2.1.3 Immunofluorescent assay: staining combination 3

The final immunofluorescent assay performed on the UCSF skin biopsy material stained for Langerhans cells, dDCs and pDCs. A representative image is shown in Figure 5.6. Langerhans cells and dDCs were identified in all sections from all UCSF skin biopsy material. pDCs were identified through the expression of the marker BDCA-2, and were a rare event. BDCA-2 expression was detected in 10 sections only from 7 different patient samples (out of 283 individual patient samples).
Figure 5.5 - Detection of CD4\(^+\) and CD8\(^+\) T cells in human skin biopsy material

Human skin biopsy samples were stained by immunofluorescence utilising primary antibodies specific for the markers CD4 and CD8 to detect CD4\(^+\) T cells (green) and CD8\(^+\) cytotoxic T cells (purple) respectively. Sections were counterstained with DAPI to show the cell nuclei. A representative section which contains all cells types examined is shown of staining on skin biopsy material - a merged image (A) and single channel images; anti-CD4 (C), anti-CD8 (C) and DAPI (D). Staining on positive control CD14\(^+\) cell pellet showed clear detection of both CD4\(^+\) and CD8\(^+\) T cells (E). Isotype staining on normal human skin (F) and background staining on uninfected HFF sections (G) were minimal.

White arrows indicate CD4\(^+\) T cell detection and orange arrows indicate CD8\(^+\) T cell detection.
Figure 5.6 - Detection of plasmacytoid dendritic cells, Langerhan cells and dermal dendritic cells in human skin biopsy material

Human skin biopsy samples were stained by immunofluorescence utilising primary antibodies specific for the markers langerin, BDCA-2 and DC-SIGN to detect Langerhan cells (red), plasmacytoid dendritic cells (pDCs) (green) and dermal dendritic cells (purple) respectively. Sections were counterstained with DAPI to show the cell nuclei. A representative section which contains all cells types examined is shown of staining on skin biopsy material - a merged image (A) and single channel images; anti-langerin (B), anti-DC-SIGN (C) and anti-BDCA-2 (D). Staining on positive control staining on a CD14+ cell pellet showed clear staining of pDCs (E). Isotype staining on normal human skin (F) and background staining on uninfected HFF sections (G) were minimal.

White arrows indicate dermal dendritic cell detection and orange arrows indicate Langerhan cell detection. pDCs detection is indicated by green arrows. pDCs were rarely detected in the skin biopsy material, however were readily observed in the positive control CD14+ cell block.
5.3.2.1.4 Microscopic analysis of immunofluorescent assays; quantitation of results

To elucidate the changes in immune cell subsets observed in the skin during the herpes zoster-associated rash and following resolution over time a quantitative analysis is required. This requires enumeration of the number of positive stained cells in each tissue sample. This involved counting the number of positive cells for each cell marker in up to three microscope images per section for each section stained. Three images per section was chosen as at the magnification utilised it covered a majority of the tissue area. The exact size of the surface area of each micrograph was calculated with the aid of the Zeiss AxioVision LE software (Carl Zeiss Pty Ltd, Australia and New Zealand). The frequency of positive cells was then obtained by dividing the number of positive cells by the total area of the tissue. This analysis was performed for all immune cells examined. T cell and pDC frequency was calculated using the entire surface area of the tissue – an example measurement shown for analysis of CD4 and CD8 expression is shown in Figure 5.7, and for dendritic cell subtypes in Figure 5.8. Langerhans cells frequency were calculated utilising the surface area of the epidermis only (Figure 5.8 A), while dDCs frequency was calculated utilising the surface area of the dermis only (Figure 5.8 B).

Once all the cell counting and analyses had been performed on individual sections, data was provided by our collaborators which identified which patient each sample belonged to. Once all sections were organised into each patient type, and immune cell phenotype over time identified, clinical data for each patient was also provided. In this way the research was performed in a completely blinded fashion at each step.

5.3.3 Patient analysis

5.3.3.1 Individual patient analysis

To establish the immune cell repertoire in skin within single patients over time following the resolution of the herpes zoster-associated rash, the median frequency of each immune cell type was graphed for each visit. Graphs for a representative patient – patient 22 – are shown in Figure 5.9. Graphs for all immune markers examined for all patients are shown in Appendix 2. CD3⁺ T cells (Figure 5.9 A) were detected in all sections examined, and CD4⁺ helper T cells (Figure 5.9 B) and CD8⁺ cytotoxic T cells (Figure 5.9 C) were detected in all sections. All T cell markers were highly variable, and there were no general increases or decreases over time. Similarly Langerhans cells frequencies within the epidermis and dDC frequencies within the dermis were also highly variable (Figure 5.9 D and E).
Figure 5.7 - Representative image of analysis of CD4 and CD8 expression in human skin biopsy material

CD4 and CD8 expression was analysed through IFA and microscopic analysis. CD4 expressing cells are shown in green, CD8 expressing cells are shown in purple and cell nuclei are labelled with DAPI (shown in blue). A merged image is shown (A), as well as single colour images for both CD4 (B) and CD8 (C).

The number of cells expressing the markers were counted, and the surface area of the material was measured with the aid of the Zeiss AxioVision LE software. An example measurement is shown - the line in red encloses the measured area, while the area is displayed in the upper left of each image (168 544 µm²).
Figure 5.8 - Representative image of analysis of langerin and DC-SIGN expression in human skin biopsy material

Langerin and DC-SIGN expression was analysed through IFA and microscopic analysis. Langerin expressing cells are shown in red (A), DC-SIGN expressing cells are shown in purple (B) and cell nuclei were labelled with DAPI (shown in blue).

The number of cells expressing the markers were counted, and the surface area of the material was measured with the aid of the Zeiss AxioVision LE software. Langerin expression was analysed using measurement of the epidermis (26 340 μm²), while DC-SIGN expression was analysed using measurement of the dermis (155 699 μm²).
Figure 5.9 - Immune cell frequencies in skin biopsy samples of patient 22

Patient skin biopsy material was examined via immunofluorescent assay and microscope analysis, and specific cell types were identified and quantified. Tissue surface area was measured, and the frequency of cell types per area was calculated. Graphs are shown for patient 22 as a representative of the patient cohort. Immune cells analysed were CD3\(^+\), CD4\(^+\) and CD8\(^+\) T cells (A-C), Langerhan cells (D), dermal dendritic cells (E) and plasmacytoid dendritic cells (F). Graphs show the median values (bar) and minimum and maximum values (points) for each biopsy sample analysed.

Biopsy site 1 was the most painful site. Biopsy site 2 was the second most painful site. Biopsy site 3 was the contralateral site to site 1. Biopsy site 4 was contralateral and distal to site 1.

Only initial patient samples were examined for the presence of CD3\(^+\) T cells.
pDC were rarely detected, and were not only restricted to the painful areas (Figure 5.9 F). pDCs were detected in 25 biopsy samples from 13 different patients. Of the twenty-five detections, ten were in biopsy samples from visit 1, three from visit 2, five from visit 3, five from visit 4 and two from visit 5. pDCs were detected in biopsies from site 1 eleven times, from site 2 seven times, from site 3 five times, site 4 one time and from an unknown site one time (data not shown).

5.3.3.2 Patient pain levels

At each visit the average, minimum and maximum pain level (as measured by VAS) were recorded (see Table 5.1). In order to perform further analyses, patients were divided into two different groups: those which continued to experience pain throughout the entire study period, and those in which the pain resolved before their final study visit. This information is shown in Table 5.5. Although PHN is defined as average daily pain ratings over the last 48 hrs of greater than zero at 6 months post-herpes zoster-rash onset, clinically significant pain levels correspond to a VAS of 30 or greater (Petersen et al., 2010). None of the patient group experienced clinically significant pain for the entire study period. Although measurements were recorded on just four different occasions, overall the median time for pain levels to reach sub-clinical levels was 39 days post-rash onset (range of 9-92 days). The median time for pain levels to reach sub-clinical levels in the “Pain resolved” patient group was 25 days post-rash onset (range 9-44 days), and in the “Pain continued” patient group was 41.5 days post-rash onset (range 16-92 days). The median time for pain to resolve in the “Pain resolved” patient group was 40 days post-rash onset (range 9-113 days).

5.3.3.3 Further analyses

5.3.3.3.1 Immune cell frequency over time

In order to examine any changes in the frequency of immune cells over time in skin biopsy material taken from the most painful area (biopsy site 1), the frequency of each immune marker was examined over time. Representative graphs displaying frequency of CD4⁺ T cells over time are shown in Figure 5.10. Values graphed are for each visit. Lines are only drawn for consecutive patient visits – when no biopsy from site 1 was provided then no value is available. Graphs for all immune markers examined are presented in Appendix 3.1. Overall there are no trends shown in the data, with highly varied distribution of immune cell frequencies observed in both the
Table 5.5 - Patient classifications utilised for further analyses. Patients were divided according to their reported pain levels at each visit, and classified as either "Pain resolved" and "Pain continued". Pain levels were classified as either clinically relevant pain (VAS > 30) or any reported pain (VAS > 0). For patients who did not report a clinically significant pain level the time is recorded as "-".

### Pain resolved

<table>
<thead>
<tr>
<th>Patient</th>
<th>Patient type</th>
<th>Last clinically significant pain level (VAS &gt; 30) Days post rash onset</th>
<th>Last VAS &gt; 0 Days post rash onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Resolved</td>
<td>V1 9</td>
<td>V1 9</td>
</tr>
<tr>
<td>2</td>
<td>Resolved</td>
<td>V1 13</td>
<td>V2 59</td>
</tr>
<tr>
<td>4</td>
<td>Resolved</td>
<td>none -</td>
<td>V1 17</td>
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### Pain continued

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Figure 5.10 - CD4⁺ T cell frequency over time

CD4⁺ T cells were identified in human skin biopsy material utilising immunofluorescence and microscopic analysis. The median CD4⁺ T cell frequency was examined in consecutive biopsy samples obtained from the most painful area of skin (biopsy site 1) in patients who experienced pain throughout the study period ("Pain continued" - A) and in those for which pain resolved within the time followed ("Pain resolved" - B).

Points are linked in consecutive biopsy samples only.
patient in whom pain continued throughout the study, and in those that pain resolved within the study period.

5.3.3.3.2 Immune cell ratio over time

In order to control for any variation between individuals a ratio was calculated using the immune cell frequency measured at biopsy site 1 to biopsy site 3 within the one patient – that is the most painful site and the contralateral control site. Representative graphs displaying this ratio of CD4⁺ T cells at biopsy site 1 and site 3 over time are shown in Figure 5.11. Lines are only drawn for consecutive patient visits – when no biopsy from site 1 or site 3 were provided then no value is available. Graphs for all immune markers examined are presented in Appendix 3.2. Overall ratios are quite varied, with no observed trend in data in either patient group.

5.3.3.3.3 Immune cell frequency compared to reported pain levels

In order to examine whether the frequency of immune cells present within the pain area of skin was related to the reported pain level, the immune cell frequency measured at biopsy site 1 (the most painful area) was graphed as a function of the reported pain level (VAS) from the 48 hours preceding the corresponding patient visit. Representative graphs for CD4⁺ T cells are shown in Figure 5.12. Graphs for all immune markers examined are shown in Appendix 3.3. There was no observed relationship between immune cell frequency and reported pain level for any immune cell marker examined. A similar analysis was performed comparing the immune cell frequencies measured at both biopsy sites 1 and 2 (the most painful and second most painful site) to the reported pain level (data not shown), and there was no observed relationship between immune cell frequency and reported pain level for any immune cell marker examined.

5.3.3.3.4 Immune cell ratio compared to reported pain levels

In order to control for any potential variation between individuals, a ratio of immune cell frequencies measured at biopsy sites 1 and 3 was calculated as above. Representative graphs for CD4⁺ T cells are shown in Figure 5.13. Graphs for all immune markers examined are shown in Appendix 3.4. There was no observed relationship between the ratio of immune cell frequencies at biopsy site 1 to biopsy site 3 and the reported pain level for any immune cell marker examined.
Figure 5.11 - Ratio of CD4+ T cell frequency at biopsy site 1 to biopsy site 3 over time

CD4+ T cells were identified in human skin biopsy material utilising immunofluorescence and microscopic analysis. The median CD4+ T cell frequency was examined in consecutive biopsy samples obtained from the most painful area of skin (biopsy site 1) and the contralateral site (biopsy site 3) in patients who experienced pain throughout the study period ("Pain continued" - A) and in those for which pain resolved within the time followed ("Pain resolved" - B). A ratio was calculated by dividing the value obtained from biopsy site 1 by biopsy site 3.

Points are linked in consecutive biopsy samples only.
Figure 5.12 - CD4⁺ T cell frequency in skin biopsy material and pain level measured at time of biopsy.

CD4⁺ T cells were identified in human skin biopsy material utilising immunofluorescence and microscopic analysis. The median CD4⁺ T cell frequency was examined in consecutive biopsy samples obtained from the most painful area of skin (biopsy site 1) for all biopsy samples. CD4⁺ T cell frequency was compared to the pain level reported by each patient. Pain level from the 48 hours preceding the time the biopsy was obtained were measured using the Visual Analogue Scale (VAS).
Figure 5.13 - Ratio of CD4⁺ T cell frequency at biopsy site 1 to biopsy site 3 and corresponding pain score measured at time of biopsy

CD4⁺ T cells were identified in human skin biopsy material utilising immunofluorescence and microscopic analysis. The median CD4⁺ T cell frequency was examined in biopsy samples obtained from the most painful area of skin (biopsy site 1) and the contralateral site (biopsy site 3) in patients who experienced pain throughout the study period ("Pain continued" - A) and in those for which pain resolved within the time followed ("Pain resolved" - B). A ratio was calculated by dividing the value obtained from biopsy site 1 by biopsy site 3. Values are plotted against the pain level measured through the Visual Analogue Scale (VAS) from the 48 hours preceding the biopsy.
5.3.3.3.5 Other analyses

A similar analysis was performed comparing the immune cell frequency calculated within the most painful and second most painful areas to the size of either the most painful area, or the size of the allo-reactive area. Graphs for all immune markers examined are shown in Appendices 3.5 and 3.6. There was no relationship observed between these variables.

Overall there was no relationship observed between immune cell frequencies, report pain level, time, or size of the painful areas.

5.3.3.4 Specific comparisons

An alternative to examining across patient cohorts is to focus on specific patient samples. Three different comparisons were selected from the patient cohort, and patients were selected to match age and sex. Patient details are shown in Table 5.6.

| Table 5.6 - Comparisons between specific patients. | Pain score (Average VAS in last 48 hr) | Time (days post herpes zoster rash onset) | Time studied |
| Age / Sex | Site of herpes zoster rash | V1 | V2 | V3 | V4 | V1 | V2 | V3 | V4 |
|-----------|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Comparison 1** | | | | | | | | | | | | | |
| Patient 15 | 77M | T4 right | 21 | 33 | 0 | 0 | 11 | 40 | 88 | 169 | 158 |
| Patient 26 | 78M | L1 left | 63 | 82 | 32 | 6 | 15 | 43 | 92 | 184 | 169 |
| **Comparison 2** | | | | | | | | | | | | | |
| Patient 10 | 70M | T10 left | 22 | 6 | 9 | 0 | 27 | 54 | 113 | 181 | 154 |
| Patient 19 | 69M | L4 left | 50 | 44 | 23 | 2 | 14 | 44 | 93 | 182 | 168 |
| **Comparison 3** | | | | | | | | | | | | | |
| Patient 2 | 73M | T4 right | 26 | 11 | 0 | 0 | 13 | 59 | 99 | 178 | 165 |
| Patient 8 | 73M | T10 right | 21 | 0 | 0 | 0 | 12 | 42 | 96 | 184 | 172 |
| Patient 22 | 73M | T4 left | 35 | 20 | 28 | 18 | 44 | 51 | 92 | 187 | 143 |

The first comparison was between patients 15 and 26. Patient 15 was a 77 year old male for whom pain resolved after his second visit. Patient 26 was a 78 year old male who experienced pain throughout the entire study, however his reported pain level dropped below clinically significant levels by his fourth visit. Both patient visits occurred at comparable intervals apart from visit 4. Immune cell frequency graphs for both patient 15 and patient 26 are shown in Figure 5.14. CD4+ T cell frequency in patient 15 was less on average less varied than that observed in patient 26. Patient
Figure 5.14 - Specific comparison of immune cell frequencies in skin biopsies from patient 15 and patient 26
Patient 15 and patient 26 were selected as they were the same sex and approximately the same age. Patient 15 experienced no pain after his second visit, while patient 26 experienced pain throughout the entire study period. Graphs show immune cell counts from patient 15 (A, C, E, G, I) are in the left column and from patient 26 (B, D, F, H, J) are in the right column. Immune cell frequencies are shown for CD4+ (A and B) and CD8+ T cells (C and D), Langerhan cells in the epidermis (E and F), dermal dendritic cells (G and H) and plasmacytoid dendritic cells (I and J).
26 had a greater frequency of CD4\(^+\) T cells present in biopsies from the most painful site (site 1) on visits 1, 2 and 3. The highest median frequency was observed in patient 26 biopsy site 2 on the first visit. CD8\(^+\) T cell frequencies were comparable at all visits. Langerhans cells and dDC were comparable at all visits. pDCs were rare in both patient types.

The second comparison was between patients 10 and 19. Patient 10 was a 70 year old male for whom pain resolved after his third visit. Patient 19 was a 69 year old male who experienced pain throughout the entire study, however his reported pain level dropped below clinically significant levels by his second visit. Both patient visits occurred at comparable intervals. Immune cell frequency graphs for both patient 10 and patient 19 are shown in Figure 5.15. There were more biopsy samples available for patient 10 – no biopsy samples were available for patient 19 visit 2. Calculated frequencies or CD4\(^+\) and CD8\(^+\) T cells, Langerhans cells and dDCs were all comparable at the visits compared. There were no pDCs detected in patient 19, and a variable amount observed in patient 10.

The final comparison was between patients 2, 8 and 22. Patient 2 was a 73 year old male for whom pain resolved after his second visit. Patient 8 was a 73 year old male for whom pain resolved after his first visit. Patient 22 was a 73 year old male who experienced pain throughout the entire study, however his reported pain level dropped below clinically significant levels by his first visit. All patient visits occurred at comparable intervals apart from visit 1 – patient 22 visited 30 days later than both patients 2 and 8. Immune cell frequency graphs for patients 2, 8 and 22 are shown in Figure 5.16. CD4\(^+\) and CD8\(^+\) T cell frequencies peaked in patient 8 at visit 1. CD4\(^+\) T cell frequencies were comparable in patients 2 and 22. CD8\(^+\) T cell frequency in patient 2 at visit 1 was double that observed in patient 22, however was comparable at all other visits. Langerhans cell and dDCs were present in comparable frequencies at all visits. On average, pDCs were only detected in patient 8, with very rare detection in both patient 2 and patient 22.

Overall there was no major difference in the frequency of immune cells within skin biopsy samples taken from patients who experienced herpes zoster-associated pain throughout the study period and those for whom pain ceased within the study period when age and sex is matched.
Figure 5.15 - Specific comparison of immune cell frequencies in skin biopsies from patient 10 and patient 19

Patient 10 and patient 19 were selected as they were the same sex and approximately the same age. Patient 10 experienced no pain after his third visit, while patient 19 experienced pain throughout the entire study period.

Graphs show immune cell counts from patient 10 (A, C, E, G, I) are in the left column and from patient 19 (B, D, F, H, J) are in the right column. Immune cell frequencies are shown for CD4+ (A and B) and CD8+ T cells (C and D), Langerhan cells in the epidermis (E and F), dermal dendritic cells (G and H) and plasmacytoid dendritic cells (I and J).
Figure 5.16 - Specific comparison of immune cell frequencies in skin biopsies from patient 2, patient 8 and patient 22

Patient 2, patient 8 and patient 22 were selected as they were the same sex and age. Patient 2 and patient 8 experienced no pain after their second and first visit respectively, while patient 22 experienced pain throughout the entire study period. Graphs show immune cell counts from patient 2 (A, D, G, J, M) in the left column, patient 8 (B, E, H, K, N) in the centre column and from patient 22 (C, F, I, L, O) in the right column. Immune cell frequencies are shown for CD4+ (A - C) and CD8+ T cells (D - F), Langerhan cells in the epidermis (G - I), dermal dendritic cells (J - L) and plasmacytoid dendritic cells (M - O).
5.4 Discussion

The focus of this study was to characterise the immune infiltration within the skin of patients following the onset of the herpes zoster-associated rash over time. Patients presented at the clinic on at least 4 separate occasions, and punch biopsies were obtained from up to 4 different sites. At each visit various clinical observations were recorded, in an effort to identify any relation between immune cell presence or viral antigen expression and clinical features including the extent, severity and persistence of pain.

This study was made possible due to the provision of precious human biopsy material by collaborators at UCSF and Albany Medical College. In total skin biopsy samples from 27 patients were completely examined by the IFA based approaches which were developed in Chapter 4.

Due to the very limited amount of tissue sections available it was not always possible to obtain a full set of biopsy samples for each patient. This restricts the ability to fully analyse all patients. For example when either biopsy site 1 or site 3 was missing, a ratio of cell frequency at site 1 to site 3 was not possible, and consequently there are missing data points in this analysis.

This study was carried out in a completely blinded fashion. At no point during the experimental process were any clinical details known. Each sample was labelled with a number only. Once the IFA staining, microscopic analysis and counting had been completed our collaborators provided the data matching biopsy sample number to the patients. Once biopsies had been sorted into patient groups, our collaborators provided the matching clinical information for each patient.

Although PHN was defined as average daily pain ratings over the last 48 hours greater than zero on the VAS at 6 months post rash onset, clinically meaningful PHN was requires a VAS of 30 or greater (Thyregod et al., 2007). Unfortunately no patient sample from the study still experienced a VAS of 30 or greater as reported on visit 4, however there were 10 patients who did report continued pain throughout the entire study period, which would allow for a diagnosis of PHN.

Despite considerable efforts there was no detection of VZV antigen positive cells within any of the skin biopsy material tested in this study. As with the last results chapter, the same two antibodies were utilised: one specific for a glycoprotein complex (gE:gI) and the other for an immediate early protein (IE63). These two
antibodies have been previously used successfully on a variety of different human tissue sections including human ganglia following natural VZV reactivation to detect VZV antigen positive cells (Steain et al., 2013). Along with this, the punch biopsy procedure utilised by our collaborators avoided any herpes zoster lesions, possibly decreasing the likelihood of detecting active viral gene expression within the painful area of skin. Although VZV antigens can be readily detected by immunohistochemistry and immunofluorescence within the skin days post herpes zoster rash onset (Huch et al., 2010), previous studies have focused on active lesions rather than surrounding skin. Consequently it is unknown whether viral antigen expression occurs in this area. Based on our findings there was no detectable VZV antigens present in the skin sites tested.

Although peripheral immune cells, including CD4⁺ and CD8⁺ T cells, have been shown to both ameliorate and contribute to pain and allodynia in animal models (Boue et al., 2011, Grace et al., 2011), it remains unknown whether they contribute to PHN. During the initial skin work presented in Chapter 4 there seemed to be an association between PHN-affected material and a greater infiltration of immune cells (and specifically T cell subsets).

The patient samples utilised in Chapter 4 were single biopsy samples from patients diagnosed with PHN at least 6 months post resolution of the herpes zoster-associated rash, while those utilised in Chapter 5 were a series of biopsies over time from patients following diagnosis of herpes zoster. The amount of patient material examined in Chapter 5 was considerably more than in Chapter 4, and consequently overall this study showed no relationship between T cell frequencies and pain levels, there was no link between the number of T cells present within the skin and either the development or pain or allodynia, or a reduction in pain reported by the patient.

The dendritic cell subsets examined in this study were Langerhans cells, dDCs and pDCs. All of these cells are major components of the body’s immunosurveillance system and play various roles in skin immunity. Langerhans cells are known to produce an important immune surveillance function and antigen presentation role (Valladeau and Saeland, 2005). Langerhans cells have also been shown to be permissive to VZV infection in vivo during both varicella and herpes zoster, and Langerhans cell frequency is significantly decreased in herpes zoster-affected skin and post-herpes zoster (Huch et al., 2010). In this study there was no observed difference in Langerhans cell number in skin biopsies taken from the painful area of skin around the herpes zoster rash and the contralateral side. In fact numbers were
also similar when compared to skin from a contralateral and distal biopsy site. Consequently the decrease in Langerhans cell numbers is possibly restricted to sites immediately surrounding herpes zoster-associated lesions. Alternatively it may be a phenotype only observed during early VZV lesion development, during which there is robust VZV infection of skin cells.

Another subset of dendritic cells present within the skin are dDCs. While these cells are present at high frequencies throughout the human dermis (Valladeau and Saeland, 2005), there is no evidence they play a role in the ongoing pathogenesis of PHN or recovery from herpes zoster. These cells were identified through the expression of DC-SIGN, which is also present on other cells such as pre-plasmacytoid dendritic cells, macrophages and a small subset of B cells (Kwan et al., 2008, Rappocciolo et al., 2006). Consequently frequencies presented in this study may be an overestimation of the true number of dDCs present in the human skin samples.

While pDCs are not present in normal human skin they are important mediators of the innate anti-viral immune response (Donaghy et al., 2009, Huch et al., 2010, Valladeau and Saeland, 2005), and have been shown to play important roles in immunity against herpesviruses in the skin (Donaghy et al., 2009, Huch et al., 2010). Of the dendritic cells examined, the pDCs were detected at the lowest frequency. They were not restricted to the area of painful skin. Nor were they present exclusively in patients whom pain continued or was resolved within the study time frame. Consequently it seems that pDCs play a limited role in the pathogenesis of VZV post-herpes zoster in the skin not immediately associated with a lesion.

This study has utilised some very rare skin biopsy material comprising of sequences of matched biopsy samples taken from patients following onset of a herpes zoster-associated rash. It has allowed for the first investigation of the kinetics of CD4+ and CD8+ T cells, and different dendritic cells as well as viral antigen expression over time within the same patient.

This study was limited in the number of sections available from each biopsy sample, and the number of biopsy samples provided from each patient. In an ideal situation there would be multiple sections from each biopsy sample that would allow the investigation of multiple immune markers with several repeats. Although there are no more sections available from the patients presented in this study, there may be more patient samples available in the future. Consequently it may be possible in the future to examine other related immune markers. It would be interesting to investigate
whether innate immune cells such as NK cells, neutrophils and mast cells play a role in the ongoing pathogenesis of PHN. As PHN is a neuropathic pain, it would also be interesting to investigate the location of any immune cells present within the tissue in relation to a nerve cell marker.

Overall there does not seem to be any relation between any of the immune cells investigated and the clinical presentation of the patients. There was no distinct pattern to the immune cell phenotype over time, nor is there a link between immune cell numbers and the reported pain level, the size of the painful or allo-reactive areas of skin based on the parameters assessed. There was no factor identified that would predispose one patient to an uncomplicated recovery from herpes zoster or a prolonged experience of allodynia or painful skin.
Chapter 6 - Final summary and relevance

Varicella zoster virus is an ubiquitous human herpesvirus that produces two distinct clinical conditions: varicella and herpes zoster. The most common complication of herpes zoster is the neuropathic pain syndrome called post-herpetic neuralgia (PHN) (Kost and Straus, 1996). Despite being the third most common cause of neuropathic pain in the United States (Bennett, 1998), resulting in significant negative impact on a person’s quality of life (Coplan et al., 2004, Davies et al., 1994, Dworkin et al., 2007b, Watson, 1998) our understanding of the underlying pathology and pathogenesis remains relatively limited.

This study performed a detailed immunohistological analysis of human ganglionic material surgically excised from a PHN-affected individual following resolution of the herpes zoster-associated rash, and compared with post mortem material taken from herpes zoster-affected and normal patients. The immunohistochemical analysis of PHN-affected ganglia samples showed a large infiltration of immune cells comprised of both CD4+ and CD8+ T cells with cytolytic potential, as well as B cells. The immune infiltration observed in the PHN-affected patient material was comparable to those seen in acute herpes zoster-reactivations. There was no detection of VZV antigen expression within the PHN-affected ganglia material. A highly sensitive real time PCR method was utilised to show that the VZV genome was present at approximately seven-fold higher levels in one of the PHN-affected samples when compared to a post-mortem ganglion sample obtained from the site of an active herpes zoster reactivation. A new quantitative RT-PCR method for examining viral nucleic acid expression was also established for use on future samples with retrievable mRNA.

This study also performed an immunohistological examination of skin biopsy samples obtained from patients suffering from PHN and normal control patients and characterised the cellular immune infiltrate in these samples. Overall there was a greater immune infiltrate present in the skin biopsy samples from PHN-affected patients, however there was no overall trend when comparing biopsies obtained from the painful and contralateral sides of PHN-affected patients. There was no VZV antigen expression detected within these tissue samples.

Finally, this study performed an immunohistological analysis of the cellular immune infiltrate and VZV antigen expression in skin biopsy samples obtained from patients
diagnosed with herpes zoster over time. There was no VZV antigen expression identified in these skin biopsy samples. Different T cell and dendritic cell subsets were examined over time, and matched with the patients’ clinical history. There was no relationship between the frequency or phenotype of immune cells examined and the clinical level of pain reported by each patient. There was no difference in the level of examined immune cell subsets between patients who reported ongoing pain and those for whom pain resolved within the time of study. There was no difference in the immune cell phenotype between patients who reported higher or lower clinical pain levels.

One major barrier to the study of PHN and VZV in general is the high species specificity of the virus, which imposes significant limitations on the use of animal models. To date there is no animal model for VZV reactivation or PHN. Consequently studies of PHN require access to appropriate human tissue samples, which are extremely difficult to obtain. This thesis has been possible through the generous provision of rare and precious clinical material, some of which has never been studied before. The PHN-affected ganglia material was surgically-excised from a PHN-affected patient in an effort to relieve their pain, after all other avenues of treatment had been exhausted. In addition, rare herpes zoster-affected and control post-mortem ganglia material formed the basis for the study and results presented in Chapter 3.

Previous studies from our research group have shown a large infiltration of immune cells within herpes zoster-affected human DRG material, comprised mainly of macrophages, natural killer cells, CD4+ and CD8+ T cells (Gowrishankar, 2008, Gowrishankar et al., 2010, Steain et al., 2013). Immunofluorescence staining for a number of immune cell markers in the ganglia samples from the PHN-affected patient again demonstrated a large infiltration of immune cells predominantly consisting of CD4+ and CD8+ T cells. Infiltration levels observed in this study were comparable to those observed in these previous studies of herpes zoster-affected ganglia material, despite the significant time between the resolution of the PHN-affected patient’s herpes zoster rash and subsequent ganglionectomy – a period of several years. This gives further evidence of an ongoing chronic inflammatory process within the DRG that may contribute to the pathogenesis of PHN.

In regards to what is driving the ongoing inflammatory response, one possibility is the presence of ongoing viral replication. To this end I performed IHC and IFA to assess
VZV antigen expression utilising anti-VZV antibodies well established to detect VZV antigens in formalin-fixed paraffin-embedded sections.

Although detection of VZV antigen expression and viral RNA transcripts within the DRG material was not possible in this study, VZV-specific DNA was detected in multiple DRG samples obtained from our PHN-affected patient. A VZV DNA quantitation method developed by our research group demonstrated a much higher VZV DNA load per cell in the PHN-associated ganglia than in material obtained from the site of reactivation in a patient who had a herpes zoster-associated rash at the time of death, as well as detectable VZV antigens present within the ganglia (Steain et al., 2013). Molecular studies also demonstrated that there was no HSV latent infection in the PHN-associated material, suggesting that any immune cells observed within the ganglia were due to the presence of VZV.

This extremely rare ganglionic material has provided us with a unique insight into the inflammatory changes present within ganglia during PHN. Clearly further study is warranted in this area in order to determine the exact cause of the chronic inflammation observed, however access to other appropriate clinical samples remains a significant obstacle.

Despite the critical role of infection of the ganglia to VZV and PHN, the skin is another important site for VZV disease. Also utilised in this thesis was rare human skin biopsy material obtained from two different patient cohorts, which were utilised for Chapters 4 and 5. The first study utilised a set of biopsies obtained from 10 different patients at least 6 months following the resolution of their herpes zoster-associated rash – 5 PHN-affected patients and 5 control patients. For each patient there was a biopsy from the affected and contralateral side.

Utilising a newly developed immunofluorescence staining technique which I established in Chapter 4, it was shown that in general there was a greater number of T cells present in the skin biopsy tissue obtained from the PHN-affected patients, however there did not seem to be a predominance of either CD4+ or CD8+ T cells. In addition, there was no clear increase in T cell number or Langerhans cell frequency in biopsies from the PHN-affected side compared to the contralateral side in this single time point “snap-shot”. PHN-affected patients experience ongoing pain and altered sensation that can last for months to years following the resolution of the herpes zoster-affected rash (Alvarez et al., 2007, Schmader, 2007, Steiner et al., 2007, Watson, 1998). Thus in
order to determine if inflammatory infiltrates observed within skin samples from PHN-affected patients changed over time from the onset of herpes zoster and associated pain through rash resolution with or without resolved pain, the second study of human skin biopsy samples, presented in Chapter 5, utilised a tissue bank of biopsies taken from multiple patients at multiple time points following diagnosis of herpes zoster. This patient material was unique in that the study examined samples taken from up to four different biopsy sites at different time points, along with detailed clinical assessments of type, extent and severity of pain for up to a year from the appearance of the herpes zoster rash.

Biopsy samples from 27 different patients were examined in a completely blinded study - representing 283 individual biopsy samples. Biopsy samples were utilised in immunofluorescent assays and imaged via fluorescent microscope. Several thousand confocal and deconvolution microscope images were obtained, and then individually analysed by manually counting and measuring tissue surface area. To ensure consistency all microscopy and analysis was performed by a single person. To the best of our knowledge, to date this is the most extensive analysis of T cell and dendritic cell frequencies in skin and corresponding clinical data over time in patient samples following herpes zoster.

There were large numbers of T cells – both CD4+ and CD8+ - detected in the skin biopsy samples, with a predominance of CD4+ T cells. Multiple dendritic cell subsets were examined; Langerhans cell and dDC numbers were found to be quite variable within and between patients, and pDCs were found to be a rare event in all biopsy samples. Despite extensive analysis examining and comparing cell frequency over time directly and indirectly, pain levels and the size of the painful and allo-reactive areas there was no obvious factors found to be specific to either the patients who experienced ongoing pain throughout the study or those in whom pain resolved before the final visit. Thus this data suggests that at the peripheral site (that is the skin) the cell types studied play a limited role in the ongoing pathogenesis of PHN.

While T cells may not be directly involved in PHN, there are still other immune mediators that remain to be investigated. Mast cells may contribute directly to the development of pain through histamine release in the skin (Baron et al., 2001). Additionally, mast cells along with macrophages and neutrophils may play a role in the generation of neuropathic pain through the release of pro-nociceptive mediators and enhance the recruitment of other immune cell types (Reviewed in Thacker et al., 2007).
In all studies performed and presented in this thesis patient sample material was very limited. Because of this restriction, a significant amount of time was spent on optimising and organising the best methods for extracting the most information from the material available. Most of the DRG samples utilised in Chapter 3 are exhausted, and no slides remain of usable areas of tissue. Likewise, there is no more material available for study from the skin biopsy material utilised in Chapter 4. While for some skin biopsy samples utilised in Chapter 5 there is 1 slide remaining, many have been completely used. However unlike the ganglia samples, there are other skin biopsy samples from new patients available for both the patient types in Chapters 4 and 5. Thus any future directions for this work will be dependent on obtaining more suitable sample material.

Overall there was a substantial amount of infiltrating immune cells present in all material from PHN-affected patients studied in this thesis. There were comparable numbers of immune cells present in both the DRG and skin samples from PHN-affected patients, however there was a clear difference between PHN and control patients observed with the human DRG material. While there were infiltrating immune cells detected in skin biopsy samples obtained from PHN-affected patients, there was no clear difference between the affected and contralateral sites within the same patient, and between those patients who experienced prolonged pain and those who experienced a relatively uncomplicated recovery.

There was a greater amount of VZV-specific DNA present in the PHN-associated ganglia material in comparison to an active case of herpes zoster. This is despite there being no clinical evidence of viral replication present in the PHN-affected patient other than the pain reported. In order to examine any VZV-specific RNA in the ganglia material, a quantitative method utilising real time RT-PCR was established. Unfortunately the ganglia material was not suitable for RNA analysis, and consequently levels of gene expression at the RNA level in these ganglia samples were not able to be analysed. This RT-PCR approach can be utilised with any suitable patient material that may be obtained in the future.

Considerable effort in this thesis was devoted to the detection of VZV antigen expression in all sample material from the PHN-affected patients. While there has been previous reported detection of VZV antigen expression in human ganglia material, there has also been some recent complications identified when using an immunohistochemical approach (Zerboni et al., 2012). Our research group has recently demonstrated limited VZV antigen detection within herpes zoster-associated
DRG (Steain et al., 2013). All attempts to detect VZV antigen expression in ganglia material presented in Chapter 3 were performed in tandem with those reported in Steain et al. (2013). Despite considerable effort in this thesis, there is currently no evidence of detectable VZV antigen expression in ganglia sample material from PHN-affected patients.

There was also no successful detection of VZV antigen expression in the skin biopsy material from both patient cohorts. In all skin material, the established biopsy protocol involved obtaining the biopsy from the painful area of skin while avoiding any active herpes zoster lesions. Previous studies of active lesions have shown VZV antigen expression from multiple kinetic classes (Huch et al., 2010). It is possible that VZV antigen expression is limited to areas immediately surrounding herpes zoster lesions. Alternatively, there may be a low level of VZV replication within the ganglia causing expression of either viral proteins or immune mediators in the skin. However it would be extremely difficult to identify the exact sites of herpes zoster lesions beyond the resolution of the herpes zoster-associated rash.

During the research conducted in this thesis, every available antibody specific for a viral protein was trialled for use on any suitable control material. However in regards to the human skin material, biopsies were obtained from areas within painful regions, and any active herpes zoster-associated lesions were avoided. Consequently we have no current access to positive control material that was an exact fixation, cryopreservation and tissue type match. While the formaldehyde-fixed and cryopreserved infHFF cell pellet utilised was useful in showing which antibodies were unsuitable for use, final selection and optimisation of antibody and staining protocol would be assisted with exact matching control material.

While this study has not been able to demonstrate VZV gene expression through viral antigen expression or RNA detection within any PHN-associated human tissue samples, there was a greater amount of VZV DNA present in one of the PHN-affected ganglia samples when compared to an active case of VZV reactivation. This was despite no visible evidence of an active VZV infection and the absence of all clinical signs other than PHN. This high level of VZV genomic material present in the PHN-associated tissue gives some evidence for an ongoing viral process that may contribute to the underlying pathogenesis of PHN.

Unfortunately there was no nucleic acid material available for skin biopsy samples, and consequently no way of examining whether higher viral DNA loads correlated with greater clinically reported pain levels, or prolonged pain.
This thesis has successfully established highly sensitive and specific molecular techniques useful in detecting the presence of VZV-specific DNA transcripts in nucleic acids from paraffin-embedded and formaldehyde-fixed ganglia material. Unfortunately the very limited amount of sample material available restricted the amount of DNA available, and the samples were not suitable for RNA analysis. However this study has established methods that can be utilised for any future sample material, and due to the quantitative nature of the analysis can be directly compared to results obtained in this study. At this point in time, nucleic acid extraction has not been attempted on any human skin material, although our current extraction protocol could be adapted if new material was provided.

Although the demonstration of RNA transcripts via real time RT-PCR is useful for analysing samples as a whole, it provides no detail on which cell types are expressing the transcripts and their location within the tissue. Initially this thesis was to incorporate an in-situ hybridisation examination of mRNA transcripts within the tissue utilising DIG-labelled riboprobes. Plasmid constructs specific for cellular and viral RNA transcripts were created, however sample material was not deemed suitable and this approach was abandoned.

Currently this study has revealed in the skin that there is no relationship between the number of infiltrating T cells, the type of dendritic cells present, the period of time in which pain is experienced, or the size of either the painful or allo-reactive areas of skin.

In regards to my prior hypotheses this about the makeup of cellular infiltrate of inflammatory cells, this thesis has shown that there is a predominance of CD4\(^+\) and CD8\(^+\) T cells present in human ganglia material obtained from a PHN-affected patient. While there are substantial numbers of T cells of both subtypes present in the painful area of skin from PHN-affected patients, there are also higher levels in the contralateral sides in comparison to skin material obtained from unaffected patients.

There was no difference in the immune cell phenotype in the skin of patients who report higher and lower clinical pain levels. However due to the very limited amount of sample material available from each patient, this thesis examined subsets of T cells and dendritic cells only. There are many other immune cells that may play an important role in the recovery from herpes zoster or the ongoing pathogenesis of PHN. Histamine release has been shown to induce a burning pain in the skin of patients suffering from PHN (Baron et al., 2001), and mast cells have been shown to be activated during peripheral nerve injury in animal models (Zuo et al., 2003). It
would be worthwhile to investigate if mast cells played any role in the underlying pathology and pathogenesis of PHN.

While T cells and dendritic cells play important roles in the adaptive immune response, to date there is limited information available on other immune cells that may contribute to the ongoing inflammation during PHN in the ganglia and inflammation in the skin. In previous ganglia studies utilising herpes zoster-associated material, macrophages and natural killer cells have been part of the infiltration identified within the tissue (Gowrishankar, 2008, Gowrishankar et al., 2010, Steain and Sutherland, 2013). Macrophages have been shown to play an important immunomodulatory role immediately following nerve injuries (Mueller et al., 2001), and NK cells play a vital role in the initial in the control of primary infection (Etzioni et al., 2005). To date their presence and number in PHN-associated material is yet to be investigated.

One of the main barriers to continued study is the lack of suitable human sample material. This thesis has had access to rare, precious, and one-of-a-kind sample material in very limited amounts. The development of an in-vitro model would allow continued research without the additional limits. One possible model to study the acute development of herpes zoster and possible latency is the two chamber model, which has been previously utilised in the study of alphaherpesvirus interaction with trigeminal ganglion neurons (Smith et al., 2001, De Regge et al., 2006b, Geenen et al., 2005, De Regge et al., 2006a). This model utilises two chambers isolated by a gel barrier through, primary human fetal explant DRG are grown within the central chamber and allowed to grow through into the outer chamber. Communication between the two chambers takes place through the neuronal processes alone. Another model is the skin raft culture, which has been previous used to study the both HSV-1 and VZV (Andrei et al., 2005, Hukkanen et al., 1999). It may even be possible to combine the two model types to investigate the interaction between DRG and skin in vitro, and would be useful in characterising the innate immune response.

This thesis has demonstrated the presence of a substantial amount of VZV DNA in the ganglia of a PHN-affected patient, at a greater level than that observed in the site of reactivation of a herpes zoster affected patient. It has also shown the presence of an ongoing chronic inflammation within the same ganglia, despite no identified stimulus or clinical presentation other than ongoing chronic pain. It was shown that there is no relationship between pain experienced, number of immune cells present in the skin or propensity to either experience prolonged pain or a speedy recovery. It
did show on average skin biopsies from PHN-affected patients had a greater frequency of immune cells present within their skin, however it was not only restricted to the painful side.

Overall this thesis provides the first evidence of an ongoing chronic inflammatory process consisting predominantly of both CD4$^+$ and CD8$^+$ T cells within DRG from a PHN-affected patient many years post resolution of the herpes zoster rash. Within this chronically inflamed tissue, there is a large amount of VZV-specific DNA. In stark contrast to this, there does not seem to be any ongoing immunohistological process within the skin of patients post-herpes zoster. These findings have increased our knowledge on the immune reaction within human ganglia and skin following herpes zoster and during PHN.
Chapter 7 - References


NCBI (2013) TIA1 - cytotoxic granule-associated RNA binding protein [Homo sapiens (human)].


The University of Sydney


SATO, H., PESNICAK, L. & COHEN, J. I. (2003c) Varicella-zoster virus ORF47 protein kinase, which is required for replication in human T cells, and ORF66 protein kinase, which is expressed during latency, are dispensable for establishment of latency. Journal of Virology, 77, 11180-5.


Appendix 1 - Primer sensitivity testing for real-time PCR primers using plasmid constructs

IE63-specific primers

Quantitation curve

Standard curve

Melt curve
gB-specific primers

Quantitation curve

Standard curve

Melt curve
**gE-specific primers**

**Quantitation curve**

![Quantitation curve graph](image1)

**Standard curve**

![Standard curve graph](image2)

**Melt curve**

![Melt curve graph](image3)
Appendix 2

Immunofluorescent staining was performed on all samples using primary antibodies which detected CD4+ T cells, CD8+ T cells, Langerhan cells, dermal dendritic cells and plasmacytoid dendritic cells (see Chapter 5). Immunofluorescent assay for using a primary antibody to detect CD3+ T cells was performed on some samples. Positive cells for each marker were counted, and the total surface of each area of tissue measured. Cell frequency per 100,000 um² was calculated, and values plotted on the graphs shown. The columns show the median value, while the minimum and maximum counts are shown by the square and triangle respectively.

**Patient 1**

**Frequency of CD3+ T cells in biopsy samples taken from patient 1**

<table>
<thead>
<tr>
<th>Site 1</th>
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**Frequency of Langerhan cells in the epidermis of biopsy samples taken from patient 1**

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**Frequency of Langerhan cells in the dermis of biopsy samples taken from patient 1**

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**Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 1**

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**Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 1**

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**Patient 2**

**Frequency of CD3+ T cells in biopsy samples taken from patient 2**

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**Frequency of Langerhan cells in the epidermis of biopsy samples taken from patient 2**

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**Patient 4**

**Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 4**

**Frequency of CD3+ T cells in biopsy samples taken from patient 4**

**Frequency of CD4+ T cells in biopsy samples taken from patient 4**

**Frequency of CD8+ T cells in biopsy samples taken from patient 4**

**Frequency of Langerhan cells in the epidermis of biopsy samples taken from patient 4**

**Frequency of Langerhan cells in the dermis of biopsy samples taken from patient 4**

**Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 4**

**Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 4**

**Patient 5**

**Frequency of CD4+ cells in biopsy samples taken from patient 5**

**Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 5**
Patient 6

Frequency of CD8+ T cells in biopsy samples taken from patient 6

Frequency of CD4+ T cells in biopsy samples taken from patient 6

Frequency of Langerhan cells in the dermis of biopsy samples taken from patient 6

Frequency of Langerhan cells in the epidermis of biopsy samples taken from patient 6

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 6

Frequency of dermal dendritic cells in the epidermis of biopsy samples taken from patient 6

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 6

Frequency of dermal dendritic cells in the epidermis of biopsy samples taken from patient 6

Frequency of dermal dendritic cells in the epidermis of biopsy samples taken from patient 6

Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 6

Frequency of CD8+ cells in biopsy samples taken from patient 5

Frequency of CD4+ cells in biopsy samples taken from patient 5

Frequency of CD3+ T cells in biopsy samples taken from patient 6

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 5

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 5

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 5

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 5

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 5

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 5

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Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 5

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 5

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 5

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 5
Patient 7

Frequency of CD3+ T cells in biopsy samples taken from patient 7

Frequency of CD4+ T cells in biopsy samples taken from patient 7

Frequency of CD8+ T cells in biopsy samples taken from patient 7

Frequency of Langerhan cells in the epidermis of biopsy samples taken from patient 7

Frequency of Langerhan cells in the dermis of biopsy samples taken from patient 7

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 7

Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 7

Patient 8

Frequency of CD4+ cells in biopsy samples taken from patient 8

Frequency of CD8+ cells in biopsy samples taken from patient 8

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 8

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 8
**Patient 9**

Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 9

![Graph showing frequency of plasmacytoid dendritic cells](image)

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 9

![Graph showing frequency of dermal dendritic cells](image)

Frequency of CD4+ cells in biopsy samples taken from patient 9

![Graph showing frequency of CD4+ cells](image)

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 9

![Graph showing frequency of Langerhan cells in epidermis](image)

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 9

![Graph showing frequency of Langerhan cells in dermis](image)

**Patient 10**

Frequency of CD4+ cells in biopsy samples taken from patient 10

![Graph showing frequency of CD4+ cells](image)

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 10

![Graph showing frequency of Langerhan cells in epidermis](image)

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 10

![Graph showing frequency of Langerhan cells in dermis](image)
Patient 13

Frequency of CD8+ cells in biopsy samples taken from patient 13

Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 13

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 13

Patient 14

Frequency of CD8+ cells in biopsy samples taken from patient 14

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 14

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 14
Patient 18

Frequency of CD4+ cells in biopsy samples taken from patient 18

Frequency of CD8+ cells in biopsy samples taken from patient 18

Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 18

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 18

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 18

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 18

Patient 19

Frequency of CD4+ cells in biopsy samples taken from patient 19

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient Z-019

Frequency of Langerhan cells in dermis of biopsy samples taken from patient Z-019

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient Z-019
Frequency of CD4+ cells in biopsy samples taken from patient 23

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 23

Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 23

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 23

Frequency of CD8+ cells in biopsy samples taken from patient 24

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 24

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 24

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 24

Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 24
Patient 25

- Frequency of CD4+ cells in biopsy samples taken from patient 25
- Frequency of CD8+ cells in biopsy samples taken from patient 25
- Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 25
- Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 25
- Frequency of Langerhan cells in dermis of biopsy samples taken from patient 25

Patient 26

- Frequency of CD4+ cells in biopsy samples taken from patient 26
- Frequency of CD8+ cells in biopsy samples taken from patient 26
- Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 26
- Frequency of Langerhan cells in dermis of biopsy samples taken from patient 26
Frequency of plasmacytoid dendritic cells in biopsy samples taken from patient 26

Frequency of CD4+ cells in biopsy samples taken from patient 27

Frequency of Langerhan cells in dermis of biopsy samples taken from patient 27

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 27

Frequency of Langerhan cells in epidermis of biopsy samples taken from patient 27

Frequency of dermal dendritic cells in the dermis of biopsy samples taken from patient 27

Frequency of CD8+ cells in biopsy samples taken from patient 27
Appendix 3.1 - Frequency of immune cells in skin biopsy material taken from the most painful area (biopsy site 1) over time

CD8 T cell frequency over time in “Pain continued” patients

CD8 T cell frequency over time in “Pain resolved” patients

Langerhan cell frequency over time in “Pain continued” patients

Langerhan cell frequency over time in “Pain resolved” patients

Dermal dendritic cell frequency over time in “Pain continued” patients

Dermal dendritic cell frequency over time in “Pain resolved” patients
Appendix 3.2 - Ratio of immune cell frequency measured at the most painful site (biopsy site 1) to the contralateral site (biopsy site 3) over time.

CD8 T cell ratio over time in “Pain continued” patients

- Patient 3
- Patient 5
- Patient 17
- Patient 19
- Patient 22
- Patient 23
- Patient 24
- Patient 26

CD8 T cell ratio over time in “Pain resolved” patients

- Patient 1
- Patient 2
- Patient 4
- Patient 6
- Patient 7
- Patient 8
- Patient 9
- Patient 10
- Patient 11
- Patient 12
- Patient 13
- Patient 14
- Patient 15
- Patient 16
- Patient 18
- Patient 20
- Patient 21

Langerhan cell ratio over time in “Pain continued” patients

- Patient 3
- Patient 5
- Patient 17
- Patient 19
- Patient 22
- Patient 23
- Patient 24
- Patient 26

Langerhan cell ratio over time in “Pain resolved” patients

- Patient 1
- Patient 2
- Patient 4
- Patient 6
- Patient 7
- Patient 8
- Patient 9
- Patient 10
- Patient 11
- Patient 12
- Patient 13
- Patient 14
- Patient 15
- Patient 16
- Patient 18
- Patient 20
- Patient 21

Dermal dendritic cell ratio over time in “Pain continued” patients

- Patient 3
- Patient 5
- Patient 17
- Patient 19
- Patient 22
- Patient 23
- Patient 24
- Patient 26

Dermal dendritic cell ratio over time in “Pain resolved” patients

- Patient 1
- Patient 2
- Patient 4
- Patient 6
- Patient 7
- Patient 8
- Patient 9
- Patient 10
- Patient 11
- Patient 12
- Patient 13
- Patient 14
- Patient 15
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- Patient 20
- Patient 21
Appendix 3.3 - Frequency of immune cells in human skin biopsy material from painful areas and corresponding reported pain score (average VAS from last 48 hours)
Appendix 3.4 - Ratio of immune cell frequency measured at the most painful site (biopsy site 1) to the contralateral site (biopsy site 3) and reported average pain level (VAS) for 48 hours preceding the biopsy date

CD8 T cell ratio and VAS pain score in "Pain continued" patients

Langerhan cell ratio and VAS pain score in "Pain continued" patients

Dermal dendritic cell ratio and VAS pain score in "Pain continued" patients

CD8 T cell ratio and VAS pain score in "Pain resolved" patients

Langerhan cell ratio and VAS pain score in "Pain resolved" patients

Dermal dendritic cell ratio and VAS pain score in "Pain resolved" patients
Appendix 3.5 - Frequency of immune cell subsets compared to the size of the most painful area of skin

**Frequency of CD4 positive T cells at biopsy site 1 and size of the most painful area in "Pain continued" patients**

- **Size of the most painful area (mm^2)**
  - Range: 0 to 300

- **CD4 positive T cell frequency per 100,000 um^2**
  - Range: 0 to 25

- Patients: 1, 2, 3, 5, 17, 19, 22, 23, 24, 26

**Frequency of CD4 positive T cells at biopsy site 1 and size of the most painful area in "Pain resolved" patients**

- **Size of the most painful area (mm^2)**
  - Range: 0 to 300

- **CD4 positive T cell frequency per 100,000 um^2**
  - Range: 0 to 25

- Patients: 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 26

**Frequency of CD8 positive T cells at biopsy site 1 and size of the most painful area in "Pain continued" patients**

- **Size of the most painful area (mm^2)**
  - Range: 0 to 300

- **CD8 positive T cell frequency per 100,000 um^2**
  - Range: 0 to 14

- Patients: 1, 2, 3, 5, 17, 19, 22, 23, 24, 26

**Frequency of CD8 positive T cells at biopsy site 1 and size of the most painful area in "Pain resolved" patients**

- **Size of the most painful area (mm^2)**
  - Range: 0 to 300

- **CD8 positive T cell frequency per 100,000 um^2**
  - Range: 0 to 30

- Patients: 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 26

**Frequency of Langerhan cells at biopsy site 1 and size of the most painful area in "Pain continued" patients**

- **Size of the most painful area (mm^2)**
  - Range: 0 to 250

- **Langerin positive cell frequency per 100,000 um^2**
  - Range: 0 to 70

- Patients: 1, 2, 3, 5, 17, 19, 22, 23, 24, 26

**Frequency of Langerhan cells at biopsy site 1 and size of the most painful area in "Pain resolved" patients**

- **Size of the most painful area (mm^2)**
  - Range: 0 to 250

- **Langerin positive cell frequency per 100,000 um^2**
  - Range: 0 to 70

- Patients: 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 21, 26
Appendix 3.5 (continued)

Frequency of dermal dendritic cells at biopsy site 1 and size of the most painful area in “Pain continued” patients:

- DC-SIGN positive cell frequency per 100,000 μm²

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<th>Patient</th>
<th>Frequency</th>
<th>Size of the most painful area (mm²)</th>
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<td>5</td>
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<tr>
<td>19</td>
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<tr>
<td>26</td>
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</table>

Frequency of dermal dendritic cells at biopsy site 1 and size of the most painful area in “Pain resolved” patients:

- DC-SIGN positive cell frequency per 100,000 μm²

<table>
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<tr>
<th>Patient</th>
<th>Frequency</th>
<th>Size of the most painful area (mm²)</th>
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</thead>
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<td>0</td>
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<tr>
<td>2</td>
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<tr>
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<td>14</td>
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</tbody>
</table>
Appendix 3.6 - Frequency of immune cell subsets compared to the size of the allodynia-affected area of skin

- Frequency of CD4 positive T cells at biopsy site 1 and size of the allo-reactive area in "Pain continued" patients
- Frequency of CD4 positive T cells at biopsy site 1 and size of the allo-reactive area in "Pain resolved" patients
- Frequency of CD8 positive T cells at biopsy site 1 and size of the allo-reactive area in "Pain continued" patients
- Frequency of CD8 positive T cells at biopsy site 1 and size of the allo-reactive area in "Pain resolved" patients
- Frequency of Langerhan cells at biopsy site 1 and size of the allo-reactive area in "Pain continued" patients
- Frequency of Langerhan cells at biopsy site 1 and size of the allo-reactive area in "Pain resolved" patients
Appendix 3.6 (continued)

Frequency of dermal dendritic cells at biopsy site 1 and size of the allo-reactive area in "Pain continued" patients:

- Patient 3
- Patient 5
- Patient 17

Frequency of dermal dendritic cells at biopsy site 1 and size of the allo-reactive area in "Pain resolved" patients:

- Patient 1
- Patient 2
- Patient 4
- Patient 6
- Patient 7
- Patient 8
- Patient 9
- Patient 10
- Patient 11
- Patient 12
- Patient 13
- Patient 14
- Patient 15
- Patient 16
- Patient 18