Induction of Programmed Cell Death in Mammalian Cells by Isolates of Ross River Virus

This thesis is presented for Honours degree in Biomedical Science at Murdoch University

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BSc. Biomedical Science & Molecular Biology
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Declaration

I declare this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary education institution.

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11 November 2011
Abstract

Arthritogenic alphaviruses, such as Ross River virus (RRV) are associated with worldwide outbreaks of human polyarthritis/arthralgia. The pathogenesis of RRV and other alphaviruses is poorly understood. Studies have shown potential links between the different strains of RRV and variation in their pathogenesis and virulence. Currently there is believed to be two circulating strains of RRV, the south western (SW) from the south west region of Western Australia and the north eastern (NE) from the east coast of Australia. Studies have suggested that the persistence of RRV may be the result of an impaired immune response. This study was designed to determine if the SW and NE isolates of RRV have the ability to induce apoptosis in DCs and fibroblasts and discover any possible variation in their apoptosis-inducing capacity. Both Vero cells and murine bone marrow DCs (BMDCs) were infected with the SW74249 (SW) and SW82627 (NE) strains of RRV. A time course analysis of two apoptotic markers and a cell viability marker for both cell types was conducted by flow cytometry. The results indicate RRV-induced apoptosis in both Vero cells and BMDCs, with RRV inducing a stronger pro-apoptotic response in BMDCs than Vero cells, 24 h after infection. Between the two strains there was little variation in the Vero cells over time. In the BMDCs there was some variation with the RRV-SW strain inducing a higher percentage of cell death than the RRV-NE strain, 24 h after infection. Collectively, the data indicates that RRV has the capacity to induce a pro-apoptotic response in DCs, with the SW presenting as more aggressive compared to the NE, potentially leading to greater virulence. This data could help to explain the mechanism of RRV persistence in vertebrate hosts, as well as the reported differences in severity and duration of human clinical symptoms. Immunotherapy aimed at correcting the patient’s dysfunctional immune system, may represent a new strategy for the successful medical treatment of RRV infection.
Acknowledgments

This has been a journey beyond anything I would have expected. This honours project saw everything from tears and frustration to happiness and excitement. If it was not for the following amazing people, I doubt I would have made it this far.

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Last but not least I would like to finish with a quote by German physicist Max Planck

*Anybody who has been seriously engaged in scientific work of any kind realises that over the entrance to the gates of the temple of science are written the words: Ye must have faith. It is a quality which the scientist cannot dispense with.*
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Abbreviations

ADE  Antibody-dependent enhancement
APC  Antigen presenting cell
ATP  Adenosine triphosphate
Apaf-1  Apoptotic protease-activating factor -1
AUS $  Australia dollar
Bak  Bcl2 antagonist/killer
Bax  Bcl2 associated X protein
B cell  Bone-marrow derived lymphocyte
Bcl-2  B-cell leukemia/lymphoma 2
BMDC  Bone-marrow derived dendritic cell
CARD  Caspase recruitment domain
CD  Cadherin domain
cDNA  Complementary DNA
CMV  Cytomegalovirus
CPE  Cytopathic effects
CPT  Camptothecin
crmA  Cytokine response modifier A
DC  Dendritic cell
DED  Death effector domain
DMSO  Dimethyl sulfoxide
DISC  Death-inducing signalling complex
dsRNA  Double stranded RNA
DNA  Deoxyribonucleic acid
DPBS  Dulbecco’s Phosphate-Buffered Saline
E1-3  E1-3 glycoprotein
FACS  Fluorescence-activated cell sorting
FADD  Fas-associated death domain
FAM  Fluorescein
FBS  Foetal bovine serum
FcR  Fc Receptor
FLICA  Flurochrome-labeled inhibitors of caspases
FLICE  FADD-like IL-1β-converting enzyme
FMK  Fluoro-methyl keton
GM-CSF  Granulocyte Macrophage Colony-Stimulating Factor
HIV  Human Immunodeficiency virus
IAP  Inhibitor of apoptosis
IFN  Interferon
IL  Interleukin
kb  Kilobase
LCMV  Lymphocytic Choriomeningitis Virus
LPS  Lipopolysaccharide
M199  Medium 199
mAb  Monoclonal antibody
mDC  Myeloid dendritic cell
MHC  Major histocompatibility complex
NE  North-East
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Nsp</td>
<td>Non-structural protein</td>
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<tr>
<td>NSW</td>
<td>New South Wales</td>
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<td>NTPase</td>
<td>RNA nucleoside triphosphatases</td>
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<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
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<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
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<td>PRRs</td>
<td>Pathogen recognition receptors</td>
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<td>PS</td>
<td>Phosphatidylserine</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>RING</td>
<td>Really interesting new gene</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RRV</td>
<td>Ross River Virus</td>
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<td>RRVD</td>
<td>Ross River Virus Disease</td>
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<td>RT</td>
<td>Room Temperature</td>
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<td>SD</td>
<td>Standard Deviation</td>
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<td>SFV</td>
<td>Semliki Forest Virus</td>
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<td>SINV</td>
<td>Sindbis Virus</td>
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<td>ssRNA</td>
<td>Single stranded RNA</td>
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<td>SW</td>
<td>South-West</td>
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<td>Tc</td>
<td>Cytotoxic T cell</td>
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<td>T cell</td>
<td>Thymus derived lymphocyte</td>
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<td>TCID$_{50}$</td>
<td>Fifty Percent Tissue Culture Infectious Dose</td>
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<td>Th</td>
<td>Helper T cell</td>
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<td>TLRs</td>
<td>Toll-like receptors</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>TNF-R</td>
<td>Tumour necrosis factor receptor</td>
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<td>TRADD</td>
<td>TNF-R-associated death domain</td>
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<td>Treg</td>
<td>Regulatory T cell</td>
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<td>VAD</td>
<td>Valylalanyl aspartic acid</td>
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<tr>
<td>vMIA</td>
<td>Viral Mitochondrial Inhibitor of Apoptosis</td>
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<td>UWA</td>
<td>University of Western Australia</td>
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<td>WA</td>
<td>Western Australia</td>
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<td>7-AAD</td>
<td>7-Aminoactinomycin D</td>
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<td>γ</td>
<td>Gamma</td>
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Chapter 1: Introduction

1.1 Ross River Virus

1.1.1 Background

The Togaviridae family of viruses is comprised of two genera, *Alphavirus* and *Rubivirus* (Rulli *et al.* 2005). The *Rubivirus* genus contains only one virus of human importance, the Rubella virus (Martinez and Zapata 2002), while the *Alphavirus* genus contains a group of viruses that are important to humans such as Sindbis Virus (SINV), Semliki Forest (SFV) and Ross River Virus (RRV) (Rulli *et al.* 2007). There are a total of 26 known members of the *Alphavirus* genus based on serological studies that are broadly divided into two groups; the New World (American) encephalitis alphaviruses and the Old World (Globally distributed) arthritogenic alphaviruses (Rulli *et al.* 2005; Ryman and Klimstra 2008). These alphaviruses have all been classified as arthropod-borne viruses (arboviruses) that are transmitted between susceptible vertebrate host and a haematophagous arthropod (Russell 2002; Rulli *et al.* 2005).

Arthritogenic alphaviruses, such as chikungunya virus, o’nyong-nyong virus and Ross River virus (RRV) are associated with worldwide outbreaks of human polyarthritis and arthralgia. Alphavirus-induced diseases are generally not life-threatening disease, however they do have a serious impact on human health, with millions of cases reported each year (Ryman and Klimstra 2008). Most recently, the re-emerging chikungunya virus was responsible for an unprecedented epidemic in a number of countries in the Indian Ocean region. It was the first time that an outbreak was associated with some severe clinical signs and lethal cases of encephalitis (Rulli *et al.* 2005; Sourisseau *et al.* 2007; Lidbury *et al.* 2011).
With various aspects of alphaviruses still unclear including their pathogenesis, the study of this virus for further understanding is becoming extremely important to the future prospects of the discovery of specific treatments or a vaccine (Bielefeldt-Ohmann and Barclay 1998; Rulli et al. 2005). This study aimed to determine the impact of RRV infection on the kinetics of apoptosis in immune and non-immune cell types, to give a greater insight into the pathogenesis of the virus.

1.1.2 Epidemiology

The first possible reports of RRV disease (RRVD) occurred in New South Wales in 1928 and the virus first was isolated from a pool of *Aedes vigilax* mosquitoes near the Ross River in Queensland in 1959 (Harley et al. 2001; Rulli et al. 2005). RRVD was first originally known as epidemic polyarthritis due to the general arthritis observed in infected patients. RRVD was later renamed when the virus was isolated from epidemic Polyarthritis patients (Rulli et al. 2005). RRV is currently endemic in Australia with strains of the virus active annually in all states. RRV is Australia’s most common arbovirus causing up to 8,000 cases annually, costing the community an estimated AUS $1,018 per patient per annum, with the costs due to diagnostics and loss in productivity (Mylonas et al. 2002; Rulli et al. 2005). Local epidemics occur in areas including the Bunbury region of South-West of Western Australia and the Griffith region of NSW (Russell 2002) in association with a range of factors including rainfall and the seasons as well as population exposure (Rulli et al. 2005). As an arbovirus, the incidence of RRV correlates with the mosquito population. Epidemics have also been recorded in a number of Pacific islands including Fiji, Samoa and the Cook Islands (Rulli et al. 2005; Rulli et al. 2007).
RRV is primarily sustained in nature by the transmission cycle between mosquitoes and susceptible vertebrates (Figure 1.1) (Rulli et al. 2005).

**Figure 1.1:** The transmission cycle of RRV

The maintenance cycle for RRV in any geographical area will only need to involve a single arthropod vector and a single vertebrate host, making these viruses enzootic (Rulli et al. 2007). Knowledge about the vertebrate range is limited, with a various range of mammals seen as possible hosts. Non-migratory native macropods are believed to be the main vertebrate hosts for RRV (Rulli et al. 2005; Rulli et al. 2007). The virus has also been isolated from other mammals including the New Holland Mouse and Flying Foxes (Russell 2002; Rulli et al. 2005). Horses, humans and mice are the only hosts of RRV that display clinical signs of infection and are classified as incidental hosts and are believed in exceptional circumstances to be involved in the maintenance cycle. (Rulli et al. 2005; Rulli et al. 2007). Horses are suspected to play an important part in the transmission of the virus from one area to another but there is a still lack of evidence (Rulli et al. 2005).

Horizontal transmission is not believed to be the only form of transmission, as transfer of the virus from mosquito to mosquito that can only be explained by vertical
transmission, has been observed. Vertical transmission could explain how RRV survives between epidemics and over the cooler months (Rulli et al. 2005).

RRV has a complex vector situation compared to other alphaviruses. The virus has been isolated from 42 mosquito species that span over seven genera: *Ochlerotatus* (*Aedes*), *Culex*, *Anopheles*, *Coquillettidia*, *Mansonia*, *Culiseta* and *Tripteroides* (Rulli et al. 2005; reviewed in Russell 2002; Ryman and Klimstra 2008). Although this is a broad vector range, only a small number of species have been accepted as significant in various regions of Australia. The relative importance of the species also varies with the season (Russell 2002; Rulli et al. 2005). The freshwater species *Cx. annulirostris* is the major vector in inland areas, although a number of other species such as floodwater *Aedes* species and *Coquillettidia* species are known to carry RRV. Both northern and southern salt marsh mosquitoes (*Ae. vigilax* and *Ae. camptorhynchus* respectively) are the major RRV vectors in coastal areas while *Ae. notoscriptus* is believed to involved in urban areas (Harley et al. 2001; Russell 2002).

Due to different vector species often involved in different environmental regions, the epidemiological range is complex with climate and environment influencing the vector abundance. Epidemics, in particular in temperate southern areas, are dependent on the summer and autumn rainfall in the inland areas, while the rainfall and/or tidal inundations affect the coastal areas (Russell and Dwyer 2000; Russell 2002; Rulli et al. 2005).

Local factors that increase the human-mosquito exposure also influence the epidemiology. Changes in viral distribution, increase in urbanisation, changes in
agricultural practices and the expanding developments of both residential and industrial areas in coastal regions are involved in the epidemiology (Rulli et al. 2005).

1.1.3 Molecular Biology

1.1.3.1 Structure and Genome

Alphaviruses are a closely related group of enveloped viruses with a single-stranded positive sense RNA genome (Strauss and Strauss 1994; Cheng et al. 1995; Rulli et al. 2005; Ryman and Klimstra 2008). The RRV has a 11.7kb genome, this can vary slightly between strains (Faragher et al. 1988; Rulli et al. 2005).

The virion expresses two glycosylated proteins: E1 (52kDa) and E2 (49kDa), while a third, E3, encoded by the genome, is not expressed (Faragher et al. 1988; Rulli et al. 2005). The icosahedral symmetrical nucleocapsid protein C (32kDa), with a diameter of 40 nm, is surrounded by a 4.2 nm thick phospholipid bilayer derived from the host. At the core of the virion is the genomic RNA (Figure 1.2 A). On the surface of the virion, 240 heterodimers of E1 and E2 form 80 trimeric spikes (Strauss and Strauss 1994; Rulli et al. 2005). In the phospholipid bilayer, these heterodimers associate one-to-one with the nucleocapsid monomers, as shown in Figure 1.2 B (Cheng et al. 1995; Rulli et al. 2005). The heterodimer undergoes irreversible conformational changes when it is exposed to a acidic fusion pH, triggering the cell/virus fusion (Strauss and Strauss 1994; Bielefeldt-Ohmann and Barclay 1998; Rayner et al. 2002; Wengler 2009).
Figure 1.2: Ross River Virus reconstructed images of (A) a cutaway of the multilayered structure of the virion and (B) a cross-section view of the overall organization within the multilayered structure. The distances from the viral centre are marked on the right. Both diagrams are coloured to represent the different viral components; the glycoprotein heterodimer(s) in blue, the phospholipid bilayer in green, the core protein in yellow and the RNA-protein interior in red. Å= 0.1 nm. [Figures originally from A) (Strauss and Strauss 1994) B) (Cheng et al. 1995) Re-coloured by (Sanders 1999)].

There are two open reading frames (ORFs) present in the RRV genome, with two-thirds transcribed from the 5’ end and the other one-third transcribed from the 3’ end of the viral RNA (Figure 1.3) (Rayner et al. 2002; Ryman and Klimstra 2008). The 5’ end encodes the non-structural proteins (nsPs) that are involved in the replication and synthesis of the genome (Rulli et al. 2005). Each of these proteins has a specific role. The first nsP (nsP1) is involved in binding and assembly of the virus replication complex to the intracellular membrane of the host cell. The second nsP (nsP2) is a RNA binding protein with NTPase and protease activity, whereby the nsPs are processed post-translationally. It is speculated that nsP2 functions as a RNA helicase. NsP3 is a phosphoprotein while nsP4 is believed to be a polymerase, based on mutagenesis studies and sequence homology with RNA-dependent RNA polymerases (Rayner et al. 2002).
The structural proteins are encoded from the 26s sub-genomic mRNA that originates from the one-third 3’ end genome. The capsid protein interacts with the RNA genome producing the nucleocapsid, while the glycoproteins form E1, E2 and E3 and the hydrophobic peptide-linker 6K protein (Rayner et al. 2002; Rulli et al. 2005). The capsid protein is autocatalytic and cleaves itself from the polyprotein transcript while the rest of the polyprotein is processed in the Endoplasmic Reticulum by cellular enzymes of the host. This digested product is cleaved in the secretory vesicles to produce the heterodimers with the cleavage of the polyprotein E2 by Furin-like proteases producing E2 and E3 (Rayner et al. 2002; Ryman and Klimstra 2008).

Each glycoprotein is involved in a specific action in the replication of the virus. E1, the haemagglutinin (Burness et al. 1988), plays a part in the fusion with the endosome.
membrane, while E2 is involved in the attachment of the virus to the host cell receptor (Strauss and Strauss 1994; Rayner et al. 2002; Ryman and Klimstra 2008). E2 has been identified to carry a major antigenic site, defined by epitopes \( a, b1 \) and \( b2 \), for antibody neutralization (Burness et al. 1988; Vrati et al. 1988; Rulli et al. 2005). These epitopes have been recognised by monoclonal antibodies (mAbs), T10C9, NB3C4 and T1E7, respectively (Vrati et al. 1988; Oliveira et al. 1997).

The E2 protein has also been suggested to play a role in RRV virulence. Vrati et al. (1986) observed in mice, a RRV mutant, which carried a 21-nucleotide deletion in the E2 gene, appeared asymptomatic compared to wild-type RRV-infected animals. Vrati et al. (1996) also observed that epitope \( b2 \) mutant variants of the T48 strain showed rapid penetration; however the growth kinetics and RNA/synthesis were impeded compared to the T48 parent. A recent study into viral determinants of alphavirus-induced rheumatic disease observed that mutations in nsP1 and PE2 play an important role in determining RRV-induced musculoskeletal inflammatory disease in a mouse model (Jupille et al. 2011). These studies indicated that the E2 (including PE2) and nsP1 proteins play a very important role in RRV pathogenesis. Virulence, genetic and antigenic variation will be discussed later in section 1.1.4.4.

### 1.1.3.2 Replication Cycle – Endosomal Pathway

The primary site for RRV replication is skeletal muscle before the virus enters the blood stream (Harley et al. 2001). There is believed to be two entry pathways into the host cells; the endosomal-based pathway (Strauss and Strauss 1994; Sharkey et al. 2001; Rayner et al. 2002; Ryman and Klimstra 2008) and the antibody-dependent
enhancement (ADE) pathway which is still a relatively new concept (Takada and Kawaoka 2003).

The endosomal pathway is common in the replication life cycles of many viruses (Brooks et al. 2007) (Figure 1.4). When the virion enters the host cell by the endosomal pathway, the E2 glycoprotein must first attach to an integrin cell surface receptor (Harley et al. 2001; Ryman and Klimstra 2008). Studies showing that both C-type lectins; (DC-SIGN and L-SIGN) (Ryman and Klimstra 2008) and α1β1 integrin receptor (Linn et al. 2005) as possible RRV receptors. The C-type lectins have been observed to bind to the E1-E2 heterodimer complex (Ryman and Klimstra 2008). Two conserved regions in the E2 glycoprotein mimic VI collagen, which the α1β1 integrin has a high affinity of binding. This would allow RRV to utilize these conserved sequences to bind to the α1β1 integrin as a receptor to enter mammalian cells (Linn et al. 2005). Human Immunodeficiency virus (HIV) vectors that express RRV envelope glycoproteins have been observed to infect cells from different species however some cells could not be infected, suggesting that the host receptors may be limited or absent on certain cell types (Strang et al. 2005).

Once attachment has occurred, the virion is enclosed into an endosome (Strauss and Strauss 1994; Rayner et al. 2002; Ryman and Klimstra 2008). Within the endosome, the acidity increases as a defence mechanism causing conformational changes in the E1-E2 heterodimer spikes (Rayner et al. 2002; Ryman and Klimstra 2008). The irreversibly changed E1 allows for the virion to fuse with the endosome’s membrane, allowing the virus to uncoat and release the nucleocaspid into the cytoplasm unharmed (Strauss and Strauss 1994; Harley et al. 2001).
The viral RNA genome is replicated and transcribed by the host cell’s mechanisms. The new synthesized RNA and caspid protein are assembled together producing the nucleocaspid of the progeny virus (Rayner et al. 2002; Ryman and Klimstra 2008). The nucleocaspid migrates to the area of the host’s cell membrane where the interaction between the cytoplasmic domain of the glycoproteins and membrane is occurring. The new mature progeny are complete once they bud from the membrane (Rayner et al. 2002).

Figure 1.4: The simplified life cycle of RRV via Endosome Pathway (Kightley 2005).

1.1.3.3 Replication Cycle- Antibody Dependent Enhancement

Antibody-dependent enhancement (ADE) activity has been observed in a number of different viruses including HIV, Dengue fever virus, Influenza and some alphaviruses (Lidbury and Mahalingam 2000). ADE is a process where at low concentrations or sub-neutralizing levels, antibodies enhance viral infection by attaching to the Fc receptors (FcR) resulting in entry via endocytosis (Mahalingam and Lidbury 2002; Takada and Kawaoka 2003; Rulli et al. 2005) (Figure 1.5 A). This is dependent on the cross-linking of complexes of virus/antibody through interactions with cellular molecules (Takada and Kawaoka 2003).
The most common form of ADE is FcR-dependent when the Fc portion of the antibody of the antibody/virus complex attaches to the Fc-γ receptor of phagocytic cells including macrophages and monocytes (Lidbury and Mahalingam 2000; Takada and Kawaoka 2003). Two concepts of ADE have been observed for RRV-ADE, 1) the entry through the attachment of the virus/antibody complex to the FcR and the involvement of a secondary co-receptor and 2) the primary FcR to catalyse membrane fusion (Takada and Kawaoka 2003) (Figure 1.5 B).

Linn et al (1996) reported the first occurrence of RRV-ADE in macrophages, which has since led to a number of significant findings including the ability of RRV to suppress or disrupt the antiviral responses of the innate immune system via ADE pathway (Mahalingam and Lidbury 2002; Suhrbier and La Linn 2003).

1.1.4 Ross River virus Disease and Pathogenesis

The pathogenesis of alphaviruses is poorly understood, in particular alphavirus-induced arthritides (Franssila and Hedman 2006) and persistence of the virus (Way et al. 2002). Small animal models are used to understand the pathogenesis...
of alphaviruses. Whilst, the majority of models focus on virus-induced neurological disease (Rulli et al. 2005), relatively little is known about virus-induced arthritides and persistence (Rulli et al. 2005; Franssila and Hedman 2006). The incubation period of the virus generally occurs for 7-9 days, however it can vary from 3-21 days (Harley et al. 2001; Franssila and Hedman 2006; Barber et al. 2009).

Relevant RRV infection mice models are used to understanding the associated pathophysiology and the mechanisms that contribute to immunity and pathogenesis (Griffin 2001; Rulli et al. 2005). Although these RRV mouse models are used, there are no known models that are 100% relevant to the natural pathogenesis of old world alphaviruses (Bielefeldt-Ohmann and Barclay 1998; Ryman and Klimstra 2008).

It is hypothesised that persistence may be the result of ineffectual clearance by poor or impaired immune responses, in particular the cell-mediated response (Linn et al. 1998; Rulli et al. 2005; Rulli et al. 2007). There are a number of viruses that are associated with a dysfunctional immune system resulting in persistence. Measles (Linn et al. 1998), strains of Lymphocytic Choriomeningitis Virus (LCMV) (Carbone and Heath 2003), Cytomegalovirus (CMV) (Andrews et al. 2001) and HIV (Knight and Patterson 1997; Whitton and Oldstone 2001) are some of the well known viruses that impair the immune system by targeting immune cells, in particular dendritic cells (Vidalain et al. 2001).

1.1.4.1 Immune Response to Viral Infection

The mammalian immune response comprises an array of protective mechanisms that aim to eliminate invading pathogens from the host. These protective mechanisms are
divided into two major branches, the non-antigen specific innate and the antigen-specific adaptive system (Chaplin 2006).

Rapid developing innate responses recognises conserved microbial components termed pathogen associated molecular patterns (PAMPs) such as dsRNA, ssRNA, lipopolysaccharide (LPS), and unmethylated CpG-DNA (Ploegh 1998; Whitton and Oldstone 2001; Barton and Medzhitov 2002; Chaplin 2006; Cunningham et al. 2010). The components of the innate response include Natural Killer cells, macrophages, dendritic cells, neutrophils, various cytokines (interferon (IFN), tumour necrosis factor (TNF), interleukin (IL)) and complement (Whitton and Oldstone 2001; Wood 2006). This response is an initial response to infection that subsequently primes the adaptive response if the infection continues to persist (Ploegh 1998; Palucka and Banchereau 1999; Turvey and Broide 2010).

Consisting of both humoral and cellular elements, the slower developing adaptive response is mediated by T and B lymphocytes which recognise specific antigen motifs or epitopes of a pathogen by their cell surface receptors. Antibodies mainly act to neutralize the infectivity of free virus by binding to the glycoproteins that interact with the host cell receptors, preventing attachment (Ploegh 1998; Whitton and Oldstone 2001; Wood 2006). Virus-specific antibodies may also agglutinate multiple virions into a group reducing infectivity or enhance antibody-dependant cell-mediated cytotoxicity (Whitton and Oldstone 2001; Wood 2006).

T cells recognise and often kill infected cells before virus can replicate, reducing the release of infectious virions. The major subtypes in the immune response to viruses are
the CD4+ T helper (Th1/Th2/Th17) and CD8+ cytotoxic T cells (Tc). CD8+ T cells produce antiviral cytokines (IFN-γ and TNF-α); induce apoptosis in infected cells by the Fas-ligand pathway or by granule exocytosis. CD4+ T cells assist in B cell maturation, antibody isotype switching and direct the adaptive response through cytokine secretion (Whitton and Oldstone 2001; Wood 2006). Th1 cells support cell-mediated response through the release of IFN-γ, while Th2 cells support humoral, anti-helminthic and allergic responses through the release of IL-4, IL-5 and IL-13. Regulatory T cells (Treg) are another class of CD4+ T cells that mediate immune regulation through the release of inhibitory signals such as IL-10 (Barton and Medzhitov 2002; Kopp and Medzhitov 2003; Chaplin 2006). Th17 cells support pro-inflammation response, through the release of IL-17 however appears to be involved in the protection in pathogens (Hou et al. 2009).

The major histocompatibility complex (MHC) proteins help in directing the type of immune response through the presentation of processed peptides to T cells, MHC I (endogenous pathogens) is expressed by all nucleated cells, while MHC II (exogenous pathogens) is only expressed by specialized antigen presenting cells (APCs). The specificity of the MHC class/peptide complex recognized by a T cell receptor and the T cell surface marker (CD4 or CD8) allows for T cells to distinguish the source of the antigen as either intracellular (e.g. virus) or extracellular (e.g. soluble protein) (Whitton and Oldstone 2001; Barton and Medzhitov 2002). CD8+ T cells recognise intracellular antigens in conjunction with MHC I complexes, while CD4+ T cells recognise exogenous antigen in conjunction with MHC II complexes (Whitton and Oldstone 2001; Wood 2006). An alternative pathway termed cross-presentation, whereby
exogenous antigen can access the intracellular MHC Class I processing pathway, has also been described (Carbone and Heath 2003).

In RRV infection, the clearance of viraemia is believed to principally mediated by antibodies (Yu and Aaskov 1994) and type I IFN (α/β) (Hwang et al. 1995). Neutralizing antibodies may be important in resolving acute togavirus infection viremia; however several reports suggest that antibodies are unable to clear residual persistent infection. The failure of neutralizing antibodies in clearance has been observed in human Rubella infections where arthritis and late-onset Rubella syndrome occur (Chantler et al. 1985; Verder et al. 1986) and in mouse brains infected with SFV or SINV (Hapel 1975; Levine et al. 1994; Amor et al. 1996).

T cell responses are also believed to be significant contributors in the elimination of RRV, where reports of CD8\(^+\) T cell activity in mouse models appeared to associate with the clearance from the peripheral blood (Linn et al. 1998; Rulli et al. 2005) and the predominance of CD4\(^+\) T cells in synovial effusions in chronic RRVD (Linn et al. 1998; Harley et al. 2001). The predominance of CD4\(^+\) T cells in the serum of chronic RRVD patients, as opposed to CD8\(^+\) T cells, could initiate a local non-specific immune response, involving an influx of mononuclear cells instead of a virus-specific cell mediated response. This suggests that the development of chronic arthritis in some patients may be due to the inability of the cell-mediated response to completely clear infection (Soden et al. 2000; Tupanceska et al. 2007). These cells appear to play a role in determining if a patient experiences rapid recovery or chronic effects of RRVD (Harley et al. 2001).
As stated previously, via the ADE pathway, RRV has the ability to negatively impact the innate antiviral responses of the host. In RRV-ADE infected macrophages several antiviral genes such as TNF-α and inducible nitric oxide synthase which have a major impact on virus survival are inhibited (Lidbury and Mahalingam 2000; Takada and Kawaoka 2003). Experiments involving LPS induced antiviral activity by Lidbury and Mahalingam (2000), observed that when ADE infection of macrophages occurred, RRV survived and was able to replicate unrestricted. Other studies demonstrate an increase in the immunosuppressive cytokine interleukin-10 (IL-10) in RRV-ADE infection. The ability of the virus to inhibit antiviral proteins and potentially manipulate the expression of others, allows the opportunity for enhanced viral pathogenesis and survival (Mahalingam and Lidbury 2002).

1.1.4.2 Dendritic Cells

Dendritic cells (DC) are specialised immune cells that originate from the bone marrow (Whitton and Oldstone 2001). These cells are found throughout the body, distributed strategically as sentinels in anatomic sites such as the skin and mucosal surfaces that are exposed to antigens and pathogens (Palucka and Banchereau 1999; Whitton and Oldstone 2001; Vermaelen and Pauwels 2005). DCs express a variety of pathogen recognition receptors (PRRs) to enable rapid recognition and binding to PAMPs at the initial site including toll-like receptors (TLRs). TLRs are a family of receptors that are able to recognise a wide variety of conserved microbial sequences. The expression of TLRs has been described in humans and a wide variety of other species (Barton and Medzhitov 2002; Finberg and Kurt-Jones 2004; Chaplin 2006; Cunningham et al. 2010). DCs consist of plasmacytoid (pDC) and myeloid (mDC) subtypes that have common and distinct functions (Palucka 2000; Vermaelen and Pauwels 2005). MDCs
which include Langerhan’s and dermal DCs, stimulate adaptive responses while pDCs stimulate the innate response through the production of IFNs (Merad et al. 2008).

DCs found in anatomic sites are termed immature due to their poor ability to stimulate naïve T cells. These immature DCs have the capacity to capture and process pathogens and antigens and sense their origin and pathogenicity through TLR binding (Palucka and Banchereau 1999; Vermaelen and Pauwels 2005). Following TLR signalling, the immature DCs undergo maturation which involves a number of dramatic changes resulting in the up-regulation of MHC, co-stimulatory molecules, the expression of cytokines and pro-inflammatory chemokines (Barton and Medzhitov 2002; Kopp and Medzhitov 2003; Vermaelen and Pauwels 2005). This combination enables the DC to activate naïve T cells and initiate adaptive immunity. The mature DCs can then migrate to the local draining lymph node, where the immune response is regulated through the production of cytokines, chemokines and presentation of processed antigen to T cells (Palucka and Banchereau 1999; Vermaelen and Pauwels 2005). Signalling through MHC, co-stimulatory molecules and TLR helps determine the nature of the T cell response, for example Th1 (cell-mediated), Th2 (humoral), Th17 (pro-inflammatory) or Treg (regulation) (Barton and Medzhitov 2002; Chaplin 2006). Tc activation by DCs can occur by endogenous or cross-presentation. DCs can activate the CD8 directly, however assistance from Th cells is generally needed (Banchereau et al. 2000).

1.1.4.3 Dendritic cells and Viruses

Viruses that infect DCs can enhance their pathogenesis by utilising and altering the specialized DC functions, by inducing marked changes (e.g. apoptosis) and/or by more widespread, subtle changes (e.g. altering cytokine production) (Palucka and
Banchereau 1999; Cunningham et al. 2010). The effects of infection are diverse, with viruses from the same and different families impacting DCs differently by either inhibiting, stimulating or having no effect on maturation, antigen presentation and cytokine/chemokine production (Cunningham et al. 2010).

HIV, measles virus, LCMV and CMV are cell known viruses that utilise DCs in their pathogenesis. HIV is believed to use DCs as a transport vehicle for virus dissemination and a secure site for virus replication (Knight and Patterson 1997; Palucka 2000). Studies on the impact of HIV infection in DCs have demonstrated that cell death by apoptosis and necrosis is induced during the fusion of HIV and pDCs (Knight and Patterson 1997; Meyers et al. 2007).

Measles virus causes immunosuppression by limiting the maturation of DCs (Carbone and Heath 2003; de Witte et al. 2006), inhibiting IL-12 secretion and inducing apoptosis (Servet-Delprat et al. 2000; de Witte et al. 2006). In co-cultures of DCs and T cells in vitro, measles virus-infected DCs fail to stimulate T cell proliferation and eventually undergo Fas-mediated apoptosis (Moss et al. 2004; de Witte et al. 2006).

LCMV stimulates the production of IL-10, inhibiting the broad-spectrum response of stimulatory cytokine production, T cell proliferation and B cell activation (Kane and Golovkina 2010). Studies on LCMV-infected DCs show a poor CD8+ T cell responses and long-term persistence (Sevilla et al. 2000)

Recent studies on CMV infection in DCs showed an impaired capacity of the cells to initiate immune responses, suggesting that the primary function was inference with DC
function. In murine cytomegalovirus infection, DCs showed an impaired capacity to completely prime T cells, with a reduced ability to secrete IL-2. Infected DC also showed a down-regulation in surface MHC and co-stimulatory molecules and a mixed phenotype of impaired endolytic capacity (Andrews et al. 2001).

In the last few decades the interactions between arboviruses, including alphaviruses and DCs have become an increasing focus. DCs have been observed as primary targets early in dengue virus infection (Palucka 2000; Wu et al. 2000; Palmer et al. 2005). The pathogenesis of dengue virus is still elusive, with studies on dengue virus-DC interactions demonstrating the implications of dengue virus on DCs. Palmer et al (2005), observed the failure of infected DCs to respond to TNF-α, a maturation stimuli producing a less mature phenotype compared to uninfected DCs, leading the cells to undergo apoptosis. These infected DCs also produced IL-10 with reduced capacity to stimulate T cells. It is possible that dengue virus impairs the antigen presentation function by the blockade of maturation and the induction of apoptosis.

Within the alphavirus genus there are known differences in natural cell tropism. An example is Venezuelan equine encephalitis being lymphotropic and transducing murine DCs (Gardner et al. 2000; MacDonald and Johnston 2000) while SFV is not lymphotropic. Studies of SINV have shown that variants of this virus differ in their ability to infect DCs. Through these studies it was observed that SINV variants infect human and murine DCs differently, suggesting that the receptor expression between the DCs differs (Gardner et al. 2000). Studies by Sourisseau et al (2007) suggested that human DC infection by SINV vector is determined by the substitution of a single amino acid in E2. Studies on SINV mosquito-cell derived virions indicate
preferentially infection of human mDC, due to the interaction between the high mannose glycans from the virion and the DC-specific DC-SIGN lectin (Shabman et al. 2007). Mosquito-derived Venezuelan equine encephalitis compared to the mammalian-derived virus exhibits enhanced infection of murine mDC (Sourisseau et al. 2007).

Shabman et al (2007) observed the interaction of mosquito and mammalian cell-derived RRV with murine and human mDCs. They noted that mosquito-cell-derived virus had a greater efficiency infecting mDCs yet were a poor inducer of type I IFN compared to the mammalian-cell-derived virus, which was a potent inducer of type I IFN. These studies suggest the virus when initially delivered by the mosquito vector is able to avoid the induction of type I IFN during the initial infection of DCs. Further studies observed that N-linked glycans that were present on the E2 glycoprotein of mammalian-RRV were required for a robust IFN-α/β induction in mDC cultures. This suggests that the mosquito- and mammalian-cell-derived viruses may be directed into different compartments within the mDC via differential interactions with mDC surface lectin receptors. It may also possible that the induction of IFN-α/β could be independent of lectin receptors, as the pattern recognition molecule might only recognise the mammalian-RRV envelope (Shabman et al. 2008).

HIV vectors pseudotyped with RRV envelope glycoproteins can infect/transduce murine DCs and a number of cell types from different species (Strang et al. 2005; Sourisseau et al. 2007). This HIV-RRV vector failed to infect/transduce human hematopoietic cells and T cells from human and murine sources (Strang et al. 2005). It appears that multiple parameters regulate the ability of alphaviruses to infect and impact DCs (Sourisseau et al. 2007).
1.1.4.3 Clinical Symptoms

The RRV disease has three major characteristic symptoms; rheumatic symptoms, rash and constitutional effects i.e. fever, fatigue, headache and myalgia. The onset of symptoms occurs suddenly with patients presenting a rash and joint pain (arthralgia) in the extremities, a couple of days post-infection. The rash only appears in two-thirds of patients (Rulli et al. 2005; Rulli et al. 2007).

The rheumatic joint pain (arthralgia) may occur in a variety of joints with the wrists, knees, toes, fingers, ankles and elbows the major sites (Rulli et al. 2005; Barber et al. 2009). The torsal joints can also be affected. The arthralgia has a broad manifestation range with some patients only having tenderness with minor restriction of movement to severe redness and swelling. The rash presents as either maculopapular, vesicular or purpuric on the torso and limbs of the patient and generally lasts for 10 days. The rash is believed to be the result of cell-mediated response towards viral antigen in the skin (Fraser et al. 1983; Rulli et al. 2005).

Although these symptoms are common for the majority, not all patients exhibit every symptom. Asymptomatic patients also occur in the population (Barber et al. 2009).

There is some discrepancy between studies in terms of the frequency, spectrum and recovery period of clinical RRVD patients (Condon and Rouse 1995). Studies on RRVD from the east coast show short recovery times with little or no persistence (Harley et al. 2001). Studies from the south of WA show that patients experience longer recovery times with persistence as well as some differences in the spectrum of the symptoms. Although an explanation for this is still undetermined, it has been
suggested that different strains may vary in their pathogenesis leading to some differences in the clinical aspect (Condon and Rouse 1995).

RRV has been observed to infect a wide range of cell types including fibroblasts, skeletal muscle, myocytes, endothelial cells, epithelial cells, macrophages and monocytes (Linn and Suhrbier 1997; Linn et al. 1998). The large range of cell types corresponds to the variety of symptoms that can be present in patients. Arthralgia/arthritis could result from the infection of synovial fibroblasts, myalgia may result from infected myocytes and the rash involves infected epithelial and epidermal cells (Linn et al. 1996). Rheumatism in patients results from the destructive and inflammatory nature of the viral cytopathic effects and the cell-mediated immune response to viral antigen. Synovial exudates from patients comprise monocytes, macrophages, B cells, Natural Killer cells and a high number of CD4+ and CD8+ T cells (Rulli et al. 2007). This composition is unlike the majority of viral associated arthritis as no immune complexes are present and neutrophils are rare, indicating that RRV associated arthritis involves a different mechanism (Rulli et al. 2005).

There is currently no treatment shown to reduce the duration or alter the course of RRVD. Symptom-based treatments such as physiotherapy and anti-inflammatory drugs have been shown to help some patients (Russell and Dwyer 2000; Barber et al. 2009).

1.1.4.4 Genetic Variation and Virulence

Distinct strains of RRV have been described that vary genetically and antigenically, supporting the theory that microevolution in separate geographical areas has occurred (Mackenzie et al. 1995; Vrati et al. 1996; Russell 2002). Isolates from different
geographic regions can differ in: virulence levels in both mice and mosquitoes: and kinetic haemagglutination and complement fixation tests (Kerr et al. 1992; Lindsay et al. 1993; Russell 2002). For example, T48 is a mouse virulent strain whereas NB5092, a Nelson Bay, NSW isolate, is a mouse avirulent strain. These strains can be serologically distinguished in neutralization tests, by TIE7 mAb, and show little divergence in sequence at the amino acid level (Faragher et al. 1988).

RRV studies at the molecular level are inconclusive. Genetic variation of alphaviruses is a slow process, as a new variant must be able to survive and replicate in both vertebrate and invertebrate hosts (Weaver et al. 1992). Extensive studies into the genomic relationship, molecular epidemiology and evolution of RRV were carried out by Lindsay et al. (1993) and Sammels et al. (1995). Lindsay et al. (1993) conducted a comparative analysis of 80 isolates from around Australia by RNase T1 oligonucleotide mapping. Results indicated the occurrence of four genotypes which associated with particular geographical regions. These distinct clusters are known as topotypes. The majority of the isolates tested could be classified into two major circulating topotypes; one from the east and the other from the west of Australia (Mackenzie et al. 1995; Oliveira et al. 1997; Russell 2002).

Sammels et al. (1995) sequenced a 505 base pair product from cloned or PCR-amplified RRV cDNA from the E2/E3 region of 56 isolates. Three genotypes were identified from this study. Genotype I contained only isolates from Queensland prior to 1967 however all isolates after 1967 are from genotype II. This genotype is believed to have originated from the east coast and dominates throughout Australia and the Pacific. Isolates from genotype III, have only been found in the South-West of WA (Mackenzie
et al. 1995; Sammels et al. 1995; Smith et al. 2008). Together these two studies both concluded the existence of two genotypes that are present in Australia, and based on their geographic origin, genotype II is better known as North-East (NE) genotype and genotype III is known as South-West (SW).

Prior to 1995, the SW genotype was the predominate genotype in the SW of WA. Since 1995, the NE genotype has increased in dominance, largely replacing the SW genotype. The cause of this shift in dominance still remains unclear (Russell and Dwyer 2000; Russell 2002; Smith et al. 2008).

A diagnostic enzyme-linked immunosorbent assay has been developed to allow for an antigenic differentiation between the two genotypes using mAb 43B2 which only tests positive to the NE isolates. Using a panel of RRV-specific mAbs, including 43B2, isolates can be antigenically identified from mosquitoes and other sources (Oliveira et al. 1995; Oliveira et al. 1997; Oliveira et al. 2006).

The involvement of genotype in pathogenesis is still unknown, however, clinical surveys from patients has suggested that genotype may have an impact. A study of patients from the South-West by Condon and Rouse (1995) found a number of differences between the frequency and spectrum of clinical symptoms, with a longer period of recovery compared to studies from elsewhere in Australia. All patients were infected during the 1988/89 outbreak in the region. Mouse model studies demonstrated differences between genotypes including the SW phenotype, which appeared more virulent with a lack of neutralizing antibody production in comparison to the levels produced towards the NE phenotype (Prow 2006).
Although there are genetic and antigenic differences present in the RRV population, further differentiation may help to explain the domination of the NE genotype and pathogenesis of RRV.

1.2 Apoptosis

1.2.1 Background

Programmed cell death, otherwise known as apoptosis, is an essential physiological process involved in development, maintenance of homeostasis and host defence in multicellular organisms (Griffin and Hardwick 1997; Chang and Yang 2000; Clayton and Hardwick 2008). Apoptotic mechanisms help eliminate potentially dangerous pathogen-infected cells, such as the auto-reactive lymphocytes during negative selection (Griffin and Hardwick 1997; Kumar et al. 2005). Dysfunction of this process has implications in a number of diseases including cancer and autoimmune disease (Chang and Yang 2000).

Apoptotic cells undergo stage-specific morphological changes, with correlating biochemical characteristic (Li and Stollar 2004) (Figure 1.6). The first signs of apoptosis include cytoplasmic and nuclear condensation followed by blebbing of the intact cytoplasmic membranes. The plasma membrane loses polarity, exposing phosphatidylserine (PS). The externalization of PS allows for the binding of Annexin V, which can be used for apoptosis analysis (Koopman et al. 1994; Vermes et al. 2000). The chromosomal DNA is cleaved by activated endonucleases into nucleosome length fragments. The cell shrinks and fragments into membrane-bound bodies, which
Figure 1.6: A depiction of the four stages of apoptosis; stimulation signal (1), the intracellular response to stimulus (2), apoptotic changes (3), and phagocytosis or persistence of inflammation through cell lysis (4). [Figure from (Afford and Randhawa 2000)]

Apoptosis involves the initiation of different apoptotic pathways; the type of apoptotic pathway is being determined by the initiation stimulus. There are two major pathways; the intrinsic or mitochondrial-mediated pathway that is triggered by intracellular events

contain cytoplasmic and nuclear material, known as apoptotic bodies (Uren and Vaux 1996; Griffin and Hardwick 1997).

The apoptotic bodies are engulfed by neighbouring cells and tissue macrophages without mediating inflammation (Li and Stollar 2004). Apoptosis is different from necrosis both biochemically and morphologically (Griffin and Hardwick 1997). Necrotic cells lose the integrity of their membrane early resulting in the spillage of the cytosol into the surrounding tissue, mediating inflammatory responses (Griffin and Hardwick 1997; McConkey 1998).
and the extrinsic or death receptor-mediated pathway that is triggered by extracellular events. An additional pathway is the endoplasmic reticulum stress pathway that is triggered by stress in the endoplasmic reticulum resulting in the release of intracellular calcium (Li and Stollar 2004).

1.2.2 Major Pathways

1.2.2.1 Intrinsic/ Mitochondrial Pathway

The intrinsic pathway initiators involve any stimuli that occur within the cell such as DNA damage caused by UV irradiation and cytotoxic drugs (Kumar et al. 2005). The intracellular stimulus activates Bcl-2 homology domain- 3 proteins only by post-transcriptional means (Urban et al. 2008). The activity of the pro-apoptotic Bcl-2 family members; Bax and Bak, trigger pores to form in the mitochondrial membrane, via an unknown mechanism, allowing for the release of cytochrome C and other apoptogenic factors (Irusta et al. 2003; Urban et al. 2008). An apoptosome complex is formed by the interactions of dATP/ATP, cytochrome C and the apoptotic protease-activating factor- 1(Apaf-1) and procaspase 9/caspase 9, which are recruited by cytochrome C, which activates the caspases 3, 6 and 7 (Kumar et al. 2005; Urban et al. 2008). These activated caspases lead to the caspase-mediated degradation of protein substrates resulting in apoptosis (LeBlanc 2003).

The intrinsic pathway is regulated by members of the Bcl-2 family (Kumar et al. 2005). The family consists of proteins that are pro-apoptotic and anti-apoptotic (Griffin and Hardwick 1997; Irusta et al. 2003). The Bcl-2 family proteins play an important role in resistance of cells to death. When the anti-apoptotic genes are over expressed or the pro-apoptotic genes are down regulated, cells become 'immortal' (Griffin and Hardwick
An example of this is the over expression of Bcl-2 in B cell lymphomas (Griffin and Hardwick 1997). Some viruses that are known to cause cell death, are unable to induce apoptosis in cells that over express Bcl-2 (Levine et al. 1993).

Figure 1.7: The Intrinsic Pathway. (Figure from (Cell Signaling Technology 2011))
1.2.2.2 Extrinsic / Death-Receptors Pathway

The extrinsic pathway is initiated by the binding of receptor specific ligand to the death receptors on the cell’s surface. The TNF gene super family receptors (TNF-R1, TNF-R2) and Fas/CD95 are the death receptors for mammalian cells and are classified as type 1 transmembrane proteins. These receptors have cysteine-rich extracellular domains and intracellular cytoplasmic death domain, which are essential for signal transduction and apoptosis to occur (Kumar et al. 2005; Urban et al. 2008).

Stimulation of the death receptors initiates the formation of the death-inducing signalling complex (DISC). In Fas-mediated caspase-dependent apoptosis the death domain of DISC binds to the death domain of the adapter Fas-associated death domain (FADD). The death domain-related death-effector domain (DED) of FADD binds to the DED present on caspase 8, resulting in caspase 8 to oligomerize into DISC. Caspase 8 cleaves caspase 3, leading to the degradation of substrates. In TNF-R1 mediated apoptosis, the death domain of TNF-R1 associates with the death domain of the intracellular TNF-R- associated death domain (TRADD) (Clayton and Hardwick 2008; Urban et al. 2008).

In DISC, TRADD recruits FADD and caspase 8, leading to the cleavage of caspase 3 (Clayton and Hardwick 2008). The extrinsic and intrinsic both converge at the activation of caspase 3. There is also some cross over between the two, when caspase 8 mediates cleavage of the Bcl-2 family member Bid into the active truncated Bid (tBid) (LeBlanc 2003)
1.2.3 Caspases

Activated cysteinyi aspartate-specific proteases, known as caspases, play a key role as the central executioners in apoptosis (LeBlanc 2003). These enzymes cleave cellular substrates after an aspartate residue, at the carboxyl end, leading to apoptosis (Li and Stollar 2004; Wang et al. 2008). The caspase family members may differ in their primary sequence and substrate specificity, however members have several common

![Death Receptor Pathway](cell_signaling_technology_2011.png)

**Figure 1.8**: The Death receptor Pathway. (Figure from (Cell Signaling Technology 2011))
features including that all the caspases are initially synthesised as inactive procaspases (zymogens) (Oliver and Vallette 2005).

Caspases, based on the sequence similarity among the protease domains, can be divided into three different groups. The first group consists of the inflammatory caspases (1, 4, 5,11,12,13 and 14), the second group consists of two caspases (2 and 9) and the third group consists of the remainder (3, 6, 7, 8 and 10) (Chang and Yang 2000; Fan et al. 2005)

The caspases are also classified into three groups based on their function; the initiator caspases, the effector/executioner caspases and the inflammatory caspases (Grandgirard et al. 1998; Fan et al. 2005; Oliver and Vallette 2005).

The initiator caspases (2, 8, 9, 10 and 12) have structurally long pro-domains and recognisable homolytic protein-protein interaction motifs, including the death effector domain (DED) and caspase recruitment domain (CARD), which contribute to the transduction of signals into proteolytic activity (Fan et al. 2005; Oliver and Vallette 2005). The initiators are activated first after a cell death initiation signal triggers apoptosis (LeBlanc 2003) resulting in proteolytic activity. The major initiator caspases are 8 and 9, that function through distinct pathways, but culminate in the activation of procaspase 3 (Oliver and Vallette 2005).

The effector or executioner caspases (3, 6 and 7) lack intrinsic enzyme activity and have short pro-domains (Oliver and Vallette 2005). These caspases are responsible for the irreversible cell damage by cleaving most of the known apoptotic substrates leading
to cell death (LeBlanc 2003; Li and Stollar 2004; Oliver and Vallette 2005). Caspases 1, 4, 5 and 11-14 are classified as inflammatory mediators and do not appear to play a role in apoptosis (Grandgirard et al. 1998; LeBlanc 2003).

Proteases are negatively regulated by different groups of inhibitors, both natural and artificial (Ekert et al. 1999; LeBlanc 2003; Wang et al. 2008). Naturally occurring inhibitors include viral and cellular genes (Ekert et al. 1999) and can protect the cell from caspase-mediated death (LeBlanc 2003). CrmA (cytokine response modifier A), from cowpox virus, is a serpin that inhibits caspases directly by targeting the active site of mature caspases. This caspase inhibitor is only limited to groups I and III caspases (excluding caspase-6). The baculovirus protein, p35, also binds to mature caspases, similarly to CrmA. This inhibitor is a broad-spectrum caspase inhibitor and does not inhibit granzyme B. Not all caspase inhibitors bind to the active site, e.g. the IAPs (Inhibitors of Apoptosis) (Fan et al. 2005). The mechanisms of these inhibitors are not completely understood, however it is thought that caspases may be inhibited through direct interaction with the caspase or through RING-dependent ubiquitination and proteasomal degradation of the caspases (Chang and Yang 2000; LeBlanc 2003).

Due to their importance in apoptosis, caspases offer an alternative in apoptosis analysis compared to gene expression. Apoptosis can be analysed through a variety of different methods such as immunoblots using caspase-specific antibodies, immunocytochemical identification of specific cleaved products, or by fluorogenic/chromogenic caspase substrates (Smolewski et al. 2002).
For phenotype analysis on a cellular level, instead of a genetic level, flurorochrome-labeled inhibitors of caspases (FLICA) are used to detect apoptosis. FLICA has three distinct components; the flurorochrome (carboxyfluorescein or fluorescein; FAM) domain, the caspase recognition domain (three- or four-amino acid peptide) and the covalent binding moiety (chloro-fluoro-methyl keton; FMK). This binding moiety binds to the cysteine of activated caspases, producing a thiomethyl ketone, resulting in the irreversible inactivation of the enzyme. FLICA is relatively nontoxic to cells and penetrates through the plasma membrane of live cells, binding to activated caspases in apoptotic cells. Unbound FLICA is removed from non-apoptotic cells via the washing buffer. The recognition peptide moiety of FLICA provides some level of specificity between ligand and particular caspases. FAM-VAD-FMK, containing valylalanyl aspartic acid, and are known as a pan-caspase markers, as the FLICA binds to numerous caspases-1,3,4,5,7,8 and 9, allowing for the analysis of apoptosis from multiple markers (Vermes et al. 2000; Smolewski et al. 2002; Grabarek and Darzynkiewicz 2003).

1.3 Viral Persistence and Apoptosis

The majority of viruses cause acute, self-limiting infections where the virus replicates rapidly then disseminates to a new host before the host clears the infection or dies. Some viruses have evolved to establish persistent infections through the manipulation of cellular mechanisms of the host in a sophisticated relationship. There are various modes of persistence e.g. non-productive, proviral integration, continuous integration (Kane and Golovkina 2010). All these modes result in the presence of infectious virus, virion components (antigen, protein, nucleic acid) and virus-specific antibodies in vertebrates after the initial infection (Kuno 2001).
Viruses may carry genes that either induce and/or prevent apoptosis. Inhibitor mechanisms can prevent the infected cells from undergoing immune response-induced apoptosis, allowing the virus to continue to replicate and infect neighbouring cells. Viruses that inhibit apoptosis may express the inhibitor mechanisms that are encoded in the genome or induce the expression of the host’s endogenous anti-apoptotic proteins (Griffin and Hardwick 1997; Li and Stollar 2004).

The CMV genome contains an IAP termed vMIA (Viral Mitochondrial Inhibitor of Apoptosis) (Irusta et al. 2003; Andoniou 2010). This particular protein can suppress apoptosis during infection by blocking the part in the apoptosis pathway where bcl-2 functions (Irusta et al. 2003). Herpes viruses also inhibit apoptosis by modulating the activation of caspase 8. The viral FLICE-inhibitory protein binds to the DED of FADD (Clayton and Hardwick 2008). The crmA protein of the cowpox and vaccina viruses encodes serine protease inhibitors (serpins) that inhibit both serine and cysteine proteases that include caspases 1,8 and 10 (Clayton and Hardwick 2008).

It has been established that persistent viral infections, target immune cells, preventing the clearance of the virus from the host. Immunosuppression may result from deregulation of molecules or dysfunction of the cells, either directly by the virus or indirectly through immunopathological mechanisms (Sevilla et al. 2000; Kuno 2001; Fahey and Brooks 2010). HIV’s impact on the immune system is well documented. HIV induces apoptosis in CD4+ T cells, leading to a decline in this T cell subset. Studies using caspase inhibitors can block HIV-induced apoptosis leading to cell survival. A side effect of this survival is increased viral replication (Chang and Yang 2000). Measles is another virus that causes immunosuppression, by inducing apoptosis.
in DCs, leading to poor stimulation of T cells (Palucka and Banchereau 1999; Carbone and Heath 2003) with impaired CD8$^+$ T cell activity, resulting in ineffective virus clearance (Carbone and Heath 2003).

It has been suggested, through various studies, that alphaviruses kill cells by inducing apoptosis or lytic necrosis (Levine et al. 1993; Strauss and Strauss 1994). Studies have shown exceptions to virus-induced death. Appel et al. (2000) observed that SINV can either produce a persistent or a lytic infection, depending on the cell type and viral strain. SINV-infected mature neurons, which over express bcl-2, have a persistent infection, while infected immature neurons die (Levine et al. 1993). Strains of SINV that differ in virulence appear to impact on whether an infection would cause apoptosis or persistence. Appel et al. (2000) observed that non-virulent SINV strains produce a non-lytic persistent infection, with an increase in bcl-2 expression. Whereas a virulent strain induced apoptosis with an increase in pro-apoptotic bax protein expression levels (Appel et al. 2000). These studies suggest that the differential expression of anti-apoptotic genes and proteins may be important in determining virulence-specific viral persistence (Levine et al. 1993; Appel et al. 2000).

It is believed that persistent infections are responsible for viral induced chronic arthritides (Linn and Suhrbier 1997; Perl 1999) with symptoms sometimes persisting for months (Perl 1999). This is a clinical feature of RRVD, with symptomatic relapses occurring months after the initial onset of infection (Way et al. 2002). The first report of persistent RRV infection was by Eaton and Hapel (1976), who observed a prolonged non-lytic infection in primary muscle cultures of mice, while Journeaux et al (1987) established a prolonged non-lytic infection in synovial cells. These two studies give
some indication that apoptosis may be influenced by RRV, similar to SINV (Eaton and Hapel 1976). More recently, a study by Way et al. (2002) into the persistence of RRV in macrophages revealed that the infection was capable of persisting up to 170 days. This persistence has only been identified in macrophages and not monocytes, but the mechanism has yet to be identified (Linn and Suhrbier 1997).

Cellular pathways involved in RRV and other alphavirus-regulated apoptosis are complex and poorly understood (Li and Stollar 2004). There are indications that caspases or the death receptor pathway may be involved (Appel et al. 2000). Further research into the mechanisms in different infected cell types may enhance the understanding of the pathogenesis of RRV and alphavirus persistence.
1.4 Hypothesis and Aims

The **underlying hypothesis** of this project is Ross River virus (RRV) isolates will induce apoptosis differently in mammalian cell types depending on the origin of the cell, the time following infection and the isolate of RRV. More specifically, it is hypothesised that cells co-ordinating immune responses to RRV will be more susceptible to apoptosis induction than non-immune cells that support viral replication.

The **overall aim** of the project was to examine the kinetics of expression markers of cell death and apoptosis by flow cytometry in mammalian cell types of immune and non-immune origins following infection with phenotypically distinct isolates of RRV.

Specifically the **study aims** were as followed:

1. Examine the expression of markers of early and late apoptosis in RRV-infected fibroblasts (Vero cells).

2. Compare the induction of apoptosis in Vero cells infected with North-East (NE) and South-West (SW) isolates of RRV.

3. Examine the expression of markers of early and late apoptosis in RRV-infected bone-marrow derived dendritic cells (BMDC).

4. Compare the induction of apoptosis in BMDCs infected with the NE and SW isolates of RRV.
Chapter 2: Materials and Methods

2.1 Cell Culture and Virus

2.1.1 Cell Lines

2.1.1.1 Vero Cells

The anchorage-dependent Vero cells are derived from kidney epithelial cells of an African green monkey (*Cercopithecus aethiops*). An initial supply of Vero cells from Dean Pemberton (Murdoch University), was cultured in Roswell Park Memorial Institute medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen, California USA) L-Glutamine (2 mM, Sigma-Aldrich), penicillin (100 U/mL, Sigma) and streptomycin (100 µg/mL, Sigma-Aldrich).

The Arbovirus surveillance unit at the University of Western Australia (UWA) supplied two batches of Vero cells, which were grown in Medium 199 (M199) (Sigma-Aldrich, Missouri, USA) supplemented with 5% heat inactivated foetal bovine serum (FBS) (Invitrogen, California USA) L-Glutamine (2 mM, Sigma-Aldrich), penicillin (100 U/mL, Sigma) and streptomycin (100 µg/mL, Sigma-Aldrich). All cells were incubated at 37°C in 5% CO₂.

2.1.1.2 Dendritic Cells

BMDCs were prepared based on the procedure developed by Lutz et al (1999). Briefly, femurs and tibiae of 7 week old male BALB/c mice (Telethon Institute for Child Health Research, Perth WA) were removed and purified from surrounding
muscle tissue. Intact bones were then incubated in 70% ethanol for 2-5 min for disinfection and washed with Dulbecco’s Phosphate-Buffered Saline (DPBS) (performed by Dr Vanessa Fear). Both ends were cut and the marrow flushed out with PBS using a syringe with a 0.45 mm diameter needle. Vigorous pipetting disintegrated clusters with the marrow suspension.

The flushed cells were plated in Iscove’s Modified Dulbecco’s medium (GIBCO) supplemented with gentamycin (50 µg/mL, Sigma), 2-mercaptoethanol (50 µmol/L, Sigma), 10% heat-inactivated FBS and 10% Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF).

BMDC cultures were phenotyped by Dr Vanessa Fear (Telethon Institute for Child Health Research) on day 8 using antibodies which included the following; MHC Class II, CD11b, CD11c, CD40 and CD80 and analysed by flow cytometry.

2.1.1.3 Subculturing

Vero cells were subcultured when the desired confluency (~80%) was reached. The growth media was removed and the monolayer was washed with DPBS (Sigma) then discarded. An appropriate amount of trypsin (3 – 5 mL) (Cellgro, Virginia USA) was added to the monolayer. The flask was incubated with trypsin to disrupt the monolayer. Once the monolayer was disrupted, the cells were resuspended with fresh growth media and aspirated. The aspirated cell solution was then aliquotted into the appropriate number of new flasks. Subculturing was used to routinely maintain the cell lines.
2.1.1.4 Cryopreservation

Stocks of Vero cells were maintained by cryopreservation. The stocks were preserved in 1:2 of M199 media and freeze mix which consisted of 15% dimethyl sulfoxide (DMSO) (Sigma) and 85% FBS. An isopropanol container was used for freezing the cryovials slowly at −80°C. The cyrovials were then placed into liquid nitrogen for long-term storage.

Resuscitated cells were defrosted quickly in a 37°C water bath and centrifuged at 20 1× g for 5 min. The supernatant was discarded and the cells were resuspended in fresh growth media. The resuspended cells were placed into a new flask and incubated at 37°C with 5% CO₂.

2.1.2 Viral Strains

The RRV strains used were SW21012, SW74249 (SW genotype), SW82627 and DC39810, (NE genotypes), originally isolated from the Peel Region of W.A. These were provided by the Arbovirology Surveillance Unit at the University of Western Australia. Each viral strain was isolated from the supernatant of infected Vero cells once the monolayer exhibited over 70% cytopathic effect (CPE). The supernatant was collected, centrifuged at 450 × g for 10 min at 4°C then aliquotted and stored at −80°C until further use.

2.1.2.1 Viral Inoculation

Vero cells were grown to 90-100% confluency and inoculated with a strain of RRV, either SW74249 (SW genotype) or SW82627 (NE genotypes). Non-inoculated control
flasks were also maintained for comparison. Growth media was aspirated and replaced with M199 media (2 mL) supplemented with 2% FBS. RRV diluted in M199 solution (50 µL) was added to the flask. To maximize absorption, cells were incubated at 37°C in 5% CO₂ for 1 h, with gentle swirling of the flask every 10 min. After 1 h, the volume of the media was increased to 10 mL. The flasks were incubated at 37°C in 5% CO₂, until 70 – 80% CPE was observed. Virus containing supernatant was harvested and stored until further use as described above (Section 2.1.2).

2.2 Determination of Viral Titres

Plaque assays were attempted originally to determine viral titre, however they were not able to be optimised in the time frame and another alternative was used.

2.2.1 Fifty Percent Tissue Culture Infectious Dose (TCID₅₀)

Stocks of the two genotypes of RRV were titrated by fifty percent tissue culture infective dose (TCID₅₀) assay. Confluent monolayers of Vero cells were grown in 48 well plates (Greiner bio-one, Germany), spent medium was removed and the monolayers inoculated with 200 µL/ well of 10-fold serial dilutions (10⁻² –10⁻⁷) of RRV. Cell controls were inoculated with 2% FBS M199 without virus. The plates were incubated at 37°C and 5% CO₂ for an h to allow viral attachment after which 200 µL of media was added. Plates were checked daily for signs of CPE, with the wells marked +/- CPE, until around 5 days. The TCID₅₀ was calculated using the Reed and Muench method (Reed and Muench 1938).
2.2.2 Temperature Stability

To test the stability of RRV at 4°C for a 7 d period, TCID$_{50}$ assays were prepared before and after storage. The values were compared to determine if the temperature had a significant impact. Both SW74249 and SW82627 RRV isolates were tested.

2.3 Flow Cytometry Analysis

2.3.1 Harvesting Method and Apoptosis Induction Optimization

2.3.1.1 Harvesting

Confluent Vero cells were harvested by different techniques to optimise flow cytometry results. Cells were harvested by cell scrapping or trypsin. Cells were harvested by cell scraper (Becton Dickinson, New Jersey, USA) in PBS (2 mL), containing 5% FBS and EDTA (1 mmol/L). Trypsinized cells were harvested by the standard cell subculturing method (Section 2.1.1.2). All harvested cells were centrifuged (201 × g, 5 min) and re-suspended in PBS containing 5% FBS, to a concentration of ~10$^6$ cells/mL. A volume of 500μL of each cell suspension was filtered through 38 μm nylon mesh (Supplied by Centre for Microscopy, Characterisation and Analysis, UWA) into 5 mL polystyrene 12 x 75 mm Falcon FACS tubes (Becton Dickinson). The forward and side scatter of the samples was measured by the BD FACSCantoII system (Becton Dickinson).

2.3.1.2 Camptothecin Positive Control Optimization

First, the optimisation of an apoptotic inducer was performed to serve as a positive control. The DNA topoisomerase I inhibitor camptothecin (CPT) (Sigma-Aldrich) was tested on different controls and analysed by PE Annexin V Apoptosis Detection Kit I
(Becton Dickinson), following a slightly modified manufacturers’ protocol to accommodate the adherent Vero cells.

Briefly, Vero cells were grown to 80-100% confluency and treated with three different concentrations of CPT: 1 µ mol/L (Hofmann et al. 1999), 5 µ mol/L and 14.5 µ mol/L (Brown et al. 2009) for 24 h. Non-treated cells were prepared for a comparison control. Cells exposed to 14.5 µ mol/L CPT or liquid nitrogen served as separate single stained positive controls. Samples were washed twice in serum-free PBS and collected by tryprsinization. Cells were then re-suspended to a concentration of ~10^6 cells/mL, with 1 X Annexin V Binding Buffer (10X buffer: 0.1 mol/L Hepes/NaOH (pH 7.4), 1.4 mol/L NaCl, 25 m mol/L CaCl_2) (Becton Dickinson). Cells (100 µL) were transferred to an individual flow cytometry tube (Becton Dickinson) through nylon mesh. PE Annexin V (5 µL) and/or 7-Amino-Actinomycin (7-ADD) (5 µL) were added to the required samples. Non-treated cells were not stained. Samples were gently vortexed then incubated at RT in the dark for 15 min. After incubation, 1 X Annexin V Binding Buffer (400 µL) was added to each sample. Cell fluorescence was measured within 1 h by the FACSCanto II flow cytometer, using standard emission filters (Table 2.1).
Table 2.1. Summary of Fluorochromes used

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Fluorescence</th>
<th>Ex-Max(nm)</th>
<th>Excitation Laser Line (nm)</th>
<th>Em-Max(nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC **</td>
<td>Green</td>
<td>494</td>
<td>488</td>
<td>519</td>
</tr>
<tr>
<td>FAM- FLICA</td>
<td>Green</td>
<td>488</td>
<td></td>
<td>530</td>
</tr>
<tr>
<td>Annexin V PE*/**</td>
<td>Yellow</td>
<td>469, 564</td>
<td>488, 532, 561</td>
<td>578</td>
</tr>
<tr>
<td>7AAD*</td>
<td>Red</td>
<td>543</td>
<td>488, 532, 561</td>
<td>647</td>
</tr>
<tr>
<td>APC **</td>
<td>Red</td>
<td>650</td>
<td>633,635, 640</td>
<td>660</td>
</tr>
<tr>
<td>Far Red Live/Dead</td>
<td>Red</td>
<td>650</td>
<td>633,635</td>
<td>665</td>
</tr>
</tbody>
</table>

Fluorochrome parameters used in Annexin V/ 7-ADD analysis (*); Fluorochrome parameters used in FLICA/ Live/Dead/ Annexin V analysis (**).

2.3.2 FLICA and Live/Dead Fixative Viability stain

FAM-VAD-FMK poly-caspase reagent was obtained from Invitrogen. This inhibitor, as a component of the Vybrant® FAM Poly Caspases Assay Kit (FLICA) (Molecular Probes, Oregon USA), was dissolved in DMSO as specified by the manufacture to obtain 150X concentrated (stock) solution. Aliquots of this solution were stored at −20°C, while protected from light. Working solution was prepared by diluting the stock solution in PBS and protected from light.

As virus-infected cells were required to be fixed before flow cytometry, a plasma membrane permeability stain was required that could be used with paraformaldehyde fixed cells. The live/dead powder from the LIVE/DEAD® Fixable Dead Cell stain kit (Molecular Probes) was dissolved in DMSO as specified by the manufacturer to obtain a reconstituted form. The dye was stored at −20°C, while protected from light.
2.3.3 Cell Staining for Apoptotic Analysis by Flow Cytometry

2.3.3.1 Vero Cells

Vero cells were grown to 90 - 100% confluency and inoculated with a strain of RRV, 14.5 µ mol/L CPT or mock infected. Both SW74249 and SW82627 strains were used. Old growth media was discarded and replaced with 2 mL of M199 media supplemented with 2% FBS.. To reduce the influence of virus concentration, the stocks were equalized by dilution using M199 based from TCID<sub>50</sub> assay results. 100 µL of RRV-containing solution was added to the respective flasks. All flasks were incubated under the same conditions as viral inoculations (Section 2.1.2.1). At 8, 24, 48 and 72 h, one flask for each experimental condition was removed and stained for flow cytometry analysis using three dyes; FLICA, Live/Dead and Annexin V. Cell culture supernatant was collected and washed with serum-free DPBS, which was also collected. Cultures were harvested by trypsinization then centrifuged with collected supernatant (290 x g, 10 min).

Supernatant was removed after centrifugation and the cells were re-suspended in serum-free DPBS to a cell concentration of ~1-2 x 10<sup>6</sup> cells/mL. All staining followed slightly modified manufacturers’ protocols as stated below (Section 2.5 and 2.6). A number of controls were produced with each staining group; unstained, and single stained samples.

2.3.3.2 Bone Marrow Derived Dendritic cells (BMDCs)

BMDCs were inoculated (100 µL) with a strain of RRV or mock infected. Viruses were adjusted to equal concentrations determined by TCID<sub>50</sub> assay. At 24 and 48 h,
cells were stained for FACS analysis using FLICA, Live/Dead and Annexin V. The growth media was collected and the plates were washed in DPBS. The collected cells were centrifuged (290 x g, 10 min) and the supernatant was removed. Cells were re-suspended in 1.5 mL DPBS.

Again, all staining followed slightly modified manufacturers’ protocols as stated below. A number of controls were produced with each staining group; unstained, and single stained samples.

2.3.3.4 FLICA and Live/Dead staining analysis

For each test sample, 300 µL of cell suspension was transferred to individual 5 mL polystyrene 12 x 75 mm Falcon FACS tube. To the required test tubes 10 µL of working solution of FAM-VAD-FMK (FLICA) were added to suspensions. The cells were protected from light and incubated for 1 h at 37°C with 5% CO₂ with gentle mixing every 20mins. The cells were washed and centrifuged twice, once with 1X Apoptosis wash buffer (supplied in the FAM Kit) and once with cold DPBS.

Samples to be stained with the Live/Dead stain (Molecular Probes) were re-suspended in 500 µL cold DPBS. After adding 0.1 µL of reconstituted Live/Dead dye, the cell suspensions were protected from light and incubated at RT for 30 min. All cell suspensions were washed, centrifuged and then re-suspended in ~500 µL of DPBS. Due to biological restrictions of RRV, all samples were fixed by adding 1:10 of fixative solution, 10% formaldehyde solution (supplied in FAM-FLICA kit), and incubated for 15 min at RT. All samples were analysed on the FACSCanto II, using standard
emission filters (Table 2.1), within 24 h post-fixation. Gating for analysis was set up was for all samples as present in Figure 2.1.

![FLICA and Live/Dead quadrant set up for apoptosis analysis.](image)

**Figure 2.1. FLICA and Live/Dead quadrant set up for apoptosis analysis.**


### 2.3.3.5 Annexin V and LIVE/DEAD staining analysis

For each test sample, 300 μL of cell suspension was transferred to individual 5 mL polystyrene 12 x 75 mm Falcon FACS tubes and then increased to 500 μL with DPBS. After adding 0.1 μL of reconstituted Live/Dead dye, the cell suspensions were protected from light and incubated at RT for 30 min. All stained samples were washed, centrifuged and then re-suspended.

The samples to be stained with Annexin V were re-suspended in 100 μL 1X Annexin V Binding Buffer. 5 μL of Annexin V was added to samples then gently vortexed and protected from light. After 20 min incubation, 400 μL of 1X Binding Buffer was
added. Due to biological restrictions of RRV, all samples were fixed using 1:10 of fixative solution. All samples were analysed on the FACSCanto II, using standard emission filters (Table 2.1), within 24 h post fixation. Gating for analysis was set up was for all samples as present in Figure 2.2.

2.4 Statistical Analysis

Differences in the TCID$_{50}$ results were assessed statistically using a Student’s t test at a significant level of 0.05. The analysis was completed in SPSS.
Chapter 3: Results

3.1 Cell Culture and Virus Strains

For the *in vitro* propagation of RRV, Vero cells were inoculated with RRV once the cells had grown to 90 – 100% confluence (Figure 3.1 A). Uninoculated control flasks were maintained for comparison. Virus inoculated cultures were harvested once the cytopathetic effects (CPE) (detachment and rounding of cells from the monolayer and clumping) reached 70 – 90% (Figure 3.1 B). Initial infections with Vero cells failed to present CPE. Reasons for the failure were unknown and Vero stocks were replaced with Vero cells that were known to be susceptible to CPE infection from the Arbovirus Surveillance Centre.

![Figure 3.1](image)

**Figure 3.1:** Vero cell and RRV – CPE culture. (A) 100% confluent Vero cells and (B) RRV infected Vero cells. (Bar =10µm).

Four RRV isolates were used; SW21012, SW74249, SW82627 and DC39810. SW21012 and SW74249 showed CPE 2 to 3 d post-inoculation while SW82627 and DC39810 showed CPE 5-7 d post-inoculation. Due to a lack of monoclonal antibody typing of SW21012; isolates SW74249 and SW82627 were chosen for experimentation. These isolates had been phenotyped using monoclonal antibody typing tests as south-west (RRV-SW) and north-east (RRV-NE) respectively by the Arbovirus Surveillance Lab (UWA).
3.2 TCID$_{50}$ and Influence of Storage Conditions

TCID$_{50}$ assays were used to ensure similar viral titres of RRV isolates were inoculated into cell cultures for subsequent experiments. To determine whether storage at 4°C for 7 d had a significant impact on the viral titre, TCID$_{50}$ assays were performed on batches of SW82627 and SW74249 at day 0 and after 7 d of storage at 4°C. Table 3.1 shows a summary of the average TCID$_{50}$/mL values. The statistical paired t-test comparison between the infectious titres of the isolates before and after storage at 4°C for 7 d resulted in insignificant $p$-values = 0.952 (RRV-SW) and 0.089 (RRV-NE). Storage for 7 d at 4°C did not appear to have a statistically significant influence on the viral titre.

Table 3.1 - Summary of TCID$_{50}$/mL average values at day 0 (A) and after 7 d storage 4°C (B) (n = 4 per strain)

<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Average TCID$_{50}$/mL ± SD</th>
<th>Average log value ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRV-NE (A)</td>
<td>$4.82 \times 10^6 \pm 4.47 \times 10^6$</td>
<td>$10^{6.7} \pm 10^{0.65}$</td>
</tr>
<tr>
<td>RRV-NE (B)</td>
<td>$3.61 \times 10^6 \pm 4.44 \times 10^6$</td>
<td>$10^{6.5} \pm 10^{0.64}$</td>
</tr>
<tr>
<td>RRV-SW (A)</td>
<td>$9.40 \times 10^5 \pm 5.98 \times 10^5$</td>
<td>$10^6 \pm 10^{5.7}$</td>
</tr>
<tr>
<td>RRV-SW (B)</td>
<td>$9.04 \times 10^5 \pm 1.13 \times 10^5$</td>
<td>$10^6 \pm 10^{6.05}$</td>
</tr>
</tbody>
</table>
3.4 Flow Cytometry Analysis

3.4.1 BMDC Phenotyping

Two BMDC cultures were phenotyped for surface expression by Dr Vanessa Fear; one was stimulated 1 µg LPS (right) to show they can be activated via TLR4, the other unstimulated (left). Cultures were assessed by flow cytometry for viable CD11c and MHC class II positive cells (Figure 3.2 A). The MHC class II and CD11c expression distinguished the cells as myeloid origin. From the gated population, further analysis was examined for the expression of CD40, CD80 and CD11b (Figure 3.2 B and C). The LPS increased the percentage of CD40+ DCs and the mean fluorescence intensity of the CD80+ DC. The increased mean fluorescence intensity of CD80 indicated an increase cell surface expression of the molecule. The expression of both CD40 and CD80 indicate that the immature mDCs were able to activate and mature.
Figure 3.2. The phenotype analysis of two BMDC cultures after 8 day culture period. 1 not simulated with LPS (left) and 1 stimulated with LPS (right). (A) The expression of MHC class II and CD11c. (B) The CD80 and CD11b expression of gated population. The CD80−/CD11b− (lower left), CD80−/CD11b+ (lower right), CD80+/CD11b+ (upper right) and CD80+/CD11b− (upper left). (C) The CD40 and CD11b expression of gated population. The CD40−/CD11b− (lower left), CD40−/CD11b+ (lower right), CD40+/CD11b+ (upper right) and CD40+/CD11b− (upper left)
3.4.2 Optimisation of Vero Cell Harvesting Methods

Two cell harvesting techniques for adherent cells, trypsinisation and cell scraping were compared to determine the best method to preserve cell viability for flow cytometry. The techniques were analysed for their forward and side scatter by flow cytometry to determine the impacts on cell dynamics. Figure 3.3 shows the forward and side light scatter profiles obtained by flow cytometry for the cells obtained by each method as indicators of cell size and granularity respectively. Both methods were successful in harvesting the Vero cells however there was variation between the methods in terms of scatter profiles produced. The trypsinised cells produced a more defined population of cells that were consistent in size and internal complexity along with a reduced amount of cell debris (Figure 3.3, left panel). The cells harvested by the scraper produced the opposite: a more heterogenous population with an increase in cell debris, reflecting that damaging nature of this harvesting method. In conclusion, the harvesting method impacts the forward and side scatter characteristics of normal Vero cells and trypsinisation was chosen as the optimum harvesting method to preserve cell viability and structure.

Figure 3.3: The comparison of Vero harvesting methods. The forward scatter indicates the size of the cell. The side scatter indicates the complexity of the cell. The trypsinized Vero cells show a defined population with similar characteristics. The scraped Vero cells show a less defined population with more variation in cellular characteristics.
3.4.3 Optimisation of Camptothecin Concentrations

CPT was chosen as an apoptotic inducing compound to act as a positive apoptosis control in subsequent experiments. Different concentrations had been used with Vero cells throughout the literature. Two concentrations were chosen from two papers (Hofmann et al. 1999; Brown et al. 2009), with a third as a middle point. The expression of Annexin V and 7–AAD (as a live/dead marker) binding at each concentration after 24 h incubation was compared (Figure 3.4). All three had an impact on cell viability. The 14.5 µ mol/L CPT provided a higher percentage of late and early apoptotic cells compared to the 1 and 5 µ mol/L experiments and was therefore was chosen for further analysis.

![Figure 3.4: Comparison of CPT concentrations on apoptosis induction. (A) An example of the 1 µ mol/L CPT FSC/SSC plot showing the selection gate which was used on all samples. (B) The basic layout for the expression of Annexin V, an apoptotic marker and 7-AAD, a necrotic marker, showing the apoptotic, necrotic and live cell quadrants. (C) Different CPT concentration-treated Vero cells analysed for PE-conjugated Annexin V and 7-AAD binding after 24 h incubation.](image)
3.4.4 FLICA and Live/Dead Stain Optimisations

3.4.4.1 FLICA

To determine if the FLICA working concentration had an impact on the fluorescence and percentages of apoptotic cells, different working concentrations were prepared and tested. (Figure 3.5 A). The FLICA positive population over all concentrations showed little change in the percentage of cells present: the 10× population presented 4.22% of cells while 15×, 20× and 30× all presented over 5% of cells (gated cells in Figure 3.5 A). However, the degree of separation between the live (FLICA-negative) population and the FLICA positive cells (gated) appeared to vary over the concentrations. The 15× and 20× populations were situated in a similar position, while the live population of the 30× was closer to the FLICA positive box. Due to these major changes between the concentrations, 15× working FLICA concentration was used for FLICA staining, allowing for one kit per cell type to be used during the time course analysis.

3.4.4.2 Live/Dead Viability Stain

Different Live/Dead viability stain volumes were tested in order to optimise the intensity of fluorescence. As shown in Figure 3.5 B, the overall fluorescence of the live and dead populations decrease with the reduction in volume used, without cell percentages varying greatly. To reduce the impact of the fluorescence on the two populations without impacting the live cells, the 0.1 µL of live/dead viability stain to 500 µL of cell suspension was chosen for the time course analysis.
3.4.4.3 Annexin V Stain

Due to no issues with the Annexin V fluorescence intensity or stain viability difficulties, the stain was used as per the manufacturers’ instructions for the project.

**Figure 3.5:** Working concentrations of FITC-conjugated FLICA and APC-conjugated Live/Dead. (A) The different working concentrations of FLICA on single stained cells exposed to 14.5µ mol/L CPT for 24 h. The gated population represents FLICA-positive cells. (B) The different volumes of Live/Dead on single stained mixed population cells. The mixed population consisted of a combination of healthy and liquid nitrogen exposed Vero cells. The dead cells (top population) fluorescence is higher than the live cells (bottom population) due to the staining of intracellular amines as well as the plasma membrane.
3.4.5 Analysis of Apoptosis in Vero Cells

Four time points were chosen for the Vero cell apoptosis analysis by flow cytometry flowing RRV-NE and RRV-SW inoculation. These time points were 8, 24, 48 and 72 h post inoculation.

3.4.5.1 FLICA and Live/Dead Stains

Mock (negative control), CPT (positive control), RRV–NE and RRV–SW samples were analysed with FLICA and Live/Dead stain expression at the individual time points in order to detect caspase activation as a measure of cell viability (Figure 3.6).

For both the mock and CPT controls, there was a slight increase in the proportion of early apoptotic cells from 8 h to 72 h, however the percentage of cells remained relatively low (Figure 3.6, upper and second rows, lower right quadrants). For late apoptotic cells, again the percentage of cells in the mock control increased slightly over the time course, while the CPT induced significant changes become after 48 h with a further increase by 72 h (Figure 3.6 upper and second rows, upper right quadrants). The two RRV isolates were similar to the mock and CPT controls at 8 h and 24 h, however a marked increase in the percentage of both early and late apoptotic cells was observed at 48 h, with further increased at 72 h (Figure 3.6, third and fourth rows).

A summary of the above data is shown in Figure 3.7. There was little variation in the percentage of late apoptotic cells and dead cells induced by the two RRV isolates in Vero cells over the time course of the study however both isolates induced apoptosis and cell death at a faster rate than the CPT control over the 24 h to 72 h period (Figure 3.7 A and B). For early apoptosis both RRV strains induced a peaked response at 48 h
that was markedly higher than the CPT control, but in this case the RRV-NE isolate induced a higher percentage of early apoptotic cells when compared to the RRV-SW isolated at the peak of the response (Figure 3.7 C).

Figure 3.6. RRV-infected Vero cells undergo apoptosis. FITC-conjugated FLICA and APC-conjugated Live/Dead of RRV-infected (NE and SW), mock-infected (uninfected) and CPT-inoculated (apoptosis induced). The percentages represent the proportion of non-apoptotic (lower left), early apoptotic (lower right), late apoptotic (upper right) and necrotic cells (upper left) after 8, 24, 48 and 72 h post-inoculation. Data represents 1 experiment (n= 16).
Figure 3.7. Percentages of (A) late apoptotic, (B) total cell death and (C) early apoptotic cells over 8-72h for the two RRV isolates (NE and SW), mock infected and CPT inoculated. Data represents 1 experiment (n= 16).
3.4.5.2 Annexin V and Live/Dead Stains

Mock (negative control), CPT (positive control), RRV–NE and RRV–SW isolates were analysed at the same time points as above and apoptosis assessed by flow cytometry using the Annexin V and Live/Dead stains as a comparison to the FLICA stain described above, in order to determine if any membrane polarity apoptotic changes were apparent. Using this approach, no considerable induction of early apoptosis was observed in any of the treatments (Figure 3.8, lower right quadrants). However, a substantial proportion of late apoptotic cells were observed in the CPT treated and the RRV-infected cells, which increased with time and were significantly increased above the mock controls (Figure 3.8, upper right quadrants).

A summary of the flow cytometry data is shown in Figure 3.9. As no significant number of early apoptotic cells was observed, only data for late apoptosis and cell death are shown. The percentage of late apoptotic cells in the mock-infected cultures showed a slight increase over time but maintained below CPT values, which increased from 24 h to 48 h (Figure 3.9 A). The percentage of dead cells showed a similar trend for the mock and CPT controls, while the percentage of RRV-induced late apoptotic and dead cells peaked higher than both controls over time, with a rapid increase after 24 h (Figure 3.9 A and B).

However, there was some variation between the two RRV isolates in terms of late apoptosis induction. The RRV-SW isolate showed a plateau effect over the 24 h period from 48 h to 72 h, while the RRV-NE isolate continued to increase (Figure 3.9A).
However, no variation between the two isolates in the percentage of dead cells was observed (Figure 3.9 B).

**Figure 3.8.** RRV-infected Vero cells undergo membrane polarity change. PE-conjugated annexin V and APC-conjugated Live/Dead of RRV-infected (NE and SW), mock–infected (uninfected) and CPT-inoculated (apoptosis induced). The percentages represent the proportion of non-apoptotic (lower left), early apoptotic (lower right), late apoptotic (upper right) and necrotic cells (upper left) after 8, 24, 48 and 72 h post-inoculation. Data represents 1 experiment (n= 16).
Figure 3.9. Percentages of (A) late apoptotic cells and (B) combined Vero cell death over 8-72 h for the two RRV isolates (NE and SW), mock infected and CPT inoculated. Data represents 1 experiment (n= 16).
3.4.6 Analysis of Apoptosis in BMDCs

Due to limited cell numbers, two time points were chosen based on the Vero cell results for the BMDCs apoptosis analysis by flow cytometry. These time points were 24 h and 48 h post inoculation. Again, due to limited availability of cells, no CPT control was used in the BMDC analysis due to time and cell yield restrictions.

3.4.6.1 FLICA and Live/Dead Stains

Mock, (negative control), RRV–NE and RRV–SW isolates were analysed with FLICA and Live/Dead stain to compare cell viability at each time point (Figure 3.10). All samples had weaker FLICA signals than expected based on the Vero results, with the clear variation in late apoptotic and dead cell numbers between the mock and RRV-infected cells at the 24 h time point.

The above data is summarised in Figure 3.11. The percentage of late apoptotic cells induced by each treatment did not differ considerably over the two time points (Figure 3.11A). The RRV-NE isolate induced the highest degree of late apoptosis compared to both the mock control and RRV-SW isolate at both time points. Further, the RRV-SW strain induced lower levels of apoptosis than the background levels observed in the mock control (Figure 3.11A). Further analysis of the combined dead cell quadrants (late apoptotic and necrotic – upper left quadrants in Figure 3.10) was also performed. Both isolates of RRV induced a higher percentage of cell death compared to the mock at both time points (Figure 3.11B). At 24 h the RRV-SW isolate had a higher percentage of cell death compared to the RRV-NE isolate. At 48 h, the reverse was observed, with the RRV-NE higher than RRV-SW. In contrast, the percentage of non-
apoptotic cells did not vary greatly (Figure 3.11 C). While the numbers of non-apoptotic cells in the RRV-NE infected cultures did not change between time points, RRV-SW at 24 h produced a low percentage of non-apoptotic cells, however at 48 h was similar to the mock and RRV-NE.

**Figure 3.10.** RRV-infected BMDCs undergo cell death. FITC-conjugated FLICA and APC-conjugated Live/dead staining of RRV-infected (NE and SW), mock–infected (uninfected). The percentages represent the proportions of non-apoptotic (lower left), early apoptotic (lower right), late apoptotic (upper right) and necrotic cells (upper left) after 24 h and 48 h post-inoculation. Data represents 1 experiment (n= 6).
Figure 3.11. Percentages of (A) late apoptotic cells, (B) combined dead cells and (C) non-apoptotic BMDCs at 24 h and 48 h post infection of the two RRV isolates (NE and SW) and mock infected. Data represents 1 experiment (n= 6).
3.4.6.2 Annexin V and Live/Dead Stains

Mock, RRV–NE and RRV–SW isolates were analysed for changes in membrane polarity induced in BMDC as based on Annexin-V expression at the 24 h and 48 h time points. All samples showed prominent staining with the Annexin V and Live/Dead markers, with cells present in all four quadrants (Figure 3.12).

As summarised in Figure 3.13 A, the mock had a lower percentage of early apoptotic cells compared to the RRV-NE and RRV-SW isolates, at both time points. The RRV-SW sample at both time points produced a higher percentage of cells compared to the RRV-NE. By 48 h, the percentage of cells in all quadrants for all samples increased. As illustrated in Figure 3.13 B, with the percentage of late apoptotic cells was higher in both RRV-NE and RRV-SW cultures when compared to the mock control at 24 h, and the RRV-SW isolate produced a higher number of late apoptotic cells compared to the RRV-NE isolate, which did not vary greatly from 24 h value. Figure 3.13 C shows the variation in overall cell death, with similar results to Figure 3.13 B for all samples. The percentage of non-apoptotic cells did not vary greatly between the samples, with the variation only noticeable at 24 h. Both RRV isolates produce a lower percentage of non-apoptotic cells compared to the mock. In BMDCs, RRV induces an apoptotic death response with some variation between isolates.
Figure 3.12. RRV-infected BMDCs undergo membrane polarity change. PE-conjugated Annexin V and APC-conjugated Live/Dead staining of RRV-infected (NE and SW), mock–infected (uninfected). The percentages represent the proportions of non-apoptotic (lower left), early apoptotic (lower right), late apoptotic (upper right) and necrotic cells (upper left) quadrants 24 h and 48 h post-inoculation. Data represents 1 experiment (n= 6).
Figure 3.13. Percentages of (A) early apoptotic, (B) late apoptotic and (C) combined dead BMDCs at 24 h and 48 h post inoculation of the two RRV isolates (NE and SW) and mock infected. Data represents 1 experiment (n= 6).
Chapter 4: Discussion

The purpose of this study was to determine if RRV infection would induce apoptosis differently in mammalian cell types from an immune and non-immune origin. This was the first study to show potential differences between the RRV phenotypes and the induction of apoptosis in dendritic cells. The overall aim of this study was to examine the kinetic expression of apoptotic and cell death markers in Vero cells and in BMDCs following infection with phenotypically distinct strains of RRV.

4.1 Cell Culture Models

The Vero cell line was selected as the non-immune cell type. This cell line is commonly used for the in vitro propagation of alphaviruses (Levine et al. 1993; Griffin 2001) and infection presents as lytic CPE within 24 h (Raghow et al. 1973). Initial Vero cell stocks from Dean Pemberton, Murdoch University, failed to produce a lytic response after inoculation with RRV. The reason for the failure of the RRV to produce CPE is unknown with further analysis needed on these stocks to confirm that the stocks are truly Vero cells and verification of RRV-infection was present. However, an alternative source of Vero cells supported lytic RRV infection and displayed expected CPE. If the original Vero cells are truly resistant to infection with RRV, further analysis is warranted, to determine any differences between the two sources of Vero cells. Such experiments would help dissect the mechanisms of RRV attachment, entry and replication in vertebrate host cells.

Initial attempts to complete plaque assays proved unsuccessful, even though they have been used to determine RRV titre previously (Lidbury and Mahalingam 2000; Lidbury
et al. 2011). The possible reason for the failure was the inability of the neutral red dye to penetrate the agar overlay and further experimentation is required to optimise the plaque assay for RRV in our laboratory. As RRV is a cytopathic virus, TCID$_{50}$ assays were performed to determine virus concentration. This assay has been validated and reproducibly used technique used for RRV by others (Kistner et al. 2007) and also for other togaviruses (Hofmann et al. 1999).

BMDCs were selected as the immune cell type. These DCs yield a higher number of cells compared to peripheral blood DCs and Langerhan’s cells (Lutz et al. 1999). The DCs collected for the study presented with a lower than expected cell yield; $1 \times 10^6$ cells/mL, after 8 days of culture with the minimum expected concentration of cells being $5 \times 10^6$ per mL (Lutz et al. 1999). The exact reason for the reduced cell yield is unknown, but may include a reduced yield of cells collection and seeding after bone marrow harvest or a deficiency with the growth factor, GM-CSF (Lardon et al. 1997; Lutz et al. 1999; Banchereau et al. 2000). BMDCs have been used in RRV studies previously (Shabman et al. 2007; Shabman et al. 2008). Due to time limitations no infection confirmation was done in this study.

*In vitro* studies involving RRV and DCs have not investigated on the impact of RRV infection on DC survival and the potential implications of immunosuppression. Previous *in vitro* studies involving RRV and DCs have targeted the type I IFN responses towards virus derived from mosquito and mammalian cell culture (Shabman et al. 2007; Shabman et al. 2008). Therefore, this is the first study to focus on the impact of RRV on DC survival.
4.2 Analysis of Preliminary Apoptosis Results

FLICA, Annexin V and Live/Dead staining was used to study the impact of RRV infection in vitro and the differences between genotypes, since apoptosis is believed to be induced in alphavirus lytic infection (Levine et al. 1993; Strauss and Strauss 1994). RRV produced an apoptotic death response in both Vero cells and BMDCs with some variation between the two isolates.

4.2.1 Analysis of Apoptosis in Vero Cells

The results in the Vero cells experiments determined that the cell death observed following inoculation with RRV was largely due to RRV, as background apoptosis of the uninfected mock was small. The rate of RRV-induced apoptosis was faster than the positive CPT control over time (Figure 3.7 A and 3.9 A). These data may suggest that the induction mechanism of RRV-apoptosis may be independent of DNA replication, and apoptosis may correlate with viral replication. Overall, the data of phosphatidylserine exposure and caspase activation in RRV infected cells, correlate with other studies that have observed apoptotic features in alphavirus infected cells indicating that RRV can induce apoptosis in cells (Levine et al. 1993).

While there was no substantial difference overall between the two isolates, subtle differences in the percentage of early and late apoptotic cells were evident and may have important virulence implications. For example, the SW isolate induced a more rapid onset of late apoptosis than NE over the Annexin V time course (Figure 3.9 A). Although the percentage of cells affected was higher in the NE isolate over time (Figure 3.7 A and 3.9 A). Furthermore, the SW induced a lower peak percentage of early apoptotic cells than the NE virus strain (Figure 3.7 C). These data may suggest
that the SW isolate could be more virulent, inducing apoptosis earlier than the NE isolate. *In vitro* studies by Prow (2006) observed no significant differences between the strains in Vero cells.

FLICA and Annexin V are widely accepted apoptotic markers for flow cytometry analysis (Vermes *et al.* 2000). In all the apoptosis experiments using Vero cells and BMDCs in this thesis both markers produced positive populations; however variation did occur between the two markers. An example is the difference in the percentage of early apoptotic signals in Vero cells (*Figure 3.6 and 3.9, lower right quadrant*). Apoptosis can be a fast process and it can be difficult to distinguish between cells that have completed apoptosis and those that are about to commence apoptosis (Gilmore and Streuli 2002). FLICA has a broad detection window compared to other apoptotic markers including Annexin V which could explain the variation (Pozarowski *et al.* 2003b). More likely, the impact of formaldehyde fixation and harvesting may explain some of these results. FLICA is an irreversible, stable, intracellular marker that can withstand cell fixation (Smolewski *et al.* 2002), while Annexin V is a reversible, extracellular based marker (Koopman *et al.* 1994; Brumatti *et al.* 2008). The majority of authors advise against fixing Annexin V stained cells due to its reversible nature, leading to either a degraded signal or non-specific Annexin V staining (Brumatti *et al.* 2008). Staining with Annexin V is not advised for adherent cells harvested with routinely applied techniques such as trypsinization or cell scraper, as membrane changes can be induced, exposing phosphatidylserine (van Engeland *et al.* 1996; van Engeland *et al.* 1998).
An alternative method for Annexin V staining of adherent cells was developed by van Engeland (1996), with labelling with Annexin V occurring prior to quantitative harvest. Alternatively an enzyme known as Accutase has been used successful in Annexin V analysis of adherent cells and this could be used in the future as an substitute for trypsin (Park et al. 2007; Weigert et al. 2007; Wang et al. 2010) allowing the multi-parameter analysis of one flask.

In addition, caution must be taken with the FLICA staining. The reason for the inconsistent staining in this study is unknown. FLICA has been compared to other methods including TUNEL (TdT-mediated dUTP Nick End Labelling) (Bedner et al. 2000; Pozarowski et al. 2003b) and immunocytochemistry (Pozarowski et al. 2003a) demonstrating that it is a proven marker of apoptosis. However, there have been reports of non-specific caspase binding by FLICA (Pozarowski et al. 2003b; Darzynkiewicz and Pozarowski 2007). Due to the potential non-specific binding, FLICA may be involved with other cell constituents which may vary between cell types and so further analysis is required to confirm in FLICA as an appropriate and reliable apoptosis marker (Pozarowski et al. 2003b). Several other methods of apoptosis analysis including TUNEL, immunohistochemistry and fluorescence microscopy (Darzynkiewicz et al. 1992; Vermes et al. 2000) could be combined with the apoptotic markers used in this study to validate the observations.

4.2.2 Analysis of Apoptosis in BMDCs

Results from the BMDCs studies found that the RRV induced a strong pro-apoptotic response in BMDCs. This conclusion was based on the data which demonstrated that uninfected cells showed a low level of apoptosis with high levels of non-apoptotic cells
in the population (**Figure 3.11 and 3.13**). Furthermore RRV induced a faster and more aggressive pro-apoptotic response in BMDCs compared to Vero cells at 24 h after inoculation. Aaskov *et al.* (1983) suggested that RRV may target APCs, like DCs before the cell can properly fulfil its function to mount an immune response. Prelimorarily data suggests that RRV could target DCs to induce apoptosis early in infection and may impact DC function. Collectively this could be important in understanding the pathogenesis and virulence of the RRV.

Results from the BMDCs study found that the SW isolate was more aggressive in DCs compared to the NE isolate. This conclusion was based on data which showed the SW isolate to have the higher apoptotic response at 24 h (**Figure 3.13 A and B**). Furthermore, the percentage of total cell death at 24 h was found to be the highest with the SW isolate (**Figure 3.11 B and 3.13 C**). At the 48 h time point, the decline in apoptotic cells for the SW isolate may be due to the engulfment of apoptotic cells by bystander DCs and macrophages present in the culture. Collectively, these data may suggest that the SW isolate could be more virulent and have a negative impact on the immune response. A study on SW and NE phenotyped isolates showed similar differences using *in vivo* suckling mouse models, with the SW presenting as the more virulent virus strain (Prow 2006). Observations of RRV isolates from different geographical regions that differ in the level of virulence have been noted in mouse and mosquito models (Kerr *et al.* 1992; Lindsay *et al.* 1993; Russell 2002). In relation to other alphaviruses, different strains of SINV that differ in virulence have been observed to have an impact on whether an infection may cause apoptosis or not (Appel *et al.* 2000).
Measles, CMV and LCMV all infect DCs and interfere with the DCs specialized functions to enhance their own pathogenesis (Carbone and Heath 2003; Cunningham et al. 2010). The inhibition of cytokine secretion (IL-12), apoptosis, limited maturation and expression of MHC Class I and II molecules lead to immunosuppression through the failure to stimulate proliferation of naïve T cells (Fugier-Vivier et al. 1997; Andrews et al. 2001; de Witte et al. 2006). In measles infection the Th responses shift from Th1, cell-mediated to Th2, humoral-mediated leading to insufficient elimination of the virus. The exact mechanism for this is unknown, but the inhibition of IL-12 is suspected to be involved (Fugier-Vivier et al. 1997).

The impacts of interference with DC function results in an impaired capacity to initiate a specific immune response towards the virus. **Figure 4.1** depicts the life cycle of a DC. If a virus infects an immature DC and suppresses its ability to mature and secrete stimulatory cytokines, activation of the viral-specific response will be impaired. The failure to activate Th1 cells will impact the Tc response, as Tc generally needs Th1 assistance for activation and results in an ineffectual cell-mediated response (Banchereau et al. 2000). Differences in the Tc responses to viruses injected into the skin have been observed which suggest that cytopathic viruses like Herpes simplex virus -1 may hamper the ability of skin DCs to prime virus specific Tc responses (Allan et al. 2003; Merad et al. 2008). By negatively impacting DCs, viruses have the potential to enhance their pathogenesis and establish a long-term persistence.
The potential implications of RRV-induced apoptosis in cells from an immune origin could support the idea that the persistence of RRV may be the result of ineffectual clearance by impaired immune responses (Linn et al. 1998; Rulli et al. 2005). If RRV interferes with DC in an immunosuppressive form it may help explain the differences between predominant CD4+ T cells in mononuclear synovial effusions of chronic RRVD patients and the almost exclusive CD8+ T cells present in the skin tissue of RRVD patients with early and complete recoveries (Fraser et al. 1983; Fraser and Becker 1984). The variation between isolates may help explain the differences in the severity and duration of RRVD across Australia.

**Figure 4.1 The life cycle of dendritic cells (DC).** Immature DCs enter the tissues. After antigen capture, the immature DCs migrate to the local lymph node where, after maturation, present the antigen to T cells via MHC molecular. This leads to the activation and co-ordination of anti-viral T cell and antibody responses [Figure from (Banchereau et al. 2000)].
The potential implications of these results on the DC-T cell interaction may explain the differences in T cell responses in patients, persistence of the virus and differences in the strains.

Studies on RRVD patients have observed a correlation between the type T cell response and the duration/ severity of the disease (Harley et al. 2001). Patients with chronic RRVD, have a predominance of CD4+ T cells in their mononuclear synovial effusions, contrasts from these observations, it suggests that individuals who develop chronic RRVD fail to generate RRV-specific CD8+ T cell response. Other studies into alphavirus-specific Tc observed that the cells were capable, in vitro, to eliminate RRV from persistently infected macrophages (Linn et al. 1998) and their activity correlated with clearance of viremia from the peripheral blood in mice models (Peck et al. 1979; Blackman and Morris 1984).

4.3 Future Directions

Even though this study provides preliminary evidence of RRV-induced apoptosis in DCs with notable differences between the two virus isolates it is far from conclusive. It is vital that research into the effects of RRV infection on apoptosis regulation in DCs is further developed. The impact of RRV on DC function requires confirmation. Further studies could employ an extended time course to determine if cell viability is alters prior to 24 h in BMDCs as well as to determine total cell numbers instead of the proportional percentage of the populations. It would be advantageous to complete further analysis on the different DC subtypes, in particular pDCs and skin DCs to study the impact on DC functions through in vivo and in vitro models, in particular DC – T cell interactions. Extending the number and genomic sequencing of isolates from different phenotypic
viruses may also provide knowledge of the differences within circulating RRV strains. These types of investigations may lead to the development of specific immunotherapeutic targets for clinical use.

4.3 Conclusions

In this study, apoptotic and cell death markers were used to investigate the impact of RRV infection in Vero cells and BMDCs using two different RRV isolates. The data obtained indicated that RRV induced apoptosis in BMDCs faster than in Vero cells, potentially enhancing virus pathogenesis. The potential implications of RRV-induced apoptosis in cells from an immune origin could support the idea that the persistence of RRV may be the result of ineffectual clearance by impaired immune responses (Linn et al. 1998; Rulli et al. 2005).

Furthermore, in BMDCs, the two isolates had different impacts on cell death; with the SW isolate inducing a faster response compared to the NE isolate, indicating a possible difference in virulence. This possible difference may help to explain the variation in the severity and duration of human clinical symptoms, linking the symptoms to the strains.
References


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