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# INHIBITION OF K-BALB MURINE TUMOURS USING SEMLIKI FOREST VIRUS AND ITS DERIVED VECTOR

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**Thesis submitted to the University of Dublin,**
**Trinity College,**
**For the Degree of Doctor of Philosophy**

*by*

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***Department of Microbiology,***
***Moyne Institute of Preventive Medicine,***
***Trinity College,***
***Dublin***

***August 2004***

*FOR MY PARENTS*

***Declaration***

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This thesis is submitted by the undersigned to the University of Dublin, Trinity College for the examination of Doctorate of Philosophy. The work herein is entirely my own work and has not been submitted as an exercise for a degree to any other university. The librarian of Trinity College Dublin has my permission to lend or copy this thesis upon request.

James Smyth

***"We are the music-makers,***
***And we are the dreamers of dreams,***
***Wandering by lone sea breakers,***
***And sitting by desolate streams.***
***World-losers and world-forsakers,***
***On whom the pale moon gleams:***
***Yet we are the movers and shakers***
***Of the world forever, it seems."***

*Arthur O'Shaughnessy, 1844-1881*

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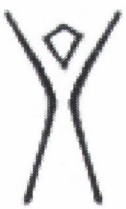

| | |
|---|---|
| aa | amino acid |
| AAV | adeno-associated virus |
| ADCC | antibody-dependent cellular cytotoxicity |
| ADP | adenosine diphosphate |
| ATCC | American Type Culture Collection |
| ATP | adenosine triphosphate |
| AIDS | acquired immunodeficiency syndrome |
| AIF | apoptosis-inducing factor |
| Apaf-1 | apoptosis protease-activating factor-1 |
| APC | antigen presenting cell |
| BC | B-cell corona |
| BHK | baby hamster kidney |
| C | SFV capsid protein |
| CAD | caspase-activated deoxyribonuclease |
| CARD | caspase-recruitment domain |
| CD | cluster of differentiation antigen |
| CDC | compliment-dependent cytotoxicity |
| CEA | carcinoembryonic antigen |
| cDNA | copy/complementary DNA |
| CNS | central nervous system |
| conA | concanavalin A |
| CPE | cytopathic effect |
| CPM | counts per minute |
| CPV | cytoplasmic vacuole |
| CTL | cytotoxic T-lymphocyte |
| dATP | deoxyadenosine triphosphate |
| DAB | diaminobezidine |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DC | dendritic cell |
| DD | death domain |
| DED | death effector domain |
| DEPC | diethylpyrocarbonate |
| DISC | death-inducing signalling complex |
| DMEM | Dulbecco's modified eagle medium |
| DNA | deoxyribonucleic acid |

| | |
|---|---|
| dsDNA | double-stranded DNA |
| dsRNA | double-stranded RNA |
| DTT | dithiotreitol |
| EBR | ELISA blocking reagent |
| EDTA | ethylenediaminetetra-acetic acid |
| EEE | Eastern equine encephalitis virus |
| EGFP | enhanced green fluorescent protein |
| EGFR | epidermal growth factor receptor |
| ELISA | enzyme-linked immunosorbant assay |
| ER | endoplasmic reticulum |
| FasL | Fas ligand |
| FADD | Fas-associated DD |
| FBS | foetal bovine serum |
| FITC | fluorescein isothiocyanate |
| FLICA | fluorochrome-labelled inhibitors of caspases |
| FLIP | Fas-associated DD-like ICE inhibitory protein |
| FMDV | foot-and-mouth disease virus |
| *g* | gravitational force |
| G | gauge |
| GAP | GTPase activating protein |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GTP | guanosine triphosphate |
| HBSS | Hank's balanced salts solution |
| HBV | hepatitis B virus |
| HCV | hepatitis C virus |
| H&E | haematoxylin and eosin |
| hepes | N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid |
| HIER | heat-induced epitope retrieval |
| HIV | human immunodeficiency virus |
| HLA | human leukocyte antigen |
| h.p.i. | hours post infection |
| HPV | human papillomavirus |
| HRP | horse radish peroxidase |
| HSV | herpes simplex virus |
| HtrA2 | high-temperature requirement protein A2 |
| IAP | inhibitor of apoptosis |

| | |
|---|---|
| i.c. | intracranial |
| ICAD | inhibitor of CAD |
| ICE | IL-converting enzyme |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| i.m. | intramuscular |
| i.n. | intranasal |
| i.p. | intraperitoneal |
| i.t. | intratumoural |
| IU | infectious units |
| i.v. | intravenous |
| MAPK | mitogen-activated protein kinase |
| MEM | minimum essential medium |
| MFI | medium for infection |
| MHC | major histocompatibility complex |
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| MS | multiple sclerosis |
| NCS | newborn calf serum |
| NDV | Newcastle disease virus |
| NF-κB | nuclear factor kappa B |
| NGS | normal goat serum |
| NK | natural killer cell |
| NO | nitric oxide |
| NRS | normal rabbit serum |
| nsP | SFV non-structural protein |
| OMM | outer mitochondrial membrane |
| OPD | *o*-phenylenediamine |
| ORF | open-reading-frame |
| PALS | periarteriolar lymphoid sheath |
| PARP | polyADP-ribose |
| PBS | phosphate buffered saline |
| PBS+ | PBS with calcium, magnesium and sodium bicarbonate |
| PBS- | PBS without calcium magnesium and sodium bicarbonate |
| PBST | PBS containing 0.05% Tween 80 |
| PCR | polymerase chain reaction |
| PDGFR | platelet-derived growth factor receptor |

| | |
|---|---|
| PFU | plaque-forming units |
| PI3K | phosphoinositide 3-kinase |
| PSA | prostate-specific antigen |
| RalGDS | Ral guanine nucleotide dissociation stimulator |
| RBC | red blood cell |
| RPV | replication proficient virus |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| rSFV | recombinant SFV |
| RSV | respiratory synctial virus |
| RT-PCR | reverse-transcription PCR |
| s.c. | subcutaneous |
| SCID | severe combined immunodeficiency |
| SDS | sodium dodecyl sulphate |
| SFV | Semliki Forest virus |
| SIV | simian immunodeficiency virus |
| SMAC | second mitochondrial activator of caspases |
| spf | specific pathogen-free |
| ssDNA | single-stranded DNA |
| SSPE | subacute sclerosing panencephalitis |
| ssRNA | single-stranded RNA |
| SV | Sindbis virus |
| TAA | tumour-associated antigen |
| TCA | trichloroacetic acid |
| TdT | terminal deoxynucleotidyl transferase |
| TGF | transforming growth factor |
| TIL | tumour-infiltrating leukocyte |
| TNF | tumour necrosis factor |
| TNFR | TNF receptor |
| TRAF | TNFR-associated factor |
| TRIP | TRAF-interacting protein |
| TUNEL | TdT-mediated dUTP-biotin nick-end labelling |
| VEE | Venezuelan equine encephalitis virus |
| VEGFR | vascular endothelial growth factor receptor |
| VLP | virus-like particle |
| VSV | vesicular stomatitis virus |
| WEE | Western equine encephalitis virus |
| WHO | World Health Organisation |

The induction of cytopathic effects in tumour cells, often by apoptosis, is the primary goal of most non-surgical cancer therapies. Cancer gene therapy represents a variety of potentially therapeutic strategies involving the introduction of new genetic information into cells with the aim of abrogating tumourigenicity or eliciting therapeutic effects. A large proportion of cancer gene therapy research is devoted to immunotherapy, which involves the induction or potentiation of host antitumour immune responses. Antigens associated with tumourigenesis have been identified and exploited in this field, but given the unstable nature of tumour cell phenotype and antigen expression, these strategies may be of limited value. Rather than restricting therapy to a specific antigen, the expression of Semliki Forest virus (SFV) antigens (predominantly E2) is employed in this study with the aim of inducing host immune responses against infected tumour cells.

The SFV vector system allows for the production of recombinant SFV (rSFV) virus-like particles (VLPs) which can achieve high levels of heterologous protein expression in target cells upon infection. The viral structural proteins are replaced with a foreign gene of choice and this rSFV RNA is co-transfected with two helper RNAs that provide the structural genes *in trans*. Only the rSFV RNA molecule encoding the foreign gene is packaged into the resulting rSFV VLP progeny, which can subsequently only undergo one round of replication as they are devoid of structural genes. The implementation of rSFV VLPs as prototype vaccines has been investigated for a variety of diseases in animal models and potent humoural and cellular immune responses against target antigens are routinely achieved. SFV and its derived vector also have the ability to induce apoptosis in a variety of mammalian cell lines, a phenomenon which occurs irrespective of the p53 status of the cell. This prompted investigation into the exploitation of rSFV VLPs as an antitumoural agent and promising results were achieved following treatment of tumour xenografts in BALB/c *nu/nu* mice. The potential application of alphavirus vectors in cancer gene therapy has become clear since their development with increasing research, not only in the exploitation of their cytopathic ability but also in the expression of high amounts of foreign (potentially therapeutic) proteins.

In this study, the potential of immune stimulation in combination with apoptosis induction SFV and its derived vector for tumour treatment was assessed. The K-BALB

murine tumour model, which readily forms tumours in BALB/c mice, was utilised as a rapidly growing poorly immunogenic model. K-BALB cells underwent apoptosis and expressed viral antigen *in vitro* when infected with the SFV4 strain of SFV, or rSFV VLPs encoding the p62-6k viral structural proteins. Apoptosis induction was confirmed by detection of active caspases and DNA fragmentation at a cellular level. VLPs were used to immunise groups of BALB/c and BALB/c *nu/nu* mice prior to subcutaneous K-BALB tumour induction and treatment. The aim of this immunisation step was to induce an immediate immune response against infected tumour cells upon initiation of treatment and also to confer protection against challenge with SFV4. Complete protection from infection with SFV4 following treatment was observed in all immunised mice and was confirmed by pathological examination of brain tissue.

Direct intratumoural injection of VLPs or SFV4 resulted in an immediate and intense inflammatory reaction in immunised groups that was not observed in naïve groups until day 5 of treatment, and was not observed in *nu/nu* groups. Histopathological examination of treated tumours, immunohistochemistry of tumour sections, and *ex vivo* immunological assays revealed that a predominantly $T_H1$-mediated inflammatory immune response was elicited. High numbers of neutrophils, macrophages and $CD4^+$ and $CD8^+$ T-lymphocytes infiltrated tumours following treatment with acute oedema also apparent. Apoptosis induction was also detected in treated tumours but bystander effects leading to the development of necrotic areas may also have played a role in tumour growth inhibition. SFV antigen expression was detectable in inflamed areas as granular extracellular deposits and within macrophage-like cells. Neither SFV4 nor rSFV VLPs appeared capable of completely disseminating through the K-BALB tumour tissue and islands of uninfected tumour cells were observed in most treated tumours.

A significantly higher level of tumour growth inhibition was observed in immunocompetent groups than in the athymic mice. SFV4 treated groups showed greater inhibition than that observed for VLP-treated groups, with immunisation prior to treatment enhancing the overall antitumour effect and immune response. SFV4 was employed in this study as a model replication-proficient SFV vector. The potential for such a vector system is clear, but anti-SFV immune responses would limit its effectiveness. Non-SFV antigens could be expressed using VLPs and a more significant response may be achieved. It is concluded that use of the inherent apoptosis-inducing capability of SFV in combination with immune stimulation, may have potential for the treatment of rapidly growing tumours.

***Presentations:***

**Apr 2003** **152nd Society for General Microbiology Meeting**
**Edinburgh, Scotland**

**Oral Presentation:** "Semliki Forest Virus and its Vector as Tumour Therapy Agents"
**J.W.P. Smyth,** B.J. Sheahan and G.J. Atkins

**Sep 2001** **5th Gene Delivery & Cellular Protein Expression Conference**
**Semmering near Vienna, Austria**

**Poster Presentation:** "Induction of antitumour immune responses by expression of viral antigens in tumour cells using the Semliki Forest Virus vector"
**J.W.P. Smyth,** B.J. Sheahan and G.J. Atkins

***Publications:***

**Smyth, J.W.P.,** M.N. Fleeton, B.J. Sheahan., and G.J. Atkins. 2004. "Treatment of rapidly growing K-BALB and CT26 mouse tumours using Semliki Forest virus and its derived vector." *Gene Therapy*. In Press

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### *Chapter 2*



### *Chapter 3*

## *Chapter 5*



## *Chapter 6*

***Chapter 9***



***Tables:***

***Chapter 9***

# 1

# GENERAL INTRODUCTION

# 1 GENERAL INTRODUCTION

With over 10 million people diagnosed with cancer each year (a figure which is estimated to rise to 15 million by 2020) (http://www.who.int/cancer/en/., 2004) the importance of scientific research into this disease and methods of treating it is unquestionable. Cancer is a molecular disease caused by the acquisition of transforming mutations by cells which result in inappropriate and uncontrolled cellular proliferation and/or a diminished rate of cell depletion (via apoptosis). Surgery is currently the primary method for treatment of malignant disease but may not be possible or entirely effective in the eradication of cancerous growths, with the occurrence of metastases often limiting its success. Chemo- and/or radiotherapy are commonly required but have well documented associated toxicities, rendering them difficult treatment regimens to administer to patients with already severely compromised health. In the search for more specific and less toxic cancer treatment strategies, cancer immunotherapy and cancer gene therapy have emerged as promising candidates.

The inherent ability of the Semliki Forest virus (SFV) vector to induce apoptosis has already been successfully exploited in the treatment of tumours in BALB/c *nu/nu* mice (Murphy *et al.*, 2000; Murphy *et al.*, 2001). In this study, the more aggressive K-BALB murine tumour model, which readily forms syngeneic tumours in immunocompetent BALB/c mice, is utilised. K-BALB cells are Kirsten murine sarcoma virus transformed BALB/3T3 mouse embryo fibroblasts which overexpress the K-*ras* oncogene. rSFV virus-like particles (VLPs) expressing the SFV antigen E2 were employed with the aim of augmenting the cytopathic antitumoural ability of the vector, through the induction of immune responses directed at infected tumour cells. In addition, the antitumoural ability of virus from the infectious clone of SFV, SFV4, was also examined as a model replication-proficient SFV vector. This study combines the induction of apoptosis in target tumour cells with the immunotherapeutic approach of attracting host immune responses to the tumour through expression of viral antigens.

## 1.1 APOPTOSIS

The term 'apoptosis' was first coined in 1972 by Kerr *et al* to describe a morphologically distinct form of cell death observed by electron microscopy which had previously been referred to as 'shrinkage necrosis'. The morphological characteristics of apoptosis include cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, and the collapse of the cell into intact vesicles (apoptotic bodies) which are phagocytosed by monocytes and neighbouring cells (Savill & Fadok, 2000). It is a discrete mechanism which occurs under the genetic control of the cell and is generally considered to be an immunologically silent phenomenon, lacking the inflammatory response typically associated with necrosis or 'accidental' cell death. Necrosis is not under cellular control and is characterised by cellular swelling and rupture with the release of cellular contents into the surrounding environment, evoking inflammation and damage to surrounding tissues (Raff, 1998).

Apoptosis is a naturally occurring process of cell suicide which is employed by metazoans during development and maintenance as a method for the efficient and discrete removal of unwanted or superfluous cells, with minimal damage to the surrounding tissue (Jacobson *et al.*, 1997; Vaux & Korsmeyer; 1999). The immune system also utilises apoptosis as a method of cell killing in cytotoxic immune responses, lymphocyte deletion during maturation, and in the resolution of immune responses through the elimination of effector cells (Henkart & Sitkovsky, 1994; Strasser, 1995). Disruption of the regulatory pathways which control apoptosis can underlie the development of a variety of disease states including; cancer, acquired immunodeficiency syndrome (AIDS), neurodegeneration, autoimmunity, and ischaemic stroke (Thompson, 1995). Necrosis, on the other hand, is believed to occur due to cell injury, for example by some physical, chemical, or thermal insult. Additional forms of cell death have been proposed, but in the interests of clarity and consistency, the definitions of apoptosis and necrosis as described above apply for the remainder of this thesis.

### 1.1.1 The coordination of apoptosis by caspases

The caspases are a family of cysteine aspartic acid-specific proteases which exist in the cytosol as inactive precursor zymogens (procaspases) that, upon activation, play a central role in the initiation and execution of apoptosis and in the processing of proinflammatory cytokines (Wolf & Green, 1999; Creagh *et al.*, 2003). Structurally,

caspases share many similarities, including an active catalytic site which is comprised of a pentapeptide sequence with the general structure QACXG (where X is R, Q, or G). The procaspase is comprised of a large subunit (containing the active site), a small subunit, and an N-terminal prodomain which is removed during activation (Thornberry *et al.*, 1997). Upon activation, the procaspase zymogen is cleaved at specific aspartate residues, separating the large and small subunits and removing the N-terminal prodomain. The cleaved subunits then form heterodimers and two of these heterodimers associate to form a tetramer with the two active catalytic sites residing at opposite ends of the complex (Walker *et al.*, 1994; Wilson *et al.*, 1994). The activating cleavage site is unique to caspases and activation occurs solely through auto-or trans-cleavage by other caspases (with the exception of granzyme B which can also activate caspases) (Lord *et al.*, 2003).

So far 14 mammalian caspases have been described, although the specific function(s) of some individual caspases have not yet been fully elucidated. Of those whose functions are known, it is possible to broadly divide the caspases into two subgroups: those that are involved in apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) and those that are activated during inflammatory responses (caspase-1, -4, -5, and -11). Caspases involved in apoptosis can be further sub-divided into initiator caspases (2, 8, 9, and 10) and effector caspases (3, 6, and 7) depending on their functional roles in the process, with initiator caspases responding to proapoptotic signals and subsequently orchestrating the activation of effector caspases (Creagh *et al.*, 2003).

Initiator caspases are structurally distinct from effector caspases in that they contain long N-terminal prodomains, termed caspase-recruitment domains (CARDs) (caspases -2 and -9) and death effector domains (DEDs) (caspases -8 and -10). These prodomains act as protein-protein interaction motifs and are instrumental in the binding of the initiator procaspases to adaptor molecules which promote caspase aggregation and activation through an autoproteolytic process termed "proximity-induced intermolecular processing" (Earnshaw *et al.*, 1999; Kumar, 1999). The activation of initiator caspases sets in motion the 'caspase cascade' through their direct activation of the effector caspases. This caspase cascade contains several positive feedback loops whereby initiator and effector caspases can activate a variety of other caspases (including those upstream of themselves), thus amplifying the death signal (Slee *et al.*, 2001).

In contrast to their initiator counterparts, effector caspases have short prodomains and rely on activation by other caspases rather than recruitment to adaptor molecules and autoproteolysis. Once activated, effector caspases orchestrate the formation of the

apoptotic phenotype through direct dismantling of cellular structures, disruption of cellular metabolism, inactivation of apoptosis inhibitory proteins, and the activation of additional destructive enzymes (Adrain & Martin, 2001; Creagh *et al.*, 2003). Examples of substrates of effector caspases include the anti-apoptotic protein Bcl-2 (Grandgirard *et al.*, 1998), the DNA repair enzyme PARP (polyADP-ribose) (Lazebnik *et al.*, 1994), and ICAD (inhibitor of caspase-activated deoxyribonuclease) (Enari *et al.*, 1998). Caspase-activated deoxyribonuclease (CAD) is normally inhibited by ICAD but cleavage of ICAD by caspase-3 enables CAD to affect the internucleosomal DNA fragmentation characteristic of apoptosis (Enari *et al.*, 1998). Caspase-3 is believed to be the dominant effector caspase, playing a central role in the demolition phase of apoptosis (Slee *et al.*, 2001).

### 1.1.2 Apoptotic pathways

The three main pathways of apoptosis induction which have been well-resolved to date are outlined in Figure 1.1 and are discussed below, these are: the death-receptor pathway, the mitochondrial pathway, and the granzyme B-initiated pathway (Creagh *et al.*, 2003). Additional pathways have been proposed such as the endoplasmic reticulum (ER) stress caspase-12-initiated pathway (Nakagawa *et al.*, 2000), and the caspase-2-initiated mitochondrial pathway (Lassus *et al.*, 2002). However, given the apparent inactivity of capsase-12 in humans (Fischer *et al.*, 2002) and redundancy of caspase-2 (O'Reilly *et al.*, 2002) these pathways will not be discussed as they remain to be fully elucidated.

#### 1.1.2.1 The death-receptor apoptotic pathway

Several receptors located in the plasma membrane belonging to the tumour necrosis factor (TNF) receptor superfamily are involved in the transduction of pro-apoptotic extracellular signals. The best characterised of these 'death receptors' are Fas/Apo1/CD95 and TNF receptor 1 (TNFR1)/p55/CD120a. The pathways of apoptosis induction differ slightly for each death receptor but typically culminate in the activation of the initiator caspase, caspase-8. The pathway described below is that of the death receptor Fas which is involved in the deletion of superfluous lymphocytes and in the killing of target cells (such as virally infected or cancer cells) by cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells (Nagata, 1997).

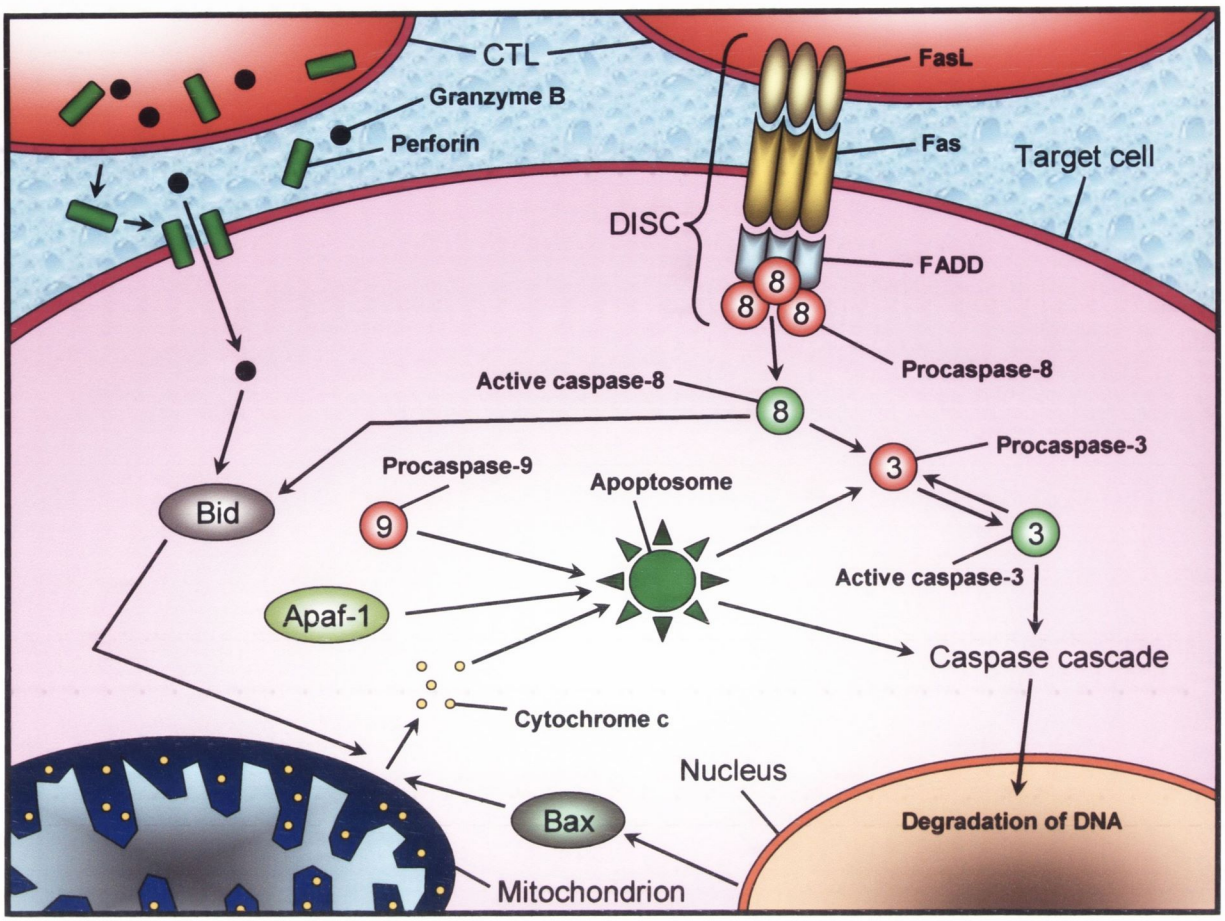


**Figure 1.1 The death receptor, mitochondrial, and granzyme B-initiated apoptotic pathways**

Binding of FasL to the Fas receptor results in trimerisation of the receptor and the recruitment of procaspase-8 via an adaptor molecule FADD. Procaspase-8 is activated due to proximity-induced intermolecular processing and subsequently activates capase-3 and the caspase cascade ensues. Alternatively, caspase-8 can also cleave Bid to tBid which induces the mitochondrial apoptotic pathway. Bid can also be activated through cleavage to gtBid by granzyme B. Translocation of Bax from the cytosol to the mitochondrial outer membrane also leads to induction of the mitochondrial apoptotic pathway. Loss in mitochondrial membrane potential leads to the release of cytochrome c into the cytosol leading to apoptosome formation from procaspase-9, Apaf-1, and cytochrome c. The apoptosome then activates the caspase cascade.

Apaf-1; apoptosis protease-activating factor-1, CTL; cytotoxic T-lymphocyte, FADD; Fas-associated death domain protein

(Original diagram based on information from: Kischkel *et al.*, 1995; Ashkenazi & Dixit, 1998; Green & Reed, 1998; Li *et al.*, 1998; Scaffidi *et al.*, 1998; Creagh *et al.*, 2003; Hill *et al.*, 2003; Lord *et al.*, 2003)

The ligand FasL binds to the extracellular domain of Fas and induces trimerisation of the death receptor. The cytoplasmic tails of death receptors contain distinct motifs termed death domains (DDs) which, upon activation and oligomerisation of the receptor, cluster together and recruit adaptor molecules which also contain DDs. In the case of Fas the adaptor molecule is termed FADD (Fas-associated death domain protein), an adaptor protein which also contains a DED motif. Procaspase-8 molecules then bind to the FADD molecules through homotypic interactions between their DEDs and are activated through self-cleavage due to proximity-induced intermolecular processing (Ashkenazi & Dixit, 1998; Muzio *et al.*, 1998). This complex is termed a death-inducing signalling complex (DISC) and is formed within seconds of receptor engagement (Kischkel *et al.*, 1995).

Following the activation of caspase-8 in the DISC, one of two possible mechanisms follows. In most cells studied, large amounts of caspase-8 are recruited to the DISC and activated which is followed by the direct activation of effector caspases and associated initiation of the caspase cascade. Other cells however, fail to activate sufficient amounts of caspase-8 in the DISC to initiate the caspase cascade. In such circumstances, proteolysis of Bid, a BH3-only member of the Bcl-2 protein family (see section 1.1.4), occurs and truncates the molecule, forming tBid. tBid then translocates to the mitochondria where it induces apoptosis through the mitochondrial pathway and the release of cytochrome c (see below) (Li *et* al., 1998; Scaffidi *et al.*, 1998).

### 1.1.2.2 The mitochondrial apoptotic pathway

The mitochondrial pathway of apoptosis can be induced by a variety of stimuli causing cell stress or damage such as cytotoxic drugs, ionising radiation, and heat-shock. Permeabilisation of mitochondrial outer membranes occurs causing the release of several proteins, most notably cytochrome c, from the mitochondrial intermembrane space into the cytoplasm (Green & Reed, 1998). The Bcl-2 family of proteins are instrumental in protection from, and induction of, this loss of mitochondrial transmembrane potential and are discussed in greater detail in section 1.1.4.

Cytoplasmic cytochrome c interacts with the C-terminus of the cytosolic protein apoptosis protease-activating factor-1 (Apaf-1). Apaf-1, together with deoxyadenosine triphosphate (dATP) and procaspase-9, forms a large caspase-activating complex initially called the aposome (Cain *et al.*, 1999) but which is now referred to as the apoptosome. Procaspase-9 is recruited to the apoptosome through interactions between

its CARD domain and a CARD domain in Apaf-1 and is subsequently activated through proximity-induced intermolecular processing (Acehan *et al.*, 2002). The apoptosome is a wheel-like structure comprised of seven molecules of Apaf-1 and seven active caspase-9 complexes which propagates the death signal through activation of downstream caspases and subsequent initiation of the caspase cascade (Hill *et al.*, 2003).

Other molecules released from mitochondria during apoptosis that are involved in cell death include: AIF (apoptosis-inducing factor) (Susin *et al.*, 1999), SMAC (second mitochondrial activator of caspases) (Du *et al.*, 2000), HtrA2 (high-temperature requirement protein A2) (Suzuki *et al.*, 2001), and endonuclease G (Li *et al.*, 2001). SMAC and HtrA2 are believed to lower the threshold for the commitment of a cell to apoptosis through the inactivation of the IAP (inhibitor of apoptosis) group of proteins which have an inhibitory effect on caspases. AIF and endonuclease G are involved in the apoptotic degradation of DNA and appear to operate through a caspase-independent pathway. However, more studies are required in order to fully resolve the roles these proteins play within the cell death machinery (Candé *et al.*, 2004; Saelens *et al.*, 2004).

#### 1.1.2.3 The granzyme B-initiated pathway of apoptosis

CTLs can induce apoptosis in target cells not only by the death receptor-mediated pathway described above, but also by the release of cytolytic molecules, particularly perforin and the serine protease, granzyme B. Perforin polymerises and forms pores in the target cell plasma membrane and endocytic vesicles which contain granzyme B. Granzyme B enters the cytosol and induces apoptosis through the activation of substrates such as procaspase-3, procaspase-8 and Bid (Lord *et al.*, 2003). Bid is cleaved by granzyme B at a site distinct from that targeted by caspase-8 (see above) producing a fragment, gtBid, which translocates to the mitochondria and promotes the release of cytochrome c and subsequent apoptosome formation (see above) (Barry *et al.*, 2000). While granzyme B has many potential targets in the cell death machinery through which it could theoretically induce apoptosis, it appears to preferentially cleave Bid and subsequently induce of the mitochondrial pathway of apoptosis (Pinkoski *et al.*, 2001).

### 1.1.3 Apoptosis regulation and oncogenesis

With a variety of potentially lethal molecules present in a dormant state in the cytosol and within mitochondria, it is not surprising that a number of stringent regulatory

mechanisms are present which are pivotal in the commitment of a cell to the apoptotic pathway. Disruption of these regulatory pathways can disturb the homeostasis within a cellular population and result in the development of a variety of diseases, including cancer, in multicellular organisms (Thompson, 1995). Several proteins are expressed which exert direct inhibitory or regulatory effects on the various pathways of apoptosis, including: IAPs (Liston *et al.*, 1996), FLIPs (Fas-associated death domain-like ICE inhibitory proteins) (Irmler *et al.*, 1997), TRIP (TNFR-associated factor (TRAF)-interacting proteins) (Lee & Choi, 1997), tumour suppressor protein p53 (Levine *et al.*, 1991), and the Bcl-2 family of proteins (Reed, 1997). Overexpression or inactivation of these proteins can result in change of a cells resistance/susceptibility to apoptosis induction and the development of cancers (Zörnig *et al.*, 2001). Bcl-2 proteins can be anti- (Bcl-2 and Bcl-$x_L$) or pro- (Bax) apoptotic and, together with p53, are among the most studied proteins involved in the regulation of apoptosis its role in the development of malignant disease (Sjöström & Bergh , 2001).

The p53 tumour suppressor protein plays a pivotal role in a cells destiny to undergo apoptosis in response to genotoxic damage. p53 arrests the cell cycle following detection of DNA damage in order to allow time for DNA repair to occur (Kastan *et al.*, 1991). Should the damage prove to be irreparable, p53 subsequently initiates apoptosis induction through upregulation of death receptors such as Fas and DR5, the proapoptotic protein Bax, and through inhibition of anti-apoptotic signals such as NF-κB (Vogelstein *et al.*, 2000). Loss of p53 or p53 function significantly contributes to tumour development through deregulation of the cell-cycle and resistance to apoptosis and is involved in over 50% of all human cancers, making it the most commonly mutated gene in human malignancies (Hollstein *et al.*, 1991; Greenblatt *et al.*, 1994; Levine, 1997)

The Bcl-2 family of proteins control the permeability of the outer mitochondrial membrane (OMM), thus regulating the release into the cytoplasm of apoptosis-related factors, such as cytochrome c, from the mitochondrial intermembrane space (Green & Reed, 1998). Family members Bcl-2, Bcl-$x_L$, Bcl-w and Mcl-1 inhibit apoptosis induction whereas Bax, Bak and Bok promote it. The BH3-only members (such as Bid) are more distantly related and serve in the promotion of apoptosis. The mechanisms by which the Bcl-2 family members control mitochondrial membrane permeability remain unclear, with a number of possible models suggested including: the formation of *de novo* protein channels, interaction with existing mitochondrial membrane pores, and the formation of lipidic pores (Sharpe *et al.*, 2004).

Bax is located predominantly in the cytosol as a monomer but upon induction of apoptosis translocation of Bax to the mitochondria occurs where it forms oligomers in the OMM, an event which is considered crucial in the permeabilisation of the mitochondrial membrane (Wolter *et* al., 1997; Antonsson *et al.*, 2000). Truncation of Bid to tBid by caspase-8 or gtBid by granzyme B results in its translocation from the cytosol to the mitochondria where it is believed to promote Bax-mediated induction of mitochondrial permeability (Eskes *et al.*, 2000; Kuwana *et al.*, 2002). Bcl-2 is present predominantly in the OMM and acts as a potent inhibitor of apoptosis through interactions with pro-apoptotic Bax, preventing its oligomerisation and subsequent induction of apoptosis (Kluck *et al.*, 1997; Gross *et al.*, 1998). Bcl-2 was initially linked to oncogenesis when its overexpression was detected in human B-cell leukaemia and follicular lymphomas, and it is from these diseases that its name is derived (Tsujimoto *et al.*, 1985). Levels of Bcl-2 are also elevated in a variety of human malignancies, including carcinomas of the breast, prostate, ovary, colon, and lung (Reed, 1995). Loss of Bax function through mutation can also lead to an oncogenic phenotype, and appears to be particularly relevant in the development of human colorectal cancers (Rampino *et al.*, 1997).

It was through the discovery of these proto-oncogenes, which contribute to malignant cell expansion through prolonging cell survival rather than increasing the rate of cellular proliferation, that the substantial role played by apoptosis in tumourigenesis gained recognition. Resistance to apoptosis not only functions in oncogenesis, but also assists in tumour development and can create difficulties in the treatment of cancer, through the development of radio- and chemoresistant phenotypes (Reed, 1995; Levine, 1997; Reed, 1997).

### 1.1.4 Viruses and apoptosis

Early induction of the apoptotic pathway in response to viral infection is likely to serve in the limitation of viral replication and the curtailment of host infection. Virally induced apoptosis, in contrast to that observed in development and in normal tissue homeostasis, is not an immunologically silent phenomenon. Cellular perturbation alone, caused by viruses can lead to attraction of the innate immune system, and cellular expression of viral proteins can induce adaptive immunity, through uptake of apoptotic bodies by professional antigen-presenting cells (APCs) for example (Everett & McFadden, 1999). Death of the host cell before the virus has sufficient time to complete

its replication cycle, together with assistance in the induction of immunity, render the induction of apoptosis in infected cells an attractive course of action in the resolution of viral infections.

Apoptosis is also believed to be of benefit to, and be exploited by, a number of viruses in the dissemination of progeny. The apoptotic process involves the packaging of cellular contents, including progeny virions, into membrane-bound vesicles which are subsequently taken up by neighbouring cells. Apart from aiding in viral spread through tissues, this may also reduce or delay inflammatory responses and protect progeny virions from aspects of the host immune system such as neutralising antibodies (Teodoro & Branton., 1997; Roulston *et al.*, 1999). Given that the ability to exploit existing cellular machinery is critical in the successful replication of a virus within a host cell, it is not surprising that many viruses have evolved an ability to manipulate the cell death programme in a bid to circumvent its abrogative effect on infection. The multi-stepped nature of the apoptotic pathways provides many targets for viruses, and only one of these may need to be disrupted in order for a virus to affect the natural process of cell death to its benefit. In fact, several viruses encode proteins which serve to both inhibit and promote apoptosis, providing time for the completion of replication before inducing cell death to aid in dissemination of progeny (Hay & Kannourakis, 2002).

Viruses which are capable of rapid replication, such as influenza virus and most other RNA viruses, can often achieve the production of high titres of viral progeny prior to the completion of the cell death programme and the mounting of effective host immune responses (Kurokawa *et al.*, 1999). While this suggests that such viruses do not necessarily manipulate the apoptotic pathway, but that they simply replicate at such a speed that cell death is not a concern, several proteins encoded by these viruses have been implicated in the induction of apoptosis (Li & Stollar, 2004). Alphavirus-induced apoptosis appears to play a role in viral dissemination and has been directly linked to neuropathogenicity, with virulent strains displaying a higher potency of apoptosis induction and more efficient spread through the central nervous system (CNS) (Ubol *et al.*, 1994; Lewis *et al.*, 1996). The mechanism by which alphaviruses induce apoptosis remains unclear, but their ability to do so in cell lines normally considered resistant to apoptosis has rendered them attractive candidates for exploitation in the field of cancer therapy (Murphy *et al.*, 2001; Yamanaka, 2004).

## 1.2 CANCER

Cancer is a term used to describe a variety of diseases which share the common feature of inappropriate and uncontrolled cell growth. Normally within multicellular organisms, there is a state of cellular homeostasis, where cells are well differentiated, are organised into complex tissue structures, and are maintained as such. Disruption of the inter- and intracellular mechanisms which are involved in the maintenance of this *status quo* can lead to the occurrence of malignant disease (Hanahan & Weinberg, 2000). The development of cancer is dependent on a series of genetic changes in somatic cells which involve: the loss of differentiation, deregulation of cell growth/death, genomic instability, and changes in the relationships of cells with surrounding tissues (Michor *et al.*, 2004).

### 1.2.1 The genes involved in malignant disease

As our understanding of the complex and dynamic molecular mechanisms involved in oncogenesis progresses, increasing numbers of genes are being implicated in the development and maintenance of cancers. A recent review by Futreal *et al* (2004) reported that 291 cancer genes have been identified to date, which accounts for over 1% of genes in the human genome. The main genes which contribute to tumourigenesis are referred to as oncogenes and tumour suppressor genes (Weinberg, 1994). In the development of cancers, oncogenes, such as *ras* and *bcl-2*, undergo a mutational event which leads to dominant gain of function, whereas tumour suppressor genes, such as *p53*, are associated with recessive loss of function due to mutation (Hanahan & Weinberg, 2000). Such mutations can confer a selective advantage on a cell within a given population, subsequently leading to cancerous growth.

### 1.2.2 The *ras* oncogene

With activating mutations detectable in approximately 30% of human cancers, the *ras* group of proto-oncogenes is among the most significant described. There are three members of the Ras protein family, H-, K-, and N-Ras, of which mutations in *k-ras* are most commonly associated with human disease (Bos, 1989). The *ras* genes encode 21 kDa guanosine triphosphate (GTP) binding proteins (p21), which function as binary switches at nodes in a variety of signal transduction pathways. Ras p21 proteins

typically cycle between an inactive guanosine diphosphate (GDP)-bound state and active GTP-bound state at the plasma membrane of normal cells (Satoh *et al.*, 1992; Boguski & McCormick, 1993). Regulation of this cycle is mediated by guanine nucleotide exchange factors (GEFs) that drive the exchange of Ras-bound GDP with GTP and GTPase activating proteins (GAPs) which in turn accelerate the rate of GTP hydrolysis to GDP by Ras, returning the protein to its inactive (GDP-bound) state (Figure 1.2) (Ellis & Clark, 2000; Midgley & Kerr, 2002). GEFs and GAPs are themselves under the influence of a variety of cell surface receptors including: platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), integrins, and cytokine receptors such as the interleukin (IL)-2 receptor and the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor (Satoh *et al.*, 1992).

Ras proteins are involved in the activation of a multitude of signalling cascades such as: the mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) pathway, and GEF pathways such as the Ral guanine nucleotide dissociation stimulator (RalGDS) pathway (Shields *et al.*, 2000; Midgley & Kerr, 2002). With such a variety of downstream effectors, Ras is pivotal in a diverse spectrum of cellular responses and influences; differentiation, apoptosis, proliferation, invasion, metastasis, and angiogenesis, to name but a few processes under its control (Shields *et al.*, 2000). Deregulation of Ras protein activity through mutation can have profound consequences, given its integral role in the transferral of information from the extracellular environment to the cell's internal response machinery (Ellis & Clark, 2000).

Activating point mutations in *ras* oncogenes are restricted to a small number of specific sites (particularly codons 12, 13, or 61), with those at codon 12 the most commonly found in Ras-associated human malignant disease (Bos, 1989). These mutations can render the Ras p21 protein constitutively active through negative effects on its intrinsic GTPase activity and a reduction in its ability to complex with GAPs (Midgley & Kerr, 2002). Given the plethora of signal transduction pathways in which Ras is involved, the consequence of the Ras binary switch being constitutively 'on' can manifest in a variety of manners which result in a malignant phenotype. The correlation between oncogenic Ras and uncontrolled cellular proliferation is well defined but other areas, like its influence on apoptosis, remain less definitive (Cox & Der, 2003). Cell-type is believed to play a major role on the influence of oncogenic Ras, with apparently contradictory effects commonly observed between different cells (Shields *et al.*, 2000; Diaz *et al.*, 2004). Oncogenic Ras proteins are crucial in mediating many malignant

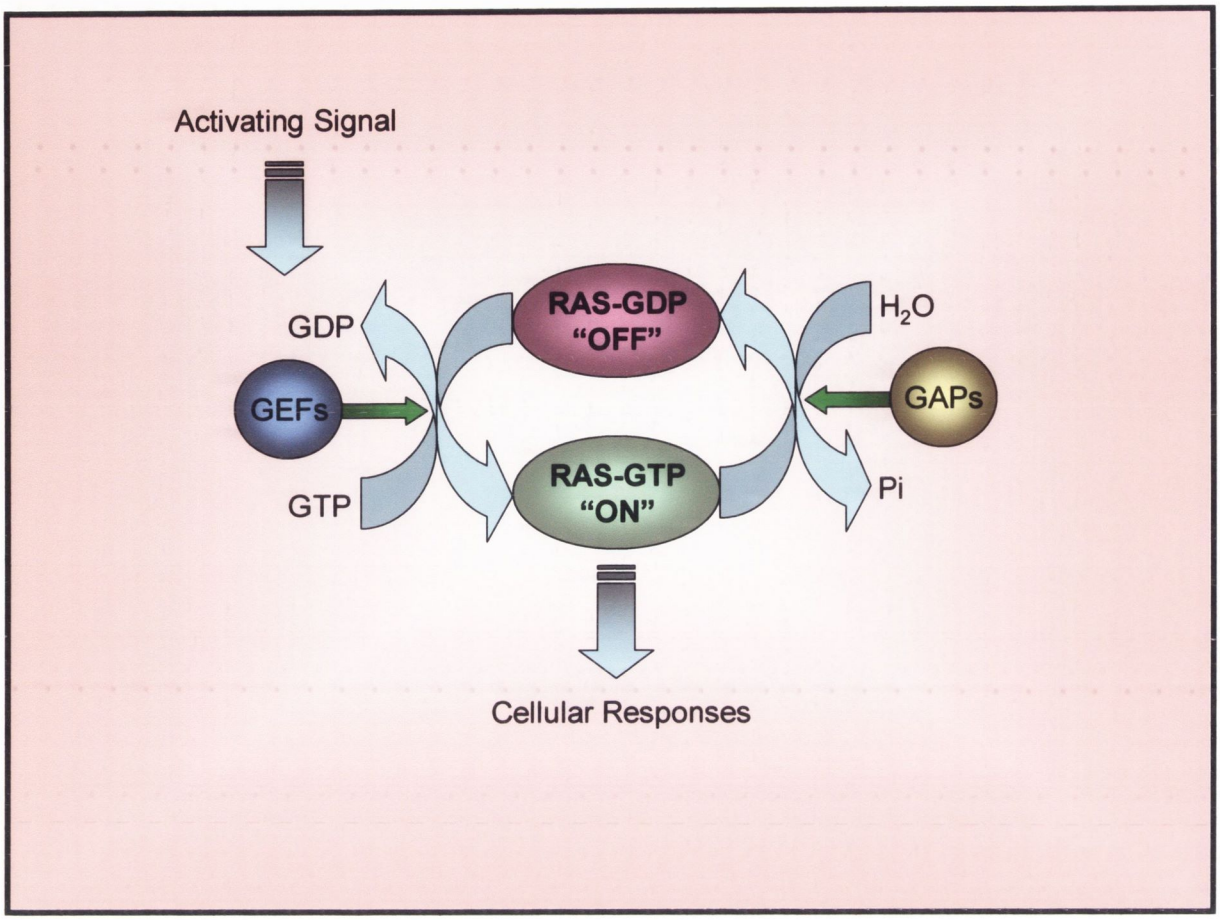


**Figure 1.2 The activation cycle of Ras proteins in signal transduction pathways**

Ras proteins cycle between an inactive GDP-bound and an active GTP-bound state at the plasma membrane of the cell. The process is mediated by GEF and GAP molecules which are, in turn, under the influence of a variety of cell-surface receptors. GEFs drive the exchange of Ras-bound GDP with GTP while GAPs accelerate the rate of GTP hydrolysis to GDP by Ras. Ras acts as a node in many signalling cascades and its activation can lead to a variety of cellular responses.

GAP; GTPase activating protein, GEF; guanine nucleotide exchange factor, GDP; guanosine diphosphate, GTP; guanosine triphosphate

(Original diagram adapted from Adjei, 2001)

characteristics of transformed cells and therefore have good potential as targets for tumour therapy (Downward, 2003).

### 1.2.3 Tumour immunology

The relationship between malignant disease and the immune system, and the potential for its exploitation in cancer therapy, has been the focus of much research for over a century (Bremers & Parmiani, 2000). The concept that the immune system is constantly surveying the body for aberrant cells and eradicating tumours prior to their clinical presentation was first suggested by Burnet in 1957 and, following further hypotheses by Thomas (1959), the term 'immunosurveillance' was coined (Burnet, 1967). Immunosurveillance has been an issue of some contention since its first description but the identification of increasing numbers of tumour-associated antigens (TAAs) and a greater understanding of the immune system have lead to increasing support for the hypothesis in recent years (Smyth *et al.*, 2001). In fact, it has been demonstrated that the immune system can play a role in sculpting tumours through the selection of tumour cells of lower immunogenicity, thus aiding tumour escape (Uyttenhove *et al.*, 1983; Shankaran, *et al.*, 2001). This has led to the proposed refinement of the term 'immunosurveillance' to the broader term 'immunoediting', given the dual host-protecting and tumour-sculpting actions of the immune system (Dunn *et al.*, 2002).

The innate immune system is now recognised as having some ability in the discrimination of tumour cells from normal cells (Diefenbach & Raulet, 2002). NK cells, γδT-cells and macrophages are believed to play an important role in the innate immune response to tumours through expression of the NKG2B receptor. The engagement of this receptor by a ligand, results in the implementation of cytolytic responses by the leukocyte (Bauer *et al.*, 1999). Ligands for NKG2B are expressed in cells in response to stress and have been detected on transformed and infected cells (Groh *et al.*, 1996). Induction of antitumour adaptive immune responses has also been demonstrated via initial recognition of such ligands by the innate immune system (Diefenbach *et al.*, 2001). NK cells are also cytotoxic towards cells which display low levels of major histocompatibility complex (MHC) class I molecules, and this pathway may be involved in the killing of tumour cells which downregulate MHC class I (see below) (Ljunggren & Karre, 1990; Garrido *et al.*, 1997).

Humoural immune responses to tumours are believed to be less potent than cellular responses, and therefore most research has concentrated on the identification of antigens recognised by T-cells (Rosenberg, 2001). It is possible however, that both cellular and humoural immune responses have roles to play in tumour cell killing (Reilly *et al.*, 2001). Antibody responses can contribute to target cell killing through complement-dependent cytotoxicity (CDC), and through antibody-dependent cellular cytotoxicity (ADCC), activating NK cells, γδT-cells and macrophages to exert their cytotoxic effects on target cells (Herlyn *et al.*, 1985; Mellstedt, 2000).

A variety of TAAs have been identified which can be recognised by T-lymphocytes (Rosenberg, 1999; Renkvist *et al.*, 2001). These antigens form a heterologous collection which can be categorised into five groups as follows: (i) overexpressed self proteins (includes cancer-testis antigens such as MAGE and GAGE), (ii) differentiation antigens (such as tyrosinase and CEA), (iii) mutated self proteins (such as caspase-8, p53, and Ras), (iv) oncogene products (such as Her2/*neu* and p53), and (v) viral antigens (includes human papillomavirus (HPV) and hepatitis B virus (HBV) antigens) (Ostrand-Rosenberg *et al.*, 2002). Many tumour antigens are endogenously synthesised and therefore presented via peptides associated with the MHC class I molecules. It is for this reason, and relative technical ease, that research has concentrated on the identification of those reactive with $CD8^+$ T-lymphocytes. Recognition of the central role played by $CD4^+$ T-lymphocytes in antitumour immunity however, has led to the identification of several MHC class II-restricted TAAs (Toes *et al.*, 1999; Rosenberg, 2001; Wang, 2003).

Many tumours persist and continue to grow despite the presence of specific host immune responses directed against them, apparently evading the immune system somehow. This has lead to the description of the phenomenon known as 'tumour escape' (Marincola *et al.*, 2000). A major factor thought to contribute to the apparently low immunogenicity of tumours, and their ability to avoid destruction by the immune system, is that of immunological tolerance (Sotomayor *et al.*, 1996). Tumour cells are host-derived and express a variety of 'self' antigens with TAA expression often described as differing from normal tissue in a quantitative, rather than qualitative manner. Also, failure in the expression of appropriate co-stimulatory or adhesion molecules can lead to the induction of T-cell anergy to tumour antigens (Baskar *et al.*, 1996; Stavely-O'Carroll *et al.*, 1998). Immunological ignorance has also been implicated, whereby antigen from peripheral tumours never reaches the secondary lymphoid organs in sufficient amounts for T-lymphocyte stimulation to occur (Melero *et al.*, 1997; Ochsenbein *et al.*, 1999).

Deficiencies in MHC class I expression are well documented as another approach employed by tumour cells in lowering their immunogenicity, effectively rendering them invisible to $CD8^+$ CTLs (Algarra *et al.*, 2000). TAAs can also be downregulated, and antigen-loss variants can arise due to the selective pressure of the immune system (Khong & Restifo, 2002).

Malignant disease, especially when advanced, has been associated with conferring a state of immunosupression on the host (Kiessling *et al.*, 1999). Apart from becoming less immunogenic as they develop, tumours can also acquire more actively immunosuppressive functions. The secretion, in particular, of cytokines such as transforming growth factor (TGF)-β and IL-10 by tumour cells are believed to have immunomodulatory and immunosuppressive effects beneficial to tumour progression and metastasis (Inge *et al.*, 1992; Yue *et al.*, 1997). Expression of FasL by tumour cells has also been implicated as a method of counter-attack by tumour cells on the immune system but has recently been contested following contradictory results in demonstrating such a mechanism of tumour escape *in vivo* (Restifo., 2000).

Taking the above into consideration, it is highly plausible that the immune system applies a selective pressure on tumours (which are known to be genetically unstable) resulting in the natural selection of less immunogenic variants. Three stages in tumour immunoediting have been proposed: *elimination*, *equilibrium*, and *escape*. Whereby: *elimination* represents the successful eradication of a tumour by the immune system without progression to the subsequent stages of immunoediting (in accordance with the original immunosurveillance hypothesis), *equilibrium* represents the containment of a (genetically unstable) tumour cell population by the immune system, and *escape* represents a phase where tumour cell variants selected during the equilibrium phase have acquired the ability to grow in a immunologically intact environment and expand in an uncontrolled manner (Dunn *et al.*, 2002; Dunn *et al.*, 2004). It is unlikely that tumour *escape* is due solely to immunoediting however, and it is important to consider tolerising events such as the induction of T-cell anergy and the peripheral, sometimes immunoprivileged, location of many tumours (Pardoll, 2003). Similarly, tumour cells which acquire functions conferring a growth advantage over other cells would be selected and possibly eventually dominate a given population based on that advantage. This correlates with the nature in which malignant disease manifests itself in the host, typically becoming more aggressive, invasive, and resistant not only to the immune system, but to clinically administered antitumour therapies (which apply their own selective pressures on the tumour cell population) as it progresses.

## 1.3 CANCER IMMUNOTHERAPY

Surgical excision of malignant tissue, where possible, is typically the primary method for the eradication of cancerous growths. Some tumours are inoperable, or surgery may not be entirely successful, and chemo- or radiotherapy must often be employed. The accompanying side-effects and toxicities of these treatment regimens are well documented (Eckhardt, 2002). Progress in cancer research is aimed at the development of more specific treatment strategies with fewer toxic side-effects.

Gene therapy refers to the introduction of heterologous genetic material into cells with the aim of treating disease. Research in the field of gene therapy has been dominated by cancer gene therapy, which is not surprising given that cancer is a molecular disease. While a large number of cancer gene therapy studies have concentrated on the correction of genetic abnormalities associated with cancer (through the ablation of activated oncogenes; Downward, 2003) or the replacement/augmentation of deleted/non-functional tumour suppressor genes (Fang & Roth, 2003), these approaches are limited, given the diversity of genetic mutations observed in cancers. Other approaches include: the expression of angiogenesis inhibitors (Feldman & Libutti, 2000), pro-apoptotic factors (Murphy *et al.*, 2001), and prodrug activation (Moolten & Mroz, 2002). The largest field of cancer gene therapy is that of immunotherapy, stimulating the induction or potentiation of host anticancer immune responses (McCormick, 2001).

### 1.3.1 Immunotherapy

Towards the end of the nineteenth century, a surgeon named William B. Coley observed regression of tumours in patients recovering from pyogenic bacterial infections of the skin. He subsequently carried out promising research by inducing such infections in cancer patients and later, using killed bacterial extracts (Coley, 1896). It was apparent that the induction of a host immune response by the bacterial toxins was indirectly having an inhibitory effect on tumours, thus bringing to light the potential of exploiting the immune system in the treatment of malignant disease. Paul Ehrlich (1909) later successfully carried out vaccinations with tumour antigens in animals, leading him to propose a role for the immune system in the routine eradication of tumours some fifty years prior to the suggestion of the immunosurveillance hypothesis by Burnet and Thomas.

It is now widely accepted that the immune system can play a significant role in the development of malignant disease through the elimination of targeted tumour cells (see above). The fact that there is a well-documented correlation between the presence of tumour-infiltrating leukocytes (TILs) and improved prognosis of disease supports immunotherapy as a viable strategy in the treatment of cancer (Clemente *et al.*, 1996; Naito *et al.*, 1998; Villegas *et al.*, 2002; Zhang *et al.*, 2003; Fukunaga *et al.*, 2004). Cancer immunotherapy is largely based on the induction or potentiation of host immune responses against malignant cells and encompasses a variety of strategies aimed at achieving this. In contrast to chemo- and radiotherapy, which are associated with severe toxicity and limited specificity (Eckhardt, 2002), immunotherapy has potential as a more natural and specific approach to the treatment of cancer.

A variety of immunotherapeutic strategies have been investigated to date with varying success. Many involve the induction or potentiation of host immune responses against specific TAAs, antigenically-undefined tumour cell preparations, or the exploitation of pro-inflammatory cytokines such as IL-2, IL-12 and GM-CSF (Rosenberg, 2001; Dranoff, 2004). Adoptive transfer of lymphocytes or dendritic cells (DCs) following *ex vivo* stimulation and/or expansion is proving to be a promising field with particular clinical relevance in the generation of tumour-specific immune responses unique to an individual's disease (Dudley & Rosenberg, 2003). The potency of DCs in the induction of immunity and their role in induction/breaking of host immune tolerance has been well established. Subsequently they have become the subject of much research in the induction of tumour-antigen specific immunity (Ardavin *et al.*, 2004; Cerundolo *et al.*, 2004). Rather than targeting the tumour cells specifically, which are genetically unstable and can prove elusive to TAA-specific immune responses, it is also possible to target antigens which are upregulated in the tumour microvasculature such as the vascular endothelial growth factor receptor (VEGFR) (Li *et al.*, 2002). Such indirect approaches are applicable to all solid tumours and are not affected by variations in TAA expression.

Following promising results in animal models, a large number of clinical trials have been completed or are underway, which implement various strategies of cancer immunotherapy. Results from these human clinical trials however, are somewhat inconsistent in comparison to those obtained in the laboratory, with a general lack of correlation between immune and clinical responses (Ko *et al.*, 2003; Marincola *et al.*, 2003). Possible explanations for these disappointing results are: genetic variation in humans in comparison to inbred animal strains, immune dysfunction in patients, a higher

tolerance of certain antigens by the human immune system than the murine, and the advanced nature of disease in patients selected for clinical trials (Ko *et al.*, 2003; Rosenberg, 2004). It is clear that more research is required in order to successfully overcome the immune escape/tolerising mechanisms employed by tumour cells.

### 1.3.2 Viral vectors for cancer therapy

As obligate intracellular parasites, viruses have evolved efficient mechanisms over millions of years for the introduction of genetic material into cells and the effective hijacking of host cell machinery, resulting in high-level expression of viral gene products and subsequent formation of daughter virions. With the development of improving techniques in recombinant gene technology, it has become possible to manipulate viruses at a molecular level and exploit them as vectors in the expression of heterologous genes in mammalian cells. Viruses have subsequently predominated over other delivery systems, such as liposomes and naked DNA vaccines, as the preferred vectors used in gene therapy research, (Vile *et al.*, 2000).

The exploitation of viruses as vectors in gene therapy typically involves the partial or complete deletion of genes involved in viral replication and their replacement with heterologous, potentially therapeutic genes. VLPs are produced by providing the deleted genes *in trans* through additional 'helper' vectors or packaging cell lines. The resulting VLPs are capable of infecting target cells and inducing expression of the heterologous protein but lack the genes necessary to replicate and so are incapable of producing any progeny virions. A variety of viruses have been adapted for gene therapy in this manner, each with its own advantages/disadvantages depending on the particular application (El-Aneed, 2004). Although the death of one patient and the development of leukaemia in two others following viral gene therapy has brought some clinical trials to a halt (see below), viruses are still the most efficient vectors for gene therapy and promising research continues in the development of more effective and safer vectors for gene delivery. The main viruses currently under examination as gene therapy vectors are discussed below.

Retroviral vectors are typically used where prolonged gene expression is desirable (such as expression of antiangiogenic factors or *ex vivo* transduction of cells for adoptive immunotherapy), as these dsRNA viruses integrate their reverse-transcribed DNA into host-cell genomes. They can only infect dividing cells, however, and it is difficult to achieve high-titres of VLPs (Miller *et al.*, 1990; Palu *et al.*, 2000). While

retrovirus vectors boast the first 'cure' of a disease using gene therapy, in the case of X-linked severe combined immunodeficiency (SCID) (Cavazzanna-Calvo *et al.*, 2000), this success is overshadowed by the fact that two patients subsequently developed leukaemia (Marshall, 2003). Lentiviral vectors, which can infect quiescent cells, have been shown to have much higher transfection efficiencies than retroviral vectors (Indraccolo *et al.*, 2002). Clinical trials with lentiviral vectors have not been yet undertaken, however, due to biosafety concerns based on the fact that the majority are derived from human immunodeficiency virus (HIV).

Adenoviruses are dsDNA viruses which replicate in the host cell nucleus, do not integrate into its genome, and can infect both dividing and non-dividing cells. High titres of recombinant adenovirus can be produced and such vectors have been studied extensively and, in some cases, successfully used as cancer gene therapy agents (Descamps *et al.*, 1996; Swisher *et al.*, 2003). The tragic death of a patient due to toxic effects following administration of high-dose adenovirus prompted considerable research in the reduction of the toxicities associated with administration of this promising gene therapy vector (Marshall, 1999; St George, 2003). Herpesviruses are large dsDNA viruses which can infect quiescent cells, such as neurons, where they establish latent infections. Herpesvirus vectors allow for the insertion of extremely large heterologous sequences, which has resulted in their use in the simultaneous delivery of several potentially therapeutic genes in cancer gene therapy (Latchman, 2001; Moriuchi *et al.*, 2002). As herpesviruses are neurotropic, their potential in the treatment of metastatic malignancies of the CNS is clear, and they have been employed in the induction of antitumour immune responses in this normally immunoprivileged area (Toda, 2003).

Adeno-associated viruses (AAVs) are non-pathogenic ssDNA defective parvoviruses with low immunogenicity that can infect both dividing and non-dividing cells (Monahan & Samulski, 2000). AAV DNA integrates into host cell genomes, but does so specifically, reducing the risk of insertional mutagenesis which has been associated with retroviral gene transfer (Samulski *et al.*, 1991). Viral titres are relatively low however, and production of AAV also requires a helper virus (such as adenovirus or herpesvirus) to provide missing structural proteins, which can lead to contamination of preparations with helper virus (Ferrari *et al.*, 1997). Poxviruses are dsDNA viruses well known for their immunogenic properties and indeed, vaccinia virus has shown good potential in a number of cancer immunotherapy studies (Hodge *et al.*, 1994; Gomella *et al.*, 2001; Qin *et al.*, 2001). Alphaviruses have also recently gained recognition as

having strong potential in the field of cancer gene/immunotherapy and are discussed in section 1.6.

## 1.4 VIRAL ONCOLYSIS

Following the regression of cervical cancers in some patients who had received live attenuated rabies virus vaccines at the beginning of the last century, the prospect of using replicating viruses in the treatment of cancer was raised (Dock, 1904; De Pace, 1912). Cases of apparently spontaneous regression of malignant disease after bouts of viral illness were reported in subsequent years, leading to experiments in the 1920's where tumour-bearing mice were treated with a variety of viruses, but inconclusive results were obtained (Gromeier, 2002). It was during the 1950s that cancer 'virotherapy' truly began to gain significant momentum, and a number of human clinical trials were undertaken with viruses such as influenza virus (Sinkovics & Horvath, 1993), Newcastle disease virus (NDV) (Flanagan *et al.*, 1955), and adenovirus (Heubner *et al.*, 1956). Similar experiments were reported up to the early 1980s by which time the oncolytic potential of mumps virus (Asada, 1974), measles virus (Bluming & Ziegler, 1971; Taqi *et al.*, 1981) and reovirus (Hashiro *et al.*, 1977) had also, amongst others, been examined (Wheelock & Dingle, 1964). Unfavourable side-effects were common during such trials, however, and tumour inhibition/regression was rarely sustained (Sinkovics & Horvath, 1993; Chiocca, 2002).

Advances in recombinant DNA technology in the 1980s allowed for the manipulation of viruses at a molecular level and their exploitation as gene delivery vectors. Replication-incompetent viral vectors subsequently dominated research involving the use of viruses in cancer therapy as the field of cancer gene therapy took shape. Clinical responses to such therapies have often failed to compare with the promising results achieved in the laboratory however, and so there has been renewed interest in the use of replication-proficient viruses/viral vectors in the field of cancer therapy (Bergsland and Venook, 2002; Ring, 2002). Such viruses/vectors have been shown to have a high therapeutic index and may affect tumour cell death simply by replicating in the tumour cells, with no need for genetic manipulation or the expression of foreign genes. However, genetic manipulation can increase their cytotoxicity and efficacy for infecting malignant tissues and limit their replication in normal, healthy tissues. As such viruses have the ability to multiply in target tissues, an amplification of the administered input dose occurs which is estimated to be in the order of a 1,000- to

10,000-fold increase, augmenting cytotoxicity, immunopotentiation, and heterologous protein expression (Kirn, 2002). Early virotherapy studies and an increased knowledge of the molecular mechanisms of viral replication and malignant disease have contributed to the development/discovery of a substantial number of replication-proficient oncolytic viruses, many of which have undergone, or are currently undergoing, human clinical trials.

### 1.4.1 Naturally occurring oncolytic viruses

Several naturally occurring viruses with an apparent innate avidity for malignant tissues have been described. The oncolytic ability of NDV has been recognised for half a century and strains of this avian virus have been employed both in the production of oncolysates (section 1.4.3) and in direct administration for the treatment of malignant disease (Flanagan *et al.*, 1955; Sinkovics & Horvath, 2000). Recently, a naturally attenuated strain of NDV (PV701) has undergone phase I human clinical trials in the treatment advanced solid tumours with encouraging results, and phase II trials are now planned (Pecora *et al.*, 2002; Lorence *et al.*, 2003). The potential of vesicular stomatitis virus (VSV) in cancer therapy has only been realised relatively recently, and several studies have demonstrated this virus's ability to selectively kill transformed cells *in vitro* and several *in vivo* animal tumour models (Stojdl *et al.*, 2000; Balachandran *et al.*, 2001; Ebert *et al.*, 2003; Shinozaki *et al.*, 2004). The oncotropism of NDV, VSV, and several other oncolytic viruses, is attributed to their exploitation of defects in antiviral interferon pathways which are often detected in tumour cells (Grander & Einhorn, 1998; Stojdl *et al.*, 2000). Reovirus is a ubiquitous human virus which has been shown to replicate preferentially in cells in which the *ras* oncogene is activated (Coffey *et al.*, 1998). As *ras* is the most common oncogene to undergo activating mutations in human cancer (Bos, 1989), the clinical potential of reovirus in the treatment of cancer has not gone unnoticed. Phase I and II clinical trials have been undertaken employing reovirus in the treatment of progressive cancers including glioblastoma multiforme, with encouraging results (Shah *et al.*, 2003; http://www.oncolyticsbiotech.com/clinical.html., 2004).

NDV, VSV and reovirus rarely cause serious disease in humans with infection typically being asymptomatic or resulting in mild, flu-like symptoms. Toxic responses to high doses of viral therapies do occur, but these were shown to be successfully reduced in the case of PV701 following prior administration of an initial 'desensitising' dose (Pecora *et al.*, 2002). Other naturally occurring oncolytic viruses have been

identified, and the number is increasing. Sindbis virus (SV) (and its derived vector), for example, recently has been shown to have an inherent avidity for malignant tissues (Tseng *et al.*, 2002; Tseng *et al.*, 2004). The oncolytic effects of the viruses discussed above can be further enhanced through genetic manipulation and the inclusion of potentially therapeutic heterologous genes, such as cytokines (Fernandez *et al.*, 2002). Apart from manipulating inherently oncolytic viruses, it is now possible to engineer viruses which do not normally display oncotropism to selectively infect and kill tumour cells.

### 1.4.2 Engineered oncolytic viruses

Sequencing of viral genomes and the identification and characterisation of specific viral genes has provided researchers with the ability to manipulate viruses at the molecular level, render them less pathogenic, and confer selective replication in malignant tissue. Selective replication can often be achieved through the deletion of viral genes which are essential for replication in normal cells but dispensable in tumour cells. The first genetically engineered oncolytic virus reported was the herpes simplex-1 virus (HSV-1) mutant *dl*sptk, which selectively replicates in dividing cells and was used to treat human malignant glioma xenografts in BALB/c *nu/nu* mice (Martuza *et al.*, 1991). Other HSV-1 oncolytic mutants, such as G207, have been described which have increased biosafety due to a hypersensitivity to certain antiviral drugs and the inactivation of virulence genes (Mineta *et al.*, 1995; Randazzo *et al.*, 1997). Clinical trials of HSV-1-derived oncolytic mutants have been carried out and have yielded promising results, both when administered alone or in combination with more conventional therapies (Prados *et al.*, 2003; Niranjan *et al.*, 2004).

The adenovirus mutant ONYX-015 (a.k.a. *dl*1520) is believed to replicate selectively in cells with altered *p53* function and has dominated clinical trials of engineered oncolytic viruses since its first description by Bischoff *et al* in 1996. Clinical trials with ONYX-015 have now entered phase III following encouraging results from previous trials during which it was found to be well-tolerated with low toxicity to patients. Responses were found to be significantly better when ONYX-015 was administered together with conventional chemotherapeutic agents than when administered alone (Khuri *et al.*, 2000; Reid *et al.*, 2002; Vile, 2001; Hecht *et al.*, 2003). A variety of oncolytic adenovirus mutants have also been described exploiting deletions in the E1A viral protein, which is involved in driving the infected cell into S-phase, and

include Δ24, KD1 and KD3 (Fueyo *et al.*, 2000; Doronin *et al.*, 2001). Placing the E1A gene under the control of a prostate-specific antigen (PSA) promoter results in an adenovirus mutant whose replication is proportional to the level of cellular PSA expression, and that has entered clinical trials in the treatment of prostate cancer (Rodriguez *et al.*, 1997; Yu *et al.*, 1999).

Another tactic employed to improve viral oncotropism which has proven to be particularly challenging is the alteration of the viral proteins involved in binding and internalisation. Targeting of viruses to EGFR, which is overexpressed in malignant tissue, is yielding promising results in the selective infection of tumour cells normally resistant to viral infection (van Beusechem *et al.*, 2003). The cytolytic potential of naturally occurring or engineered oncolytic viruses can be further enhanced through the expression of heterologous genes encoding; cytotoxic proteins, drug-sensitising factors, or cytokines (Ring, 2002). In conclusion, the advent of recombinant DNA technology has provided researchers with a means to enhance existing oncotropic and oncolytic abilities of specific viruses and to confer these traits on previously non-tumour selective viruses. These oncolytic viruses have shown promising clinical potential, particularly when employed in tandem with chemo- or radiotherapy.

### 1.4.3 Exploiting the antiviral immune response in oncolytic virotherapy of cancer

One apparent disadvantage in the use replicating viral systems in cancer therapy is the induction of neutralising host immune responses and subsequent inhibition of viral replication and dissemination. This can be of particular relevance when using viruses to which patients may have pre-existing immunity, a factor also of importance to replication-incompetent viral vectors. It has also been reported, however, that the induction of host immune responses by virally infected malignant tissue can actually aid in the induction or potentiation of antitumour immune responses directed against TAAs. Oncolysates, prepared from tumour cells infected with viruses *ex vivo*, are well established as being more potent cancer vaccines than uninfected tumour cell lysates (Cassel & Garrett, 1966; Sinkovics, 1991). Toda *et al* (1999, 2003) have described the use of the HSV-1 mutant G207 in the induction of specific systemic cytotoxic antitumour immune responses. The expression, therefore, of viral antigens in tumour cells can aid in the eradication of tumour cells and in the induction or potentiation of host immune responses directed against TAAs following an initial immune response directed against virally infected tumour cells.

## 1.5 SEMLIKI FOREST VIRUS

SFV is a member of the *Togaviridae* family belonging to the large genus of arthropod-borne viruses *Alphavirus*. SFV was first isolated in the Semliki rainforest of Uganda in 1944 by Smithburn & Haddow and its natural vertebrate hosts are wild birds and small rodents. Alphaviruses are not major human pathogens, with infection usually resulting in subclinical or mild febrile illness. It was originally believed that SFV was non-pathogenic in humans but an outbreak of SFV was reported by Mathiot *et al* in 1990 that occurred in the Central African Republic in 1987 with mild symptoms including fever, persistent headache, myalgia and arthralgia. There are alphavirus strains, however, which can cause severe (and sometimes fatal) encephalitis in humans such as Eastern equine encephalitis virus (EEE) and Western equine encephalitis (WEE). SV and Venezuelan equine encephalitis virus (VEE), together with SFV, have provided much of the information known today regarding Alphaviruses.

### 1.5.1 The SFV virion

SFV is a spherical enveloped virus which has a diameter of approximately 69 nm. Virions are composed of an envelope of host-derived membrane studded with 80 spikes (comprised of E1, E2 and E3 proteins) which tightly surrounds a nucleocapsid made up of 240 copies of capsid (C) protein in association with a single positive-sense ssRNA molecule of approximately 11.4 kb (Figure 1.3). Submolar quantities of an additional viral protein termed 6k, which is involved in viral budding, are also present in SFV virions (Loewy *et al.*, 1995; Strauss and Strauss, 1994).

The spike proteins of the envelope are composed of three copies each of the E1, E2 and E3 glycoproteins, with three heterotrimers forming each spike. An icosahedral lattice with T=4 symmetry is formed by the 80 spikes, which is characteristic of alphaviruses (Vogel *et al.*, 1986). The E1 glycoprotein is comprised of 438 amino acids (aa) and has a molecular mass of 49 kDa. It is responsible for viral penetration and fusion and is anchored to the viral membrane via a hydrophobic region and 2 arginine residues at its C-terminus (Walhberg *et al.*, 1992). The E2 glycoprotein consists of 422 aa, has a molecular mass of 52 kDa, and contains a large ectodomain at its N-terminus which serves as a receptor binding subunit. A 31 aa C-terminal tail region, which is separated from the ectodomain by a hydrophobic transmembrane region, resides within the viral membrane where it has been shown to interact with the nucleocapsid (Metsikko

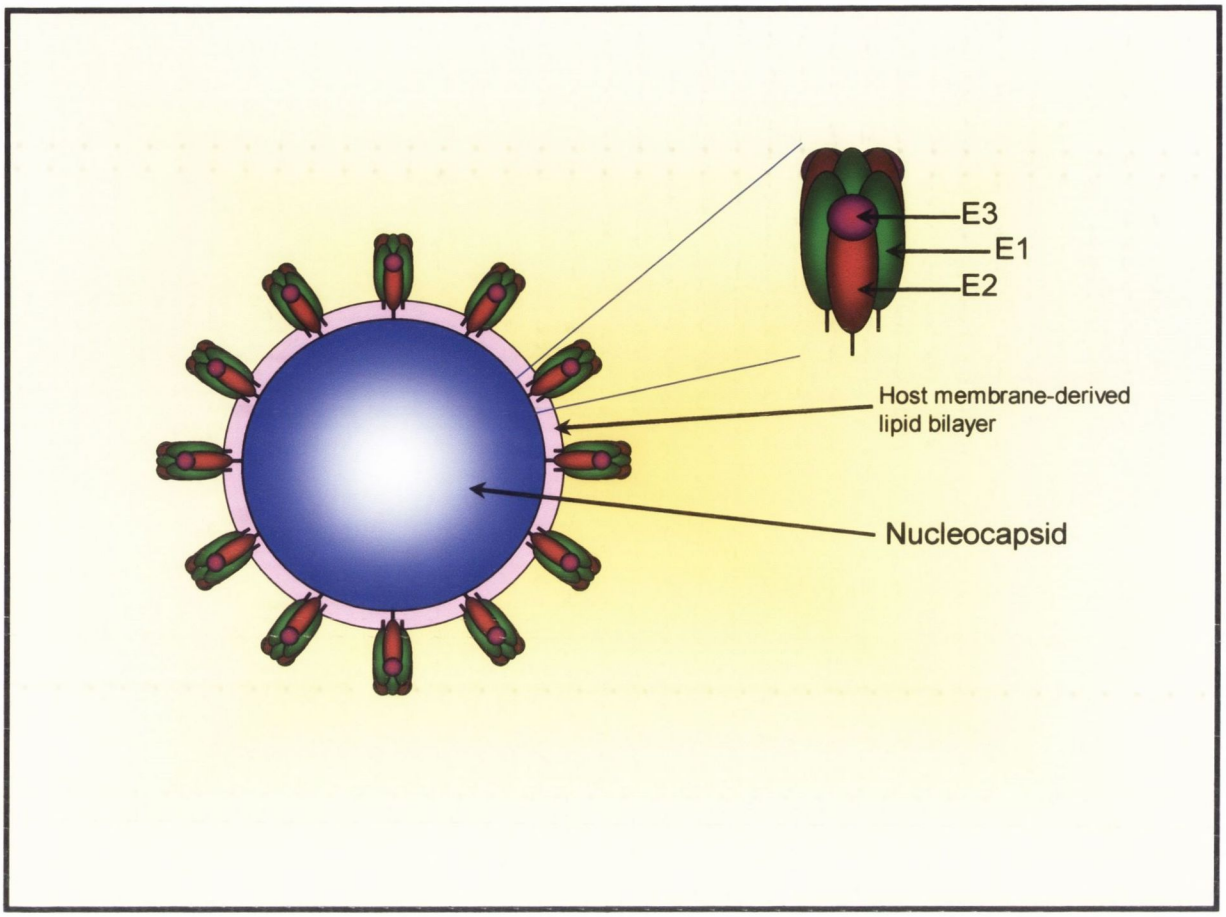


**Figure 1.3 Structure of the SFV virion**

The SFV virion is comprised of a nucleocapsid, made up of 240 copies of a capsid protein in association with a single positive-sense ssRNA molecule, encased in a host membrane-derived lipid bilayer which is studded with 80 viral spike complexes. Each viral spike complex is made up of three copies each of the E1, E2 and E3 glycoproteins. Only the C-terminus of the E2 protein is in contact with the nucleocapsid. (Original diagram based on information from: Strauss & Strauss, 1994)

& Garoff, 1990; Skoging *et al.*, 1996). The E2 and E3 proteins are derived from a common precursor, p62, which is cleaved during virus maturation. E3 remains non-covalently associated with the mature virion spike protein of SFV, but is shed into the culture fluid upon maturation of other alphaviruses such as SV (Garoff *et al.*, 1974; Mayne *et al.*, 1984). The function of E3 remains to be completely elucidated, although it has been established that its N-terminus acts as a signal sequence for p62 during viral replication (Lobigs *et al.*, 1990).

The nucleocapsid has a diameter of 38 nm and C proteins are arranged in a T=4 icosahedral array, as with the envelope proteins (Choi *et al.*, 1991). The fenestrated structure of SFV nucleocapsids renders the encapsidated RNA sensitive to RNase, and they have also been shown to shrink at acidic pH, resulting in loss of viral RNA (Soderlund *et al.*, 1979). The C protein is comprised of 267 aa and has a molecular mass of 30 kDa. A conserved C-terminal chymotrypsin-like serine protease region facilitates autocleavage from the nascent structural polypeptide and RNA binding is mediated by the N-terminal domain (Melancon & Garoff, 1987; Owen & Kuhn, 1996).

### 1.5.2 The SFV replication cycle

#### 1.5.2.1 Viral entry

Alphaviruses infect cells by a process termed receptor-mediated endocytosis which is normally reserved by the cell as an uptake mechanism for receptor-ligand complexes. The virus binds to a receptor on the cell surface and, following internalisation of virus in vesicles and transfer to endosomes, fusion of viral envelope with the endosome membrane occurs and the nucleocapsid gains entry to the cytoplasm (DeTulleo & Kirchausen, 1998). Several proteins have been proposed as possible functional receptors for SFV, including human leukocyte antigen (HLA)-A and HLA-B (Helenius *et al.*, 1978), but this was later disputed by Oldstone *et al* (1980). Laminin receptor and heparin sulphate have been identified as putative mammalian receptors for SV (Byrnes & Griffin, 1998; Wang *et al.*, 1992). Given the extremely broad host range of alphaviruses which comprises both invertebrates and vertebrates and their ability to infect a variety of diverse cell-types, it is highly probable that these viruses exploit a variety of receptors with varying affinities in order to gain entry to cells (Strauss & Strauss, 1994).

The E2 subunit of the viral spike has been demonstrated to interact with the cell surface and facilitate viral entry (Dubuisson & Rice, 1993). Viruses bind in clathrin-coated pits and are internalised in similarly coated vesicles and subsequently delivered to intracellular endosomes (DeTulleo & Kirchausen, 1998). The reduced pH in the endosome induces a conformational change in the viral spike proteins which culminates in the fusion of viral and endosome membranes. The E1-E2 heterooligomers dissociate and the individual subunits undergo conformational changes. E1 homotrimers then form and create pores in the virion membrane which is followed by fusion of the viral and endosome membranes and the release of the nucleocapsid into the cytoplasm (Dick *et al.*, 1996; Kielien & Helenius, 1985; Wahlberg *et al.*, 1992; Gibbons *et al.*, 2003). Disassembly of the nucleocapsid is believed to be mediated by host cell ribosomes (Singh & Helenius, 1992).

#### 1.5.2.2 Viral RNA replication

The SFV genome consists of a single positive-sense ssRNA molecule of approximately 11.4 kb which has a sedimentation coefficient of 42S. The 5' end is capped with a 7-methylguanosine residue and a polyadenylated tail is located at the 3' end, allowing the genome to function directly as mRNA upon introduction to the cytosol (Clegg & Kennedy, 1974). The genome can be divided into two major regions, the two-thirds at the 5' end encode non-structural proteins (nsP1, nsP2, nsP3 and nsP4), and the remaining one third towards the 3' end encodes the structural proteins (C, P62, 6k, and E1) (Strauss & Strauss, 1994).

Replication of RNA occurs exclusively in the cytosol within 0.6-2.0 μm-wide type-1 cytoplasmic vacuoles (CPV-1) which are derived from endosomes and lysosomes (Froshauer *et al.*, 1988; Peränen & Kääriänen, 1991). Translation is initiated at the 5' open-reading-frame (ORF) which encodes the non-structural precursor polyprotein P1234 (Lehtovaara *et al.*, 1980, Takkinen *et al.*, 1991). P1234 is then autoproteolytically cleaved into the four non-structural proteins nsP1, nsP2, nsP3 and nsP4 which form the viral replicase complex (Liljeström & Garoff, 1991). The replicase is responsible for the synthesis of minus-strand 42S RNA species from the positive-strand and the subsequent generation of positive-strand daughter RNA molecules, with the minus-stand RNAs acting as templates (Strauss & Strauss, 1994). A smaller positive-strand 26S RNA species that encodes the structural proteins is also transcribed, under the control of an internal subgenomic promoter, from these minus-strand RNA templates. Daughter RNA

molecules are capped and polyadenylated by the replicase complex, after which full-length 42S RNAs are predominantly encapsidated into new virions and subgenomic 26S RNAs serve as mRNA for the translation of a structural precursor polyprotein (Figure 1.4) (Sawiki *et al.*, 1978; Welch *et al.*, 1981).

### 1.5.2.3 Synthesis of viral structural proteins and viral release

The structural polyprotein precursor translated from the 26S subgenomic RNA is comprised of all the viral structural proteins in the order $NH_2$-C-p62-6k-E1-COOH. The C protein contains an intrinsic serine-protease catalytic site and cleaves itself from the nascent polypeptide chain co-translationally (Aliperti & Schlesinger, 1978; Melancon & Garoff, 1987). Following autoproteolysis, C proteins form multimers and associate with genomic 42S RNA in the cytoplasm, forming nucleocapsids. The packaging signal sequence is located in the *nsP2* region of the SFV genome where it is believed the binding of C proteins initiates the binding of additional C protein molecules to form the final structure of the nucleocapsid in the cytoplasm (Forsell *et al.*, 1995; Strauss *et al.*, 1995; White *et al.*, 1998).

Cleavage of the C protein exposes an N-terminal signal sequence at the p62 ectodomain region of the precursor polyprotein which directs translocation into the lumen of the ER (Garoff *et al.*, 1990). The ER signal peptidase cleaves E1 and 6k from the polyprotein and p62 and E1 oligomerise into heterodimers following the insertion of E1 into the ER membrane (Barth *et al.*, 1995). The cleavage of p62 into E2 and E3 occurs post-translationally and most probably by a host cell furin in the trans-golgi (de Curtis & Simons, 1988; Sariola *et al.*, 1995). This cleavage event is crucial in virus maturation as it is essential for membrane fusion and host-cell binding (Keranen and Kaariainen, 1975). The E1-E2-E3 heterotrimers trimerise into homotrimers which constitute mature SFV spike complexes that that are transported to the plasma membrane. Interactions occur between C proteins in mature nucleocapsids and the cytoplasmic tails of E2 proteins in mature spike complexes, but preformed nucleocapsids are not essential in the budding process (Figure 1.4) (Suomalainen *et al.*, 1992; Forsell *et al.*, 1996).

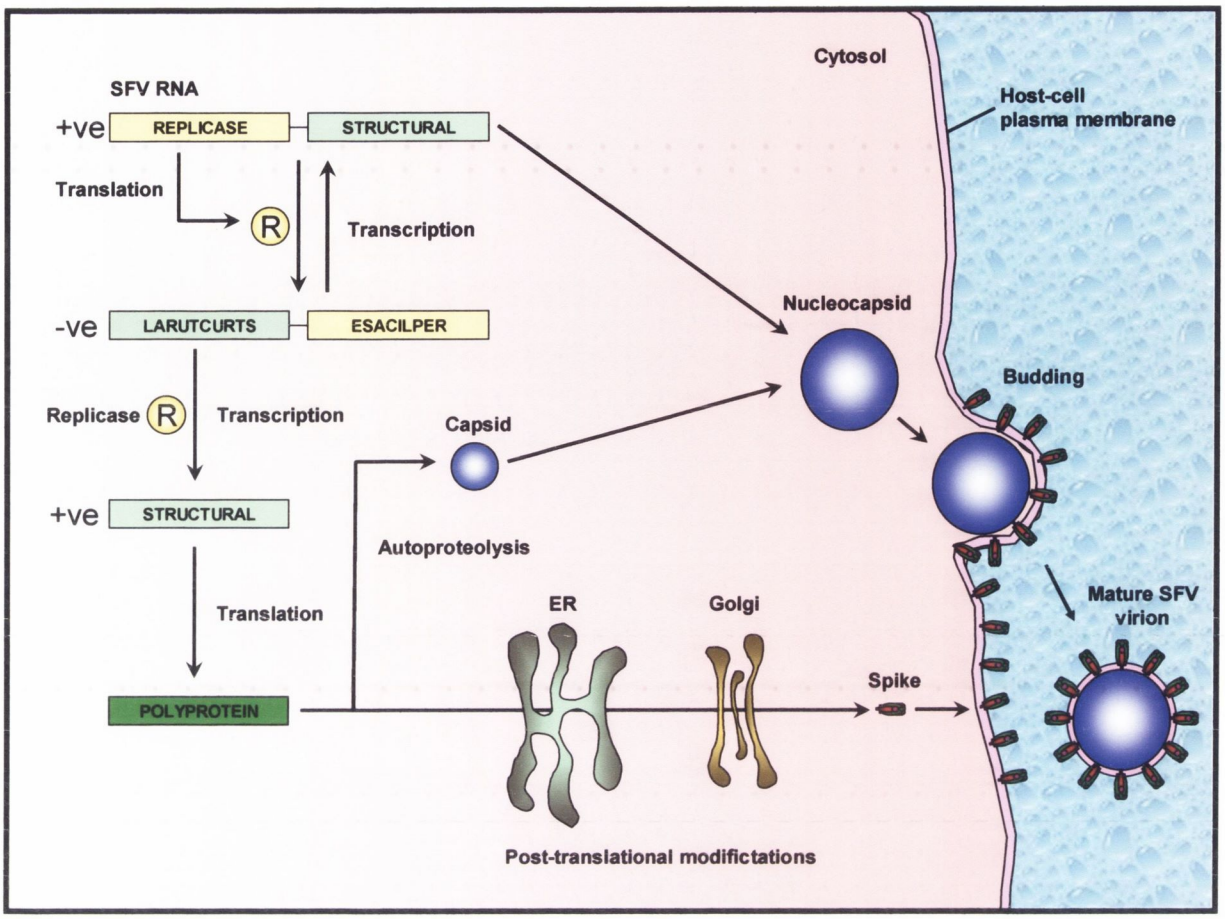


**Figure 1.4 SFV RNA replication and release of progeny virions from infected cells**

Following infection of a cell, SFV RNA can act directly as mRNA and the non-structural replicase complex is translated from this 42S molecule. The replicase then syntheses 42S minus-strand RNA molecules which act as templates for smaller positive-strand 26S RNA molecules encoding the structural genes only, and more full-length positive-strand 42S RNA molecules. The structural polyprotein is translated from the subgenomic 26S RNAs and the capsid protein autoproteolytically cleaves itself from the nascent polypeptide chain. Capsid proteins then complex with genomic 42S positive strand RNAs to form mature nucleocapsids. The remaining structural polyprotein is cleaved into E1, p62, and 6k in the ER and p62 is cleaved into E2 and E3 in the trans-golgi. Mature spike complexes form and are then transported to the host cell plasma membrane. Interactions occur between capsid proteins in mature nucleocapsids and the cytoplasmic tails of the E2 proteins in mature spike complexes and SFV virions then bud from the infected cell.

ER; endoplasmic reticulum

(Original diagram based on information from: Strauss & Strauss, 1994)

### 1.5.3 Effects of SFV infection in host cells

The lifecycle of alphaviruses requires that they infect and replicate in both arthropod and vertebrate hosts. Once infection is established in the arthropod (most commonly mosquito) host, viral replication occurs primarily in the fat body and salivary glands, where a persistent state of infection is established for life (Mims *et al.*, 1966; Scott & Weaver, 1989). A persistent state of infection of mosquito cells grown in culture has also been demonstrated in the absence of gross cytopathic effects (CPE) (Davey & Dalgarno, 1974; Tooker & Kennedy, 1981). Upon infection of a permissive vertebrate host, through the bite of an infected arthropod, a substantial viraemia develops which facilitates vector-borne transmission prior to viral clearance by the host immune system (Strauss & Strauss, 1994). In culture, infection of vertebrate cells typically results in rapid viral replication with substantial CPE and rarely in the establishment of a persistent infection (Glasgow *et al.*, 1997).

Alphaviral infection of permissive vertebrate cells such as BHK-21 cells (baby hamster kidney) results in the rapid take-over of host-cell translational machinery by viral mRNAs with only viral structural proteins being synthesised by 6 hours post infection. Approximately $10^4$ viral particles are produced per cell in 24 hours, during which time cells undergo CPE and ultimately die by 72 hours post infection (Liljeström & Garoff, 1991; Glasgow *et al.*, 1997). This CPE is thought to be predominantly due to the induction of apoptosis in the majority of vertebrate cells studied, with the exception of cells naturally resistant to apoptosis induction such as mature neurons and fully differentiated macrophages (which undergo necrosis) (Balluz *et al.*, 1993; Glasgow *et al.*, 1997; Scallan *et al.*, 1997). The structural proteins of SV (specifically the E2 glycoprotein) have been demonstrated to enhance, but are not essential for, the induction of apoptosis in the BHK-21 cell line (Frolov & Schlesinger., 1994). Cytopathic effects however, are not influenced by the deletion of the structural proteins of SFV, suggesting a role for viral RNA replication in apoptosis induction. In fact, deletion of most of the nsP2 gene of SFV has been shown to abrogate RNA synthesis and also the induction of apoptosis (Glasgow *et al.*, 1998).

A variety of cellular factors have been suggested in the modulation of the outcome of alphaviral infection, but the exact pathway of apoptosis induction remains unclear. The expression of the anti-apoptotic gene *bcl-2* has been shown to restrict SFV and SV replication as well as delaying the induction of apoptosis in the rat prostate cancer cell line AT3 (AT3-Bcl-2) (Levine *et al.*, 1993; Scallan *et al.*, 1997; Murphy *et*

*al.*, 2001). Grandgirard *et al*, (1998) later demonstrated that SFV and SV can overcome the protective effect of *bcl-2* overexpression and induce apoptosis via caspase-3-mediated cleavage of the bcl-2 protein following infection. Other cellular factors which have been demonstrated to influence alphavirally-induced apoptosis include, amongst others, NF-κB activation (Lin *et al.*, 1998), Ras (Joe *et al.*, 1996) and protein kinase C (Zrachia *et al.*, 2002). SFV-induced apoptosis can also occur in the absence of p53, as was demonstrated in the human lung carcinoma cell line H358a which contains a homozygous deletion of the *p53* gene (Glasgow *et al.*, 1998). To date, evidence exists for the involvement of mitochondrial pathways and the death receptor pathway in the induction of apoptosis due to alphavirus infection (Li & Stollar, 2004). It is likely that alphavirus-induced apoptosis is a multifaceted phenomenon in which differing cell death pathways may be activated depending on cellular factors, and on the individual species of alphavirus.

### 1.5.4 Pathogenesis of SFV in mice

For many years, SFV has been employed as a model for neuropathogenesis in mice with a variety of pathologies described, ranging from asymptomatic infection to fatal encephalitis. Route of administration, age of host and the particular strain of SFV all play significant roles in the outcome of infection. As mentioned previously, Smithburn & Haddow first isolated SFV from mosquitoes in Uganda in 1944. This original isolate was designated L10 and is neurovirulent causing fatal encephalitis in neonatal and adult mice when administered either peripherally or intracranially (i.c.). McIntosh *et al* described another strain of SFV in 1961, A7, which was isolated from mosquitoes in Mozambique and was avirulent in adult mice, but fatal in neonatal mice. Selection for avirulence by passaging of A7 in primary chick cells generated the strain A7(74) (Bradish *et al.*, 1971). The original cDNA infectious clone of SFV, derived from L10, is designated pSP6-SFV4 and has lost some of its virulence, most probably due to passage of the prototype strain in cell culture. Transcription from this infectious clone yields the virus SFV4 which shows complete virulence in both neonatal and adult mice when administered intranasally (i.n.). Only 60-70% of adult mice die, however, when the same dose of virus is administered by the intraperitoneal route (i.p.) (Glasgow *et al.*, 1991).

Upon infection of a mouse with SFV through the bite of a mosquito for example, the site of primary replication is typically the local skeletal muscle. Virus can also be

detected in Langerhans cells which may transport it to the draining lymph nodes. Viraemia ensues and virus can spread and replicate in other permissive tissues prior to entering the CNS (Griffin, 2001). While the precise mechanism by which encephalitic alphaviruses enter the CNS following peripheral administration is yet to be fully elucidated, the A7(74) strain of SFV has been demonstrated to infect vascular endothelial cells of the CNS causing increased vascular permeability and providing the most likely route for viral entry (Khalili-Shirazi *et al.*, 1988; Soilu-Hänninen *et al.*, 1994). A more direct route of administration to the CNS, which yields more consistent results than i.p., is i.n. infection. This method targets the olfactory bulb as the point of entry to the CNS from where axonal transport and macrophages have been implicated in the transport of virus from nerve endings in the nasal mucosa (Sheahan *et al.*, 1996; Sammin *et al.*, 1999).

The ability of a particular strain of SFV to replicate in neurons, and the efficiency at which it does so, is directly related to its virulence. Virulent strains have been shown to multiply faster and to a higher titre in cultures of rat cerebellar granule cells (neurons which have been differentiated in culture) and in the murine CNS in comparison to avirulent strains (Gates *et* al., 1985; Atkins *et al.*, 1990). Following peripheral administration, A7(74) and L10 generate similar levels of viraemia in the murine host. Upon entering the CNS, regardless of route of administration, a different pattern is observed where brain viral titres gradually decrease over time for A7(74) but continue to rise for L10 until death (Balluz *et al.*, 1993; Fazakerley *et al.*, 1993). Similarly, A7(74) is only detected as localised foci surrounding cerebral capillaries while L10 disseminates more efficiently and rapidly through the CNS, causing extensive neuronal damage (Oliver *et al.*, 1997). Death of the host occurs due to a lethal threshold of damage to neurons being exceeded prior to intervention and viral clearance by the host immune system (Atkins *et al.*, 1999).

A complex interplay exists between host cell and virally encoded factors in the mechanisms determining virulence of individual alphaviruses. As neurons mature and differentiate they become increasingly resistant to alphavirus-induced apoptosis which is apparent upon infection of neonatal mice which display an extensive induction of apoptosis in the CNS and succumb to infection with either avirulent or virulent strains. Adult mice do not succumb to avirulent infection and display a form of neuronal cell death which more closely resembles necrosis and is more extensive for virulent strains (Oliver *et al.*, 1997; Griffin, 1998; Oliver & Fazakerley, 1998; Sammin *et al.*, 1999). A single amino acid change in the E2 glycoprotein of SV (which is known to be involved

in the induction of apoptosis) has been shown to overcome Bcl-2 inhibition of apoptosis and confer increased neurovirulence, further implicating a correlation between alphavirus-induced apoptosis and neurovirulence (Ubol *et al.*, 1994; Lewis *et al.*, 1996). Also, studies involving the non-structural region of the SFV genome, have demonstrated a major role for the nsP2 and nsP3 (particularly nsP3) genes in determining the neurovirulence of the virus (Fazakerley *et al.*, 2002; Tuittila and Hinkkanen; 2003).

SFV preferentially replicates in the oligodendrocytes of primary rat mixed glial cell cultures (which contain few neurons), inducing apoptosis and eventually infecting astrocytes (Bruce *et al.*, 1984). Adult mice infected with A7(74) display a subacute immune-mediated demyelinating disease with destruction of oligodendrocytes by either SFV-induced apoptosis or targeting by cytotoxic T-lymphocytes following viral infection (Kelly *et al.*, 1982; Atkins, 1990). Demyelination occurs as discrete plaques and infection of mice with avirulent strains of SFV has been suggested as a possible model for the study of demyelinating diseases in humans such as multiple sclerosis (MS) and subacute sclerosing panencephalitis (SSPE). Virulent strains also induce demyelination, but this is obscured by death of the host (Fazakerley & Buchmeier, 1993; Atkins *et al.*, 1994; Atkins *et al.*, 1999). Virulent and avirulent strains of SFV can also be transmitted transplacentally and are teratogenic in mice. The virus replicates first in the placenta itself, before spreading to the embryo where a lethal threshold of damage is exceeded resulting in foetal death before intervention by the maternal humoural immune response (Atkins *et al.*, 1999).

### 1.5.5 Host immune responses to SFV infection

The early innate immune response to viral infection can play an important role in mediating disease through the secretion of the cytokines IFN-α and IFN-β, which limit viral replication and activate NK cell-mediated cytotoxicity prior to the mounting of specific, adaptive immune responses (Biron, 1998). Such (type-1) IFNs are produced by many cell types upon viral infection but are thought to be predominantly secreted by macrophages and DCs in lymphatic tissues. In alphaviral infection, IFN-α/β production has been shown to be dependant on the formation of dsRNA and is proportional to the amount of virus produced (Griffin, 2001).

The adaptive immune response to SFV infection, which is detectable by day 5 following infection, encompasses both neutralising and non-neutralising antibodies, together with T-cell mediated immunity (Amor *et al.*, 1996). The humoural response is

believed to be essential in the recovery from disease with the appearance of antibody coinciding with the cessation of viraemia (Griffin, 2001). The E2 glycoprotein of SFV is known to contain B- and T-cell epitopes, and it is against this protein that the majority of neutralising antibodies are directed. Effective peptide vaccines developed using these epitopes as templates were shown to be effective in protecting mice against SFV infection (Snijders *et al.*, 1992; Fernández *et al.*, 1993). Non-neutralising antibodies, directed against either E1 or E2, have also been shown to confer protection against SFV infection (Boere *et al.*, 1984). Other mechanisms, such as inhibition of virus maturation through binding of antibodies to immature glycoproteins and compliment-mediated lysis may also be involved in protection in such cases (Strauss & Strauss, 1994). Parsons and Webb (1992) identified the predominant subclasses of IgG in response to SFV infection to be initially 2a and 2b with levels of anti-SFV IgG1 dominating only after viral clearance. Seropositivity is maintained for long periods in mammals, conferring protection from reinfection (Griffin, 2001).

While the role of cellular immune responses elicited by SFV infection in the recovery from disease has not been fully elucidated, such responses have been implicated as necessary for the clearance of residual persistent infections in the murine CNS (Amor *et al.*, 1996). Antiviral T-cells exhibit cytotoxicity and secrete IFN-$\gamma$ in response to SFV antigens *ex vivo* from 5 days post infection, coinciding with clearance of viraemia and establishment of humoural immune responses (Blackman & Morris, 1984). The induction of $CD8^+$ CTL responses has been found to be essential in the development of neuropathologic changes associated with the demyelinating disease associated with avirulent (A7(74)) SFV infection (Subak-Sharpe *et al.*, 1993). It is likely that the humoural immune response to SFV infection is predominantly responsible for recovery from disease, while cellular responses appears to be involved in the eradication of virus from tissues harbouring persistent infections and in the development of demyelinating disease.

## 1.6 THE SEMLIKI FOREST VIRUS VECTOR SYSTEM

### 1.6.1 Alphaviruses as expression systems

The generation of infectious clones of SV (Rice *et al.*, 1987), SFV (Liljeström *et al.*, 1991), and VEE (Davis *et al.*, 1989) has allowed for the development of expression vectors from these three alphaviruses. *In vitro* transcription of these plasmids generates full-length infectious viral RNA, as they contain the viral cDNA under the control of a prokaryotic DNA-dependant RNA polymerase promoter, such as SP6 (SV and SFV) or T7 (VEE). Transfection of cells, usually by electroporation, with these infectious RNA transcripts results in intracellular replication and the propagation of progeny virus particles.

There are several characteristics of alphaviruses which make them attractive candidates for the development as expression vectors. Firstly, their genome is relatively small, easily manipulated, and functions directly as mRNA upon introduction to the cellular cytosol where it replicates exclusively. Secondly, alphaviral RNA essentially hijacks host cell macromolecular synthesis with virtually all proteins synthesised by infected cells encoded by the subgenomic viral RNA. Thirdly, the gene products of the subgenomic RNA are not involved in intracellular RNA replication, thus allowing for the manipulation or replacement of this region without affecting the replicative and expressive capacity of the system.

### 1.6.2 The development of the SFV vector system

Early studies in the implementation of alphaviruses as expression vectors involved the production of double subgenomic vectors where heterologous genes were inserted under the control of an additional 26S subgenomic promoter either 3' or 5' to the structural protein genes (Raju & Huang, 1991). Such replicons expressed the alphavirus structural genes as well as the foreign gene of interest and gave rise to infectious virus which could be passaged *in vitro*. Apart from raising biosafety issues due to the production of infectious virus, these vectors proved to be unstable, with the heterologous genes being readily deleted (Pugachev *et al.*, 1995).

In 1991, Liljeström and Garoff reported the development of the SFV vector system which allows for the expression of foreign proteins in mammalian cells by replacing the viral structural protein genes with a short polylinker sequence to facilitate

the insertion of heterologous genes. Upon transfection of cells with recombinant RNA by electroporation high levels of the foreign protein are expressed. It was also possible to produce rSFV VLPs with this system using a helper RNA molecule which provides the structural proteins *in trans* when co-transfected with the recombinant RNA. This helper RNA contained the complete structural protein sequences and the 26S subgemonic promoter but lacked the non-structural region of the genome which encodes the viral replicase and contains the packaging signal. Thus, upon cotransfection, only the recombinant RNA molecule (which contains the full non-structural region and packaging signal) is encapsidated into infectious VLPs. As the resulting VLP progeny contain only the recombinant RNA and lack the viral structural genes, they are capable of only one round of replication. Similar vector systems were also described for SV (Bredenbekk *et al.*, 1993) and VEE (Pushko *et al.*, 1997).

The generation of infectious replication proficient virus (RPV), presumably due to recombination events, was detected in all three alphaviral vector systems however, and this drove more research into improving the biosafety (Berglund *et al.*, 1993; Raju *et al.*, 1995; Pushko *et al.*, 1997). Berglund *et al* (1993) introduced a new helper RNA which contained mutations in the *p62* gene rendering the immature form of the viral spike protein resistant to intracellular cleavage by host cell furin-like proteases into E2/E3. The resulting VLP progeny were incapable of infecting cells without prior incubation with chymotrypsin *in vitro* to cleave p62. While this strategy reduced the probability of RPV generation to $2 \times 10^{-6}$, reversion or suppressor mutations in the altered *p62* gene could potentially generate infectious RPVs (Tubulekas & Liljeström, 1998). The method also proved to be cumbersome and with an additional risk of host proteases activating recombinant RPVs *in vivo*, biosafety concerns remained. Smerdou and Liljeström (1999) described an improved SFV vector system employing two helper RNA molecules from which the generation of RPVs has never been demonstrated and which has been employed since as the preferred vector system (see below). Split-helper vector systems have also been developed using SV (Frolov *et al.*, 1997) and VEE (Pushko *et al.*, 1997).

Studies in the expression of proteins using SFV and SV vector systems revealed that expression levels were significantly higher from vectors which included the C protein encoding sequence. The discovery of a translational enhancer sequence in the N-terminal region of the C protein which is active only in the context of the alphaviral replicon shed some light on this phenomenon (Frolov & Schlesinger, 1994; Sjöberg *et al.*, 1994; Sjöberg & Garoff, 1996). Foreign sequences were expressed as fusion

polypeptides with the complete C-protein, which autoproteolytically cleaves itself from the nascent protein, and expression levels were increased by up to 10-fold. Only the 48 N-terminal aa of the C protein are required for translation enhancement however, and vectors encoding this minimal enhancer sequence express the foreign gene as a fusion protein. In order to obtain high-levels of expression of heterologous protein without the additional N-terminal C residues, the 2A autoprotease from foot-and-mouth disease virus (FMDV) has been inserted as a linker between enhancer and foreign gene sequences (Smerdou & Liljeström, 1999).

The structural proteins were separated into two helper RNA molecules, one encoding the envelope proteins E1, p62 and 6k (pSFV-HelperS2) and the other encoding the C protein (pSFV-CS219A). A mutation abolishing the autoproteolytic capability of the C protein was introduced as a further precaution so that, in the unlikely event of a full recombinant RNA molecule forming, it would not be possible for viral particles to assemble in infected cells. This strategy has reduced recombination frequencies between the three transfected RNA species to an estimated $10^{-12}$. The capsid translational enhancer sequence was also included upstream of the envelope proteins (together with the 2A autoprotease of FMDV) in the pSFV-HelperS2 construct so as to ensure comparable levels of expression of the envelope and C proteins (Smerdou & Liljeström, 1999). This SFV vector system along with the process of producing rSFV VLPs is outlined in Figure 1.5.

### 1.6.3 Applications of the SFV vector system

Initially, the SFV vector system was used primarily as an expression vector and as a prototype vaccine where its ability to achieve transient high-level protein production were favourable. Unlike viral vectors derived form DNA viruses, for example, the SFV vector system does not appear to persist in the host. Murine studies employing RT-PCR analysis revealed that rSFV VLPs do not disperse throughout other organs of the body with detectable rSFV RNA persisting only at the site of injection for 7 days and in lymphoid organs for up to 24 h post inoculation (Morris-Downes *et al.*, 2001). This, together with the ability of the VLPs to induce potent humoural and cellular immune responses against specific antigens, and the lack of anti-SFV antibodies in humans, highlights the potential of the vector system as a prototype vaccine (Zhou *et al.*, 1995). SFV-based vaccines against viral pathogens have shown promising results with the induction of strong and specific immune responses, conferring significant protection

**Figure 1.5 Production of rSFV VLPs using the split-helper SFV vector system**

(i) Plasmids are grown up in *E. coli* and are linearised through digestion with *Spe* I.

(ii) Linearised plasmids are cleaned up and RNA produced by Sp6 *in vitro* transcription.

(iii) The three RNA species are electroporated into BHK-21 cells.

(iv) Following electroporation, only recombinant RNA molecules are packaged into VLP progeny. Helper RNAs provide the structural protein information but contain no packaging signal and so remain in the mother cell. rSFV VLPs are released into the supernatant which can then be used to infect target cells. Infected target cells express the foreign protein encoded by the recombinant but produce no viral progeny.

Amp; Ampicillin resistance gene for selection in *E coli*, ori; *E. coli* origin of replication

(Original diagram based on information from: Smerdou & Liljeström, 1999)

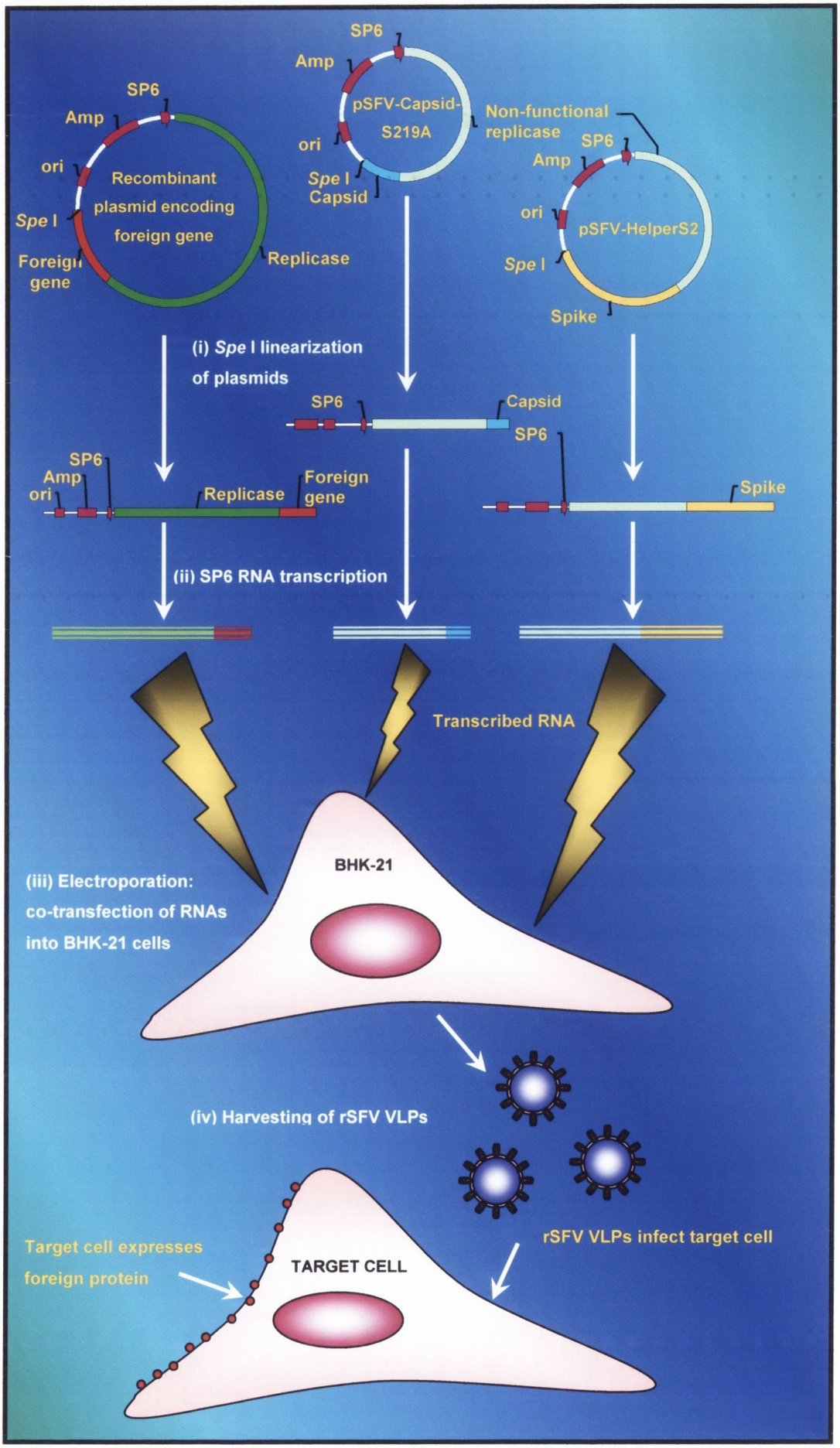


**Figure 1.5 Production of rSFV VLPs using the split-helper SFV vector system**

against challenge following immunisation. Prototype vaccines studied include those aimed at: HIV (Hanke *et al.*, 2003), respiratory synctial virus (RSV) (Chen *et al.*, 2002), hepaptitis C virus (HCV) (Brinster *et al.*, 2002), Louping-ill virus (Fleeton *et al.*, 1999; Morris-Downes *et al.*, 2001), simian immunodeficiency virus (SIV) (Nilsson *et al.*, 2001), influenza virus (Fleeton *et al.*, 2001), and Murray Valley virus (Colombadge *et al.*, 1998).

The SFV vector system has also shown promise in the field of cancer gene therapy. While the cytopathic effect of many viral vectors employed in gene therapy is often believed to be unfavourable, it is the inherent ability of the SFV vector system to induce apoptosis in a variety of cell types that has led to its exploitation as a prototype cancer therapy agent. The fact that deletion of the viral structural proteins does not abrogate the vector's cytopathic effect allows for the introduction of therapeutic genes to augment any antitumour response achieved (Glasgow *et al.*, 1998). The cytopathic effect of the vector alone was successfully employed in the treatment of human non-small cell lung carcinoma xenografts (H358a) in BALB/c *nu/nu* mice by direct intratumoural (i.t.) injection with rSFV VLPs expressing enhanced green fluorescent protein (EGFP) (Murphy *et al.*, 2000). This effect was shown to be enhanced in a more aggressive tumour model (AT3-Bcl-2) through the expression by the rSFV VLPs of the pro-apoptotic gene *bax* (Murphy *et al.*, 2001). Recombinant SFV vectors have also been successfully employed in the inhibition of tumour growth in murine models through the expression of anti-angiogenic factors such as IL-12 and endostatin (Asselin-Paturel *et al.*, 1999; Yamanaka e*t al.*, 2001).

The ability of rSFV to elicit strong immune responses against target antigens has also lead to investigation into its potential in the field of in cancer immunotherapy (Ying *et al.*, 1999). rSFV VLPs expressing the P815A TAA were shown to confer protective immunity against P815 tumour induction in mice and were also shown to be effective in inhibiting tumour growth (predominantly through induction of antitumour immune responses) following direct i.t. injection, as were rSFV VLPs expressing IL-12 (Colmenero *et al.*, 1999; Colmenero *et al.*, 2002). Similarly, rSFV expressing HPV (associated with the development of cervical carcinoma) antigens E6 and E7 has been demonstrated to induce antitumour immunity and protect mice against challenge with HPV-transformed cells (Daemen *et al.*, 2000; Daemen *et al.*, 2002; Daemen *et al.*, 2003). The expression of GM-CSF by SFV vectors has also been exploited in treatment of a murine ovarian cancer model and has shown potential in the production of autologous tumour cell vaccines *ex vivo* (Klimp *et al.*, 2001; Withoff *et al.*, 2001). Yamanaka and

colleagues have carried out numerous immunotherapy studies (2000; 2002; 2002; 2003) utilising DCs pulsed with rSFV cDNA illustrating the potential of the vector in DC-based tumour vaccines. Phase I and II clinical trials are now also being developed with the aim of employing liposomally-encapsulated SFV VLPs expressing IL-12 in the treatment of glioblastoma multiforme (Ren *et al.*, 2003). Other alphavirally derived vectors are also showing promise in the field of cancer gene therapy with a VEE replicon recently demonstrated to break tolerance to a tumour 'self' antigen in a rat mammary tumour model (Nelson *et al.*, 2003) and SV vectors displaying an apparent tropism for malignant tissues (Tseng *et al.*, 2002; Tseng *et al.*, 2004).

The neurotropism of SFV has been exploited in the application of rSFV VLPs as a vector for the CNS. Administration of rSFV VLPs expressing the cytokine IL-10 by the non-invasive i.n. route was shown to have a therapeutic effect on the murine multiple sclerosis model, experimental autoimmune encephalomyelitis (Jerusalmi *et al.*, 2003). A replication-proficient SFV vector derived from the avirulent strain A7(74) termed VA7 may also have good potential as a CNS vector which would be capable of higher levels of heterologous gene expression than the non-replicative rSFV VLPs (Vaha-Koskela *et al.*, 2003). Overall, it would appear that the SFV vector system has many potential clinical applications for vaccine use, cancer gene therapy, and as a CNS vector.

## 1.7 OBJECTIVES OF STUDY

The application of rSFV VLPs in the inhibition of tumour growth *in vivo* through their inherent cytopathic effect and also as potent inducers of humoural and cellular immunity against target antigens are combined in this study. The aim of this research was to exploit these two abilities in the inhibition of the aggressive murine tumour model K-BALB. Several plasmids with specific deletions have been produced from the pSP6-SFV4 infectious clone of SFV (Barth and Garoff, 1997). pSFV-p62-6k contains deletions of the capsid and E1 genes, and is used in this study to express the E2 protein of SFV and hence stimulate immunity. pSFV-p62-6k was used in combination with pSFV-HelperS2 and pSFV-CS219A to produce rSFV-p62-6k VLPs as illustrated in Figure 1.5. The recombinant SFV constructs used in this study are illustrated along with the wild-type SFV4 infectious clone of SFV in Figure 1.6.

**The prime objectives of this study were as follows:**

1) To assess the cytopathic effect of rSFV-p62-6k VLPs and SFV4 on K-BALB cells *in vitro* and to ascertain if any CPE observed was due to apoptosis induction. BALB/3T3 cells were used as non-tumourigenic control cells and BHK-21 cells were employed as a positive control for SFV-associated CPE and apoptosis induction.
2) To examine the ability of rSFV-p62-6k VLPs to inhibit the growth of K-BALB tumours in BALB/c mice through induction of CPE and host immune responses against SFV antigens. BALB/c *nu/nu* mice were also employed as a control immunocompromised model.
3) To examine the ability of SFV4 to inhibit the growth of K-BALB tumours *in vivo* and determine if this replication-proficient virus was superior in tumour growth inhibition than the replication incompetent vector system.
4) To determine if prior immunisation with rSFV-p62-6k VLPs significantly enhanced any inhibition of K-BALB tumour growth observed following treatment with either rSFV-p62-6k VLPs or SFV4.
5) To characterise the cellular infiltrate observed through histopathological and immunohistochemical analysis of tumour sections.

It is hoped that this information will enable advances in the exploitation of this tumour treatment strategy as well as the further development of rSFV VLPs, and possibly a replicating SFV vector, as viable tumour therapy agents.

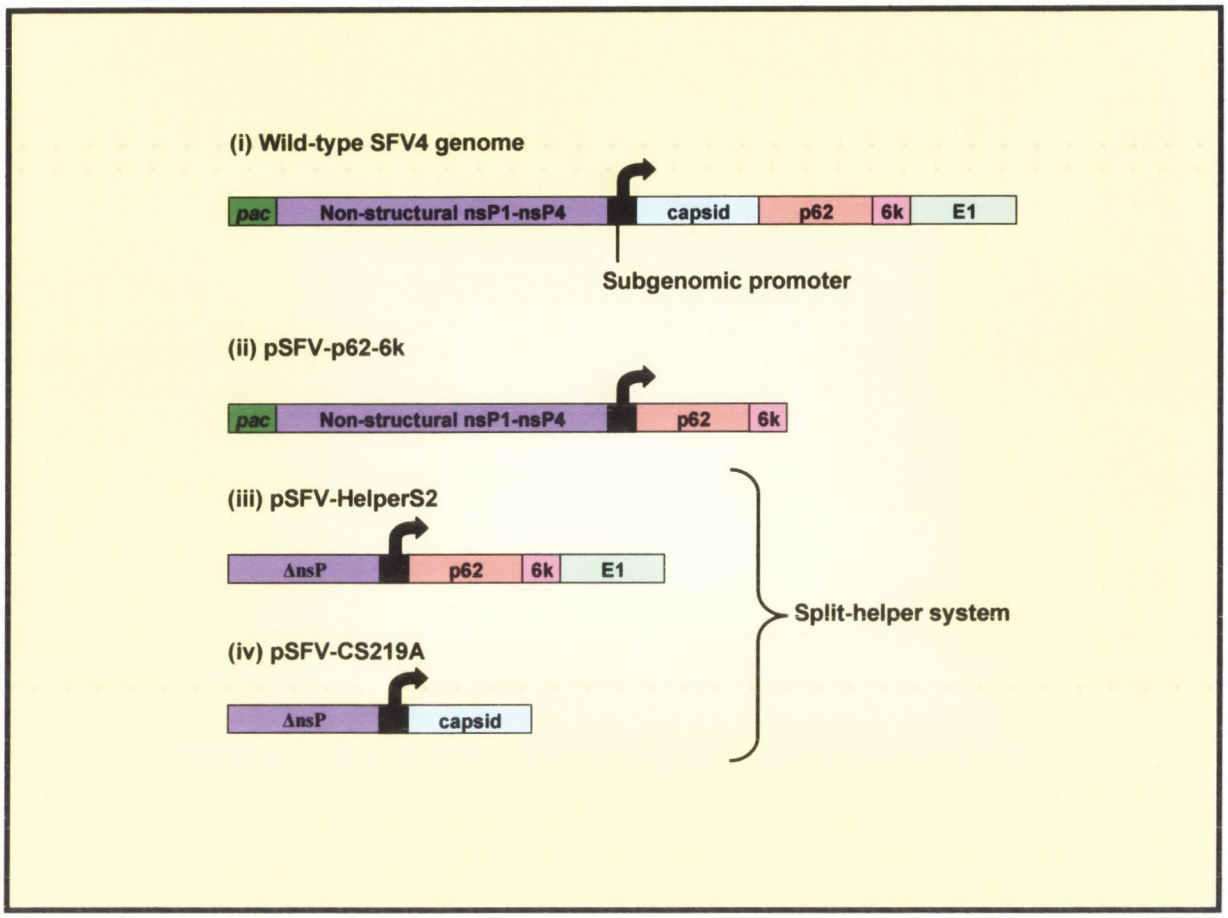


**Figure 1.6 Schematic representation of recombinant SFV constructs used in this study**

(i) Representation of the linearised cDNA from the SFV4 infectious clone of SFV, which is used to produce infectious SFV4 virus and from which all other constructs were derived.

(ii) The pSFV-p62-6k construct, from which the capsid and E1 genes have been deleted.

(iii) pSFV-HelperS2 which encodes the spike structural proteins E1, E2, and E3 as well as 6k. Note the absence of the packaging signal and substantial deletions in the non-structural region.

(iv) pSFV-CS219A which encodes the capsid protein. Note the absence of the packaging signal and substantial deletions in the non-structural region.

pSFV-p62-6k, pSFV-HelperS2 and pSFV-CS219A were used to produce rSFV-p62-6k VLPs.

*pac*; packaging signal, ΔnsP; substantially deleted non-functional replicase genes

(Original diagram based on information from: Barth & Garoff, 1997; Smerdou & Liljeström 1999)

# 2

# MATERIALS AND METHODS

## 2.1 SFV4 AND rSFV VLP PRODUCTION

### 2.1.1 Cell culture

#### 2.1.1.1 BHK-21 cell line

The Baby Hamster Kidney 21 (BHK-21) cell line was obtained from the American Type Culture Collection (ATCC, USA) and was used for SFV4 virus and rSFV VLP production. As it has already been established that these cells are susceptible to SFV-induced apoptosis upon infection with SFV and its derived vector (Glasgow *et al.*, 1998) they were also employed as a positive control for *in vitro* assays examining apoptosis induction and cell viability.

Cells were cultured in 25 ml BHK-21 medium (Glasgow MEM), supplemented with 5% (v/v) newborn calf serum (NCS), 5% (v/v) tryptose phosphate broth (Invitrogen, UK), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Sigma, USA), and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ in 150 $cm^2$ cell culture flasks (Iwaki, Japan). Cells were typically sub-cultured as follows: confluent monolayers were washed twice with Dulbecco's phosphate buffered saline without calcium, magnesium and sodium bicarbonate (PBS-) (Invitrogen, UK) and incubated with 0.5% Trypsin 5.3mM EDTA (Invitrogen, UK) at 37 °C until detachment was evident at which point supplemented BHK-21 medium was added to terminate trypsinisation. The resulting cell suspension was then aspirated several times to break up cell clumps and split into fresh 150 $cm^2$ flasks containing 25 ml BHK-21 medium. All BHK-21 cells used in *in vitro* assays were passaged no more than 12 times and typically sub-cultured at a ratio of 1:3.

#### 2.1.1.2 sBHK cell line

The cell line sBHK (origin unknown) was a gift from P. Liljeström (Microbiology and Tumorbiology Centre, Karolinska Institute, Stockholm, Sweden) and was used to produce and titrate rSFV VLPs. Cells were cultured in 25 ml BHK-21 Medium, supplemented with 10% (v/v) foetal bovine serum (FBS), 20 mM Hepes buffer (Invitrogen, UK), 5% (v/v) tryptose phosphate broth, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ in 150 $cm^2$ cell culture flasks. As this cell line grows extremely

rapidly, they were typically sub-cultured at a ratio of 1:5 or higher as necessary in the same manner as described in section 2.1.1.1.

#### 2.1.1.3 BALB/3T3 cell line

BALB/3T3 cells (clone A31, ATCC) are contact-inhibited mouse embryo fibroblasts and were employed as a non-tumourigenic control cell line for *in vitro* assays. Cells were cultured in 25 ml Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, UK), supplemented with 10% (v/v) NCS, 1 mM sodium pyruvate (Invitrogen, UK), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ in 150 $cm^2$ cell culture flasks. Unlike BHK-21 and sBHK, BALB/3T3 cells were not allowed to reach confluence prior to sub-culturing as this can select for non-contact-inhibited mutants. Cells were typically sub-cultured at a ratio of 1:3 or higher in the same manner as described in section 2.1.1.1.

#### 2.1.1.4 K-BALB cell line

K-BALB cells (ATCC) are non-producing Kirsten murine sarcoma virus transformed BALB/3T3 cells which readily form tumours in BALB/c mice upon sub-cutaneous (s.c.) injection. Cells were cultured in 25 ml supplemented DMEM as described in section 2.1.1.3 and confluent monolayers were sub-cultured at a ratio of 1:4 or higher as described in section 2.1.1.1. BALB/3T3 and K-BALB cells used in *in vitro* assays were passaged no more than 12 times.

### 2.1.2 SFV4

#### 2.1.2.1 Production of SFV4 working stock

The infectious clone of SFV, pSP6-SFV4, contains the full coding and non-coding genomic cDNA sequence of virulent SFV (Liljeström *et al.*, 1991). Plaque purified SFV4 seed stock was allowed to adsorb onto subconfluent BHK-21 cells in a 75 $cm^3$ cell culture flask (Iwaki, Japan) for 1 h at 37 °C in a humidified atmosphere of 5% $CO_2$ with rocking every fifteen minutes. After 1 h the inoculum was removed and 20 ml fresh BHK-21 medium was added before incubation at 37 °C in a humidified atmosphere of 5% $CO_2$ for 24 h. The supernatant was then decanted, centrifuged at 3,000 *x g* for 10

min to remove cellular debris, and filtered through a 0.2 μm filter. The resulting clarified supernatant, now termed SFV4 working stock, was stored at -70 °C in 1 ml aliquots.

### 2.1.2.2 Titration of SFV4 working stock by plaque assay

The medium for infection (MFI) used in all *in vitro* assays involving infection of cells with either SFV4 or rSFV VLPs was composed of Minimum Essential Medium (MEM) supplemented with 0.2% (v/v) bovine serum albumin (Invitrogen, UK), 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

BHK-21 cells were seeded into twelve 60 $mm^2$ cell culture dishes (Iwaki, Japan), incubated at 37 °C in a humidified atmosphere of 5% $CO_2$, and allowed to reach subconfluence. Doubling serial dilutions of SFV4 working stock were prepared in MFI from $10^{-2}$ to $10^{-10}$. Medium was decanted from the dishes after which 500 μl inoculum from each dilution was added in duplicate and the dishes then incubated for 1 h at 37 °C in a humidified atmosphere of 5% $CO_2$ with rocking every fifteen minutes. Two dishes were mock-infected with 500 μl MFI alone and acted as negative controls.

After removal of the inoculum, equal amounts of 1.8% noble agar (Difco, USA) at 45 °C and overlay medium (2X MEM supplemented with 10% NCS, 10% tryptose phosphate broth, 200 U/ml penicillin, 200 μg/ml streptomycin and 4 mM L-glutamine) at 37 °C were mixed and added to the dishes to a final volume of 3 ml per dish. When the agar had solidified (~10 min at room temperature) the plates were incubated for 48 h at 37 °C in a humidified atmosphere of 5% $CO_2$. After 48 h dishes were flooded with neutral buffered formalin (BDH, UK) and allowed to fix for approximately twenty minutes, after which the neutral buffered formalin and agar were removed under running tap water. The dishes were left to dry, plaques counted against a dark background and viral titre expressed as plaque forming units (PFU)/ml.

### 2.1.2.3 Preparation of purified SFV4

Six confluent 75 $cm^3$ flasks of BHK-21 cells were infected with SFV4 seed stock and incubated for 24 h as described above. Supernatants were pooled and aliquoted into six Ultra-Clear SW40Ti centrifuge tubes (Beckman, USA). The six tubes were balanced to within 0.05 g and centrifuged at 100,000 x *g* for 2 h at 4 °C in a Beckman L8-60M ultracentrifuge in order to pellet the virus. The supernatant was decanted from each tube

and the pelleted virus resuspended by thorough vortexing. Sucrose gradients (20% to 65% (w/v) sucrose in TNE buffer [50 mM Tris-HCl (BDH, UK), pH 7.4, 100 mM NaCl (Merck, Germany), 0.1 mM EDTA (Sigma, USA)] were prepared in two SW40Ti centrifuge tubes and a Pasteur pipette was used to layer the resuspended virus on top of the gradients. The tubes were balanced as before and centrifuged at 100,000 x *g* overnight at 4 °C. SFV4 virus was clearly visible as an opaque band in each the gradient which was collected using a peristaltic pump (Amersham Pharmacia Biotech, Sweden) and added to 12ml PBS. Virus was pelleted by centrifugation at 100,000 x *g* for 2 h at 4 °C and supernatant decanted. The now purified SFV4 was resuspended by thorough vortexing and 4 ml PBS was added before storage at -70 °C in 500 µl aliquots.

#### 2.1.2.4 Production of rabbit SFV4 antiserum

Two New Zealand white rabbits (Harlan, UK) were bled to obtain pre-immune serum prior to inoculation with SFV4. Blood was incubated at 4 °C overnight and centrifuged at 700 *x g* to obtain clarified serum which was stored at – 70 °C in 50 µl aliquots. Each rabbit received one 500 µl intravenous (i.v.) and two 250 µl sub-cutaneous (s.c.) injections of purified SFV4. Three weeks later each rabbit was boosted with two further 250 µl s.c. injections of purified SFV4. Both rabbits were euthanised and bled by cardiac puncture two weeks post-boosting. Serum was prepared as described above and stored at – 70 °C in 50 µl aliquots.

#### 2.1.2.5 Detection of SFV antigen by immunocytochemistry and immunofluorescence

##### 2.1.2.5.1 Preparation of BHK-21 cells expressing viral antigen

Twenty six sterile 22 $mm^2$ coverslips (Chance Propper, UK) were individually placed in the wells of six-well cell culture dishes (Beckton Dickenson, USA) and BHK-21 cells were seeded onto them at 5 X $10^5$ cells/well in a final volume of 2 ml/well. The plates were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ until the cells reached ~80% confluence.

Medium was removed from the wells and the monolayers were washed twice with 2 ml Dulbecco's PBS with calcium, magnesium and sodium bicarbonate (PBS+). Thirteen wells were infected with 500 µl SFV4 working stock at 2 X $10^5$ PFU/ml in MFI and incubated with rocking every fifteen minutes for 1 h at 37 °C in a humidified

atmosphere of 5% $CO_2$. The remaining thirteen wells were simultaneously mock-infected with 500 µl MFI alone. After 1 h and removal of inoculum, each well was washed twice with 2 ml PBS+ before the addition of 2 ml fully supplemented BHK-21 growth medium. Dishes were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ for 24h, at which point CPE was apparent in infected monolayers.

#### 2.1.2.5.2 Titration of rabbit SFV4 antiserum

Medium was removed from infected and mock-infected wells and monolayers were fixed for 10 min with neutral buffered formalin at a final volume of 2 ml/well. Coverslips were then removed, placed in a holder and washed twice for 5 min in PBS (Oxoid, UK). Post-fixation was performed for 5 min at -20 °C ethanol/acetic acid (2:1) (BDH, UK) before a another two 5 min PBS washes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (BDH, UK) in PBS for 10 min at room temperature and was followed by a further two 5 min PBS washes. Thirteen plain glass microscope slides were coated with parafilm (Pechiney, USA) and two 150 µl drops of PBS containing (v/v) 1.5% normal goat serum (NGS) (Vector Laboratories, USA) were pipetted onto the surface. Coverslips were placed monolayer-down onto these drops and incubated in a humidified chamber at 37 °C for 20 min in order to block non-specific binding sites.

Dilutions of the rabbit pre-immune and SFV4 antiserum (section 2.1.2.4) at 1/50, 1/100, 1/500, 1/1000, 1/1500 and 1/2000 were prepared in PBS containing 5% NGS. One infected and one mock-infected coverslip was incubated with 150 µl of each dilution of rabbit pre-immune and SFV4 antiserum as described above for 1 h at 37 °C. The remaining one infected and one mock-infected coverslips were incubated with PBS containing 5% NGS alone simultaneously under the same conditions.

Coverslips were placed back in the holder and washed twice for 10 min in PBS containing 0.05% Tween 80 (BDH, UK) (PBST) and twice for 5 min in PBS. After washing, the coverslips were incubated as above for 30 min at room temperature with PBS containing 0.15% (v/v) NGS and 0.5% (v/v) goat anti-rabbit IgG (Vector Laboratories, USA).

After incubation, coverslips were placed back in the holder and washed twice for 10 min in PBST and twice for 5 min in normal PBS. Prior to washing, the horse-radish peroxidase (HRP) Vectastain *Elite* ABC reagent (Vector Laboratories, USA) was prepared in PBS as described by the manufacturer (2% (v/v) Reagent A and 2% (v/v)

Reagent B) and allowed to stand at room temperature for 30 min (during the wash steps above). Coverslips were placed back in the holder and incubated with the Vectastain *Elite* ABC reagent for 30 min at room temperature as previously described.

After washing the coverslips for 5 min in PBS, SigmaFast diaminobenzidine (DAB) (Sigma, USA) was prepared according to the manufacturers instructions in 5 ml $dH_2O$. Coverslips were incubated with 200 µl DAB for exactly 2 min before being placed back in the holder and washed under gently running tap water for 5 min. At this point, coverslips were counterstained in Harris' Haematoxylin (BDH, UK) for 20 sec and cleared under running tap water. Coverslips were differentiated in acid alcohol (1% conc. HCl (BDH, UK) in 70% ethanol (BDH, UK)), rinsed in tap water and 'blued-up' by dipping three times in ammonia water (3% ammonia solution (BDH, UK) in tap water). After rinsing again in tap water, the coverslips were placed for 2 min each in 70%, 95% and 100% ethanol followed by three 2 min steps in xylene before mounting using DPX (BDH, UK).

#### 2.1.2.5.3 Routine detection of SFV antigen by immunocytochemistry and immunofluorescence

Detection of SFV antigen in infected cells by immunocytochemistry was performed as described in section 2.1.2.5.2 using the rabbit SFV4 antiserum prepared in section 2.1.2.4 at a dilution of 1/1000 at 37 °C for 1 h (Figure 2.1). For immunofluorescence, this primary antibody step was performed at the same dilution but at 4 °C for 18 h, also, endogenous peroxidases were not quenched with hydrogen peroxide. In place of the HRP Vectastain *Elite* ABC reagent, a fluorescent avidin conjugate was employed; either rhodamine avidin DCS or fluorescein avidin DCS (Vector Laboratories, USA) at a dilution of 1/200 in high-salt buffer (0.5 M NaCl, 10 mM Hepes) for 30 min at room temperature. Coverslips/slides were then washed twice in high-salt buffer before being dipped in alcohol and mounted using Vectashield mountant containing 4',6-diamidino-2-phenylindole (DAPI) (Vector laboratories, USA). Immunofluorescent slides were examined with fluorescent microscopy using a Nikon eclipse E400 microscope and appropriate filters (DAPI; DAPI filter, excitation 340-380 nm, rhodamine; G-2A filter; excitation 510-560 nm, FITC and fluorescein; B-2E filter; excitation 450-490 nm).

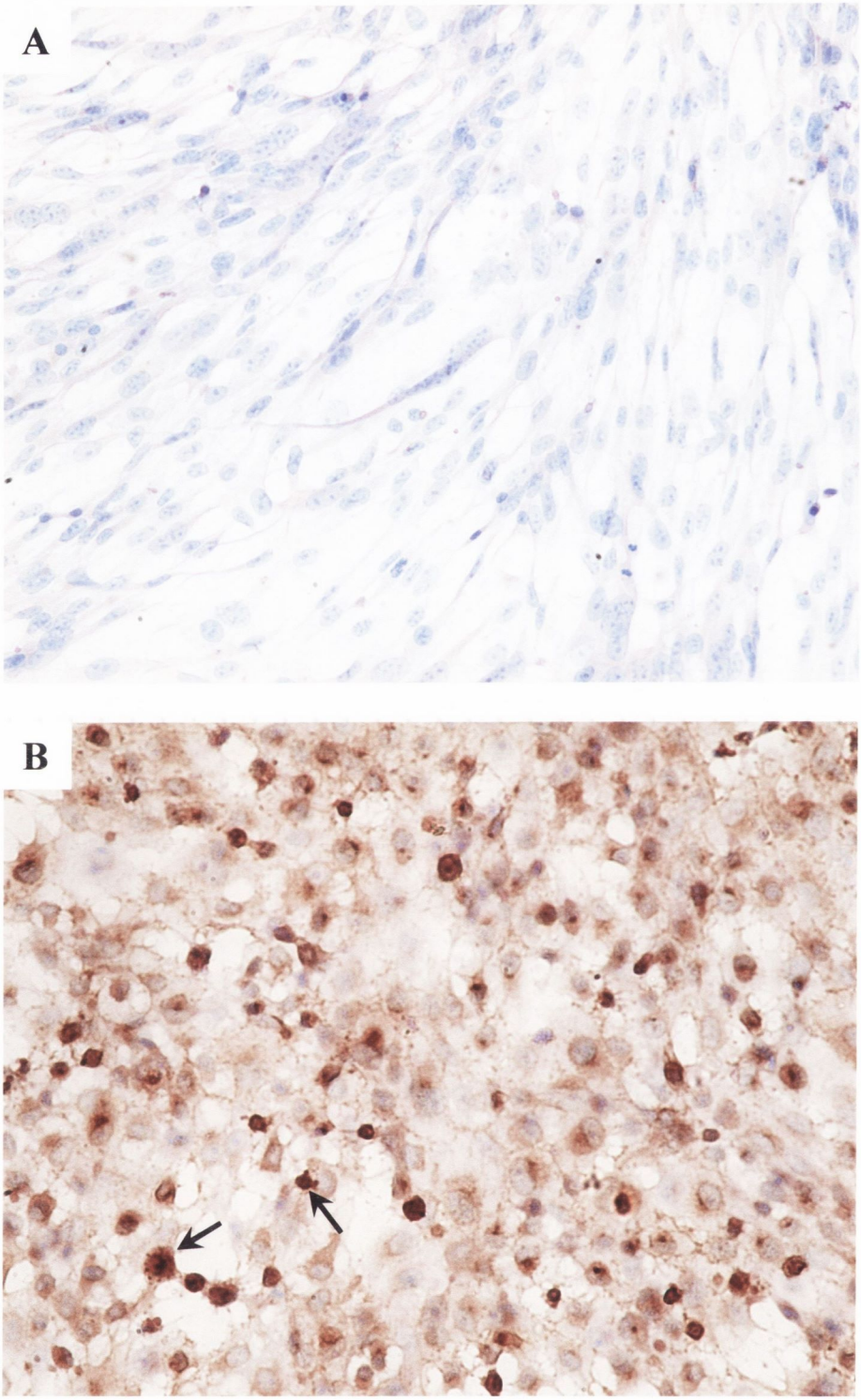


**Figure 2.1 Detection of SFV4 antigen expression in BHK-21 cells by immunohistochemistry 24 hour post infection**

**(A)** Mock-infected BHK-21 cells devoid of detectable SFV4 antigen, 200x

**(B)** SFV4 infected cells positive for SFV4 antigen (brown) and displaying morphological characteristics of apoptosis induction such as cell shrinkage and membrane blebbing (*arrows*), 200x

### 2.1.3 rSFV-p62-6k VLP production

#### 2.1.3.1 Expression Vectors

The SFV split-helper vector system was kindly provided by Professor P. Liljeström which encompassed the packaging vectors pSFV-HelperS2 (encoding the envelope proteins) and pSFV-CS219A (encoding the capsid protein). The pSFV-p62-6k plasmid was obtained from Professor H. Garoff (Department of Biosciences, Karolinska Institute, Novum, Huddinge, Sweden) and was employed together with pSFV-HelperS2 and pSFV-CS219A to produce rSFV-p62-6k VLPs (Figure 1.5 & Figure 1.6).

#### 2.1.3.2 Preparation of competent *E. coli* DH5α cells

*Escherichia coli* strain DH5α (New England Biolabs, USA) was employed for the propagation of the two helper SFV plasmids and the pSFV-p62-6k construct. A 2 ml overnight culture was prepared in L-broth which was used to inoculate a further 400 ml of fresh L-broth in a 2 L baffled flask. This culture was incubated at 37 °C with shaking (200 rpm) until the cells reached the mid-log phase of growth ($OD_{600}$= 0.4-0.5). Cells were then incubated on ice for 1 h before being harvested by centrifugation at 3,000 x *g* for 10 min at 4 °C in a Sorval RC 5C Plus centrifuge in 2 x 200 ml volumes. The supernatant was discarded and the cells were resuspended gently in 2 x 30 ml ice-cold 100 mM $MgCl_2$ (BDH, UK) and pelleted as above. Pellets were resuspended gently in 2 x 100 ml of ice-cold 100 mM $CaCl_2$ (Merk, Germany) and placed on ice for 1 h. Cells were pelleted as above and resuspended gently in 2 x 20 ml ice-cold 100 mM $MgCl_2$. 80 % glycerol (BDH, UK) was then added drop-wise with gentle swirling to a final concentration of 10% w/v. This cell-glycerol suspension was then aliquoted into 0.5 ml quantities on ice and snap-frozen in liquid nitrogen before storage at -70 °C.

#### 2.1.3.3 Transformation of *E. coli* DH5α cells

An aliquot of competent *E. coli* DH5α prepared in section 2.1.3.2 was thawed quickly and placed on ice. The cells were then aliquoted in 100 µl volumes into five sterile eppendorf tubes on ice. 1 µl of plasmid pSFV-p62-6k, pSFV-HelperS2, or pSFV-CS219A was added to three of the tubes, leaving two as controls. Cells were incubated on ice for 1 h with gentle mixing every 15 min. The tubes were then heat-shocked at 42

°C for 2 min in a waterbath before being replaced on ice for 10 min. The cells were then added to 1 ml L-broth in test tubes and incubated at 37 °C with shaking for 1 h. In order to select for transformants, 1 µl of 100 mg/ml ampicillin (Sigma, USA) was added to all tubes bar one of the controls and cells were incubated for a further 1 h at 37 °C with shaking. Serial dilutions of each were prepared in L-broth to $10^{-2}$. 200 µl from each dilution of the ampicillin-free control was plated out on L-agar and 200 µl from each dilution of the remaining cultures was plated out on L-agar containing 1mg/ml ampicillin. Plates were incubated at 37 °C overnight and examined for colonies.

### 2.1.3.4 Isolation of plasmid DNA from *E. coli* cells

Plasmid DNA was purified from *E. coli* DH5α cells using the *Qiagen Hi-speed Plasmid Purification kit* (Qiagen, UK) according to manufacturer's instructions. This yielded approximately 1 ml of each plasmid at a concentration of 2 µg/ml in $dH_2O$. Plasmid concentrations were estimated using a GeneQuant DNA/RNA spectrophotometer and visualised by gel electrophoresis as follows. A 25 ml 0.8% TBE (Promega, USA) agarose (Roche, Germany) gel containing 2.5 µl 10 mg/ml ethidium bromide (BioRad, USA) was prepared and 2 µl of each plasmid preparation was mixed with 14 µl TBE buffer and 4 µl 6X blue/orange loading dye (Promega, USA). Samples were loaded in 5 µl and 10 µl amounts and run at 75 mA along with 5 µl and 10 µl *Lambda* molecular weight marker (Promega, USA) (5 µl Lambda *Hind* III, 17 µl loading dye and 78 µl TBE). Gels were run for approximately 30 min at which point they were visualised using a BioRad Gel Doc 2000 and accompanying Multi-Analist (version 1.1) software.

### 2.1.3.5 Linearisation of plasmid DNA with *Spe* I

Each of the SFV plasmids contains a unique *Spe* I restriction site preceding the non-structural protein genes and SP6 promotor which was exploited in order to linearise plasmids prior to *in vitro* RNA transcription. A total of 20 µg plasmid DNA was linearised in two reaction volumes of 50 µl containing 5 µl NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), and 20 U *Spe* I (New England Biolabs, USA). Following 16-18 h digestion in a waterbath at 37 °C, cut and uncut plasmids were visualised by gel electrophoresis along with *Lamda* molecular weight marker as described in section 2.1.3.4 (Figure 2.2a). Linearised plasmids were then

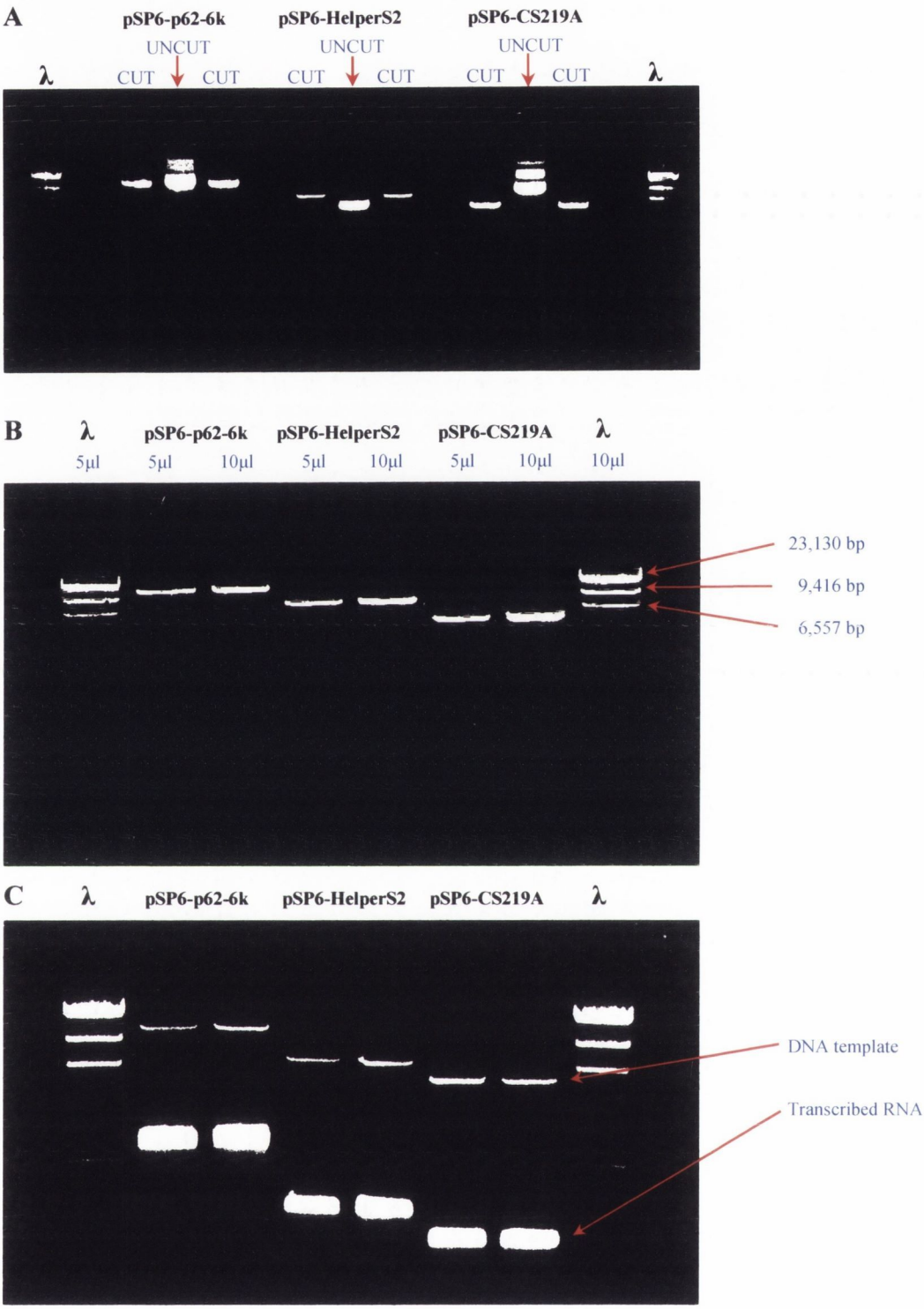


**Figure 2.2 Linearisation, cleanup and *in vitro* RNA transcription from pSFV-p62-6k, pSFV-HelperS2, and pSFV-CS219A as visualised by agarose gel electrophoresis**

**(A)** Intact purified plamids (*uncut*) alongside linearised plasmids (*cut*) following *Spe* I digestion, 0.8% agarose

**(B)** Linearised plasmids following cleanup with the *Qiagen nucleotide extraction kit*, 0.8% agarose

**(C)** DNA template and transcribed RNA products following *in vitro* transcription reaction, 0.6% agarose

cleaned using the *Qiagen nucleotide extraction kit* (Qiagen, UK) and resuspended to a final volume of 50 µl sterile nuclease-free $dH_20$ (Promega, USA). It was also possible at this point to estimate plasmid concentrations by comparing band intensities to those of the *Lambda* marker bands (which were of known concentrations) upon visualisation by gel electrophoresis as described in section 2.1.3.4 (Figure 2.2b).

### 2.1.3.6 *In vitro* SP6 RNA transcription

1.5 µg of each linearised and cleaned plasmid DNA (section 2.1.3.5) was used as a template in 50 µl SP6 *in vitro* RNA transcription reactions containing 1 x SP6 Buffer (40 mM Hepes-KOH pH 7.4, 6 mM MgOAc, 2 mM spermidine-HCl) (Sigma, USA), 1 mM $m^7G(5')ppp(5')G$ (CAP) (Amersham Pharmacia Biotech, Sweden), 5 mM DTT (Sigma, USA), 1 mM each rATP, rCTP, rUTP, 500 µM rGTP (Amersham Pharmacia Biotech, Sweden), 60 U RNasin (Promega, USA), and 34 U SP6 RNA polymerase (Amersham Pharmacia Biotech, Sweden) in nuclease free water. Reactions were incubated for 2 h at 37 °C and placed on ice. In order to confirm transcription, a 0.6% TBE agarose gel was prepared using diethylpyrocarbonate (DEPC) (Sigma, USA) treated $H_2O$ and 2 µl reaction product was mixed with 14 µl DEPC TBE and 4 µl loading dye. Samples and *Lambda* marker were loaded and run in the same manner as described in section 2.1.3.4 (Figure 2.2c). Transcribed RNAs were stored at -70 °C.

### 2.1.3.7 pSFV-p62-6k RNA electroporation and detection of antigen expression

Before producing rSFV-p62-6k VLPs it was necessary to confirm the ability of pSFV-p62-6k transcribed RNA to induce expression of antigenic SFV protein upon introduction to the cellular cytosol. Two 75 $cm^3$ flasks of BHK-21 cells were propagated and trypsinised as described in section 2.1.1.1. Cells were centrifuged at 400 x *g* for 10 min at room temperature and the pellets were gently resuspended in 6 ml PBS. The cell suspensions were centrifuged again as above and the pellets gently resuspended in final volumes of 700 µl PBS. pSFV-p62-6k RNA (section 2.1.3.6) was thawed and added to one of the cell suspensions (the other acting as a negative control) which was then mixed by gentle aspiration and both suspensions were added to 0.4 µm electroporation cuvettes (BTX, USA). Electroporation was then performed in two brief pulses at 0.85 kV and 25 µF capacitance, using a BioRad Gene Pulser II. Cells were then immediately and gently resuspended in 20 ml BHK-21 medium at 37 °C each and seeded onto 22 $mm^2$ coverslips

in 6-well cell culture dishes (2 ml/well). After incubation for 18 h at 37 °C in a humidified atmosphere of 5% $CO_2$, supernatants were aspirated and the coverslips fixed in 10% formalin and SFV antigen expression was detected by immunocytochemistry as described in section 2.1.2.5.3 (Figure 2.3).

### 2.1.3.8 rSFV-p62-6k VLP production

In order to produce rSFV-p62-6k VLPs for *in vitro* studies (titre of approximately 1-5 x $10^8$ IU/ml) a flask of ~80% confluent BHK-21 cells was propagated and trypsinised as described in section 2.1.1.1 and electroporation was performed as described in section 2.1.3.7 with the following modifications; *in vitro* transcribed RNA from pSFV-p62-6k and the two helper reactions was pooled and added to the PBS cell suspension and mixed by gentle aspiration before addition to a 0.4 μm gap electroporation cuvette. After electroporation with two pulses, cells were immediately and gently resuspended in 20 ml BHK-21 medium at 37 °C and incubated for 36 h at 33 °C in a humidified atmosphere of 5% $CO_2$. The culture supernatant (containing the rSFV-p62-6k VLPs) was removed, centrifuged (3000 x *g*, 15 min, 4 °C) in order to remove cellular debris and placed on ice. Aliquots of 1 ml were made into cryotube vials (Nunc, Denmark) and snap-frozen in liquid nitrogen before storage at -70 °C.

### 2.1.3.9 rSFV-p62-6k VLP titration

A 75 $cm^3$ flask of ~80% confluent sBHK cells was propagated as described in section 2.1.1.2, trypsinised and seeded onto 22 $mm^2$ coverslips in a six-well cell culture dish and incubated until confluent. Serial dilutions (10 μl, 1 μl, 0.1 μl) of rSFV-p62-6k VLPs were prepared on ice and used to infect, in duplicate, successive wells of the confluent sBHK monolayers in a total volume of 500 μl MFI for 1 h, with rocking every 15 min. The inocula were then aspirated and the monolayers washed twice with 1 ml PBS before the addition of 2 ml fresh BHK-21 medium at 37 °C to each well. Following 18 h incubation at 37 °C, medium was removed and coverslips were fixed and SFV antigen expression was detected by immunofluorescence as described in section 2.1.2.5.3 (Figure 2.4). Positive cells were enumerated and VLP titres were calculated at a magnification of x 400 and expressed as IU/ml.

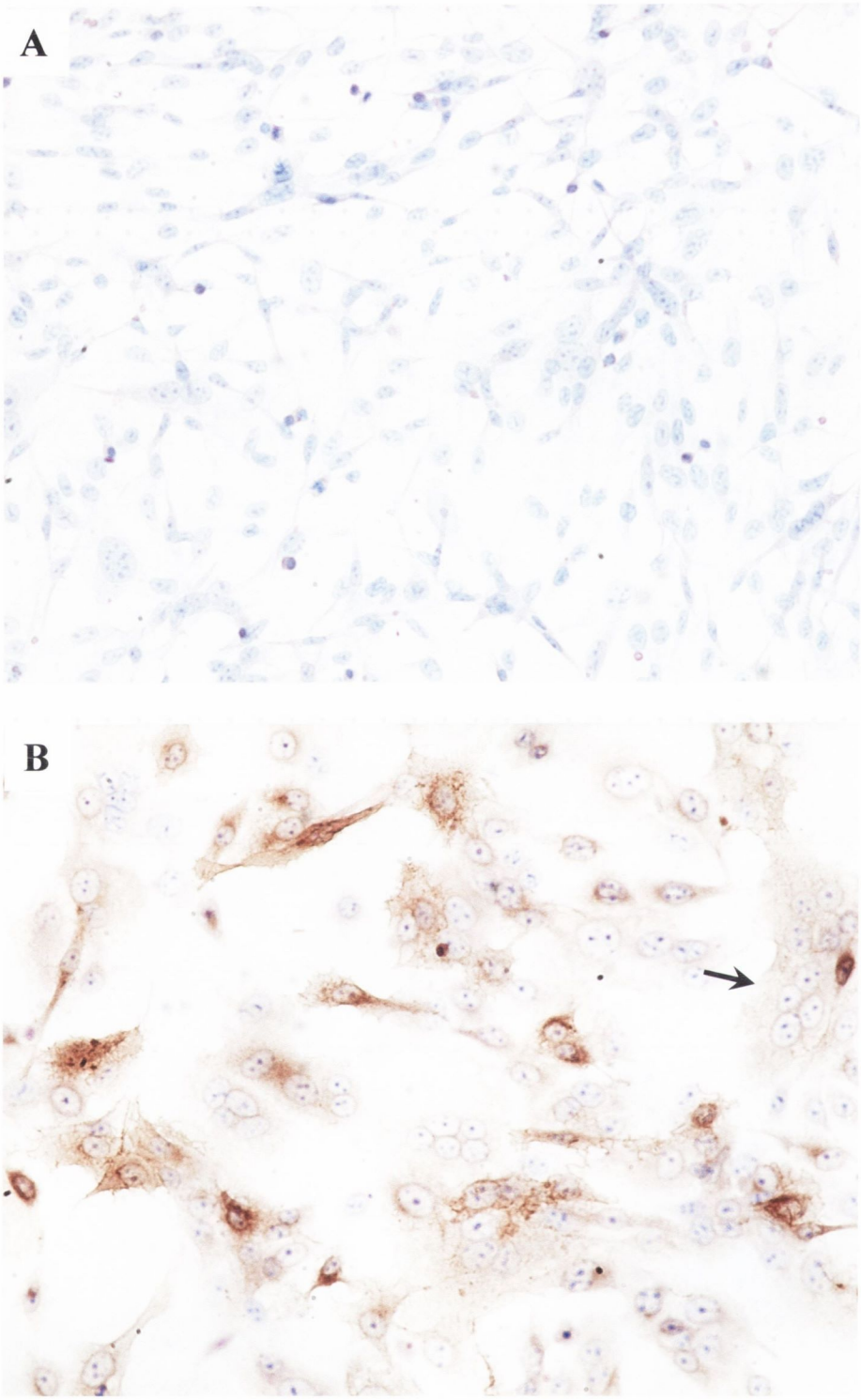


**Figure 2.3 Detection of SFV antigen expression of BHK-21 cells by immunohistochemistry 24 h following electroporation**

**(A)** BHK-21 cells electroporated with PBS devoid of detectable SFV antigen, 200x

**(B)** BHK-21 cells electroporated with rSFV-p62-6k RNA positive for SFV antigen expression (brown) with giant-cell synctia formation (*arrow*), 200x

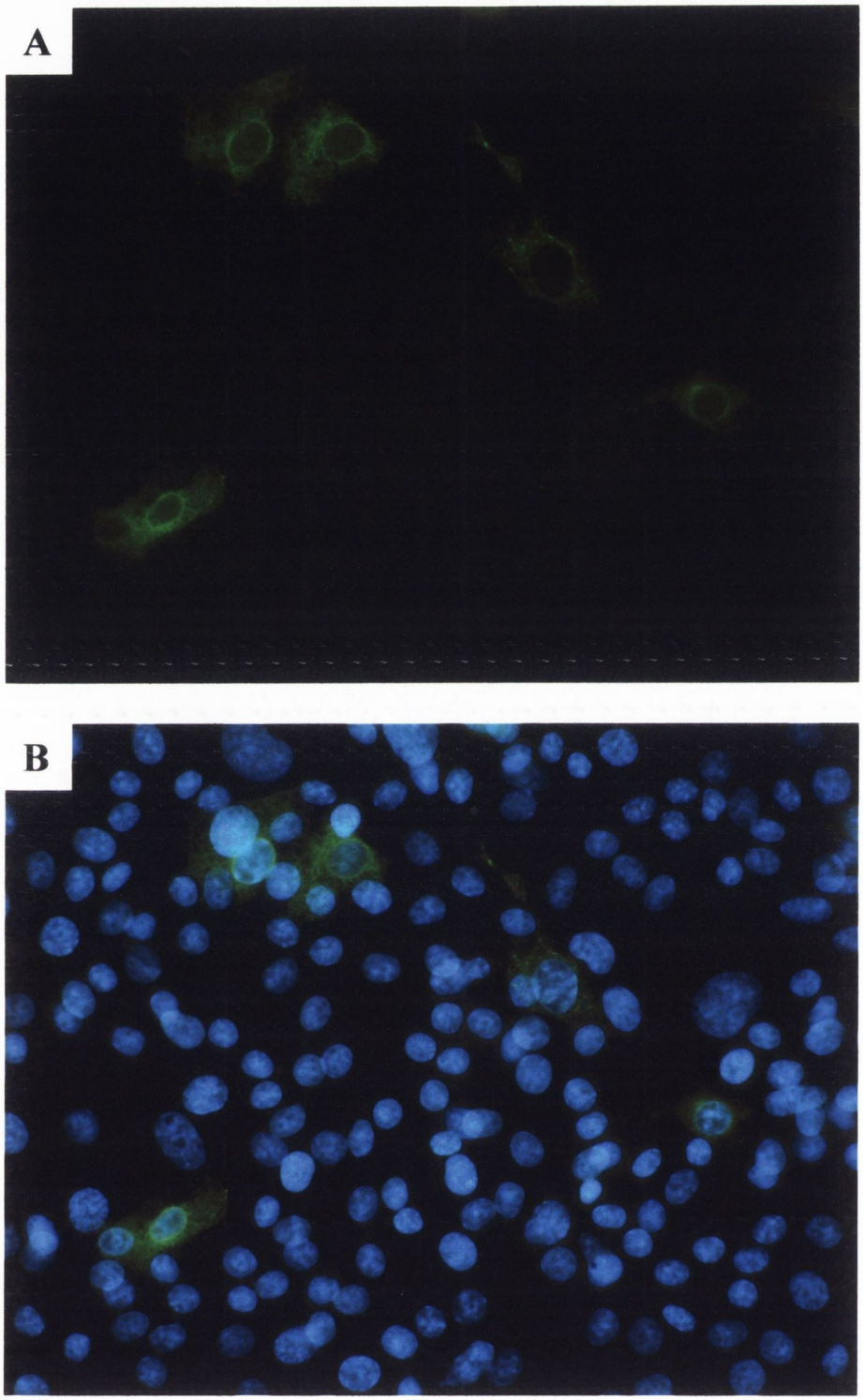


**Figure 2.4 Titration of rSFV-p62-6k VLPs by immunofluorescence**

**(A)** sBHK cells infected with rSFV-p62-6k VLPs positive for SFV antigen labelled with FITC (green), 400x

**(B)** DAPI (blue) was employed as a nuclear fluorescent counterstain (same field), 400x

### 2.1.4 Concentrated SFV4 and high-titre rSFV-p62-6k VLP production for use in *in vivo* studies

In order to achieve a more significant response to treatment of K-BALB tumours *in vivo*, it was necessary to concentrate SFV4 and the rSFV-p62-6k VLPs to titres of ~$10^{10}$ IU/ml. The techniques described in sections 2.1.2 and 2.1.3 were modified as described below.

#### 2.1.4.1 Production of concentrated SFV4

Four 150 $cm^3$ flasks of BHK-21 cells were grown to 80% confluence as described in section 2.1.1.1 and infected with SFV4 seed stock as described in section 2.1.2.1 with a total volume of 25 ml medium per flask.

##### 2.1.4.1.1 Concentration of virus by ultracentrifugation

Supernatants were pooled (100 ml) and clarified by centrifugation at 6,000 x *g* for 30 min at 4 °C in a Sorval Rc 5C Plus centrifuge. Supernatants were then decanted into fresh centrifuge tubes and re-centrifuged under the same conditions for 15 min. Clarified supernatants were then pooled and aliquoted into four Ultra-Clear SW28 centrifuge tubes (Beckman, USA) in 25 ml amounts. A 25% sucrose (v/v) solution was prepared in TNE buffer and 5 ml of this was carefully added to the base of each tube using a Pasteur pipette to form a sucrose cushion. Tubes were then filled to within 2-3 mm from the top with BHK-21 medium and balanced before being spun at 100,000 x *g* for 2 h at 4 °C in a Beckman L8-60M ultracentrifuge. Supernatants and sucrose cushions were removed using a pipette and a sterile cotton swab was utilised to remove residual sucrose from the sides of the tubes.

In order to resuspend the viral pellets, 250 µl TNE buffer was added to the base of each tube and aspirated gently. Tubes were then covered with parafilm and incubated on ice overnight after which the TNE was aspirated, removed and pooled on ice. A further 250 µl TNE buffer was added to each of the tubes which were then vortexed for 1 min and incubated on ice for 1 h. The TNE was again removed and pooled on ice and 500 µl TNE was used to wash each tube in succession by aspiration before being pooled along with the other viral suspensions to a final volume of ~2,500 µl. The virus

suspension was then aliquoted into 50-200 µl volumes on ice and snap-frozen in liquid nitrogen.

#### 2.1.4.1.2 Plaque assay of concentrated SFV4

Concentrated SFV4 viral titre was calculated using plaque assay as described in section 2.1.2.2 but doubling serial dilutions were prepared from $10^{-6}$ to $10^{-14}$ in order to accommodate the substantially higher viral titres generated.

### 2.1.4.2 Production of high titre rSFV-p62-6k VLPs

#### 2.1.4.2.1 *In vitro* SP6 RNA transcription

The DNA plasmids pSFV-p62-6k, pSFV-HelperS2 and pSFV-CS219A were propagated, linearised and cleaned as described in sections 2.1.3.3-2.1.3.5. For *in vitro* SP6 RNA transcription, reactions were made up in the same manner as section 2.1.3.6 but at six times the volume of each reagent, with a final volume of 300 µl. Reactions were split into 100µl aliquots in three eppendorf tubes and incubated for 2 h at 37 °C after which appropriate aliquots were re-pooled and placed on ice. Transcription was confirmed by gel electrophoresis as described in section 2.1.3.6 and samples were then stored at -70 °C.

#### 2.1.4.2.2 Electroporation

Six ~80% confluent flasks of BHK-21 were propagated as described in section 2.1.1.1, trypsinised, pooled and centrifuged at 400 x *g* for 15 min at room temperature. Pellets were gently resuspended in 15 ml PBS by aspiration and centrifuged again as described above. The cells were then resuspended in a total volume of 4.2 ml PBS by gentle aspiration. The *in vitro* transcribed RNA from pSFV-p62-6k, pSFV-HelperS2 and pSFV-CS219A (section 2.1.4.1) was pooled and added to the cell suspension which was then mixed gently by aspiration. The RNA-cell suspension was then added to six 0.4 µm gap electroporation cuvettes in 850 µl amounts and pulsed as described in sections 2.1.3.7-2.1.3.8. The cuvettes were resuspended in 2 x 50 ml volumes of BHK-21 medium at 37 °C in two groups of three which were then added to two 150 $cm^3$ cell culture flasks and incubated at 33 °C for 36 h in a humidified atmosphere of 5% $CO_2$.

#### 2.1.4.2.3 Harvesting high titre rSFV-p62-6k VLPs

Supernatants form both flasks were pooled (100 ml) and rSFV-p62-6k VLPs were concentrated by ultracentrifugation and resuspended in the same manner as described in section 2.1.4.1.1.

#### 2.1.4.2.4 Titration of high titre rSFV-p62-6k VLPs

The titration of high titre particles was performed in much the same way as described in section 2.1.3.9 but with higher serial dilutions (5 µl, 0.5 µl, 0.05 µl, and 0.005 µl) in order to accommodate the substantially higher concentrations of VLPs and therefore facilitate clearer and more accurate counts.

## 2.2 *IN VITRO* ASSAYS

### 2.2.1 Infection of cell lines with SFV4 and rSFV-p62-6k VLPs

#### 2.2.1.1 SFV4 growth curves

In order to assess the efficiency of SFV multiplication in K-BALB cells compared to their non-tumourigenic counterpart BALB/3T3 as well as BHK-21 cells, viral growth curves were generated over a 5 day period. Cell lines were seeded into 30 wells of 24-well cell culture dishes (Nunc, Denmark) at a concentration of 5 x $10^4$ cells/well in maintenance medium (DMEM supplemented with 2% (v/v) NCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine) and allowed to adhere for ~12 h. Monolayers were then washed once with 1 ml PBS+ (Invitrogen, UK) and infected with working stock SFV4 (section 2.1.2.1) at a multiplicity of infection (MOI) of 100 or 0.1 PFU/cell in a total volume of 200 µl MFI per well for 1 h at 37 °C with rocking every 15 min. After 1 h, the inoculum was removed, monolayers washed twice with 1 ml PBS+ to remove any unbound virus and 1 ml of fully supplemented DMEM at 37 °C was added to each well. Plates were incubated at 37 °C for 5 days during which time supernatants were sampled in triplicate at 12, 24, 36, 48, 72, 96 and 120 h.p.i., clarified by centrifugation (3,000 x *g* for 10 min, 4°C), aliquoted and stored at –70 °C. In order to quantify the amount of SFV4 virus at each timepoint, plaque assays were carried out on thawed samples as described in section 2.1.2.2 and plotted as viral growth curves.

#### 2.2.1.2 rSFV-p62-6k VLP infection efficiency

To determine the infection efficiency of the rSFV-p62-6k VLPs, K-BALB, BALB/3T3 and BHK-21 cells were seeded in 24-well cell culture dishes onto 13 mm round coverslips (Chance Propper, UK) at a concentration of 5 x $10^3$ cells/well in 1 ml maintenance medium and allowed to adhere for ~12 h. Cells were then washed with 1 ml PBS+ and infected with rSFV-p62-6k VLPs in triplicate at a variety of MOIs (0, 1, 10, 50, 100, 500, and 1000) in 200 µl MFI for 1 h at 37 °C with rocking every 15 min. After two 1 ml PBS+ washes, 1 ml maintenance medium at 37 °C was added to each well and cells were maintained for 18 h at 37°C in a humidified atmosphere of 5% $CO_2$. Supernatants were removed, cells were fixed with 10% formalin and SFV antigen

expression was detected by immunofluorescence as described in section 2.1.2.5.3. Infection efficiencies were calculated as the number of SFV antigen-positive cells as a percentage of the total number of DAPI-positive cells in each microscopic field.

### 2.2.2 Cell viability and proliferation assays

In order to assess the effect of SFV4 and rSFV-p62-6k infection on viability and proliferation of cultured cells *in vitro*, K-BALB, BALB/3T3, and BHK-21 cells were seeded at a concentration of 5 x $10^5$ cells/well in 6-well cell culture dishes in 2 ml maintenance medium and allowed to adhere for ~12 h. Monolayers were washed once with 1 ml PBS+ and infected in triplicate with MFI alone, SFV4 or rSFV-p62-6k VLPs at a MOI of 100 in 500 µl MFI. After 1 h incubation at 37 °C with rocking every 15 min, the inocula were removed and monolayers were washed twice with 1 ml PBS+ before the addition of 2 ml fully supplemented DMEM at 37 °C and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$. Cells were analysed for cellular viability by trypan blue exclusion (section 2.2.2.1) and for proliferation by [$^3$H]-*methyl* thymidine incorporation (section 2.2.2.2).

#### 2.2.2.1 Trypan blue exclusion assay

The viability of mock-infected, SFV4 and rSFV-p62-6k VLP infected cells was analysed by trypan blue exclusion at 12 h intervals over a 5 day period. Upon sampling, supernatants were removed and monolayers were washed twice with 2 ml PBS and trypsinised in 500 µl trypsin. 500 µl fully supplemented DMEM was then added, aspirated until a homogenic cell suspension was achieved from which 100 µl was removed and mixed by aspiration with 100 µl trypan blue solution (Sigma). This cell suspension was then loaded onto a haemocytometer and viable (trypan blue-excluding) cells were enumerated.

#### 2.2.2.2 [$^3$H]-*methyl* thymidine incorporation assay

The proliferation rate of mock-infected, SFV4 and rSFV-p62-6k infected cells was assessed by [$^3$H]-*methyl* thymidine incorporation at 24 h intervals over a 5 day period. 24 h prior to sampling, 2 µl [$^3$H]-*methyl* thymidine (specific activity: 3.22 TBq/mmol, 87.0 Ci/mmol, Amersham Pharmacia Biotech, Sweden) was added to

appropriate wells resulting in a final concentration of 1 μCi/ml. Upon sampling, supernatant was removed and monolayers were washed twice with 2 ml PBS+ before the addition of 2 ml 1% (v/v) sodium dodecyl sulphate (SDS) in PBS to lyse the cells. The SDS cell lysate was aspirated to ensure all cells were lysed and was then transferred to 5 ml glass bijous and stored at -70 °C. In order to process the samples for scintillation, 10% ice cold trichloroacetic acid (TCA) was added to each thawed sample and incubated on ice for 15 min to precipitate large molecules and cellular DNA. Precipitates were collected on glass fibre filter mats (Whatmann, USA) using a manifold (Millipore, USA), washed twice with ice-cold 5% TCA followed by 100% ethanol and air-dried. Filter mats were then placed in plastic scintillation vials into which 4 ml scintillation fluid (ICN, USA) was added prior to counting using a Tricarb 1500 scintillation counter (3 min count/sample). Counts per minute (CPM) were directly proportional to the rate of proliferation of cell in culture.

### 2.2.3 Detection of apoptosis induction and SFV antigen expression

In order to simultaneously detect the induction of apoptosis and the expression of viral antigen following infection with SFV4 or rSFV-p62-6k VLPs, two approaches exploiting epifluorescence were employed. Statistical comparisons were performed with Graphpad Prism 4.00 using a two-way repeated measures ANOVA with the Bonferroni post-test comparing individual groups to each other.

#### 2.2.3.1 TUNEL with anti-active caspase-3 and anti-SFV4 immunofluorescence

Fluorescent labelling of DNA fragmentation which is often associated with apoptosis induction was achieved using the Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling (TUNEL) technique (Gavrieli *et al*, 1992) along with the detection of active caspase-3 and SFV antigen expression by immunofluorescence. K-BALB, BALB/3T3, and BHK-21 cells were seeded at a concentration of 5 x $10^5$ cells/well in 6-well cell culture dishes in 2 ml maintenance medium per well and allowed to adhere for ~12 h. Monolayers were then washed once with 2 ml PBS+ and infected in triplicate with MFI alone, SFV4 or rSFV-p62-6k VLPs at a MOI of 100 for 1 h at 37 °C with rocking every 15 min. Cells were then washed twice with PBS+, 2 ml fully supplemented DMEM at 37 °C was added and cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$. Upon sampling,

monolayers were scraped into the medium using cell scrapers (Sarstedt, Germany) and homogenous cell suspensions were obtained by aspiration. Cell suspensions were then enumerated (viable and non-viable cells were counted) utilising a haemocytometer and adjusted to 3 x $10^5$ cells/ml with fully supplemented DMEM. Cells from each sample were spun onto poly-L-lysine coated slides in duplicate in 200 µl volumes using a cytospin III (Shandon, USA) at 700 rpm for 2 min. One set of slides was fixed in 10% formalin and SFV antigen was detected by immunofluorescence as described in section 2.1.2.5.3 and percentage positive cells calculated with DAPI as counterstain. The remaining slides were fixed in acetone at -20 °C for 10 min, allowed to air-dry and cytospins were outlined with a DAKO Cytomation pen before a 5 min wash step in PBS. Cells were then permeabilised at 4 °C for 2 min in a 0.1% solution of triton-X 100 in 0.1% sodium citrate and washed twice in PBS for 5 min. 50 µl fluorescein conjugated TUNEL reaction mixture (Roche, Germany) was applied to each cytospin and incubated in a humidified chamber for 1 h at 37 °C. After a further 3 x 5 min PBS washes, 100 µl 1.5% NGS in PBS was added and slides were incubated at room temperature for 30 min. Following 2 x 10 min PBST and 2 x 5 min PBS washes, 100 µl rabbit anti-active caspase-3 (BD Pharmingen, UK) at a dilution of 1/200 in PBS containing 5% NGS was applied to the slides which were then incubated for 18 h at 4 °C in a humidified chamber. Slides were washed again in PBST and PBS as above and 100 µl rhodamine avidin DCS at a dilution of 1/200 in high-salt buffer was applied to each before incubation at room temperature for 30 min. Following 2 x 5 min washes in high-salt buffer, slides were dipped in $dH_2O$ and mounted using Vectashield containing DAPI. Positive cells were visualised using fluorescent microscopy and percentage TUNEL (green) and active caspase-3 (red) positive cells calculated using DAPI as counterstain. It was also possible to assess cell viability by nuclear morphology with apoptotic nuclei displaying condensation and fragmentation and complete nuclear degradation resulting in anuclear 'ghost' cells.

### 2.2.3.2 FLICA and anti-SFV4 immunofluorescence

Fluorochrome-labelled inhibitors of caspases (FLICA) have been developed more recently as a method of detecting and quantifying active caspases at a cellular level (Bedner *et* al., 2000) and were employed in combination with anti-SFV4 immunofluorescence as an additional assay to study rSFV-p62-6k and SFV4 induced apoptosis. K-BALB, BALB/3T3 and BHK-21 cells were seeded at a concentration of 5

x $10^4$ cells/well in 24-well cell culture dishes in a total volume of 1 ml maintenance medium per well and allowed to adhere for ~12 h. Monolayers were washed once with 1ml PBS+ and infected in triplicate with MFI alone, SFV4 or rSFV-p62-6k VLPs at a MOI of 100 in 200 µl MFI for 1 h at 37 °C with rocking every 15 min. Cells were then washed twice with 1 ml PBS+ before the addition of 1 ml fully supplemented DMEM at 37 °C and incubated at 37 °C in a humidified atmosphere of 5% $CO_2$. Cells were sampled at 6, 12, 24, 36, 48 and 72 h.p.i. and analysed as follows. FITC-VAD-FMK (Oncogene, USA) was added to each well at a concentration of 3.3 µl/ml as per manufacturers recommendations and cells were incubated for 1 h at 37 °C in a humidified atmosphere of 5% $CO_2$. Supernatants were removed and added to 2 ml microtubes. Monolayers were then washed once with 400 µl PBS- (which was subsequently added to supernatants in 2 ml tubes) and trypsinised in 400 µl trypsin EDTA before aspiration and addition to supernatant and wash PBS in the appropriate 2 ml tubes. Cells were pelleted at 850 x *g* in a microfuge for 5 min, supernatants were discarded and cells were resuspended in 1 ml wash buffer (oncogene, USA) and vortexed to ensure homogenic resuspension. This washing step was repeated twice after which the cell pellet was resuspended in 300 µl 2% paraformaldehyde (BDH, UK) in PBS and tubes were stored at 4 °C in the dark overnight. Fixed cell suspensions were then spun onto slides by cytospin as described in section 2.2.3.1 and SFV antigen detected by immunofluorescence using a rhodamine avidin DCS as described in section 2.1.2.5.3. Slides were mounted with Vectashield mountant containing DAPI, positive cells counted using fluorescence microscopy and percentages calculated.

## 2.3 TREATMENT OF K-BALB TUMOURS *IN VIVO* WITH rSFV-p62-6k VLPS AND SFV4

### 2.3.1 Immunisation of BALB/c and BALB/c *nu/nu* mice

#### 2.3.1.1 Mice

Specific pathogen-free (spf) 40-60 day old BALB/c and BALB/c *nu/nu* mice (Harlan, UK) were maintained in a sterile (negative pressure) environment at 23 °C with sterilised food and water supplied *ab libitum*. Mice were allowed to acclimatise for 1 week prior to initiation of any *in vivo* studies. BALB/c *nu/nu* mice were not included in SFV4 treatment groups as it was assumed that these mice would succumb to SFV4 infection.

#### 2.3.1.2 Immunisation with rSFV-p62-6k VLPs

For immunisation, rSFV-p62-6k VLPs were produced as described in section 2.1.3.8 and adjusted to 2 x $10^7$ IU/ml in TNE buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1 mM EDTA). Groups of mice to be immunised received a 50 µl intramuscular (i.m.) injection of the rSFV-p62-6k VLPs in TNE at a concentration of 2 x $10^7$ IU/ml in the left *tibialus anterior* and were boosted two weeks later with the same injection in the right *tibialus anterior*. As naïve controls, groups of mice were simultaneously mock-immunised with TNE alone in the same manner.

### 2.3.2 K-BALB tumour induction and treatment *in vivo*

#### 2.3.2.1 Assessment of K-BALB tumour formation *in vivo*

Prior to the initiation of studies examining the treatment of K-BALB tumours *in vivo*, it was first necessary to study the normal growth rate and histology of these tumours in BALB/c mice. Eleven (naïve) BALB/c mice were used and prior to tumour induction their right flanks were shaved in order to facilitate tumour induction and measurement. K-BALB cells were propagated in 150 $cm^3$ cell culture flasks as described in section 2.1.1.4 and harvested by trypsinisation when in the exponential phase of growth. Cells were centrifuged at 400 x *g* at 4 °C and resuspended by gentle

aspiration in non-supplemented DMEM. Cells were centrifuged and resuspended as before, adjusted to a concentration of 1 x $10^7$ cells/ml and injected subcutaneously (s.c.) into the right flank of each mouse using a 1 ml syringe (Beckton Dickenson, UK) and 21 G needle in 100 µl amounts. Mice were examined daily for signs of s.c. tumour formation and when detected, tumour diameters were measured using a linear callipers in 2 perpendicular diameters with the average tumour diameter calculated as the square-root of the product of cross-sectional diameters, assuming spherical shape. Three mice were used for tumour measurement and the remaining 8 were sampled in duplicate for histology at 3, 6, 9 and 12 days post tumour formation. Upon sampling, mice were euthanised by halothane overdose, perfused with formal saline and tumours processed for paraffin embedding as described in section 2.4.1.1 before staining and histological examination as described in sections 2.4.2.1 and 2.4.2.2. The remaining mice were euthanised when tumours reached an average diameter of 20 mm.

#### 2.3.2.2 K-BALB tumour induction in BALB/c and BALB/c *nu/nu* mice

Two weeks after boosting (section 2.3.1.2), the right flank of each BALB/c mouse was shaved in order to facilitate tumour induction and measuring, this was not necessary for BALB/c *nu/nu* mice which are naturally almost completely hairless. K-BALB cells were propagated and tumours were induced as described in section 2.3.2.1. Tumour diameters were measured daily and treatment was initiated once tumours reached an average diameter of 4 mm.

#### 2.3.2.3 Treatment of K-BALB tumours with rSFV-p62-6k VLPs *in vivo*

High titre rSFV-p62-6k VLPs were prepared as described in section 2.1.4.3, titrated (section 2.1.4.4), and adjusted to a concentration of 1 x $10^{10}$ IU/ml in TNE buffer. Mice received 6 x i.t. injections of 50 µl TNE buffer alone (control groups) or the TNE containing high-titre rSFV-p62-6k VLPs using a 0.5 ml insulin microsyringe (Beckton Dickenson, UK). Injections were directed towards the centre of each tumour and administered slowly to facilitate the absorption of the fluid by the tumour and to avoid the surrounding normal tissue. A total of six injections were administered to each tumour with treatment every second day for a total of twelve days and groups of five mice were used per treatment group. Tumours were measured daily as described in

section 2.3.2.1 and mice were euthanised by halothane (Rhône Mérieux, UK) overdose and cervical dislocation when tumours approached 20 $mm^2$.

### 2.3.2.4 Treatment of K-BALB tumours with SFV4 *in vivo*

Concentrated SFV4 virus was prepared as described in section 2.1.2.6, titrated by plaque assay (section 2.1.2.2), and adjusted to a concentration of 1 x $10^{10}$ PFU/ml in TNE buffer. Mice received 6 x i.t. injections of TNE buffer alone or of concentrated SFV4 in the same manner as described in section 2.3.2.2 with five mice per treatment group.

#### 2.3.2.4.1 Detection of replicating SFV4 in tumours and brains

As SFV4 is a neurotropic virus which can cause disease in BALB/c mice, brains and tumours of mice treated with SFV4 were sampled in order to assess the multiplication of the virus in the tumours and whether or not it was successfully infecting the central nervous system of the mice. Groups of three mice were sampled on days 1, 5, 11, and 15 post initiation of treatment at which point they were euthanised by halothane overdose and cervical dislocation. Using aseptic technique, tumours and brains were carefully excised, weighed and 10% (w/v) homogenates were prepared in MFI using glass hand homogenisers. Homogenates were then clarified by centrifugation at 3,000 x *g* for 10 min at 4 °C, aliquoted and stored at -70 °C. Viral titres were determined from thawed samples by plaque assay as described in section 2.1.2.2 and expressed as PFU/mg tissue.

### 2.3.3 Immunological studies

#### 2.3.3.1 Harvesting of serum and splenocytes

Groups of BALB/c mice were sampled in triplicate from all groups on days 0, 1, 5, 11, and 15 post initiation of treatment to assess the humoural and cellular immune responses to SFV antigens. Being athymic, BALB/c *nu/nu* mice were not sampled throughout the course of the experiment but rather at experiment end at which point serum and splenocytes were sampled and analysed in the same manner as with BALB/c mice in order to confirm the absence of an anti-SFV immune response. Mice were

euthanised by halothane overdose and cervical dislocation after which the abdomen was opened aseptically and the hepatic artery severed. A Pasteur pipette was used to harvest blood from the mice into an eppendorf tube which was subsequently incubated at 4 °C for 18 h. The then clotted blood was centrifuged at 400 x *g* for 10 min at 4 °C and the serum was decanted and aliquoted before storage at -70 °C.

Immediately after harvesting blood, spleens were excised aseptically and placed in 15 ml ice cold Hank's balanced salts solution (HBSS) (Invitrogen, UK) in groups of three. Spleens were then teased through a cell strainer (Falcon) into 50 ml tubes (Falcon) in order to obtain a single-cell suspension of splenocytes which was then centrifuged at 400 x *g* for 10 min at 4 °C. Pellets were resuspended in 15 ml ice-cold red blood cell (RBC) lysis buffer (eBiosciences, UK) and incubated on ice for 15 min with mixing by gentle inversion every 5 min. In order to stop the reaction, 35 ml ice-cold HBSS was added and tubes were centrifuged at 400 x *g* for 10 min at 4 °C. Pellets were resuspended in 5 ml ice cold cryopreservation medium (90% heat inactivated FBS and 10% RPMI medium (Invitrogen, UK)) and passed through a cell strainer to remove debris and obtain a homogenous suspension of splenocytes. Viable cells were enumerated using trypan blue and a haemocytometer and suspensions were adjusted to a concentration of 1 x $10^6$ cells/ml. 1 ml aliquots were then frozen using a "Mr Frosty" cryopreservation container (Nalgene, USA) according to manufacturer's instructions and stored under liquid nitrogen to await analysis. Tumours from mice sampled for immunological studies were excised and processed for cryosectioning (section 2.4.1.2).

### 2.3.3.2 Detection of humoural anti-SFV immune response

#### 2.3.3.2.1 Production of positive and negative anti-SFV reference sera

In order to set-up and optimise an ELISA for the detection of mouse anti-SFV IgG it was first necessary to obtain positive and negative reference sera to act as standards and quality controls. Negative reference serum was prepared by euthanising five BALB/c mice and pooling their serum which was harvested from blood as described in section 2.3.3.1. Positive reference serum was generated by immunising ten BALB/c mice as described in section 2.3.1.2 and challenging two weeks post boost with 1 x $10^6$ PFU purified SFV4 (section 2.1.2.3) in 500 µl PBS i.p.. Three weeks post challenge serum was harvested and pooled as above and both reference sera were then aliquoted and stored at -70 °C. Splenocytes were also harvested and frozen from the above mice as

described in section 2.3.3.1 to act as negative and positive controls in splenocyte stimulation assays (section 2.3.3.3.1).

#### 2.3.3.2.2 "Checkerboard" optimisation of mouse anti-SFV IgG ELISA

Concentrated SFV4 was prepared as described in section 2.1.4 along with control BHK-21 antigen which was prepared from concentrated supernatants of mock-infected flasks of BHK-21 in tandem with SFV4 concentration. The concentrated SFV4 was titrated by plaque assay as described in section 2.1.2.2 and UV-inactivated before storage at –70 °C. Two Nunc maxisorp 96-well plates were coated with eight serial doubling dilutions (1/100 to 1/12,800, left to right) of UV-inactivated concentrated SFV4 in 100 µl 0.1 M sodium carbonate coating buffer (pH 9.5) for 18 h at 4 °C and two additional plates were coated with serial dilutions of BHK-21 control antigen in the same manner. Plates were washed 3 x 4 min 250 µl/well PBST and blocked for 15 min (sealed) at room temperature using 200 µl/well ELISA blocking reagent (EBR) (Roche, Germany). Following 3 x 4 min PBST washes, serial doubling dilutions of positive reference serum (1/50 to 1/12,800, top to bottom) were applied to one SFV4- and one BHK-21 control antigen-coated plate diluted in EBR at a volume of 100 µl/well. Negative control serum was applied to the remaining SFV4- and BHK-21 control antigen-coated plates in the same manner. Plates were sealed and incubated at room temperature for 1 h after which they received 3 x 4 min PBST washes. HRP-conjugated Rabbit anti-mouse IgG secondary antibody (DAKO, Denmark) was then added to each well in 100 µl EBR and plates were sealed and incubated for 1 h at room temperature. Following 3 x 4 min PBST washes 100 µl/well 0.4 mg/ml SigmaFast *o*-phenylenediamine (OPD) was added and plates were incubated in the dark for 10 min. The reaction was then stopped with the addition of 50 µl/well 2N $H_2SO_4$ and plates were read using a Thermo Labsystems Multiscan RC ELISA plate reader (492 nm). The optimum dilutions chosen were those with absorbances below 0.2 on BHK-21 control antigen plates for all sera and above 1.2 for positive reference serum and below 0.2 for negative control serum on the SFV4 plate (concentrated SFV4; 1/3,200, serum; 1/400 to 1/800).

#### 2.3.3.2.3 Mouse anti-SFV IgG ELISA

Nunc maxisorp 96-well plates were coated with UV-inactivated purified SFV4 at a dilution of 1/3,200 in a volume of 100 µl/well in 0.1 M sodium carbonate coating buffer sealed and incubated at 4 °C for 18 h. Plates then received 3 x 4 min 250 µl/well PBST washes and were blocked for 15 min at room temperature (sealed) with 200 µl/well EBR. Following a further 3 x 4 min PBST washes serum samples were diluted 1/600 in EBR and added to triplicate wells at a final volume of 100 µl/well. Positive and negative sera (section 2.3.3.2.1) were also applied in the same manner as samples to each plate to act as quality controls. Serial doubling dilutions of positive reference serum ranging from 1/50 to 1/102,400 in EBR and added to duplicate wells to generate a reference curve. Plates were sealed and after 1 h incubation at room temperature received 3 x 4 min PBST washes. HRP-conjugated Rabbit anti-mouse IgG was diluted 1/2,000 in EBR and applied in a volume of 100 µl/well, plates were sealed and incubated at room temperature for 1 h. After washing again as above OPD substrate was added to each well in a volume of 100 µl/well, incubated for 10 min in the dark and the reaction was stopped with the addition of 50 µl/well 2 N $H_2SO_4$. Plates were read as above and Graphpad Prism 4.0 software was used to generate a sigmoidal standard curve from the positive reference serum serial doubling dilutions from which unknown samples were assigned arbitrary anti-SFV4 IgG units to a maximum of 1,000. Quality controls were compared between plates and any plates falling outside a standard deviation threshold of 15% plate-to-plate variance were discounted and serum samples re-assayed.

### 2.3.3.3 Detection of cellular anti-SFV immune response

#### 2.3.3.3.1 Splenocyte stimulation assay

In the following assay, splenocytes were cultured in RPMI medium supplemented with 10% heat-inactivated FBS, 20 mM Hepes buffer, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, 50 µM 2-mercaptoethanol in U-bottomed 96-well cell culture dished (Nunc, Denmark). Splenocytes (sections 2.3.3.1 and 2.3.3.2.1) were thawed rapidly and added to 10 ml fully supplemented RPMI at 37 °C, centrifuged for 10 min at 170 x *g* and resuspended in 2 ml fully supplemented RPMI at 37 °C. Viable cells were enumerated on a haemocytometer and splenocytes were cultured in triplicate wells in the presence of medium alone, 5 µg/ml concanavalin A (conA) (Sigma, USA) or $10^5$

PFU UV-inactivated purified SFV4 (sections 2.1.2.3 and 2.3.3.2.2) in a total volume of 200 µl/well and at a concentration of 2 x $10^5$ cells/well for 72 h at 37 °C in a humidified atmosphere of 5% $CO_2$. After 72 h, plates were spun at 170 x *g* at 4 °C for 10 min and supernatants were carefully collected, aliquoted and stored at -70 °C.

#### 2.3.3.3.2 Cytokine capture ELISAs

In order to characterise the cellular immune response, cytokine release by splenocytes stimulated with medium alone, conA and SFV4 was analysed using capture ELISA. The OptEIA IFN-γ capture ELISA kit (BD Pharmingen, UK) was employed to analyse supernatant samples from section 2.3.3.3.1. Samples were diluted 1/50 in assay diluent (10% (v/v) heat-inactivated FBS in PBS). Capture ELISAs were performed according to manufacturer's instructions and stimulation indices were calculated by dividing cytokine production by cells stimulated with SFV4 by cytokine production by cells stimulated with medium alone.

## 2.4 HISTOPATHOLOGY

### 2.4.1 Sampling of mice for histopathology

#### 2.4.1.1 Perfusion of mice, processing, paraffin embedding and sectioning of tissue

For general pathology and immunohistochemical detection of CD45R, CD3, and active caspase-3, mice were sampled in triplicate at day 0, 1, 5, 11, and 15 post initiation of treatment. Mice were heavily anaesthetised with halothane and perfused through the left ventricle with PBS followed by 10% formal saline for 5 min and allowed to fix overnight at 4 °C. Tumours, and (in the case of SFV4 treated groups) brains, were excised, placed in 5% formal saline and processed for paraffin embedding. Processing of tissues, paraffin embedding and sectioning of sampled tumours and brains was performed by Ms. Alex Whelan and Ms. Marie Moore (Veterinary Pathology Laboratory, University College Dublin). Fixed brains were divided coronally at the levels of the olfactory cortex, optic nerves and pons/cerebellum whereas tumours were processed whole. Samples were dehydrated through a series of graded alcohol washes as follows; 50% (v/v) alcohol for 1 h, 70% (v/v) alcohol for 1 h and 90% (v/v) alcohol for 1 h, before 2 x 40 min absolute alcohol washes. Samples were then immersed for 1 h in an absolute alcohol and xylene (1:1) solution before 3 x 40 min absolute xylene washes. This was followed by 4 x 40 min immersions in paraffin wax and subsequent mounting onto blocks. For routine histology and immunohistochemistry, 3 μm sections were cut using a microtome and dried overnight at 37 °C in an oven.

#### 2.4.1.2 Preparation of K-BALB tumour cryosections

As many epitopes can be irretrievably 'masked' by formalin fixation, tumours from mice sampled for immunological studies (section 2.3.3.1) were excised for the immunohistochemical and immunofluorescent detection of CD4, CD8, CD11c, CD19, F4/80, SFV antigen and active caspase-3. Excised tumours were embedded in OCT compound (TissueTec, USA) on cork disks (Lamb, UK), snap-frozen in liquid nitrogen-cooled isopentane and stored at -70 °C. A Leica CM 1900 cryostat was used to prepare 6 μm thick sections at –15 °C which were placed on poly-L-lysine coated glass slides, fixed in acetone at -20 °C for 5 min, air-dried overnight and stored in airtight containers at -70 °C.

### 2.4.2 Histopathology and immunohistochemistry

#### 2.4.2.1 Haematoxylin and eosin staining for routine histology

Sections of paraffin embedded tumours and brains (section 2.4.1.1) were examined routinely using the haematoxylin and eosin (H&E) staining method. Dried sections were dewaxed with 3 x 10 min washes in 100 % xylene and subsequently rehydrated through 100%, 95%, 70% ethanol for 5 min each followed by $dH_2O$. Sections were then placed in Harris' haematoxylin (BDH, UK) for 10 min, rinsed under running tap water until cleared, differentiated in 1% acid alcohol and 'blued-up' by dipping three times in 3% ammonia water. After a subsequent wash under running tap water, sections were counterstained in 1% dichromate eosin (BDH, UK) for 2 min and washed under running tap water for a further 5 min or until cleared. Stained sections were then dehydrated and mounted using DPX as described in section 2.1.2.5.2. H&E stained slides were prepared from two separate sections of each tumour to provide a more representative depiction of the microscopic changes.

#### 2.4.2.2 Routine histology

Coded H&E stained tumour sections were examined blind by light microscopy and relevant histological details were noted such as: tumour cell morphology, mitotic index, invasion of surrounding tissues by tumour cells, development of areas of necrosis within tumours, thromboses and haemorrhages, presence of TILs and their location in the tumour microenvironment. Coded H&E stained brain sections were examined blind for the presence of lesions characteristic of SFV4 infection.

#### 2.4.2.3 Immunohistochemical analysis of paraffin embedded tumour sections

To further characterise the cellular infiltrates and to detect apoptosis the following immunohistochemical methods were utilised: CD45R (B-cells), CD3 (T-cells) and active caspase-3 (apoptosis). Sections were dewaxed and rehydrated to $dH_2O$ as described in section 2.4.2.1 and placed in PBS. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS for 10 min at room temperature after which slides were washed twice for 5 min in PBS.

Antigen retrieval was performed by immersing slides in boiling unmasking buffer (see below) in a pressure cooker (Prestige, UK) for 3 min at 121 °C and 15 psi followed by 20 min cooling at room temperature. For the detection of CD45R and active caspase-3, citrate buffer (10 mM citric acid (BDH, UK), pH 6.0) was used as unmasking buffer and for the detection of CD3, 1mM EDTA (Promega, USA) was used as unmasking buffer. After antigen retrieval and cooling, slides were washed twice for 5 min in PBS, sections were outlined using a DAKO Cytomation pen and non-specific binding sites were blocked using 5% normal rabbit serum (NRS) in PBS for CD45R and CD3 detection or 5% NGS in PBS for active caspase-3 detection at room temperature for 30 min in a humidified chamber. After a brief wash in PBS, endogenous avidin and biotin was blocked using the Vector avidin/biotin blocking kit (Vector laboratories, USA) according to manufacturer's instructions and slides were again washed briefly in PBS.

Primary antibodies were incubated as follows: CD3 (NovaCastra, UK) 1/25 in 5% NRS overnight at room temperature, CD45R (BD Pharmingen, UK) 1/150 in 5% NRS overnight at 4 °C and active caspase-3 (Cell signalling technologies, USA) 1/1000 in 5% NGS overnight at 4 °C. Slides were then washed twice for 10 min with PBST followed by two 5 min washes in PBS. A 0.5% dilution of biotinylated goat anti rabbit IgG (active caspase-3) or mouse-adsorbed biotinylated rabbit anti rat IgG (Vector laboratories, USA) (CD3 and CD45R) was then prepared in 0.15% NGS or 0.15% NRS respectively, applied to slides, and incubated at room temperature for 30 min in a humidified chamber. Slides were washed with PBST and PBS as above and incubated with the Vectastain *Elite* ABC standard HRP reagent (section 2.1.2.5.2) for 30 min at room temperature. After a single 5 min wash in PBS sections were incubated at room temperature for 2 min with the reagent DAB and subsequently placed under running water for five minutes. Slides were counterstained, dehydrated and mounted with DPX as described in section 2.1.2.5.2. Sections of mouse spleen acted as positive control slides for the detection of CD3 and CD45R (Figure 2.5).

### 2.4.2.4 Immunohistochemical analysis of frozen tumour sections

Frozen tumour sections were used for detection of F4/80 (macrophage) (Abcam, UK), SFV antigen expression, CD19 (B-cells), CD4 and CD8 (T-cells) (BD Pharmingen, UK) as these antigens were more difficult to detect consistently in paraffin embedded tissues. Immunohistochemistry was performed in the same manner as in section 2.4.2.3 but rehydration and antigen retrieval were not necessary. F4/80 (Abcam, UK), CD4 and

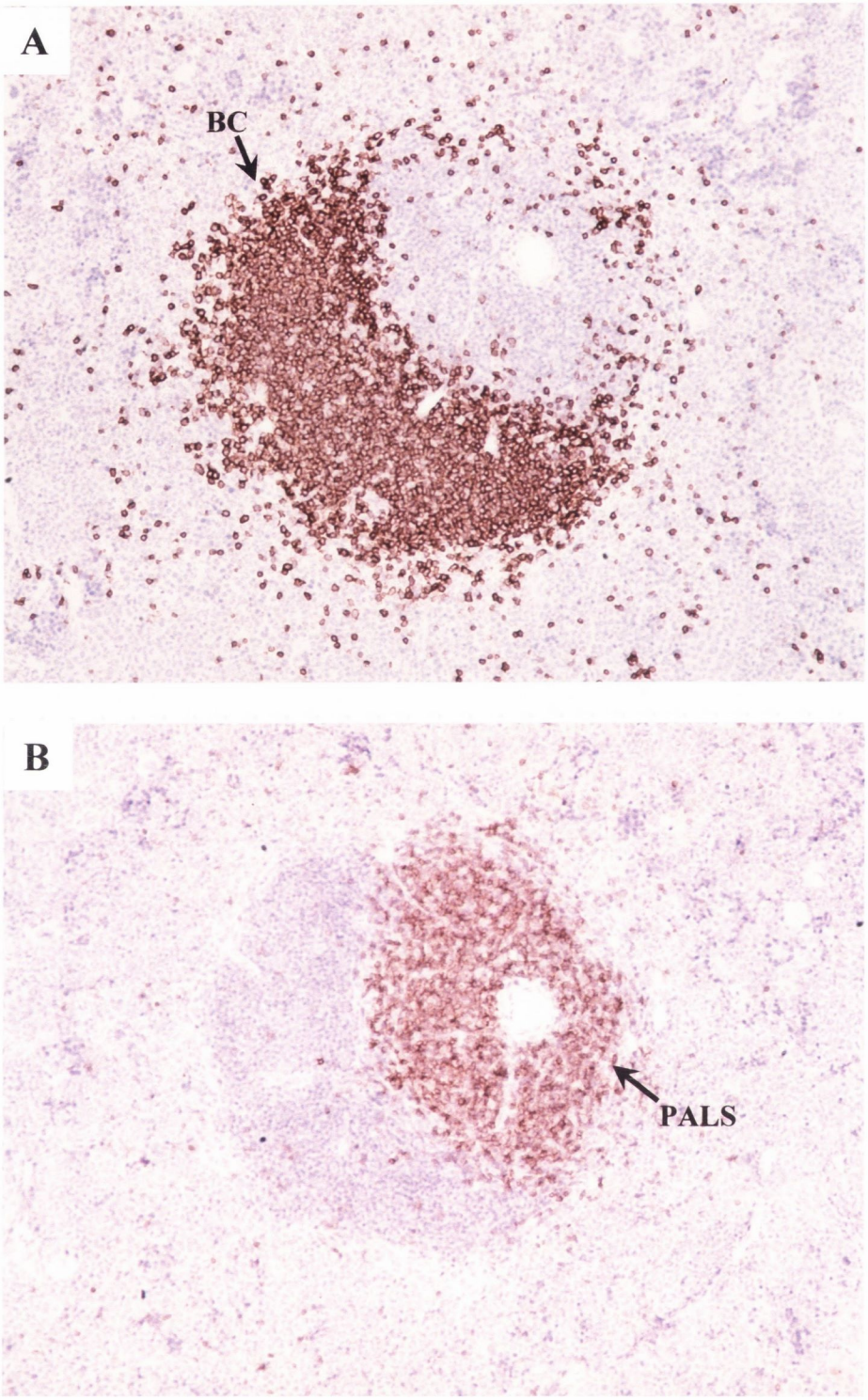


**Figure 2.5 Detection of CD45R$^+$ and CD3$^+$ lymphocytes localised at a lymphoid follicle in mouse spleen by immunohistochemistry**

**(A)** CD45R$^+$ B-lymphocytes localised in the B-cell corona (BC), 200x

**(B)** CD3$^+$ T-lymphocytes surrounding the central arteriole in the periarteriolar lyphoid sheath (PALS), 200x

CD8 (BD Pharmingen, UK) antibodies were raised in rat and diluted 1/500, 1/20 and 1/20 respectively. Sections of mouse spleen were employed as a positive control for CD4 and CD8 (Figure 2.6) and sections of mouse brain known to be positive for SFV4 antigen and macrophages were kindly provided by Prof. Brian Sheahan (Department of Veterinary Pathology, University College Dublin) as positive controls for SFV antigen and F4/80 detection (Figure 2.7).

Immunofluorescence was also employed for the demonstration of SFV antigen in conjugation with the above leukocyte markers. Immunofluorescence was undertaken in the same manner as immunohistochemistry with rhodamine or fluorescein avidin DCS replacing the Vectastain *Elite* ABC peroxidase step as in section 2.1.2.5.3. For double-labelling, the Vector avidin/biotin blocking kit was re-applied between each immunofluorescent labelling procedure. Routinely, SFV4 was labelled with rhodamine and leucocytes with fluorescein with Vectashield mountant containing DAPI providing a nuclear counterstain.

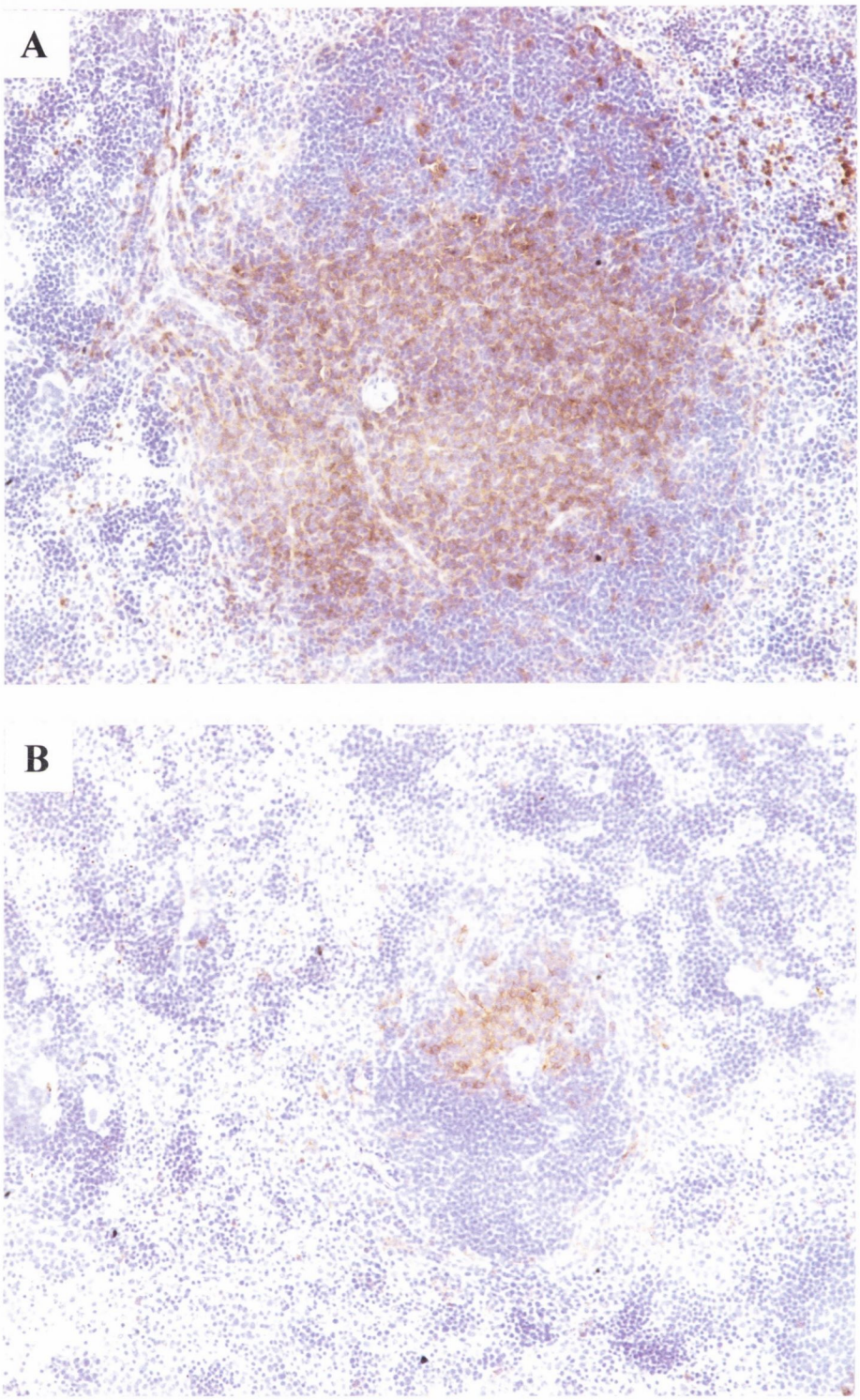


**Figure 2.6 Detection of $CD4^+$ and $CD8^+$ T-lymphocytes localised at lymphoid follicles in a mouse spleen by immunohistochemistry**

**(A)** $CD4^+$ T-lymphocytes localised predominantly within the PALS surrounding the central arteriole, 200x

**(B)** $CD8^+$ T-lymphocytes also concentrated within the PALS, 200x

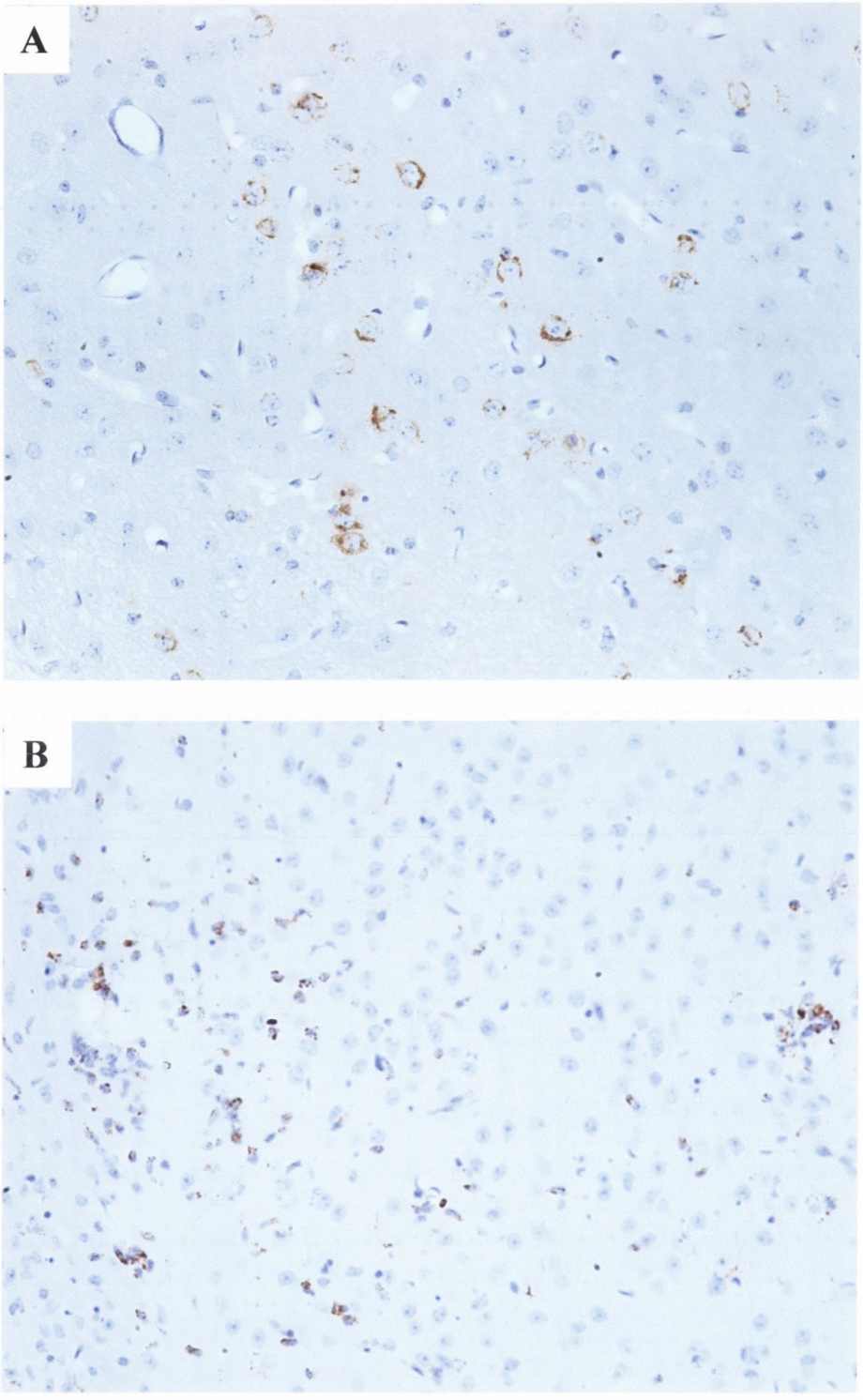


**Figure 2.7 Detection of SFV4 antigen and F4/80$^+$ macrophages by immunohistochemistry in brains of mice infected with SFV4**

**(A)** SFV4 antigen localised in neurons of an infected mouse brain, 200x

**(B)** F4/80$^+$ macrophages detected within an SFV4 infected mouse brain, 200x

# 3

# *IN VITRO* ANALYSIS OF THE EFFECTS OF INFECTION WITH rSFV-p62-6k VLPs AND SFV4 ON BHK-21, BALB/3T3 AND K-BALB CELL LINES

## 3.1 INTRODUCTION

The ability of SFV to induce apoptosis in a variety of cell lines has been well established (Glasgow *et al.*, 1997; Scallan *et al.*, 1997). This capacity to induce apoptosis is also shared by the SFV vector and rSFV VLPs and occurs independently of viral structural proteins and the status of cellular p53 (Glasgow *et al.*, 1998; Murphy *et al.*, 2000). Given the previous success in exploiting this cytopathic effect by treating tumour xenografts in BALB/c *nu/nu* mice with rSFV VLPs (Murphy *et al.*, 2000; Murphy *et al.*, 2001), it was decided to investigate the possibility of recruiting the immune system by the expression of the highly antigenic viral antigen E2 (as well as E3 and 6k) in order to enhance the antitumoural effect of rSFV VLPs. The potential for a replicating SFV vector in cancer therapy was also investigated using the replication proficient infectious clone SFV4 as a model.

The Ras family of proteins act as binary switches at nodes in signal transduction pathways and are involved in cellular differentiation, proliferation and survival. Mutations in *ras* genes are involved in approximately 30% of all human malignancies, and of these, K-*ras* is the most common. A point mutation in codon 12, 13, or 61 of a *ras* gene can render its protein product constitutively active and result in a malignant phenotype (Bos, 1989). The murine tumour model used in this study is the Kirsten murine sarcoma transformed BALB/3T3 cell line, K-BALB, which overexpresses K-*ras* and forms fast growing syngeneic tumours in immunocompetent BALB/c mice upon s.c. injection (Aaronson & Weaver, 1971; Stephenson & Aaronson, 1972).

In this chapter, the cytopathic effect of rSFV-p62-6k VLPs and SFV4 on cultured cells was examined along with the expression of viral antigen by infected cells and growth of SFV4 *in vitro*. It has been previously demonstrated that rSFV VLPs and SFV4 infect BHK-21 cells efficiently and rapidly induce apoptosis upon infection (Glasgow *et al.*, 1997). Therefore, the BHK-21 cell line was chosen to serve as a positive control for the viability, proliferation and apoptosis studies undertaken. BALB/3T3 cells were also included as a non-tumourigenic equivalent to K-BALB cells in order to assess what influence K-*ras* overexpression has on cell survival upon infection with rSFV-p62-6k VLPs or SFV4.

The well established trypan blue exclusion (Phillips & Terryberry, 1957) and [$^{3}$H-*methyl*] thymidine incorporation (Firket & Verly, 1958) assays were employed to quantify cellular viability and proliferation respectively. As activated Ras is thought to play a role in whether or not a cell undergoes apoptosis (Downward, 1998) the

mechanism of cell death in all three cell lines was examined in order to determine what influence, if any, K-*ras* overexpression has on SFV-induced apoptosis. Caspase-3 has been implicated previously in alphaviral infection of cells (Grandgirard, 1998) and so immunofluorescence was employed to detect active caspase-3 at a cellular level in tandem with the TUNEL assay. TUNEL detects fragmented DNA generated by the downstream apoptosis effector nucleases with the addition of fluorescein-dUTP to 3'OH ends of double- and single- stranded DNA fragments by the enzyme terminal deoxynucleotidyl transferase (TdT) (Gavrieli *et al.*, 1992). Fluorochrome-labelled inhibitors of caspases (FLICA) provide a faster and more straightforward method of detecting apoptosis. FLICA involve the exploitation of cell permeable, non-toxic specific peptides (in this case VAD-FMK) which bind specifically to activated caspases and are conjugated to a fluorochrome (Bedner, 2000). FITC-VAD-FMK was used to quantify apoptosis induction by SFV4 and rSFV-p62-6k VLPs in all three cell lines in this study. Viral antigen expression was also detected by employing double-labelling immunofluorescence concurrently with the above apoptosis assays.

Despite a relatively low infection efficiency it was found that K-BALB cells in culture are indeed susceptible to SFV4 and rSFV-p62-6k VLP cytopathic effect and efficiently express viral antigen as early as 6 h post infection. Furthermore, K-BALB cells were shown to undergo apoptosis upon infection with SFV4 or rSFV-p62-6k VLPs despite the overexpression of K-*ras*, indicating the potential of this virus and its derived vector as cytotoxic cancer therapy agents.

## 3.2 RESULTS

### 3.2.1 Growth of SFV4 in cell culture

BHK-21, BALB/3T3 and K-BALB cells were seeded into 24-well cell culture dishes at a concentration of 5 x $10^4$ cells/well and infected with SFV4 at a MOI of either 0.1 or 100 PFU/cell. Plates were incubated and supernatants were sampled over a five-day period for plaque assay as described in section 2.2.1.1 and viral growth curves plotted accordingly.

BHK-21 cells are used routinely in the laboratory to propagate SFV4 and the virus grew at the greatest speed and to the highest levels in this cell line. When cells were infected at a MOI of 0.1, viral titres increased in a linear fashion to a peak of 1.2 x $10^8$ PFU/ml at 48 h.p.i. before dropping to 6 x $10^7$ PFU/ml at 72 h.p.i. and continuing to fall to 4.3 x $10^5$ PFU/ml at 120 h.p.i.. Infection at the higher MOI of 100 resulted in an earlier peak in viral growth at 36 h.p.i. of 7 x $10^7$ PFU/ml followed by a linear drop in viral titre over time to 4 x $10^3$ PFU/ml at experiment end (Figure 3.1a). Monolayers were completely obliterated at 36 h.p.i. when infected at MOI = 100 and at 48 h.p.i. when infected at the lower MOI of 0.1 with no cells surviving past 48 h.p.i. in either case.

SFV4 grew to significantly lower levels in BALB/3T3 cells reaching a peak of 4.6 x $10^6$ PFU/ml at 72 h.p.i. when infected at a MOI of 0.1 before falling to 4.1 x $10^4$ PFU/ml at 120 h.p.i.. An earlier but lower peak in viral growth was observed when cells were infected at a MOI of 100 which reached 1.1 x $10^6$ PFU/ml at 36 h.p.i. and was followed by a drop in viral titres to 3.9 x $10^3$ PFU/ml at 96 h.p.i. (Figure 3.1b). Monolayers showed high levels of cytopathic effect and only occasional cells survived after 36 h.p.i. when infected at a MOI of 100. Cytopathic effect peaked at 72 h.p.i. when BALB/3T3 cells were infected at a MOI of 0.1 and some sparse cells were still seen to be surviving at 120 h.p.i..

K-BALB cells showed substantially higher levels of SFV4 production than BALB/3T3 cells when infected at a MOI of 0.1 which peaked at 72 h.p.i. at 5.1 x $10^7$ PFU/ml and dropped to 9.9 x $10^6$ at experiment end. Infection of K-BALB cells at a MOI of 100 resulted in an earlier and lower peak in SFV4 growth of 3.5 x $10^6$ PFU/ml at 36 h.p.i. and a more gradual decrease over time than that observed in BALB/3T3 cells to 3.9 x $10^3$ PFU/ml at 120 h.p.i. (Figure 3.1c). Again, cytopathic effect was observed to reach a peak at the same timepoint as SFV4 viral titre and infection with a MOI of 100

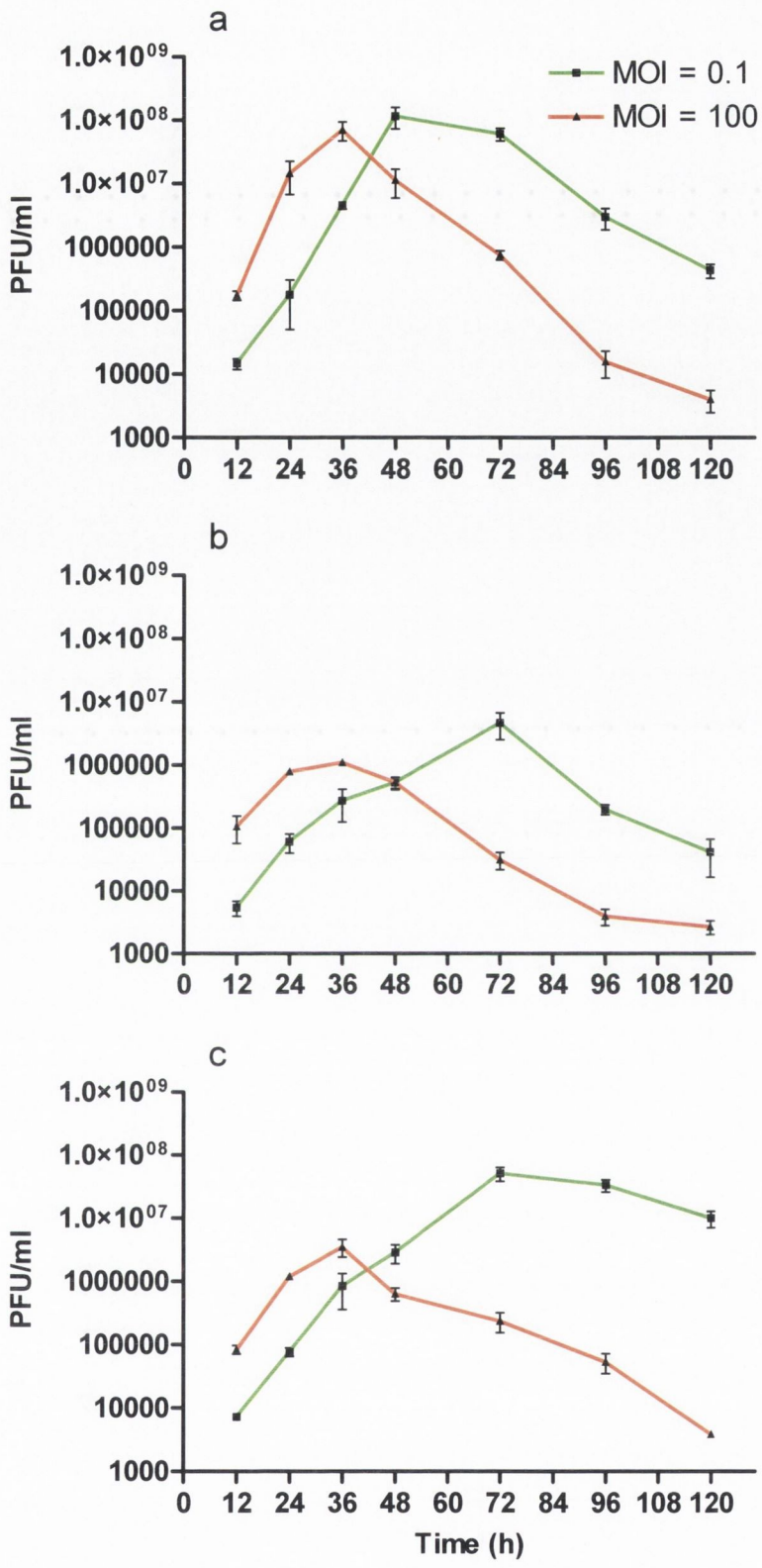


**Figure 3.1 Growth of SFV4 virus *in vitro*.** BHK-21 (a), BALB/3T3 (b), and K-BALB (c) cells were seeded into 24-well cell culture dishes at a concentration of 5 x $10^4$ cells/well and infected with SFV4 virus at a MOI of 0.1 or 100 PFU/cell. Supernatants were sampled over a five-day period and viral titres assessed by plaque assay. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments.

resulted in complete obliteration of the monolayers. A higher number of cells survived to 120 h.p.i. however than observed with BALB/3T3 infection when infected at the lower MOI of 0.1 and it is possible that these populations had become persistently infected as viral titres were maintained at higher levels from 72 h.p.i. onwards.

### 3.2.2 Infection efficiency of rSFV-p62-6k VLPs in cell lines

As different cell lines display varying infection efficiencies to viral vectors, it was necessary to assess the infection efficiency of K-BALB in comparison to its non-tumourigenic predecessor BALB/3T3 and the easily infectable BHK-21 cell lines. BHK-21, BALB/3T3 and K-BALB cells were seeded onto coverslips in 24-well cell culture dishes at a concentration of 5 x $10^3$ cells/well, infected with rSFV-p2-6k VLPs at increasing MOIs as described in section 2.2.1.2, and incubated for 18 h. Coverslips were then fixed and stained for SFV antigen, detected using immunofluorescence as described in section 2.1.2.5.3, allowing the percentage positive cells to be calculated for each MOI using DAPI as a counterstain (Figure 3.2).

BHK-21 cells were infected most efficiently with a clear linear relationship observed between the percentage of cells positive for SFV antigen and the MOI of rSFV-p62-6k VLPs used up to MOI = 100 at which point 73% of cells were positive. Infection efficiencies then began to reach maximum levels with 91% and 96% of cells positive at MOI = 500 and MOI = 1000 respectively.

BALB/3T3 and K-BALB cells displayed similar patterns of infection efficiencies to each other over the range of MOIs tested which were significantly lower than those observed with BHK-21 cells. Again, a linear relationship was observed between the MOI and percentage of SFV antigen-positive cells up to MOI = 100 where the percentage positive BALB/3T3 cells reached 32% and the number of positive K-BALB cells was slightly higher at 37%. The number of positive BALB/3T3 cells increased only slightly to 42% at MOI = 500 and 45% at MOI = 1000. Similarly, the number of positive K-BALB cells increased to 39% at MOI = 500 and 48% at MOI = 1000.

### 3.2.3 Growth and viability of cell lines following infection with rSFV-p62-6k VLPs and SFV4

BHK-21, BALB/3T3 and K-BALB cell lines were seeded at a concentration of 5 x $10^5$ cells/well in 6-well cell culture dishes and infected with MFI alone, rSFV-p62-6k

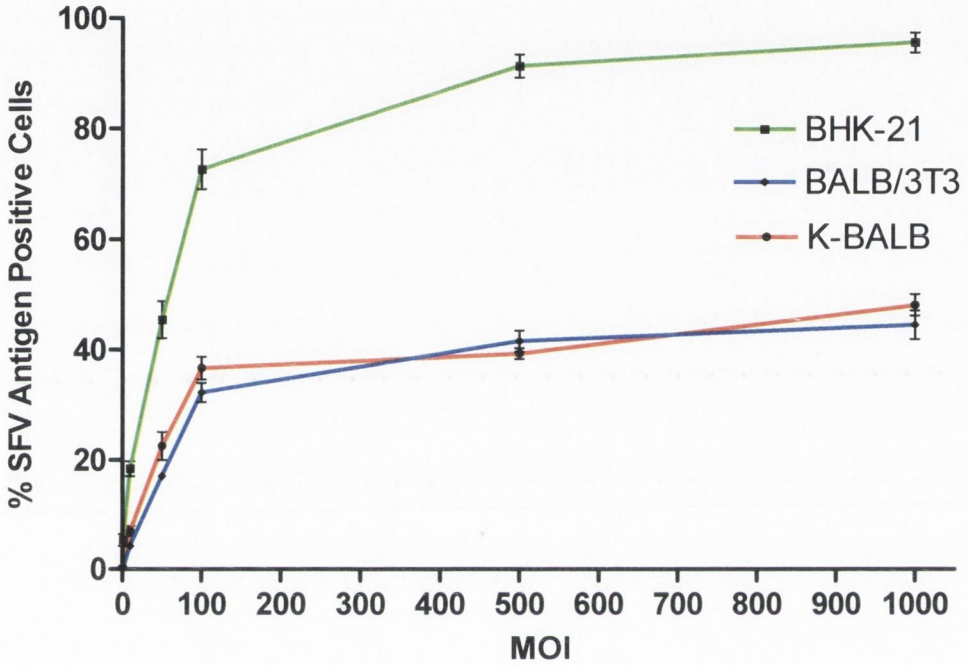


**Figure 3.2 Infection efficiency of rSFV-p62-6k VLPs in BHK-21, BALB/3T3 and K-BALB cells.** Cells were seeded onto 13 $mm^2$ coverslips in 24-well cell culture dishes and infected with rSFV-p62-6k VLPs at a variety of MOIs. After incubation for 18 h cells were fixed and expression of SFV antigen was detected by immunofluorescence. The percentage of cells positive for detectable SFV antigen was calculated from total cell numbers in each field obtained using DAPI as a nuclear counterstain. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

VLPs or SFV4 (MOI = 100) as described in section 2.2.2. Viability was assessed using the trypan blue exclusion method, and proliferation was quantified using [*methyl*-$^3$H] thymidine incorporation as described in sections 2.2.2.1 and 2.2.2.2 respectively. Statistical comparisons were performed using a two-way repeated measures ANOVA with Bonferroni post-tests. Results were considered statistically significant when $P < 0.05$.

Infected cells began to show early signs of cytopathic effect such as vacuolisation between 6 and 12 h.p.i. in all three cell lines and morphological changes associated with apoptosis were apparent by 18 h.p.i.. Cells were observed to round-up and nuclear and cellular shrinkage was noted. Between 36 and 48 h.p.i. BHK-21 monolayers infected with either rSFV-p62-6k VLPs or SFV4 were completely obliterated with only occasional surviving cells still adhering after this point in rSFV-p62-6k VLP infected wells. SFV4 infection of BALB/3T3 and K-BALB cells resulted in a similar effect but a low number of sparsely distributed cells survived to experiment end. The cytopathic effect of rSFV-p62-6k VLPs on BALB/3T3 and K-BALB cells peaked between 36 and 60 h.p.i. but healthy, apparently uninfected, cells remained and continued to proliferate which resulted in monolayer recovery by 120 h.p.i..

Numbers of viable BHK-21 cells dropped to 55% and 41% of controls at 12 h.p.i. when infected with rSFV-p62-6k VLP and SFV4 respectively ($P < 0.01$; $P< 0.001$). Viability was observed to drop steadily and reach levels which remained below 5% of controls from 48 h.p.i. onwards and showed no significant recovery ($P < 0.001$). The number of viable cells in control wells increased in a linear fashion over time up to 96 h.p.i. after which a drop in viability was observed (Figure 3.3a). The [*methyl*-$^3$H] thymidine incorporation assay concurred with these results (Figure 3.3b) showing a decrease in proliferation in the first 24 h.p.i. followed by no detectable proliferation in infected monolayers after this time. Control cells continued to proliferate for the duration of the experiment.

Because BALB/3T3 cells are contact inhibited (Aaronson & Todaro, 1968) and are slower growing than K-BALB and BHK-21 cells, numbers of cells in control wells peaked at 72 h.p.i. at only $1.7 \times 10^6$ viable cells/well compared with cell numbers of $3.4 \times 10^6$ and $4 \times 10^6$ viable cells/well for K-BALB and BHK-21 respectively at this timepoint. Similarly, control BALB/3T3 cells displayed the lowest levels of [$^3$H-*methyl*] thymidine incorporation out of the three cell lines which peaked at 72 h.p.i. and dropped after this point, unlike BHK-21 which continued to proliferate throughout the experiment. By 24 h.p.i. percentage viability in VLP infected wells had dropped to 36%

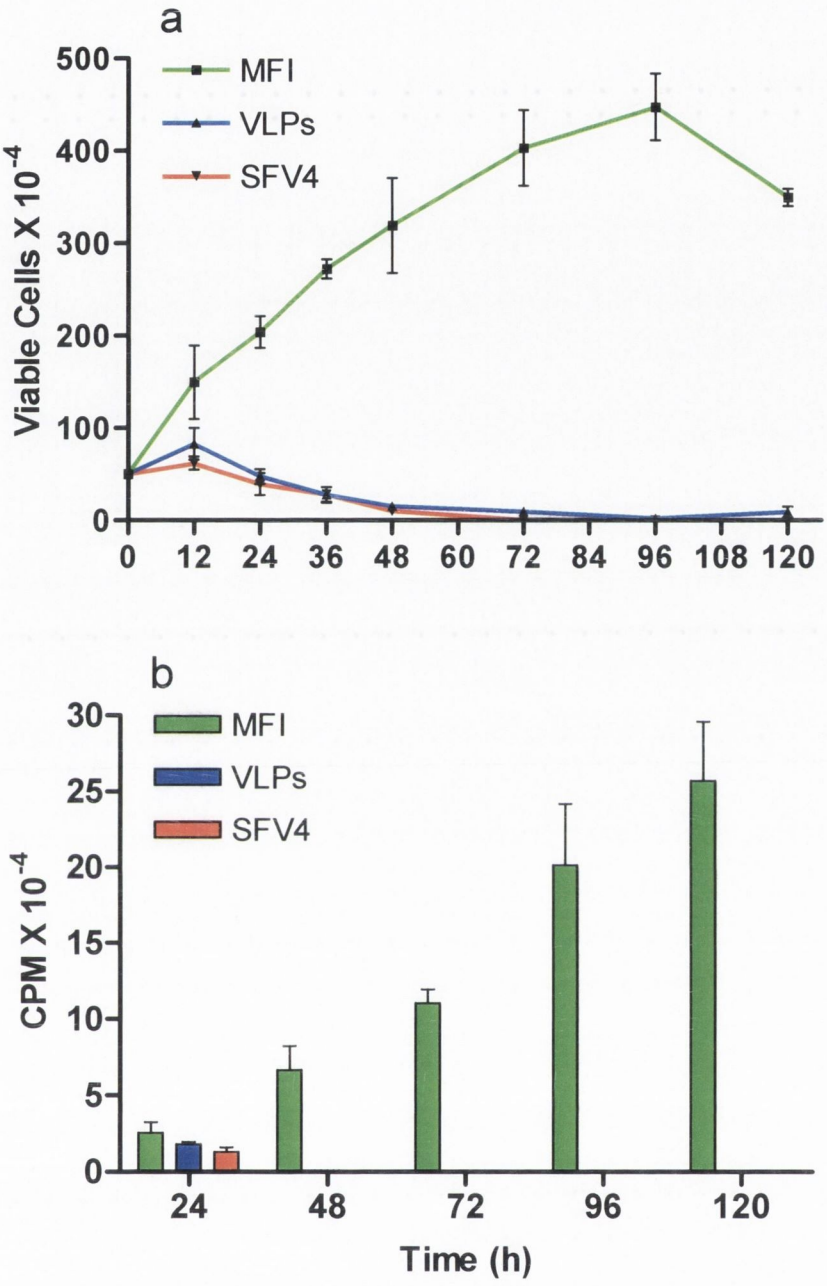


**Figure 3.3 Viability and proliferation of BHK-21 cells following infection with rSFV-p62-6k VLPs and SFV4, as determined by trypan blue exclusion and [$^3$H-*methyl*] thymidine incorporation.** BHK-21 cells were seeded into 6-well cell culture dishes at a concentration of 5 x $10^5$ cells/well and infected with MFI alone, rSFV-p62-6k VLPs or SFV4 (MOI = 100). Cells were sampled over a five-day period assessed for; viability using the trypan blue exclusion method (a) and proliferation employing [$^3$H-*methyl*] thymidine incorporation (b). *Points*; mean of three replicates, *columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

($P < 0.001$) but remained above 20% for the following 72 h ($P < 0.001$) after which monolayers began to recover reaching 68% of control wells by 120 h.p.i. ($P < 0.05$). SFV4 infection resulted in a similar drop in viability to 32% of controls at 24 h.p.i. ($P < 0.01$) which then continued to drop steadily over time to just above 2% at 72 h.p.i. ($P < 0.001$) after which some recovery was observed but was significantly lower than rSFV-p62-6k VLP infection ($P < 0.01$) (Figure 3.4a). Both rSFV-p62-6k and SFV4 infection resulted in proliferation dropping to almost undetectable levels between 24 and 48 h.p.i. with rSFV-p62-6k VLP infected monolayer proliferation increasing slowly to just below half that detected in control wells. Proliferation of SFV4 infected monolayers was shown to recover slightly from 48 h.p.i. but to a much lower and insignificant extent (Figure 3.4b).

Control K-BALB cells grew rapidly in a similar manner to BHK-21 cells for the initial 48 h.p.i. after which the viable cell numbers remained somewhat constant until experiment end. These cells showed significantly higher levels of [$^3$H-*methyl*] thymidine incorporation in the first 72 h.p.i. than BHK-21 and BALB/3T3 cells but proliferation dropped in a linear fashion after a peak at 48 h.p.i.. The viability of rSFV-p62-6k infected K-BALB monolayers dropped to just under 9% at 48 h.p.i. ($P < 0.001$) after which monolayers began to recover and reached 39% of control wells by 120 h.p.i. ($P < 0.01$). While this percentage recovery is lower than that observed in BALB/3T3 cells it is important to note at this point that this is due to the significantly lower number of control BALB/3T3 cells which had dropped in numbers from 72 h.p.i. onwards. K-BALB cells displayed the highest level of recovery in terms of cell numbers due to their more rapid rate of proliferation and lack of contact inhibition. SFV4 infected monolayer viability had dropped to 4% of control wells by 72 h.p.i. ($P < 0.001$) after which some recovery was observed which reached almost 7% of controls at 120 h.p.i. ($P < 0.001$) (Figure 3.5a). Infected monolayers showed a maximum inhibition of [$^3$H-*methyl*] thymidine incorporation between 48 and 72 h.p.i. ($P < 0.001$) after which rSFV-p62-6k infected monolayers began to recover to significant levels between 96 and 120 h.p.i. ($P < 0.01$). SFV4 infected monolayers displayed a much lower level of proliferation between 96 and 120 h.p.i. in comparison to that observed in monolayers infected with rSFV-p62-6k VLPs (Figure 3.5b).

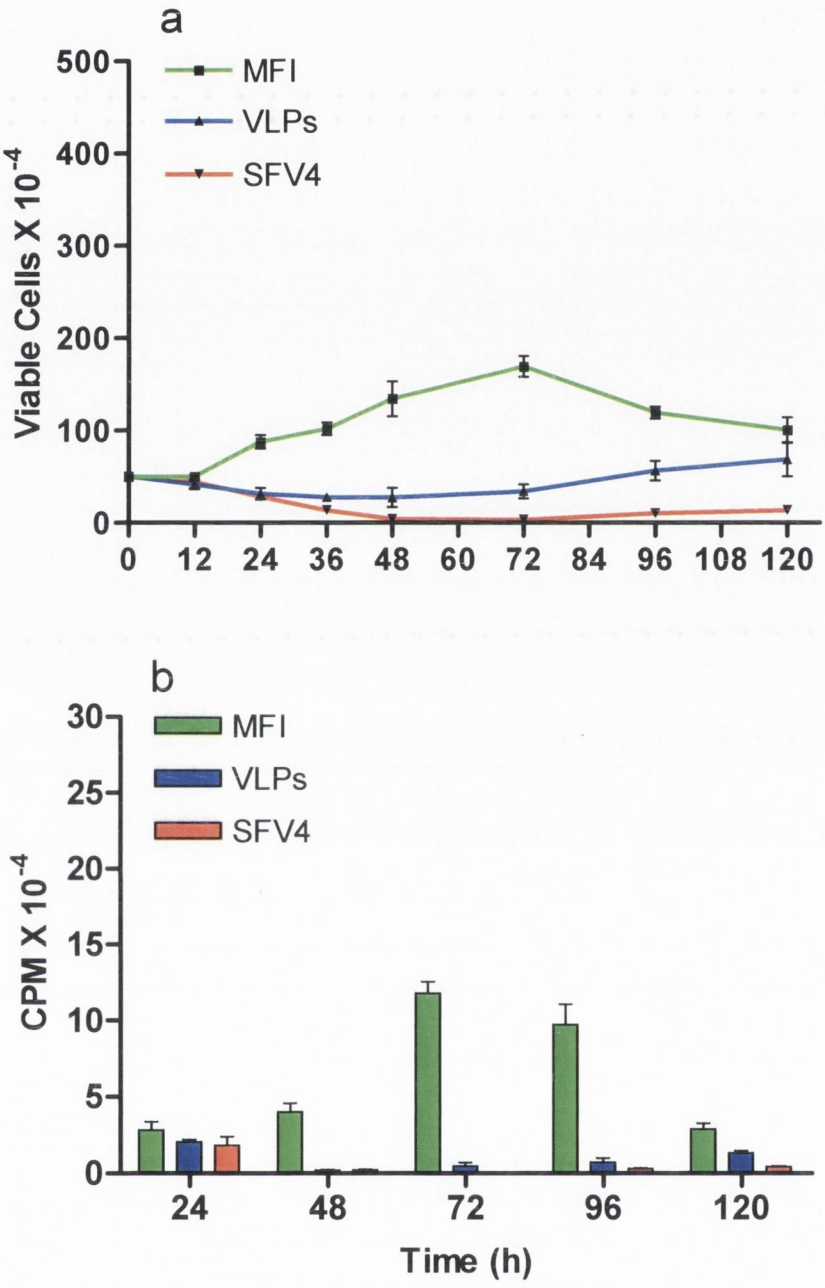


**Figure 3.4 Viability and proliferation of BALB/3T3 cells following infection with rSFV-p62-6k VLPs and SFV4, as determined by trypan blue exclusion and [$^3$H-*methyl*] thymidine incorporation.** BALB/3T3 cells were seeded into 6-well cell culture dishes at a concentration of 5 x $10^5$ cells/well and infected with MFI alone, rSFV-p62-6k VLPs or SFV4 (MOI = 100). Cells were sampled over a five-day period assessed for; viability using the trypan blue exclusion method (a) and proliferation employing [$^3$H-*methyl*] thymidine incorporation (b). *Points*; mean of three replicates, *columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

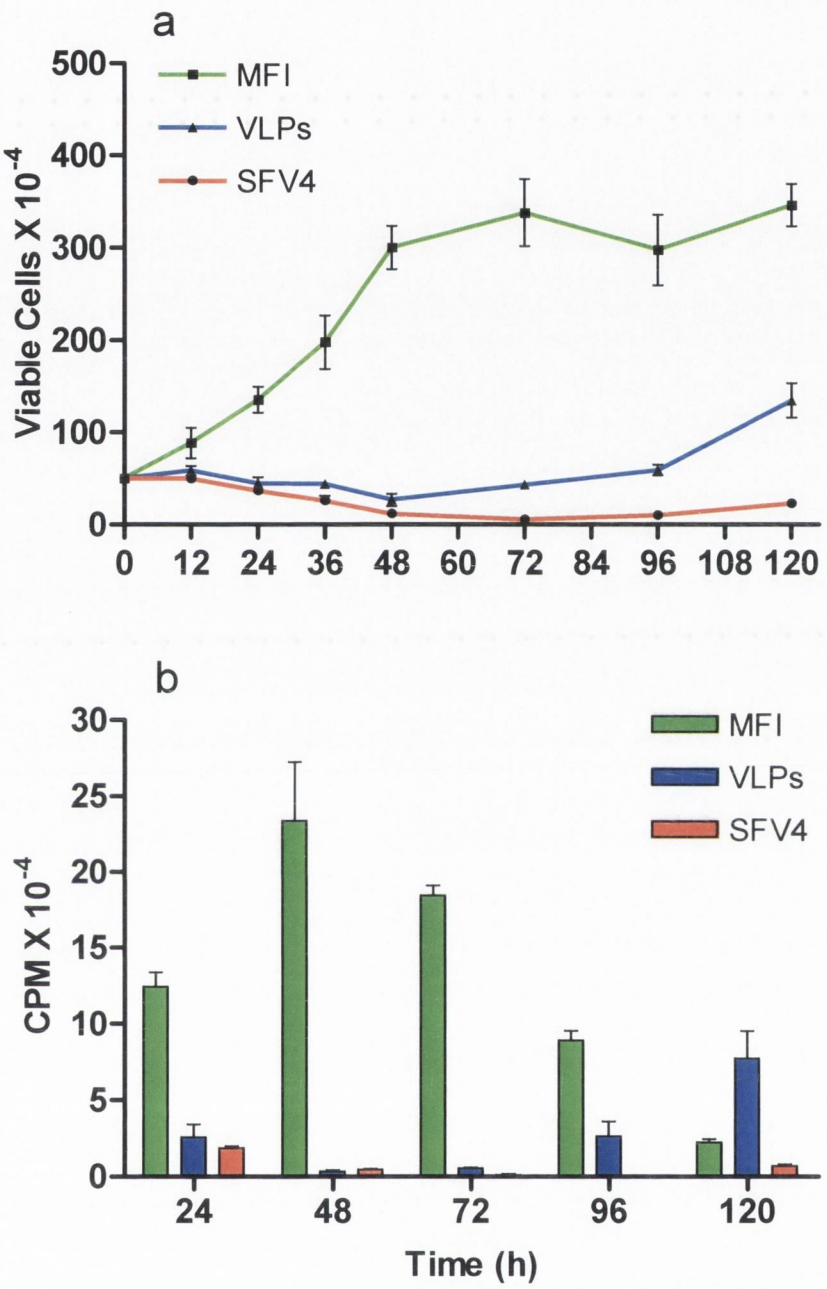


**Figure 3.5 Viability and proliferation of K-BALB cells following infection with rSFV-p62-6k VLPs and SFV4, as determined by trypan blue exclusion and [$^3$H-*methyl*] thymidine incorporation.** K-BALB cells were seeded into 6-well cell culture dishes at a concentration of 5 x $10^5$ cells/well and infected with MFI alone, rSFV-p62-6k VLPs or SFV4 (MOI = 100). Cells were sampled over a five-day period assessed for; viability using the trypan blue exclusion method (a) and proliferation employing [$^3$H-*methyl*] thymidine incorporation (b). *Points*; mean of three replicates, *columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

### 3.2.4 Induction of apoptosis and expression of SFV antigens in cells following infection with rSFV-p62-6k VLPs and SFV4

Two procedures involving fluorescent double labelling of individual cells were employed to examine the induction of apoptosis and expression of SFV antigens in the cell lines following infection with rSFV-p62-6k VLPs or SFV4.

#### 3.2.4.1 TUNEL with anti-active caspase-3 and anti-SFV4 immunofluorescence

BHK-21, BALB/3T3 and K-BALB cells were seeded into 6-well cell culture dishes at a concentration of 5 x $10^5$ cells/well and infected with MFI alone, rSFV-p62-6k VLPs or SFV4 (MOI = 100). Cells were sampled every 24 h and apoptosis detected by TUNEL (fluorescein) and anti-active caspase-3 (rhodamine) immunofluorescence as described in section 2.2.3.1. Cytospins of the same cell populations were also stained for expression of SFV antigen using immunofluorescence as described in section 2.1.2.5.3 and DAPI was employed as a counterstain to provide total cell counts and a method of assessing nuclear viability. Viable nuclei were well defined and easily distinguishable from condensed or fragmented non-viable nuclei (Figure 3.6). Active caspase-3 and SFV antigen positive cells (stained on different slides) showed bright red fluorescence in the cytoplasm and on the cell surface respectively. TUNEL positive nuclei displayed bright green fluorescence and anuclear 'ghost' cells were observed increasingly over time where cellular DNA had been completely degraded (Figure 3.7).

Statistical comparisons were performed on the results obtained using a two-way repeated measures ANOVA with Bonferroni post-tests. This method of statistical analysis allowed for all groups to be compared to eachother and yielded probability values for each individual timepoint. The following results are presented below: probability values comparing mock-infected controls to infected cultures of the same cell line (section 3.2.4.1.1); probability values comparing rSFV-p62-6k VLP infected cultures to SFV4 infected cultures of the same cell line (section 3.2.4.1.2); and probability values comparing rSFV-p62-6k VLP and also SFV4 infection between different cell lines (section 3.2.4.1.3). Results were considered to be statistically significant when $P < 0.05$.

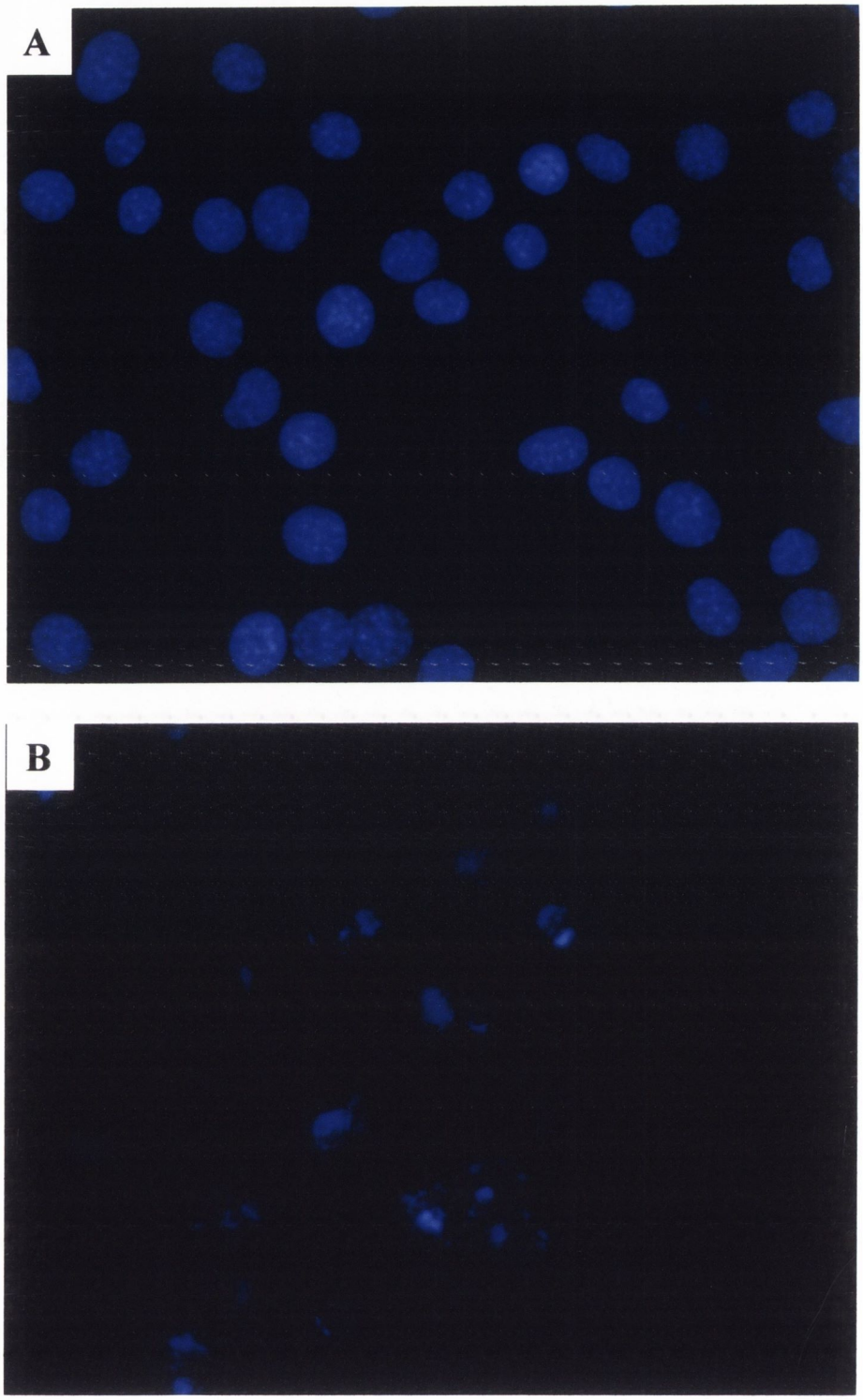


**Figure 3.6 Assessment of nuclear viability of K-BALB cells at 48 h.p.i. using the fluorescent nuclear stain DAPI**

**(A)** Mock-infected K-BALB cells displaying well-defined viable nuclei, 400x

**(B)** Condensed and fragmented nuclei/nuclear debris of K-BALB cells infected with SFV4, typical of apoptosis induction, 400x

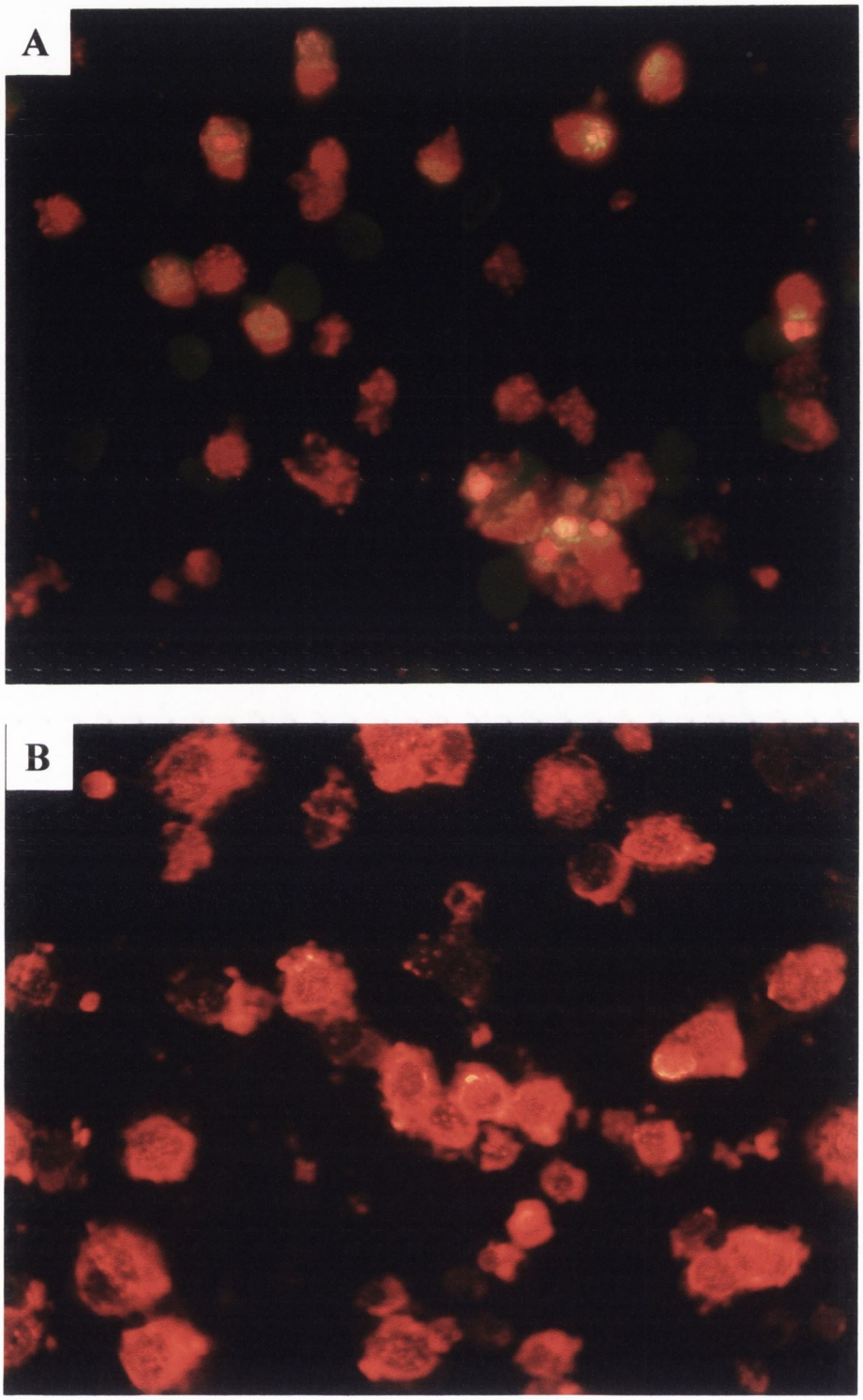


**Figure 3.7 Demonstration of apoptosis induction by TUNEL and immunofluorescent detection of active caspase-3 and SFV antigen in BHK-21 cells 48 h.p.i. with SFV4**

**(A)** TUNEL positive condensed nuclei (green/yellow) in BHK-21 cells also positive for active caspase-3 (red), 400x

**(B)** SFV antigen (red) in the cytoplasm and on the surface of infected cells, 400x

#### 3.2.4.1.1 Nuclear viability, activation of caspase-3, TUNEL and SFV antigen expression in individual cell lines

BHK-21 cells were shown to be highly susceptible to rSFV-p62-6k VLP and SFV4-induced apoptosis and showed a high level of SFV antigen expression upon infection. Mock-infected control cells showed a low-level of background apoptosis which rose gradually over the course of the experiment and was accompanied by an expected gradual drop in nuclear viability. rSFV-p62-6k VLP infection resulted in a drop of 14% in nuclear viability in the first 24 h followed by a steeper drop from 86% to 36% at 48 h.p.i. ($P < 0.001$) after which a steady fall is observed to just above 2% viable nuclei at 120 h.p.i. ($P < 0.001$). Expression of SFV antigen had reached 53% at 24 h.p.i. ($P < 0.001$) and rose to 78% by 48 h.p.i. ($P < 0.001$) after which it remained somewhat constant. Apoptosis induction was apparent with an increase in activation of caspase-3 before a significant increase in TUNEL was observed. The numbers of active caspase-3 positive cells peaked at 67% at 48 h.p.i. ($P < 0.001$) and decreased slowly to 56% at 120 h.p.i. ($P < 0.001$) whereas the levels of TUNEL positive cells did not reach significant levels until 48 h.p.i. and subsequently peaked at 48% at 72 h.p.i. ($P < 0.001$) before decreasing to 30% at experiment end ($P < 0.001$). Infection of BHK-21 cells with SFV4 resulted in a more substantial effect with numbers of SFV antigen positive cells above 95% from 48 h.p.i. onwards ($P < 0.001$). Activation of caspase-3 peaked at 48 h.p.i. with 74% of cells positive and this level was essentially maintained until experiment end ($P < 0.001$). The numbers of TUNEL positive cells were not significant until 48 h.p.i. and appeared to peak above 55% sometime between 48 and 72 h.p.i. ($P < 0.001$) and subsequently drop to 44% at 120 h.p.i. ($P < 0.001$) (Figure 3.8).

BALB/3T3 cells were also shown to undergo apoptosis and express SFV antigen upon infection with rSFV-p62-6k VLPs or SFV4 but to a lower extent than that which was observed in the BHK-21 cell line. Mock-infected cells showed a low level of background apoptosis increasing gradually over time and an accompanying drop in nuclear viability to a similar level of that observed in BHK-21 cells. Infection with rSFV-p62-6k VLPs resulted in a rapid and linear drop in viability to 40% at 48 h.p.i. ($P < 0.001$) followed by a gradual recovery to 58% at experiment end ($P < 0.001$). This was reflected in expression of viral antigen and apoptosis induction with a peak in SFV antigen and active caspase-3 positive cells at 48 h.p.i. of 74% and 63% respectively ($P < 0.001$ ; $P < 0.001$) followed by a drop over time to 35% and 22% respectively at 120 h.p.i. ($P < 0.001$ ; $P < 0.001$). The number of TUNEL positive cells was not significant

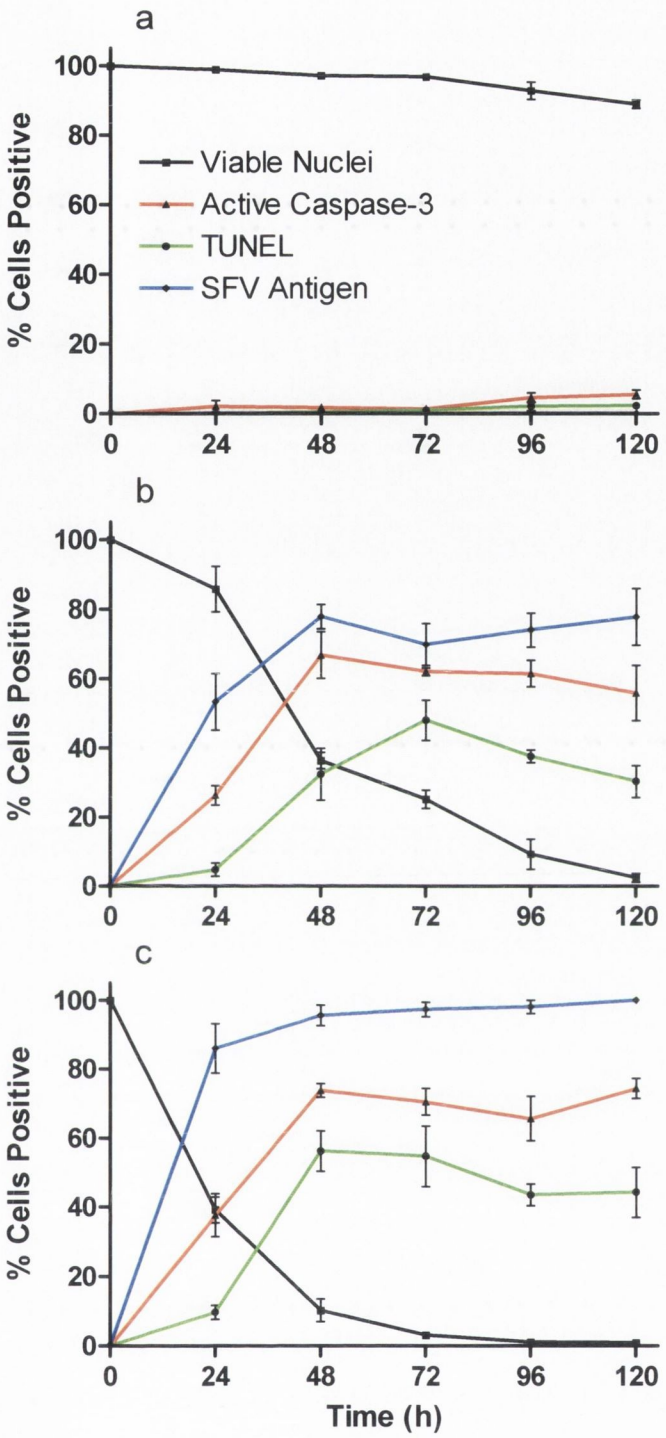


**Figure 3.8 Apoptosis induction and expression of SFV antigen in BHK-21 cells infected with rSFV-p62-6k VLPs and SFV4, as determined by nuclear viability, TUNEL and immunofluorescent detection of active caspase 3 and SFV antigen.** BHK-21 cells were seeded into 6-well cell culture dishes at a concentration of 5 x $10^5$ cells/well and infected with MFI alone (a), rSFV-p62-6k VLPs (b) or SFV4 (c) at a MOI of 100. Cells were sampled over a five-day period and cytospins were assessed for; nuclear viability using DAPI, DNA fragmentation using the TUNEL assay, activation of caspase 3 using immunofluorescence and expression of SFV antigens using immunofluorescence. Positive cells were counted and expressed as a percentage of the total number of cells in each field. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments.

until 48 h.p.i. and peaked 24 h later at 72 h.p.i. to 36% ($P < 0.001$) before subsequently falling to 9% at experiment end ($P > 0.05$). SFV4 infection resulted in a steady drop in nuclear viability over time to just above 1% at 120 h.p.i. ($P < 0.001$). SFV antigen expression reached 75% by 24 h.p.i. ($P < 0.001$) and remained above 90% from 72 h.p.i. onwards. Apoptosis induction was less sustained than that observed in SFV4 infected BHK-21 cells and peaked at 67% at 48 h.p.i. ($P < 0.01$) for active caspase-3 before dropping to 49% at experiment end ($P < 0.001$) and TUNEL positive cell numbers were not significant until 48 h.p.i. after which they peaked at 55% at 72 h.p.i. ($P < 0.001$) before dropping to 28% at 120 h.p.i. ($P < 0.001$) (Figure 3.9).

K-BALB cells were also susceptible to apoptosis induction when infected with rSFV-p62-6k VLPs or SFV4 but the effect was delayed and observed at a lower extent than in BHK-21 and BALB/3T3 cell lines. Mock-infected cells showed a low level of background which appeared earlier than in the other two cell lines but remained constantly below 5% for the duration of the experiment. rSFV-p62-6k VLP infection resulted in a rapid and linear drop in nuclear viability to 39% at 48 h.p.i. ($P < 0.001$) which was followed by a linear increase in viability that reached 88% at 120 h.p.i. ($P > 0.05$). Unlike the previous two cell lines, a statistically significant increase in caspase-3 activation was not observed until 48 h.p.i. at which point the number of positive cells peaked at 53% ($P < 0.001$) before dropping again to 11% at experiment end ($P > 0.05$). SFV antigen expression peaked at 48 h.p.i. at 73% ($P < 0.001$) and fell to 15% by 120 h.p.i. ($P < 0.05$). The number of TUNEL positive cells did not reach significant levels until 48 h.p.i. and peaked 24 h later reaching 42% at 72 h.p.i. ($P < 0.001$) before dropping to 9% by 120 h.p.i. ($P > 0.05$). Infection with SFV4 resulted in a drop in nuclear viability resembling a sigmoidal curve and reaching 7% at 120 h.p.i. ($P < 0.001$). At 24 h.p.i. the number of SFV antigen positive cells was 75% and remained above 87% for the duration of the experiment ($P < 0.001$). Cells positive for active caspase-3 were detected in significant numbers from 24 h.p.i. and increased in a linear fashion to 54% at 48 h.p.i. ($P < 0.001$) after which they remained somewhat constant from this point on. Again, numbers of TUNEL positive cells were insignificant until 48 h.p.i. and subsequently peaked at 72 h.p.i. to 38% ($P < 0.001$) before dropping to 19% at experiment end ($P < 0.05$) (Figure 3.10).

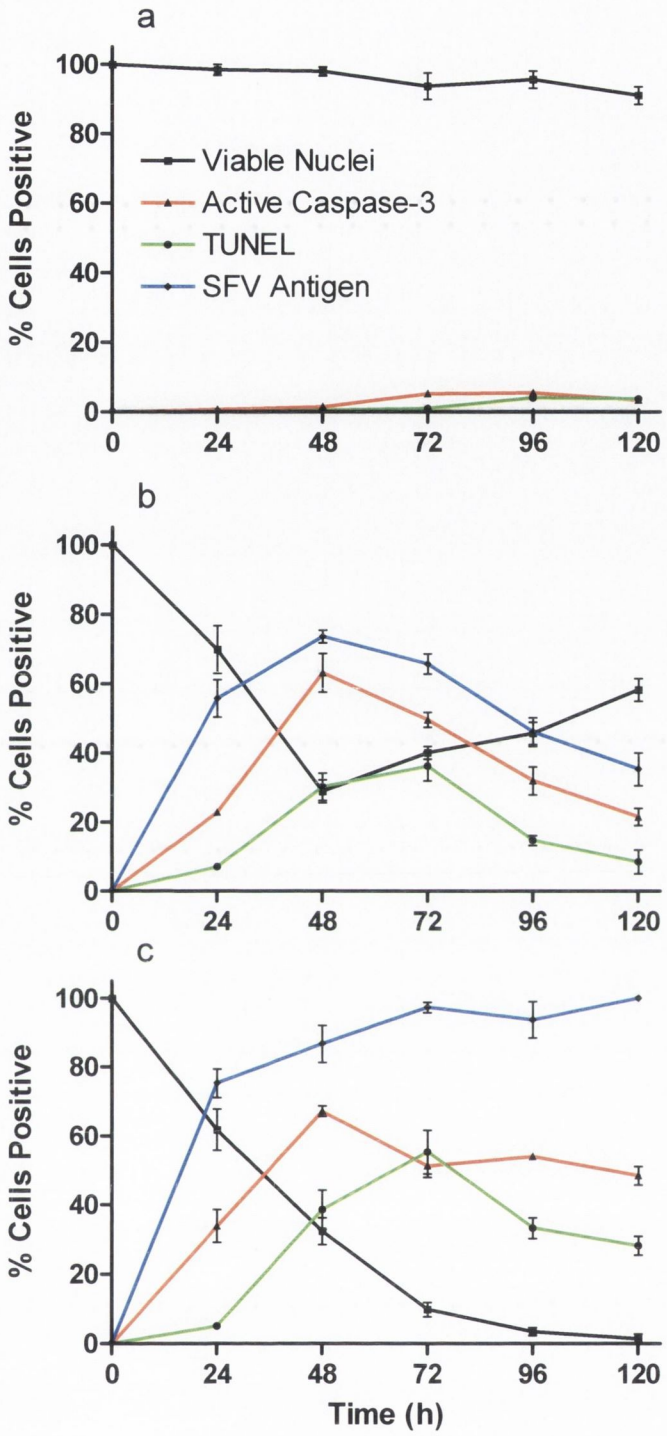


**Figure 3.9 Apoptosis induction and expression of SFV antigen in BALB/3T3 cells infected with rSFV-p62-6k VLPs and SFV4, as determined by nuclear viability, TUNEL and immunofluorescent detection of active caspase 3 and SFV antigen.** BALB/3T3 cells were seeded into 6-well cell culture dishes at a concentration of 5 x $10^5$ cells/well and infected with MFI alone (a), rSFV-p62-6k VLPs (b) or SFV4 (c) at a MOI of 100. Cells were sampled over a five-day period and cytospins were assessed for; nuclear viability using DAPI, DNA fragmentation using the TUNEL assay, activation of caspase 3 using immunofluorescence and expression of SFV antigens using immunofluorescence. Positive cells were counted and expressed as a percentage of the total number of cells in each field. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments.

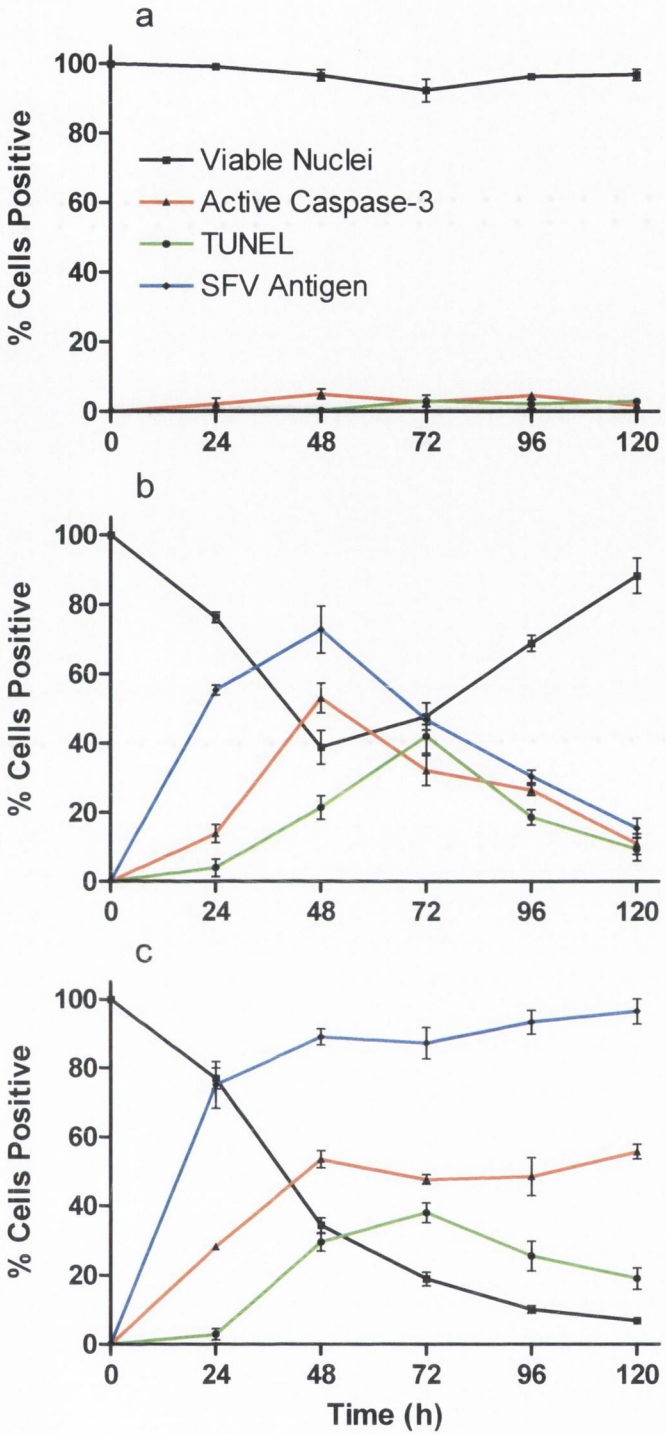


**Figure 3.10 Apoptosis induction and expression of SFV antigen in K-BALB cells infected with rSFV-p62-6k VLPs and SFV4, as determined by nuclear viability, TUNEL and immunofluorescent detection of active caspase 3 and SFV antigen.** K-BALB cells were seeded into 6-well cell culture dishes at a concentration of 5 x $10^5$ cells/well and infected with MFI alone (a), rSFV-p62-6k VLPs (b) or SFV4 (c) at a MOI of 100. Cells were sampled over a five-day period and cytospins were assessed for; nuclear viability using DAPI, DNA fragmentation using the TUNEL assay, activation of caspase 3 using immunofluorescence and expression of SFV antigens using immunofluorescence. Positive cells were counted and expressed as a percentage of the total number of cells in each field. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments.

#### 3.2.4.1.2 Apoptosis induction and SFV antigen expression by cells infected with rSFV-p62-6k VLPs compared to cells infected with SFV4

Infection of BHK-21 cells with SFV4 resulted in a significantly higher loss of nuclear viability in the first 72 h.p.i. ($P < 0.01$) than that observed in BHK-21 cells infected with rSFV-p62-6k VLPs. By 96 h.p.i. however, the difference between the viability of the two infected cultures was not statistically significant and remained so for the duration of the experiment ($P > 0.05$). In contrast to this, BALB/3T3 and K-BALB cells showed no significant difference in the nuclear viability of cells infected with rSFV-p62-6k VLPs and those infected with SFV4 until 72 h.p.i. ($P < 0.001$). This extremely significant difference was maintained until experiment end as monolayers infected with rSFV-p62-6k recovered, in contrast to SFV4 infected cells.

No significant difference in caspase-3 activation between cells infected with rSFV-p62-6k VLPs and those infected with SFV4 was observed in BHK-21 cells until 120 h.p.i. ($P < 0.05$). BALB/3T3 cells showed and maintained a significantly higher level of cells positive for active caspase-3 in cultures infected with SFV4 from 96 h.p.i. ($P < 0.01$). The findings were similar for K-BALB cells, where this significance had increased somewhat by 120 h.p.i. ($P < 0.001$). Few significant differences were observed in the numbers of TUNEL positive cells infected with rSFV-p62-6k VLPs and those infected with SFV4.

The numbers of cells expressing detectable SFV antigen was significantly higher in cells infected with SFV4 for all three cell lines. This was less significant in BHK-21 cells which were more efficiently infectable with rSFV-p62-6k VLPs and was most significant in K-BALB cells. Overall, SFV4 infection resulted in a more substantial and sustained induction of apoptosis and expression of SFV antigen in all three cell lines when compared to rSFV-p62-6k VLP infection but particularly in the BALB/3T3 and K-BALB cell lines.

#### 3.2.4.1.3 Comparison of apoptosis induction and SFV antigen expression between cell lines

BHK-21 and BALB/3T3 cell lines showed no significant difference in nuclear viability until 96 h.p.i. ($P < 0.001$) when infected with rSFV-p62-6k VLPs and this was the case with active caspase-3 ($P < 0.001$), TUNEL ($P < 0.05$), and SFV antigen expression ($P < 0.01$) with BHK-21 cells being more susceptible to the cytopathic effect

of the VLPs. When infected with SFV4 these cell lines showed no significant difference in numbers of TUNEL positive cells or SFV antigen expression at any time point. A significantly higher loss in nuclear viability was observed in BHK-21 cells in the first 48 h.p.i. ($P < 0.01$) but both cells lines succumbed to infection after this time. Regarding caspase-3 activation, BHK-21 cells showed higher levels at 72 h.p.i. ($P < 0.05$) and at 120 h.p.i. ($P < 0.01$).

No significant difference in nuclear viability was observed in BHK-21 or K-BALB cells infected with rSFV-p62-6k VLPs until 72 h.p.i. ($P < 0.01$). This significance increased at 96 h.p.i. ($P < 0.001$) and was maintained until experiment end as K-BALB monolayers recovered from infection, in contrast to BHK-21 monolayers. Although no significant difference was observed in the numbers of TUNEL positive cells between these cell lines, a significantly higher level of caspase activation was observed in BHK-21 cells from 72 h.p.i. ($P < 0.001$). Similarly, a significant difference in detectable SFV antigen expression was not observed until 72 h.p.i. ($P < 0.05$) which was sustained at a higher level in BHK-21 cells until experiment end ($P < 0.001$). When infected with SFV4, BHK-21 and K-BALB cell lines showed no significant difference in SFV antigen expression but a significantly higher loss in nuclear viability was observed in BHK-21 cells in the first 48 h.p.i. ($P < 0.001$) before both cell lines succumbed to SFV4 infection. From 48 h.p.i. ($P < 0.05$) onwards BHK-21 cells showed higher levels of caspase-3 activation and at 48 h.p.i. ($P < 0.01$) and 120 h.p.i. ($P < 0.01$) regarding numbers of TUNEL positive cells.

K-BALB and BALB/3T3 cell lines displayed a significant difference in nuclear viability from 96 h.p.i. to experiment end ($P < 0.001$) when infected with rSFV-p62-6k VLPs as K-BALB cells have a higher proliferation rate which accommodated a faster recovery from infection. No significant difference was observed in numbers of TUNEL positive cells at any timepoint and not until 120 h.p.i. for caspase-3 activation ($P < 0.001$) and detectable SFV antigen expression ($P < 0.05$) both of which were higher in BALB/3T3 cells. Upon infection with SFV4, no significant difference between K-BALB and BALB/3T3 cell lines was observed at any timepoint in any assay.

### 3.2.4.2 FLICA and anti-SFV4 immunofluorescence

BHK-21, BALB/3T3 and K-BALB cells were seeded at a concentration of 5 x $10^4$ cells/well in 24-well cell culture dishes and infected with either MFI alone, rSFV-p62-6k VLPs or SFV4 (MOI = 100). Cells were sampled at 6, 12, 24, 36, 48 and 72

h.p.i. at which point they were incubated with FITC pan-caspase FLICA, spun onto slides and labelled for SFV antigen using immunofluorescence as described in section 2.2.3.2. Cells positive for active caspases displayed bright green fluorescence in the cytoplasm at all time points and localised in the nuclei at early time points. SFV antigen expression was observed as bright red fluorescence on the cell surface (Figure 3.11). As with the previous experiment (section 3.2.4.1), low levels of background fluorescence were observed in all control mock-infected cultures and remained below 4% in all cases.

Statistical comparisons were performed using a two-way repeated measures ANOVA with Bonferroni post-tests which yielded: probability values comparing mock-infected controls to infected cultures of the same cell line (section 3.2.4.2.1); probability values comparing rSFV-p62-6k VLP infected cultures to SFV4 infected cultures of the same cell line (section 3.2.4.2.2); and probability values comparing rSFV-p62-6k VLP and also SFV4 infection between different cell lines (section 3.2.4.2.3). Results were considered to be statistically significant when $P < 0.05$.

#### 3.2.4.2.1 Caspase activation and expression of SFV antigen in individual cell lines

Upon infection with rSFV-p62-6k VLPs, BHK-21 cells showed an initial rise in caspase positive cells of 13% at 6 h.p.i. ($P < 0.05$) which increased to 28% at 24 h.p.i. ($P < 0.001$), peaked at 54% at 48 h.p.i. ($P < 0.001$) and decreased to 47% at 72 h.p.i. ($P < 0.001$). SFV antigen positive cells increased over time to 83% at 36 h.p.i. ($P < 0.001$) and subsequently fell slightly to 77% at 72 h.p.i. ($P < 0.001$). Infection with SFV4 resulted in a higher and faster increase in caspase positive cells at earlier timepoints which peaked at 56% at 48 h.p.i. ($P < 0.001$) and remained essentially constant through to experiment end ($P < 0.001$). SFV antigen positive cells were also detected in higher numbers and at earlier timepoints than in VLP infected cultures with 66% of cells positive as early as 6 h.p.i. ($P < 0.001$). Over 95% of cells were positive from 36 h.p.i. onwards ($P < 0.001$) (Figure 3.12).

BALB/3T3 cells infected with rSFV-p62-6k VLPs showed a gradual increase in active caspase positive cells to 20% at 24 h.p.i. ($P < 0.001$) followed by a steeper increase to a peak of 39% at 48 h.p.i. ($P < 0.001$) followed by a decrease to 33% at 72 h.p.i. ($P < 0.001$). SFV antigen expression peaked at 77% at 36 h.p.i. ($P < 0.001$) and then gradually fell to 60% at experiment end ($P < 0.001$). SFV4 infection showed a similar pattern of caspase activation to VLP infection which peaked at 48 h.p.i. at 46% ($P< 0.001$) and dropped to 35% at 72 h.p.i. ($P < 0.001$). SFV antigen expression reached

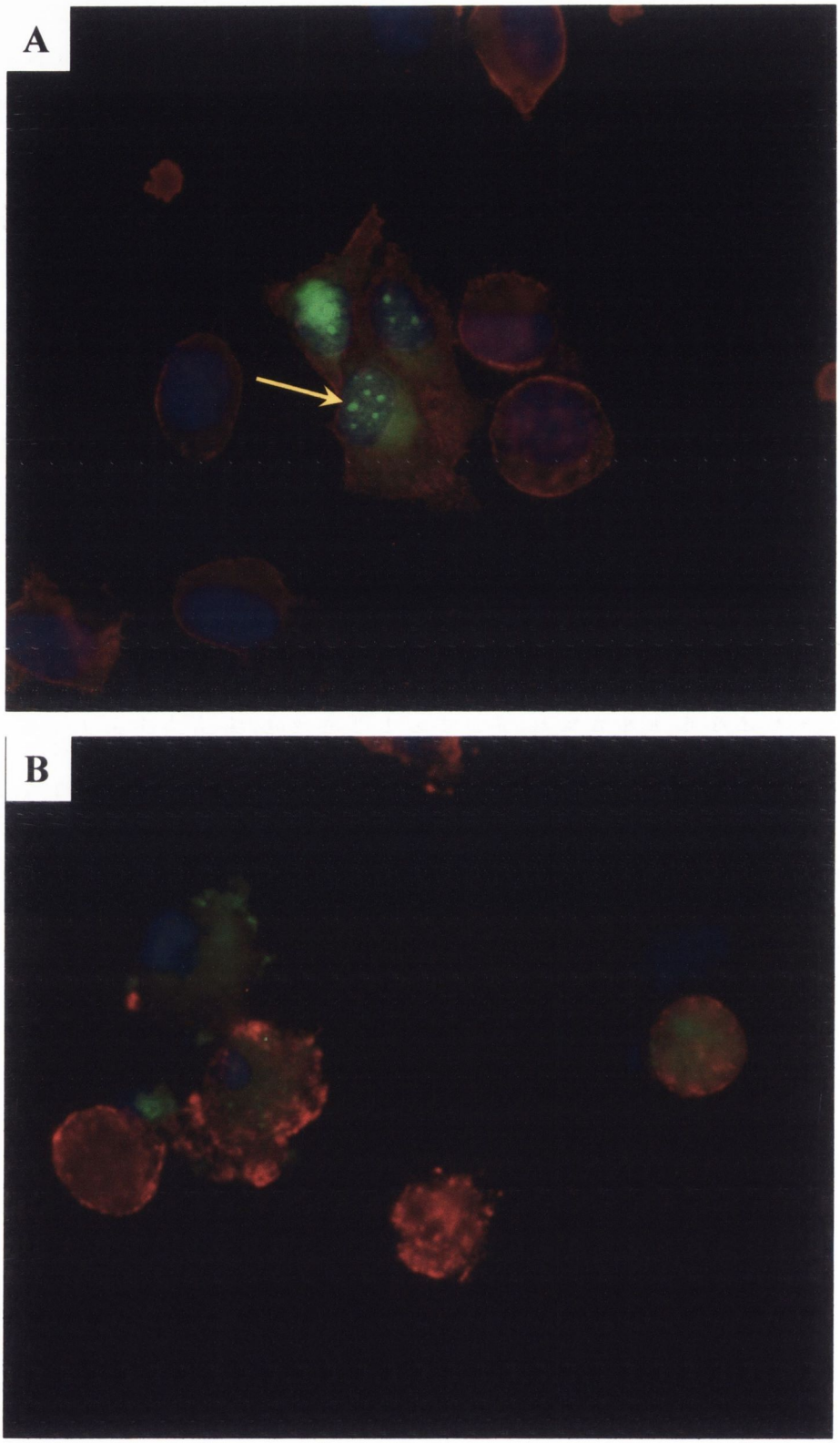


**Figure 3.11 Detection of active caspases by FLICA (green) and SFV antigen by immunofluorescence (red) in K-BALB cells following infection with rSFV-p62-6k VLPs**

**(A)** Colocalisation of active caspases with apparently viable nuclei (blue) of infected cells at 6 h.p.i. (*arrow*), 800x

**(B)** Cell shrinkage and membrane blebbing apparent in K-BALB cells at 48 h.p.i. with rSFV-p62-6k VLPs, 800x

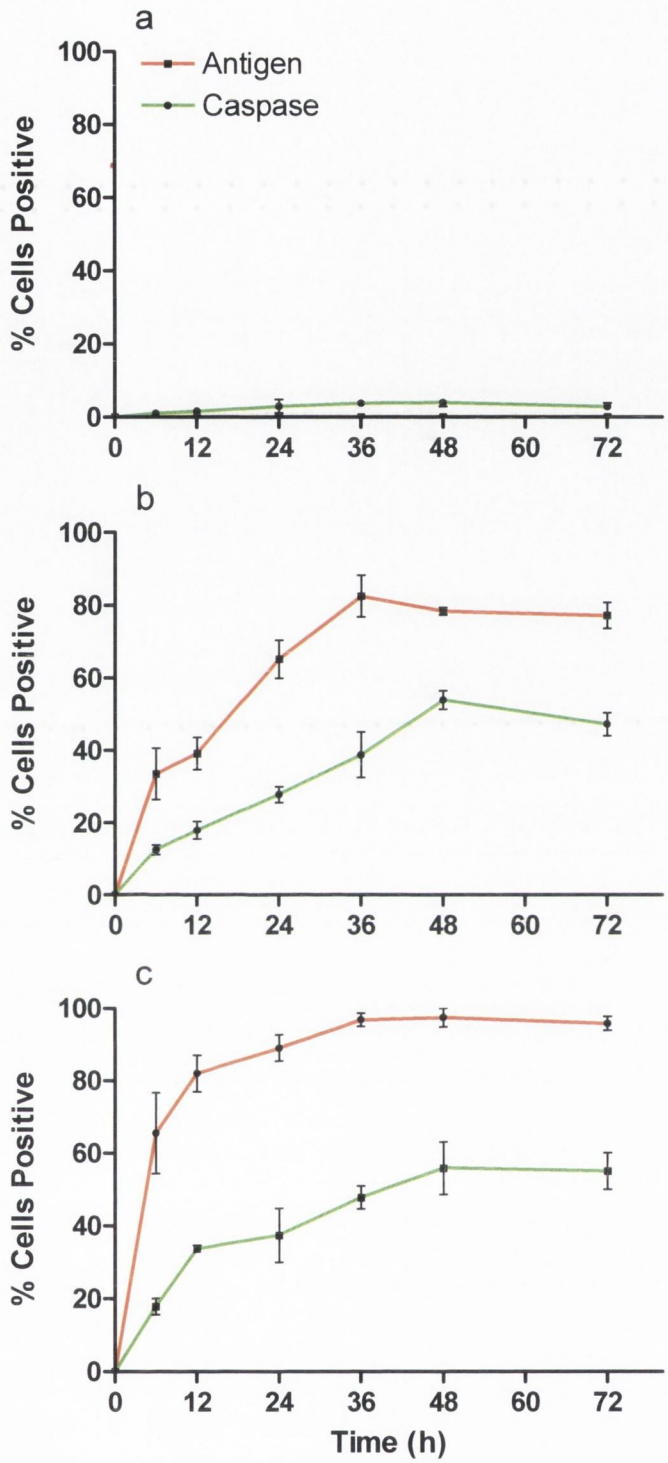


**Figure 3.12 Apoptosis induction and expression of SFV antigen in BHK-21 cells infected with rSFV-p62-6k VLPs and SFV4, as determined by FLICA and immunofluorescence respectively.** BHK-21 cells were seeded into 24-well cell culture dishes at a concentration of 5 x $10^4$ cells/well and infected with MFI alone (a), rSFV-p62-6k VLPs (b) or SFV4 (c) at a MOI of 100. Cells were sampled over a three-day period and assessed for activation of caspases using FLICA and for SFV antigen expression using immunofluorescence with DAPI as a nuclear counterstain. Positive cells were counted and expressed as a percentage of the total number of cells in each field. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments.

89% by 24 h.p.i. ($P < 0.001$) and remained above 95% from 48 h.p.i. onwards ($P < 0.001$) (Figure 3.13).

rSFV-p62-6k infected K-BALB cells did not show a significant increase in caspase activation until 36 h.p.i. and showed a peak of 43% at 48 h.p.i. ($P < 0.001$) which then dropped to 22% at 72 h.p.i. ($P < 0.001$). SFV antigen expression was observed to increase in a linear fashion to 87% at 36 h.p.i. after which the number of cells expressing SFV antigen fell to 50% at experiment end ($P < 0.001$). SFV4 infection resulted in higher levels of antigen expression with 58% of cells positive for SFV antigen at 6 h.p.i. ($P < 0.001$) and above 85% positive from 12 h.p.i. onwards ($P < 0.001$). Significant numbers of active caspase positive cells were observed as early as 6 h.p.i. with 13% of cells positive ($P < 0.05$) after which numbers reached 37% at 36 h.p.i. ($P < 0.001$) and remained somewhat constant after this time (Figure 3.14).

#### 3.2.4.2.2 Caspase activation and expression of SFV antigen in cells infected with rSFV-p62-6k VLPs compared with cells infected with SFV4

A significantly higher level of antigen expression was observed in all three cell lines when infected with SFV4 as opposed to rSFV-p62-6k VLPs but this was not reflected to the same level in the numbers of cells positive for active caspases. BHK-21 cells displayed significantly higher levels cells positive for active caspases at 12 h.p.i. ($P < 0.01$) and BALB/3T3 at 12 and 48 h.p.i. ($P < 0.05$) only. K-BALB cells infected with SFV4 showed significantly higher levels of caspase positive cells at 48 ($P < 0.05$) and 72 h.p.i. ($P < 0.01$) when compared to the same cell line infected with rSFV-p62-6k VLPs.

#### 3.2.4.2.3 Comparison of caspase activation and SFV antigen expression between cell lines

Significant differences between BHK-21 and BALB/3T3 cells infected with rSFV-p62/6k VLPs were only observed at 72 h.p.i. ($P < 0.05$) for detectable SFV antigen expressing cells and from 48 h.p.i. onwards ($P < 0.01$) for cells positive for active caspases, with BHK-21 cells displaying higher levels in both cases. Antigen expression in these two cell lines was similar when infected with SFV4 and the only significant difference was observed at 6 h.p.i. ($P < 0.05$). Caspase activation was higher, however, in BHK-21 cells and significantly so at 12, 14, 36 ($P < 0.05$) and 72 h.p.i. ($P < 0.001$).

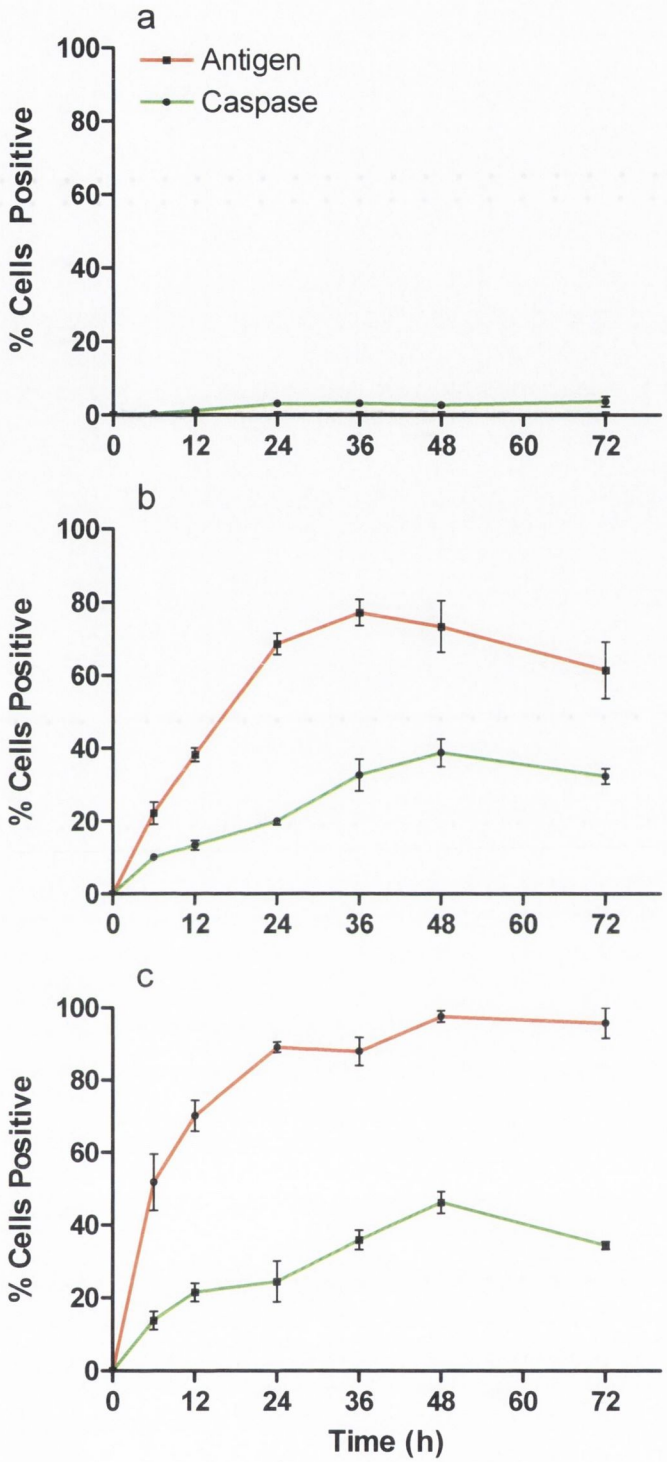


**Figure 3.13 Apoptosis induction and expression of SFV antigen in BALB/3T3 cells infected with rSFV-p62-6k VLPs and SFV4, as determined by FLICA and immunofluorescence respectively.** BALB/3T3 cells were seeded into 24-well cell culture dishes at a concentration of 5 x $10^4$ cells/well and infected with MFI alone (a), rSFV-p62-6k VLPs (b) or SFV4 (c) at a MOI of 100. Cells were sampled over a three-day period and assessed for activation of caspases using FLICA and for SFV antigen expression using immunofluorescence with DAPI as a nuclear counterstain. Positive cells were counted and expressed as a percentage of the total number of cells in each field. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments.

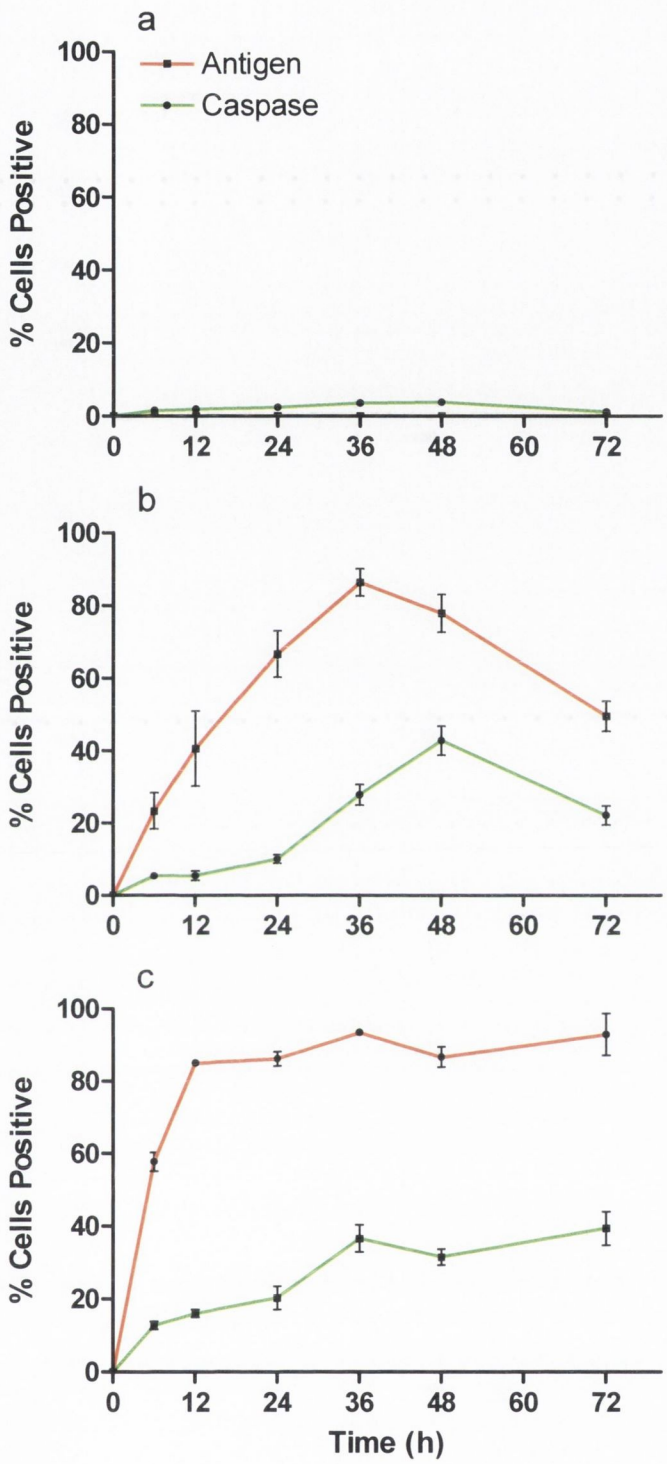


**Figure 3.14 Apoptosis induction and expression of SFV antigen in K-BALB cells infected with rSFV-p62-6k VLPs and SFV4, as determined by FLICA and immunofluorescence respectively.** K-BALB cells were seeded into 24-well cell culture dishes at a concentration of 5 x $10^4$ cells/well and infected with MFI alone (a), rSFV-p62-6k VLPs (b) or SFV4 (c) at a MOI of 100. Cells were sampled over a three-day period and assessed for activation of caspases using FLICA and for SFV antigen expression using immunofluorescence with DAPI as a nuclear counterstain. Positive cells were counted and expressed as a percentage of the total number of cells in each field. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments.

BHK-21 and K-BALB cells showed no significant difference in antigen expression levels until 72 h.p.i. ($P < 0.001$) when infected with rSFV-p62-6k VLPs as K-BALB monolayers began to recover from infection and BHK-21 monolayers did not. The numbers of BHK-21 cells positive for active caspases were significantly higher from 12 h.p.i. ($P < 0.05$) and this significance was maintained through to experiment end ($P < 0.001$). No significant difference was observed in detectable SFV antigen expression when cells were infected with SFV4. BHK-21 cells displayed a significantly higher level of caspase activation from 12 h.p.i. and this significance was sustained to experiment end ($P < 0.001$).

No significant difference was observed between K-BALB and BALB/3T3 cell lines when infected with rSFV-p62-6k VLPs and very few significant differences were observed between these two cell lines when infected with SFV4 (SFV antigen 12 h.p.i. ($P < 0.05$); active caspases 48 h.p.i. ($P < 0.001$)).

## 3.3 DISCUSSION

Cells often undergo apoptosis in response to viral infection in what is widely believed to be an attempt to abrogate both viral replication and spread within the host (Everett & McFadden, 1999). Several viruses have evolved mechanisms of preventing or delaying apoptosis induction in order to allow completion of their replication cycle and/or establish a state of persistent infection in a cell population. Apoptosis can also be exploited by viruses, however, and many induce it either upon infection or at a later stage in their lifecycle as a means of infecting neighbouring cells and avoiding host immune responses (Hay & Kannourakis, 2002; Teodoro & Branton, 1997).

The ability of alphaviruses to induce apoptosis in mammalian cell culture is well established and has been directly correlated with their pathogenicity *in vivo* (Fazakerley, 2002; Lewis *et al.*, 1996). It is this inherent capacity to induce apoptosis which makes the SFV vector system attractive as a tumour therapy agent. While the structural proteins of SV have been shown to induce apoptosis (Joe *et al.*, 1998), the structural proteins of SFV are not required for apoptosis induction (Glasgow *et al.*, 1998; Murphy *et al.*, 2000), although it is possible that they could enhance cytopathic effect. This allows for other therapeutic genes to be cloned into the SFV vector system to compliment its 'built-in' apoptosis induction mechanism (Murphy *et al.*, 2001). rSFV VLPs encoding the SFV protein E2 (rSFV-p62-6k) which has B and T cell epitopes (Snijders *et al.*, 1992, Strauss & Strauss, 1994) were employed in an attempt to augment the antitumoural effect of rSFV VLPs by attracting an immune response directed at infected cells. The potential for a replicating SFV vector as a tumour therapy agent was also investigated using the replication proficient infectious clone SFV4 as a model.

K-BALB cells, which express oncogenic K-*ras*, are the mouse tumour model employed in this study and form rapidly growing syngenic tumours in immunocompetent BALB/c mice upon s.c. injection (Aaronson & Weaver, 1971; Stephenson & Aaronson, 1972). The relationship between oncogenic Ras and apoptosis is complicated and not clearly understood. Ras proteins act as binary switches at nodes in several signalling transduction pathways and the correlation between oncogenic Ras and uncontrolled cellular proliferation is well defined. It would appear, however, that its influence on a cell's destiny to undergo apoptosis is less definitive with oncogenic Ras either protecting from, or sensitising cells to, apoptosis. Cell type plays a major role in the effects of Ras on apoptosis with studies indicating that activated Ras promotes apoptosis in fibroblasts

and lymphocytes but has a protective effect on epithelial cells and myeloid cells (Arber, 1999; Cox & Der, 2003; Downward, 1998).

Prior to undertaking *in vivo* studies it was necessary to assess the ability of rSFV-p62-6k VLPs and SFV4 to successfully infect the K-BALB cell line and to examine the effect of infection on cellular proliferation and viability. Given the association of activated K-*ras* with increased resistance/susceptibility to apoptosis, the induction of apoptosis by rSFV-p62-6k VLPs and SFV4 was also examined along with the expression of SFV antigen in infected cells. As the non-tumourigenic predecessor of K-BALB, the BALB/3T3 cell line was included in all experiments as a control of the same cell type in which oncogenic K-*ras* was not expressed. BHK-21 cells are known to be highly susceptible to SFV infection and the associated induction of apoptosis (Glasgow *et al.*, 1997) and so were employed as a positive control in the assays in this chapter.

The growth of SFV4 in the cell lines over a five-day period was examined and as expected the virus grew at the fastest rate and to the highest titres in the BHK-21 cell line. The virus grew to significantly lower levels in BALB/3T3 and K-BALB cells and a more delayed peak in viral titre was observed when infected at the low MOI of 0.1 PFU/cell than in BHK-21 cells. This was most probably due to a lower infection efficiency of these cell lines which would increase the time taken for the cell population to succumb to infection. K-BALB cells have a higher proliferation rate than BALB/3T3 and this could account for the higher peak in SFV4 replication at 72 h.p.i. as well as the higher number of cells surviving to experiment end. It is possible for K-BALB cell cultures to become persistently infected with SFV4 (Glasgow, 1986; unpublished data) and given the relatively high titres of SFV4 detected from 72 h.p.i. onwards in cultures infected at a MOI of 0.1 PFU/cell and the presence of apparently healthy cells, this could indeed be the case. Although SFV4 grew more efficiently in cultures of K-BALB cells than in BALB/3T3 cells, it is more likely that this was due to the higher proliferation rate of uninfected cells than any resistance to SFV-associated cytopathic effect conferred by the overexpression of K-*ras*. The constant proliferation of uninfected cells would provide a supply of healthy cells for the virus to infect and replicate in, delaying a complete abolition of the monolayer to SFV4 infection as observed with BHK-21 and BALB/3T3 and the subsequent drop in viral titres.

The infection efficiency of rSFV-p62-6k VLPs was then assessed by infecting the three cell lines at a variety of MOIs and detecting SFV antigen expression by immunofluorescence. Over 90% of BHK-21 cells were positive for SFV antigen at MOIs of 500 IU/cell and above whereas BALB/3T3 and K-BALB were not as easily

transduced with just below 50% of cells positive when infected at a MOI of 1000 IU/cell. Very little difference was observed between the infection efficiencies of BALB/3T3 and K-BALB cells which ruled out any influence activation of K-*ras* might have on the infectability of the cell lines. This low infection efficiency is likely to also hinder SFV4 infection of cells and so correlated with the results obtained from SFV4 growth curves in the cell lines discussed above.

Infection with rSFV-p62-6k VLPs and SFV4 resulted in a rapid inhibition in cellular viability and proliferation in all three cell lines as determined by trypan blue exclusion and [$^{3}$H-*methyl*] thymidine incorporation respectively. Despite their low infection efficiencies, BALB/3T3 and K-BALB monolayers were more susceptible to rSFV-p62-6k VLP-induced cytopathic effect than expected. This phenomenon could be due to hypothetical events such as the transfer of viral RNA to neighbouring cells or uptake of apoptotic bodies containing viral RNA by neighbouring cells, thus creating an *in vitro* 'bystander' effect. It is also feasible that monolayers are more efficiently infected than the sparsely distributed populations of cells studied in the infection efficiency assay. BALB/3T3 and K-BALB monolayers recovered from rSFV-p62-6k VLP infection by 120 h.p.i. as infected cells died off and the remaining uninfected cells proliferated. BHK-21 monolayers were completely obliterated by rSFV-p62-6k VLP and SFV4 infection again underlining the susceptibility of this cell line to SFV infection and its high infection efficiency.

All three cell lines presented morphological changes associated with apoptosis such as rounding up of cells, cell shrinkage, chromatin condensation and membrane blebbing. It was, however, necessary to confirm the mechanism of cell death and in doing so it was also possible to compare the sensitivity and effectiveness of three methods of detecting apoptosis at a cellular level: immunofluorescent detection of active caspase-3, TUNEL labelling of fragmented DNA and FLICA detection of active caspases. Expression of SFV antigen by infected cells was also examined alongside these assays by immunofluorescence. From the results of these assays it was possible to conclude that the main mechanism of cell death observed in all three cell lines was indeed apoptosis and that each cell line was also efficiently expressing SFV antigen upon infection with either rSFV-p62-6k VLPs or SFV4.

Examining caspase-3 activation and TUNEL assays it was found that BHK-21 cells were the most susceptible to SFV-induced apoptosis and so were effective in their role as positive control for these experiments. A similar pattern was observed to that in the viability and proliferation assays discussed above with BHK-21 cells showing a

sustained effect in both rSFV-p62-6k VLP and SFV4 infection, an effect which was only seen in BALB/3T3 and K-BALB cells infected with SFV4. Both K-BALB and BALB/3T3 cells displayed a peak and fall in apoptosis induction and SFV antigen expression which was mirrored by a loss and gain in nuclear viability when infected with rSFV-p62-6k VLPs. The numbers of cells positive for SFV antigen were higher than expected, given the relatively low transfection efficiencies of these cell lines. This could be attributed to the presence of an *in vitro* bystander effect as discussed above. Cytopathic effects and antigen expression consistently peaked between 36 and 72 h.p.i. with cells expressing antigen at different stages despite the fact that all cells were infected at the same timepoint. The mitotic status of individual cells at the time of infection may influence this and provide a possible explanation for the time differences observed in antigen expression and apoptosis induction between cells from the same population.

In all cases TUNEL labelling tended to peak 24 h later than caspase activation which concurs with the activation of caspases prior to DNA fragmentation. Active effector caspases have been implicated in the cleavage of proteins such as PARP and ICAD into inactive subunits which allows for the degradation of DNA (Lazebnik *et al.*, 1994; Enari *et al.*, 1998). The relatively low levels of TUNEL positive cells observed in all cases can be attributed to the appearance of anuclear 'ghost' cells at later timepoints in which cellular DNA had been completely degraded. Such cells/debris remained positive for active caspases and SFV antigen.

In most assays K-BALB cells displayed lower numbers of apoptosis-positive cells than BHK-21 and BALB/3T3 cells. In the FLICA assay specifically it appeared that rSFV-p62-6k VLP-induced apoptosis was delayed as a significant increase in positive cells was not observed until 36 h.p.i., 12 h later than in BALB/3T3 cells. While it is tempting to speculate a possible role for activated K-*ras* in this delayed and lower level of apoptosis, it is important to consider that this may have resulted from the faster proliferation rate of uninfected cells. This was the case with rSFV-p62-6k VLP infected monolayers but with SFV4 infection an almost complete obliteration of all three cell lines was observed and very little difference is observed in the numbers of cells positive for apoptosis induction between the BALB/3T3 and K-BALB cell lines. It can be concluded that while it is possible that activation of K-*ras* may delay rSFV-p62-6k VLP-induced apoptosis it does not confer resistance to it in the fibroblastic cell line K-BALB and appears to have very little influence on induction of apoptosis by SFV4.

The FLICA assay generated similar results but the numbers of cells positive for active caspases were substantially lower than those detected utilising an anti-active caspase-3 antibody. This was surprising as a pan-caspase FLICA was employed whereas the immunofluorescent technique was restricted to a single effector caspase. The most likely explanation for the increased sensitivity of the immunofluorescent assay is that the immunofluorescent technique utilises a biotin-avidin amplification step. This allows four avidin-fluorescein molecules to bind to each biotin molecule of the biotinylated antibodies which have bound indirectly to active caspase-3 whereas each FLICA molecule only binds one FITC molecule to each active caspase molecule. The FLICA assay did, however, provide a better method of localising active caspases within cellular compartments over time. Active caspases were observed in the cytoplasm but also concentrated in the nuclei prior to nuclear condensation. This translocation of active caspases to the nucleus has been well documented in the literature (Benchoua *et al.*, 2002; Ramuz *et al.*, 2003; Zhivotovsky *et al.*, 1999) and indicates that FLICA detection by confocal microscopy could provide a relatively simple method of tracking active caspases within cells.

SFV4 infection resulted in a faster and more sustained expression of SFV antigen and induction of apoptosis in all three cell lines than that observed with rSFV-p62-6k VLP infection. SFV4, being replication proficient, can overcome the low infection efficiency of BALB/3T3 and K-BALB cell lines whereas rSFV-p62-6k VLPs cannot as they undergo only one round of replication. Although this was not a quantitative assay in terms of amount of SFV antigen expressed, it was also clear that SFV4 infected cells were expressing higher amounts of antigen than cells infected with rSFV-p62-6k VLPs. It also needs to be considered that SFV4 infection results in the expression of the full compliment of SFV antigens including the E1 glycoprotein which rSFV-p62-6k VLPs do not encode.

In conclusion, both rSFV-p62-6k VLPs and SFV4 were shown to successfully infect K-BALB cells and induce apoptosis as well SFV antigen expression *in vitro*. Although the cells displayed a relatively low transfection efficiency upon infection with rSFV-p62-6k VLPs, monolayers still displayed high levels of cytopathic effect before recovery. SFV4 infection resulted in a more sustained cytopathic effect and expression of SFV antigen due to the fact that it is replication proficient. Overall, this provides good justification for assessing the potential of rSFV-p62-6k VLPs and SFV4 as tumour therapy agents in the K-BALB mouse tumour model.

# 4

# TREATMENT OF K-BALB TUMOURS *IN VIVO* WITH rSFV-p62-6k VLPs

## 4.1 INTRODUCTION

The precedence of activating mutations in *ras* oncogenes in human malignancies, particularly K-*ras*, and their apparent essential role in tumour maintenance (Bos, 1989; Chin *et al.*, 1999), has stimulated a large amount of anticancer research targeted either at Ras directly, or at the various signalling pathways in which it plays an important role (Adjei, 2001). There is evidence supporting Ras proteins as having multiple, and sometimes seemingly contradictory, roles as regulators of cellular proliferation, differentiation, apoptosis and angiogenesis which can also differ depending on cell type (Downward, 1998; Rak *et al.*, 2000; Shields *et al.*, 2000). In chapter 3, the susceptibility of the murine tumourigenic cell line K-BALB (which overexpresses the K-*ras* oncogene) to SFV-induced apoptosis was demonstrated, providing a foundation for the premise that a possible antitumoural effect would be observed upon treating K-BALB tumours with rSFV VLPs *in vivo*.

A previous study has successfully exploited the inherent ability of rSFV VLPs to induce p53-independent apoptosis in the treatment of H358a human lung carcinoma xenografts in BALB/c *nu/nu* mice (Murphy *et* al., 2000). An antitumoural effect also observed in xenografts of the rat prostate cancer cell line AT3-*bcl*-2, was enhanced with the use of rSFV VLPs expressing the pro-apoptotic gene *bax*, but was not as effective as that observed in the slower growing H358a xenografts (Murphy *et al.*, 2001). K-BALB cells form rapidly growing syngeneic tumours in immunocompetent BALB/c mice upon s.c. injection (Stephenson and Aaronson, 1972) and in order to enhance any antitumour effect observed due to rSFV VLP cytotoxicity it was decided to recruit the host immune system by way of expression of the SFV antigen E2. E2 is a highly antigenic envelope protein of SFV which is known to contain both B- and T-cell epitopes and is encoded by the p62 gene of SFV along with the E3 protein (Snijders *et al.*, 1992; Strauss & Strauss, 1994). Several plasmids with specific deletions have been produced from the pSP6-SFV4 infectious clone of SFV (Barth and Garoff, 1997). pSFV-p62-6k contains deletions of the capsid and E1 genes, and is used in this study to express the E2 protein and hence stimulate immunity.

In this chapter, the ability of rSFV-p62-6k VLPs to inhibit the growth of s.c. K-BALB tumours and elicit anti-SFV immune response by expression of the E2 antigen was assessed in BALB/c *nu/nu* and immunocompetent BALB/c mice. Groups of mice were also vaccinated prior to tumour induction with rSFV-p62-6k VLPs in an attempt to enhance any anti-SFV immune response generated. Anti-SFV humoural and cell-

mediated immune responses were assessed by ELISA and *ex vivo* splenocyte stimulation assays respectively. BALB/c *nu/nu* mice are athymic and therefore cannot generate a normal mature T-lymphocyte population and subsequently mount significant cell-mediated immune responses or humoural immune responses requiring $CD4^+$ helper T cells (Flanagan, 1966; Pantelouris, 1968). Hence, BALB/c *nu/nu* groups acted as controls in this study where no anti-SFV immune response was detected and any inhibition of tumour growth observed was most probably solely due to the cytopathic effect of the rSFV-p62-6k VLPs.

H&E staining of paraffin-embedded formalin fixed tissue sections is the most widely used stain for routine histopathology. Haematoxylin is a basic stain which preferentially reacts with nucleic acids and acidic groups of proteins and eosin is an acidic stain which is employed in H&E staining as a pinkish red cytoplasmic counterstain. In this study, H&E staining of paraffin-embedded formalin fixed tumour sections was used for the routine examination of tumour tissue. A significant inhibition of K-BALB tumour growth was observed upon treatment with rSFV-p62-6k VLPs in immunocompetent BALB/c mice. Strong inflammatory immune responses were apparent in treated tumours 1 day after initiation of treatment in immunised groups but not until day 5 in naïve groups, although the difference overall between naïve and immunised groups was not significant. BALB/c *nu/nu* groups displayed some inhibition of tumour growth but this was found to be insignificant. The results presented here clearly demonstrate that expression of SFV antigen enhances the cytotoxic effect of rSFV VLPs on K-BALB tumours *in vivo* and highlight the future potential for such immunotherapeutic strategies.

## 4.2 RESULTS

### 4.2.1 Growth of K-BALB tumours *in vivo*

Prior to initiating studies assessing the treatment of K-BALB tumours *in vivo* it was necessary to examine the ability of K-BALB cells to form s.c. tumours in BALB/c mice and to examine the normal growth rate and pathology of these tumours. K-BALB tumours were induced in BALB/c mice and sampled over time for pathological studies as described in section 2.3.2.1. Separate tumours were also measured using a linear callipers and average tumour diameter was plotted against time until tumours reached an average diameter of 20 mm, at which point mice were euthanised (Figure 4.1). Tumours were detectable 24 h post induction and grew exponentially over the following 20 days by which time they had reached an average diameter of 20 mm and mice were euthanised.

Pathological examination of formalin-fixed H&E stained sections of K-BALB tumours revealed highly cellular tumours with microvascular proliferation and invasion of local muscular tissue. At 3 days post formation, tumours were composed of sheets of fibroblastic 'spindle' cells with well-defined cell borders similar in morphology to K-BALB cells *in vitro*. At later timepoints tumour cells began to regress to a less-differentiated and more densely packed polyhedral phenotype and by day 9, tumours were composed of pale cells with finely vacuolated cytoplasm and hyperchromatic nuclei with coarsely clumped chromatin and multiple nucleoli (Figure 4.2). Mitotic figures and binucleated forms were common throughout and pleomorphic tumour cells containing multiple nuclei were occasionally observed. Variable-sized areas of necrosis and haemorrhage were apparent in most tumours and included fibrin thrombi with aggregates of neutrophils mostly located at the interface with the viable tumour cells (Figure 4.3a). Small numbers of neutrophils were loosely distributed in the surrounding connective tissue stroma and were only observed infiltrating the tumour tissue at the necrotic regions mentioned above. Occasional lymphocytes and macrophages were also noted in some perivascular and perineural areas but no significant immune response to the K-BALB tumours was apparent pathologically (Figure 4.3b).

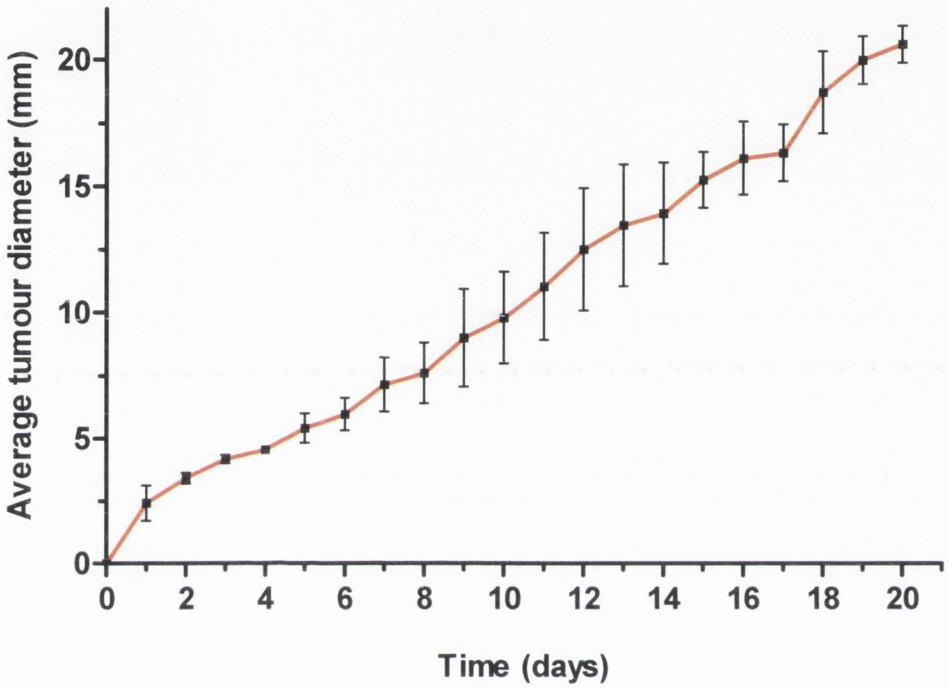


**Figure 4.1 K-BALB tumour formation *in vivo*.**

Three female BALB/c mice received a 100 µl s.c. injection of K-BALB cells in non-supplemented DMEM at a concentration of 1 x $10^7$ cells/ml. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements, assuming spherical shape. Mice were euthanised when tumour average diameter reached 20 mm. *Points*; mean of three replicates, *bars*; +/- SEM. Results are representative of a single experiment.

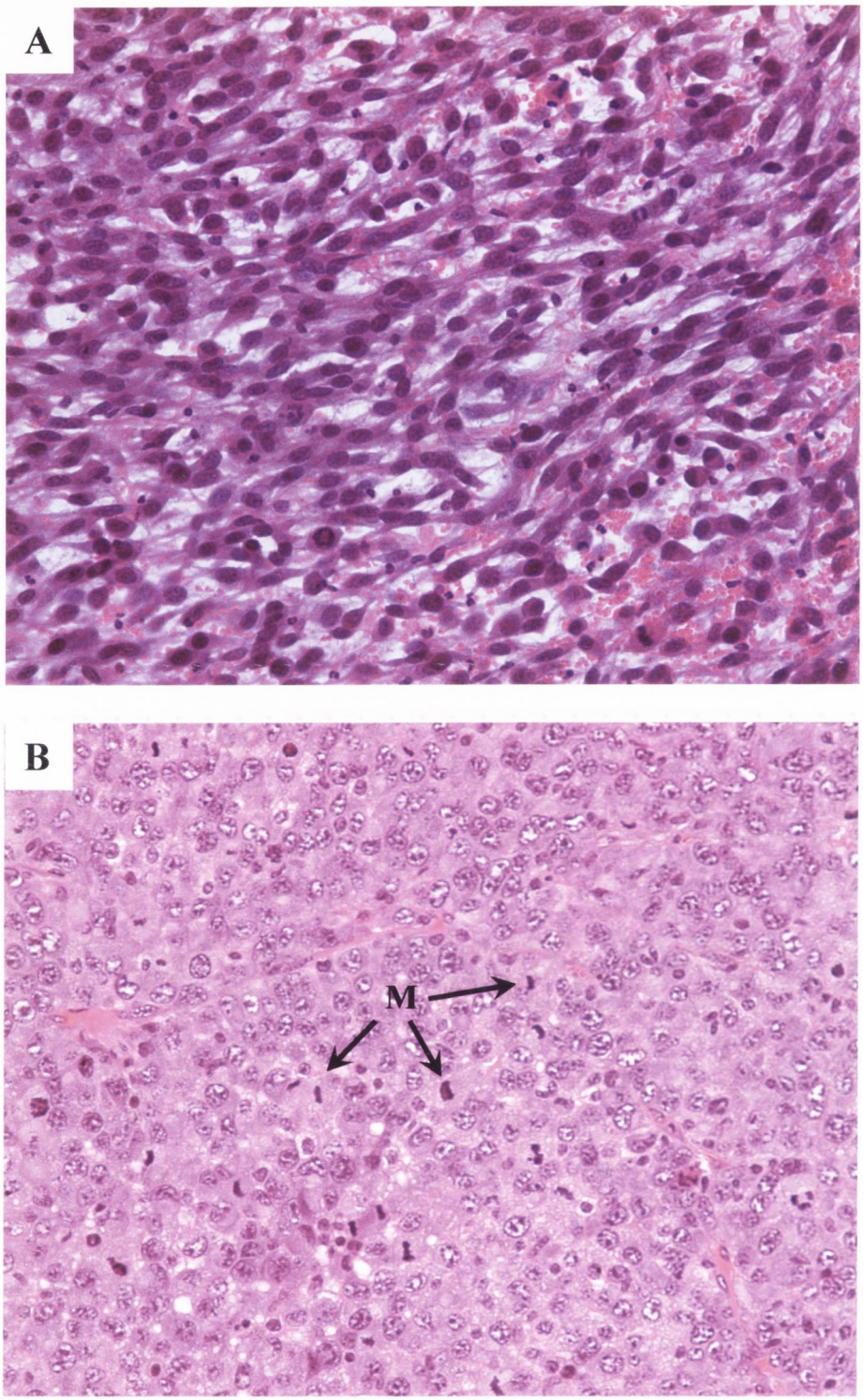


**Figure 4.2 Changes in morphology of K-BALB tumour cells in BALB/c mice over time following formation, stained using H&E**

**(A)** Predominantly spindle-shaped tumour cells 3 days post formation with well-defined cell borders, 200x

**(B)** At 9 days post formation, tumour cells show a poorly differentiated, densely-packed polyhedral phenotype, with pale finely vacuolated cytoplasm. Mitotic figures (M) are common, 200x

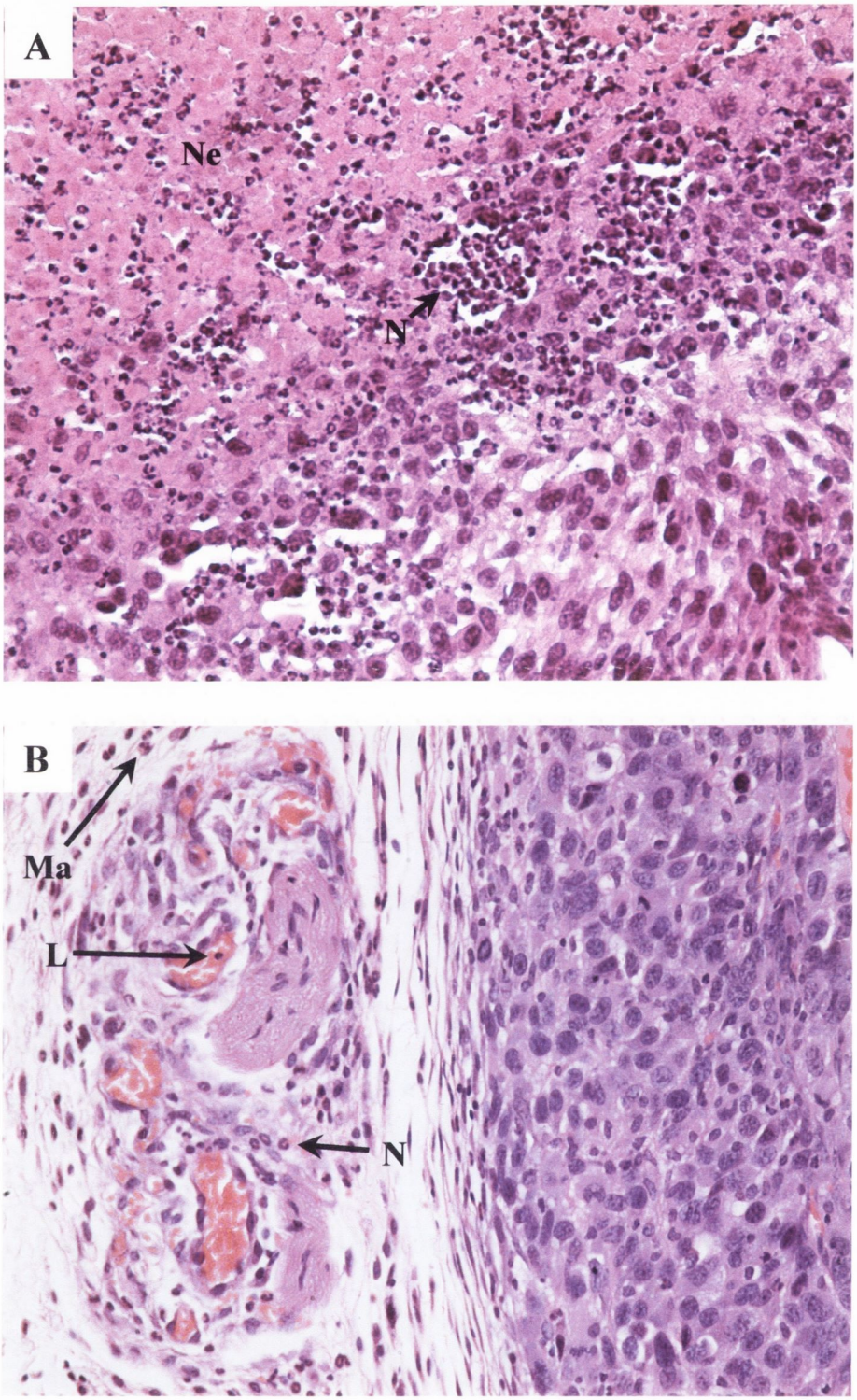


**Figure 4.3 Histology of untreated K-BALB tumours 11 days following induction**

**(A)** Area of necrosis (Ne) infiltrated by neutrophils (N), 200x

**(B)** Occasional neutrophils (N), macrophages (Ma) and lymphocytes (L) at the tumour periphery in perivascular and perineural spaces, 200x

### 4.2.2 Treatment of K-BALB tumours in BALB/c *nu/nu* mice with rSFV-p62-6k VLPs

Groups of 5 BALB/c *nu/nu* mice were immunised with rSFV-p62-6k VLPs or mock-immunised with TNE as described in section 2.3.1.2 and s.c. K-BALB tumours were induced as described in section 2.3.2.2. Treatment was initiated when tumours reached an average diameter of 4 mm at which point high titre rSFV-p62-6k VLPs (1 x $10^{10}$ IU/ml) or TNE buffer alone were administered by an i.t. injection. A total of 6 injections were administered to each tumour with treatment every second day over an 11 day period and tumours were measured daily before injection (section 2.3.2.3). Approximately one third of mice developed tumours with an average diameter of 4 mm 24 h post induction and by 96 h post induction all mice showed tumours of this size.

Control (TNE-treated) tumours grew in the same manner observed in untreated immunocompetent BALB/c mice. All five control tumours had reached an average diameter of 20 mm by day 25 and mice were euthanised accordingly. Tumours treated with rSFV-p62-6k VLPs displayed some inhibition of tumour growth and this was more apparent in those mice which had been previously immunised with rSFV-p62-6k VLPs than in naïve mice. Tumour diameters in immunised mice were 77.7% of control tumours at day 15 compared with naïve tumours which were 92.8% (Figure 4.4). Upon statistical analysis using a one-way repeated measures ANOVA with Tukey's multiple comparison post test, this inhibition of tumour growth was found to be insignificant in comparison to control tumours and no significant difference was noted between naïve and immunised groups (Appendices 9.2; comparison of each group to control at day 15, and 9.3; comparison of all groups over entire experiment). It can therefore be concluded that rSFV-p62-6k VLPs were incapable of significantly inhibiting K-BALB tumour growth in the T-lymphocyte deficient BALB/c *nu/nu* mouse model.

### 4.2.3 Treatment of K-BALB tumours in immunocompetent BALB/c mice with rSFV-p62-6k VLPs

K-BALB tumours were induced in groups of 5 rSFV-p62-6k VLP immunised and TNE buffer mock-immunised immunocompetent BALB/c mice in exactly the same manner as BALB/c *nu/nu* mice described in sections 2.3.1.2 and 2.3.2.2. Treatment with high titre rSFV-p62-6k VLPs (1 x $10^{10}$ IU/ml) or TNE medium commenced when tumours reached an average diameter of 4 mm as described above and in section 2.3.2.3.

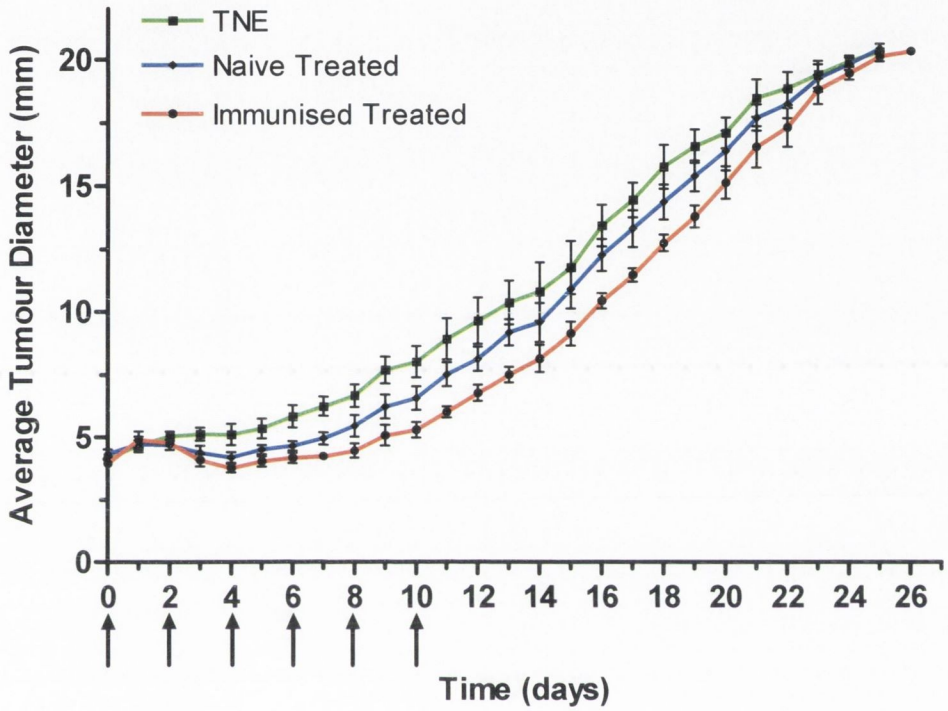


**Figure 4.4 Treatment of K-BALB tumours in BALB/c *nu/nu* mice with rSFV-p62-6k VLPs.** K-BALB tumours were induced in naïve and rSFV-p62-6k VLP immunised female BALB/c *nu/nu* mice by s.c. injection of 100 µl of K-BALB cell suspension in non-supplemented DMEM at a concentration of 1 x $10^7$ cells/ml. Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and groups of five mice were used per treatment group. Tumours received six 50 µl intratumoural injections of TNE alone or TNE containing rSFV-p62-6k VLPs at a concentration of 1 x $10^{10}$ IU/ml which were administered every other day over an eleven day period. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised when average tumour diameter reached 20 mm. *Points*; mean of five replicates, *bars*; +/- SEM, *arrows*; days of treatment. Results are representative of two individual experiments.

Mice developed tumours at the same rate as the BALB/c *nu/nu* mice with 100% of mice having developed tumours with an average diameter of 4 mm by 96 h post induction.

Control (TNE treated) tumours grew rapidly and at the same rate as control tumours in BALB/c *nu/nu* mice with tumours reaching an average diameter of 20 mm between days 22 and 25. Tumours in immunised mice treated with rSFV-p62-6k VLPs swelled to approximately twice their original size by 24 h post injection before returning to an average diameter of approximately 4 mm by 48 h post injection, when the next treatment was administered (Figure 4.5a). This phenomenon was consistently observed after each injection and was not apparent in the naïve group until after the third injection (day 5).

Following cessation of treatment, tumour swelling subsided by day 12 and a significant inhibition of tumour growth was noted in both the naïve and immunised groups with average tumour diameters of 51.2% and 43.7% of control tumours respectively at day 15 ($P < 0.001$; $P < 0.001$). All five tumours in the naïve group had begun to regrow by day 16 and tumours reached an average diameter of 20 mm between days 27 and 35. Tumours from immunised mice began to regrow between days 16 and 22, with one tumour regressing to an average diameter of 2 mm on day 21 before starting to regrow. Tumours reached an average diameter of 20 mm between days 29 and 40 (Figure 4.5b). The two groups were not found to be significantly different however, despite the higher level of growth inhibition observed in the immunised group. Both groups displayed a significantly higher level of tumour growth inhibition than their BALB/c *nu/nu* counterparts, however, and so it can be concluded that rSFV-p62-6k VLPs are capable of significantly inhibiting the growth of K-BALB tumour growth in the immunocompetent BALB/c mouse model (Appendices 9.2 and 9.3).

### 4.2.4 Detection of anti-SFV immune responses in mice treated with rSFV-p62-6k VLPs

Groups of additional immunocompetent BALB/c mice were immunised, induced with K-BALB tumours and subsequently treated with rSFV-p62-6k VLPs or TNE buffer in tandem with (and in exactly the same manner as) the above tumour growth studies. Mice were sampled in triplicate on days 1, 5, 11 and 15 for serum and splenocytes as described in section 2.3.3.1. BALB/c *nu/nu* mice were sampled at experiment end for serum and splenocytes as no significant anti-SFV immune response was expected in

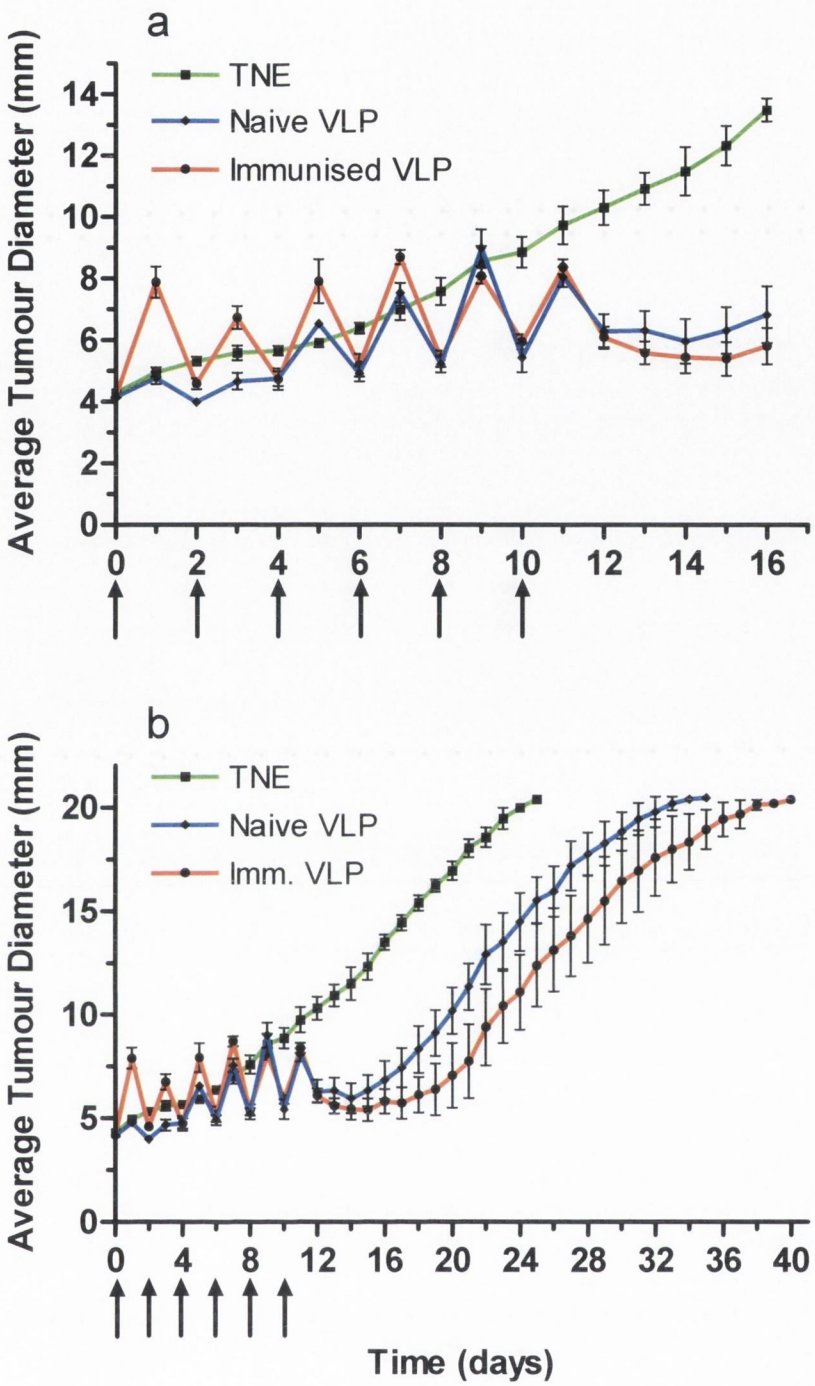


**Figure 4.5 Treatment of K-BALB tumours in immunocompetent BALB/c mice with rSFV-p62-6k VLPs.** K-BALB tumours were induced in naïve and rSFV-p62-6k VLP immunised female immunocompetent BALB/c mice by s.c. injection of 100 µl of K-BALB cell suspension in non-supplemented DMEM at a concentration of 1 x $10^7$ cells/ml. Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and groups of five mice were used per treatment group. Tumours received six 50 µl intratumoural injections of TNE alone or TNE containing rSFV-p62-6k VLPs at a concentration of 1 x $10^{10}$ IU/ml which were administered every other day over an eleven day period. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised when average tumour diameter reached 20 mm. *Points*; mean of five replicates, *bars*; +/- SEM, *arrows*; days of treatment. Results are representative of two individual experiments.

these groups. Two-way repeated measures ANOVAs with Bonferroni post tests were employed for statistical analyses.

#### 4.2.4.1 Detection of humoural anti-SFV immune responses in mice treated with rSFV-p62-6k VLPs

Serum samples were analysed by ELISA and assigned arbitrary anti-SFV4 IgG units by comparison to a reference curve of known positive serum as described in section 2.3.3.2.3. Samples falling below a cut-off absorbance of 1.5 times that of the negative control were deemed seronegative and not assigned anti-SFV4 IgG units. All samples from BALB/c *nu/nu* groups were found to be seronegative illustrating the apparent inability of these athymic animals to produce anti-SFV humoural immune responses in reaction to rSFV-p62-6k VLPs. The naïve (TNE-treated) control group also remained seronegative for the duration of the experiment. On day 1 following initiation of treatment, immunised groups showed a high level of anti-SFV4 IgG with no detectable humoural immune response in naïve mice treated with tSFV-p62-6k VLPs. By day 5 however, a detectable level of anti-SFV4 IgG was present in naïve mice ($P < 0.001$) and was comparable with that present in immunised groups by 11 days post initiation of treatment. The control immunised group maintained a high and somewhat even level of anti-SFV4 IgG throughout the course of the experiment. Immunised mice treated with rSFV-p62-6k VLPs also maintained a high anti-SFV4 IgG level but this was seen to increase to near-maximum levels from day 5 onwards (Figure 4.6) although these values were not significantly higher than those in the control immunised group at any time point. Serum samples from mice sampled at experiment end (when tumours reached 20 mm) maintained comparable levels of anti-SFV4 units where expected (not shown).

#### 4.2.4.2 Detection of cellular anti-SFV immune responses in mice treated with rSFV-p62-6k VLPs

Splenocytes were incubated in the presence of conA, medium alone, or purified UV-inactivated SFV4 for 72 h after which supernatants were analysed for secreted IFN-γ as described in section 2.3.3.3. Incubation with conA, which was intended as a positive control, resulted in the secretion of relatively high levels of IFN-γ. Figure 4.7 shows amounts of IFN-γ secreted by splenocytes and Figure 4.8 expresses these data as stimulation indices which were calculated by dividing amounts of IFN-γ in SFV4

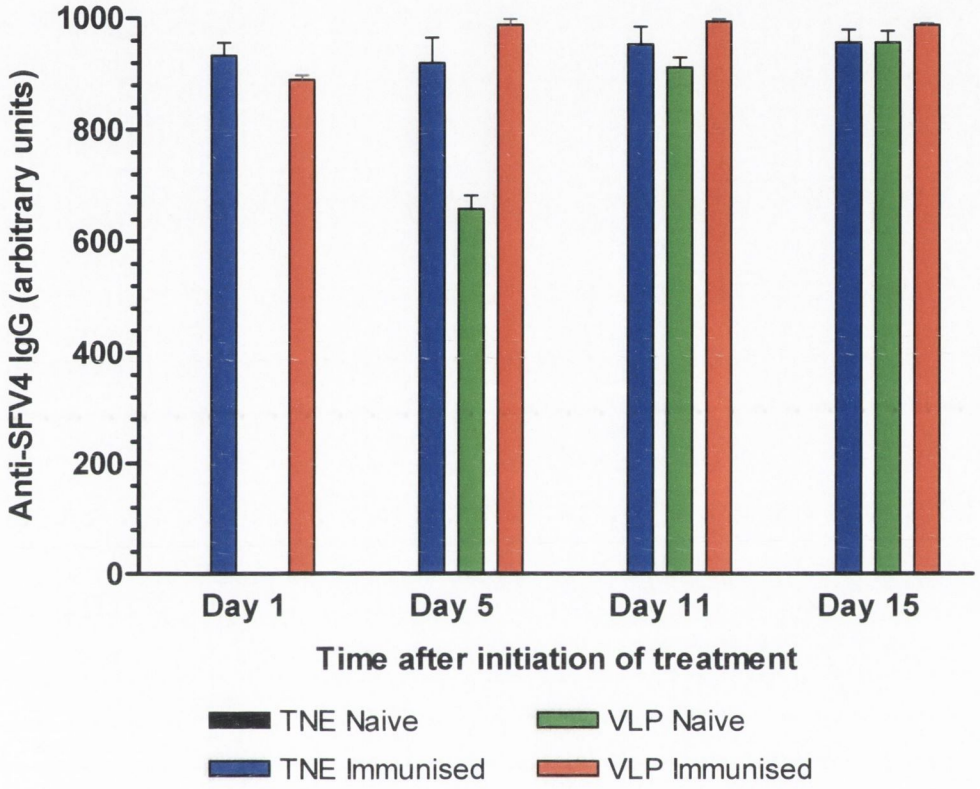


**Figure 4.6 Anti-SFV humoural immune responses in immunocompetent BALB/c mice treated with rSFV-p62-6k VLPs as determined by anti-mouse IgG ELISA.** Serum samples were collected in triplicate from groups of euthanised immunocompetent BALB/c mice on days 1, 5, 11 and 15 post initiation of intratumoural treatment with rSFV-p62-6k VLPs. Samples were assayed utilising an anti-SFV4 IgG ELISA and assigned arbitrary anti-SFV4 IgG units from a reference curve of positive serum. All samples below 1.5 times the negative control were deemed seronegative and not signed anti-SFV4 IgG units. *Columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

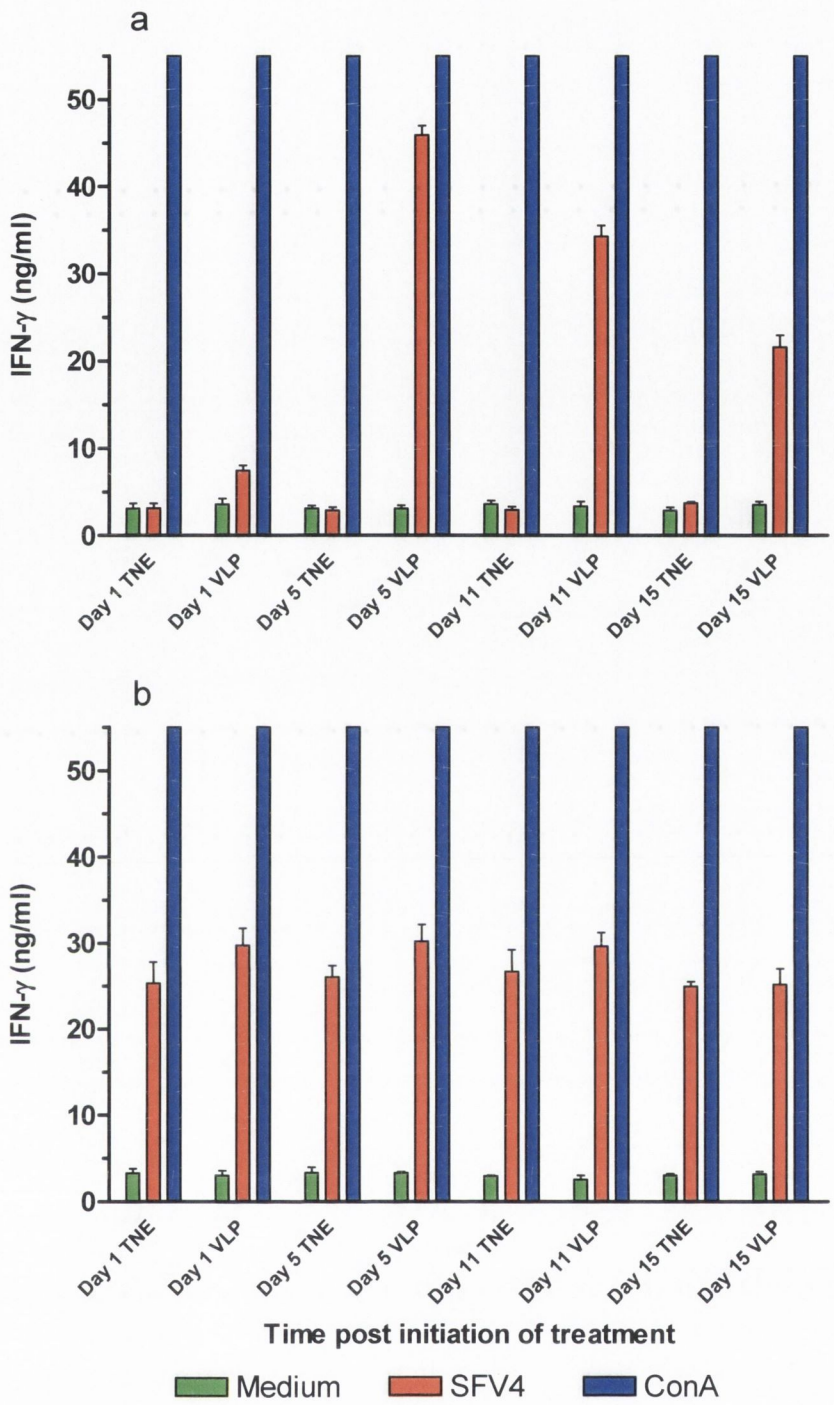


**Figure 4.7 IFN-γ secretion by splenocytes, as determined by capture ELISA, from naïve (a) and immunised (b) immunocompetent BALB/c mice treated with rSFV-p62-6k VLPs cultured *ex vivo* in the presence of medium alone, UV-inactivated SFV4 or ConA.** Splenocytes were harvested from immunocompetent BALB/c mice in triplicate at days 1, 5, 11 and 15 post initiation of treatment and cultured in the presence of medium alone, UV-inactivated SFV4 (1 x $10^5$ PFU/well) or ConA (5 μg/ml) for 72 h. Supernatants were clarified by centrifugation and concentrations of IFN-γ determined by capture ELISA. *Columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

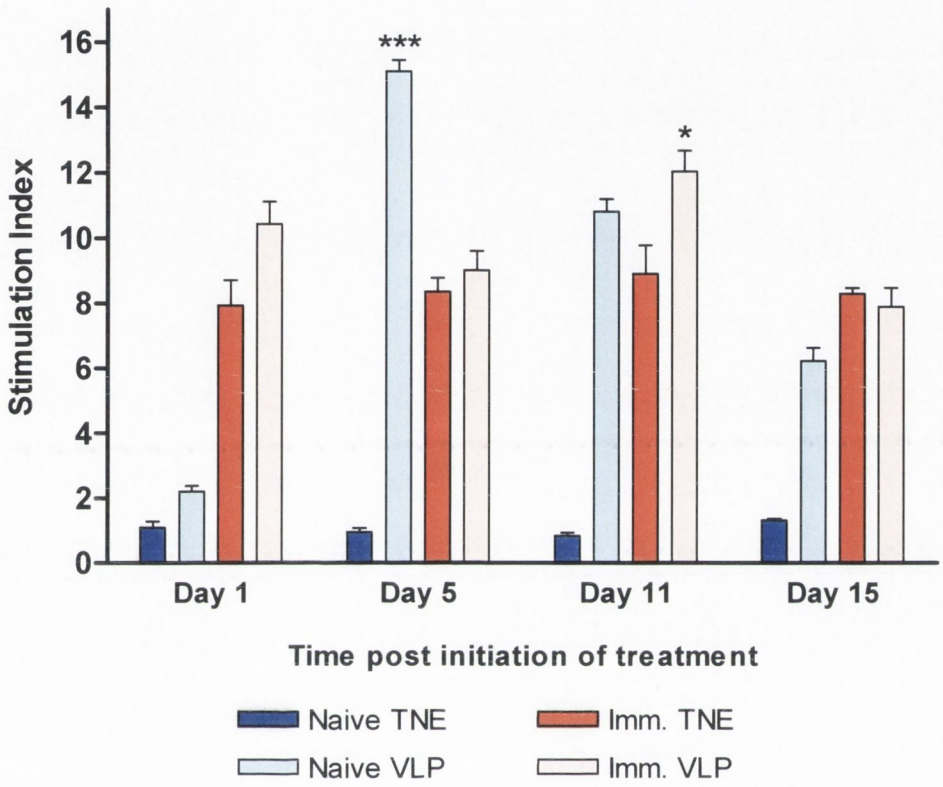


**Figure 4.8 Stimulation indices of splenocytes from immunocompetent BALB/c mice treated with rSFV-p62-6k VLPs to SFV4 antigens.** Splenocytes were incubated in triplicate with UV-inactivated SFV4 for 72 h and levels of IFN-γ secretion were determined by capture ELISA. Stimulation indices were calculated by dividing amounts of IFN-γ in SFV4 stimulated wells by the amount secreted in negative control wells. *Columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments. * = $P < 0.05$, *** = $P < 0.001$, VLP treated versus appropriate TNE control.

stimulated wells by the amount secreted in negative control wells. Stimulation indices were significantly higher in naïve mice treated with rSFV-p62-6k than in naïve control (TNE-treated) mice from day 5 onwards. A clear peak in stimulation was observed in naïve mice treated with rSFV-p62-6k VLPs at day 5 ($P < 0.001$) which then dropped to lower but still significant levels by day 15 ($P < 0.01$). Both immunised control and immunised rSFV-p62-6k VLP treated groups showed significant levels of stimulation with no obvious peak. A significant difference between treated and control immunised groups was only noted at day 11 when treated mice showed a higher level of stimulation ($P < 0.05$).

### 4.2.5 Pathology of paraffin embedded, formalin fixed tumour tissue

The histological appearance of control tumours was similar to that described in section 4.2.1. Whereas tumours from day 1 were composed of spindle-shaped cells, tumours from day 5 onwards mostly comprised the less differentiated polyhedral phenotype. Intratumoural injection with TNE buffer resulted in no significant morphological changes.

Tumours from immunised mice sampled at day 1 following treatment with rSFV-p62-6k VLPs showed islands of tumour cells separated by oedema, fibrin, neutrophils and macrophages (Figure 4.9a). Lymphocytes and plasma cells were clustered in perivascular and perineural spaces at the periphery of the tumour cell islands (Figure 4.9b). Tumours from naïve mice treated with rSFV-p62-6k VLPs showed less severe inflammatory oedema and lower levels of lymphocytic infiltration than tumours from immunised mice sampled at the same time point.

Increased levels of lymphocytic infiltration were apparent in tumours from immunised mice sampled at days 5 and 11 following treatment with rSFV-p62-6k VLPs (Figure 4.10). Lymphocytes, neutrophils and macrophages were located among the tumour cells and lymphocytes, together with plasma cells, were clustered in perivascular and perineural spaces towards the periphery of the tumour cell islands. Tumours from naïve mice sampled at day 5 following treatment showed severe inflammatory oedema and neutrophil exudation similar to that seen in the immunised group at day 1 following treatment. Levels of lymphocytic infiltration in tumours from naïve mice sampled at day 11 following treatment were lower than in immunised mice. Tumours from immunised and naïve mice treated with rSFV-p62-6k VLPs and sampled at days 5 and 11 following treatment were mostly composed of spindle-shaped cells.

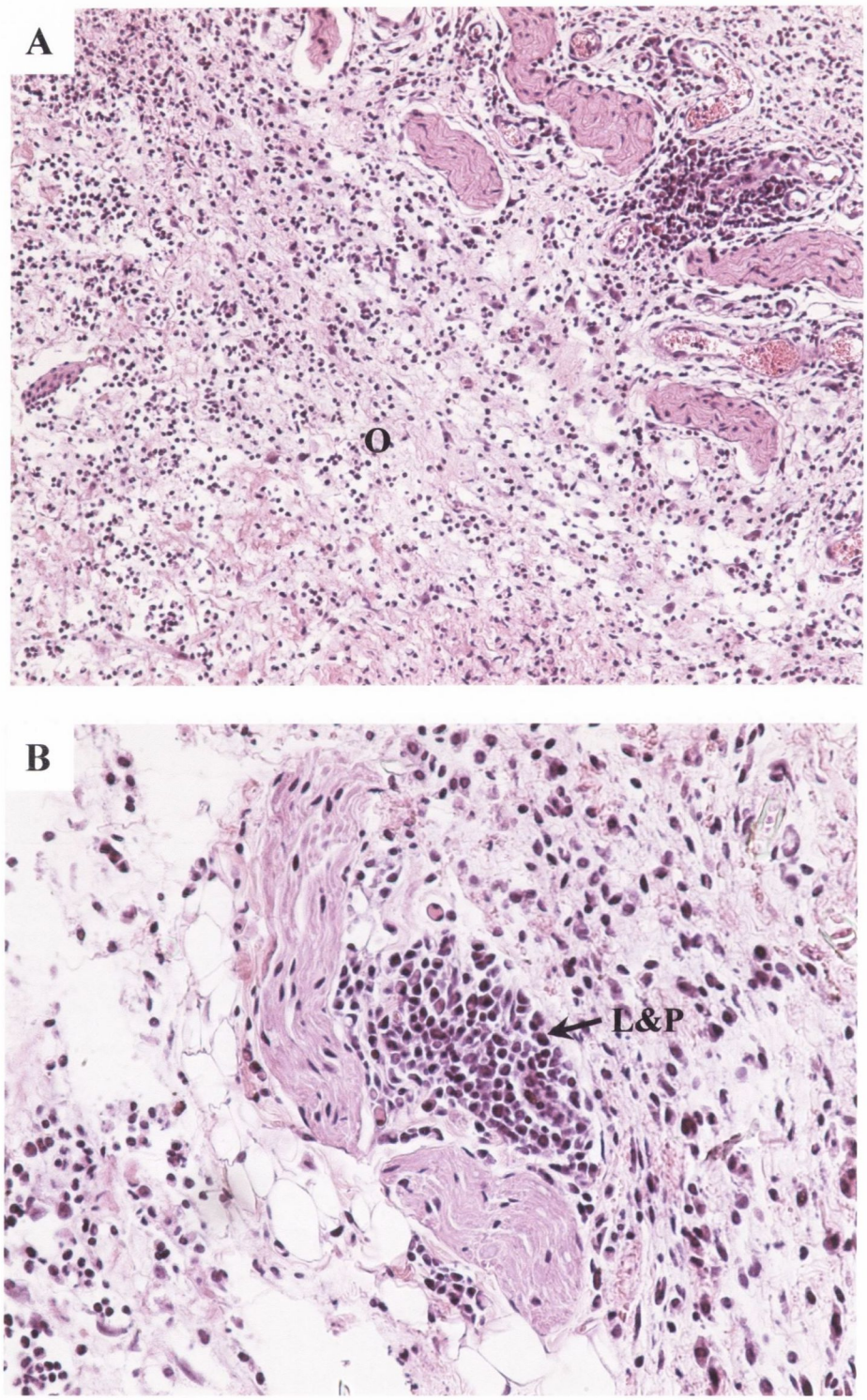


**Figure 4.9 Histology of K-BALB tumours from immunised BALB/c mice 1 day post initiation of treatment with rSFV-p62-6k VLPs**

**(A)** Acute inflammatory oedema (O) with fibrin and neutrophils, 100x

**(B)** Lymphocytes and plasma cells (L&P) concentrated around blood vessels and nerve fibres at the periphery of the tumour, 200x

***Corresponding control tumours were reminiscent of those shown in figures 4.2 & 4.3***

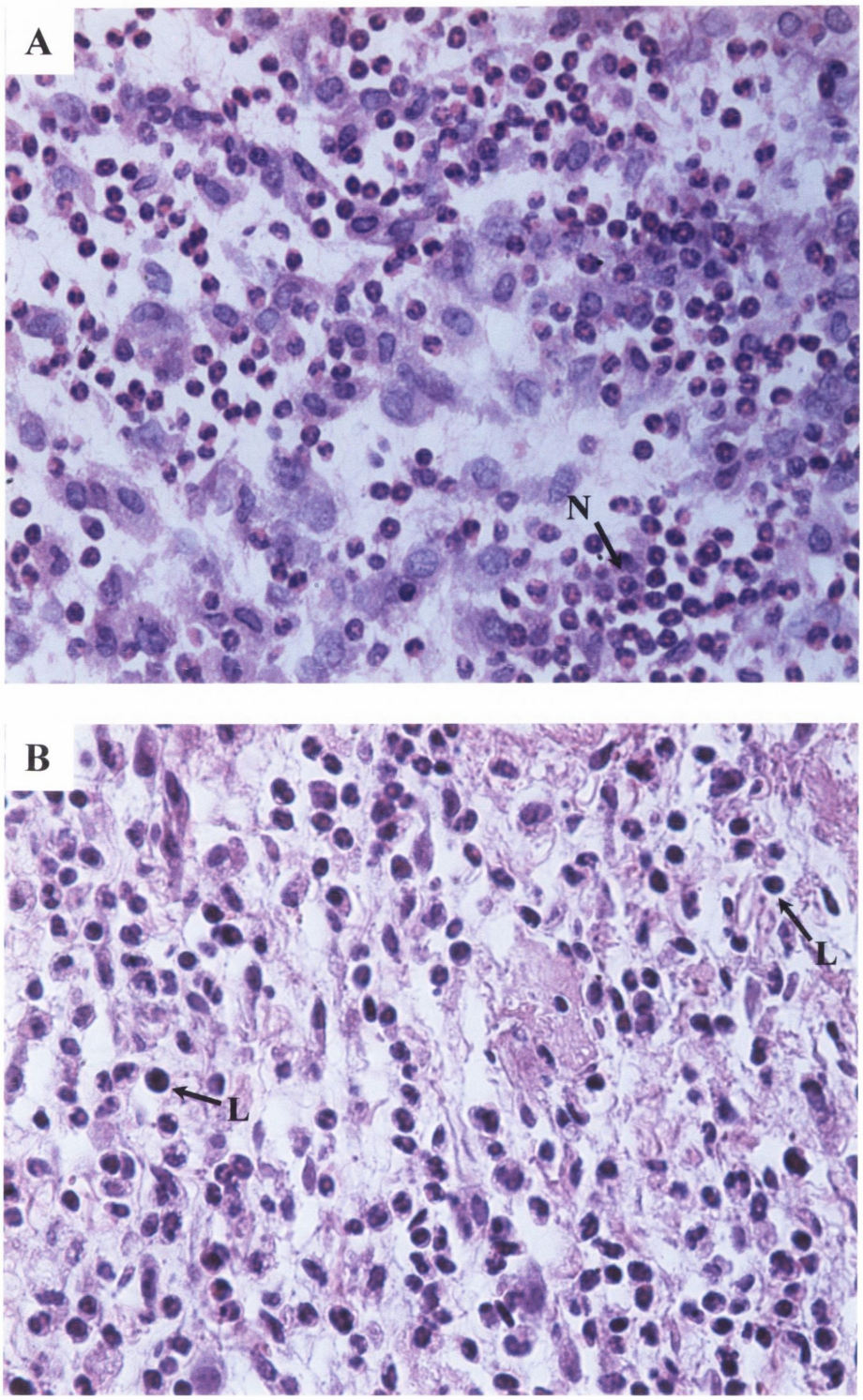


**Figure 4.10 Composition of inflammatory infiltrate at 1 and 5 days post initiation of treatment of tumours in immunised mice with rSFV-p62-6k VLPs**

**(A)** Cellular infiltrate at 1 day post initiation of treatment composed predominantly of neutrophils (N), 400x

**(B)** Five days post initiation of treatment, increasing numbers of lymphocytes (L) were apparent, 400x

***Corresponding control tumours were reminiscent of those shown in figures 4.2 & 4.3***

Tumours from immunised mice sampled at day 15 following treatment with rSFV-p62-6k VLPs showed higher levels of lymphocytic infiltration than tumours from similarly-treated naïve mice sampled at this time point (Figure 4.11a). Plasma cells clustered in perivascular and perineural spaces at the periphery of the tumour cell islands were more numerous in immunised mice than in naïve mice and frequently had a reactive appearance with large eosinophilic intracytoplasmic inclusions (Russel Bodies) (Figure 11b; Figure 4.12a). Mast cells were more numerous in peritumoural areas than in controls (Figure 4.12b).

It was concluded that intratumoural injection of K-BALB tumours with rSFV-p62-6k VLPs results in severe inflammation within the tumour microenvironment and that the inflammatory response occurs more rapidly and is more sustained in immunised mice than in controls. Maintenance of the tumour cell phenotype as a more differentiated spindle-shaped phenotype was associated with lymphocytic infiltrates (Figure 4.11a). Further studies relating to characterisation of the intratumoural lymphocytes and localisation of SFV antigen and apoptotic forms are detailed in chapter 6.

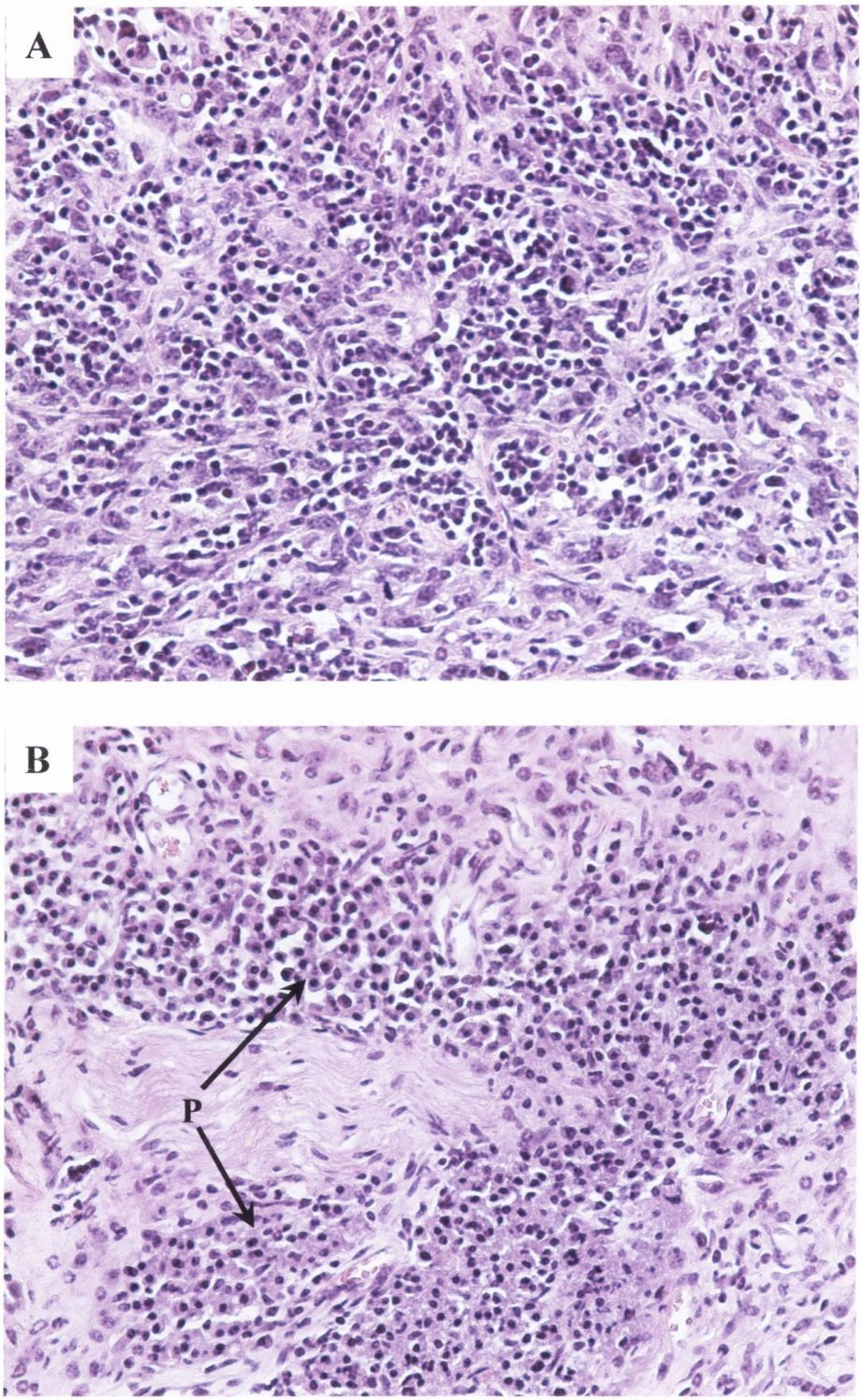


**Figure 4.11 Cellular infiltrate at 15 days post initiation of treatment of K-BALB tumours in immunised mice with rSFV-p62-6k VLPs**

**(A)** Lymphocytes, neutrophils and macrophages among spindle-shaped tumour cells, 200x

**(B)** Sheets of plasma cells (P) surrounding blood vessels and nerve fibres, 200x

***Corresponding control tumours were reminiscent of those shown in figures 4.2 & 4.3***

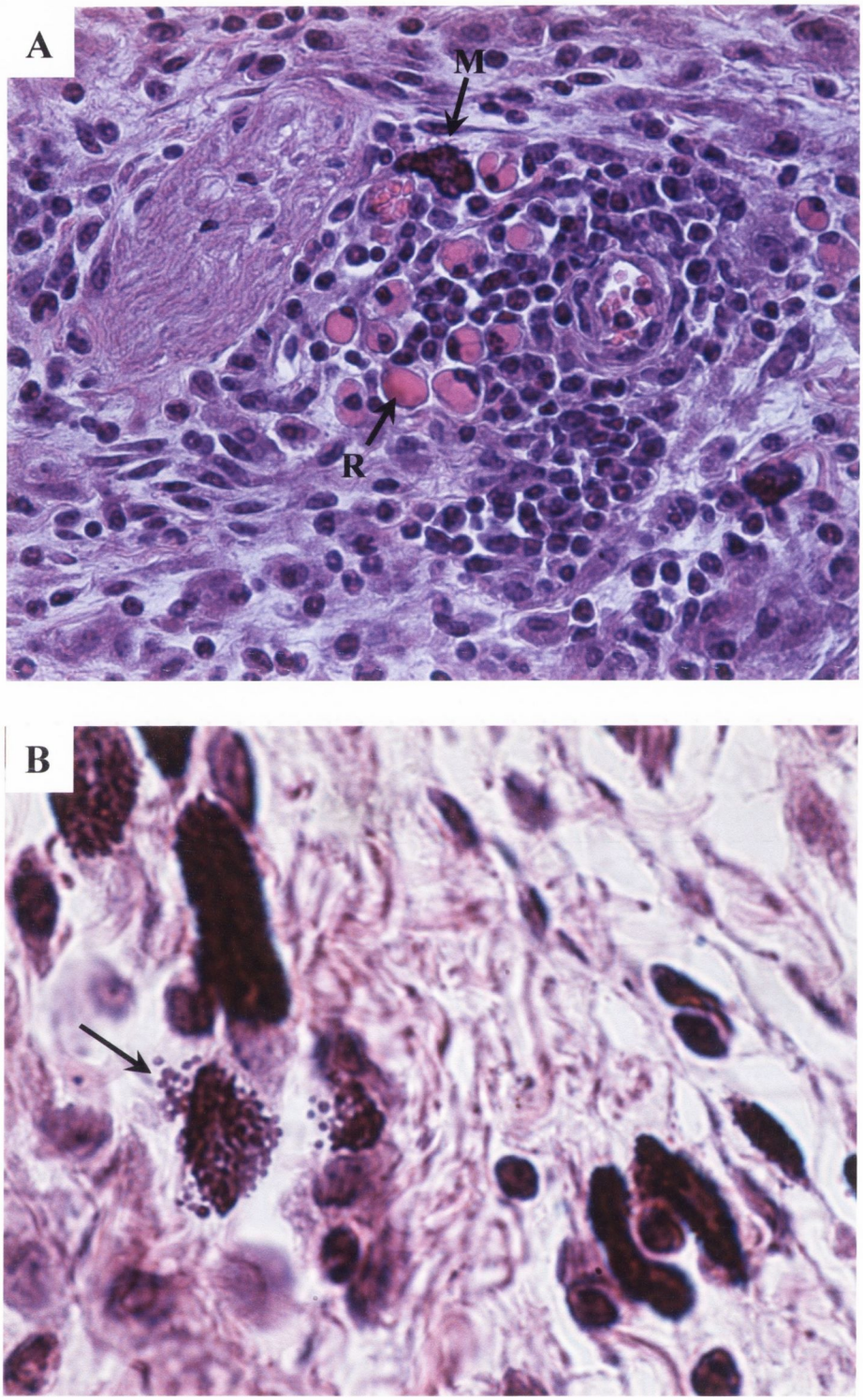


**Figure 4.12 Plasma cells containing Russell bodies and mast cells in tumours of immunised mice 11 days post initiation of treatment with rSFV-p62-6k VLPs**

**(A)** Perivascular and perineural accumulation of lymphocytes, plasma cells, Russell bodies (R) and mast cells (M), 400x

**(B)** Several mast cells in inflamed area of tumour with some apparently actively releasing granules (*arrow*), 1000x

***Corresponding control tumours were reminiscent of those shown in figures 4.2 & 4.3***

## 4.3 DISCUSSION

Several strategies in the exploitation of both viral and non-viral vectors for cancer therapy have been investigated ranging from specific approaches such as oncogene inactivation, to less direct methods such as the inhibition of angiogenesis. Induction of apoptosis in tumour cells is a goal common to many cancer treatment regimens and gene therapy is no exception, with much promising research in the introduction of pro-apoptotic factors to the tumour microenvironment (Hughes, 2004; Waxman & Schwartz, 2003). The SFV vector system encodes its own apoptosis induction mechanism which, in itself, can be exploited for tumour therapy, effectively eliminating the need for the expression of pro-apoptotic genes by the vector (Murphy *et al.*, 2000). This provides an opportunity for the expression of other potentially therapeutic genes by the vector system to compliment its inherent antitumoural effect. The expression of the anti-angiogenic cytokine IL-12 and of the pro-apoptotic gene *bax* by rSFV VLPs have both been employed successfully in the treatment of murine tumour models in this manner (Asselin-Paturel *et al.*, 1998; Murphy *et al.*, 2001).

The field of cancer immunotherapy, which involves the induction or potentiation of host immune responses against tumour cells, is evolving into a promising area of research both in the laboratory and clinically (Whelan *et al.*, 2003; Rosenberg, 2001). Development of prototype cancer vaccines and their use in immunotherapy has largely involved the exploitation of TAAs (Finn, 2003; Kuroki *et al.*, 2003). Because such antigens are associated with only a minority of tumours and, given the dynamic nature of tumour cell phenotype and surface antigen expression, these strategies may be of limited value (Dunn *et al.*, 2002; Khong & Restifo, 2002). Rather than restricting therapy to a specific TAA, expression of viral or other antigens to effectively flag tumour cells is a strategy applicable to a much broader spectrum of tumours and is clearly preferable.

A large amount of promising research aimed specifically at oncogenic Ras or its gene products, has involved the exploitation of ribozyme and RNA interference technologies as well as the utilisation of mutated *ras* as a target for cancer immunotherapy/vaccination (Abrams *et* al., 1996; Duursma & Agami, 2003; Kijima *et al.*, 2004). It was shown in chapter 3 that the activation of K-*ras* in the K-BALB cell line did not confer resistance to SFV-induced apoptosis, thus eliminating any requirement for targeting the oncogene and providing an opportunity for the expression of SFV antigens using VLPs encoding the p62-6k genes. Infection of cells with rSFV-p62-6k VLPs results in the expression of the structural proteins E2, E3 and 6k, of which

E2 is known to be highly antigenic. It was hoped that the expression of the E2 antigen would elicit an immune response against infected tumour cells/debris thus enhancing the antitumoural effect of the vector.

K-BALB cells were found to form rapidly-growing tumours upon s.c. injection in both immunocompetent BALB/c and BALB/c *nu/nu* mice. No difference was noted in the rate of tumour growth between immunocompetent and athymic mice and pathological examination confirmed the low-immunogenicity of this syngeneic murine tumour model. Leukocytes belonging to the innate immune system such as neutrophils and macrophages predominated and were restricted to peripheral regions of tumours, infiltrating areas of necrosis, haemorrhage and thrombosis. Lymphocytes were noted only occasionally in perivascular and perineural areas and were rarely observed infiltrating the tumour tissue.

As the cell line displayed a low infection efficiency and also because of the low volume used for direct i.t. injection, rSFV-p62-6k VLPs were concentrated by ultracentrifugation to maximise the response to treatment. Groups of mice were also immunised with rSFV-p62-6k VLPs prior to tumour induction and treatment in order to bolster and accelerate the anti-SFV immune response. Upon direct i.t. injection with high-titre rSFV-p62-6k VLPs, tumours in the immunised immunocompetent group swelled to twice their original size due to acute inflammation and oedema as revealed by histopathology. The delayed inflammatory response in the naïve immunocompetent group at day 5 post initiation of treatment coincided with the development of detectable humoural and cellular immune responses *ex vivo*. No such reaction was observed in BALB/c *nu/nu* groups which failed to mount an adaptive immune response against SFV antigens.

Inhibition of K-BALB tumour growth was observed in all groups treated with rSFV-p62-6k VLPs but was only statistically significant in immunocompetent groups. A higher level of inhibition was noted in immunised groups when compared with naïve mice but this difference was statistically insignificant. Immunisation also resulted in a more immediate, intense and sustained immune response with higher numbers of TILs in the immunised immunocompetent group than in naïve mice. No anti-SFV humoural or cellular immune responses were detected in naïve or immunised BALB/c *nu/nu* mice and it was concluded that the inhibition observed in those groups was due to the cytopathic effect of the rSFV-p62-6k VLPs. The therapeutic potential of rSFV VLPs as tumour therapy agents for rapidly growing tumours of low infection efficiency therefore seems limited when relying solely on the inherent cytotoxicity of the system. The significantly

higher level of inhibition of tumour growth achieved in immunocompetent groups when compared to athymic mice suggests that the antitumoural effect of rSFV-p62-6k VLPs was successfully augmented by the induction of anti-SFV host immune responses against infected cells.

A strong anti-SFV $T_H1$ cellular immune response was detected in *ex vivo* splenocyte stimulation assays by quantification of secreted IFN-γ. The histopathological studies indicated that the induction of this anti-SFV $T_H1$ cellular immune response and the secretion of $T_H1$-associated cytokines played a major role in the induction of cytotoxic immune responses and subsequent inhibition of tumour growth. The humoural immune response detected was probably predominantly of the IgG2a isotype typical of rSFV VLP-induced $T_H1$-mediated immunity (Berglund *et al.*, 1999; Fleeton *et al.*, 1999). While the generation humoural immune responses can be counterproductive when using viral vectors as therapeutic agents, it is clear that rSFV-p62-6k VLPs were still capable of infecting cells at the site of injection despite the presence of a strong humoural anti-SFV immune reaction. This anti-SFV humoural immune response may have enhanced the antitumoural effect through complement activation against infected tumour cells and the binding of antibody to Fc receptors on accessory effector cells such as macrophages, neutrophils, mast cells and NK cells (Reilly *et al.*, 2001). The cellular infiltrate observed in pathological studies was further characterised by immunohistochemistry and is discussed in greater detail in chapter 6.

The change in morphology observed over time from fibroblastic 'spindle' cells to polyhedral pleomorphic cells was representative of the loss in differentiation associated closely with malignant tissue. As tumours grew the cells reverted to a more poorly differentiated phenotype, a process which was delayed by treatment with rSFV-p62-6k VLPs. Disruption of tumour vasculature through the cytotoxicity of the rSFV-p62-6k VLPs and the repeated inflammatory oedema may also have played an indirect role in the inhibition of tumour cell growth observed.

Despite the significant levels of tumour growth inhibition and inflammation, all tumours began to regrow in the days following cessation of treatment. Immunised immunocompetent mice showed a more sustained inhibition of growth with one tumour actually regressing to an average diameter of 2 mm before beginning to regrow. Pathological studies revealed islands of apparently healthy uninfected tumour cells in all cases. It is possible that the inability of the rSFV-p62-6k VLPs to eradicate the tumour, even after multiple treatments, may be overcome through the introduction of cytotoxic or anti-angiogenic factors (including cytokines) into the vector which would be secreted by

infected cells and diffuse through the tumour (Asselin-Paturel *et al.*, 1998; Yamanaka *et al.*, 2001). It is also possible that the strong anti-SFV humoural immune response generated by rSFV-p62-6k VLPs may have had a negative effect on subsequent administrations of VLPs through the presence of neutralising antibodies. This effect could be partially abrogated by the use of VLPs expressing a non-viral antigen but such particles would still contain viral antigen and continue to induce a neutralising immune response. In this study the use of rSFV-p62-6k VLPs provided a good comparison to the replication proficient SFV4, which is examined in the next chapter (5) as a model for a replicating vector system.

The SFV vector system, which allows for transient high-level expression of foreign proteins, has been employed successfully in the induction of cytotoxic and humoural immune responses against a variety of antigens as a prototype vaccine and in cancer immunotherapy (Berglund *et al.*, 1999; Colmenero *et al.*, 2002; Zhou *et* al., 1995). Murine studies employing RT-PCR analysis revealed that rSFV VLPs do not disperse throughout other organs of the body with detectable rSFV RNA persisting only at the site of injection for 7 days and in lymphoid organs for up to 24 h post inoculation (Morris-Downes *et al.*, 2001). While vector persistence can be beneficial in some areas of gene therapy, this transience of the SFV vector system reduces some of the biosafety concerns associated with other (DNA-based) vector systems.

In conclusion, the expression of SFV antigens in K-BALB tumour cells successfully augmented the inherent antitumoural effect of rSFV VLPs through the induction of an acute inflammatory immune response. The ability to generate high-titres of rSFV VLPs and their localised action with apparent negligible toxicity to surrounding tissues makes them an attractive candidate for the treatment of solid tumours. Phase I and II clinical trials for liposome-encapsulated SFV vectors expressing IL-12 for the treatment of glioblastoma multiforme were recently proposed (Ren *et al.*, 2003).

# 5

# TREATMENT OF K-BALB TUMOURS *IN VIVO* WITH SFV4

## 5.1 INTRODUCTION

While the use of replication-incompetent viral vectors as tumour therapy agents has produced promising results in many cases, the limitations of such systems are becoming apparent and an interest in the therapeutic potential of replication-competent viruses is increasing. The replication of oncolytic viruses in tumour tissue can allow for a 1,000 to 10,000-fold amplification of the input dose at the tumour site and a subsequent increase in cell-killing, immunopotentiation, or expression of heterologous therapeutic protein. Several viruses which display a natural tropism for tumour tissues have been described and with the advent of recombinant DNA technology it has been possible to engineer viruses with increased oncolytic and/or oncotropic capacities (Bergsland & Venook, 2002; Kirn, 2002). The potential antitumoural effect of a replicating SFV vector is assessed in this chapter, using the infectious clone SFV4 (from which pSFV-p62-6k was derived) as a model. It is not yet known if SFV replicates preferentially in tumour tissue but recent studies with closely related SV vectors have revealed a tropism for a variety of tumour models *in vivo* (Tseng *et al.*, 2002; Tseng *et al.*, 2004).

The susceptibility of the K-BALB cell line to SFV4-induced apoptosis was demonstrated in chapter 3 and a more sustained cytopathic effect was observed than that induced by rSFV-p62-6k VLP expression. A higher proportion of cells were also found to express SFV antigen and at earlier timepoints when infected with the replication-proficient SFV4 than with rSFV-p62-6k VLPs. SFV4 is virulent in BALB/c mice, killing 60-70% when administered i.p. ($1 \times 10^6$ PFU) (Glasgow *et al.*, 1991). Therefore immunisation of mice with rSFV-p62-6k VLPs prior to tumour induction and treatment acted not only to bolster the anti-SFV immune response against infected tumour cells, but also to effectively vaccinate mice against potentially lethal infection. Given the inability of BALB/c *nu/nu* mice to clear SFV infection (Jagelman *et al.*, 1978) it was decided that their inclusion in this study would not be justified.

Treatment of K-BALB tumours with SFV4 resulted in a significant inhibition of tumour growth which was significantly enhanced by prior immunisation of mice with rSFV-p62-6k VLPs. Immunisation also conferred protection against challenge with SFV4 with no immunised mice presenting clinical signs or pathological lesions of SFV4 infection. Inflammatory responses comparable to those observed in chapter 4 were also noted, although higher levels of TILs persisted.

## 5.2 RESULTS

### 5.2.1 Treatment of K-BALB tumours in BALB/c mice with SFV4

Groups of 5 BALB/c mice were immunised with rSFV-p62-6k VLPs or mock-immunised with TNE as described in section 2.3.1.2 and s.c. K-BALB tumours were induced as described in section 2.3.2.2. Treatment was initiated when tumours reached an average diameter of 4 mm at which point concentrated SFV4 (1 x $10^{10}$ IU/ml) or TNE buffer alone was administered by direct i.t. injection. A total of 6 injections were administered to each tumour with treatment every second day over an 11 day period and tumours were measured daily prior to treatment (section 2.3.2.4). Approximately one third of mice developed tumours with an average diameter of 4 mm 24 h post induction and by 96 h post induction all mice had presented tumours of this size.

Control (TNE treated) tumours grew rapidly and reached an average diameter of 20 mm between days 22 and 25. Tumours responded to treatment with SFV4 in a manner similar to that observed in those treated with rSFV-p62-6k VLPs (chapter 4). Upon examination 24 h after the initial i.t. injection with SFV4, tumours from immunised groups had swelled to approximately twice their original size. Swelling subsided by 48 h post injection at which point the next i.t. injection of SFV4 was administered. Tumours swelled and subsided in the same manner following each of the 6 injections administered. This phenomenon was not observed in naïve mice until after their third i.t. injection with SFV4 (day 5) (Figure 5.1a).

The last injection of SFV4 was administered on day 10 and tumour swelling had subsided by day 12. Significant inhibition of tumour growth was noted in both naïve and immunised mice following this with average tumour diameters of 43.8% and 45.6% of control tumours at day 15 respectively ($P < 0.001$; $P < 0.001$). Two tumours from the naïve group did not begin to regrow until days 20 and 23 post initiation of treatment whereas the remaining three had started to regrow by day 16. Tumours from naïve mice reached an average diameter of 20 mm between days 29 and 37. Complete tumour regression was noted in one mouse from the immunised group at day 19 post initiation of treatment. This tumour began to regrow on day 35 when a 2 mm diameter nodule was detected which continued to grow before reaching an average diameter of 20 mm at 58 days post initiation of treatment. Of the remaining four tumours in immunised mice, two began to regrow at day 16 and the remaining two on days 17 and 21 post initiation of

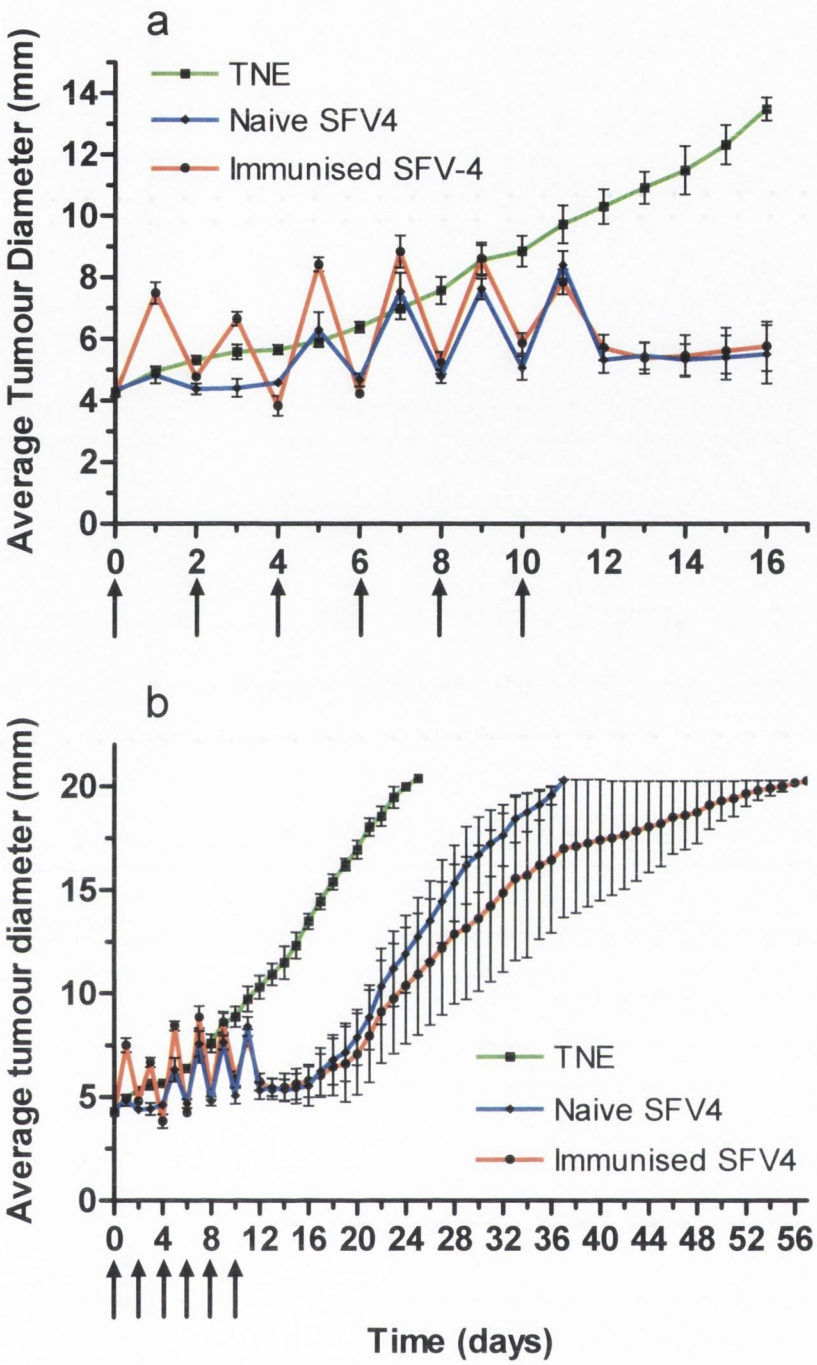


**Figure 5.1 Treatment of K-BALB tumours in immunocompetent BALB/c mice with SFV4.** K-BALB tumours were induced in naïve and rSFV-p62-6k VLP immunised female immunocompetent BALB/c mice by s.c. injection of 100 µl of K-BALB cell suspension in non-supplemented DMEM at a concentration of 1 x $10^7$ cells/ml. Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and groups of five mice were used per treatment group. Tumours received six 50 µl intratumoural injections of TNE alone or TNE containing SFV4 at a concentration of 1 x $10^{10}$ PFU/ml which were administered every other day over an eleven day period. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised when average tumour diameter reached 20 mm. *Points*; mean of five replicates, *bars*; +/- SEM, *arrows*; days of treatment. Results are representative of two individual experiments.

treatment before reaching an average diameter of 20 mm between days 32 and 37 (Figure 5.1b).

A significantly higher level of inhibition of tumour growth was achieved in the rSFV-p62-6k VLP immunised group than that observed in naïve mice ($P < 0.05$). Appendix 9.1 plots the effect of SFV4 treatment on K-BALB tumour growth along with the results from rSFV-p62-6k VLP treatment in chapter 4. Treatment of tumours in naïve mice with rSFV-p62-6k VLPs showed the lowest level of inhibition while treatment with rSFV-p62-6k VLPs in immunised mice and treatment with SFV4 in naïve mice displayed almost identical inhibition of tumour growth. These three groups were found not to be significantly different from each other. Treatment of immunised mice with SFV4 however, resulted in a significantly higher level of inhibition of K-BALB tumour growth than that observed in all other groups (Appendices 9.2 and 9.3).

### 5.2.2 Detection of anti-SFV immune responses in mice treated with SFV4

Serum and splenocytes (as described in section 2.3.3.1) were sampled in triplicate on days 1, 5, 11, and 15 from additional mice immunised with rSFV-p62-6k VLPs following induction with K-BALB tumours and subsequent treatment with SFV4 or TNE buffer.

#### 5.2.2.1 Detection of humoural anti-SFV immune responses in mice treated with SFV4

Serum samples were analysed by ELISA and assigned arbitrary anti-SFV4 IgG units by comparing absorbance values to a reference curve of known positive serum as described in section 2.3.3.2.3. Samples which fell below a cut-off absorbance of 1.5 times that of the negative control were deemed seronegative and were not assigned anti-SFV4 IgG units. Naïve (TNE treated) control mice were found to be seronegative for the duration of the experiment. On day 1 post initiation of treatment, control and SFV4-treated immunised mice displayed high levels of anti SFV4 IgG units with no detectable response in the naïve SFV4-treated group. Naive mice treated with SFV4 began to show extremely significant levels of anti-SFV4 IgG units ($P < 0.001$) at day 5 post initiation of treatment which reached comparable levels with immunised groups by day 11 (Figure 5.2). Mice were also sampled for serum from tumour growth experiments at experiment

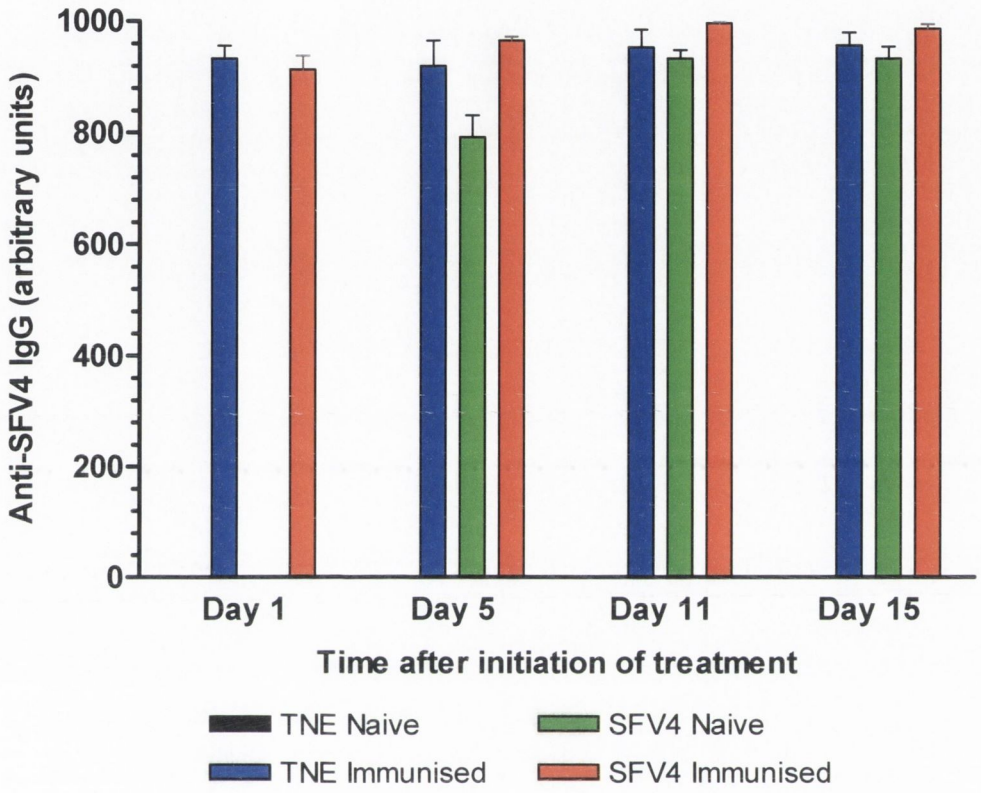


**Figure 5.2 Anti-SFV humoural immune responses in immunocompetent BALB/c mice treated with SFV4 as determined by anti-mouse IgG ELISA.** Serum samples were collected in triplicate from groups of euthanised immunocompetent BALB/c mice on days 1, 5, 11 and 15 post initiation of intratumoural treatment with SFV4. Samples were assayed utilising an anti-SFV4 IgG ELISA and assigned arbitrary anti-SFV4 IgG units from a reference curve of positive serum. All samples below 1.5 times the negative control were deemed seronegative and not signed anti-SFV4 IgG units. *Columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

end (when tumours reached an average diameter of 20 mm) and these were found to have maintained comparable levels of anti-SFV4 IgG units (not shown).

#### 5.2.2.2 Detection of cellular anti-SFV immune responses in mice treated with SFV4

Splenocytes were incubated in the presence of conA, medium alone, or purified UV-inactivated SFV4 for 72 h after which supernatants were analysed for secreted IFN-γ as described in section 2.3.3.3. Incubation with conA resulted in the secretion of relatively high levels of IFN-γ in the splenocyte populations examined. Figure 5.3 shows the amounts of IFN-γ secreted by splenocytes and Figure 5.4 expresses these data as stimulation indices which were calculated by dividing amounts of IFN-γ in SFV4 stimulated wells by the amount secreted in negative control wells. Naïve mice treated with SFV4 showed a peak in stimulation at day 5 which then dropped off to a lower but still significant level at day 15 ($P < 0.001$). Immunised mice treated with TNE maintained a significant level of stimulation throughout the experiment ($P < 0.001$). Significantly higher levels of stimulation were noted in immunised mice treated with SFV4 than in immunised control mice at days 1 and 11 post initiation of treatment ($P < 0.005$).

### 5.2.3 Detection of infectious SFV4 virus in tumours and brains and pathological examination of brain tissue from mice treated with SFV4

As SFV4 is a neurotropic virus which is virulent in BALB/c mice, it was of interest to monitor the possible spread of the virus into the central nervous system and the appearance of any clinical or pathological changes associated with SFV4 infection. Groups of mice were immunised and induced with K-BALB tumours which were subsequently treated with SFV4 or TNE buffer and sampled in triplicate on days 1, 5, 11 and 15 post initiation of treatment. Brains and tumours were homogenised and clarified by centrifugation as described in section 2.3.2.4.1, and titres of SFV4 determined by plaque assay of resulting supernatants as described in section 2.3.2.4.1. Brains from mice used in the pathological studies were examined histologically for the presence of lesions characteristic of SFV4 infection as described in section 2.4.1.1. No immunised mice presented clinical signs of SFV4 infection whereas two of a total of 35 naïve mice treated with SFV4 displayed dullness and anorexia commencing on day 4 post initiation

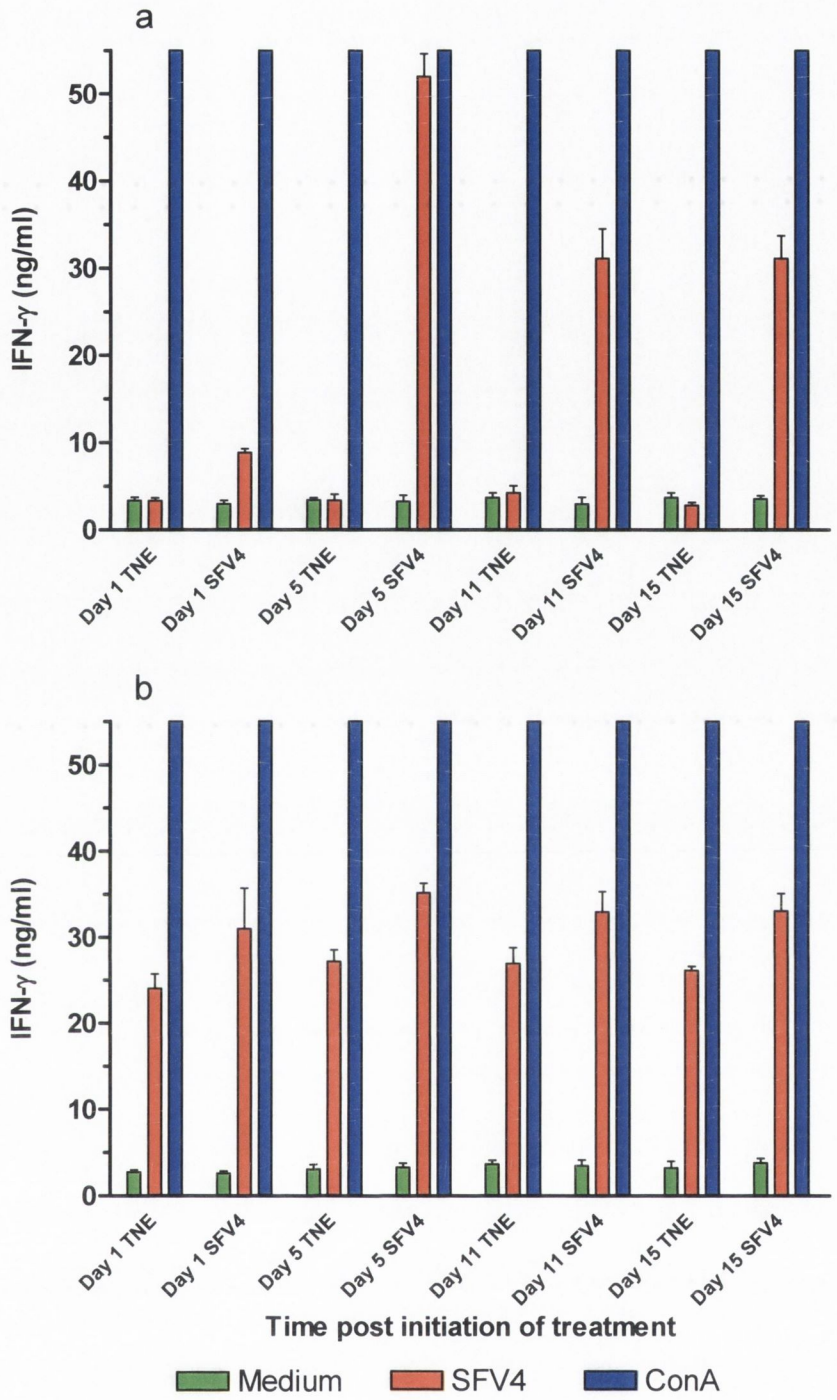


**Figure 5.3 IFN-γ secretion, as determined by capture ELISA, by splenocytes from naïve (a) and immunised (b) immunocompetent BALB/c mice treated with SFV4 cultured *ex vivo* in the presence of medium alone, UV-inactivated SFV-4 or ConA.** Splenocytes were harvested from immunocompetent BALB/c mice in triplicate at days 1, 5, 11 and 15 post initiation of treatment and cultured in the presence of medium alone, UV-inactivated SFV4 (1 x $10^5$ PFU/well) or ConA (5 μg/ml) for 72 h. Supernatants were clarified by centrifugation and concentrations of IFN-γ determined by capture ELISA. *Columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments.

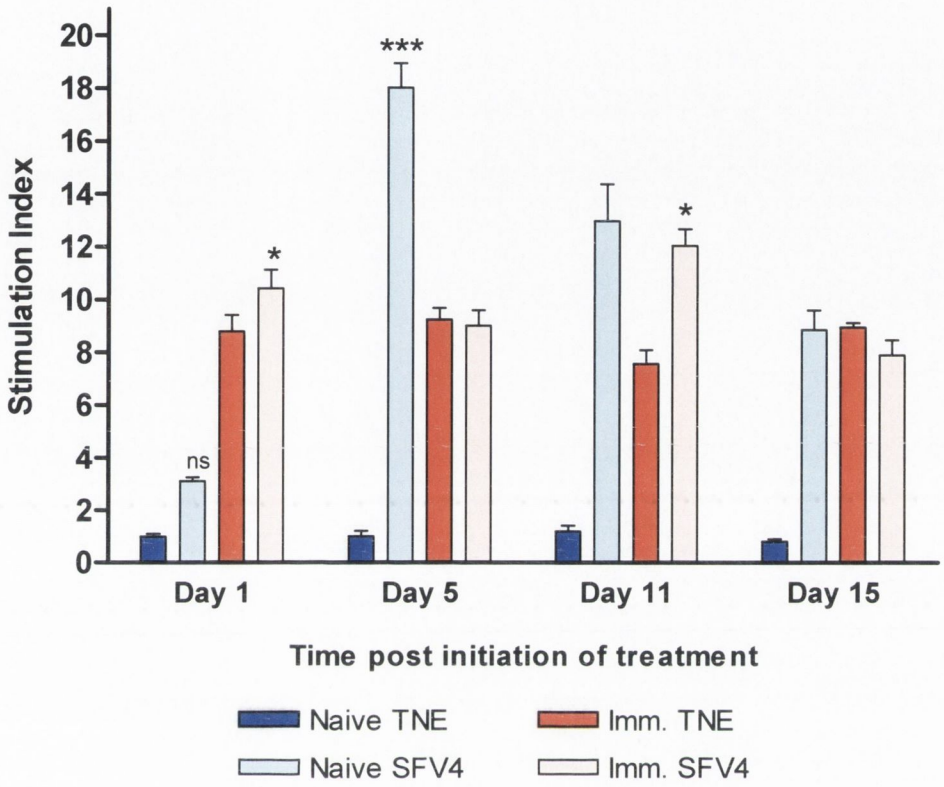


**Figure 5.4 Stimulation indices of splenocytes from immunocompetent BALB/c mice treated with SFV4 to SFV4 antigens.** Splenocytes were incubated in triplicate with UV-inactivated SFV4 for 72 h and levels of IFN-γ secretion were determined by capture ELISA. Stimulation indices were calculated by dividing amounts of IFN-γ in SFV4 stimulated wells by the amount secreted in negative control wells. *Columns*; mean of three replicates, *bars*; +/- SEM. Results are representative of three independent experiments. Ns = not significant, * = $P < 0.05$, *** = $P < 0.001$, SFV-4 treated versus appropriate TNE control.

of treatment. These two mice were in groups sampled for brain pathology and detection of replicating SFV4 and were sampled accordingly on day 5.

All tumours sampled at day 15 were negative for infectious virus, whereas virus was detected in all tumours sampled prior to this time, with titres typically between 1 x $10^4$ and $10^5$ PFU/mg. No significant difference was noted between naïve and immunised groups (not shown). All brain homogenates from immunised groups were negative for infectious virus. For the two mice from the naïve group which showed clinical signs of SFV4 infection prior to sampling, one was positive for SFV4 replicating virus and one showed brain lesions characteristic of SFV4 infection which were confirmed positive by immunohistochemical detection of SFV4 antigen as described in section 2.4.2.3 (Figure 5.5). Brain samples from all other mice appeared normal and homogenates were negative for infectious virus. All 35 SFV4-treated immunised mice from tumour growth, immunological, infectious virus and pathological studies were apparently fully protected against challenge with SFV4 by prior administration of rSFV-p62-6k VLPs.

### 5.2.4 Pathology of paraffin embedded, formalin fixed tumour tissue

Histological examination of H&E stained sections of paraffin embedded tumours revealed comparable results with those observed with rSFV-p62-6k treatment discussed in chapter 4 (section 4.2.5). Control tumours were initially composed of sheets of spindle cells, regressing over time to the poorly differentiated polyhedral phenotype as previously described (section 4.2.1).

Tumours from immunised mice sampled on day 1 displayed acute inflammation and necrosis. Islands of spindle-shaped tumours cells were separated by oedema, fibrin and large numbers of infiltrating neutrophils and macrophages. Lymphocytes and plasma cells were localised in clusters in perivascular and perineural areas. Lymphocytes were also apparent in perivascular and perineural areas in tumours excised from naïve mice and an increase in the neutrophilic infiltrate was also apparent, but the inflammation and oedema described above was not observed.

By day 5 tumours from immunised mice were characterised by leukocytic infiltrates composed of lymphocytes, neutrophils and macrophages. Lymphocytes and plasma cells were clustered in perivascular and perineural areas and islands of tumour were loosely infiltrated with leukocytes (Figure 5.6). Tumours from naïve mice appeared similar to those present in immunised mice on day 1, as described above.

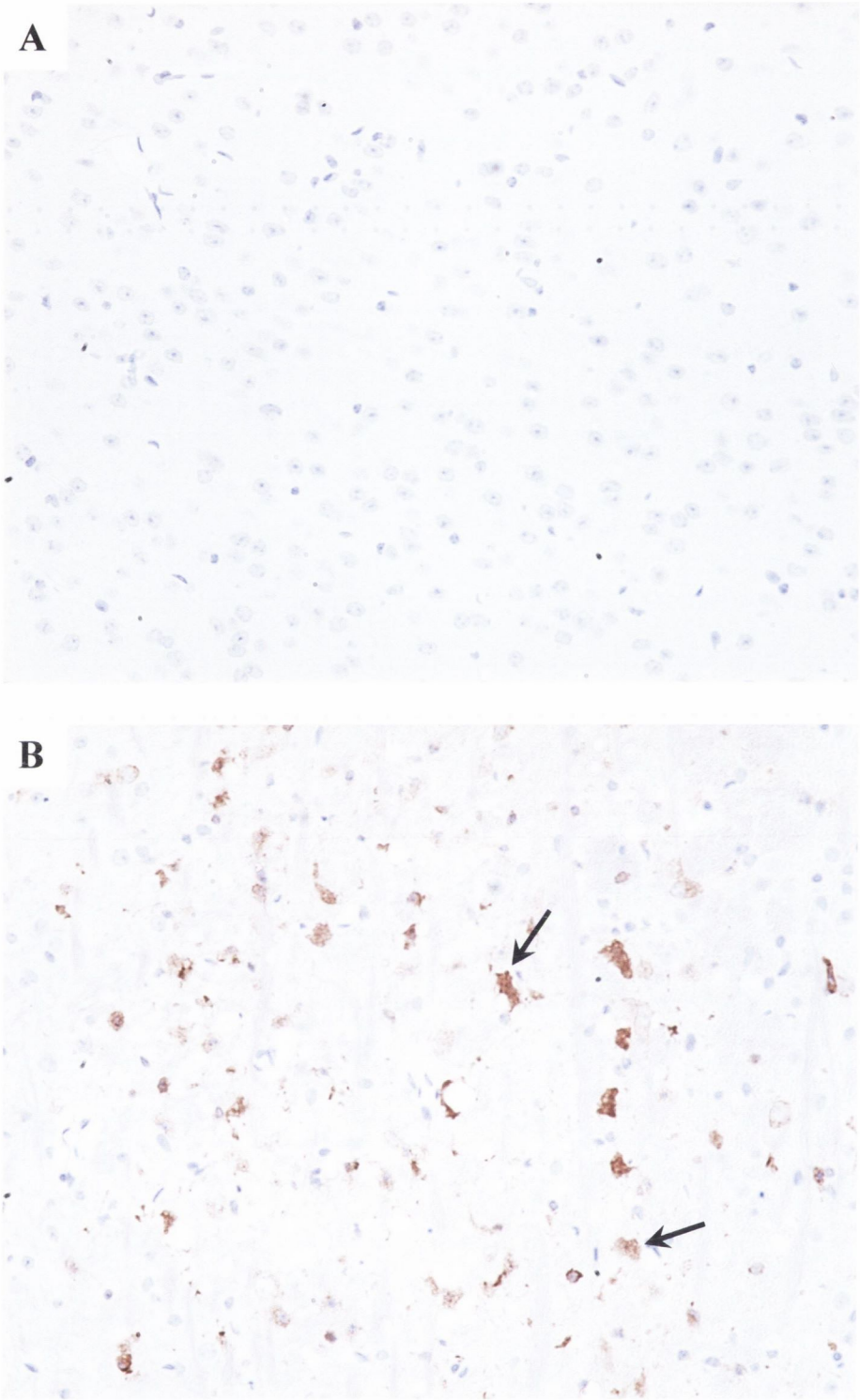


**Figure 5.5 Immunohistochemical detection of SFV4 antigen in brains of BALB/c mice 5 days post initiation of treatment with SFV4**

**(A)** Brain from an immunised mouse which was histologically normal and negative for SFV4 antigen, 200x

**(B)** Brain from naïve mouse which showed clinical signs of SFV4 infection displaying SFV4 antigen localised in neurons (*arrows*), 200x

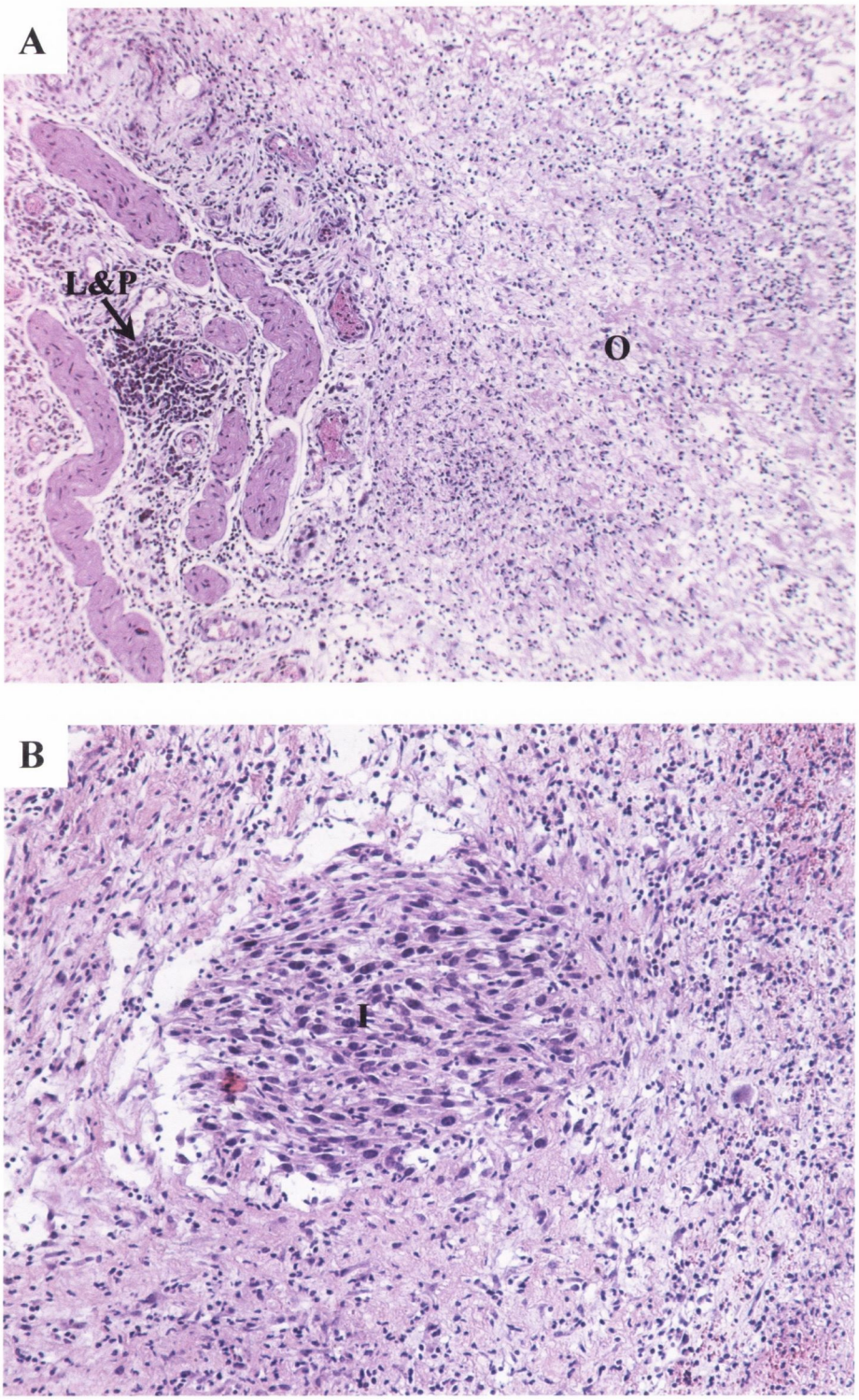


**Figure 5.6 Inflammation in tumours of immunised mice 5 days post initiation of treatment with SFV4**

**(A)** Acute inflammatory oedema (O) with fibrin and neutrophils; lymphocytes and plasma cells (L&P) are concentrated around blood vessels and nerve fibres towards the periphery of the tumour, 100x

**(B)** An island of apparently uninfected tumour cells (I) surrounded by inflammation and oedema (B), 200x

***Corresponding control tumours were reminiscent of those shown in figures 4.2 & 4.3***

Treated tumours from immunised mice maintained their spindle morphology at day 11 in contrast to control tumours which had regressed to pleomorphic poorly-differentiated phenotypes by this time. Sheets of plasma cells were present in perivascular and perineural spaces in immunised groups along with large numbers of lymphocytes. The leukocytic infiltrate in inflamed areas was comprised of high numbers of lymphocytes with neutrophils and macrophages. Treated tumours from naïve mice displayed a similar pattern of inflammation but with fewer plasma cells in perineural and perivascular areas.

Oedema had subsided completely by day 15 at which point spindle shaped tumour cells were still heavily infiltrated with lymphocytes, neutrophils and macrophages (Figure 5.7a). Sheets of plasma cells encircled nerves and blood vessels and large numbers of lymphocytes were distributed among the tumour cells in tumours from both immunised and naïve mice (Figure 5.7b). Russell bodies were frequently observed in reactive plasma cells and increased numbers of mast cells were common.

Overall, studies revealed pathology comparable to that observed in tumours treated with rSFV-p62-6k VLPs (section 4.2.5). Inflammation did appear, however, to be more intense in tumours treated with SFV4, particularly at day 15 where higher numbers of TILs were present (Figures 4.11a and 5.7a). The cellular infiltrate is further characterised in chapter 6 along with detection of SFV antigen and apoptotic cells within tumours using immunohistochemistry and immunofluorescence. To conclude, treatment of K-BALB tumours in BALB/c mice with SFV4 resulted in inflammatory changes similar to those described in chapter 4 following treatment with rSFV-p62-6k VLPs. Neutrophils and macrophages were among the first leucocytes to infiltrate the tumours and were followed later by lymphocytes and plasma cells. Plasma cells did not infiltrate among the tumour cells to the same extent as other leukocytes and remained in perivascular and perineural locations. Prior immunisation with rSFV-p62-6k VLPs resulted in a more immediate and sustained response than that observed in the naïve group.

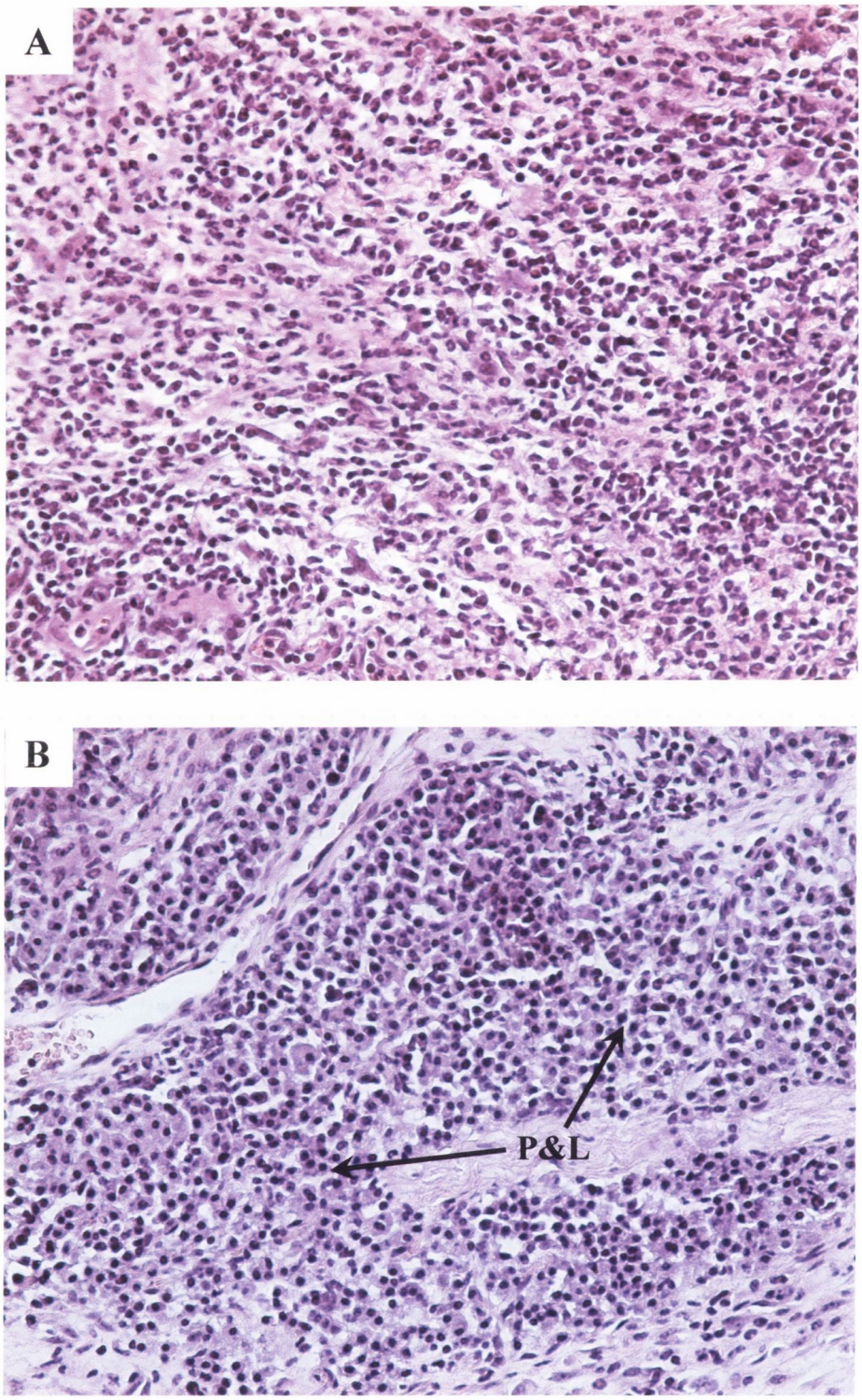


**Figure 5.7 Cellular infiltrate at 15 days post initiation of treatment of K-BALB tumours in immunised mice with SFV4**

**(A)** Lymphocytes were observed in blood vessels and among spindle-shaped tumours cells, 200x

**(B)** Dense sheets of plasma cells and lymphocytes (P&L) around nerve fibres and blood vessels, 200x

***Corresponding control tumours were reminiscent of those shown in figures 4.2 & 4.3***

## 5.3 DISCUSSION

The potential for the exploitation of viruses in the treatment of cancer first came to light a century ago when cases of regression in cervical cancers were noted following administration of attenuated rabies vaccine (Dock, 1904; De Pace, 1912). Natural viral infections have also been associated with remissions in malignancies and several clinical trials of 'virotherapy' were carried out during the 1950s using a variety of wild-type strains of viruses, but side-effects were common and tumour inhibition/regression rarely sustained (Newman & Southam, 1954; Huebner *et al.*, 1956). Several viruses with apparent natural oncotropic and/or oncolytic activity have been described including NDV, reovirus and more recently, VSV. In recent years, clinical trials with reovirus and a naturally attenuated vaccine strain of NDV (PV701) are yielding promising results with limited virus-associated toxicities (Shah *et al.*, 2003; Lorence *et al.*, 2003).

The advent of recombinant DNA technology and an increasing understanding of the molecular mechanisms involved in carcinogenesis and viral replication has allowed for the further development of replicating viruses as tools in the treatment of malignancies. Strategies have involved the deletion of specific viral genes and the isolation of oncotropic mutants but it has also become possible to enhance the oncolytic abilities of viruses with the expression of cytotoxic proteins, drug sensitivity genes and cytokines (Bergsland & Venook, 2002; Ring, 2002). Adenoviruses, such as *dl*1520 (a.k.a. ONYX-015) have dominated clinical trials with engineered/mutated oncolytic viruses and have progressed to phase III trials which are currently underway with the *dl*1520 mutant. Other viruses which have been engineered for oncolytic applications have been derived from HSV, measles, and vaccinia viruses (Vile *et al.*, 2002).

Recent studies illustrating the apparent oncotropic capabilities of SV vectors provide encouraging data for the use of (closely related) SFV vectors in cancer therapy (Tseng *et al.*, 2002; Tseng *et al.*, 2004). The potential for a replicating SFV-derived vector was examined in this chapter using the infectious clone SFV4 as a model. The fact that SFV is a neurotropic virus raises biosafety concerns which must be addressed in the development of such a vector for clinical applications. rSFV-p62-6k VLPs conferred protection against challenge with SFV4 administered by i.t. injection, with no replicating virus or lesions associated with SFV infection detected in brains. Only 2 of 35 naïve mice treated with SFV4 were found to have succumbed to infection, a relatively low number considering the viral load administered, and previous studies of SFV4 virulence in mice (Glasgow *et al.*, 1991). This could be due to the (essentially peripheral) route of

administration and possible preferential growth of the virus within the tumour microenvironment. It is now accepted that the non-structural region of the SFV genome, (specifically the nsP2 and nsP3 regions) plays a major role in the virulence of the virus (Fazakerley *et al.*, 2002; Tuittila and Hinkkanen; 2003). Apart from vaccination, manipulation of this region of the genome could possibly yield an avirulent replicating vector with low/no neuropathogenicity.

Treatment of K-BALB tumours with SFV4 resulted in a significant inhibition of tumour growth and the induction of an acute inflammatory response comparable to that observed with rSFV-p62-6k treatment as reported in chapter 4. Apart from conferring protection against SFV4 infection, prior immunisation with rSFV-p62-6k VLPs resulted in a significant enhancement of the antitumoural effect. Inflammatory responses manifested macroscopically as tumour swelling which was apparent at 1 day post initiation of treatment in immunised mice and at 5 days post initiation of treatment in naïve groups. Pathological studies revealed this swelling to be due to acute inflammation and oedema with accompanying high numbers of TILs. It was concluded that immunisation results in a more immediate, intense, and sustained inflammatory reaction directed at SFV antigens (and the cells expressing them) in the tumour microenvironment, successfully augmenting the effect of the treatment.

Humoural and cellular immune responses detected *ex vivo* using ELISA and splenocyte stimulation assays respectively revealed strong immune responses to SFV4 from the outset in immunised mice and from 5 days post initiation of treatment in naive mice. Similar temporal differences relating to the development of inflammation in tumours from naïve and immunised were apparent in the histological studies. The composition of the cellular infiltrate was comparable to that observed in rSFV-p62-6k treatment studies (chapter 4) but higher numbers of TILs were apparent, particularly at later timepoints. The high levels of IFN-γ produced by SFV4-stimulated splenocytes and the severe inflammation and oedema observed provide evidence for a strong $T_H1$-mediated immune response. It is possible that cellular and humoural immune responses were both instrumental in tumour cell destruction and the overall inhibition of tumour growth achieved by the treatment (Reilly *et al.*, 2001). The cellular infiltrate observed in pathological studies was further characterised by immunohistochemistry and is discussed in greater detail in chapter 6.

Complete tumour regression was noted in one out of the five immunised mice treated with SFV4. The mouse remained tumour-free for over two weeks but some tumour cells must have survived, however, as the tumour then regrew. This underlines

the difficulties experienced in the treatment of cancer as theoretically even one surviving cell has the potential to form a new tumour. Although inhibition of tumour growth was significantly higher than that observed with rSFV-p62-6k VLPs (Appendices 9.1, 9.2 and 9.3) the failure of SFV4 to fully disseminate through tumours and achieve total cell killing was somewhat surprising given that it is replication competent. It is likely that the low infection efficiency of the K-BALB cell line, together with the presence of neutralising antibodies, hindered the replication and spread of the virus.

While the studies described in this chapter do provide evidence for the potential of a replicating SFV vector as a cancer therapy agent, the inhibition of tumour growth achieved was lower than expected. The expression of cytotoxic factors could enhance the antitumoural effect or cytokine expression could possibly be employed in order to modulate immune responses against the vector. As SFV is an RNA virus, a derived replicating vector would not persist in the same manner as those derived from DNA viruses. This characteristic, together with its inherent cytopathic ability, render a replicating SFV vector a good candidate for the field of cancer therapy but improving the efficacy and ensuring the biosafety of the vector system requires additional research.

# 6

# CHARACTERISATION OF CELLULAR INFILTRATE AND DEMONSTRATION OF APOPTOSIS AND SFV ANTIGEN EXPRESSION *IN VIVO*

## 6.1 INTRODUCTION

As mentioned previously, H&E staining of paraffin embedded formalin-fixed tissue sections is routinely used for histopathology. A variety of structures and cell-types can be identified with confidence by this method but further and more definitive characterisation is often necessary, and can usually be achieved by employing immunohistochemical techniques. Formaldehyde-based fixatives are advantageous in that they penetrate specimens well and provide good preservation of morphological detail, when compared with other fixatives such as alcohol and acetone. This serves well for routine staining of tissues but difficulties can arise when analysing these tissues immunohistochemically, as many epitopes are often masked by such fixation. It is now possible to detect many of these masked antigens through a process termed heat-induced epitope retrieval (HIER), a technique first described by Shi *et al* in 1991. Since then the use of different buffers and temperatures in HIER has enabled the detection of a broad array of previously undetectable antigens. The precise mechanisms by which formalin fixation and HIER occur remain unclear, but heat-dependant hydrolysis of adducts formed by formalin fixation with re-naturation of protein has been suggested (Shi *et al.*, 2001). Although many antigens are now detectable in formalin-fixed tissues some, such as murine CD4 and CD8, remain either completely undetectable or only detectable at low, unsatisfactory levels. Acetone-fixed cryostat sections of snap-frozen tissue provide excellent preservation of immunoreactive sites (including leukocyte markers) but this is accompanied by a loss in morphological detail and resolution.

Treatment of K-BALB tumours with rSFV-p62-6k VLPs or SFV4 resulted in an acute inflammatory reaction detectable at day 1 post initiation of treatment in groups previously immunised with rSFV-p62-6k VLPs and at day 5 post initiation of treatment in naïve groups (chapters 4 and 5). Although SFV4 treated tumours displayed a more intense and sustained response, the composition of the inflammatory infiltrate was comparable between groups treated with rSFV-p62-6k VLPs and those treated with SFV4. For this reason, both groups will be discussed together and referred to collectively as 'treated' in this chapter. Due to the extremely high numbers of TILs observed and density of many of the leukocytic clusters, it was not possible to accurately enumerate individual cell types and so results are based on qualitative, rather than quantitative, observations.

In this chapter, immunohistochemistry was employed in order to further characterise the cellular infiltrate observed. The following markers were identified in

formalin-fixed, paraffin-embedded sections following HIER: T-lymphocyte marker CD3, B-lymphocyte (and plasmacytoid dendritic cell) marker CD45R, and active caspase-3 as a marker for apoptosis. Acetone-fixed frozen sections were employed for the detection of the T-lymphocyte subset markers CD4 and CD8, B-lymphocyte marker CD19, macrophage marker F4/80, and SFV antigen. Acetone-fixed frozen sections and immunofluorescence were utilised in the detection of cells double-labelled with SFV antigen and either CD4, CD8, or F4/80 leukocyte markers. Large numbers of $CD4^+$ T-lymphocytes were detected among the leukocytic infiltrates along with $CD8^+$ cells. $CD45R^+$ and $CD19^+$ B-lymphocytes were present but only in occasional clusters. The high numbers of T-lymphocytes, neutrophils and macrophages, together with oedema, suggest that a $T_H1$-mediated inflammatory immune response predominated.

## 6.2 RESULTS

### 6.2.1 Demonstration of apoptosis in K-BALB tumour tissue by detection of active caspase-3

K-BALB tumours from perfused mice treated with rSFV-p62-6k VLPs, SFV4 or TNE alone were excised and processed for paraffin embedding as described in section 2.4.1.1. Active caspase-3 was detected by performing immunohistochemistry on tumour sections utilising HIER to unmask epitopes (section 2.4.2.3). Cells positive for active caspase-3 were concentrated at the interface between viable tumour cells and areas of necrosis, indicating a possible role for apoptosis in the development of necrosis and thrombosis within the tumours (Figure 6.1a). Occasional individual immunopositive cells were also noted throughout the tumour tissue. Cells positive for active caspase-3 typically displayed morphological characteristics associated with apoptosis such as nuclear condensation and cell shrinkage with the formation of apoptotic bodies (Figure 6.1b). As areas of necrosis were present in most tumours, control and treated, it was not possible to determine quantitatively whether there was an increase in apoptosis tumours due to treatment with rSFV-p62-6k VLPs or SFV4. Inflamed areas of treated tumours, however, were scattered with active caspase-3-positive cells and cellular debris separated by TILs and oedema (Figure 6.2).

### 6.2.2 Characterisation of cellular infiltrate in K-BALB tumours treated with rSFV-p62/6k VLPs and SFV4

K-BALB tumours from perfused mice treated with rSFV-p62-6k VLPs, SFV4 or TNE alone were excised and processed for paraffin embedding as described in section 2.4.1.1. Immunohistochemistry with HIER was employed to detect CD3 and CD45R antigens in sections of these paraffin embedded formalin-fixed tumours (section 2.4.2.3). Acetone-fixed cryosections of snap-frozen tumours were used for the immunohistochemical detection of CD4, CD8, CD19, F4/80 and SFV antigens (sections 2.4.1.2 and 2.4.2.4).

Low numbers of leukocytes positive for the above markers were located primarily in perivascular spaces at the periphery of control tumours at all time points. Of these, B-lymphocytes ($CD45R^+$, $CD19^+$) and macrophages ($F4/80^+$) were most common

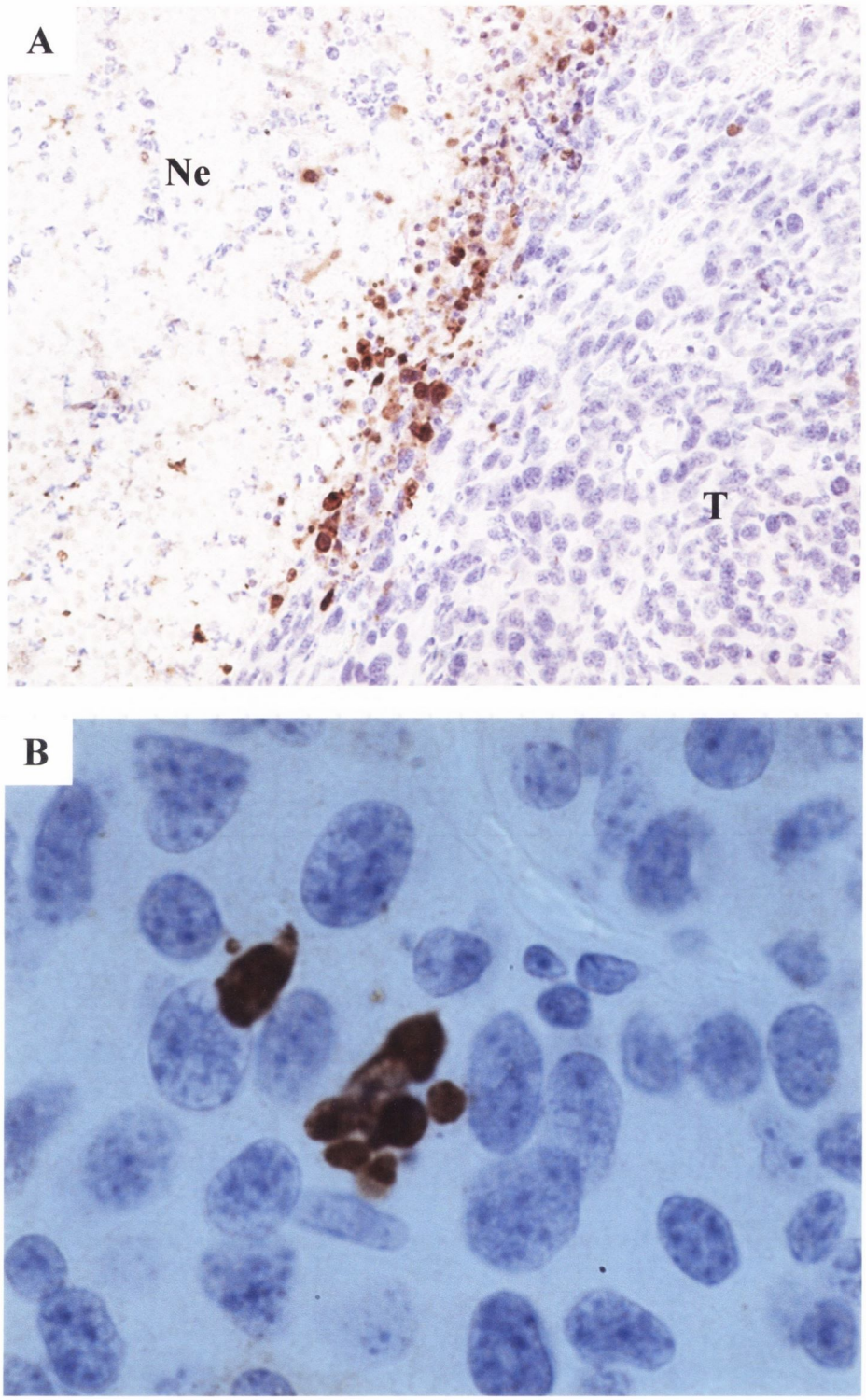


**Figure 6.1 Demonstration of apoptotic forms in control K-BALB tumours by immunohistochemical detection of active caspase-3**

**(A)** Apoptotic cells (brown) concentrated at the interface between viable tumour cells (T) and necrotic areas (Ne), 200x

**(B)** Cells positive for active caspase-3 showing shrinkage and compartmentalisation into apoptotic bodies, 1000x

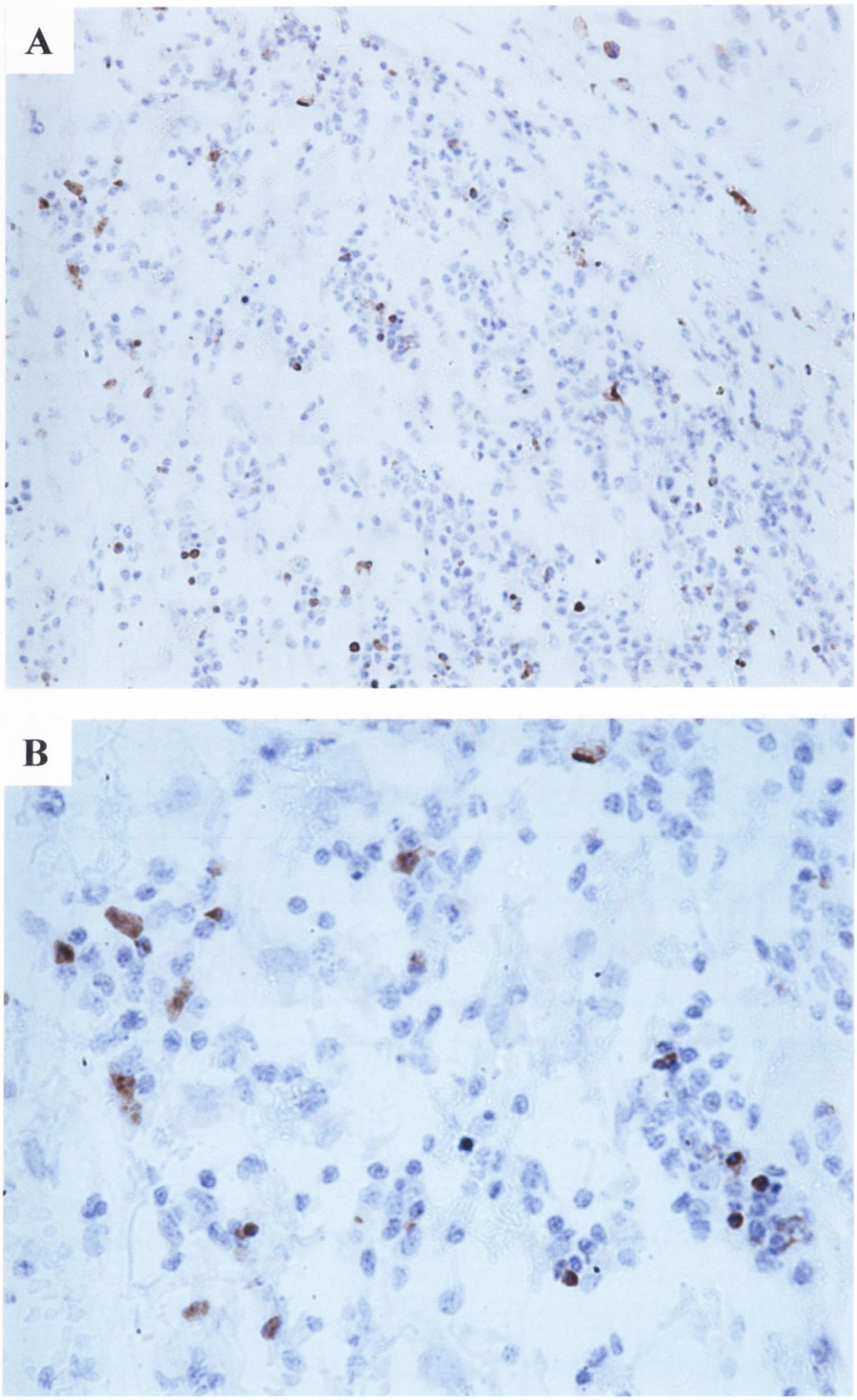


**Figure 6.2 Demonstration of apoptotic forms in rSFV-p62-6k VLP treated K-BALB tumours from immunised mice at day 5 post initiation of treatment**

**(A)** Apoptotic cells positive for active caspase-3 (brown) separated by TILs and oedema, 200x

**(B)** Higher magnification of above, 400x

***Corresponding control tumours were reminiscent of those shown in figure 6.1***

with only very occasional cells positive for the T-lymphocyte markers CD3, CD4 and CD8 (Figure 6.3).

At the initial onset of inflammation in treated tumours (day 1 in immunised groups, day 5 in naïve groups), a major increase in $CD3^+$ cells was observed in perivascular and perineural areas and in areas of inflammatory oedema. These T-lymphocytes were predominantly $CD4^+$ and were accompanied by lower numbers of $CD8^+$ cells. As mentioned previously, neutrophils predominated as the major TIL at this point (sections 4.2.5 and 5.2.4) along with $F4/80^+$ macrophages which also occurred in high numbers. An increase in B-lymphocytes ($CD45R^+$, $CD19^+$) was also apparent but to the level of increase was lower than that observed for T-lymphocytes.

$CD3^+$ T-lymphocytes joined neutrophils and $F4/80^+$ macrophages as the main TILs infiltrating treated tumours from day 5 in immunised groups and day 11 in naïve groups (Figure 6.4a). Clusters of B-lymphocytes ($CD45R^+$, $CD19^+$) were present at perivascular and perineural areas but did not infiltrate among the tumour cells in the same manner as $CD3^+$ T-lymphocytes (Figure 6.4b). Sheets of $CD3^+$ T-lymphocytes, of which the majority were $CD4^+$, encircled blood vessels and nerves (Figure 6.5a). $CD8^+$ cells were also increased in number and were located in perivascular and perineural areas as well as throughout the tumour tissue (Figure 6.5b). A clear difference was noted between naïve and immunised groups regarding the relative distribution of T-lymphocytes, with a much denser $CD4^+$ infiltrate detected in immunised groups (Figure 6.6). $F4/80^+$ macrophages were detected in high numbers infiltrating throughout the tumour tissue and clustered with lymphocytes in perivascular and perineural areas in all inflamed tumours (Figure 6.7). Inflammation was sustained in treated tumours from immunised mice sampled at day 11, with comparable numbers of $CD4^+$ and $CD8^+$ cells present throughout the tumours from this point onwards.

While oedema had subsided in tumours sampled at day 15 post initiation of treatment, a high number of TILs remained (Figure 4.12a). These cells were predominantly T-lymphocytes ($CD3^+$) with neutrophils and macrophages ($F4/80^+$) present, but at lower levels. B-lymphocytes were also present, but at similar levels to those observed at previous timepoints. Treated tumours from immunised mice displayed higher numbers of TILs at this point than treated tumours from naïve groups. Overall, T-lymphocytes, neutrophils, and macrophages predominated as the major TILs in treated tumours with B-lymphocytes present, but at a substantially lower level. Plasma cells were apparent in high numbers at the later timepoints, as discussed in chapters 4 and 5. The T-lymphocyte population was initially composed mainly of cells belonging to the

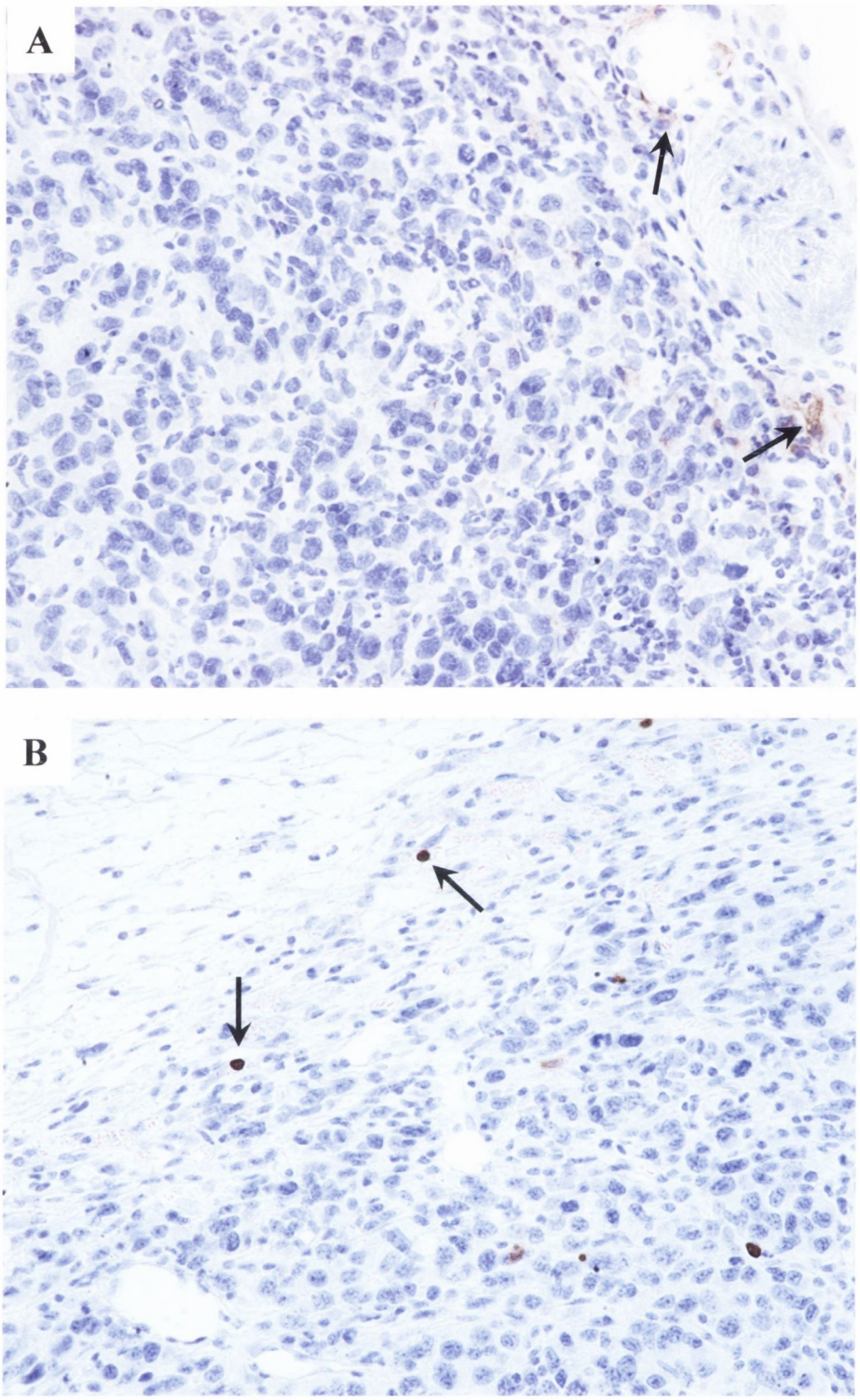


**Figure 6.3 Demonstration of F4/80$^+$ macrophages and CD45R$^+$ B-lymphocytes in control K-BALB tumours 5 days post initiation of treatment**

**(A)** Occasional F4/80$^+$ macrophages (*arrows*) in perineural and perivascular areas, 200x

**(B)** Occasional CD45R$^+$ B-lymphocytes (*arrows*) at the periphery of the tumour, 200x

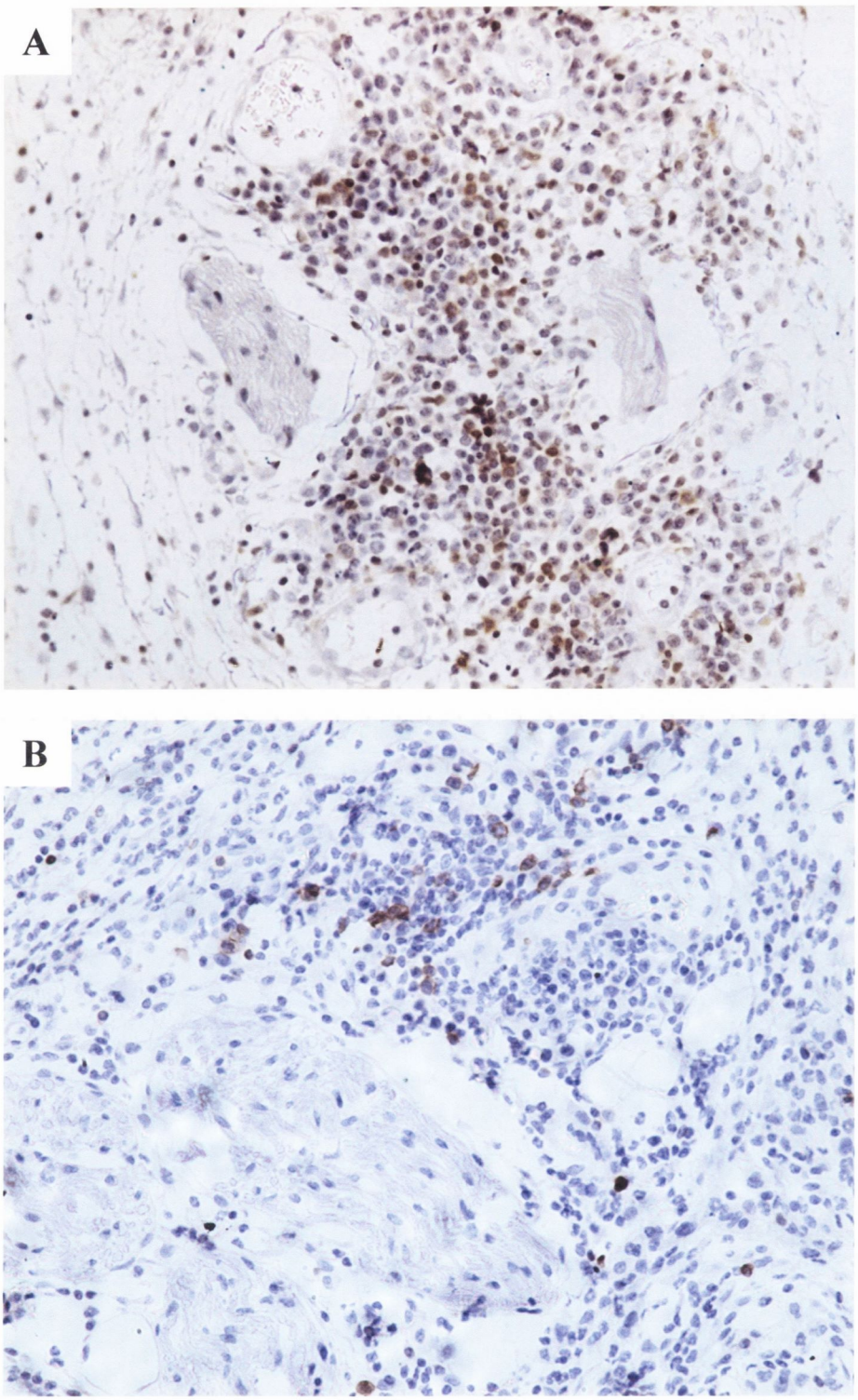


**Figure 6.4 Demonstration of $CD3^+$ T-lymphocytes and $CD45R^+$ B-lymphocytes in SFV4 treated K-BALB tumours from naïve mice at day 5 post initiation of treatment**

**(A)** Large numbers of $CD3^+$ T-lymphocytes (brown) in perivascular and perineural areas at the periphery of the tumour, 200x

**(B)** Moderate numbers of $CD45R^+$ B-lymphocytes (brown) in perivascular and perineural areas at the periphery of the tumour, 200x

***Corresponding control tumours were reminiscent of those shown in figure 6.3***

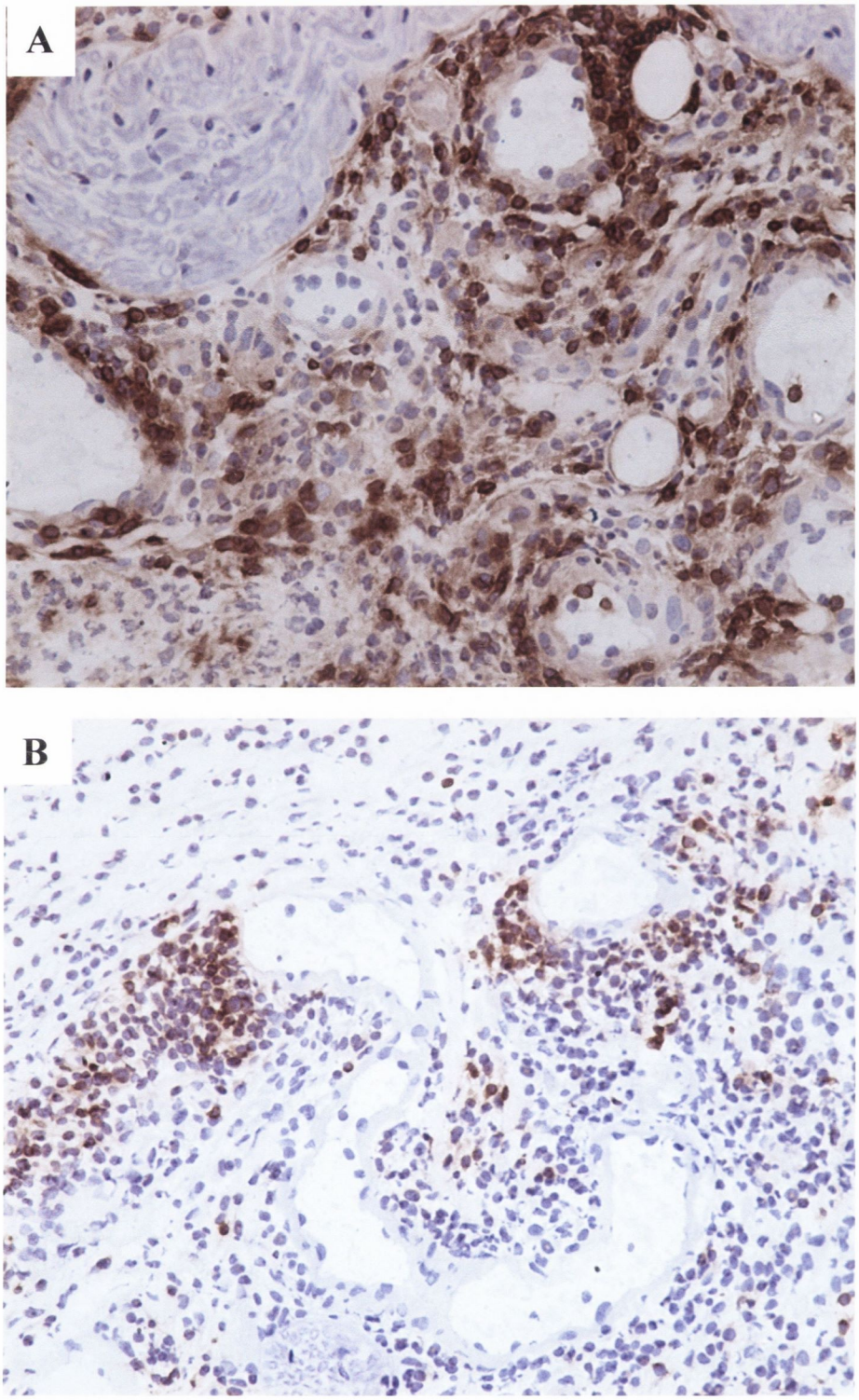


**Figure 6.5 Demonstration of $CD4^+$ and $CD8^+$ T-lymphocytes in rSFV-p62-6k VLP treated K-BALB tumours from immunised mice 5 days post initiation of treatment**

**(A)** Large numbers of $CD4^+$ T-lymphocytes (brown) in perivascular and perineural areas at the periphery of tumour, 200x

**(B)** Large numbers of $CD8^+$ T-lymphocytes (brown) in perivascular and perineural areas at the periphery of tumour, 200x

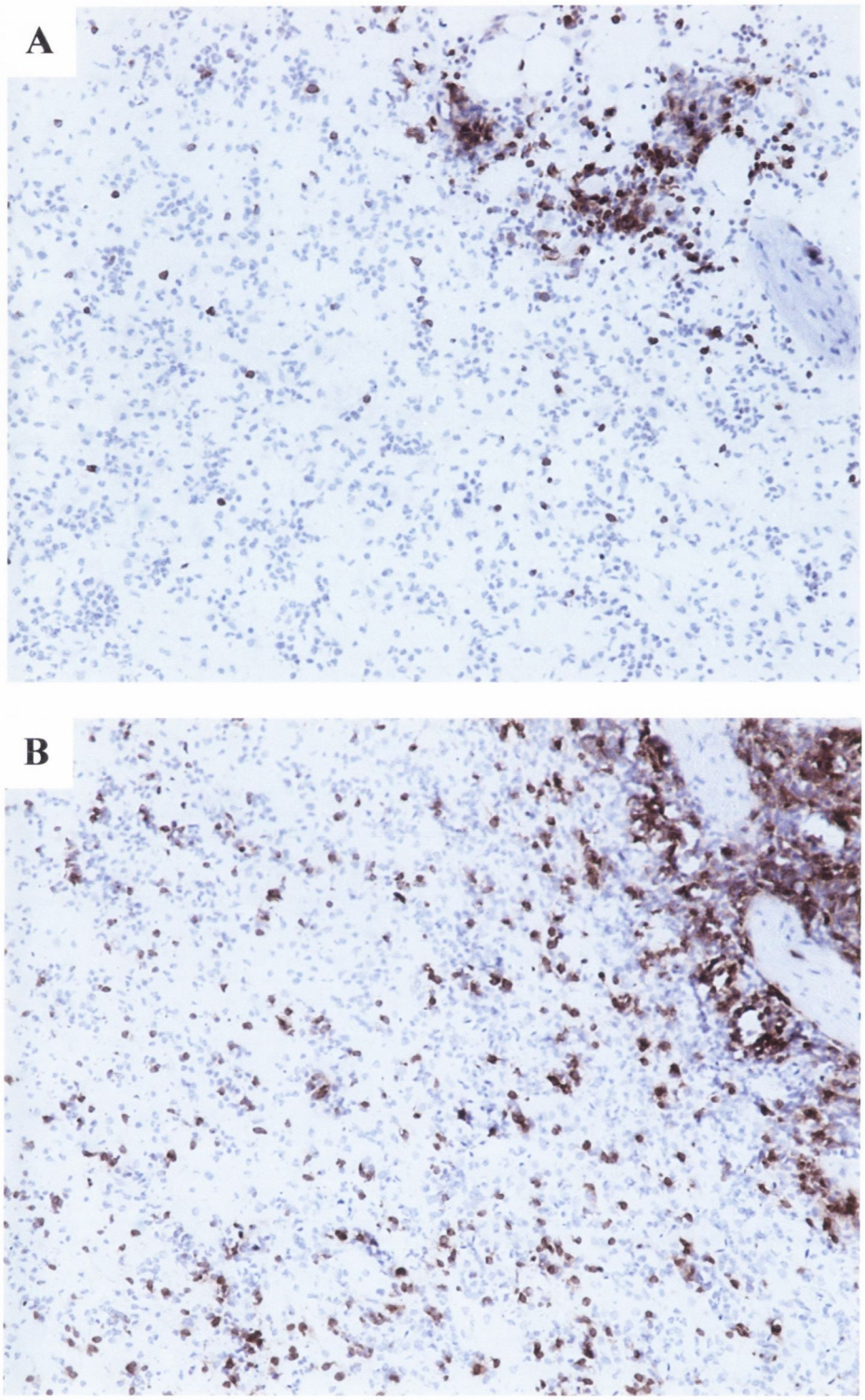


**Figure 6.6 Relative distribution of $CD4^+$ T-lymphocytes in tumours from naïve and immunised mice at day 5 post initiation of treatment with rSFV-p62-6k VLPs**

**(A)** Tumour from naïve mouse, moderate numbers of $CD4^+$ T-lymphocytes (brown) mostly localised at the periphery of the tumour, 100x

**(B)** Tumour from immunised mouse, large numbers of $CD4^+$ T-lymphocytes (brown) at the periphery of the tumour (right) and randomly distributed among the tumour cells, 100x

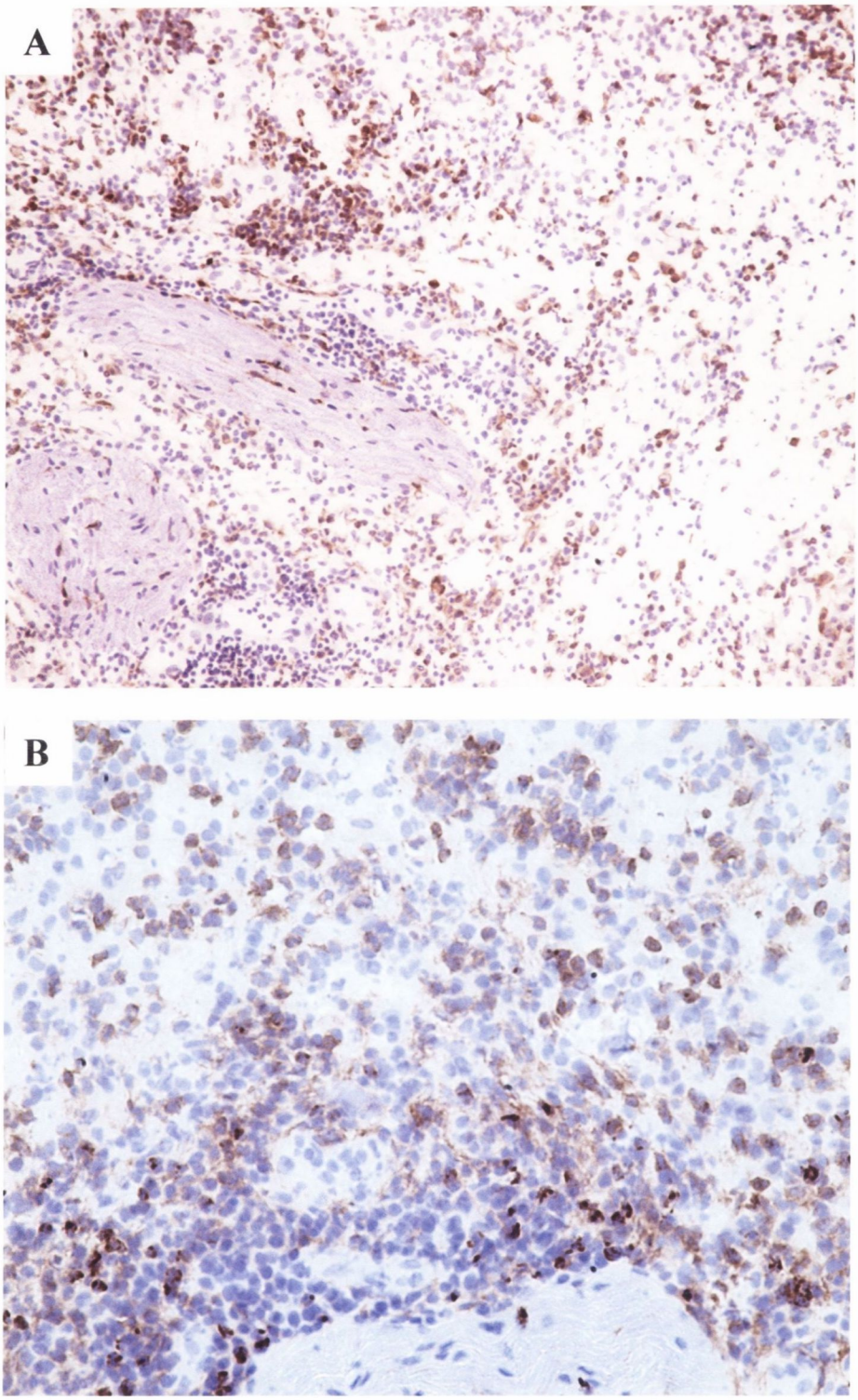


**Figure 6.7 Distribution of F4/80$^+$ macrophages in a K-BALB tumour treated with SFV4 from a naïve mouse at day 5 post initiation of treatment**

**(A)** F4/80$^+$ macrophages (brown) in perivascular and perineural areas and infiltrating throughout the tumour cells, 100x

**(B)** Higher magnification of above, 200x

***Corresponding control tumours were reminiscent of those shown in figure 6.3***

CD4 subset, but comparable numbers of $CD8^+$ T-lymphocytes were present as the inflammatory response developed.

### 6.2.3 Demonstration of SFV antigen in K-BALB tumours and immunofluorescent double-labelling of leukocyte markers in tandem with SFV antigen

Acetone-fixed cryosections were of snap-frozen tumours directly excised from mice (section 2.4.1.2) were used for the immunohistochemical detection of SFV antigen. Double immunofluorescent labelling of SFV antigen along with the leukocyte markers CD4, CD8, and F4/80 was also employed as described in section 2.4.2.4. SFV antigen was detected exclusively in inflamed areas of treated tumours and occurred as granular extra-cellular deposits as intracytoplasmic deposits in macrophage-like cells (Figure 6.8). Cells positive for SFV antigen were separated by oedema and TILs and concentrated areas of SFV antigen expression were not observed. Macrophages ($F4/80^+$) with intracytoplasmic deposits of SFV antigen were demonstrated using double-labelling immunofluorescence (Figure 6.9). $CD8^+$ lymphocytes commonly occurred adjacent to SFV $antigen^+$ cells/debris (Figure 6.10). Islands of viable tumour cells negative for detectable SFV antigen occurred in most treated tumours.

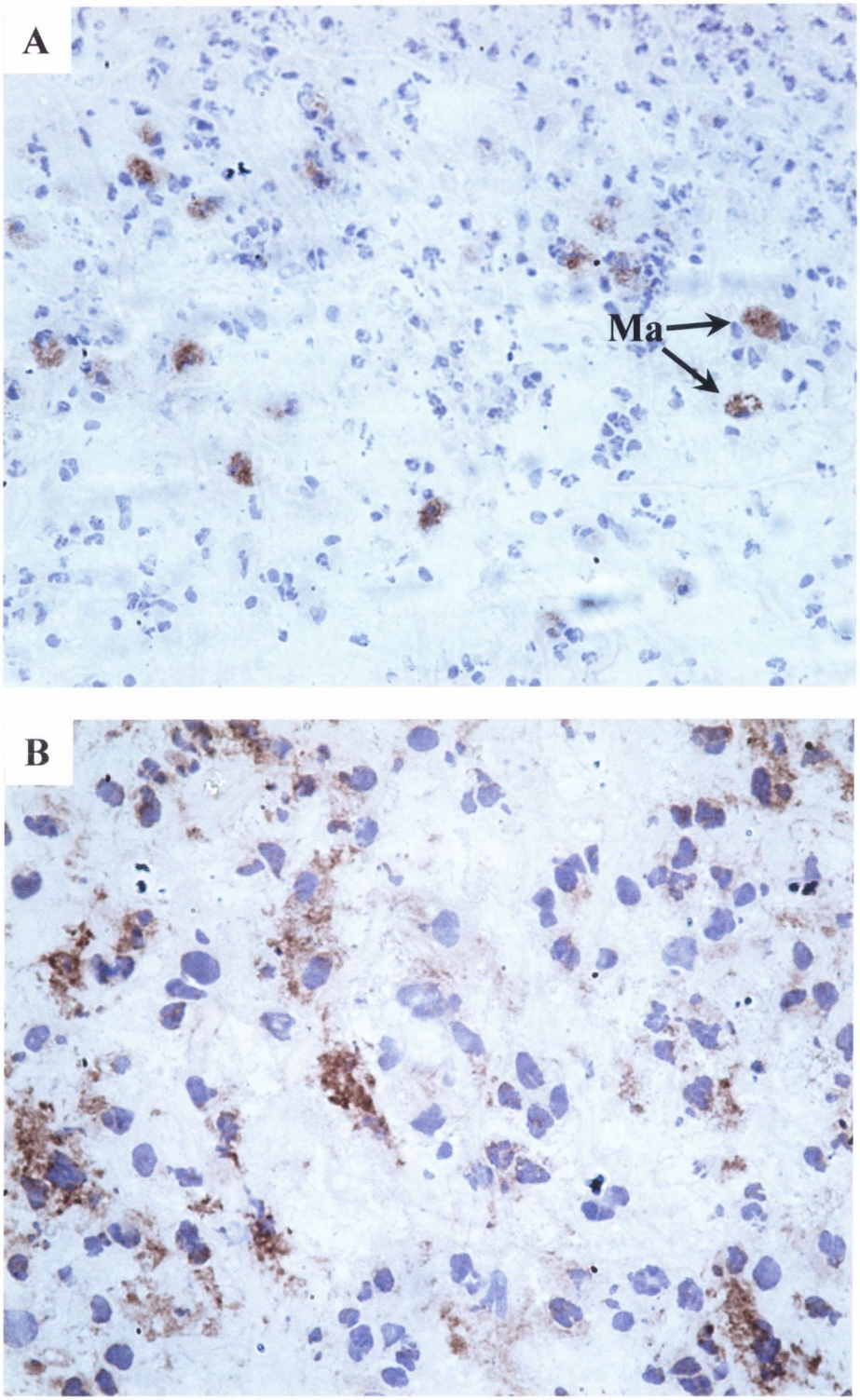


**Figure 6.8 Demonstration of SFV antigen in a K-BALB tumour treated with SFV4 from an immunised mouse at day 5 post initiation of treatment**

**(A)** Granular extracellular deposits of SFV antigen (brown) and intracellular deposits in macrophage-like cells (Ma), 200x

**(B)** Higher magnification of above, 400x

***Control tumours were devoid of detectable SFV antigen***

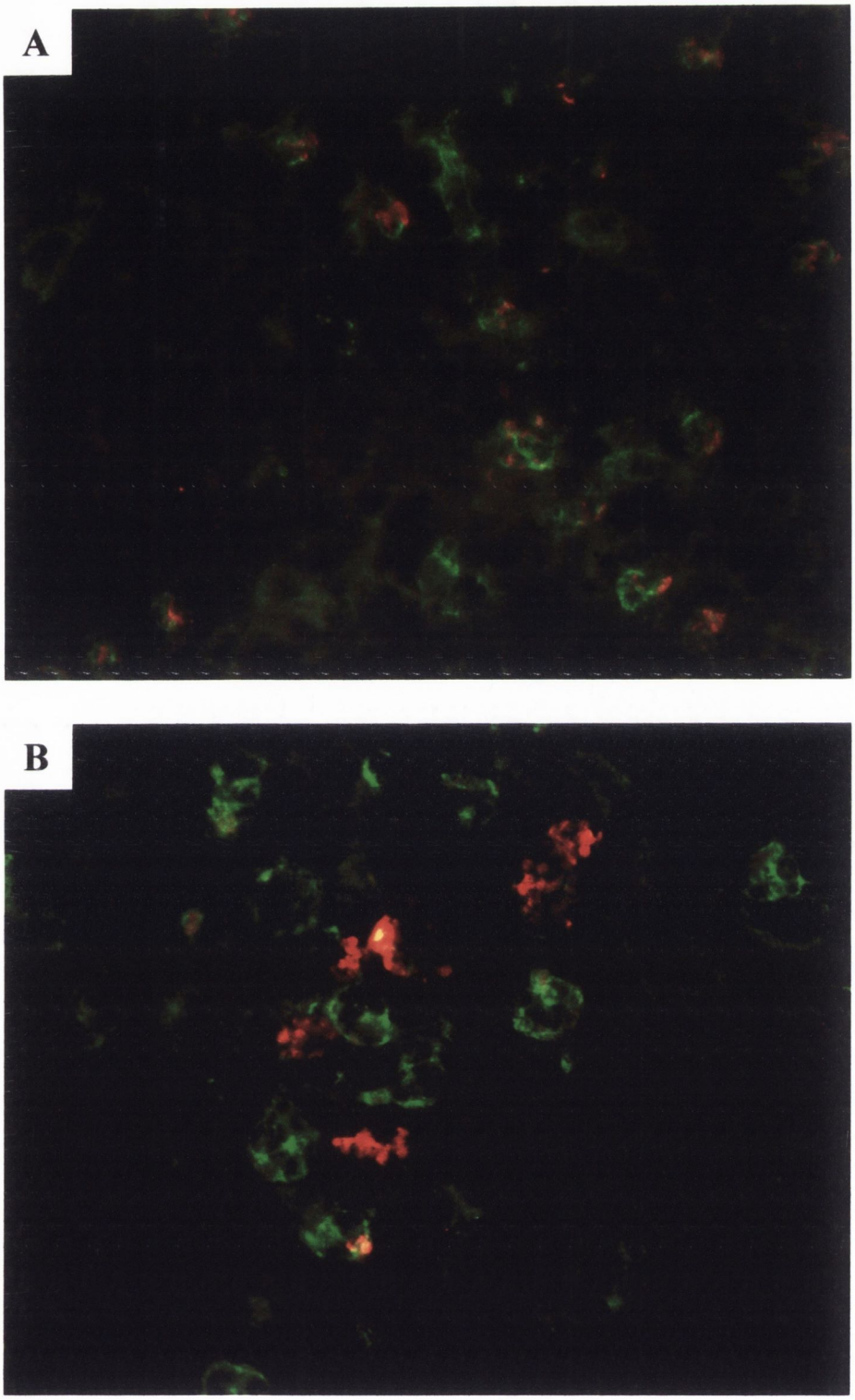


**Figure 6.9 Demonstration of SFV antigen and F4/80$^+$ macrophages in a K-BALB tumour from an immunised mouse treated with rSFV-p62-6k VLPs 5 days post initiation of treatment**

**(A)** F4/80$^+$ macrophages (green) containing intracellular deposits positive for SFV antigen (red), 400x

**(B)** SFV antigen$^+$ apoptotic cells and debris (red) surrounded by F4/80$^+$ macrophages (green), 800x

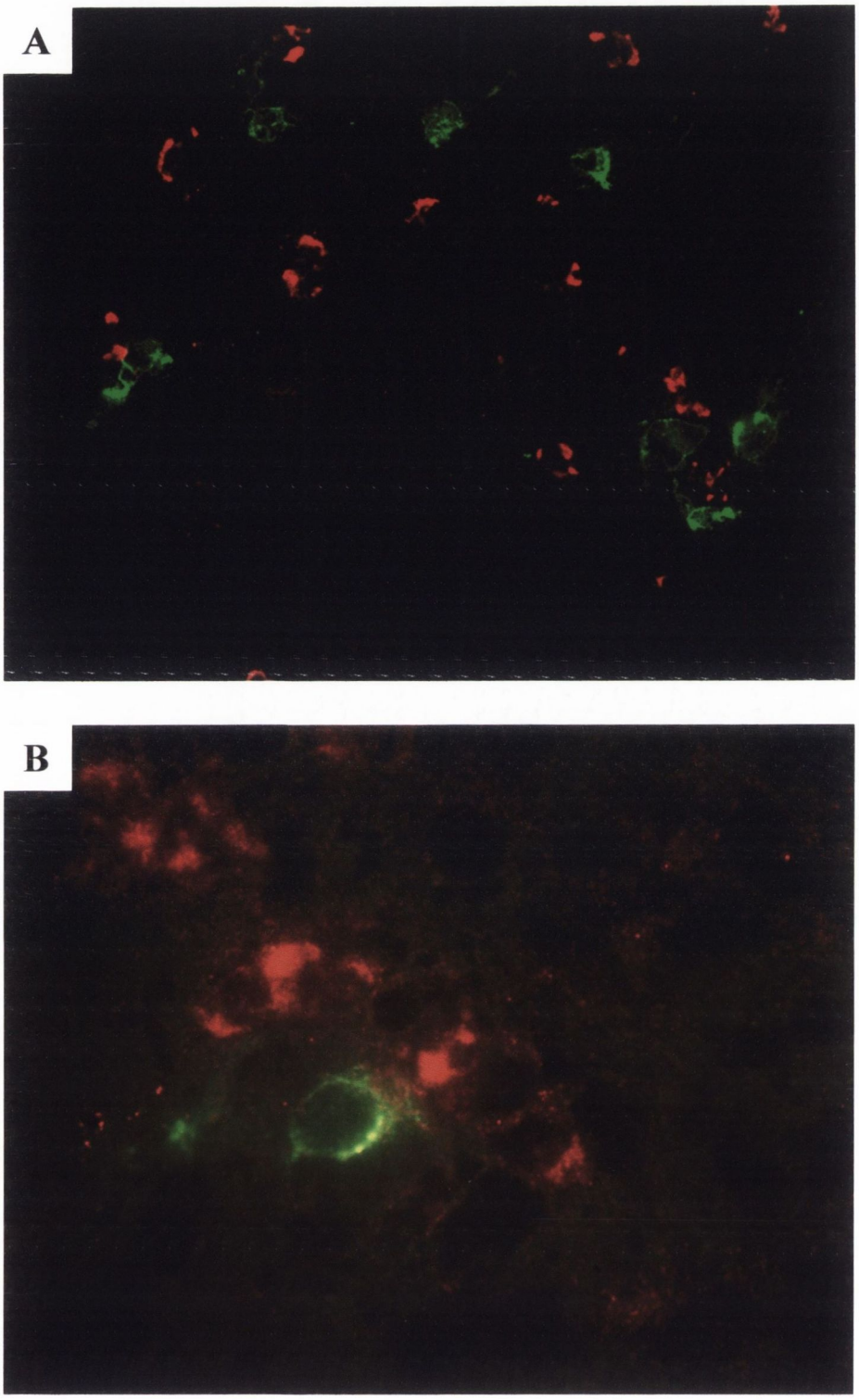


**Figure 6.10 Demonstration of SFV antigen and $CD8^+$ T-lymphocytes in a K-BALB tumour from an immunised mouse treated with rSFV-p62-6k VLPs 5 days post initiation of treatment**

**(A)** Co-localisation of $CD8^+$ T-lymphocytes (green) and SFV $antigen^+$ (red) cells and debris, 400x

**(B)** Higher magnification of above, 1000x

## 6.3 DISCUSSION

Treatment of K-BALB tumours with rSFV-p62-6k VLPs or SFV4 resulted in the induction of a strong anti-SFV immune response which, when established, manifested as acute inflammation as described in chapters 4 and 5 respectively. In this chapter, the cellular infiltrate was further characterised in order to better elucidate the nature of the immune response. Localisation of SFV antigen expression in the tumour microenvironment was also examined and apoptotic cells were detected using an antibody specific for active caspase-3.

Infiltrating lymphocyte populations in treated tumours were composed predominantly of $CD3^+$ T-lymphocytes with $CD45R^+$ B-lymphocytes restricted to occasional clusters in perivascular and perineural areas. Most T-lymphocytes were found to belong to the $CD4^+$ subset at earlier timepoints with numbers of $CD8^+$ cells increasing to comparable levels by treatment end. Necrotic and haemorrhagic regions were observed in the majority of tumours examined, and were often infiltrated with neutrophils and macrophages. High numbers of neutrophils and $F4/80^+$ macrophages were observed in all treated tumours, predominating as the chief cellular infiltrates at earlier timepoints. Neutrophils are typically the earliest phagocytic cells to be recruited to inflammatory sites and are followed by monocytes which mature into macrophages (Janeway *et al.*, 1999).

Macrophages play a major role in the removal of cellular debris in areas of tissue damage and $F4/80^+$ macrophages were frequently found to be positive for SFV antigen in treated tumours having apparently phagocytosed debris from lysed infected tumour cells. While macrophages are known to also behave as professional APCs, DCs are more efficient in the induction of host immune responses and are likely to play a major role in the induction of the anti-SFV immune response (Banchereau & Steinman. 1998; Chung *et al.*, 2004). Phagocytosis of SFV antigen-positive apoptotic bodies and cellular debris from infected K-BALB cells by professional APCs, such as macrophages and DCs, and the subsequent activation of lymphocytes through co-stimulation and cross-priming is, most probably, the predominant pathway in the induction of the anti-SFV immune responses observed, given the inefficiency with which SFV and rSFV infect these cell types directly (Behrens *et al.*, 2004; Huckriede *et al.*, 2004).

Previous studies have shown that rSFV vectors expressing viral antigens *in vivo* have the ability to elicit strong cytotoxic and humoural immune responses (Zhou *et al.*, 1995). Later studies, investigating the potential of rSFV VLPs as prototype vaccines,

revealed significantly higher levels of serum IgG2a than IgG1, indicating the predominance of a $T_H1$-mediated immune response (Berglund *et al.*, 1999; Fleeton *et al.*, 1999). Humoural immune responses to infection with replication proficient SFV also appear to be mainly $T_H1$-like with IgG2 subclasses predominating in sera and preceding the appearance of detectable IgG1 (Parsons & Webb, 1992). Studies by Keogh *et al* (2002, 2003) have illustrated that $T_H1$-mediated immune responses are important for clearance of SFV from the CNS of mice and in the development of protective immunity. Cytokine profiling, however, revealed no bias towards a $T_H1$- or $T_H2$-mediated response in the development of demyelinating disease following avirulent SFV infection (Morris *et al.*, 1997). Given that high numbers of neutrophils, $F4/80^+$ macrophages, $CD4^+$ T-lymphocytes and $CD8^+$ T-lymphocytes infiltrated treated tumours, it would appear that treatment of normally poorly immunogenic K-BALB tumours with rSFV-p62-6k VLPs or SFV4 resulted in the induction of a predominantly $T_H1$-mediated inflammatory immune response. Cellular immune responses are rarely exclusively $T_H1$ or $T_H2$ and it is likely that a local infiltration with $CD4^+$ $T_H2$ cells also played a role in the inhibition of K-BALB tumour growth.

The central role of $CD4^+$ T-lymphocytes in the orchestration of antitumoural immune responses has been well established (Hung *et al.*, 1998; Pardoll & Topalian, 1998; Toes *et al.*, 1999). $CD4^+$ T-lymphocytes are involved in a diverse range of anticancer immune responses, including: cytokine-mediated inhibition of angiogenesis and cancer cell proliferation, activation of macrophages and granulocytes, priming of antigen-specific $CD8^+$ CTLs, maturation of DCs, and the exertion of direct cytotoxic effects on target cells under certain circumstances (Toes *et al.*, 1999; Dranoff, 2004; Porakishvili *et al.*, 2004). Activation of macrophages by armed $CD4^+$ $T_H1$ cells (mediated by IFN-$\gamma$, TNF-$\alpha$ and CD40L expression) results in the secretion of toxic factors including proteases, oxygen radicals and nitric oxide (NO). Secretion of these substances by activated macrophages is somewhat indiscriminate and often leads to damage in surrounding tissues (Janeway *et al.*, 1999). The induction of such indirect non-specific effects can be of benefit in tumour therapy where they can lead to possible bystander effects through uninfected tumour cell killing and disruption of the tumour microvasculature.

The generation and maintenance of antigen-specific $CD8^+$ CTL responses is dependant on $CD4^+$ $T_H1$ cells (Behrens *et al.*, 2004), and is of significant importance in the development of strategies for cancer immunotherapy. The presence of $CD8^+$ T-lymphocytes in treated K-BALB tumours is indicative of the successful induction of

such a cytotoxic immune response directed against cells positive for SFV-antigen. Double fluorescent labelling revealed $CD8^+$ cells adjacent to SFV-antigen$^+$ cells and debris where CTL-mediated cell killing may have been exerted. Antigen-specific $CD8^+$ CTLs are known to be efficient killers of specific target cells through induction of apoptosis via the Fas death receptor- or granzyme-B-initiated pathways. Primed $CD8^+$ CTLs also contribute to $T_H1$-mediated immune responses through the secretion of ($T_H1$-like) cytokines such as IFN-γ, TNF-α and TNF-β, and subsequent activation of macrophages (Janeway *et al.*, 1999).

SFV antigen expression in treated K-BALB tumours was demonstrated by immunohistochemistry as granular extracellular deposits and as intracytoplasmic deposits within large macrophage-like cells. SFV antigen$^+$ cells/debris were separated by oedema and cellular infiltrate and no concentrated areas of SFV antigen expression were noted. The results suggest that areas of K-BALB tumours successfully infected by treatment underwent significant disruption due to oedema and infiltration of high numbers of leukocytes. As mentioned above, double labelling immunofluorescence studies confirmed $F4/80^+$ macrophages to be positive for SFV antigen. The presence of islands of apparently uninfected tumour cells negative for SFV-antigen in most treated tumours, indicated that both rSFV-p62-6k VLPs and replication-proficient SFV4 were incapable of disseminating completely throughout K-BALB tumour tissue. It is possible that the anti-SFV immune responses elicited, together with the low infection efficiency of the K-BALB cell line restricted, the spread of rSFV VLPs and the replicative potential and spread of SFV4 within the tumour.

Inflamed areas of treated tumours were scattered with cells and cellular debris positive for active caspase-3. The frequency of apoptotic cells within both control and treated tumours rendered accurate assessment of any increase in apoptosis due to treatment unfeasible. The intensity of the inflammatory response and associated oedema, together with high numbers of neutrophils and macrophages would suggest that these phagocytic cells were highly activated and producing cytotoxic factors as mentioned above. These insults, coupled with possible disruption of the tumour microvasculature and high levels of cytokine production may have overwhelmed the integrity of the tumours and contributed to necrosis as well as apoptosis induction. While apoptosis is the primary mechanism observed *in vitro* by which K-BALB cells infected with rSFV-p62-6k VLPs or SFV4 die, the tumour microenvironment is far more complex and it is likely that both apoptosis and necrosis played a role in the inhibition of K-BALB tumour growth observed.

Prior immunisation of mice with rSFV-p62-6k VLPs resulted in the rapid induction of anti-SFV immune responses directed against infected tumour cells detectable at day 1. Naïve tumours did not display similar cellular infiltrates until day 5 post initiation of treatment, at which point high numbers of $CD8^+$ T-lymphocytes had infiltrated throughout tumours of immunised mice. At day 11 post initiation of treatment, the composition of the cellular infiltrates in both naïve and immunised groups was comparable. No obvious difference was noted in the composition of the cellular infiltrate of tumours treated with rSFV-p62-6k and those treated with SFV4; this highlights the potency of rSFV VLPs in the induction of host immune responses through expression of viral antigens despite their inability to replicate.

# 7

# GENERAL DISCUSSION

# 7 GENERAL DISCUSSION

The use of viruses in the treatment of cancer is somewhat ironic, given that they are pathogens themselves and that approximately 15% of human cancers are believed to be caused by virus infection (Ring, 2002). Viruses have evolved over millions of years alongside their hosts to become extremely efficient in the introduction and expression of their genetic information in target cells. The harnessing of this ability and its exploitation for therapeutic means through manipulation of viral genomes has aided the field of cancer gene therapy to become increasingly clinically viable. Additionally, growing numbers of naturally occurring viruses are being described and engineered which replicate selectively in transformed cells (Chiocca, 2002). Our increased understanding of the molecular mechanisms behind oncogenesis and viral replication has allowed for the development oncolytic viruses and viral vectors as feasible cancer therapy agents of great potential.

Since their development, alphavirus vectors have gained much recognition as prototype vaccines and as promising cancer gene therapy agents (Yamanaka, 2004). Alphaviruses have a broad host range and can infect a variety of cell types irrespective of mitotic status, unlike other viruses such as retroviruses (Strauss & Strauss, 1994). While persistent expression of heterologous proteins can be favourable, the transient high level of expression achieved using alphaviral vectors is particularly suited to cytotoxic and immunostimulatory cancer gene therapy, were prolonged expression is not necessarily desirable. The transient nature of the system is also beneficial in terms of biosafety, and as RNA replication occurs exclusively within the cytosol, insertional mutagenesis (which can occur with some DNA virus vectors such as those derived from retroviruses) is not a concern. The rSFV vector RNA persists at the site of injection for up to 7 days and for only 24 h in lymphoid organs (Morris-Downes *et al.*, 2001). Using the split-helper vector system, rSFV VLPs can be routinely produced at relatively high titres (>1 x $10^{10}$ IU/ml) and the risk of RPV generation is negligible. Another advantage over some other viral vector systems is that most mammalian species have no pre-existing immunity to alphaviruses (Atkins *et al.*, 1999).

Given their inherent ability to induce apoptosis in a variety of cell lines and to elicit strong immune responses when employed as prototype vaccines, the potential for the implementation of rSFV VLPs in immunotherapy of cancer is clear (Berglund *et al.*, 1999; Fleeton *et al.*, 1999; Murphy *et al.*, 2000). Similarly, a replication proficient SFV derived vector could also be implemented in cancer therapy where it would potentially

affect more widespread cytotoxicity and higher levels of heterologous (therapeutic) protein expression. In this study, rSFV-p62-6k VLPs encoding the SFV structural protein E2 (aswell as E3 and 6k) were employed in the treatment of K-BALB tumours in BALB/c mice. It was hoped that the expression of viral antigens by the VLPs would augment their antitumoural effect through the induction of host immune responses directed at infected tumour cells. The antitumoural potential of the infectious clone of SFV, SFV4, was also examined as a model replication proficient SFV vector in comparison to the rSFV VLPs, which are capable of only one round of replication.

One limiting factor in the effectiveness of viral vectors in gene therapy is the efficiency by which they can successfully infect target cells and therefore achieve their desired therapeutic effect. Cell type has a major influence on viral infection efficiency, with viruses typically displaying tropism for particular tissues where they replicate most efficiently. Tumour cells can present altered and unstable surface protein profiles and cells of transformed phenotype can display differing infection efficiencies in comparison to their non-cancerous counterparts. The infection efficiency of rSFV-p62-6k VLPs was determined in three cell lines: the tumourigenic K-BALB cells, their non-tumourigenic predecessors BALB/3T3, and the highly infectable BHK-21 cell line (*chapter 3*). A relatively low infection efficiency was observed in K-BALB cells and also in BALB/3T3 cells, indicating that the reduction in infectability was due to cell type rather than transformation. While lower infection efficiencies often have a negative influence on treatment, bystander effects can be observed *in vivo*, where cells can undergo 'sympathetic' cell death along with dying neighbouring cells following treatment. Another more indirect bystander effect observed is believed to be due to disruption of tumour vasculature which can lead to amplified cytotoxic responses to treatment.

SFV4 growth curves were also generated for each cell line by performing plaque assays on supernatants from infected monolayers over a 5 day period. The virus was found to replicate fastest and to the highest levels in BHK-21 cells in comparison to BALB/3T3 and K-BALB cells. Infection at a MOI of 0.1 resulted in a peak in SFV4 titre 24 h later in BALB/3T3 and K-BALB cells than that observed in BHK-21 cells. It was deduced that the apparent delayed replication and lower viral titres were most probably due to the lower infection efficiency of the K-BALB and BALB/3T3 cell lines.

Prior to undertaking any *in vivo* studies, the effect of infection with rSFV-p62-6k VLPs and SFV4 on cellular viability and proliferation was examined by employing the trypan blue exclusion and [$^{3}$H-*methyl*] thymidine incorporation assays respectively (*chapter 3*). BHK-21 cell monolayers were completely decimated following infection

with rSFV-p62-6k VLPs or SFV4 whereas BALB/3T3 and K-BALB monolayers began to recover from VLP infection by 72 h.p.i.. The recovery of BALB/3T3 and K-BALB monolayers was attributed to their relatively low infection efficiency. Oncogenic K-*ras* often influences cell survival and plays a role in apoptosis induction/inhibition which prompted further investigation into the type of cell death induced by infection of K-BALB cells with rSFV-p62-6k VLPs and SFV4. DNA fragmentation was detected using the TUNEL assay and by microscopic evaluation of nuclear integrity using DAPI as a fluorescent nuclear stain. As necrosis can also lead to degradation of cellular DNA, active caspases were demonstrated at a cellular level using immunofluorescent detection of active caspase-3 and pan-caspase FLICA in order to confirm the induction of apoptosis. Activation of caspases and degradation of nuclear DNA was demonstrated in all three cell lines following infection with rSFV-p62-6k VLPs and SFV4, and it was concluded that apoptosis was the predominant form of cell death observed. Apoptosis induction was delayed in K-BALB monolayers infected with rSFV-p62-6k VLPs in comparison to the other cell lines, but this was not the case with cells infected with SFV4. Oncogenic K-*ras* therefore, does not confer resistance to SFV-induced apoptosis in the K-BALB cell line but may delay the onset of the cell death pathway in those cells infected with rSFV VLPs.

Many of the genetic mutations leading to oncogenic phenotypes are associated with increased resistance to apoptosis (Igney & Krammer, 2002), but none have yet been demonstrated to completely abrogate the cytopathic effects of infection with SFV or its derived vector. SFV-induced apoptosis is p53 independent and is delayed, but not prevented, by overexpression of the *bcl-2* oncogene (Glasgow *et al.*, 1998; Grandgirard *et* al., 1998; Murphy *et al.*, 2000; Murphy *et al.*, 2001). The potential for SFV and its derived vector as cytopathic therapeutic agents in the treatment of cancers is therefore clear, given that tumour cells often acquire resistance to apoptosis induction by more conventional therapies such as chemo- and radiotherapy through selection of resistant mutants. SFV structural proteins are superfluous to the induction of apoptosis, providing the vector system with the advantage of inherently inducing cell death and allowing for the expression of other therapeutic proteins in their place (Glasgow *et al.*, 1998). The precise apoptotic pathway induced by SFV remains to be fully elucidated, with the mitochondrial and death receptor pathways both implicated at this point (Li & Stollar, 2004). Alphavirus-induced apoptosis is most probably a multifaceted phenomenon in which differing cell death pathways may be activated depending on cellular factors, and on the individual species of alphavirus.

Expression of SFV antigens in cells infected with rSFV-p62-6k VLPs and SFV4 was assessed at a cellular level alongside the apoptosis assays. All three cell lines were demonstrated to efficiently express SFV antigen upon infection with rSFV-p62-6k VLPs or SFV4. The numbers of positively infected cells (expressing SFV antigen) in VLP infected BALB/3T3 and K-BALB monolayers were higher than that anticipated based on infection efficiency studies, as was the cytopathic effect observed. This may be due to an *in vitro* bystander effect as discussed in chapter 3 whereby the possible uptake of apoptotic bodies containing infectious RNA, for example, may facilitate infection of neighbouring cells. Mitotic status at time of infection may also have an influence on the rate of antigen expression and apoptosis induction, as some cells appear to express antigen and die faster than others within a given population.

*In vivo* studies were undertaken in immunocompetent BALB/c mice and BALB/c *nu/nu* mice following the *in vitro* studies described above, which confirmed K-BALB cells were susceptible to SFV-induced apoptosis and successfully expressed SFV antigens upon infection with rSFV-p62-6k VLPs or SFV4. Tumours were treated with either TNE buffer alone, rSFV-p62-6k VLPs, or SFV4. BALB/c *nu/nu* mice were not included in the SFV4 studies and served as an immunocompromised control in rSFV-p62-6k VLP treatment studies. Some groups of mice were immunised with rSFV-p62-6k VLPs prior to treatment of K-BALB tumours with rSFV-p62-6k VLPs or SFV4. In the case of VLP treatment, prior immunisation was aimed at further augmenting the antitumoural effect through eliciting an immediate immune response upon treatment with the VLPs. In the case of SFV4 treatment, prior immunisation protected mice against the potentially lethal effects of infection with this virulent strain, as well as priming the immune response to the treated tumour as with the VLP treatment.

K-BALB cells readily form syngeneic tumours of low immunogenicity in immunocompetent BALB/c mice following s.c. injection (Aaronson & Weaver 1971; Stephenson & Aaronson, 1972). Histopathology performed on K-BALB tumour sections suggested that the tumours were not significantly immunogenic with leukocytic infiltrates confined to the tumour periphery (*chapters 4 & 5*). The leukocytes in the tumour periphery were most probably present in response to the invasive nature of the tumour and pressure on surrounding tissues rather than any specific antitumour immune response. Additionally, there was no significant difference in the rate of growth of K-BALB tumours between immunocompetent BALB/c and the immunocompromised BALB/c *nu/nu* groups.

A previous study successfully exploited the inherent cytopathic effect of rSFV VLPs alone in the treatment of the H358a tumour model in BALB/c *nu/nu* mice, resulting in complete tumour regression in some cases (Murphy *et al.*, 2000). Significant inhibition of tumour growth was also observed in treatment of a more aggressive tumour model overexpressing *bcl*-2 and was enhanced through the expression of (pro-apoptotic) *bax* by rSFV VLPs, but the antitumoural effect was limited in comparison to the slower growing H358a tumour model (Murphy *et al.*, 2001). K-BALB tumour growth was successfully and significantly inhibited following treatment with rSFV-p62-6k VLPs (*chapter 4*) and SFV4 (*chapter 5*) in immunocompetent BALB/c mice. The inhibition of tumour growth following treatment with rSFV-p62-6k VLPs was found to be statistically insignificant in immunocompromised BALB/c *nu/nu* mice however, illustrating the need to augment the inherent cytopathic effect of rSFV VLPs when dealing with comparably aggressive and rapidly growing tumour models such as K-BALB.

A significant inhibition of tumour growth was observed for both SFV4 and rSFV-p62-6k VLP treated naïve BALB/c mice which was associated with a strong anti-SFV immune response, detectable by day 5 of treatment. This inflammation coincided with the development of detectable anti-SFV humoural and cellular immune responses which were demonstrated by ELISA and *ex vivo* splenocyte stimulation assays respectively. Tumours swelled considerably due oedema and the influx of large numbers of neutrophils, macrophages and lymphocytes as demonstrated by histopathology (*chapters 4, 5 & 6*). By immunising mice with rSFV-p62-6k VLPs prior to tumour induction and treatment, this immune response was present from initiation of treatment and the overall inhibition of tumour growth was greater than in naïve groups. Immunisation with rSFVp62-6k VLPs also conferred protection against SFV4, which is pathogenic in mice (Atkins *et al.*, 1999). No SFV4 virus or lesions associated with SFV4 infection were found in the brains of immunized mice treated with SFV4. Treatment of K-BALB tumours in BALB/c *nu/nu* mice did not result in effective inhibition of tumour growth for this rapidly growing tumour, so it can be concluded that the host immune system played a substantial role in the reduction of tumour volume in the immunocompetent groups.

The presence of large numbers of macrophages, $CD4^+$, and $CD8^+$ T-lymphocytes among the leukocytic infiltrates indicated that a $T_H1$ inflammatory response played a more significant role in the antitumour response than the $T_H2$ reaction. Previous studies in the exploitation of rSFV VLPs as prototype vaccines identified the predominant IgG isotype as IgG2a, which is indicative of a $T_H1$-mediated immune response (Berglund *et*

*al.*, 1999; Fleeton *et al.*, 1999). While specific IgG isotypes were not examined in this study, the high levels of IFN-γ secreted by splenocytes from treated mice in response to SFV antigens, together with the histopathological evidence described above, further support the conclusion that a predominantly $T_H1$-mediated immune response was elicited. A $T_H2$-mediated response is also likely to have played some role in the inhibition of K-BALB tumour growth, as cellular immune responses are rarely exclusively $T_H1$ or $T_H2$. Areas of suppurative necrosis containing elevated numbers of macrophages and neutrophils were also apparent, indicating that a non-specific immune response was also contributing to the overall reaction.

The humoural immune response to tumours was traditionally thought of as less potent than cytotoxic cellular responses and counterproductive when exploiting viral vectors, due to the generation of neutralising antibodies. It is possible, however that humoural and cellular responses may compliment each other in cancer immunotherapy (Reilly *et al.*, 2001). These studies also show the successful infection of K-BALB tumour cells by rSFV VLPs and SFV4 at the site of injection and the subsequent inhibition of tumour growth despite the presence of a strong anti-SFV humoural immune response. It is also possible that the anti-SFV immune response served to contain SFV4 in the tumour locality, and prior immunisation could be considered as a method of indirectly targeting the virus to the site of injection by preventing its spread to neighbouring tissues. The induction of antiviral immune responses through the expression of structural proteins is a limitation of all replicating viral vectors, but this study has demonstrated that it can be exploited as an immunotherapeutic strategy.

A change in morphology of K-BALB tumour cells was observed over time in control tumours whereby tumour cells gradually lost their fibroblastic spindle-cell morphology and reverted to a more poorly differentiated pleomorphic phenotype. Treatment of tumours with rSFV-p62-6k VLPs or SFV4 and the subsequent infiltration of leukocytes was associated with preservation of the spindle-cell morphology. Several factors are likely to have contributed to this, such as changes in the tumour microvasculature and cytokine profile possibly slowing the rate of tumour cell proliferation. Given the sheer numbers of infiltrating leukocytes, which included many immune effector cells, treated tumours are likely to have contained high levels of cytokines, such as IL-12 and IFN-γ which are antiangiogenic, exerting direct and indirect effects on the tumour microenvironment (Dranoff, 2004).

*In vitro* studies demonstrated that apoptosis was the predominant form of cell death induced in K-BALB cells following infection with rSFV-p62-6k VLPs or SFV4.

The *in vivo* tumour microenvironment is much more complex than the *in vitro* monolayer, however, and it is likely that apoptosis was not the only form of cell death occurring within K-BALB tumours following treatment. All tumours contained areas of necrosis which were surrounded by cells positive for active caspase-3. Such necrotic areas most probably came about due to hypoxia as the aggressive growth of the K-BALB tumour model frequently leads to haemorrhage and thrombosis formation. Disruption of tumour vasculature through endothelial cell killing and the generation of thromboses following treatment would also lead to the development of additional areas of necrosis within tumours. Also, the mechanical effect of repeated swelling and shrinkage would have disruptive effects on the tumour architecture. Cells and debris positive for active-caspase-3 were detected throughout inflamed areas of treated tumours, presumably due to infection or cell-mediated cytotoxic effects. Cytokines, mentioned above, and additional cytotoxic factors secreted by activated neutrophils and macrophages would also have contributed to tumour cell killing by apoptosis and necrosis.

The induction of immune responses against viral antigens for immunotherapy of cancer typically involves targeting those viral antigens which have also been identified as TAAs. Such strategies have been dominated by the E6 and E7 HPV oncoproteins associated with uterine and cervical tumours (Tindle, 1997; Daemen *et al.*, 2003) and viral antigens associated with hepatocarcinomas following HBV and hepatitis C virus (HCV) infection (Chun *et al.*, 2003). In this study, the viral antigen expressed by rSFV VLPs is not a TAA, but the highly antigenic SFV spike protein E2 (along with the E3 and 6k proteins). rSFV VLPs expressing a model TAA have been employed previously in the immunotherapy of the P815A murine tumour model (Colmenero *et al.*, 1999; Colmenero *et al.*, 2002). The use of a non-TAA in immunotherapy is aimed, in this case, at bypassing some of hurdles facing such cancer immunotherapy strategies due to the selection and proliferation of antigen-loss variants through immunoediting and the induction of immunotolerance to TAAs (Pardoll, 2003; Dunn *et al.*, 2004). Also, the specific immunogenicity of cancers, when possible to define, varies with disease and between individual patients. Rather than restricting therapy to a specific TAA, utilising the expression of viral or other antigens to effectively flag tumour cells is a strategy applicable to a much broader spectrum of tumours. The induction of inflammatory responses at the site of the tumour following the expression of viral antigens and the cytopathic effect of the VLPs or SFV4 would also aid in the presentation of TAAs to the immune system and possibly overcome immunological ignorance which can occur due to the peripheral location of many tumours (Melero *et al.*, 1997; Ochsenbein *et al.*, 1999).

Despite the presence of a strong anti-SFV immune response, both rSFV-p62-6k VLPs and SFV4 were capable of infecting cells at the site of injection and significantly inhibiting tumour growth. However, neither the VLPs nor SFV4 were capable of eradicating all the tumour cells, even after multiple treatments. This effect could possibly be overcome with the introduction of cytotoxic or anti-angiogenic factors (including cytokines) into the vector which would be secreted by infected cells and diffuse through the tumour (Asselin-Paturel *et al.*, 1999; Withoff *et al.*, 2001; Yamanaka *et al.*, 2001; Colmenero *et al.*, 2002). Combined therapy with conventional chemo- or radiotherapy may also be of benefit as such strategies have yielded superior results than gene therapy alone with other viral vector systems (Khuri *et al.*, 2000; Hecht *et al.*, 2003; Quist *et al.*, 2004).

Two recent studies by Tseng *et al* (2002; 2004) have demonstrated selective targeting of tumour tissues by SV vectors, possibly due to the expression of high levels of high-affinity laminin receptors which are believed to act as receptors for SV (Wang *et al.*, 1992; Strauss *et al.*, 1994). The prospect of administering oncotropic or targeted viral vectors for cancer therapy systemically is advantageous in comparison to those which must be administered by intratumoural injection and are therefore restricted to readily accessible tumours. This oncotropism has not been demonstrated in SFV VEE vectors to date, but its application may be limited as multiple treatments may lead to the induction of antiviral immunity and reduce the effect of the vector. Neutralising antibodies would deactivate the virions systemically, whereas ironically, the immunosuppressed tumour microenvironment may allow viral vectors to infect cells locally following intratumoural injection.

In this study, rSFV VLPs expressing SFV antigens were administered which may have reduced the effect of subsequent VLP administrations through the induction of neutralising immune responses. This effect could be partially abrogated by the use of rSFV VLPs expressing a non-SFV antigen. However, such VLPs would contain viral antigen and may continue to induce a neutralising immune response. For this study, rSFV-p62-6k VLPs provided a good comparison to the replication proficient SFV4, and prior administration of these VLPs was necessary to protect against the possible pathogenic effect of SFV4. Treatment with SFV4 was significantly more successful than with the VLPs, and the cellular immune response was more sustained. While the induction of anti-SFV immune responses was most probably responsible for hindering the efficient spread of the virus, prior vaccination with rSFV-p62-6k VLPs did enhance tumour growth inhibition. In a clinical situation, it may be possible to utilise rSFV VLPs

expressing antigens to which a patient already has immunity, or to vaccinate the patient during an initial phase of conventional chemo- or radiotherapy.

Toda *et al* (1999; 2003) have demonstrated that the induction of antiviral immune responses following treatment of murine tumours with G207 HSV-1 mutant can in turn lead to induction of antitumour immune responses which can inhibit metastases and other non-treated tumours. The use of virally infected tumour cell debris, or oncolysates, has long been known as a method of inducing immunity to TAAs which had previously avoided recognition by the immune system (Cassel & Garret, 1966; Sinkovicks; 1991). Therefore the strategy undertaken in this study may also aid in the induction of tumour-specific immune responses. K-BALB tumours regrew at the same rate as control tumours following treatment, and so it is unlikely that specific immune responses were generated against this lowly immunogenic cell line (Stephenson & Aaronson, 1972). It is possible that such responses could be achieved utilising a more antigenic cell line and/or a different immunotactic strategy such as the expression of non-SFV antigens or cytokines as mentioned above.

Recent years have seen increased interest in viral oncolysis by replication-proficient viruses as the limitations of non-replicative systems become apparent (Ring, 2002). This study investigated the potential of SFV4 in the treatment of K-BALB tumours through the induction of cytopathic effects and immune responses against infected tumour cells. Higher levels of tumour growth inhibition were achieved than with non-replicative rSFV VLPs, but greater inhibition of tumour growth had been anticipated, given the replicative capacity of SFV4. The expression of cytotoxic factors could enhance the antitumoural effect or cytokine expression could possibly be employed in order to modulate immune responses against the vector. As SFV is an RNA virus, a derived replicating vector would not persist in the same manner as those derived from DNA viruses. It is concluded that there is potential for a replication proficient SFV vector in cancer therapy but anti-viral immune responses may hinder its success. Given the comparable inflammatory responses to both treatments (as demonstrated by histopathology) there is clearly still great potential for rSFV VLPs in cancer therapy.

Cancer immunotherapy has undergone somewhat of a revival in recent years with the continuing identification of novel TAAs and an increased understanding of the immune system and cancer immunology. Several immunotherapeutic strategies have progressed to clinical trials, but success in the laboratory has proven difficult to reciprocate in human disease with immune responses rarely correlating with clinical responses (Ko *et al.*, 2003). The strategy employed in this study does not rely on any

specific TAA but instead induces potent immune responses in the tumour microenvironment directed against virally infected tumour cells. Also, this study does not depend solely on cytotoxic antitumour immune responses, as the rSFV VLPs and SFV4 are inherently capable of affecting significant cytotoxic effects in tumour cells through the induction of apoptosis. It is unlikely that the immunosuppressive functions of tumours could overcome the substantial specific and non-specific immune responses which were elicited in this study in response to tumour cell killing and viral antigen infection following infection with either rSFV-p62-6k VLPs or SFV4.

To conclude, viral immunotherapy of cancer is an area of gene therapy with great potential. Broader strategies which do not depend on specific TAAs are applicable to a wider variety of malignancies and may also overcome problems caused by immunoediting, tolerance induction, and immunological ignorance. Promising studies utilising rSFV VLPs expressing the cytokine IL-12 are currently underway where total regression of K-BALB tumours has been observed in 100% of treated mice. Preliminary studies also indicate that specific immune responses to K-BALB tumours have been induced in such mice as re-challenge with the tumour cells does not result in tumour formation. Additionally, the prospect of administering the SFV vector via *in vivo* electroporation of tumours is also under investigation. This strategy allows for the expression of cytotoxic or proapoptotic factors such as Bax without the need for VLP production, as only the recombinant RNA is electroporated *in vivo*. This overcomes problems associated with constructs encoding such factors, as the accelerated induction of death in VLP producing cells significantly reduces the VLP titres achieved (Murphy *et al.*, 2001).

The number of clinical trials involving virally based cancer treatments (either alone or combined with conventional therapies) is increasing with each year and, although results may not be as successful as was initially hoped, the field is progressing as limitations are exposed and addressed. Phase I and II clinical trials for liposomally encapsulated rSFV VLPs expressing IL-12 were recently proposed for the treatment of glioblastoma multiforme, but such a trial has yet to be undertaken (Ren *et al.*, 2003). The potential of a replication-proficient SFV vector in gene therapy remains to be fully explored as does any oncotropism it may possess. The work presented here shows that SFV4 shares the antitumoural abilities of its derived vector, but anti-viral immunity is a limiting factor. rSFV VLPs have been implemented successfully in a variety of strategies in the treatment of murine tumour models ranging from immunotherapy (Colmenero *et* al., 1999; Colmenero *et al.*, 2001) and anti-angiogenesis (Asselin-Paturel

*et al.*, 1999; Yamanaka *et al.*, 2001) to the exploitation and enhancement their inherent cytopathic effect (Murphy *et al.*, 2000; Murphy *et al.*, 2001). It is hoped that the research carried out in this study further illustrates the potential of SFV and its derived vector for the treatment of malignant disease in humans.

# 8

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# 8 REFERENCES


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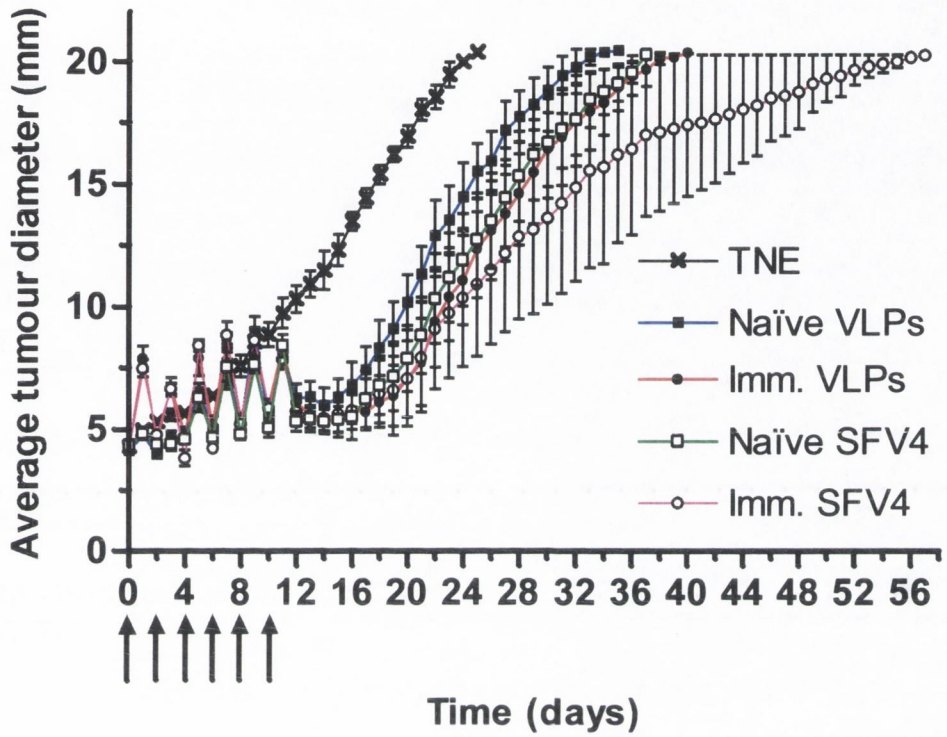
**Web pages cited:**

**Oncolytics**
http://www.oncolyticsbiotech.com/clinical.html

**World Health Organisation (WHO)**
http://www.who.int/cancer/en/

# 9

# APPENDICES




**Appendix 9.1 Treatment of K-BALB tumours in immunocompetent BALB/c mice with rSFV-p62-6k VLPs and SFV4. Compilation of tumour growth data from chapters 4 & 5.** K-BALB tumours were induced in naïve and rSFV-p62-6k VLP immunised female immunocompetent BALB/c mice by s.c. injection of 100 µl of K-BALB cell suspension in non-supplemented DMEM at a concentration of 1 x $10^7$ cells/ml. Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and groups of five mice were used per treatment group. Tumours received six 50 µl intratumoural injections of TNE alone, TNE containing rSFV-p62-6k VLPs or TNE containing SFV4 at a concentration of 1 x $10^{10}$ PFU/ml which were administered every other day over an eleven day period. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised when average tumour diameter reached 20 mm. *Points*; mean of five replicates, *bars*; +/- SEM, *arrows*; days of treatment. Results are representative of two independent experiments.

| *Group*[a] | *Median survival (days)*[b] | *Average tumour diameter (mm)* | *% of control (day 15)* | *Probability*[c] |
|---|---|---|---|---|
| TNE control *nu/nu* | 23 | 11.75 | - | - |
| VLP *nu/nu* naïve | 23 | 10.90 | 92.75 | ns[d] |
| VLP *nu/nu* immunised | 25 | 9.13 | 77.65 | ns |
| TNE control BALB/c | 23 | 12.32 | - | - |
| VLP BALB/c naïve | 31 | 6.31 | 51.21 | < 0.001 |
| VLP BALB/c immunised | 32 | 5.39 | 43.74 | < 0.001 |
| SFV4 BALB/c naïve | 33 | 5.40 | 43.84 | < 0.001 |
| SFV4 BALB/c immunised | 33 | 5.62 | 45.64 | <0.001 |

**Appendix 9.2 Overall survival and tumour size at day 15 after initiation of treatment. Compilation of data from chapters 4 & 5.** [a]Five mice were used per treatment group, and tumour treatment by direct injection (virus or TNE buffer as control) commenced when tumours reached an average diameter of 4 mm. [b]Mice were euthanised when the tumour reached 20 mm in diameter, and overall survival is based on time taked to reach this size. Overall median survival of mice from treatment groups was determined by Kaplan Meier survival curves. [c]A one-way repeated measures ANOVA was performed on all groups and Tukey's multiple comparison post test was used to compare each to its appropriate TNE control group. [d]ns; not significant. Results are representative of two independent experiments.

| *Group*[a] | TNE control *nu/nu* | VLP *nu/nu* naïve | VLP *nu/nu* immunised | TNE control BALB/c | VLP BALB/c naïve | VLP BALB/c immunised | SFV4 BALB/c naïve | SFV4 BALB/c immunised |
|---|---|---|---|---|---|---|---|---|
| **TNE control *nu/nu*** | - | **ns**[b] | **ns** | **ns** | nd[c] | nd | nd | nd |
| **VLP *nu/nu* naïve** | **ns** | - | **ns** | nd | **P < 0.01** | **P < 0.001** | nd | nd |
| **VLP *nu/nu* immunised** | **ns** | **ns** | - | nd | **ns** | **P < 0.001** | nd | nd |
| **TNE control BALB/c** | **ns** | nd | nd | - | **P < 0.001** | **P < 0.001** | **P < 0.001** | **P < 0.001** |
| **VLP BALB/c naïve** | nd | **P < 0.01** | **ns** | **P < 0.001** | - | **ns** | **ns** | **P < 0.001** |
| **VLP BALB/c immunised** | nd | **P < 0.001** | **P < 0.001** | **P < 0.001** | **ns** | - | **ns** | **P < 0.05** |
| **SFV4 BALB/c naïve** | nd | nd | nd | **P < 0.001** | **ns** | **ns** | - | **P < 0.05** |
| **SFV4 BALB/c immunised** | nd | nd | nd | **P < 0.001** | **P < 0.001** | **P < 0.05** | **P < 0.05** | - |

**Appendix 9.3 Overall probabilities of statistically significant differences between all groups treated with rSFV-p62-6k VLPs and SFV4. Compilation of data from chapters 4 & 5.** A one-way repeated measures ANOVA was performed on all groups and Tukey's multiple comparison post test was used to compare groups to eachother. [a]Five mice were used per treatment group, and tumour treatment by direct injection (virus or TNE buffer as control) commenced when tumours had reached 4 mm in diameter. [b]ns; not significant, [c]nd; not done. Results are representative of two independent experiments.