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TUMOUR THERAPY USING CYTOKINE-EXPRESSING SEMLIKI FOREST VIRUS VECTORS

A thesis submitted to the University of Dublin, Trinity College

For the Degree of Doctor of Philosophy

By

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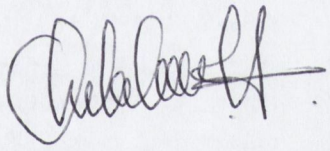

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Ad - adenovirus

a.a. - amino acid

ADP - adenosine diphosphate

APC - antigen presenting cell

ASO - antisense oligonucleotides

ATCC - American type culture collection

ATP - adenosine triphosphate

BHK - baby hamster kidney

bp - base pair

CD - cluster of differentiation antigen

CEA - carcinoembryonic antigen

CMV - cytomegalo virus

CNS - central nervous system

ConA - concanavalin A

CPE - cytopathic effect

CTL - cytotoxic T-lymphocyte

DAB - diaminobenzidine

DAPI - 4, -6, diamidino-2-phenylindole

dATP - deoxyadenosine triphosphate

DC - dendritic cell

DEPC - diethylpyrocarbonate

DMEM- Dulbecco's modified eagle medium

DNA - deoxyribonucleic acid

dNTP - deoxynucleoside triphosphates

dsDNA - double-stranded DNA

DTT - Dithiotreitol

EBV - Epstein Barr virus

EDTA- ethylenediaminetetra-acetic acid

EGFP - Enhanced green fluorescent

EGFR - epidermal growth factor receptor

ELISA - enzyme linked immunosorbent assay

ER - endoplasmic reticulum

FADD - Fas-associated death domain

FBS - foetal bovine serum

FCS - foetal calf serum

FITC - fluorescein isothiocyanate

FMDV - foot-and-mouth disease virus

G - gauge

g - gravitational force

GM-CSF - granulocyte-macrophage colony-stimulating factor

H & E - Haematoxylin and Eosin

h.p.i. - hours post infection

HBSS - Hank's balanced salts solution

HEPES - N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid

HIV - human immunodeficiency virus

HLA - human lymphotrophic antigen

HPV - Human papilloma virus

HRP - horseradish peroxidase

i.m. - intramuscular

i.n. - intranasal

i.p. - intraperitoneal

i.t. - intratumoural

i.v. - intravenous

IFN - interferon

Ig - immunoglobin

IL - interleukin

IPTG – isopropyl-β-D-galactopyranoside

IU - infectious units

M - molar

MCS - multiple cloning site

MEM - minimum essential medium

MHC - major histocompatibility complex

MOI - multiplicity of infection

mRNA - messenger ribonucleic acid

NK - Natural killer cells

NSCLC - non-small cell lung cancer

nsp - SFV non-structural protein

NTR - nontranslated region

p.i. - post-infection

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PFU - plaque forming units

PKR - interferon-inducible dsRNA-activated protein kinase

r - recombinant

RBC - red blood cell

RNase - ribonuclease

rpm - revolutions per minute

rSFV- EGFP - recombinant EGFP-expressing SFV virus particles

RT-PCR - reverse transcription PCR

s.c - subcutaneously

SCLC - small cell lung cancer

SFV - Semliki Forest virus

spf - specific pathogen-free

SV - Sindbis virus

TAA - tumour associated antigen

TCA - trichloroacetic acid

TGF- transforming growth factor

TILs - tumour-infiltrating leukocytes

TNF - Tumour necrosis factor

TNFR - TNF receptor

TRAF - TNF receptor associated factor

VEE - Venezuelan equine encephalitis

VEGFR - vascular endothelial growth factor receptor

SUMMARY

Semliki Forest Virus (SFV) vector is a transient RNA based suicidal expression vector system and has been previously used as a potential anti-cancer agent. Recently, a new enhanced SFV vector has been developed, pSFV10-E. Cells transfected with this vector yield up to ten times more foreign protein than cells transfected with original (non-enhanced) expression vector pSFV10. The two IL-12 gene subunits were cloned from mouse splenocytes and inserted into the pSFV10-E and pSFV10 vectors. Both the pSFV-mIL-12 constructs were characterised for their expression levels qualitatively and quantitatively in BHK-21 and K-BALB cells. The secretion of biologically active mIL-12 was confirmed by inducing splenocytes for IFN-γ production using IL-12 expressed by BHK-21 and K-BALB cells. K-BALB, CT26, and 4T1 tumours are the experimental tumour models in our study.

Subcutaneous K-BALB tumours in BALB/c mice were treated with six intratumoural injections of rSFV10-E-mIL-12 VLPs in an increasing order of titres at $4x10^7$, $4x10^8$, and $4x10^9$,i.u/injection. Administration of high titre rSFV10-E-IL12 VLPs to treat K-BALB and CT26 tumours in BALB/c mice demonstrated complete and permanent tumour regression in comparison to control or rSFV10-IL12 treated groups. Inhibition of primary tumour growth and lung metastases of a metastatic (4T1) tumour model indicated the potential of high titres of rSFV10-E-IL12 particles as an efficient anti-tumour therapeutic agent. Histopathology and immuno-histochemistry of tumours revealed tumour necrosis in addition to aggressive influx of CD4+ and CD8+ T-cells and other immune cells. Higher levels of an antiangiogenic factor IP-10 were detected in IL-12 treated animals compared to control treated groups indicating antiangiogenic effect induced by IL-12.

Another *in vivo* tumour treatment study using rSFV10-E replicons expressing IL-18 at a titre of $4x10^9$ IU/injection on K-BALB and CT26 tumours investigated antitumour activity of IL-18 *in vivo*. Although IL-18 induced tumour inhibition, the overall treatment effect was not as effective as IL-12 treatment.

Presentations & Publications

Presentations:

Oral Presentation ;

1. **The third European Union SFVectors meeting during 1st to 4th March 2004, in Arona Gran hotel, Los Cristianos, Tenerife Islands.**

   'Tumour therapy using SFV vectors expressing IL-12'.
   Chikkanna-Gowda CP, Fleeton MN, Sheahan BJ, Atkins GJ.

Poster presentation ;

1. **96th AACR meeting, April 15-20th, 2004. Anaheim, CA, USA**

   'Regression of mouse tumours and inhibition of metastases following administration of Semliki Forest virus vector with enhanced expression of IL-12'.
   Chikkanna-Gowda CP, Marina N Fleeton, Brian J Shehan, Gregory J Atkins.

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2. **Chikkanna-Gowda CP**, Sheahan BJ, Fleeton MN, Atkins GJ. "Inhibition of K-BALB and CT26 tumours using an enhanced Semliki Forest virus vector expressing cytokine IL-18." Manuscript in preparation.

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GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Cancer is generally regarded as a molecular disease of uncontrolled cell growth caused by the acquisition of transforming mutations by cells. A number of properties distinguish tumour from normal cells, including their invasiveness, loss of growth contact inhibition and their lack of response to regulation. The origin and host response to tumours is currently the focus of extensive basic and clinical research. Among more than 200 different types of malignancies, all share common features of uncontrolled growth and lack of differentiation. Currently, surgery is the primary method for treatment of cancers but is a less effective method for complete eradication of cancerous growths or in preventing the development of metastases. In addition, even though chemo- and/or radiotherapy have provided the only alternative or adjuvant to surgery, the associated toxicity with these regimes is closely related to their inability to effectively target the tumour. In the search for more specific and less toxic cancer treatment strategies, cancer immunotherapy and cancer gene therapy have emerged as promising candidates. Research on cancer gene therapy has largely focussed on induction of apoptosis in cancer cells or on stimulation of immune mechanisms to eliminate cancer cells. Many investigations have used viral vectors such as retro, adeno, adeno-associated, herpes viruses, and poxviruses or alphavirus vectors (Karlsson, 2004; Lundstrom, 2001). Recently, there has been much interest in the use of replication competent viruses in the treatment of cancer, because of their greater oncolytic potential (Tanaka *et al.*, 2003; Daemen *et al.*, 2000). Generally, another approach to using vectors for tumour treatment, which has recently been developed, is to target the tumour vasculature.

Alphavirus expression systems are based on infectious clones, which are grown as plasmids in bacteria. These plasmids are transcribed *in vitro* to produce vector RNA that can be transfected into cells (Berglund *et al.*, 1993). Alphavirus suicide vectors allow only transient expression of foreign genes and limited penetration of tissue because they can only undergo one round of replication and no infectious progeny are produced (Liljestrom *et al.*, 1991).

A study of the treatment of two model tumours in mice was carried out more recently to assess the potential of immune stimulation in combination with apoptosis induction by Semliki Forest virus (SFV) and its derived vector for tumour treatment (Smyth *et al.*, 2004). The inherent ability of the 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). It has been shown that the tumour vasculature can be disrupted following administration of cytokines such as IL-12 and IL-18 (Coughlin *et al.*, 1998; Asselin-Paturel *et al.*, 1999; Chada *et al.*, 2003). Immuno-gene therapy with cytokines is one of the strategies being developed for the activation of immune cells to enhance anti-tumour responses and inhibition of tumour angiogenesis. These and other studies have indicated that the SFV vector is an effective tumour therapy agent (Atkins *et al.*, 2004). More recently a new high-level SFV expression vector (pSFV10-E) has been constructed which gives expression levels up to 10-fold higher than the standard expression vector (pSFV10) (Sjoberg *et al.*, 1994).

In this study, we constructed pSFV10-IL12 (normal expression of murine IL-12) and pSFV10-E-IL12 (enhanced expression) vectors and examined the anti-tumour effect of rSFV-IL12 constructs in three different tumour models to demonstrate the consistency of IL-12 therapeutic efficacy. K-BALB cells are murine sarcoma virus transformed mouse fibroblasts that overexpress the K-*ras* oncogene and form aggressive localised syngeneic tumours in immunocompetent BALB/c mice on subcutaneous (s.c.) injection (Aaronson *et al.*, 1971; Stephenson *et al.*, 1972). CT26 cells are murine colon adenocarcinoma cells, which form localised tumours of low immunogenicity in BALB/c mice after s.c. injection (Brattain *et al.*, 1980). To examine the effect of treatment on a metastasising tumour model, we have selected the highly malignant, spontaneously metastasising 4T1 mouse mammary carcinoma model that also readily develops into tumours on s.c. implantation (Aslakson *et al.*, 1992; Pulaski and Ostrand-Rosenberg, 1998). In addition, the antitumoural ability of SFV expressing cytokine IL-18 was examined. IL-12-induced specific immunological memory has been reported, making IL-12 a promising candidate for permanent and specific tumour eradication. This study combines the immunotherapeutic approach of attracting host immune responses to the tumour and inhibition of tumour angiogenesis through expression of cytokine IL-12, and has demonstrated IL-12 induced anti-metastatic activity.

1.1 CANCER

Cancer is a collective term for malignant neoplasms arising in the body, amounting to hundreds of different disease states. It is the second leading cause of death in the world (Futreal *et al.*, 2004). Cancer is distinguished by renegade cells with no functions other than to proliferate, spread, and forge a destructive path through healthy tissue. The command of this unrestrained, potentially deadly growth is dispatched by genes rendered abnormal perhaps by viruses, radiation, environmental poisons, defective genes inherited from parents, wrong dietary choices or a combination of all the above. Tumours can be divided into two main groups: benign or malignant. Benign tumours grow within a well-defined capsulated structure, which limits the tumour spread in to the circulatory system, maintain the characteristics of the cell of origin, and are usually well differentiated. Cancer, malignant tumour, is distinguished from benign tumours by the properties of dedifferentiation, invasiveness, and often the ability to metastasise. 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).

1.1.1 Genesis of a cancer cell

Cancer development is the result of a multi stage process, and cancer cells have acquired a number of genetic alterations by the time they become invasive and metastatic (Hanahan and Weinberg, 2000). A normal cell turns malignant because of mutations in its DNA, which can be inherited or acquired. There are, however, two main categories of genetic change that lead to cancer: (i) inactivation of tumour suppressor genes, and (ii) activation of proto-oncogenes to oncogenes. Oncogenes confer malignancy on a cell. Proto-oncogenes normally control cell division and differentiation, but ultimately can be converted into oncogenes as a result of point mutations, gene amplification, chromosomal translocation, or the action of viruses.

Oncogenes not only interfere with cellular differentiation but also can confer autonomy of cell growth by affecting one or more of the signal transduction mechanisms for cell division by producing abnormalities in; (i) the production of autocrine growth factors; (ii) the receptors for growth factors; (iii) receptor linked

signalling pathways: the cytosolic and nuclear transducers; and (iv) cell cycle transducers such as cyclins and cyclin dependent kinases.

1.1.2 Oncogenes

Alterations in the cellular genome affecting the expression or function of genes controlling cell growth and differentiation are considered to be the main cause of cancer. Molecular cancer research aims at identifying the genes that are altered in the various tumour types and elucidating the role of these genes in carcinogenesis. A recent review by Futreal *et al* (2004) reported that 291 cancer genes have been identified to date, which accounts for over 1% of the genes in the human genome. The 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). 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 and its role in the development of malignant disease (Sjöström & Bergh, 2001).

1.1.2.1 *Ras* oncogenes

A family of genes that is frequently found to harbour mutation in human tumours is that of *ras* genes. This family consists of three functional genes, *H-ras, K-ras,* and *N-ras,* which encode similar proteins with molecular weights of 21kDa (Si'lvia Guerrero *et al.*, 2002). Mutations in *K-ras* are most commonly associated with human disease (Bos, 1989). K-BALB tumours that express *K-ras* oncogene are recently used as an experimental tumour model in tumour studies experiment (Smyth *et al.*, 2004). Mutated *ras* genes were first identified by their ability to transform NIH/3T3 cells after DNA transfection. Subsequent analysis of a variety of tumour samples revealed that in some human tumours one of the three *ras* genes harboured a

point mutation; as a result, the protein product has an altered amino acid at one of the critical positions 12, 13 or 61. The *ras* genes, which encode 21 kDa guanosine triphosphate (GTP) binding proteins (p21), 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 an active GTP-bound state at the plasma membrane of normal cells (Satoh *et al.*, 1992; Boguski & McCormick, 1993). The functional and structural resemblance of the *ras* proteins with G-proteins controlling adenylate cyclase has led to the proposal that normal p21*ras* proteins are involved in the transduction of external stimuli induced by growth factors or factors involved in cell differentiation.

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 (Diaz *et al.*, 2004). The highest incidence is found in tumours from the exocrine pancreas, where more than 80% of the tumours harbour a mutated *K-ras* gene. Oncogenic *ras* proteins are crucial in mediating many malignant characteristics of transformed cells and therefore have good potential as targets for tumour therapy (Downward, 2003).

1.1.2.2 Apoptosis regulatory genes

Apoptosis or programmed cell death, first described in 1972, is a series of genetically controlled events which result in the removal of unwanted cells without disruption of tissues (Kerr *et al.*, 1972). As with differentiation or proliferation, apoptosis is an important method of cellular control responsible for the maintenance of the state of homeostasis (Wyllie *et al.*, 1972). Apoptosis differs from necrosis or accidental cell death in a number of important ways. Firstly, it is an active process as opposed to an unplanned process induced by cell injury. Secondly, apoptotic cells are recognised by phagocytes and removed before they disintegrate. Apoptotic cells show condensation of nuclear, heterochromatin, cell shrinkage and loss of positional organisation of organelles in the cytoplasm. In contrast, in necrosis, cells become leaky, release macromolecules and rapidly disintegrate, thereby inducing inflammation (Renehan *et al.*, 2000; Sperandio *et al.*, 2000).

Several proteins are expressed which exert direct inhibitory or regulatory effects on the various pathways of apoptosis, including: IAPs (inhibitor of apoptosis) (Liston *et al.*, 1996), FLIPs (Fas-associated death domain-like ICE [IL-converting enzyme] inhibitory proteins) (Irmler *et al.*, 1997), TRIP (TNFR [TNF-receptor] -associated factor, TRAF [TNFR-associated factor] -interacting proteins) (Lee & Choi, 1997), tumour suppressor protein *p53* (Levine *et al.*, 1991), and the *Bcl-2* family of proteins (Reed, 1997).

One of the most important controls of cell cycle progression and apoptosis induction is the tumour suppressor *p53* gene product. The *p53* protein has been defined as a nuclear phosphoprotein which functions as a transcription factor for the activation of a variety of cell cycle- and apoptosis-related genes (Levine *et al*, 1991). As a consequence, pertubations in *p53* expression and function are associated with a broad array of malignancies (Hollstein *et al.*, 1991). *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). Genotoxic stress inducers of apoptosis, including UV- and γ-irradiation, and chemotherapeutic drugs, are dependent on *p53* induction (Kuerbitz *et al.*, 1992; Lowe *et al.*, 1993). In addition, when cells are induced to proliferate by deregulated expression of certain viral and cellular oncogenes, they undergo *p53*-dependent apoptosis, unless rescued by pro-survival *Bcl-2*-like gene products, which act to block apoptosis specifically (Symonds *et al.*, 1994; Levine, 1997).

The *Bcl-2* family of proteins, potent repressors of apoptosis, 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). The *Bcl-2* gene was localised to chromosome 18, at the site of one of the breakpoints of a reciprocal translocation, also involving chromosome 14, in B-cell follicular lymphomas (Tsujimoto *et al.*, 1985). Family members *Bcl-2*, *Bcl-x_L*, *Bcl-w* and *Mcl-1* inhibit apoptosis induction whereas *Bax*, *Bak* and *Bok* promote it. The *Bcl-2* protein was defined as unique, because it contributed to malignant cell expansion primarily by

prolonging cell survival, rather than by increasing the rate of cellular proliferation (Yang & Korsmeyer, 1996).

Inhibition of cell death, either by suppression of those genes which induce cell death, or by activation of those genes which cause cell survival, therefore contributes to the development of tumours. There is considerable interest in this area not just to gain a better understanding of tumourigenesis but also because it may in the near future reveal novel targets for tumour therapy.

1.1.3 Tumour immunology

Successful immunotherapy of cancer will ultimately require understanding the natural relationship between the immune system and tumours as they transform, invade, and metastasise. Given the vast number of genetic and epigenetic changes associated with carcinogenesis, it is clear that tumours express many neoantigens. A central question in the immunology is whether recognition of tumour antigens by the immune system leads to activation (surveillance) or tolerance. Immune surveillance is a concept that envisages prevention of the development of most tumours through early destruction of abnormal cells by the host's immune system. Ever since its proposal by Thomas (1959) and then Burnet (1970), the immune surveillance hypothesis has come under relentless attack. The hypothesis is that tumours arise with similar frequency to infection with pathogens and that the immune system constantly recognises and eliminates these tumours based on their expression of tumour- associated antigens (TAAs) (Ehrlich, 1909). A corollary to the original immune surveillance hypothesis is that progressor tumours in animals as well as clinically progressive spontaneous tumours in all species, are not eliminated because they develop active mechanisms of either immune escape or resistance (Uyttenhove *et al.*, 1983; Shankaran, *et al.*, 2001). The mechanisms by which tumour cells may escape killing by the immune system include: (i) Induction of tolerance to tumour antigens; (ii) Development of tumour cells lacking antigens to which the immune system has responded; (iii) Modulation of tumour antigen expression. (iv) Suppression of anti-tumour immunity; (v) Poor immunogenicity of the tumour perhaps resulting from lack of expression of MHC class I; (vi) Expression of Fas ligand (FasL) on tumours, which may induce apoptosis of effector cells. The emerging evidence for immune surveillance systems of

carcinogenic events is counterbalanced by evidence that the normal immune response to tumour antigens is tolerance induction rather than activation.

Oncogenic DNA and RNA viruses code for both cell surface and nuclear viral antigens, which become expressed by the tumour, and are shared by all tumours induced by the same virus. However, because of random mutagenesis of DNA, chemically induced tumours often express antigens unique to the individual tumour. An antigen such as the carcinoembryonic antigen (CEA), a cell surface glycoprotein with a putative role in cell adhesion, is expressed at very low levels in normal adult tissues, but is overexpressed in the foetus and in 90% of colorectal, lung, ovarian and pancreatic cancers (Chung-Faye *et al.*, 2000). Alpha-fetoprotein (AFP) is highly associated with germ cell derived tumours and hepatomas. The antigens are not unique to tumour cells since they are also found in normal cells during embryonic development and at low levels in normal human serum.

In addition, there is considerable evidence for quantitative and qualitative differences in antigens associated with normal vs tumour cells. These antigens can be divided into tumour specific antigens (TSA), those unique to tumour cells, or TAAs also found on some normal cells. Another classification system is based on the origin or nature of the antigens and includes viral, chemical, oncofetal and differentiation antigens. 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).

The innate immune system is now recognised as having some ability to discriminate 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). Specific antitumour immunity appears to develop in tumour-bearing patients in much the same way as it does to pathogens or foreign antigens. Both TSA and TAA associated with tumour cells appear to be processed and presented in association with MHC class I molecules, making them potential targets for cytotoxic T cells. NK cells kill tumour cells not expressing MHC class I molecules and this pathway may be involved in the killing of tumour cells which down-regulate MHC class I molecules (Ljunggren & Karre, 1990; Garrido *et al.*, 1997). Many tumour antigens are endogenously synthesised and therefore presented via peptides associated with the MHC class I molecules. It is for this reason, and the 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).

1.2 CANCER THERAPY

Recently, advances in molecular cancer research have revealed numerous new therapeutic targets, some of which have already been tested in clinical settings. However, the clinical management of cancer patients is still based on surgical and/or radiological eradication of the primary solid tumour and chemical/radiological anti-proliferative/cytotoxic treatment of the disseminated disease. Despite a great deal of improvement in the control of benign disease, only slow progress has been seen in malignant disease. As a result, mortality of cancer patients is still high due almost exclusively to the development of metastases. One obvious explanation for this discrepancy could be a difference between the molecular pathways controlling tumour growth and tumour progression. Clinical evidence has clearly established that chemotherapy and radiotherapy exert only limited efficacy in the majority of malignant diseases (Eckhardt, 2002)

The potential targets for tumour therapy include the oncogenes themselves, their RNA transcripts, and their protein products. Several approaches to the therapeutic uses of oncogenes are worth considering: (i) antibodies can be used against growth factors and other factors associated with the transformed phenotype, such as enzymes or proto-oncogene products like *ras* p21. This includes the use of

antibodies to focus toxic agents or cells of the immune system on cancer cells; (ii) oncogenic nucleic acid sequences could be targeted by anti-sense nucleotides of DNA or RNA, or by nucleotide anti-metabolites such as dideoxy- or methyl phosphonate-modified nucleotides; (iii) a mutated oncogene might be directly replaced by its non-mutated normal counterpart by gene therapy. 'Naked' nucleic acid vaccines are potentially useful candidates for the treatment of patients with cancer, but their clinical efficacy has yet to be demonstrated (Liu *et al.*, 1998; Zhang *et al.*, 2005). Efforts have already been made to enhance the efficacy of nucleic acid vaccines by using 'self-replicating' RNA as a cancer vaccine (Ying *et al.*, 1999). 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 (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).

In addition, anti-*p53* antibodies in the sera of patients with a variety of cancers have been demonstrated (Gumus *et al.*, 2004; Limpaiboon *et al.*, 2005) and also the *in vitro* induction of cytotoxic T-cells specific for wild type and mutant *p53* peptides (Lee *et al.*, 2004; Hoffmann *et al.*, 2002). The demonstration that the immunisation of the patients with a vaccinia virus vector containing a CEA peptide can induce cytotoxic T cells which recognise MHC restricted binding motifs in CEA (Tsang *et al.*, 1995), indicating that it is possible to generate immune responses to the 'self-antigens' expressed by tumour cells that offers an additional potential avenue for therapeutic intervention in the future.

1.2.1 Gene therapy

Gene therapy shows considerable promise as a treatment for patients with advanced or resistant carcinoma. In gene therapy, well-characterised genes, and their regulatory elements are introduced into genes of the patient's cells using vectors. It is assumed that gene therapy will ultimately be used in association with other treatment

for advanced tumours where presently no effective treatment can be advocated. Gene therapy modulates chemotherapy and is used as adjuvant therapy after surgery. In patients with high risk of tumour recurrence and also in situations with no clinically detectable disease, it may also be effective. However, it is limited in cancer patients by difficulty in achieving efficient gene delivery into tumour cells. Despite the current uncertainties, gene therapy could hold exciting prospects for better treatment for cancer. The current strategies employed in gene therapy of malignant disease include targeting of oncogenes, drug resistant gene therapy, targeted gene therapy, genetic immunomodulation and gene silencing by antisense small interfering RNAs using both viral and non-viral vectors as gene carriers.

Tumour suppressor gene therapy: In tumour cells, the activities of oncogenes and tumour suppressor genes (constituents of normal genomes) are either activated or suppressed. In tumour gene therapy, clinical protocols have been designed either to inhibit the expression of mutant oncogene *L-ras*, p21 or to replace the defective anti-oncogene *p53* using intratumoural injection of viral vectors expressing normal *k-ras* or *p53* respectively. Viral vectors have the potential for delivering these genes. This approach is suitable for respiratory, digestive tract and some metastatic tumours because high concentrations of the non-toxic agents can be achieved through local injections and these agents warrant great promise providing a high therapeutic index and as adjuvant therapy to surgery for patients with early stage cancer.

Drug resistant gene therapy: Several genes confer drug resistance to hematopoietic progenitor cells. One of the first cancer gene therapy strategies proposed, and the most frequently used, were designed to kill cells directly using toxic or prodrug therapy (Davis, 1996). Suicide genes, the genes that sensitise cells to drugs that are normally non-toxic, are an alternative approach to the development of selective therapy (Moolten *et al.*, 1994). The principal suicide gene currently under study is the thymidine kinase gene of herpes simplex viruses (HSV-TK) (Jia *et al.*, 1994). The HSV-TK phosphorylates the systematically delivered antiviral drug, gancyclovir into an intermediary metabolite, which is then converted by cellular kinases into a potent inhibitor of DNA polymerase, leading to cell death (Huang *et al.*, 2003). Normal cells remain unaffected by the drug. Several trials have used this approach to treat glioblastomas (Freeman *et al.*, 1993). Investigators have recently used recombinant viral vectors to deliver high levels of HSV-TK to tumours such as

lung carcinoma (Osaki *et al.*, 1994), melanoma (Vile *et al.*, 1994), mesothelioma (Hwang *et al.*, 1995) and squamous cell carcinoma of the head and neck (O'Malley *et al.*, 1995). Antitumour immune responses are produced in a minority of patients, with mixed clinical response in melanoma and kidney cancers. Current protocols are attempting to introduce drug resistance genes into hematopoietic cells to provide increased protection from commonly used myelosuppressive chemotherapeutic agents (Sorrentino *et al.*, 1992). Gene transfer of multidrug resistance gene (MDR)-1 allows for the production of P-glycoprotein, which functions as a cellular efflux pump, and expels a range of toxic metabolites from the cell. Mickisch *et al.*, (1992), and other investigators showed that MDR-1 gene transfer provided resistance to a wide range of chemotherapeutic agents, including paclitaxel, anthracyclines and bisanthrene. Other potential genes include dihydrofolate reductase (May *et al.*, 1995) and O^6-methylguanine-DNA methyltransferase, an efficient DNA repair enzyme, which was shown to protect mouse hemotopoietic cells from nitrosourea-induced toxicity (Moritz *et al.*, 1995).

Targeted gene therapy: Several genes inhibit tumour neovascularisation, an important step in tumour progression and metastasis. The majority of cancer gene therapy protocols have been aimed at immunostimulation (Ponsaerts *et al.*, 2003; Henderson *et al.*, 2005). Tumour associated antigens (TAAs) can be targeted for cancer vaccination. TAA can elicit specific antitumour immune responses; vaccination with vaccinia-CEA (carcinoembryonic antigen) in breast and colon adenocarcinoma has been investigated (Conry *et al.*, 1995). MUC-1, a TAA and erb B2/neu, a receptor for a growth factor, are also being considered for breast cancer. Several tumour vaccine trials involving melanoma are underway following the recent cloning of melanoma-associated antigen encoding genes. Many cancers can be recognised and destroyed by a tumour-specific immune response, usually mediated through cytotoxic T cells or natural killer (NK) cells (Durrant *et al.*, 1995). The activation of this process requires three synergistic signals; (i) presentation of tumour-associated antigens to T cells in conjugation with MHC class I and MHC class II molecules, which transport the tumour antigens to the cell surface, (ii) additional costimulatory molecules, such as B7-1 and B7-2, which are required for effective induction of the efferent arm of the immune response, and (iii) cytokines secreted by the helper T cell population following stimulation by the antigen presenting cell

(APC). Many cancers may be defective in any one of the above pathways, which may contribute to T cell anergy to the tumour antigens (Harding *et al.*, 1992).

Antisense/ribozymes; Ribozymes are RNA molecules that function as catalysts for other RNAs (Gibson *et al.*, 2000). The identification of specific genes that contribute to the development of cancer, including dominant protooncogenes and tumour suppressor genes, presents an opportunity to use these genes as prevention and treatment targets. Based on this realisation, the introduction of a functional tumour suppressor gene, or the downregulation of an activated oncogene, could lead to the destruction of the malignant cell. The use of ribozymes in cancer has focussed mainly on the inhibition of tumour specific oncogene expression. Ribozymes have the added advantage that following degradation of the target mRNA molecule, the molecule becomes free to bind to another molecule, thereby increasing the levels of oncogene ablation. Hammerhead ribozymes specific for *Bcl-2* mRNA have been shown to sensitise prostate cancer cells to apoptotic cell death induction (Dorai *et al.*, 1997). Antisense technology involves the introduction into the cell of a gene construct that has a base sequence complementary to the RNA sequence targeted for inhibition. The advantage of this is the potential to achieve ablation of oncogene activity at the proximal level of mRNA splicing, transport or translation. This strategy has been used effectively for the downregulation of mutant *k-ras* protein in NSCLC cell lines (Georges *et al.*, 1993; Mukhopadhyay *et al.*, 1991). *K-ras*, a member of the GTPase family of oncoproteins, is frequently mutated early in lung, colorectal and other cancers (Marshall, 1991). In colon cancer cell lines, antisense oligonucleotides (ASO's) complementary to K-*ras* inhibited cell growth, colony formation, and K-*ras* expression in a dose-dependent manner (Sakakura *et al.*, 1995). Expression of *Bcl-*X_L, a close anti-apoptotic homologue of *Bcl-2*, was similarly downregulated using a 2'methoxy-ethoxy-modified ASO in a number of small cell lung cancer cell lines (Leech *et al.*, 2000). Other oncogenes targeted include the cAMP-dependent protein kinase A (Nesterova *et al.*, 1995), and TGFα (Laird *et al.*, 1994). Cleavage of the human papillomavirus oncogenes involved in cervical cancer, by ribozymes *in vitro* has been reported (Takahashi *et al.*, 1989). As *p53* is inactivated in up to 70% of human lung malignancies, a significant number of wt-*p53* gene replacement protocols have been applied to lung cancers (Fujiwara *et al.*, 1994). More recently however, this strategy has been applied to a myriad of other human cancer cell lines, including,

squamous head and neck (Liu *et al.*, 1995; Frederick *et al.*, 1999), ovarian (Hamada *et al.*, 1996), colorectal (Harris *et al.*, 1996), prostate and nasopharyngeal carcinomas (Li *et al.*, 1997), with the successful induction of programmed cell death in culture and in subcutaneous mouse xenograph and orthotopic (intratracheal) cancer models (Fujiwara *et al.*, 1994). *Bax* overexpression may also facilitate apoptosis induction in more resistant cell lines; particularly those that overexpress key apoptosis inhibitors, such as *Bcl-2* (Kagawa *et al.*, 2000; Xiang *et al.*, 2000). These studies suggest that there are many appropriate targets for ribozyme strategies in cancer therapy.

Currently, the genes most frequently used in gene therapy trials are HLA B7/B2M (to co-stimulate the macrophages with tumour antigen), IL-2 (to stimulate cellular immunity against cancer cells), thymidine kinase (to activate gancyclovir in the cancer cells) and GM-CSF (to stimulate the macrophages). Other trials use TAAs such as CEA (an antigen present on adenocarcinoma), *p53* (an anti-oncogene), MART-1 (a TAA present on melanoma), and interferon gamma IFN-γ (a lymphokine secreted by T-lymphocytes to activate macrophages).

RNA interference is considered to have begun as an evolutionarily ancient mechanism for protecting organisms from viruses. Many viruses have RNA, rather than DNA, as their genetic material and go through at least one stage in their life cycle in which they make double stranded RNA. Perhaps not surprisingly, all multicellular organisms have evolved a well conserved protein apparatus that destroys double stranded RNA but this has also been found to play a role in maintenance of the organism's own genome stability by suppressing the movement of mobile genetic elements, such as transposons and repetitive sequences.

The gene silencing process of RNA interference (RNAi) involves the manufacture of short double stranded RNA molecules by an enzyme called DICER, which cleaves RNA duplexes into 21–26 base pair oligomers. These small interfering RNAs (siRNA) cause sequence specific, post-transcriptional gene silencing by guiding an endonuclease, the RNAi induced silencing complex (RISC), to mRNA. This process has been seen in a wide range of organisms such as Neurospora fungus (in which it is known as quelling), plants (post-transcriptional gene silencing), and mammalian cells (RNAi). Downregulation of target gene expression has been found to involve interactions at multiple levels. Where there is complete or near complete sequence complementarity between the small RNA and the target, the Argonaute 2

component of RISC mediates cleavage of the target transcript (Liu et al., 2004; Meister et al., 2004). In contrast, where there is sequence mismatch between the miRNA and the target transcript, the mechanism appears to involve repression of translation predominantly (Kim et al., 2004). More recently, it has been recognised that siRNA molecules can induce transcriptional silencing through promoter methylation (Kawasaki et al., 2004; Morris et al., 2004).

The major challenge in turning RNA interference into an effective therapeutic strategy is the delivery of RNA interference agents, whether they are synthetic, short double stranded RNAs or viral vectors directing production of double stranded RNA, to the target cells within the body. While siRNA technology has proven extremely powerful and robust for cell culture work, translating this success reliably to animals or humans is proving very difficult, due to insufficient bioavailability of the compounds.

1.2.2 Immunotherapy

Immunotherapy is an attractive strategy for cancer treatment. However, self-tolerance is one of the major mechanisms that dampen immune responses against self-tumour antigens (Sotomayor *et al.*, 1996; Morgan *et al.*, 1998). It is believed that the effective T cell immunity is a critical component of the immune response to a growing tumour. With the identification of TAAs (Van der Bruggen *et al.*, 1991; Vanden Eynde *et al.*, 1997), immunotherapeutic strategies designed to induce cellular immune responses have received much attention as a promising approach for the treatment of many types of cancer. In this context, immunotherapy, which has been amply validated as an effective therapeutic strategy in animal model systems (Yu *et al.*, 2002), has attracted much attention in recent years. The concept of immunotherapy is based on the immunosurveillance theory (Thomas 1959; Burnet 1970). According to this theory, the host's immune system monitors and eliminates tumour cells because of their expression of TAAs. Interest in the clinical application of immunotherapy as a treatment regimen has been rekindled in the last two decades by (i) revival of the immunosurveillance theory, (ii) availability of well defined and structurally characterised human TAAs, (iii) progress in our understanding of the molecular pathways required for the induction and maintenance of an immune response, and (iv) advances in methodologies for the development of specific

immunological probes in the form of TAA-specific cytotoxic T lymphocytes (CTLs) and monoclonal antibodies (mAbs). Many studies have generated much information, and have highlighted some of the major challenges that face tumour immunologists today. Contrary to the results obtained in animal model systems (Yu *et al.*, 2002; Milstein *et al.*, 1999), the clinical outcomes of immunotherapy of malignant diseases have been generally disappointing. In particular, there has been a lack of correlation between immune and clinical response in patients treated with immunotherapy (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). A variety of immunotherapeutic strategies has 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). Tumour cells or accessory cells genetically engineered to produce IL-12 or IL-18 have also been used in animal models of cancer immunotherapy (Yamanaka *et al.*, 2002, 2003). Immunotherapy has been used to target TAAs by either passive or active therapeutic modalities. The former strategy has utilised TAA-specific antibodies and/or CTLs, while the latter strategy has relied on the induction of TAA-specific cellular and/or humoural immune responses in situ. The adoptive transfer of immune cell populations can also implement passive immunotherapy. Many investigators have aimed to enhance the ability of adoptively transferred T cells, particularly CTLs, to target tumours (Kessels *et al.*, 2002). For cell-based immunotherapy, T cells can be transfected with T-cell receptor (TCR) genes specific for the TAA of interest, or chimeric constructs containing antibody variable regions with defined specificities, to increase tumour targeting (Schumacher, 2002; Sadelain *et al.*, 2003). Because of the requirement for constant infusions to maintain therapeutic efficacy, passive immunotherapy may not represent a practical long-term therapeutic modality.

The majority of the previous studies have relied exclusively on the administration of a single immunotherapeutic agent. An alternative approach has been to combine passive and active immunotherapeutic modalities in order to elicit

multiple immune effector functions concurrently. In a HER-2/neu transgenic mouse model, a combination of potent humoural and cellular immunity to the same TAA target elicited higher numbers and enhanced the function of TAA-specific CTLs, and increased tumour-free survival compared with either therapy alone (Wolpoe *et al.*, 2003). As immunologists uncover more of the molecular mechanisms underlying normal immune function, it is possible to rationally design immunotherapeutic approaches to address the current limitations. By taking advantage of the growing interest in the implementation of combination therapeutic strategies, it may be possible to expand the role of immunotherapy as a treatment modality for malignant diseases.

1.2.3 Oncolytic virotherapy

The resistance of cancers to conventional treatments has stimulated the search for novel approaches. Replication competent viruses offer great promise for cancer treatment because of their ability to amplify themselves and spread within the tumour mass. Furthermore they are able to express foreign proteins that enhance their own inherent cytolytic potential. Observations made in the early 1920s indicated that viruses replicated in and lysed murine and other experimental human carcinoma after inoculation of the patient with attenuated rabies vaccine (Dock, 1904; De Pace, 1912). Oncolytic viruses, which have been tested as cancer therapeutics, have either been naturally selected or have been genetically engineered to grow specifically in and kill tumour cells. Specificity to cancer is derived by exploiting cell surface or intracellular aberrations in gene expression that rise in malignancies during tumour evolution (Bell *et al.*, 2003). Adenoviruses are the most widely studied engineered oncolytic viruses in the clinic. These adenoviral constructs include Onyx 015 (Bischoff *et al.*, 1996; Heise *et al.*, 1997), CG7060 (Yu *et al.*, 1999), CG7870 (Yu *et al.*, 1999), dl922-947 (Heise *et al.*, 1997), Ad5-CD/tk-rep (Freytag *et al.*, 1998), Ad-delta24 (Fueyo *et al.*, 2000), Ad DF3-E1 (Kurihara *et al.*, 2000), Onyx 411 (Johnson *et al.*, 2002), OAV001 (Hallenbeck *et al.*, 2002), KD3 (Doronim *et al.*, 2000), and 01/PEME (Ramachandra *et al.*, 2001). Studies in the 1950s with Egypt 101 virus revealed anticancer activity in the form of transient tumour necrosis in patients. However, further studies were discontinued when immune compromised patients developed encephalitis (Southam *et al.*, 1952; Huebner *et al.*, 1956; Russel *et al.*, 1994). Later attenuated mumps virus

was tested in cancer patients and more impressive anticancer activity was observed. Responses, however, were generally transient, and they were limited to the site of injection (Asada, 1974; Yamanishi *et al.*, 1970).

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). Recently, a naturally attenuated strain of NDV (PV701) has undergone phase I human clinical trials in the treatment of advanced solid tumours with encouraging results, and phase II trials are now planned (Pecora *et al.*, 2002; Lorence *et al.*, 2003). A number of articles report the potentiation of cytotoxic effects by viruses expressing drug susceptibility genes while co-administrating the appropriate cytotoxic drug (Aghi *et al.*, 1999; Chase *et al.*, 1998; Freytag *et al.*, 1998). Enhanced cytotoxic effects have also been achieved by the expression of IL-4 and IL-12. Moreover, antitumour immunity has been demonstrated following administration of an IL-12 expressing HSV (Wong *et al.*, 2001). This is particularly noteworthy because stimulation of an antitumour immune response is likely to be critical to the long-term success of the treatment.

An ideal anti-cancer virus would be based upon a highly lytic virus that has been modified so that it would only replicate in tumour cells (by manipulating the viral attachment proteins and/or the use of tumour-specific promoter/enhancer elements). Bergman *et al.* (2001) have recently demonstrated that influenza A NS1 knockout virus replicates selectively in oncogenic *ras*-expressing cells and suggested that the virus represents an attractive candidate for the therapy of tumours exhibiting an activated *ras*-signalling pathway. The E1B and E1A-deleted adenoviral vectors provide selective replication capacity and can be utilised as gene delivery vehicles to potentiate the viral oncolytic effect (Johnson *et al.*, 2002). Interestingly, in Freytag's work (1998), the prodrug anticancer effect was greater than the direct viral cytolytic effect when the prodrug was administered early. Other viruses have also been

explored preclinically as selective cancer therapeutics (e.g. poliovirus (Dobrikova *et al.*, 2003), vesicular stomatitis virus (Fernandez 2002), influenza virus (Bergmann *et al.*, 2003), and measles virus (Peng *et al.*, 2003). 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. Changing the surface components, such as the fiber and knob, to alter cancer binding, particularly when Coxsachic Adenovirus Receptors (CAR) expression is reduced or not displayed has also demonstrated improved anticancer activity (Akiyama *et al.*, 2004). Furthermore, containment of viral particles in liposomes or polymer-coated ligands may improve viral uptake particularly when administered systemically (Fisher *et al* 2003). Clinical investigation of conditional replicating oncolytic viral therapies continues to be pursued in oncology. There is a high degree of confidence now in the safety and selectivity of a variety of oncolytic viruses. 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). Furthermore, understanding of viral biology has led to the modification of the viral genome in order to address therapeutic options and safety issues. In addition, work now being carried out in addressing optimal viral surface coat components, systemic clearance factors, potency, replication capacity, oncolytic capacity, and gene delivery will begin, as more is understood.

1.2.4 Tumour vaccination

Numerous approaches are being used to develop vaccines for the treatment of cancer. Prophylactic approaches focus on the use of vaccines that induce immunity to viruses known to be associated with the development of a tumour. Work on the use of alphavirus vectors as tumour vaccines has concentrated on three mouse models of human tumours, all of which are weakly antigenic and express known tumour-associated antigens. These are the P815 mastocytoma (Colmenero P *et al.*, 1999), Human papilloma virus (HPV) associated proteins E6 and E7, which are associated with cervical carcinoma, and the breast and ovarian carcinoma antigen HER2/neu

(Daemen *et al.* 2000; 2002; 2003). Vaccines developed against specific viral proteins of HPV are currently in clinical trial. Sindbis Virus (SV) vectors expressing E7 fused to the herpes simplex tegument protein VP22, which enhances spread to neighbouring cells, have also been shown to have an enhanced anti-tumour effect (Cheng *et al.*, 2002). Similar results have been obtained by Cassetti *et al.* (2004) for a vector based on VEE, and used to treat 3 different mouse tumour models; in this case mutated E6 and E7 genes lacking oncogenic potential were used. Vaccination with a SV layered DNA-RNA vector expressing the antigen HER2/neu inhibited the growth of a mouse breast carcinoma cell line and reduced the incidence of metastases (Lachman *et al.*, 2001).

A variety of tumour vaccine approaches has been explored for inducing or enhancing a patient's immunity to their tumour. These include injecting killed or irradiated tumour cells from the patients, an approach which has had little success. The identification of appropriate TAAs (those expressed at low levels on normal cells and high levels on tumours), and their potentially immunogenic peptides has resulted in their use in vaccines to focus the patient's immune system to respond to antigens that are primarily tumour associated. As with the immunisation using whole cells, these antigens would most likely induce a T helper cell rather than a more desirable CTL response, as they would enter APCs by an exogenous pathway and be presented on MHC class II molecules. However, it is now clear that the APC presenting antigen to the CTL needs first to be conditioned by interaction with a T helper cell before it can effectively induce a CTL response. Moreover, antigens entering by the exogenous pathway may in some instances be presented on MHC class I molecules and initiate a CTL response.

Since most types of tumour cells do not express the co-stimulatory molecules B7.1 and B7.2, which are important for the induction of an immune response, studies were carried out to determine if transfecting tumour cells with these molecules would enhance their immunogenicity (Tao *et al.*, 2002). B7 transfected tumour cells induced a strong CTL response against the tumour. Furthermore, these CTLs were sometimes able to lyse parent tumour cells not expressing B7, because once activated, CTLs do not need the B7 co-stimulatory signals to kill. Another approach involves transfecting tumour cells with a cytokine gene, as certain cytokines expressed by the tumour may attract, expand, and activate cells of the immune system and induce or enhance immunity to tumour antigens. In experimental models, tumour cells transfected with

cytokine genes such as IL-2 (Ge *et al.*, 2003), IL-12 (Weber *et al.*, 1999), IFN-γ (Yang *et al.*, 1999) and GM-CSF (Zhou *et al.*, 2005) are able to induce immunity to the tumour resulting in its regression or rejection. A very active area of tumour vaccine research involves loading of the patient's dendritic cells (DCs) *in vitro* with TAA and re-injection of these cells into the patient. Since immature DCs are best able to ingest antigen and mature DCs are best at presenting antigen, loading of immature DCs followed by cytokine induced differentiation of these cells to mature DCs is more readily accomplished *in vitro* than *in vivo*. These mature, loaded APCs are then reintroduced into the patient, fully able to stimulate T cells. Considerable effort is directed at determining optimal conditions for loading and maturing DCs so they induce strong CTL antitumour responses when introduced into the patients.

1.2.5 Targeting tumour vasculature

The central concept that tumour growth is angiogenesis dependent (Folkman, 1990) is well accepted today, with more than 2500 scientific reports showing angiogenesis linked to tumour growth. Stated concisely, that it is every increment of tumour growth requires an establishment of vascular growth (Folkman, 1986). It is recognised that the endothelial cell, by paracrine mechanisms, produces growth factors that stimulate the proliferation of the tumour cell population. Thus, there is a bi-directional reciprocal signalling of endothelial and tumour cell growth. Overall, angiogenesis can be viewed as the result of the complex balance of tightly regulated oncogenes and suppressor genes, stimulatory and inhibitory peptides, proteases and endogenous inhibitors, and micro-environmental factors such as the level of oxygen or copper ions. Experiments where tumours were transplanted into an avascular environment prove that angiogenesis is a control point in tumour growth. Tumours deprived of angiogenesis remained dormant indefinitely; rapid logarithmic growth followed the acquisition of the blood supply (Brem *et al.*, 1976). Recent data suggest that anti-angiogenesis not only will be useful in the control of solid tumours (Folkman, 1972) but also may be valuable as therapy for patients with leukaemia and myeloma (Bikfalvi *et al.*, 1994; Bellamy *et al.*, 1999). Angiogenic inhibitors suppress endothelial cell proliferation (Matsubara *et al.*, 1989; Brem *et al.*, 1990) but another important control mechanism is the induction of endothelial cell apoptosis.

Furthermore, even without changes in microvascular density, apoptosis of tumour cells occurs in proximity to endothelium following treatment with angiogenesis inhibitors (Bergers *et al.*, 1999). Recent attention has been focussed on the role of the *p53* tumour suppressor gene in angiogenesis. Exogenous expression of wt-*p53* inhibits angiogenesis *in vivo* resulting in the formation of dormant tumours (Holmgren *et al.*, 1998). The principal growth and regulatory factors driving angiogenesis are vascular endothelial growth factor (VEGF), bFGF, hepatocyte growth factor, angiopoietin-1, angiotropin, angiogenin, epidermal growth factor, granulocyte-CSF, IL-1, IL-6, IL-8, platelet-derived growth factor (PDGF), and tumour necrosis factor-alpha TNF-α (Fan *et al.*, 1992; Benjamin *et al.*, 1999; Schmidt *et al.*, 1999).

Among the most promising of exciting new gene therapies are the regulators of angiogenesis. It has been demonstrated that the transfection of antisense-VEGF-cDNA results in down-regulation of the endogenous VEGF and suppresses the ability of glioma cells to form tumours in mice (Fueyo *et al.*, 1999). Many workers have shown that the tumour vasculature can be disrupted following administration of cytokines such as IL-12 and IL-18 (Coughlin *et al.*, 1998; Chada *et al.*, 2003). Central to the antitumoural capacity of these cytokines is the induction of strong IFN-γ responses leading to production of the chemokines MIG (monokine induced by IFN-γ) and IP-10 (IFN-γ inducible protein-10). In clinical trials both cytokines have shown potent antitumour properties; however, systemic administration of either cytokine has been associated with severe toxic side effects (Dranoff, 2004). In contrast, local administration of vectors coding for IL-12 or IL-18 is well tolerated. Intratumoural injection of rSFV-IL-12 into implanted B16 melanomas inhibited tumour growth. This was not associated with an increase in cytotoxic T-cell or natural killer (NK) cell activity, but was associated with inhibition of neovascularisation of the tumour (Asselin-Paturel *et al.*, 1999).

Currently, there are about thirty-five antiangiogenesis therapies being evaluated in clinical trials but so far there is no drug approved by the Food and Drug Administration (FDA) for antiangiogenesis. As we learn more about the fundamental mechanisms of angiogenesis, there are many opportunities for drug discovery and development of effective methods of cancer control will be implemented.

1.3 CYTOKINES

Cytokines are small molecules that signal between cells, inducing growth, differentiation, chemotaxis, activation, enhanced cytotoxicity and/or regulation of immunity (Berkow *et al.*, 1997). They have autocrine and paracrine functions, so that they function locally or at a distance to enhance or suppress immunity. Cytokines regulate the innate immune system: natural killer (NK) cells, macrophages, and neutrophils. They also regulate the adaptive immune system, the T and B cell immune responses. They are referred to as interleukins if produced primarily by leukocytes, monokines if produced by myeloid cells, lymphokines if produced by lymphocytes. The term interleukin (IL) is often used to describe cytokines produced by leukocytes. A group of small heparin binding cytokines, chemokines, has recently been recognised which direct cell migration and may also activate cells in response to infectious agents or tissue damage. Interferons, secreted by a variety of cells, are involved in defence against viral infection and in activation and modulation of immunity. The term type-1 cytokine refers to cytokines produced by T_H1 T-helper cells while type-2 cytokines are those produced by T_H2 T-helper cells.

More than 40 cytokines have been discovered in recent times and are grouped into i) growth factors, ii) interleukins, iii) interferons, iv) chemokines, v) haematopoietic and other cytokines. Today, by using genetic recombinant methods, 11 cytokines have been prepared for therapeutic applications out of which haematopoietic growth factors, granulocyte-monocyte colony stimulating factors and granulocyte-colony stimulating factors have successfully been used to stimulate the production of red blood cells, granulocytes and monocytes. All these colony-stimulating factors are of therapeutic importance in the treatment of anaemia and in the elevation of the levels of granulocytic and monocytic leucocytes in some diseases that involve bone marrow disorders. After many years of clinical trials, the use of alpha, beta and gamma interferons are limited to the treatment of infections like hepatitis B and/or C and to some types of cancer such as hairy cell leukaemia and chronic granulocytic leukaemia. Other cytokines such as the interleukins IL-2, IL-3, IL-4 and IL-6 and monocyte colony-stimulating factor have had therapeutic benefits in some clinical trials. It is hoped that in the future, some of them in combination with other immunological activators may show synergistic biological effects and be used in

the treatment of diseases like acquired immune deficiency syndrome or malignant tumours, but further studies are required.

1.3.1 Cytokines in tumour therapy

Immunogenetherapy with cytokines is one of the promising strategies for malignant disease and aims at the activation of tumour specific T-lymphocytes and/or natural killer (NK) cells capable of killing tumour cells in patients with low tumour burden after surgical tumour removal, or at protecting patients from recurrence after initial response to treatment (Mach *et al.*, 2000). Cytokines previously used for immunotherapy of cancer include IL-2, IL-4, IL-7, IL-12, interferon-γ, GM-CSF, and TNF-α, alone or in combination with co-stimulatory molecules, such as B7-1 or CD40 (Niranjan *et al.*, 2000; Mackensen *et al.*, 1997; Qian *et al.*, 2002). Theoretically, cytokine immunotherapy aims at actively stimulating CTLs and/or NK cells to achieve systemic and/or local cellular anti-tumour responses. Immunotherapy in murine tumour models with low immunogenicity using cytokines has demonstrated regression of tumours and in some instances immunological memory has been achieved for a tumour challenge (Boulikas, 1997 & 1998). Overall, cytokines are substances that appear to have application in the treatment of haematological malignancies or immunogenic tumours. A multifaceted cellular and humoural reaction might be most likely to overcome the immuno-evasive properties and many apoptotic defects that are intrinsic to cancer cells (Curcio *et al.*, 2003).

The aim of this study is to evaluate safety, maximum tolerated dose and antitumour efficacy of recombinant Semliki Forest virus with enhanced expression ability carrying the murine interleukin-12 gene in three different tumour models, to investigate IL-12 therapeutic efficacy. Some experiments were also carried out with IL-18.

1.3.2 Tumour treatment with IL-12

Antitumour effects mediated by the administration of recombinant cytokines, including IL-1 (Ciolli *et al.*, 1991), IL-2 (Rosenberg *et al.*, 1985), IL-4 (Bosco *et al.*, 1990), IFN-α (Brunda *et al.*, 1987; Sayers *et al.*, 1990), IFN-γ (Giovarelli *et al.*, 1986), and TNF-α (Mule` *et al.*, 1988), have been investigated in mice bearing transplantable tumours, as well as in patients with cancer. These cytokines have had limited antitumour effects with significant side effects. Recently, IL-12 has been shown to be effective in the treatment of murine tumours with minimal side effects (Brunda *et al.*, 1993; Nastala *et al.*, 1994). In these studies, the antitumour effects of IL-12 on cancer are mediated by NK cells, CD4+, CD8+ T cells, and IFN- γ (Brunda *et al.*, 1996).

The cytokine interleukin-12 (IL-12) is a disulphide linked hetero-dimeric protein, comprising an α-chain (p35 subunit) and a β-chain (p40 subunit) to form a biologically active 74 kDa protein (Figure 1.1). IL-12 is produced by antigen presenting cells (APC) such as dendritic cells (DC), macrophages, B-cells and possibly by other accessory cells following encounter with infectious agents (Trinchieri, 1995). The cells that produce the biologically active IL-12 heterodimer secrete β-chain in free form in a 10-100-fold excess over the IL-12 heterodimer (D'Andrea *et al.*, 1992). Disulfide linked homodimers of β-chain are produced in the mouse which have the ability to block IL-12 functions *in vitro* and *in vivo* (Gillessen *et al.*, 1995) whereas the physiological relevance of human β-chain homo-dimers is still debated (Ling *et al.*, 1995; Carra *et al.*, 2000). The two IL-12 receptor (IL-12R) subunits designated as IL-12R β1 and β2 are primarily expressed on activated T and NK cells (Gately *et al.*, 1998), which belong to the gp130 subgroup of the cytokine receptor super-family. They are type I transmembrane glycoproteins, which occur as dimers/oligomers (Gately *et al.*, 1998). Even though co-expression of both of the IL-12R subunits is required for the generation of human high affinity IL-12 binding sites, recent evidence suggests that expression of both the human and mouse IL-12R β2 proteins may be confined to Th1 cells and correlates with IL-12 responsiveness in these cells (Szabo *et al.*, 1997). IL-12 is a major cytokine to induce the differentiation of T cells (CD4+ and CD8+) cells, which are potential producers of IFN-γ production (Brunda *et al.*, 1993) (Figure 1.2). It has been reported that local and/or systemic

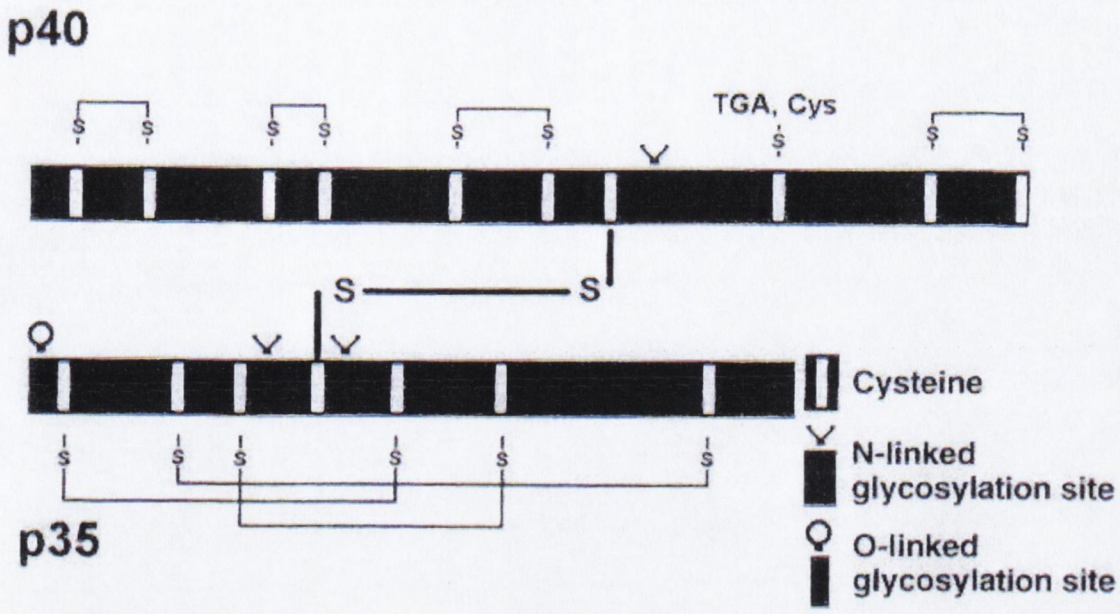


Figure 1.1 Schematic model of the interleukin-12 molecule, a disulfide linked heterodimer formed by a heavy chain (p40, above) and a light chain (p35, below)

Source: *The Cytokine Networks and Immune Function. Oxford University press. Jacques Theze. Third edition. 1996.*

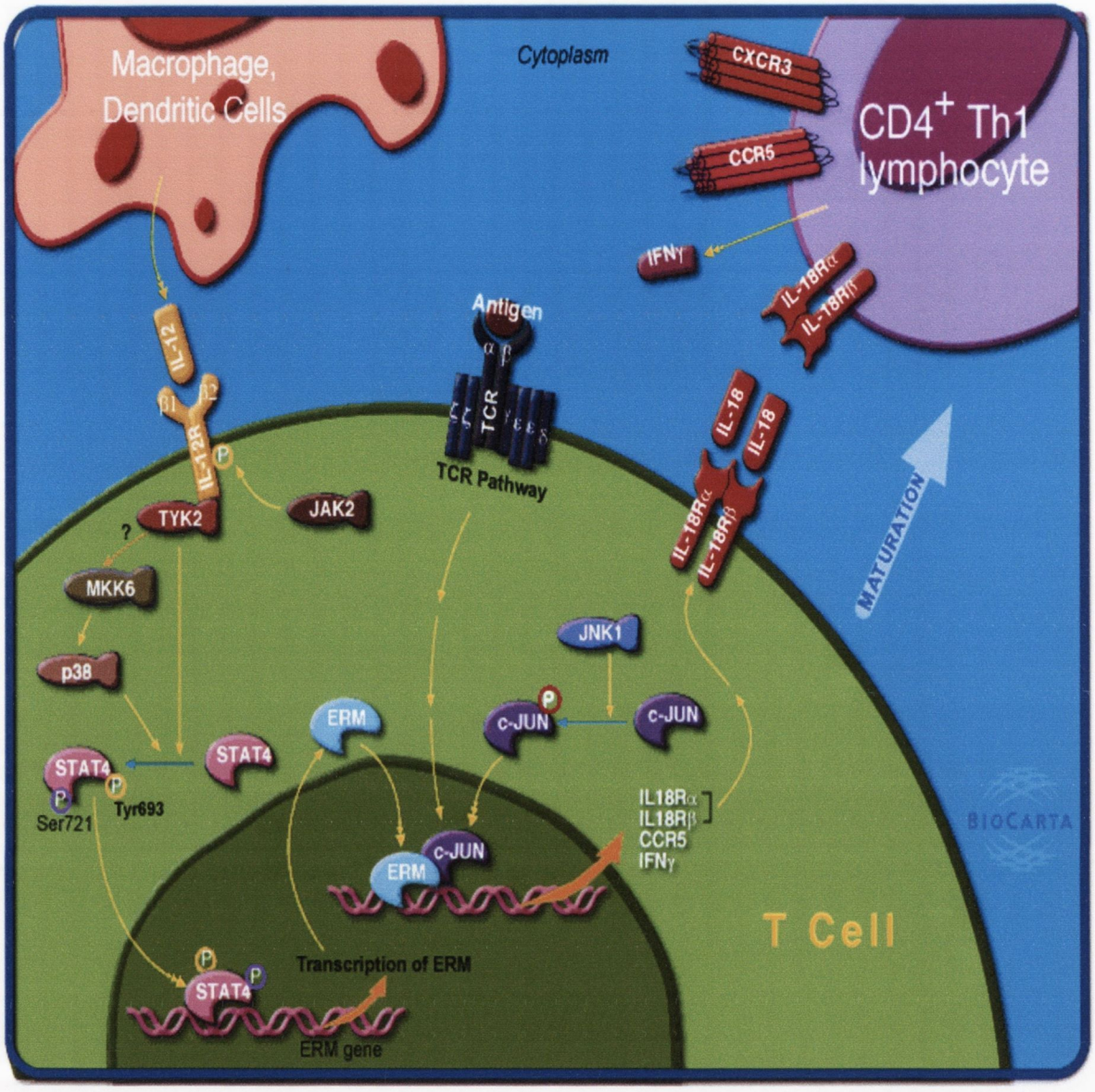


Figure 1.2 IL-12 and Stat-4 Dependent Signalling Pathway in Th1 Development

Interleukin-12 (IL-12) promotes cell-mediated immunity by inducing Th1 cell differentiation and activation of both T cells and NK cells. Dendritic cells and macrophages in peripheral tissues act as antigen presenting cells and secrete IL-12 as one component of the antigen response, Th1 differentiation. The role of IL-12 in cellular immunity is largely mediated by the STAT-4 transcription factor. STAT-4 is essential for IL-12 activity and the phenotype of mice lacking STAT-4 is very similar to the phenotype of mice lacking the IL-12 receptor or IL-12. The role of IL-12 in Th1 differentiation may not be to induce the Th1 cell fate, but to stimulate growth of

cells determined for the Th1 cell fate by the T-bet transcription factor. Several signaling pathways contribute to IL-12 activation of STAT-4 to regulate cell-mediated immune responses. The JAK kinases such as JAK2 and TYK2 interact with the activated IL-12 receptor and tyrosine phosphorylates the IL-12 receptor and STAT-4. IL-12 also activates a map kinase pathway activating the map kinase kinase MKK6 and p38. Phosphorylation of STAT-4 on serine 721 by p38 contributes to the full transcriptional activation of genes by STAT-4. Some of the events downstream of IL-12 appear to include genes activated indirectly by STAT-4, such as genes activated by the transcription factor ERM. ERM is in the Ets family of transcription factors, is activated by IL-12 and activates IL-12 inducible genes such as interferon-gamma that are not activated by STAT-4 itself. Interferon-gamma transcription in T cells is also activated by other signals such as from the T cell receptor. Other proteins activated transcriptionally downstream of IL-12 and STAT-4 include the chemokine receptor CCR5 and IL-18 and its receptor. Some viruses, including HIV, repress cell-mediated immunity by blocking IL-12 signalling.

Source: ***http://www.biocarta.com/pathfiles/h_IL12Pathway.asp***

Contributor: Glenn Croston, PhD.

tumour treatment using IL-12 induces IFN-γ and chemokines such as IP-10 and MIG at the tumour site, contributing to the antitumour effects through the direct toxic effect on tumour cells, or by activating the potent anti-angiogenic mechanisms (Sgadari *et al.*, 1996). IL-12 treatment was shown to almost completely inhibit corneal neo-vascularisation in C57BL/6, SCID and beige mice (Voest *et al.*, 1995). The role of angiogenesis inhibition in the anti-tumour activity of IL-12 was also demonstrated in K1735 murine melanomas (Gee *et al.*, 2003). The IL-12 induced anti-angiogenic mechanisms are complex and dependent both on the direct effect of the pro-inflammatory cytokines/chemokines on endothelial cells and by the recruitment of effector NK and T-cells. In other instances, IL-12 induced specific immunological memory has been reported reflecting the potency of IL-12 as a promising candidate for specific tumour eradication (Colmenero *et al.*, 2002). Several other reports have shown that IL-12 has a strong antitumour effect against other tumour models such as B16 melanoma (Nanni *et al.*, 1998), C26 colon carcinoma (Chiodoni *et al.*, 2001), TSA mammary carcinoma (Cavallo *et al.*, 1997; Di Carlo *et al.*, 2001), SCK mammary carcinoma and K1735 melanoma (Coughline *et al.*, 1995).

However, it has been shown that the amount of IL-12 at the tumour site is critical for tumour regression and expressing IL-12 locally can avoid complications such as toxic effects caused by high-level systemic IL-12 concentrations (Colombo *et al.*, 1996). In order to sustain high IL-12 concentration at the tumour site approaches such as frequent intra-tumoural injections with IL-12 expressing agents may be required. Recently, Rodriguez-Madoz *et al.*, (2005) have demonstrated that an SFV vector with an enhanced ability to express IL-12 has induced complete tumour regressions in murine colon adenocarcinomas. The promising data obtained in the pre-clinical models of antitumour immunotherapy have raised much hope that IL-12 could be a powerful therapeutic agent against cancer. Clinical protocols that minimise toxicity without affecting the anti-tumour effect of IL-12 need to be planned.

1.3.3 Tumour treatment with IL-18

A number of investigators have reported the potential of introduction of genes encoding cytokines into tumour cells as an antitumour strategy; tumour cells engineered to secrete cytokines inhibit tumour growth *in vivo* and induce systemic immune reaction against the untreated parental tumours (Colombo *et al.*, 1991;

Botazzi *et al.*, 1992; Yamanaka *et al.*, 1999; Xiang *et al.*, 2001, etc.). Among this form of tumour vaccination therapy, transduction of the IL-18 gene has been shown to exert marked anti-tumour effects (Hara *et al.*, 2000; Hara *et al.*, 2001). IL-18, produced by the monocyte lineage, was originally identified as an IFN-γ inducing factor, and exhibits powerful antitumour activity *in vivo* by the augmentation of NK cell activity as well as the production of IFN-γ by stimulated T-cells (Okamura *et al.*, 1995; Micallef *et al.*, 1997). Although IL-18 shares some structural similarities with IL-1 (Okamura *et al.*, 1995), IL-18 appears to have its own receptor (Akita *et al.*, 1997). Furthermore, several studies have demonstrated the synergistic effect of IL-12 and IL-18 on IFN-γ production by T-cells (Oshikawa *et al.*, 1999; Tatsumi *et al.*, 2003). Although IL-12 and IL-18 synergise in IFN-γ production, their receptors and signal transduction pathways appear to be different (Kohno *et al.*, 1997).

IL-18 binding protein (IL-18BP) is a recently discovered inhibitor of IL-18 that is distinct from the IL-1 and IL-18 receptor family (Novick *et al.*, 1999). IL-18BP has been shown to block IFN-γ production induced by LPS, IL-18, or IL-12 and also may play a central role in the control of Th1 immune responses by limiting the availability of IL-18 to activate lymphocytes in conjunction with IL-12 or IL-2. In addition, it has been shown that those cytokines capable of synergising with IL-18 to stimulate IFN-γ production, most notably IL-12, are the primary inducers of IL-18BPa in monocytes. The induction of this inhibitor of IL-18 by IL-12 is IFN-γ dependent, suggesting that IL-18BPa forms part of a negative feedback loop designed to limit immune activation by IL-12 (Veenstra *et al.*, 2002).

1.4 VECTORS USED FOR TUMOUR THERAPY

The concept of using viruses as oncolytic agents dates back nearly a century. The recent advances in the fields of molecular biology, genetics, and virology have enabled investigators to engineer viruses with greater potency and tumour specificity. Further, developments in this approach involve arming the vectors with therapeutic transgenes, combining the traditional chemo- or radiotherapies with oncolytic viral therapy and modulating the immune responses to reduce antiviral immunity while at the same time maximising antitumour immunity. A variety of viruses has been adapted for gene therapy in this manner, each with its own advantages/disadvantages

depending on the particular application (El-Aneed, 2004). 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 in several *in vivo* animal tumour models (Stojdl *et al.*, 2000; Balachandran *et al.*, 2001; Ebert *et al.*, 2003; Shinozaki *et al.*, 2004). The two main types of gene delivery vector for cancer therapy are viral and non-viral vectors. There are a number of naturally occurring viruses that are tumour selective in replication and cytolysis but it is unlikely that all these viruses will demonstrate the degree of tumour selectivity required for the safe routine treatment of cancer patients. Viral vectors are modified viruses in which the genes required for viral replication have been either partially or completely removed, and replaced with the therapeutic gene(s). The viral proteins, which are encoded for by the missing viral genes, are supplied *in trans*, using a 'helper' virus(es) (Smerdou & Liljeström, 1999) or a packaging cell line (Markowitz *et al,*, 1988). The main viral vectors currently under examination as gene therapy vectors are discussed below.

1.4.1 Adenovirus vectors

Adenovirus (Ad) is a non-enveloped linear double stranded DNA virus of approximately 38kb genome size, which is capable of infecting dividing and non-dividing cells. Adenoviral vectors allow local, high efficiency, but transient transgene expression. The majority of cancer therapy studies that have given the most encouraging results to date have utilised this Ad system as the delivery agent (Sobol & Scanlon, 1995). Adenoviral-based cytokine gene therapy has many advantages over other forms of cytokine delivery systems (Arthur *et al.*, 1997). Transgenic mice developing spontaneous breast tumours given a single intratumoural injection of AdVmIL-12 showed tumour regressions in 75% of treated mice (Bramson *et al.*, 1995, 1996, 2002). Most first generation Ad vectors were based on the Ad5 serotype (Li *et al.*, 1993), since this vector was capable of transducing cells at very high efficiency, regardless of their mitotic status, to give high levels of expression of the therapeutic gene (Mehtali *et al.*, 1996). Since Ad is a human pathogen (Horwitz, 1990), causing mild upper and lower respiratory tract infections, toxic effects in patients have been reported after administration of an Ad vector expressing *wt-p53* genes (Mountain, 2000). One adenovirus construct ONYX-015, exhibits marked

cytopathic effects in *p53* mutant cancer cells, but only limited cytotoxicity in normal fibroblasts and endothelial cells with normal *p53* function (Hann *et al.*, 2003; Bischoff *et al.*, 1996). However, in another Phase I/II dose-escalation trial of intra-arterial ONYX-015 administration to patients with colorectal carcinoma liver-metastases, patients who received the highest doses experienced better survival than those patients treated with lower doses (Reid *et al.*, 2002). To summarise, steps should be taken to reduce the toxicity associated with the administration of Ad vectors to increase the treatment efficacy.

1.4.2 Retrovirus vectors

Retroviral vectors (Rv) are being used in a wide range of clinical trials, which have induced apoptosis and tumour regression following Rv-mediated exogenous *wt-p53* delivery (Fujiwara *et al.*, 1994; Roth *et al.*, 1996). One of the advantages of using these retroviruses is their ability to integrate their reverse-transcribed DNA into the host cell genome and maintain prolonged expression (Miller *et al.*, 1990) but at much reduced levels. 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). In contrast to Ad systems, most first generation Rv have limited host range, but do not express residual viral proteins and thus are much less immunogenic than Ad (Roth & Cristiano, 1997; Ghazizadeh *et al.*, 1997). For cancer gene therapy, Rv has been most useful where prolonged gene expression was required, such as the delivery of antisense mRNAs or anti-angiogenic factors (Chung-Faye *et al.*, 2000). However, given recent reports, the risk of oncogenesis induced by retroviral integration is much higher than with any other vector (Marshall, 2003). These risks must therefore be reduced by strategies such as targeting integration to inactive regions of the host genome.

1.4.3 Adeno-associated virus vectors

Adeno-associated viruses (AAVs) are small linear ssDNA defective non-pathogenic parvoviruses having several advantages for gene therapy, including low pathogenicity, a broad host range and stable expression of foreign proteins (Monaghan & Samulski, 2000). AAV vectors expressing *wt-p53* have shown great

promise in mediating human lung tumour regression both *in vitro* and *in vivo* (Quazilbash *et al.*, 1997), and have been utilised in various other anti-cancer strategies for exogenous expression of anti-angiogenic and pro-apoptotic factors (Nguyen *et al.*, 1998). The AAV vector is known to be particularly useful for long-term expression of transgenes in muscle, liver, and brain cells (Chintala *et al.*, 1997; Yanamandra *et al.*, 2005). Noro *et al* (2004) have demonstrated that a classical AAV serotype 2 vector expressing endostatin achieves a state of antiangiogenesis in model animals and also have shown that the route of vector administration influences the efficacy of AAV-mediated endostatin expression. AAV has an integrative capacity for a specific region of chromosome 19 (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). All these data suggest that AAV-mediated long-term expression of therapeutic genes may have clinical utility against the recurrence of cancer after primary therapies and may present rational adjuvant therapies in combination with radiation or chemotherapy.

Other Viral vectors; In addition to the above mentioned virus vectors, certain herpes simplex virus (HSV) vectors appear to be active and selective against malignant tissue. The replication competent oncolytic HSV is also a popular vector currently in use in several clinical trials of cancer gene therapy (Latchman, 2001; Moriuchi *et al.*, 2002). This vector has a wide cell tropism and remains extrachromosomal, therefore minimizing the risk of insertional mutagenesis. Recently, investigators have demonstrated oncolytic activity of reovirus against human ovarian and colon cancer cells lines carrying a high percentage of *k-ras* mutations implanted in mice (Robert *et al.*, 1999). Many studies have used vaccinia virus, a poxvirus as an immunotherapeutic agent. Administration of mutant vaccinia virus has resulted in conditional replication in cancer cells but not in normal cells (Naik *et al.*, 2001). Whether using mutant vaccinia viruses or wild type vaccinia virus tumour lysates as antitumour vaccines (Wallack *et al.*, 1992; Scoggin *et al.*, 1992), vaccinia virus appears to offer multiple potential ways to treat cancer. Similarly, Newcastle disease virus (NDV), a paramyxovirus has been used in clinical trials as an oncolysate tumour vaccine (Pecora *et al.*, 2002; Batliwalla *et al.*, 1998). Baculovirus, an insect virus (Lentolainen *et al.*, 2002; Forstova *et al.*, 1995), and lentiviral vectors, such as Simian Immunodeficiency virus (SIV) and HIV (Trono, 2000) are also being

considered for gene therapy, primarily due to high levels of protein expression. A huge amount of work is yet to be done to improve the safety of the viral vectors. With the efficient monitoring of trials and continuous improvement of viral vectors, gene therapy may still represent an important addition to conventional therapies for a range of diseases.

1.4.4 Non-viral vectors

Non-viral therapies were initially developed due to their ease of manufacture and lower toxicity and immunogenicity compared with their viral counterparts (Ledley, 1994). *In vivo* electroporation is a safe, non-toxic delivery system and has been used for efficient delivery of chemotherapeutic agents and plasmid DNA including plasmids encoding IL-12 (Lucas *et al.*, 2000 & 2002) and GM-CSF or IL-2 (Heller *et al.*, 1999 & 2000). However, naked DNA results in only very transient gene expression, is rapidly degraded and effects are only limited to the site of injection (Li & Hunag, 2000). Intratumoural injection of naked plasmids leads to accumulation of considerable amounts (50%) of DNA within the tumour and plasmid-derived protein was detected in one study (Nomura *et al.*, 1997). A study has investigated the treatment of human and murine melanomas in mice by intratumoural application of IL-12 encoding plasmid DNA *in vivo* and observed potent anti-tumour effects (Heinzerling *et al.*, 2002). This technology has been applied in three clinical protocols for the generation of vaccines against colon cancer and melanoma (Sobol & Scanlon, 1995). It would be interesting in future to encapsulate the non-viral delivery systems in the liposomal encapsulated form, which restricts the early recognition and degradation of the delivery systems by the immune system.

1.4.5 Alphavirus vectors

Alphaviruses are enveloped, positive single-stranded RNA viruses that are proving their value as expression vectors (Karlsson *et al.*, 2004; Lundstrom, 2001; 2003a). Over the past 10 years, the alphavirus RNA replication and packaging machinery has been adapted for expression of heterologous genes (Lundstrom, 2003a; Berglund *et al.*, 1993). The alphaviruses are arthropod-borne, neurotropic viruses, which may also infect mammalian and avian species. Through recombinant

technology, the alphavirus RNA replication machinery has been engineered for high-level expression of the proteins. As a transient expression system, alphavirus vectors offer several advantages such as (i) a broad range of host cells (ii) high levels of RNA and protein expression without splicing or integrating into the host genome, (iii) full length infectious RNA transcripts can be generated by *in vitro* transcription, (iv) rapid high-titre production.

The three alphaviruses that are now serving as vectors are Sindbis virus (SV), Semliki Forest virus (SFV), and Venezuelan equine encephalitis (VEE) virus. The generation of infectious clones of SV (Rice *et al.*, 1987), SFV (Liljeström *et al.*, 1991), and VEE (Davis *et al.*, 1989) has allowed the development of expression vectors from these three alphaviruses by cloning viral cDNA under the control of a prokaryotic DNA-dependent RNA polymerase promoter, such as SP6 or T7, to allow viral mRNA to be transcribed. SV and SFV have recently been developed as prototype anticancer agents whereas VEE, being a human pathogen, is a potential biological weapon. SV (and its derived vector) has been shown to have an inherent avidity for malignant tissues (Tseng *et al.*, 2004). Alphavirus vectors, particularly those based on the replicon of Semliki Forest virus, have shown great potential as gene delivery vehicles for various applications in cancer gene therapy (Atkins *et al.*, 2004). SFV-based vectors are also being developed as prototype vaccines (Morris-downess *et al.*, 1999) and as vectors for the central nervous system (Jerusalmi *et al.*, 2003). The rapid production of high titre recombinant SFV particles, which show high transduction rates in various mammalian cells lines *in vitro* and *in vivo*, results in high levels of transgene expression. Additionally, the inherent apoptosis induction by SFV vectors in transduced host cells can further increase their efficiency in tumour therapy. Tumour vaccine approaches have been taken by injection of SFV vectors as naked RNA molecules, DNA plasmids or recombinant particles to achieve both therapeutic and prophylactic efficacy. The continuous improvement of alphavirus vectors will further expand the application range in the future.

1.5 SEMLIKI FOREST VIRUS

The 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 from mosquitoes in the Semliki rain forest, western Uganda in 1944 (Smithburn and Haddow 1944). It is naturally transmitted to vertebrates (avian and mammalian hosts) by mosquitoes, creating a life cycle between the two. Most human infections of SFV are subclinical; however, there has been one death suspected to be due to laboratory infection by SFV (Willems *et al,* 1979). A second set of cases were reported in 1987 when SFV was isolated from serum samples of individuals in the Central African Republic with fever, persistent headache, myalgias, and arthralgias (Mathiot *et al,* 1990). The original isolates of SFV are designated as (i) L10, neurovirulent for mice causing lethal encephalitis; (ii) an avirulent strain A7. Most of the SFV strains used for laboratory studies are derived from these two isolates.

1.5.1 The structure of the SFV virion

SFV is a spherical enveloped small virus, measuring 69 nm in diameter. It is a relatively simple virus, encoding only nine functional proteins, four of which are nonstructural proteins that are concerned with viral RNA synthesis and the remaining are the structural proteins which form the capsid (C) protein and the envelope 6K (E1, E2, and E3) proteins. SFV virions are composed of an envelope of host-derived membrane studded with 80 spikes, which tightly surrounds a nucleocapsid made up of 240 copies of capsid (C) protein (Vogel *et al.*, 1986) in association with a single positive-sense ssRNA molecule of approximately 11.4 kb. Each viral spike consists of three copies each of the glycoproteins E1/E2/E3 (Garoff *et al.*, 1974). The distance between each spike structure ranges from 4 to 6 nm. The E1-E1 interactions are responsible for maintaining spike stability, as suggested by cross-linking studies (Wahlberg *et al,* 1992). The nucleocapsid is organised into T=4 icosahedral lattices (Choi *et al*, 1991, von Bonsdorff and Harrison 1975) (Figure1.3). The C protein (approx. 30 kDa) contains an N-terminal RNA-binding domain and a conserved chymotrypsin-like serine protease COOH terminal domain, which facilitates capsid autocleavage from the nascent viral polypeptide (Melancon and Garoff, 1987; Owen and Kuhn, 1996).

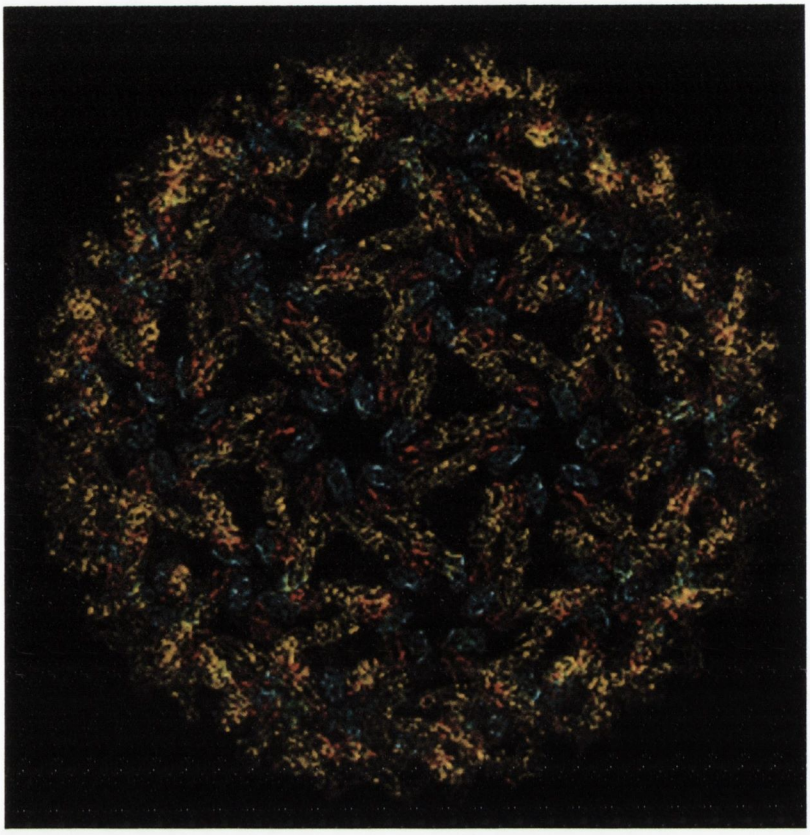


Figure 1.3 The T=4 icosahedral protein layer (envelope shell) formed by E1 on the Semliki Forest Virus surface.

Source: *The European Synchroton Radiation Facility (ESRF).*
www.esrf.fr/.../2001/ life-sciences/LS3.html

The C protein has 267 amino acids (a.a.) and an apparent molecular weight of 30 kDa. It contains an RNA-binding domain residing in the N-terminus and a conserved C-terminal domain similar to chymotrypsin-like serine proteases (Choi *et al,* 1991, Melancon and Garoff 1987, Weiss *et al,* 1989). The E1 glycoprotein is comprised of 438 amino acids (a.a.) 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 a.a, has a molecular mass of 52 kDa, and contains a N-terminal ectodomain (Simons & Garoff, 1980), a hydrophobic transmembrane region that serves as a receptor binding subunit and a large, 31 a.a. C-terminal sequence, which resides within the viral membrane and interacts with the virion nucleocapsid (Skoging *et al.*, 1996; Metsikko & Garoff, 1990). E2 is formed from a precursor, p62, which also contains a smaller 66 a.a. glycoprotein E3. The p62 protein forms a heterodimer with E1 via its E3 domain in the ER (Lobigs *et al,* 1990, Wahlberg *et al,* 1989). 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). Finally, there is a small 66 aa hydrophobic peptide, 6K, found in submolar quantities in the SFV virion. Although of unknown function, it may be involved in viral budding (Liljeström *et al.*, 1991; Lusa *et al.*, 1991).

1.5.2 The SFV replication cycle

1.5.2.1 Viral entry

Alphaviruses enter cells by cell fusion, and/or receptor-mediated endocytosis which is normally reserved by the cell as an uptake mechanism for receptor-ligand complexes. Different proteins have been suggested as functional receptors for SFV. Early studies first suggested the HLA and the H2 histocompatibility antigens as cell surface receptors (Helenius *et al,* 1978); however, it was later proved that the presence of these antigens is not essential for infectivity (Oldstone *et al,* 1980).

The viral protein E2 is responsible for the interactions with cell receptors (Dubuisson and Rice 1993). The virus binds to a receptor on the cell surface and the virus in vesicles is transferred to endosomes; fusion of viral envelope with the endosome membrane then occurs (DeTulleo & Kirchausen, 1998). A reduction in endosomal pH induces a conformational change in the viral spike proteins, resulting in E1-E2 heterooligomeric dissociation as well as conformational changes within individual E1 and E2 subunits (Wahlberg *et al.*, 1992). E2 dissociation stimulates E1 homotrimerisation at the centre of the spike. This E1 homotrimer directly mediates fusion of the viral membrane with the endosomal membrane (Wahlberg *et al.*, 1992). This fusion requires the presence of cholesterol and sphingolipid in the target membrane (Nieva *et al,* 1994, Phalen and Kielian 1991, Smit *et al,* 1999). Following fusion, the nucleocapsid is released into the cytoplasm where it is uncoated by host cell ribosomes (Singh & Helenius, 1992). This helps in the release of viral RNA into the cytoplasm.

1.5.2.2 Viral RNA replication

SFV replication occurs in the cytoplasm of infected cells, more specifically in 0.6-2.0 2 μm wide cytoplasmic vacuoles (CPV-1) derived from endosomes and lysosomes (Froshauer *et al*, 1988; Peränen & Kääriäinen, 1991). The SFV RNA genome is 11,442 nt long with positive polarity (5' capped and 3' polyadenylated) which functions directly as mRNA (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) which comprise the viral replicase, and the remaining one third towards the 3' end encodes the structural proteins (C, p62, 6K, and E1) (Strauss & Strauss, 1994).

Replication begins with translation of the 5' two-thirds of the viral genome, coding for the non-structural polyprotein. Protein nsP1 encodes a methyl transferase responsible for virus-specific capping activity and initiation of minus strand synthesis (Mi *et al,* 1989, Mi and Stollar 1991). The nsP4 protein is responsible for cleaving the polyprotein into its four subcomponents (Ding and Schlesinger 1989, Hardy and Strauss 1989). The nsP2 performs helicase and protease function during the viral replication; nsP3 is a phosphoprotein involved in RNA replication (La Starza *et al,*

1994, Peränen *et al.*, 1991); however, it may be required in some capacity for RNA synthesis (LaStarza *et al,* 1994). The SFV RNA polymerase activity is located in the protein nsP4 (Hahn *et al,* 1989, Sawicki *et al,* 1990). The replicase is responsible for the synthesis of minus-strand 42S RNA species from the positive-strand (Strauss and Strauss, 1994). This intermediate RNA species serves as the template for synthesis of new positive-strand 42S RNA, and for the production of subgenomic 26S RNA, by virtue of its internal subgenomic promoter (Sawicki *et al.*, 1978). Subgenomic 26S RNAs serve as mRNA for the translation of structural precursor polyproteins, which are cleaved to yield viral structural proteins (Figure 1.4) (Welch and Sefton, 1980; Welch *et al.*, 1981). Daughter RNA molecules are capped and polyadenylated by the replicase complex, after which full-length 42S RNAs are predominantly encapsidated into new virions (Sawiki *et al.*, 1978).

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 (Garoff *et al,* 1982). All structural proteins are translated from the 26S subgenomic RNA early (2 to 3 hours post infection) in the replication cycle. The efficiency of translation is explained by the secondary structure of the 5' NTR, and a translational enhancer sequence present in the first 102 nucleotides of the capsid-encoding region (Frolov and Schlesinger, 1994; Sjöberg and Garoff, 1996). Once synthesised, the C protein folds to create at its COOH-terminus, a serine-protease catalytic site, which is autoproteolytically cleaved off the nascent polypeptide chain; it multimerises and associates with genomic 42S RNA in the cytoplasm, forming nucleocapsids (Aliperti and Schlesinger 1978, Hahn and Strauss 1990, Melancon and Garoff 1987). This occurs in association with the large ribosomal subunits (Ulmanen *et al.*, 1976). Within the *nsP2* gene, an encapsidation signal is responsible for viral RNA packaging (White *et al.*, 1998).

Cleavage of the C protein from the polypeptide exposes the N-terminus of the p62 polypeptide precursor, which functions as a signal sequence to facilitate translocation into the endoplasmic reticulum (ER) (Garoff *et al.*, 1990). The ER signal peptidase cleaves E1 and 6K from the polyprotein. The p62 and E1 become glycosylated, and associate to form a heterodimer complex in the ER following the

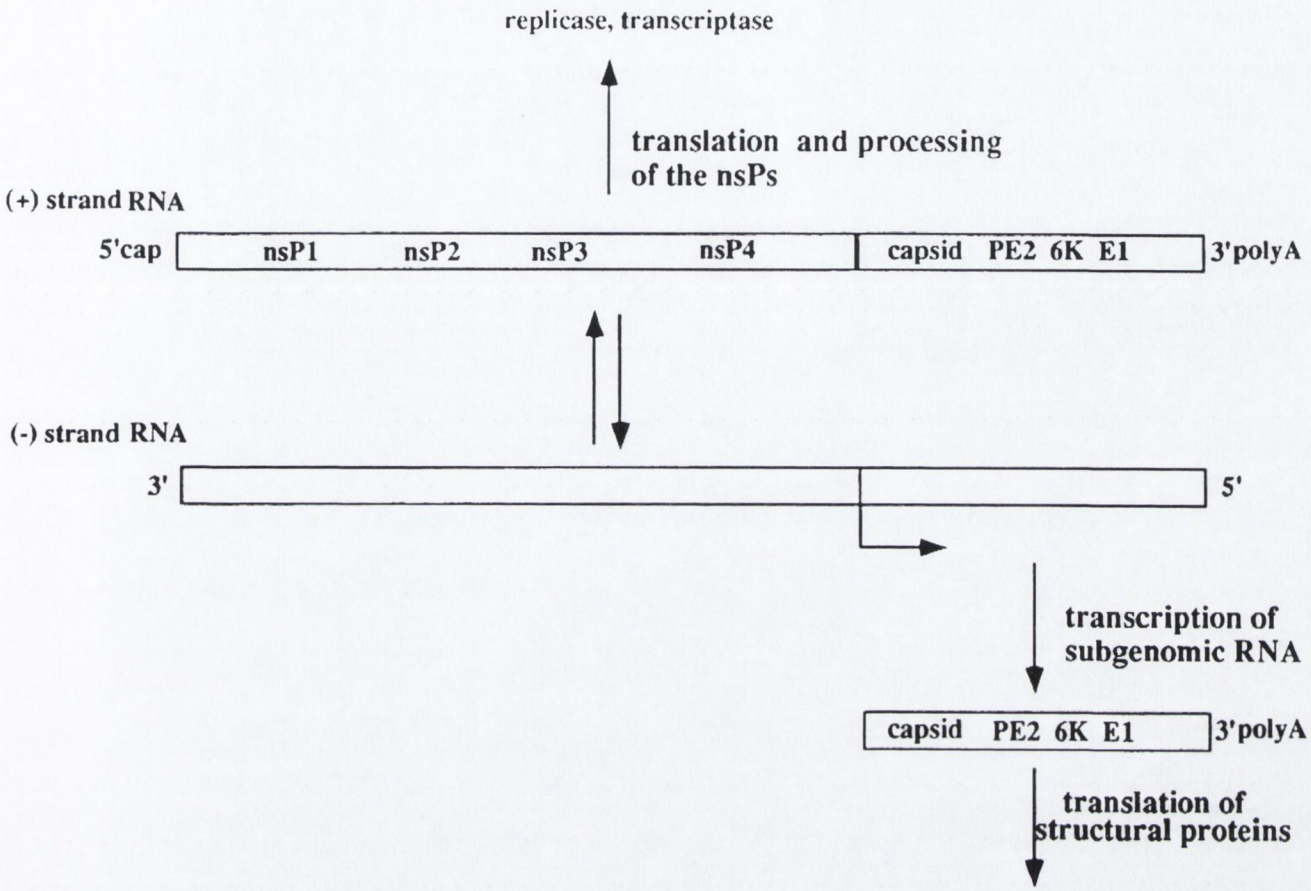


Figure 1.4 SFV replication and transcription

insertion of E1 into the ER membrane (Barth *et al.*, 1995). This complex, resistant to low pH, interacts with 6K and is transported out to the plasma membrane, via the Golgi apparatus, where 6K dissociates and virus budding takes place through spike nucleocapsid interactions (Lusa *et al,* 1991, Wahlberg *et al,* 1989). Due to a requirement for cholesterol in viral assembly, 6K may function to select specific lipids for insertion into the membrane bilayer, to facilitate efficient viral budding (Marquart *et al.*, 1993) and perhaps to promote the correct folding of the E1 protein in its heterodimeric form (Strauss and Strauss, 1994). The E1-E2-E3 heterotrimers trimerise into homotrimers, which constitute mature SFV spike complexes that are transported to the plasma membrane. Budding of virus particles occurs at the plasma membrane, driven by interactions between the capsid proteins of the nucleocapsid and the cytoplasmic tail of E2 (Garoff & Simons, 1974; Suomalainen *et al.*, 1992). A schematic representation of the processing of structural proteins and viral release is shown in Figure 1.4.

1.5.3 Effects of SFV infection on host cells

Alphaviruses infect vertebrate cells leading to rapid viral replication, and death of most cells results from induction of apoptosis or programmed cell death (Strauss & Strauss, 1994, Glasgow *et al.*, 1997); however, cells naturally resistant to apoptosis, such as fully differentiated macrophages, muscle cells and neurons are killed by necrosis (Atkins *et al,* 1990, Balluz *et al,* 1993, Frolov and Schlesinger 1994, Glasgow *et al,* 1997). In contrast, infection of invertebrate cells leads to a persistent infection, with no significant cell damage. The mechanism underlying the persistent infection in mosquito cell lines is not known (Brown and Condreay 1986, Karpf and Brown 1998).

A number of cellular factors have been suggested as crucial in modulating the outcome of alphaviral infection, including *ras* (Hsu *et al.*, 1995), caspases (Nava *et al.*, 1998), nitric oxide (Tucker *et al.*, 1996) and NF-κB (Lin *et al.*, 1998; Lin *et al.*, 1995). SFV may also indirectly trigger the cell death pathway as a consequence of viral replication and the induction of dsRNA-dependent protein kinase (PKR) (Favre *et al.*, 1996; Grandgirard *et al.*, 1998), which has been shown to mediate a number of pro-apoptotic effects in virally-infected cells, including the inhibition of protein

synthesis and upregulation of the Fas/TNFR pathways (Balachandran *et al.*, 1998; Lee & Esteban, 1994). 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). SFV-induced apoptosis has also been shown to occur independently of *p53* expression, as H358 cells, containing a homozygous deletion in the *p53* gene, readily undergo apoptosis following SFV infection (Glasgow *et al.*, 1998). It appears that alphaviral-induced apoptosis is multifaceted, involving the interaction of several apoptotic proteins and pathways, which may or may not be mutually exclusive. Structural proteins are produced in very high amounts during the first 24 h of infection, and about 10^4 viral particles are released per infected BHK-21 cell. After 6 h of infection, only viral proteins are produced, with host cell synthesis being completely shut down (Liljestrom and Garoff 1991a). An extensive cytopathic effect is observed in infected cells, characterized by cell rounding, shrinkage, and cytoplasmic blebbing (Levine *et al,* 1993). Since synthesis of viral structural proteins is not required for cytopathic effect (CPE), it is mediated by viral RNA replication (Frolov and 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). 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).

1.5.4 Pathogenesis of SFV

Depending on the type of infection they generate, SFV strains can be classified as virulent (the original strain L-10 and its derivative SFV4) or avirulent (A7 and its derivatives) strains. Route of administration, age of host and the particular strain of SFV all play significant roles in the outcome of infection. Intraperitoneal infection with both A7 and L10 strains of SFV result in similar levels of viremia, attributed mainly to replication within mouse muscle, which subsequently leads to CNS invasion (Fazakerley *et al.*, 1993). Avirulent strains multiply slower than virulent strains, causing less neuronal damage, and as a result, are cleared from the

CNS by the immune system (Atkins and Sheahan 1982, Atkins *et al,* 1990, Balluz *et al,* 1993, Fazakerley *et al,* 1993, Gates *et al,* 1985).

The direct cytocidal effects of SFV in culture underly alphaviral pathogenesis *in vivo,* which appears to be mediated by a complex interplay of host cell and virally encoded factors. 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). The pro- and anti-apoptotic factors may function to modulate neuronal infection and cell death (Levine *et al.*, 1993; Griffin & Harwick, 1997; Levine *et al.*, 1996; Griffin, 1998). Replication of A7 in the brains of adult mice appears to occur predominantly in oligodendrocytes, resulting in subacute demyelinating disease (Amor *et al.*, 1996). Both virulent and avirulent strains induce demyelination, which is obscured by death for virulent strains (Atkins *et al.*, 2000). Virulent and avirulent strains of SFV can also be transmitted transplacentally and are teratogenic in mice resulting in foetal death before intervention by the maternal humoural immune response (Atkins *et al.*, 1999).

1.5.5 Immune response 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). 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 recovery from disease with the appearance of antibody coinciding with the cessation of viraemia (Griffin, 2001). It is likely that the humoural immune response to SFV infection is predominantly responsible for recovery from disease, while cellular responses appear to be involved in the eradication of virus from tissues harbouring persistent infections and in the development of demyelination.

1.6 THE SEMLIKI FOREST VIRUS VECTOR SYSTEM

1.6.1 rSFV vector system

The generation of infectious clones of SV (Rice *et al.*, 1987), SFV (Liljeström *et al.*, 1991), and VEE (Davis *et al.*, 1989) has allowed 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-dependent 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. The system is capable of producing particles of very high titre up to 10^{10} IU.

The SFV vector system induces high-level transient RNA-based suicidal expression, and is derived from a full-length infectious clone of SFV (Liljeström *et al.*, 1991). This system consists of an expression vector in which foreign genes can be expressed, and two helper vectors that encode the structural protein genes (Figure 1.5). The vector RNA lacks the structural protein genes, which are supplied (in trans) by a split helper system (Smerdou & Liljestrom et al., 1999). The replicon vector contains the replicase genes, the sub-genomic promoter followed by a multiple cloning site where a heterologous gene could be inserted, and the 5' and 3' ends of the genome required for replication (Figure 1.6). The helper vectors contain the complete sequence present in the viral sub-genomic RNA, as well as the 3' and 5' replication signals from the genomic RNA, but do not encode the viral replicase. They supply the packaging system needed for vector production. Co-transfection of all three RNA transcripts into mammalian cells results in release of recombinant virus-like particles (VLPs) coding for the foreign gene (Figure 1.7) (Liljestrom and Garoff 1991, Smerdou and Liljeström 1999). As the resulting VLP progeny contains only the recombinant RNA and lack the viral structural genes, they are capable of only one round of replication. Such recombinant particles (rSFV) are "suicide" particles in that they undergo one round of multiplication on infection but are unable to proceed further because the structural genes are missing and the RNA cannot be encapsidated. Similar vector systems were also described for SV (Bredenbekk *et al.*, 1993) and VEE (Pushko *et al.*, 1997).

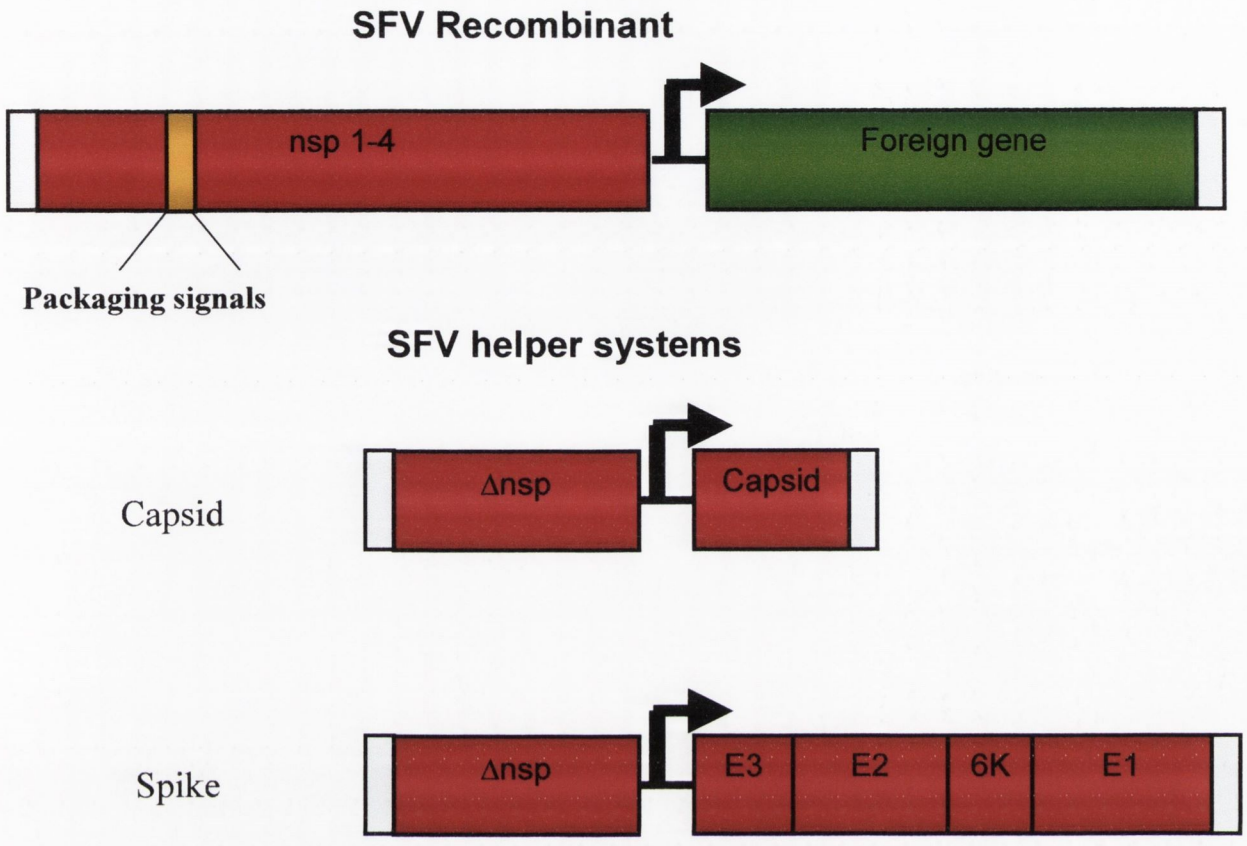


Figure 1.5 Suicidal SFV-Vector Systems

Source: *www.sfvectors.ed.ac.uk/* ***EU*** *Fifth Framework*

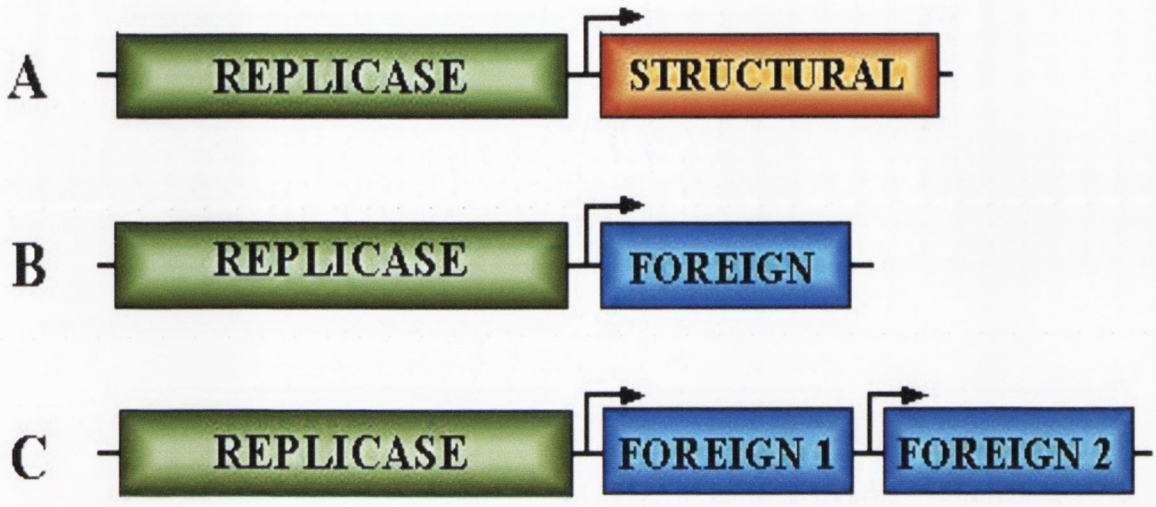


Figure 1.6 Expression of foreign genes in SFV vector system

The SFV vector family is derived from infectious cDNA copy of the SFV RNA genome (A). Replacement of the structural genes of SFV with a polylinker allows insertion of foreign coding sequences thus creating general expression vectors (B). Due to the self replicative nature of such recombinant RNAs, this constitutes an efficient technique of expressing foreign sequences in transfected animal cells. The system can be adapted to express two foreign genes or gene subuinits (C).

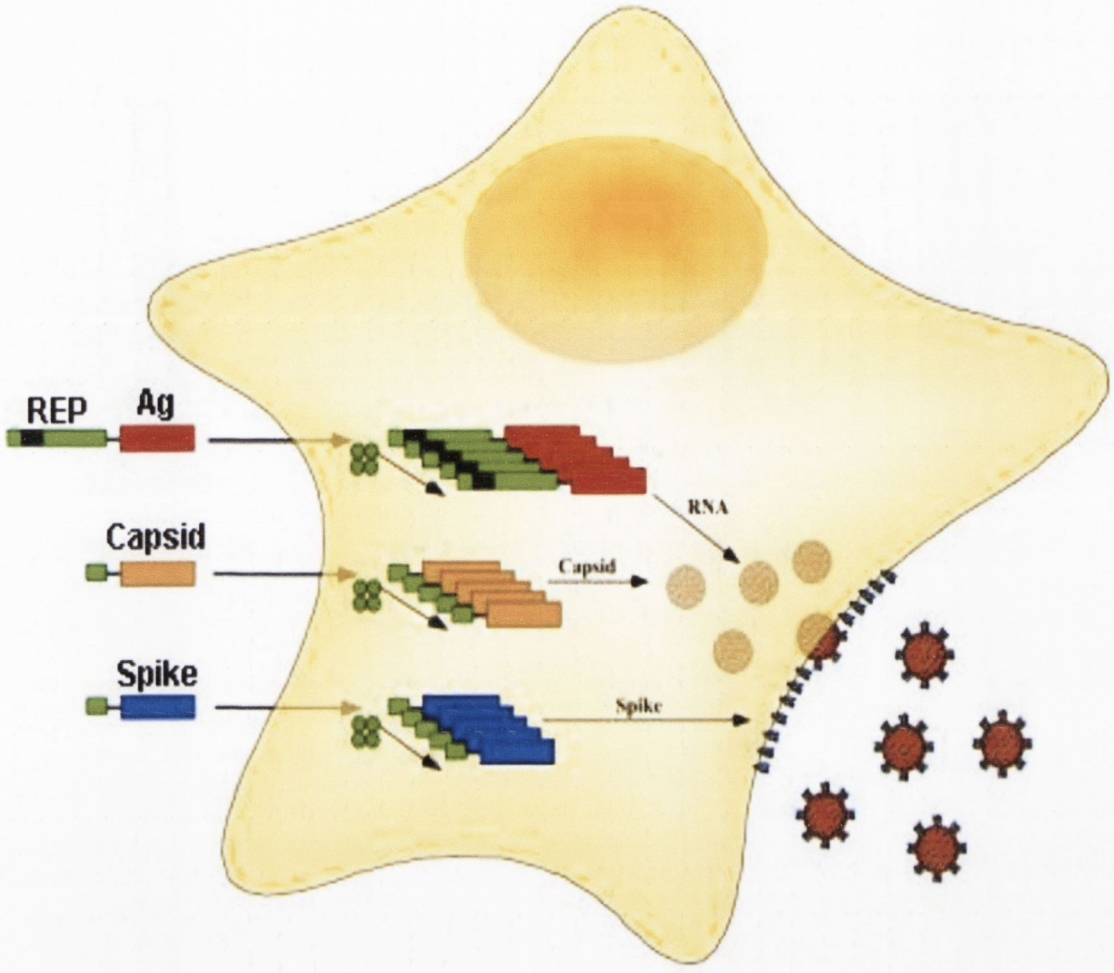


Figure 1.7 Production of rSFV-Virus like particles (VLPs)

Recombinant Virus like particles are generated by co-transfection of cells with three plasmids coding for (1) the replicase-foreign gene (2) the capsid protein and (3) the spike glycoproteins. This allows assembly of virus particles. These are identical to SFV particles except for the content of the packaged RNA. In the virus all of these genetic elements are linked together on one RNA molecule. In the virus like particles only the replicase-foreign gene RNA transcripts are packaged.

Source: *www.sfvectors.ed.ac.uk/* ***EU*** *Fifth Framework*

A DNA-RNA layered vector has also been constructed with sequences encoding the alphavirus replicon under the transcriptional control of the CMV immediate-early promoter, followed by an SV40-derived transcription termination region. When transferred into the nucleus of a cell, transcription of the vector produces self-amplifying alphavirus replicons (Berglund *et al.*, 1998). A modification of this layered vector based on the SV replicon has been described which gives higher level expression and may be useful for tumour treatment (Yamanaka *et al.*, 2004).

Recently, a new SFV-based expression vector system, SFV10-E (Figure 2.3) has been developed. Cells transfected with this vector have been shown to produce up to 10 times more foreign protein than the original SFV expression vector. This vector contains the N terminal 34 amino acid sequence of the SFV capsid enhancer protein 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 and Liljeström, 1999). 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 to ensure comparable levels of expression of the envelope and C proteins (Smerdou and Liljeström, 1999).

1.6.2 Potential applications of the SFV vector system

SFV vectors have several advantages in different areas, such as new prototype vaccines, cancer gene therapy, cancer immunotherapy and vectors for CNS diseases (Zhou *et al.*, 1994; Atkins *et al.*, 1996; Tubulukas *et al.*, 1997; Berglund *et al.*, 1998; Smerdou and Liljeström, 1999). Since SFV RNA replication is cytoplasmic, there is no risk of viral genome integration into the host genome and due to infected cell death (apoptosis), the virus genome does not persist in the tissue. 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 adjacent lymphoid organs for up to 24 h post inoculation (Morris-Downes *et al.*, 2001). In addition, most humans and animals have no pre-existing immunity against the vector and SFV has a broad host range, infecting most types of cell (Zhou *et al.*, 1995). The ability of the SFV VLPs to induce potent humoural and cellular

immune responses against specific antigens confers significant protection against challenge following immunisation. Prototype vaccines studied include those aimed at: HIV (Hanke *et al.*, 2003), respiratory syncitial virus (RSV) (Chen *et al.*, 2002), hepaptitis C virus (HCV) (Brinster *et al.*, 2002), the structural prME proteins of louping-ill virus (Fleeton *et al.*, 1999; Morris-Downes *et al.*, 2001), the envelope glycoproteins of simian immunodeficiency virus (SIV) (Berglund *et al.*, 1997; Nilsson *et al.*, 2001), HA and NP proteins of influenza virus (Zhou *et al.*, 1995; Berglund *et al.*, 1999), and Murray Valley virus (Colombadge *et al.*, 1998).

SFV-induced immune stimulation is generally strong, which has led to an interest in the utilisation of SFV vectors as anti-tumour vaccines. Tumour vaccines based on alphaviruses can be divided into those designed to be prophylactic vaccines, i.e. vaccines stimulating preventive immunity, and those used in the therapy of established tumours, and designed to stimulate or augment an immune response against existing tumour cells. 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 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 *et al.*, 2001).

SFV-vectors have been successfully used in cell culture and *in vivo* expression of a number of human or murine genes such as cytokines (Zhang *et al.*, 1997; Yamanaka *et al.*, 2003), tumour associated antigens (TAA) (Colmenero *et al.*, 2002), vaccine studies (Morris-Downes *et al.*, 2001; Fleeton *et al.*, 2000), tumour related genes (*Bcl-2*) (Lundstrom *et al.*, 1997) and delivery of therapeutic genes to the central nervous system (Jerusalmi *et al.*, 2003). It has been shown that recombinant SFV (rSFV) replicons can efficiently infect tumour cell lines, and that intra-tumoural treatments with rSFV and its derived virus particles resulted in the induction of *p53*-independent apoptosis, leading to significant tumour inhibition (Glasgow *et al.*, 1998).

It was recently shown in animal models of malignant glioma that immunisation of mice with rSFV infected dendritic cells (DC) lead to enhanced immune responses against the encoded (TAA) antigen and subsequent tumour rejection (Colmenero *et al.*, 2002). The previous anti-tumour treatment work on mice vaccinated with recombinant rSFV replicons encoding the P815A tumour antigen (rSFV/E-P1A) showed protection against lethal challenge with the P815 tumour by antigen specific cytotoxic lymphocyte (CTL) responses (Colmenero *et al.*, 1999). In another instance, tumour inhibition was induced on treating K-BALB tumours in pre-vaccinated BALB/c nu/nu mice with an SFV-antigen expressing replicon (SFV-P62 antigen) (Smyth *et al.*, 2004). It has been reported that SFV vectors expressing the cytokine interleukin-12 have resulted in enhanced tumour regression and inhibition of angiogenesis (Asselin-Paturel *et al.*, 1999). Currently, clinical trials (Phase I and II) have been undertaken to use liposome-encapsulated SFV vectors in the treatment of glioblastoma multiforme (Ren *et al.*, 2003). These, and other studies have indicated that the SFV vector is a strong and potent tumour therapeutic agent.

The neurotropism of SFV has been exploited in the application of rSFV VLPs as a vector for the CNS. Extensive research on the neuropathogenesis of SFV has demonstrated that following i.n. infection, the virus follows axonal transport along olfactory tracts from nerve endings in the olfactory mucosa, reaching the CNS via the olfactory bulb (Sammin *et al,* 1999, Sheahan *et al,* 1981, Sheahan *et al,* 1996). Administration of rSFV VLPs expressing the cytokine IL-10 by the non-invasive i.n. route has been shown to have a therapeutic effect on a 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). Thus, there is a great potential for this system to be used in the treatment of CNS diseases.

1.7 OBJECTIVES OF THIS STUDY

Prior experiments using the pSFV vector expressing cytokines have given promising results for tumour therapy. Much research using the cytokine IL-12 expression at the tumour site has shown that the amount of IL-12 available at the tumour site is critical for tumour regression (Colombo *et al.*, 1996). A major drawback of systemic treatment of IL-12 is its short plasma half-life and the toxicity associated with high systemic peak concentrations. However, local concentration of cytokine at the tumour site increases the therapeutic efficacy and reduces the systemic side effects (Trinchieri, 1995). Hence approaches like frequent intratumoural injections of enhanced IL-12 expression systems are required to ensure the long lasting and high concentration of cytokine release. A new enhanced vector pSFV10-E that has been developed recently, which expresses foreign genes at levels up to ten times higher than the original vector, offered a potential approach to cancer gene therapy. In addition, due to the known potential of the cytokine IL-12 as an antitumour agent, the pSFV10-E vector has been used in this study to achieve the maximum therapeutic efficacy.

The main objectives of this study were as follows:

- To the clone murine IL-12 (mIL-12) gene subunits into pSFV10-E (enhanced) and pSFV10 (normal) expression vectors and *in vitro* characterization of these constructs for their ability to express and secrete biologically active murine IL-12.
- To compare the ability of the rSFV10-E-mIL12 and rSFV10-mIL12 replicons to inhibit K-BALB and CT26 tumours in BALB/c mice
- To study the ability of high titre rSFV10-E-IL12 VLPs to inhibit lung metastasis of a metastasising 4T1 tumour in BALB/c mice.
- To examine the cellular infiltrate and vascular density by histopathological and immunohistochemical analysis of tumour sections and also to examine the IL-12 induced antiangiogenic factors in the blood.
- To compare the treatment effect of rSFV10-E-IL18 with that of rSFV10-E-IL12 replicons on K-BALB and CT26 tumours.

2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Lines

The Baby Hamster Kidney 21 (BHK-21), K-BALB, CT26 and 4T1 cell lines were obtained from the American Type Culture Collection (ATCC) (Maryland, USA). The BHK cell line, sBHK, was a gift from Prof. P. Liljeström, (Microbiology and Tumorbiology center, Karolinska Institute, Stockholm, Sweden). BHK medium, RPMI-1640 medium, DMEM medium, newborn calf serum (NCS), fetal calf serum (FCS), tryptose phosphate broth, HEPES, sodium pyruvate and trypsin EDTA were from Gibco BRL (UK), Penicillin-streptomycin-L-glutamine solution was from Sigma (UK). Dulbecco's phosphate buffered saline (PBS-) was from Invitrogen (UK). 75 cm^3 and 150 cm^3 cell culture flasks were from Iwaki (Japan). Hank's Balanced Salt Solution (HBSS) (Gibco),

2.1.2 Expression Vectors

The SFV expression vectors, pSFV-SP6-helper-S2 and pSFV-SP6-CS219A (Figure 2.1), pSFV10 (Figure 2.2), pSFV10-E (Figure 2.3), pSFV-EGFP, and helper vectors, were a gift from Prof. P. Liljeström. A pSFV10-E vector expressing IL-18 was constructed by Ms. Susan McNally (project student, Dept of Microbiology, Trinity college).

2.1.3 Molecular Biology Reagents

TRIzol Reagent used for RNA extraction was purchased from Invitrogen. First strand cDNA synthesis was carried out using Reverse Transcription Kit (Promega) from the RNA. Primers used for PCR amplification were as follows:
All the following primers were obtained from MWG-Biotech AG (Germany).
(a) Gene amplification primers:

Mouse GAPDH F	5'-ACCACCATGGAGAAGGCTG-3'
Mouse GAPDH R	5'-CTCAGTGTAGCCCAGGATGC-3'
IL-12 p35 (F)	5'CCCAAGCTTATGTGCAATCACGCTACCTC3'
IL-12 p35 (R)	5'ATAAAGCTTTCAGGCGGAGCTCAGATAGCC3'

IL-12 p40 (F) 5'CCCAAGCTTATGTGTCCTCAGAAGCTAACC3'

IL-12 p40 (R) 5'ATAAAGCTTCTAGGATCGGACCCTGCAGG3'

(b) Sequencing primers:

IL-12 p40 PCR (F) 5' TGCAGAGAAGGTCACACTGGAC 3'

IL-12 p40 PCR (R) 5' ATCCTCCTGGCAGGACACTGAA 3'

PSFV10-E nsP3 (F) 5' TGGCTCCCGTCGTCAATTTTGA 3'

PSFV10-E nsP3 (R) 5' ACTAGTGAACTCGAGTTCCCGG 3'

The restriction enzymes *EcoRI, NruI, HindIII, SmaI, PvuII, DraI,* DNA PolymeraseI Large fragment (Klenow), T4 DNA Ligase, *XbaI, SphI , BamHI , SpeI,* Bovine serum albumin and quick ligation kit were from New England Biolabs (NEB) (Massacheussets, USA), recombinant RNAsin ribonuclease inhibitor, Reverse transcriptase, Oligo(dT) primer, dNTP mixture, $MgCl_2$, reverse transcriptase buffer, nuclease-free water, PCR molecular weight markers and 10X loading dye were from Promega (Wisconsin, USA). The 100 bp DNA ladder was from NEB. Shrimp Alkaline Phosphatase (SAP), Liberase Blendzymes and Elastase were from Roche (Penzberg, Germany). The PCR clean up, nucleotide extraction, gel extraction and plasmid purification kits for mini- and midi-preps were from Qiagen Ltd. (West Sussex, UK). SP6 RNA polymerase and $m^7G(5')ppp(5')G$ were from Amersham Pharmacia Biotech (Uppsala, Sweden). Pfu DNA polymerase was from Stratagene (California, USA), *Lambda* molecular weight marker from Promega, Red Blood Cell (RBC) lysis buffer eBioscience (California, USA).

2.1.4 Equipment

A Sorvall RC 5C plus centrifuge with a SS-28 rotor, and a Beckman L8-M ultracentrifuge were used for centrifugation during Virus like particle (VLP) production. Ultracentrifuge tubes and the SW40Ti rotor and swing buckets were from Beckman-Coulter Instruments Inc. (CA, USA). A refrigerated tabletop centrifuge for spinning bacterial cultures was from (IEC Micromax). A CM 1900 cryostat from Leica Microsystems (Nussloch, Germany) was used for cutting tumour sections for histopathological studies. Electroporation cuvettes were from BTX (San Diego, USA). A Nikon Eclipse E400 Epiflourescence microscope was used for both bright field and fluorescence microscopy. The following Nikon filters were used for fluorescence detection: GFP filter at 460-500 nm, DAPI filter at 340-380 nm, and

G2A filter at 510-560 nm. ELISA plates were rinsed on a MultiWash II washer from Tri Continent, and absorbance read using a Multiskan RC reader from Thermo Labsystems. All micropipettes used were from Gilson. The following equipment was used: BOD incubator for cell culture from Revco Ultima, FastPrep Instrument (FP 120, Anachem) was used during total RNA extraction. GeneQuant DNA/RNA spectrophotometer (Thermo Labsystems). PCR machine (Hybaid), water bath (Memmert), BioRad Gel Doc 2000 and accompanying Multi-Analist (version 1.1) software, heating block (Grants instruments (Cambridge) Ltd, England), BioRad Gene Pulser II, electronic multi-channel pipette (Biohit-Proline), Multiscan RC ELISA plate reader (Thermo Labsystems), haemocytometer (Neubauer-Blaubrand, Germany), Vortex-2-Gene (Scientific Industries), Chemical fumehood (Chemical Systems control Ltd. Ireland), multispeed refrigerated centrifuge (Medical Supply Ireland), a basic pH meter (Denver Instruments, USA).

2.1.5 Mice

Specific pathogen-free (spf) 4-6 weeks old female BALB/c mice (Harlan, UK) were maintained in accordance with the principles outlined in S1 17/94 European Communities regulations 1994, for care and use of laboratory animals. Syringes (1.0 ml and 0.5 ml microfine insulin) and needles (21G and 29G), were from Becton Dickenson (Le pont de Claix, France), halothane from Rhône Mérieux, UK.

2.1.6 Antibodies

Normal goat and rabbit serum were from were from Vector Laboratories Inc. (CA, USA). Purified rat anti-mouse IL-12 (p40/p70) (C15.6 and C17.8) monoclonal antibody, purified rat anti-mouse IL-18 monoclonal antibody, purified rat IgG1 were obtained from BD PharMingen (BD Biosciences), FITC-conjugated rabbit anti-rat immunoglobulins from Dako (Denmark). The VectaStain Elite ABC kit containing blocking serum, biotinylated antibody anti-rat IgG, VECTASHIELD hardest mounting medium with DAPI and VECTASTAIN reagent was from Vector Laboratories Inc. CD31 antibodies were form Pharmingen.

2.1.7 Histological and pathological studies

Tissue-Tek OCT compound (TissueTec, Fisher scientific USA), DABCO, and Diamino-2-phenylindole (DAPI) were from Sigma. Isopentane, paraformaldehide (PFA), haematoxylin Harris, eosin aqueous solution, dichromate eosin and DPX solutions were from BDH Ltd, UK. Cork discs were purchased from Raymond Lamb (East Sussex, UK). Mowiol was from Calbiochem Novabiochem (Nottingham, UK). Dako pens were from Dako (Denmark).

2.1.8 Enzyme Linked Immunosorbant Assay

The OptEIA mouse IL-12 (p70) set, OptEIA mouse IL-18 set and the OptEIA mouse IFN-γ set were from BD Biosciences PharMingen (USA). The Quantakine Mouse IP-10/CRG-2/CXCL10 immunoassay kit was from RandD systems. The ELISA substrate was 3,3',5'5-Tetramethylbenzidine (TMB) from Sigma.

2.1.9 Miscellaneous

Sucrose was from BDH Ltd (Poole, UK). hydrogen peroxide (H_2O_2) was from BDH. Trypan blue, Tween 20, 3,3'-diaminobenzidine tetrahydrochloride (DAB), and ampicillin were from Sigma (USA). phenol: chloroform: isoamyl alcohol and agarose were from Promega. TNE buffer: 50 mM Tris, 0.1 M NaCl and 1 mM EDTA were dissolved in 800 ml distilled water, pH adjusted to 7.4 and volume to 1 litre. Phosphate buffered-saline (PBS) was from Gibco, $MgCl_2$, glycerol (BDH, UK) and crystal violet from ClinTech. (Essex, UK), lysing matrix D tube was from Q-Biogen (UK). Noble agar was from Difco (Detroit, USA). Tween 80 was from Merk (Germany). 5-bromo-4-chloro-3-indoyly-β-D-galactopyranoside (X-gal) was from Biosynth AG (Switzerland). The suppliers of other materials were as follows: Ethidium bromide ((BioRad, USA)), eppendorf tubes (Axygen), TBE (Promega, USA) agarose (Roche, Germany), blue/orange-loading dye (Promega, USA), cryotubes (Nunc, Denmark), SW-28 ultra centrifuge-tubes (Ultra-clear, Beckman), flat-bottomed 96 wells ELISA plate (NUNC, Denmark), 70μm cell strainer (Falcon), 6-Thioguanine (2-amino-6-mercaptopurine) (Sigma),

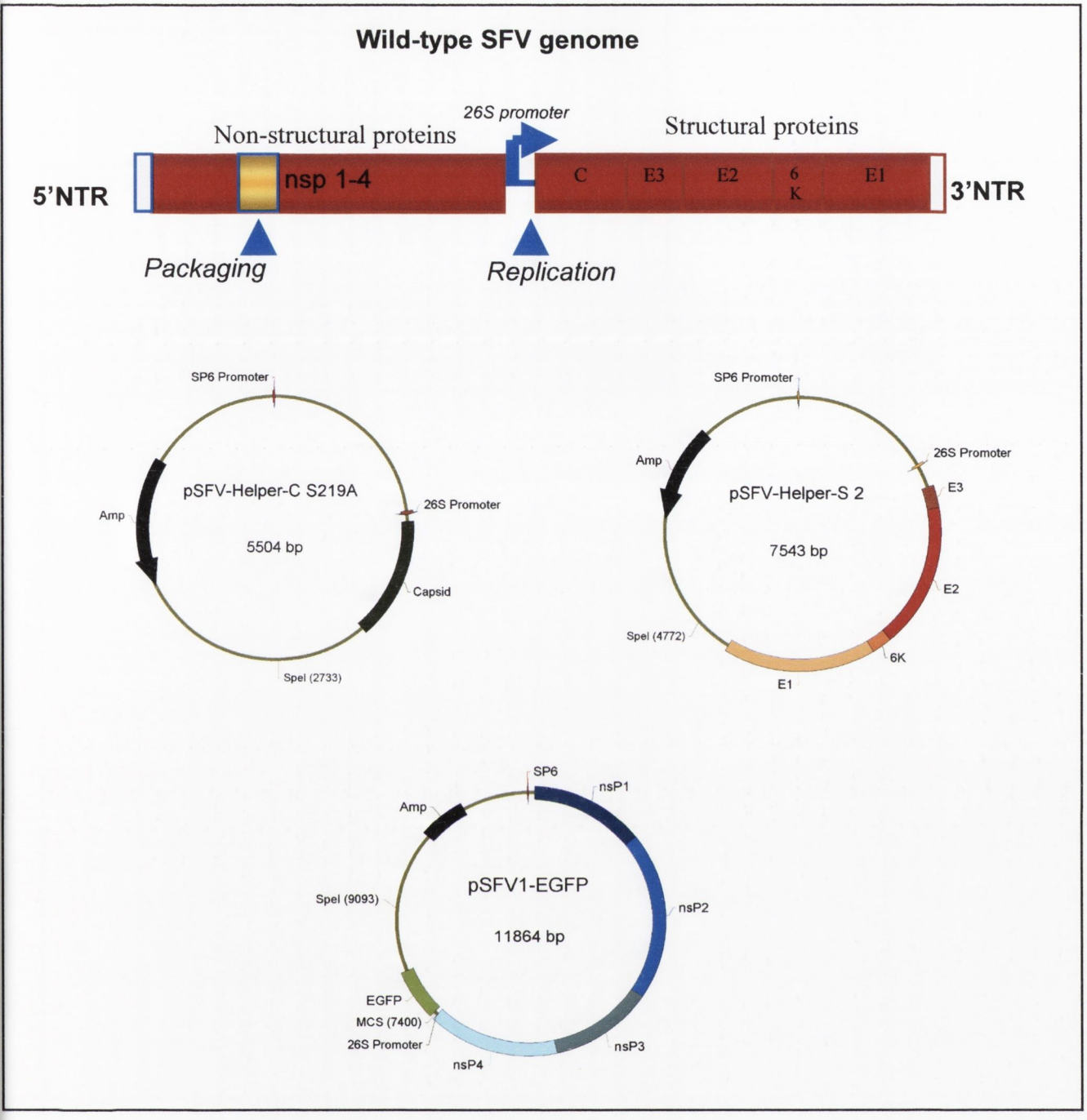


Figure 2.1 Maps of Semliki Forest virus vectors

The recombinant vector pSFV-EGFP as well as helper vectors, pSFV-Helper S2 and pSFV-Helper CS219A are shown. The recombinant vector incorporates the four genes encoding the SFV replicase complex (nsP1-4), but lacks the structural genes (C, p62, 6K, E1), which are located on the helper vectors. All plasmids contain the unique *SpeI* site used for linearisation, and SP6 promoter, which drives *in-vitro* transcription.

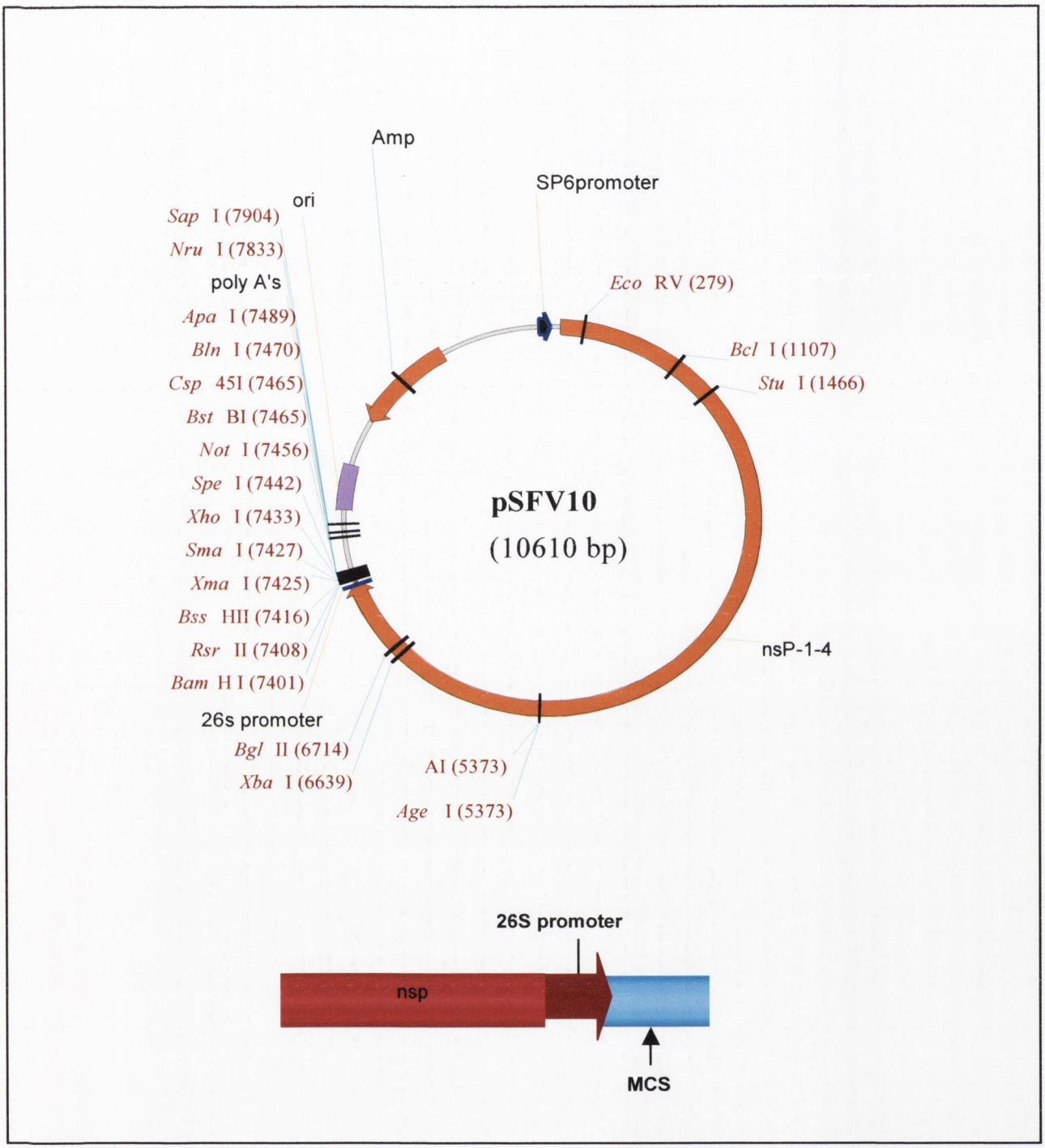


Figure 2.2 Map of pSFV10 expression vector showing restriction sites in MCS

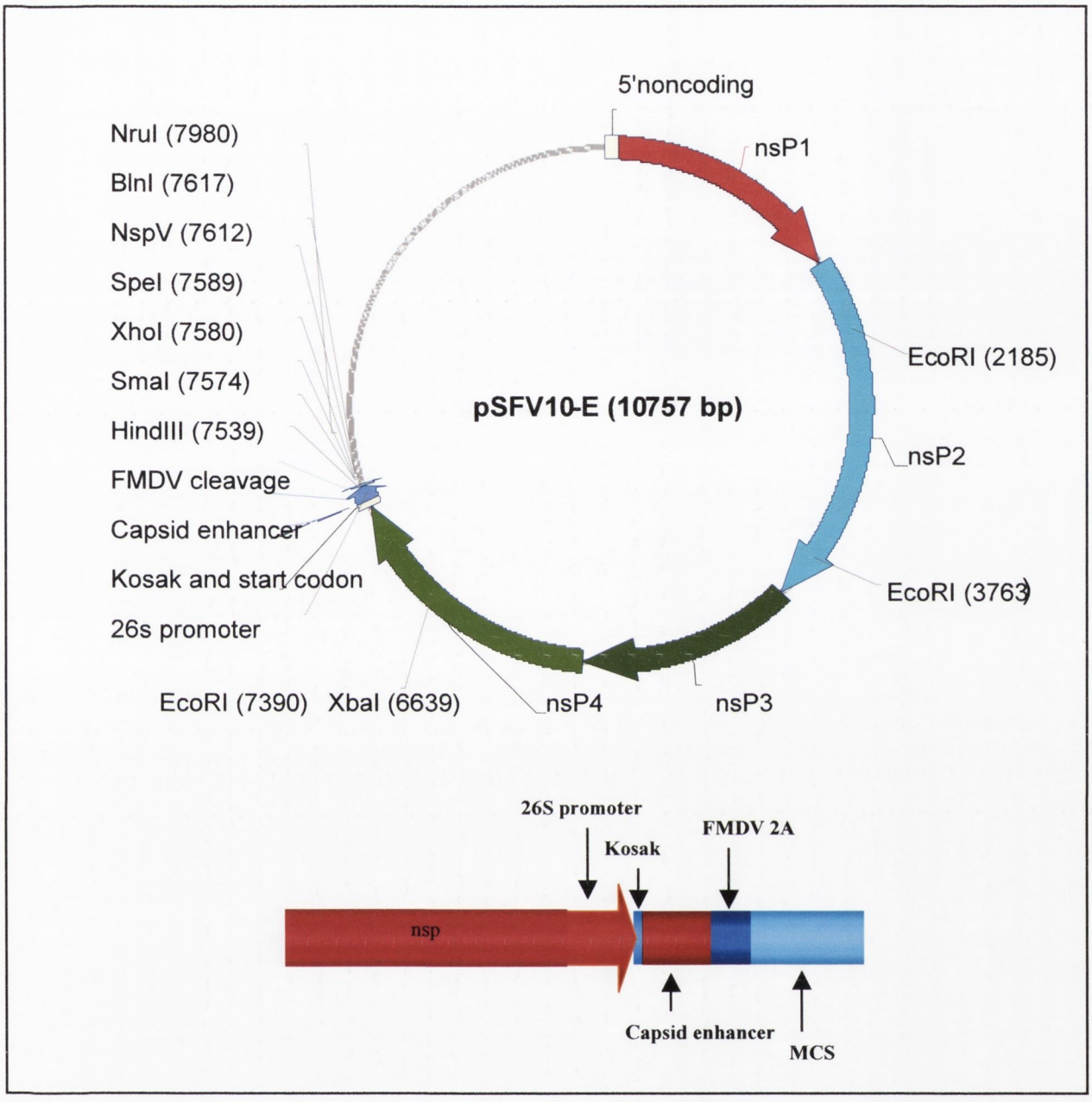


Figure 2.3 Map of the enhanced-expression vector pSFV10-E showing restriction sites in MCS

2.2 METHODS

2.2.1 Cell Culture

Baby hamster kidney cell strain 21 (BHK-21) and transformed mouse embryo fibroblast K-BALB tumour cells were used for virus particle titration and *in vitro* studies. Since it has already been established that BHK-21 cells are susceptible to SFV-induced apoptosis upon infection with SFV and its derived vector (Glasgow *et al.*, 1998) they were employed as a positive control for *in vitro* assays. Swedish BHK (sBHK) cells were used for the production of high titre virus-like particles (VLPs).

BHK-21 and sBHK cells were grown in 150 cm^3 cell culture flasks containing 25 ml of BHK medium supplemented with 5% new born calf serum, 10% tryptose phosphate broth, 20 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-Glutamine and maintained at 37 °C in a humidified atmosphere of 9% CO_2.

Similarly, K-BALB and CT26 tumour cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) new born calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM sodium pyruvate and maintained at 37 °C in a humidified atmosphere of 9% CO_2 in 75cm^3 or 150 cm^3 cell culture flasks.

Splenocytes were grown in RPMI-1640 containing 10% foetal bovine serum (FBS), 10 mM HEPES, 2 mM glutamine, 100 U Penicillin 100 μg/ml streptomycin, 10 mM sodium pyruvate, 1% non-essential amino acids, 50 μM β-Mercaptoethanol and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in 75 cm^3 or 150 cm^3 cell culture flasks.

4T1 tumour cells were grown in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 10 mM sodium pyruvate and 1% non-essential amino acids and maintained at 37 °C in a humidified atmosphere of 9% CO_2 in 75 cm^3 or 150 cm^3 cell culture flasks.

Confluent monolayers of BHK-21, sBHK, K-BALB, CT26 or 4T1 cells were detached using Trypsin-EDTA. The confluent monolayers were washed with Dulbecco's phosphate buffered saline without calcium, magnesium and sodium bicarbonate (PBS-) and incubated with 0.5% Trypsin 5.3mM EDTA at 37 °C until cell detachment was observed and their specific cell culture medium was added to terminate trypsinisation. The BHK-21 cells used in *in vitro* assays were typically sub-cultured at a ratio of 1:3 but due to the rapidly growing nature of sBHK, K-BALB, CT26 and 4T1 cells, they were also sub-cultured at a ratio of 1:4 or higher depending on confluency. Cell lines used in *in vitro* and *in vivo* assays were passaged not more than 12 times.

Viral infection medium was composed of Eagles Minimum Essential Medium (MEM) supplemented with 0.2% bovine serum albumin, 2 mM L-glutamine, and 20 mM HEPES buffer.

Stocks of cells were prepared by slow freezing (at the rate of -1°C/min) to -70°C in medium containing 10% dimethylsulfoxide (Aldrich) and subsequently stored in liquid nitrogen.

2.2.2 Cloning of rSFV10-E -IL12 and rSFV10-IL12

For cloning the IL-12 gene subunits into the SFV expression vectors, pSFV10-E and pSFV10, a strategy was designed using the unique restriction sites in the MCS of both the vectors. This strategy is illustrated in Figures 2.4 & 2.5.

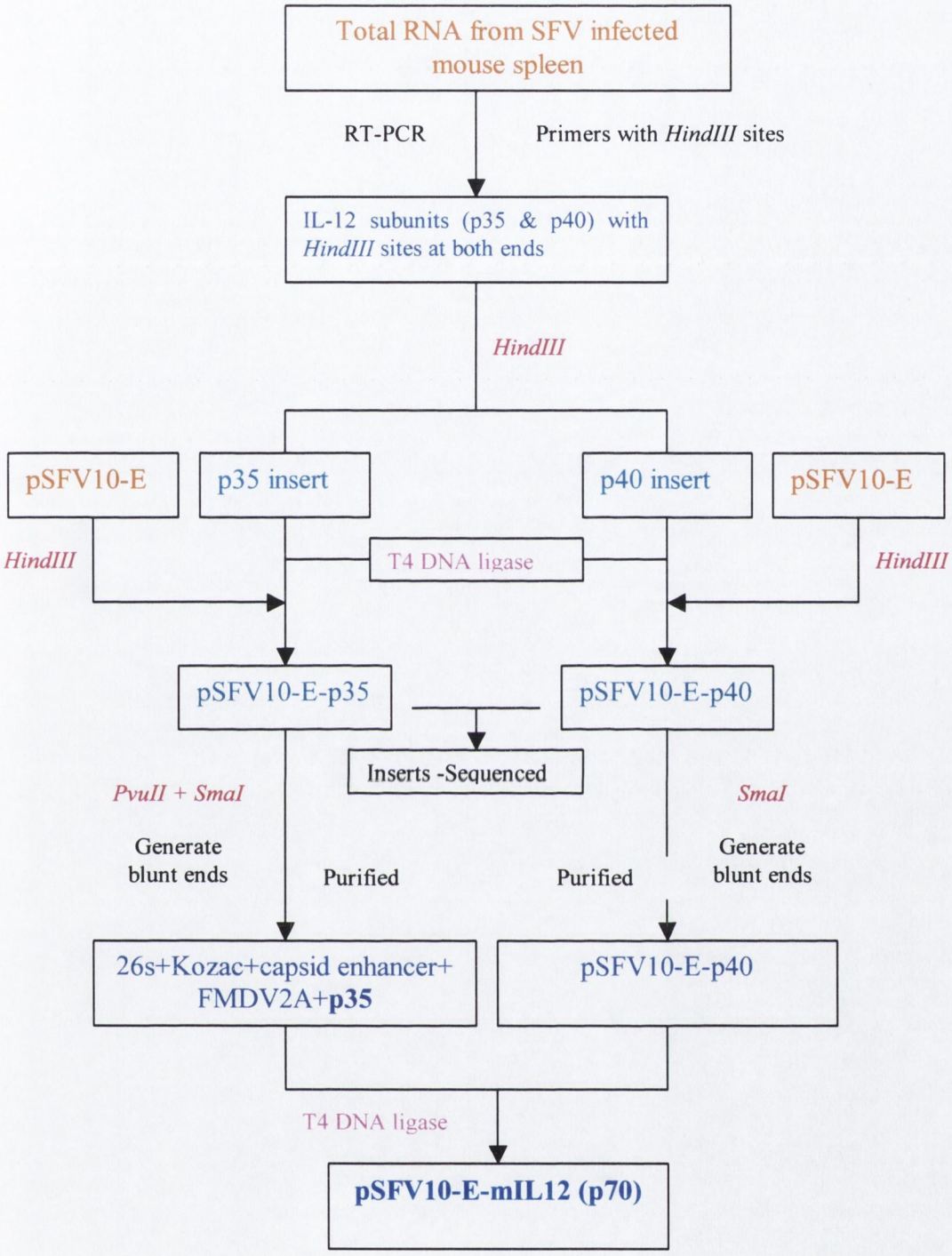


Figure 2.4 Schematic representation of pSFV10-E-IL12 cloning strategy.

RNA from spleen of SFV-A7 infected mouse was extracted. RT-PCR with primers specific for the coding region of IL-12 subunits including the restriction site *HindIII* was carried out. The amplified IL-12 subunits p35 and p40 were cloned into pSFV-10-E. The resultant construct was screened for presence and orientation of the inserts by restriction enzyme analysis, and the correct clones sent for sequencing.

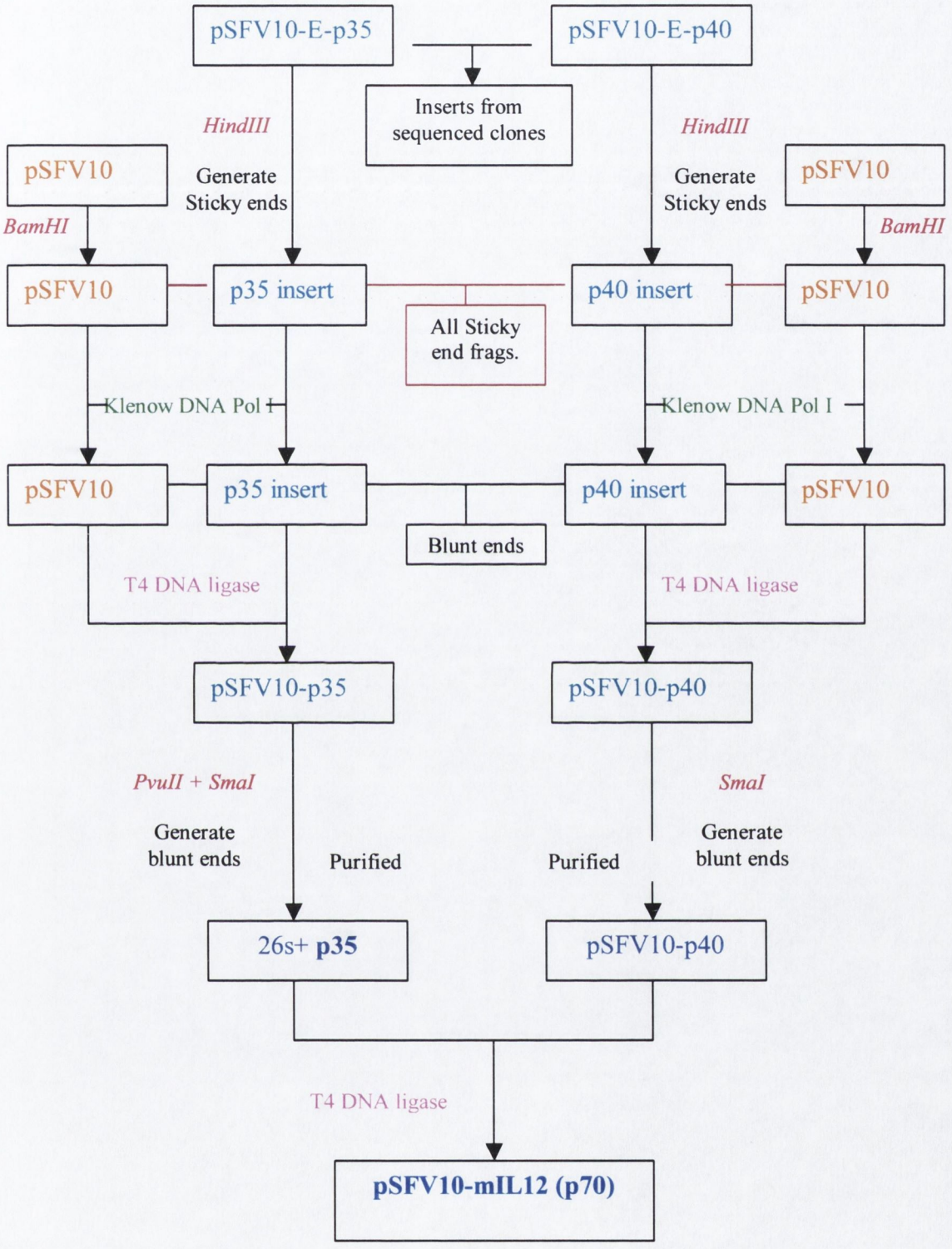


Figure 2.5 Schematic representation of pSFV10-IL12 cloning strategy.

The sequenced p35 and p40 inserts from pSFV10-E-p35 and pSFV10-E-p40 constructs were cloned into pSFV10 to obtain pSFV10-p70. The expression of p35 and p40 subunits by pSFV10-IL-12 were controlled separately with their own 26S sub-genomic promoters.

2.2.2.1 Total RNA extraction from mouse splenocytes

Total RNA was isolated from spleens that were taken from BALB/c mice infected i.p. with avirulent SFV-A7 virus four days after the virus injection. The spleens were mixed with Trizol Reagent in a lysing matrix D tube. For every 50-100 mg of tissue, 1 ml of Trizol Reagent was added; while 1.5 ml were added for 75-150 mg. The spleen tissue was homogenized in a FastPrep Instrument at 5.5 pulse for 45 sec (homogenized twice), and then incubated on ice for 10 min. The mixture was then centrifuged (12,000 g, 10 min, 4°C) and the supernatant transferred to a microfuge tube. After 5 min incubation at room temperature (RT), 200 µl of chloroform (per ml of Trizol Reagent used) was added to the tube, and the mixture was centrifuged (12,000 g, 15 min, 4°C). The colourless aqueous phase was transferred to a new tube, mixed with 500 µl of isopropanol (per ml of Trizol Reagent), and incubated at RT for 10 min. The mixture was centrifuged (12,000 g, 10 min, 4°C), the supernatant was removed and the pellet washed with 1 ml of 75% ethanol (-20°C) by vortex. The mixture was again centrifuged (12,000 g, 10 min, 4°C), ethanol was removed and RNA resuspended in 20 µl of nuclease-free water before storage at -70°C. One µl of the extracted RNA was analysed on a 0.6% (wt/v) agarose gel and the RNA concentration was measured on a GeneQuant DNA/RNA spectrophotometer.

2.2.2.2 Isolation of murine IL-12 gene (p35 and p40 subunits) by RT-PCR

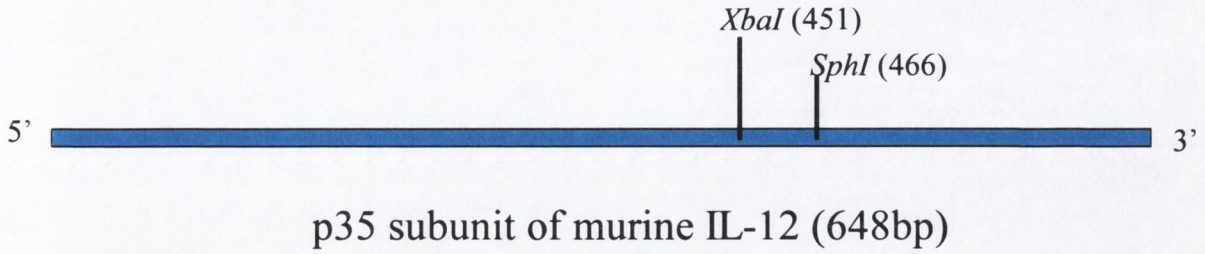
The integrity of the extracted total RNA was confirmed using specific primers for mouse GAPDH by polymerase chain reaction (PCR) as described below. To amplify the IL-12 gene subunits, p35 and p40 (Figure 2.6 & 2.7), primers were designed using MW Genosys Oligo calculation program to obtain the amplified subunits of IL-12 containing *HindIII* restriction enzyme sites at their ends. The primer sequences used for the amplification are listed in section 2.1.3.

First strand cDNA was synthesised from 1 µg mouse RNA using the reverse transcription kit (Promega). Each reaction contained 0.5 µg reverse-oligo (dT) primers of p35 or p40 with 1 µg of total RNA in separate reactions, incubated at 70°C for 5 min, 4°C for 5 min and held on ice. To this mixture 5 mM $MgCl_2$, 1X reverse transcription buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100), 10

mM each dNTP, 1 U RNAsin (ribonuclease inhibitor), and 15 U high concentration reverse transcriptase (RT) was added. After mixing the ingredients, the tubes were incubated on PCR machine programmed to run at an initial primer annealing temperature of 25°C for 5 min and extension temperature at 42°C for 60 min. Reverse transcriptase activity was heat inactivated by incubating the tubes at 70°C for 15 min. The controls for the RT-PCR procedure included samples with either no RNA or no RT added. Oligonucleotide primers that produce a 508 bp product of the GAPDH gene were used to demonstrate the integrity of the DNA. One µl cDNA of both p35 and p 40 subunits along with the controls were analysed on a 0.8% agarose gel stained with ethidium bromide. A 25 ml 0.8% TBE agarose gel containing 2.5 µl 10 mg/ml ethidium bromide was prepared and 2 µl of each DNA preparation was mixed with 14 µl TBE buffer and 4 µl 6X blue/orange loading dye. Samples were loaded in 5 µl and 10 µl amounts and run at 75 mA along with 5 µl and 10 µl 1Kb molecular weight marker (5 µl 1Kb marker, 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-Analyst (version 1.1) software.

2.2.2.3 PCR amplification of p35 and p40 cDNA

The cDNA (20 µl) of p35 and p40 subunits was set up in 100 µl PCR reaction tubes separately at the following final concentrations: 10 mM each dNTP, 2 mM $MgCl_2$, 1X reverse transcription buffer, 0.1 µM forward and reverse primers of p35 or p40, and 1.5 U of Pfu DNA polymerase. The following temperature profile was used in the PCR reaction: 45 s at 94°C followed by 35 cycles of incubation at 94°C for 45 s, 63°C for 45 s 72°C for 60 s with a final extension of 10 min at 72°C. Negative control samples contained cDNA from RNA without Pfu DNA polymerase enzyme.

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  1  atgtgtcaat cacgctacct cctcttttg gccacccttg ccctcctaaa
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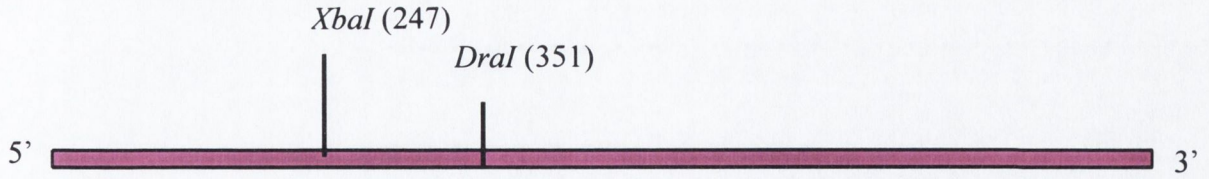
101  ttagccagtc ccgaaacctg ctgaagacca cagatgacat ggtgaagacg
151  gccagagaaa aactgaaaca ttattcctgc actgctgaag acatcgatca
201  tgaagacatc acacgggacc aaaccagcac attgaagacc tgtttaccac
251  tggaactaca caagaacgag agttgcctgg ctactagaga gacttcttcc
301  acaacaagag ggagctgcct gcccccacag aagacgtctt tgatgatgac
351  cctgtgcctt ggtagcatct atgaggactt gaagatgtac cagacagagt
401  tccaggccat caacgcagca cttcagaatc acaaccatca gcagatcatt
451  ctagacaagg gcatgctggt ggccatcgat gagctgatgc agtctctgaa
501  tcataatggc gagactctgc gccagaaacc tcctgtggga gaagcagacc
551  cttacagagt gaaaatgaag ctctgcatcc tgcttcacgc cttcagcacc
601  cgcgtcgtga ccatcaacag ggtgatgggc tatctgagct ccgcctga
```

Figure 2.6 The sequences of the coding region of p35 subunit of murine IL-12

The coding region of the p35 subunit (which codes for the expression and secretion of the subunit) of the mouse IL-12 gene is a 648 bp (codes for 216 amino acids) out of a total of 774 bp. The starting codon of p35 is located at 127th bp and stop codon at 774th bp of the p35 cDNA. The forward primer was designed to include the first 21 nucleotides of the coding region and a *HindIII* restriction site. The reverse primer was designed to include the final 21 nucleotides of the coding region of the p35 subunit, and a *HindIII* restriction site. The restriction sites *XbaI* and *SphI* on the coding sequences of p35 subunit at the position 451 and 466 respectively were used for the analysis of the PCR amplified p35 subunit.



p40 subunit of murine IL-12 (1008bp)

```
   1  atgtgtcctc agaagctaac catctcctgg tttgccatcg ttttgctggt
  51  gtctccactc atggccatgt gggagctgga gaaagacgtt tatgttgtag
 101  aggtggactg gactcccgat gcccctggag aaacagtgaa cctcacctgt
 151  gacacgcctg aagaagatga catcacctgg acctcagacc agagacatgg
 201  agtcataggc tctggaaaga ccctgaccat cactgtcaaa gagtttctag
 251  atgctggcca gtacacctgc cacaaaggag gcgagactct gagccactca
 301  catctgctgc tccacaagaa ggaaaatgga atttggtcca ctgaaatttt
 351  aaaaaatttc aaaaacaaga ctttcctgaa gtgtgaagca ccaaattact
 401  ccggacggtt cacgtgctca tggctggtgc aaagaaacat ggacttgaag
 451  ttcaacatca agagcagtag cagttcccct gactctcggg cagtgacatg
 501  tggaatggcg tctctgtctg cagagaaggt cacactggac caaagggact
 551  atgagaagta ttcagtgtcc tgccaggagg atgtcacctg cccaactgcc
 601  gaggagaccc tgcccattga actggcgttg gaagcacggc agcagaataa
 651  atatgagaac tacagcacca gcttcttcat cagggacatc atcaaaccag
 701  acccgcccaa gaacttgcag atgaagcctt tgaagaactc acaggtggag
 751  gtcagctggg agtaccctga ctcctggagc actccccatt cctacttctc
 801  cctcaagttc tttgttcgaa tccagcgcaa gaaagaaaag atgaaggaga
 851  cagaggaggg gtgtaaccag aaaggtgcgt tcctcgtaga gaagacatct
 901  accgaagtcc aatgcaaagg cgggaatgtc tgcgtgcaag ctcaggatcg
 951  ctattacaat tcctcatgca gcaagtgggc atgtgttccc tgcagggtcc
1001  gatcctag
```

Figure 2.7 The sequences of the coding region of p40 subunit of murine IL-12

The coding region of the p40 subunit of the mouse IL-12 gene is a 1008 bp (codes for 336 amino acids) out of a total of 1840 bp. The starting codon of p40 is located at 35th bp and stop codon at 1042nd bp of the p40 cDNA. The forward primer was designed to include the first 21 nucleotides of the coding region and a *HindIII* restriction site. The reverse primer was designed to include the final 20 nucleotides of the coding region of the p40 subunit, and a terminal *HindIII* restriction site. The restriction sites *XbaI* and *DraI* on the coding sequences of p40 subunit at the position 247 and 351 respectively were used for the analysis of the PCR amplified p40 subunit.

The amplified products were analysed with 1 Kb DNA marker on 1% agarose gels stained with ethidium bromide as outlined in 2.2.2.2.

2.2.2.4 Analysis of the amplified IL-12 subunits

The products of separate RT-PCR reactions were purified using the QIAGEN PCR purification kit. To each PCR reaction mix, 5 volumes of buffer PB were added and mixed (500 µl of buffer to 100 µl PCR reaction). This buffer allows for binding of the PCR product and removal of primers of up to 40 nucleotides. Samples were added to QIAquick columns (4 PCR reactions per column) and centrifuged (10,000g, 1 min) to allow for binding of DNA. The columns were then washed with 750 µl of buffer PE to remove unwanted primers and impurities, and centrifuged (10,000g, 1 min) twice. Finally the DNA was eluted in 50 µl of nuclease-free water per column by centrifugation (10,000 g, 1 min).

The purified PCR amplified products of both the IL-12 subunits were analysed by unique restriction enzyme digestion and observed for their characteristic digested band size on agarose gel. *XbaI* and *SphI* enzymes were used for the analysis of the p35 subunit whereas *XbaI* and *DraI* were used for the p40 subunit analysis. One µg of each DNA was used for restriction digestion analysis. The following reaction mixture was double digested at 37°C for 2 h: one µg of each p35 or p40 DNA, 5 µl nuclease free water, 5 µl 10X NEBuffer2 (150 mM NaCl, 10 mM Tris-Hcl, 10 mM $MgCl_2$, 1 mM dithiothreitol pH 7.9), and a combination of 1 U of each restriction enzyme (*XbaI* and *SphI* or *XbaI* and *DraI*). The digested products were analysed on a 1.5% agarose gel as described in 2.2.2.2. After confirming the specific IL-12 gene subunits by restriction digestion, the purified PCR amplified DNA was mixed with 35 µl of loading dye, and run on a 1.0% (wt/vol) agarose gel and placed on a UV trans-illuminator. The bands of the correct size DNA fragments of p35 and p40 in the gel were excised using a clean sharp blade and purified by using the QIA Quick Gel extraction kit. The excised gel bands were weighed and mixed with buffer QG at a rate of 3 volumes of buffer to 1 volume of gel (300 µl of buffer to 100 µg of gel). The mixture was incubated for 10 min at 50°C (tubes mixed every 2-3 min). This buffer solubilizes the agarose gel slice, and provides appropriate conditions for binding of DNA to the silica membrane. Once the gel was completely dissolved, isopropanol

was added and mixed (1 volume per volume of gel). The mixture was added to 2 QIA Quick columns and centrifuged (10,000 g, 1 min). The columns were washed with 500 µl of buffer QG to remove all traces of agarose, and centrifuged (10,000 g, 1 min). They were then further washed with 750 µl of buffer PE and centrifuged (10,000 g, 1 min) twice. Purified DNA was eluted in 30 µl of nuclease-free water per column, and pooled (Figure 3.4B). This kit relies on the selective binding properties of a silica-gel membrane in a spin-column to bind the DNA. The concentration of both the purified p35 and p40 DNA was estimated on a gel by comparing one µl of sample DNA with 0.5µg of 100 bp DNA ladder (NEB).

2.2.2.5 Preparation of pSFV10-E and IL-12 gene inserts for cloning

The 10.75 kbp plasmid pSFV10-E vector containing a MCS for use in the construction of recombinant plasmids, and an ampicillin resistance gene for bacterial screening was used to prepare the SFV expression vector.

The purified stocks of the IL-12 subunits and pSFV10-E vector were digested using *HindIII* restriction enzyme in separate reaction sets. Each of the digestion reaction mixture contained 10 µg of p35 or p40 or 5 µg pSFV10-E vector DNA, 10 µl 1x NEBuffer (150 mM NaCl, 10 mM Tris-Hcl, 10 mM $MgCl_2$, 1 mM dithiothreitol pH 7.9), 10 µl of 10x BSA and 20 U *HindIII* enzyme. The reaction mixtures were incubated at 37°C in a water-bath overnight and the reaction was arrested by heating at 65°C for 20 min.

HindIII digested p35 and p40 fragments from different digested reaction mixtures were pooled and cleaned using QIAquick Nucleotide Removal Kit. Pooled digested DNA was mixed with 800 µl buffer PN, which promotes the absorption of oligonucleotides longer than 17 bases and DNA fragments up to 10 kb to the silica membrane. The mixture was added to a MinElute column, and centrifuged (6,000 g, 1 min) to allow binding of DNA. The column was then washed with buffer PE and centrifuged (6,000 g, 1 min), then further centrifuged (10,000 g, 1 min). The purified p35 or p40 or pSFV10-E DNA was eluted in 10.5 µl of nuclease-free water. The final product was analysed and quantified by electrophoresis of a 0.5 µl aliquot on a 1.2% (wt/vol) agarose gel as outlined in 2.2.2.2 (Figure 3.10).

To remove the phosphate group from the 5' end of the *HindIII* linearised pSFV10-E vector DNA, shrimp alkaline phosphatase (SAP) was used, thus preventing the vector from self-ligating. 1 U of SAP and 0.9 µl 10X SAP buffer was mixed to the *HindIII* digested pSFV10-E DNA and incubated at 37°C for 30 min and the reaction was arrested by incubating the tubes at 65°C for 15 min. The dephosphorylated DNA was cleaned from the SAP reaction mixture using the QIAquick Nucleotide Removal Kit as described above and eluted in 20 µl of DNAse free water. 1 µl of this sample was analysed on a 0.8% agarose gel.

2.2.2.6 DNA ligation

The p35 and p40 subunits of IL-12 were ligated to the pSFV10-E vector separately. The ligation mix contained 50ng of vector DNA with three molar excess of p35 or p40 (in separate tubes) inserts, 10 µl of 2x quick ligation buffer (132 mM Tris-HCl, 20 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP 15% polyethylene glycol (PEG 6000) pH 7.6) and 1µl (10U) of Quick T4 DNA ligase. The mixture was mixed thoroughly, centrifuged briefly, incubated at room temperature for 2-5 min, and chilled on ice and stored at -20°C. A control ligation was carried out using only pSFV10-E DNA.

2.2.2.7 Transformation

2.2.2.7.1 Preparation of competent *E. coli* DH5α cells

Cells used for transformation of rSFV10-E , rSFV10 and 'helper' SFV plasmids were *Escherchia coli* strain DH5α (New England Biolabs, USA). 500 ml of *E. coli* cells were grown in a 2 litre baffled flask containing L broth incubated at 37 °C with shaking (200 rpm) until the cells reached the mid-log phase of growth (OD. 600= 0.4-0.5). The culture was then chilled on ice for 2 h after which it was centrifuged at 800g for 20 min at 4°C. 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 $CaCl_2$ (Merc, 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.

2.2.2.7.2 Transformation of *E. coli* DH5α cells

Competent DH5α cells were transformed with ligated rSFV10-E-p35 or rSFV10-E-p40 mixture separately by incubating 200 µl of competent cells with each plasmid DNA for 1 h on ice (mixed every 10 min). The cells were transformed by heat shock at 42°C for 2 min and placed on ice for a further 10 min. Transformed cells were incubated in L-broth and allowed to grow for a further 1 h with the addition of 100 µg ampicillin. Transformed cells were plated onto ampicillin L-agar plates containing 1mg/ml ampicillin. The plates were incubated upside down overnight at 37°C and observed for colony growth.

2.2.2.8 Screening of the plasmid DNA from the transformed colonies

A few isolated transformed colonies of recombinant plasmids were inoculated in 15 ml of L-broth containing 100 µg/ml ampicillin separately. The cell cultures were grown for 18 h shaking at 37°C. From each culture, a 5 ml aliquot was removed and mixed with 15% (wt/vol) 80% glycerol. These were then stored at -70°C in 1 ml aliquots. The remaining culture was used to purify the plasmid, using the QIAGEN Miniprep Plasmid Purification Kit that is based on the modified alkaline lysis protocol. Cells were harvested by centrifugation (1000 g, 10 min, 4°C). Each pellet was resuspended in 250 µl of buffer P1 (10 mM EDTA, 100 µg/ml RNAse A, 50 mM Tris-HCL, pH 8.0), and transferred to an eppendorf tube. Cells were lysed by the addition of 250 µl of buffer P2 (200 mM NaOH, 1% (wt/vol) SDS) by inverting the tube 5-6 times. 350 µl of buffer N3 was added, and mixed gently prior to centrifugation (10,000 g, 10 min). The supernatant was applied to a QIAprep column and further centrifuged (10,000 g, 1 min). The column was then washed with 500 µl of buffer PB, to remove all trace of nuclease activity. The column was again washed

with 750 µl of buffer PE, and centrifuged twice (10,000 g, 1 min). Clean DNA was eluted in 30 µl of nuclease free water by centrifugation (10,000 g 1 min).

Each plasmid DNA from different transformed colonies was screened for the presence of insert (p35 or p40) and its orientation by confirmed restriction digestion analysis. To test the presence of the insert 1 µl of each DNA was digested for 1 h at 37°C with *EcoRI* enzyme in the presence of 10x BSA and 1x *EcoRI* buffer. The digested products for each DNA samples were analysed on 1% agarose gel by comparing with control vector digested by *EcoRI* and 1Kb DNA. The plasmid DNA carrying the insert in each of the rSFV10-E-p35 and rSFV10-E-p40 were selected and the orientation of the insert tested. The positive clones were analysed using *XbaI* restriction digestion separately in the presence of NEBuffer 2 and the digested fragments were analysed on a 0.8% agarose gel by comparing with a 1Kb DNA ladder ladder (Figure 3.11-14). After confirming the correctly oriented insert (p35 or p40) in rSFV10-E constructs, 1 ml aliquots of the original culture were re inoculated into fresh ampicillin L-broth in sterile baffled flasks and plasmids were extracted using QIAPrep miniprep kit as described before. Finally, positive clones with correct orientation of inserts in pSFV10-E-p35 and pSFV10-E-p40 were re-analysed by restriction analysis and sent for sequencing.

2.2.2.9 Sequencing of the inserts in pSFV10-E-p35 and pSFV10-E-p40

Four clones with the correct insert in each of the IL-12 subunit constructs were sent for sequencing to LARK technologies Inc. (UK) using insert primers. As the p40 subunit is about 1kb long, it was necessary to include multiple primers for the efficient amplification of the insert. Oligonucleotide primers for sequencing the insert p40 were designed to amplify the insert along with a small portion of the non-structural region (nsP4) of SFV vector from both ends of the insert. The same primers that were used for cDNA amplification of p35 were employed for sequencing purposes (refer section 2.1.3). The sequences obtained was blasted against the NCBI Gene database, and then paired to the original sequence for the coding region of p35 and p40 subunits of IL-12 obtained from the GenBank database. A glycerol stock was prepared from the correct clone as described in section 2.2.2.7.1.

2.2.2.10 Preparation of SFV10 and IL-12 gene inserts for cloning

Stocks of pSFV10-E-p35 and pSFV10-E-p40 were made by the QIAGEN Miniprep Plasmid Purification Kit as described in the section 2.2.2.6. The p35 and p40 genes from the pSFV10-E constructs were released by *HindIII* digestion and the genes were purified by using the QIA quick Gel extraction kit (Qiagen) as described in section 2.2.2.2. The *HindIII* digested sticky ends of the inserts were blunted (filled-in) using DNA Polymerase I, Large fragment (Klenow). 2-3μg of each gene subunit was used in the reaction mixture with 3μl EcoPol 10x buffer (10mM Tris-Hcl, 5mM $Mgcl_2$, 7.5 mM dithiothreitol, pH 7.5 at 25°C), 10.5μl of dNTP mix (10mM) and 5U of Klenow in a total 30μl volume.

Similarly, 1 μg of pSFV10 was linearised using 1.5μl of *BamHI* restriction enzyme and the digested sticky ends were blunted using Klenow with the above reaction conditions. The 100 ng of Klenowed vector pSFV10 was dephosphorylated by 2μl of SAP as described in the section 2.2.2.5. The blunt ended pSFV10 vector and p35 and p40 inserts were cleaned up using QIAquick Nucleotide Removal Kit and were analysed on a gel as described in 2.2.2.4.

2.2.2.11 Ligation and Transformation

The p35 and p40 subunits of IL-12 were ligated to linearised SFV10 vector separately at the molar ratio of 1:3 vector to insert as described before in section 2.2.2.6. Transformation of the ligated DNA into competent *E. coli* DH5α cells and plating were performed as outlined in section 2.2.2.7.

2.2.2.12 Screening

A few isolated transformed colonies of recombinant plasmids were grown in sterile ampicillin (100 mg/ml) L-broth. The plasmid DNA was extracted from the harvested cells using QIAprep Miniprep Kit (Qiagen) as described in section 2.2.2.6. The restriction enzyme *XbaI* was used to screen the plasmids to test the presence and orientation of the insert (p35 or p40) in rSFV10 by restriction digestion analysis. The correct oriented positive clones of rSFV10-p35 and rSFV10-p40 were selected as outlined in section 2.2.2.8.

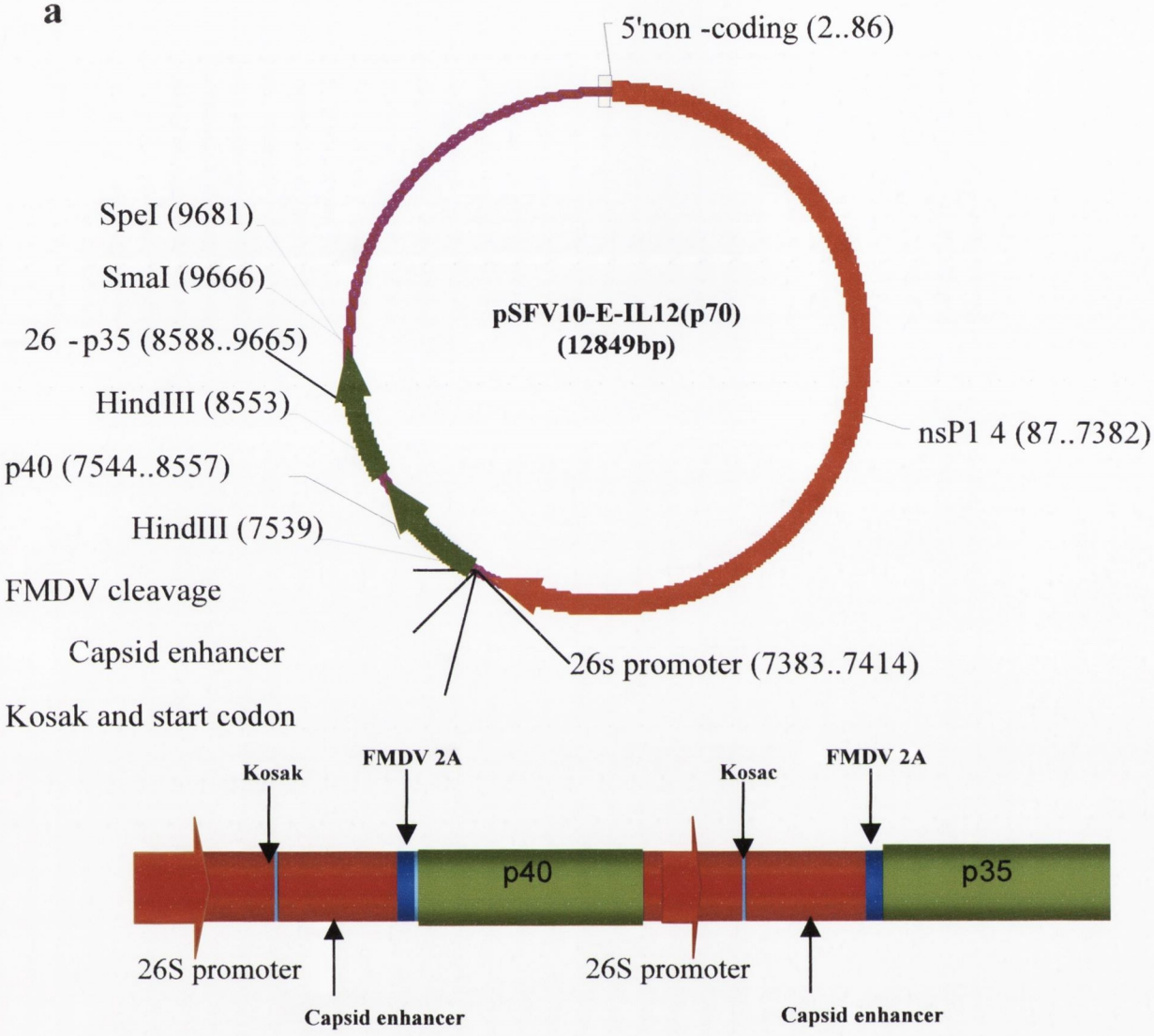
2.2.2.13 Construction of pSFV10-E-IL12 (p70)

Finally, both subunits of IL-12 were cloned into pSFV10-E to obtain pSFV10-E-IL12. Separate 26S subgenomic promoters controlled these subunits in pSFV10-E-IL12 construct (Figure 2.8). The pSFV10-E-p40 vector was linearised by *SmaI* restriction (blunt end) digestion. The p35 fragment along with the 26S subgenomic promoter from the pSFV10-E-p35 construct was cut using blunt end digestion by *PvuII* and *SmaI* restriction enzymes. The digestion reaction mixture for vector DNA contained 5 µl of 10x BSA (NEB), 5 µl of NEB-4 buffer and the reaction was carried out at 25^0C for 2 h in 50 µl total reaction volume. The digested plasmids were analysed on 0.8% agarose gel. The digested 26S-p35 fragment was excised from the gel, purified using the Qia-Gel extraction Kit as described in the section 2.2.2.2 and kept ready for ligating in to the linearised pSFV10-E-p40 construct.The linearised pSFV10-E-p40 vector DNA (50 ng) was dephosphorylated using Shrimp Alkaline Phosphatase as described before in the section 2.2.2.5. The 26S-p35-fragment obtained from the digestion of the pSFV10-E-p35 construct was ligated into dephosphorylated pSFV10-E-p40 using Quick T_4 DNA Ligase under theconditions described previously (2.2.2.6). The ligated DNA was transformed and plated as before and incubated overnight at 37°C. The plasmids from the transformed colonies were isolated using Qiagen Miniprep Kit. The presence and orientation of 26S-p35 fragments in the pSFV10-E-p40 construct was tested by *XbaI* digestion and analysed on a 0.8% agarose gel. A few positive clones of pSFV10-E-p70 were selected for further study.

2.2.2.14 Construction of pSFV10-IL12 (p70)

The p35 fragment along with 26S subgenomic promoter from the SFV10-p35 construct was separated using blunt end digestion by *PvuII* and *SmaI* restriction enzymes and ligated into *SmaI* linearised pSFV10-p40 vector DNA as described in section 2.2.2.10 to ultimately obtain pSFV10-IL12 (Figure 2.9). The presence and orientation of 26S-p35 fragments in the pSFV10-p40 construct was tested by *XbaI* and analysed on a 0.8% agarose gel.

a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SmaI (9666)
26 -p35 (8588..9665)
HindIII (8553)
p40 (7544..8557)
HindIII (7539)
FMDV cleavage
Capsid enhancer
Kosak and start codon
pSFV10-E-IL12(p70)
(12849bp)
nsP1 4 (87..7382)
26s promoter (7383..7414)
Kosak
FMDV 2A
p40
Kosac
FMDV 2A
p35
26S promoter
Capsid enhancer
26S promoter
Capsid enhancer

b

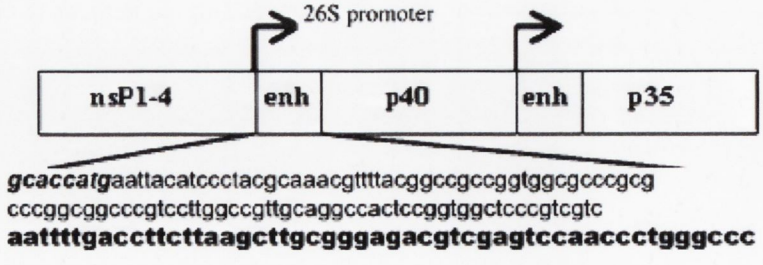

26S promoter
nsP1-4
enh
p40
enh
p35
gcaccatgaattacatccctacgcaaacgttttacggccgccggtggcgcccgcg
cccggcggcccgtccttggccgttgcaggccactccggtggctcccgtcgtc
aattttgaccttcttaagcttgcgggagacgtcgagtccaaccctgggccc

Figure 2.8 Schematic representation of pSFV10-E-mIL12.

(a) The higher expression vector pSFV10-E showing murine IL-12 subunits p40 and p35 in the multiple cloning site (MCS) of pSFV10-E with their own 26S sub-genomic promoters. The construction of this vector was carryout as described in the section 2.2.2. To construct pSFV10-E-mIL12, cDNA of p35 and p40 were amplified from total RNA of mouse splenocytes. Initially p35 and p40 subunits were ligated into MCS of two separate pSFV10-E vectors to get pSFV10-E-p35 and pSFV10-E-p40. Then p35 subunit along with 26s sub-genomic promoter, Kosak, capsid enhancer and FMDV 2A cleavage-site sequences from pSFV10-E-p35 was cut and subsequently cloned into *SmaI* restriction site just after p40 subunit in pSFV10-E-p40 to get pSFV10-E-p70.

(b) The nucleotide sequence of the enhancer element is shown in the inset. Italics indicate the Kozak and methionine initiation sequences, standard typeface codons for amino acids 2–34 of the capsid protein, and bold codons for 17 amino acids of the FMDV2A protein; cleavage occurs before the last amino acid (proline).

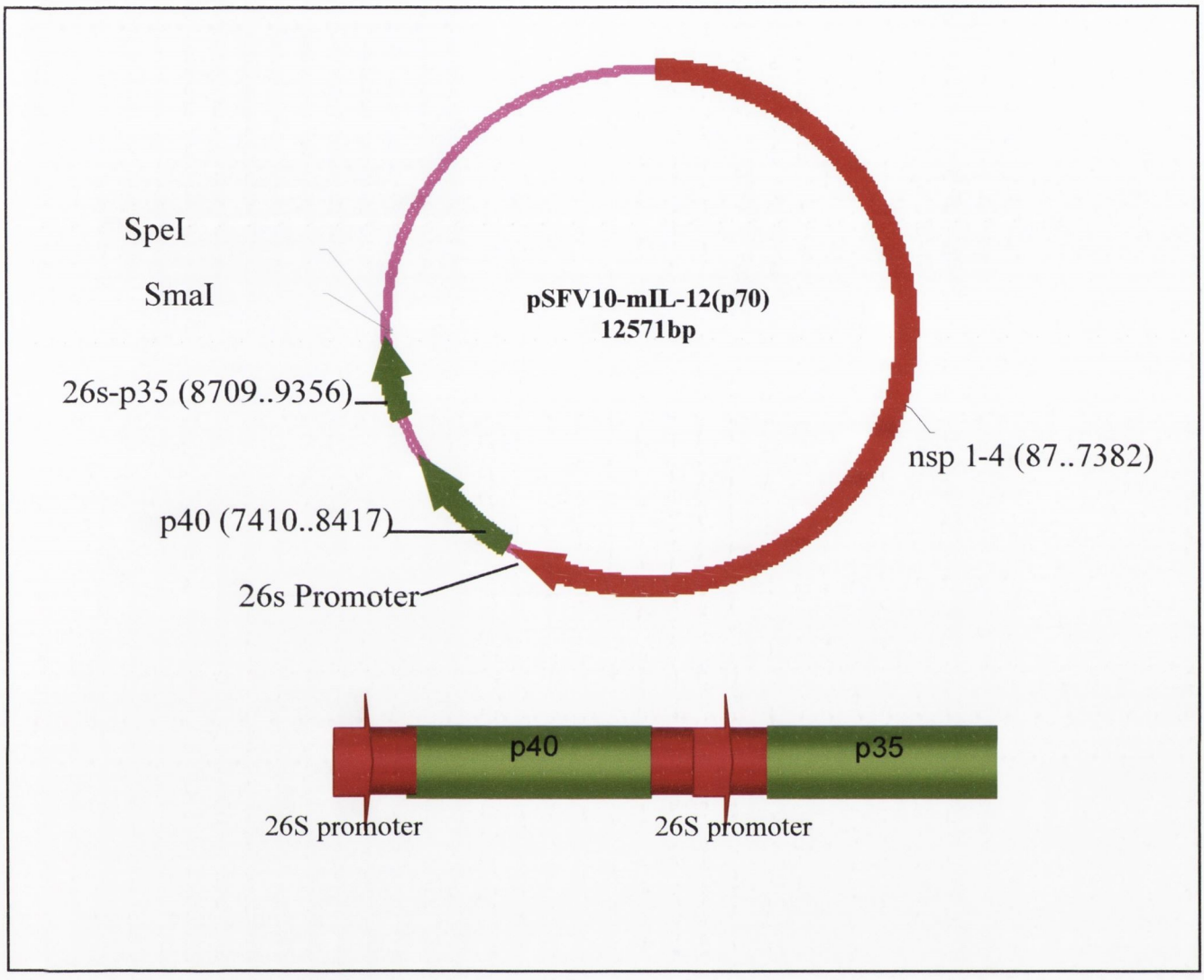


Figure 2.9 Schematic representation of pSFV10-mIL12.

The construction of this vector was carried out as described in the section 2.2.2.10-13. The p35 and p40 subunits from pSFV10-E-p35 and pSFV10-E-p40 constructs were released by *HindIII* restriction digestion. The pSFV10 vector was linearised using of *BamHI* (MCS), and the generated sticky ends of the inserts and vector were blunted using Klenow-large (DNA PolymeraseI). The linearised vector was dephosphorylated using Shrimp Alkaline Phosphatase. Blunted p35 and p40 subunits were ligated into linearised pSFV10 separately to get pSFV10-p35 and pSFV10-p40. Then the p35 subunit along with 26S sub-genomic promoter from pSFV10-p35 was cut and cloned into *SmaI* restriction site just after p40 subunit in pSFV10-p40 to get pSFV10-p70.

2.2.3 Recombinant Semliki Forest virus like particle (VLP) production

2.2.3.1 Expression Vectors

The SFV split-helper vector system that encompasses the packaging vectors pSFV-HelperS2 (encoding the envelope proteins) and pSFV-CS219A (encoding the capsid protein), and pSFV-EGFP were a gift from Professor P. Liljeström. A pSFV10-E vector expressing IL-18 was constructed by a project student Ms Susan McNally at the Dept of Microbiology, Trinity College. The constructed pSFV10-E-IL12 or pSFV10-IL12 plasmid was employed together with pSFV-HelperS2 and pSFV-CS219A to produce rSFV-IL12 VLPs.

2.2.3.2 Preparation of recombinant and helper SFV plasmids

Competent DH5α cells were transformed with pSFV-IL12, pSFV10-E-IL18, pSFV-EGFP, pSFV-Helper S2 and pSFV-Helper CS219A separately as outlined in the section 2.2.2.5. Transformed pure single colonies were then grown in L-broth separately for each plasmid DNA. Each of the plasmid DNAs was purified from *E. coli* DH5α cells using the QIAGEN Miniprep Plasmid Purification Kit as described in the section 2.2.2.6. Plasmid concentrations were estimated using a DNA/RNA spectrophotometer and visualised by gel electrophoresis as described in 2.2.2.2.

2.2.3.3 Linearisation of plasmid DNA for in vitro transcription

For the production of both helper and recombinant SFV viral RNA, each plasmid was linearized using an unique restriction enzyme. In each of the SFV plasmids a unique restriction site preceding the non-structural protein genes and SP6 promotor was selected. The *Spe* I site was used for pSFV-EGFP, and helper SFV plasmids to linearise whereas the *NruI* site was used for pSFV-IL12 and pSFV10-E-IL18 plasmids. A total of 10 µg plasmid DNA was linearised in a 50 µl volume containing 5 µl NEBuffer 2 or *NruI* -unique buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9), and 20 U *Spe I* or *NruI*. Following 16-18 h digestion in a waterbath at 37 °C, cut and uncut plasmids were visualised by gel

electrophoresis along with a λ- molecular weight marker as described in section 2.2.2.2. Linearised plasmids were then cleaned using the Qiagen nucleotide extraction kit and resuspended in a final volume of 50 µl sterile nuclease-free water. The concentrations of the linearised plasmid DNA were estimated on a 0.8% agarose by comparing band intensities with those of 0.5 µg Lambda (*HindIII*) DNA molecular weight markers.

2.2.3.4 *In vitro* SP6 RNA transcription

1.5 µg of each linearised plasmid DNA (section 2.2.3.4) was used as template in 50 µl SP6 Polymerase RNA transcription reaction containing SP6 buffer [40 mM HEPES-KOH pH 7.4, 6 mM MgOAc, 2 mM Spermidne-HCl], 1 mM $m^7G(5')ppp(5')G$, sodium (CAP), 5 mM dithiothreitol (DTT), 1 mM each rATP, rCTP, rUTP, 500 µM rGTP, 60 U RNasin and 34 U SP6 RNA polymerase in nuclease free water. This reaction was carried out in 1.5 ml eppendorf tubes separately for each DNA sample. The tubes were vortexed, centrifuged briefly and incubated at 37°C for 1 h 50 min on a heating block. Each of the RNA transcripts of rSFV10-E-IL12, rSFV10-IL12, rSFV10-E-IL18, rSFV-EGFP, rSFV-Helper S2 and rSFV-Helper CS219A were analysed by running 1µl on 0.6% (w/v) agarose gel as described in the section 2.2.2.2 and stored at -70°C.

In vitro transcription of each of the IL12 constructs and the helper vectors pSFV-S2 spike and pSFV-C219A capsid were made in 300 µl volume. For each stock of VLPs. three 150 cm^3(or six 75 cm^3) flasks of confluent Swedish-BHK cells per IL12 construct were prepared for electroporation.

2.2.3.5 High titre rSFV-IL12 VLP production

2.2.3.5.1 Electroporation of RNA

In order to produce rSFV-IL12 VLPs, three 150cm^3 tissue culture flasks of ~80% confluent sBHK cells was propagated, washed and trypsinised as described in section 2.2.1. The cells were resuspended in 10 ml of fresh BHK medium. Cells were centrifuged at 400 x *g* for 10 min at room temperature and the pellets were gently resuspended in 10 ml PBS and spun again. The cells of all the the confluent flasks

were resuspended finally in 4 ml of PBS and kept on ice. The *in vitro*-transcribed RNA reactions were divided into six 150 µl aliquots, with each tube containing 50 µl each recombinant SFV RNA (pSFV10-E-IL12 or SFV10-IL12 or pSFV10-E-IL18 or SFV-EGFP), SFV-Helper S2 RNA and SFV-Helper CS219A RNA. A control tube with 150µl PBS was mixed with 650µl of the cell suspension in separate tubes. 650 µl of cell suspension was added to the 150 µl RNA mixture and kept on ice for 5 min prior to loading into a 4 mm electroporation cuvette. The cell suspension was then pulsed twice at 0.85 kV and 25 µF capacitance with time constants ~0.4 seconds using a Gene Pulser II. A total of six separate electroporations were carried out in quick succession for each batch of VLP stock. The cell suspensions of all the six electroporations were mixed with 100 ml of fresh BHK medium and divided into two $150cm^3$ flasks (50ml/flask). The cells were incubated at 33°C in a humidified atmosphere with 5% CO_2 for 36-48 h. The culture supernatant (containing the rSFV-IL-12 VLPs) was collected and centrifuged at 18,000 rpm for 15 min twice (Sorvall RC 5C Plus) in order to remove cellular debris and placed on ice.

2.2.3.5.2 Harvesting high titre rSFV particles

The clarified supernatant was aliquoted into three SW-28 ultracentrifuge tubes with 5ml of 20% sucrose (in TNE buffer) as cushion beneath the virus supernatant. The tubes were placed into SW28 ultracentrifuge buckets and all the tubes were balanced by using another mimic tube. The buckets were fixed to the SW28 rotor and centrifuged at 25000 rpm for 2 h at 4°C in the Beckman ultracentrifuge. Following ultracentrifugation, the supernatant was immediately removed, the sides of the tubes dried with sterile cotton tips, and each pellet was resuspended with 400 µl TNE buffer (50 mM Tris-HCL, pH 7.4, 100 mM NaCl, 0.1 mM EDTA) per tube and incubated on ice for 2-3 hours to overnight. The pellet was resuspended with gentle pipetting and the virus like particles (rSFV10-E-IL12 or rSFV10-IL12 or rSFV-EGFP or rSFV10-E-IL18) were collected. A further 400 µl of TNE was added to each tube, which were then incubated on ice for 1 h and vortexed briefly and collected into the previously harvested TNE to a final volume between 2-3 ml. The resuspended VLPs were aliquoted in 100 µl into cryotubes and were snap frozen using dry ice cooled methanol or liquid nitrogen before storage at -70°C.

The production of high-titre stocks of rSFV10-E-IL-12, rSFV10-IL12, rSFV10-E-IL18 and rSFV-EGFP, was performed as described above by utilizing the plasmids pSFV10-E-IL12, pSFV10-IL12, pSFV10-E-IL18 and pSFV-EGFP and stored frozen at -70°C.

2.2.3.5.3 High titre rSFV particle titration

2.2.3.5.3.1 Immunoflourescence

BHK-21 cells were grown confluently on 22x22 mm glass cover slips in a 6-well plate. Serial dilutions (1 µl, 0.1 µl, 0.01 µl, 0.001 µl, and 0.0001 µl) of virus stock were made using a total volume of 500µl medium for infection (2.2.1). The cells were infected with 500µl of virus dilutions for one hour at 37°C by rocking every 10 min. 2.5 ml fresh BHK medium was added to each well and incubated at 37°C in a humidified atmosphere with 5% CO_2.

After 16-18 h immunofluorscence (IF) was carried out. The cells were fixed in 2 ml of 4% paraformaldehyde (PFA) in PBS^- for 20-30 min. The PFA was removed and cells were incubated in acetic acid : ethanol (1:2) at -20°C for 5-10 min. The cells were blocked with 1ml rabbit serum blocking buffer containing 0.5% of 7.5% BSA (Sigma) and 1ml rabbit serum in 50ml PBS for 30 min. The coverslips were incubated with 50µl primary (IL-12) antibody (1:50) (purified Rat anti-mouse IL-12 p-70) on slides covered with Parafilm for 1 h and with 50µl secondary antibody (biotinylated rabbit anti rat-IgG; mouse adsorbed) for 30 min. After washing, the cells were incubated with streptavidin/FITC (1:100 in HEPES-NaCl buffer) for 30 min and washed twice in high-salt HEPES-NaCl buffer (0.5 M NaCl, 10 mM HEPES) for 30 min at room temperature. The cells on the coverslips were mounted on slides with hardest vectashield mounting medium (DAPI) and examined using a fluorescent microscope. The average number of positive cells from 20 random fields at a magnification of 400x was used to calculate VLP titres and expressed as infectious units (IU) per ml. Similarly, the titres of IL12 VLPs were calculated on K-BALB cells by IF.

The rSFV-EGFP VLPs were titrated by observation in the fluorescent microscope. The titration of rSFV10-E-IL18 VLPs was carried out using IL-18

antibodies (purified Rat anti-mouse IL-18) (1:50) by immunofluorescence as described above.

2.2.3.5.3.2 Immunocytochemical staining

The BHK-21 and K-BALB cells were infected and fixed as above. The cells were quenched in 3% H_2O_2 (in PBS) (Sigma) for 10 min. Then cells were blocked, treated with primary and secondary antibodies, and washed as described above. The cells were treated with Vectastain (Vector) for 30 min, 2 min in DAB (Sigma) solution and 20 seconds in Harris' haematoxylin stain (BDH). Cells were examined using bright field microscopy; the positive cells were easily distinguished (clearly brown stained) from uninfected cells.

2.2.4 *IN VITRO* ASSAYS

2.2.4.1 Comparative analysis of rSFV10-E-IL12 and rSFV10-IL12 for the expression of IL12 by VLPs

Analyses of expression and secretion levels of IL12 were carried out using BHK-21 and K-BALB cells. The cells were grown confluent by seeding $2.5x10^5$ cells /well in a 24-well plate. To achieve complete infection, the cells were infected in duplicate with an MOI (multiplicity of infection) of 10 VLPs (rSFV10-E-IL12 or rSFV10-IL12) and incubated at 37°C in 9% CO_2. The supernatants of the infected cells were collected at different time points (12 h, 24 h, 48 h and 72 h) and centrifuged 10,000 rpm for 10 min to remove the cell debris.

The level of secreted IL-12 protein (1:20, 1:200 and 1:250) in triplicate for each time point sample was estimated by capture ELISA using OptEIA™ Set Mouse IL-12p (70) Kit in flat-bottomed 96-well ELISA plates. Capture ELISAs were performed according to the kit manufacturer's instructions. Wells of a 96-well plate were coated with 100 µl/well of capture antibody diluted in coating buffer (1:250 dilution), and incubated overnight at 4°C. Wells were washed 3 times with 300 µl/well of wash buffer, and plates blocked for 1 h at RT with 200 µl/well of assay diluent. Wells were again washed 3 times with wash buffer, and incubated sealed for

2 h at RT with 100 µl/well of either standard (serial dilutions in assay diluent) or samples. Plates were washed as before with 5 total washes, and then incubated sealed for 1 h at RT with 100 µl/well of working detector solution. Plates were again washed with 7 total washes. 100 µl/well of TMB substrate was added, and plates incubated at RT in the dark for 30 min, before addition of stop solution (2N H_2SO_4). Absorbances were read using a Multiscan RC ELISA plate reader at 450 nm within 30 min of adding stop solution. The values were plotted to see the difference in the levels of IL12 secreted by rSFV10-E-IL12 or rSFV10-IL12 in both BHK-21 and K-BALB cell lines.

The difference in the IL-12 expression levels from rSFV10-E-IL12 and rSFV10-IL12 VLPs were tested in BHK-21 and K-BALB cells qualitatively by immunofluorescence as described in section 2.2.3.5.3.1.

2.2.4.2 Testing biological activity of the IL-12 secreted by the rSFV-IL-12 VLPs

Biologically active IL-12 induces splenocytes (T-cells) to secrete interferon-γ (IFN-γ). A spleen from BALB/c mouse was collected in Hank's Balanced Salt Solution (HBSS) on ice and chopped with scissors. The tissue in HBSS was smashed with a syringe plunger and passed through a 70 µm cell strainer into a 50ml Falcon tube. The cells were spun at 1500 rpm for 10 min at 4°C. After discarding the supernatant, 5ml of 1x red blood cell (RBC) lysis buffer was added and incubated for 2 min at room temperature. The tubes were topped up with HBSS and spun as before twice. The supernatant was discarded and the cells were resuspended in 20 ml RPMI-1640 containing 10% heat inactivated foetal bovine serum (FBS), 20 mM HEPES buffer, 100µg/ml streptomycin 2 mM L-Glutamine, 1% Sodium pyruvate, 1% non-essential amino acids and 50 µM β-Mercaptoethanol. The cells in the suspension were counted by tryphan blue exclusion assay using a haemocytometer as follows: from the cell suspension, 20 µl was mixed well with 20 µl tryphan blue and loaded into the gap between the haemocytometer and cover slip. The clear viable (tryphan blue excluding) cells distinguished from those of dead (blue) and red blood (dark) cells were counted in all the four squares and the average number of viable cells was calculated. The cell suspension was spun again and resuspended with medium to get $1x10^6$/250 µl ($8x10^7$ cells/20ml). 0.5 ml of the cells per well were dispensed into two

24 well plates. 100µl (neat) or1µl (1:100) of the supernatant from the VLP-infected (rSFV-EGFP or SFV-IL12 or infection medium) BHK-21 or K-BALB cells were added to the splenocytes in the wells and volume was made up to 1ml in each well. Naïve, concavalin A (ConA) (1 µg/ml) (250 µl of 4x/well) and rSFV-EGFP supernatant were used as controls. The plates were incubated at 37°C and the supernatants were harvested at different time points (24 h, 48 h, and 72 h). The cells were separated by centrifuging the harvested supernatant from the splenocytes at 10,000 rpm and stored at -20°C for further analysis.

A capture ELISA test (OptEIA™ Set Mouse IFN-γ kit), was used to measure the production of IFN-γ from murine splenocytes induced with the supernatants containing IL-12 secreted by rSFV-IL12 VLP infected BHK-21 and K-BALB cells. The capture ELISAs were performed according to the manufacturer's instructions. The absorbances were read at 450 nm and mean concentrations at each time point were plotted on a graph for rSFV10-E-IL12 and rSFV10-IL12 VLPs.

2.2.4.3 *In vitro* characterization of rSFV10-E -IL18 VLPs

Expression and secretion levels of IL18 were tested in BHK-21 and K-BALB cells and were estimated by the capture ELISA test using OptEIA™ Set Mouse IL-18 kit as outlined in the section 2.2.4.1. Biologically active IL-18 is known to induce splenocytes to secrete IFN-γ. Hence the biological activity of the secreted IL18 from both the cell lines was tested using a capture ELISA test using the OptEIA™ Set Mouse IFN-γ kit as described in section 2.2.4.2.

2.2.5 *IN VIVO* TUMOUR TREATMENT STUDIES USING rSFV VLPs

2.2.5.1 Induction of tumours (K-BALB, CT26 or 4T1) *in vivo*

BALB/c mice were used for *in vivo* experimentation, which were maintained as described in section 2.1.5. Six female BALB/c mice of 6-8 weeks age were used for each treatment group throughout the tumour treatment experiments. Prior to tumour induction their right flanks were shaved in order to facilitate tumour induction and measurement of the growth. Tumour (K-BALB or CT26 or 4T1) cells were grown in 150 cm^3 cell culture flasks as described in section 2.2.1 and harvested by

trypsinisation during their log phase of growth. Cells were centrifuged at 400 x *g* at 4 °C followed by washing with Hanks balanced salt solution (HBSS). The cells were resuspended gently to a final concentration of 1 x 10^7 cells/ml in non-supplemented DMEM or HBSS, viability having been determined using the trypan blue exclusion assay as described in section 2.2.5.1. The resuspended cells were injected subcutaneously (s.c.) ($1x10^6$ /mouse in 100 µl) into the right flank of each mouse using a 1 ml syringe and 23G needle. Mice were examined daily for signs of s.c. tumour formation and when detected, tumour diameters were measured using a linear caliper in 2 perpendicular diameters with the average tumour diameter calculated as the square-root of the product of cross-sectional diameters, assuming spherical shape. Tumours of 4 mm diameter were seen at 3-4 days post injection in case of K-BALB and 4T1 tumours and at 5-6 days with the CT26 model.

2.2.5.2 Treatment of tumours (K-BALB, CT26 or 4T1) *in vivo* with rSFV VLPs

High titre stocks of rSFV10-E-IL12, rSFV10-IL12, rSFV10-E-IL18, and rSFV-EGFP VLPs were prepared as described in section 2.2.3.5, and titrated (section 2.2.3.5.3). The titre of rSFV10-E-IL12 was adjusted to a concentration of 4 x 10^7 IU/ml, $4x10^8$ IU/ml and $4x10^9$ IU/ml in TNE buffer. The treatment was started when the tumour size reached 4 mm. Mice received a total of six intratumoural (i.t.) injections each on every alternate day, of 50 µl TNE buffer alone (control groups) or TNE containing rSFV-EGFP VLPs (as virus control groups) or the TNE containing high-titre rSFV-IL12/IL18 VLPs using a 0.5 ml insulin microsyringe. Each set of tumour treatment experiments in BALB/c mice was carried out separately for different titres ($4x10^7$ IU/ml or $4x10^8$ IU/ml or $4x10^9$ IU/ml) of rSFV VLPs. 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. Tumours were measured daily as described in section 2.2.5.1 and mice were euthanised by halothane overdose and cervical dislocation when tumours approached 15 mm^2. Two sets of animals for each treatment group were used for all the different tumour cell lines. The first group was assayed for survival and the second group assayed for pathology, serology, immuno-histochemistry, and lung

metastases (4T1 tumour model). The schematic representation of treatment strategy is given in the Figure 2.10.

K-BALB tumours in BALB/c mice were treated using rSFV10-E-IL12 VLPs at $4x10^7$ IU/ml, $4x10^8$ IU/ml and $4x10^9$ IU/ml titres in ascending order in separate sets of experiments for each viral titre. CT26 tumours were treated with rSFV10-E-IL12 VLPs at $4x10^8$ IU/ml and $4x10^9$ IU/ml titres whereas 4T1 tumours were treated using $4x10^9$ IU/ml. To examine and compare the *in vivo* treatment efficacy of rSFV10-E-IL12 and rSFV10-IL12 VLPs, K-BALB tumours were treated at $4x10^9$ IU/ml titre as described before.

2.2.5.3 Treatment of K-BALB and CT26 tumours using rSFV10-E-IL18 VLPs

An *in vivo* tumour treatment study was carried out on K-BALB and CT26 tumours using rSFV10-E-IL18 ($4x10^9$ IU/injection) at a titre of $4x10^9$ iu/injection as outlined in section 2.2.5.1 and 2.2.5.2.

2.2.5.4 Treatment of 4T1 tumours with rSFV10-E-IL12 VLPs

4T1 tumours were induced subcutaneously on the right flank of each mouse by injecting 10^6 cells in 100 µl Hank's balanced salt medium (HBSS) with a 29G 0.5ml syringe as outlined in section 2.2.5.1. When the size of the 4T1 tumours reached $4mm^2$, they were treated with TNE, or $4x10^9$ IU/injection rSFV-EGFP, or rSFV10-E-IL12 VLPs, as described in section 2.2.5.2.

2.2.5.5 Immunological studies

2.2.5.5.1 Harvesting of serum from K-BALB tumour bearing mice

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 clotted blood was centrifuged at 400 *g* for 10 min at 4 °C and the serum was decanted and aliquoted before storage at -70 °C.

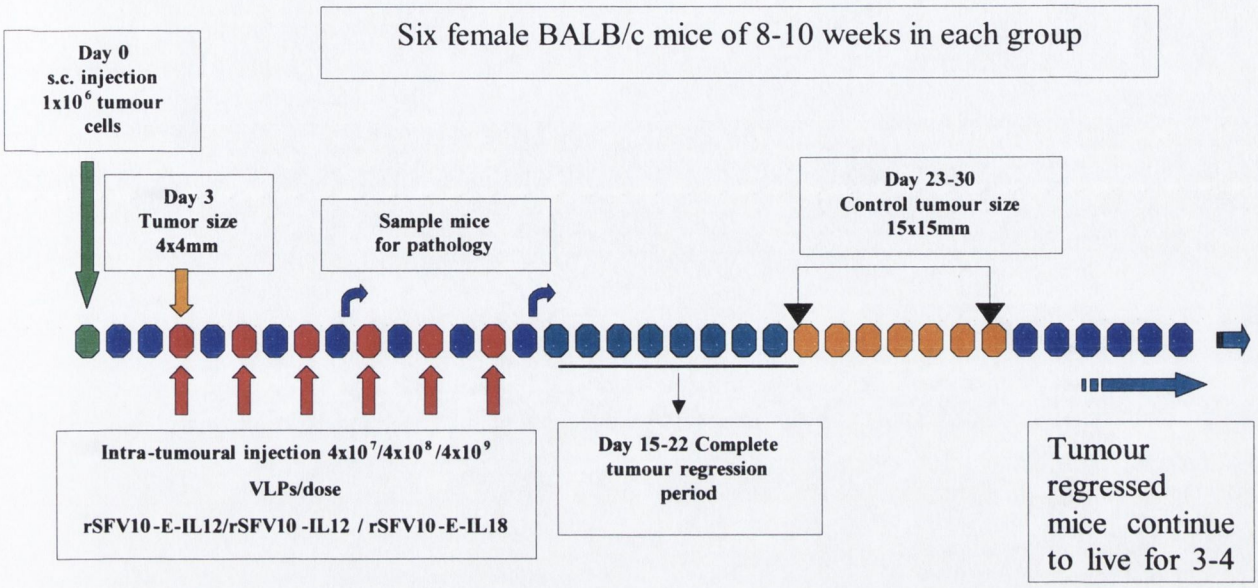


Figure 2.10 General strategy for *in vivo* treatment of K-BALB, CT26 or 4T1 tumours in immuno-competent BALB/c mice

2.2.5.5.2 Serum analysis for antiangiogenic factor IP-10

Blood serum from three K-BALB tumour bearing mice from each group that were treated with $4x10^9$ IU/injection of rSFV-IL12 VLPs, rSFV-EGFP or TNE was collected 3 days after the 6th treatment as outlined in section 2.2.5.6.1. An assay for IP-10 in the sample blood serum in triplicate of each treated group was quantified by using Quantikine mouse IP-10/CXCL10 (R&D Systems) immunoassay kit. The sample dilutions along with the standard and controls were incubated in a flat bottomed 96 well ELISA plate pre-coated with antibodies raised aginst *E. coli*-expressed recombinant mouse IP-10. The protocol was followed according to the manufacturer's instructions. The absorbance of the samples was read at 450 nm.

2.2.6 HISTOPATHOLOGY

2.2.6.1 Sampling of mice for histopathology

2.2.6.1.1 Sampling and processing of tumour and lung samples

For general pathology and immuno-histochemical detection of CD8+, CD4+, and CD31, mice were sampled in triplicate on the next day after the 3rd and the 6th treatment and at the end of the treatment i.e., when the tumour size reached 15 mm^2. Mice were sacrificed by a quick cervical dislocation. Tumours were excised, placed in 5% formal saline and processed for pathological studies.

Similarly, lungs from 4T1 tumour bearing mice at day 24 post-tumour induction were collected in 5% formal saline to examine the 4T1 lung metastases. Processing of tissues, and sectioning of sampled tumours for general pathology was performed by Ms. Alex Whelan (Veterinary Pathology Laboratory, University College Dublin). 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 3x40 min xylene washes. This was followed by 4x40 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.

2.2.6.1.2 Preparation of K-BALB tumour cryosections

For immuno-histochemical examination, the resected tumour samples were embedded in Tissue-Tek OCT compound on cork disks, snap-frozen in liquid nitrogen-cooled isopentane and stored at -70 °C. A Leica CM 1900 cryostat was used to prepare 4-6 μm thick sections at –20 °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.

2.2.6.2 Histopathology and immunohistochemistry

2.2.6.2.1 Haematoxylin and eosin (H & E) staining for routine histology

Sections of paraffin embedded tumours and lungs 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 distilled water. Sections were then placed in Harris' haematoxylin 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 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 by covering the section with a coverslip without air bubbles. H&E stained slides were prepared from three separate sections of each tumour or lung to provide representative pathological observations.

2.2.6.2.2 Routine histology

Coded H&E stained tumour sections without any pre-knowledge of the treatment given were examined 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, tumour ulceration, presence of TILs and their location in the

tumour microenvironment. Coded H&E stained lung sections were examined blind for the presence of 4T1 tumour cells.

2.2.6.2.3 Immunohistochemical analysis of frozen tumour sections

Frozen tumour sections were used for immunohistochemical staining to detect the influx of immune cells such as CD4+ and CD8+ into the tumour. In brief, the sections were fixed in a series of different percentage ethanol, quenched with 0.3% hydrogen peroxide, blocked in 5% rabbit serum for 30 min at room temperature and blocking endogenous avidin and biotin using the avidin/biotin blocking kit. The sections were treated with primary antibody, either rat anti mouse CD4 (1:20) or CD8 (1:20) diluted in 5% rabbit serum at room temperature for 1 hour. Secondary antibody, biotinylated rabbit anti-rat antibody, (1:1000) was used on the sections at room temperature for 45 min. The primary-secondary antibody complexes were detected by using Vector Elite ABC standard HRP kit according to the manufacturer's instructions with 3,3-Diaminobenzidine (DAB) as substrate. The sections were washed between each step with PBS and finally counterstained with haemotoxylin. Sections of mouse spleen were employed as a positive control for CD4 and CD8. A negative control was used for each type of cell by substituting primary antibodies with 5% normal rabbit serum. The stained sections were mounted and observed by light microscopy for positive cells stained light or dark brown.

2.2.6.2.4 Assay for vascular density

Frozen tumour sections sampled after the 6th treatment were examined for the morphology of the tumour vasculature by immunohistochemistry staining as mentioned above. The endothelial cells of blood capillaries were stained using 5% goat serum as blocking buffer, rat anti-mouse CD31 as primary antibody, and biotinylated goat anti-rat IgGs as secondary antibodies as outlined in section 2.2.6.2.3.

2.2.6.2.5 Detection of lung metastases by clonogenic assay

Lungs were examined for the 4T1 metastases as described by Pulaski BA *et al.*, 1998. 4T1 cells are resistant to 60 μM 6-Thioguanine, but this drug kills other cells. Briefly, the lungs sampled from all the treated and control mice groups (2.4.1.1) were subjected to clonogenic assay. Lungs were treated with Elastase and Liberase blendzymes (1:2) and the cells were harvested by filtering through a 70 μm nylon cell strainer after washing with HBSS. Lung cells were diluted and plated in tissue culture complete RPMI-1640 medium (2.2.1) containing 60 μM 6-thioguanine (2-amino-6-mercaptopurine). The 4T1 cells which are resistant to 60 μM thioguanine were allowed to grow for 10-12 days and the clonogenic colonies of 4T1 cells were fixed in methanol and stained with crystal violet for quantitation of clonogenic lung metastases.

2.2.7 Statistical analysis of the data

The statistical significance of difference in the tumor size among the different experimental treatment groups was analysed by one-way ANOVA. Tukey's multiple comparisons post-test was used to compare the probability (P) value of each group with its appropriate control TNE treated group. Kalpan-Meir survival curves were used to analyse the overall median survival of mice and survival proportions in each treatment group. The P value significance of the lung metastasized clonogenic colonies was analysed using Dunn's Multiple Comparison post-test. All the statistical analyses were performed using GrahPad Prism 4 program and a P value less than 0.05 was considered significant.

3

CLONING AND *IN VITRO* CHARACTERISATION OF IL-12 IN pSFV EXPRESSION SYSTEMS

3.1 INTRODUCTION

Replication-deficient alphavirus vectors, specifically Semliki Forest virus vectors, have demonstrated transient expression in a variety of mammalian cell types. SFV-vectors have been successfully used for cell culture and in vivo expression of a number of human or murine genes such as IL-12 (Asselin-Paturel *et al.*, 1999, Colmenero *et al.*, 2002, Ren *et al.*, 2003), p53 (Glasgow *et al.*, 1998) or IL-18 (Yamanaka *et al.*, 2003). It has been shown that recombinant SFV (rSFV) replicons can efficiently infect rat prostrate cancer cell lines (Murphy *et al.*, 2001). It has also been shown that SFV vectors can efficiently infect and apoptosis was rapidly induced in majority of cells infected with SFV-LacZ virus (Hardy *et al.*, 2000). Intratumoural injections of SFV expressing the cytokine IL-12 showed significant tumour regression and inhibition of tumour angiogenesis in a mouse B16 melanoma model (Zhang *et al.*, 1997, Yamanaka *et al.*, 2000). It was also shown recently in animal models of malignant glioma that SFV infection of autologous DC is providing a potential mechanism for enhanced immunogenisity and tumour rejection (Yamanaka *et al.*, 2001). Prior experiments using SFV-LacZ virus particles showed no detectable side effects in the CNS (Lundstrom *et al.*, 2003) whereas rSFV-IL12 was shown to cause significant cytotoxicity in solid tumours in pre-clinical animal models of human breast, lung, prostate and pancreatic cancer in mice (Murphy *et al.*, 2000; Hardy *et al.*, 2000). In addition, SFV seems to be particularly suitable for induction of systemic immune responses against tumour cells and against the transgene proteins carried by the vector. Expression of one such tumour-associated antigen (TAA), the P1A gene from the SFV vector resulted in induction of tumour immunity against P815 cells *in vivo* (Colmenero *et al.*, 1999). These and other studies have indicated that rSFV is an expression vector with strong antitumour effect and that it could be a potentially useful for cancer vaccines.

Several studies in animal models have indicated that the expression of various cytokine genes in tumour cells generally results in a dramatic alteration of tumour cell growth and in induction of tumour specific immunity (Rakhmilevich *et al.*, 1997). Furthermore, use of various viral vectors has demonstrated in murine models that gene transfer of IL-12 promoted tumour regression and inhibition of metastases, making the use of this cytokine particularly relevant in cancer therapy (Lasek *et al.*, 1997, Ren *et al.*, 2003). However, it has been shown that the IL-12 concentration at

the tumour site is critical for tumour regression (Colombo *et al.*, 1996). In order to sustain high IL-12 concentration at the tumour site approaches such as frequent intratumoural injections with IL-12 encoding virus particles that have enhanced expression may be required.

This chapter describes how p35 and p40 subunits of murine IL-12 were cloned into two different SFV vectors with different expression ability (pSFV10-E and pSFV10), and experiments to *in vitro* characterise them for expression and secretion of biologically active murine IL-12, following infection of BHK-21 and K-BALB tumour cells. Murine splenocytes were used in this study to examine the biological function of the secreted IL-12 by rSFV-IL12 constructs.

3.2 RESULTS

3.2.1 Construction of pSFV10-E-mIL12

Murine IL-12 (mIL-12) gene subunits were amplified from total RNA of SFV-A7 infected mouse splenocytes using reverse transcriptase. PCR amplified p35 (648bp) (Figure 3.1) and p40 (1008bp) (Figure 3.2) subunits of mIL-12 were cloned into *HindIII* digested pSFV10-E (Figure 3.3) separately to obtain pSFV10-E-p35 and pSFV10-E-p40. Pfu DNA-polymerase amplified p35 and p40 subunits in separate pSFV10-E, were analysed for correct orientation by *EcoRI* and *XbaI* digestion (Figure 3.4 & 3.5).

The p35-subunit was cut along with its subgenomic promoter 26S from pSFV10-E-p35 and inserted into the *SmaI* restriction site just after the p40 subunit in pSFV10-E-p40, which resulted in pSFV10-E-p70 (pSFV10-E-mIL12). This construct was analysed on a gel by *XbaI* restriction digestion (Figure 3.8).

3.2.2 Construction of pSFV10-mIL12

IL-12 genes were excised from the pSFV10-E-p35 and pSFV10-E-p40 constructs and cloned in to pSFV10 separately to get pSFV10-p35 and pSFV10-p40 constructs. These constructs were analysed to confirm the correctely-orientated subunits using XbaI digestion (Figure 3.6 and 3.7). The p35-subunit was cut along with its subgenomic *26S* promoter from pSFV10-p35 and inserted into the *SmaI* restriction site just after the p40 subunit in pSFV10-p40, which resulted in pSFV10-p70 (pSFV10-mIL12). This construct was analysed on a gel by *XbaI* restriction digestion. Restriction digested pSFV10-E-mIL12 and pSFV10-mIL12 constructs were analysed on gel and correctly orientated pSFV-mIL12 constructs were selected for further in vitro expression studies (Figure 3.8).

3.2.3 Sequencing of p35 in pSFV10-E-p35 and p40 in pSFV10-E-p40 constructs

A few of the correctly orientated clones in each construct were verified for right sequences in order to compare and confirm the pfu amplified sequences with GeneBank data base sequences of IL-12 gene subunits. Nucleotide sequence obtained

from a few clones in each of the constructs was blasted against the NCBI database, and an E-value of 0.0 was obtained for a match to the mouse IL-12 gene (Figures 3.9 and 3.10).

3.2.4 *In vitro* transcription

Helper and recombinant (rSFV-IL12) plasmids were linearized prior to transcription using the unique restriction site SpeI found on helper SFV plasmids and NruI on recombinant SFV-IL12 plasmids and analysed on gel (Figure 3.11). These linearised helper and recombinant (rSFV10-E-IL12) plasmids were *in vitro* transcribed and analysed on a 0.8% agarose gel (Figure 3.12).

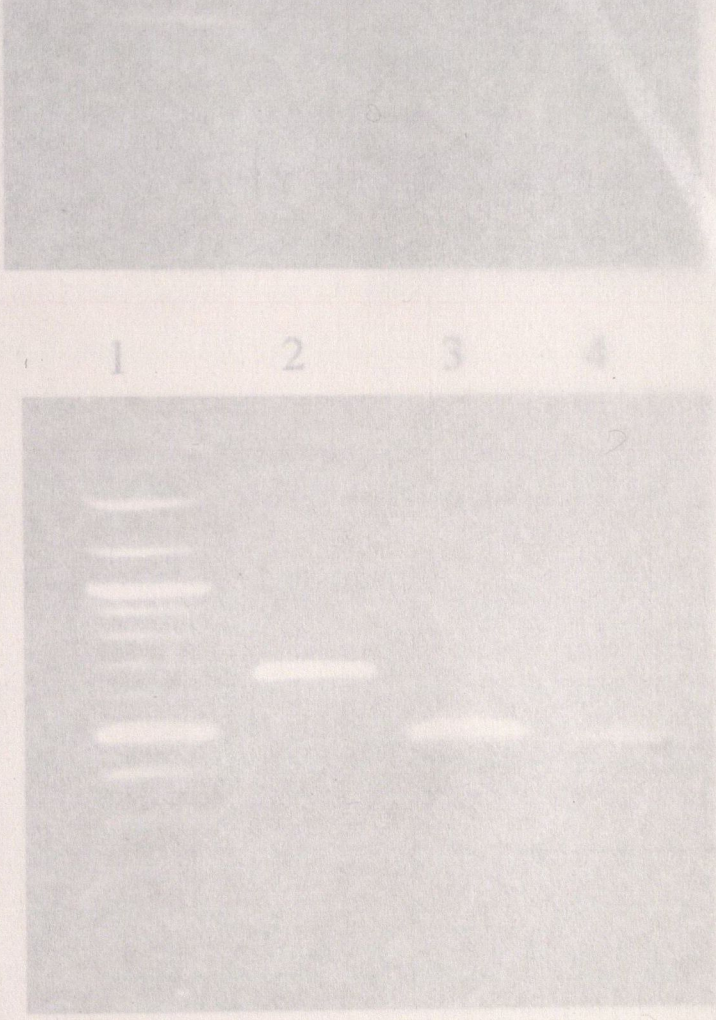
3.2.5 Comparative analysis of mIL-12 expression levels

The difference in expression levels of murine IL-12 (mIL-12) between the two constructs rSFV10-E-IL12 and rSFV10-IL12 was examined by qualitative and quantitative assays in BHK-21 and K-BALB cells. The qualitative difference in expression levels between these IL-12 constructs was observed by the staining intensities of the infected BHK-21 positive cells when examined under microscopy (Figure 3.13, 3.14 & 3.15). The cells infected with rSFV10-E-IL12 VLPs were stained brightly due to high level expression compared to rSFV10-IL12 infected cells.

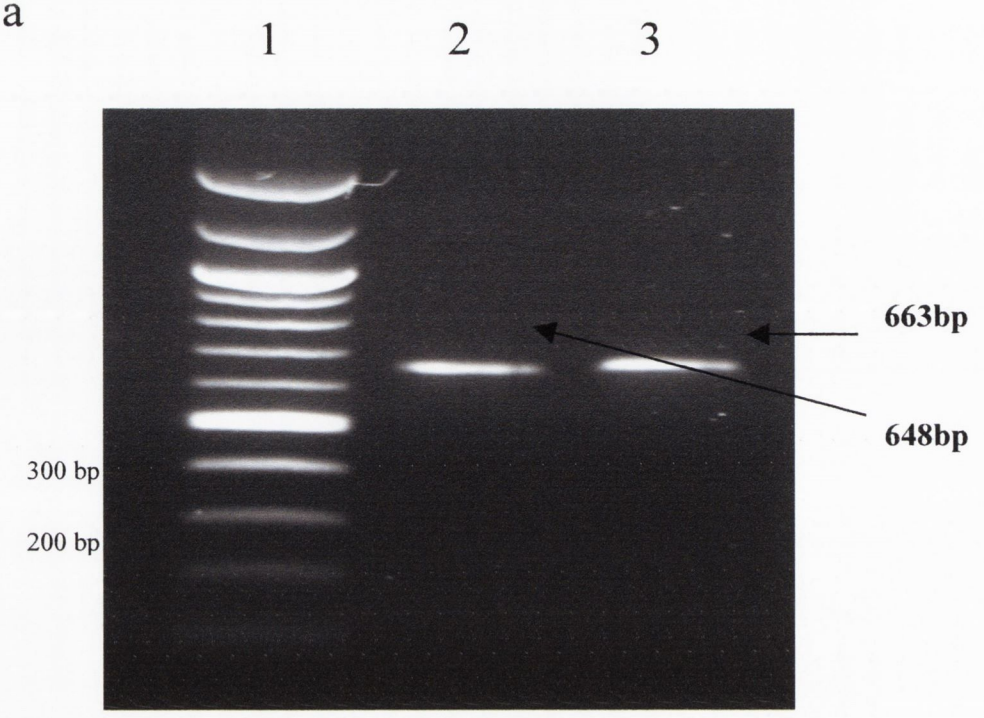
BHK-21 and K-BALB cells grown in 6-well plates were infected with VLPs of rSFV10-E-mIL12 and rSFV10-mIL12 at MOI of 10. The supernatants of the infected cells were collected at different time points (12 h, 24 h, 48 h and 72 h). An ELISA estimated the expressed IL-12 protein in the supernatants of both cell lines. The results were plotted to measure the difference in the expression levels of IL-12 by rSFV10-E-IL12 or rSFV10-IL12 in both BHK-21 and K-BALB cell lines. Higher concentrations of secreted levels of IL-12 from infected BHK-21 and K-BALB cells were found between 24 and 48 hours. The levels of IL-12 expression by rSFV10-E-IL12 in BHK-21 cells were 3-4 times higher than those of rSFV10-IL12 VLPs whereas in K-BALB cells, 10-fold higher expression was found. Mock-infected control BHK-21 and K-BALB cells supernatant showed no IL-12 expression (Figure 3.16 & 3.17).

3.2.5 Testing biological activity of secreted murine IL-12

Biologically active IL-12 induces T-cells to secrete interferon-γ (IFN-γ). An ELISA measured the production of IFN-γ from the 24 h supernatants of murine splenocytes treated with IL-12 supernatants of VLP infected BHK-21 cells or K-BALB cells. The result showed that the supernatant of rSFV-IL12 replicons infected BHK-21 and K-BALB cells stimulated splenocytes to produce almost similar levels of IFN-γ as positive control (Con A) treatment and indicated that the secreted IL-12 is biologically functional (Figure 3.18).



a



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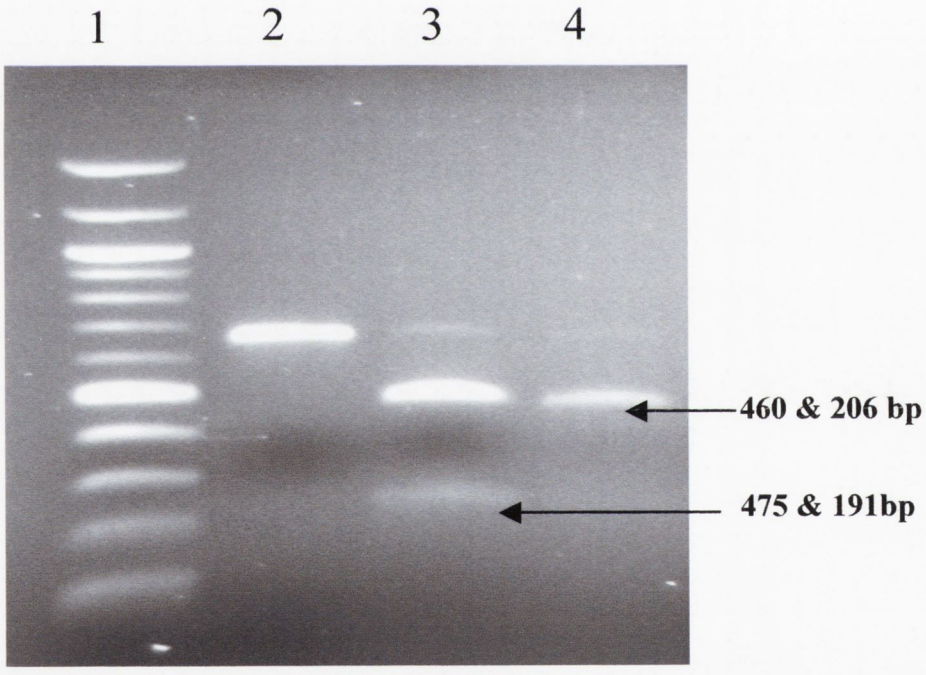


Figure 3.1 Gel analysis of PCR amplified p35 subunit

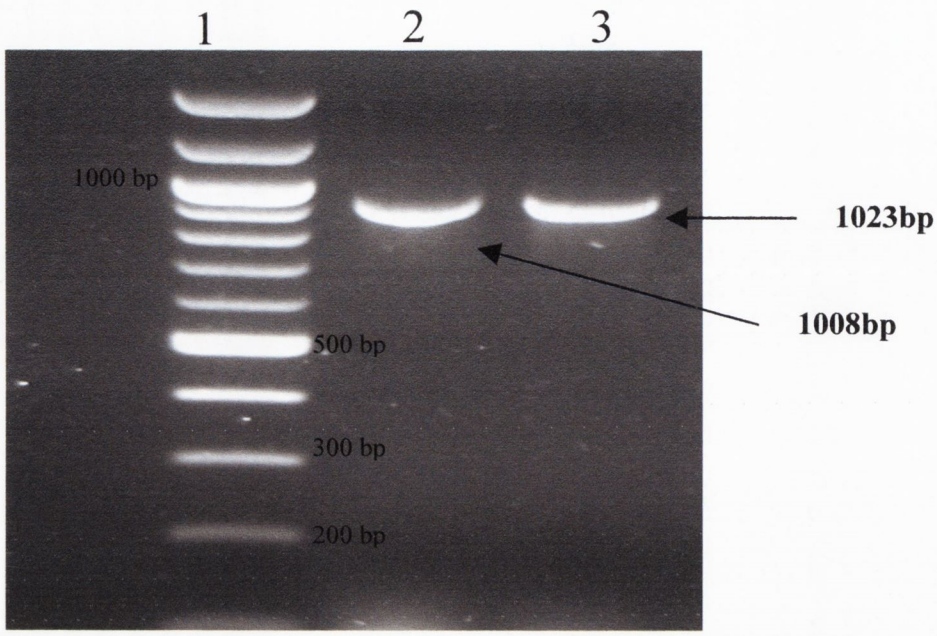
Total RNA was isolated from spleen of BALB/c mice infected i.p. with SFV-A7 at 4 dpi. The integrity of this RNA was checked by RT-PCR using primers specific for mouse β-actin and mouse GAPDH (not shown). The PCR amplified p35 (648bp) using pfu polymerase was analysed by electrophoresis of a 10 µl aliquot on a 1.0% (wt/vol) agarose gel (a) and further confirmed by restriction digestion using *SphI* & *XbaI* (b)

(a) *Lane 1;* 100 bp DNA ladder. *Lane 2*: Undigested p35 PCR product. *Lane 3*: *HindIII* digested PCR product of p35 subunit (648 bp) and *Lane 4;* Purified p35 PCR product (648bp + 3 random bp + 6 bp × 2 *HindIII* site sequences = 663bp).

(b) *Lane 1;* 100 bp DNA ladder. *Lane 2*: *SphI* digested p35 fragments: 475 & 191 bp *Lane 3*: *XbaI* digested p35 fragments: 460 & 206 bp

Higher amounts of IL-12 subunits, p35 and p40 were detected in samples taken from spleens at 4 dpi.

a


1
2
3
1000 bp
500 bp
300 bp
200 bp
1023bp
1008bp

b

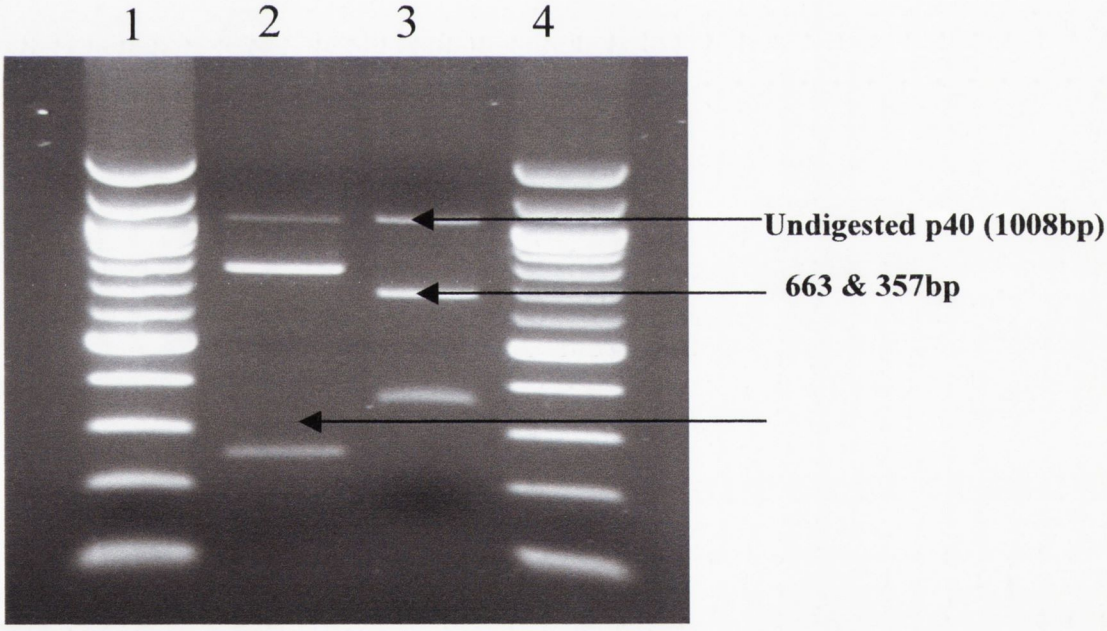

1
2
3
4
Undigested p40 (1008bp)
663 & 357bp

Figure 3.2 Gel analysis of PCR amplified p40 subunit

The PCR amplified p40 (1008bp) using pfu polymerase was analysed by electrophoresis of a 10 µl aliquot on a 1.0% (wt/vol) agarose gel (a) and further confirmed by restriction digestion using *DraI* and *XbaI* (b)

(a) *Lane 1;* 100 bp DNA ladder. *Lane 2*: *HindIII* digested PCR product of p40 subunit (1008 bp) and *Lane 3;* Purified p40 PCR product (1008bp + 3 random bp + 6 bp×2 *HindIII* site sequences = 1023 bp).

(b) *Lane 1;* 100 bp DNA ladder. *Lane 2*: *XbaI* digested p40 fragments: 766 & 256 bp (1023 bp). *Lane 3*: *DraI* digested p40 fragments: 663 & 357 bp

The PCR amplified p35 and p40 subunits of IL-12 were confirmed by restriction digestion and sequence analysis.

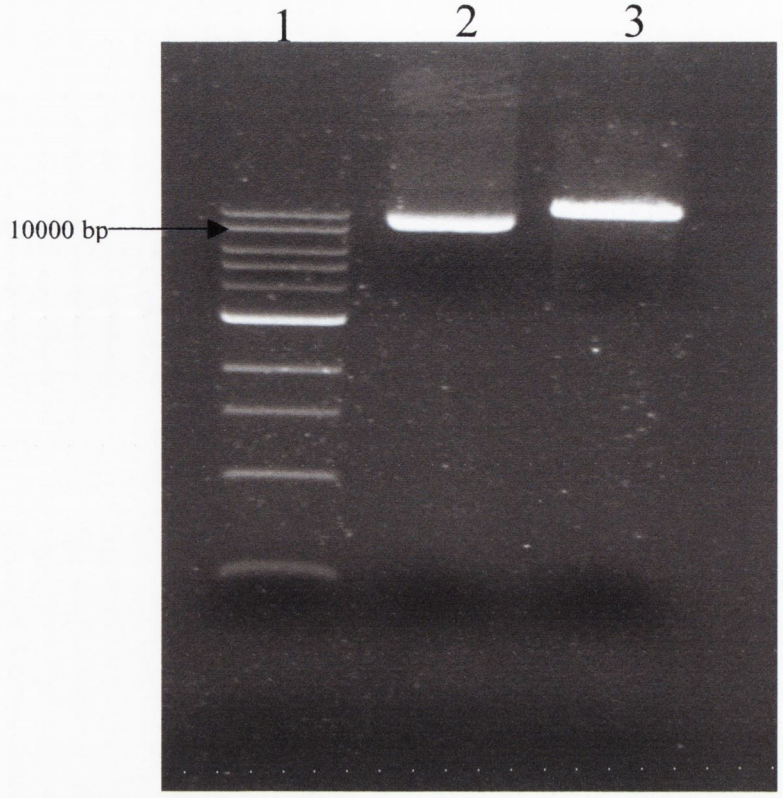
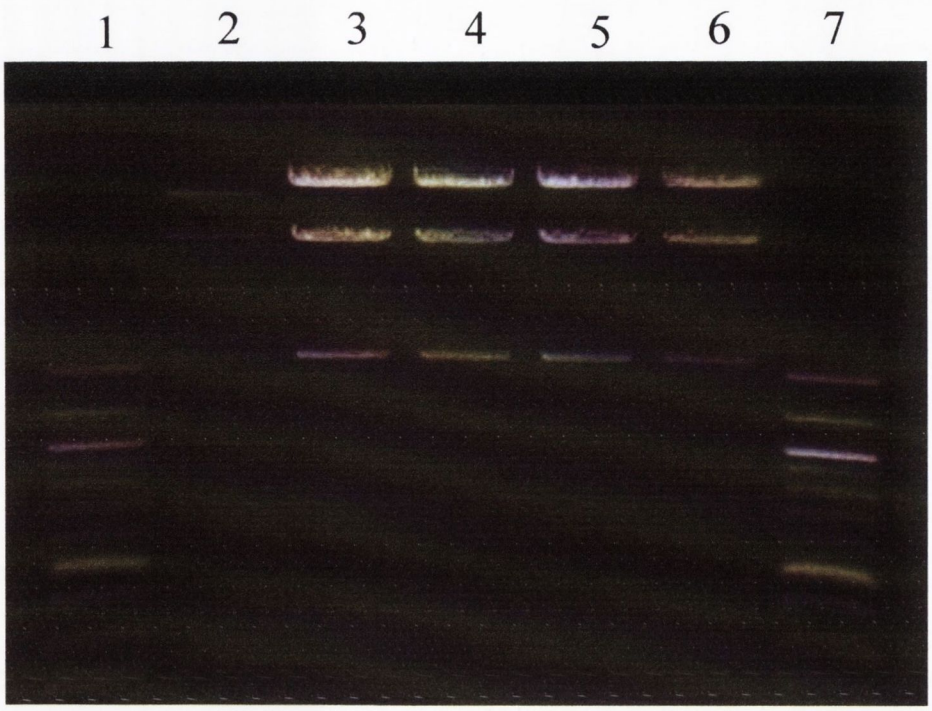


Figure 3.3 Purified pSFV10-E plasmids

The enhanced expression vector pSFV10-E plasmid was linearised for cloning using *HindIII* restriction enzyme and purified using the QIAGEN DNA elution kit as described in section 2.2.2.4. The amount of linearised plasmid needed for cloning was calculated based on band intensity. *Lane 1*; 1 μg of 1kbp DNA molecular weight marker. *Lane 2*; 1.0 μl of undigested pSFV10-E. *Lane 3*; 1.0 μl of *HindIII* digested (linearised) pSFV10-E.

0.8% (wt/vol) agarose gel, stained with ethidium bromide was used for the analysis.

a



b

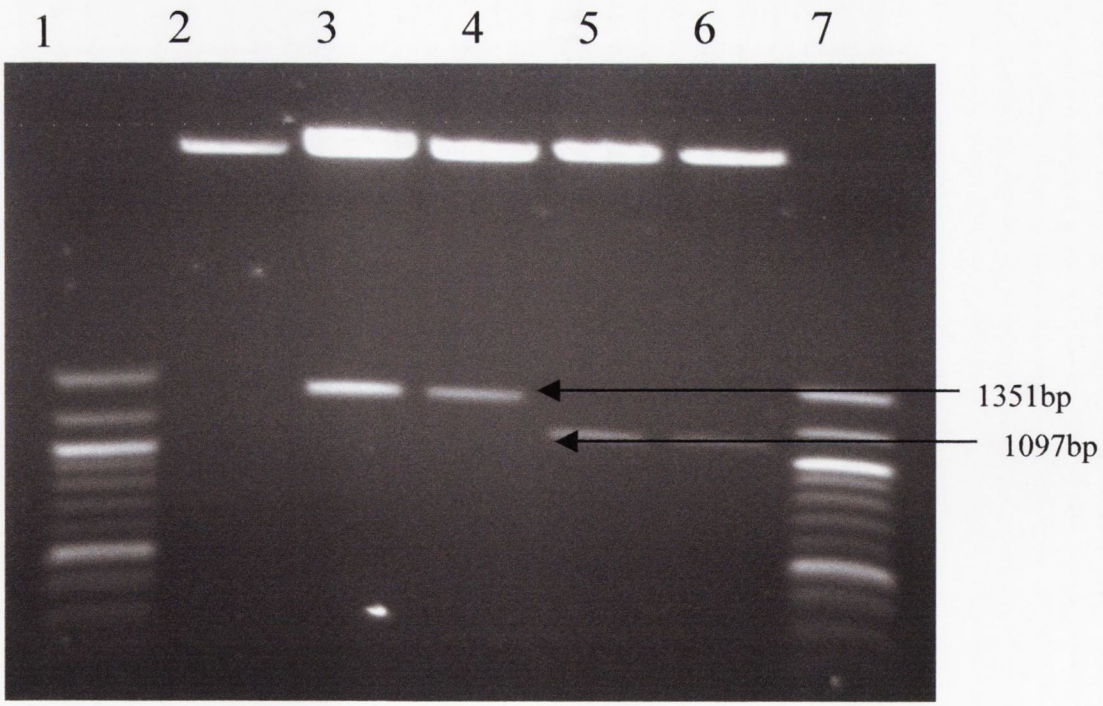


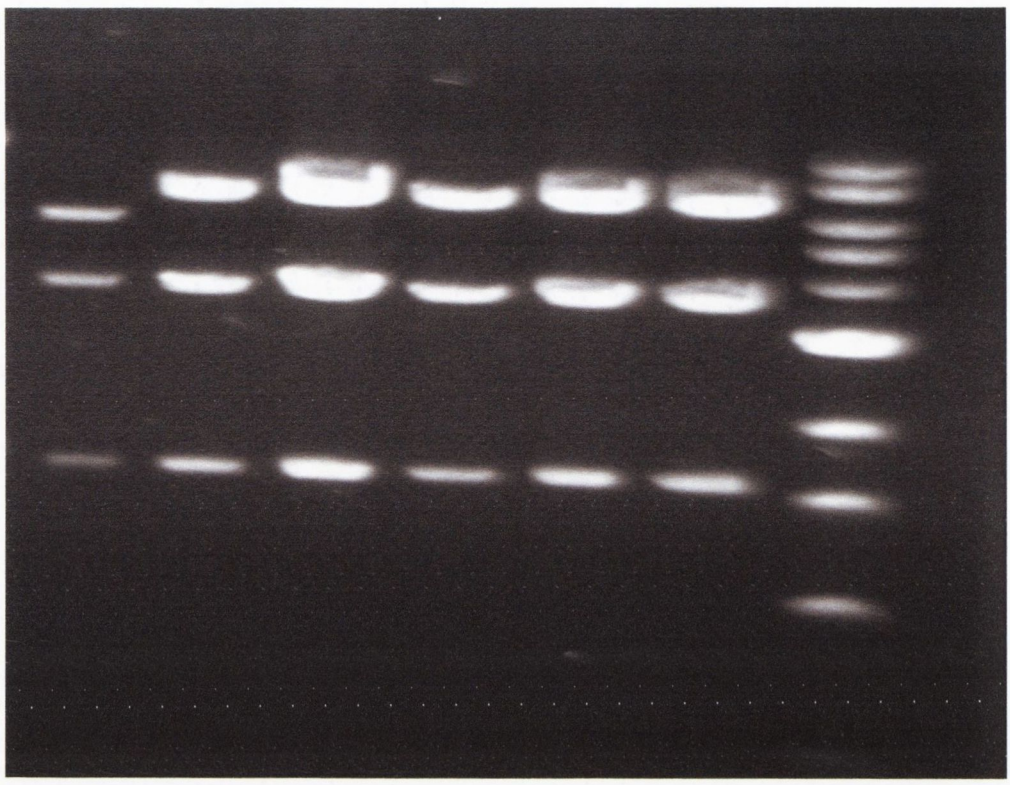
Figure 3.4 Screening for pSFV10-E-p35 clones by restriction analysis.

The presence and orientation of the insert p35 in pSFV10-E-p35 was analysed using restriction enzymes *EcoRI* and *XbaI*. 1.0 μg of pSFV10-E-p35 construct was digested using *EcoRI* and *XbaI* in separate reactions. 1.0 μl-digested product was analysed on 1% agarose gel and observed for specific size of fragments. Digested empty pSFV10-E vector was used as control DNA along with 100bp molecular weight DNA marker.

(a) *EcoRI* cut the vector pSFV10-E at 3 points yielding 3 fragments of size 1578bp, 3627 bp and 5552 bp = 10757 bp. The presence of insert p35 in the vector is digested into 3 fragments of size 1578bp, 3627bp & (5552+648 = 6200bp) = 11405bp.
Lane 1 and 7; 100 bp DNA ladder. *Lane 2*: *EcoRI* digested pSFV10-E (without insert). *Lane 3-6; EcoRI* digested pSFV10-E-p35 (four different clones with insert).

(b) A unique restriction site *XbaI* in the pSFV10-E vector (at 6639 bp) and p35 (at 451bp) were selected to analyse the orientation of the insert in pSFV10-E-p35. The difference between the *XbaI* site on the vector and the first nucleotide sequence of p35 in the construct was 900 bp. The presence and correct orientation of p35 insert in pSFV10-E-p35 after *XbaI* digestion should produce a fragment size of 1351 bp (900+451). Incorrectly orientated p35 in the construct should release a fragment of size 1097bp (900+(648-451=197 bp). Four positive clones were analysed to determine the orientation of p35.
Lane 1 and 7; 100 bp DNA ladder. *Lane 2*: *XbaI* digested pSFV10-E (without insert). *Lane 3-6; XbaI* digested pSFV10-E-p35 (with insert): the samples in the Lane 3 and 4 were confirmed as correctly orientated insert whereas lane 5 and 6 are incorrectly orientated insert in pSFV10-E-p35. Control empty vector sample digested using *XbaI* in the lane 2 confirmed *XbaI* as the unique site in the vector.

a



b

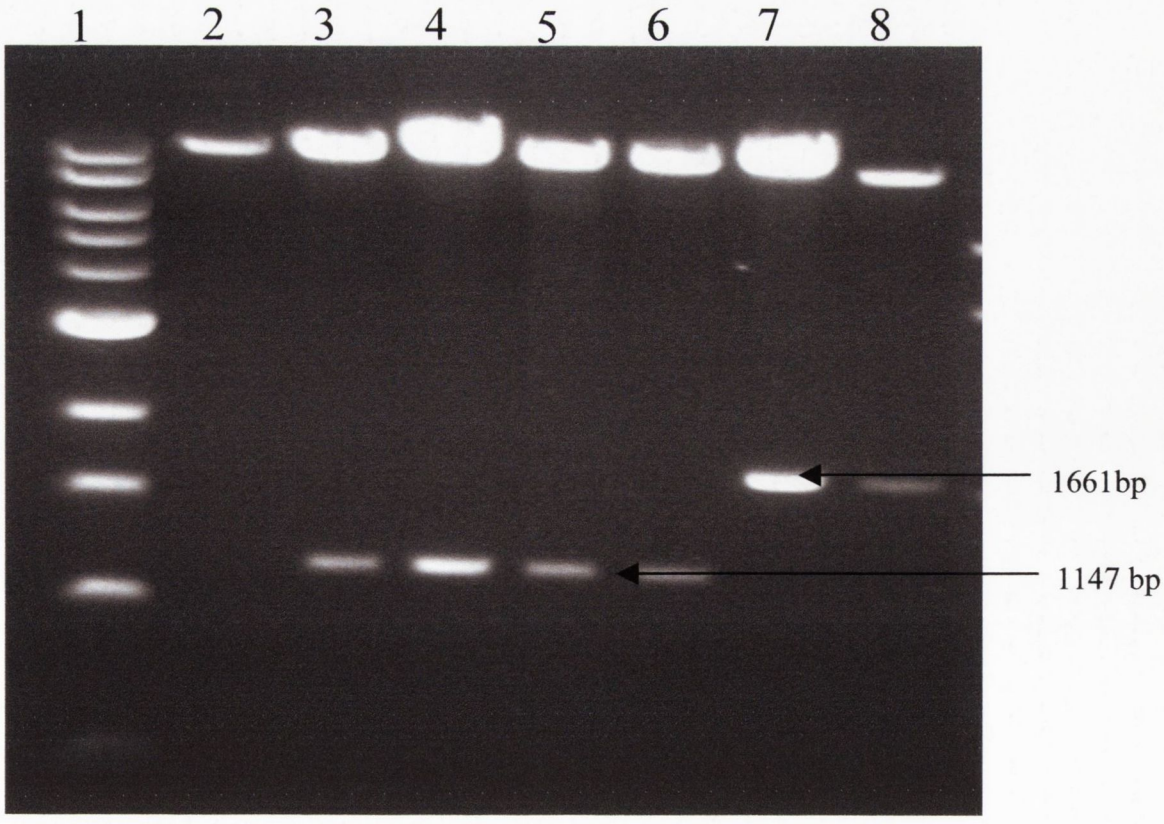


Figure 3.5 Screening for pSFV10-E-p40 clones by restriction analysis.

The presence and orientation of the insert p40 in pSFV10-E-p40 was analysed using restriction enzymes *EcoRI* and *XbaI*. 1.0 μg of pSFV10-E-p40 construct was digested using *EcoRI* and *XbaI* in separate reactions. 1.0 μl-digested product was analysed on 1% agarose gel and studied for specific size fragments. Digested empty pSFV10-E was used as control DNA along with 1kbp molecular weight DNA marker.

(a) *EcoRI* cut the vector pSFV10-E at 3 sites yielding 3 fragments of size 1578bp, 3627bp & 5552bp = 10757 bp. The vector with the insert p40 is digested into 3 fragments of sizes 1578 bp, 3627 bp and (5552+1008=6560 bp) =11765 bp.
Lane 1; EcoRI digested pSFV10-E (without insert). *Lane 2-6; EcoRI* digested pSFV10-E-p40 (five different clones with insert). *Lane 7;* 1 Kbp DNA ladder

(b) A unique restriction site *XbaI* on pSFV10-E (at 6639 bp) and p40 (at 247 bp) were selected to analyse the orientation of the insert in pSFV10-E-p40. A difference of 900 bp was found between the *XbaI* site on the vector and the first nucleotide sequence of p40 (*HindIII* site) in the construct. The presence and correct orientation of p40 in pSFV10-E-p40 after *XbaI* digestion should produce a fragment size of 1147 bp (900+247). Incorrectly orientated p40 in the construct should release a fragment of size 1661 bp (900+(1008-247 = 761). Six positive clones were analysed to determine the orientation of the insert.
Lane 1; 1 kbp DNA ladder. *Lane 2*: *XbaI* digested pSFV10-E (without insert). *Lane 3-8; XbaI* digested pSFV10-E-p40 (with insert): the samples in the Lane 3-6 were confirmed as the constructs with correctly orientated insert whereas in the lanes 7 and 8 the orientation of the insert was incorrect. Control empty vector sample digested with *XbaI* in lane 2 confirmed *XbaI* as the unique site on vector.

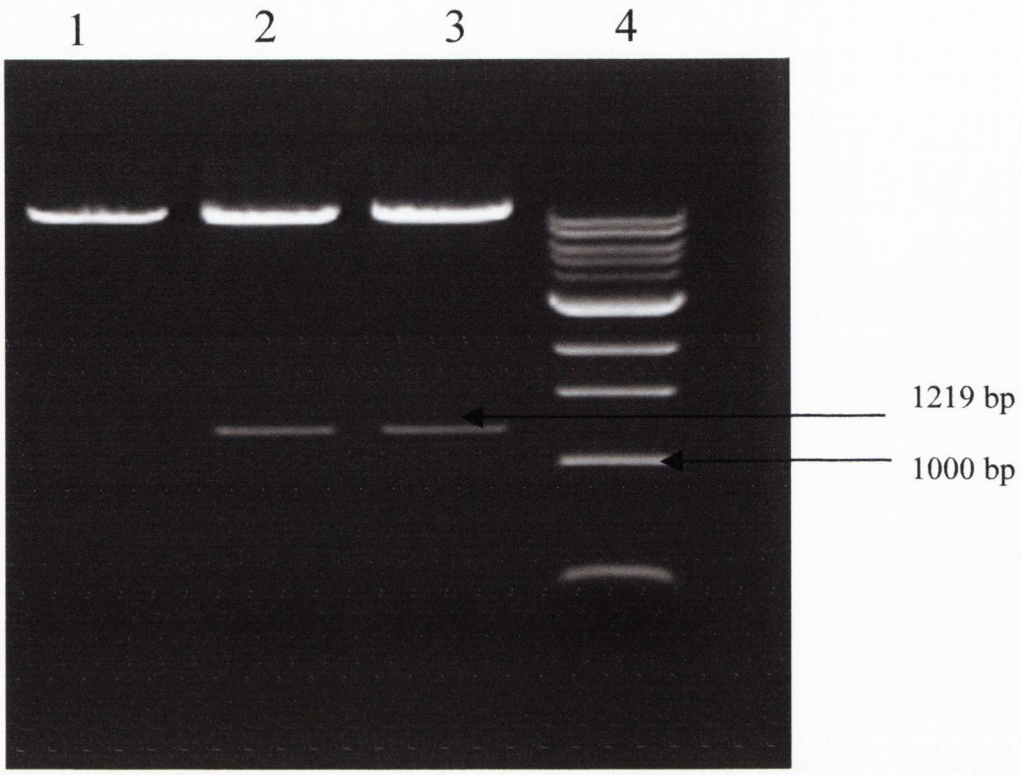


Figure 3.6 Screening for pSFV10-p35 clones by *XbaI* restriction digestion.

A unique restriction site *XbaI* in the pSFV10 (at 6639 bp) and p35 (at 451 bp) was selected to analyse the presence and orientation of the insert in pSFV10-p35. The difference between the *XbaI* site (at 6639 bp) on the vector and the first nucleotide sequence of p35 (*BamHI* site at 7401 bp) in the construct was 762 bp. The presence and correct orientation of p35 insert in pSFV10-p35 after *XbaI* digestion should produce a fragment of size 1219 bp (762+451+6 (*HindIII*-site). Incorrectly orientated p35 in the construct should release a fragment of size 965 bp (762+203). Two positive clones were analysed to determine the orientation of p35 insert in the construct.

Lane 1; XbaI digested pSFV10 (without insert). *Lanes 2 and 3*: *XbaI* digested pSFV10-p35 (positive clones). *Lane 4;* 1 kbp DNA ladder: the samples in the Lanes 2 and 3 were confirmed as the constructs with correctly orientated insert p35. Control empty vector sample digested using *XbaI* in the lane 1 confirmed *XbaI* as the unique site in the vector. Two positive clones of pSFV10-p35 had correctly orientated insert.

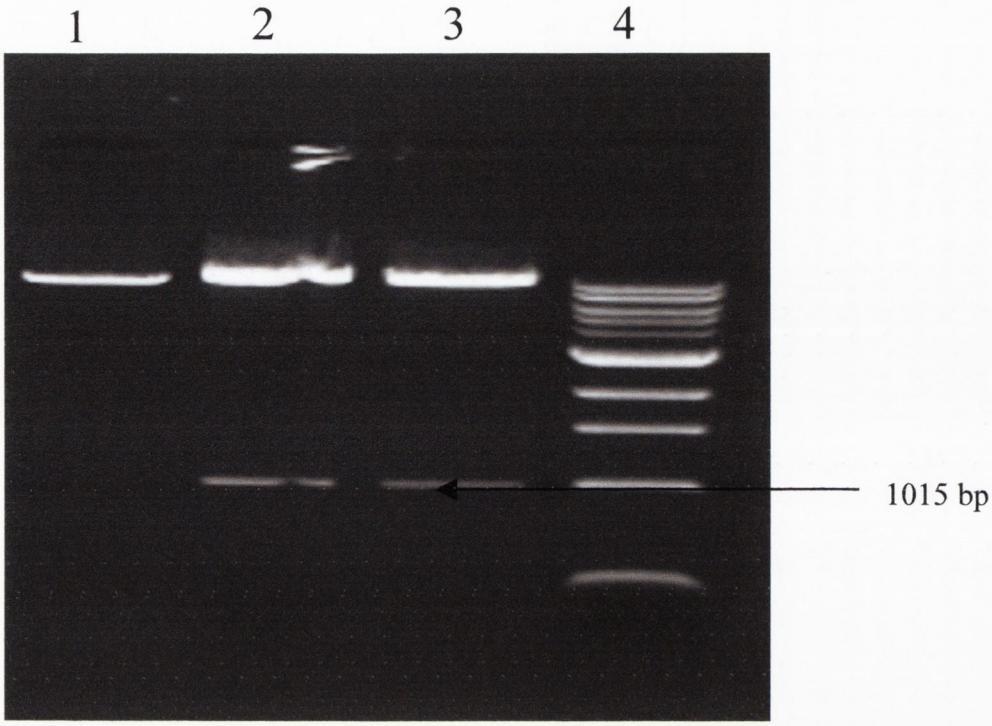


Figure 3.7 Screening for pSFV10-p40 clones by *XbaI* restriction digestion.

A unique restriction site, *XbaI* in the pSFV10 (at 6639bp) and p40 (at 247bp) was selected to analyse the presence and orientation of the insert in pSFV10-p40. A difference between the *XbaI* site (at 6639 bp) on the vector and the first nucleotide sequence of p40 (*BamHI* site at 7401 bp) in the construct was 762bp. The presence and correct orientation of p40 insert in pSFV10-p40 after *XbaI* digestion should produce a fragment of size of 1015 bp (762+247+6 (*HindIII*-site). Incorrectly orientated p40 in the construct should release a fragment of size 1530 bp (762+761+6). Two positive clones were analysed to determine the orientation of the p40 insert.

Lane 1; XbaI digested pSFV10 (without insert). *Lanes 2 and 3*: *XbaI* digested pSFV10-p40 (positive clones). *Lane 4;* 1 Kbp DNA ladder: the samples in Lane 2 and 3 had correctly orientated insert in pSFV10-p40.

The position of *Xba*I on p40 in pSFV10-E-p70 is at 7790 bp

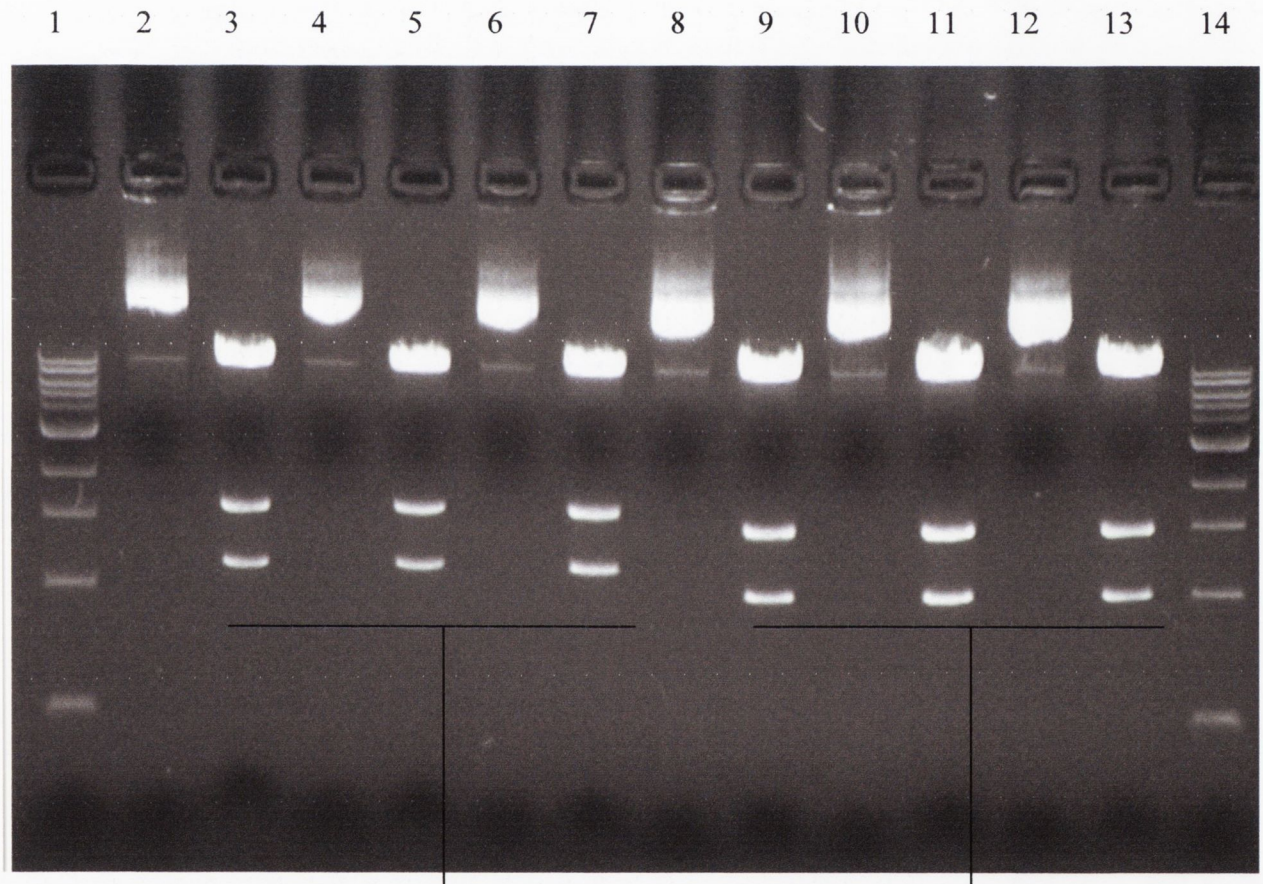
The position of *Xba*I on p35 in pSFV10-E-p70 is at 9432 bp

The position of *Xba*I on pSFV10-E in pSFV10-E-p70 is at 6639 bp

The position of *Xba*I on p40 in pSFV10-p70 is at 7656 bp

The position of *Xba*I on p35 in pSFV10-E-p70 is at 9159 bp

The position of *Xba*I on pSFV10-E in pSFV10-E-p70 is at 6639 bp



10056bp
1642bp
1151bp

Total size of pSFV10-E-p70	**12849 bp**

10051bp
1503bp
1017bp

Total size of pSFV10-p70	**12571 bp**

Figure 3.8 Restriction analysis pSFV10-E-IL 12 and pSFV10-IL12 constructs

pSFV10-E-p40 and pSFV10-p40 constructs were linearised using *SmaI* restriction enzyme and dephosphorylated using Shrimp Alkaline phosphatase. p35 fragment along with 26S sub-genomic promoter from pSFV10-E-p35 and pSFV10-p35 were digested using *PvuII* and *SmaI* restriction enzymes (generates blunt ends). The 26S-p35 fragment of pSFV10-E- p35 was ligated into linearised pSFV10-E- p40 and 26S-p35 fragment of pSFV10-p35 was ligated into linearised pSFV10-p40 vector using Quick T_4 DNA Ligase to obtain pSFV10-E-p70 and pSFV10-p70.

Positive clones of pSFV10-E-p70 and pSFV10-p70 were analysed by *XbaI* digestion to confirm the presence and orientation of 26S-p35 fragments in pSFV10-E- p40 and pSFV10-p40 constructs. The presence and correct orientation of 26S-p35 insert in pSFV10-E-p40 and pSFV10-p40 was analysed using *XbaI* digestion.

Lane 1 and *14;* 1 kbp DNA ladder. *Lanes 2, 4 and 6*: Undigested pSFV10-E-p70. *Lanes 3, 5 and 7; XbaI* digested pSFV10-E-p70 clones. *Lanes 8, 10 and 12*; Undigested pSFV10-p70. *Lanes 9, 11 and 13*; *XbaI* digested pSFV10-p70 clones. Three positive clone samples for each of the IL-12 constructs were analysed to determine the correct orientation of p35 and p40 inserts (data not shown).

Blast 2 Sequences results

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.4 [Aug-26-2002]

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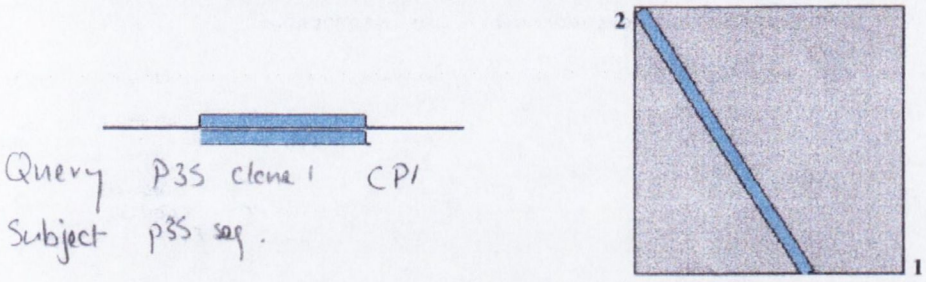

NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

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Sbjct: 590 gtgaagcaggatgcagagcttcattttcactctgtaagggtctgcttctcccacaggagg 531

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Sbjct: 50  taggagggcaagggtggccaaaaagaggaggtagcgtgattgacccat 3

CPU time:     0.06 user secs.          0.03 sys. secs          0.09 total secs.

Lambda      K       H
    1.33     0.621      1.12

Gapped
Lambda      K       H
    1.33     0.621      1.12
```

Figure 3.9 p35 subunit of IL-12 in pSFV10-E-p35 sequence analyses

Obtained p35 sequence from pSFV10E-p35 (using insert primers) was blasted against the original mouse p35 sequence of IL-12 from the GenBank database. A complete homology of sequence was found with an expected value of 0.0, indicating a perfect match with correct orientation in the vector. This was also confirmed by restriction analysis.

Blast 2 Sequences results

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.4 [Aug-26-2002]

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x_dropoff: 50 expect: 10.000 wordsize: 11 Filter ☑ Align

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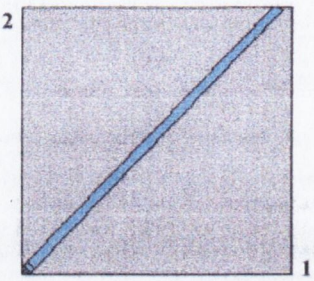

NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

NOTE: If protein translation is reversed, please repeat the search with reverse strand of the query sequence

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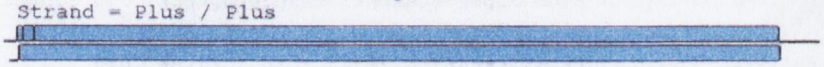

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Figure 3.10 p40 subunit of IL-12 in pSFV10-E-p40 sequence analyses

Obtained p40 sequence from pSFV10E-p40 (using insert primers) was blasted against the original mouse p40 sequence of IL-12 from the GenBank database. A complete homology of sequence was found with an expected value of 0.0, indicating a perfect match with correct orientation in the vector. This was also confirmed by restriction analysis.

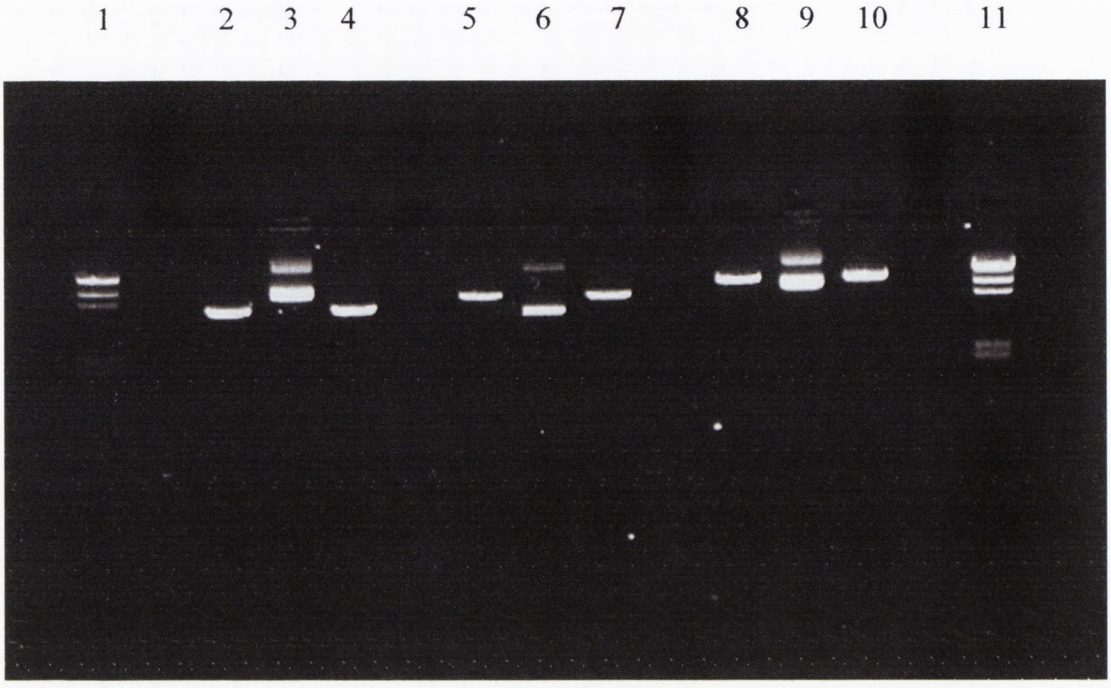


Figure 3.11 Linearised recombinant IL-12 and helper SFV plasmids

Helper and recombinant (rSFV-IL12) plasmids were Linearised prior to transcription using the unique restriction site SpeI found on helper SFV plasmids and NruI on recombinant SFV-IL12 plasmids.. Linearised plasmid concentrations were calculated using the band intensity from the gel used for analysis and were calculated the amount of DNA needed for transcription. *Lane 1 and 11*; 0.125 µg and 0.25 µg Lambda (*HindIII*) DNA molecular weight marker. *Lane 2, 4 and 3*; 0.5 µl, 1.0 µl of digested pSFV- Helper CS219A DNA and undigested control, respectively. *Lane 5, 7 and 6*; 0.5 µl, 1.0 µl of digested pSFV- Helper S2 DNA and undigested control, respectively. *Lane 8, 10 and 9*; 0.5 µl, 1.0 µl of digested pSFV10-E-mIL12 vector DNA and undigested control, respectively. 0.8% (wt/vol) agarose gel, stained with ethidium bromide.

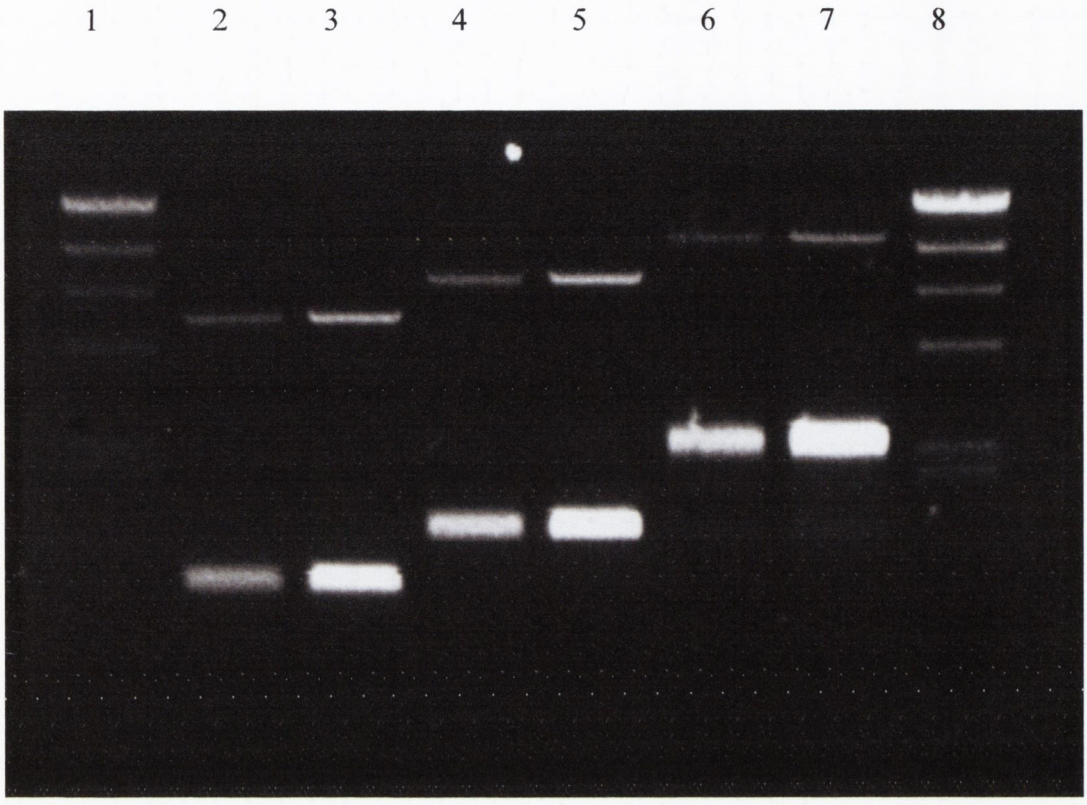
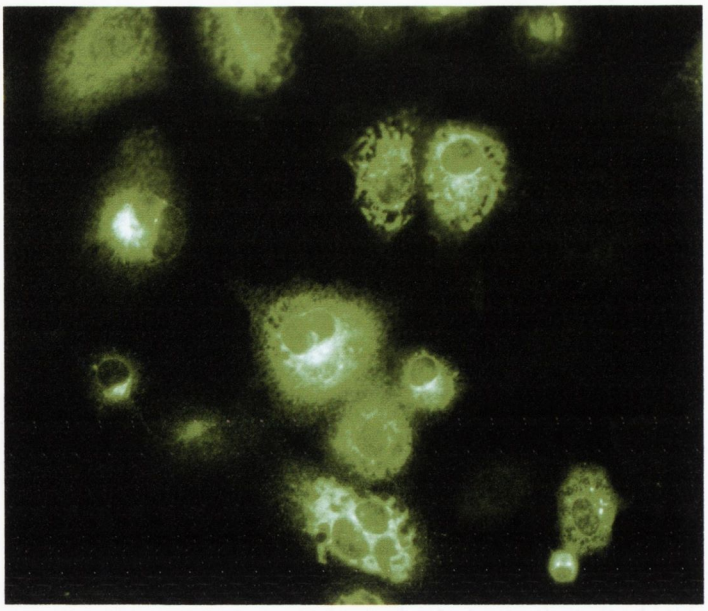


Figure 3.12 ***In vitro*** **SP6 RNA transcription**

Helper and recombinant (rSFV10-E-IL12) plasmids were *In vitro* transcribed and analysed on a 0.8% agarose gel. *Lanes 1 and 8*; 0.125 µg and 0.25 µg Lambda (*HindIII*) DNA molecular weight marker. *Lanes 2 and 3*; 0.5 µl and 1.0 µl each of rSFV-Helper CS219A RNA. Lanes *4 and 5*; 0.5 µl and 1.0 µl each of rSFV-Helper S2 RNA. *Lane 6 and 7*; 0.5 µl and 1.0 µl each of rSFV10-E-IL12 RNA. 0.8% agarose gel, stained with ethidium bromide was used for the analysis. The concentration of the *In vitro* transcribed RNAs was approximately estimated by considering the staining intensity of the RNA bands.

a



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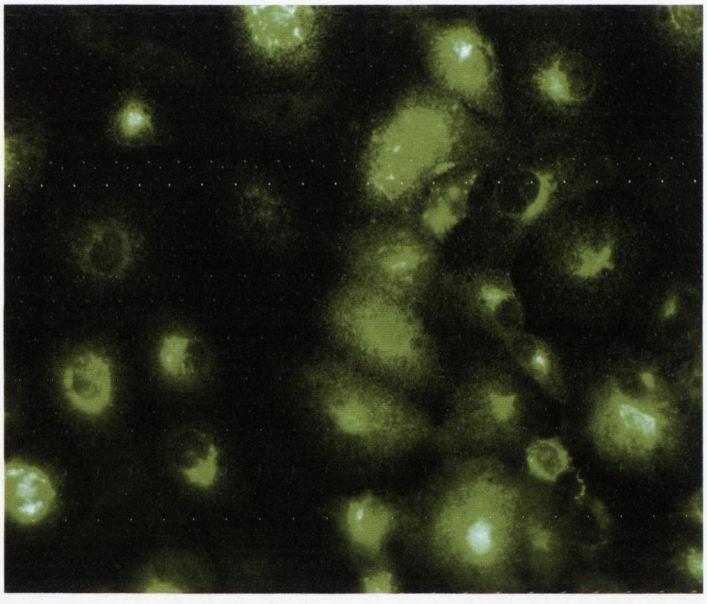
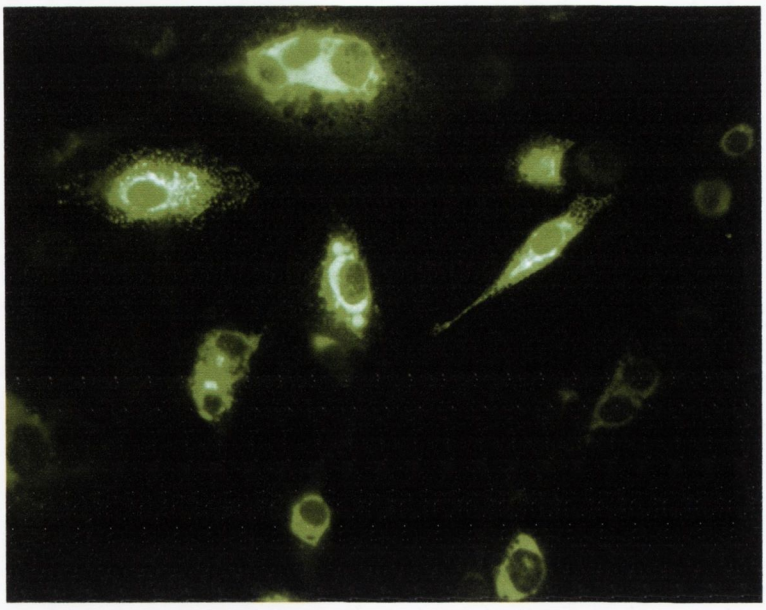


Fig 3.13 Qualitative analysis of IL-12 expression levels in Swedish BHK cells by Immunofluorescence.

The difference in expression levels of IL-12 between the two constructs was qualitatively examined by immunofluorscence (IF) staining of sBHK cells infected by VLPs of rSFV10-E-IL12 and rSFV10-IL12. The difference in expression levels between the mIL-12 constructs was compared by the intensities of IF staining under constant visual parameters. The cells infected with rSFV10-E-IL12 VLPs (a) were stained brightly due to high level expression compared to the rSFV10-IL12 (b) infected cells. IF was absent in mock infected cells (not shown). Original magnification 200X.

a



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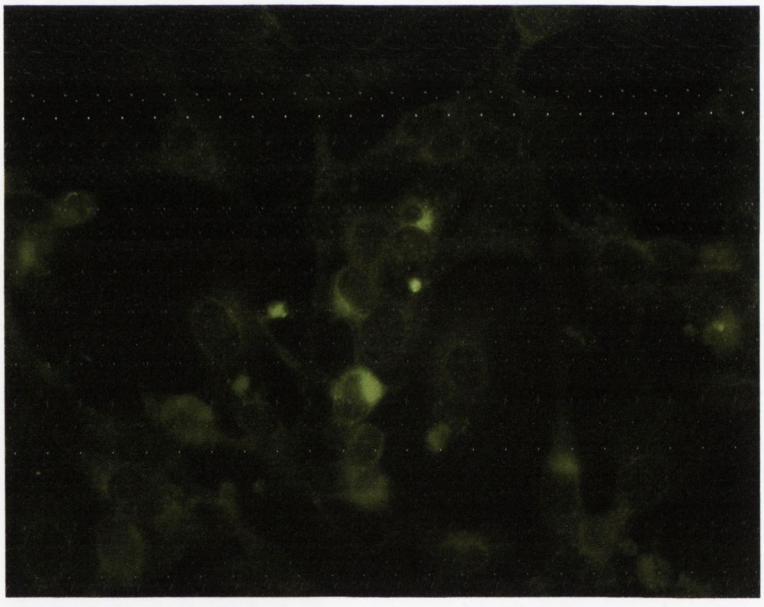
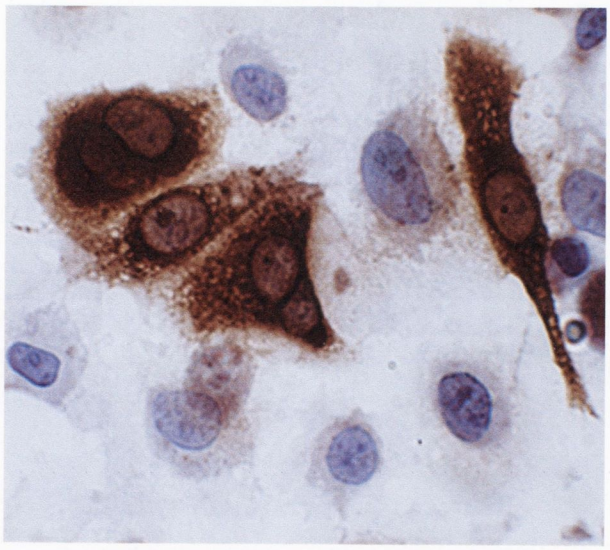


Fig 3.14 Qualitative analysis of IL-12 expression levels in K-BALB cells by Immuno-fluorescence.

The difference in expression levels of mIL-12 between the two mIL-12 constructs was further examined by immunofluorscence-staining of rSFV-IL12 VLPs infected K-BALB cells. The difference in expression levels was compared by the intensities of IF staining under constant visual parameters. The cells infected with rSFV10-E-IL12 VLPs (a) were stained brightly due to high level expression **compared to the** rSFV10-IL12 (b) infected cells. IF was absent in mock infected cells (not shown). Original magnification x200.

a



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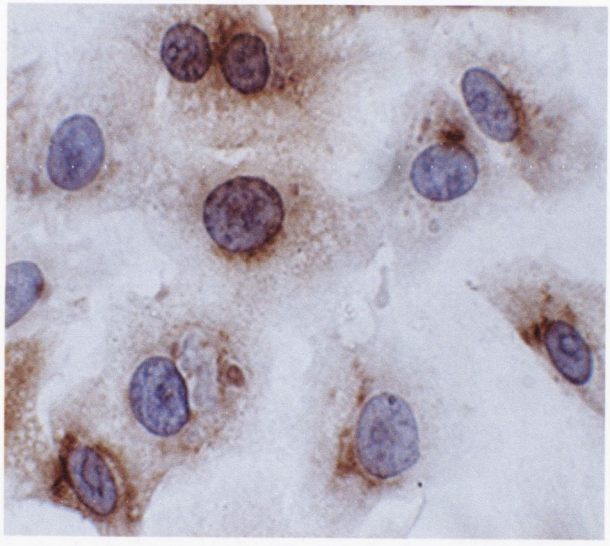


c

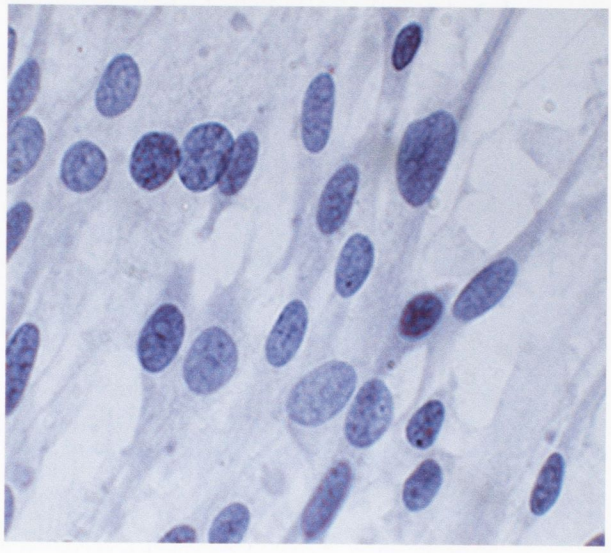


Figure 3.15 Immunocytochemical staining for IL-12.

The difference in expression levels of mIL-12 between the two constructs was further examined by immunocytochemical staining of BHK-21 cells infected by VLPs of rSFV10-E-IL-12 and rSFV10-IL-12. The infected cells were stained at 24h post infection using di-amino benzidine (DAB) solution, and Harris' Haematoxylin stain. The stained cells were examined using bright field microscopy at a magnification x200. The expression levels were compared by the intensities of DAB staining (brown). The expression of higher levels of IL-12 in the positive cells infected with rSFV10-E-IL12 (a) was found compared to those cells infected with rSFV10-IL12 (b) where as no expression was observed in the mock-infected cells (c).

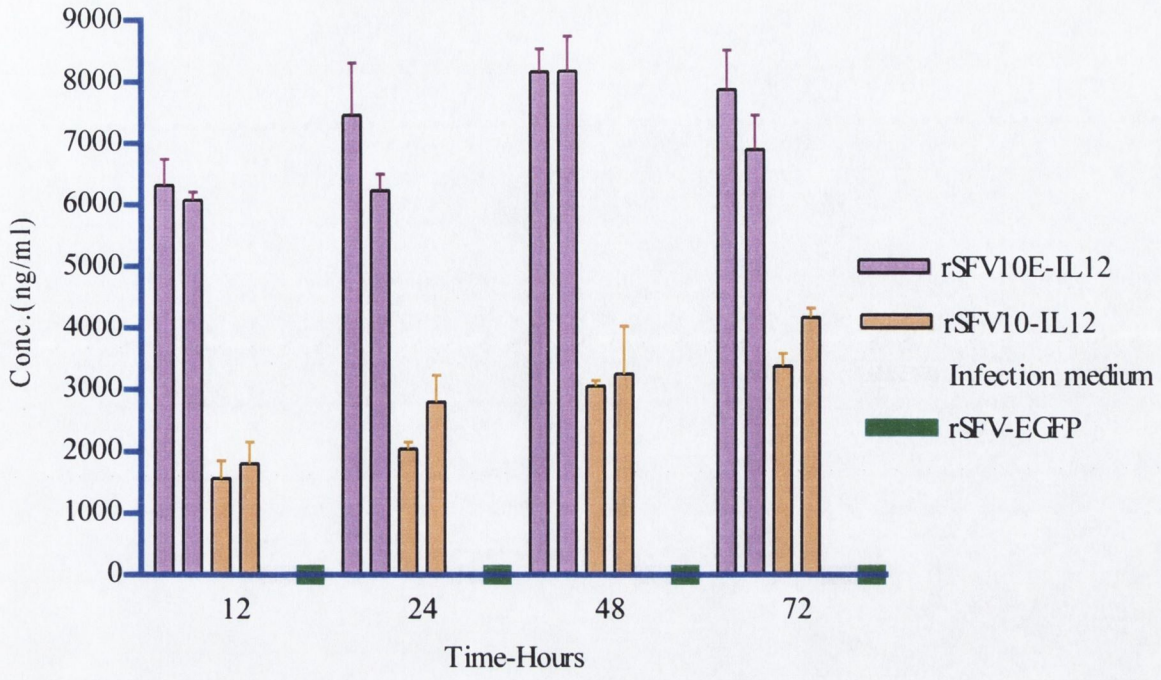


Figure 3.16 Quantitative analysis of IL-12 expression levels in the supernatants of rSFV-mIL-12 VLPs infected BHK-21 cells

The difference in expression levels of murine IL-12 by pSFV10-E-IL12 and pSFV10-IL12 was examined in BHK-21 and K-BALB cells. An ELISA measured the difference in expression levels at different time points in supernatants of VLPs infected BHK-21 cells at MOI of 10. The levels of secreted IL-12 in the supernatants of infected BHK-21 cells by rSFV10-E-IL12 were 3-4 times higher than by rSFV10-IL12. The supernatant from mock-infected control BHK-21 cells showed IL-12 expression. The values in the graph represent IL-12 secretion from 5x105 cells. Two different positive clones of each construct were used for the in vitro expression assays.

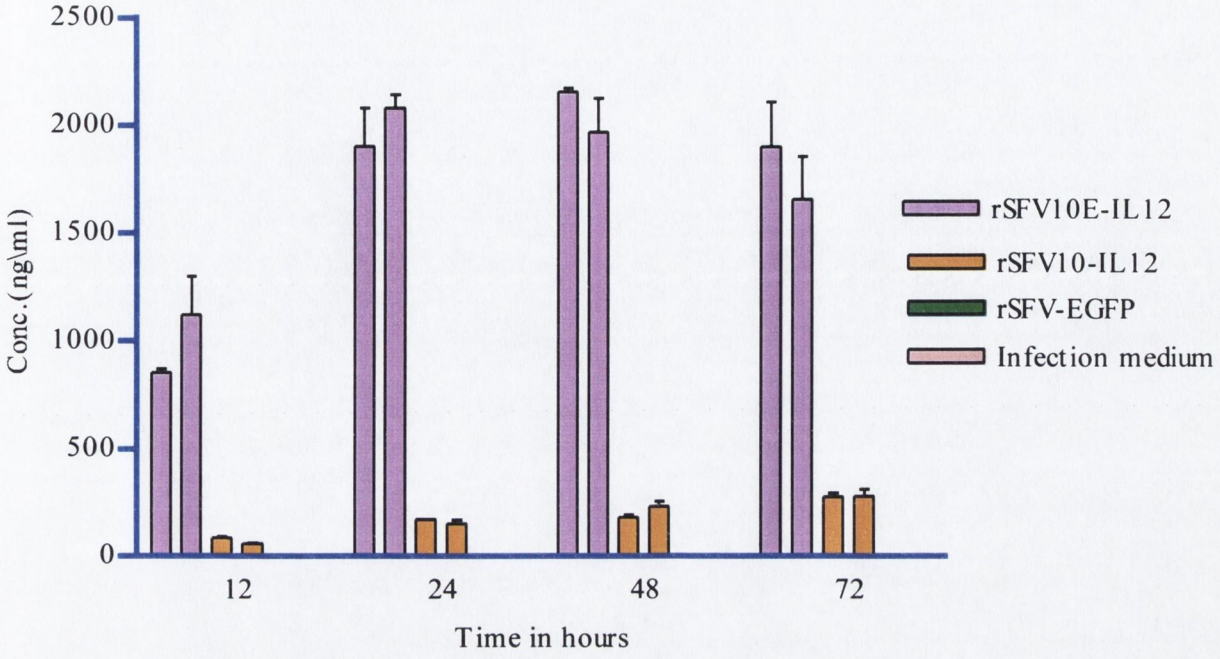
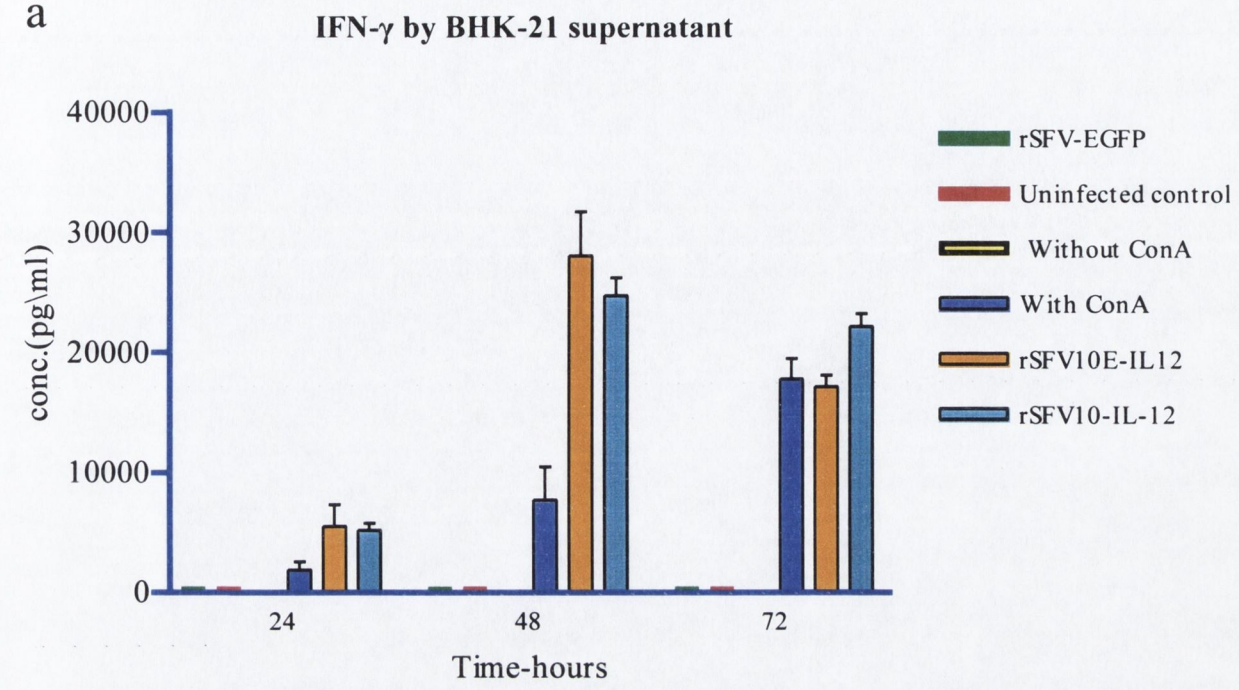


Figure 3.17 Comparative analysis of expression and secretion levels of IL-12 in K-BALB cells

K-BALB cells were infected with VLPs of rSFV10-E-IL12 and rSFV10-IL12 at MOI of 10. ELISA quantified the levels of secreted IL-12 from the supernatants of infected K-BALB cells. Two different positive clones of each construct were used for the in vitro expression assay. The expressed levels of IL-12 in K-BALB cells infected with rSFV10-E-IL12 were ten-times higher than rSFV10-IL12 at any given point of time. The supernatant from rSFV-EGFP or mock-infected control K-BALB cells had no IL-12 expression. The values shown in the graph represent IL-12 secretion from $5x10^5$ cells.

a


IFN-γ by BHK-21 supernatant
40000
30000
20000
10000
0
conc.(pg\ml)
24
48
72
Time-hours
rSFV-EGFP
Uninfected control
Without ConA
With ConA
rSFV10E-IL12
rSFV10-IL-12

b

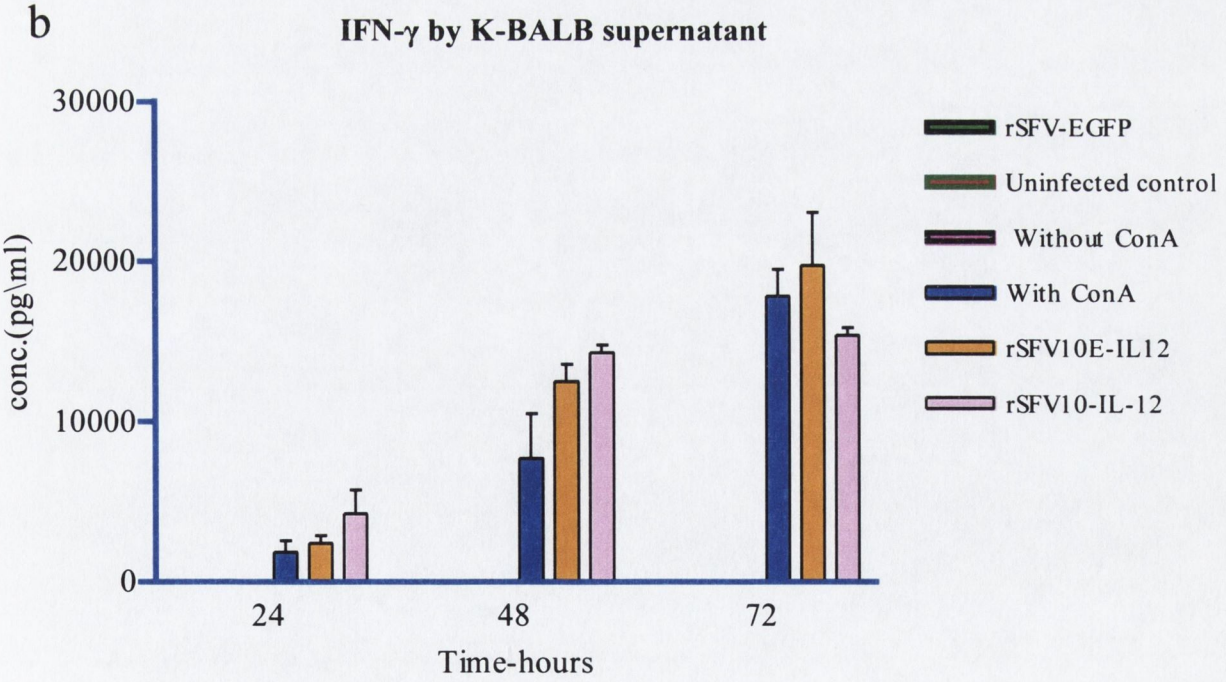

IFN-γ by K-BALB supernatant
30000
20000
10000
0
conc.(pg\ml)
24
48
72
Time-hours
rSFV-EGFP
Uninfected control
Without ConA
With ConA
rSFV10E-IL12
rSFV10-IL-12

Figure 3.18 Testing biological activity of secreted murine IL-12

Biologically active IL-12 induces T-cells to secrete interferon-γ (IFN-γ). An ELISA measured the production of IFN-γ from murine splenocytes (1x106) treated with 100μl of 24h naive IL-12 supernatants of VLP infected BHK-21 cells (a) or K-BALB cells (b). This showed that supernatants of rSFV-IL12 infected BHK-21 and K-BALB cells, containing IL-12, stimulated splenocytes to produce similar levels of IFN-γ as positive control Con A treatment. These results confirmed the expression and secretion of biologically active IL-12 from both pSFV10-E-IL12 and pSFV10-IL12 vectors. Supernatants from control groups (rSFV-EGFP, uninfected, and without conA) were unable to induce splenocytes to produce IFN-γ.

3.3 DISCUSSION

The use of immuno-stimulatory cytokines such as IL-2, IL-4, IL-12, IL-18 or IL-21 as tumour therapeutic agents has become a promising strategy for cancer therapy. Strategies that provide high levels of immunostimulatory cytokines locally at the site of the tumour have already demonstrated therapeutic efficacy in different tumour models.

Interleukin-12, a multifunctional cytokine is a heterodimer composed of two subunits, p35, and p40, encoded by separate genes. The expression of biologically active IL-12 requires co-ordinated expression of both subunits in equal proportion. If the two subunits are expressed at different levels, biologically inactive p40 homodimers are produced. Murine p40 homodimers bind to the IL-12Rβ1 receptor-chain with an affinity similar to that of the heterodimers thus effectively blocking the function of IL-12. Dendritic cells and other antigen presenting immune cells that are normally the first to encounter and react to foreign, disease-causing agents produce IL-12. IL-12 serves as the bridge between the natural or innate immune response and the adaptive immune response mediated by T-cells. Previous studies have demonstrated that IL-12 promotes the activity of T cells and NK cells that can directly attack cancer cells. This cytokine induces the production of interferon-γ, another biological response modifier, which augments the antitumour response through anti angiogenic effects (Shibuya *et al.*, 2003).

Successful gene therapy mainly depends on properties of gene delivery systems such as its antigenicity, bio-safety and its ability to express a functionally active product. The Semliki Forest virus vector is a transient suicidal expression vector system with low immunogenicity. The bio-safety of SFV has been improved by modifying the original vector into a split vector system, which allows the vector to undergo only one round of replication in the host cells (Smerdou *et al.*, 1999). This vector has been previously used in vaccine studies to induce protection against louping-ill virus (Fleeton *et al.*, 2000) and in the expression of many other proteins including IL-12 (Asselin-Paturel *et al.*, 1999). The development of pSFV10-E, a new SFV based expression vector that has the ability to express foreign protein up to ten-fold higher than the original pSFV10 has provided a valuable tool to examine the anti-tumour effects of IL-12 expression locally at the tumour site. Since a previous study has demonstrated that the amount of IL-12 available at the tumour site is critical for

the tumour regression, availability of pSFV10-E has enabled us to enhance IL-12 expression locally at the tumour site.

Hence, we constructed pSFV10-E-IL12 and pSFV10-IL12, and compared the expression of IL-12 in standard BHK-21 cells and the murine tumour K-BALB cell line. To avoid the possible antagonism of p40 homodimers against the biologically functional heterodimeric IL-12 molecule, we cloned the component p40 and p35 subunits into a single vector, each expressed from separate *26S* promoters. The difference in the expression levels among the IL-12 constructs was qualitatively confirmed by immunofluorescence and cytochemical staining of infected BHK-21 and K-BALB cells. To examine the ability of rSFV10-E to express and secrete IL-12, the supernatant of infected cells was analysed by an IL-12 ELISA test. These measurements also confirmed the higher expression level from the rSFV10-E vector compared to rSFV10. The difference in the expression levels of IL-12 in BHK-21 and K-BALB cells using a constant MOI of VLPs may reflect the variability in infection and expression with different cell lines.

Since the mIL-12 (p70) specific antibodies used in the assay also recognise the p40 homodimers, it was essential to test the biological function of the secreted IL-12. To confirm the biological activity, we measured the ability of supernatants from rSFV-IL12 infected cells to induce IFN-γ production by mouse splenocytes that were cultured in the presence of supernatants. An ELISA test using specific IFN-γ antibodies estimated the amount of IFN-γ produced by the splenocytes. IFN-γ levels in the supernatants of splenocytes induced by rSFV10-E-IL12 and rSFV10-12 were similar. This may be due to saturated levels of IL-12 in the supernatants produced by a limited number of splenocytes. As this assay was only to test the biological functionality of the secreted IL-12 by rSFV-IL12 constructs, the amount of secreted IFN-γ was not considered to comparatively characterise the rSFV-IL12 constructs.

4

TREATMENT OF K-BALB AND CT26 TUMOURS WITH rSFV-IL12 VLPs

4.1 INTRODUCTION

The use of immunostimulatory molecules has proven efficient at enhancing anti-tumour immune response through the modulation of the tumour microenvironment and activation of tumour lytic cells. Interleukin-12 is an important immunoregulatory cytokine, which enhances the function of cytotxic immune cells including CTL and NK cells, and possesses potent IFN-γ dependent therapeutic activity (Wigginton *et al.*, 2001). IL-12, secreted mainly by macrophages, monocytes and dendritic cells (Macatonia *et al.*, 1995), activates CTLs and NK cells (Kobayashi *et al.*, 1989) for IFN-γ secretion and promotes Th1 differentiation (Manetti *et al.*, 1994) that contributes to tumour cell killing. The anti-tumour effect of IL-12 has been predicted to be more efficient compared with other tested cytokine genes such as IL-2, IL-4, IL-6, IFN-γ, TNF-α or GM-CSF (Boulikas *et al.*, 1998, Colombo *et al.*, 2002). In a recent study, mice bearing GL-26 gliomas were treated with direct intratumoural administration of adenovirus carrying the IL-12 gene. Survival was significantly prolonged in IL-12 treated animals, and immunohistochemistry demonstrated increased CD4+ and CD8+ T cell infiltration of the tumour compared to the controls (Liu Y *et al.*, 2002).

The efficiency of alphavirus replication has been exploited for the development of expression vectors based on Semliki forest, Sindbis and VEE (Smerdou *et al.*, 1999). SFV is RNA virus from the alphavirus family, which is non-pathogenic to humans. SFV vectors have already been successfully used in cancer gene therapy applications as well as for vaccine productions (Lundstrom., 2000). 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). Expression of the P1A gene from recombinant SFV vectors resulted in induction of tumour immunity (Colmenero *et al.*, 1999). The transfer of the IL-12 gene using the SFV vector has induced tumour (B16) regression and also inhibited insitu neovascularisation within the tumour without affecting the resistance index of pre-existing intratumoural blood flows (Asselin-Paturel *et al.*, 1999). A phase I/II

clinical study in adult human patients with recurrent glioblastoma multiforme (GBM) is planned which is aimed at evaluating biological safety, maximum tolerated dose and antitumour efficacy of genetically modified replication-disabled SFV carrying the human IL-12 gene encapsulated in cationic liposomes (Ren *et al.*, 2003).

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 the enhanced expression of IL-12 using a recently developed SFV vector with enhanced expression ability. A previous study has assessed the anti-SFV humoural and cell-mediated immune responses by ELISA and *ex vivo* splenocyte stimulation assays respectively (Smyth *et al.*, 2004). Hence, in this study we concentrated on characterizing the recently developed pSFV10-E vector for the expression of IL-12 and its ability to induce anti-tumour immunity in comparison to the original SFV10- IL12. In order to examine the consistency of the *in vivo* treatment effect of rSFV10-E-IL12, three different tumour cell lines (K-BALB, CT26 and 4T1) have been used in immunocompetent female BALB/c mice. Treatment of K-BALB and CT26 tumours with rSFV10-E-IL12 at a titre of $4x10^9$ IU/dose resulted in complete tumour regression, which was significantly enhanced in comparison to the original rSFV10-IL12 VLPs.

In this study, H&E staining of paraffin-embedded formalin fixed and immunohistochemistry of frozen tumour sections were used for the routine examination of tumour tissue and strong anti-tumour immune responses were apparent in IL-12 treated tumours. The results presented here clearly demonstrate and highlight the future potential of rSFV10-E-IL12 for cancer gene therapeutical applications.

4.2 RESULTS

4.2.1 Intra-tumoural treatment of K-BALB and CT26 tumours using rSFV replicons expressing murine IL-12

Initially, experiments were performed to optimise the amount IL-12 available at the tumour site by varying the titres of VLPs to get the maximum treatment effect used for treatment per dose. K-BALB tumours were induced subcutaneously in 4-6 weeks old immunocompetent female BALB/c mice. An average tumour diameter of 4 mm was reached 4 days post induction of K-BALB or CT26 tumours and the treatment was started at this point. The K-BALB tumours were treated with different titres of rSFV10-E-IL12 VLPs in ascending order ($4x10^7$ IU/ dose, $4x10^8$ IU/ dose, and $4x10^9$ IU in 50 µl / dose). The treatment of the CT26 tumour was carried out using rSFV10-E-IL12 VLPs at the titre that induced maximum anti-tumour effect on the K-BALB tumour (i.e., $4x10^9$ IU in 50 µl / dose). A total of six intratumoural injections on every alternate day were given.

In these experiments, when the K-BALB tumours were treated with $4x10^7$ IU / dose of rSFV10-E-IL12, one mouse at day 19 had complete tumour regression and the average tumour growth showed a statistically significant difference ($P<0.01$) after the second treatment compared to either the control TNE buffer or rSFV-EGFP treated groups. The smaller error bar for each group in the graph shows the reproducibility of the results. In an another experiment K-BALB tumours were treated with $4x10^8$ IU/dose of rSFV10-E-IL12; three out of 6 mice (50%) showed complete tumour regression (between 15 to 21 days post tumour induction) with P value less than 0.01, the median survival time increased (49 days) compared to the control groups. Finally, all six mice (100% with P value less than 0.001) bearing K-BALB tumours showed complete tumour regression when treated with six intra-tumoural injections of rSFV10-E-IL12 at $4x10^9$ IU VLPs. None of the rSFV10-IL-12 treated mice showed complete tumour regression although there was a significant growth inhibition compared to the TNE treated groups (Figure 4.1 - 4.4). In these experiments, none of the TNE treated mice showed tumour inhibition whereas rSFV-EGFP showed some inhibitory effect on tumour growth during the treatment stage but later the tumour grew back at a similar rate as TNE treated tumours. This initial slower tumour growth

rate may be due to the inherent ability of rSFV VLPs to induce apoptosis in tumour cells (Atkins *et al.*, 2004).

A similar treatment protocol was performed on the CT26 tumour using rSFV10-E-IL12 at $4x10^9$ IU VLPs. This dose induced complete tumour regression in all the six mice used (100%) (Figure 4.5). A significant difference in the treatment effect was found compared to the control groups ($P<0.001$) (Table1). There was no statistical difference of the treatment effect between TNE and rSFV-EGFP treated K-BALB or CT26 tumours ($P>0.05$). All tumours that received rSFV VLPs expressing either EGFP or IL-12 showed tumour swelling during the treatment stage which was an indication of inflammatory reaction (Smyth *et al.*, 2004). The K-BALB and CT26 tumour regressed mice were monitored for three months to ensured no tumour re-growth, indicating that the regressions were permanent.

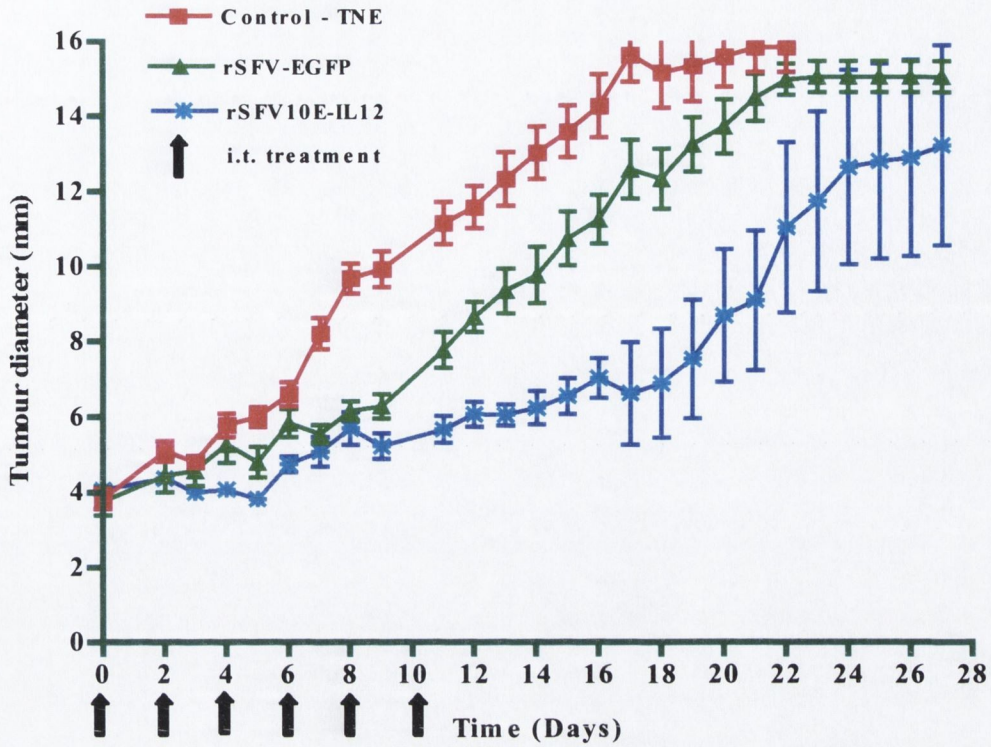
4.2.2 Tumour pathology and immunohistochemistry

The formalin fixed paraffin embedded tumour sections from one day after the sixth treatment were stained using haematoxylin and eosin (H&E) and examined histologically to evaluate the influx of immune cells into the tumour. The sections of K-BALB tumours treated with TNE were composed of intact polyhedral and spindle shaped cells characterised by well-defined cell borders with bizarre mitotic forms. A small number of neutrophils were sparsely distributed throughout the tumours. Areas of massive necrosis with dense infiltrates of lymphocytes and large number of plasma cells in the surrounding connective tissue were detected at the tumour site for tumours treated with rSFV-EGFP replicons. No intact tumour cells were seen in most of the area examined but occasional intact tumour cells at the periphery were observed. Histopathological analysis of rSFV10-E-IL12 treated tumours showed complete tumour necrosis with substantial neutrophils, lymphocytes, and macrophage infiltration. In most of the IL-12 treated tumour sections, no tumour cells were seen other than nuclear and cell debris, and deformed tumour vasculature (fibrin thrombi), representing the antiangiogenic effects of IL-12. Examination of CT26 tumour sections showed a similar pattern of histopathology to that of K-BALB tumours showed in Figures 4.9 & 4.10.

For immunohistochemistry, frozen tumour sections were used to further examine the anti-tumour immune response and tumour vasculature in rSFV10-E-IL12

and mock treated K-BALB and CT26 tumours after the sixth treatment. The sections were stained using CD4 and CD8 antibodies to detect the type and number of infiltrating lymphocytes. We found higher numbers of CD4+ (MHC class II restricted) and CD8+ (MHC class I restricted) cells located in perivascular spaces, necrotic areas, and the tumour nodular margins of IL-12 treated tumours. In contrast, mock treated tumours showed no infiltrating lymphocytes except occasional neutrophils, indicating the recruitment of immune cells in the IL-12 treated tumours but not in untreated tumours. In most of the IL-12 treated sections, greater populations of CD4+ cells were observed compared to CD8+, but overall the quantitative difference was not significant. CD8+ cells were mostly observed in necrotic areas whereas CD4+ were found especially at the interface between the necrotic areas and viable cells (Figures 4.11 & 4.12). As IL-12 has been reported to exhibit anti-angiogenic activity, frozen K-BALB and CT26 tumour sections were analysed for intratumoral vascular density using CD31 antibodies, a marker specific for vascular endothelial cells. The examination of IL-12 treated tumour sections showed the restriction of endothelial cell density to the non-necrotic areas of the tumours, and showed a poorly developed or damaged vasculature pattern. In contrast, mock treated sections showed intact and well-defined tumour vasculature throughout the tumour area (Figures 4.13 & 4.14). However, attempts were not made to quantitate vascular density between the control and treated groups as the IL-12 treated tumours had large necrotic areas. These results therefore indicate a cumulative anti-tumour effect of the IL-12 expressing enhanced SFV vector that may involve several mechanisms.

a


Control - TNE
rSFV-EGFP
rSFV10E-IL12
i.t. treatment
Tumour diameter (mm)
Time (Days)
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2
4
6
8
10
12
14
16
18
20
22
24
26
28

b

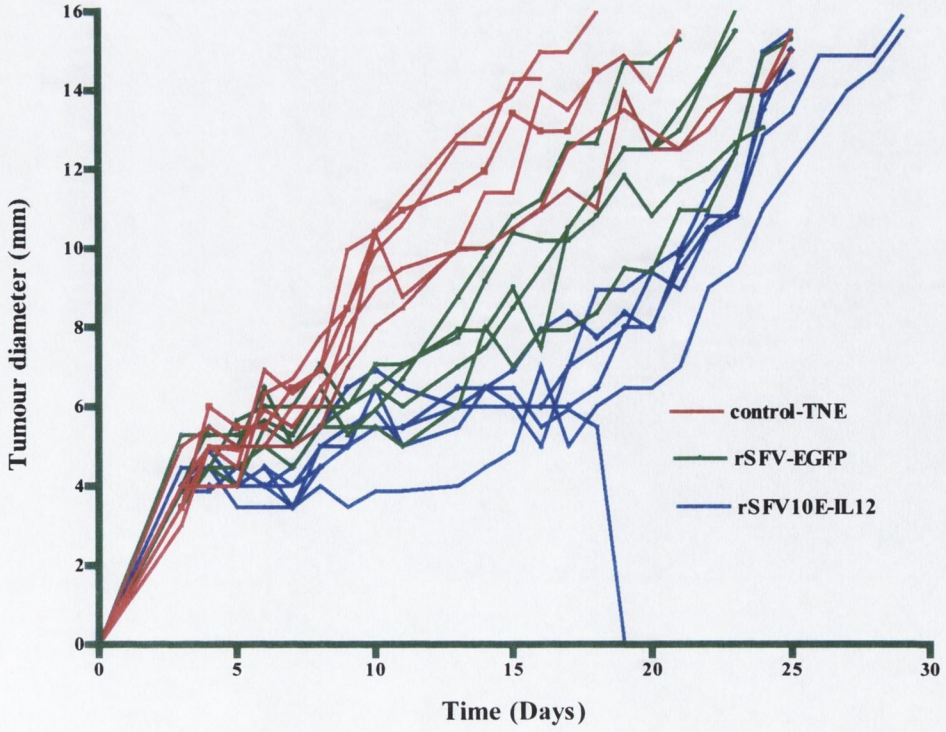
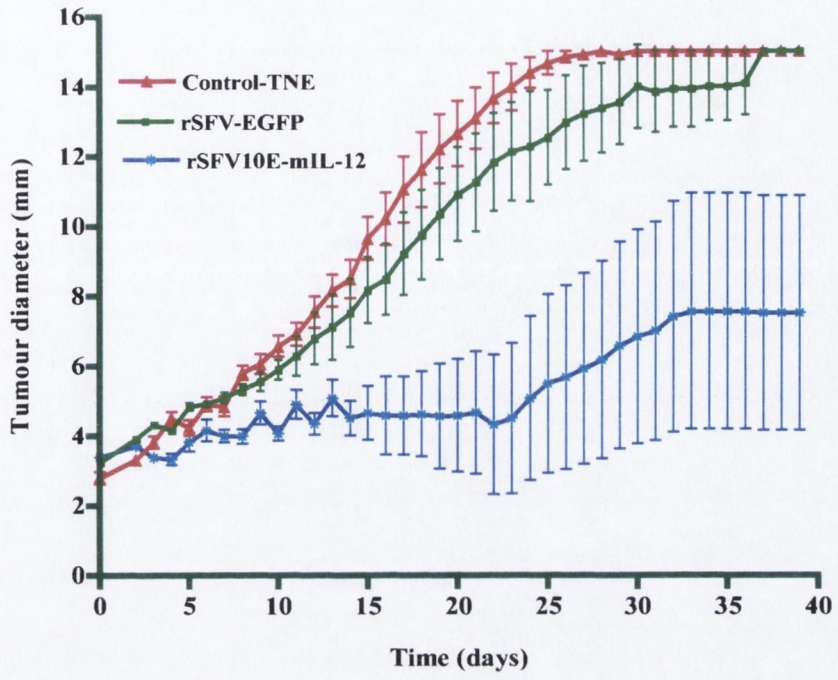

Tumour diameter (mm)
Time (Days)
control-TNE
rSFV-EGFP
rSFV10E-IL12
0
5
10
15
20
25
30

Figure 4.1 Treatment of K-BALB tumours in immunocompetent BALB/c mice with rSFV10-E-IL12 VLPs ($4x10^7$ VLPs/dose):

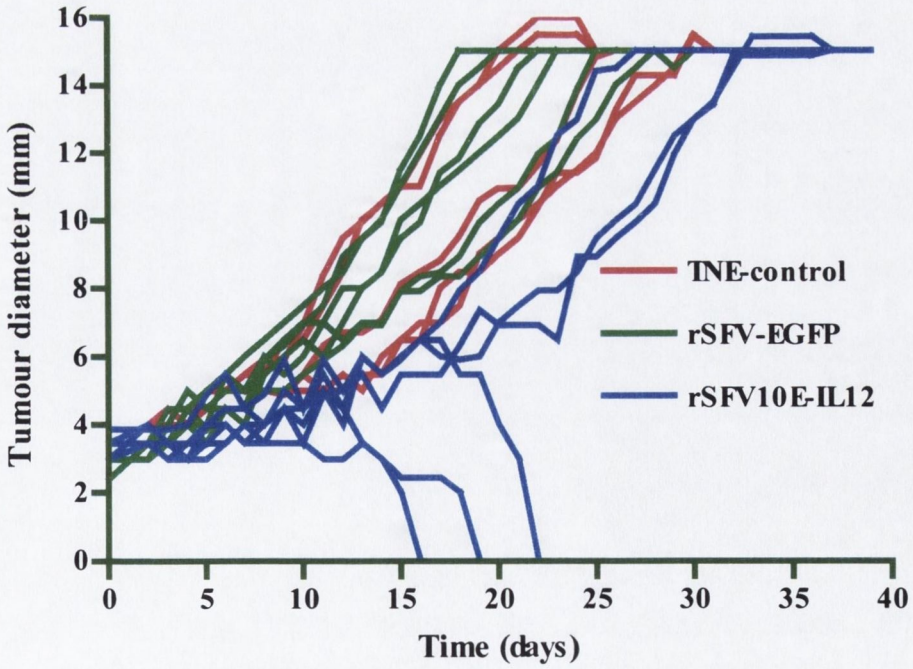
K-BALB tumours were induced on the right abdominal flank of female BALB/c mice by s.c. injection of 100 µl of K-BALB cell suspension in non-supplemented DMEM at a concentration of 1 x 10^7 cells/ml. Treatment was initiated in individual tumours at an average tumour diameter of 4 mm. A group of six mice were used per treatment group. Animals received six 50µl intratumoural injections of TNE alone, TNE containing rSFV-EGFP or rSFV10-E-IL12 VLPs at a concentration of $4x10^7$ IU/dose. Intratumoural injections were administered every other day over an eleven-day period. Treatment with rSFV-EGFP was used in the study as virus control. 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 15 mm. Inhibition of tumour growth in the rSFV10-E-IL12 VLPs treated mice observed compared to control treated groups (a); the tumour growth inhibition was found statistically significant ($P<0.01$) when analysed using using Tukey's multiple comparison test by one-way ANOVA. One mouse out of six rSFV10-E-IL12 treated mice had complete tumour regression at day 19 (a few days after the sixth treatment) (b).

Points; mean of six replicates, *bars*; +/- SEM, *arrows*; days of treatment. This graph represents results of a single experiment.

a


Control-TNE
rSFV-EGFP
rSFV10E-mIL-12
Tumour diameter (mm)
Time (days)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30
35
40

b


TNE-control
rSFV-EGFP
rSFV10E-IL12
Tumour diameter (mm)
Time (days)
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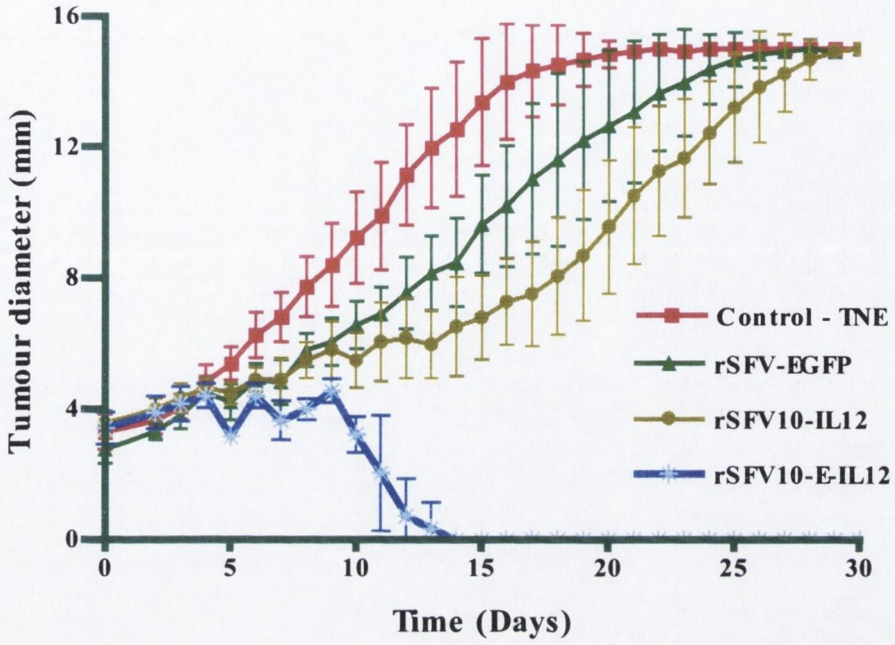
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30
35
40

Figure 4.2 Treatment of K-BALB tumours in immunocompetent BALB/c mice with rSFV10-E-mIL12 VLPs ($4x10^8$ VLPs/dose):

K-BALB tumours were induced on the right abdominal flank of female BALB/c mice as described previously. A group of six mice was used per treatment. When the treatment was initiated, animals received six intratumoural injections of TNE alone, TNE containing rSFV-EGFP, or rSFV10-E-IL12 VLPs at a concentration of $4x10^8$ IU/dose in 50 µl volume on each alternate day. Treatment using rSFV-EGFP was used in the study as a control. Tumour growth was monitored by daily measuring the tumour diameter and mice were euthanised when average tumour diameter reached 15 mm. Inhibition of tumour growth in the rSFV10-E-IL12 VLPs treated mice compared to control treated groups (a); the tumour growth was found statistically significant ($P<0.01$) when analysed using Tukey's multiple comparison test by one-way ANOVA. Three mice out of six rSFV10-E-IL12 treated mice had complete tumour regression few days after the sixth treatment (b).

Points; mean of six replicates, *bars*; +/- SEM. This graph represents results of a single experiment.

a


Tumour diameter (mm)
16
12
8
4
0
0
5
10
15
20
25
30
Time (Days)
Control - TNE
rSFV-EGFP
rSFV10-IL12
rSFV10-E-IL12

b

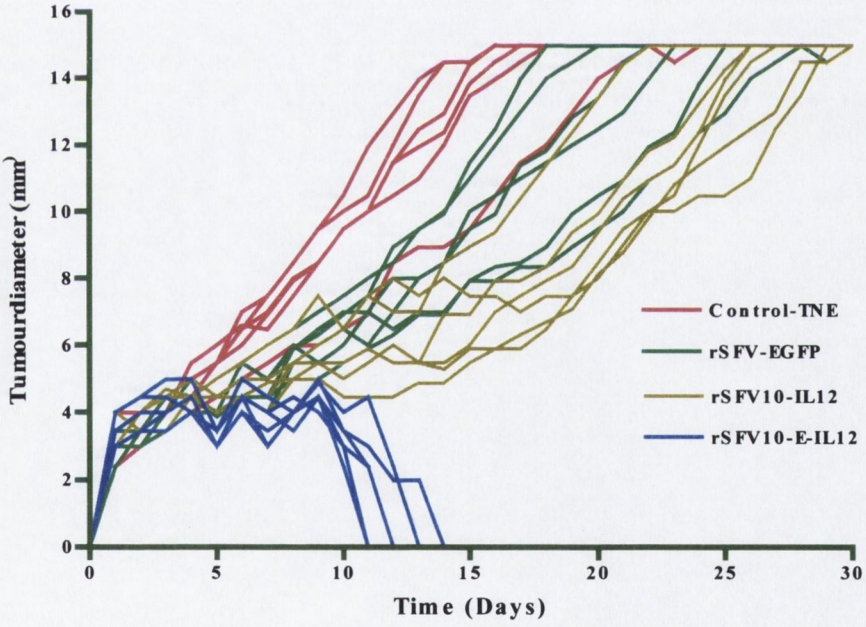
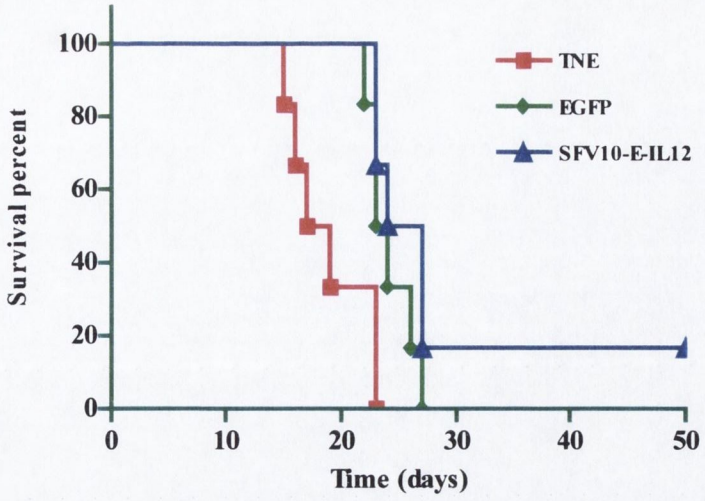

Tumourdiameter (mm)
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
30
Time (Days)
Control-TNE
rSFV-EGFP
rSFV10-IL12
rSFV10-E-IL12

Figure 4.3 Treatment of K-BALB tumours in immunocompetent BALB/c mice with rSFV10-E-IL12 VLPs ($4x10^9$ VLPs/dose):

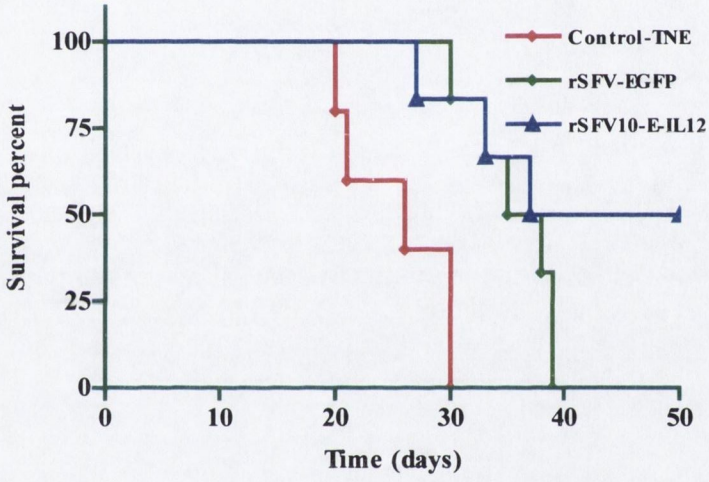
K-BALB tumours were induced on right abdominal flank of female BALB/c mice as described previously. A group of six mice was used per treatment group. Treatment was initiated at an average tumour diameter of 4mm. Tumours received six intratumoural injections of TNE alone, or TNE containing rSFV-EGFP or rSFV10-E-IL12 or rSFV10-IL12 VLPs at a concentration of 4×10^9 IU/dose in 50 µl volume, on each alternate day. Treatment using rSFV-EGFP was used in the study as a control. Tumour growth was monitored by daily measuring the tumour diameter and mice were euthanised when average tumour diameter reached 15 mm. All six mice bearing K-BALB tumours showed complete tumour regression (a & b) whereas none of the rSFV10-IL12 treated mice showed complete regression, although there was a significant growth inhibition compared to the TNE treated group. In these experiments, rSFV-EGFP showed some inhibitory effect on tumour growth during the treatment stage due to the inherent ability of rSFV VLPs to induce apoptosis in tumour cells. Inhibition of tumour growth in the rSFV10-E-IL12 VLPs treated mice was found statistically significant ($P<0.001$) when analysed using Tukey's multiple comparison test by one-way ANOVA.

Points; mean of six replicates, *bars*; +/- SEM. This graph represents results of a single experiment.

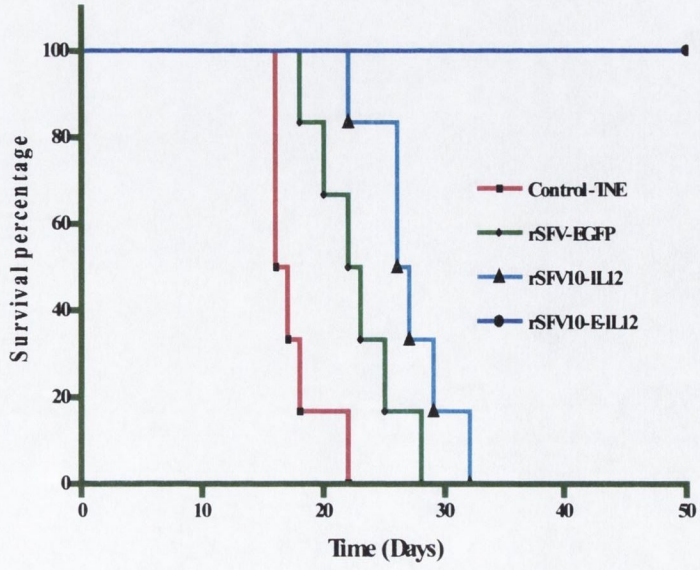
a


Survival percent
100
80
60
40
20
0
0
10
20
30
40
50
Time (days)
TNE
EGFP
SFV10-E-IL12

b


Survival percent
100
75
50
25
0
0
10
20
30
40
50
Time (days)
Control-TNE
rSFV-EGFP
rSFV10-E-IL12

c


Survival percentage
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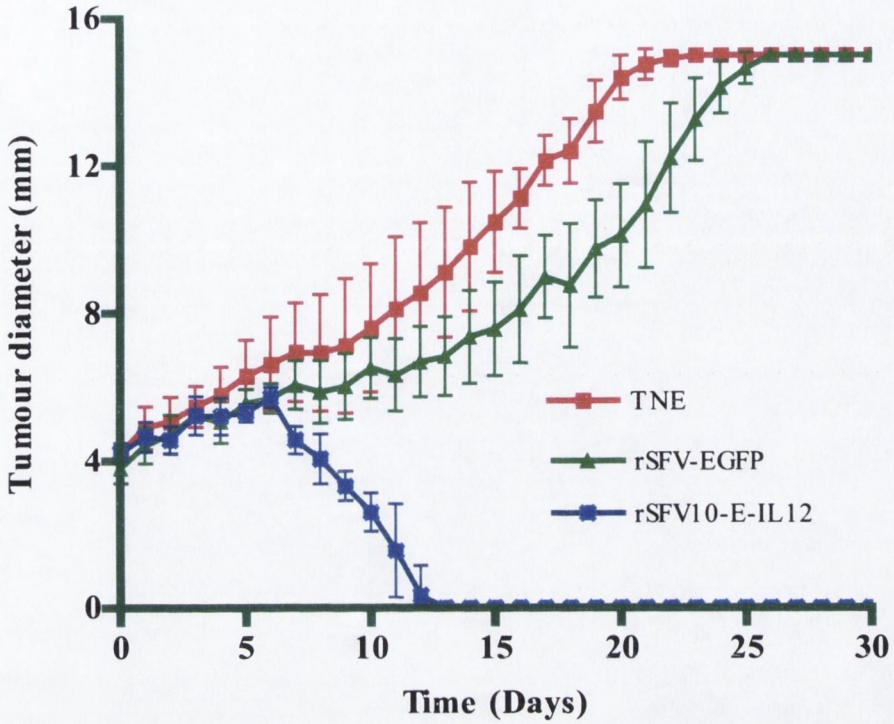
80
60
40
20
0
0
10
20
30
40
50
Time (Days)
Control-TNE
rSFV-EGFP
rSFV10-IL12
rSFV10-E-IL12

Figure 4.4 Survival proportions of the mice bearing K-BALB tumours treated with rSFV10-E-IL12

Mice were sacrificed when the tumour size reached 15mm diameter. The average time taken to reach this size for a group of mice is the survival time. The total number of mice, which had complete and permanent tumour regression, is considered as number of survivors. The survival proportions of the mice bearing K-BALB tumour was 16.66% when treated with a total of six intratumoural injections of rSFV10-E-IL12 at a titre of $4x10^7$ IU/dose (a). The survival percentage increased to 50% (b) and 100% (c) when tumours were treated using six intratumoural injections of rSFV10-E-IL12 at a viral titre of $4x10^8$ and $4x10^9$VLPS/dose respectively. The tumour regressions in the IL-12 treated mice were complete and permanent. Control (TNE or rSFV-EGFP) treated groups used along with the three different K-BALB tumour treatment experiments at different viral titres had almost similar tumour growth pattern with 0% survival proportions at the end of the treatment experiment. A significant difference in the number of survival days between rSFV10-E-IL12 and rSFV10-IL12 treated groups was observed but none of the mice survived in the rSFV10-IL12 treated group (c).

a


16
12
8
4
0
Tumour diameter (mm)
0
5
10
15
20
25
30
Time (Days)
TNE
rSFV-EGFP
rSFV10-E-IL12

b

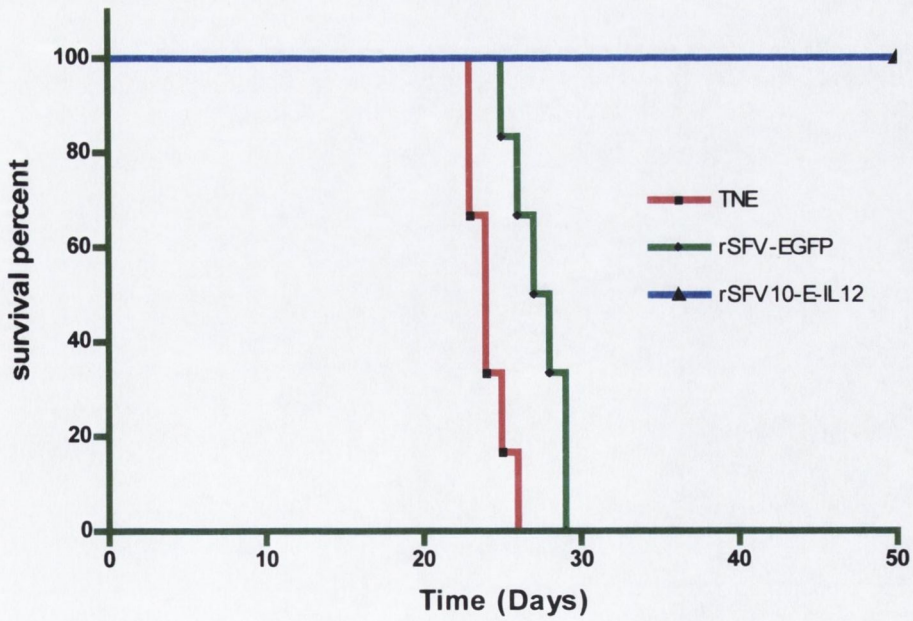

100
80
60
40
20
0
survival percent
0
10
20
30
40
50
Time (Days)
TNE
rSFV-EGFP
rSFV10-E-IL12

Figure 4.5 Treatment of CT26 tumours in immunocompetent BALB/c mice with rSFV10-E-IL12 VLPs ($4x10^9$ IU/dose):

CT26 tumours were induced on the right abdominal flank of female BALB/c mice as described previously. A group of six mice were used per treatment group. An average tumour diameter of 4mm was reached at 4 days post induction for CT26. Treatment was initiated at an average tumour diameter of 4 mm. Tumours received six intratumoural injections of TNE alone or TNE containing rSFV-EGFP or rSFV10-E-IL12 VLPs at a concentration of $4x10^9$ IU/dose in 50 µl volume, on each alternate day. Tumour growth was monitored by daily measuring the tumour diameter and mice were euthanised when average tumour diameter reached 15 mm. All six mice bearing CT26 tumours (100%) showed complete tumour regression (a & b) whereas none of the control treated mice showed tumour regression, although there was some inhibition of tumour growth in the rSFV-EGFP treated group compared to the TNE treated group. CT26 tumour regression in the rSFV10-E-IL12 VLPs treated mice was statistically significant ($P<0.001$) when analysed using Tukey's multiple comparison test by one-way ANOVA.

Points; mean of six replicates, *bars*; +/- SEM.

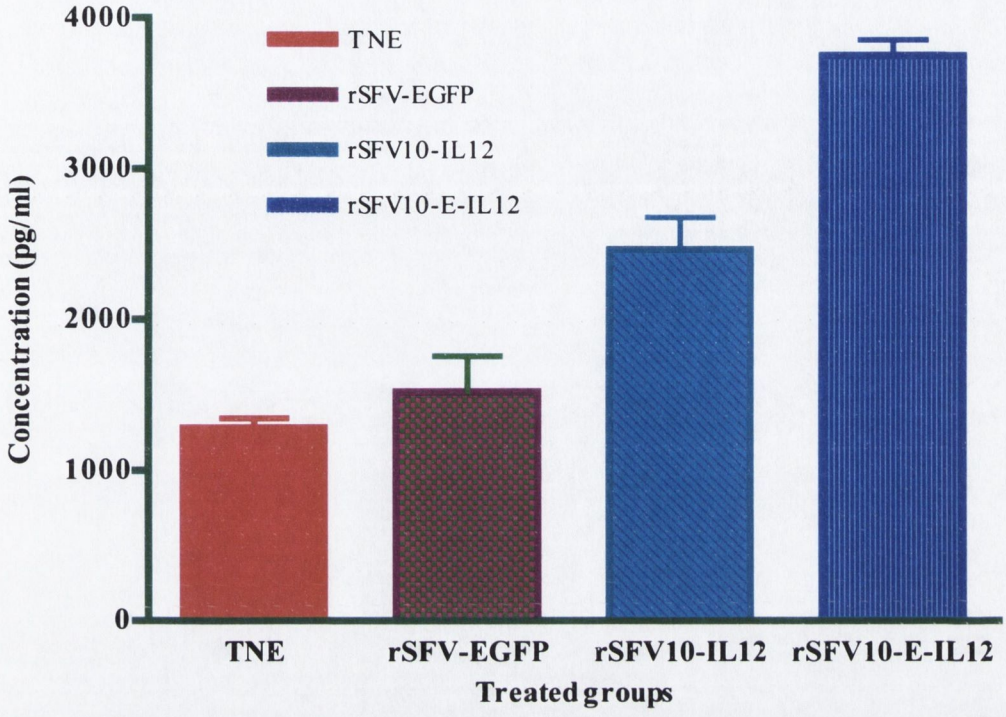


Figure 4.6 Serum levels of antiangiogenic factor IP-10

The inducible protein-10 (IP-10), a chemokine, is known to mediate the anti-angiogenic activity of IL-12. Serum samples of three K-BALB tumour-bearing mice was collected 3 days following the sixth treatment to estimate the serum IP-10 concentration by ELISA. The serum from rSFV10-E-IL12 ($4x10^9$ IU in 50µl/dose) treated animals had high levels of average IP-10 (3679 pg/ml) compared to the control TNE (1278 pg/ml) or rSFV-EGFP (1522 pg/ml) groups. On an average, IP-10 concentration in IL-12 treated serum sample was found to be 2-3 fold higher than TNE or EGFP (control) treated groups ($P<0.01$). The serum concentration of IP-10 was also found considerably higher than the original SFV vector expressing IL-12. The actual mechanism of antiangiogenic effects induced by IP-10 still remains as unclear. These results correlate with the enhanced antitumour effect obtained in the animals treated with rSFV10-E-IL12 VLPs.

a

b

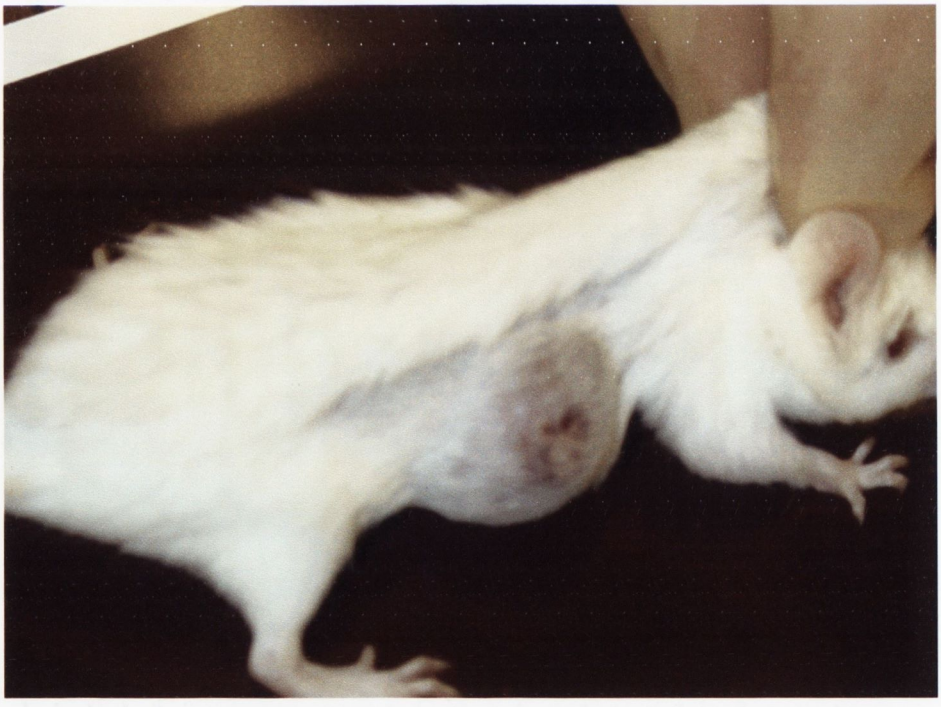


Figure 4.7 BALB/c mice bearing K-BALB tumours, 20 days post tumour induction.

(a) TNE treated K-BALB tumour (15mm diameter) after six intratumoural injections. Mice were culled when the tumours reached 10% of body weight.

(b) rSFV-EGFP treated K-BALB tumour. The tumour is of similar size to the TNE-treated tumours.

a

b

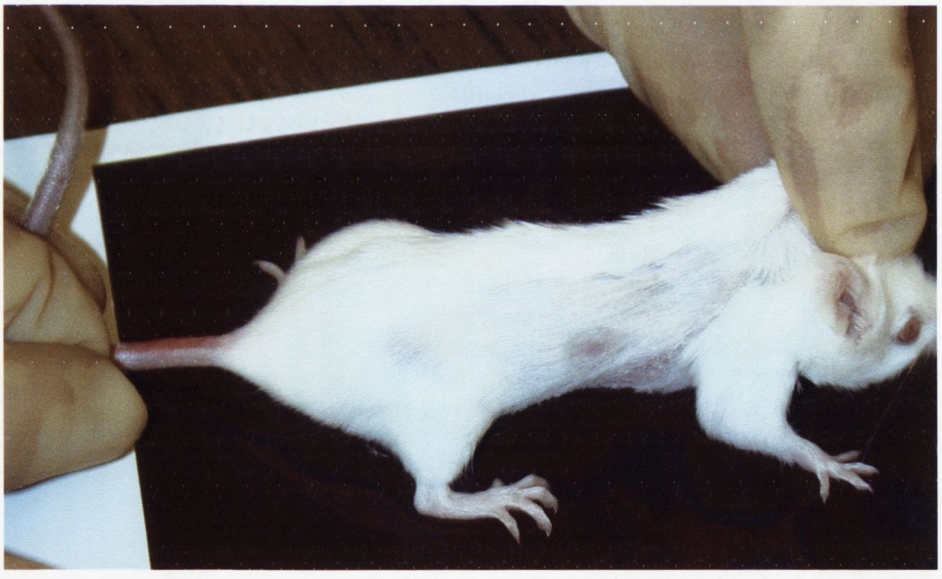
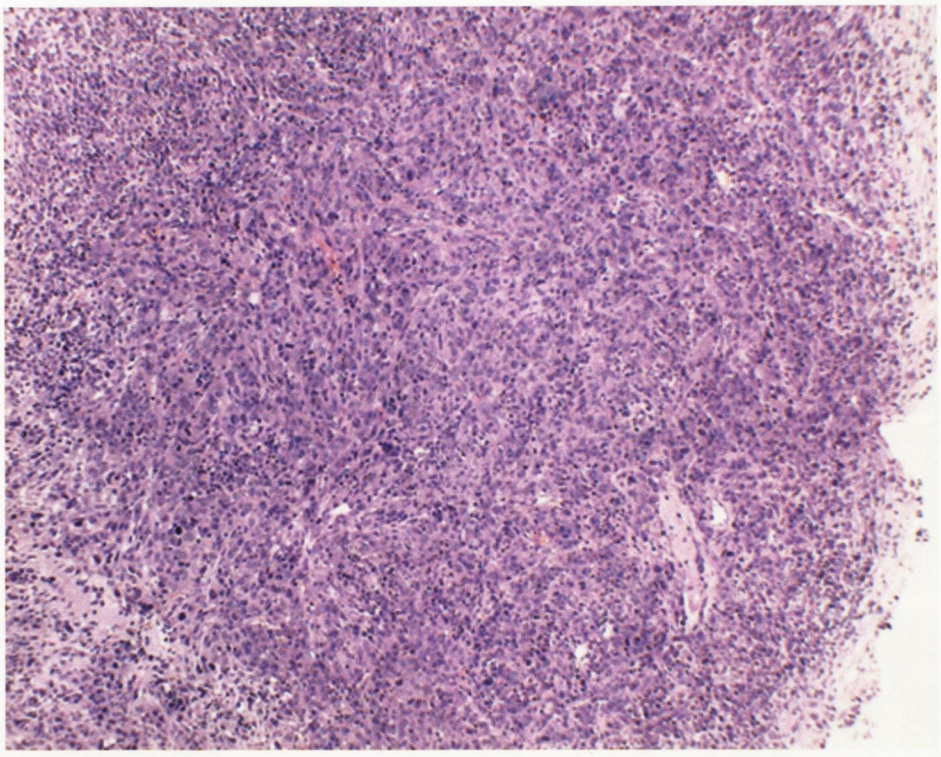


Figure 4.8 rSFV10-E-IL12 treated BALB/c mice showing complete K-BALB tumour regression.

(a) No tumour visible, 20 days post tumour induction.

(b) No tumour re-growth, 90 days post tumour induction indicating permanent tumour regressions.

a



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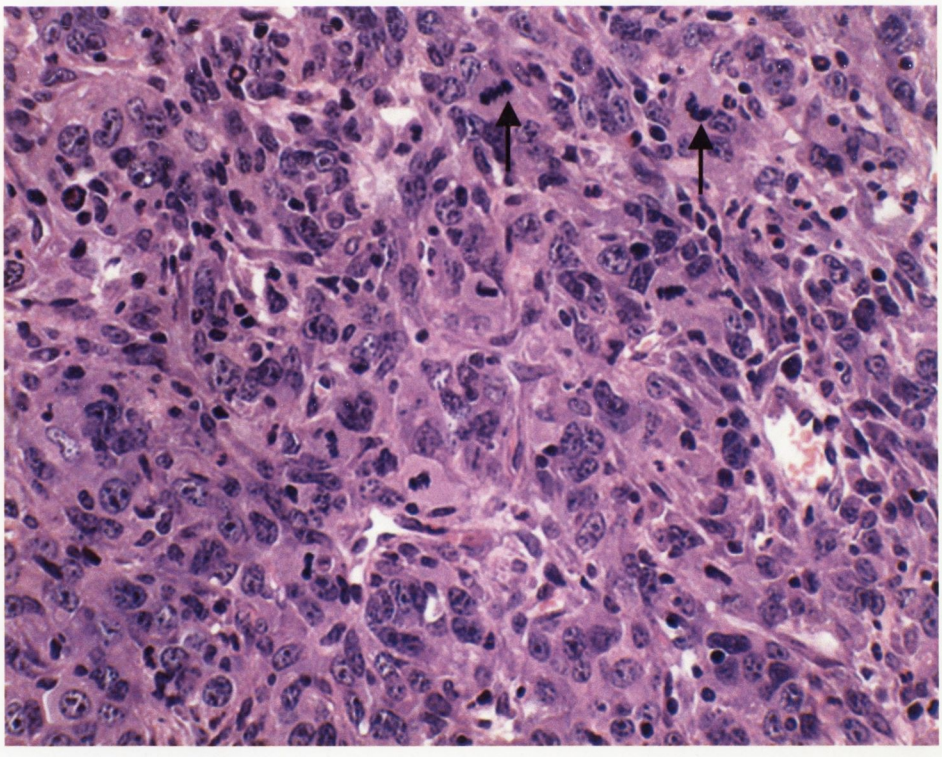
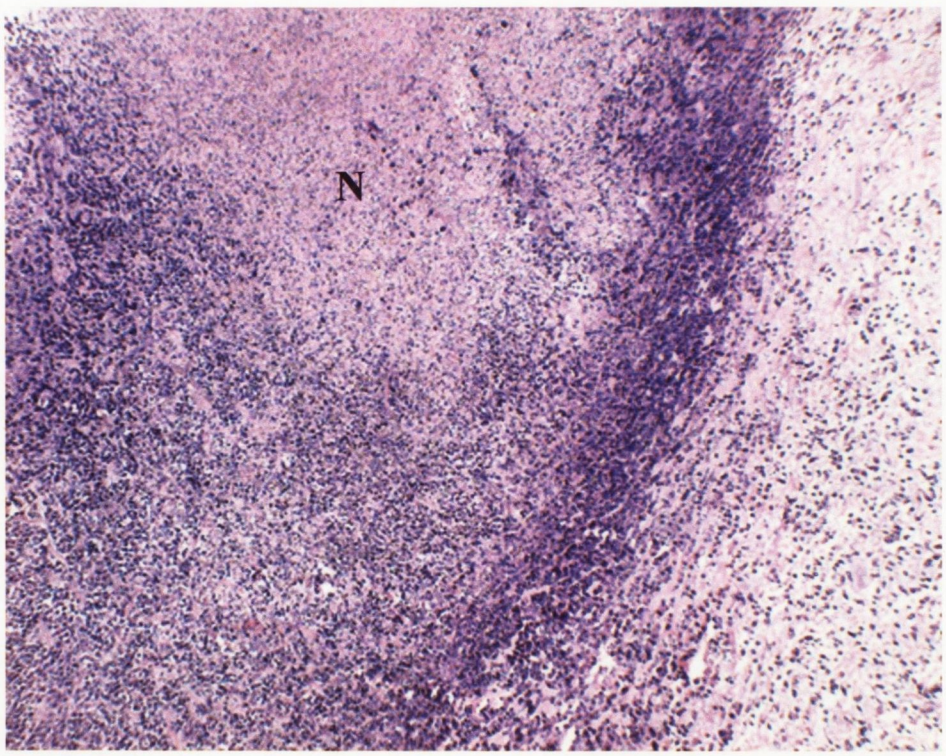


Figure 4.9 K-BALB tumours, one-day post treatment with TNE.

(a) Small numbers of lymphoid cells and neutrophils among the tumour cells and at the periphery. H&E, x 40.

(b) Polyhedral and spindle-shaped tumour cells with well-defined cell borders and bizarre mitotic forms (arrows). H&E, x 200.

a



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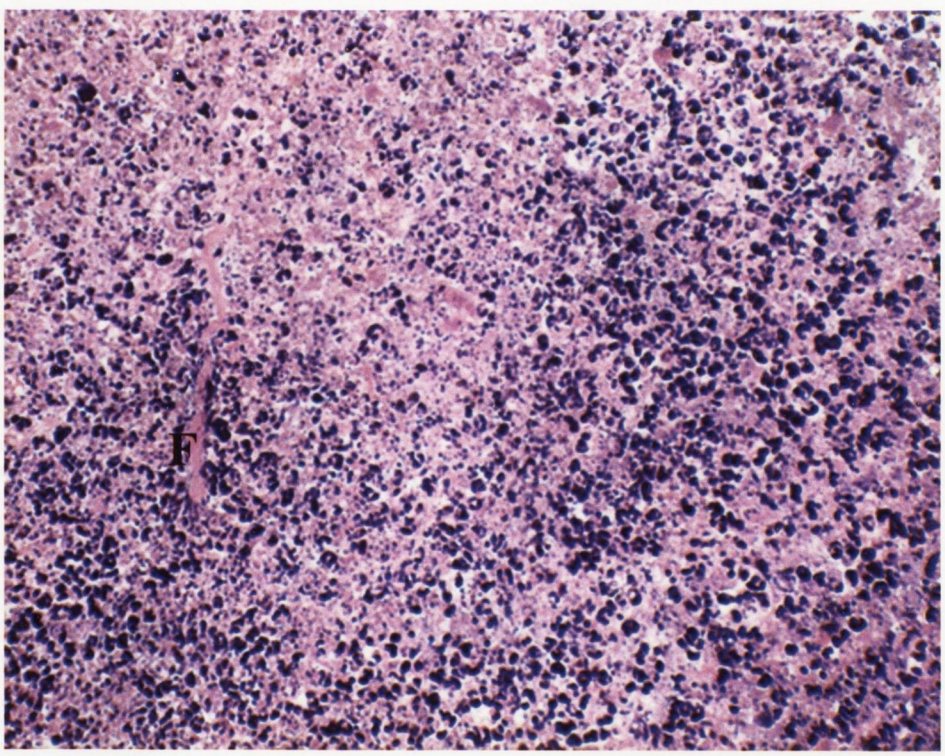
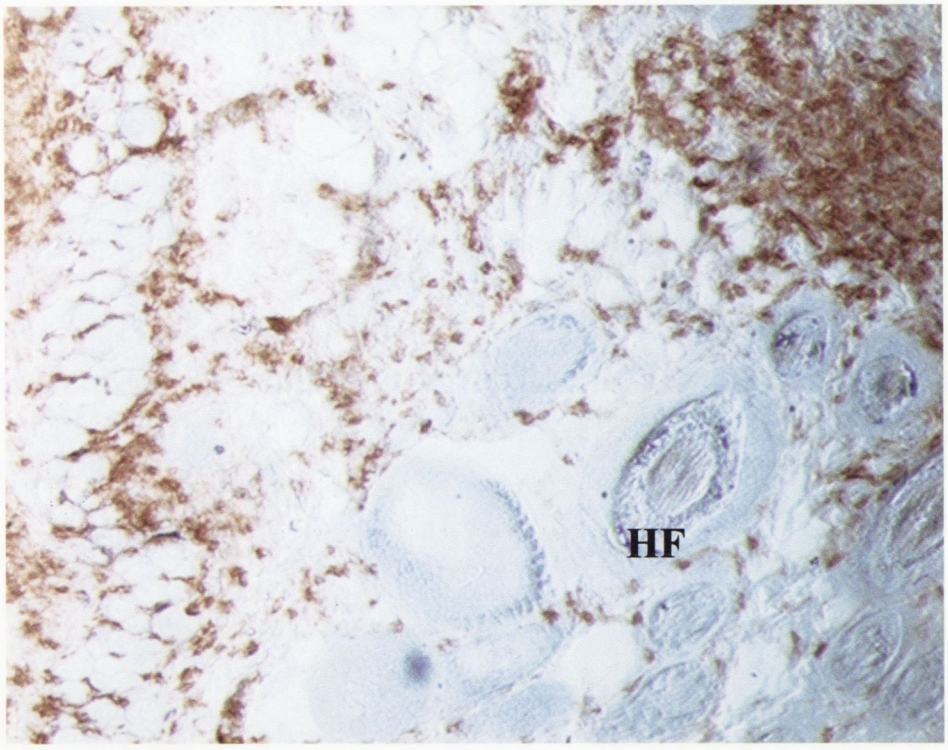


Figure 4.10 K-BALB tumour, one-day post treatment with rSFV10-E-IL12.

(a) Large area of necrosis (N) circumscribed by leucocyte infiltrates. H&E, x 40.

(b) Higher magnification of (a) showing details of the necrotic cell death, with neutrophils and fibrin thrombi (F). Intact tumour cells are absent. H&E, x 200.

a



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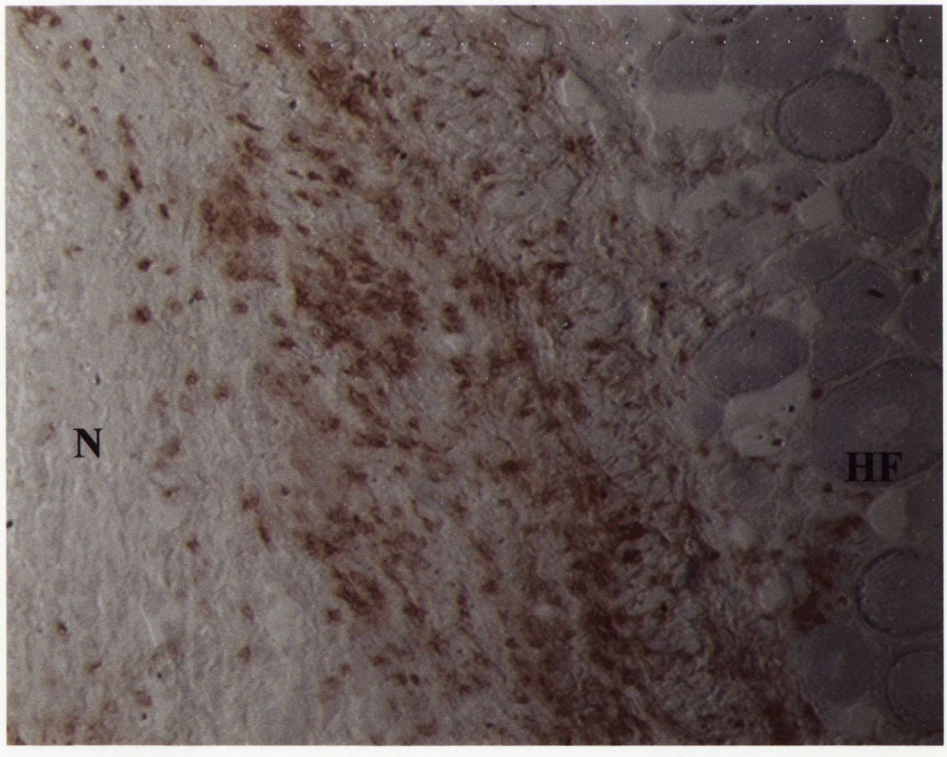
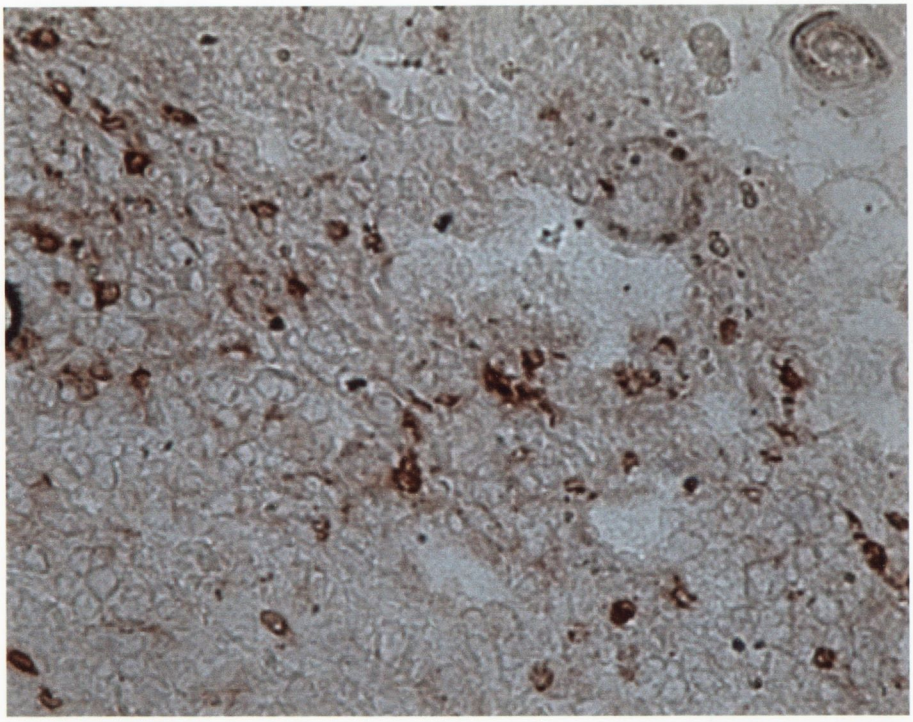


Figure 4.11 CD4+ immunohistochemistry.

(a) K-BALB tumours one-day post tumour treatment with rSFV10-E-IL12. CD4+ T-lymphocytes in the peritumoural connective tissue. Hair follicles (HF) are indicated. x 400.

(b) CT26 tumours one-day post tumour treatment with rSFV10-E-IL12. CD4+ T-lymphocytes at the interface between necrotic area (N) and hair follicles (HF). X 400.

a



b

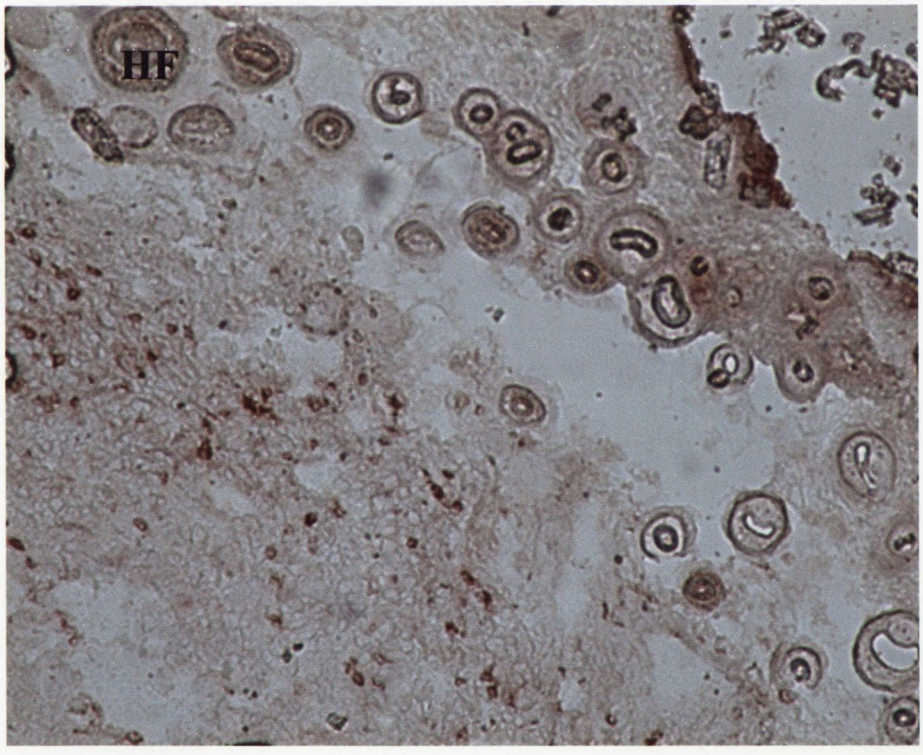
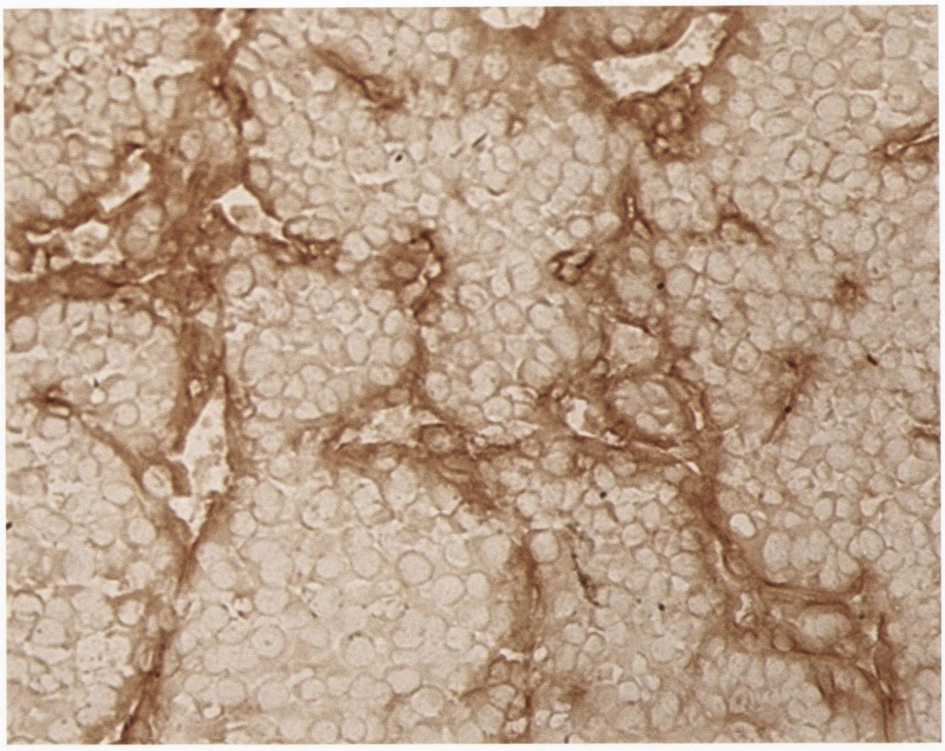


Figure 4.12 CD8+ immunohistochemistry.

(a) K-BALB tumours one-day post tumour treatment with rSFV10-E-IL12. A small numbers of CD8+ T-lymphocytes in an area of oedema and necrosis. x 400.

(b) CT26 tumours one-day post tumour treatment with rSFV10-E-IL12. CD8+ T-lymphocytes showing similar distribution to that in figure (a). Hair follicles are indicated (HF). x 400.

a



b

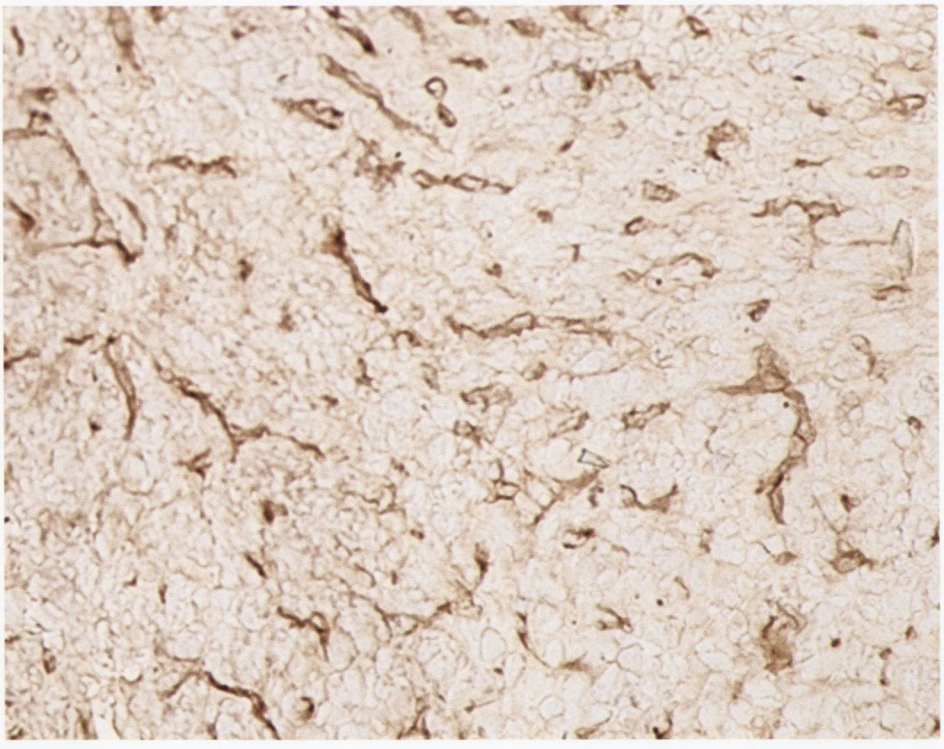


Figure 4.13 K-BALB tumour microvasculature labelled with CD31 antibodies using immunohistochemistry.

(a) TNE-treated tumour with well-defined capillary network uniformly distributed between the tumour cells. x 200.

(b) rSFV10-E-IL12 treated tumour with poorly-developed microvasculature. x 200.

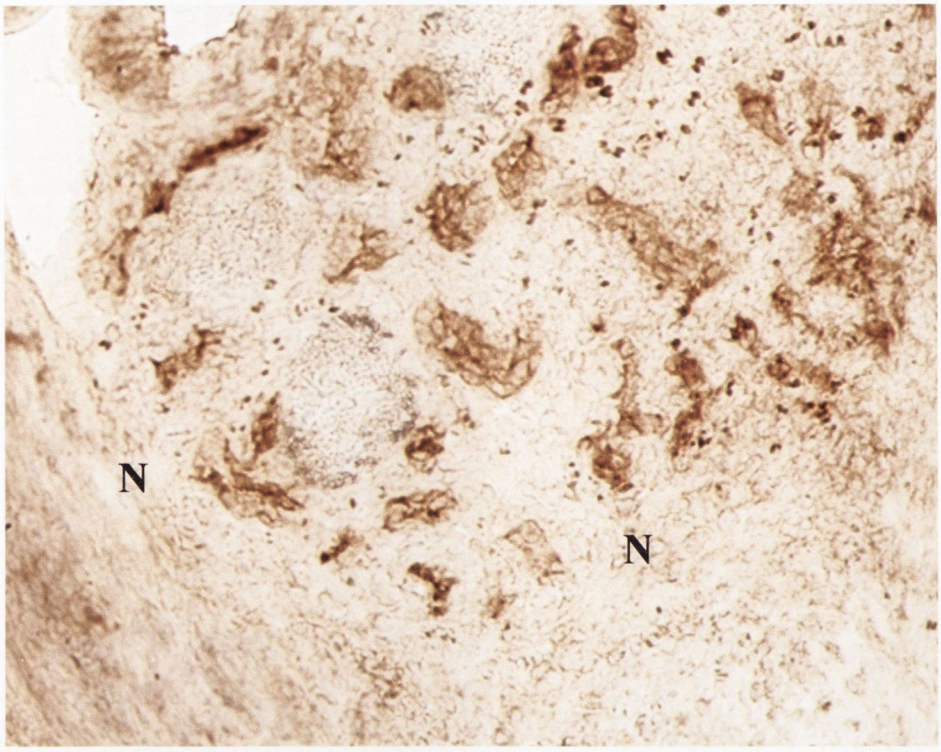


Figure 4.14 K-BALB tumour microvasculature labelled with CD31 antibodies using immunohistochemistry.

Poorly developed microvasculature adjoining an area of necrosis (N). x 200.

Table 1 Inhibition of K-BALB and CT26 tumour growth by rSFV-IL12 treatment

Tumour Model	*Treatment groups*[a]	*Median survival (days)*[b] *(No. of survivors*[c]*)*	*Average. tumour diameter*[d] *(mm) (Std.error)*	*Probability*[h]
K-BALB	TNE[e]	22 (0)	11.9 (+/- 0.66)	
	rSFV-EGFP ($4x10^7$IU/dose)	24 (0)	10.0 (+/- 0.80)	>0.05
	rSFV-EGFP ($4x10^8$IU/dose)	24 (0)	11.4 (+/- 0.57)	>0.05
	rSFV-EGFP ($4x10^9$IU/dose)	23 (0)	11.4 (+/- 0.65)	>0.05
	rSFV10-IL12 ($4x10^9$IU/dose)	27 (0)	10.5 (+/- 0.66)	>0.05
	rSFV10-E-IL12 ($4x10^7$IU/dose)	26 (1)	7.3 (+/- 0.58)	>0.05
	rSFV10-E-IL12 ($4x10^8$IU/dose)	49 (3)	5.9 (+/- 0.20)	>0.05
	rSFV10-E-IL12 ($4x10^9$IU/dose)	Undefined (6)[f]	1.0 (+/- 0.23)	<0.001
CT26	TNE	24 (0)	11.0 (+/- 0.67)	
	rSFV10-EGFP ($4x10^9$IU/dose)	28 (0)	9.8 (+/- 0.69)	>0.05
	rSFV10-E-IL12 ($4x10^9$IU/dose)	Undefined (6)[f]	1.5 (+/- 0.33)	<0.001

[a]Six Balb/c mice per treatment group were used for all experiments.

[b]Mice were sacrificed when the tumour reached 15 mm diameter and the time taken to reach this size is the survival time.

[c]Mice which had complete and permanent tumour regression; in a few cases a residual small nodule remained that was composed entirely of non-malignant scar tissue.

[d]Average tumour diameter for each group was calculated at their appropriate median survival time.

[e]Average of the control (TNE) treated groups in the three different K-BALB tumour treatment experiments with different titres of VLPs.

[f]Since all mice showed complete tumour regression, the median survival time cannot be calculated by Kaplan-Meier analysis.

[g]Two mice were culled due to the appearance of clinical signs before the termination of the experiment and before maximum tumour size was attained.

[h]The probability value is based on the average tumour diameter at their median survival time compared with that of the TNE treated (control) groups.

4.3 DISCUSSION

Our in vivo tumour treatment studies involved the intratumoural treatment of fast growing, poorly immunogenic localised K-BALB and CT26 tumours in immuno-competent female BALB/c mice. Currently there is much interest in IL-12, because of its potent antitumour and anti-metastatic activitites in different tumour models (Brunda *et al.*, 1993). Previous work has shown that dramatic antitumour effects can be achieved by transient IL-12 delivery instead of continuous high doses (Mu *et al.*, 1995, Rakmilevich *et al.*, 1996). It has also been shown that the amount of IL-12 at the tumour site is a key factor for tumour regression rather than systemic presence. Based on these concepts, we have used a new vector, pSFV10-E, that has enhanced expression ability to express murine IL-12.

Previous studies on different tumour models using SFV vector have demonstrated the low immunogenisity of the vector as it undergoes only one round of replication in host cells (Zhou *et al.*, 1995). The data in our treatment experiments clearly shows that six intra-tumoural treatments of K-BALB tumours using lower titres of rSFV10-E-IL12 replicons ($4x10^7$ IU/dose) resulted in significant tumour growth inhibition including complete regression in one out of six mice (16.66%). Further, an improved treatment effect was found when three out of six mice (50%) had complete regression at $4x10^8$ IU/dose of VLPs. Finally, complete and permanent K-BALB tumour regressions were found in all six animals (100%) treated with $4x10^9$ IU/dose of rSFV10-E-IL12 replicons compared to original rSFV10 expressing murine IL-12. This is in agreement with Colombo *et al* (1996), indicating that an increased anti-tumour effect correlated with increased levels of IL-12 expressed locally at the tumour site. The consistency of this treatment effect was confirmed on CT26 colon carcinoma, another localised tumour, with complete and permanent tumour regressions in all 100% of the treated animals. These results showed that an efficient anti-tumour effect could be achieved using rSFV10-E-IL12 replicons. The findings of a recent study by Rodriguez-Madoz et al. (2005) have also indicated enhanced effect using IL-12 expressed locally at the tumour site by SFV10-E. These results are in agreement with the antitumour effects of IL-12 obtained from our studies. It was evident from the immunohistochemistry that the rSFV10-E-IL12 has induced cytotoxic activities of B- and T-lymphocytes that are crucial for anti-tumour effects.

Detection of massive numbers of CD4+ and CD8+ T-lymphocytes compared to the control tumours accounts for the induced tumour targeted immune response. Similar histopathological observations in K-BALB and CT26 tumours suggest the requirement of T-lymphocytes to promote more efficacious antitumour function. Variations in the tumour size (tumour swelling) during the treatment might be due to the influx of a wide range of immune cells leading to inflammatory swelling of the IL-12 treated tumours. In addition, detection of increased serum IP-10 concentrations in animals treated with rSFV10-E-IL12 replicons after the treatments (Figure 4.7) also accounts for the anti-tumour effect through the inhibition of angiogenesis. IP-10 is an IL-12 -induced downstream product that is responsible for antiangiogenic activity. This conclusion is in agreement with the previous results showing that IP-10 was a downstream molecule primarily responsible for IL-12 mediated tumour vasculature inhibition (Luster *et al.*, 1995, Sgadari *et al.*, 1996). The IL-12 induced anti-angiogenic effect was further detected by staining IL-12 treated tumour sections for endothelial cells of the tumour vasculature using anti-mouse CD-31 antibodies. The observations show poorly developed or completely damaged blood-capillaries in the IL-12 treated tumour sections compared to the control treated tumours. Increased density of intact and healthy blood-capillaries was observed in the control treated tumours. This represents morphological evidence for IL-12 induced antiangiogenesis. The IL-12 induced anti-angiogenic effect may have been the cause for tumour necrosis that was observed in most of the IL-12 treated sections.

These results suggest that it is likely that the potent anti-tumour activity of the IL-12 expressing SFV vector results from a combination of mechanisms. However, complete regression of established K-BALB and CT26 tumours was found to be permanent. This was concluded after the disease-free survival of the tumour regressed animals for 90 days. Although, tumour therapy using rSFV-EGFP replicons (virus control) showed significant tumour growth inhibition compared to TNE treated groups, no complete regressions were reported. This inhibitory effect of rSFV-EGFP replicons during the treatment stage indicates the viral oncolytic (inherent ability to induce apoptosis) property and confirms that the inherent oncolytic property of the virus alone is not sufficient for complete tumour eradication.

vectors into the other organs such as liver kidney, brain etc, has been well established by a previous study by Morris-Downes in 1999.

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5

TREATMENT OF METASTASES IN THE 4T1 TUMOUR MODEL WITH rSFV10-E-IL12 VLPs

5.1 INTRODUCTION

Interleukin-12 (IL-12) is a multifaceted antitumour agent that promotes cellular immune responses (Trinchieri *et al.*, 1998) and through IFN-γ and other mediators, mediates tumour cell apoptosis and inhibits tumour angiogenesis (Gee *et al.*, 1999). IL-12 has been shown to be one of the most potent anti-tumour cytokines in experimental models (Brunda *et al.*, 1993, Mu *et al.*, 1995). Furthermore, a significant antitumour effect of the localized IL-12 gene delivery protocol was shown against distant solid tumours and spontaneous metastases in immunogenic murine tumour models (Rakhmilevich *et al.*, 1997, 2000). It has been shown previously that expression of tumour associated antigens (MUC-1, HER-2/neu etc.) on human breast tumour cells can be recognized by the immune system and immunotherapeutic strategies for treatment have been studied for potential clinical application (Apostolopoulos *et al.*, 1995). The development and metastasis of primary tumours mainly depends on the development of adequate vasculature (McNamara *et al.*, 1998). These key findings have lead to strategies for tumour treatment involving inhibition of tumour angiogenesis. This is considered to be a promising therapeutic strategy based on the results of animal studies, which have shown that angiogenesis inhibitors can reduce metastases and shrink established experimental tumours (Skobe *et al.*, 1997, Boehm *et al.*, 1997). A tumour treatment study by Rakhmilevich *et al.*, 2004 using IL-12 in combination with anti-vascular endothelial growth factor receptor-2 monoclonal antibodies has resulted in the inhibition of 4T1 adenocarcinoma and was also effective against lung metastases. But in most of the previous studies where angiogenesis inhibitors were used, residual primary tumours or dormant metastases began to grow following the discontinuation of the treatment (Holmgren *et al.*, 1998). These and other studies support IL-12 gene therapy for metastatic breast cancer and other malignancies and also suggest the development of a clinical strategy.

To examine the treatment effect of rSFV10-E-12 on a metastasising tumour model, we have selected a highly malignant, spontaneously metastasising 4T1 mouse mammary carcinoma model which readily develops into tumours upon s.c., injection (Aslakson *et al.*, 1992, Pulaski *et al.*, 1998). The 4T1 cell line was originally established from a spontaneous moderately differentiated mammary adenocarcinoma growing in BALB/c mice (Aslakson *et al.*, 1992). The 4T1 mammary carcinoma

model has been already used in immunotherapy studies (Majumdar *et al.*, 2000). In this study, we treated the animals bearing the 4T1 tumour using rSFV10-E-12 at a titre that induced complete tumour regression of K-KBAB and CT26 tumours ie., $4x10^9$ IU/dose. In this model, micrometastases develop in lung and lymphnodes around 20 days post tumour implantation. Our results demonstrated that intratumoural administration of rSFV10-E-IL12 replicons resulted in complete tumour regression and a striking inhibition of metastatic tumour growth in lungs. These observations indicate that IL-12 can induce a curative immune response, even in the face of an aggressive micrometastasising tumour and also add to a growing list of evidence for the antitumour and antimetastatic effects of IL-12.

5.2 RESULTS

5.2.1 Effect of rSFV10-E-IL12 treatment of 4T1 mammary carcinoma

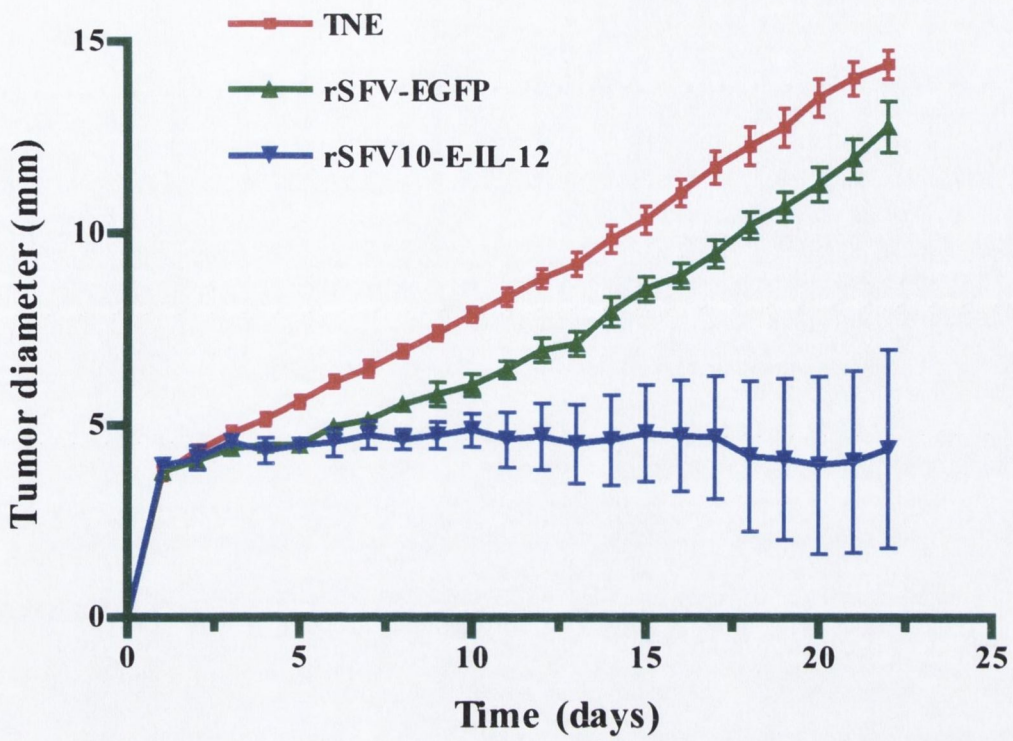
Since the treatment efficacy of the rSFV10-E-IL12 replicon was verified using two different localised tumour models, we next examined its potential to inhibit the growth of established lung metastases in the 4T1 mammary tumour model. 4T1 tumours were established subcutaneously on the abdominal right flank of BALB/c mice and treated with six intra-tumoural injections of $4x10^9$ IU/dose in 50µl on every alternate day. Four out of six treated mice had complete tumour regression between days 18 to 22 post tumour induction (Figure 5.1) and the remaining two mice grew tumours at the same rate as control groups during the post treatment period ($P<0.01$). All the control (TNE and rSFV-EGFP) treated tumours were sampled when the size reached 15mm diameter for immuno-histochemistry. The solid 4T1 tumour nodules were found with local tumour spread after repeated intra-tumoural treatment. This may be due to the pressure of the intra-tumoural injections that was damaging the intact nodular shape. Graphical representation of the 4T1 tumour growth shows a growth pattern that is different from K-BALB or CT26 tumours. The overall treatment summary of K-BALB and CT26 tumour models is presented in Table 1 and for 4T1 tumour in Table 2.

5.2.2 rSFV10-E-IL12 therapy reduces the number of 4T1 lung metastases

The 4T1 breast carcinoma formed solid tumours of 4mm diameter upon s.c injection day 4 post-induction. A large difference in lung metastases between the rSFV10-E-IL12 and control treated groups was found when the lungs were examined for visible tumour nodules at day 22 post-tumour induction. The average number of tumour nodules on the TNE and rSFV-EGFP treated lungs were 16 and 13.2 respectively compared to the IL-12 treated groups which had the lowest average number of 1.5 (Figure 5.2). Previous studies have demonstrated metastasis in the lungs best represents the extent of 4T1 cell metastasis throughout the body. The number of 6-thioguanine resistant colonies in the lungs of the 4T1 tumour bearing

BALB/c mice indicated the extent of 4T1 lung metastases. The lungs of tumour bearing mice, which received TNE or rSFV-EGPP tumours, had a mean of 35644 or 22572 metastatic colonies, respectively, showing no significant difference between them ($P>0.05$). However, the lungs of the rSFV10-E-IL12 treated animals with a mean of 3039 metastatic colonies showed a significant difference from the control treated groups ($P<0.01$) (Table 2). These results indicate that animals treated with rSFV10-E-IL12 are associated with increased survival time, which results from inhibition of primary 4T1 tumour growth and lung metastasis. The observation of no metastatic colonies in the lungs of complete tumour regressed mice indicated a strong correlation between tumour size and the number of metastatic colonies for each mouse, which is consistent with other studies using the 4T1 tumour model (Darby *et al.*, 2003).

a


TNE
rSFV-EGFP
rSFV10-E-IL-12
Tumor diameter (mm)
15
10
5
0
0
5
10
15
20
25
Time (days)

b

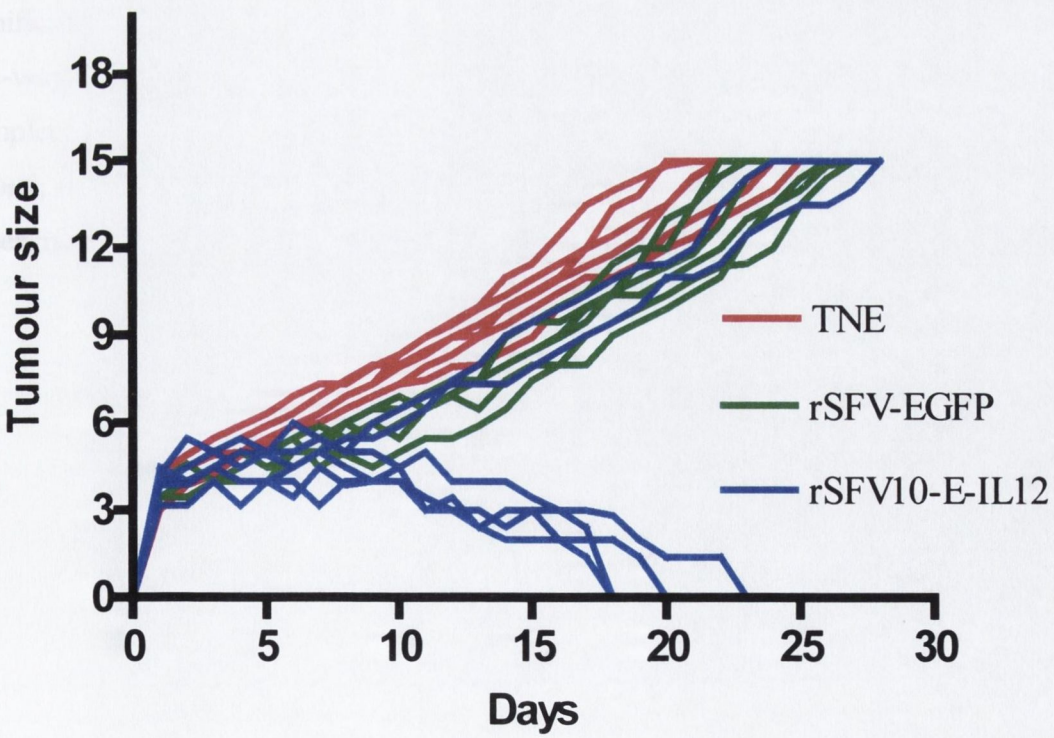
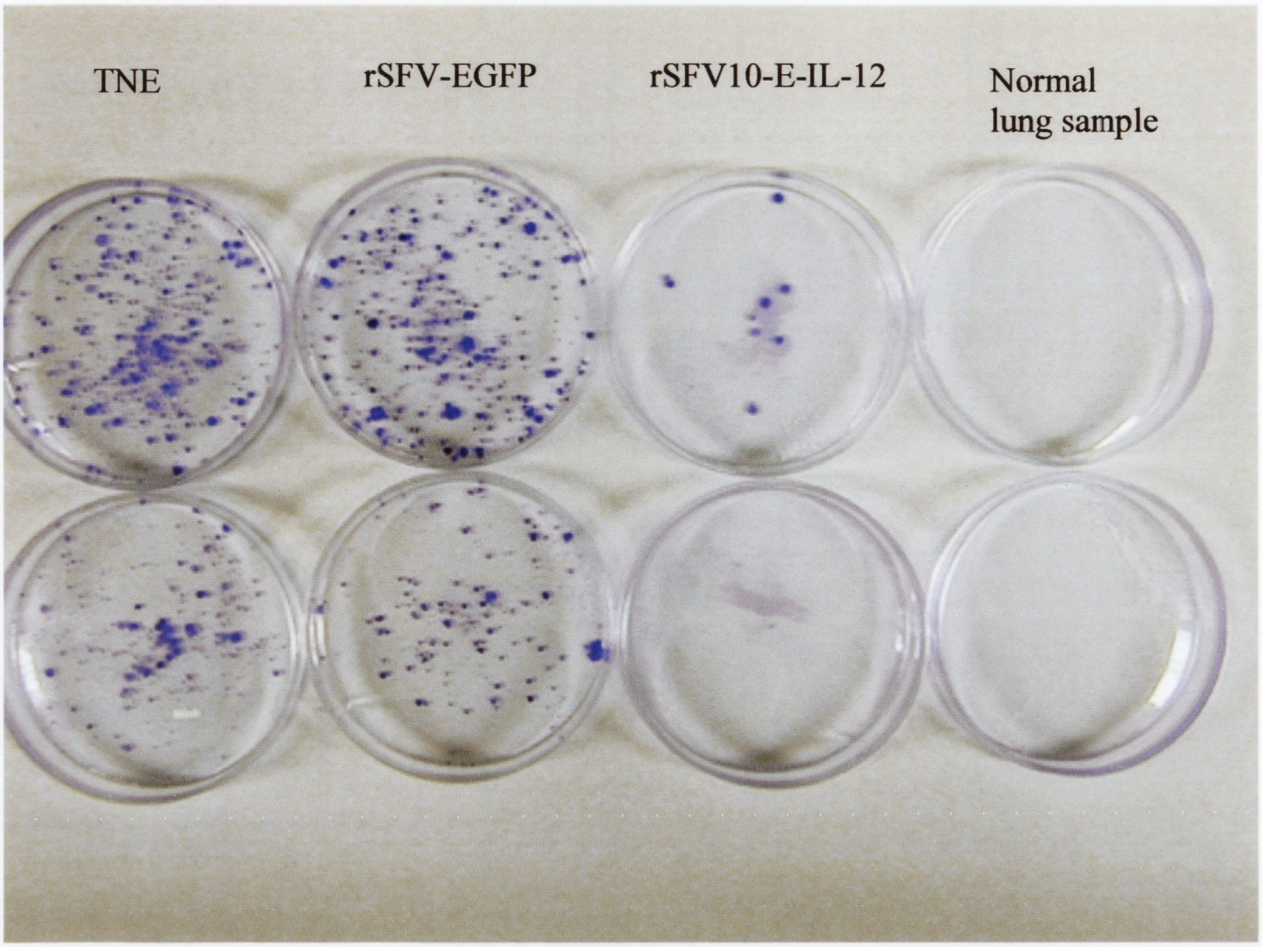

Tumour size
18
15
12
9
6
3
0
0
5
10
15
20
25
30
Days
TNE
rSFV-EGFP
rSFV10-E-IL12

Figure 5.1 Treatment of 4T1 tumours in BALB/c mice with rSFV10-E-IL12 VLPs ($4x10^9$ VLPs/dose):

4T1 tumours were induced on the right abdominal flank of female BALB/c mice by s.c. injection of 100 µl of 4T1 cell suspension in non-supplemented DMEM at a concentration of 1 x 10^7 cells/ml. Treatment was initiated in individual tumours after an average tumour diameter of 4 mm was reached. A group of six mice were used per treatment group. (a) Tumours received six 50 µl intratumoural injections of TNE alone or TNE containing rSFV-EGFP or rSFV10-E-IL12 VLPs at a concentration of 4 x 10^9 IU/dose which were administered every other day over an eleven day period. Treatment using rSFV-EGFP was used as a control group. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised on day 22 post-tumour induction. Inhibition of tumour growth in the rSFV10-E-IL12 VLPs treated mice compared to control treated groups was found statistically significant ($P<0.01$) when analysed using using Tukey's multiple comparison test by one-way ANOVA. **(b)** Four mice out of six rSFV10-E-IL12 treated mice got complete tumour regression at day 19 (a few days after the sixth treatment).
Points; mean of six replicates, *bars*; +/- SEM, This graph represents results of a single experiment.

a


TNE
rSFV-EGFP
rSFV10-E-IL-12
Normal lung sample

b

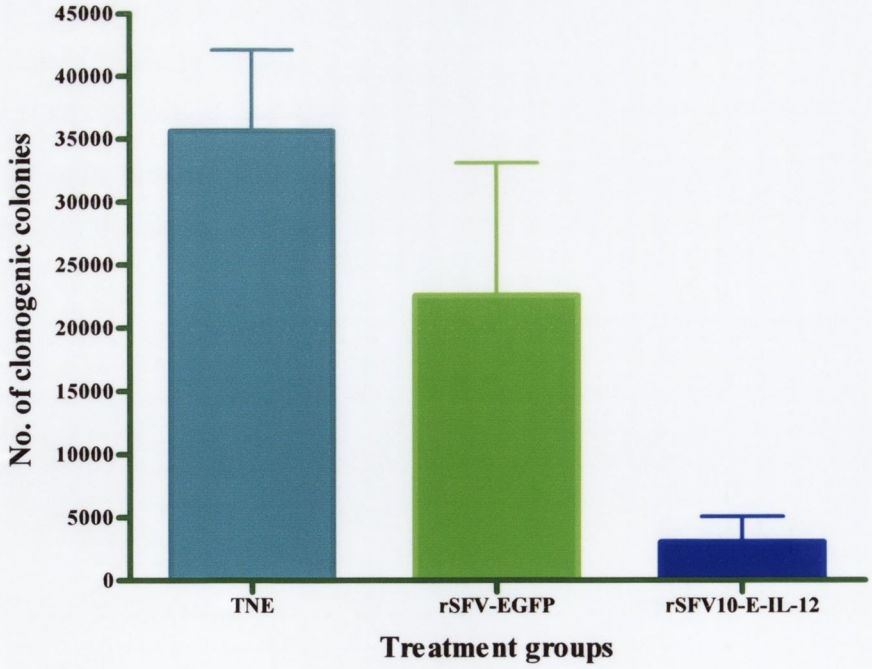

45000
40000
35000
30000
25000
20000
15000
10000
5000
0
No. of clonogenic colonies
TNE
rSFV-EGFP
rSFV10-E-IL-12
Treatment groups

Figure 5.2 rSFV10-E-IL-12 therapy reduces the number of 4T1 lung metastases

Following s.c. injection, 4T1-breast carcinoma cells formed solid tumours of 4mm diameter by day 4 post induction. A large difference in lung metastases between the rSFV10-E-IL12 and control treated groups was found when the lungs were examined for visible tumour nodules at day 22 post tumour induction. The average numbers of tumour nodules in the TNE and rSFV-EGFP treated lungs were 16 and 13 respectively compared to the rSFV10-E-IL12 treated groups which had an average of 1.5 (Table 2).

(a) The number of 6-thioguanine resistant colonies in the lungs of the 4T1 tumour bearing BALB/c mice indicated the extent of 4T1 lung metastases (Table 2). Lung cells were cultured in the presence of 6-thioguanine and the number of drug resistant colonies were stained and counted after incubating the plates for 10-14 days at 37^0C.

(b) The lungs of tumour bearing mice that received TNE or rSFV-EGFP had a mean of $4x10^4$ metastatic colonies. However, the lungs of the rSFV10-E-IL12 treated animals with a mean of $3x10^3$ metastatic colonies showed significantly less than both control groups (Table 2). These results indicate that treatment of 4T1 tumours with rSFV10-E-IL12 is associated with increased survival time, which results from inhibition of both primary 4T1 tumour growth and the growth of metastases. The graph represents the extent of lung metastases.

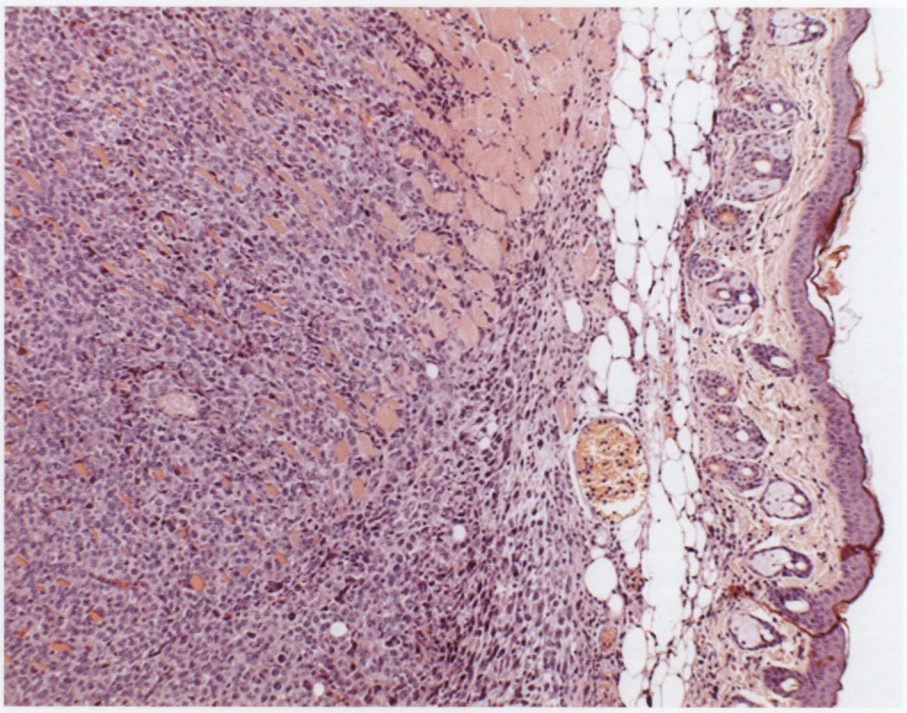
5.2.3 Pathology and immunohistochemistry

The formalin fixed paraffin embedded tumour and lung sections from day 22 post-tumour induction were stained using haematoxylin and eosin (H&E). These sections were examined histologically to evaluate the influx of immune cells into the tumour and metastases of 4T1 tumour into the lungs.

The examination of lungs of the mock (TNE) treated mice showed sub-pleural aggregates of 4T1 metastatic spindle shaped cells characterised by well-defined cell borders (Figure 5.5). No tumours were detected in the lungs of the tumour regressed mice treated with rSFV10-E-IL12 replicons and were characterised by large accumulations of neutrophils in alveolar capillaries and larger blood vessels with small numbers in alveolar spaces (Figure 5.6). Control treated 4T1 tumour sections (H&E stained) showed a sparse number of infiltrating lymphocytes. A high degree of cell proliferation and compression of panniculus muscle fibres indicating the aggressive growth of tumours were observed in large areas of mock treated sections. Histopathological analysis of rSFV10-E-IL12 treated tumour sections showed a large number of lymphocytes with suppurative necrotic area circumscribed by intact tumour cells and lipid vacuoles were prominent. (Figures 5.3 & 5.4).

To detect the type and number of infiltrating lymphocytes, frozen tumour sections were stained by immunohistochemistry using CD4 and CD8 antibodies. Higher numbers of CD4+ and CD8+ cells were located in perivascular spaces, necrotic areas, and the tumour nodular margins of IL-12 treated tumours (Figure 5.7). CD4+ cells were found especially at the interface between the necrotic areas and viable cells.

a



b

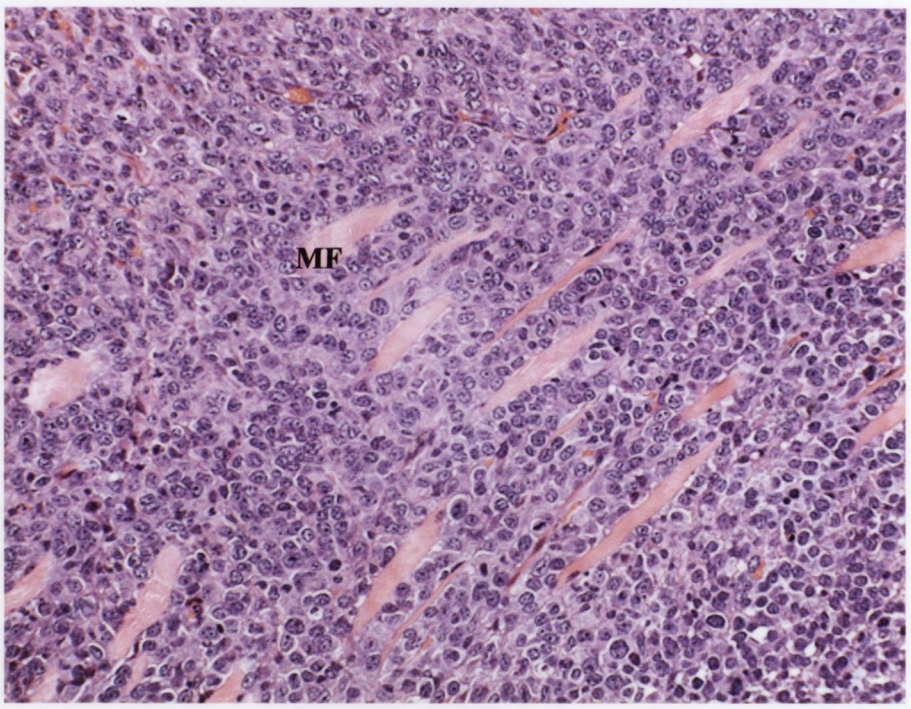
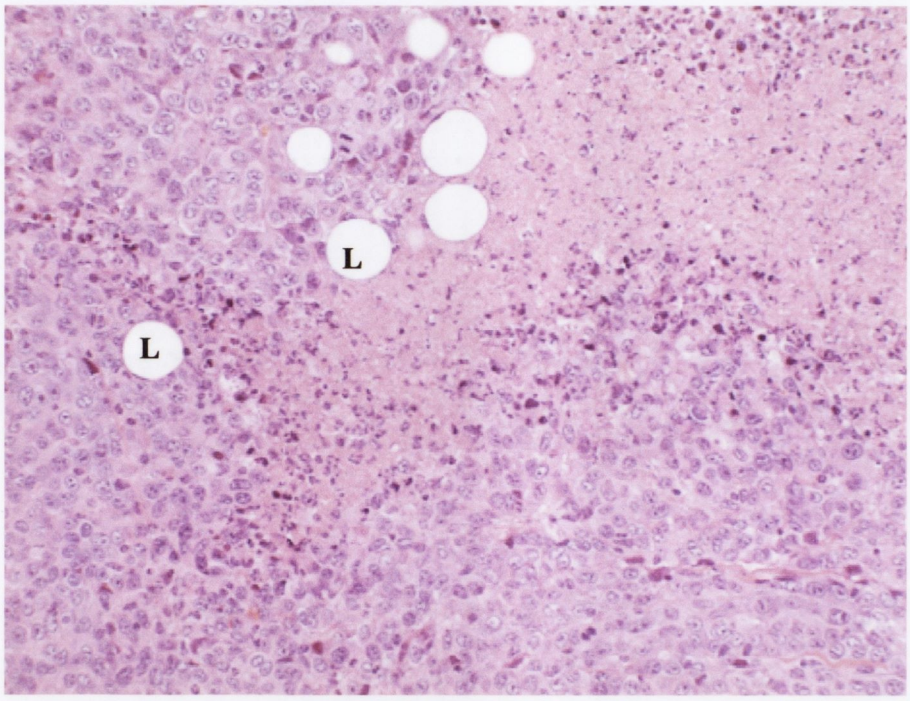


Figure 5.3 TNE treated 4T1 tumour histopathology

(a) 4T1 tumour 22 days following induction and 8 days following cessation of treatment with TNE. Tumour proliferation and invasion towards subcutis by the compression of panniculus muscle fibres. H&E, x 40

(b) Higher magnification of (a) showing an area of compression of muscle fibres (MF). H&E, x 200

a



b

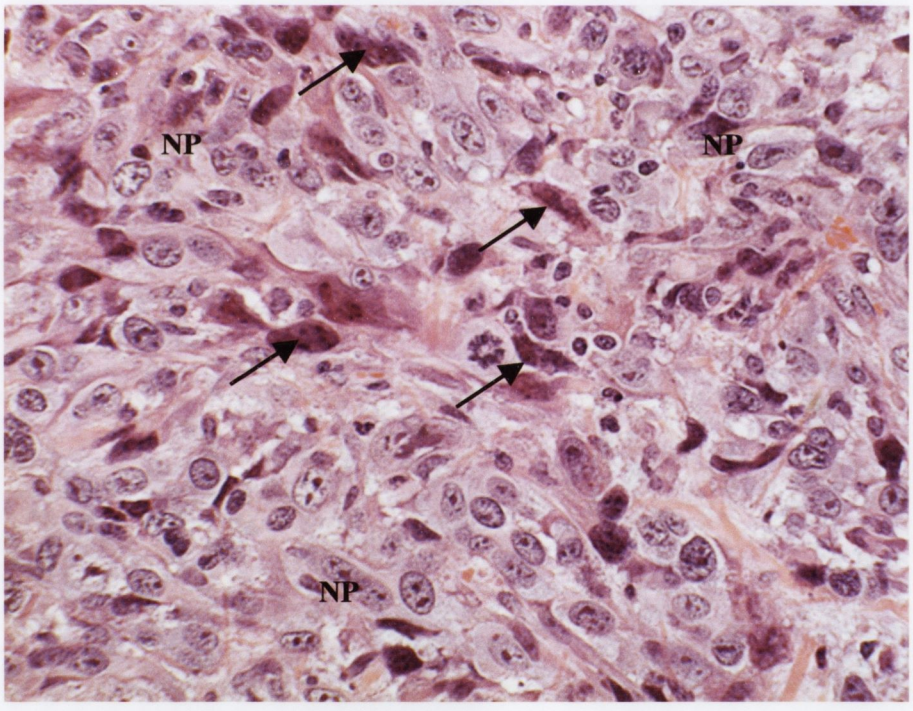


Figure 5.4 rSFV10-E-IL12 treated 4T1 Tumour histopathology

(a) 4T1 tumour 22 days following induction and 8 days following cessation of treatment with rSFV10-E-IL12. Tumour necrosis, and neutrophil infiltration circumscribed by intact tumour cells. Lipid vacuoles (L) are indicated. x 200.

(b) rSFV10-E-IL12 treated 4T1 tumour showing features of high malignancy such as nuclear pleomorphism (NP) and bizarre mitotic figures (arrows). x 400.

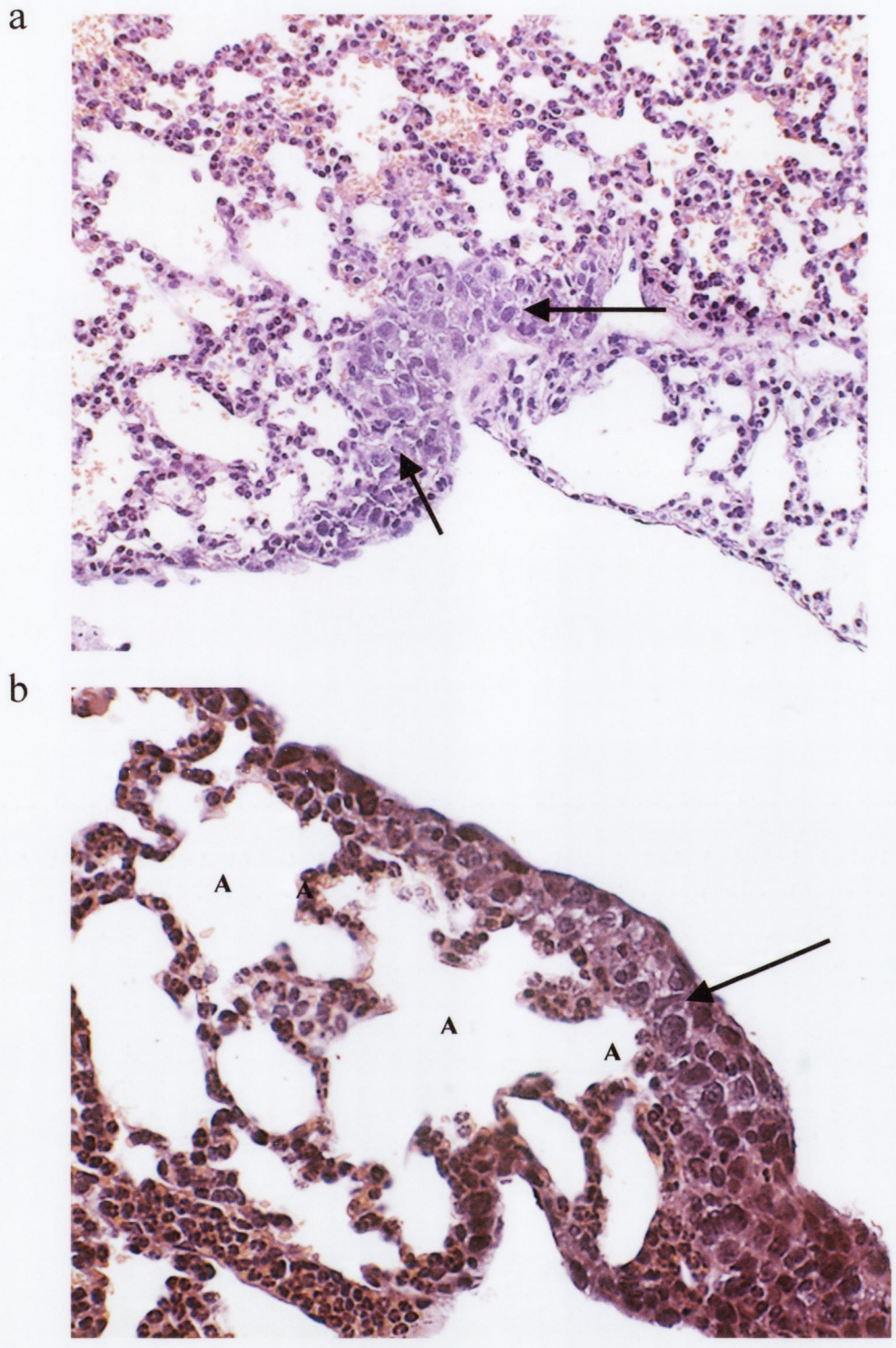


Figure 5.5 Histopathology of control treated 4T1 lung metastasis.

TNE (a) and rSFV-EGFP (b) treated mouse, 22 days post tumour induction and 8 days following treatment cessation showing subpleural aggregates of neoplastic cells (arrows). Alveolar spaces are indicated (A). H&E. x 200

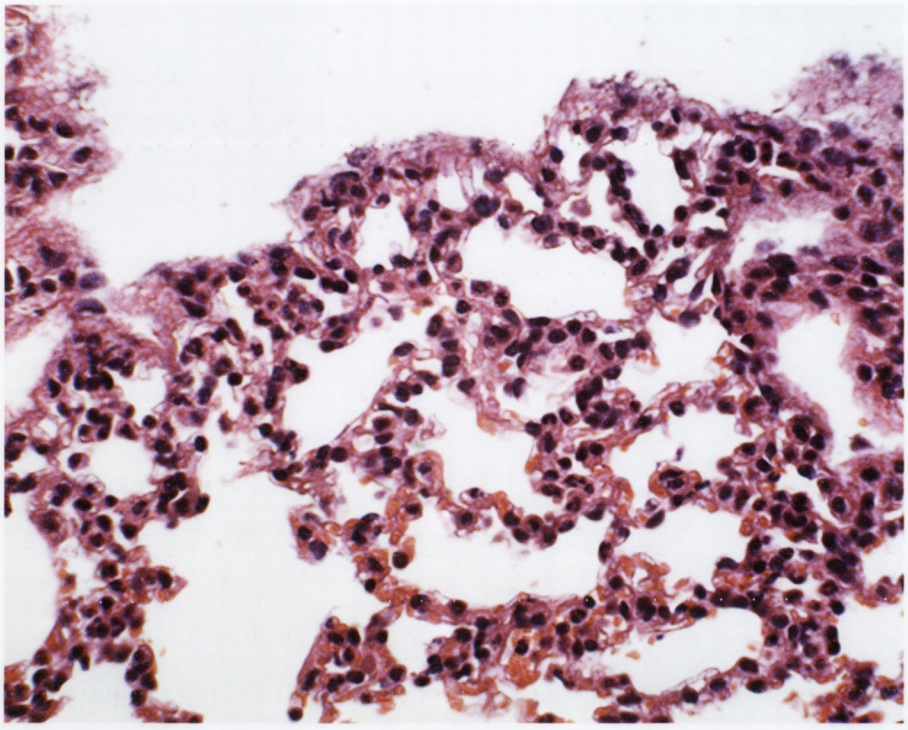
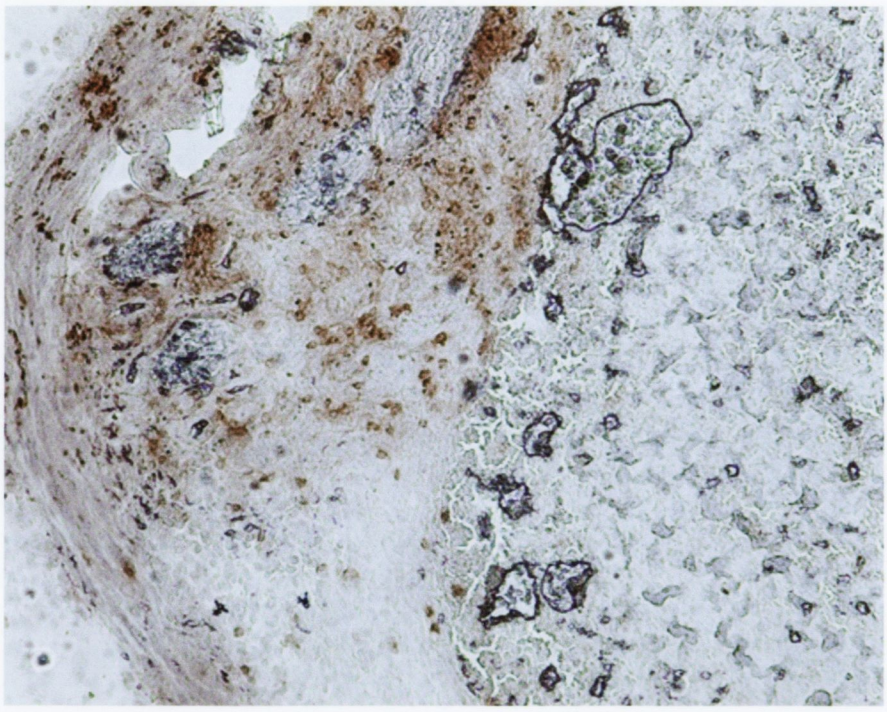


Figure 5. 6 Histopathology of rSFV10-E-IL12 treated 4T1 lung metastasis. rSFV10-E-IL12 treated mouse, 22 days post tumour induction and 8 days following treatment cessation showing absence of 4T1 lung metastases. Alveolar spaces are indicated (A). H&E, x 200.

a



b

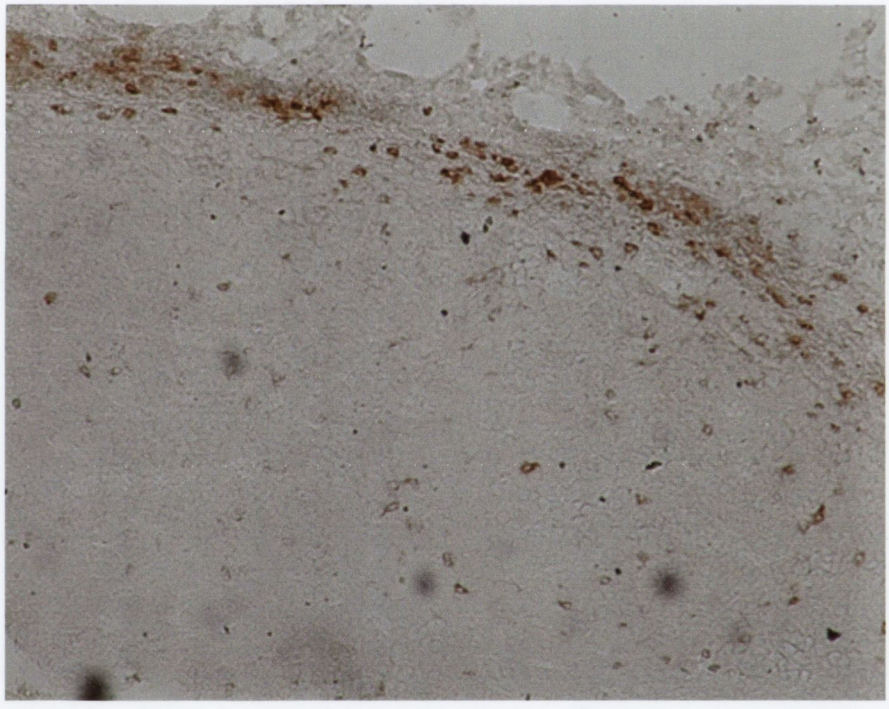


Figure 5.7 Detection of $CD4^+$ and $CD8^+$ T-lymphocytes in rSFV10-E-IL12 treated 4T1 tumours.

(a) CD4+ T-lymphocytes are located in the peritumoural stroma and necrotic areas.

(b) Small numbers of CD8+ T-lymphocytes are confined to the peritumoural stroma

Table 2 Inhibition of 4T1 tumour lung metastases following treatment with rSFV10-E-IL12

Treatment[a]	*Mouse No./ Tumour size (mm)*	*Lung metastases (No. of nodules)*[b] *= Avg.*	*No.of clonogenic lung metastases*[c]	*Average no. of lung metastases ± Std. error*	*P- Value*
TNE (Contol)	1/15	14	34747	35644 ± 6445	-
	2/15	23	35933		
	3/13	5 = 16	21432		
	4/15	17	50707		
	5/15	28	55723		
	6/13	9	15321		
rSFV10-EGFP	1/14	12	21796	22572 ± 10536	>0.05
	2/12	21	31646		
	3/11	3 = 13	1915		
	4/11	6	2006		
	5/15	25	69494		
	6/13	12	8572		
rSFV10-E-L12	1/11	4	6931	3039 ± 2004	<0.01
	2/0	0	0		
	3/0	0 = 2	0		
	4/13	5	11308		
	5/0	0	0		
	6/1	0	0		

[a]Mice received six intratumoral injections of rSFV VLPs ($4x10^9$ IU/dose) and were sacrificed on day 22 post-tumour induction; lungs were then analysed for metastases.

[b]Visible tumour nodules on the lung surface were counted and the average number was calculated for each group.

[c]The number of 6-thioguanine-resistant 4T1 lung metastasised colonies stained and counted for each mouse.

5.3 DISCUSSION

Previous reports have demonstrated the anti metastatic effect of IL-12 in several tumour models (Brunda *et al.*, 1993, Nastala *et al.*, 1994). One primary interest of this study was to determine whether IL-12 treatment is consistent in its antitumour effects in a metastasisng tumour model. The continued investigations of our study examined the anti-tumour effect of rSFV10-E-IL12 on a metastatic tumour model, 4T1 mammary carcinoma and also its ability to inhibit lung metastases through its known antiangiogenic effects. The 4T1 mammary carcinoma model has been already used in immunotherapy studies (Majumdar *et al.*, 2000).

We treated the established subcutaneous 4T1 tumour using six i.t injections of rSFV10-E-IL12 ($4x10^9$ IU/dose) and found complete tumour regression in four out of six mice (66.66%) indicating promising therapeutic effects. However, the reduced survival percentage of 4T1 tumour bearing mice compared to K-BALB or CT26 tumour bearing mice may be due to the reduced infectivity or replication kinetics of rSFV10-E-IL12 in 4T1 cells. This speculation is based on the previous results, which has demonstrated that due to the poor replication of HSV-1 1716 in 4T1 cells, the treatment was found moderately effective (Darby *et al.*, 2003). 4T1 cells are known to be very selective to host a replicating agents (Toda *et al.*, 1998). In addition, due to the pressure created by repeated intra-tumoural injections into a tumour of smaller size, leakage or the marginal transfer of tumour cells along with the injected material was found. Due to this, the tumour nodular structure was found distorted leading to local-tumour spread accounting for tumour escape from the treatment. Absence of lung metastases in the rSFV10-E-IL12 treated animals may show the antiangiogenic effects of IL-12 since an established vasculature is required for metastases to occur. Inhibition of metastases is associated with inhibition of vasculature development by antiangiogenic agents. Several studies show that angiogenesis inhibitors induce cell apoptosis associated with limited supply of nourishments and other cell survival factors derived from endothelial cells (Rak *et al.*, 1995).

Our immunohistochemical analyses have revealed that a massive accumulation of CD4+ and CD8+ T-cells was detected around the regressive 4T1 tumour mass. The observations of large necrotic areas in the IL-12 treated 4T1 tumour sections compared to control treated tumours also suggest the possibility angiogenesis inhibition.

Upon the examination of lungs to detect 4T1 metastases, a large number of 6-thioguanine resistant 4T1 colonies in the control treated animals were detected compared to rSFV10-E-IL12 treated animals, indicating the extent of established tumour vasculature among both the group of animals. Another observation during our study was that the number of lung metastases was correlated with the size of the tumour. This observation was confirmed when the lungs of large tumour bearing mice were detected with the maximum number of clonogenic metastatic 4T1 colonies where as IL-12 treated mice with smaller 4T1 tumours were found with very few lung metastases (Table 2). The visible tumour nodules on the lung surface indicated the advanced stage of the disease, which was also evidenced by the clinical signs of the animals.

Our results obtained in a murine spontaneous metastasis model illustrate that IL-12 expressed by the enhanced vector pSFV10-E is effective in inhibiting the development of micrometastasis. Thus, the present study could provide support for clinical application of the IL-12 immunotherapy.

6

IN VITRO CHARACTERISATION OF rSFV10-E-IL18 AND TREATMENT OF K-BALB AND CT26 TUMOURS

6.1 INTRODUCTION

Recently, IL-18 has been shown to be effective in the treatment of murine tumours with minimal side effects. This cytokine was initially termed IFN-γ-inducing factor based on high level IFN-γ secretion from NK and T cells at levels substantially greater than that observed with IL-12 (Okamura *et al.,* 1995). IL-18 is produced by cells of monocyte lineage, augments NK cytolytic activity, and enhances proliferation of T cells (Robinson *et al.,* 1997). IL-18 also promotes NK and T cells to secrete IFN-γ as well as granulocyte-macrophage CSF (GM-CSF) (Micaleff *et al.,* 1996). Although IL-18 and IL-12 synergise in IFN-γ production, their receptors and signal-transduction pathways appear to be different (Kohno *et al.,* 1997). These findings strongly suggest that IL-18 has significant immunoregulatory functions that could be modulated further using strategies that may involve site of expression, expression levels, or in combination with other therapeutic agents. The antitumour effect of IL-18 has been demonstrated in murine models of breast cancer, renal cell cancer, and melanoma (Tan *et al.,* 1998, Tanaka *et al.,* 2000) and is greatly enhanced by the addition of IL-12 or IL-2 (Nagai *et al.,* 2000). The induction of tumour regression by the combination of IL-18 and IL-12 has been shown to occur through an antiangiogenic mechanism that is IFN-γ dependent (Coughlin *et al.,* 1998).

Recently, IL-18 binding protein (IL-18BP), an inhibitor of IL-18 that is distinct from IL-18 receptor families, has been discovered. This protein has been shown to block IFN-γ production by IL-18 or IL-12 (Novick *et al.,* 1999). It is believed that IL-18 BP may play an important role in controlling immune responses by limiting the availability of IL-18 to activate lymphocytes in conjunction with IL-12. So far nothing is known regarding the control of IL-18BP expression by cytokines or the cell types responsible for the synthesis of IL-18BP. Two isoforms of murine IL-18BP have been isolated (Kim *et al.,* 2000). Furthermore, Korina G Veenstra *et al* (2002) have shown that those cytokines capable of synergising with IL-18 to stimulate IFN-γ production especially IL-12, are the primary inducers of IL-18BPa, an isoform of IL-18BP in monocytes. In addition, they have demonstrated that the induction of IL-18BPa by IL-12 is IFN-γ dependent, suggesting that IL-18BPa forms part of a negative feedback loop to limit immune activation by IL-12. In our study, we have examined the antitumour effect of rSFV10-E-IL18 on K-BALB and CT26 tumours.

The murine IL-18 gene was cloned into the enhanced expression vector pSFV10-E (Figure 6.1) by Ms. Susan McNally and was characterized in BHK-21 and K-BALB cells.

6.2 RESULTS

6.2.1 Quantitative analysis of IL-18 expression by rSFV10-E-IL18 in BHK-21 and K-BALB cells.

BHK-21 and K-BALB cells grown in 6-well plates were infected with rSFV10-E-IL18 replicons at an MOI of 10. The supernatants of the infected cells were collected at different time points (12 h, 24 h, 48 h and 72 h). An ELISA estimated the expressed IL-18 protein in the supernatants of both the cell lines. The results were plotted to measure the difference in the IL-18 expression levels by rSFV10-E-IL18 in both BHK-21 and K-BALB cell lines. The levels of IL-18 expression by rSFV10-E-IL18 in BHK-21 cells were found to be 2-3 times higher than K-BALB cells. Mock-infected control BHK-21 and K-BALB cells supernatant had no IL-18 expression (Figure 6.2).

6.2.2 Testing biological activity of secreted murine IL-18

Biologically active IL-18 induces T-cells to secrete interferon-γ (IFN-γ). An ELISA measured the production of IFN-γ from the 24 h supernatants of murine splenocytes treated with IL-18 supernatants of rSFV10-E-IL18 infected K-BALB cells. The result showed that the supernatant of K-BALB cells infected with rSFV10-E-IL18 replicons stimulated splenocytes to produce considerably higher levels of IFN-γ as the positive control, Concavalin A treatment, indicating the expression of functionally active IL-18 by rSFV10-E-IL18 replicons (Figure 6.3).

6.2.3 Intra-tumoural treatment of K-BALB and CT26 tumours using rSFV10-E-IL18 replicons.

K-BALB and CT26 tumours were induced on the right abdominal flank of female BALB/c mice. A group of six mice were used per treatment group. Treatment was initiated at an average tumour diameter of 4 mm. Tumours received six intratumoural injections of TNE alone, or TNE containing rSFV-EGFP or rSFV10-E-IL18 or rSFV10-IL12 VLPs at a titre of $4x10^9$ IU in 50µl/dose, on each alternate day. Treatment using rSFV-EGFP was used in the study as a control. Tumour growth was monitored by daily measuring the tumour diameter and mice were euthanised when average tumour diameter reached 15 mm. In this experiment, rSFV-EGFP showed some inhibitory effect on tumour growth during the treatment stage due to the inherent ability of rSFV VLPs to induce apoptosis in tumour cells. Two mice had complete tumour regressions in the IL-18 treated groups in both of the tumour models. None of the control treated groups showed complete tumour regression (Figures 6.4-6.7). Inhibition of tumour growth in the rSFV10-E-IL18 VLPs treated mice was not statistically significant ($P>0.05$) when analysed using Tukey's multiple comparison-test by one-way ANOVA (Table.3).

6.2.4 Tumour histopathology

Histopathological examination of H & E stained sections of control treated tumours were composed of densely cellular, expansile masses extending to the margins of the samples with no evidence of encapsulation. Invasion of subcutis muscle fibres was prominent (Figure 6.8). rSFV10-E-IL18 treated tumours showed areas of avascular and suppurative necrosis that are present in all of the tumours with substantial infiltrations of neutrophils, lymphocytes, and macrophages. Small foci of chronic inflammation and fibrosis in the subcutis were common (Figures 6.9 and 6.10).

pSFV10-E-IL18

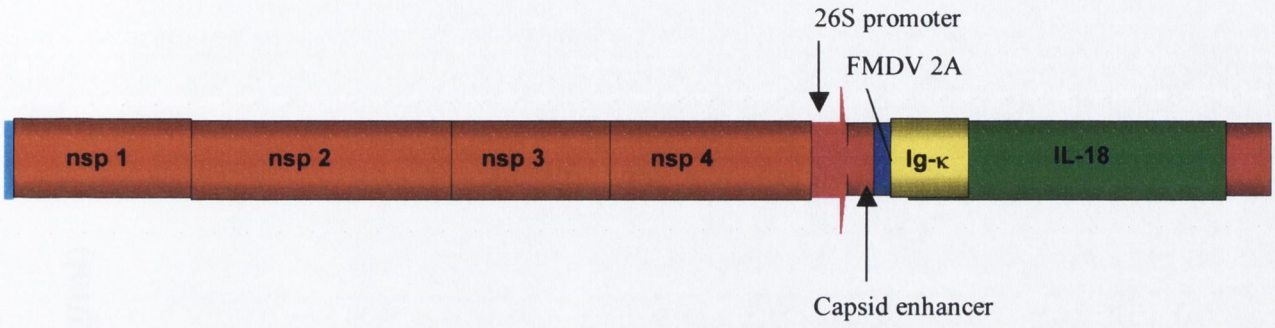


Figure 6.1 Schematic representation of the pSFV10-E-IL18 construct.

The coding sequences of IL-18 gene with upstream Ig-kappa transport signal sequences were cloned into the *HindIII* site in the MCS of pSFV10-E. The expression and secretion of IL-18 was regulated by the 26S sub-genomic promoter, Kosak, capsid enhancer and FMDV 2A cleavage-site of pSFV10-E. The data on the construction of pSFV10-E-IL18 is not shown in this report as Ms Susan McNally constructed it.

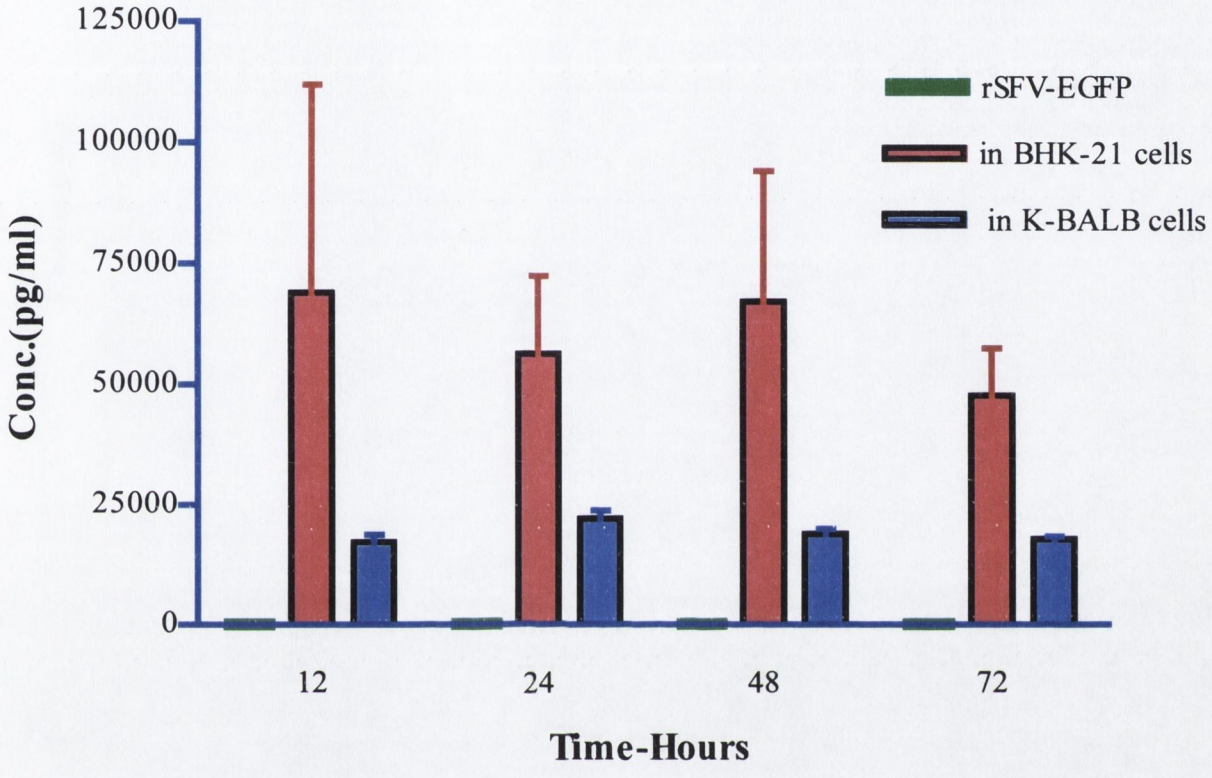


Figure 6.2 Quantitative analysis of IL-18 expression levels in the supernatants of rSFV10-E-IL18 VLPs infected BHK-21 and K-BALB cells

The expression levels of murine IL-18 by pSFV10-E-IL18 was examined in BHK-21 and K-BALB cells. An ELISA measured the expression levels at different time points in supernatants BHK-21 and K-BALB cells that were infected at an MOI of 10. The levels of IL-18 in the supernatants of infected BHK-21 cells were at higher levels than those of K-BALB cells. The expression levels of IL-18 in BHK-21 cells by rSFV10-E-IL18 were 2-3 times higher than in K-BALB cells. The supernatants from rSFV-EGFP or mock-infected BHK-21 or K-BALB cells showed no IL-18 expression. The values shown represent IL-18 secretion from $2.5x10^5$ cells.

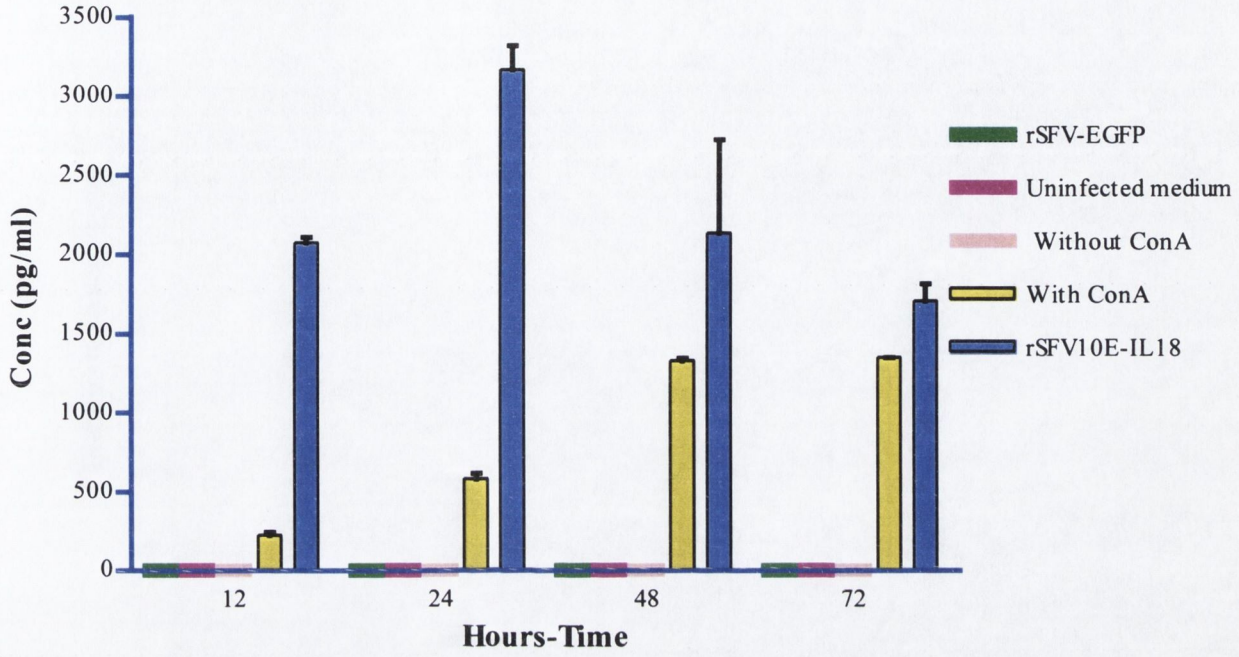
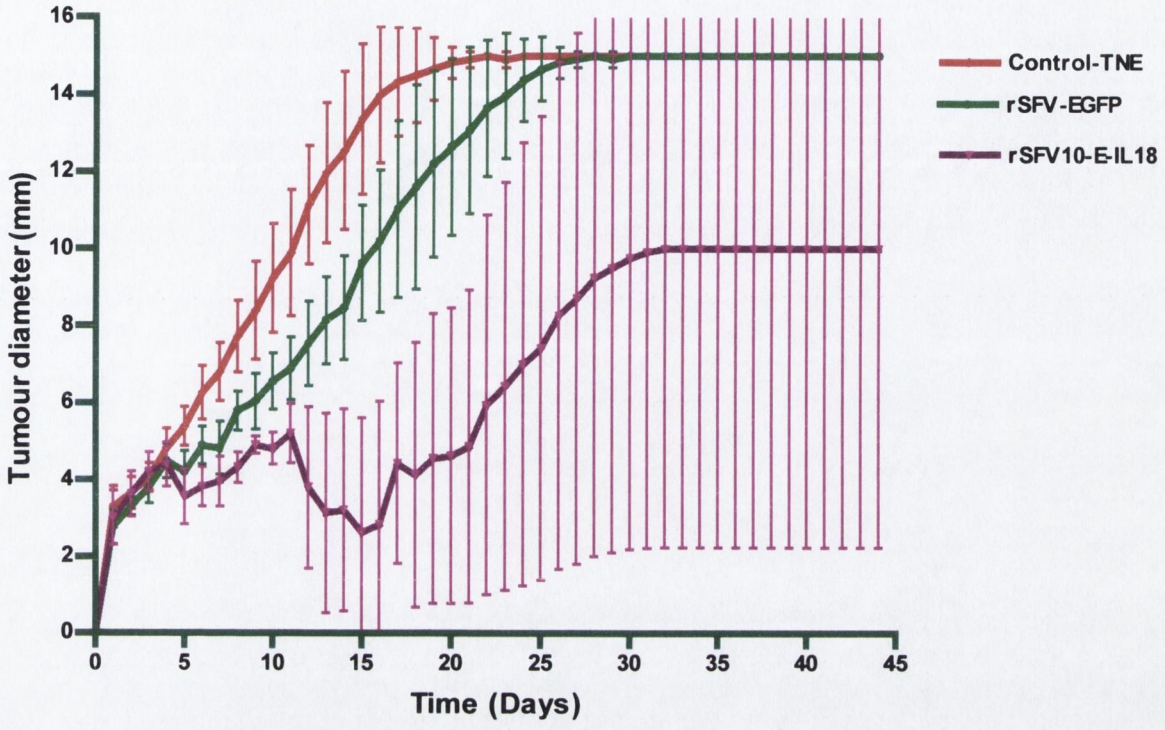


Figure 6.3 Biological activity of murine IL-18 secreted by K-BALB cells

Biologically active IL-18 is known to induce T-cells to secrete IFN-γ. An ELISA measured the production of IFN-γ from murine splenocytes ($1x10^6$) treated with 100 µl of 24 h supernatants from rSFV10-E-IL18 infected K-BALB cells. This showed that supernatants of infected K-BALB cells, containing IL-18, stimulated splenocytes to produce higher levels of IFN-γ than the positive Concavalin A (ConA) treated control. These results confirmed the expression and secretion of biologically active IL-18 from rSFV10-E-IL18. Supernatants from control groups (rSFV-EGFP, uninfected, and without conA) were unable to induce IFN-γ production from splenocytes.

a


Tumour diameter (mm)
Time (Days)
Control-TNE
rSFV-EGFP
rSFV10-E-IL18

b

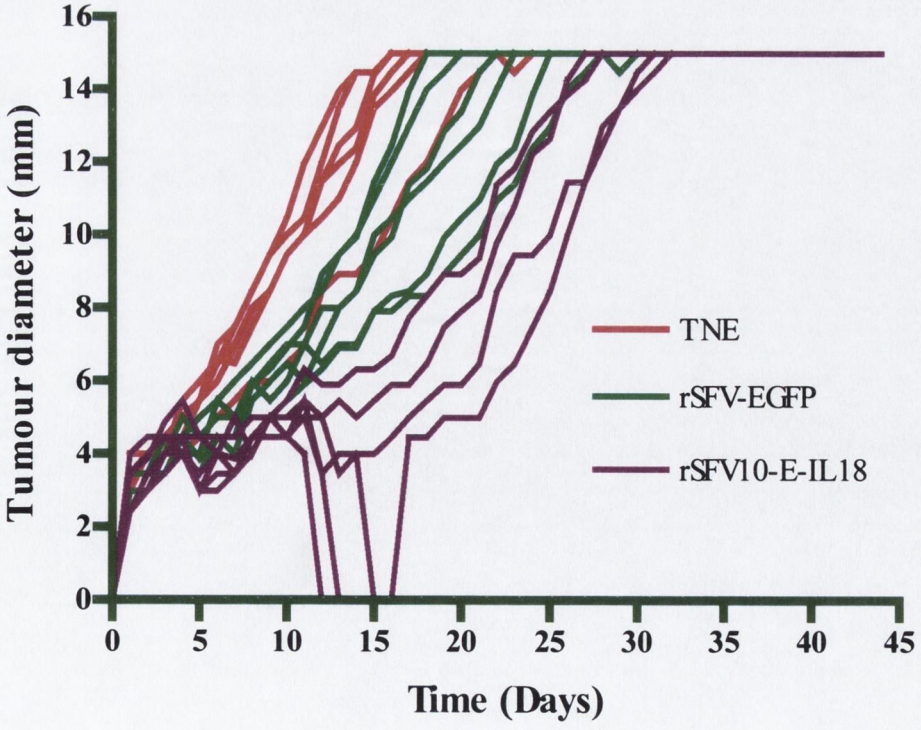

Tumour diameter (mm)
Time (Days)
TNE
rSFV-EGFP
rSFV10-E-IL18

Figure 6.4 Treatment of K-BALB tumours in BALB/c mice using rSFV10-E-IL18 replicons ($4x10^9$ IU/dose).

K-BALB tumours were induced on the right abdominal flank of female 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 at an average tumour diameter of 4 mm. Six mice were used per treatment group. Animals received six 50 µl intratumoural injections of TNE alone, or TNE containing rSFV-EGFP or rSFV10-E-IL18 at a concentration of $4x10^9$ IU /dose which was administered on each alternate day. Treatment using rSFV-EGFP was used in the study as a control group. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice in all the groups were euthanised when average tumour diameter reached 15mm.

The inhibition of tumour growth and complete regression of K-BALB tumours in two mice by rSFV10-E-IL18 treatment was statistically significant compared to the control treated groups.

Although three out of six mice among rSFV10-E-IL18 treated group had complete tumour regression, one of them re-grew its tumour 3 days following the primary tumour regression (indicated by the larger error bars). These results show that the rSFV10-E-IL18 can be used as antitumour agent (Figure 6.4).

Points; mean of six replicates, *bars*; +/- SEM.

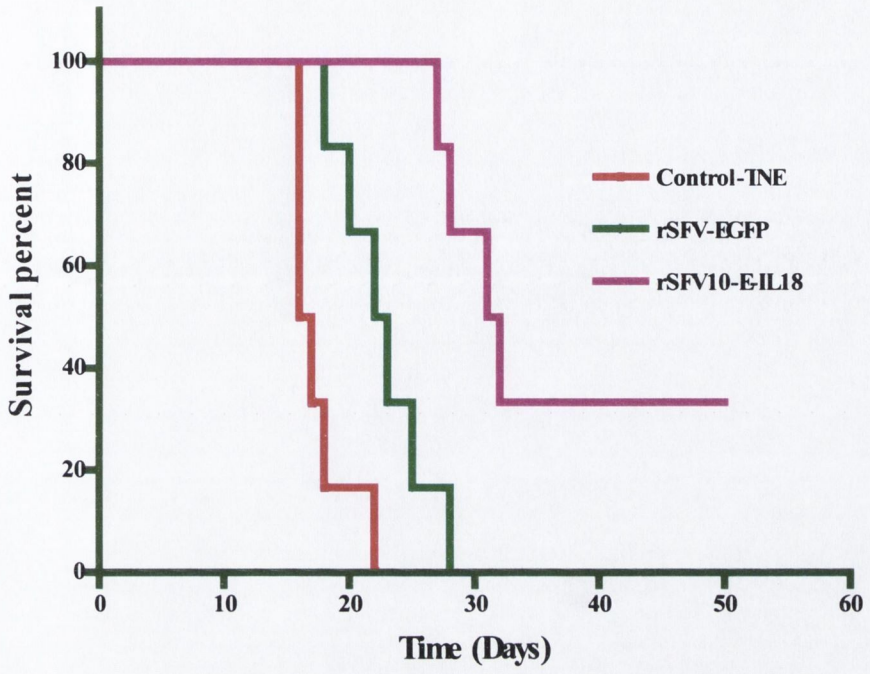
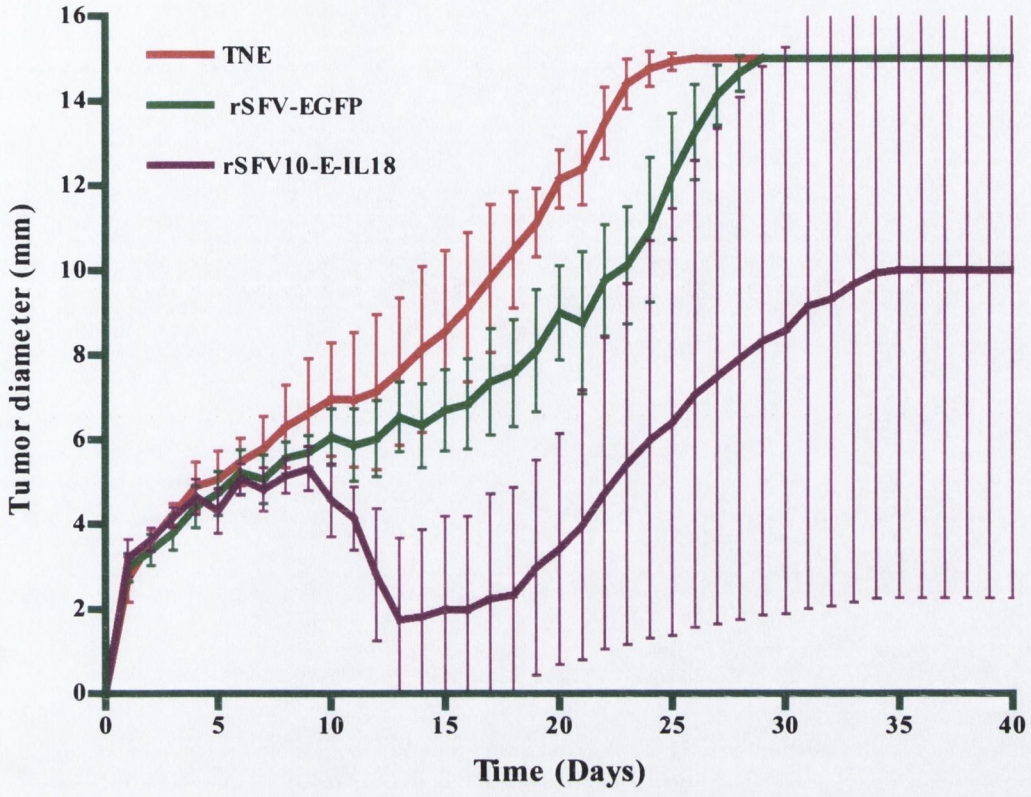


Figure 6.5 Survival proportions of the mice bearing K-BALB tumours treated using rSFV10-E-IL18 at $4x10^9$ IU/dose

Mice were sacrificed when the tumour size reached 15mm diameter. The average time taken to reach this size for a group of mice is the survival time. Mice, which had complete and permanent tumour regression are considered survivors. The survival of mice bearing K-BALB tumours was 33.3% when tumours were treated using six intratumoural injections of rSFV10-E-IL18. None of the control (TNE or rSFV-EGFP) treated animals showed tumour inhibition and had 0% survival proportions at the end of the treatment experiment.

b


TNE
rSFV-EGFP
rSFV10-E-IL18
Tumor diameter (mm)
Time (Days)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30
35
40

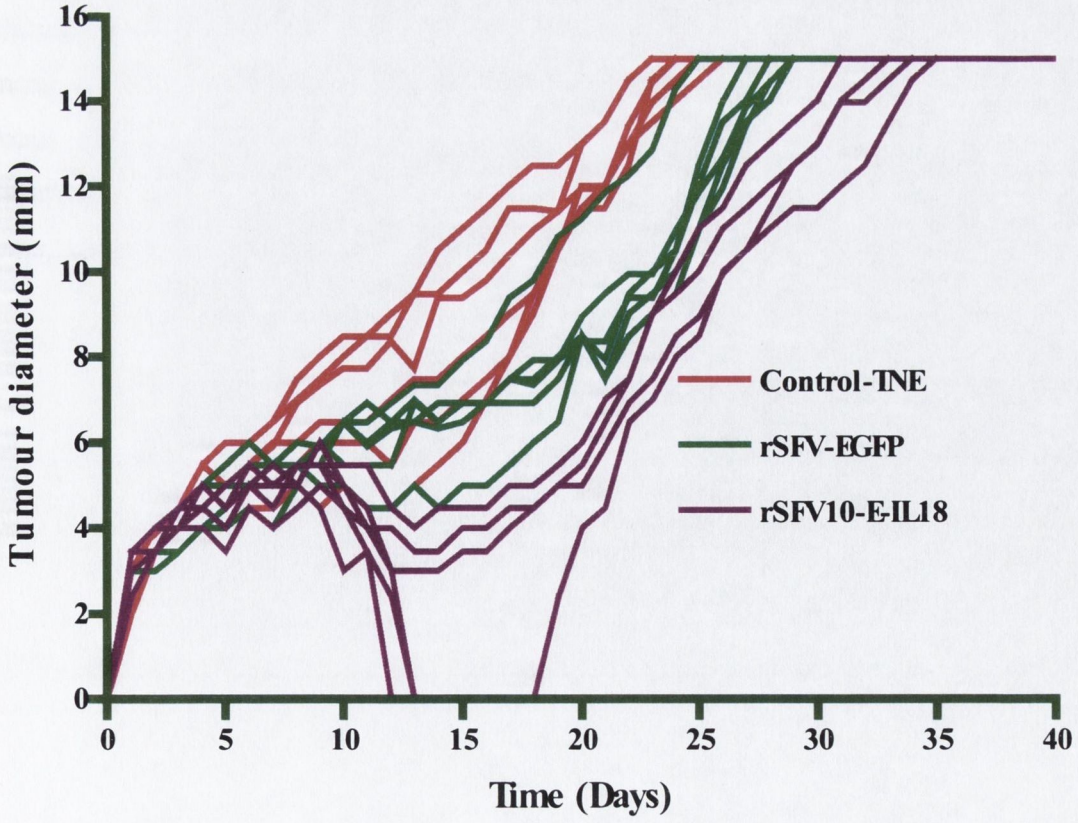

Control-TNE
rSFV-EGFP
rSFV10-E-IL18
Tumour diameter (mm)
Time (Days)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30
35
40

Figure 6.6 Treatment of CT26 colon carcinoma in BALB/c mice using rSFV10-E-IL18 replicons ($4x10^9$ IU/dose).

CT26 tumours were induced on right abdominal flank of female BALB/c mice by s.c. injection of 100 µl of CT26 cell suspension in non-supplemented DMEM at a concentration of 1 x 10^7 cells/ml. Treatment was initiated in individual tumours after an average tumour diameter of 4 mm was reached. A group of six mice was used per treatment group. Tumours received six 50 µl intratumoural injections of TNE alone, or TNE containing rSFV-EGFP, or rSFV10-E-IL18 at a concentration of $4x10^9$ IU /dose which was administered on each alternate day. Treatment using rSFV-EGFP was used in the study as a control group. Mice in all the groups were euthanised when average tumour diameter reached 15 mm.

The inhibition of tumour growth and complete regression CT26 tumours in two mice by rSFV10-E-IL18 treatment compared to control treated groups was observed. Although three out of six mice among rSFV10-E-IL18 treated group had complete tumour regression, one of them re-grew its tumour few days following the primary tumour regression. These results show that the rSFV10-E-IL18 can be used as antitumour therapeutic agent (Figure 6.6)

Points; mean of six replicates, *bars*; +/- SEM.

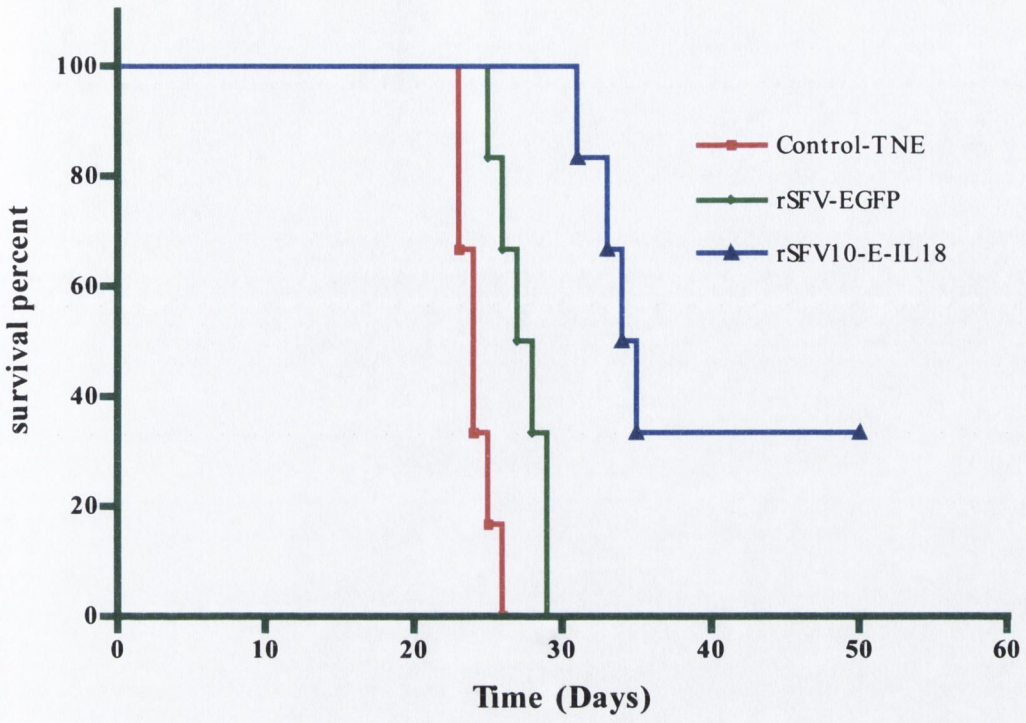


Figure 6.7 Survival proportions of the mice bearing CT26 tumours treated using rSFV10-E-IL18 at $4x10^9$ IU/dose

Mice were sacrificed when the tumour size reached 15mm diameter. The average time taken to reach this size for a group of mice is the survival time. Mice, which had complete and permanent tumour regression are considered survivors. The survival of mice bearing CT26 tumours that were treated with IL-18 was 33.3% (similar to K-BALB tumour survivors) whereas control (TNE or rSFV-EGFP) treated groups had almost similar tumour growth with 0% survival proportions at the end of the treatment experiment.

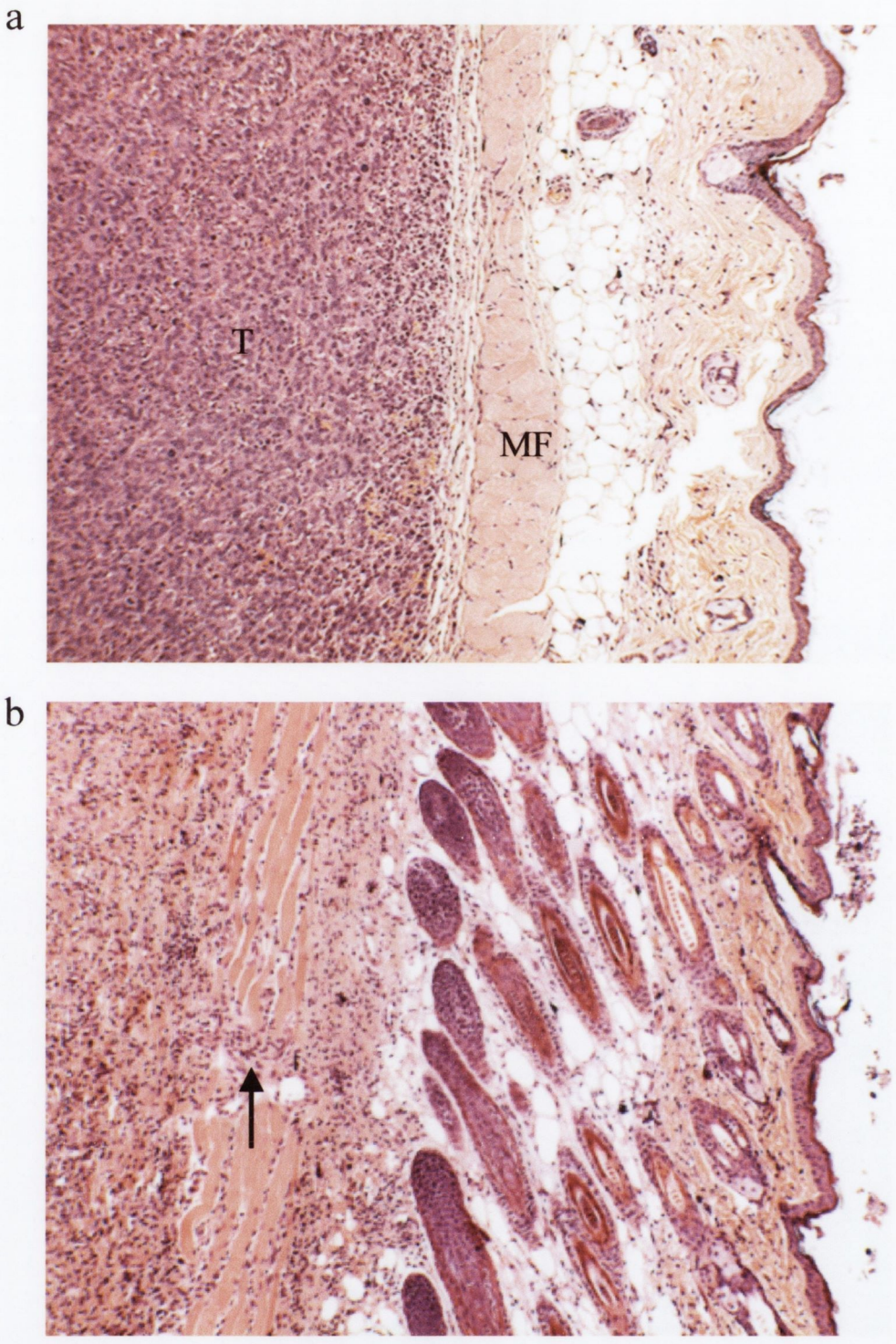
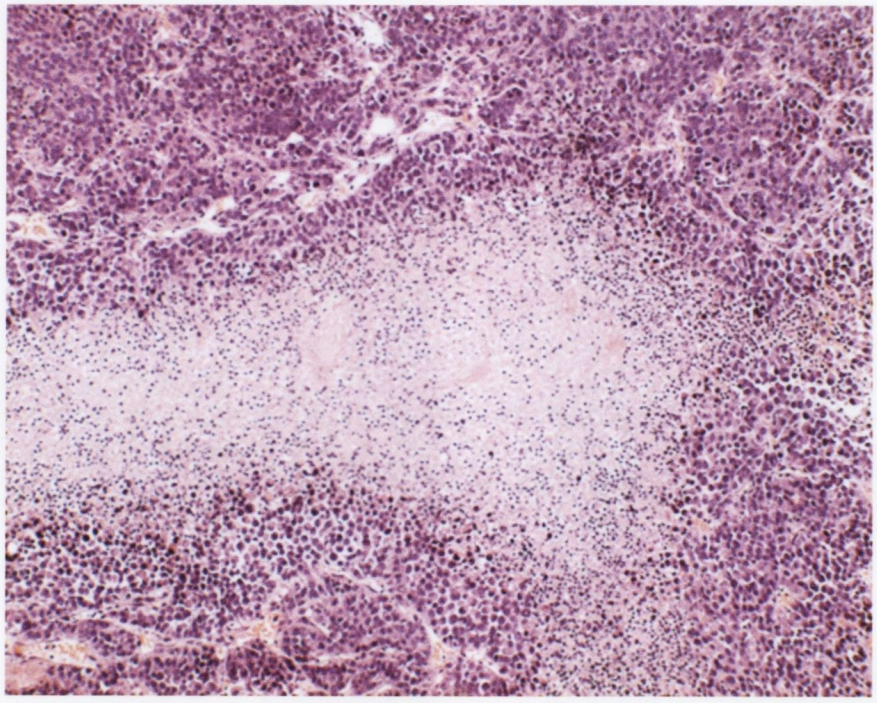


Figure 6.8 K-BALB tumours, one-day post treatment with TNE.

(a) Migration of tumour (T) cells towards the subcutis through the intact layers of epidermal tissue. Muscle fibre (MF) layer is indicated. H&E, x 100.

(b) Aggregation of tumour cells surrounding fibrovascular connective tissue in the cuticular region. Tumour cells passing through compressed panniculus muscle fibres are shown (arrow). H&E, x 100.

a



b

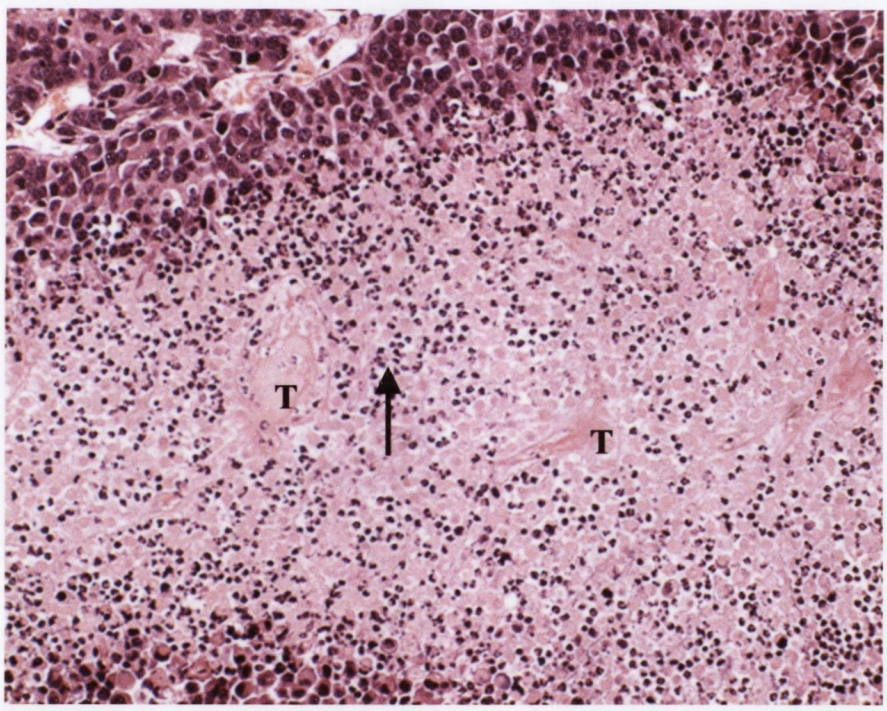


Figure 6.9 CT26 tumours, one-day post treatment with rSFV10-E-IL18.

(c) Tumour with area of avascular and suppurative necrosis. H&E, x 100.

(d) Higher magnification of (a) showing area of suppurative necrosis, throbosis (T), and densely infiltrated with neutrophils (arrow). H&E, x 200.

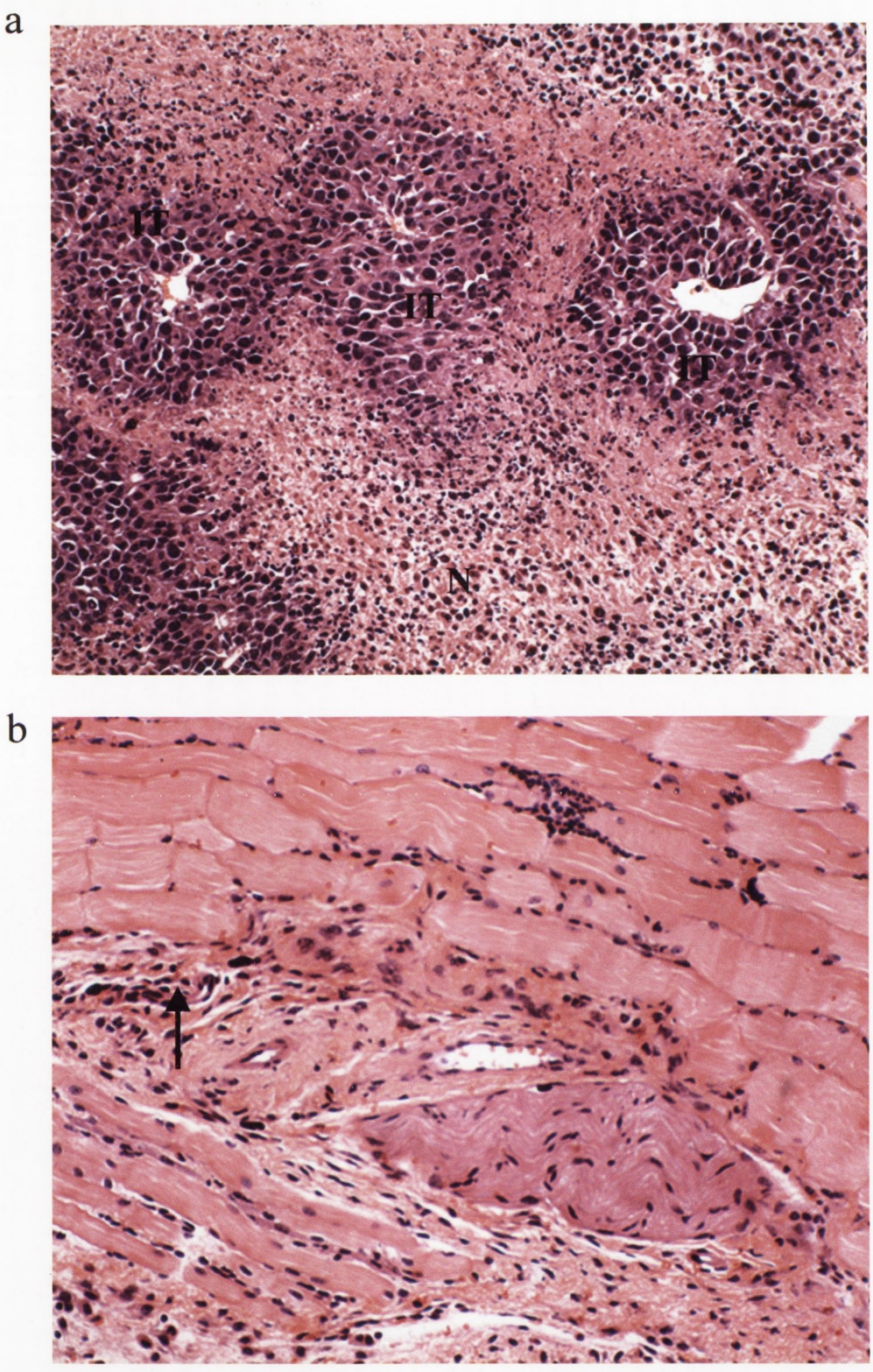


Figure 6.10 K-BALB tumours, one-day post treatment with rSFV10-E-IL18.

(a) Islands of tumour (IT) cells surrounding blood capillaries. The tumour cells well nourished by the blood capillaries where as marginal cells are undergoing necrosis. (b) Small foci of chronic inflammation and fibrosis in subcutis (arrow). No tumour cells seen. H&E, x 200.

Table 3 Inhibition of tumour growth by rSFV10-E-IL18 treatment

Tumour Model	*Treatment groups*[a]	*Median survival (days)*[b] *(No. of survivors*[c]*)*	*Average. tumour diameter*[d] *(mm) (Std.error)*	*Probability*[g]
K-BALB	TNE[e]	22 (0)	11.9 (+/- 0.66)	
	rSFV-EGFP ($4x10^9$IU/dose)	23 (0)	11.4 (+/- 0.65)	>0.05
	rSFV10-E-IL18 ($4x10^9$IU/dose)	32 (2)	6.3 (+/- 0.42)	>0.05
CT26	TNE	24 (0)	11.0 (+/- 0.67)	
	rSFV10-EGFP ($4x10^9$IU/dose)	28 (0)	9.8 (+/- 0.69)	>0.05
	rSFV10-E-IL18 ($4x10^9$IU/dose)	35 (2)	5.9 (+/- 0.45)	>0.05

[a]Six BALB/c mice per treatment group were used for all experiments.

[b]Mice were sacrificed when the tumour reached 15 mm diameter and the time taken to reach this size is the survival time.

[c]Mice which had complete and permanent tumour regression; in a few cases a residual small nodule remained that was composed entirely of non-malignant scar tissue.

[d]Average tumour diameter for each group was calculated at their appropriate median survival time.

[e]Average of the control (TNE) treated groups in the three different K-BALB tumour treatment experiments with different titres of VLPs.

[g]The probability value is based on the average tumour diameter at their median survival time compared with that of the TNE treated (control) groups.

6.3 DISCUSSION

Several cytokine genes introduced intratumourally in mice have shown antitumour effects. The strong antitumour property of IL-12 and IL-18 has been previously reported using murine renal cell carcinoma and bladder cancer models (Yamanaka *et al.,* 1999, Hara *et al.,* 2000). Powerful antitumour effects of combination therapy of the IL-12 gene introduced cells and systemic administration of recombinant IL-18 against established tumours have been reported suggesting synergistic antitumour effects. In our study we analyzed the antitumour effect of a rSFV10-E-IL18 replicons in order to examine and compare the antitumour efficacy of IL-12 and IL-18 expressed by rSFV10-E separately or in combination, the data of which is not shown.

For an effective IL-18 gene therapy it may be essential to construct a vector that secretes high levels of bioactive IL-18. IL-18 is first synthesised as a leaderless precursor, and it requires a conventional signal sequence for extra cellular secretion of mature IL-18. Ms. Susan McNally, a project student, constructed a pSFV10-E-IL-18 vector that contains the Igk (Immunoglobulin Kappa) leader sequence, which regulates the expression of mature IL-18. It has been reported that the dose of IL-18 administered correlates with the resulting level of serum IFN-γ and the strength of its antitumour activity (Osaki *et al.,* 1998). Direct injection of a mature IL-18 adenoviral vector into mice with MCA205 elicited an effective immune response (Osaki *et al.,* 1999). In this study we have *in vitro* characterized rSFV10-E-IL18 replicons to examine the expression levels of IL-18 in BHK-21 and K-BALB cells. The levels of IL-18 secreted in the supernatants of infected BHK-21 cells were found at higher levels than those of K-BALB cell supernatant. This difference in expression levels might be attributed to variability in infection expression and extracellular secretion of the expressed protein with different cell lines. To determine the bioactivity of the secreted IL-18, we induced splenocytes for IFN-γ production and the results confirm that IL-18 produced by rSFV10-E-IL18 replicons in K-BALB cells is biologically functional. The IL-18 construct with enhanced expression ability, which contains the Ig/kappa leader sequence induced secretion of large amounts of IFN-γ (approximately 1500 pg/ml). These observations are in agreement with the findings of a previous

study that has used a non-enhanced expression vector along with pro-IL18 sequences that contains the Ig-kappa leader sequence (Yoshimura *et al.*, 2001).

A previous study has demonstrated that the local secretion of IL-12 or IL-18 could lead to the complete tumour rejection as well as metastases inhibition (Satoko *et al.*, 2004). In this study, we examined the *in vivo* antitumour effect of IL-18 alone or in combination with IL-12 in two different tumour models. The results of our experiments in immunocompetent BALB/c mice have showed a similar antitumour effect in the animals treated with IL-18 alone or in combination with IL-12. The tumour inhibition due to the treatment effect between these two groups was statistically not significant.

In addition, we showed that the treatment of K-BALB and CT26 tumours using rSFV10-E-IL18 replicons induced tumour growth inhibition, which is statistically significant compared to the control groups. Although three out of six mice had complete tumour regression in the IL-18 treated group, the data on survival proportions is statistically not significant. Perhaps, these results could be further analysed by increasing the number of animals used per treatment group.

Further, histopathological analysis of IL-18 treated tumours showed no comparable difference to IL-12 treated tumours. The recurrence of a tumour in one completely regressed mouse indicated the incomplete clearance of the tumour even after six intratumoural treatments. In order to achieve an efficient antitumour effect, the optimization of IL-18 concentrations at the tumour site may needs to be optimized in further. The observations of this study support the previous reports that IL-12 and IL-18 molecules perform similar biological functions.

7

GENERAL DISCUSSION

7.1 GENERAL DISCUSSION

Gene therapy has potentially expanded the limits of tumour therapy by targeting tumour angiogenesis inhibition or recruiting immune cells. This can be achieved by the expression of high or critical concentrations of therapeutic agents within the tumour by repeated intra-tumoural administrations. In *ex vivo* gene therapy, gene transfer is performed in cells that have been removed from the patient. Once the therapeutic gene has been introduced into these cells they are transferred back to the patient. Cytokine expression at the tumour site has allowed better anti-tumour effects with reduced cytotoxicity (Gately *et al.,* 1992). The use of immuno-stimulatory cytokines as tumour therapeutic agents has become a promising strategy for cancer therapy. Strategies that provide high levels of immunostimulatory cytokines locally at the site of the tumour have already demonstrated therapeutic efficacy (Rodriguez-Madoz *et al.,* 2005).

The cytokine IL-12 has given many hopes as a tumour therapeutic agent because of its demonstrated potent antitumour and antimetastatic activities (Brunda *et al., 1993)* in different tumour models. It has been shown that IL-12 promotes the activity and differentiation of T-cells and NK-cells to induce an aggressive anti-tumour immune response. In addition, another effective mode of tumour inhibition by IL-12 is in its ability to induce IFN-γ production by T-cells, which in turn mediates anti-angiogenic effects (Sgadari *et al.,* 1996). A previous study has shown that the amount of IL-12 available at the tumour site is critical for tumour regression and evidence has been provided showing that maximum anti-tumour effects can be achieved by transient IL-12 expression systems instead of prolonged high-dose treatment (Colombo *et al.,* 1996).

Successful gene therapy mainly depends on the properties of gene delivery systems mainly its antigenicity, bio-safety and its ability to express a functionally active product. The Semliki Forest virus vector is a transient suicidal expression vector system with less immunogenicity (Lundstrom *et al.,* 2001). The bio-safety of SFV has been improved by modifying the original vector into a split vector system, which restricts the vector to undergo only one round of replication in the host cells and reduces the frequency of recombination between helper and vector (Liljeström *et al.,* 1999). This vector has been previously used in vaccine studies to induce

protection against louping ill virus (Fleeton *et al.*, 2000) and in the expression of many other proteins including IL-12 (Colmenero *et al.*, 1999, Asselin-Paturel *et al.*, 1999).

The recent development of pSFV10-E, a new SFV based expression vector system with enhanced expression of foreign protein (up to ten fold higher than the original pSFV10 vector) has provided a valuable tool to examine the anti-tumour effects of IL-12 expression locally at the tumour site. Hence, we started this study with the construction of pSFV10-E-IL12 and pSFV10-IL12. Since our study included the mouse tumour models, we isolated the IL-12 gene subunits from mouse splenocytes. IL-12 is a heterodimer composed of two subunits, p35, and p40, encoded by separate genes. The expression of biologically active IL-12 requires co-ordinated expression of both subunits in equal proportions. If both subunits are expressed at different levels, biologically inactive p40 homodimers may be produced. Normally cells producing IL-12 secrete more free p40 chain than biologically active p70 heterodimer. This can range up to 100-1000 times more. Secretion of recombinant p40 IL-12 either as disulfide-bonded homodimer or as a monomer in mouse has been observed whereas no p40 homodimers have been reported so far in the human. Murine p40 homodimers bind to the IL-12Rβ1 chain with an affinity similar to that of the heterodimers for binding to the IL-12 receptors, effectively blocking the biological functions of IL-12 on murine cells. Thus, in mouse IL-12 p40-homodimers may represent a physiological antagonist of IL-12 p70. To avoid this possible antagonism of p40-homodimers against the biologically functional heterodimeric IL-12 molecule, we cloned the component p40 and p35 subunits into single vectors (pSFV10-E and pSFV10), each expressed from separate 26S promoters to achieve the equal proportionate expression of both the subunits.

Expression and secretion levels of IL-12 by pSFV10-E and pSFV10 constructs were tested *in vitro* in BHK-21 and K-BALB cells using the infectious virus like particles from these constructs. When the cells were infected at a multiplicity of infection (MOI) of 10, expression levels of IL-12 from rSFV10-E-IL12 infected K-BALB cells was almost a ten-fold higher than that of rSFV10-IL12 infected cells, whereas in BHK-21 cells about a 3-4-fold difference was observed. These results confirm the enhanced expression ability of pSFV10-E compared to original pSFV10 vector. This was also confirmed qualitatively by staining intensities of the infected cells by immunofluorescence and DAB staining. Although SFV replicons have the

capacity to infect a variety of cell lines *in vitro*, very little is known about their infectivity *in vivo* (Lundstrom *et al.*, 1999, Wahlfors *et al.*, 2000). A difference in the expression levels in K-BALB and BHK-21 cells reflects the infection variability of the replicons with different cell lines and/or the expression ability of the cell lines. This observation was in agreement with a previous study on K-BALB cells and supports previous observations (Smyth *et al.*, 2004).

As the mIL-12 specific antibodies may also recognise the P40 homodimers, it was essential to test the biological function of the secreted IL-12. The biologically active IL-12 induces T-cells to produce IFN-γ. To confirm secretion of the expressed protein and its biological activity, we measured the ability of supernatants from VLP infected cells to induce IFN-γ production by mouse splenocytes along with a positive control Con A (a known inducer of IFN-γ production), before employing these constructs for the *in vivo* tumour treatment studies. The expressed IL-12 in the supernatant by the infectious virus like particles of both the constructs in BHK-21 and K-BALB cells induced splenocytes for IFN-γ production that was quantified by an ELISA, indicating the expression and secretion of functionally active IL-12 molecule. IFN-γ levels induced by rSFV10-E-IL12 and rSFV10-IL12 infected K-BALB and BHK-21 supernatants were found to be similar. This may be due to saturated levels of IL-12 in the supernatant that was used to induce a limited number of splenocytes. As this assay was only to test the bioactivity of the secreted IL-12 by the rSFV-IL12 constructs, the amount of secreted IFN-γ was not critical to differentiate the expression levels of these two IL-12 constructs.

Our *in vivo* tumour treatment studies involved the intra-tumoural treatment of fast growing, poorly immunogenic localised K-BALB and CT26 tumour models in immuno-competent BALB/c mice using rSFV10-E replicons expressing murine IL-12. Further, we also examined the treatment effect on a lung metastatic 4T1 tumour model. The in vivo antitumour effect of rSFV10-E-IL12 and rSFV10-IL12 on the K-BALB tumour was compared in immunocompetent BALB/c mice and confirmed the enhanced antitumour effect associated with the enhanced expression vector.

It should be noted that the majority of the previous studies have used exclusively the administration of a single immunotherapeutic agent. Even after the gene, vector and expression system have been optimized, the route through which a gene therapeutic is administered influences the therapeutic effect of the product.

Dosing regimens for gene therapy products are likely to require treatment every few weeks or every few months. Recent studies have demonstrated that multi-modality therapeutic approaches may yield additive or synergistic antitumour effects, because of their increased ability to stimulate immune functions required for tumour targeting and/or counteract mechanisms utilised by tumour cells to escape from immune recognition and destruction. Our data clearly shows that six intra-tumoural treatments of K-BALB tumours using lower titres of rSFV10-E-IL12 replicons resulted in significant tumour growth inhibition. An improved effect, however was found using an increased dose. Thus, there is a progressive anti-tumour effect with increased concentrations of IL-12 replicons. Since a high systemic concentration of IL-12 has been reported to have lethal toxic effects in animals (Sarmiento *et al.,* 1994), it was essential for us to optimise the threshold concentrations of IL-12 to treat the animals. In our study, some clinical symptoms (shivering and fever) were observed with animals that were treated with high doses of rSFV10-E-IL12 VLPs after five intratumoural injections. These observations restricted our study from investigating the antitumour effects of systemic treatment with rSFV10-E-IL12 VLPs. Oshikawa *et al.* recently found that IL-12 cDNA delivered s.c. into the opposite lateral side of the tumour site, was less efficient in inducing tumour regression than local treatment. Complete and permanent K-BALB tumour regression was found in all the six mice (100%) treated with rSFV10-E-IL12 replicons at a titre of $4x10^9$ IU/dose, indicating the increased levels of IL-12 at the tumour site induce an efficient anti-tumour effect compared to rSFV10-IL12, without inducing lethal toxic effects. The consistency of the above treatment effect was demonstrated using another localised tumour model, the CT26 colon carcinoma.

IL-12 promotes the activity and differentiation of T-cells and NK-cells to induce an aggressive anti-tumour immune response. It has been suggested that IL-12 promotes the development of Th1 cells (Hsieh *et al.,* 1993), and these in turn may positively regulate the expansion and/or activation of other lymphoid cell populations. In addition, a further anti-tumour activity of IL-12 is its ability to induce IFN-γ production by T-cells, which in turn mediates anti-angiogenic effects (Manetti *et al.,* 1994, Sridhar *et al.,* 2004). It is likely that the potent anti-tumour activity of the IL-12 expressing SFV vector results from a combination of mechanisms. SFV vectors have an inherent p53-independent apoptosis inducing capability (Glasgow *et*

al., 1998), which has been successfully used to treat tumours in nude mice (Toda *et al.*, 1998, Murphy *et al.*, 2000) and it is likely that this operates also for the vectors used in this study. However, the presence of the enhancer in rSFV10-E-IL12 is unlikely to increase this inherent apoptosis induction since all cells infected by the unenhanced vector undergo apoptosis and the vector does not spread intercellularly (Glasgow *et al.*, 1998, Toda *et al.*, 1998). The enhanced ability of this vector to destroy tumour cells must therefore result from the induction of enhanced IL-12 secretion. IL-12 also has a potent pro-inflammatory activity that may also contribute to the anti-tumour effect. Another anti-tumour mechanism that may operate is the inhibition of tumour angiogenesis by IL-12, which has been demonstrated in a previous study of tumour treatment by an IL-12 expressing SFV vector (Asselin-Paturel *et al.*, 1999).

It was evident from the tumour pathology and immunohistochemistry that the intratumoural IL-12 expression using rSFV10-E-IL12 replicons has induced the cytotoxic activities of B- and T-lymphocytes that are considered as crucial for IL-12 induced anti-tumour effects. Detection of an increased number of infiltrating CD4+ and CD8+ T-lymphocytes in the IL-12 treated K-BALB and CT26 tumours compared to the control tumours demonstrates a tumour targeted immune response induced by IL-12 treatment and strongly suggests the critical requirement of T-lymphocytes to promote more efficacious antitumour function. Histopathological examination of tumour sections during the treatment stage showed an intact tumour nodule with actively growing tumour cells in control treated mice whereas in most of the IL-12 treated K-BALB and CT26 tumours, massive areas of necrosis throughout the tumour nodules were observed. Variations in the tumour size (tumour swelling) during the treatment stage may correlate with the influx of a wide range of immune cells leading to inflammatory swelling of the IL-12 treated tumours. Detection of increased IP-10 concentrations in the blood serum of the animals treated with rSFV10-E-IL12 just after the last treatment may account for an anti-tumour effect through the inhibition of angiogenesis. This conclusion was in agreement with previously demonstrated results showing that IP-10 is a downstream molecule primarily responsible for IL-12 mediated tumour vasculature inhibition (Luster *et al.*, 1995, Sgadari *et al.*, 1996). The IL-12 induced anti-angiogenic effect was further detected by staining the control or Il-12 treated tumour sections for endothelial cells of the tumour vasculature using anti-mouse CD31 antibodies. The observation of damaged and reduced numbers of blood

capillaries in the IL-12 treated tumour sections compared to the increased density of intact and healthy vasculature of control treated tumours demonstrated morphological evidence of IL-12 induced anti-angiogenesis. The induced anti-angiogenic effects of IL-12 provides the reason for the presence of large necrotic areas observed in the IL-12 treated tumours that might have resulted from undernourishment of the tumour. However, complete regression of established K-BALB and CT26 tumours was permanent, which was indicated by the disease-free survival of the animals for 60-90 days. Therefore, it is concluded that the success of IL-12 treatment depends on the number of activated T-lymphocytes and NK cells that infiltrate into the tumour and also its ability to induce an anti-angiogenic effect at the tumour site. However, further investigation is required to elucidate the precise mechanisms that are involved in the antitumour activity of IL-12.

Interestingly, tumour therapy using rSFV-EGFP replicons that were used as virus control showed significant tumour inhibition compared to the control (TNE buffer) treated groups. These observations were also reported in a previous study in which human lung carcinoma cell growth was inhibited by apoptosis induction using Semliki Forest virus recombinant particles (Murphy *et al.*, 2000). In our study, the enhanced treatment effect observed in IL-12 treated animals compared to rSFV-EGFP treated groups with the tumour cell lines indicates that the viral oncolytic (inherent ability to induce apoptosis) property that was observed in the above study alone is not sufficient for complete tumour eradication and strongly supports the IL-12 induced antitumour effect.

We also examined the anti-tumour effect of rSFV10-E-IL12 on a metastatic 4T1 tumour model and its ability to induce lung metastasis inhibition. The antitumour mechanisms of IL-12 against some poorly immunogenic tumours have been attributed to antiangiogenic effects (Voest *et al.*, 1995). Absence of lung 4T1 metastases in the rSFV10-E-IL12 treated animals compared to the lungs of control treated animals may indicate failure of tumour vasculature establishment and inhibition of metastases may be associated with vascular disruption by antiangiogenic agents. Several studies show that angiogenesis inhibitors induce cell apoptosis associated with limited supply of nutrients and other cell survival factors derived from endothelial cells (Rak *et al.*, 1995). The number of lung metastases was also correlated to the size of the tumour at the time of metastastic assay. The lungs of large tumour bearing mice had the maximum number of clonogenic metastatic 4T1 colonies compared to the lungs of IL-

12 treated animals which had smaller 4T1 s.c. tumours (Table 2). Advanced techniques or assays need to be developed to estimate more accurately the number of lung metastases, as the clonogenic assay is not an efficient technique to accurately measure the number of lung metastases.

All these results have shown that the cumulative effects of intratumoral treatment with rSFV10-E-IL12 replicons effectively inhibit tumour growth in three murine tumour models. These results confirm those of other studies that have used IL-12 expressing SFV vectors for the treatment of other tumour models (Zhang *et al.*, 1997, Colmenero *et al.*, 2002, Yamanaka *et al.*, 2003). However, we have also shown that a combination of the use of high titre vector preparations and a vector expressing high levels of IL-12 can result in complete and permanent tumour regression. This treatment also inhibits the growth of lung metastases in a metastatic tumour model.

In this study, we also studied the *in vivo* antitumour effect of IL-18 expressed by rSFV10-E on K-BALB and CT26 tumour models. The biological function of secreted IL-18 was assayed using mouse splenocytes. A previous study has demonstrated that the local secretion of IL-18, IL-12 or both could lead to the complete tumour rejection as well as metastases inhibition (Satoko *et al.*, 2004).

Complete regression of K-BALB and CT26 tumours in two out of six mice following intratumoural treatment using rSFV10-E-IL18 at a titre of $4x10^9$ IU/dose, indicated the antitumour effect of IL18. We also studied the antitumour effect of IL-18 in combination with IL-12 in K-BALB and CT26 tumour models, but the tumour inhibition in combination treated animals showed no statistical difference compared to the IL-12 treatment (data not shown). Histopathological examination of IL-18 treated tumours showed similar observations to that of IL-12 treated tumours indicating similar biological functions of these molecules. However, this study demonstrated that rSFV10-E-IL12 is more efficient antitumour agent than IL-18 when expressed by rSFV10-E.

7.2 Future work and directions

The results of our experiments support and add to the body of existing data on the *in vivo* bio-safety and anti-tumour therapeutic efficacy of locally administered rSFV10-E replicons expressing the cytokine IL-12. In designing treatment strategies for clinical trials in humans, the use of the high titre and high expression recombinant particle system used in our study should be considered in order to achieve an efficacious antitumour effect. The inherent ability to induce apoptosis is advantageous in the effective eradication of tumours. Further, this enhanced expression SFV vector could be redesigned to include tumour specific envelope proteins, to specifically target tumour cells. In addition, it could be further exploited in antitumour vaccine studies to express tumour-associated antigens and to target angiogenesis. This enhanced expression SFV vector system could be used as a safer alternative to the existing DNA vaccines due to its transient expression ability. We also believe that this enhanced expression system could be effectively used in multimodality treatments with other therapeutic agents to synergise the therapeutic efficacy for many diseases including cancer. In general, this vector could emerge as a novel therapeutic agent provided the required alterations are made to increase the specificity and the biosafety of the vector.

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9

APPENDICES

Group[a]	TNE control	rSFV-EGFP ($4x10^7$IU/dose)	rSFV-EGFP ($4x10^8$IU/dose)	rSFV-EGFP ($4x10^9$IU/dose)	rSFV10-E-IL12 ($4x10^7$IU/dose)	rSFV10-E-IL12 ($4x10^8$IU/dose)	rSFV10-E-IL12 ($4x10^9$IU/dose)	rSFV10-IL12 ($4x10^9$IU/dose)
TNE control	-	**ns**[b]	**ns**	**ns**	**P < 0.001**	**P < 0.001**	**P < 0.001**	**P < 0.05**
rSFV-EGFP ($4x10^7$IU/dose)	**ns**	-	nd[c]	nd	**P < 0.05**	nd	nd	nd
rSFV-EGFP ($4x10^8$IU/dose)	**ns**	**nd**	-	nd	**nd**	**P < 0.01**	nd	nd
rSFV-EGFP ($4x10^9$IU/dose)	**ns**	nd	nd	-	**nd**	**nd**	**P < 0.001**	**ns**
rSFV10-E-IL12 ($4x10^7$IU/dose)	**P < 0.01**	**P < 0.05**	**nd**	**nd**	-	**nd**	**nd**	**nd**
rSFV10-E-IL12 ($4x10^8$IU/dose)	**P < 0.001**	**nd**	**ns**	**nd**	**nd**	-	**nd**	**nd**
rSFV10-E-IL12 ($4x10^9$IU/dose)	**P < 0.001**	nd	nd	**ns**	**nd**	**nd**	-	**P < 0.001**
rSFV10-IL12 ($4x10^9$IU/dose)	**P < 0.05**	nd	nd	**ns**	**nd**	**nd**	**P < 0.001**	-

Appendix 9.1 Probabilities of differences between groups treated with TNE or rSFV-IL12 or rSFV-EGFP VLPs for K-BALB tumours.

A one-way repeated measures ANOVA was performed on all treated groups and Tukey's multiple comparison post-test was used to compare groups to each other. [a]Six 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 (i.e., P>0.5), [c]nd; not done. Results are representative of three independent experiments.

a

Group[a]	TNE control	rSFV-EGFP ($4x10^9$IU/dose)	rSFV10-E-IL12 ($4x10^9$IU/dose)
TNE control	-	ns[b]	P < 0.001
rSFV-EGFP ($4x10^9$IU/dose)	ns	-	P < 0.001
rSFV10-E-IL12 ($4x10^9$IU/dose)	P < 0.001	P < 0.001	-

b

Group[a]	TNE control	rSFV-EGFP ($4x10^9$IU/dose)	rSFV10-E-IL12 ($4x10^9$IU/dose)
TNE control	-	ns[b]	P < 0.001
rSFV-EGFP ($4x10^9$IU/dose)	ns	-	P < 0.001
rSFV10-E-IL12 ($4x10^9$IU/dose)	P < 0.001	P < 0.001	-

Appendix 9.2 Probabilities of differences between groups treated with TNE or rSFV10-E-IL12 or rSFV-EGFP VLPs at $4x10^9$IU/dose for CT26 (a) and 4T1 (b) tumours.
A one-way repeated measures ANOVA was performed on all treated groups and Tukey's multiple comparison post-test was used to compare groups to each other. [a]Six 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 (i.e., P>0.5). Results are representative of two independent experiments.

Group[a]	TNE control	rSFV-EGFP ($4x10^9$IU/dose)	rSFV10-E-IL18 ($4x10^9$IU/dose)
TNE control	-	**ns**[b]	**$P < 0.01$**
rSFV-EGFP ($4x10^9$IU/dose)	**ns**	-	**$P < 0.01$**
rSFV10-E-IL18 ($4x10^9$IU/dose)	**$P < 0.01$**	**$P < 0.01$**	-

Appendix 9.3 Probabilities of differences between groups treated with TNE, rSFV-EGFP VLPs or rSFV10-E-IL18 at a titre of $4x10^9$IU/dose for K-BALB and CT26 tumours.

A one-way repeated measures ANOVA was performed on all treated groups and Tukey's multiple comparison post-test was used to compare groups to each other. [a]Six 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 (i.e., $P>0.05$). The data for both K-BALB and CT26 tumours were similar for all the groups. Results are representative of two independent experiments.

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RESEARCH ARTICLE

Regression of mouse tumours and inhibition of metastases following administration of a Semliki Forest virus vector with enhanced expression of IL-12

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The Semliki Forest virus (SFV) vector is an RNA-based suicide expression vector that has been used experimentally for tumour therapy. Recently, a new enhanced vector pSFV10-E has been developed that expresses foreign genes at levels up to 10 times higher than the original vector. Interleukin-12 (IL-12), an immunomodulatory cytokine, plays a key role in the induction of T-helper1 responses. The two IL-12 gene subunits were cloned from mouse splenocytes and inserted into the pSFV10-E and pSFV10 (nonenhanced) vectors. Both constructs expressed and secreted biologically active murine IL-12. Administration of high titre rSFV10-E-IL12 particles intratumourally to treat implanted K-BALB tumours in BALB/c mice demonstrated complete tumour regression in comparison to control or rSFV10-IL12 treated groups. High titre rSFV10-E-IL12 particles were also effective in the CT26 tumour model. Histological and immunohistochemical studies revealed tumour necrosis in addition to aggressive influx of CD4+ and CD8+ T cells and other immune cells. Furthermore, inhibition of primary tumour growth and lung metastases of a metastatic (4T1) tumour model indicated the potential of high titres of rSFV10-E-IL12 particles as an efficient antitumour therapeutic agent.
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Keywords: *interleukin-12; Semliki Forest virus vectors; antiangiogenesis; tumour models; metastasis inhibition; cancer gene therapy*

Introduction

Research on cancer gene therapy has largely focused on induction of apoptosis in cancer cells or on stimulation of immune mechanisms to eliminate cancer cells. Many investigations have used viral vectors such as retro, adeno, adeno-associated, or alphavirus vectors. Alphavirus vectors demonstrate high-level expression of heterologous proteins in a broad host range of cells[1–5] and have been shown to induce apoptosis in infected cells.[6,7]

Semliki Forest virus (SFV), an alphavirus, is an enveloped positive-sense RNA virus of the family Togaviridae; it has been developed into a transient RNA-based suicidal expression vector system.[8] This system consists of a vector in which foreign genes can be expressed, and two helper vectors that encode the structural protein genes. Cotransfection of all three vectors into cells results in release of recombinant virus-like particles (VLPs) coding for the foreign gene.[9] The SFV vector system has several advantages over other vector systems in its broad host range and inherent ability to induce apoptosis.[10]

SFV vectors have been successfully used to induce *in vivo* expression of a number of genes such as cytokines,[11–13] tumour-associated antigens (TAA),[13,14] and vaccine antigens,[15,16] and to deliver therapeutic genes to the central nervous system.[17] It has been shown that recombinant SFV (rSFV) particles can efficiently infect tumour cells and that intratumoural treatment with rSFV particles results in the induction of p53-independent apoptosis leading to significant tumour inhibition. However, in order to achieve complete regression in at least a proportion of treated mice, multiple treatments at relatively high doses are needed.[7] SFV vectors expressing the cytokine interleukin-12 (IL-12) induce tumour regression mainly by inhibition of angiogenesis.[18] Clinical trials (Phase I and II) have been planned to use liposome-encapsulated SFV vectors expressing IL-12 in the treatment of several tumours, including glioblastoma multiforme.[19] These and other studies have indicated that the SFV vector is an effective tumour therapy agent.[10]

An enhanced SFV expression vector system, pSFV10-E, has been developed which has been shown to produce up to 10 times more foreign protein than the original SFV vector. This vector contains the N-terminal 34 amino acids of the SFV capsid protein followed by the foot and mouth disease virus (FMDV) 2A cleavage sequence (Figure 1). The capsid enhancer drives the enhanced expression of the foreign genes[20] and the FMDV protease cleaves the expressed protein from the capsid enhancer.

Immuno-gene therapy with cytokines is one of the strategies being developed for the activation of

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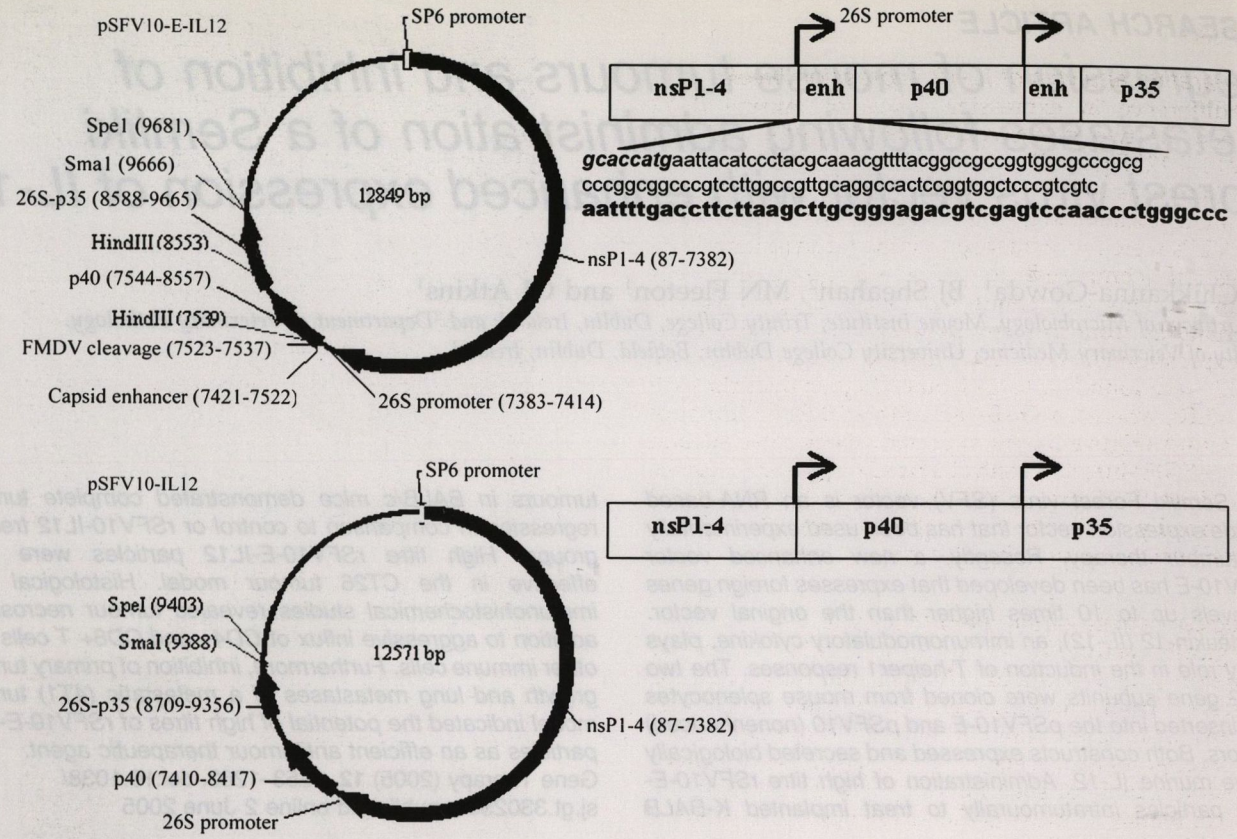

Figure 1 *The SFV vectors. The standard SFV vector SFV10 and the enhanced vector pSFV-E, both expressing the IL-12 subunits p40 and p35 from separate 26S promoters. The plasmid used to transcribe the vector RNA from the SP6 promoter (after linearization with SpeI) is shown on the left and the transcribed RNA (incorporating the enhancer element (enh) in the case of rSFV-E) on the right. Recombinant VLPs were produced using the split helper system.[9] The nucleotide sequence of the enhancer element is shown in the inset. Italics indicate the Kozak and methionine initiation sequences, standard typeface codons for amino acids 2–34 of the capsid protein, and bold codons for17 amino acids of the 2A protein; cleavage occurs before the last amino acid (proline).*

immune cells to enhance antitumour responses. Previous experiments using SFV vectors expressing cytokines have given promising results.[11–13] It has been shown that the amount of IL-12 available at the tumour site is critical for tumour regression. A major drawback of systemic treatment with IL-12 is its short plasma half-life and the toxicity associated with high systemic concentrations. However, concentration of cytokine at the tumour site increases therapeutic efficacy and reduces the systemic side effects.[21,22]

The cytokine IL-12 is a disulphide linked heterodimeric protein, comprising a 35 kDa subunit and a 40 kDa subunit to form a biologically active p70 complex. It is produced by antigen-presenting cells such as dendritic cells, macrophages, B-cells, and possibly other accessory cells, after exposure to infectious agents. IL-12 induces the differentiation of T-helper1 (Th1) CD4+ cells, which are inducers of IFN-γ production.[23] It has been reported that tumour treatment using IL-12 is associated with the expression of the cytokine IFN-γ and chemokines such as IP-10 at the tumour site, inducing the antitumour effect through a direct toxic effect on the tumour cells or by activating potent antiangiogenic mechanisms.[24] The induced antiangiogenic mechanisms are complex and dependent both on the direct effect of the proinflammatory cytokines/chemokines on endothelial cells and the recruitment of effector NK and T-cells. IL-12-induced specific immunological memory has been reported, making IL-12 a promising candidate for permanent and specific tumour eradication.[25]

In this study, we constructed pSFV10-IL12 (normal expression of murine IL-12) and pSFV10-E-IL12 (enhanced expression) vectors and examined the antitumour effect of these constructs. Three different tumour models were utilised to demonstrate the consistency of IL-12 therapeutic efficacy. K-BALB cells are murine sarcoma virus transformed mouse fibroblasts that overexpress the K-ras oncogene and form aggressive localized syngeneic tumours in immunocompetent BALB/c mice on subcutaneous (s.c.) injection.[26,27] CT26 cells are murine colon adenocarcinoma cells which form localized tumours of low immunogenicity in BALB/c mice after s.c. injection.[28–30] To examine the effect of treatment on a metastasizing tumour model, we have selected the highly malignant, spontaneously metastasising 4T1 mouse mammary carcinoma model that also readily develops into tumours on s.c. implantation.[31–33] Complete and permanent *in vivo* tumour regression was found in all three tumour models treated with high titre rSFV10-E-IL12 particles, and in the case of the 4T1 model, inhibition of metastasis formation also occurred.

Results

Comparative analysis of IL-12 expression levels

The difference in expression levels of murine IL-12 between the two vector constructs pSFV10-E-IL12 and pSFV10-IL12 (Figure 1) was examined by qualitative and quantitative assays in BHK-21 and K-BALB cells. By immunohistochemistry, cells infected with rSFV10-E-IL12 VLPs were more intensely stained due to high-level expression compared to rSFV10-IL12-infected cells (Figure 2). This was confirmed by an ELISA assay of supernatants from infected BHK-21 and K-BALB cells. The expression levels of IL-12 in BHK-21 cells by rSFV10-E-IL12 were found to be 3–4 times higher than rSFV10-IL12 VLPs, whereas in K-BALB cells, a 10-fold difference was found (Figure 3). As expected, mock-infected and rSFV10-EGFP-infected control BHK-21 and K-BALB cells showed no IL-12 expression. Expression levels of IL-12 were broadly similar to those obtained for adeno[34] and herpes[35] vectors expressing the same cytokine.

Biological activity of secreted murine IL-12

Biologically active IL-12 induces T cells to secrete interferon-γ (IFN-γ). An ELISA measured the production of IFN-γ from supernatants of murine splenocyte cultures 24 h after stimulation with supernatants of VLP-infected BHK-21 cells or K-BALB cells. This showed that supernatants of such infected BHK-21 and K-BALB cells, containing IL-12, stimulated splenocytes to produce similar levels of IFN-γ to the positive Concavalin A (ConA)-treated control (Figure 4). These results confirmed the expression and secretion of biologically active IL-12 from both rSFV10-E-IL12 and rSFV10-IL12 vectors. However, the stimulation of IFN-γ was not directly related to the amount of IL-12 present in the cell supernatants; this may reflect a quantal production of IFN-γ on IL-12 stimulation at a threshold level.

Treatment of K-BALB, CT26, and 4T1 tumours using rSFV particles

Initially, experiments were performed to optimize the amount of IL-12 available at the tumour site by varying the titres of VLPs administered. K-BALB tumours were treated with six injections of rSFV10-IL12 or rSFV10-E-IL12 VLPs at 4×10^7, 4×10^8, and 4×10^9 IU in 50 µl/dose, whereas CT26 and 4T1 tumours were treated only with a titre of 4×10^9 IU (Figure 5, Table 1). An average tumour diameter of 4 mm was reached at 4 and 6 days postinduction of K-BALB, or CT26, and 4T1 tumours, respectively. When K-BALB tumours were treated with a 4×10^7 IU/dose of rSFV10-E-IL12, one mouse showed complete tumour regression at day 19 but average tumour growth did not show a statistically significant difference compared to either the control TNE buffer or rSFV10-EGFP-treated groups (Table 1). For K-BALB tumours treated with 4×10^8 IU/dose of rSFV10-E-IL12, three out of six mice showed complete tumour regression and increased median survival time compared to the controls (Table 1). Finally, all six mice bearing K-BALB tumours showed complete tumour regression when treated with six intratumoural injections of 4×10^9 IU (Figure 5a, Table 1). None of the rSFV10-IL12-treated mice showed regression, and there was no overall significant growth inhibition compared to the TNE-

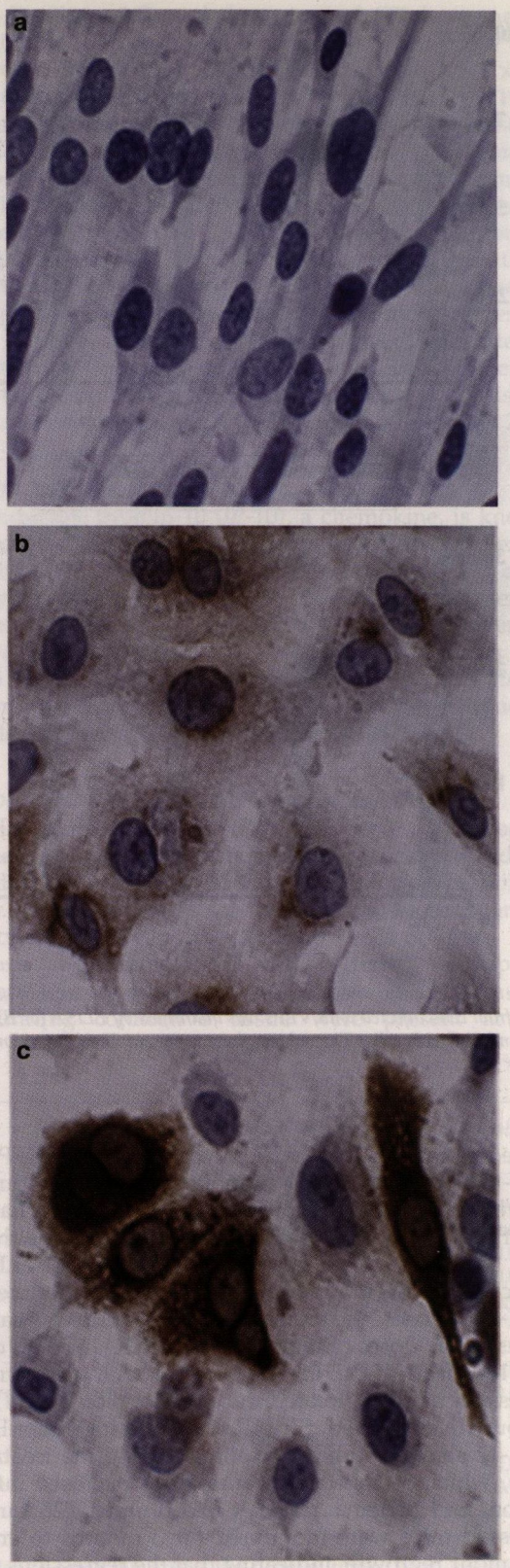

Figure 2 *Immunocytochemical staining for IL-12. BHK-21 cells were mock infected (a) or infected with rSFV-IL12 replicons and examined at 24 h postinfection by immunocytochemical staining for IL-12 (brown stain). The expression levels of IL-12-positive cells infected with rSFV10-IL12 (b) were distinguished from rSFV10-E-IL12-infected (c) cells by lower staining intensities. Original magnification* × *200.*

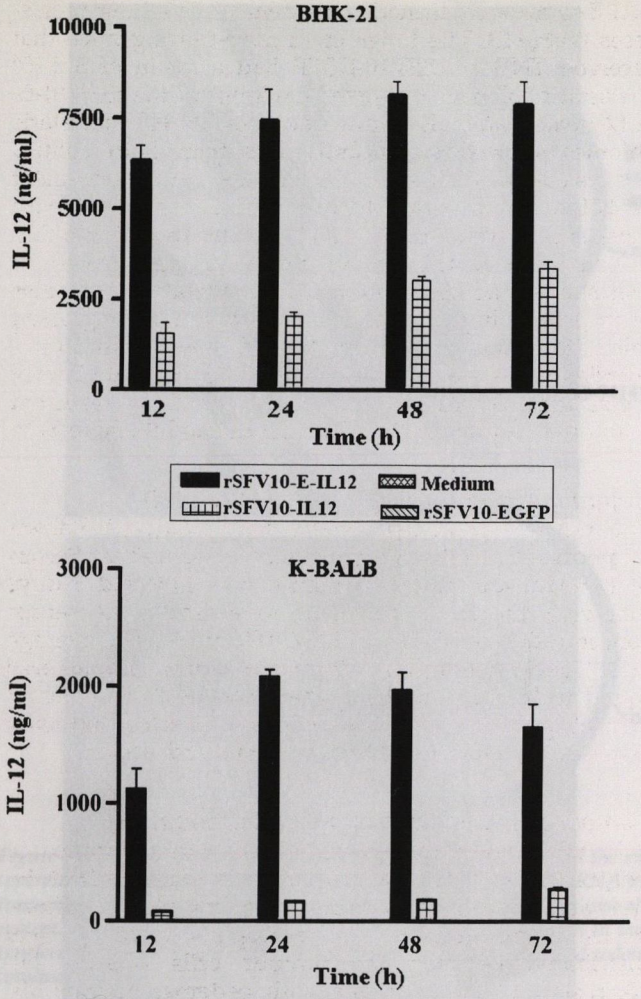

Figure 3 *Comparative analysis of IL-12 expression. The level of secreted IL-12 from cells infected with VLPs was quantified by ELISA for BHK-21 cells and K-BALB cells. The values given represent IL-12 secretion from 5×10^5 cells.*

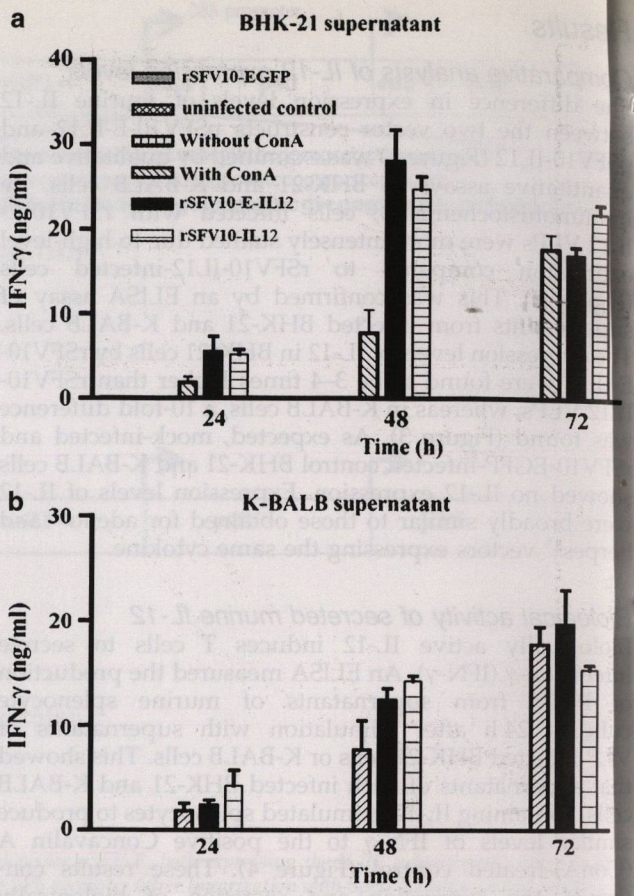

Figure 4 *IFN-γ ELISA. An ELISA was used to measure the production of IFN-γ by murine splenocytes induced by supernatants of BHK-21 cells (a) and K-BALB cells (b) infected by VLPs.*

treated group. In these experiments, rSFV10-EGFP and rSFV-IL12 showed some inhibitory effect on tumour growth during the treatment stage, but overall had no significant effect. This initial slower tumour growth rate may be due to the inherent ability of rSFV VLPs to induce apoptosis in tumour cells.[10] Based on these results for K-BALB cells, treatment of CT26 tumours was carried out using six doses of high titre (4×10^9 IU in 50 µl/dose) rSFV10-E-IL12 VLPs; this dose induced complete tumour regression in all six treated mice (Figure 5b, Table 1). All tumours that received rSFV VLPs expressing either EGFP or IL-12 showed tumour swelling during the treatment stage, which related to intratumoural necrosis, inflammation, and oedema.[30] The K-BALB and CT26 tumour regressed mice were monitored for 3 months to ensure no tumour regrowth, indicating that the regressions were complete.

Since the treatment efficacy of the rSFV10-E-IL12 replicon was verified using two different localized tumour models, we next examined its potential to inhibit the growth of established lung metastases in the 4T1 mammary tumour model. Established s.c. 4T1 tumours on the right abdominal flank of BALB/c mice were treated with six injections of 4×10^9 IU/dose in 50 µl on alternate days. Four out of six mice showed complete tumour regression between days 18 and 22 (Table 2) and the remaining two mice grew tumours at the same rate as control groups during the post-treatment period. All control (TNE and rSFV10-EGFP) and IL-12-treated tumours were sampled for histopathology and immunohistochemistry when the size reached 15 mm diameter (around day 22). Local spread of 4T1 tumour nodules was noted in control mice after repeated injections.

rSFV10-E-IL12 therapy reduces the number of 4T1 lung metastases

Following s.c. injection, 4T1-breast carcinoma cells formed solid tumours of 4 mm diameter by day 6 postinduction and frequent lung metastases by day 22 postinduction. A large difference in numbers of lung metastases between the rSFV10-E-IL12 and control treated groups was found when the lungs were examined at day 22 post tumour induction. The average number of grossly visible tumour nodules in the TNE and rSFV10-EGFP-treated lungs were 16 and 13.2, respectively, compared to the IL-12-treated groups, which had an average of 1.5 (Table 2). These findings were supported by histological studies (below). Using the clonogenic assay, the number of 6-thioguanine-resistant colonies in the lungs of the 4T1 tumour-bearing

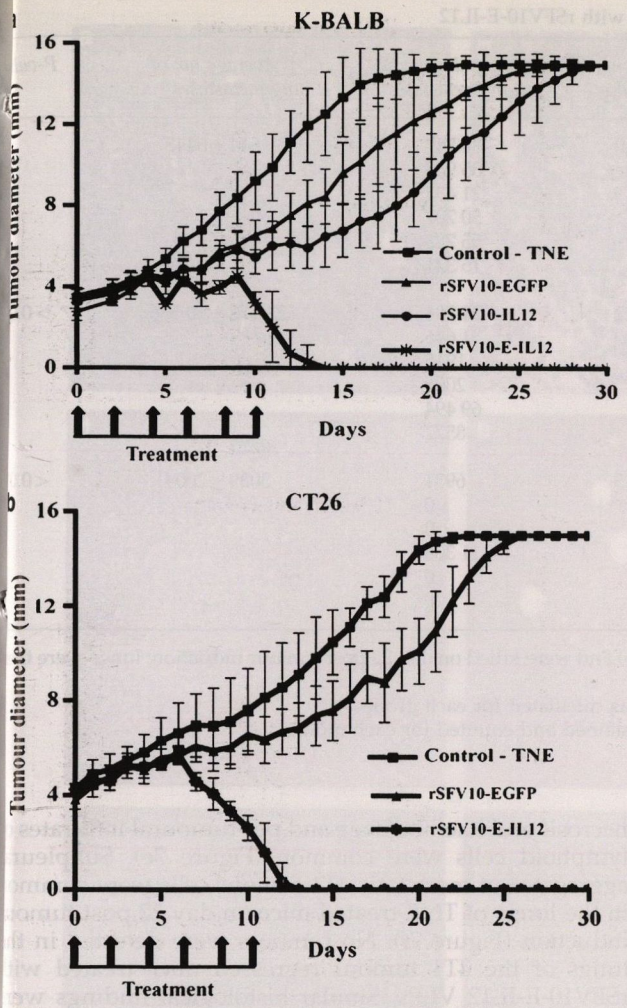

Figure 5 Tumour treatment. Effect of six injections of VLPs (high titre, 4×10^9 IU/dose) on K-BALB (a) and CT26 (b) tumour growth.

BALB/c mice confirmed the extent of 4T1 lung metastases (Table 2). The lungs of tumour-bearing mice that received TNE or rSFV10-EGFP had a mean of 3×10^4 metastatic colonies. However, the lungs of the rSFV10-E-IL12-treated animals with a mean of 3×10^3 metastatic colonies showed significantly less than both control groups (Table 2). These results indicate that the treatment of 4T1 tumours with rSFV10-E-IL12 is associated with increased survival time, which results from inhibition of both primary 4T1 tumour growth and the growth of metastases. The observation that no metastatic colonies were present in the lungs of complete tumour regressed mice (four out of the six treated mice) indicates a correlation between tumour size and the number of metastatic colonies for each mouse, which is consistent with other studies of the 4T1 tumour model (Table 2).[33]

Serum levels of antiangiogenic factor IP-10

The inducible protein-10 (IP-10), a chemokine, is known to mediate the antiangiogenic activity of IL-12.[24] Serum of K-BALB tumour-bearing mice was collected 3 days following the sixth treatment to estimate the serum concentration of IP-10 by ELISA (Figure 6). The average IP-10 concentration in IL-12-treated serum samples was found to be significantly higher than in TNE or EGFP control groups, with the enhanced vector inducing significantly more IP-10 than the standard vector.

Tumour pathology and immunohistochemistry

K-BALB tumours treated with TNE were composed of polyhedral and spindle-shaped cells characterized by well-defined cell borders and frequent bizarre mitotic forms. Small numbers of neutrophils were sparsely distributed between the tumour cells (Figure 7a). Necrosis and haemorrhage with oedema, neutrophils, macrophages, lymphocytes, and plasma cells in the surrounding connective tissue were present at the

Table 1 Inhibition of tumour growth by rSFV treatment

Tumour model	*Treatment groups*[a]	*Median survival (days)*[b] *(No. of survivors)*[c]	*Average tumour diameter (mm)*[d]	*Probability*[e]
K-BALB	TNE[f]	22 (0)	11.9 (±0.7)	
	rSFV10-EGFP (4×10^9 IU/dose)	23 (0)	11.4 (±0.8)	>0.05
	rSFV10-IL12 (4×10^9 IU/dose)	27 (0)	10.5 (±0.7)	>0.05
	rSFV10-E-IL12 (4×10^7 IU/dose)	26 (1)	7.3 (±0.6)	>0.05
	rSFV10-E-IL12 (4×10^8 IU/dose)	49 (3)	5.9 (±0.2)	<0.001
	rSFV10-E-IL12 (4×10^9 IU/dose)	Undefined (6)[g]	1.0 (±0.2)	<0.001
CT26	TNE	24 (0)	11.0 (±0.7)	
	rSFV10-EGFP (4×10^9 IU/dose)	28 (0)	9.8 (±0.7)	>0.05
	rSFV10-E-IL12 (4×10^9 IU/dose)	Undefined (6)[g]	1.5 (±0.3)	<0.001
4T1	TNE	22 (0)	10.2 (±0.8)	
	rSFV10-EGFP (4×10^9 IU/dose)	22 (0)	8.9 (±0.6)	>0.05
	rSFV10-E-IL12 (4×10^9 IU/dose)	22 (4)[h]	4.5 (±0.3)	<0.001

[a] Six Balb/c mice per treatment group were used for all experiments.
[b] Mice were killed when the tumour reached 15 mm diameter and the time taken to reach this size is the survival time.
[c] Mice which had complete and permanent tumour regression; in some cases a residual small nodule remained that was composed entirely of scar tissue.
[d] Average tumour diameter for each group was calculated at the median survival time (±standard error).
[e] The probability is based on the average tumour diameter compared to that of the TNE-treated (control) groups over the survival time of the mice.
[f] Average of the control (TNE)-treated groups in the three different K-BALB tumour treatment experiments with different titres of VLPs.
[g] Since all mice showed complete tumour regression, the median survival time cannot be calculated by Kaplan–Meier analysis.
[h] All mice were culled at day 22; two mice had tumours of maximum size.

Table 2 Inhibition of 4T1 tumour lung metastases following treatment with rSFV10-E-IL12

Treatment[a]	*Mouse no./tumour size (mm)*	*Lung metastases (no. of nodules)*[b]	*Avg. no. nodules*	*No. of clonogenic lung metastases*[c]	*Average no. of lung metastases ± s.e.*	*P-value*
TNE (Control)	1/15	14	16.0	34 747	35 644 ± 6445	
	2/15	23		35 933		
	3/13	5		21 432		
	4/15	17		50 707		
	5/15	28		55 723		
	6/13.5	9		15 321		
rSFV10-EGFP	1/14.5	12	13.2	21 796	22 572 ± 10 536	>0.05
	2/12	21		31 646		
	3/11	3		1915		
	4/11.4	6		2006		
	5/15	25		69 494		
	6/12.8	12		8572		
rSFV10-E-IL12	1/11.5	4	1.5	6931	3039 ± 2004	<0.01
	2/0	0		0		
	3/0	0		0		
	4/13.5	5		11 308		
	5/0	0		0		
	6/1.4	0		0		

[a]Mice received six intratumoural injections of rSFV VLPs (4×10^9 IU/dose) and were killed on day 22 post-tumour induction; lungs were then analysed for metastases.
[b]Visible lung tumour nodules were counted and the average number was calculated for each group.
[c]The number of 6-thioguanine-resistant 4T1 lung metastasized colonies stained and counted for each mouse.

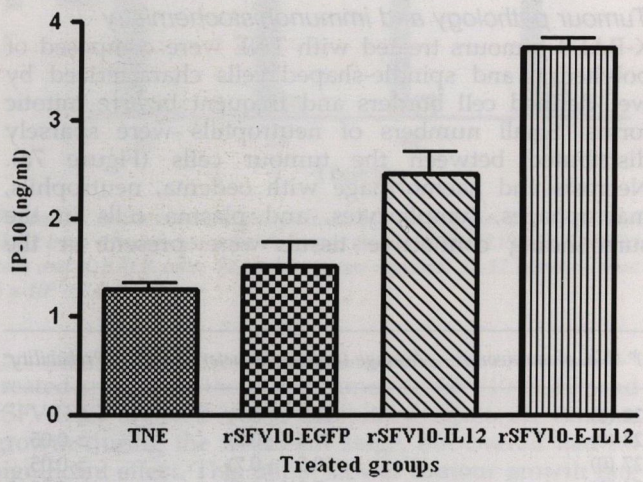

Figure 6 *Serum IP-10 levels. Serum from K-BALB tumour-bearing mice collected 3 days following the sixth treatment was analysed by ELISA.*

tumour sites following treatment with rSFV10-EGFP replicons (Figure 7b). Occasional intact tumour cells were located at the periphery of the necrotic areas. For K-BALB tumours treated with IL-12, intact tumour cells were absent and necrosis appeared complete with dense infiltrates of neutrophils, macrophages and lymphoid cells (Figure 7c). Fibrin thrombi were common in blood vessels. CT26 and 4T1 tumours treated with TNE and IL-12 showed similar patterns of change to those seen in K-BALB tumours. Actively growing 4T1 tumours located in the subcutis of TNE-treated mice were composed of polyhedral cells with well-defined cell borders, round to oval nuclei and pale vacuolated cytoplasm (Figure 7d). Nuclear pleomorphism, mitotic forms, focal areas of necrosis and haemorrhage and peritumoural infiltrates of lymphoid cells were common (Figure 7e). Subpleural aggregates of metastatic 4T1 tumour cells were common in the lungs of TNE-treated mice on day 22 post tumour induction (Figure 7f). No tumours were detected in the lungs of the 4T1 tumour regressed mice treated with rSFV10-E-IL12 VLPs. Similar histological findings were obtained for all three mice examined from each treatment group.

The cellular response and tumour microvasculature in IL-12 and mock-treated K-BALB and CT26 tumours were further examined using immunohistochemistry. Higher numbers of CD4+ (MHC class II restricted) and CD8+ (MHC class I restricted) cells were located in perivascular spaces at the margins of IL-12-treated tumours than in those treated with TNE (Figure 7g, h). For most of the IL-12-treated tumours, numbers of CD4+ cells exceeded those of CD8+ cells, but overall the quantitative difference was not significant (data not shown). Using anti-CD31, an antibody specific for vascular endothelial cells, mock-treated K-BALB tumours showed a well-defined capillary network uniformly distributed between the tumour cells (Figure 7i). Vascular endothelial cells in IL-12 treated tumours were restricted to the non-necrotic areas of the tumours. These results indicate a cumulative antitumour effect of the IL-12-expressing vector, which may involve several mechanisms.

Discussion

In this study, we have shown that intratumoural treatment with SFV vectors expressing IL-12 effectively inhibits tumour growth in three murine tumour models. These results confirm those of other studies that have used IL-12-expressing SFV vectors for the treatment of

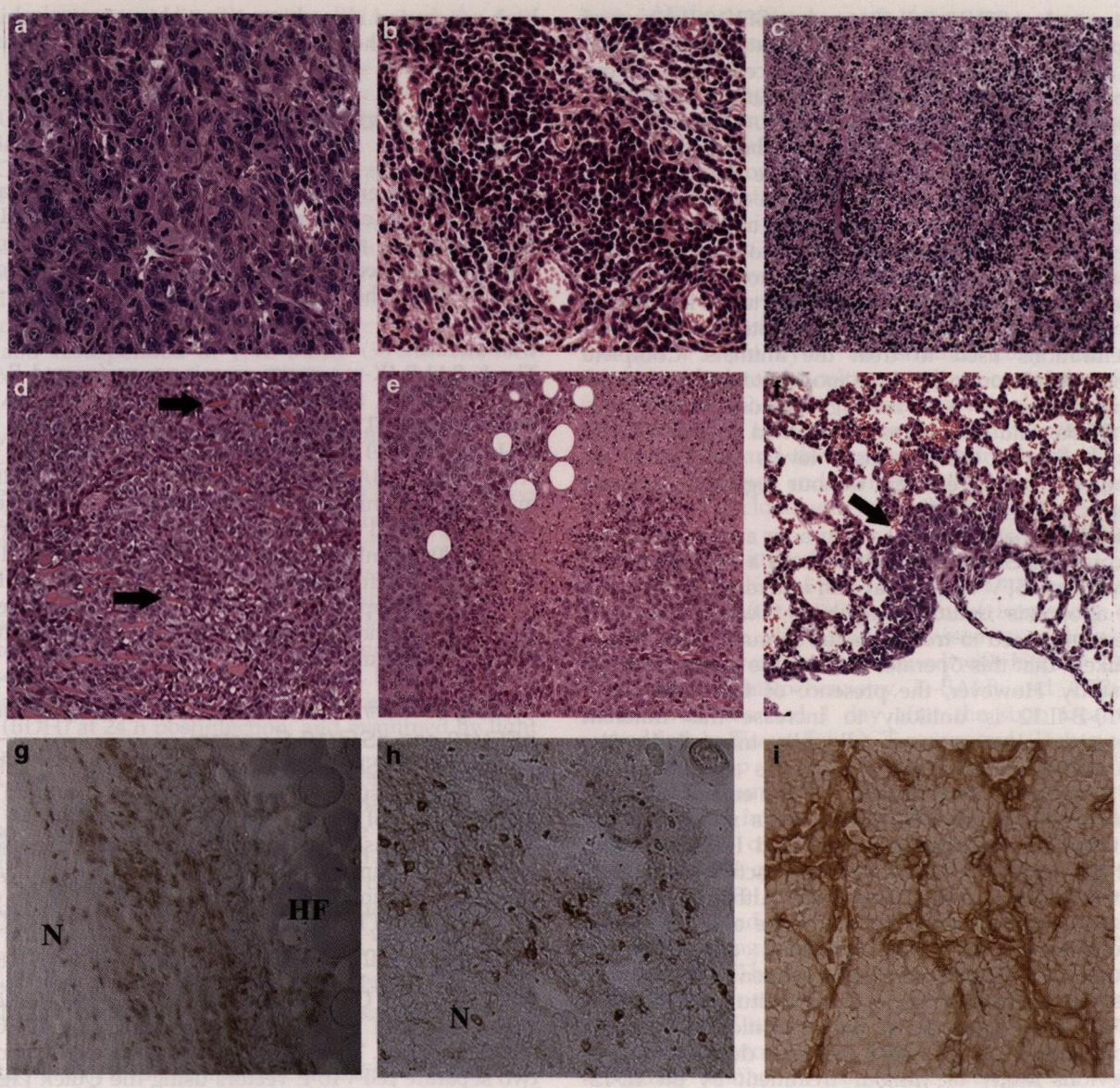

Figure 7 Examples of tumour histopathology and immunohistochemistry. (a) K-BALB tumour, mock-treated (TNE). Polyhedral tumour cells and neutrophil infiltration. H&E, × 200. (b) K-BALB tumour, mock-treated. Lymphocytes and plasma cells in the peritumoural stroma. H&E, × 200. (c) K-BALB tumour, IL-12 treated. Area of necrosis densely infiltrated with neutrophils, macrophages and lymphocytes. H&E, × 200. (d) 4T1 tumour, mock-treated. Compression of panniculus muscle fibres (arrows) by polyhedral tumour cells. H&E, × 200. (e) 4T1 tumour, mock-treated (TNE). Area of necrosis circumscribed by tumour cells and lipid vacuoles. H&E, × 200. (f) Subpleural aggregate of metastatic tumour cells (arrow) in the lung of a mock-treated (TNE) mouse bearing a 4T1 tumour. H&E, × 200. (g) CT26 tumour, IL-12-treated. CD4+ cells (brown) interposed between an area of necrosis (N) and hair follicles (HF). ABC, × 200. (h) CT26 tumour, IL-12 treated. CD8+ cells adjoining an area of necrosis (N). ABC, × 200. (i) K-BALB tumour, mock-treated (TNE). Well-defined capillary network expressing CD31 (brown) uniformly distributed between the tumour cells. ABC, × 200.

other tumour models,[11–14] and indeed results obtained for adeno[34] and herpes vectors[35] expressing IL-12. However, we have also shown that a combination of the use of high titre vector preparations and a vector expressing high levels of IL-12 can result in complete tumour regression. This treatment also inhibits the growth of lung metastases in a metastatic tumour model.

The cytokine IL-12 has potential as a tumour therapy agent because of its antitumour and antimetastatic activity.[36] IL-12 promotes the activity and differentiation of T cells and NK cells to induce an aggressive antitumour immune response. In addition, a further antitumour activity of IL-12 is its ability to induce IFN-γ production by T cells, which in turn mediates antiangiogenic effects.[23–25] Previous studies have shown that the amount of IL-12 available at the tumour site is critical for tumour regression, since it determines the number and type of infiltrating leucocytes.[21] It may also mediate the antiangiogenic effect of IL-12,[18] although the relative contribution of these two effects to the antitumour activity of IL-12 is not clear.

The development of pSFV10-E, a new SFV-based expression vector system with enhanced expression of foreign protein (up to 10-fold more than the standard pSFV10 system), has enabled us to enhance IL-12 expression locally at the tumour site. Hence, we

constructed pSFV10-E-IL12 and pSFV10-IL12, and compared the expression of IL-12 in standard BHK-21 cells and the murine tumour K-BALB cell line. To avoid the possible antagonism of p40 homodimers against the biologically functional heterodimeric IL-12 molecule, we cloned the component p40 and p35 subunits into single vectors, each expressed from separate 26S promoters. To confirm the biological activity and secretion of the expressed protein, we measured the ability of supernatants from vector-infected cells to induce IFN-γ production by mouse splenocytes. Since high systemic concentrations of IL-12 have been reported to have lethal toxic effects,[22] it was essential to optimize the IL-12 concentrations used to treat the animals. Complete tumour regression in all three models tested was found in animals treated with the highest dose of rSFV10-E-IL12 VLPs, and the animals remained free of clinical signs, indicating that increased levels of IL-12 at the tumour site induce efficient tumour regression without systemic lethal toxic effects.

It is likely that the potent antitumour activity of the IL-12-expressing SFV vector results from a combination of mechanisms. SFV vectors have an inherent p53-independent apoptosis inducing capability,[6] which has been successfully used to treat tumours in nude mice,[7,37] and it is likely that this operates also for the vectors used in this study. However, the presence of the enhancer in rSFV10-E-IL12 is unlikely to increase this inherent apoptosis induction since all cells infected by the unenhanced vector undergo apoptosis and the vector does not spread intercellularly.[6,7,37] The enhanced ability of this vector to destroy tumour cells must therefore result from the induction of enhanced IL-12 secretion. IL-12 has a potent proinflammatory activity that may contribute to the antitumour effect. Although K-BALB cells are syngeneic with BALB/c mice and are poorly immunogenic,[27] influx of lymphoid cells into the peritumoural tissue following treatment was demonstrated by histopathology. A third antitumour mechanism that may operate is the inhibition of tumour angiogenesis by IL-12, which has been demonstrated in a previous study of tumour treatment by an IL-12-expressing SFV vector.[18] In the present study, the increased levels of the antiangiogenic chemokine IP-10 in the blood of treated mice, allied to the frequent occurrence of thrombosis and haemorrhage in the treated tumours, suggests that inhibition of angiogenesis also played a role in our model system.

The antiangiogenic action of IL-12 may also be responsible for inhibition of the growth of metastases in the 4T1 tumour model, since angiogenesis inhibitors are known to induce tumour cell apoptosis by limiting the supply of nutrients and other cell survival factors derived from endothelial cells.[38] It is also possible that inhibition of the growth of the primary tumour restricts tumour mass so that fewer metastases are formed. However, the latter explanation does not explain the total absence of metastases in animals where the primary tumour had totally regressed.

A phase I/II clinical trial has been proposed for the use of an SFV vector expressing IL-12 for the treatment of glioblastoma multiforme, an incurable human brain tumour, by direct perfusion of the tumour with liposome-encapsulated recombinant particles.[19] In designing this and similar clinical trials, the use of the high titre and high expression recombinant particle system used in the present study should be considered.

Materials and methods

Mice

Specific pathogen-free immuno-competent 4–6-week-old female BALB/c mice were obtained from Harlan (Bicester, UK) and maintained under pathogen-free conditions in accordance with the principles set down in SI 17/94 of the European Union.

Cell culture

The K-BALB (K-ras overexpressing transformed BALB/3T3), CT26.WT (colon adeno carcinoma from BALB/c mouse), and 4T1 (metastasing breast carcinoma from BALB/c mouse) tumour cell lines were obtained from ATCC. The K-BALB, CT26, and 4T1 cells were cultured in Dulbecco's minimal essential medium supplemented with 10% foetal bovine serum and antibiotics (streptomycin 100 µg/ml and penicillin 100 U/ml). BHK-21 cells were obtained from ATCC and were propagated in BHK-21 medium supplemented with 5% foetal bovine serum, 10% tryptose phosphate broth, 2 mM L-glutamine, 20 mM HEPES, and antibiotics.

Cloning of murine IL-12 subunits p35 and p40 into pSFV10 and pSFV10-E vectors

To construct pSFV10-IL-12 vectors (Figure 1), initially cDNA of IL-12 subunits p35 and p40 was amplified from the total RNA of BALB/c mouse splenocytes using PFU-DNA polymerase. The following primers were used to amplify the dimeric subunits of IL-12, each having *Hind*III restriction enzyme sites (bold) at their ends: 5′CCC **AAG CTT** ATG TGT CAA TCA CGC TAC CTC3′ (p35, sense), 5′-ATA **AAGCTT** TCA GGC GGA GCT CAG ATA GCC-3′ (p35, antisense), 5′-CCC **AAG CTT** ATG TGT CCT CAG AAG CTA ACC-3′ (p40, sense), 5′-ATA **AAG CTT** CTA GGA TCG GAC CCT GCA GG-3′ (p40, antisense). The p35 and p40 genes were ligated into two separate pSFV10-E vectors using the Quick Ligation Kit (NEB), which resulted in pSFV10-E-p35 and pSFV10-E-p40. The p35 and p40 genes from their respective pSFV10-E constructs were released by *Hind*III (NEB) digestion and the generated ends of the subunits were blunted using DNA polymerase I large fragment (Klenow, NEB). The pSFV10 vector was linearized using *Bam*HI and the sticky ends blunted and dephosphorylated with shrimp alkaline phosphatase (Roche). Blunted p35 and p40 subunits were ligated into linearized pSFV10 separately to obtain pSFV10-p35 and pSFV10-p40 and the orientation checked. The p35 subunit was then removed together with its subgenomic 26S promoter from the pSFV10-E-p35 and pSFV10-p35. The 26S-p35 fragment was subsequently cloned into the *Sma*I restriction site just downstream of the p40 subunit in the pSFV10-E-p40 and pSFV10-p40 constructs to obtain the pSFV10-E-IL12 and pSFV10-IL12 constructs (Figure 1).

Production of rSFV-IL-12 virus-like particles

The SFV split-helper vector system was obtained from P Liljeström (Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden) and VLPs produced as previously described.[9] Briefly, BHK-21 cells

were coelectroporated with vector RNA and the two helper RNAs: rSFV-CS219A (encoding the capsid protein) and rSFV-HelperS2 (encoding the envelope proteins). After the cells were incubated for 36 h at 33°C in 5% CO_2, the medium containing the VLPs was harvested, clarified by centrifugation, aliquoted, and stored at −70°C. Titration was performed by infecting monolayers of BHK-21 cells with serial dilutions of the IL-12-expressing VLPs and immunofluorescence was carried out using rat anti-mouse IL-12 p-70 (Pharmingen) 18 h postinfection. For *in vivo* studies, VLPs were concentrated by ultracentifugation through a 20% w/v sucrose cushion (Beckman SW28 rotor; 28 000 r.p.m. for 2 h at 4°C) and resuspended in TNE buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.1 mM EDTA).

Analysis of IL-12 expression by rSFV10-E-IL12 and rSFV10-IL-12 VLPs

Expression and secretion levels of IL-12 from infected BHK-21 and K-BALB cells were analysed using the ELISA OptEIA™ Set Mouse IL-12 (p70) Kit (BD Biosciences Pharmingen). Cell supernatants were collected at 12, 24, 48, and 72 h after mock (TNE) or rSFV-IL12 VLP infection at a multiplicity of infection (MOI) of 10. Infected BHK-21 cells were also stained using rabbit anti-mouse IL-12 (p70) antibody (Vectastain), DAB (Diaminobenzidine, Sigma) solution, and Harris haematoxylin stain (BDH) at 24 h postinfection, and examined by light microscopy.

An assay for the biological activity of secreted IL-12 from both the rSFV constructs was performed using mouse T cells. Mouse splenocytes were incubated with supernatants (24 h after infection) from mock or rSFV-IL12 VLP-infected BHK-21 or K-BALB cells. Naïve and Concavalin A (ConA) (1 μg/ml)-treated splenocytes were used as controls. The supernatant was collected at 24, 48, and 72 h after infection and an IFN-γ assay was carried out. This consisted of an ELISA test using OptEIATM Set Mouse IFN-γ (BD Biosciences).

Establishment and treatment of K-BALB, CT26, and 4T1 tumours with rSFV-IL12 VLPs

Female 6–8-week-old BALB/c mice were used in all the tumour treatment experiments. Tumours were induced subcutaneously on the right flank of each mouse by injecting 10^6 cells in 100 μl Hank's balanced salt medium with a 29 G 0.5 ml syringe. Tumours were measured daily using a linear caliper and average tumour diameter was calculated as the square root of the product of two perpendicular measurements, assuming spherical shape.[7] The established tumours were treated when the size reached 4 mm diameter. In all tumour treatment experiments, mice were culled once the tumour size reached 10% of body weight (15 mm). Six mice were used in each of the treatment groups. The tumours were treated on alternate days with a total of six intratumoural injections of 50 μl of TNE buffer alone, TNE buffer containing rSFV10-EGFP,[7] rSFV10-E-IL12 or rSFV10-IL12. VLPs were administered at varying titres (4×10^7, 4×10^8 or 4×10^9 IU/ml). CT26 and 4T1 tumours were treated only with TNE, or 4×10^9 IU/ml rSFV-EGFP or rSFV10-E-IL12 VLPs. Two sets of animals were used for each treatment; the first group was assayed for survival and the second group assayed for pathology, serology, immunohistochemistry, and lung metastases (4T1 tumour model only).

Detection of lung metastases

Lungs were examined for 4T1 metastases as previously described.[33] Briefly, mice were killed at day 24 post-tumour induction and the lungs removed for gross examination, histopathology, and clonogenic assay. The right lungs were immersion fixed in formol saline, sliced, and processed to paraffin wax; sections 4 μm thick were cut and stained with haematoxylin and eosin (H&E). The left lungs were treated with Elastase and Liberase blendzymes (Roche) and the cells harvested by filtering through a 70 μm nylon cell strainer. Lung cells were diluted and plated in culture medium containing 60 μM 6-thioguanine (2-amino-6-mercaptopurine, Sigma). The 4T1 cells (which are resistant to thioguanine) were grown for 10–12 days and the clonogenic colonies of 4T1 cells fixed in methanol and stained with crystal violet for quantitation of clonogenic lung metastases.

Pathology and immunohistochemistry

For pathology, K-BALB, CT26, and 4T1 tumours were resected 1 day after the sixth treatment, immersion fixed in formal saline, sliced and processed to paraffin wax; 4 μm sections were cut and stained with H&E.

For immunohistochemistry, K-BALB and CT26 tumours were resected 1 day after the sixth treatment, embedded in Tissue-Tek OCT compound (Fisher scientific), and snap frozen in isopentane. Tumours were sectioned at 6 μm in a cryostat microtome. The sections were used for immunohistochemical staining to detect CD4 and CD8 cells in the tumour. The sections were fixed in ethanol, quenched with 0.3% hydrogen peroxide, blocked in 5% rabbit serum for 30 min at room temperature, and endogenous avidin and biotin blocked using a blocking kit (Vectorstain ABC Kit Elite, Vector Laboratories). The sections were treated with primary antibody, either rat anti-mouse CD4 (1:20, Pharmingen) or CD8 (1:20, Pharmingen) diluted in 5% rabbit serum (Vector Laboratories) at room temperature for 1 h. Secondary antibody and biotinylated rabbit anti-rat antibodies, (1:1000, Vector Laboratories) were used at room temperature for 45 min. For detection of vascular endothelial cells, sections of tumours were labelled using 5% goat serum as blocking buffer, rat anti-mouse CD31 (1:50, Pharmingen) as primary antibody, and biotinylated goat anti-rat IgGs as secondary antibody. The primary–secondary antibody complexes were detected using the Vector Elite ABC standard HRP kit according to the manufacturer's instructions, with DAB as substrate. The sections were washed between each step with PBS and counterstained with haematoxylin. Mouse spleens were used as positive controls; a negative control was used in each assay by substituting the primary antibody with 5% normal rabbit serum. The labelled sections were mounted and observed by light microscopy for positive cells stained brown.

Serum analysis for antiangiogenic factor IP-10

Blood serum from three mice from each group was collected 3 days after the sixth treatment from the K-BALB tumour bearing mice. Levels of IP-10 in the sample blood serum in triplicate were quantified using the Quantikine mouse IP-10/CXCL10 (R&D Systems)

immunoassay kit, according to the manufacturer's instructions. The absorbance of the samples was read at 450 nm.

Statistical analysis

The statistical significance of tumour size among the different experimental treatment groups was analysed by one-way ANOVA, using Tukey's multiple comparison post-test to compare the probability (*P*) value of each group with its appropriate control TNE-treated group. Kaplan–Meier survival curves were used to analyse the overall median survival of mice and survival proportions in each of the treatment groups. The *P*-value significance of the lung metastasized clonogenic colonies was analysed using Dunn's multiple comparison post-test. All the statistical analyses were performed using the GraphPad Prism 4 programme and a *P*-value less than 0.05 was considered significant.

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