

Inhibition of murine K-BALB and CT26 tumour growth using a Semliki Forest virus vector with enhanced expression of IL-18

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Abstract. The enhanced Semliki Forest virus vector (SFV10-E), an RNA-based suicide expression vector system, expresses foreign genes at levels up to 10x higher than the original SFV10 vector. This vector has been used previously to express interleukin-12 for a tumour treatment study in a BALB/c murine model. Interleukin-18, an IFN- γ -inducing cytokine, plays a key role in the early induction of T helper1 (Th1) cell-mediated immune responses in addition to anti-angiogenic activity. In this study, the murine IL-18 gene along with an Ig-kappa leader sequence was cloned into the SFV10-E vector. The pSFV10-E-IL-18 construct was characterised *in vitro* for levels of expression and secretion, and the production of biologically active IL-18 was confirmed. An *in vivo* tumour treatment study using high titre rSFV10-E-IL-18 virus-like particles to treat subcutaneous K-BALB and CT26 tumours in BALB/c mice demonstrated therapeutic efficacy including the disappearance of tumour cells in a minority of treated animals. Tumour regression was associated with induction of avascular and suppurative necrosis.

Introduction

IL-18 belongs to the IL-1 family of cytokines and shares structural homology with IL-1. Like IL-1 it lacks a conventional signal sequence but has its own receptor. It is produced intracellularly as an inactive 24 kDa precursor and must be cleaved by caspase-1 to form the mature bioactive molecule. A wide range of cells including activated macrophages, dendritic cells, Kupffer cells, osteoblasts and epithelial cells produces the mature 18.3 kDa-protein. IL-18 promotes NK and T cells to secrete IFN- γ and the granulocyte-macrophage colony stimulating factor (GM-CSF) (1,2).

IL-18 has been shown to be effective in the treatment of a variety of murine tumours, either alone or in combination with IL-12. Transduction of the IL-18 gene induces anti-tumour activity by the augmentation of NK-cell activity, the production of IFN- γ by activated T-cells, and the inhibition of angiogenesis (3-12).

IL-18 is associated with Th1 response and, as such, can be a mediator of tissue injury and pathology (2). Local administration of the cytokine mediated by vectors, including viruses, dendritic cells and plasmids, has proved successful in mediating anti-tumour activity (9,10), but the systemic administration of IL-18 has been shown to be associated with toxicity (2,12).

The Semliki Forest virus (SFV) vector system induces high-level transient RNA-based suicidal expression and, like other alphaviruses, has recently been developed both as a cytolytic anti-cancer agent and for the construction of prototype anti-tumour vaccines (13-15). This system consists of an expression vector RNA with which foreign genes can be expressed, and two helper vectors that encode the structural protein genes. Co-transfection of all three RNA transcripts into mammalian cells results in the release of recombinant virus-like particles (VLPs) coding for the foreign gene (16-18). As the resulting VLP progeny contain only the recombinant RNA and lack the viral structural genes, they are capable of only one round of replication. 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 encapsulated. SFV also induces apoptosis of infected cells (19), which means that the virus does not persist in tissue after infection (20).

Recently, a new SFV-based enhanced expression vector, SFV10-E, has been developed. Cells transfected with this vector have been shown to produce up to 10x more foreign protein than the original SFV expression vector. This vector was used in our previous study to express IL-12 for tumour treatment in mouse models (21). This enhanced expression 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) was inserted as a linker between the enhancer

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and foreign gene sequences. 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 (21).

In this study, we cloned the murine IL-18 gene into SFV-10-E to produce a biologically active mature protein and measured expression of the protein in cultured BHK-21 and K-BALB cells. We examined the anti-tumour effect of pSFV10-E-IL-18 in two different tumour models in immunocompetent BALB/c mice. BHK-21 and K-BALB fibrosarcoma cells were used for virus particle titration and cell culture studies. 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 (22,23). CT26 cells are murine colon adenocarcinoma cells, which form localised tumours of low immunogenicity in BALB/c mice after s.c. injection (24). We showed that the treatment of K-BALB and CT26 tumours with rSFV10-E-IL-18 VLPs induced significant inhibition of tumour growth. Regression of tumour growth was associated with increased levels of avascular and suppurative necrosis. Tumour cells were absent at sites of tumour regression in a minority of treated animals.

Materials and methods

Cell culture. The BHK-21, K-BALB, and CT26 cell lines were obtained from the American Type Culture Collection (MD, USA). The BHK cell line, sBHK, was a gift from Professor P. Liljeström (Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden). BHK-21 and sBHK cells were cultured in BHK-21 medium supplemented with 5% newborn calf serum, 10% tryptose phosphate broth, 20 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. K-BALB and CT26 tumour cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM sodium pyruvate. Splenocytes were cultured in RPMI-1640 medium containing 10% foetal bovine serum (FBS), 10 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM sodium pyruvate, 1% non-essential amino acids, 50 µM β-mercaptoethanol.

Mice. Specific pathogen-free 4 to 6-week old female BALB/c mice (Harlan, UK) were maintained in accordance with the principles outlined in S1 17/94 European Communities regulations 1994, for the care and use of laboratory animals.

Construction of the pSFV10-E-IL-18 vector. To construct the pSFV10-E-IL-18 vector, IL-18 cDNA was amplified from total RNA of BALB/c mouse splenocytes using oligonucleotide primers and a reverse transcription kit (Promega, UK). The primers had terminal HindIII restriction enzyme sites (bold) and the sequences were: 5'**CCC AAG CTT AAC TTT GGC CGA CTT**3' (forward) and 5'**CCC AAG CTT CTA ACT TTG ATG TAA G3**' (reverse). Amplification was performed using the TripleMaster Taq PCR system

(Eppendorf, UK). The PCR product was cloned into the pSecTag2 B expression vector (Invitrogen, UK). The pSecTag2 B plasmid was used to add Ig-kappa signal sequence to the N-terminus of the IL-18 gene to induce the expression and secretion of the functional IL-18 protein. The IL-18 gene along with the leader sequence from the pSecTag2 B-IL18 construct was PCR amplified using the following primers: 5'TTA TAA TAC CCG GGA TGG AGA CAG ACA CA3' (forward) and 5'CTA TAC CCG GGA AGC TTC TAA CTT TGA TGT A3' (reverse). The pSFV10-E vector and the Ig-κ-IL-18 insert were digested with XmaI and the insert was ligated into the XmaI linearised vector using T4 DNA Quick Ligase to obtain pSFV10-E- Igκ-IL-18.

Production of rSFV10-E-IL-18 virus-like particles. 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 and pSFV10-E, was obtained from Professor P. Liljeström. For the production of rSFV10-E-IL-18 VLPs, the methods described previously were used (21). *In vitro* transcripts of the IL-18 construct and the helper vectors (rSFV-Helper S2 and rSFV-Helper CS219A) were co-electroporated into sBHK cells. After incubating the cells for 36 h at 33°C in 5% CO₂, the medium containing the VLPs was harvested, clarified by centrifugation, aliquoted, and stored at -70°C. Titration was performed by infecting the monolayers of BHK-21 cells and immunofluorescence was carried out using rat anti-mouse IL-18 antibodies (Pharmingen) 18 h post infection. To obtain high titer stocks of rSFV10-E-IL-18 VLPs, the supernatant was concentrated by ultracentrifugation through a sucrose cushion as described previously (21).

Secretion of IL-18 by cultured cells infected with rSFV-10-E-IL-18. Secretion levels of IL-18 from infected BHK-21 and K-BALB cells were analysed by ELISA using the OptEIA™ Set Mouse IL-18 kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. The cell supernatants were collected 12, 24, 48, and 72 h after mock (TNE) infection, rSFV-EGFP or rSFV10-E-IL-18 VLP infection at a multiplicity of infection (MOI) of 10.

The biological activity of the secreted IL-18 from both cell lines was assayed using mouse splenocytes (T cells). The splenocytes were incubated with supernatants of mock or rSFV-IL-18 VLP-infected BHK-21 or K-BALB cells collected at 24 h. Naïve and Conavalin A (Con A) (1 µg/ml) treated splenocytes were used as controls since Con A is an inducer of IFN-γ by T-cells. The supernatant from the splenocytes was collected at 12, 24, 48, and 72 h and assayed for IFN-γ as before (21).

Immunofluorescence of rSFV10-E-IL-18 VLP-infected BHK-21 cells (18 h post-infection) was carried out using goat anti-rat IL-18 antibody (R&D Systems, UK).

Treatment of K-BALB and CT26 tumours using rSFV10-E-IL-18 VLPs. Tumours were induced subcutaneously into the right flank by injecting 10⁶ cells as described previously (21). Tumours were measured daily using a linear caliper and average tumour diameter was calculated as the square root of

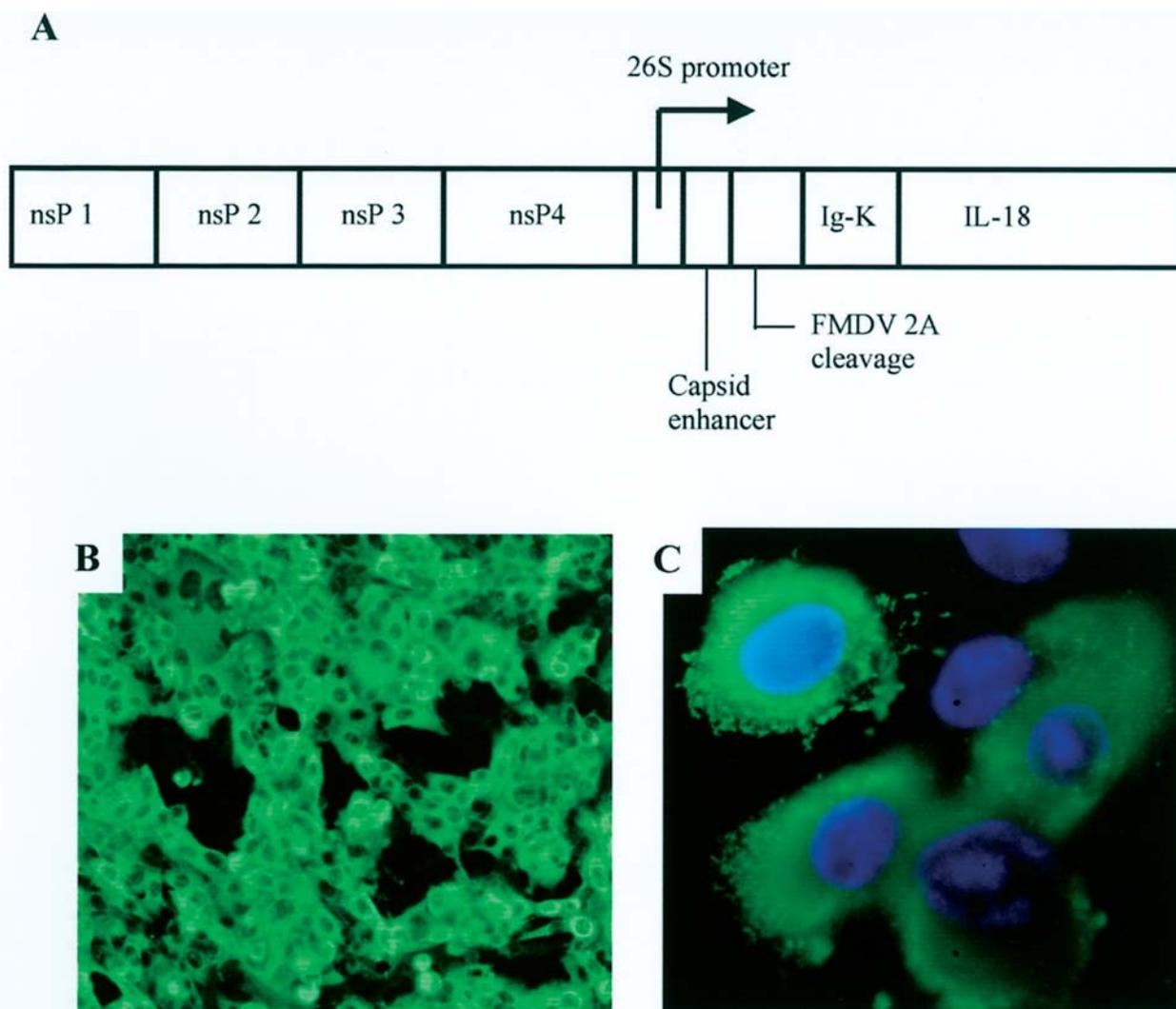


Figure 1. (A) Schematic representation of the pSFV10-E-IL-18 construct. The coding sequences of IL-18 gene with upstream Ig-kappa transport signal sequences were cloned into the MCS of pSFV10-E. The 26S sub-genomic promoter, Kozak, the capsid enhancer and FMDV 2A cleavage-site of pSFV10-E regulated the expression of IL-18. Original magnification x40. (B and C) Immunofluorescence staining for IL-18. BHK-21 cells were infected with rSFV10-E-IL-18 and examined 18 h post infection. Original magnification x40 (B), x200 (C).

the product of two perpendicular measurements, assuming spherical shape. Treatment began when the size of the established tumours reached 4 mm diameter. The tumours were treated every alternate day with a total of six intratumoural injections of 50 μ l of TNE buffer alone, TNE buffer containing rSFV-EGFP (virus control), and rSFV10-E-IL-18 VLPs at a titre of 4×10^9 IU for each injection. Efficient therapeutic effects were obtained using this regimen in our previous tumour study on IL-12 (21). Two sets of animals for each treatment group were used for each tumour cell line. The first group was assayed for survival and the second group assayed for histopathology. Mice were euthanised one day following the final treatment or when the tumour size reached 10% of body weight (15 mm).

Histopathology. For histopathology, 3 μ m sections of formaldehyde-fixed, paraffin-embedded tumours were stained routinely with H&E. Separate sections containing three different areas in each tumour were examined in a

blinded manner and the following parameters were assessed: Tumour cell morphology, heterogeneity, mitotic index, infiltration, ulceration, necrosis, haemorrhage, thrombosis, and leucocytic infiltrates.

Statistical analysis. The statistical data analysis of tumour treatment groups was performed by one-way repeated measures ANOVA. The probability (P-value) of each group was calculated using Tukey's multiple comparison post-test. All statistical analyses were made using the GraphPad Prism4 program and a P-value <0.05 was considered significant.

Results

IL-18 expression in BHK-21 and K-BALB cells. The murine IL-18 gene was cloned into the enhanced expression vector pSFV10-E to obtain the pSFV10-E-IL-18 vector that contains the Igk leader sequence (Fig. 1A). Immuno-

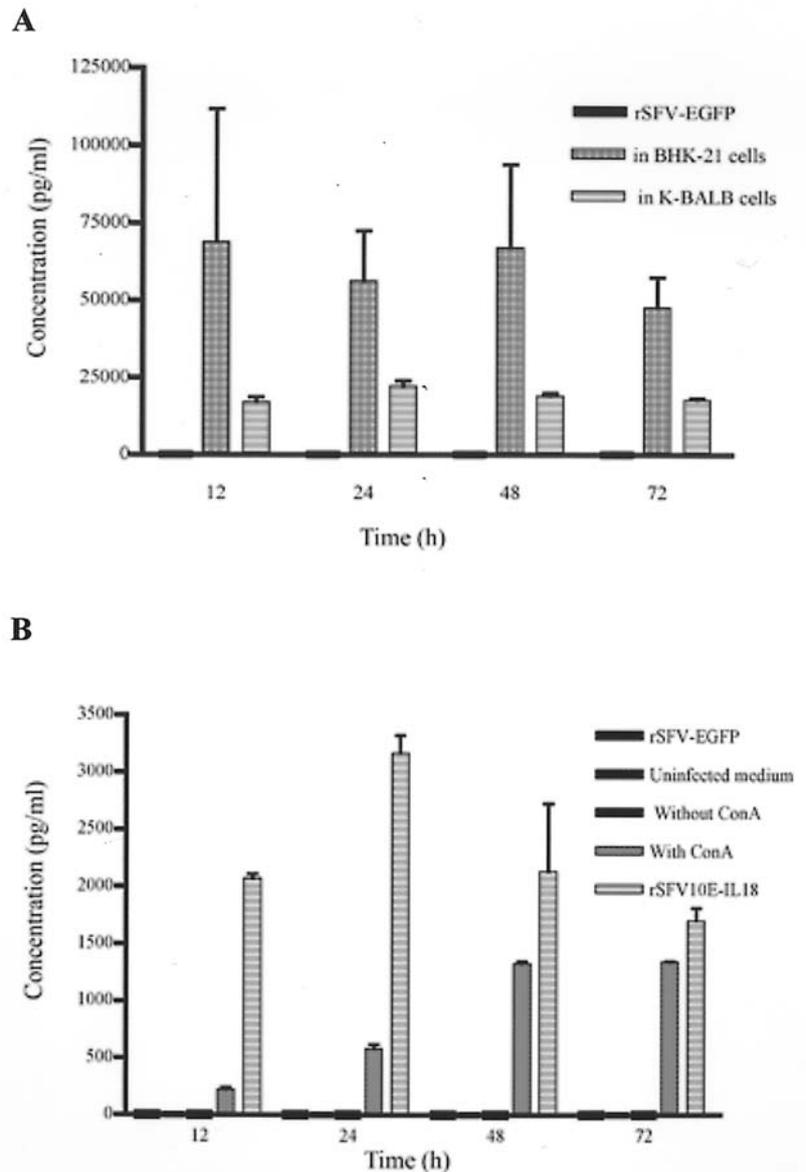


Figure 2. (A) IL-18 expression levels in the supernatants of rSFV10-E-IL-18 infected BHK-21 and K-BALB cells. The values shown represent IL-18 secretion from 2.5×10^5 cells as measured by ELISA. (B) Biological activity of murine IL-18 secreted by K-BALB cells. Using ELISA the production of IFN- γ from murine splenocytes (10^6 cells) treated with supernatants from rSFV10-E-IL-18 infected K-BALB cells was measured. Supernatants from control groups (rSFV-EGFP, uninfected, and without Con A) were unable to induce IFN- γ production.

fluorescence staining of rSFV10-E-IL-18-infected BHK-21 cells using IL-18 specific antibody qualitatively confirmed IL-18 expression (Fig. 1B). Estimation of IL-18 in the supernatants of BHK-21 and K-BALB cells infected with rSFV10-E-IL-18 was carried out at 12, 24, 48 and 72 h after infection using ELISA (Fig. 2A). The levels of IL-18 expression were 2-3x higher in BHK-21 cells than in K-BALB cells. Overall expression levels were similar to those recorded in our previous study using IL-12 (21). IL-18 expression was absent in the supernatants from mock-infected control BHK-21 and K-BALB cells.

Biological functionality of secreted IL-18. Using ELISA the production of IFN- γ from the 24 h supernatants of murine splenocytes treated with supernatants from rSFV10-E-IL-18-infected K-BALB cells was measured. The results showed

that the supernatants of K-BALB cells infected with rSFV10-E-IL-18 stimulated splenocytes to produce higher levels of IFN- γ than Concanavalin A treatment, indicating the production of functionally active IL-18 (Fig. 2B).

Treatment of K-BALB and CT26 tumours using rSFV10-E-IL-18. Tumour growth was monitored daily and mice were euthanised one day following final treatment or when the average tumour diameter reached 15 mm. The average time taken to reach this size for a group of mice represented the survival time and mice showing complete tumour regression were considered survivors.

The growth of K-BALB and CT26 tumours was inhibited following treatment with rSFV10-E-IL-18 and the levels of growth inhibition were significant compared to the controls. A complete regression of tumours and survival occurred in 2

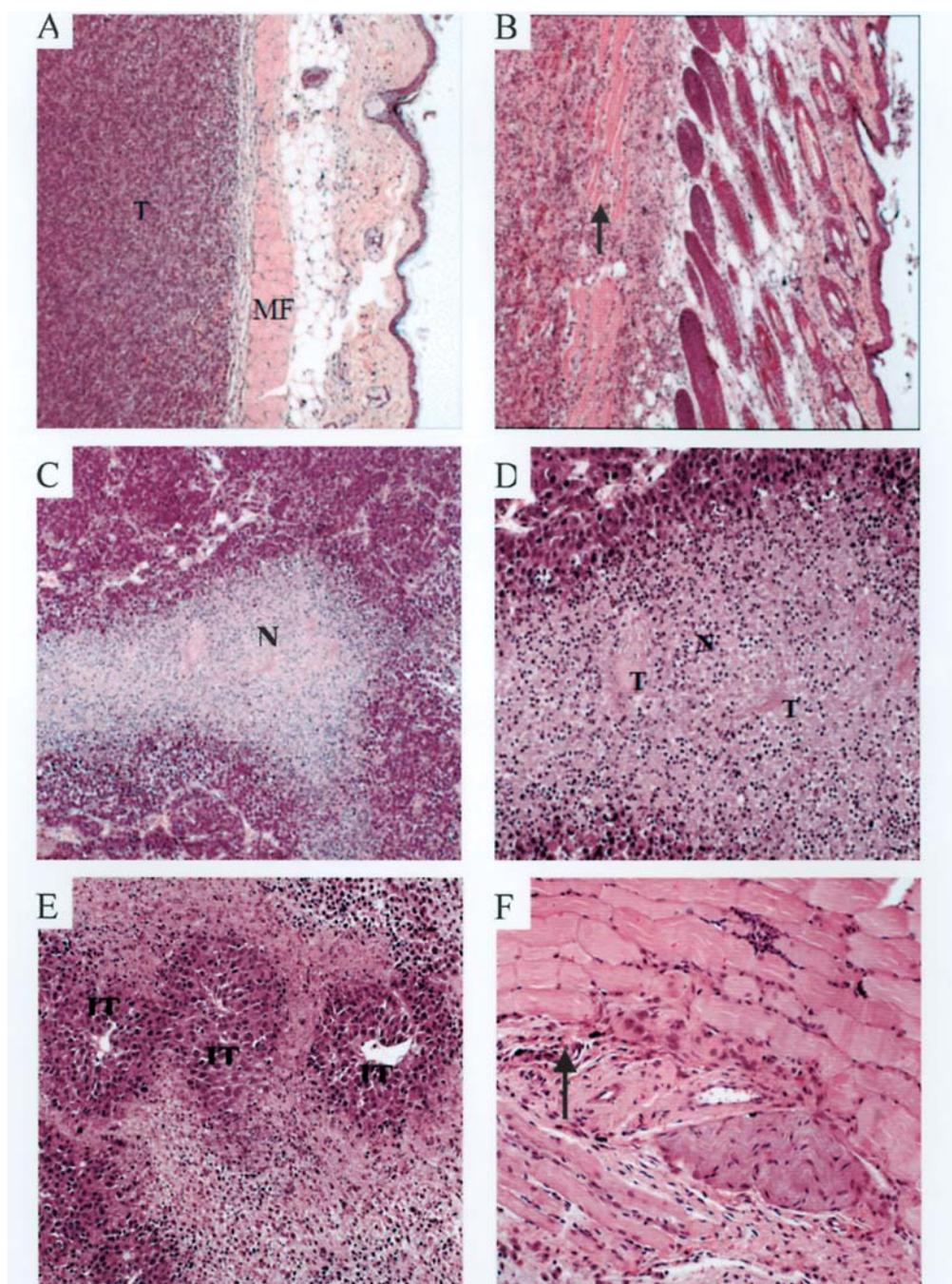


Figure 3. (A and B) K-BALB tumours, one day following final treatment with TNE. (A) Dense aggregate of tumour cells (T) in the subcutis. The epidermis, dermis and panniculus muscle fibres (MF) are intact. H&E, x100. (B) Invasion of tumour cells through the panniculus muscle (arrow) into the deep dermis. H&E, x100. (C and D) CT26 tumours, one day following final treatment with rSFV10-E-IL-18. (C) Centrally located area of suppurative necrosis (N). H&E, x100. (D) Higher magnification of (C) showing fibrin thrombi (T) and neutrophils. H&E, x200. (E and F) K-BALB tumours, one day following final treatment with rSFV10-E-IL-18. (E) Islands of intact tumour cells (IT) around thin-walled blood vessels and avascular necrosis in marginal areas. H&E, x100. (F) Fibrous scarring, low-grade infiltrates of lymphocytes and macrophages (arrow) and regenerating skeletal muscle myofibers. No tumour cells are visible. H&E, x200.

of 6 mice with K-BALB tumours and in 2 of 6 mice with CT26 tumours. The control (TNE or rSFV-EGFP) treated groups showed a similar tumour growth with no survivors (Table I).

Histopathology. The control tumours were located in the subcutis and were composed of densely cellular, expansive masses with no evidence of encapsulation. Focal necrosis, ulceration and invasion of panniculus muscle fibres were

common (Fig. 3A and B). Areas of avascular and suppurative necrosis with fibrin thrombi and haemorrhage were more prominent in rSFV10-E-IL-18 treated tumours than in the control tumours. Neutrophils and macrophages were located in areas of necrosis and lymphocytes were sparsely distributed in peripheral areas at the interface with surrounding tissues (Fig. 3C-E). Tumour cells were not detected at the sites of induction of CT26 tumours in 2 mice following local treatment with rSFV10-E-IL-18 where small

Table I. Inhibition of tumour growth by rSFV10-E-IL-18 treatment.

Tumour model	Treatment group ^a	Median survival (days) ^b (No. of survivors) ^c	Average tumour diameter ^d (mm) (std. error)	Probability ^f
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)	<0.01
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)	<0.01

^aSix BALB/c mice per treatment group were used for all experiments; ^bmice were sacrificed when the tumour reached 15 mm diameter and the time taken to reach this size is the survival time; ^cmice which had complete and permanent tumour regression; ^daverage tumour diameter for each group was calculated at their appropriate median survival time; ^eaverage of the control groups treated with TNE buffer only; ^fthe probability value is based on the average tumour diameter at their median survival time compared with that of the TNE treated (control) groups.

cutaneous nodules were characterised by fibrosis, chronic inflammation and atrophic and regenerative changes in skeletal muscle myofibers (Fig. 3F).

Discussion

Several cytokine genes introduced intratumourally in mice have shown anti-tumour effects. The strong anti-tumour properties of IL-12 and IL-18 have been previously reported using murine renal cell carcinoma and bladder cancer models (4,5). We have previously shown the anti-tumour effect of IL-12-expressing SFV VLPs in K-BALB and CT26 murine models (21); in this study extended this to IL-18.

IL-18 is first synthesised as a leaderless precursor, and it requires a signal sequence for extracellular secretion of the mature protein. We constructed a pSFV10-E-IL-18 vector containing the Igk leader sequence to regulate the expression of mature IL-18. It has been reported that direct injection of an IL-18 adenoviral vector into mice with MCA205 sarcoma elicits an effective immune response. The dose of IL-18 administered correlated with the resulting level of serum IFN- γ and the level of anti-tumour activity (25). The rSFV10-E-IL-18 vector constructed in the present study showed higher levels of expression in the supernatants of infected BHK-21 cells than in the supernatants of infected K-BALB cells. These differences probably reflected different levels of infectivity for the two cell lines and varying abilities to secrete the expressed protein. To determine the bioactivity of secreted IL-18, we induced mouse splenocytes (T-cells) for IFN- γ production and the results confirmed that the IL-18 produced by rSFV10-E-IL-18 in K-BALB cells is biologically functional. The IL-18 construct with enhanced expression ability, which contains the Igk leader sequence, induced secretion of large amounts of IFN- γ . These observations are consistent with a previous study where successful immunogene tumour therapy was achieved using a plasmid expression vector along with pro-IL-18 sequences that also contained the Igk leader sequence (26).

In this study we showed that the treatment of K-BALB and CT26 tumours with rSFV10-E-IL-18 induced a significant inhibition of tumour growth. Complete tumour regression occurred in a minority of treated animals. The findings resemble those obtained in a previous study of K-BALB and CT26 tumours treated with rSFV10-E-IL-12 (21) and showed that IL-18 and IL-12 have potent anti-tumour effects when expressed from the enhanced expression SFV vector.

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