

**CELLULAR IMMUNOTHERAPY FOR
MURINE NEUROBLASTOMA**

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ABBREVIATIONS

Ad	Adenovirus
APC	Antigen presenting cell
β 2m	Beta-2-microglobulin
BM	Bone marrow
bp	base pair
BSA	Bovine serum albumin
CAR	Coxsackie-adenovirus receptor
CNS	Central nervous system
Con A	Concanavilin A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte associated protein 4
dH ₂ O	Distilled water
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPE	Dioleoylphosphatidylethanolamine
DOTMA	Dioleoyloxypropyltrimethylsmmonium chloride
DTH	Delayed-type hypersensitivity
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay

ELISpot	Enzyme linked immunosorbent (spot assay)
FACS	Fluorescent activated cell sorting
FADD	Fas-associated death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-like IL-1 β -converting enzyme
FLIP	FLICE-inhibitory protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
IP-10	Interferon-gamma-inducible protein 10
kb	kilobase pairs
kDa	kilo Daltons
LB	Luria-Bertani broth
LPD	Lipid-cationic targeting peptide- DNA complexes
MDR1	Multi drug resistance gene
MHC	Major histocompatibility complex
MRP	Multi drug resistance-related protein
NB	Neuroblastoma
N2a	Neuro-2a murine NB cell line
NK	Natural killer cell
PBS	Phosphate buffered saline

PBMC	Peripheral blood mononuclear cell
PD	Cationic targeting peptide-DNA complex
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
RNA	Ribonucleic acid
RT	Room temperature
SCID	Severe combined immunodeficiency
STAT (4)	Signal transducer and activator of transcription (4)
TAA	Tumour associated antigen
TAE	Tris acetate EDTA
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TE	Tris-EDTA buffer
TGF- β	Transforming growth factor beta
TNF- α	Tumour necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
Tris	2-amino-2-[hydroxymethyl]-1,3 propandiol
Tween 20	Polyethylene-sorbitan monolaurate
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
w/v	Weight per volume

AMINO ACIDS

Full name	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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ABSTRACT

Neuroblastoma (NB) is the most common extracranial paediatric tumour, and patients with disseminated disease have a poor long-term prognosis. Due to the significant mortality rate, alternative treatments to conventional therapies are continually sought. Using the A/J mouse model it has previously been demonstrated that a cellular vaccine of the syngeneic Neuro-2a NB cell line modified to express IL-2 and IL-12 abrogated the tumourigenicity of Neuro-2a cells, and mediated regression of established tumours. However, establishing cultures of primary NB cells can be problematic, making this approach difficult to implement clinically.

This thesis describes the development of an alternative cellular vaccine to treat murine NB using a synthetic vector. Firstly, transfection of primary dendritic cells was optimised. Dendritic cells are potent antigen presenting cells and studies have shown them to generate anti-cancer responses. Optimal transfection levels of 5% were obtained but antigen presentation by these cells was limited. Therefore, an alternative approach was developed using fibroblasts engineered to express IL-2 and IL-12. Cytokine-expressing fibroblasts could be used in place of transfected tumour cells to provide sustained, high-level cytokine expression in the tumour locale. Transfection of syngeneic and allogeneic murine fibroblasts was optimised *in vitro* to produce therapeutic levels of IL-2 and IL-12. Cytokine-transfected fibroblasts were compared with cytokine-transfected Neuro-2a cells to prevent engraftment of wild-type Neuro-2a cells *in vivo*. The allogeneic cells prevented tumour engraftment in a non-specific, cytokine-independent manner. Syngeneic fibroblasts expressing IL-2 and IL-12 inhibited tumour engraftment as effectively as cytokine expressing Neuro-2a cells, and this rejection was cytokine-dependent. The cytokine-transfected syngeneic fibroblasts induced protective immunity against rechallenge with wild-type Neuro-2a cells as effectively as cytokine-transfected-Neuro-2a cells. Intratumoural vaccination of cytokine-transfected syngeneic fibroblasts also demonstrated therapeutic efficacy against Neuro-2a-derived established tumours. Splenocytes from vaccinated mice demonstrated increased IL-2 and IFN- γ expression and cytotoxicity compared with controls when co-cultured with wild-type Neuro-2a cells *in vitro*. Vaccinated tumours showed decreased vascularity and increased infiltration of CD45⁺ cells compared with controls. Therefore, cytokine-transfected syngeneic fibroblasts are a viable potential alternative vaccine for the treatment of minimal residual NB.

CHAPTER 1

AIMS AND INTRODUCTION

1.1 NEUROBLASTOMA

1.1.1 Disease features

Neuroblastoma (NB) is the commonest extracranial solid tumour of childhood, affecting one in 7000 children. The tumours arise from any site where the neural crest cells are located, the most frequent locations being the adrenal medulla and the sympathetic ganglia. This tumour accounts for 8% to 10% of all cancers of childhood and for 15% of cancer related deaths in children (Young & Miller, 1975). An internationally accepted system for classifying tumour stage and facilitating comparison was agreed in 1988, then further modified in 1993 (Table 1.1). NB frequently presents as an aggressive malignant tumour, but is also distinctive, as the human tumour to most frequently undergo spontaneous regression. Alternatively, NB may differentiate into benign ganglioneuromas, and both features are most prevalent in stage 4-S tumours. The clinical prognosis of the disease depends on a number of factors. Age of diagnosis is critical, with infants diagnosed at <12 months having a generally promising prognosis, even if they are suffering from metastatic disease. Those diagnosed over 1 year of age have a much more dismal outlook and often fail to respond to even the most aggressive combinations of therapy (Evans *et al.*, 1987).

NB is characterised by several features. The tumours metabolise catecholamines, resulting in the excretion of metabolites (Voorhess & Gardner, 1961) which are used as markers of tumour activity. These include normetanephrine (NME), vanillylmandelic acid (VMA) and homovanillic acid (HVA) (Nakano *et al.*, 1976; Brewster & Berry, 1979). The ratios between these two compounds can denote tumour differentiation (Fitzgibbon & Tormey, 1994) and combined determination of NME with either VMA or HVA further enhances

Table 1.1 International Neuroblastoma Staging System (INSS) (Brodeur *et al.*, 1993)

Stage 1	Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral and contralateral lymph nodes negative for tumour microscopically.
Stage 2a	Localised tumour with incomplete gross excision; representative ipsilateral and non-adherent lymph nodes negative for tumour microscopically.
Stage 2b	Localised tumour with complete or incomplete gross excision; with ipsilateral and non-adherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes must be negative microscopically.
Stage 3	Unresectable unilateral tumour infiltrating across the midline with or without regional lymph node involvement; or localised unilateral tumour with contralesional regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement.
Stage 4	Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined in stage 4S)
Stage 4S	Localised primary tumour (as defined in stage 1, 2a or 2b) with dissemination limited to skin, liver and/or bone marrow (limited to infants less than one year old). Bone marrow involvement should be minimal; i.e. <10% of nucleated cells identified as malignant.

their diagnostic power (Monsaingeon *et al.*, 2003). It is these compounds that are analysed in population screening. Critical analysis of several such programs has shown that screening does not confer any benefit to patient outcome and may even be detrimental, leading to unnecessary treatment of stage 1 or 2 tumours and the morbidity and mortality associated with therapy of tumours that may well have regressed spontaneously (Schilling *et al.*, 2002).

1.1.2 Genetic features

1.1.2.1 Chromosome 1 and chromosome 17

Genetic anomalies in chromosome 1 include rearrangements or deletions, leading to loss of material from the short arm (1p) or addition of segments to the long arm (1q) (Brodeur *et al.*, 1977). Genetic loss in 1p is one of the most common chromosomal rearrangements, seen in ~33% of tumours, and indicates the loss of function of yet unidentified tumour suppressor genes (Caron *et al.*, 1996). Screening regions of 1p has identified putative candidate genes (Krona *et al.*, 2003), and the *p53* homologue *p73* has also been localised to 1p36, which is frequently deleted in NB.

Gain of 17q is the most common cytogenetic abnormality in NB, seen in over 50% of NB tumours, and is also strongly associated with other risk factors including age >1 year, unfavourable ploidy and *N-myc* amplification (Bown *et al.*, 1999). The status of 17q is a powerful prognostic factor in NB; the five year survival of patients with 17q gain was ~31%, compared with 86% for patients with normal 17q (Bown *et al.*, 1999). The correlation between 1p loss, 17q gain and *N-myc* amplification is highly significant, as *N-myc* amplification is never seen in the absence of 17q gain or deletion of 1p (Caron *et al.*, 1996; Bown *et al.*, 1999).

1.1.2.1 N-Myc

Many genetic markers that predict prognosis can reflect the tumour's ability to proliferate or differentiate. *N-myc* amplification is the best prognostic indicator in NB (Corvi *et al.*, 1997), and is often found in the more aggressive stage 3 and 4 tumours. *N-myc* is a member of a small closely related family of genes and was first recognised by its similarity to *c-myc* and amplification in NB. The oncogene is located at chromosome 2p23-24, and encodes a 64-kDa DNA-binding transcriptional regulator that together with the 22-kDa Max protein forms N-myc/Max heterodimers (Facchini & Penn, 1998). The gene is expressed during normal embryogenesis and is involved with neuronal cell migration, early phases of ganglionic neural crest differentiation (Wakamatsu *et al.*, 1997). Human embryonal sympathetic ganglia express *N-myc* up to week 8.5 (Jogi *et al.*, 2002).

N-myc copy number appears to be constant throughout the tumour's history; from tumour presentation to either regression or patient death (Brodeur *et al.*, 1987) and many prognostic factors are correlated with *N-myc* amplification. Reduced expression of *p73* is significantly correlated with *N-myc* overexpression. The *p73* gene is deleted ~33% of NB tumours, while many other NB tumours expressed reduced levels of p73 protein (Zhu *et al.*, 2002). A variety of other factors in poor prognoses NB are correlated with *N-myc* amplification, including silencing of CD44 expression (Combaret *et al.*, 1996; Yan *et al.*, 2003), increased blood plasma levels of the growth factor midkine (Ikematsu *et al.*, 2003), inactivation of caspase-8 (Teitz *et al.*, 2000), high expression of TRKB, the receptor for brain derived neurotrophic factor (BDNF), (Nakagawara *et al.*, 1994) and high telomerase activity (Hiyama *et al.*, 1995).

Furthermore, *N-myc* amplification correlates with the angiogenic activity of NB. Amplified tumours promoted the development of significantly higher numbers of vessels in

angiogenesis assays (Ribatti *et al.*, 2002a). Overexpression of *N-myc* down regulates interleukin-6 (IL-6) and leukaemia inhibitory factor (LIF) expression (Hatzi *et al.*, 2002a; Hatzi *et al.*, 2002b). IL-6 and LIF do not directly inhibit NB cell growth, but inhibit endothelial cell proliferation and vascular endothelial growth factor (VEGF)-induced angiogenesis. Therefore down-regulation of IL-6 and LIF removes these inhibitory stimuli, resulting in increased angiogenesis and enhancement of tumour growth.

Up to 60% of primary NB tumours express the anti-apoptotic protein Bcl-2, conferring resistance to chemotherapy and radiation treatment (Noujaim *et al.*, 2002). Co-expression of Bcl-2 and *N-myc* up-regulates expression and activation of matrix metalloproteinase-2 (MMP-2) (Noujaim *et al.*, 2002). MMPs are vital for tumour metastasis by either invading into surrounding tissues or intravasion through endothelial cells into the bloodstream (Stamenkovic, 2000) and increased MMP activity levels play a role in many cancers (Kelly *et al.*, 2000; Kanayama, 2001).

Unusually for cancer, *p53* mutations in NB are rare (Moll *et al.*, 1995), and the vast majority of primary tumours contain wild-type *p53*. However, *p53* was sequestered in the cytoplasm rather than the nucleus in many undifferentiated NB tumours, preventing it from functioning as a tumour suppressor.

1.1.3 Current NB Therapy

The current treatment for NB depends on the stage of the disease (Table 1.2). For patients with severe disease, induction chemotherapy is usually given, using a cocktail of several agents with different targets, followed by surgical resection, then high dose chemotherapy and stem cell rescue (Ninane & Pearson, 1997). One of the main barriers

Table 1.2 Current Therapeutic Classification System (Ninane & Pearson, 1997)

Group	Tumour stage	Patient age (years)	Therapy
A	1, 2, 4S	Any	Surgery (Emergency therapy if symptomatic)
B	3*	Any	Conventional chemotherapy \pm radiotherapy
	4	<1	Surgery
C	4	>1	Intensive chemotherapy, surgery, high dose chemotherapy with haematopoietic stem cell rescue

* Patients with stage 3 tumours with *N-myc* amplification should be treated as stage 4 >1yr

to chemotherapy, particularly to repeated courses of treatment, is the induction of drug resistance. The best characterised mechanisms of multidrug resistance are the *MDR1* gene, encoding P-glycoprotein (Pgp) and the multidrug resistance-associated protein (*MRP*) gene. Both of these proteins encode ATP-dependent membrane transport proteins, which render cells resistant to treatment with a variety of chemotherapeutic agents (Grant *et al.*, 1994). Analysis of pre- and post-therapy patient-derived cell lines showed drug resistance to increase by up to 1000-fold in the post-therapy cell lines against certain agents following chemotherapy (Keshelava *et al.*, 1997).

In addition to chemotherapy, retinoids are also used in the treatment of several malignancies, including NB. A recent study demonstrated the efficacy of an intensive high-dose triple-tandem chemotherapy regimen in combination with peripheral blood stem cell rescue, and a proportion of the patients also received 13-*cis*-retinoic acid, showing a 3-year survival rate of 79% (Kletzel *et al.*, 2002).

Retinoids are derivatives of vitamin A and are signalling molecules that regulate cell growth and differentiation, or can induce apoptosis. They mediate their effects at transcription level by binding to dimers of retinoic acid receptors (RAR) which function as transcription factors, resulting in the production of many intracellular and secreted growth inhibitors (Roninson & Dokmanovic, 2003). Prevention of tumour growth by retinoids has also been shown to be due to senescence rather than differentiation. However, as with so many drugs, tumour cells can become resistant to effects of retinoids and this is due to the inactivation of the RAR (Altucci & Gronemeyer, 2001).

Alternative retinoids have different effects; 9-*cis*-retinoic acid has been shown to be more effective than other isomers in inducing RAR expression. Additionally, certain RAR antagonists inhibit the expression of RAR- β in response to all-*trans*- but not 9-*cis*-retinoic acid, indicating different isomers have alternative mechanisms of gene induction (Lovat *et al.*, 1997). The 9-*cis* isomer has also shown superior inhibition of differentiation and sustained differentiation of human NB cells *in vitro*. Due to the significant mortality rate still seen in NB, alternatives are continually being sought to improve patient prognosis.

1.2 Mechanisms of tumour progression

1.2.1 Angiogenesis and tumour growth

Angiogenesis is the process of forming new blood vessels and capillaries from pre-existing vasculature. This is an elaborate cascade of events, beginning with degradation of the basement membrane of the existing vessel. Endothelial cells are then able to migrate from the vessel and invade the extracellular matrix. Here they proliferate, ultimately resulting in the formation of the lumen of new capillaries. This process of neovascularisation occurs

in normal physiological situations, such as wound healing, but is also found in pathological conditions. In the formation of solid tumours, the switch from the avascular to the vascular stage is seen as a specific clinical turning point in disease development (reviewed in Bergers & Benjamin, 2003). Once this blood supply is formed, oxygen and nutrients can be supplied and the tumour can grow almost indefinitely. Primary NB and metastases are well vascularised, thus angiogenesis plays a vital role in tumour development.

1.2.1.1 Pro-angiogenic factors

In order for this process to take place there is a requirement for the tumour cells to produce endothelial cell-specific growth factors, such as VEGF, which stimulate the development of host blood vessels (Senger *et al.*, 1993). VEGF signals through two tyrosine kinase receptors, Flt-1 and Flk-1 (Terman *et al.*, 1991).

Analysis of clinical NB samples showed that VEGF expression by NB cells was significantly higher in tumours with unfavourable histology. Increased Flk-1 expression in NB cells and vascular endothelia was also related to unfavourable histology (Fukuzawa *et al.*, 2002). VEGF can also protect NB cells *in vitro* from apoptosis induced by serum starvation or the presence of tumour necrosis factor- α (TNF α), by up regulating Bcl-2 production (Beierle *et al.*, 2002). VEGF may also confer protection to cytotoxic drugs by inducing expression of multidrug-resistance proteins (Zhang *et al.*, 2001) or the anti-apoptotic factor, survivin (Tran *et al.*, 2002).

However, despite the presence of many angiogenic factors, tumours often have an insufficient blood supply, selecting for tumour cells that thrive under hypoxic conditions. This is clinically relevant because cells under hypoxia are less accessible to cytotoxic drugs or immunotherapeutic modulators and less susceptible to ionising radiation (reviewed in

Rabatti *et al.*, 2002b). Hypoxia has also been shown to result in a higher mutation rate and increased metastasis; the frequency of mutations in cells within tumours can be up to 5-fold higher than otherwise identical cells grown in culture (Reynolds *et al.*, 1996).

1.2.1.2 Antiangiogenic therapy

Antiangiogenic strategies have been developed to treat many tumours. These are aimed at inhibiting the growth of endothelial cells rather than inhibiting the tumour cells directly. Endothelial cells are genetically stable and therefore do not develop drug resistance in the same manner as rapidly mutating tumour cells. Many antiangiogenic strategies have been investigated in the treatment of NB. Combination therapy of experimental NB using an anti-VEGF antibody and low-dose high-frequency chemotherapy has been shown to significantly reduce tumour mass and vascularity (Kim *et al.*, 2002). Cytokines such as IL-12 and interferon- γ -inducible protein-10 (IP-10) (Feldman *et al.*, 2002) also display antiangiogenic activity.

1.2.2 Tumour mechanisms of immune escape

There is evidence from experimental tumours generated under certain conditions to support the theory of immunosurveillance in inhibiting the growth of very early tumours; tumours chemically-induced in immunodeficient mice are consistently less tumourigenic when transplanted into syngeneic immunocompetent mice (reviewed in Khong & Restifo, 2002). The initial lesions that develop into aggressive tumours have “escaped” immune recognition, and the immunological phenotype of the tumour has been “shaped” by the surveillance process.

There is evidence that surveillance against tumours fails due to immunological ignorance. One study revealed that tumours would grow if small pieces were transplanted, but if the

equivalent cell number was transferred as a single cell suspension, a protective cytotoxic T lymphocyte (CTL) response developed. Tumour growth was tightly correlated with the failure of tumour cells to reach the draining lymph nodes and the absence of primed CTLs. It was also shown that a response could be generated by direct immunisation into lymphoid tissue, hence CTLs were neither tolerant nor deleted (Ochsenbein *et al.*, 1999). Tumours are inherently genetically unstable, and those that possess mutations that enhance proliferation or survival will have a growth advantage over other cells. Tumour escape is a passive process of natural selection where tumour subclones with favourable characteristics will outgrow others in response to conditions in the tumour microenvironment such as nutrient and oxygen supply and growth factors, in the setting of untreated tumours and those exposed to immuno- or chemotherapy. Malignant cells have evolved many mechanisms to escape immune detection, involving multiple cellular pathways.

1.2.2.1 Death Receptors

Death receptors are transmembrane proteins that belong to the TNF receptor family. Six have been identified so far in humans, and all contain a cytoplasmic “death domain” sequence which couples caspase cascades essential for the induction of apoptosis to each receptor (French & Tschopp, 1999).

1.2.2.1.1 Fas/Fas ligand

Various lymphocytes have been reported to mediate tumour immunity, such as CTL, natural killer (NK) and lymphokine activated killer (LAK) cells. These cells induce apoptosis of tumour cells by two methods; the granzyme/perforin pathway, which induces pores in the target cell membrane through which granzymes are secreted leading to

apoptosis, and the Fas/Fas ligand (FasL) pathway. Fas (CD95) is a death receptor expressed by the target tumour cell which interacts with FasL expressed on the surface of the killer cell. The Fas-associated death domain (FADD) then activates caspase-8, which subsequently activates itself and cleaves other downstream members of the caspase cascade such as caspase-3, -6 and -7 (Salvesen & Dixit, 1999).

Experiments in perforin-deficient mice have demonstrated the role of Fas in tumour rejection, where it was observed that *in vitro*-Fas-negative tumour cell lines upregulated Fas when the cells were vaccinated *in vivo*. Tumour cells transfected with a Fas anti-sense construct grew faster in the initial stages of tumour growth compared to Fas-expressing cells, although both groups of mice eventually succumbed to disease, suggesting a role for Fas in tumour immunity in the early stages of tumour growth (Rosen *et al.*, 2000). At more advanced stages of malignancy, tumour cells have been demonstrated to become resistant to Fas-mediated apoptosis by down-regulation of Fas expression or function (Hahne *et al.*, 1996), lack of surface expression due to sequestration of Fas in the cytoplasm (Nambu *et al.*, 1998) or by Fas splicing variants that result in truncated Fas lacking the intracellular death domain (Cascino *et al.*, 1996).

Tumour cell Fas expression has been shown to be upregulated by irradiation of tumour cells *in vitro* and subcutaneous tumours *in vivo*, sensitising the tumour cells to apoptosis by antigen-specific CTLs. Combination treatment of experimental murine colon carcinoma with tumour irradiation and CTL adoptive transfer resulted in significantly decreased growth rate and tumour volume of established tumours compared to either treatment alone or controls (Chakraborty *et al.*, 2003). Transducing tumour cells to express higher levels of Fas-L, has also been shown to increase the level of induced apoptosis (Shimizu *et al.*, 1999), demonstrating an important role of Fas in tumour cell rejection. CTL-mediated

cytotoxicity via the perforin pathway can also be blocked by tumours through the overexpression of a serine protease inhibitor that inactivates granzyme B known as SPI-6 (Medema *et al.*, 2001).

1.2.2.1.2 FLIP

Murine models have directly shown that cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein (cFLIP) expression results in escape from T-cell immunity *in vivo*. Tumour cells with elevated cFLIP expression are selected for *in vivo* and blockade of Fas-dependant apoptosis can result in tumour escape even when the perforin pathway is operational (Medema *et al.*, 1999). Furthermore, it has recently been shown that cFLIP can inhibit T-cell activation, proliferation and IL-2 expression (Tai *et al.*, 2004). Several viruses, including oncogenic human herpesvirus-8 and Kaposi's sarcoma-associated herpesvirus, express viral FLIP which mediates resistance to FasL (French & Tschopp, 1999).

cFLIP is also an inhibitor of caspase-8, a cysteine protease associated with apoptosis that signals through Fas/FasL (Irmeler *et al.*, 1997), while inactivating mutations of caspase-10 have been detected in lymphoma patients (Shin *et al.*, 2002).

1.2.2.1.3 TRAIL

TNF-related apoptosis-inducing ligand (TRAIL) is cancer cell-specific, killing many transformed cells but sparing most normal cells, sparking a great deal of interest in the use of TRAIL as a cancer therapeutic. TRAIL binds its receptor, DR5, which mediates its apoptotic effects through a FADD and caspase-8 dependent pathway (Bodmer *et al.*, 2000). TRAIL is highly expressed on activated NK cells, T-cells, CD11c⁺ dendritic cells (DC) and monocytes, and TRAIL-knock out mice have shown a direct role for NK cell-TRAIL in natural suppression of tumour metastases.

A clear role for TRAIL in T-cell mediated immune defence against cancer has been shown in allogeneic haematopoietic cell transplantation. The donor T-cells exhibit anti-tumour effects, (graft-versus-tumour (GVT)), which have been postulated to provide the greatest indication of T-cell-mediated, clinically significant, anti-tumour activity, and TRAIL has been shown to be necessary for optimal donor T-cell GVT activity. As GVT activity is stimulated by detection of tumour-associated antigens or alloantigens, the main complication of this treatment is graft-versus-host-disease (GVHD), resulting in systemic illness and organ damage. However, TRAIL plays little or no role in GVHD, selectively killing malignant cells over normal cells, making TRAIL an exciting prospect for immunotherapy (Schmaltz *et al.*, 2002).

Interferon- γ (IFN- γ) is also linked with TRAIL-mediated immunosurveillance, through the regulation of effector cell TRAIL expression and by augmenting the responsiveness of tumour cells to TRAIL-mediated cytotoxicity (reviewed in Smyth *et al.*, 2003). Neutralisation of TRAIL promotes methylcholanthrene- (MCA)-induced tumour development in mice, and p53^{+/-} mice were more prone to spontaneous sarcoma development when TRAIL-depleted (Takeda *et al.*, 2002). Studies in TRAIL-deficient, IFN- γ -deficient or NK depleted mice also showed the development of TRAIL-sensitive MCA-induced and spontaneous sarcoma cells is favoured, indicating that during tumour development, immunoselective pressure rejects TRAIL-sensitive cells (Takeda *et al.*, 2002). Despite the therapeutic potential of TRAIL, cells from many leukaemia patients are insensitive to TRAIL *in vitro* (Wuchter *et al.*, 2001). Tumour resistance to apoptosis mediated by TRAIL can be caused by loss of expression due to mutation or chromosomal loss, FADD mutation, post-transcriptional regulation leading to low expression of death receptors (Hersey & Zhang, 2001) or mutations in the TRAIL receptor (El-Deiry, 2001).

1.2.2.2 Secreted Immunosuppressive Factors

Tumours secrete a variety of immunosuppressive cytokines that negatively effect immune cell maturation and function, allowing them to escape detection by the immune system. One such cytokine is transforming growth factor- β (TGF- β), and production of high levels of TGF- β by tumours inhibits lymphocyte proliferation and activation (Fontana *et al.*, 1989) and has been correlated with progressive disease (Gorsch *et al.*, 1992). TGF- β expression has also been linked to poor clinical and immunological responses following immunotherapy (Doran *et al.*, 1997).

Prostaglandin E₂ secretion by tumours decreases IL-12 production while enhancing the production of IL-10 in macrophages (Huang *et al.*, 1998). The serum concentration of IL-10 is often elevated in cancer patients and IL-10 has been shown to inhibit the differentiation of stem cell precursors into DC and impede their subsequent activation and function (Almand *et al.*, 2000). IL-12 production, antigen presentation and T_H1 responses *in vivo* are also down regulated by IL-10 (Sharma *et al.*, 1999), while spontaneous apoptosis of DC is increased (Ludewig *et al.*, 1995), all of which contribute to an anti-inflammatory tumour environment.

Macrophage migration inhibitory factor (MIF) is an immunosuppressive cytokine produced by tumours, which can also directly inhibit NK cell activation. Both the murine Neuro-2a NB cell line, and patient derived cell lines have been shown to produce MIF, which can activate tumour cell migration (Bin *et al.*, 2002). Many tumours express VEGF, which in addition to its angiogenic properties also suppresses DC differentiation and

maturation through inhibition of the transcription factor NF- κ B in haematopoietic stem cells (Oyama *et al.*, 1998).

Gangliosides are another group of molecules with immunosuppressive functions. They are carbohydrate molecules containing ceramide and their overexpression is a feature of many tumours. They are shed into the circulation from tumour cells, inhibiting DC generation from precursors and attenuating DC function (Shurin *et al.*, 2001). Gangliosides also inhibit the functional activity of T and NK cells, contribute to tumour-induced bone marrow suppression and cause multiple alterations of haematopoiesis, resulting in pancytopenia (Dumontet *et al.*, 1994; Niethammer & Handgretinger, 1995).

1.2.2.3 Loss of MHC I expression

The major histocompatibility complex (MHC) molecules are the major antigen presenting molecules. Antigen presentation in the context of MHC provides the first signal for the priming of naïve T-cells. They are divided into two classes; MHC class I (MHC I) are found on many cells types, while MHC class II (MHC II) are restricted to specialised antigen presenting cells (APCs) such as DC and macrophages. The MHC molecule binds peptides in an intracellular location and delivers it to the cell surface where the combined ligand is recognised by a T-cell via the T-cell receptor (TCR).

Proteins found in the cytosol of virally infected cells, or proteins from certain bacteria that infect the cytosol are degraded by the proteasome into peptide fragments of 8-10 amino acids and presented on MHC I, which are recognised and eliminated by CD8⁺ CTLs. Tumour cells also present tumour antigens through MHC I to CD8⁺ CTLs, resulting in the elimination of the tumour cells.

As a means of escaping recognition by the immune system, many tumours down regulate MHC I expression by inactivation of proteins essential for antigen processing such as TAP-1 and 2 (Transporters associated with Antigen Processing-1 and 2) (Ritz *et al.*, 2001) or β 2-microglobulin (β 2m) (Cabrera *et al.*, 2003).

IL-10 secretion by tumours has been shown to cause MHC loss by downregulating TAP-1 and 2 (Zeidler *et al.*, 1997), while defective expression of TAP subunits and β 2m have been shown to contribute to MHC I loss in NB cell lines (Corrias *et al.*, 2001). MHC I loss in these cases is due to transcriptional dysregulation, and expression can be restored following treatment with IFN- γ (Propper *et al.*, 2003; Corrias *et al.*, 2001). *N-myc* is known to contribute to loss of MHC I expression in NB by inhibiting nuclear factor binding to the enhancer-A element in the MHC I promoter. The p50 subunit of NF- κ B has been shown to be suppressed by *N-myc*, and MHC I expression is downregulated by suppression of p50 expression (van't Veer *et al.*, 1993).

However, mutations in the genes for structural elements of MHC I result in irreversible decrease or total loss of MHC I expression. Mutations in the β 2m gene are frequent in melanoma and in some cases have been shown to be the result of successive mutational events affecting both copies of the β 2m gene (Paschen *et al.*, 2003). MHC I loss can be greater than 50% in some cancers (Cabrera *et al.*, 1996), and is associated with invasive and metastatic lesions. Some tumours selectively down regulate expression of MHC I haplotypes, and *c-myc* overexpression in melanoma patients has been correlated with HLA-B loss (Versteeg *et al.*, 1989) and block of MHC I expression is a barrier to immunotherapy in a number of cancer patients.

NK cells recognise MHC I by killer inhibitory receptors (KIRs), preventing NK cells from killing normal cells. However, as NK cells normally lyse cells that do not express MHC, how do MHC-negative tumour cells escape recognition by NK cells? One hypothesis is the down regulation of NK-activating stress-induced ligands (MICA and MICB) by tumours, but this has not yet been demonstrated in humans (Garrido & Algarra, 2001). An alternative theory is the lack of pro-inflammatory mediators within the tumour, so NK cells are not activated (Gerosa *et al.*, 2002).

1.2.2.4 Down-Regulation of Tumour Antigens

Tumours can suppress the expression of immunodominant antigens independently of MHC I down regulation, resulting in a poorly immunogenic tumour not readily detected by the immune system. Expression of the melanoma differentiation antigens gp100, MART-1 and tyrosinase is down regulated during disease progression (d’Vries *et al.*, 1997), while antigen expression can also decrease following peptide vaccination therapy. Following immunotherapy with a gp100 peptide vaccine, the gp100 expression in tumours reduced from 47% to 38%, while other tumour antigens remained unchanged (Riker *et al.*, 1999), probably due to selective pressure allowing gp100-negative cells to outgrow the vaccine-targeted gp100-positive cell population.

Antigenic drift is another mechanism for tumour escape. This phenomenon was first identified in the selection of viral escape variants and occurs by mutations that cause replacement of residues within the antigenic epitope. It has recently been shown that adoptive transfer of both polyclonal and monoclonal CTLs specific for a particular antigen selects for multiple antigenic epitope mutations, resulting in greatly reduced T-cell recognition of the tumour antigen. This affects a variety of mechanisms including

MHC:peptide interaction modulation and TCR binding to the MHC:peptide complex (Bai *et al.*, 2003), leading to reduced immune recognition.

1.2.2.5 T-Cell Anergy

Activation of naïve T-cells requires two independent stimuli. The first stimulus is binding of the peptide:MHC complex to the antigen-specific TCR and CD4 or CD8 co-receptors. The second stimulus is the ligation of co-stimulatory molecules (CD80 and CD86) on the APC to CD28 on the T-cell. Both stimuli must be delivered by the same APC, and therefore, only APCs can activate and differentiate naïve T-cells into effectors and induce clonal expansion. T-cell anergy is a state where the T-cell becomes refractory to activation by a specific antigen, even when the antigen is presented by an APC.

Antigen binding to the T-cell receptor in the absence of co-stimulation causes the anergic state, which is augmented by immunomodulatory cytokines such as IL-10 and TGF- β that are secreted by tumours into their microenvironment, and the lack of expression of co-stimulatory molecules by tumour cells. Self-reactive T-cells, such as those specific for tumour antigens, can be functionally silenced as a result of TCR and/or co-receptor downregulation caused by persistent antigen exposure (Schönrich *et al.*, 1991). Recently it has been shown that the growth of solid tumours can result in antigen-specific CD4⁺ T-cell tolerance. Cross-presentation of tumour antigens by bone marrow-derived APCs results in T-cell anergy during solid tumour growth, and this anergy to tumour antigens can limit the efficacy of therapeutic vaccination (Cuenca *et al.*, 2003). Anergy abrogates IL-2 production, resulting in defective T-cell proliferation (Telander *et al.*, 1999) and anergic T-cells can also inhibit the antigen presenting function of DC, mediating down regulation of CD80, CD86 and MHC II (Vendetti *et al.*, 2000), collectively resulting in an immunosuppressed tumour environment. Providing IL-12 has been shown to reverse the

anergic phenotype *in vivo* (Grohmann *et al.*, 1997) and IL-2 stimulation rectifies anergy *in vitro* (Beverly *et al.*, 1992).

1.2.2.6 Role of Suppressor T-Cells

During negative selection in the thymus, some self-reactive T-cells will escape. When these cells encounter self-antigens following infection or tissue injury, a means of limiting the activation of these cells is required and evidence suggests that immunoregulatory CD4⁺ CD25⁺ cells involved. Inhibition of the activation of autoreactive T-cells by peripheral tolerance also influences the induction of T-cell responses to tumour antigens, many of which are self-antigens.

CD4⁺ CD25⁺ regulatory T-cells comprise 5-10% of CD4 T-cells in humans and rodents and have been shown to prevent autoimmunity, playing a vital role to the tolerance of self-antigens. While their mechanism of action is unclear, depletion of these CD25⁺ cells can mediate autoimmune destruction of the mucosal lining of the stomach and small intestine, thyroid gland and accelerate diabetes in non-obese diabetic mice (Sakaguchi *et al.*, 2001). CD25⁺ depletion also induces rejection of many transplantable murine tumour lines (reviewed in Gallimore & Sakaguchi, 2002). CD25⁺ regulatory cell suppression is generally considered to be antigen non-specific, indicating that responses to a spectrum of tumour antigens could be uncovered following the depletion of the regulatory cells. Additionally, these cells produce IL-10 and TGF- β , compounding the already immunosuppressed state of the tumour environment (Powrie *et al.*, 1996; Asseman *et al.*, 1999).

CD4⁺ NK1.1⁺ T-cells (NKT-cells) are another subset implicated in immunosuppression. These cells are decreased or absent in mice that suffer spontaneous autoimmune disease,

and CTL-mediated tumour rejection by IL-13 via the IL-4R-STAT6 (signal transducers and activators of transcription6) pathway is inhibited by NKT-cells (Terabe *et al.*, 2000).

1.3 Immunotherapy for Cancer

The basic principle of tumour immunotherapy is to enable the immune system to recognise the tumour as non-self, rather than tolerate it. Numerous strategies are being developed for the treatment of cancer, which attempt to augment antigen presentation and increase the immunostimulatory capacity in the tumour microenvironment, with the aim of achieving a systemic response. Immunotherapy aims to exploit the differences between malignant and normal cells, such as the expression of mutated antigens or antigens that are of embryonic origin so are not present on differentiated tissues. This has been achieved by a variety of approaches that have been successful in many murine tumour models and shown efficacy in human cancers.

1.3.1 MHC and Co-stimulatory Molecules

As discussed above, MHC I and MHC II are the major antigen presenting molecules and they provide the first stimulus to prime naïve T-cells. Expression of both MHC I (Prezioso *et al.*, 1995; Mandelboim *et al.* 1995) and MHC II (Armstrong *et al.* 1998; Leach & Callahan, 1995) on tumour cells has successfully generated tumour immunity.

Co-stimulatory molecules expressed on the surface of APCs provide the second stimulus for priming naïve T-cells. The co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) are homodimeric members of the immunoglobulin superfamily. Both CD80 and CD86 interact with CD28 on naïve T-cells. Once T-cells are activated they express cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4/CD152), which binds CD80 and CD86 with much higher avidity than CD28 and delivers an inhibitory signal, limiting the proliferative

response of activated T-cells (Chambers *et al.*, 2001). CTLA-4 knock-out mice develop massive proliferation of lymphocytes and fatal multi-organ degeneration, demonstrating the importance of CTLA-4 regulation (Tivol *et al.*, 1995).

Expression of co-stimulatory molecules on tumour cells can augment antitumour immune responses, and this approach has shown efficacy in both murine models and human patients. Direct injection of adenovirally-encoded CD80 into rat osteosarcoma tumours demonstrated curative immunity of the primary tumour and lung metastases, provided protective immunity against rechallenge with parental CD80-negative osteosarcoma cells and activated regional lymph node CD4⁺ T-cells (Tsuji *et al.*, 2002). Similarly, lung tumour cells adenovirally modified to express CD80 were able to generate effector lymphocytes that were capable of lysing parental CD80-null lung cancer cells (Iwakami *et al.* 2001). This approach was also used in a phase I clinical trial of metastatic renal carcinoma, where tumour cells transduced to express CD80 were delivered in conjunction with systemic IL-2. Immunological and clinical responses were observed (Antonia *et al.* 2002), demonstrating the efficacy of co-stimulatory molecules in generating antitumour immune responses.

Delivery of costimulatory molecules with cytokine genes has also been effective tumour immunotherapy. *In vivo* gene delivery of CD80 and IL-12 encoded in a single adenoviral vector to murine mammary adenocarcinomas resulted in regression of 70% of primary tumours and the cured mice were immune to further tumour challenges (Pützer *et al.*, 1997). Similarly, a bicistronic retroviral construct, encoding CD80 and IFN- γ , was used to treat a murine model of human ovarian cancer, markedly reducing the tumorigenicity of the transduced tumour cells (Qian *et al.*, 2002). In a variation of this approach, experimental NB has been successfully treated with adoptive transfer of CTL generated

from CD86-expressing NB cells, and these were able to suppress multi-organ metastases *in vivo* (Enomoto *et al.* 1997). The use of co-stimulatory molecules has been particularly effective when co-transduced with MHC II genes (Heuer *et al.*, 1996), showing that with the appropriate signals, T-cells can be effectively primed against tumour cells.

1.3.2 CD40 Ligand

CD40-ligand (CD40L/CD154) is a cell surface molecule important in T-cell activation. Its receptor, CD40, is found on B-cells and APCs. When CD40L binds CD40, it activates the CD40⁺ cells, inducing surface expression of CD80 and CD86. This enhances T-cell responses, making CD40L a potentially useful molecule in immunotherapeutic applications. Primary NB cells have been shown to express CD40, and incubation *in vitro* with rCD40L induced the cells into apoptosis, suggesting that CD40 could be targeted therapeutically in NB (Airoldi *et al.*, 2003).

CD40L expression has also shown efficacy *in vivo*. CD40-L-transduced tumour cells have been used to treat experimental bladder cancer (Loskog *et al.*, 2001) and murine lung cancer (Noguchi *et al.*, 2001), showing increased survival of mice with established tumours and giving protective immunity to challenge with parental cells in syngeneic mice. This antitumour effect was further enhanced when co-inoculated with IFN- γ -transduced cancer cells *in vivo* (Noguchi *et al.*, 2001). CD40L gene therapy has also been used to treat chronic lymphocytic leukaemia (CLL) patients, where autologous CLL cells transduced to express CD40L increased the number of leukaemia-specific T-cells and reduced lymph node size and leukaemic cell count. Enhanced expression of immune accessory molecules on bystander non-infected CLL cells, and increased serum levels of IL-12 and IFN- γ was also observed, with no dose-limiting toxicity (Wierda *et al.*, 2000).

1.3.3 Tumour Associated Antigens

Tumour associated antigens (TAA) are divided into two categories. The first category consists of antigens selectively expressed on tumour cells and is composed of mutated antigens and virus derived tumour antigens, while the second category of antigens are present on tumour cells and normal tissues.

1.3.3.1 Tumour restricted antigens

Targeting mutated antigens would be advantageous as there would be minimal cross-reaction with normal tissue. However, most mutated antigens are unique to each cancer patient, requiring the production of patient tailored vaccines. Viral antigens are an attractive target as they are often shared between many patients; 95% of human cervical carcinomas express HPV-16 E7 antigen (zur Hausen, 2000), and this has been exploited as a TAA. Cells transfected with a construct encoding the E7 antigen, or DCs pulsed with lysates of such transfected cells, exhibited enhanced MHC I antigen presentation. Moreover, vaccination of mice with this construct resulted in a striking increase in E7-specific CTLs and significant reduction of tumour development compared to treatment with wild-type E7 DNA (Hung *et al.*, 2001). However, only a small minority of cancers are associated with viral infection, limiting the application of such a strategy.

1.3.3.2 Shared antigens

The second antigen category is non-mutated antigens, shared with normal tissues, and the majority of tumour antigens characterised thus far belong to this group. The potential usefulness of these antigens depends on their tissue distribution and pre-existing tolerance. Because these antigens are shared with normal tissue, they are also expressed in the

thymus, so self-tolerance severely impacts on the T-cell repertoire's capacity to respond satisfactorily towards these TAAs. Therefore, the understanding of why certain tissue-specific antigens induce tolerance while others do not is vital to the design of tumour vaccines (reviewed in de Visser *et al.*, 2003).

Some cancers such as melanoma and renal cell carcinoma express well-defined antigens that may elicit a spontaneous immune response (reviewed in Jager *et al.*, 2001). However, most TAA do not induce immunity in this manner, but expression by APCs and subsequent antigen presentation can provoke immune responses against such TAAs. This approach requires identification and sequencing of the TAA gene, which should ideally be tumour restricted. The TAA gene is then used to transduce APCs, so the antigen is optimally processed and epitopes presented in the context of MHC, inducing T-cell responses against tumour cells expressing these antigens. Examples include MUC1, found on various human cancer cells, making it a therapeutic target for many types of cancer (Maruyama *et al.*, 2001), or antigens with restricted expression patterns such as prostate-specific antigen (Willis *et al.*, 2001).

This approach has shown efficacy *in vivo*. DC transduced with the murine melanoma antigen tyrosinase-related protein-2 demonstrated protective immunity against tumour challenge and mediated regression of established tumours (Metharom *et al.*, 2001). In another melanoma model, cotransduction of DC with the antigen gp100 and the chemokine lymphotactin was able to significantly inhibit tumour growth and increase IL-2 and IFN- γ production more efficiently than either gene alone (Xia *et al.*, 2002). However, for many cancers there are no identified TAAs, or the antigen may be up-regulated on tumours but also present on other tissues, albeit at lower levels. These TAA are not ideal, as there is the danger of generating an autoimmune response.

1.3.4 Antibody therapy

1.3.4.1 Anti-tumour cell antibodies

Targeted antibodies have become an important approach in the treatment of cancer due to their high affinity and specificity towards cells from primary and metastatic lesions, resulting in antibody-dependent cell-mediated cytotoxicity and complement-mediated cytolysis of tumour cells. By late 2002, six antibodies had been approved for use in the United States to treat both solid and haematologic neoplasms (Ross *et al.*, 2003). These include Rituximab, which targets CD20, found on many B-cell non-Hodgkin's lymphomas (Grillo-Lopez *et al.*, 2002), and Trastuzumab, which targets the HER-2/neu growth factor receptor upregulated in many breast cancers (Ross & Fletcher, 1999). Many others antibodies are in stage II/III clinical trials.

1.3.4.2 Conjugated antibodies

Antibodies conjugated to cellular toxins or radioisotopes target the therapy to tumour cells, thus minimising damage to surrounding tissues, are also involved in clinical trials, and two have been approved in the US. Ibritumomab tiuxetan is the murine version of rituximab covalently linked to a metal chelator, allowing stable binding of yttrium 90 (^{90}Y) to produce improved targeted cytotoxicity.

Initial trials using murine monoclonal antibodies (mAb) were of limited success due to the production of human anti-mouse antibodies, which severely attenuated the half-life of the administered mAb and prevented repeat administration. Chimeric antibodies were subsequently developed, containing human IgG constant domains fused to murine variable regions, which reduced, but did not eradicate, the production of anti-mouse

antibodies. Partially humanised antibodies and “primatised” antibodies have enabled long-term therapy with repeat dosing (Ross *et al.*, 2003) and recently, fully human antibodies have been developed using transgenic procedures and murine sources (Reff *et al.*, 2002).

Antibodies targeting NB cells have been conjugated to various molecules to induce antitumour responses. GD₂-expressing NB cells have been successfully targeted *in vitro* using the catalytic domain of diphtheria toxin (which inhibits protein synthesis) fused to a single-chain anti-GD₂ Ab, which showed selective toxicity to GD₂⁺ cells only (Thomas *et al.*, 2002). Alternatively, GD₂-targeted liposomes containing *c-myc* antisense oligodeoxynucleotides reduced NB cellular proliferation by inhibiting gene expression (Pagnan *et al.*, 2000). NB has also been targeted *in vitro* with a single-chain antibody against neural cell adhesion molecule (NCAM) conjugated to granulocyte colony-stimulating factor (GM-CSF), which could selectively induce growth inhibition of a human NB cell line (Dehal *et al.*, 2002).

1.3.4.3 ADEPT

An alternative technique using antibodies for cancer therapy is antibody-directed enzyme prodrug therapy, where an antibody-bound enzyme is targeted to tumour cells, permitting selective activation of a non-toxic prodrug to a cytotoxic agent at the tumour site. This approach has reached clinical trials for colorectal carcinoma using an antibody to carcinoembryonic antigen conjugated to a carboxypeptidase, which converts a benzoic acid mustard-glutamate prodrug into a cytotoxic molecule (Napier *et al.*, 2000). A variation is to exploit an enzyme within the cancer cells; a peptide covalently linked to the chemotherapy agent doxorubicin considerably reduces the toxicity of the drug, but is hydrolysable by prostate specific antigen (PSA), a serine protease, releasing the highly cytotoxic doxorubicin. Therefore, only PSA-expressing cells are exposed to the toxicity of

the drug, increasing the maximum tolerated dose. Nude mice inoculated with PSA-positive cells and treated with the drug-peptide conjugate showed considerable reductions in tumour burden (DeFeo-Jones *et al.*, 2000).

1.3.4.4 Antibody therapy for NB

A truly tumour specific antigen has not yet been identified for NB, although the disialoganglioside GD₂ is highly upregulated on many NBs. Gangliosides are sialic acid-containing glycosphingolipids that are mainly detected in the cell surface membrane and GD₂ is highly expressed on embryonic tissues. It is down regulated on differentiated tissues such as cerebellum and peripheral nerves, but there may still be a basal level of expression. Although not all NBs express GD₂, preventing it from being used as a universal target in all patients, it is currently the best characterised and widely studied NB antigen and has been targeted in many clinical trials.

Patients with GD₂ positive tumours were treated in a phase I trial with the anti-GD₂ murine monoclonal antibody 14.G2a in combination with recombinant IL-2 with some success (Frost *et al.*, 1997). The murine antibody was well tolerated and displayed some antitumour activity, although some patients developed human antimouse antibodies. A different anti-GD₂ murine monoclonal IgG3 antibody, 3F8, in conjunction with GM-CSF in a phase II trial, showed the treatment was promising for minimal residual disease and was well tolerated (Kushner *et al.*, 2001). To reduce antimouse antibody responses, a chimeric anti-GD₂ antibody, ch14.18, was used in another trial in conjunction with chemotherapy. Limited levels of toxicity were observed, although no survival advantage was seen over those patients receiving chemotherapy alone (Kletzel *et al.*, 2002).

1.3.5 Dendritic Cells in Immunotherapy

Manipulating cells of the immune system to recognise tumour cells and elicit an immune response may be another way to combat cancer. As discussed above, the tumour environment is often immunosuppressive, resulting in defective immune responses. DC are the most potent professional APCs. They act as sentinels of the immune system and are unique in their ability to stimulate primary naïve T-cells with antigen presented on MHC, and also provide the necessary co-stimulation signals to activate the cytotoxicity activity of T-cells (Banchereau & Steinman, 1998).

DC exist as a small fraction of the peripheral blood mononuclear cell population (PBMC) in an immature form. As immature DC, they have a high endocytic capability, and express low levels of activation markers such as CD40, CD80, CD86, MHC II, Dec-205 and are CD83-negative. Upon encountering antigens or pathogens, they endocytose and process the antigen and present fragments in the peptide-binding groove of MHC class II. During antigen-processing, the cell develops a mature phenotype; it loses the ability to endocytose further material, and shows a marked increase in the surface expression of CD40, CD80, CD86, MHC Class II, Dec-205 and becomes CD83^{high} (Banchereau *et al*, 2000). The cells become motile and migrate from the tissues into the circulation and home to lymph nodes, where they present their antigen to T-cells. Peptide presented in this way interacts with the TCR. Mature DC also secrete high levels of IL-12 (Reis e Sousa *et al*, 1997).

1.3.5.1 DC and tumour peptide antigens

Exposing DC to peptide *in vitro* results in the peptide being endocytosed, processed and antigen being presented to T-cells in the context of MHC molecules. This approach has been used in a clinical trial for stage IV melanoma by pulsing DC with identified individual TAA peptides. The majority of patients showed an expansion of antigen-specific CTL

precursors, regression of metastases was observed in over half the patients, and CTL infiltration was demonstrated in lesions in two patients (Thurner *et al.*, 1999). Due to their high endocytic capacity, co-culturing DC with whole TAA proteins may allow endocytosis, processing and presentation of optimal epitopes, but this approach is limited to only a few antigens, as the full sequence of most TAAs is not known.

1.3.5.2 DC and whole tumour lysates

Tumour lysate-pulsing of APC is an alternative strategy that can be used when there are no identified TAA. A surgical biopsy is used to make a lysate, and DC are exposed to the unfractionated antigen mix (Schott *et al.*, 2001). This approach has the advantage of producing a polyclonal effector T-cell population, therefore reducing the possibility of tumour escape variants, for example, by antigenic drift (Bai *et al.*, 2003), which can be a problem with the single TAA approach. It also requires no prior knowledge of any TAA. A comparison of apoptotic and necrotic tumour cells showed that, at least in mice, both populations have equivalent capacity to mature DC and both resulted in immune priming and anti-tumour immunity *in vivo* (Kotera *et al.*, 2001).

Success with the lysate-pulsing method has been demonstrated *in vitro* with pancreatic carcinoma cell lines (Schnurr *et al.*, 2001), with *in vivo* murine models of sarcoma or breast carcinoma (Fields *et al.*, 1998), and in melanoma therapy clinical trials (Nestle *et al.*, 1998). This approach has also reached clinical trials in the treatment of solid paediatric malignancies (Geiger *et al.*, 2001), where cellular immunity against autologous tumour was demonstrated in half the patients treated. Systemic administration of cytokines has also been shown to augment primary CD8⁺ responses following vaccination with peptide-pulsed DC. In mice, adoptive transfer of antigen-specific T-cells and antigen-pulsed DC

plus systemic IL-15 demonstrated a significant increase of antigen specific-T-cells compared to pulsed DC alone (Rubinstein *et al.*, 2002).

Combining administration of immature DC with targeted γ -irradiation of a tumour is another antitumour application of DC, where growth of experimental murine sarcoma was significantly inhibited and tumour-specific T-cell responses increased by combination therapy (Nikitina & Gabrilovich, 2001).

An adaptation of the antigen-pulsing technique that produces anti-tumour responses is transfecting DC with RNA encoding whole antigens (Koido *et al.*, 2000) or unfractionated RNA from tumour cell lysates (reviewed in Mitchell & Nair, 2000). The latter approach has been shown to generate antigen-specific T-cells responses that lyse autologous primary tumour cells *in vitro* (Nair *et al.*, 2002).

1.3.5.3 DC and pro-inflammatory cytokines

An alternative application of DC in tumour immunotherapy is expressing cytokine transgenes. Two murine lung carcinoma models have been successfully treated with DC transduced with IL-7, generating systemic antitumour responses, eradicating established tumours, and significantly increasing IFN- γ and GM-CSF secretion from splenocytes in response to tumour target cells (Miller *et al.*, 2000). Furthermore, complete regression of murine NB tumours was observed following treatment with IL-12-transduced DC and isolated splenocytes had greater cytolytic activity *in vitro* compared to controls. Tumour infiltration of DC and T-cells was increased, and these cells were protected from tumour-induced apoptosis (Shimizu *et al.*, 2001).

However, DC differentiated from the bone marrow of NB tumour-bearing mice and transduced to express IL-12 resulted in reduced anti-tumour responses when adoptively transferred into tumour bearing hosts compared to IL-12 transduced DC from naïve mice. The greater tumour burden of the donor mice, the less therapeutic effect of the transduced DCs in the host mice, despite comparable phenotype and effector cell activation *in vitro* with DC from naïve mice (Redlinger *et al.*, 2003a). The duration of tumour exposure appeared to be related to anti-tumour activity loss, so this feature may be important when differentiating DC from tumour bearing individuals for *ex vivo* modification.

A slightly different approach for the treatment of experimental prostate carcinoma used a DC cell line stably transfected with IL-12, then pulsed with tumour lysate. Tumour engraftment was delayed, tumour growth reduced by up to 80% compared with controls, and tumour-infiltrating mononuclear cells induced tumour cell apoptosis and necrosis. This study also showed that route of administration had a marked effect on vaccine efficacy, and intramuscular injection was more effective than the intraperitoneal or subcutaneous routes (Zhang *et al.*, 2003a). Vaccines to treat murine tumours have also been produced by the fusion of DC with tumour cells. Transgenic mice expressing the human MUC1 antigen were unresponsive irradiated carcinoma cells expressing MUC1, but immunisation of these cells fused to DC resulted in regression of MUC1-positive metastases (Gong *et al.*, 1998). Similarly, mice immunised with DC fused to NB Neuro-2a tumour cells, were protected from challenge with wild-type Neuro-2a cells, and T-cells responses were induced *in vitro* and *in vivo* (Orentas *et al.*, 2001).

1.3.6 Cytokine Therapy

Cytokines are small soluble proteins that affect the behaviour or properties of the secreting cell itself (autocrine) or other cells (paracrine). Cytokines can be delivered systemically as a

bolus injection of protein, elevating the serum concentration, and this has shown efficacy in murine models. The major drawback with this method is that many cytokines are toxic when delivered at a concentration high enough to elicit antitumour effects (Marshall, 1995; Smith, 1997). Thus, other strategies have been developed to reduce the toxicity.

The alternatives include local injection of the cytokine directly into the tumour, but this tends to result in only a short period of therapeutic efficacy. Gene therapy vectors allow longer-term expression of cytokines, and initially vectors were injected directly *in vivo*. However, anti-viral immune responses, particularly to adenovirus limited this approach (Thomas *et al.*, 2003). A more successful approach has been to modify cells *ex vivo* and then return them to the tumour microenvironment. The advantages of this approach are that only the target cells are modified to express the transgene, and anti-vector immunity is reduced. Many clinical trials of tumour cells modified to express immunostimulatory cytokines have shown therapeutic antitumour responses. NB has been treated in clinical trials by IFN- γ transduced allogeneic tumour cells (Seeger *et al.*, 1998) or autologous NB cells adenovirally transduced with IL-2 and the chemokine lymphotactin (Brenner *et al.*, 2000)

1.3.6.1 Interleukin 2

Interleukin-2 (IL-2) was first identified in 1976 (Morgan *et al.*, 1976) in the supernatant of activated peripheral blood lymphocytes as a substance capable of driving lymphocyte growth and proliferation. Human IL-2 is a 15.5kDa protein composed of 133 amino acids, which can cross-react with mouse cells. The IL-2 gene, located on chromosome 4, encodes a globular protein composed of two sets of α -helical domains, positioned perpendicular to one another (Brandhuber *et al.*, 1987). Mutations in the IL-2 gene have

been linked with autoimmune diabetes, and it has been suggested that dysregulation of IL-2 signalling has a pathogenic role in adult T-cell leukaemia caused by HTLV-1.

IL-2 is secreted by activated T-lymphocytes, particularly CD4⁺ T-helper cells, and induces T-cell proliferation (**Figure 1.1**). It also acts as an immune modulator and stimulates T-cell activation causing them to secrete the immunostimulatory cytokine IFN- γ . IL-2 plays a role in maintaining an immune response and activates many cell types in the immune system, including CD4⁺ T-helper cells, CD8⁺ cytotoxic T-cells, B cells NK cells and macrophage-monocyte cells (reviewed in Church, 2003).

The IL-2 receptor (IL-2R) is composed of three subunits, designated IL-2R α , IL-2R β and common γ -chain (γ c). IL-2R β is shared with the IL-15R complex, while γ c is shared with the receptors for IL-4, IL-7, IL-9, IL-15 and IL-21. The IL-2R α chain (CD25) is unique to the IL-2R. This subunit sensitises cells to physiological levels of IL-2, although it does not contribute directly in IL-2 signalling due to the extremely short cytoplasmic tail. Upregulation of IL-2R α is also important for physiological concentrations of IL-2 to induce T-cells to proliferate and become sensitive to activation induced cell death (Nguyen *et al.*, 2000). Conversely, both the IL-2R β and γ c chains are required for signalling. IL-2R β and γ c also compose the signalling fraction of the IL-15R, and therefore IL-2 and IL-15 initiate very similar signalling cascades (Gaffen, 2001). The gene for γ c is located on the X chromosome, and loss of expression of this protein leads to X-linked SCID due to a phenotypic loss of all the cytokine receptors listed above.

As with many cytokine receptors, binding of the cytokine induces oligomerisation of the IL-2R subunits to initiate signalling. Although the IL-2R α subunit itself does not signal

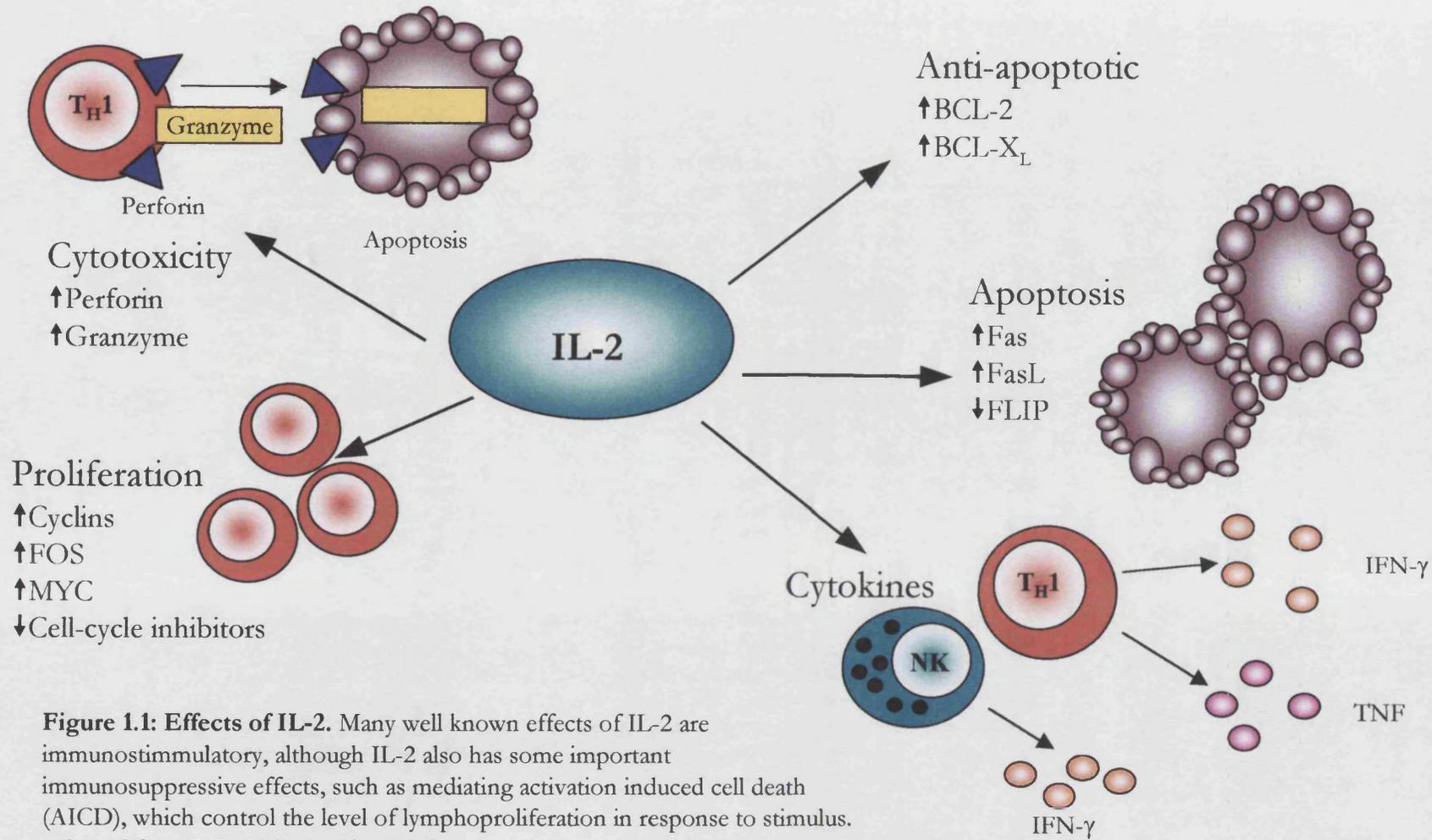


Figure 1.1: Effects of IL-2. Many well known effects of IL-2 are immunostimulatory, although IL-2 also has some important immunosuppressive effects, such as mediating activation induced cell death (AICD), which control the level of lymphoproliferation in response to stimulus. Adapted from J. J. O'Shea *et al.*, (2002)

intracellularly, it has been shown that loss of IL-2R α is functionally equivalent to absence of IL-2 (Willerford *et al.*, 1995). IL-2R β and γ c monomers have very little affinity for IL-2, but as a heterodimer form an “intermediate affinity” receptor, which is able to deliver signals. IL-2R α expression is very tightly regulated, and has binding affinity for IL-2, although the monomer cannot take part in signalling. However, the heterotrimeric receptor is the classical, high affinity IL-2R that is able to efficiently transduce signals at physiological concentrations of IL-2 (Gaffen, 2001).

The effects of IL-2 are mediated through a series of phosphorylation events. Binding of IL-2 induces the subunits to form the IL-2R oligomer. This oligomerisation causes the phosphorylation of tyrosine residues on Janus Kinase (JAK)1, which binds to IL-2R β , and JAK3 which binds to γ c, resulting in a significant increase in their catalytic activity. The JAKs phosphorylate tyrosine residues on the cytoplasmic tails of IL-2R β and γ c, which subsequently act as docking sites for signalling molecules containing phosphotyrosine-binding domains (reviewed in Gaffen, 2001).

IL-2 has been used clinically to enhance T-cell activity in HIV patients (Jacobsen *et al.*, 1996) and in the treatment of many cancers. Also, antibodies to IL-2R have been used to treat lymphoma and in the prevention of transplant rejection (Waldmann & O’Shea, 1998). However, systemic administration of high concentrations of rIL-2 often results in detrimental side effects which include high fever, extreme flu-like symptoms, capillary leak with hypotension and renal failure (Jacobsen *et al.*, 1996).

Combination of lower doses of IL-2 with other cytokines or local IL-2 delivery has been shown to mediate beneficial effects with much lower toxicity. IL-18 is an IFN γ -inducing

factor that acts on T and NK cells. When used with IL-18, a 100-fold lower concentration of IL-2 could induce greater levels of cytolytic activity and proliferation of human lymphocytes compared with IL-2 alone (Son *et al.*, 2001). Intratumoural vaccination of recombinant IL-18 and IL-2 has been successful in treating established murine sarcoma, showing induction of memory responses, enhanced proliferation of NK cells and upregulation of CD25, as well as synergistically stimulating NK activity and IFN γ production (Son *et al.*, 2003). Systemic IL-2 has also been used in combination with tumour cells expressing Flt-3 ligand, a key regulator of DC, in a murine colon carcinoma model. Mice treated with combination therapy had significantly decreased tumour burden and survived significantly longer compared with controls (Sivanandham *et al.*, 2002), while IL-2-transduced human melanoma cells have been shown to efficiently stimulate lysis by both MHC-restricted and -unrestricted autologous lymphocytes (Arienti *et al.*, 1994).

1.3.6.2 Interleukin-12

Interleukin-12 (IL-12) is a potent proinflammatory cytokine secreted primarily by activated APCs such as DC and macrophages and forms a link between innate resistance and adaptive immunity (reviewed in Trinchieri, 2003). IL-12 is a heterodimeric protein of 70kDa (p70) composed of two covalently linked glycoprotein chains of 35kDa (p35) and 40kDa (p40). The human p35 gene is located on chromosome 3p12-q13.2, and encodes a 219aa protein, while the gene for human p40 is located on chromosome 5q31-33 and encodes a 328aa protein. Expression of both subunits in the same cell is necessary for secretion of biologically active IL-12p70. Human IL-12 has no activity on mouse cells, but murine IL-12 is able to elicit effects on both human and mouse cells.

Most cell types express low levels of p35 transcripts, although the protein is not secreted in significant amounts. The expression of p40 is much more tightly regulated than p35

and is only detected in cells that are able to secrete IL-12. All cells that secrete IL-12p70 produce a large excess of free p40 (Trinchieri, 1995). p40 homodimers are produced in mice, and these can block the *in vitro* and *in vivo* functions of IL-12p70. In contrast, human cells do not endogenously produce p40 homodimers, and therefore p40 homodimers are not physiological antagonists of IL-12p70 in humans (Trinchieri, 2003) and the significance of the excess p40 is not clear.

IL-12-mediated innate responses include stimulation of NK cytotoxicity and the ensuing activation of neutrophils and macrophages, leading to production of superoxides and nitric oxide (NO) which have been shown to contribute to control of tumour growth in animal models (Wigginton *et al.*, 1996). IL-12 also induces proliferation and activation of lymphokine-activated killer cells and T cells, promoting the differentiation of naïve T_H0 cells down the T_H1 pathway.

The IL-12 receptor is composed of two subunits, IL-12R β 1 and IL-12R β 2, both of which bind IL-12 with low affinity and are unable to transmit signals as monomers. When these subunits form a heterodimer, they constitute the functional receptor and bind IL-12 with high affinity. IL-12R is not detected on resting T-cells, but activation through the TCR induces expression of both chains, but particularly the β 2 chain. IL-12R expression is enhanced by T_H1 cytokines, including IL-12 itself, and co-stimulation through CD28, but inhibited by T_H2 cytokines (Gollob *et al.*, 1997, Trinchieri, 2003). The IL-12R activates the JAK-STAT signal transduction pathway, causing phosphorylation of JAK2 and TYK2, and the specific effects of IL-12 are mainly due to STAT4 activation through phosphorylation (Thierfelder *et al.*, 1996).

Since Brunda *et al.*, (1993) first showed that IL-12 had potent anti-tumour immunity, many tumour therapy studies incorporating IL-12 have been undertaken using murine models and some have progressed to clinical trials. It is well established that the anti-tumour activity of IL-12 is due to IFN- γ induction and effector NK and CD8⁺ T-cell activation (reviewed in Colombo & Trinchieri, 2002) (**Figure 1.2**). However, like IL-2, high systemic doses of IL-12 are toxic, and despite the initial promising results of murine tumours treated with systemic IL-12, the first clinical trial of IL-12 protein therapy was halted after the death of two patients and severe toxic effects in 15 others (Marshall, 1995). Therefore, much work has focused on combination cytokine therapy, enabling the use of lower doses of IL-12 while still retaining therapeutic efficacy, or local delivery of IL-12 where the cytokine level is high in the tumour locale but is not elevated systemically.

IL-12 has been delivered to tumours by a number of mechanisms. Viral delivery, including intraperitoneal administration of vaccinia virus or adenovirally-transduced DC (Tsung *et al.*, 1997; Redlinger *et al.*, 2003b), or non-viral delivery of IL-12 using a gene gun (Rakhmievich *et al.*, 1997) or a polyvinyl polymeric vector (Mendiratta *et al.*, 1999) have been shown to elicit potent anti-tumour effects against many established murine tumours including sarcoma, NB, renal carcinoma and colon carcinoma. The antitumour effects are mediated by a variety of mechanisms, particularly increased expression of inducible nitric oxide synthase (iNOS) in activated macrophages enhancing NO production (Tsung *et al.*, 1997), NK and CD8⁺ mechanisms (Redlinger *et al.*, 2003b), augmented CD8⁺ anti-tumour cytotoxicity and generation of immune memory (Rakhmievich *et al.*, 1997; Mendiratta *et al.*, 1999).

Co-transduction of IL-12 with the costimulatory molecule CD80 directly into tumours *in vivo* showed increased efficacy compared to either transgene alone, mediating regression of

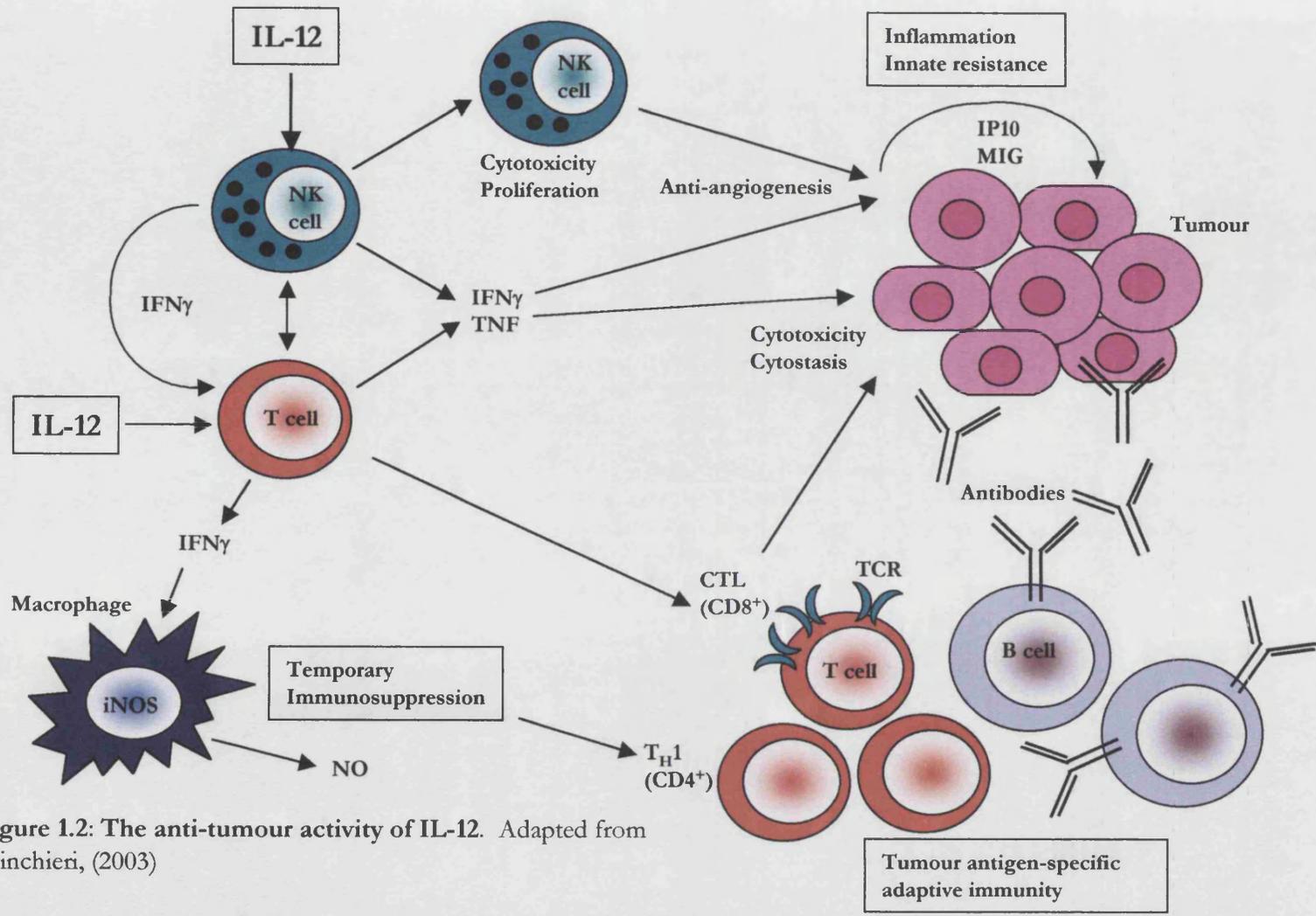


Figure 1.2: The anti-tumour activity of IL-12. Adapted from Trinchieri, (2003)

established tumours and induction of immune memory. This vaccine was most effective when using one virus encoding both molecules, rather than expressing the genes from separate vectors, as high level IL-12 in the vicinity of CD80⁺ tumour cells was required for maximal synergy and separate vectors may not infect the same cell (Pützer *et al.*, 1997).

Like IL-2, IL-12 stimulates IFN- γ production but it also has a potent antiangiogenic effect. Studies have shown IL-12 can mediate an angiostatic effect dependent on IFN- γ but independent of T and NK cells, by the antiangiogenic effect of IL-12 secreting fibroblasts on tumours engrafted into NK-depleted SCID mice (Duda *et al.*, 2000). The antiangiogenic function of IL-12 does not seem to require recognition of tumour antigens, thus it has been suggested that this function should be considered an innate resistance mechanism (Colombo & Trinchieri, 2002). This antiangiogenic effect of IL-12 has largely been attributed to IP-10 (Coughlin *et al.*, 1998), and experiments using different proportions of IP-10-transduced and null-transduced human melanoma cells in nude mice showed a direct association between the fraction of IP-10 cells present with tumour growth inhibition and microvessel density. Indeed, significant reduction of tumour growth was observed when only 50% of melanoma cells expressed IP-10, indicating a substantial paracrine effect (Feldman *et al.*, 2002). IP-10 has also been shown to be essential for IL12-mediated antiangiogenesis, T-cell chemotaxis and the induction of tumour-protective CD8⁺-mediated immune memory in murine NB; depletion of IP-10 with mAb during immunisation abrogated systemic immunity, but IP-10 depletion in the effector phase had little effect on immune memory (Pertl *et al.*, 2001).

IL-12 also enhances IL-2 function by inducing IL-2R α expression, which is essential for high affinity binding of IL-2 at physiological concentrations. This upregulation is mediated through a p38 MAP kinase pathway (Nguyen *et al.*, 2000). Furthermore, IL-2 has

been demonstrated to upregulate both subunits of the IL-12R, and subsequently maintains expression of STAT4, a protein critical in IL-12 signalling in NK cells (Wang *et al.*, 2000). The synergistic action of combining these two cytokines has been observed in the treatment of murine NB. Co-transfecting NB cells to express both IL2 and IL-12 significantly reduced the growth of established NB tumours (Siapati *et al.*, 2003), while vaccinating with IL-12-transfected NB cells together with administration of booster injections of an anti-GD₂ mAb-IL-2 fusion protein that targets the IL-2 to the site of the tumour cells gave lasting protective immunity. The latter treatment also prevented the appearance of liver and bone marrow metastases following a lethal challenge of parental NB cells and generated CD8⁺ T-cell activation and MHC I-restricted tumour target lysis *in vitro* (Lode *et al.*, 1999).

IL-12 used in combination with other cytokines can further enhance its effects. The combination of IL-12 and IL-18 greatly enhances T-cell production of IFN- γ , which was shown to correlate with the IL-12 mediated upregulation of the IL-18R (Ahn *et al.*, 1997). However, excessively increased serum levels of IFN- γ as a result of systemic IL-12 and IL-18 can result in lethal lymphoproliferation in mice, so the combination of cytokines must be carefully considered (Nakamura *et al.*, 2000).

1.3.6.3 Other cytokines

GM-CSF plays an important role in the maturation and functions of specialised APCs (Pardoll, 1995). Elevated levels of GM-CSF in the tumour locale results in a proinflammatory microenvironment and GM-CSF has been used to modify both autologous (Moret *et al.*, 2001; Sandler *et al.*, 2003) and allogeneic tumour cells (Thomas *et al.*, 1998), with both approaches demonstrating antitumour efficacy *in vivo*. Furthermore, when linked with diphtheria toxin, GM-CSF has been shown to reduce the number of

leukaemic colony forming units by monocytes from myelomonocytic leukaemia patients (Frankel *et al.*, 1998).

A further related cytokine is macrophage colony-stimulating factor, and the membrane form (mM-CSF) has been used to treat a murine model of hepatocellular carcinoma. Mice vaccinated with hepatoma cells transduced to express mM-CSF developed protective immunity against wild type hepatoma cells that was mediated by macrophages and CD8⁺ CTLs (Dan *et al.*, 2001).

IFN- γ is a proinflammatory cytokine that has been widely used in cancer immunotherapy and has reached clinical trials for the treatment of malignant melanoma with intratumoural adenoviral delivery (Khorana *et al.*, 2003) or intratumoural retroviral transfer, which resulted in clinical responses plus significantly elevated levels of anti-melanoma-antigen antibodies in patients receiving multiple treatment doses (Fujii *et al.*, 2000). An alternative strategy is to increase the level of the IFN- γ receptor, which improved the response of murine tumours transfected with an adenovirus encoding IFN- γ R to recombinant IFN- γ *in vivo* (Nishida *et al.*, 2002). Increased IFN- γ results in upregulation of further downstream mediators such as IP-10, but has also been demonstrated to inhibit the expression of many other chemokines (Baggiolini, 1998).

As well as mediating immune effects, IFN- γ has also been demonstrated to inhibit the growth of murine melanoma by non-immune mechanisms, where IFN- γ was shown to directly inhibit the proliferation of the tumour cells (Yu & Thomas-Tikhonenko, 2001).

1.3.6.4 Chemokines

Chemokines are small, secreted proteins which mediate leukocyte transport regulation by influencing the adhesion of leukocytes to endothelial cells, the initiation of migration across the endothelial layer and tissue invasion. They can also affect non-haematopoietic cells such as stromal and tumour cells (Homey *et al.*, 2002). GTP-binding protein-coupled receptors mediate the effects of chemokines, and the change in cell morphology after just a few seconds of adding attractant to leukocytes is their most striking effect. Activation of leukocytes by chemokines also results in a rise of intracellular free calcium, production of microbicidal oxygen radicals and bioactive lipids and release of cytoplasmic storage granule contents, including proteases from neutrophils and monocytes and cytotoxic proteins from eosinophils (reviewed in Baggiolini, 1998).

IP-10 is an immunomodulatory chemokine that is a chemoattractant for stimulated T-cells combined with potent antiangiogenic activity, which has demonstrated potent reduction of tumour growth and microvessel density when expressed by transduced tumour cells *in vivo* (Feldman *et al.*, 2002). This approach has been successful in treating murine NB (Shimizu *et al.*, 2001), with regression of primary tumours and lasting memory responses that rejected subsequent tumour rechallenge. Co-administration of IP-10 and other proinflammatory cytokines has also been successful; adenoviral transfer of IP-10 and IL-18 directly into murine myeloma tumours synergistically reduced tumour volume and activated tumour specific CTLs (Liu *et al.*, 2002).

Another chemokine that has shown anti-tumour effects is lymphotactin (Lptn), which specifically regulates NK and T-cell migration. Experiments *in vivo* showed that injection of Lptn into the peritoneum of mice resulted in large influx of T-cells and NK cells, and

that activated NK cells also produce Lptn themselves, perhaps suggesting a self-regulatory migration mechanism (Hedrick *et al.*, 1997).

Due to its T-cell attracting property, Lptn has been investigated alone and in combination with other cytokines. Fibroblasts transduced with Lptn alone mediated little anti-tumour activity, but co-vaccination of fibroblasts modified to express IL-2 and Lptn significantly suppressed tumourigenicity and growth of established tumours in a murine leukaemia model (Dilloo *et al.*, 1996). Another study showed that co-transduction of Lptn+IL-2 or Lptn+IL-12 significantly enhanced the anti-tumour effect of both cytokines in murine breast cancer models, inducing long lasting protective immunity (Emtage *et al.*, 1999).

Lptn has also been used in combination with TAA; mice vaccinated with DC co-transduced with Lptn and the melanoma antigen gp100 showed increased resistance to tumour challenge and superior regression of established murine melanomas compared to singly transduced DC. Production of IL-2 and IFN- γ , and CTL and NK cytotoxicity were significantly increased, and tumour necrosis and infiltration of lymphocytes was also greater in mice receiving co-transduced DC (Xia *et al.*, 2002).

1.3.7 Fibroblasts in Cancer Immunotherapy

The major drawback to modifying tumour cells from surgical biopsies is the difficulty of manipulating primary tumour cells *in vitro*. This is true for NB (E. Siapati, personal communication) and other cancers such as breast cancer (de Zoeten *et al.*, 1999). Model systems using transfected cell lines are very effective, and many murine models of disease have been successfully treated including fibrosarcoma (Leach & Callahan, 1995), NB (Shimizu *et al.*, 1999) and hepatocellular carcinoma (Dan, *et al.*, 2001). However, when the system is implemented with clinical samples, the results tend to be poor. The cells often

do not proliferate and die after fairly short culture periods. Furthermore, some tumours may be unresectable, or insufficient tumour may be available in cases of minimal residual disease.

One approach to overcome these problems is to use allogeneic tumour cells, with mismatched MHC. Murine melanoma has been successfully treated with GM-CSF-transduced allogeneic melanoma cells, and this vaccine also induced systemic immunity against autologous tumour (Thomas *et al.*, 1998), while NB has been treated in phase I trials with allogeneic tumour cells modified to express IFN- γ (Seeger *et al.*, 1998).

An alternative strategy is the use of autologous dermal fibroblasts as a vehicle for cytokine gene expression. These are relatively easy to establish and expand in culture from skin explants, and studies using retroviral transfer of cytokine genes have resulted in high-level gene expression. The rationale behind this method is that like transfected tumour cell lines, the fibroblasts secrete sustained high levels of cytokines in a localised area. However, as primary tumour cells are difficult to culture, the intratumourally injected fibroblasts offer an alternative way to provide local sustained cytokine secretion.

An advantage with fibroblasts is that a large culture can be established and transduced, then aliquots stored for future doses. IL-12-expressing fibroblasts successfully generated protective immunity and were used to treat established murine sarcoma by vaccination at the tumour site and a distant site, mediating tumour regression and infiltration of CD4 and CD8 T-cells and macrophages. This strategy also significantly reduced lung metastases when delivered with low dose systemic IL-2 (Zitvogel *et al.*, 1995). Transduced fibroblasts have also successfully treated experimental tumours with IL-2 (Fakhrai *et al.*, 1995).

Fibroblasts retrovirally transduced to express IL-2 were used in a phase I trial for colorectal carcinoma and showed a 5-fold increase in tumour-specific CTL precursors in some patients and delayed-type hypersensitivity (DTH) responses were seen at the vaccination sites, implying the induction of immune memory (Sobol *et al.*, 1999). Another phase I trial used autologous fibroblasts transduced with IL-12 to treat a variety of solid tumours (Kang *et al.*, 2001) that resulted in clear, although transient reductions in tumours in nearly half of patients and CD8⁺ cells were observed infiltrating tumours. A further phase I trial is underway using IL-4 transduced fibroblasts in conjunction with irradiated autologous glioma cells and dendritic cells to treat malignant glioma (Okada *et al.*, 2001).

Gene-modified fibroblasts have also been efficacious in treating other conditions, including experimental arthritis. Syngeneic fibroblasts transfected with a non-viral vector encoding IL-4 were used to treat collagen-induced arthritis and demonstrated significant clinical and histological improvement in reducing joint inflammation compared with sham-transfected controls or allogeneic IL-4 secreting fibroblasts (Bessis *et al.*, 2002).

Some evidence suggests that cytokine-transfected syngeneic fibroblasts are superior to cytokine-transfected allogeneic fibroblasts at priming lymph node cells for *in vivo* anti-tumour activity (Aruga *et al.*, 1997). However, other publications report the antitumour efficacy of allogeneic tumour cells (Kayaga *et al.*, 1999), and allogeneic cytokine-expressing tumour cells have been used in clinical trials (Jaffee *et al.*, 1998; Bowman *et al.*, 1998). Thus, the efficacy of autologous compared to allogeneic cells may depend on the tumour being studied and the particular functional assays studied as a measure of vaccine efficacy.

Another application of fibroblasts in experimental cancer therapy has been to transfect semiallogeneic IL-2-secreting fibroblasts with DNA from breast cancer cells (de Zoeten *et*

al., 1999). Tumour bearing mice that received this vaccine, expressing the weakly immunogenic breast cancer antigens on the more immunogenic fibroblasts, survived significantly longer than controls. It has even been suggested that fibroblasts can act as APCs (Kundig *et al.*, 1995) and there is some evidence they express the co-stimulatory molecule CD80 (Kim *et al.*, 1995).

1.3.8 Immunotherapy-induced autoimmunity

One question that has been raised is the potential problem of autoimmunity. All the immunotherapy strategies described above rely on generating host immune responses to previously ignored/tolerated host antigens, either by increasing the proinflammatory potential of the tumour environment or directly exposing APCs with tumour peptides or lysates, and many of these antigens are also present on normal tissues. However, in the clinical trials that have used these approaches, no reactions resulting in morbidity or mortality have been observed (Holdt *et al.*, 1999) although vitiligo has been reported in patients treated for melanoma with lysate pulsed DC (Jager *et al.*, 2001). In this case the consequence was not a serious condition, but does demonstrate that induction of an immune response is being targeted against a normal cellular protein.

However, in a murine model severe autoimmune disease was observed when peptide-pulsed dendritic cells were directed against tumour antigens shared with normal host cells, which had previously been immunologically ignored, and CTL activity was maintained for a prolonged period of time. Transgenic mice expressing the model tumour antigen in pancreatic β cells developed fatal diabetes or mice that expressed the model tumour antigen in cardiomyocytes and arterial smooth muscle developed severe myocarditis and arteritis and eventually dilated cardiomyopathy where cardiomyocytes had been destroyed and replaced by fibrotic tissue (Ludewig *et al.*, 2000). Although an artificial system, this

study highlights the potential dangers of targeting antigens that are not truly tumour specific.

1.4 Gene Therapy Vectors

The process of gene transfer into mammalian cells is generally divided into two groups; viral and synthetic methods. Most viral gene delivery is mediated by Herpes simplex virus, retroviruses, lentiviruses, adenoviruses or adeno-associated viruses and each has merits for use with certain cell types. The amount of transgene that can be delivered is limited and varies between viruses (**Table 1.3**). Other viruses have been investigated, including vaccinia virus, but not as widely as the five types above. Influenza viruses have shown some promise (Strobel *et al.*, 2000), but generation of recombinant influenza vectors is hampered by the virus's segmented RNA genome.

Synthetic vectors initially consisted of naked DNA, but have become more sophisticated, employing DNA-condensing agents and cell-targeting moieties or ligands (**Table 1.4**). Viruses are incredibly effective at entering and delivering nucleic acid to cells, and therefore many gene delivery vectors attempt to mimic this process. One of the main difficulties is delivering the vector to the target cells, especially in a population of mixed cell types, thus much work has been undertaken on the specific targeting of vectors for gene therapy.

Vector safety is a crucial issue, and in the case of viruses it is essential that the vectors do not recombine and become replication-competent (reviewed in Thomas *et al.*, 2003). Another problem that may limit gene delivery efficiency is the host immune response to the vector. Vector immunogenicity results in cellular and humoral immunity, preventing repeated administration of the vector and mediating destruction of transduced cells.

Vector	Genetic Material	Packaging Capacity	Tropism	Inflammatory Potential	Vector Genome Forms	Main Limitations	Main Advantages
<i>Enveloped</i>							
Retrovirus	RNA	8kb	Dividing Cells Only	Low	Integrated	Only transduces dividing cells; integration might induce oncogenesis in some applications	Persistent gene transfer in dividing cells
Lentivirus	RNA	8kb	Broad	Low	Integrated	Integration might induce oncogenesis in some applications	Persistent gene transfer in most tissues
HSV-1	dsDNA	40kb (replication defective) 150kb (amplicon)	Strong for neurons, but will infect other tissues	High	Episomal	Inflammatory: transient transgene expression in cells other than neurons	Large packaging capacity; strong tropism for neurons
<i>Non-enveloped</i>							
AAV	ssDNA	<5kb	Broad, with the possible exception of haematopoietic cells	Low	Episomal (>90%) Integrated (<10%)	Small packaging capacity	Non-inflammatory; non-pathogenic
Adenovirus	dsDNA	8kb (replication defective) 30kb (helper dependent)	Broad	High	Episomal	Capsid mediates a potent inflammatory response	Extremely efficient transduction of most tissues

Table 1.3 The main groups of viral vectors for gene therapy. Adapted from Thomas *et al.*, 2003.

Vector	Constituents	Main Limitations	Main Advantages
Naked DNA	Plasmid DNA	Very low gene transfer levels Only been shown to work <i>in vivo</i> when injected directly into muscle	Gene expression can persist for several months in muscle
Gene Gun	Gold particles coated with plasmid DNA transferred into cells by high pressure	Difficult to treat internal organs Transient in dividing cells	Transfect a wide variety of cells <i>in vivo</i> High-level gene transfer
Lipoplex Vectors	Plasmid DNA and cationic lipids	Short-term gene expression No targeting specificity of gene transfer	Non-immunogenic. Limits the level of endosomal degradation of vector
Polyplex Vectors	Plasmid DNA and condensing agents such as poly-L-lysine or PEI	Rapidly cleared from circulation <i>in vivo</i> by complement fixing No targeting specificity	Positive charge increases contact with cells. Biodegradable variants have reduced immunogenicity and cytotoxicity
Lipopolyplex Vectors	Plasmid DNA, cationic lipid and condensing agent, often with receptor targeting capacity	Transient gene expression Transfection efficiency decreased compared with viral vectors	Target specific cell populations Generally higher transfection efficiency than non-specific synthetic vectors Addition of certain viral proteins can increase transfer of vector into the nucleus

Table 1.4 The main groups of synthetic vectors for gene therapy.

1.4.1 Viral Vectors

1.4.1.1 Adenoviral vectors

Adenovirus (Ad) is a non-integrating double stranded DNA virus, which is maintained episomally. Most vectors are based on serotype-5, and due to their broad tropism and high transduction level, Ad are used extensively in clinical trials (Somia & Verma, 2000). Ad vectors have been widely used in the treatment of cancer, delivering cytokine genes such as IL-12 or IFN- γ , genes encoding TAAs and immunostimulatory molecules such as MHC and CD80/CD86. Another application in cancer therapy is the use of selectively replicating viruses that only replicate in tumour cells in which the missing function is supplied. The conditionally replicating adenovirus ONYX-015 has shown antitumour efficacy when used with chemotherapy in phase II trials, and has shown tumour selectivity in patients with advanced head and neck cancer (Nemunaitis *et al.*, 2000).

Ad vectors target the coxsackie-adenovirus receptor (CAR) and interaction of the penton base with cell surface integrins enables internalisation into endosomes (Wickham *et al.*, 1993), but infection is less efficient in cells that do not express CAR. Pseudotyping Ad-5 vectors with alternative fibre proteins creates chimeric viruses; this strategy generated a virus that could efficiently transduce CD34⁺ HSCs, by incorporating the Ad-35 fibre into an Ad-5 capsid (Shayakhmetov *et al.*, 2000).

An alternative approach is to genetically modify the fibre protein; replacing the CAR binding sequence with an integrin-targeting motif increased gene transfer to CAR-negative cells *in vitro* (Hidaka *et al.*, 1999). Cell-specific binding peptides can be identified by phage panning with specific cell types, then the virus fibre protein engineered to express the phage-isolated motifs. This not only increases transduction efficiency, but can also reduce non-specific transduction in a mixed cell population.

One major problem with Ad vectors is their inherent immunogenicity, which can prevent repeated dosing of the vector. First generation vectors induced strong CTL responses that resulted in elimination of transgene expression. Second and third generation vectors are less immunogenic and have reduced toxicity. Ad vectors can induce many components of the immune system, including CTL responses against viral gene products or “foreign” transgene products. The viral capsid itself induces release of inflammatory cytokines and humoral responses, even in the absence of any gene expression. The anti-vector response lead to the death of one patient in a phase I clinical trial. An 18 year old, suffering from a partial deficiency of ornithine transcarbamylase (OTC) was the first patient whose death was directly attributed to the gene therapy vector. He received 3.8×10^{13} second generation recombinant Ad particles via intrahepatic administration, but within hours experienced a massive inflammatory response leading to intravascular coagulation, respiratory distress and multiorgan failure, and died four days later (Marshall, 1999), underlining how severe anti-viral responses can be.

However, helper-dependent vectors with all the viral genes deleted are considerably less immunogenic (Morsy & Caskey, 1999). Their increased transgene expression is more applicable to long-term gene therapy, and such vectors have shown life-long phenotypic correction in murine models of Duchenne muscular dystrophy (DelloRusso *et al.*, 2002).

1.4.1.2 Adeno-Associated Virus Vectors

Adeno-associated virus (AAV) is a small single stranded replication-deficient non-enveloped DNA parvovirus, which requires adenovirus or herpes simplex virus to replicate. AAV is composed of an icosahedral protein capsid and a genome of 4.7kb, which encodes *rep* and *cap*, flanked at both ends by inverted terminal repeats (ITR)

(Rabinowitz & Samulski, 2000). Most AAV vectors are based on serotype-2 (AAV-2) and are promising for gene therapy, as they are not known to cause any human disease. However, only 4.5kb of DNA can be inserted into AAV-2, so small packaging capacity is a limitation. Wild-type AAV-2 can integrate into chromosome 19q of the host cell, although AAV vectors appear to integrate randomly (Russell *et al.*, 1994). Integration of recombinant AAV genomes into host chromatin is associated with chromosomal rearrangements, including deletions and translocations (Miller *et al.*, 2002).

AAV-2 has a broad tropism, infecting liver, retina, brain, muscle and heart as it binds to ubiquitously expressed heparin sulphate proteoglycans (Summerford & Samulski, 1998). However, it does not infect all cells efficiently and pseudotyping AAV-2 with capsids from other serotypes with different tissue tropisms is one method of vector retargeting (Büning *et al.*, 2003). Alternatively, insertion of ligand coding sequences into the capsid genes (Girod *et al.*, 1999) or using an intermediate molecule to interact between the target cell and the vector, such as a bispecific antibody (Bartlett *et al.*, 1999) can retarget the vector.

In cancer therapy AAV vectors have delivered the angiostatic molecule angiostatin in a murine model of metastatic liver cancer, significantly suppressing angiogenesis and growth and increasing survival time (Xu *et al.*, 2003). AAV has also been used in hybrid vectors, which combine the best features of different viruses. An Ad/AAV hybrid vector has been used to deliver antisense telomerase RNA to breast cancer cells, enhancing apoptosis and significantly suppressing their malignant phenotype *in vitro* (Zhang *et al.*, 2003b).

1.4.1.3 Retroviral Vectors

Retroviral (RV) vectors integrate the transgene stably into the host genome, such that the transferred gene is passed on and expressed in all daughter cells. Retroviruses consist of

two copies of a single stranded RNA genome encoding sequences called *gag*, which encodes structural proteins, *pol*, which encodes reverse transcriptase (RT) and integrase, and *env*, which encodes the envelope glycoprotein that binds receptors on the cell surface to gain entry and infect the target cell. Once in the cell, the single stranded RNA is converted to dsDNA by RT, and integrase inserts the viral DNA into the host genome (reviewed in Kurian *et al.*, 2000; Hu & Pathak, 2000).

The most frequently used RV vectors are based on oncogenic viruses and Moloney Murine Leukaemia virus is used most often. Pseudotyping these vectors with surface proteins of other viruses has improved the host range and titre. Pseudotyping with VSV-G (the glycoprotein of vesicular stomatitis virus), which targets ubiquitously expressed phosphatidyl serine residues, has been particularly successful as these vectors have a broad host range. VSV-G has also enabled higher viral titres, as it is more stably associated with virions than many RV envelope proteins, allowing efficient concentration by ultracentrifugation or anion-exchange purification (Yee *et al.*, 1994, Scherr *et al.*, 2002).

Self-inactivating vectors with deletions in the U3 region of the 3' LTR have further improved vector safety. After one round of replication, these deletions are copied into the 5' and 3' LTRs, producing inactive provirus (Yu *et al.*, 1986). The use of tissue-specific promoters has also been used to restrict expression to target cell populations.

Severe combined immunodeficiency (SCID)-X1 has been treated with an MLV-based RV vector delivering γc to CD34⁺ stem cells, resulting in functional reconstitution of immunity (Cavazzana-Calvo *et al.*, 2000). However, there is concern that the gene could be integrated into a proto-oncogene, resulting in malignancy. Indeed, this has resulted in two of fourteen patients in the SCID-X1 trial developing T-cell lymphomas. But these are just

two patients from the hundreds treated with RV vectors for various diseases (Thomas *et al.*, 2003). At present, there are no efficient methods for targeted integration. Integrating viruses are preferable if stable genetic alteration is to be maintained in dividing cells. However, integration does not guarantee stable transcription as transgene expression can gradually be silenced over time (Pannell & Ellis, 2001).

RV vectors can also deliver two genes on one plasmid with the use of an internal ribosomal entry site (IRES) and this approach has been used in cancer therapy, using one vector to deliver CD80 and IFN- γ (Qian *et al.*, 2002). This allows the ribosome to bind the middle of an mRNA and translate a gene far from the 5' end of the message, although generally the gene expressed with the IRES is translated less efficiently than the gene at the 5' end of the mRNA. RV vectors only transduce dividing cells, making them an attractive target for cancer gene therapy as they may target rapidly dividing malignant cells *in vivo* (Roth *et al.*, 1996). However, most RV modification occurs *ex vivo*, and the requirement for mitosis limits their use.

1.4.1.4 Lentiviral Vectors

Lentiviral (LV) vectors are complex retroviruses typically associated with the infection of macrophages and lymphocytes. LV vectors are able to infect non-dividing cells by interacting with the nuclear import machinery, and the preintegration complex is actively transported through the nucleopores (Bukrinsky *et al.*, 1993). Most LV vectors are based on HIV-1 although other lentiviruses have been investigated such as HIV-2 and equine infectious anaemia. The endogenous HIV-1 envelope protein (gp120) has a very narrow tropism, targeting only CD4 and a co-receptor (CCR5) *in vivo*, although variants can evolve to use CXCR4 as a co-receptor (Clapham & McKnight, 2001), and therefore they are often pseudotyped with VSV-G to increase their tropism.

Many other viral proteins are also deleted, including *vif*, *vpr*, *vpu*, and *nef*, which all contribute to viral pathogenesis (Naldini & Verma, 1999). LV vectors need to express accessory proteins for regulation of their replication so additional *cis*-acting elements are incorporated into LV plasmids, including the Rev response element which permits transport of viral RNA to the cytoplasm. Addition of the woodchuck hepatitis post-transcriptional regulatory element (WPRE) has also been shown to enhance gene expression by stabilising mRNA and allowing increased translation (Zufferey *et al.*, 1999). Another element that enhances efficient LV transduction is the central polypurine tract, which is necessary for second strand DNA synthesis, and is also thought to enhance the nuclear transport of the provirus (Zennou *et al.*, 2000).

LV vectors efficiently transduce non-dividing cells including adult neurons (Naldini *et al.*, 1996) and human DC and macrophages differentiated from peripheral blood (Schroers *et al.*, 2000). LV vectors also integrate into the host genome, although this is not as random as previously thought. Analysis of hundreds of HIV integration sites showed that the viral genome preferentially integrates into transcriptionally active genes (Schroder *et al.*, 2002).

LV vectors have been used to treat primate models of Parkinson's disease with the delivery of glial derived neurotrophic factor due to their ability to transduce neurons (Kordower *et al.*, 2000). They are also effective in cancer therapy, as demonstrated in a murine melanoma model, delivering a tumour antigen which resulted in regression of established tumours and protective immunity against rechallenge (Metharom *et al.*, 2001).

Another genus of the retrovirus family are the foamy viruses (spumaviruses), which are distinct from both RV and LV vectors. They are not known to cause any disease in the

host, which has prompted interest in their use for gene delivery (Linial, 1999). Preliminary work has shown that foamy viruses transduce CD34⁺ human HSCs with high efficiency, equivalent to HIV-1-based LV vectors (Leurs *et al.*, 2003)

1.4.1.5 Herpes Simplex Virus

Herpes simplex virus (HSV) is a non-integrating, enveloped, double stranded DNA virus with a genome of 152kb. Wild-type HSV infects the sensory ganglia of peripheral nerves, where it can progress to a latent phase or lytic phase. Many viral genes can be deleted and complemented *in trans*, allowing for their replacement with large or multiple transgenes.

HSV has many advantages as a gene delivery vector, including broad host range, high infectivity, the ability to infect non-dividing cells, relatively long-term gene expression and low risk of insertional mutagenesis (reviewed in Burton *et al.*, 2002). However, the vector is episomal so it may be diluted out in dividing cells. HSV vectors are divided into two categories: recombinant HSV vectors and HSV amplicons.

The recombinant vectors consist of a wild-type HSV genome rendered replication-defective by deletion or disruption of essential genes, and can incorporate ~30kb of insert DNA. Amplicons are eukaryotic expression plasmids that contain a virus-derived origin of replication and the cleavage/packaging sequence. These can be modified to include up to ~130kb of foreign DNA, enabling the inclusion of promoters, enhancers and transgenes, possibly in their genomic form. This was recently achieved using an amplicon to deliver and express *in vitro* the complete genomic human hypoxanthine phosphoribosyltransferase locus of 115kb (Wade-Martins *et al.*, 2001).

HSV has a natural tropism for neural cells where it can establish a latent infection for the duration of the host's lifetime, thus is being considered as a vector for many neurological diseases and neurodegeneration, including peripheral neuropathy (Goins *et al.*, 1999; Chattopadhyay *et al.*, 2002) and chronic pain management (Goss *et al.*, 2001). HSV vectors have also been used to treat cancers of the CNS. Malignant glioma is a fatal, but normally localised, CNS tumour and has been treated *in vivo* with γ -irradiation and a HSV vector encoding TNF α and HSV-TK in combination with gancyclovir (Niranjan *et al.*, 2000).

HSV-1 amplicons have also been used in immunotherapy of murine tumours. Cytokine therapy of murine melanoma by intratumoural inoculation with a HSV-1 amplicon encoding the GM-CSF gene was able to significantly inhibit tumour growth in a dose dependent manner (Toda *et al.*, 2000).

1.4.2 Synthetic Gene Therapy Vectors

The first experiments were performed with naked plasmid DNA injected into muscle (Wolff *et al.*, 1990). The level of gene transfer was extremely low but gene expression persisted for months under certain conditions. Since then non-viral vectors have become more sophisticated and more efficient. They are considered a safer alternative to viruses as they are less immunogenic, permitting repeated administration (Jenkins *et al.*, 2000) and there is no risk of infection. They have fewer size constraints on the amount of DNA they can deliver to cells, being able to transfer up to 200kb, thus several genes can be delivered at once. However, as they are non-integrating, the transgene is diluted out of rapidly dividing cells. This is not necessarily a disadvantage, as some applications may only require transient expression.

Bacterial plasmid DNA incorporated in non-viral vectors is more straightforward to construct and produce than the packaging and purification of modified viruses. Immune responses triggered against viral proteins can limit the expression from some vectors and prevent the re-administration of the vector. Many patients have pre-existing antibodies against viral coat proteins due to previous natural exposure to the virus; over 70% of the population may carry antibodies to Ad and AAV (Somia & Verma, 2000), which may prevent infection and therefore expression of the transgene. Expression of viral proteins may stimulate B-cells to generate anti-viral antibodies and CTLs to kill transfected cells.

Plasmids grown in bacteria are associated with innate immune responses to palindromic sequences surrounding unmethylated CpG motifs present in bacterial DNA. These sequences are down-regulated in vertebrate DNA, and 75% of these dinucleotides are methylated. The differences in sequence in bacterial DNA activate components of the innate immune system that are an evolutionarily-conserved host defence mechanism that recognises conserved molecules restricted to micro-organisms (reviewed in Spack & Sorgi, 2001) and Toll-like receptor 9 (TLR9) has been shown to be responsible for CpG-DNA-mediated signal transduction (Hemmi *et al.*, 2000). The immunostimulatory properties of CpG have been exploited in experimental NB therapy and when CpG oligonucleotides were inoculated in conjunction with GM-CSF-secreting irradiated tumour cells, tumour-free mice were also resistant to further tumour challenge, indicating development of memory responses (Sandler *et al.*, 2003).

Gene gun technology uses a shock wave generated by high pressure helium to force DNA-coated micrometer-sized gold particles to cells or tissues and this method has been used in the treatment of experimental cervical cancer, to transfer DNA encoding HPV-16 E7 antigen (Hung *et al.*, 2001). Murine sarcoma has also been successfully treated by gene

gun-mediated transfer of IL-12 to the area of skin overlying the tumour, eradicating established tumours at the site of gene transfer, tumours at distant sites and rejecting later rechallenge with unmodified tumour cells, demonstrating systemic effect and immune memory (Rakhmilevich *et al.*, 1997).

The main disadvantage of non-viral gene transfer is the low transfection efficiency compared to viruses, resulting in much lower transgene expression. Nevertheless, 100% transfection efficiency is not necessarily required, as secreted factors can mediate effects through paracrine mechanisms (Feldman *et al.*, 2002).

1.4.2.1 Lipoplex Vectors

DNA is negatively charged and when mixed with a cationic lipid, forms a lipoplex due to electrostatic interactions. Many reagents have been developed which aim to increase the cellular uptake of plasmid DNA. Several of these are cationic lipids that are thought to aid entry to the cytoplasm by fusing with the cellular membrane, releasing the DNA into the cytoplasm and avoiding degradation via the lysosomal pathway (Felgner *et al.*, 1999). Several lipids are regularly used, including DOTMA (dioleoyloxypropyltrimethylammonium chloride), which had a single positive charge, and DOPE (dioleoylphosphatidylethanolamine), which is neutral.

Synthetic vectors have been successful in murine tumour models in treating established disease and generating memory responses; intratumoural delivery of IL-12 using a polyvinyl non-condensing polymeric vector demonstrated potent antitumour immunity against renal and colon carcinoma, and induced protective immune memory. Importantly, this formulation did not generate any anti-vector immune responses (Mendiratta *et al.*, 1999). It has been shown that DNA uptake is quite efficient, but two steps limit

expression; the routing of DNA through endosomes and lysosomes, and the nuclear purging of foreign DNA. Cell lines which are readily transfected demonstrated nuclear stability of DNA, while primary cells that show much lower transfection efficiency rapidly excluded DNA from the nucleus (Coonrod *et al.*, 1997).

Lipoplexes have been used in experimental cancer models, including murine colon carcinoma; a plasmid containing the IFN- γ gene complexed with the lipid DC-chol gave a greater tumour inhibitory effect than naked plasmid DNA alone, and also generated protective immunity against further tumour challenge (Nomura *et al.*, 1999). In another murine model, intravenous administration of lipid-DNA complexes encoding IL-2 or IL-12 significantly inhibited the growth of advanced lung metastases, and increased numbers of NK and CD8⁺ cells and IFN γ production within lung tissues (Dow *et al.*, 1999).

1.4.2.2 Polyplex Vectors

Condensing agents are widely used to package plasmid DNA into smaller particles, such as polyethylenimine (PEI) and poly-L-lysine, which can be used alone or in combination with lipid formulations. The positive charge of these compounds increases the contact between the DNA complex and the negatively charged cell membrane, and cells transfected by lipofection show a significant accumulation of DNA at the cell surface (Coonrod *et al.*, 1997), although this non-specific interaction results in binding to any type of cell.

In vivo, polyplex vectors have shown efficacy, with aerosol delivery of a PEI-p53 gene complex in a murine melanoma lung metastasis model inhibiting development of tumour foci and metastasis to lymph nodes, and increasing survival time (Gautam *et al.*, 2000). However, direct injection of polyplex vectors into the circulation is often ineffective. In mice, this is mediated by rapid clearance from the blood due to fixing by complement,

limited solubility and removal in the liver. *In vitro* analysis with human blood also showed complexes are fixed by complement and IgG. Furthermore, it has also been shown that the polycation molecular weight and the type of DNA (circular plasmid or linear) can affect the rate at which complexes are cleared from the circulation (Ward *et al.*, 2001). A variant of polylysine, which is biodegradable due the replacement of amide linkages with ester linkages, showed efficacy in a murine cancer model. Due to its biodegradable nature, it is thought to be nonimmunogenic, and has low cytotoxicity, allowing repeat dosing (Maheswari *et al.*, 2000).

PEI-condensed DNA has also been used in combination with a replication-deficient Ad vector; the transgene is not encoded by the virus, but the virus aids endosomal release and transport of the plasmid DNA to the nucleus (T. Gust, personal communication). A variation of this is the use of adenovirus hexon protein in combination with a PEI/DNA vector. Covalently linking hexon protein to PEI demonstrated 10-fold greater transgene expression *in vitro* than PEI/DNA alone, and injection into *Xenopus* oocytes also showed a 10-fold increase in transgene expression compared to PEI/DNA, without increasing vector-uptake. This improved gene expression was due to increased nuclear transfer efficiency by means of the nuclear pore complex (Carlisle *et al.*, 2001).

Another cationic peptide, CL22, contains an influenza nucleoprotein sequence and has shown increased transfection activity that is thought to result from favourable intracellular trafficking, DNA uncoating or expression (Haines *et al.*, 2001). The DNA-binding protein, histone H2A, has also been used in transient transfections to increase DNA uptake. Gene transfer of IL-2 and IL-12 using histone H2A resulted in antitumour immunity to murine neuroblastoma involving both NK and T-cells. Gene transfer was shown to not rely

merely on positive charge alone, but on other molecular characteristics of H2A, possibly including a nuclear localisation signal (Balicki *et al.*, 2000).

One of the main problems with synthetic vectors is the degradation of internalised vector, usually in the acidic endosomes. Endosomal buffering agents, such as chloroquine, have shown enhanced transgene expression, which is thought to be due to decreased acid degradation of complexed DNA after uptake into endosomes. Chloroquine also induces osmotic swelling of endosomes, resulting in endosome destabilisation and release of the internalised DNA (Wagner *et al.*, 1994).

A novel strategy to aid endosomal escape is the use of bacterial toxin translocation domains, such as *Pseudomonas aeruginosa* exotoxin A domain II (ETA), which facilitates translocation of DNA from endosomes/lysosomes to the cytoplasm. Expression of a tumour antigen linked to ETA enhanced MHC I presentation of the tumour antigen to CD8⁺ CTLs (Hung *et al.*, 2001). Similarly, the C-terminal B fragment of diphtheria toxin also contains a translocation domain that facilitates endosomal escape, and modular DNA-carrier proteins that employ the natural endosome escape mechanism of the toxin are able to transport DNA into cells. A chimeric diphtheria toxin molecule constructed by replacing the cell recognition function with tumour-specific ligands and replacing the enzymatic A fragment with a DNA-binding domain have been used to target gene delivery to cancer cells (Uherek *et al.*, 1998).

Another strategy to enhance uptake of DNA is to complex the plasmid with molecules that target cell surface receptors (reviewed in Cotton & Wagner, 1999). Asialoorosomuroid covalently linked to polylysine was the first targeting ligand used to target asialoglycoprotein receptors on hepatocytes (Wu & Wu, 1987). The transferrin receptor

was also exploited due to its rapid internalisation and ubiquitous distribution across many types of cell (Wagner *et al.*, 1990). Since this initial work many ligands have been integrated into transfection vectors, including antibody fragments, synthetic peptides and carbohydrates. Generally, transgene expression is required in only one or a few cell types, so targeting of these complexes is important.

1.4.2.3 LPD Vector

The vector used in this project contains targeting cationic peptides (P) together with a cationic lipid formulation (L) and plasmid DNA (D), known as a LPD vector. A panel of peptides containing a variety of different binding motifs has been isolated by phage panning on different cell types for superior binding ability or transgene expression when incorporated into the LPD vector, plus a range of ~10 peptides have been synthesised with motifs that target α_5 , α_4 , or α_v integrins (Table 2.1, Materials and Methods). Integrins are surface molecules involved in adherence that are exploited as a means of entering the cell by a variety of viruses. This suggests that they are a good target for gene therapy vectors (Hart, 1999). The binding motif is flanked by cysteine residues that form a disulphide bond, resulting in a constrained cyclic head group. The targeting head group is linked to a 16-residue polylysine tail, resulting in a molecule that targets cell-surface molecules at one end and has strong DNA condensing properties at the other end.

At physiological salt and pH, the three reagents spontaneously form a complex through electrostatic interactions. It is thought that the DNA and polylysine part of the peptide form the core of the complex and the cyclic head groups displayed on the complex surface with the lipid forming an outer layer (Figure 1.4). Receptor-mediated endocytosis increases the level of cellular uptake and the lipid component aids endosomal escape of the DNA once it has entered the cell (Figure 1.5). This vector has been successfully used to

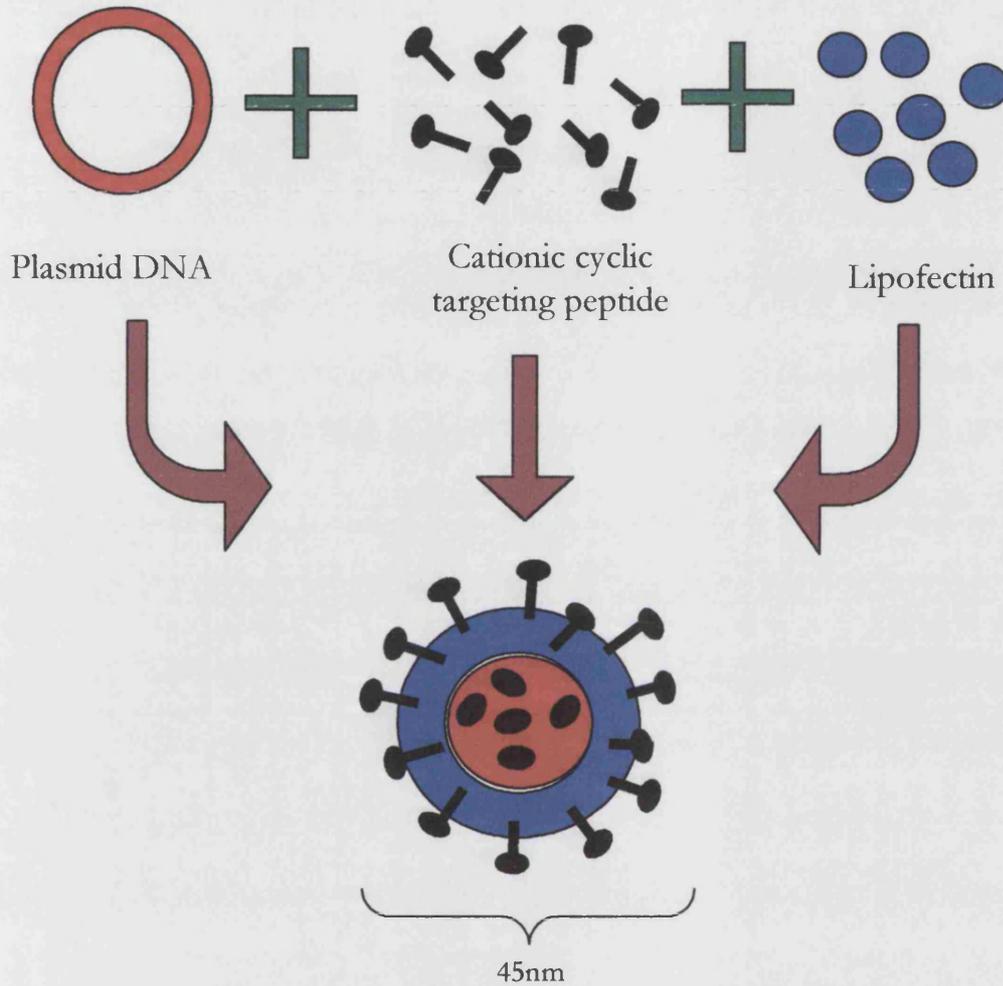


Figure 1.4: The lipid, peptide and DNA components of the vector spontaneously combine to form particles. The DNA condenses with the positively charged polylysine tail of the peptide, while the cyclic targeting head group is displayed in the outer surface of the vector. Different peptides can be used to target different cell surface molecules. The lipid is thought to coat the complex. The size of the complex has been determined by atomic force microscopy (Hart *et al*, 1998).

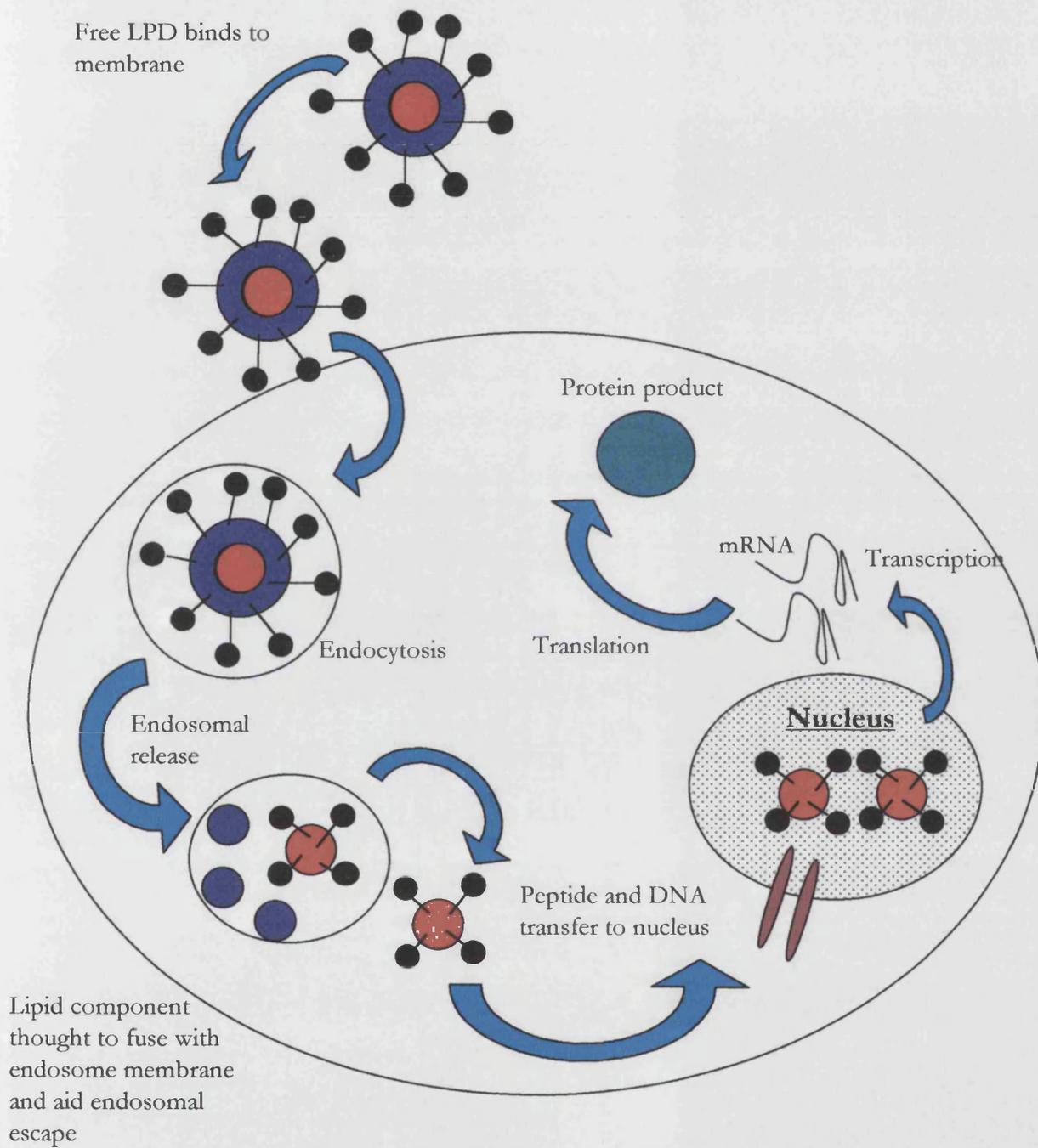


Figure 1.5: Proposed mechanism of LID uptake and trafficking through the cell

treat murine NB in our lab by the transfection of a murine NB cell line with cytokine genes (Siapati *et al.*, 2003). However, due to the problems associated with culturing primary tumour cells, this model is difficult to transfer to the clinic. In this project, I will be developing a more clinically-applicable cellular vaccine for the treatment of experimental murine NB.

1.5 Murine NB

The model of NB used in the project is the A/J mouse and the syngeneic Neuro-2a (N2a) neuroblastoma cell line. Originally, C1300-NB appeared spontaneously from the spinal cord of strain A mice about 50 years ago. The A/J mouse and N2a cell line is a subclone of C1300, is widely used by many researchers working on NB and is considered a good model of the disease (Ziegler *et al.*, 1986). The N2a cell line is syngeneic, and therefore is used to engraft tumours into A/J mice. Untreated, they form locally invasive, tumours which grow progressively until the death of the animal. This allows a variety of experiments to be conducted, such as tumourigenicity experiments (preventing engraftment of tumours) or eradication experiments (treatment of established tumours). For this project, the tumours are engrafted subcutaneously in the flank, making them accessible for measurement and treatment. The tumours in this model do not readily metastasise, making measurement easier. An alternative syngeneic clone, TBJ-NB, is characterised by increased invasive and aggressive growth, and readily forms metastases. *N-myc* mRNA is expressed at much higher levels in the metastatic TBJ-NB clone than in the locally-growing C1300 and its derivatives (Ziegler *et al.*, 1997), consistent with what is observed in human NB.

Murine NB displays many of the properties of primary human NB tumours. Like human NB, the majority of cells in solid primary or metastatic murine tumours are in the resting

stage (Hayes & Mauer, 1976), and both human and murine cells express only low levels of MHC I (Lampson *et al.*, 1983; Katsanis *et al.*, 1995). Similar to human NB, increased concentrations of catecholamine metabolites have been detected in the urine of tumour-bearing mice (Pons *et al.*, 1982).

Murine NB also shows differences to the human disease. Primary human NB typically shows aggressive growth of primary tumour followed by metastasis to the regional lymph nodes and liver, and then in more advanced disease to the bone cortex or bone marrow. In the C1300-derived model of NB, regional lymph node metastasis may be seen in the later stages of tumour growth, but metastasis to central organs is not observed. The spontaneous regression seen in some stage IV-S human NB is very rarely observed in murine tumours. Human tumours typically form rosettes of well-oriented neuroblasts, which are not observed in murine NB (Ziegler *et al.*, 1997).

NK cell activity against NB can be enhanced in both humans (Alvarado *et al.*, 1989) and mice (Reynolds *et al.*, 1989) by administration of recombinant interferon- γ or recombinant IL-2. Analysing the TCR repertoire of the infiltrating lymphocytes of human tumours has demonstrated T-cells with activity against NB, and clonal expansion has also been observed (Valteau *et al.*, 1996). CTL activity has also been documented in murine NB, and there is some evidence that this activity is augmented by abrogation of the activity of suppressor cells (Choi *et al.*, 1989). NB cells engineered to express IL-2 have shown reduced tumorigenicity, which was shown principally to be mediated by CD8⁺ T-cells (Katsanis, *et al.*, 1994), and this has also been shown in murine NB (Siapati *et al.*, 2003).

1.6 AIM

Previous work has shown N2a-derived tumours in the A/J mouse can be effectively treated by N2a tumour cells engineered to express IL-2 and IL-12 (Siapati *et al.*, 2003). However, due to the difficulty of culturing primary NB cells from patients, this strategy may not be feasible to transfer to the clinic. The aim of this project was to create an alternative immunotherapeutic strategy, developing a cellular vaccine to treat murine NB in A/J mice which would be more applicable in a clinical setting.

The two cell types investigated were DC and fibroblasts. The first approach was the optimisation of a synthetic gene transfer protocol using the LPD vector to transfect DC. DC are the most potent APC of the immune system, activating naïve T-cells and being essential for the maintenance of memory T-cells. Transfer of genes encoding immunostimulatory molecules or model tumour antigens into DC using a synthetic vector could be a novel method to treat murine NB tumours. DC transduced with adenoviral vectors encoding IL-12 have previously been shown to mediate therapeutic effects against murine NB (Redlinger *et al.*, 2003b; Shimizu *et al.*, 2001). However, despite optimisation of many parameters, DC appeared refractory to transfection with the LPD vector.

Therefore, an alternative immunotherapeutic strategy was developed using fibroblasts transfected with the LPD vector to express IL-2 and IL-12, analogous to the cytokine-expressing N2a cells studied previously. Previous studies have shown that cytokine-expressing fibroblasts are effective against murine tumours (Lichter *et al.*, 2002; Zitvogel *et al.*, 1995; Fakhrai *et al.*, 1995), and have also been used in clinical trials for the treatment of solid tumours (Sobol *et al.*, 1999; Kang *et al.*, 2001). Cytokine-transfected fibroblasts were compared with cytokine-transfected N2a cells for their potential to abrogate the tumorigenicity of wild-type N2a cells and for therapeutic efficacy against established

tumours. *In vitro* assays were performed to investigate the cellular antitumour immune response generated against N2a cells in vaccinated mice and tumour sections were examined to assess the effect of vaccination on tumour histology, and whether vaccination increased the presence of tumour infiltrating leukocytes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

All cell culture media, penicillin/streptomycin, sodium pyruvate and non-essential amino acids are manufactured by Life Technologies (Paisley, UK). All foetal calf serum (FCS) used in cell culture is manufactured by the Sigma Chemical Company (Dorset, UK) unless otherwise stated. All tissue culture plastics were manufactured by Helena Biosciences, Sunderland, UK) unless otherwise stated.

2.1.1 Murine DC Culture

Bone marrow was obtained by flushing out the hind tibia and femur from A/J mice and resuspended in HEPES-buffered RPMI1640 + Glutamax-1 supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin (complete media) and murine granulocyte-macrophage colony-stimulating factor (mGM-CSF - gift from S. de Noronha). Cells were maintained at 37°C in 5% CO₂ for 6 days. The source of GM-CSF was the supernatant from a plasmacytoma cell line transfected with an expression vector (Karasuyama *et al.*, 1990) containing the PCR-derived mouse GM-CSF gene (Zal *et al.*, 1994). The optimal concentration of GM-CSF-containing supernatant had been previously titrated to 5% of the final culture volume (S. de Noronha, unpublished).

Total bone marrow from one mouse was cultured in a total of 60 ml media, distributed evenly between 2 x 6-well plates. To obtain pure populations of DC for transfection experiments, cells were positively selected using MACS mouse CD11c (N418) microbeads (Miltenyi Biotech, Surrey, UK). Briefly, cells were washed from 6-well plates, spun down at 240 x *g* and resuspended in 400 µl MACS buffer (0.1% BSA in PBS). 100 µl of MACS beads were added and the cells incubated on ice for 15 minutes. Unbound beads were diluted out with 10 ml MACS buffer, and the cells spun down and resuspended in 500 µl

MACS buffer. A MACS MS column was equilibrated with 500 μ l MACS buffer and the cells applied to the column. The column was washed three times with 500 μ l MACS buffer, then 1 ml of complete media added and the bound cells were pushed off the column with the syringe plunger. This was repeated with another 1 ml of complete media. The cells were counted, spun down and resuspended at the appropriate concentration. An aliquot of cells was tested for purity by FACS analysis of the %-CD11c⁺ population.

2.1.2 Human DC Culture

Venous blood was venisected into heparinised tubes and diluted 1:1 in HEPES-buffered RPMI1640 + Glutamax-1 before layering onto Lymphoprep (Axis-Shield, Oslo, Norway) and spinning at 1100 x g with brake off for 25 minutes. The PBMC layer was aspirated from the lymphoprep/serum interface and washed in HEPES-buffered RPMI1640 + Glutamax-1 twice to remove platelets, then PBMC were plated into 6-well dishes (Falcon, Becton Dickinson, Oxford, UK) in HEPES-buffered RPMI1640 + Glutamax-1, antibiotics and 10% low endotoxin FCS (Life Technologies, Paisley, UK) at 3.4×10^6 cells/ml, 3 ml per well. Monocytes were allowed to adhere for 2h, after which the floating fraction of cells was removed and media replaced. Cytokines were then added; 10 ng/ml rhGM-CSF (Leucomax, Novartis, Spain) and 7.5 ng/ml rhIL-4 (Peprotech, London, UK). Cells were maintained in 5% CO₂ at 37°C for 5-6 days before use.

On day 5-6 cells were immunodepleted with CD3 and CD19 magnetic beads (Dynabeads, DYNAL Biotech, Wirral, UK) to removing any remaining T and B cells respectively. Briefly, the floating fraction of the cells was removed and pelleted. The cells were resuspended in 2 ml complete media and split between two 1.5 ml tubes. 10 μ l each of CD3 and CD19 beads were added to each tube on ice then transferred to a rotating mixer at 4°C for 20 minutes. The tubes were then placed on a magnet and B and T cells bound to the beads

were pulled to the side of the tube. The supernatant was pipetted off and transferred to a clean tube and placed back on the magnet to remove any remaining beads. The cells remaining in the supernatant were used for experiments. A small aliquot was retained for determining cell purity by FACS with anti CD11c-PE (Pharmingen, BD Biosciences, Oxford, UK).

2.1.3 Mouse Primary Skin Fibroblasts

Skin was removed from A/J mice, the fur trimmed off and digested in trypsin-EDTA overnight at 4°C. The dermis was then peeled away from the epidermis and digested in 1mg/ml Liberase CI (Boehringer Mannheim, Lewes, UK) in DMEM (no FCS) at 37°C for 2 h. The Liberase was inactivated by diluting 1:1 with FCS and the suspension passed through a cell strainer. Cells were pelleted by centrifugation (240 x g), washed twice with DMEM with 50% FCS to inactivate any remaining Liberase and plated out in 6-well plates in DMEM with 20% FCS and antibiotics and maintained at 37°C in 5% CO₂. Cells were used at passage 3-5.

2.1.4 Mouse Embryonic Fibroblast culture

MEF cultures were prepared by adaptation of the original 3T3 protocol of Todaro & Green (1963) and expertise within the Institute of Child Health (M. Hubank, personal communication). Fifteen 13.5dpc A/J embryos were dissected into PBS and the crania and red organs removed, leaving the limb buds and spinal region. Each embryo was kept as a separate culture. This remaining tissue was minced using scalpels and digested in trypsin at 37°C for 30 mins. This was passed through a 16G needle to break up the tissue and then passed through a cell strainer. The cells were then counted and plated out into a 75 cm² flask in DMEM + 10% FCS and antibiotics. Three days later the cells were trypsinised, counted and 50,000 cells put in a 25 cm² flask, and the process repeated after

another 3 days. After 2-3 passages most of the cultures became senescent, and at this stage, were monitored every few days without passaging of the cells. After ~14-20 days, ten of the cultures began growing again. Of these ten, two had a fibroblast like morphology, and were used in initial transfections. Of these two, one had superior levels of transgene expression and the cells grew more rapidly. This culture, hereafter termed AJ3.1, was used in all subsequent MEF experiments.

2.1.5 Human Primary Fibroblasts

Human dermal primary fibroblasts (gift from Dr Mike Hubank, Institute of Child Health) and maintained in DMEM with 10% FCS and antibiotics and passaged once a week. Two cultures of fibroblasts were obtained from skin biopsies from two different normal healthy donors. The cells had equivalent morphology and growth characteristics and were used in transfections were between passages 13-22.

2.1.6 Cell Line Culture

The mouse fibroblast line NIH-3T3, mouse neuroblastoma line Neuro-2a (N2a), human airway epithelia (HAE), human embryonic kidney epithelial line (293) and human cervical adenocarcinoma epithelial line (HeLa) were obtained from the ATCC and maintained in DMEM with antibiotics, 10% FCS, 1% sodium pyruvate (v/v) and 1% non-essential amino acids (v/v). The T-cell CD4⁺ hybridoma, A18, was a gift from B. Stockinger, (NIMR, London, UK) maintained in RPMI + Glutamax-1 with 5% FCS, 50 μ M β -mercaptoethanol (Sigma Chemical Co, Poole, UK) and antibiotics. All cells were maintained at 37°C with 5% CO₂

2.2 Plasmid Preparation

Plasmids pCI (Promega, WI, USA) pCI-Luc, pCI-hIL-2, pCI-mIL-12 (gifts from E. Siapati, Institute of Child Health, London), pCMV-C5 (gift from B. Stockinger, NIMR, London, UK) and pEGFP-N1 (Clontech, CA, USA) were maintained in *E.coli* strains DH5 α or JM109 (Promega, Southampton, UK). See appendix 1 for plasmid maps. All plasmids contained the ampicillin resistance gene, except pEGFP-N1, which contained the kanamycin resistance gene. All genes in all plasmids were under the control of the CMV-intermediate-early (CMV i.e.) promoter and the plasmids purified with the EndoFree Mega Kit (Qiagen, Crawley, UK). Briefly, 500 ml LB broth (Invitrogen, Paisley, UK) containing 100 μ g/ml ampicillin or 50 μ g/ml kanamycin was inoculated with a 1 ml starter culture and grown overnight at 37°C with shaking. The following day the cells were pelleted at 6000 $\times g$ at 4°C and lysed by alkaline lysis. The lysate was neutralised and passed through a filter column using vacuum filtration. Endotoxin removal buffer was added to the filtrate and incubated on ice for 30 mins, then the solution passed through an anion-exchange resin column. The DNA was eluted by 15% isopropanol in 1.6 M NaCl, pH7 and precipitated by the addition of 0.7-volumes 100% isopropanol. The solution was decanted into endotoxin-free baked glass Corex centrifuge tubes and spun at 15,000 $\times g$ for 30 mins at 4°C. The supernatant was removed and the pellet washed with 70% EtOH in endotoxin-free water and spun at 15,000 $\times g$ for 15 minutes.

The pellets were resuspended in 1 ml endotoxin-free TE buffer and DNA quantitated by absorbance at 260 nm with a GeneQuant spectrophotometer (Pharmacia, Buckingham, UK). The ratio of 260 nm/ 280nm was used to determine the protein contamination, and all plasmid solutions had an absorbance ratio of 1.8 ± 0.1 , indicating minimal protein. Digestions with restriction enzymes (all Promega, WI, USA) were performed to check

plasmid integrity. 0.5 µg of plasmid DNA was digested with 1 µl enzyme for one hour at 37°C in a total volume of 50 µl of 1x restriction buffer (Promega) and analysed on 1% agarose gels stained with ethidium bromide. Fragment sizes were established by comparison with 1 kb ladder (Bioline, London, UK).

2.3 “Helper” Adenovirus Production

Replication-deficient E1-deleted adenovirus (Ad) containing the LacZ reporter gene was used to infect 293 cells at an MOI of 1. The cells were grown until they were forming rounded up plaques and just beginning to detach from the plates, then washed off the plates, spun down and resuspended in 10 ml Ad freezing buffer (0.1 M Tris, 5 mM MgCl₂, pH8). The cells were freeze-thawed three times at -80°C, and the lysate spun at 2750 x g for 10 mins to remove cell debris. The density of the supernatant was adjusted to 1.40 g/ml with CsCl. This was layered onto a 1.5 g/ml CsCl cushion, and the tubes (Ultra Clear, Beckman Coulter, High Wycombe, UK) topped up with more 1.4 g/ml CsCl and spun at 300,000 x g in an ultracentrifuge (Beckman Coulter) at 15°C for 21-24 hours with the brake off.

The virus band was removed by piercing the tube with an 18G needle and carefully drawing the virus into a 5 ml syringe. This was resuspended in a total volume of 6 ml 1.4 g/ml CsCl, layered onto another 1.5 g/ml CsCl cushion, topped up with 1.4 g/ml CsCl and spun again at 300,000 x g 15°C for 21-24 hours. The band of virus was again removed and transferred to a Slide-A-Lyser dialysis cassette (Pierce, IL, USA). The CsCl was dialysed out overnight in 4 l Ad dialysis buffer (10 mM Tris, 5 mM MgCl₂, 2.3% sucrose, pH8, 4°C). The virus was titred on HeLa cells in 96-well plates, 40,000 cells per well with serial dilutions of virus. 1 µl of virus stock was diluted in 1 ml DMEM, and 100 µl of this

was added to 900 μ l DMEM as the most concentrated sample. 100 μ l of this was then used to make the next dilution and so on, until there was a range of 10-fold serial dilutions from 10^{-3} to 10^{-9} . 50 μ l of each dilution was added to the HeLa cells in triplicate and incubated for 2-3 h, then another 200 μ l DMEM added to each well and incubated for a further 48 h. The media was then removed from the cells and the cells fixed with 0.5% gluteraldehyde in PBS. The cells were washed then 50 μ l 1 mg/ml X-gal added to each well and incubated at 37°C, checking regularly for blue cells. The viral titre was determined by the limiting dilution that produced blue colonies by X-Gal staining and calculating the number of infectious particles per ml. This value is used to calculate the multiplicity of infection (MOI) in transfection experiments.

2.4 Phage panning and isolation of binding peptides

Peptides that bound to particular cell types with high affinity were identified by panning with phage display libraries (M. Writer, in press and unpublished, A. Kritz, unpublished). These peptides were then engineered into the cationic peptide component of the LPD vector, by flanking the phage-isolated motif with cysteine residues which cyclise at physiological pH, and attaching a chain of 16 lysine residues to the N-terminus.

2.5 LPD Transfections

2.5.1 Calculation of charge ratio of complexes

The charge ratio of the LPD vector was determined from the net charge and molecular weight of plasmid DNA and each targeting peptide (see **Table 2.1**). The charge of DNA is calculated from the single negative charge of the phosphate on each DNA base. Thus, 1 μ g of pEGFP-N1 (4700 bp, $M_r = 3.11 \times 10^6$) contains 0.32 pmol of DNA which contributes $(0.32 \text{ pmol} \times 4700 \times 2) = 3.0 \text{ nmol}$ of negative charge. As all plasmids contain

Table 2.1 Peptides used in LPD transfections

Peptide	Molecule targeted	Sequence	Net charge	Mass of peptide (μg) for a 7:1 charge ratio with $1\mu\text{g}$ DNA
Integrin targeting peptides				
1	α_v, α_5	[K] ₁₆ GACRGDMFGCA	+16	4
6	α_5	[K] ₁₆ GACRRETAWACG	+17	4
6J	Scrambled	[K] ₁₆ GACATRWARECG	+17	4
7	α_5	[K] ₁₆ GAGPEILDVPST	+14	4.6
8	α_4	[K] ₁₆ GACQIDSPCA	+15	4
10	α_4	[K] ₁₆ GACWLDVCA	+16	3.85
11	None	[K] ₁₆ GACRGEMFGCA	+16	4.05
12	α_5	[K] ₁₆ XSXGACRRETAWACG	+17	4.39
14	α_5	[K] ₁₆ [XSX] ₃ GACRRETAWACG	+17	5
K16	None	KKKKKKKKKKKKKKKKKK	+16	2.6
Peptides isolated by phage display				
VYAR	Unknown	[K] ₁₆ GACVYARSMNCG		4
VNLQ	Unknown	[K] ₁₆ GACVNLQNPYCG		4
P	Unknown	[K] ₁₆ GACLPHKSMPCG		4
E	Unknown	[K] ₁₆ GACSERSMNFCG		4
A	Unknown	[K] ₁₆ GACSHVKLNSCG		4
B	Unknown	[K] ₁₆ GACAPSNSTACG		4

the same charge per base, 1 μg of any plasmid will contribute 3.0 nmol of negative charge. The charge of the peptide depends on the sequence, with glutamic acid (E) and aspartic acid (D) each containing a charge of -1 and lysine (K) and arginine (R) each containing a charge of +1. Lipofectin contains only one positive charge per molecule (**Figure 2.1A**), so is considered insignificant when calculating LPD vector charge ratios.

2.5.2 DC and Primary Fibroblast Transfections

Cells were seeded into 48-well plates at 50,000 cells per well, and allowed to adhere for 1 h. LPD complexes were prepared by combining 1 mg/ml Lipofectin (Invitrogen, Paisley, UK) with 100 $\mu\text{g}/\text{ml}$ peptide (Zinsser Analytic, Slough, UK), then 10 $\mu\text{g}/\text{ml}$ plasmid DNA was added to the lipid/peptide mixture. The order in which the vector components are added together has previously been shown to be essential to transfection (Hart *et al.*, 1998). For transfections using an Ad helper virus, the virus was added to the plasmid DNA, then the Ad/DNA mix was added to the lipid/peptide combination.

Peptides and DNA were diluted to a working concentration in OptiMEM serum-free media (Life Technologies, Paisley, UK), and LPD complexes were also formulated in OptiMEM. The ratio of Lipofectin to DNA remained constant at 0.75 μg Lipofectin per 1 μg DNA, while charge ratio, amount of DNA, targeting peptide (see **Table 2.1**) and length of transfection period were investigated. Total transfection and culture time before analysis was 24 h for luciferase assays and up to 14 days for GFP analysis and supernatant samples for ELISA, with samples collected over 24 h periods. Each transfection condition was performed in triplicate.

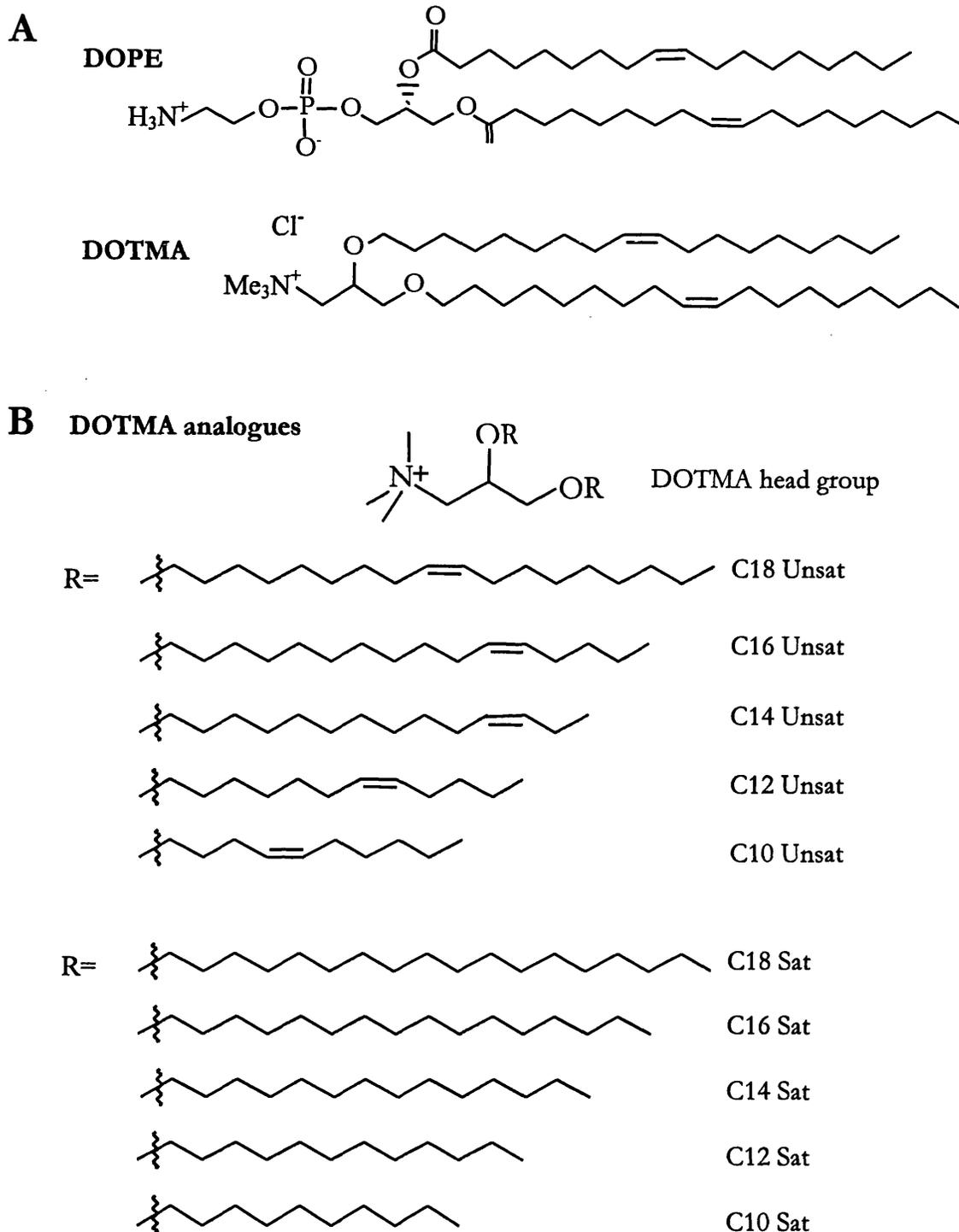


Figure 2.1 Structures of lipids used for transfection (A) Structures of DOPE and DOTMA, which are combined at an equimolar ratio to form Lipofectin. DOPE is a neutral lipid. DOTMA has a net charge of +1 and is composed of two C18 chains, each containing one unsaturated bond, covalently linked to the charged head group.

(B) Structures of DOTMA analogues, which were formulated with and without DOPE. The analogues contain the same charged head group as DOTMA, but the C18 chains are replaced with lipid chains from the saturated or unsaturated series. Each analogue is composed of two identical chains.

For human fibroblasts, all transfections to test the DOTMA analogues (**Figure 2.1B**) were performed in 96-well plates using peptide 6 at a charge ratio of +7, 0.25 μg DNA per well and were transfected for 4 h. Lipofectin was used at a weight ratio of 0.75 μg per 1 μg DNA, while the DOTMA analogues were used at weight ratios of 2 μg or 4 μg per 1 μg DNA. Each lipid was assayed in six replicates. Transfections to test the peptides isolated by phage display, and transfections with cytokine genes were performed in 48-well plates using peptides at a charge ratio of +7, 1 μg DNA per well for 4 h. As before, the vector components were always added in the order of lipid, then peptide, then DNA.

2.5.3 Cell Line Transfections

Initial transfections were performed in 48-well plates with 25,000 cells per well. Each well was transfected in 500 μl OptiMEM containing LPD vector formed with 2 μg DNA at a charge ratio of +7 and Lipofectin at a ratio of 0.75 μg per 1 μg of DNA. Cells were transfected for 4 h at 37°C, after which the complexes were removed and replaced with complete media. Luciferase assays were performed 24 h after transfection and GFP transfections were analysed after 48 h. Cells transfected with cytokines for ELISA samples were cultured in 1 ml complete medium. Supernatant was collected at 24 h intervals and stored at -80°C until analysis by ELISA.

2.6 Retargeted Adenovirus

Retargeted serotype 5 adenovirus a gift from Dr Dan Von Seggern, (The Scripps Research Institute, La Jolla, CA, USA). The HI loop of the adenovirus fibre protein, which is responsible for binding to CAR (**Figure 2.2A**) was retargeted with the peptides A and B identified by phage display. Briefly, a plasmid containing the gene for the fibre protein, in which the wild-type HI loop was deleted and replaced with the coding sequence for

peptides A or B, was used to transfect 293 cells. The following day, an Ad-5 vector mutant that does not express the fibre protein was used to co-infect the same 293 cells (Jakubczak *et al.*, 2001). The retargeted fibre assembles in the virions, and the virus harvested ~3 days later contains the fibre protein with the retargeted HI loop (**Figure 2.2B**).

2.6.1 DC Transduction

GFP-expressing Ad5 vectors encoding containing the fibre protein retargeted with peptide A or B were compared with fibre-deleted Ad5 or Ad5 expressing the wild-type fibre protein. 50,000 MACS-separated CD11c⁺ were plated into 24-well plates in 1 ml of complete media, and Ad5 virus was added at 100,000 particles per cell (~MOI 100). Cells and virus were incubated together for 24 h at 37°C, then cells were fixed in 1% paraformaldehyde (PFA) and analysed by flow cytometry. All transductions were performed in triplicate.

2.6.2 Stem Cell Transduction

Bone marrow was flushed from the hind tibia and femur of A/J mice and resuspended in 20 ml RPMI, spun down and washed with 20 ml PBS containing 1% BSA. Cells were spun down again and resuspended in 4.5 ml PBS/BSA plus 100 µl of mouse stem cell antigen-1 (Sca-1) MACS microbeads (Miltenyi Biotech) and incubated at 4°C for 20 minutes. 20 ml PBS/BSA was then added to wash off unbound beads, spun down and resuspended in 1 ml PBS/BSA. The suspension was applied to a MACS MS column and unbound cells allowed to pass through. The bound cells were removed from the column with 2 x 1 ml washes of RPMI containing 30% FCS and counted. 50,000 cells were plated out in 24-well plates in RPMI with 30% FCS containing 50 ng/ml mSCF, 20 ng/ml IL-6 and 10 ng/ml Flt-3L (all Peprotech, London, UK). Retargeted, wild type or knock-out

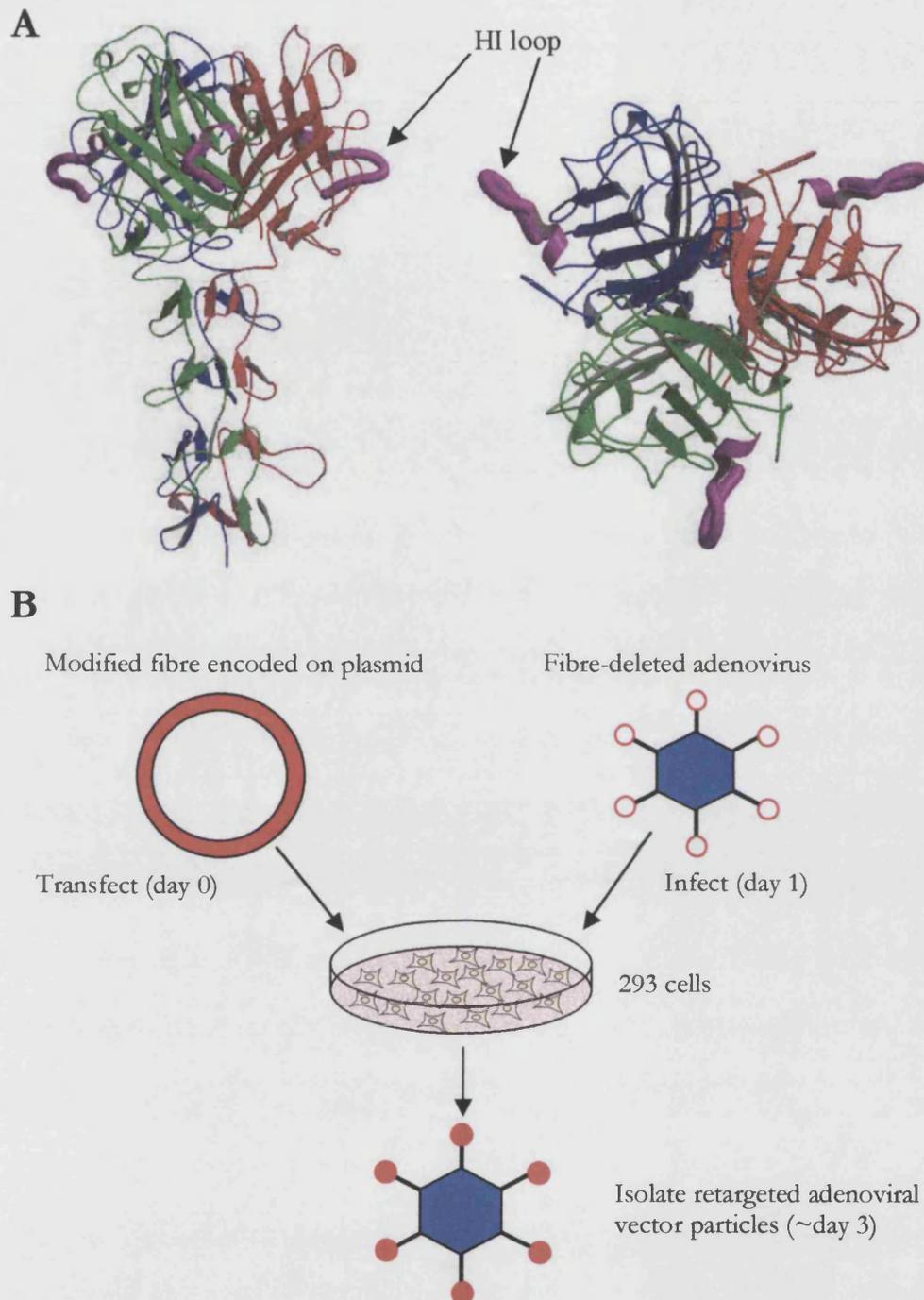


Figure 2.2 Retargeting of adenovirus fibre protein (A) Side and top views of the structure of the fibre protein of adenovirus serotype 5 showing the position of the HI loop. (Courtesy of D. von Seggern). **(B)** Overview of production of retargeted adenovirus. 293 cells are transfected with a plasmid encoding the modified fibre protein, and the following day infected with an Ad5 vector that does not express any fibre protein. The new modified fibre assembles on the virus in the 293 cells, and the retargeted virus containing the new fibre is isolated around three days later.

adenovirus expressing GFP was added as above, at 100,000 particles per cell (~MOI 100) and incubated overnight at 37°C and analysed by flow cytometry 24 h later.

2.7 Assessment of transfection

2.7.1 Luciferase Assay

Luciferase transfections were assayed using Luciferase Assay System (Promega, WI, USA). Media was aspirated from cells, and cells washed once in PBS. 100 µl of Reporter Lysis Buffer (RLB) was added per well and the cells lysed by freeze-thawing at -80°C. Each well was gently scraped to ensure complete lysis of all the cells and transferred to a V-bottomed 96-well plate. The lysates were spun at 900 x *g*, 10 minutes, 4°C to pellet the cell debris, then 20 µl of the supernatant from each well was transferred to a white 96-well plate. The plate was read by an Anthos Lucy-1 luminometer (Labtech) and the relative light units emitted by each well was determined 10 seconds after the addition of 100 µl of luciferase substrate. The protein concentration of the cell lysate was measured using BioRad Protein Assay Reagent (BioRad, Hemel Hempstead, UK) and measuring the absorbance at 590 nm and comparing to a standard curve prepared from a range of BSA concentrations. The protein values were used to convert the luciferase assay values to relative light units per mg of protein (RLU/mg).

2.7.2 Confocal Microscopy

50,000 human DC or HAE cells were plated onto sterile glass cover slips in 24-well plates and allowed to adhere. LPD complexes were prepared containing either 1% Oregon Green labelled DOTMA, 5% Texas Red labelled peptide 6 (both gifts from Dr. Hailes, Department of Chemistry, UCL) or Cy5 labelled pCI-Luc (Mirus LabellIT, Panvera, CA, USA), then transfected at a 3:1 charge ratio. This ratio was chosen as it minimises the

amount of free peptide in the transfection complexes and it is vital there is no free peptide which could be falsely identified as LPD vector. Cells were fixed with 4% PFA at appropriate time points and stained with either Alexa488-phalloidin or Rhodamine-phalloidin (both Molecular Probes, OR, USA) as an actin cytoskeletal counterstain, then mounted on microscope slides and visualised with a TCS NT Leica confocal scanning laser microscope (Leica Inc., St. Gallen, Switzerland). Images were analysed using Adobe PhotoShop (Adobe Systems, CA, USA).

2.7.3 Cytokine ELISA

Sandwich ELISAs were performed to quantitate supernatant concentrations of cytokine secreted by transfected cells with hIL-2 DuoSet and mIL-12p70 DuoSet ELISA kits (R&D Systems, Oxford, UK). 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μ l 4 μ g/ml purified capture antibody in PBS at room temperature. The following day the plates were washed four times with wash buffer (0.05% Tween 20 (Sigma) in PBS) then blocked with blocking buffer (1% BSA, 5% sucrose, 0.05% NaN_3 in PBS) for 1 h. Plates were washed and appropriate dilution of samples and standards added in DMEM and incubated for 2 h at room temperature. Samples were removed and plates washed four times and 100 μ l of biotinylated detection antibody added (50 ng/ml of anti-hIL-2 and 400 ng/ml anti-mIL-12p70) and incubated at room temperature for 2 h. Plates were then washed, and 100 μ l of 1:200 dilution of streptavidin-HRP (horseradish peroxidase) in PBS added for 30 mins at room temperature. This was washed off, and 100 μ l of substrate added (Colour Reagent Substrate, R&D Systems) and the colour allowed to develop. When the colour had developed sufficiently, 50 μ l of 2N H_2SO_4 was added to halt the reaction and the plates read at 450nm blanked against 570nm. Cytokine secretion was calculated as ng/24h/ 10^6 cells.

2.8 ELISpot Assays

All ELISpot assays were performed on 96-well MultiScreen Immobilon-P plates (Millipore, MA, USA) coated with 1 µg/ml anti-mouse IL-2 (clone JES6-1A12, Pharmingen, Oxford, UK) or 1 µg/ml anti-mouse IFN-γ (clone XMG1.2, Bioscience, San Diego, USA) and incubated at 37°C for 2 h. Plates were then washed three times with RPMI and blocked with 50 µl FCS per well for 30 mins. Following another three washes with RPMI, cells were incubated on the plates for 24 h, then plates washed three times with wash buffer (PBS + 0.05% Tween-20 (Sigma Chemical Co, Dorset, UK)). Biotinylated anti-IL-2 (clone JES6-5H4, Pharmingen) or biotinylated anti-IFN-γ (clone R4-6A2, Bioscience) was diluted to 2 µg/ml in wash buffer and incubated on plates for 2 h at 37°C, followed by three washes with wash buffer. A streptavidin-alkaline phosphatase (AP) conjugate (ExtrAvidin, Sigma, Dorset, UK) was diluted 1:1000 and incubated on wells for 30 mins. After three washes, spots were developed using AP-substrate. The reaction was stopped by washing plates in water, and when plates were dry they were read on a plate reader (Bioreader, Biosys, Germany).

2.8.1 Antigen presentation ELISpot Assay

DC were transfected for 90 mins with the LPD vector incorporating peptide 1 at a charge ratio of +7 with pCMV-C5, which expresses the full-length cDNA for murine complement protein 5 (C5), then incubated with complete media overnight at 37°C. The T-cells used in this assay were a CD4⁺ hybridoma, A18, which is derived from a clone isolated from an A/J mouse immunised with C5 protein that expresses a TCR specific for one epitope of mC5 (residues 106-121: VVSKHFSSKSKKIPITY) (Zal *et al.*, 1994). The following day, ratios of DC to A18 CD4⁺ hybridoma cells from 1:1 to 1:300 were

incubated for 24 h on plates previously coated with anti-IL-2 then blocked. Cells were washed off and plates developed as above.

2.8.2 Splenocyte ELISpot

Spleens were removed from mice and passed through a cell strainer with RPMI. Cells were spun down and resuspended in 1 ml Red Blood Cell Lysing Buffer (Sigma) and incubated on ice for 5 mins. Tubes were then topped up to 50 ml with RPMI to dilute out the lysis buffer and cells spun down and resuspended in complete media (CM) at 10^6 /ml, and 5×10^5 , 2.5×10^5 , 1×10^5 and 5×10^4 splenocytes were plated out in triplicate for each mouse in 96-well U-bottomed tissue culture plates. 5×10^3 live N2a cells were also added to each test well, giving effector:target ratios of 100:1, 50:1, 20:1 and 10:1.. Positive control wells contained 50,000 splenocytes in CM + 5 μ g/ml Concanavilin A (Sigma), and negative control wells contained 50,000 splenocytes in CM only. All wells contained a volume of 200 μ l and plates were incubated overnight at 37°C then the contents of each well was transferred onto ELISpot plates pre-coated with anti-mIFN γ or anti-mIL2. Plates were incubated for a 24 h then developed the following day.

2.9 Lactate Dehydrogenase Release Assay

Spleens were passed through cell strainers with RPMI to form single cell suspensions, spun down, and red cells lysed in 1 ml Red Blood Cell Lysing Buffer (Sigma) on ice for 5 mins. Tubes were filled to 50 ml to dilute out lysis buffer, cells pelleted and resuspended in RPMI-Glutamax-1 with 10% FCS and antibiotics at 10^6 /ml and incubated in the presence of 60 IU/ml rhIL-2 (Peprotech, London, UK). Irradiated (2500rads - 25 Gy from a cesium-137 source) N2a were added at an effector:target ratio (E:T) of 50:1, and

cells cocultured for 6 days at 37°C. On day 6 the non-adherent CTL effector fraction was removed and resuspended at 10⁷/ml.

LDH is released from lysed cells and is proportional to the number of cells lysed. LDH assay (CytoTox96 Non-Radioactive Cytotoxicity Assay, Promega, Southampton, UK) was used to determine the percentage cytotoxicity generated against N2a cells by the splenocytes. CTL effector cells were co-cultured in quadruplicate in RPMI-Glutamax-1 without phenol red containing 5% FCS and antibiotics in U-bottomed 96-well tissue culture plates with 5x10³ viable N2a cells at E:T ratios between 27:1 to 1:1 in a total volume of 200 µl. Controls were also set up in quadruplicate in the plate: culture media only background, spontaneous target lysis, maximum target lysis and volume control. Spontaneous effector release controls were also set up in quadruplicate for every effector concentration cocultured with targets. Plates were incubated for 4 h at 37°C, then centrifuged and 50 µl supernatant transferred to a flat-bottomed 96-well plate. 50 µl assay substrate was added to each well and plates incubated in the dark for 30 mins before halting the reaction with 50 µl stop solution. Plates were then read at 492 nm and % cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = \frac{[(\text{Experimental release} - \text{Effector spontaneous release} - \text{Target spontaneous release}) \div (\text{Target maximum release} - \text{Target spontaneous release})] \times 100}$$

2.10 Flow Cytometry

All flow cytometry was performed using an EPICS XL cytometer (Beckman Coulter, High Wycombe, UK) and data was analysed using Expo 32 software (Beckman Coulter). All cells suspended in FACS wash buffer (FWB: 0.1% BSA, 0.005% NaN₃ in PBS) and were excited with a 488 nm argon laser. Positive events were captured using a band pass filter

at 525nm (FL1), 575nm (FL2) or 675nm (FL3). Forward and side scatter characteristics were used to gate the live cell population, and 10,000 events from the live gate were collected.

2.10.1 Phenotype staining of transfected DC

Murine DC were stained for surface markers using the following monoclonal antibodies anti-CD11c-biotin or anti-CD11c-PE, anti-CD40-biotin, anti-CD80-FITC, anti-CD86-FITC, anti-I-A^K α -FITC, anti-I-A^K β -FITC, IgG1-FITC isotype, IgG2-FITC isotype and streptavidin-Cy (all Pharmingen, BD Biosciences, Oxford, UK). The polyclonal Dec-205 antibody was a gift from S. De Noronha. All antibodies were used at 1 μ g/10⁶ cells, except the Dec-205 antibody, which was used at a 1:5 dilution. All antibodies were incubated with cells at 4°C for 1 h in a total volume of 100 μ l, then unbound antibody was removed by two washes of 2 ml FWB, before fixing in 300 μ l FWB containing 1% PFA and storage at 4°C until analysis.

2.10.2 GFP Analysis

GFP transfections were analysed by flow cytometry. Cells were excited with a 488 nm laser and GFP⁺ events captured with a 525 nm band pass filter. Transfected cells and an untransfected negative control were removed from wells, washed in FWB then resuspended and fixed in 300 μ l 1% PFA in FWB. 10,000 total events were counted and the cells gated to include only viable cells.

2.11 Animal procedures

All animal procedures were approved and licensed by the Home Office and performed to the standards required by the UKCCCR (Workman *et al.*, 1998). Male 5-7 week old A/J

mice, syngeneic with the N2a NB cell line, were purchased from Harlan Laboratories (UK) and were housed at the Institute of Child Health according to the Animals (Scientific Procedures) Act (1986).

2.11.1 Transfection of cells for injection

Transfections of AJ3.1, NIH-3T3 and N2a cells for injection were carried out in 150 cm² flasks. Cells were seeded in flasks at a density of 3x10⁶ (AJ3.1) or 4x10⁶ (NIH-3T3 and N2a) and cultured overnight. The following day cells were transfected in a volume of 25 ml with LPD complexes containing a total of 80 µg (40 µg each of pCI-hIL-2 and pCI-mIL-12) plasmid DNA with Lipofectin at a ratio of 0.75 µg per 1 µg DNA and at a charge ratio of +7. Peptide VYAR was used to transfect AJ3.1 cells, and peptide 6 was used to transfect NIH-3T3 and N2a cells. Cells were transfected for 4 h, then replenished with complete media and cultured for a further 24 h before being trypsinised, washed three times to remove FCS and resuspended in RPMI at 10⁷/ml for injection. All transfected cells were assayed for cytokine expression by ELISA in triplicate.

2.11.2 Tumourigenicity Experiments

Tumourigenicity experiments were performed to determine whether cytokine-transfected fibroblasts were capable of preventing engraftment of N2a cells. NIH-3T3, AJ3.1 and N2a cells were transfected as described above then washed three times and resuspended at 10⁷/ml in RPMI. Unmodified N2a cells were also trypsinised, washed and resuspended at 10⁷/ml in RPMI.

For the experiments using allogeneic fibroblasts, 10⁶ live wild-type N2a alone or in combination with 10⁶ NIH-3T3 cells transfected with cytokine genes, empty vector or untransfected were mixed together in a total volume of 200 µl. Mice (n=6 per group)

were then inoculated s.c. in the left posterior flank and tumour growth monitored every 2-3 days. Tumour volume was calculated by the formula:

$$\text{Tumour volume (mm}^3\text{)} = (\text{shortest axis}^2 \times \text{longest axis}) \times 0.52$$

In experiments where irradiated cells were required, cells were irradiated with 2500rads (25 Gy from a cesium-137 source) following transfection.

For experiments with syngeneic fibroblasts, mice (n=6 per group) were inoculated s.c. in the left posterior flank with 10^6 wild-type N2a cells alone or in combination with 10^6 wild-type AJ3.1, 10^6 AJ3.1-IL2-IL12 or 10^6 N2a-IL2-IL12 in a volume of 200 μ l and tumour growth monitored every 2-3 days. In the second tumourigenicity experiment, transfected AJ3.1 were irradiated as described above. Mice (n=6 per group) were inoculated s.c. with 10^6 wild-type N2a alone or in combination with 10^6 irradiated AJ3.1-IL2-IL12 or 2×10^6 irradiated AJ3.1-IL2-IL12. Tumour growth was monitored every 2-3 days.

2.11.3 Rechallenge Inoculations

To determine if systemic immunity had been established in tumour-free mice from the tumourigenicity experiments with syngeneic fibroblasts, mice were rechallenged at least 7 weeks later with 10^6 wild-type N2a cells suspended in a volume of 100 μ l RPMI inoculated s.c. in the right flank. Tumour development was monitored every 2-3 days.

2.11.4 Eradication Experiments Using AJ3.1

Tumours were engrafted in the left flank of A/J mice with 10^6 wild-type N2a cells and palpable masses were present after 5 days. Mice were allocated to different experimental groups such that there was a range of tumour sizes in each group (~1x1 mm to 5x5 mm). Another group of six mice was engrafted with a tumour on each flank.

Transfected cells for vaccinations were prepared as above and DC were cultured and purified as described in 2.1.1. Mice (n=6 per group) received either 10^6 N2a-IL2-IL12 intratumourally (i.t.), 10^6 AJ3.1-IL2-IL12 i.t., 10^6 AJ3.1-IL2-IL12 + 10^6 DC i.t., or 10^6 AJ3.1-IL2-IL12 + 10^6 irradiated N2a cells in the opposite flank. In the group of mice with two tumours, one tumour was treated with 10^6 AJ3.1-IL2-IL12 i.t., and the other was left untreated. Control mice were vaccinated with RPMI i.t and all vaccinations were prepared in a volume of 100 μ l. Tumour growth was monitored every 2-3 days.

2.11.5 Cytokine Dosing Experiments

A single vaccine was administered to mice (n=6 per group) s.c. in the left flank containing 10^6 wild-type N2a cells plus N2a-IL2-IL12 in a range of doses between 10^6 - 10^3 cells in a total of 200 μ l. A control group of mice received 10^6 wild-type N2a only. Tumour growth was monitored every 2-3 days.

The transfected cells were analysed by ELISA for cytokine expression, and the amount of cytokine expressed by each cell dose was calculated from the ELISA data. The number of tumour-free mice in each group was used to determine an approximation of the minimum dose of cytokine necessary to prevent tumour engraftment.

2.12 Immunohistochemistry

2.12.1 Embedding and sectioning tumour tissue

Tumours were removed from the flank of sacrificed A/J mice, washed in PBS and fixed overnight in 4% PFA at 4°C. Tissue was then serially dehydrated in alcohol from 50% ethanol in PBS through 70%, 85% and 95% ethanol in water to 100% ethanol, 30 mins per

dehydration, before 2 x 45 mins incubations in HistoClear (National Diagnostics, Yorkshire, UK). Tumours were then incubated in 2 x 45 mins incubations in paraffin wax (Raymond Lamb, East Sussex, UK), before finally embedding in paraffin wax and set over night. Tissue was then sectioned on a microtome (Microm HM325 Rotary Microtome, Raymond Lamb) and subbed onto polylysine coated glass microscope slides (BDH, Poole, UK), air dried overnight at 37°C and stored at 4°C until use.

2.12.2. Histology

Staining with haematoxylin and eosin was performed to study tumour tissue morphology. Wax was removed from sections by 2 x 3 mins washes in HistoClear. Sections were then rehydrated by 3 mins washes in 100%, then 95% and 70% ethanol, followed by rinsing in dH₂O. Slides were then stained with Ehrlich's haematoxylin (BDH) for 10 minutes followed by rinsing in dH₂O and rapid immersion in acid alcohol (1% concentrated HCl in 70% ethanol (v/v)). Slides were rinsed again in dH₂O before staining in a 1% aqueous solution of eosin (Raymond Lamb) and finally rinsing in dH₂O. Sections were then dehydrated by washes in 70%, then 95% and 100% ethanol and air-dried. Slides were mounted with DPX mountant (BDH) using glass coverslips. Slides were analysed using a AxioPhot 2 microscope (Zeiss, Welwyn Garden City, UK) attached to a ProgRes 3012 digital camera (Kontron Electronic, Chichester, UK) and images were analysed using Adobe Photoshop (Adobe Systems, CA, USA).

2.12.3 Immunohistochemical staining

Wax was again removed from sections by 2 x 3 mins incubations in HistoClear, rehydration in 3 mins washes in 100%, then 95% and 70% ethanol in dH₂O, followed by 50% ethanol in PBS and rinsing in dH₂O. Sections were then microwaved for 10 mins in antigen unmasking solution (3.75 ml in 400 ml dH₂O, Vector Laboratories, CA, USA), followed by

rapid cooling in two washes of dH₂O. Endogenous peroxidase activity was blocked (0.3% H₂O₂ (v/v), 0.1% NaN₃ (w/v) in PBS) for 15 mins, then slides were washed in PBS twice. PBS containing 10% rabbit serum (v/v) (Vector Laboratories) was applied to the sections to block non-specific antibody binding and incubated at RT°C for 30 mins. Excess block was removed from slides, then a 1:20 dilution of unconjugated rat anti-mouse CD45 (pan-leukocyte marker, Pharmingen) in PBS/1% BSA was applied. Sections were incubated overnight in a humid chamber at 4°C. Control slides were incubated with 10% rabbit serum in PBS. Sections were washed in PBS three times, and then the 2^o rabbit anti-rat biotinylated antibody (Dako, Glostrup, Denmark) was added at a 1:250 dilution in PBS and incubated at RT°C for 30 mins. Slides were washed twice then a streptavidin-biotinylated peroxidase complex was applied and incubated for 30 mins (ABC Reagent, Vector Laboratories). Positively stained cells were identified with 3,3'-Diaminobenzidine (Sigma FAST DAB, Sigma Chemical Co.) and the reaction was halted by rinsing in PBS. Sections were counterstained in Methyl Green (Vector Laboratories) for 10 mins, washed twice in dH₂O, then dehydrated in butan-1-ol by 2 x 10 immersions. Finally, slides were washed in fresh butan-1-ol for a further 30 secs, then incubated in Histoclear for 10 mins and allowed to air dry. Slides were mounted with glass coverslips using DPX mountant and analysed as before.

2.13 Statistical analysis

The two-sample t-test was used to assess the significance between experimental animal groups and the paired t-test was used to assess the difference between tumours in the mice engrafted with a tumour in each flank.

2.14 Buffers

Buffers were prepared in dH₂O and autoclaved, except antibiotics, which were added to solutions <50°C.

LB broth	10 g/l tryptone + 5 g/l yeast extract (both Becton Dickinson) + 10 g/l NaCl (BDH) or 20 g/l LB broth premix (Invitrogen). 15 g/l agar was added to LB for solid growth media for culture plates.
Ampicillin	4 x 25 mg tablets (Stratagene) per litre autoclaved LB broth, giving a working concentration of 100 µg/ml.
Kanamycin	Stock solution of 50 mg/ml, diluted in autoclaved LB giving a working concentration of 50 µg/ml.
TE Buffer	10 mM Tris.Cl, pH 8.0; 1 mM EDTA
TAE (50x)	242 g Tris base, 57.1 ml Glacial acetic acid, 100 ml 0.5M EDTA per litre
PBS	Phosphate Buffered Saline tablets (Oxoid, Basingstoke, UK) were dissolved in dH ₂ O, 10 tablets/litre
Ad freezing buffer	0.1 M Tris, 5 mM MgCl ₂ , pH8
Ad dialysis buffer	10 mM Tris, 5mM MgCl ₂ , 2.3% sucrose, pH8
MACS buffer	1xPBS + 0.1% BSA, sterile filtered through 0.22 µm filter.
FACS wash buffer	1xPBS + 0.1% BSA + 0.005% (w/v) NaN ₃
ELISA blocker	1xPBS + 1% BSA + 5% sucrose + 0.05% NaN ₃ , sterile filtered through 0.22 µm filter
ELISA/ ELISpot wash buffer	1xPBS + 0.05% Tween 20 (Sigma)

CHAPTER 3

OPTIMISATION OF DC TRANSFECTION

3.1 INTRODUCTION

Most applications of gene therapy vectors require the maximal output of transgene expression. Therefore, transfection efficiency and total transgene production from the transfected cell population are very important, and although not absolutely correlated, higher transfection efficiency generally leads to higher total transgene expression.

Previous work with human DC has shown that the transfection process can activate the cells (H. Truman, unpublished), and in the case of a cancer vaccine this is advantageous as the cells are in a state to generate proinflammatory immune responses. Activation of DC increases the expression of surface molecules involved in antigen presentation and T-cell activation, thus their maturation state is critical to their use in immunotherapy. If the cells are immature, antigen presentation will not be maximised and the cells will not activate T-cells efficiently. However, if the cells are in the late stages of maturity, they will lose their migratory capacity and will not travel to lymph nodes.

The lipid and peptide components of the LPD vector components have been shown to contribute to activation (H. Truman, unpublished), as well as the CpG oligonucleotides in bacterial plasmid DNA. Internalisation and acidification of DNA in endosomes is required for CpG-mediated activation of leukocytes and is coupled to the production of reactive oxygen species, leading to NF- κ B activation and cytokine expression (Yi *et al.*, 1998). However, other reports have shown that CpG can bind-cells surface receptors and transduce stimulatory signals (Liang *et al.*, 2000). More recently, CpG-mediated signal transduction has been shown to be dependent on Toll-like receptor-9 (TLR9), and the presence of CpG contributes to DC activation, inducing IL-12 production and upregulation of CD40, CD80, CD86 and MHC II (Hemmi *et al.*, 2000).

Optimal proportions of each vector component should be determined for murine DCs. Incubation time of the complexes with the cells also requires optimisation to maximise binding and internalisation. However, incubation for excessive periods may be detrimental due to serum-starvation. The following experiments were performed to determine the optimal combination of targeting peptide, charge ratio, amount of DNA, duration of transfection, and the effect of transfection on the phenotype of the cells.

3.2 RESULTS

3.2.1 Optimisation of Targeting Peptide

DC transfections with a luciferase reporter gene were carried out in serum-free OptiMEM to optimise the LPD transfection conditions in murine DC. The first experiment determined the optimal targeting peptide that gave the highest luciferase expression. A range of integrin-targeting peptides were screened, all containing the cationic K₁₆ tail and a cyclic head group, held in this conformation by a disulphide bond (Table 2.1, Materials and Methods). Only integrin-targeting peptides were available at this stage of the project, hence only results with these peptides are shown. Previous work has shown that the peptides have strong DNA-condensing properties, but have little transfection ability when complexed with DNA alone. The incorporation of Lipofectin into the vector is necessary for efficient transfection and a ratio of 0.75µg:1µg DNA is optimal (Hart *et al.*, 1998). The charge ratios used in this experiment were +7, as this was determined as optimal in other cell types, and +3 and +1 to get a better idea of targeting specificity. 50,000 CD11c⁺ DC were transfected for 4 hours in 48-well plates at 37°C then incubated overnight in complete media before analysis and this experiment was repeated at least once.

Pep1 was found to consistently result in the highest luciferase expression at all charge ratios (**Figure 3.1**). This peptide contains an RGD motif which is known to bind $\alpha 5$ integrins on the cell surface. Peptide11 also showed good levels of luciferase expression at all charge ratios. Peptide 11 has the same charge and sequence as peptide 1 except that the RGD motif is changed to a non-specific RGE, suggesting transfection is not mediated through $\alpha 5$ integrins in this case.

This result is different to those obtained previously with cell lines and human DC, which have generally found superior levels of luciferase expression given by peptide 6 and peptide 8 respectively, and this is likely due to different integrin expression on murine DC. K16, which contains no targeting group, showed decreased transgene expression as the charge ratio was reduced, suggesting the luciferase expression observed at +1 charge ratio with peptides 1 and 11 is mediated specifically through surface receptor targeting. Peptides 7 and 10 gave minimal levels of transfection, despite their positive charge.

3.2.2 Optimisation of Charge Ratio

The next condition optimised was charge ratio, which was varied by altering the amount of cationic peptide, while the amount of DNA and Lipofectin was held constant. As Lipofectin only has one positive charge per molecule of DOTMA and DOPE is neutral, it is considered insignificant relative to the peptide component of the vector. Charge ratios between +1 and +10 were prepared with peptide 1 and cells transfected for 4 hours, incubated for 24 hours and analysed by luciferase assay.

The results show that +7 was the optimal charge ratio (**Figure 3.2**). Reporter gene expression is increased by 1.75-fold as the charge ratio increases from +3 up to +7, due the increasing attraction between the negatively charged cell membrane and the positively

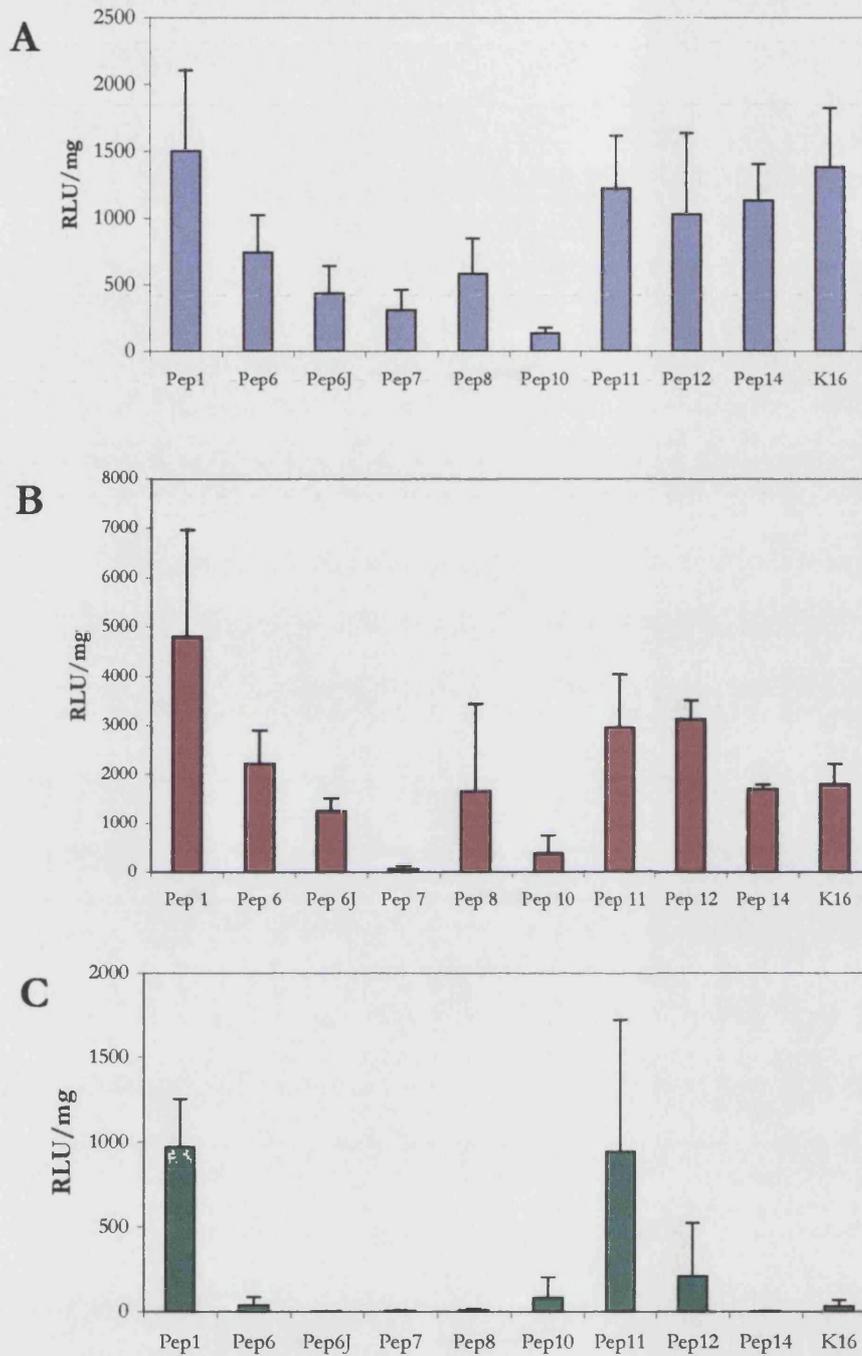


Figure 3.1 Optimisation of targeting peptide for LPD vector. Murine bone marrow-derived DC were transfected with LPD complexes incorporating different targeting peptides at a charge ratio of +7 (A), +3 (B) or +1 (C). Luciferase expression was analysed 24 hours after transfection and error bars represent standard deviations from triplicate transfections. The experiment was repeated at least once and representative data is shown.

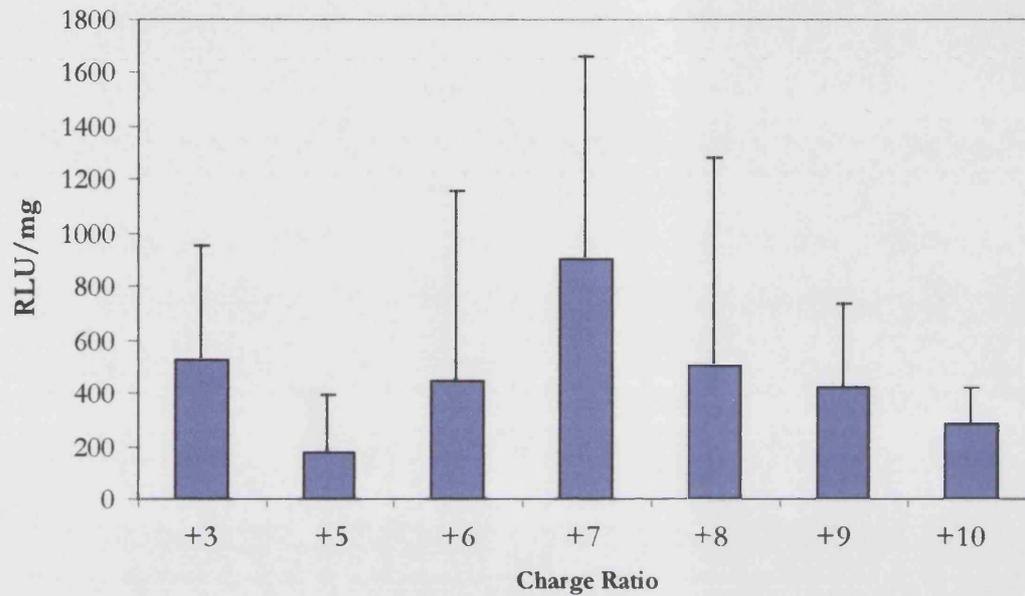


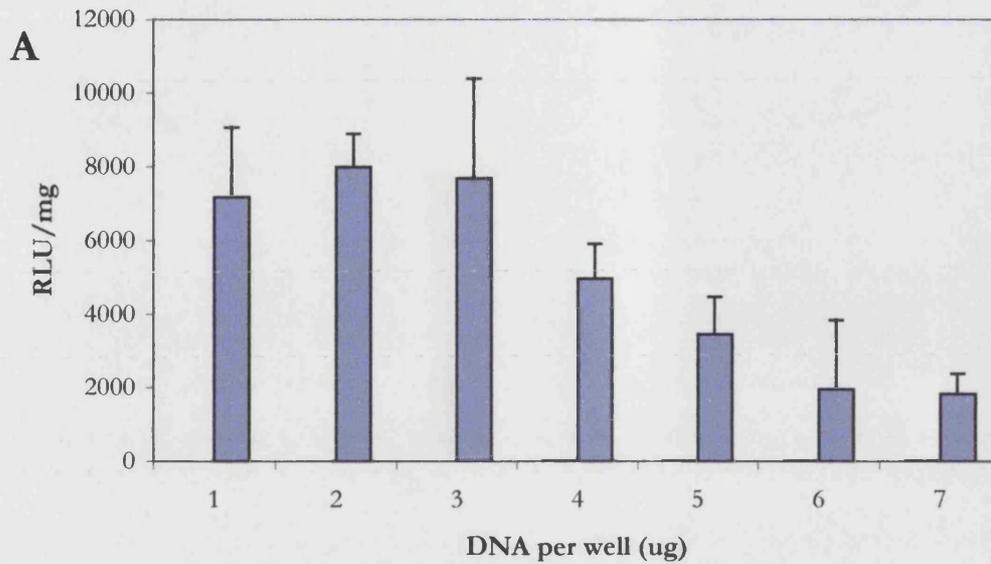
Figure 3.2 Optimisation of charge ratio of LPD vector. The charge ratio was varied by altering the amount of cationic targeting-peptide (peptide 1) whilst keeping the amount of DNA and Lipofectin constant. DC were transfected with $2\mu\text{g}$ DNA per well, and luciferase expression analysed 24 hours after transfection. Error bars represent standard deviations of triplicate transfections. Experiments were repeated at least once and representative data is shown.

charged complexes. This is consistent with what has been observed in other cell types, including human DC and cell lines. Further increase of the charge ratio above +7, up to +10 caused a 3-fold drop luciferase expression, so subsequent experiments were performed with a +7 charge ratio.

3.2.3 Optimisation of DNA Content of Complexes

The amount of DNA used to transfect each well was varied between 1 μ g to 7 μ g. The charge ratio of peptide:DNA, and lipid:DNA ratio was kept constant, so the absolute amounts of these components varied, but were kept constant in relation to the amount of DNA. Generally, there was an inverse relationship between the amount of DNA used to transfect the cells and the level of luciferase expression. Expression peaked at 2 μ g/well (7990 \pm 910 RLU/mg) and then dropped in a dose-dependent manner, such that luciferase expression with 7 μ g/well of DNA was nearly 4.5-fold less (1830 \pm 550 RLU/mg) (**Figure 3.3A**).

This drop in luciferase expression was most likely due to the toxicity of high levels of complex on the cells. Morphologically, the cells looked more apoptotic with membrane blebbing as the amount of DNA was increased (data not shown). This was also evident from the scatter of the cells when analysed by FACS, which appeared very granular when transfected with higher amounts of DNA. The gated viable cell population was assessed by forward scatter and side scatter characteristics, and decreased with increasing amounts of DNA. A dose-dependent decrease in viable cells was observed, from 47.5% viable cells in the population transfected with 1 μ g DNA, decreasing to 19.4% of cells transfected with 7 μ g DNA. The viable cell population in the untransfected control was 48.7% (**Figure 3.3B**).



B

Amount of DNA	GFP positive live cells (%)	viable cells (%)
Untransfected control	<1	48.7
1 μ g	3.3	47.5
2 μ g	3.4	41.3
3 μ g	3.6	31.4
4 μ g	3.7	27.8
5 μ g	3.1	25.2
6 μ g	2.9	22.5
7 μ g	3.7	19.4

Figure 3.3 Optimisation of amount of DNA in LPD vector. Transfection of DC with LPD vector with varying amounts of DNA while maintaining the charge ratio at +7 and Lipofectin:DNA weight ratio at 0.75:1. **(A)** Luciferase expression was analysed 24 hours after transfection and error bars represent standard deviations of triplicate transfections. Experiment was repeated at least once and representative data is shown. **(B)** Transfection efficiency of the viable cell population was assessed by GFP expression 24 hours after transfection. Viable cells were determined by forward scatter and side scatter characteristics.

3.2.4 Optimisation of Length of Transfection Period

Finally the transfection incubation period was investigated. Cells were transfected with vector complexes containing peptide 1 at a +7 charge ratio with 2 μ g DNA per well for varying amounts of time between 30 mins and 4 h, after which the complexes were replaced with complete media. All the cells were incubated at 37°C for a total of 24 h and analysed by luciferase assay. Interestingly, the level of luciferase expression rose over 3-fold from 30 mins to 90 mins, before dropping slightly then levelling out (**Figure 3.4A**). This is likely to be the optimum balance between the time the complexes had to bind to the cell surface and be internalised, and the minimum serum-starvation time and time the cells are exposed to the lipofection process. Thus 90 mins was considered the best transfection period for all subsequent experiments.

DC were then transfected with the optimised LPD vector using a GFP reporter gene to determine transfection efficiency. Cells were analysed by FACS and showed an average of <5% GFP positive cells (**Figure 3.4B**). This is much lower than generally found in cell lines, but this level of transfection in DC may still be therapeutic, as one DC is capable of activating 100-3000 T-cells (Banchereau & Steinman, 1998). The phenotype of transfected and untransfected DC was compared by staining for surface activation markers. This confirmed that the transfection process causes murine DC to mature, with increased surface expression of activation makers such as Dec205, CD80, CD86 and MHC class II (**Figure 3.5**).

3.2.5 Adenovirus as a Helper Virus

To try improving the transfection efficiency adenovirus (Ad) was added as a “helper virus”. This approach has previously been demonstrated to increase the transfection in human DC when formulated as an Ad/PEI/DNA complex (Diebold *et al.*, 1999), or as an

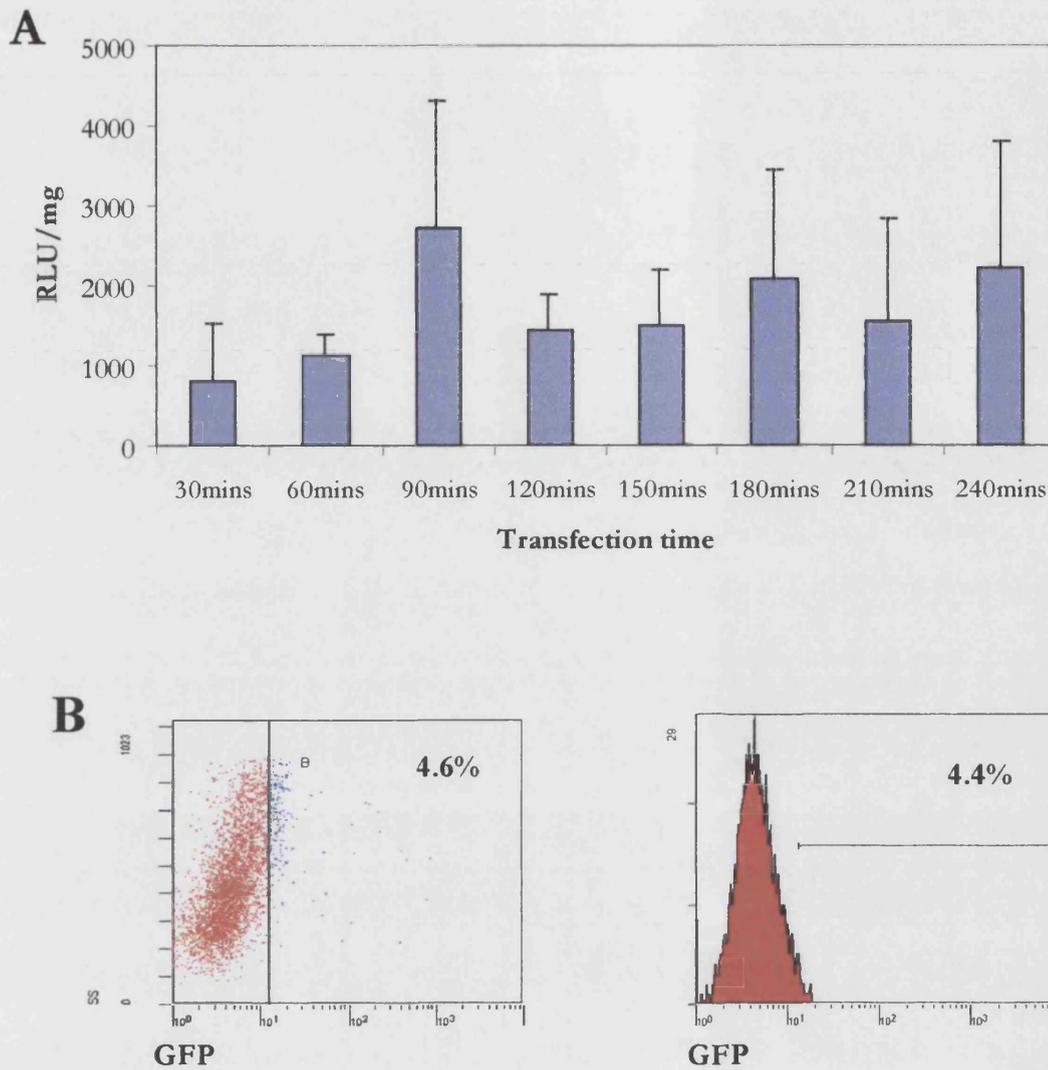
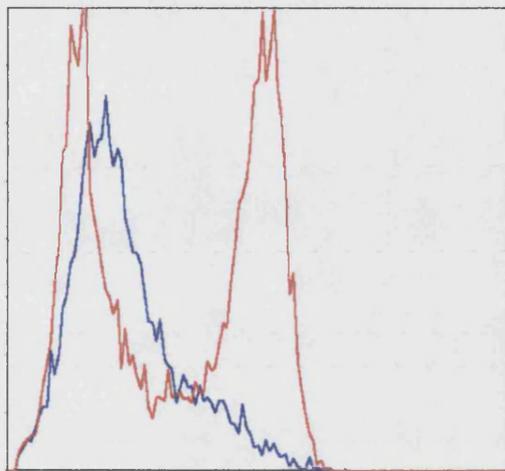
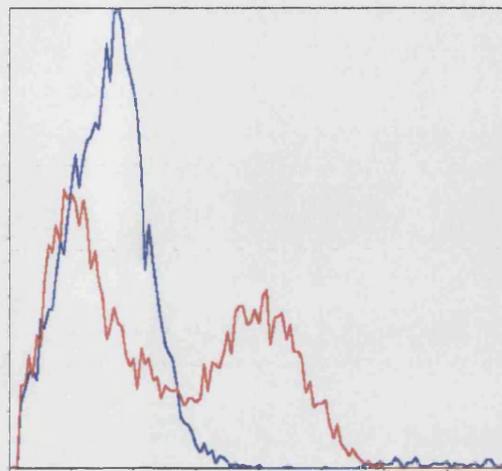
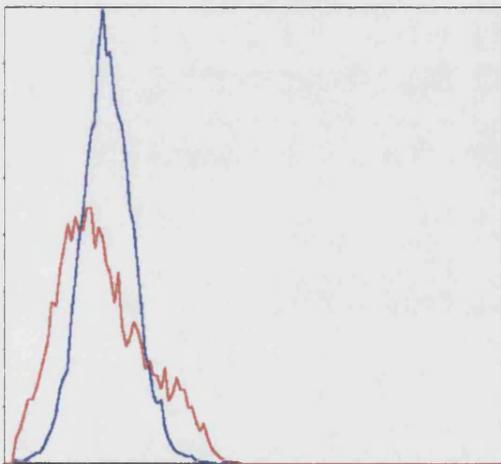


Figure 3.4 Optimisation of transfection time with the LPD vector

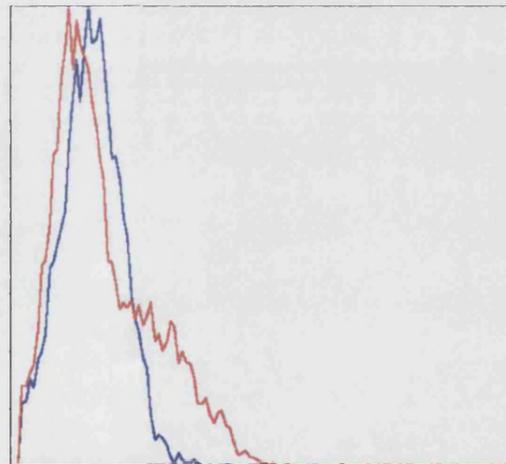
A) Murine DC were transfected with peptide 1 at a charge ratio of +7 with 2 μ g DNA per well for varying times and luciferase expression analysed 24 hours after transfection. Error bars represent the standard deviation of triplicate transfections and one representative experiment is shown. **(B)** Murine DC were transfected with the optimal LPD vector for the optimal time of 90 minutes with pEGFP-N1 to determine maximal transfection efficiency. Cells were analysed by FACS 24 hours after transfection and 10,000 events were collected. The scatter plot shows that a few of the cells have a high fluorescence intensity, but the percentage of transfected cells was very low.

MHC Class II (I-A^k)

CD86



CD80



DEC-205

Figure 3.5 Analysis of transfected DC phenotype by FACS. The phenotype of transfected and untransfected DC was compared by staining for the presence of surface activation markers. Blue lines represent untransfected cells, and red lines represent transfected cells. Viable cells were gated and 10,000 events from the live gate are shown and representative data is shown.

Ad/PD complex (H. Truman, unpublished) probably by augmenting endosomal escape of the complex, reducing DNA degradation. The virus used was an E1-deleted replication-deficient serotype 5 virus carrying the LacZ reporter gene, which allows virally infected cells to be identified and aids titration. The virus was produced as according to materials and methods and gave a titre of 5×10^8 infectious units/ml when titred on HeLa cells.

A range of MOI values were tested with GFP and luciferase. While the addition of virus increased the level of luciferase expression in some experiments, this improvement was not consistent as expression fluctuated in repeats of the experiment. An experiment using Ad/PD (no lipofectin) was performed, as this has been shown to give higher expression in human DC, but did not enhance the transfection of murine DC (**Figure 3.6**). Increasing the viral MOI resulted in no increase in transfection efficiency as assessed by GFP transfection of DC and analysis by FACS. X-Gal staining showed the level of virally infected cells was low (<5%; data not shown), so it was concluded that Ad did not enhance the percentage transfection efficiency or the level of transgene expression, as assessed by luciferase assay, of murine DC.

3.2.6 Antigen Presentation Assay

Although transfection efficiency was very low, it was still important to investigate whether antigen presentation was sufficient to activate T-cells. To test the antigen presentation ability of transfected DC an ELISpot assay was performed. DC were transfected with pCMV-C5 which encodes full-length murine complement 5 protein (mC5); this protein was chosen because A/J mice are C5-negative so mC5 could potentially be used as a model antigen *in vivo*. Also, a CD4⁺ T-cell hybridoma (A18) was available which specifically recognises one epitope of mC5 and these were used as the target cells for antigen presentation. These cells lose their CD4 expression in culture fairly quickly, so

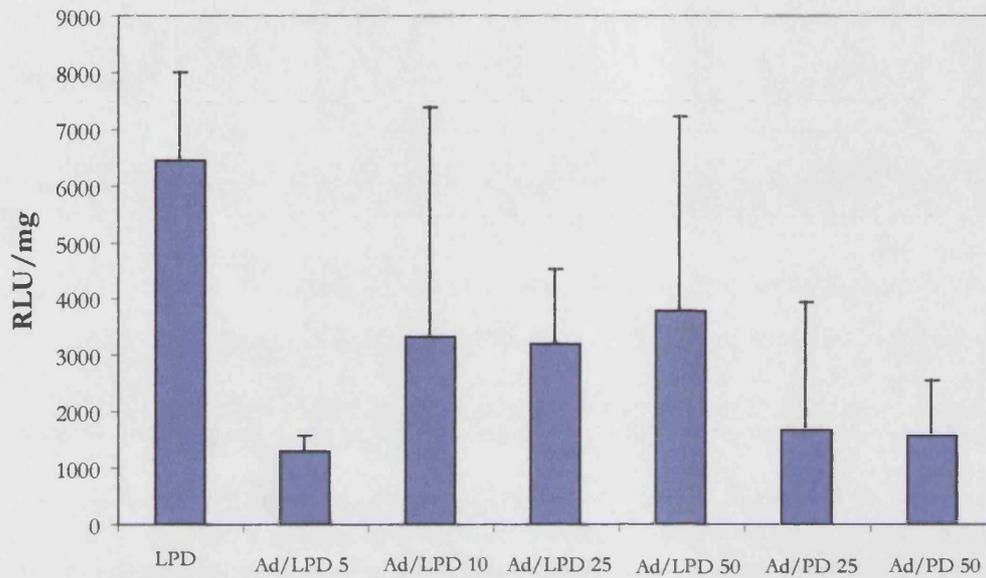


Figure 3.6 Comparison of luciferase expression between the LPD vector, Ad/LPD and Ad/PD. Murine DC were transfected with pCI-Luc included in either LPD vector alone, LPD vector incorporating Ad-lacZ or PD complexes incorporating Ad-lacZ at a range of MOIs and cells were analysed 24 hours after transfection. Error bars represent the standard deviation of triplicate transfections and one representative experiment is shown.

freshly sorted cells were used for the assay. Hybridomas do not secrete IFN- γ , so an ELISpot to measure IL-2 was performed. The assay was performed three times, and no IL-2 secretion could be detected on any of the plates (data not shown), suggesting antigen was not being presented to the A18 cells.

3.2.7 Confocal Microscopy of Transfected Cells

Confocal microscopy of human DC transfection using labelled components of the LPD vector was used to investigate where barriers to transfection were occurring. Human DC were used, as these were previously shown to transfect with peptide 6 more efficiently than murine DC, although still at a very low level (~5%) and this was the only labelled peptide available. Fluorescently tagged transfection reagents were used to track the progress of the complex through the cell, and to establish which steps were limiting.

These images show that the complexes efficiently adhered to the DC surface within 30 mins and remained adhered to the cell surface for many hours (**Figure 3.7, Figure 3.8**). Vector internalisation could not be detected by 30 minutes post-transfection (**Figure 3.7C**), but a few examples of intracellular vector could be detected after 2 h of transfection by examining individual sections taken through cells in the XY plane and Z-axis (**Figure 3.8 C&D**). If the internalisation had been high, but the complexes had remained in the cytoplasm due to low nuclear access, it may have been feasible to try a cytoplasmic expression system. However, the internalisation of the complexes was so low, it was concluded that this vector system is not optimal for use with these cells.

Confocal imaging of HAE cells was used to investigate the internalisation of LPD by cells that transfect with high efficiency. HAE cells transfected with LPD vector containing peptide 6 (labelled with Texas Red) and DNA (labelled with Cy5) demonstrated that the

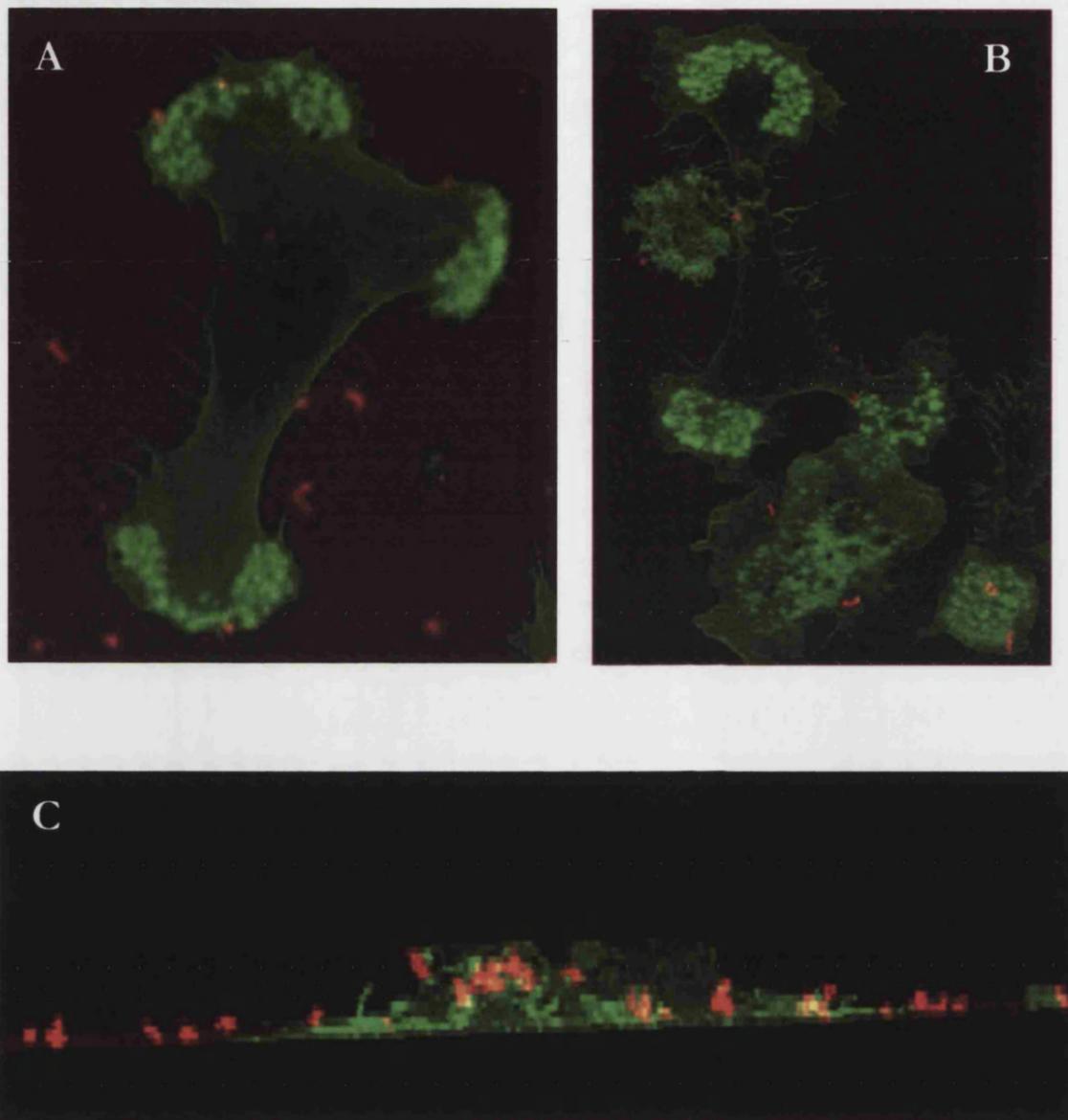


Figure 3.7 Confocal microscopy of human DC 15-30 minutes after transfection. Cells were transfected on glass coverslips with LPD vector containing Texas Red-labelled peptide 6 (red) and were fixed and counter-stained with Alexa488-phalloidin (green). (A&B) DC after 15 minutes of transfection, showing morphological characteristics of immature DC. (C) Z-section of DC 30 minutes after transfection, showing labeled complexes adhered to the coverslip and cell surface. Between 10-12 sections were taken through each cell, and red and green images overlaid. Representative data is shown.

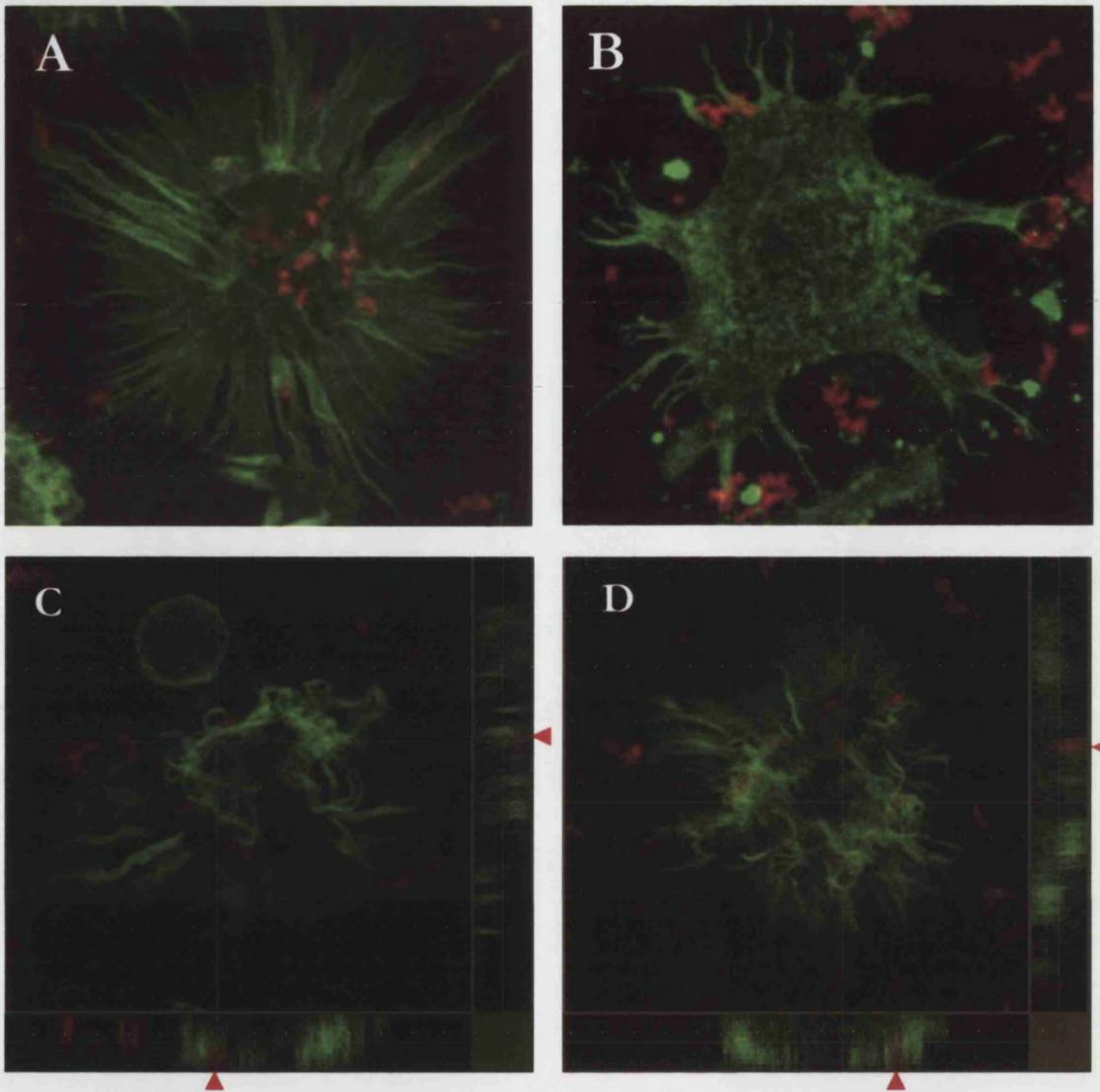


Figure 3.8 Confocal microscopy of human DC 90 minutes - 4h after transfection.

Cells were transfected on glass coverslips with LPD vector containing Texas Red-labelled peptide 6 (red) and were fixed and counter-stained with Alexa488-phalloidin (green). After 90 minutes (**A**) 2 hours (**C&D**) and 4 hours (**B**) of transfection the morphology of the DC has changed to that of a mature phenotype. Podosomes are no longer present and the cells have a stellate appearance with many membrane spikes and ruffles. Z-sections taken through cells after 2 hours of transfection (**C&D**) shows some labelled vector that appears to be intracellular, indicated by red arrow heads. Between 10-12 sections were taken through each cell, and red and green images overlaid. Representative data is shown

peptide and DNA remain tightly complexed, shown by pink staining, until the DNA enters the cell (**Figure 3.9**). Here it appeared that some DNA had separated from the peptide, which was shown by diffuse blue staining, and examination of the individual sections through the cells confirmed this diffuse DNA staining was intracellular. DNA was only observed to separate from the peptide once inside the cell; no separation of components was seen with vector bound to the cell surface or adhered to the glass coverslip.

Further images with HAE cells, transfected with Cy5-labelled DNA and Oregon Green-labelled lipid, showed that the lipid dissociated from the intracellular complexes within 4 h (data not shown) and remained perinuclear up to 24 h afterwards (**Figure 3.10**). Lipid staining was shown to be intracellular by analysing individual sections and in some cells was quite punctate. This would suggest an endosomal location and is consistent with the lipid's proposed role of aiding the endosomal escape of the complexes. The lipid fluorescence was not as strong as the DNA or peptide, and the lipid and DNA stain did not appear to co-localise as much as the peptide and DNA stain. However, even the HAE cells, which have a high transfection efficiency, showed significant amounts of complex adhered to the cell surface which still was not internalised after 24 h. A considerable amount of complex also adhered to the glass coverslips due to the electrostatic charge of the vector.

The confocal images also showed the effect that LPD transfection had on the morphology of the DC, which still displayed the immature characteristics of podosomes (dots of actin involved in cell adhesion) and flat, fairly polarised cells 15 mins after transfection (**Figure 3.7 A&B**). Within 90 mins, podosomes were no longer present and the cells

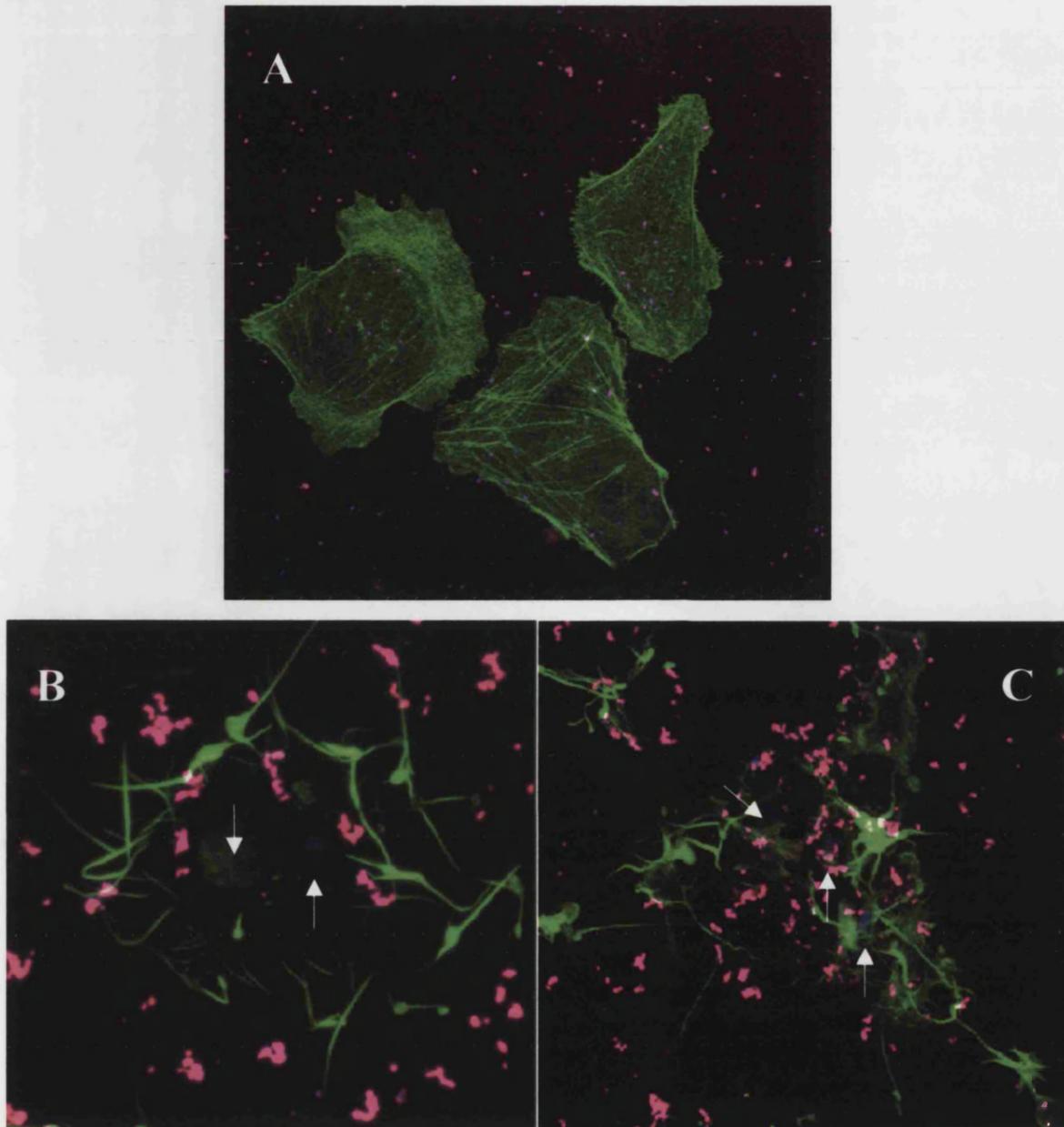


Figure 3.9 Confocal microscopy of HAE cells transfected with labeled peptide and DNA. (A) HAE cells were transfected on glass coverslips with LPD vector containing Texas Red-labeled peptide 6 (red) and Cy5-labelled DNA (blue) and fixed and counter-stained with Alexa-488 phalloidin (green) after 15 minutes of transfection. The pink colour shows co-localisation of peptide and DNA. (B & C) HAE cells were transfected as above and fixed and counter-stained 24 hours after transfection. Between 10-12 sections were taken through each cell, and red, blue and green images overlaid. The arrows indicate diffuse blue staining where the DNA appears to have dissociated from the peptide. Individual sections confirmed this staining was intracellular.

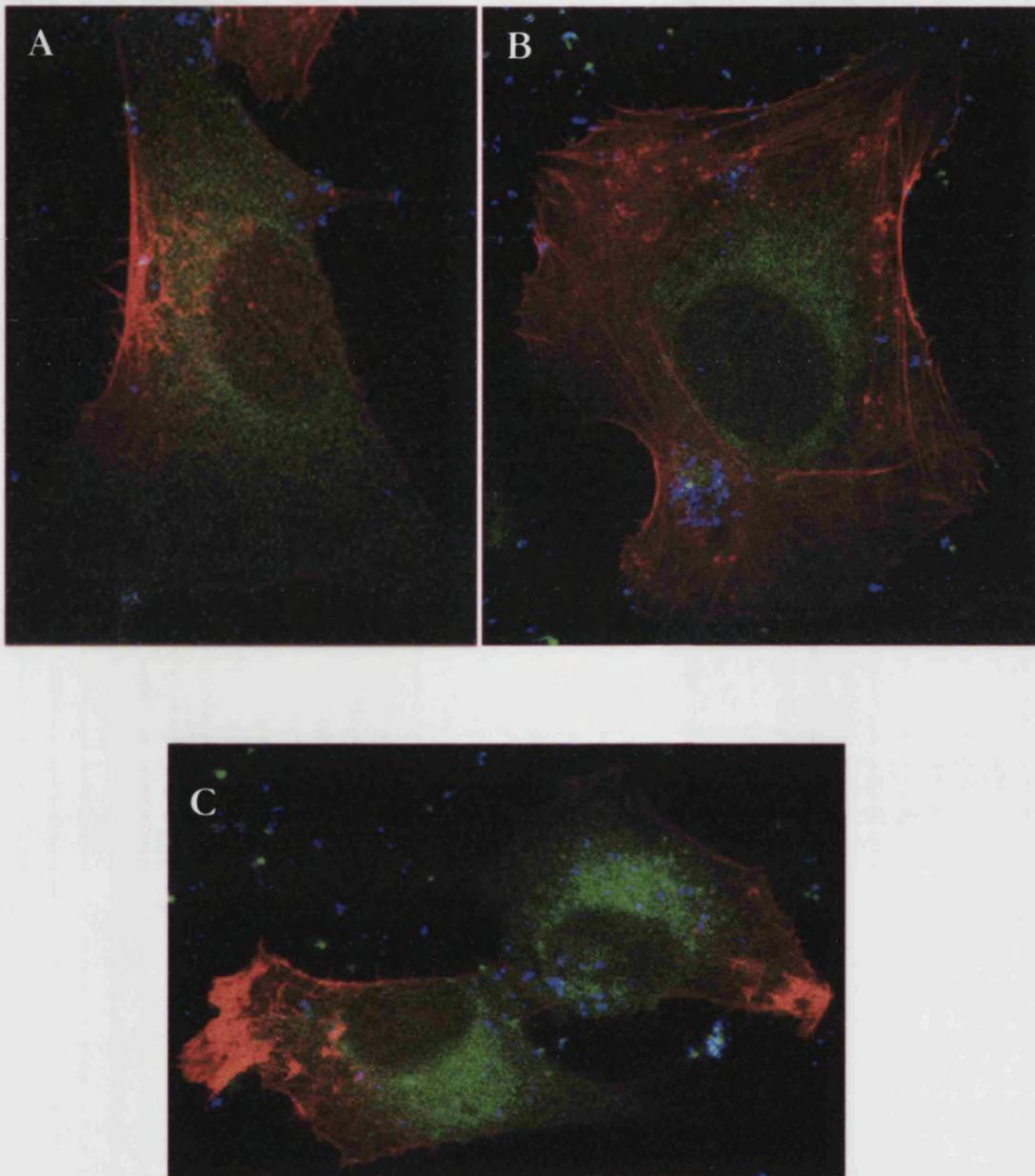


Figure 3.10 Confocal microscopy of HAE cells transfected with labelled lipid and labelled DNA. (A-C) HAE cells were transfected on glass coverslips with LPD vector containing Oregon Green labelled-lipid (green), Cy5 labelled-DNA (blue) for 4 hours, then replenished with complete media. Cells were fixed after a further 20 hours incubation, then fixed and counter-stained with rhodamine-phalloidin (red). Between 10-12 sections were taken through each cell, and red, blue and green images overlaid.

became more rounded, developing a stellate appearance with membrane ruffles and spikes that is characteristic of a mature phenotype (Figure 3.8 A-D).

3.2.8 Retargeted Adenovirus Vectors

The previous experiments suggest that DC appear refractory to transfection with the LPD vector. An alternative would be to use a viral vector that transfects non-dividing cells, such as an adenovirus. Adenoviruses efficiently transfect a variety of cell types and binding and internalisation is often mediated by the coxsackie-adenovirus receptor (CAR) (Wickham *et al.*, 1993). Cells that do not express CAR are still permissive to transfection, albeit at lower levels. Retargeting the Ad vectors to target surface molecules other than CAR is one way of increasing transfection efficiency in CAR-negative cell types (Hidaka *et al.*, 1999). Alternatively, pseudotyping with fibre proteins from different Ad serotypes may also enhance the specificity of vector uptake to certain cell populations (Shayakhmetov *et al.*, 2000).

Adenoviral vectors been shown to successfully transfect human (Diao *et al.*, 1999; Lundqvist *et al.*, 2002) and murine DC (Korst *et al.*, 2002; Song *et al.*, 1997), although these cells do not express CAR. Advances in vector technology have allowed the genetic manipulation of the adenoviral fibre protein, which is responsible for binding to host cells and subsequent internalisation. Changes in the fibre protein sequence can result in Ad vectors targeted towards specific cell populations, such as DC, mediating increased transfection. The increased transfection efficiency may allow lower MOIs to be used, which could reduce vector toxicity and anti-vector immune responses, permitting repeated administration.

3.2.8.1 Transfection of murine DC with retargeted adenovirus

The Ad-lacZ vector used in section 3.2.5 did not appear to augment LPD-mediated transfection of murine DC, and no expression of Ad vector-encoded lacZ could be detected. However, murine DC have been shown to be permissive to transfection with Ad vectors (Korst *et al.*, 2002; Xia *et al.*, 2002). Transfection efficiency in murine DC may be improved by manipulating the sequence of the fibre protein to a motif that is known to bind DC. Phage panning was used to identify two peptide sequences that bound human DC with high affinity, and these are designated peptide A and peptide B (M. Writer, unpublished) (Table 2.1, Materials and Methods). The HI loop of the fibre protein of a GFP-encoding E1 and E3 deleted adenoviral vector was engineered to contain the sequence of peptide A or peptide B. A knock-out Ad vector was also constructed, containing a HI loop with no CAR binding domain. The Ad-peptide A, Ad-peptide B and knock-out Ad vectors were compared with a virus containing a wild-type fibre protein for their ability to transfect murine CD11c⁺ DC. GFP⁺ cells were analysed by flow cytometry.

The knock-out Ad vector mediated only negligible transfection of DC with 1.5% GFP⁺ DC, while the wild-type Ad vector had a transfection efficiency of 15%, which is superior to what was achieved with the LPD vector. However, the Ad vectors retargeted with the DC binding peptides showed a massive increase in transfection efficiency. Transfection with Ad-peptide A resulted in 79% GFP⁺ DC, and Ad-peptide B resulted in 61% GFP⁺ DC (Figure 3.11), demonstrating the contribution of the fibre protein sequence to vector binding and internalisation.

3.2.8.2 Transfection of murine stem cells with retargeted adenovirus

The same Ad vectors were also used to transfect Sca-1⁺ stem cells isolated by positive selection from the bone marrow of A/J mice. Stem cells are an attractive target due to

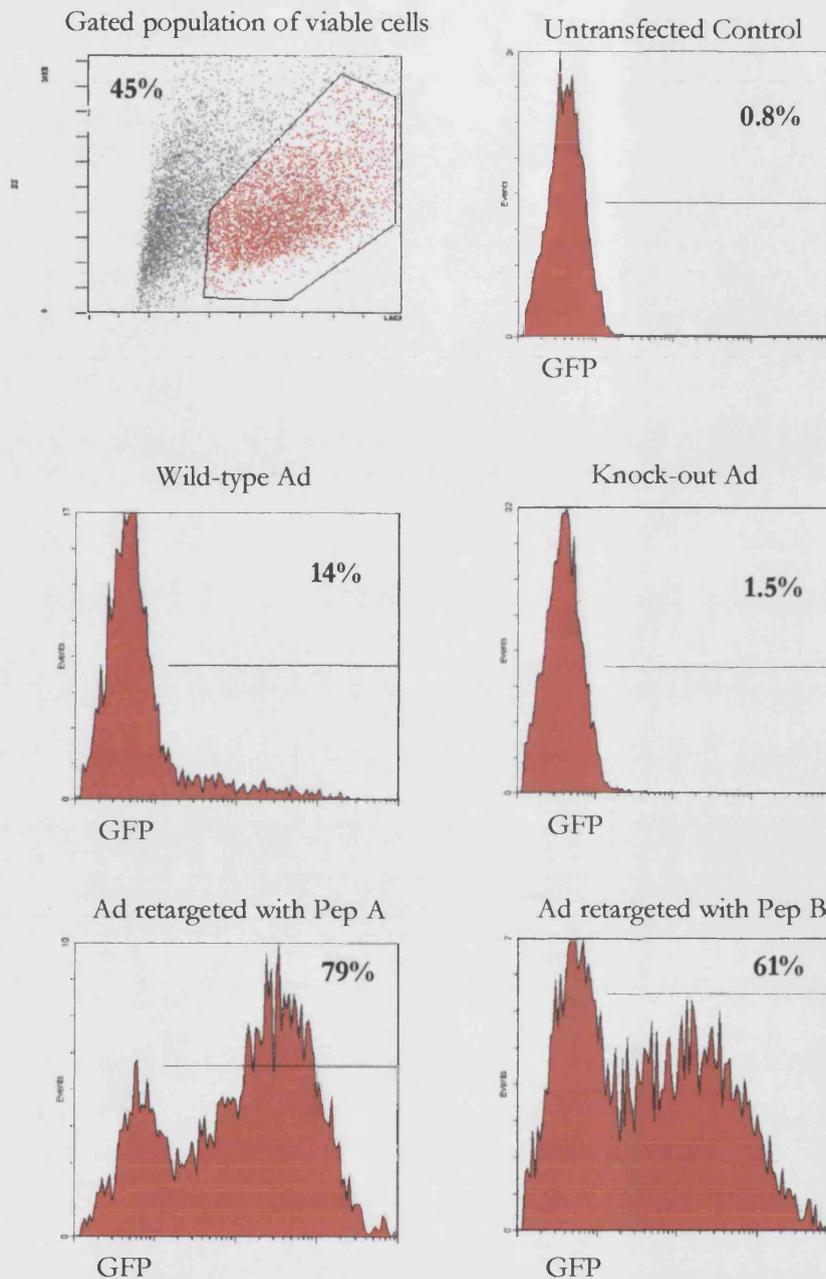


Figure 3.11 Improvement of transfection efficiency of murine DC with retargeted adenovirus. CD11c⁺ DCs were transfected with 100,000 Ad-GFP particles per cell (~MOI 100) in complete media. 24 hours after transfection cells were analysed by FACS. The scatter plot in the top left corner shows the gated population of viable cells with the scatter characteristics of DC, and 10,000 events were collected from this gate are shown in the histograms. Representative data is shown.

their ability to differentiate into all hematopoietic lineages. Transfection of stem cells was investigated because there would be many advantages to transferring genes to these cells in their quiescent state, without pushing them into the cell cycle, where they start to lose their early precursor characteristics. Although current Ad vectors are non-integrating, vectors are being developed with the ability to integrate into the cell genome, preventing vector loss as the cell divides (Murphy *et al.*, 2002; Mitani & Kubo, 2002).

The knock-out vector mediated negligible transfection that was equivalent to background untransfected control. Transfection efficiency of murine stem cells was much lower than achieved in murine DC, with only 2.5% GFP⁺ stem cells following transfection with wild-type Ad, and 6% and 5.5% GFP⁺ stem cells following transfection with Ad-peptide A and Ad-peptide B respectively (Figure 3.12). Although the overall level of transfection is low, the retargeted vectors still demonstrate increased transfection efficiency compared to the wild-type vector.

3.3 DISCUSSION

The aim of the section of work was to optimise the transfection of murine bone marrow-derived DC using the synthetic LPD vector to provide a quick and straightforward method of efficient gene transfer to these cells. Transfection of murine DC with the LPD vector mediated gene transfer efficiency of ~5%. This is comparable with the transfection efficiency achieved in murine primary DC transfected with another cationic peptide, CL22, which contains a sequence from influenza nucleoprotein. 1% of DC were transfected using CL22, and this was shown to be sufficient for antigen-specific responses (Irvine *et al.*, 2000). However, CL22 achieved 17% transfection of human DC, compared to 1% of murine primary DC, suggesting murine DC may be intrinsically more difficult to transfect than human DC (Irvine *et al.*, 2000; Haines *et al.*, 2001). Other non-viral vectors used to

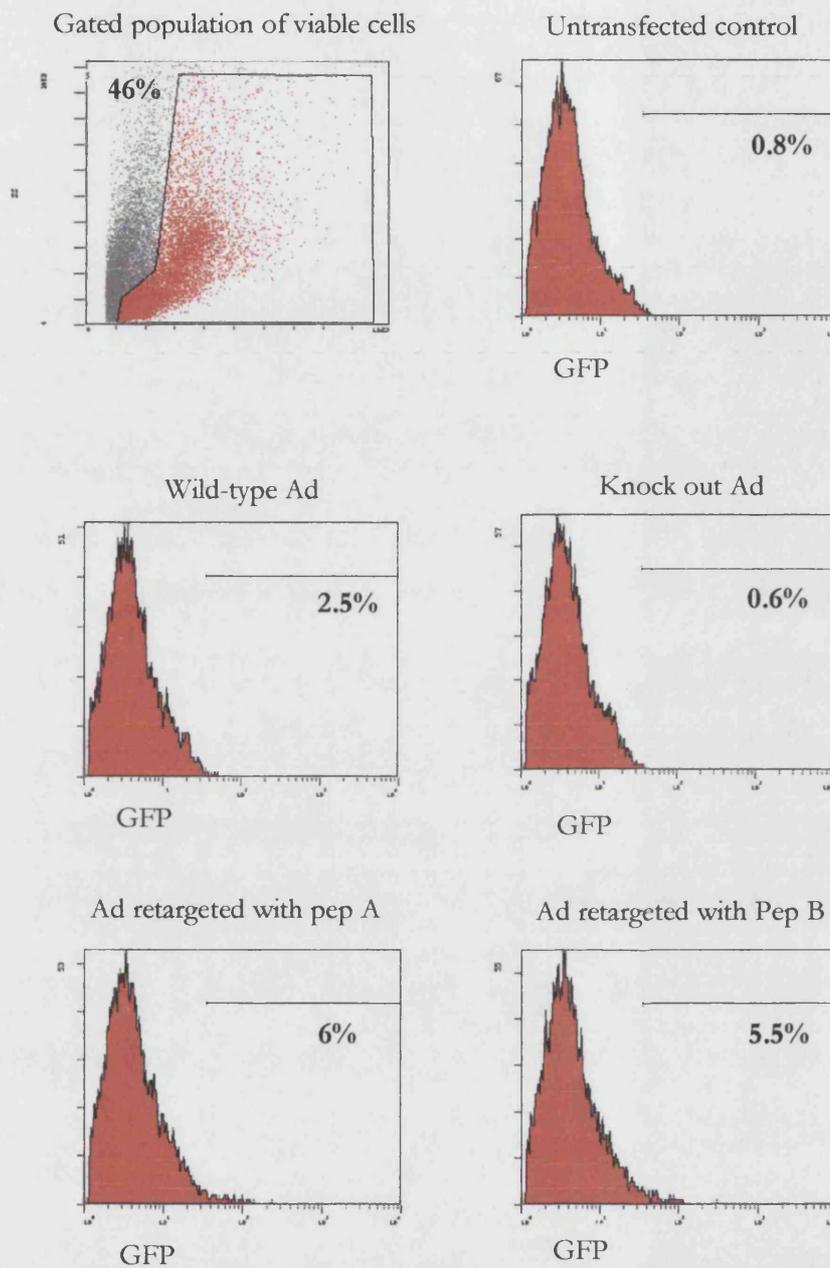


Figure 3.12 Transfection of murine Sca-1⁺ haematopoietic stem cells with retargeted adenovirus. Sca-1⁺ stem cells were transfected with 100,000 Ad-GFP particles per cell (~MOI 100) in complete media supplemented with Flt-3 ligand, mSCF and IL-6. 24 hours after transfection cells were analysed by FACS. The scatter plot in the top left corner shows the gated population of viable cells with the scatter characteristics of stem cells, and 10,000 events were collected from this gate are shown in the histograms. Representative data is shown.

transfect DC include PEI/DNA vectors which incorporated an Ad helper virus, which were shown to mediate transfection of ~10% (Diebold *et al.*, 1999), while cationic peptides composed of ornithine and histidine repeats mediated 3-fold higher transgene expression than lysine-based peptides (Chamarthy *et al.*, 2003). The highest level of non-viral transfection of DC has been achieved using the recently developed nucleoporation technology, mediating transfection of up to 60%, although loss of cell viability has hampered this approach (Lenz *et al.*, 2003).

The LPD vector used here is composed of plasmid DNA, Lipofectin and a receptor targeting cationic peptide. Incorporation of the targeting peptide has been shown to mediate enhanced transfection in many cell types compared to lipid-DNA complexes or untargeted peptide-lipid-DNA complexes (Hart *et al.*, 1998), so was therefore a good candidate for transfection of DC. The peptide has two functions; the chain of 16 lysine residues has a high positive charge that has DNA condensing properties, while the receptor targeting part of the molecule contains a motif that targets cell surface molecules, and is held in a cyclic conformation by two cysteine residues flanking the motif, forming a disulphide bond. The LPD vectors used here contained integrin-targeting motifs. It is proposed that once the vector binds to the cell, it enters the cell through receptor-mediated endocytosis. The lipid component of the vector is thought to fuse with the endosomal membrane and facilitate release of the peptide/DNA components of the vector into the cytoplasm, reducing acidic degradation of the vector. The plasmid component of the vector is transported to the nucleus, probably still bound to the peptide, although it is not clear how this occurs. Once in the nucleus, the gene is transcribed by the host cell's transcriptional machinery.

The individual LPD components were optimised for transfection of mouse bone marrow-derived DC. The optimal combination was determined of peptide 1 at a charge ratio of +7, 2µg DNA in a volume of 500µl, and a transfection period of 90 minutes.

Peptide 1 contains an RGD motif that targets many integrins including α_v and α_5 (Parkes & Hart, 2000), and RGD motifs can be found in proteins involved in cell-cell or cell-matrix interactions, such as fibronectin, vitronectin and thrombospondin (Humphries *et al.*, 2000). Peptide 11 contains the RGE motif so is not integrin-specific, yet both peptide 1 and peptide 11 resulted in high luciferase expression at all charge ratios tested. Peptide 11 may mediate internalisation by receptors other than integrins or part of the head group other than the RGD/RGE motif may be targeting molecules on the cell surface, as the rest of the sequence is identical in both peptides, leading to similar luciferase expression. The untargeted K16 peptide also showed comparable levels of transfection with peptide 1 at a charge ratio of +7, indicating that LPD vector internalisation is not mediated entirely by integrin binding, but also by the positive charge of the complexes.

A charge ratio of +7 consistently gave the highest transgene expression in conjunction with peptide 1, probably due to maximal binding to the cell surface and internalisation. The further the charge ratio was increased above +7, the less luciferase expression was observed, which may be due to some peptide not being incorporated into the complexes. Free peptide may be competing with the LPD vector for binding to integrins, blocking the binding of complexes to the cell and decreasing the amount of internalised vector.

Confocal microscopy with fluorescently-tagged vector components was performed to visualise the complexes in the cells and ascertain where the block to transfection was occurring. Potential barriers to efficient transfection were limited internalisation of the

LPD vector, endosomal degradation of the vector, and prevention of the plasmid DNA released into the cytoplasm from translocating to the nucleus. Luciferase transfections showed that LPD vector incorporating the labelled peptide mediated comparable transfection to unlabelled LPD vector (see appendix 3).

DC transfected with labelled peptide revealed that the LPD complexes adhered to the cell surface and remained in contact with the cell for many hours. This has also been observed in primary human fibroblasts transfected by lipofection, where substantial amounts of complex remained adhered to the cell surface several hours after transfection (Coonrod *et al.*, 1997). However, LPD complexes were only observed inside very few DC.

Human airway epithelial (HAE) cells were used in comparison to DC as these are known to transfect with high efficiency, and could therefore demonstrate more clearly the cellular trafficking of the LPD vector. Cy-5-labelled DNA was also used for these images, and this showed that the peptide and DNA components of the vector remain bound together when adhered to the cell surface. HAE cells transfected with labelled lipid and labelled DNA showed perinuclear localisation of the lipid after four hours of transfection, with punctate staining consistent with an endosomal location, suggesting that the complexes are internalised via the endosomal pathway. This is consistent with the model of the lipid layer being shed in the endosome and staining with endosomal markers to detect co-localisation with lipid could confirm this. The only DNA that did not appear to be bound to peptide was observed intracellularly, as shown by sections taken through the cell. The intracellular DNA may be located in the nucleus, and co-localisation with a nuclear counter-stain could determine whether the labelled DNA was intranuclear.

Comparison of DC and HAE cells showed the main barrier to transfection in DC was limited vector internalisation. Many HAE cells showed internalised LPD by the presence of lipid in a location consistent with endosomes, and the identification of DNA located within the cells. In DC, vector adhered to the cell but very little was shown to internalise.

To further investigate the nature of the receptors for the LPD vector, a panel of antibodies targeting different integrins could be used to characterise the surface expression of integrins on murine DC. Blocking antibodies against integrins or small soluble peptides which compete for binding sites on the integrin molecule with the LPD vector could determine the specificity of the integrins targeted. Alternatively, vector internalisation may be improved by identifying peptides that bind with a higher affinity, increasing the contact time between the vector and the cell surface.

When using DC as a cancer immunotherapy vaccine, ideally the cells should transfect with high efficiency and also display high levels of immunostimulatory surface markers. Comparison of the phenotype of untransfected and transfected cells showed an increase in the level of activation markers following transfection. However, when murine C5 was used as a model antigen in an antigen presentation assay no activation of antigen-specific CD4 T-cell hybridoma target cells (A18) was observed. The A18 cells recognise only one epitope of murine C5 protein (Žal *et al.*, 1994), so it is possible that the DC were presenting other epitopes, which were not recognised by the A18 cells. To test the A18 cells, ConA, PHA and PMA, which non-specifically activate T-cells, were added to the positive control wells, but still no IL-2 secretion was seen, suggesting that the A18 cells were not responding to stimulation. Therefore, it was difficult to assess the antigen presenting capacity of the DC due to the low stimulation capability of the A18 cells.

An alternative method of testing the ability of DC to present transfected antigen C5 as a model antigen could be to immunise A/J mice with C5 peptide. The splenocytes from these primed mice could be used in an ELISpot with C5-transfected DC to determine the ability of the DC to re-stimulate C5-specific splenocytes.

Many publications concerning gene transfer to primary DC use adenoviral vectors, which can achieve transfection efficiency of up to 85% or 90% in murine DC (Korst *et al.*, 2002; Xia *et al.*, 2002), and >70% in human DC (Diao *et al.*, 1999), while HSV-1 amplicons and retroviral vectors have achieved a 70% transduction efficiency in human and murine DC (Willis *et al.*, 2001; Specht *et al.*, 1997; Movassagh *et al.*, 1999). Recently reports using lentiviral vectors have been published demonstrating transfection efficiencies of 35% in human DC (Schroers *et al.*, 2000) or up to 80% in murine DC (Metharom *et al.*, 2001), demonstrating that further work is needed to increase the level of transfection with synthetic vectors up to that achieved with viral gene transfer systems.

The addition of Ad-lacZ vector to the LPD vector as a helper virus had little enhancement of transfection. However, the adenoviral vectors with fibre proteins retargeted to express DC-binding peptides resulted in high-level transfection efficiency and gene expression in murine DC. This demonstrates the potential of vector retargeting to augment gene transfer into target cells. Ad vectors retargeted to DC could prove very useful in mediating high level transgene expression, and transfection with adenoviruses has been shown to induce an activated phenotype in both human and murine DC (Korst *et al.*, 2002; Lundqvist *et al.*, 2002) which is critical to their antigen presenting capacity. Further work would be necessary to characterise the phenotype of murine DC transfected with these retargeted viruses, and to confirm the transfected DC are functional.

In conclusion, the optimal LPD vector formulation was able to mediate only low-level transfection of DC, with a transfection efficiency of <5%. An antigen presentation assay showed that transfected DC did not appear to be presenting antigen to target cells. Confocal work demonstrated that low internalisation of the vector was the major barrier to transgene expression in DC, as the cells appear to bind high levels of LPD complex but little was taken up into the cell. The studies discussed above have reported that gene transfer into DC using synthetic vectors can be achieved, although these levels are lower than attained with viral systems. Therefore, further LPD vector development to enhance vector uptake should improve the transfection efficiency and transgene expression in primary DC.

CHAPTER 4

OPTIMISATION OF FIBROBLAST TRANSFECTION

4.1 INTRODUCTION

Vaccination with DC modified to express tumour antigens (Metharom *et al.*, 2001) or tumour lysate-pulsed DC (Fields *et al.*, 1998) is one approach to generating a local pro-inflammatory environment. An alternative means of increasing the immunogenicity of the tumour microenvironment is to increase the concentration of pro-inflammatory cytokines. Proinflammatory cytokines in the tumour locale have been shown to generate cellular and humoral adaptive immune responses against established tumours (Son *et al.*, 2003; Fujii *et al.*, 2000). Systemic administration of inflammatory cytokines is toxic at the concentrations required for tumour regression, and intratumoural injection of recombinant proteins results in only short-term cytokine exposure. Transfecting tumour cell lines with cytokine genes and injecting them into mice gives localised, high-level cytokine secretion for several days, and has been shown to effectively retard tumour growth *in vivo* in murine tumour models (Moret *et al.*, 2001). This approach has reached clinical trial for some malignancies, including NB using viral gene transfer methods (Seeger *et al.*, 1998; Brenner *et al.*, 2000).

However, this method is not always appropriate to transfer to a clinical setting due to technical difficulties with the culture and manipulation of primary tumour cells from surgical specimens (E.Siapati, personal communication; de Zoeten *et al.*, 1999). Additionally, there may be insufficient tumour material available from which to culture primary cells. Conversely, primary dermal fibroblasts are readily cultured from skin biopsies and when transfected with cytokine genes, may be used as a cellular vaccine to give the same localised cytokine expression.

This approach has already been used in phase I trials for a variety of solid tumours, using viral transfer of IL-2 (Sobel *et al.*, 1999) or IL-12 (Kang *et al.*, 2001). Both of these studies used autologous fibroblasts and therapeutic effects were seen with these vaccines. These

phase I clinical trials using autologous cells mentioned above used a retroviral vector to transfer the cytokine genes to the fibroblasts. While very efficient, there are potential problems associated with retroviral vectors, including the possibility of generating replication-competent virus or aberrant integration into the host genome. Synthetic vectors are much quicker and simpler to prepare and are considered a safer alternative to viral vectors. Provided that therapeutic levels of transgene can be produced, they may become an attractive alternative in clinical applications.

The aim of this section of work was to evaluate murine fibroblasts transfected to express IL-2 and IL-12 as a tumour vaccine. Therefore transfection of autologous and allogeneic murine fibroblasts using the synthetic LPD-vector was optimised for maximal cytokine expression.

4.2 RESULTS

4.2.1 Optimisation of transfection

4.2.1.1 Transfection of Murine Dermal Fibroblasts

Mouse primary dermal fibroblasts were prepared from skin explants of A/J mice by enzyme digestion to produce single cell suspension, and were expanded *in vitro*. These cells were transfected with the LPD vector using the previously optimised transfection conditions of a charge ratio of +7, 2 μ g DNA per well, 0.75 μ g Lipofectin per 1 μ g DNA and a transfection time of four hours. Therefore the only condition specifically optimised for primary A/J fibroblasts was the targeting peptide and the presence/absence of Ad helper virus.

Experiments using a luciferase reporter gene showed that the LPD vector incorporating peptide 6 and Ad helper virus generally had the highest reporter gene expression (Figure 4.1), although the Ad-mediated enhancement of transfection over LPD vector alone was inconsistent, possibly due to differences in cell viability between different cell preps. The Ad/PD vector, which has shown superior transfection in human DC (H. Truman, unpublished), did not result in superior transfection in murine primary fibroblasts. The problems with using these cells were the slow growth and poor viability of these cells, which could only be maintained for a few passages. This would be a major limiting factor in generating the large numbers of cells required for *in vivo* procedures with several experimental groups of mice.

4.2.1.2 Generation of a syngeneic MEF line

Due to the difficulties of using primary dermal fibroblasts detailed above, a fibroblast cell line syngeneic with A/J mice was used to overcome these problems. However, no such line is commercially available so a syngeneic fibroblast cell line was developed from 13.5dpc A/J embryos in a variation of the original method of Todaro and Green (1963) and advice available in the institute (Dr M. Hubank, personal communication).

Briefly, the crania and red organs were removed and the remaining tissue digested to form a single cell suspension. This suspension was plated in a 75cm³ flask for 2 days, then cells were split and plated out at a density of 50,000 per 25cm³ flask and passaged every ~3 days until cells reached senescence. This allows the fibroblasts to out-grow any other cell types, most of which will not establish in culture. After 10-14 days, a few cells were observed to spontaneously immortalise and started growing at an increased rate. Of the 15 embryos cultured, 2 gave rise to cells with fibroblast-like morphology. Cells were passaged 5-7 times to ensure a uniform population of immortalised cells, then used in reporter gene

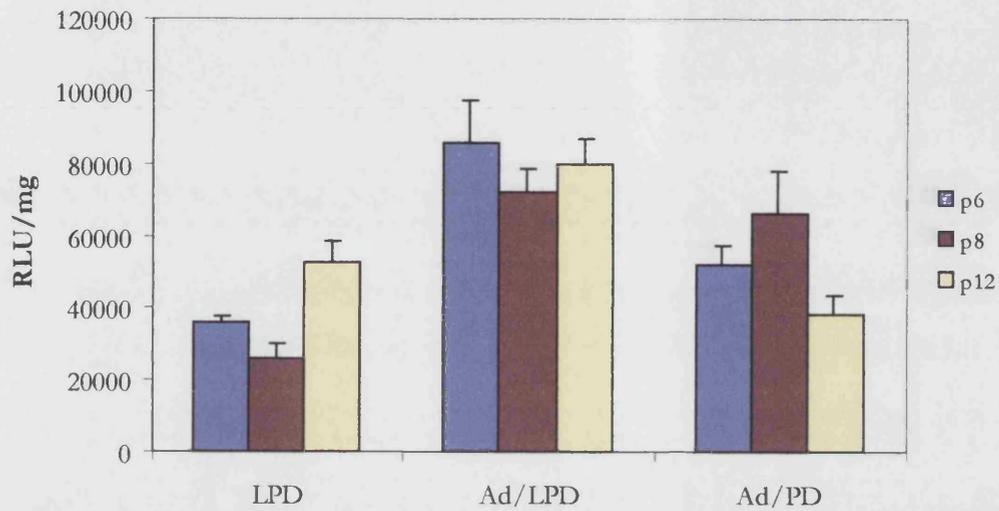


Figure 4.1 Reporter gene expression in primary A/J dermal fibroblasts.

Cells were transfected with LPD vector, Ad/LPD or Ad/PD incorporating peptide 6, peptide 8 or peptide 12 and analysed by luciferase assay 24 hours after transfection. Error bars denote the standard deviation of triplicate transfections and representative data is shown.

transfections. Initial transfections showed one to consistently give higher transgene expression levels (see appendix 4) and this culture was used in all subsequent experiments, hereafter referred to as AJ3.1 (Figure 4.2A).

4.2.1.3 Optimisation of AJ3.1 transfection

As with the primary dermal fibroblasts, a charge ratio of +7, 2µg DNA per well and a transfection time of 4 h was used. At this stage in the project, peptides isolated by phage display were available to compare with the integrin-targeting peptides. The two peptides with the highest binding on fibroblasts (VNLQNPY and VYARSMN; Table 2.1), the two peptides with the highest binding to HAE cells (pep P and E; Table 2.1) and the two peptides with the highest binding to human DC (pep A and B; Table 2.1) to the integrin-targeting peptide 6.

Both VNLQNPY and VYARSMN gave consistently higher levels of luciferase expression than peptide 6 (Figure 4.2B). The DC-binding peptide A and HAE-binding peptide E gave consistently elevated luciferase expression compared to peptide 6, but lower than the expression mediated by the fibroblast-specific peptides. Peptide B and peptide P did not enhance luciferase expression compared to peptide 6. Luciferase expression from transfected AJ3.1 was consistently more than 25-fold higher than observed in A/J primary dermal fibroblasts.

4.2.1.4 Optimisation of Transfection of Allogeneic NIH-3T3 Fibroblasts

NIH-3T3 fibroblasts were chosen as the allogeneic cells. These cells are derived from NIH-Swiss mice, which is an outbred strain and therefore they do not express a defined MHC. The phage-isolated peptides were not available at this stage in the project. 50,000 NIH-3T3 cells were transfected using 2µg DNA per well, with peptide 6 or peptide 1 at

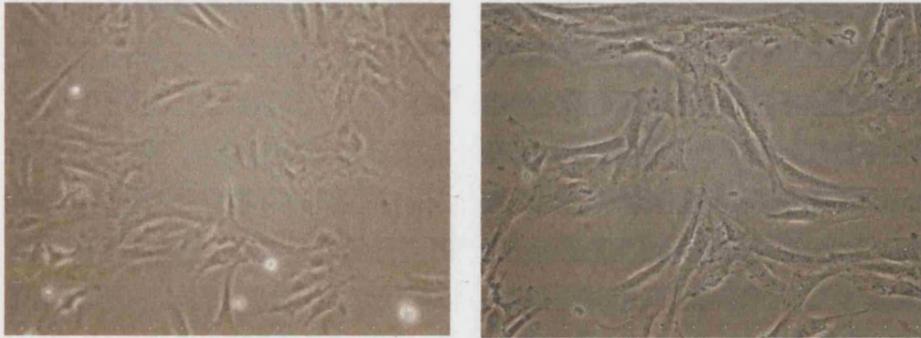
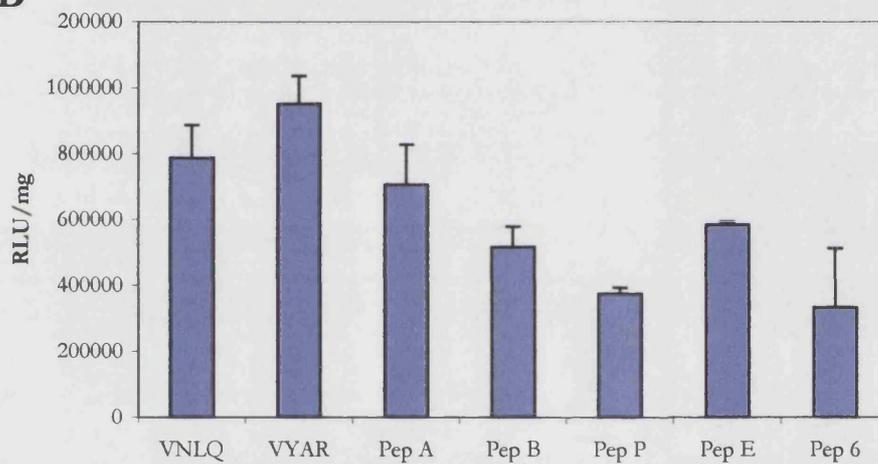
A**B**

Figure 4.2 Generation and optimisation of transfection of AJ3.1 cells. (A) Phase contrast microscopy of AJ3.1. These images show passage 12 AJ3.1 cells, demonstrating typical fibroblast morphology and a uniform population. Representative images are shown of a series of frames captured.

(B) Optimisation of targeting peptide with AJ3.1. Cells were transfected with pCI-Luc using the LPD vector incorporating different targeting peptides at a charge ratio of +7. Cells were analysed by luciferase assay 24 hours after transfection and error bars denote the standard deviation from triplicate transfections. Representative data is shown.

+3 or +7 charge ratio for 4 hours at 37°C. Cells were analysed 24 h after transfection by luciferase assay (**Figure 4.3**) which showed either peptide at a charge ratio of +7 to give substantially greater luciferase expression than vectors at a charge ratio of +3. Peptide 6 showed slightly higher expression than peptide 1, so it was decided to use a LPD vector containing peptide 6 at a charge ratio of +7 for further work.

Comparison of luciferase expression from the transfected fibroblasts shows that transfection of both fibroblast cell lines is far superior to the level of transfection obtained in primary dermal fibroblasts. Luciferase expression in AJ3.1 fibroblasts is ~30-fold higher than in A/J primary dermal fibroblasts. There is also little difference in luciferase expression between either fibroblast cell line and the Neuro-2a (N2a) neuroblastoma cell line, which are known to transfect with high efficiency and express high levels of transgene using a LPD vector containing peptide 6 (**Figure 4.4**).

4.2.1.5 Transfection efficiency of fibroblasts with LPD vectors

Transfection with a GFP reporter gene using the optimal LPD vector for each cell type was performed to determine transfection efficiency. Primary dermal A/J fibroblasts were transfected with peptide 6 and showed very low transfection efficiency of $\leq 5\%$ (**Figure 4.5A**). AJ3.1 cells transfected with the LPD vector incorporating peptide VYARSMN showed the transfection efficiency was consistently $>20\%$ (**Figure 4.5B**) indicating that high level reporter gene expression was achieved with the LPD vector incorporating VYARSMN. Transfection of NIH-3T3 cells with the LPD vector incorporating peptide 6 resulted in a transfection efficiency of 45% (**Figure 4.5C**).

These results suggest the A/J primary dermal fibroblasts are not suited to transfection with this vector, and this is probably related to their poor viability in culture. The

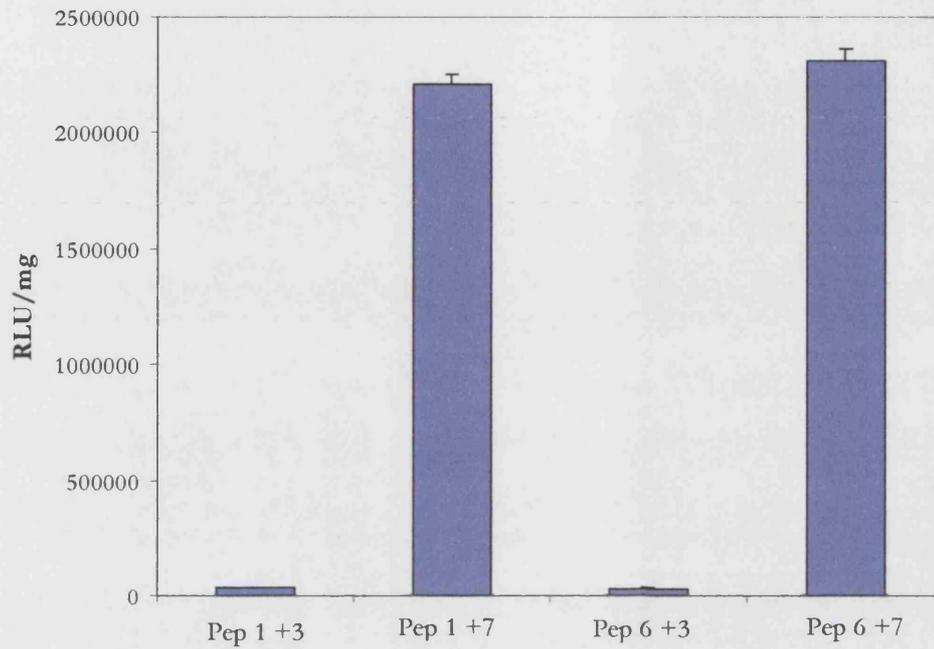


Figure 4.3 Luciferase expression in NIH-3T3 cells. Cells were transfected with pCI-Luc by LPD vector incorporating peptide 6 or peptide 1 at a charge ratio of +3 or +7 and analysed by luciferase assay 24 hours after transfection. Error bars represent standard deviations of triplicate transfections and representative data is shown.

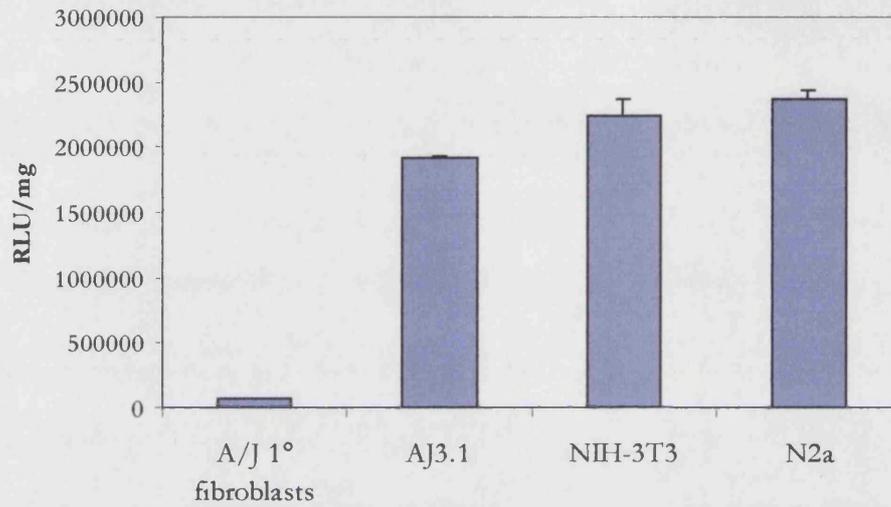


Figure 4.4 Comparison of luciferase expression in different fibroblast types.

The luciferase expression mediated by the optimal transfection formulation for A/J primary dermal fibroblasts, AJ3.1 fibroblasts and NIH-3T3 fibroblasts was compared with transfection of the A/J derived neuroblastoma cell line (N2a) cells, which are known to transfect with high efficiency. Cells were analysed by luciferase assay 24 hours after transfection and error bars represent standard deviation of triplicate transfections.

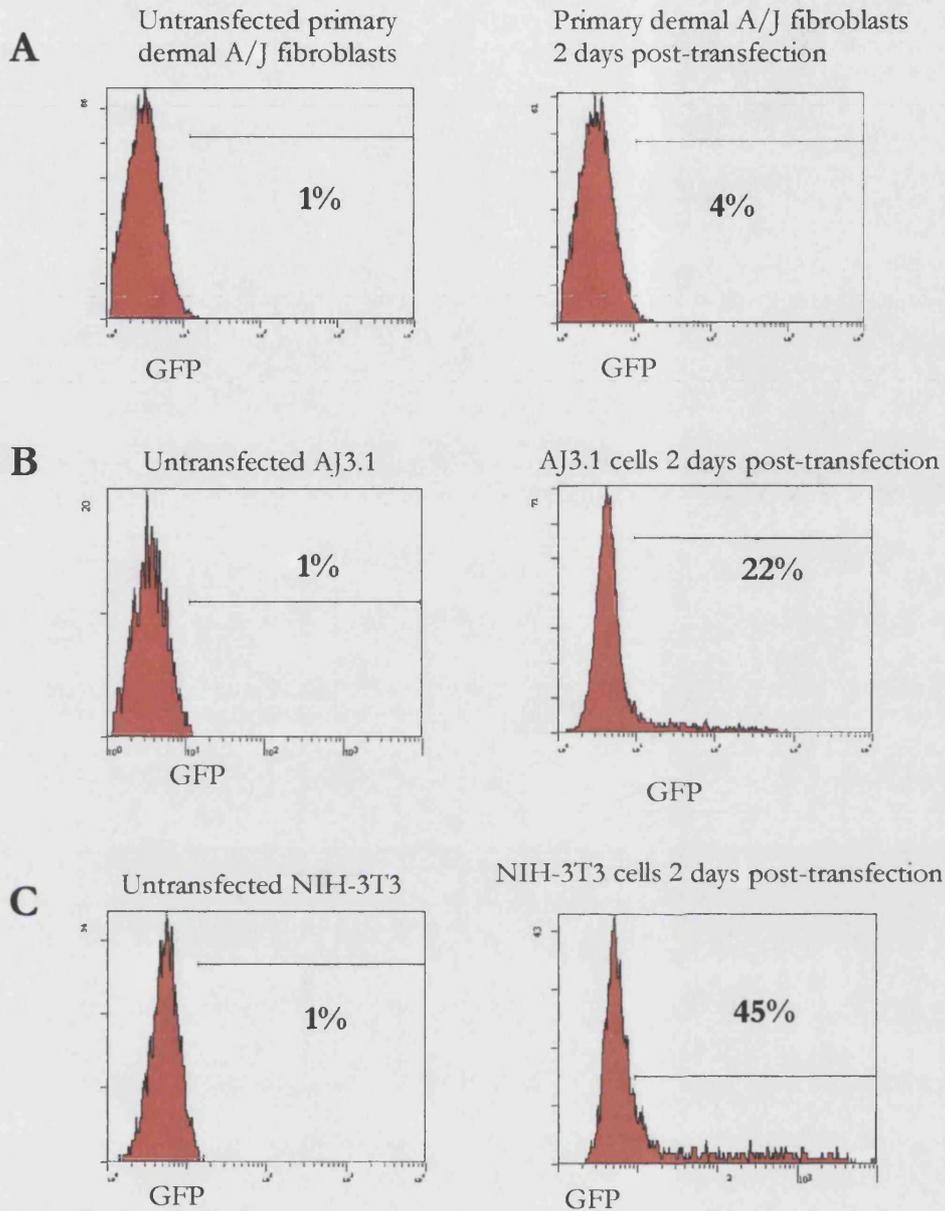


Figure 4.5 Comparison of transfection efficiency of syngeneic and allogeneic fibroblasts. Cells were transfected with their respective optimal LPD vector at a charge ratio of +7 incorporating the plasmid pEGFP-N1 and analysed by FACS 48 hours after transfection. Primary dermal A/J fibroblasts were transfected with peptide 6 (**A**), AJ3.1 fibroblasts were transfected with peptide VYARSMN (**B**) and NIH-3T3 cells were transfected with peptide 6 (**C**). Viable cells were gated on the basis of forward scatter and side scatter characteristics, and 10,000 events collected from this gate are shown in the histograms. Representative data is shown.

syngeneic AJ3.1 and allogeneic NIH-3T3 fibroblast cell lines both demonstrated a high transfection efficiency with their respective optimal LPD vectors. Therefore the level of cytokine expression that could be achieved in these cells by transfection with pCI-IL-2 and pCI-IL-12 genes using the LPD vector was determined

4.2.2 Cytokine expression of transfected fibroblasts

4.2.2.1 Cytokine expression by primary dermal fibroblasts

Murine primary dermal fibroblasts were transfected with pCI-IL12 using the LPD vector and culture supernatant analysed by ELISA (**Figure 4.6**). This showed the level of secreted cytokine was ~100-fold lower than typically observed in transfected N2a cells (**Figure 4.7**), and expression rapidly dropped below the level of detection. Transfection using Ad/LPD or different targeting peptides did not improve cytokine secretion levels, and peak expression was 12ng/24h/10⁶ cells on day 1 using Ad/LPD 12. Cytokine expression also decreased rapidly, although very low level IL-12 secretion was detectable on day 11. IL-2 secretion by transfected fibroblasts was below the level of detection. Repeats of this experiment in primary cells showed this level of secretion to be typical.

It was decided not to progress further with these cells for *in vivo* work for several reasons. Poor cell viability, as mentioned previously, was limiting the number of cells that could be maintained in culture, so could subsequently restrict the *in vivo* use of these cells. Variability between cultures was also a problem, particularly as reproducibility of the transfected cell vaccine is vital to its *in vivo* application. Finally, low level of secreted cytokine by transfected primary dermal A/J fibroblasts is unlikely to confer any therapeutic benefit, being ~100-fold lower than obtained with transfected N2a cells that have mediated eradication of established tumours (Siapati *et al.*, 2003).

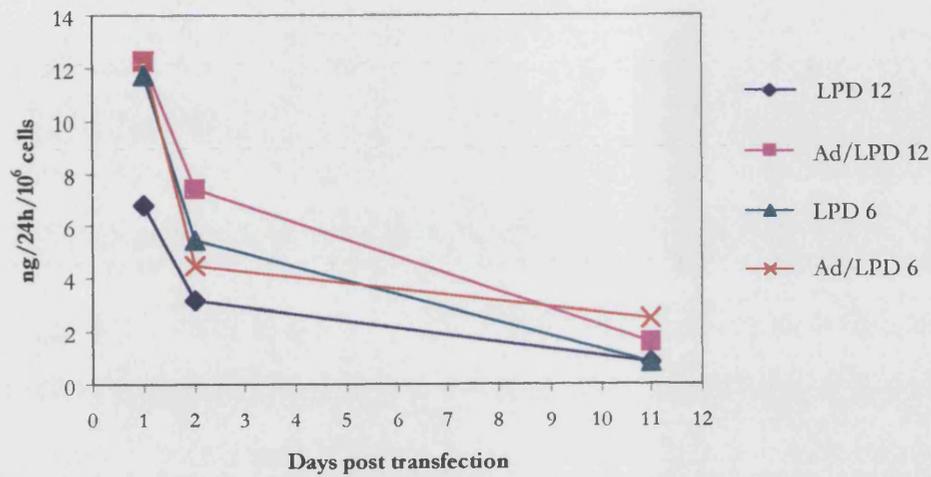


Figure 4.6 Secretion of mIL-12p70 by transfected primary dermal A/J fibroblasts. Cells were transfected with pCI-scIL-12 using LPD or Ad/LPD vector incorporating peptides 6 or 12. Supernatant was harvested and IL-12p70 expression determined over a 24-hour period using a sandwich ELISA. Representative data is shown.

4.2.2.2 Cytokine expression by transfected AJ3.1

AJ3.1 cells were transfected individually with pCI-IL2 or pCI-mIL12, or co-transfected with both plasmids using peptide VYARSMN and supernatant harvested every 24 hours. Cytokine expression was determined by sandwich ELISA and compared to the expression from N2a cells co-transfected with both cytokine plasmids. Expression of IL-2 and IL-12 was very similar between singly transfected and co-transfected cells. In one representative experiment, IL-12 expression of transfected AJ3.1 cells peaked two days after transfection at 1370ng/24h/10⁶ cells (single transfection) or 1100ng/24h/10⁶ cells (co-transfection) compared to 1350ng/24h/10⁶ N2a cells (Figure 4.7A). Five days after transfection, IL-12 secretion by AJ3.1 cells was 480ng/24h/10⁶ cells (single transfection) or 300ng/24h/10⁶ cells (co-transfection), compared with N2a cells at 430ng/24h/10⁶ cells.

Peak expression of IL-2 by AJ3.1 cells was 190ng/24h/10⁶ cells (single transfection) or 230ng/24h/10⁶ cells (co-transfection), while N2a cells expressed 146ng/24h/10⁶ cells. Five days after transfection, AJ3.1 cells expressed 9ng/24h/10⁶ cells (single transfection) or 12ng/24h/10⁶ cells (co-transfection), while N2a cells expressed 71ng/24h/10⁶ cells (Figure 4.7B). While there was some variation between experimental replicates, AJ3.1 cells demonstrated expression of both IL-2 and IL-12 that was high and comparable with the levels obtained from transfected N2a cells. This level of cytokine secretion was known to be in the therapeutic range from previous experiments with N2a cells (Siapati *et al.*, 2003), so was suitable for investigation *in vivo*.

4.2.2.3 Cytokine expression by transfected NIH-3T3 cells

25,000 3T3 cells were transfected with cytokine genes in 24-well plates in triplicate. Cells were co-transfected with 1µg each of pCI-IL-2 and pCI-scIL-12 using peptide 6 at a

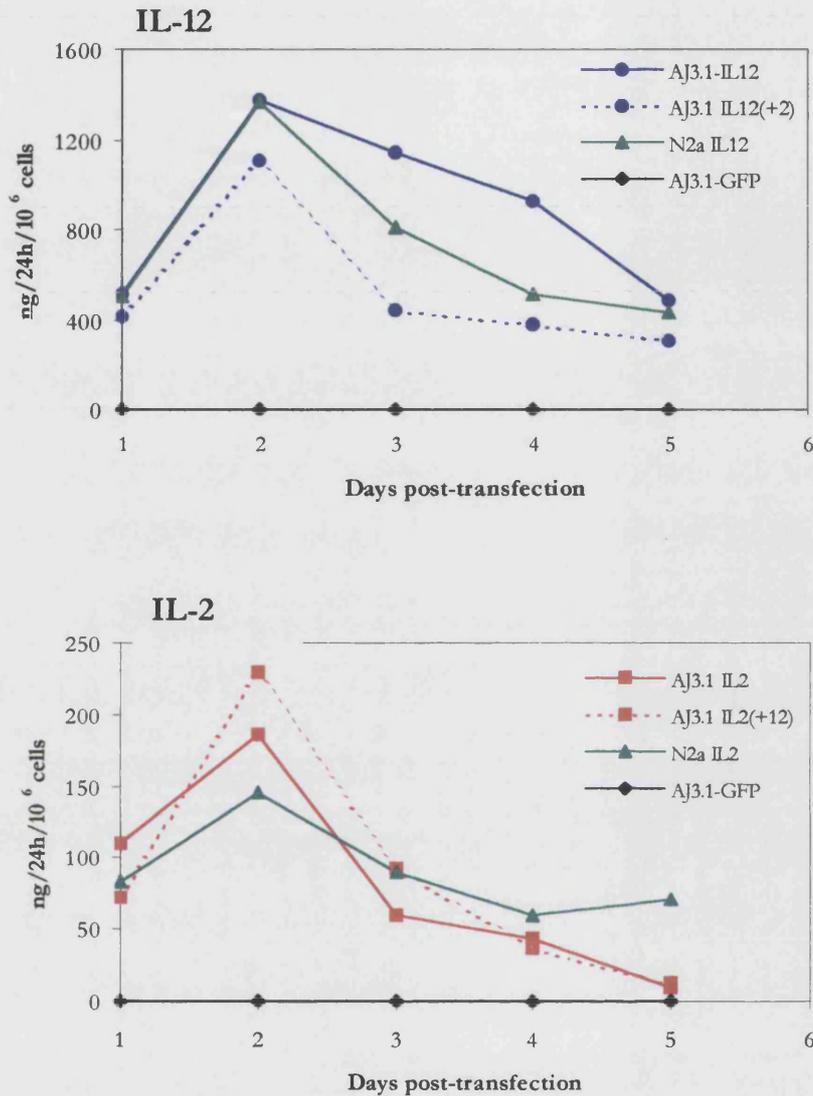


Figure 4.7 Cytokine production by transfected AJ3.1 cells compared with transfected N2a cells. AJ3.1 were transfected with LPD vector containing VYARSMN and N2a cells were transfected with LPD vector containing peptide 6. AJ3.1 cells were transfected singly with pCI-IL-2 or pCI-scIL-12 (solid lines) or co-transfected with both plasmids (dotted lines) and cytokine expression was compared with co-transfected N2a cells (green lines) or control (GFP) transfected cells (black lines). Supernatant was collected at 24-hour intervals and cytokine concentration was assayed by sandwich ELISA. Representative data is shown.

charge ratio of +7. Supernatant samples that were conditioned for 24 hours were collected after 1, 2 and 7 days and cytokine secretion analysed by ELISA (**Figure 4.8**).

The levels of IL-2 and IL-12 secreted by transfected 3T3 cells were comparable with those secreted by transfected N2a cells, which have been successfully used in our lab for *in vivo* tumour eradication experiments. Expression of IL-12 peaked at 1392ng/24h/10⁶ cells and IL-2 expression peaked at 760ng/24h/10⁶ cells. Cytokine expression was still detectable 7 days after transfection with IL-12 levels of 66ng/24h/10⁶ cells and IL-2 levels of 45ng/24h/10⁶ cells. This level of cytokine expression was in the therapeutic range achieved with N2a cells, so cytokine expression was considered sufficient for *in vivo* experiments. No cytokine expression was observed in GFP-transfected control cells.

4.2.3 Human Primary Fibroblasts

Assessment of primary dermal human fibroblasts was performed *in vitro*. Although these cells could not be used with the mouse model, it was still of great interest to optimise transfection, as these cells are analogous to what could potentially be used clinically as the NB vaccine. Normal human primary dermal fibroblasts were obtained from two healthy donors by skin biopsy (Dr Mike Hubank, personal communication), and the following experiments were performed on cells below passage 22.

4.2.3.1 Optimisation of Targeting Peptide

25,000 cells were transfected with LPD at a +7 charge ratio, a Lipofectin:DNA ratio of 0.75:1 and 2µg DNA per well for 4 hours. Peptide 6 was again compared to the peptides obtained with the phage display libraries (**Figure 4.9**). As with the AJ3.1 cells, the peptides that had been isolated on NIH-3T3 cells, VYARSMN and VNLQNPY, mediated the highest luciferase expression, which was ~3-fold higher than achieved with peptide 6.

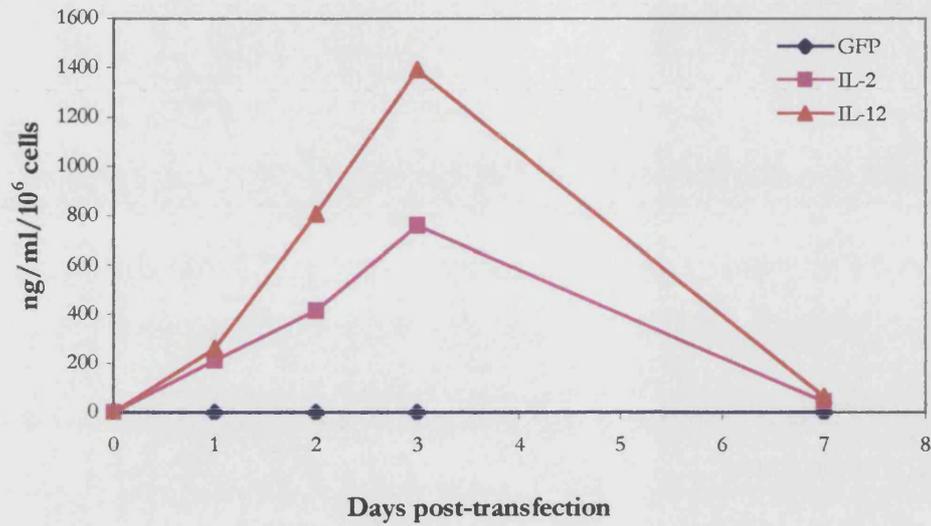


Figure 4.8 Cytokine expression of transfected NIH-3T3 cells. Cells were co-transfected with pCI-IL2 and pCI-IL12 using peptide 6 at a charge ratio of +7. Conditioned supernatant was collected for 24-hour periods and analysed by sandwich ELISA. Representative data is shown.

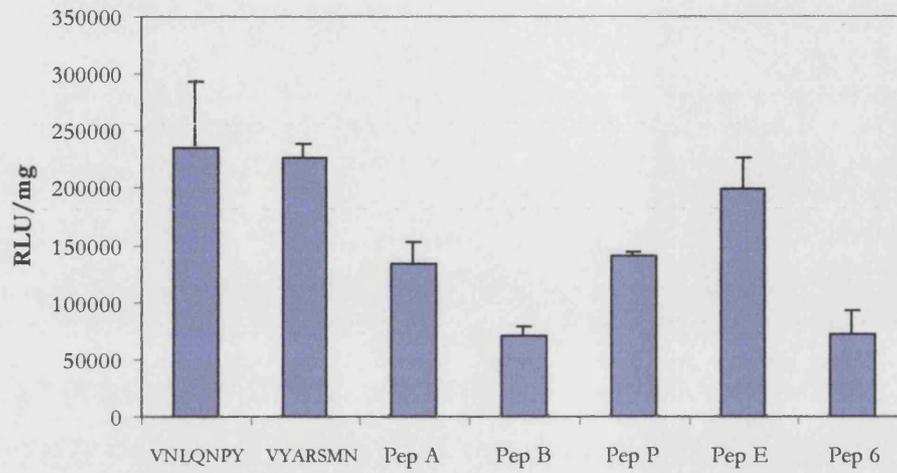


Figure 4.9 Optimisation of targeting peptide on human primary fibroblasts. Cells were transfected with LPD complexes incorporating different targeting peptides. The charge ratio (+7) and amount of DNA (1 μ g/well) remained constant. Cells were analysed 24 hours after transfection. Error bars represent the standard deviation from triplicate transfections and representative data is shown.

There was little difference between VYARSMN and VNLQNPY in many replicates of the experiment, but overall VYARSMN mediated slightly superior levels of transgene expression. The HAE-binding peptide, E, also gave high levels of luciferase expression. Peptides A and P showed a slight improvement of luciferase expression compared to peptide 6, while peptide B did not show any enhancement of transgene expression over peptide 6.

4.2.3.2 Optimisation of Lipid

A series of DOTMA analogues became available through a collaboration (Chemistry Department, UCL) to compare with Lipofectin for improvement of transgene expression. The lipid normally used in the LPD vector is Lipofectin, a commercially prepared equimolar solution of DOPE, a neutral lipid, and DOTMA, a lipid chain of 18 carbon atoms containing one unsaturated bond, with a positively charged head group. The analogues consisted of two series of lipids with a tail of 10-18 carbon atoms (C10-C18); one series contained a single unsaturated double bond, while the other series was composed of different length chains composed entirely of saturated bonds (see **Figure 2.1**, Materials and Methods). All lipids contained the same head group as DOTMA and each lipid was formulated with and without DOPE. Previous preliminary work had shown that these lipids had improved transfection when used at higher weight ratios than that used with Lipofectin. Therefore, lipid:DNA weight ratios of 2:1 or 4:1, were compared with the standard Lipofectin:DNA weight ratio of 0.75:1.

Luciferase assay revealed that the C16 lipid with the unsaturated bond (C16UN) in combination with DOPE had greatly superior transfection compared to all other formulations (**Figure 4.10**), and gave ~20-fold better higher transgene expression than Lipofectin. In virtually all formulations, the presence of DOPE enhanced transfection.

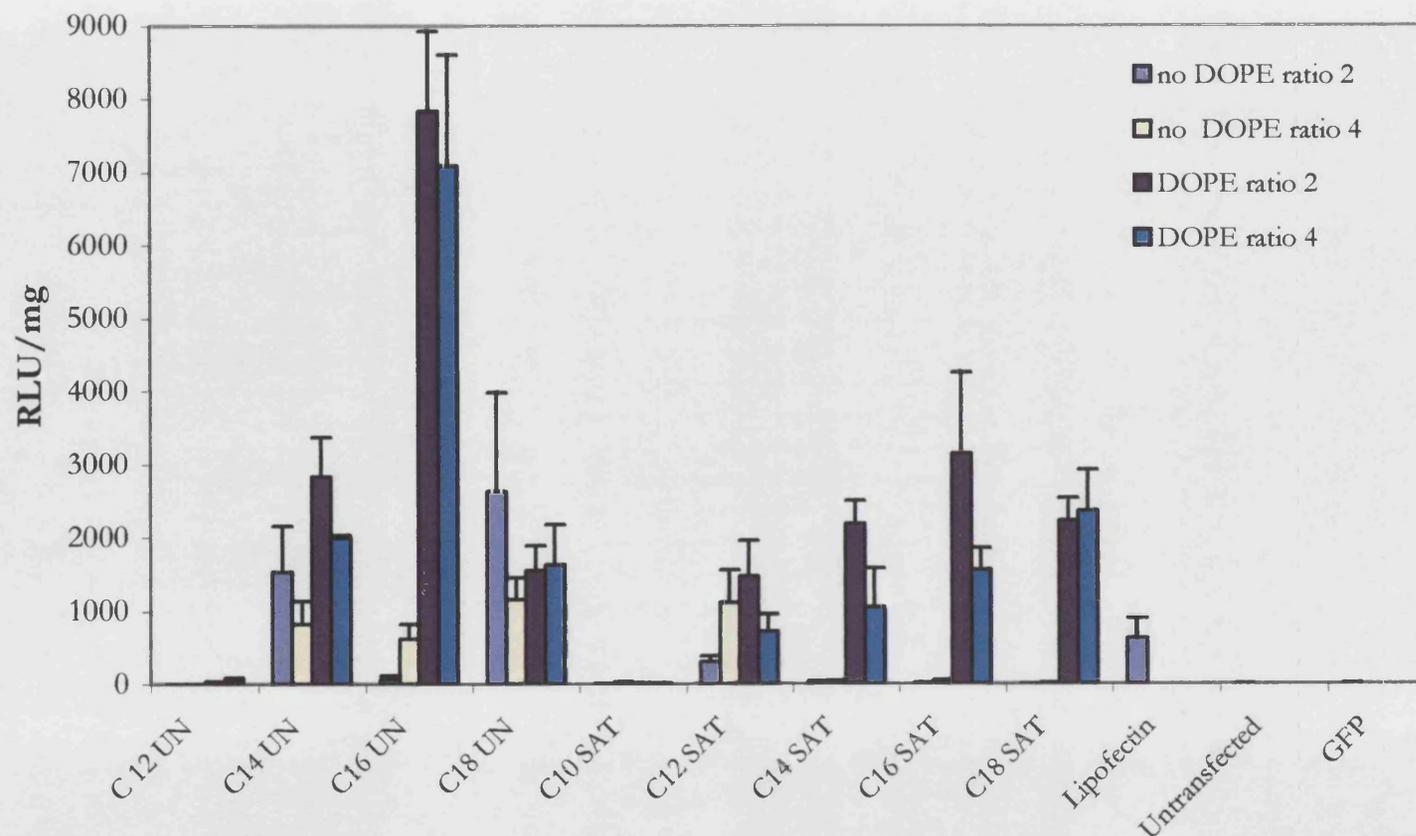


Figure 4.10 Screening of DOTMA analogues on human primary fibroblasts. Cells were transfected with pCI-Luc and peptide 6 at +7 charge ratio. A series of DOTMA analogues with chain lengths of 10-18 carbon atoms, with or without an unsaturated double bond at position X, and with or without the neutral lipid DOPE, were compared with Lipofectin. The Lipofectin:DNA ratio was 0.75:1, while the lipid:DNA weight ratio of the analogues was 2:1 or 4:1. Cells were analysed by luciferase assay 24 hours after transfection and the error bars represent the standard deviation from six transfection wells. Representative data is shown.

The unsaturated lipids in the presence of DOPE showed increasing transfection with increasing chain length between C12 to C16, but transgene expression reduced when chain length was extended to C18. In the absence of DOPE, the unsaturated lipids still mediated some transgene expression, but the presence of DOPE generally further enhanced transfection. C16UN plus DOPE at a lipid:DNA ratio of 2:1 repeatedly gave the highest luciferase expression.

The series of saturated lipids in the presence of DOPE showed a trend of increasing transfection with increasing chain length. In the presence of DOPE, a lipid:DNA ratio of 2:1 generally gave higher luciferase expression than a lipid:DNA ratio of 4:1. Luciferase activity was virtually undetectable using the saturated lipids in the absence of DOPE except in the C12SAT lipid.

However, when C16UN + DOPE was compared with Lipofectin some weeks later, the same 20-fold increase in luciferase expression could not be observed over several experimental replicates, and transgene expression was equivalent to that mediated by Lipofectin. This could be due to the lipid oxidising, so new batches of C16UN + DOPE were tested. Again, it was not possible to replicate the 20-fold improvement of transfection that had been observed previously, and only marginal enhancement of luciferase expression was achieved compared to Lipofectin. Due to this inconsistency, it was decided to continue using Lipofectin for the following experiments.

4.2.3.3 Maintenance of Transgene Expression

Transfection with synthetic vectors is inherently transient, as the gene is not incorporated into the host genome. One factor determining how rapidly transgene expression is lost is the rate at which the cells divide, as the amount of plasmid will be shared between each

daughter cell. Primary human fibroblasts divide more slowly than many immortalised cell lines, so it was of interest to investigate the duration of transgene expression, which was determined by transfecting the cells with GFP and analysis by FACS.

Human fibroblasts were transfected with pEGFP-N1 using Lipofectin and peptide VYARSMN at a charge ratio of +7. Transfected cells were analysed by FACS, which showed the transfection efficiency to be 34% 48 hours post-transfection (**Figure 4.11A**). This is high for primary cells transfected with a synthetic vector, when compared with <5% for primary A/J DC or primary A/J dermal fibroblasts or 40% for primary human umbilical vascular endothelial cells (M. Jacobsen, personal communication). It is also higher than the efficiency achieved in some cell lines such as the human SHSY5Y NB cell line that demonstrated 25% transfection efficiency (E. Siapati, PhD thesis, 2001). Eight days after transfection 28% of human fibroblasts were GFP-positive, and 14 days post-transfection, 19% were GFP-positive.

4.2.3.4 Cytokine Expression by Transfected Human Fibroblasts

To determine if cytokine expression could be maintained, cells were transfected with pCI-IL-2 or pCI-IL-12 alone, or co-transfected with both plasmids using peptide VYARSMN and Lipofectin at a charge ratio of +7. Conditioned supernatant was harvested over 24-hour periods and analysed by ELISA (**Figure 4.11B**). Expression of both cytokines was maximal one day after transfection. The levels of IL-12 expression were similar between singly and co-transfected cells. IL-12 expression peaked at $\sim 155\text{ng}/24\text{h}/10^6$ cells 24 hours after transfection, then fell steadily to $11.5\text{ng}/24\text{h}/10^6$ cells and $9.6\text{ng}/24\text{h}/10^6$ cells for singly and co-transfected cells respectively 14 days after transfection.

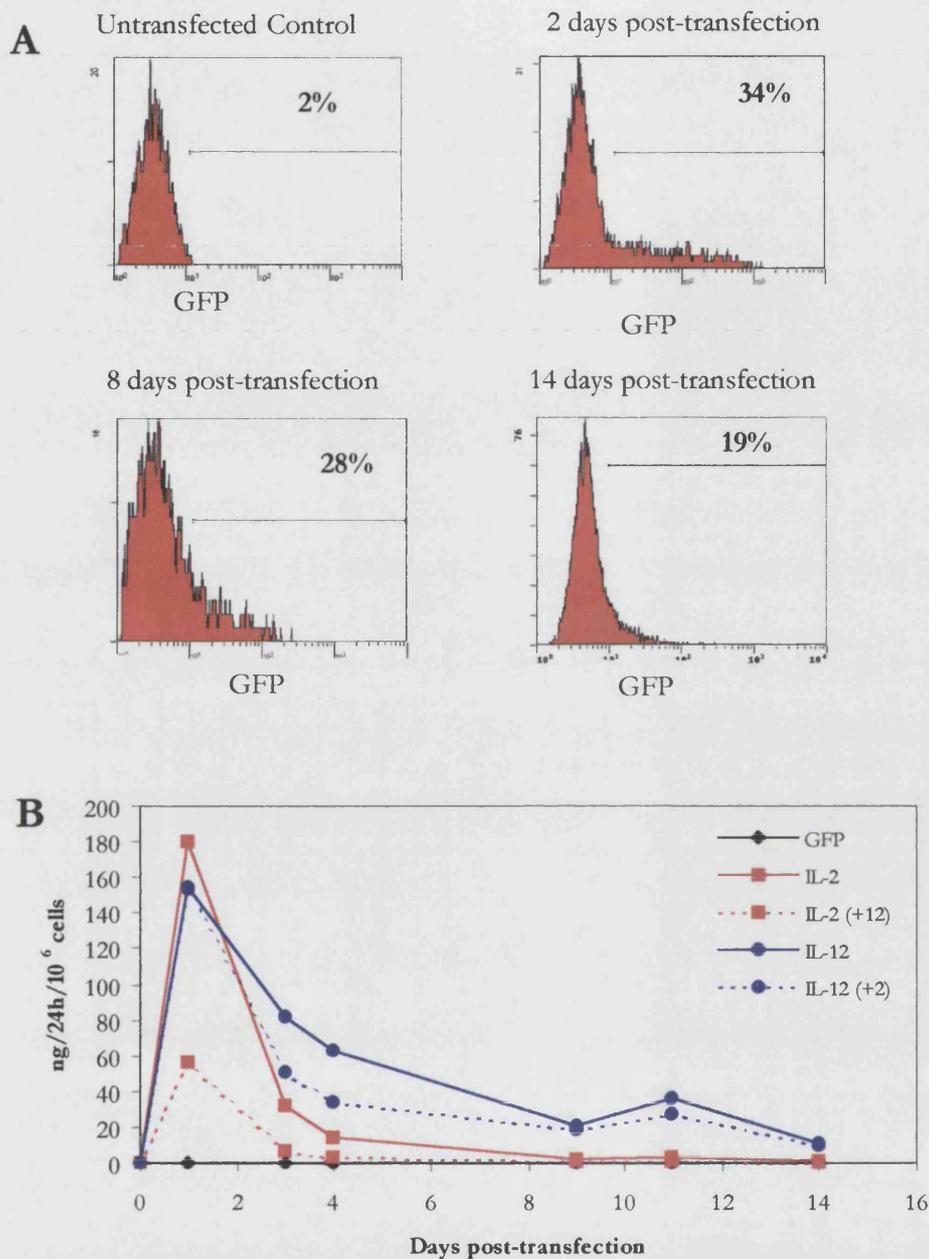


Figure 4.11 Maintenance of transgene expression by primary human dermal fibroblasts. (A) Cells were transfected with pEGFP-N1 using peptide VYARSMN at a charge ratio of +7 and analysed by FACS 2, 8 and 14 days post-transfection. Viable cells were gated on forward and side scatter characteristics and 10,000 live events were captured. (B) Cells were transfected with pCI-IL2 or pCI-IL12 alone (solid lines), or co-transfected with pCI-IL2 and pCI-IL12 (dotted lines). Conditioned supernatant was harvested over 24-hour periods and analysed by sandwich ELISA. Representative data is shown.

Expression of IL-2 from singly transfected cells peaked at 180ng/24h/10⁶ cells, while co-transfected cells peaked at 56ng/24h/10⁶ cells. Expression levels declined rapidly to <3ng/24h/10⁶ cells for either singly or co-transfected cells by 9 days after transfection, and trace levels could just be detected 14 days following transfection (1.2ng/24h/10⁶ cells for singly transfected cells and 0.52ng/24h/10⁶ cells for co-transfected cells).

4.3 DISCUSSION

Primary autologous tumour cells from patient biopsies can be difficult to manipulate in culture or sufficient tumour cells may not be available, limiting their use as cancer vaccines. An alternative approach would be to use fibroblasts transfected to express pro-inflammatory cytokines. Therefore, the aims of the work in this chapter were to optimise the LPD vector for the transfection of murine and human fibroblasts and determine the expression of IL-2 and IL-12 mediated by the optimised vector.

Two syngeneic types of fibroblast were considered for potential use in a NB vaccine; primary A/J dermal fibroblasts and a mouse embryonic fibroblast line, AJ3.1, which was established from a 13.5dpc A/J embryo. The allogeneic fibroblasts assessed as a vaccine were NIH-3T3 fibroblasts, which are derived from out-bred NIH-Swiss mice.

Initially transfection of A/J primary fibroblasts, AJ3.1 and NIH-3T3 cells with the LPD vector was optimised and compared with N2a cells. Peptide 6 was the optimal peptide for A/J primary fibroblasts and NIH-3T3 cells. For transfection optimisation of AJ3.1 cells, a series of targeting peptides identified by phage display was available to compare with the integrin-targeting peptide 6. These new peptides were incorporated into the cyclic head group of the targeting peptide attached to the [K]₁₆-DNA-condensing tail. A novel

peptide, VYARSMN, which targets an unknown receptor, mediated optimal transfection in AJ3.1 cells, and was used in subsequent transfections.

Transfections with GFP determined the transfection efficiency of the optimal LPD vector in each fibroblast population. A/J primary dermal fibroblasts had a transfection efficiency of <5%. In comparison the fibroblast cell lines showed consistently much higher transfection efficiencies of >20% for AJ3.1 cells and ~45% for NIH-3T3, which is consistent for other cell lines transfected with the LPD vector. The human NB lines SHSY5Y and IMR-32 showed transfection efficiencies of 25% and 55% respectively, and N2a cells transfect with an efficiency of 40% (E. Siapati, PhD thesis, 2001).

An advantage of the LPD vector is that transfection of two genes can be easily achieved by one vector. Fibroblasts were co-transfected with the IL-2 and IL-12 genes using their respective optimal LPD formulations, and cytokine expression was compared with transfected N2a cells, which express quantities of cytokine that mediate antitumour effects against established NB tumours (Siapati *et al.*, 2003). Autologous A/J primary dermal fibroblasts expressed undetectable levels of IL-2 and very low amounts of IL-12 that were substantially below the levels secreted by cytokine-transfected N2a cells. However, the AJ3.1 fibroblasts and NIH-3T3 fibroblasts expressed high levels of cytokine when transfected with pCI-IL2 and pCI-IL12, which were equivalent to the cytokine-transfected N2a cells, and were higher than mediated by other synthetic vectors (Chong *et al.*, 1998; Lode *et al.*, 1999; Larchian *et al.*, 2000; Pertl *et al.*, 2001). Furthermore, cytokine expression was superior to retroviral and HSV expression systems (Duda *et al.*, 2000; Tahara *et al.*, 1995; Arienti *et al.*, 1994; Toda *et al.*, 2000) and comparable to the high levels achieved with adenoviral vectors (Emtage *et al.*, 1999).

Transfection of human primary dermal fibroblasts showed that, as with the murine AJ3.1 fibroblasts, peptide VYARSMN consistently gave the highest luciferase expression. Cell surface variation between individuals may affect the targeting and transfection of the LPD vector. However, transfection efficiency of primary fibroblasts from two donors was similar, suggesting the LPD vector is not specific to individuals and will transfect cells from different donors, which is important in the development of autologous vaccines.

The next parameter optimised was the lipid component. At this point in the project, a series of DOTMA analogues became available as part of a collaboration (H. Hailes, Department of Chemistry, UCL), and these were compared to Lipofectin. The C16UN+DOPE formulation at a 2:1 weight ratio with DNA initially showed the highest luciferase expression, ~20-fold higher than Lipofectin at the standard 0.75:1 DNA weight ratio. Under identical experimental conditions, this result was replicated, although in following experiments, results were inconsistent. However, it shows in principle that different lipids have the potential to considerably improve transfection and further lipid development may further enhance transgene expression by LPD vectors. The C16UN+DOPE lipid's initial improvement of transfection may be mediated by reducing endosomal DNA degradation, perhaps by fusing more rapidly with the endosome membrane than Lipofectin, releasing the peptide and DNA before it has been degraded.

Transfection efficiency of human primary fibroblasts was assessed by transfection with GFP, and peaked at >30% two days after transfection. 19% of cells remained GFP-positive 14 days post-transfection, demonstrating that a non-integrating synthetic vector can mediate sustained gene expression in human primary fibroblasts.

IL-2 and IL-12 production by human fibroblasts peaked 24 hours after transfection, and encouragingly, expression could still be detected 14 days later. The amount of IL-2 secreted by these cells compared well with retrovirally transduced autologous dermal fibroblasts used in a phase I trial that delivered up to 800 units (up to 30.4ng) of IL-2 (Sobol *et al.*, 1999). Increases in tumour CTL precursors were observed in some patients (Sobol *et al.*, 1999), suggesting that the IL-2 levels achieved by LPD transfection are sufficient to mediate immunological responses. The IL-12 levels achieved in human fibroblasts with LPD transfection were also equivalent to those observed in primary human fibroblasts retrovirally transduced to express IL-12 (Park *et al.*, 1997). The subsequent phase I trial by the same group administered fibroblasts expressing doses of IL-12 of up to 5000ng /24h by increasing the number of fibroblasts inoculated (Kang *et al.*, 2001), a strategy easily applied to LPD-transfected fibroblasts.

Although IL-2 and IL-12 expression could be detected for 14 days in the LPD-transfected fibroblasts, after 4 days secretion of IL-2 decreased to around a tenth of peak expression, while IL-12 expression decreased by more than 50%. There is evidence that IL-2 can switch off some viral promoters, including CMV, (Spack and Sorgi, 2001) and thus highly active mammalian promoters such as the chicken β -actin (CBA) promoter may prolong expression of the transfected cytokine genes. Alternatively, changes to the cytokine gene itself may augment expression. The 3' UTR of most cytokine genes contain AU-rich elements, which confer mRNA instability. Deletion of this region was shown to significantly increase expression of several human and murine cytokines in murine cell lines, murine skin and serum, and human primary lymphocytes (Yang *et al.*, 2004).

Autologous dermal fibroblasts retrovirally-transduced to express cytokines have been used in phase I trials to treat adult solid malignancies (Sobol *et al.*, 1999; Kang *et al.*, 2001) as an

alternative cytokine delivery “vehicle” to gene-modified primary tumour cells. The availability of a population of pure primary tumour cells, together with the difficulty of expanding and manipulating these cells in culture limits their use in vaccines. However, dermal fibroblasts may overcome the problems associated with primary tumour cells. They are straightforward to culture from skin explants, and as shown in these experiments are readily transfected with synthetic vectors to co-express two cytokines at levels comparable to viral gene transfer systems.

Clinically, it may be advantageous to develop cytokine-expressing bystander allogeneic cell lines to treat all patients, removing the need to culture cells from individual patients. These bystander lines could be mixed with unmodified autologous tumour, circumventing the need to modify autologous cells, while providing pro-inflammatory cytokines in the presence of tumour antigen. The allogeneic cells themselves may mediate inflammatory reactions, enhancing tumour rejection. A phase I trial of GM-CSF-expressing allogeneic pancreatic tumour cells to treat pancreatic adenocarcinoma showed systemic antitumour immune responses against autologous tumour and increased disease-free survival time in some patients (Jaffee *et al.*, 2001). However, the host adaptive immune response against the allogeneic cells may prevent repeated dosing, limiting the use of this method.

In conclusion, human primary dermal fibroblasts can be transfected with high efficiency, using the LPD vector and cytokines are expressed at comparable levels to cells transduced with retroviral vectors. LPD-transfected syngeneic and allogeneic murine fibroblast lines expressed levels of cytokine that have previously been shown to mediate therapeutic immunological effects *in vivo* and cytokine expression was maintained for several days in both cell types. Furthermore, these levels are equivalent or superior to those mediated by several types of viral vector. Therefore they may provide a suitable alternative to

autologous cytokine-transfected tumour cell vaccines and will be tested in the treatment of experimental murine NB tumours *in vivo* using the A/J mouse model.

CHAPTER 5

IN VIVO *IMMUNOTHERAPY*

5.1 INTRODUCTION

The model of neuroblastoma used in this project is the A/J mouse and syngeneic Neuro-2a (N2a) neuroblastoma cell line, and is acknowledged to be a good model of the disease, as described in chapter 1 (section 1.7).

The therapeutic effectiveness of IL-2 and IL-12 expressing N2a cells, reducing tumourigenicity and mediating regression of established tumours with this model has been demonstrated previously (Siapati *et al.*, 2003). The following experiments were conducted to compare the antitumour efficacy of N2a cells and fibroblasts, each co-transfected with IL-2 and IL-12. Initially, the amount of IL-2 and IL-12 required to prevent tumour engraftment was titrated *in vivo* using transfected N2a cells. This will also clarify whether the cytokine-transfected murine fibroblasts are expressing sufficient IL-2 and IL-12 to mediate therapeutic effects.

Tumourigenicity experiments were performed to investigate the ability of cytokine-transfected fibroblasts (allogeneic and syngeneic) to inhibit the development of wild-type NB tumours, and eradication experiments studied whether cytokine-transfected fibroblasts (syngeneic only) were able to mediate regression of established NB tumours. *In vitro* investigation of immune memory responses was also explored.

5.2 RESULTS

5.2.1 *In vivo* cytokine dosing

IL-2 and IL-12 transfected N2a cells express levels of cytokine that prevent engraftment of N2a tumour cells in A/J mice (Siapati *et al.*, 2003). However, the actual threshold of IL-2 and IL-12 required to prevent tumour engraftment in this model had not been

established. Determination of these levels would confirm whether cytokine production from the murine fibroblasts is above the threshold required for stimulation of immune responses *in vivo*.

5.2.1.1 Effect of cytokine dose on N2a tumourigenicity

To determine the cytokine concentration necessary to prevent tumour engraftment, a tumourigenicity experiment was performed. Groups of mice were inoculated subcutaneously with 10^6 wild type N2a together with 10^3 - 10^6 cytokine-transfected N2a as a single vaccine (n=6 per group). A control group (n=6) received wild type N2a only. The maximum amount of cytokine expressed was 2350ng IL-12 + 400ng IL-2/24h from 10^6 N2a transfectants, down to a minimum of 2.3ng IL-12 + 0.4ng IL-2/24h by a dose of 10^3 transfected N2a cells. Mice were monitored every 2-3 days for tumour engraftment.

The mice receiving the highest dose of 10^6 transfected cells (2350ng IL-12 + 400ng IL-2/24h) were fully protected against tumour engraftment for over 30 days. The mice receiving 10^5 cells corresponding to 235ng IL-12 + 40ng IL-2/24h, and therefore only one tenth of the amount of each cytokine, remained tumour free for up to 30 days, when one of six mice developed a tumour (**Figure 5.1A**). Mice receiving 5×10^4 transfected N2a cells corresponding to 118ng IL-12 + 20ng IL-2/24h protected 83% (5 of 6) mice from tumour engraftment until day 30, when a second mouse developed a tumour. Even a dose of 23.5ng IL-12 + 4ng IL-2/24h, which is 100-fold less than the maximum dose, was sufficient to prevent tumour engraftment in 50% of mice for over 30 days. All control mice had developed tumours within 11 days.

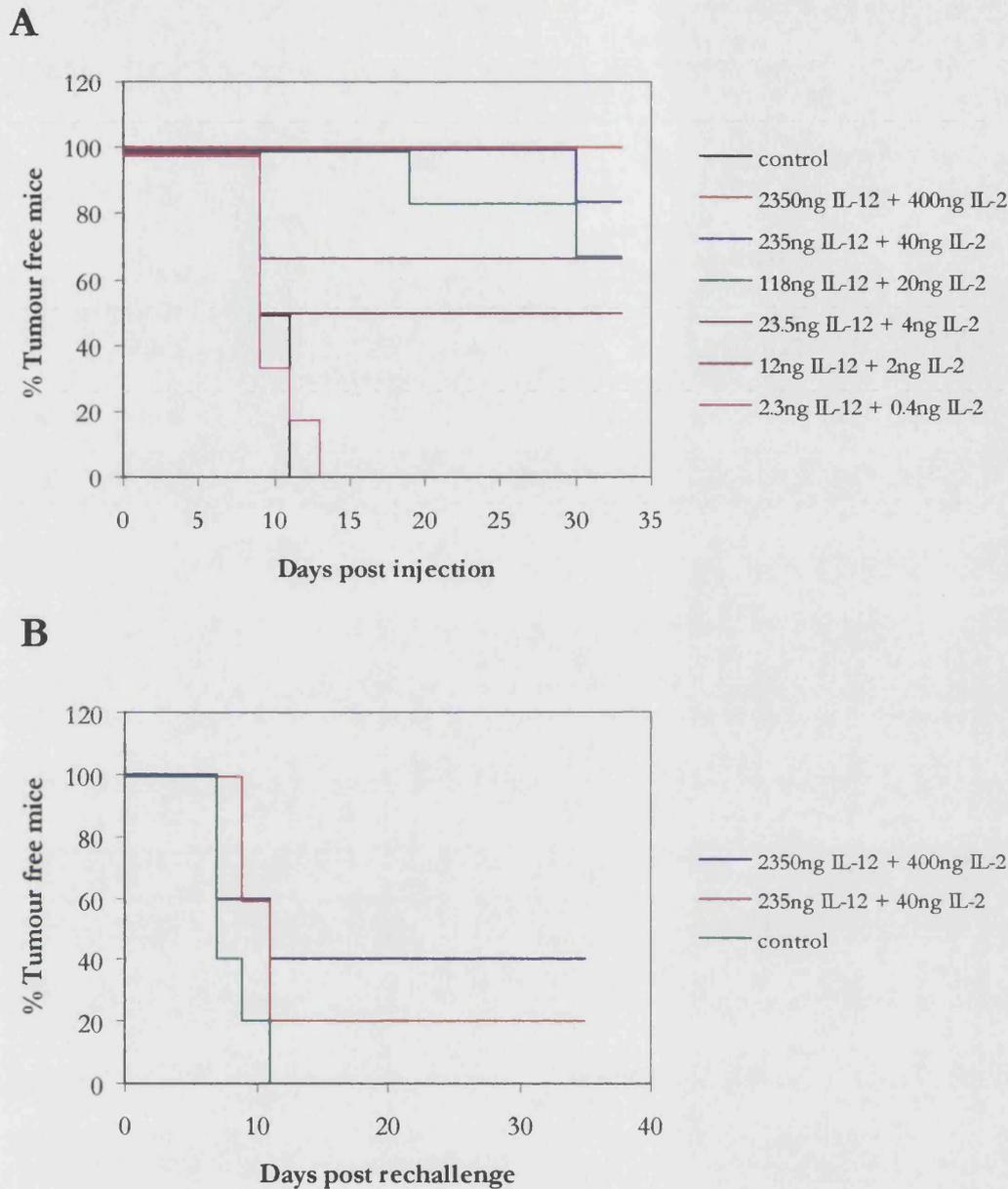


Figure 5.1 *In vivo* titration of cytokine dose required to inhibit tumour engraftment. **(A)** Mice ($n=6$ per group) received one vaccination s.c. into the left flank containing 10^6 wild type N2a cells in combination with 10^6 - 10^3 cytokine-transfected N2a cells corresponding to different doses of cytokine, as shown in the graph legend. Control mice received N2a cells only. Mice were checked for tumour engraftment every 2-3 days and a dose-dependent response can be observed.

(B) Five tumour free mice from the two highest cytokine dose groups were subsequently rechallenged with 10^6 wild type N2a cells in the opposite flank from the original vaccination. Control mice received N2a cells only and mice were checked for tumour growth every 2-3 days. Mice receiving the highest cytokine dose appeared to have slightly superior systemic antitumour immunity.

5.2.1.2 Effect of cytokine dose on systemic immunity

Five of six mice in each group that received the two highest doses of IL-2 and IL-12-expressing N2a cells remained tumour free seven weeks after inoculation. These tumour-free mice were rechallenged with 10^6 wild type N2a cells in the opposite flank to determine if systemic immunity to N2a cells had been established. Two of five mice (40%) which initially received the highest cytokine dose remained tumour free. One of five mice (20%) receiving the next highest cytokine dose also remained tumour free (**Figure 5.1B**) demonstrating that antitumour immunity had been established in a proportion of the mice. These mice remained tumour free for over 35 days. Mice that developed tumours did not show any substantial delay in tumour engraftment or growth compared to controls.

5.2.2 Allogeneic Fibroblasts

5.2.2.1 Tumourigenicity experiment using irradiated NIH-3T3 cells

Previous work has shown that injection of 10^6 wild-type N2a cells consistently results in tumour engraftment in 100% of animals in the absence of any therapeutic intervention (E. Siapati, PhD thesis, University of London, 2001), and this cell dose was used for all *in vivo* experiments. Live N2a cells in RPMI were injected alone or in combination with either 10^6 irradiated wild-type NIH-3T3 cells, 10^6 irradiated NIH-3T3 cells transfected with empty vector (pCI) or 10^6 irradiated NIH-3T3 cells transfected with pCI-IL2 and pCI-scIL12 s.c. Cytokine expression of transfected cells is shown in appendix 5A.

It was hypothesised that all the mice except those receiving the NIH-3T3-IL2-IL12 cells would develop tumours. However, all the groups of NIH-3T3 cells appeared to exert a non-specific antitumour effect whether the NIH-3T3 cells were untransfected, transfected with empty vector or transfected with cytokine genes as virtually all the mice receiving

these cells failed to develop tumours. All control mice receiving only N2a cells developed aggressive tumours within 10 days (Figure 5.2).

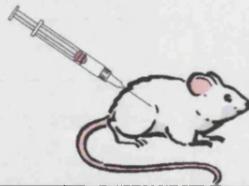
83% of mice receiving irradiated NIH-3T3 cells and 100% of mice receiving NIH-3T3-pCI and NIH-3T3-IL2-IL12 remained tumour-free for over 40 days (Figure 5.3). Any mass that was palpable under the skin was investigated by dissection, and appeared to be avascular fibrous, scar-like tissue. No tumour tissue was detectable in the mice injected with the NIH-3T3 vaccines, with the exception of one mouse in the untransfected NIH-3T3 group. The growth of this tumour was delayed by 14 days.

5.2.2.2 Tumourigenicity experiment using live NIH-3T3 cells

To establish if the presence of apoptotic irradiated NIH-3T3 cells caused the failure of engraftment of the N2a cells, the experiment was repeated, with live, non-irradiated NIH-3T3 cells. Again, all mice were inoculated with 10^6 live wild type N2a cells in combination with 10^6 cytokine-transfected N2a, 10^6 unmodified NIH-3T3 cells, 10^6 cytokine-transfected NIH-3T3 cells. Control mice received N2a only. The cytokine-transfected N2a group was included as a control for tumour rejection. The NIH-3T3-pCI group was omitted to reduce the number of mice required.

All mice in the control group that received N2a cells only rapidly developed aggressive tumours. The mice which received N2a cells plus unmodified NIH-3T3 cells did not develop tumours, but had small lumps under the skin at the injection site which were revealed by dissection to resemble fibrous scar tissue. This fibrous tissue was also present in the mice receiving the cytokine-transfected NIH-3T3 cells, except for one mouse which developed a highly-vascularised, wild-type tumour similar to that observed in control mice. All of the mice receiving the combination of wild-type N2a plus cytokine-transfected N2a

A



Day 0: Transfect NIH-3T3 cells with empty pCI vector or IL-2 and IL-12

Day 1: Co-inject transfected irradiated NIH-3T3 cells with live wild-type N2a cells s.c.

Monitor Tumour Growth

B

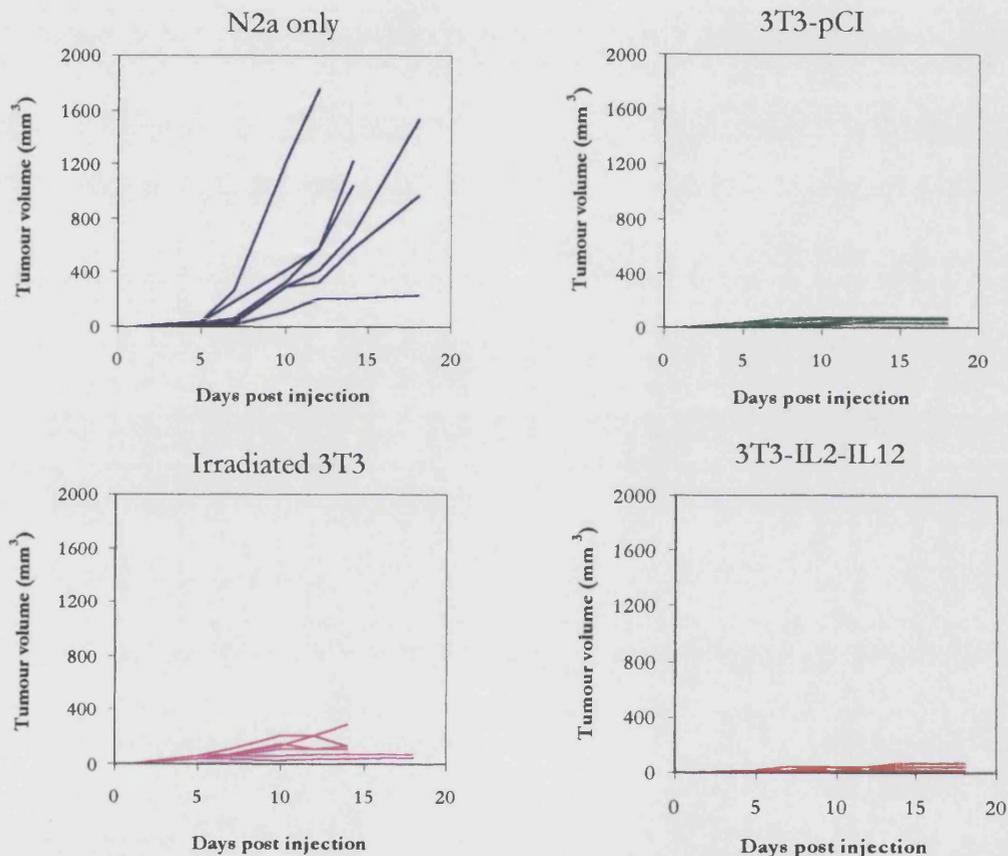


Figure 5.2 Tumourigenicity of N2a cells when co-administered with irradiated allogeneic NIH-3T3 fibroblasts. (A) Schematic representation of experimental protocol. NIH-3T3 cells were transfected *in vitro* with pCI-IL2 and pCI-IL12 or empty pCI vector. The following day animals (n=6 per group) were injected s.c. in the left flank with 10⁶ live wild-type N2a plus 10⁶ irradiated transfected or untransfected NIH-3T3 cells. Control animals received 10⁶ live N2a only.

(B) Tumour growth curves for each experimental group. Each line represents one mouse. Tumours were measured every 2-3 days with calipers and tumour volume calculated from two perpendicular axes.

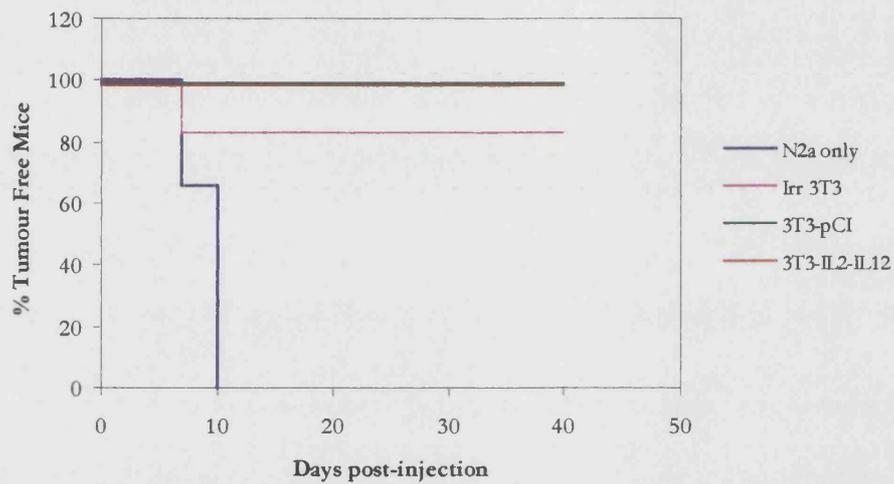


Figure 5.3 Pooled data of experimental groups in the irradiated allogeneic NIH-3T3 tumorigenicity experiment. Percentage of tumour free mice in each experimental group (n=6). All control animals developed tumours by day 10, while 83% of mice receiving irradiated untransfected NIH-3T3 and 100% of mice receiving irradiated NIH-3T3 cells transfected with cytokines or empty plasmid remained tumour free for over 40 days.

cells failed to develop any tumours (**Figure 5.4**). One mouse that received cytokine-transfected NIH-3T3 cells developed a tumour on day 13, although the onset of tumour engraftment was delayed compared with control mice which had developed tumours by day 8 (**Figure 5.5**). The remaining 83% (5 out of 6) mice in this group, and 100% of mice in the untransfected NIH-3T3 group and cytokine-transfected N2a group remained tumour free for over 40 days.

The tumour rejection pattern showed many similarities to the previous experiment, with virtually all mice vaccinated with NIH-3T3 failing to engraft N2a-derived tumours, regardless of whether or not the NIH-3T3 cells were secreting cytokines. This makes the use of allogeneic fibroblasts a poor model for therapeutic treatment of murine NB, as it is not possible to delineate any effect of the cytokines from the non-specific allogeneic effect.

5.2.3 Syngeneic Fibroblasts

5.2.3.1 Tumourigenicity experiment using live transfected AJ3.1

As allogeneic fibroblasts were not suitable for demonstrating a cytokine-mediated anti-tumour immune effect, it was decided to proceed with syngeneic fibroblasts. For these experiments the AJ3.1 cell line derived from A/J embryos was used, which was shown to secrete high amounts of cytokine when transfected with the LPD vector. To determine whether cytokine-expressing AJ3.1 fibroblasts could prevent engraftment of N2a derived tumours, tumourigenicity experiments were performed (**Figure 5.6A**). Mice ($n=6$ per group) received one subcutaneous injection containing 10^6 wild-type N2a together with 10^6 wild-type AJ3.1, 10^6 AJ3.1-IL2-IL12 or 10^6 N2a-IL2-IL12. Control mice received 10^6 N2a only. The untransfected AJ3.1 group was included to determine whether the fibroblasts themselves exerted a protective effect, and the transfected N2a group was included as a

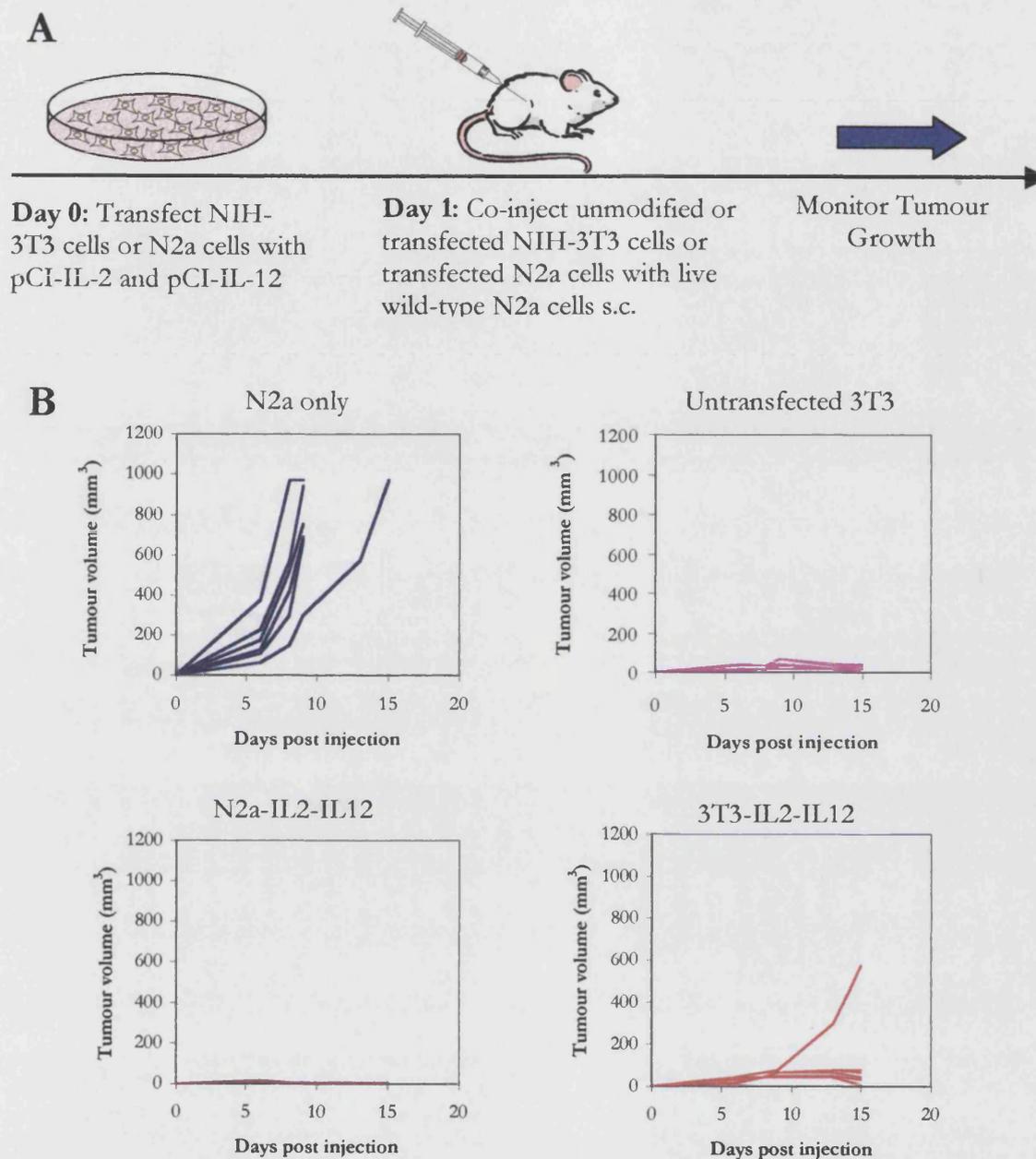


Figure 5.4 Tumourigenicity of N2a cells when co-administered with live allogeneic NIH-3T3 fibroblasts. (A) Schematic representation of experimental protocol. NIH-3T3 cells or N2a cells were transfected in vitro with pCI-IL2 and pCI-IL12. The following day animals ($n=6$ per group) were injected s.c. in the left flank with 10^6 live wild-type N2a plus 10^6 live cytokine-transfected N2a cells, 10^6 live cytokine-transfected NIH-3T3 or 10^6 live wild-type NIH-3T3 cells. Control animals received 10^6 wild-type N2a only. (B) Tumour growth curves for each experimental group. Each line represents a single mouse. Tumours were measured every 2-3 days with calipers and tumour volume calculated from two perpendicular axes.

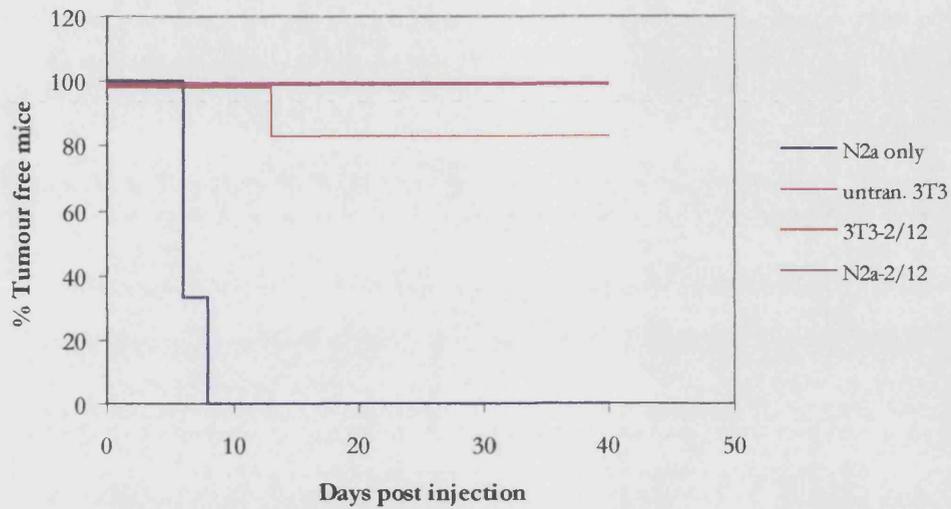


Figure 5.5 Pooled data of experimental groups from the live allogeneic NIH-3T3 tumourigenicity experiment. Percentage of tumour free mice in each experimental group (n=6). All control animals had developed tumours by day 8, while 83% of mice receiving cytokine-transfected NIH-3T3 cells and 100% of mice receiving untransfected NIH-3T3 cells or cytokine-transfected N2a cells remained tumour free for over 40 days.

control for prevention of engraftment. Cytokine expression of transfected cells used for these vaccinations is shown in appendix 5C.

The vaccines containing cytokine-transfected AJ3.1 or cytokine-transfected N2a cells prevented tumour engraftment in 5 of 6 (83%) mice in each group, demonstrating the AJ3.1 fibroblast vaccine was as effective as the N2a tumour cell vaccine in inhibiting tumour engraftment (**Figure 5.6B, Figure 5.7**). Aggressive tumours developed rapidly in all mice in the N2a-only control group, and all mice in the N2a + wild-type AJ3.1 group showed equivalent rapid development of tumours, demonstrating that fibroblasts alone did not have a protective effect. Tumour engraftment in the single mouse that developed tumour in the AJ3.1-IL2-IL12 group was delayed until day 22, compared with tumour engraftment by day 7 in control mice. In the N2a-IL2-IL12 group, a single mouse developed a tumour by day 30, compared with day 7 in control mice. The remaining 83% of mice in each of these groups remained tumour free for over 40 days.

5.2.3.2 Tumourigenicity experiment using irradiated transfected AJ3.1

Many clinical protocols state that cytokine-transfected cells would be irradiated before being administered to the patient. To establish if the tumour protective effect accomplished with live cells could be achieved with irradiated cells, the tumourigenicity experiment was repeated using irradiated cytokine-transfected AJ3.1 cells. Irradiation with 2500 rads, has previously been shown to halt the growth of cytokine-transfected N2a cells, while cytokine expression is maintained until the cells undergo apoptosis after ~7 days (E. Siapati, PhD thesis, University of London, 2001). Therefore, the same dose of γ -radiation was administered to cytokine-transfected AJ3.1 cells. Preliminary *in vitro* assay showed that irradiated cytokine-transfected AJ3.1 expressed equivalent levels of cytokine to transfected live AJ3.1 (appendix 6). Also, as one mouse in each therapeutic group in the previous

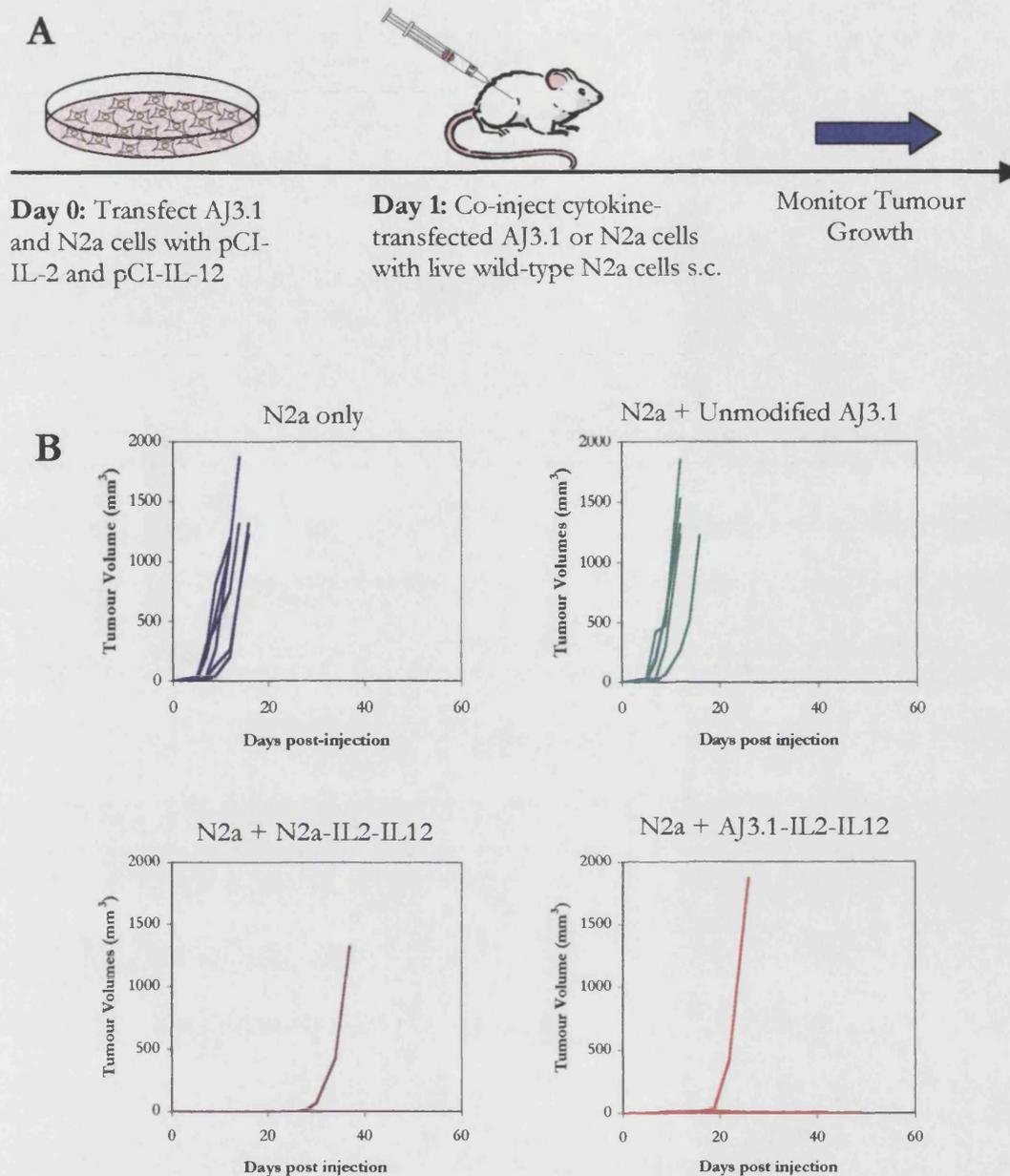


Figure 5.6 Tumourigenicity of N2a cells when co-administered with live syngeneic AJ3.1 fibroblasts. (A) Schematic representation of experimental protocol. AJ3.1 and N2a cells were transfected *in vitro* with pCI-IL2 or pCI-IL12. The following day animals ($n=6$ per group) were injected s.c. in the left flank with 10^6 live wild-type N2a plus either 10^6 cytokine-transfected N2a cells or 10^6 unmodified or cytokine transfected AJ3.1 cells. Control animals received 10^6 live N2a cells only. **(B)** Tumour growth curves for each experimental group. Each line represents one mouse. Tumours were measured every 2-3 days with calipers and tumour volumes calculated from two perpendicular axes.

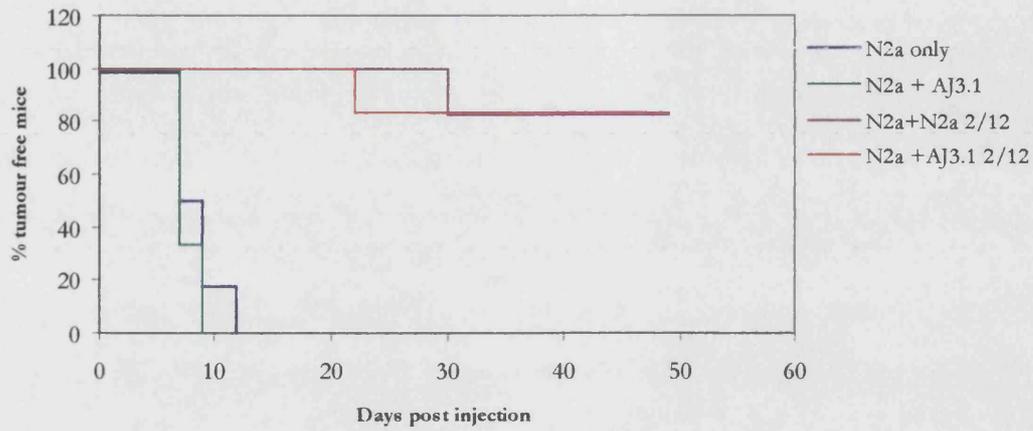


Figure 5.7 Percentage tumour free mice when wild-type N2a cells are co-administered with live AJ3.1 syngeneic fibroblasts. Percentage of tumour free mice when live N2a cells were co-administered with wild-type AJ3.1, cytokine-transfected AJ3.1 or cytokine-transfected N2a cells (n=6 per group).

experiment using live AJ3.1 cells had succumbed to a growing tumour, a group receiving a higher dose of transfected cells was included to see if this could prevent tumour engraftment in 100% of vaccinated mice.

In this second tumourigenicity experiment, mice (n=6 per group) received 10^6 wild type N2a together with either 2×10^6 irradiated AJ3.1-IL2-IL12 or 10^6 irradiated AJ3.1-IL2-IL12 in a single injection inoculated s.c. into the left flank (**Figure 5.8A**). Control mice received 10^6 wild-type N2a only. Cytokine expression by transfected cells is shown in appendix 5D.

Figure 5.8B shows that the mice receiving cytokine-expressing irradiated AJ3.1 cells demonstrated a substantial reduction in tumour engraftment compared to control mice, which all rapidly developed aggressive tumours. There also appeared to be a dose effect, as 4 of 6 mice receiving 1×10^6 AJ3.1-IL2-IL12 developed tumours, while 2 of 6 mice in the group receiving 2×10^6 AJ3.1-IL2-IL12 developed tumours. Once established, these tumours grew aggressively, with similar kinetics to those in control mice. However, the engraftment of tumours in mice receiving cytokine-transfected AJ3.1 cells was delayed compared to control mice receiving N2a cells only (**Figure 5.9**). All mice in the therapeutic groups remained tumour-free until 14 days following engraftment, while 5 of 6 control mice (83%) had developed tumours 14 days after engraftment. On day 14, 2 of 6 mice in the 1×10^6 AJ3.1-IL2-IL12 group, and 1 of 6 mice in the 2×10^6 AJ3.1-IL2-IL12 group developed a tumour, which grew rapidly requiring culling of the animals. No more tumours developed until 35 days after engraftment, when another 2 mice in the 1×10^6 AJ3.1-IL2-IL12 group developed tumours, and 41 days after engraftment, one mouse in the 2×10^6 AJ3.1-IL2-IL12 group developed a tumour. Again, once engrafted, these tumours developed aggressively, requiring culling of the animals.

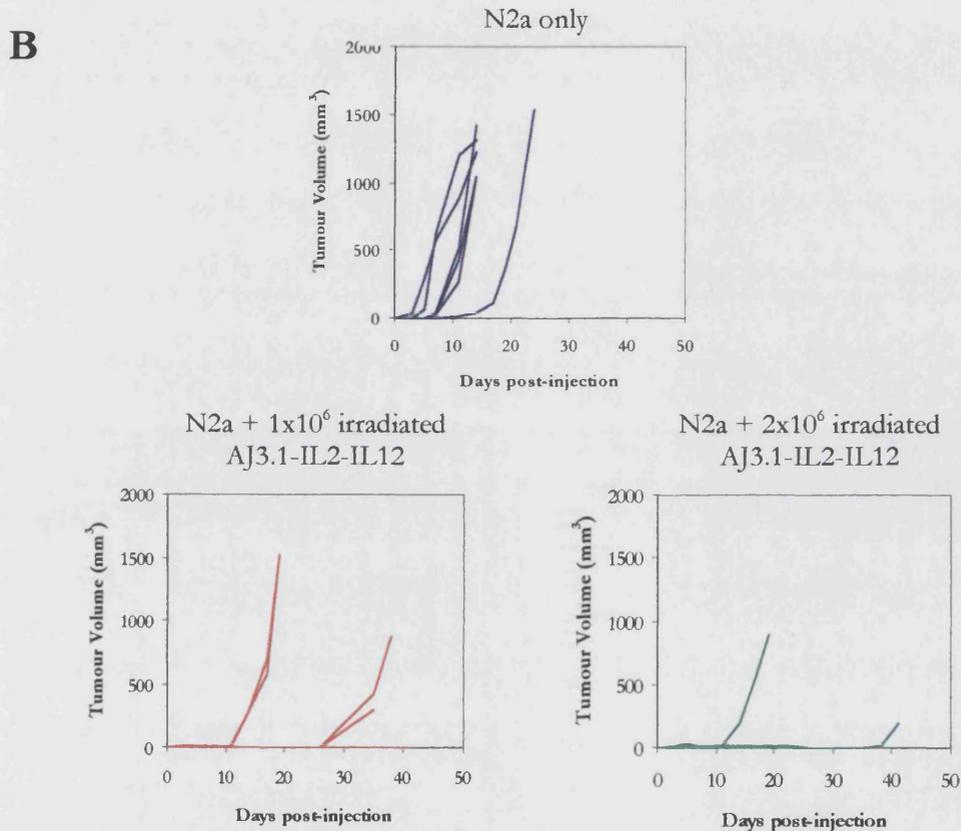
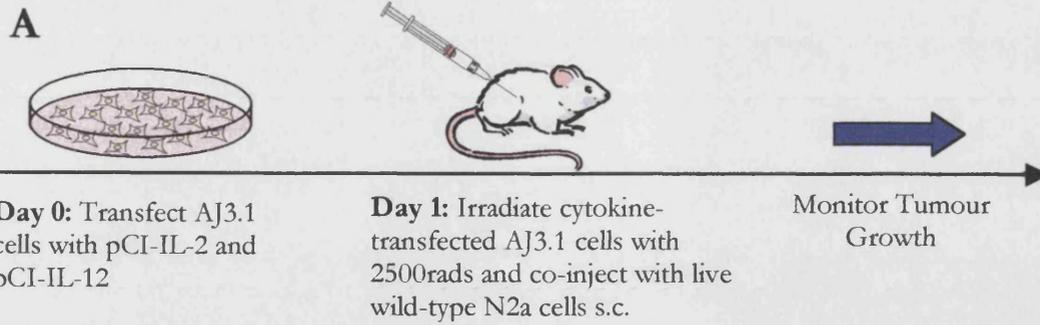


Figure 5.8 Tumorigenicity of N2a cells when co-administered with irradiated syngeneic AJ3.1 fibroblasts. (A) Schematic representation of experimental protocol. AJ3.1 cells were transfected *in vitro* with pCI-IL2- and pCI-IL12. The following day the AJ3.1 cells were irradiated with 2500 rads and animals ($n=6$ per group) were injected s.c. in the left flank with 10^6 live wild-type N2a cells plus 1×10^6 or 2×10^6 irradiated cytokine-transfected AJ3.1 cells. Control animals received 10^6 live N2a cells only.

(B) Tumour growth curves for each experimental group. Each line represents one mouse. Tumours were measured every 2-3 days with calipers and tumour volumes calculated from two perpendicular axes.

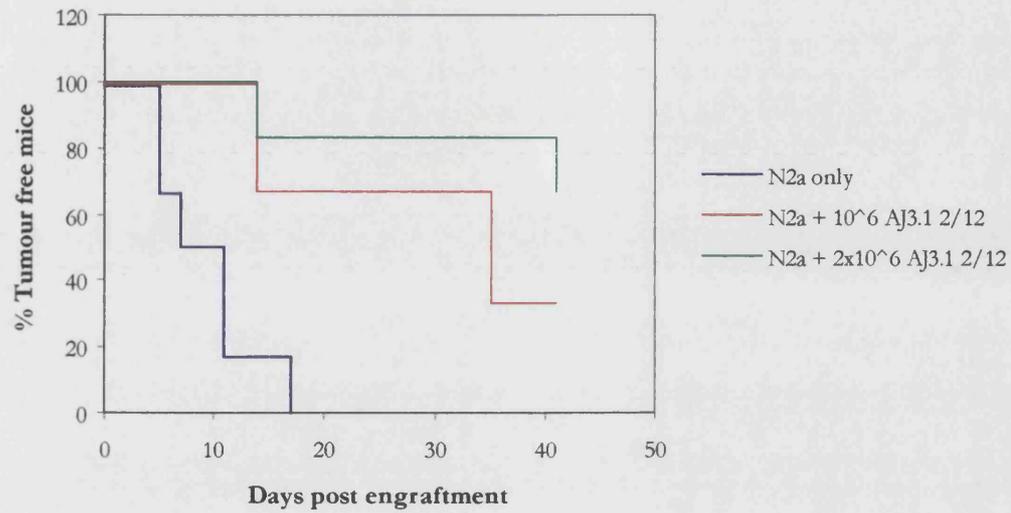


Figure 5.9 Percentage tumour free mice when wild-type N2a cells are co-administered with irradiated AJ3.1 syngeneic fibroblasts. Percentage of tumour free mice when live N2a cells were co-administered with two different doses of IL-2 and IL-12 co-transfected AJ3.1 cells. (n=6 animals per group).

The irradiated vaccine did not appear to be as effective as the live vaccine as 4 of 6 mice receiving 10^6 irradiated AJ3.1-IL2-IL12 developed tumours (**Figure 5.8B**), compared with 1 of 6 mice developing a tumour in the group receiving 10^6 live AJ3.1-IL2-IL12 in the previous experiment (**Figure 5.6**). Moreover, 2 of 6 mice receiving 2×10^6 irradiated AJ3.1-IL2-IL12 developed tumour, compared with 1 of 6 mice developing a tumour in the group receiving 10^6 live AJ3.1-IL2-IL12 in the previous experiment.

5.2.3.3 Rechallenge with wild-type N2a cells

It was important to determine if long-term systemic immunity to N2a-derived tumours had been established in the mice that had rejected N2a cells in the tumourigenicity experiments. There is a clinical relevance to the ability of mice to reject tumour cells when treatment is no longer being administered, as one goal of immunotherapy is to establish systemic protective immunity which can recognise and destroy tumour cells to prevent the formation of solid tumours. This experiment is a model for the reoccurrence of malignant cells at a site distant to that of the primary tumour.

The five remaining tumour free mice from the N2a-IL2-IL12 and AJ3.1-IL2-IL12 groups in the first AJ3.1 tumourigenicity were rechallenged seven weeks after the initial vaccination with 10^6 wild type N2a in the opposite flank to see if immune memory had been established. Naive mice ($n=5$) which had no prior exposure to tumour cells were included as a control for the engraftment potential of the wild-type N2a cells.

Two out of five mice (40%) from each of the N2a-IL2-IL12 and AJ3.1-IL2-IL12 groups remained tumour free for four weeks after the rechallenge vaccinations (**Figure 5.10A**), suggesting evidence of systemic immune memory. The three mice from each group that did develop tumours showed similar kinetics of tumour growth to the naïve mice

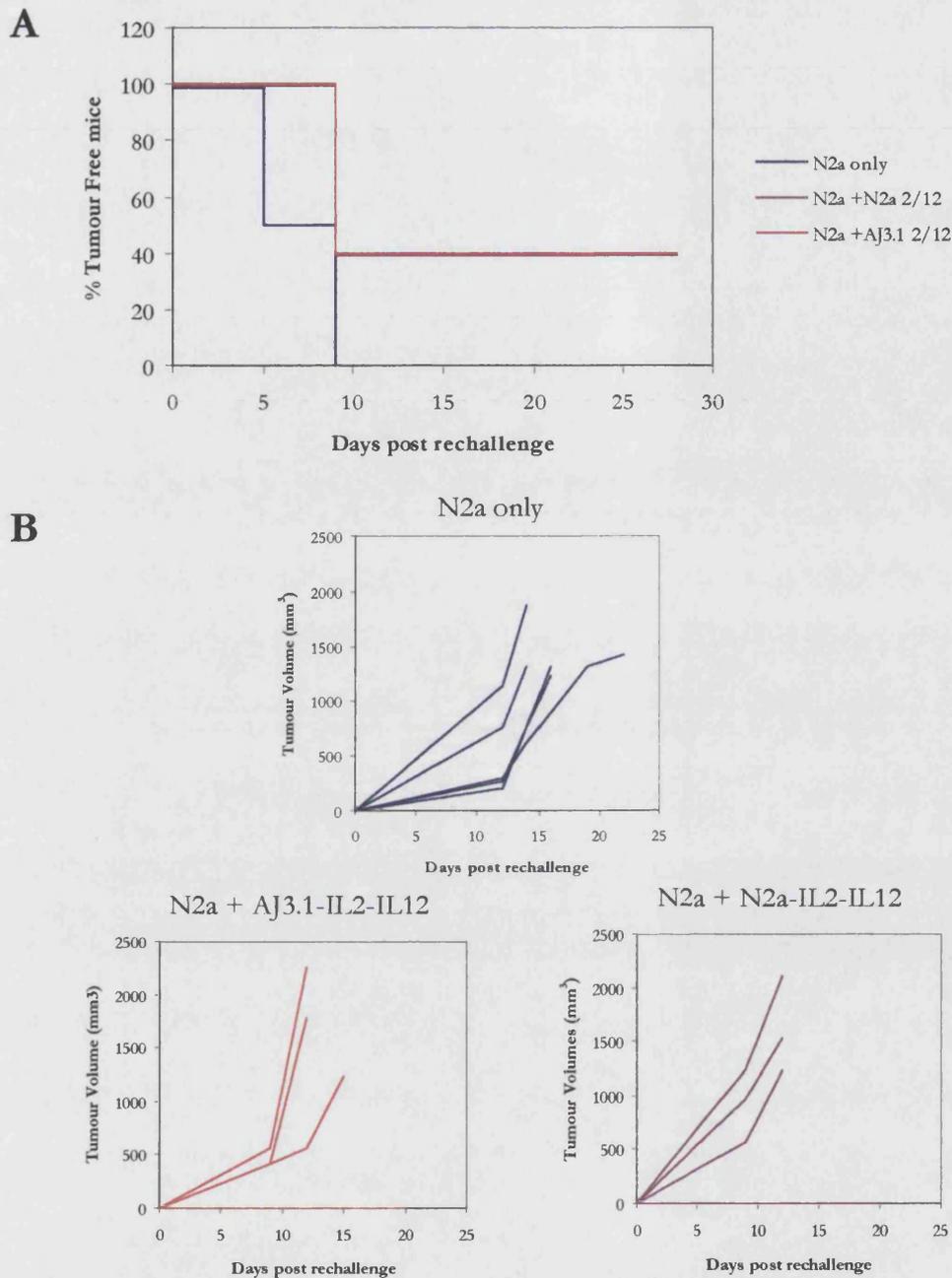


Figure 5.10 Effect of cytokine-transfected fibroblasts compared with cytokine-transfected N2a cells to generate systemic anti-tumour immunity against wild-type N2a cells. **(A)** Mice that remained tumour free 7 weeks after the vaccination with cytokine-transfected AJ3.1 or cytokine-transfected N2a cells were rechallenged with 10^6 wild-type N2a in the opposite flank from the initial vaccination. Control naïve mice received N2a cells only ($n=5$ animals per group). Tumour progression was monitored.

(B) Tumour growth curves for each experimental group. Each line represents one mouse. Tumours were measured every twice per week with calipers and tumour volumes were calculated from two perpendicular axes.

engrafted with wild type N2a cells (Figure 5.10B), suggesting insufficient systemic immunity.

5.2.3.4 *In vitro* evidence of immune memory

To characterise the antitumour immunity in the tumour-free mice, *in vitro* assays were performed to investigate cellular immune responses against wild-type N2a cells. The two mice from each group (two from the N2a-IL2-IL12 group and two from the AJ3.1-IL2-IL12 group) that remained tumour free four weeks after rechallenge were sacrificed, and splenocytes used in ELISpot assays to assess the production of pro-inflammatory cytokines in response to N2a cells. Lactate dehydrogenase (LDH) release assays were performed to assess cytotoxic responses against wild-type N2a cells. The responses of these mice were compared with two mice that had not developed a tumour after the primary immunisation, but had succumbed to secondary tumour after rechallenge. Further comparisons were made with two mice which developed primary tumours following engraftment with wild-type N2a cells and two naïve control mice that had no prior exposure to N2a cells.

5.2.3.4.1 ELISpot

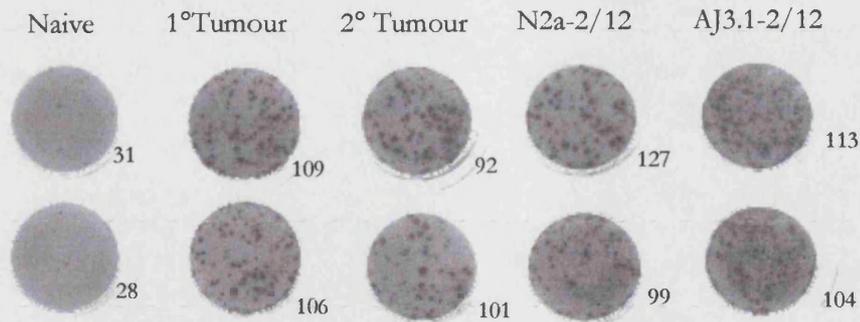
ELISpot assays to detect IL-2 and IFN- γ secreting splenocytes were performed to determine if splenocytes produced pro-inflammatory cytokines in response to co-culture with syngeneic wild-type N2a cells. Splenocytes were co-cultured with irradiated N2a in 96-well plates cells at effector (E):target(T) ratios of 100:1, 50:1, 20:1 and 10:1 in triplicate where sufficient cells were available. All experimental wells contained 5×10^3 N2a cells. Positive control wells contained splenocytes co-cultured with $5 \mu\text{g/ml}$ Concanavalin A (ConA), negative wells contained splenocytes only. Cells were incubated overnight at

37°C before being transferred to ELISpot plates pre-coated with capture antibody and incubated for a further 24 h at 37°C. Plates were developed and analysed.

Due to high background staining on the IFN- γ plate, quantification using the plate reader was not possible. The IL-2 plate had clearer, well-defined staining that was quantified using a plate reader. The positive control wells showed very dense spots, confirming the splenocytes were functional, and very few spots were present in negative control wells, indicating there was little non-specific splenocyte activation. The E:T ratio of 100:1 gave the clearest result for every animal tested, so all results discussed refer to this E:T ratio.

A striking difference was seen between the IL-2 production from naïve mice and those that had rejected tumour (**Figure 5.11A**). The number of spots present in the wells containing splenocytes from naïve mice were ~3-fold lower (32 ± 6.8 spots) than any wells that contained splenocytes from mice that had been exposed to tumour cells. The number of spots from mice that had developed primary tumours following engraftment with wild-type N2a cells (105 ± 11.6 spots) was equivalent to those that had rejected engraftment of wild-type N2a cells when co-administered with AJ3.1-IL-2-IL12 but succumbed to secondary tumour following rechallenge with wild-type N2a cells only (100 ± 7.2 spots). Mice that had rejected the primary challenge of wild-type N2a cells co-administered with AJ3.1-IL2-IL12 or N2a-IL2-IL12 and also the subsequent challenge of wild-type N2a cell only, also showed equivalent numbers of spots, with 109 ± 11.1 spots from mice receiving the N2a-IL2-IL12 vaccine and 109 ± 6.7 spots from mice receiving the AJ3.1-IL2-IL12 vaccine (**Figure 5.11B**). All mice exposed to tumour cells had significantly greater IL-2 expression compared to naïve control mice ($p < 0.001$ for all groups).

A



B

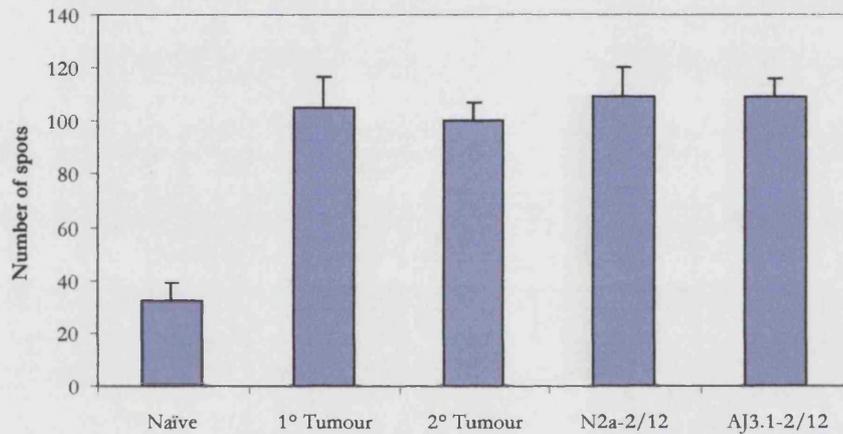


Figure 5.11 *In vitro* evidence of immune memory in vaccinated mice with IL-2 ELISpot. Wild-type N2a cells were co-cultured with splenocytes isolated from naïve mice, control tumour bearing mice (1° tumour), mice which had developed tumour following rechallenge (2° tumour) and tumour-protected mice which rejected rechallenge (N2a-2/12 and AJ3.1-2/12). **(A)** Numbers of spots representing IL-2 secreting splenocytes were quantified using a plate reader. Wells shown are of an effector:target ratio of 100:1 and are representative for each experimental group. **(B)** Average number of spots in each experimental group at the 100:1 effector:target ratio. Error bars represent standard deviations of six experimental wells. All mice exposed to tumour cells had significantly greater IL-2 expression compared to naïve control mice ($p < 0.001$ for all groups, calculated by t-test).

5.2.3.4.2 LDH Release Cytotoxicity Assay

Splenocytes from groups of mice detailed above were co-incubated with irradiated N2a at a E:T ratio of 50:1 for 6 days in the presence of 60IU rhIL-2. CTL effectors were then harvested and co-incubated with live N2a at ratios of 27:1, 9:1, 3:1 and 1:1 for 5 h and LDH release from N2a target cells quantified and used to calculate % cytotoxicity.

Results from the 27:1 and 9:1 E:T ratios gave the clearest and most consistent results and are shown in **Figure 5.12**. Naïve mice showed <2% cytotoxicity above background, and mice which had developed primary tumours also showed low levels of N2a lysis, with $5 \pm 6\%$ cytotoxicity at a E:T ratio of 9:1 and $6 \pm 8\%$ cytotoxicity at a E:T ratio of 27:1. Mice which had rejected their first tumour but had developed tumour following rechallenge had an increased level of cytotoxicity with $12 \pm 13\%$ at a E:T ratio of 9:1 and $14 \pm 6\%$ at a E:T ratio of 27:1. Mice which rejected two tumour challenges showed the highest level of cytotoxicity against N2a cells. Mice vaccinated with N2a-IL2-IL12 showed target cell lysis of $36 \pm 34\%$ at a 9:1 E:T ratio and $34 \pm 47\%$ at a 27:1 E:T ratio. Mice vaccinated with AJ3.1-IL2-IL12 showed lysis of target cells $29 \pm 30\%$ at a 9:1 E:T ratio and $39 \pm 41\%$ at a 27:1 E:T ratio (**Figure 5.12**). Due to the large standard deviations, the difference in splenocyte cytotoxicity against N2a cells between mice that rejected two tumour challenges and those that developed primary tumours ($p > 0.1$) or naïve mice ($p < 0.1$ but > 0.05) does not reach significance.

Encouragingly however, mice vaccinated with cytokine-transfected AJ3.1 fibroblasts mediated equivalent levels of cytotoxicity against wild-type N2a cells as the mice that received cytokine-transfected N2a tumour cells, demonstrating that syngeneic fibroblasts are capable of generating CTL responses against wild-type N2a cells.

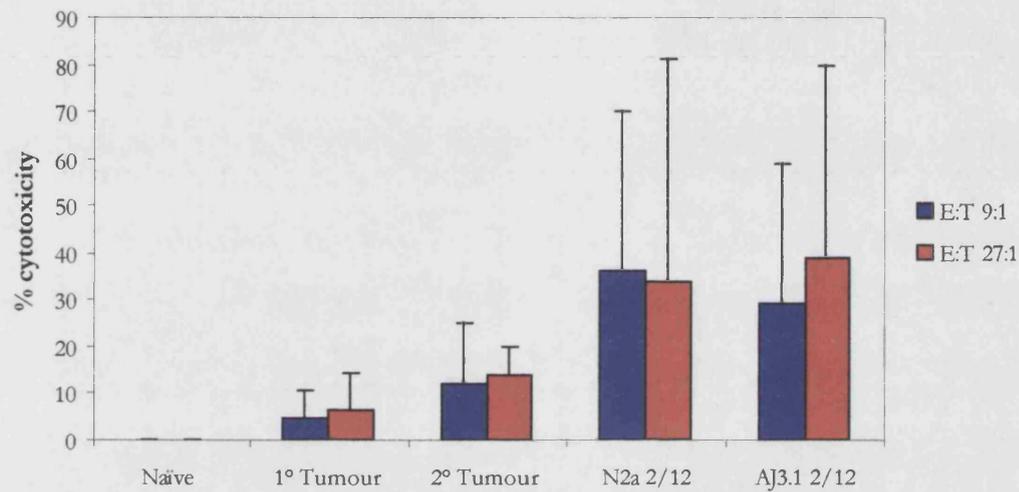


Figure 5.12 LDH release assay to show *in vitro* splenocyte-mediated cytotoxicity against N2a target cells. The ability of splenocytes to lyse N2a targets was measured by LDH release from N2a cells following co-culture with CTL effectors. Data showing average cytotoxicity values of two different effector:target ratios is representative for each experimental group. Error bars represent standard deviations of quadruplicate samples.

5.2.3.5 Treatment of established N2a tumours with transfected AJ3.1

The following experiment was performed to investigate the anti-tumour efficacy of cytokine-transfected AJ3.1 fibroblasts compared with cytokine-transfected N2a cells when multiple doses were administered to mice with established N2a-derived tumours. Due to the large numbers of cells required over a three day period for this experiment, large batches of transfected cells were prepared to be stored at -80°C . The appropriate number of cells was then thawed and cultured overnight before vaccination. Previous preliminary assays had shown that cytokine expression was maintained following freezing down and subsequent reconstitution in culture (see appendix 7).

Mice ($n=30$) were engrafted with 10^6 wild type N2a cells s.c. in the left flank. A further group of mice ($n=6$) was engrafted with 10^6 wild type N2a cells s.c. in both flanks. Tumour engraftment was checked 4 days after inoculation, and transfected cells were thawed out from storage at -80°C . On day 5, tumours were $\sim 3\text{-}4$ mm in diameter (range between $1\times 1\text{mm}$ to $5\times 5\text{mm}$) and the 30 mice engrafted with one tumour were divided into five groups of six mice, with a range of tumour sizes in each group.

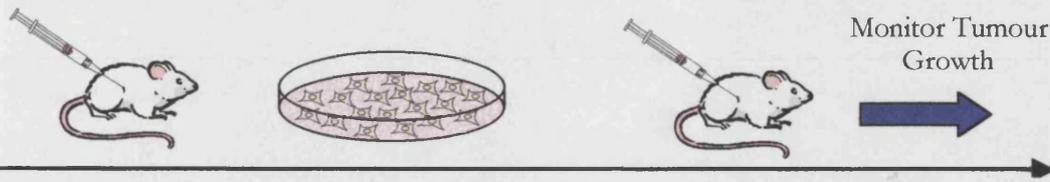
Mice ($n=6$ per group) received either 10^6 N2a-IL2-IL12 i.t., 10^6 AJ3.1-IL2-IL12 i.t., 10^6 AJ3.1-IL2-IL12 + 10^6 DC, or 10^6 AJ3.1-IL2-IL12 + 10^6 irradiated N2a. Another group was engrafted with a tumour on each flank, of which one was treated with 10^6 AJ3.1-IL2-IL12, to investigate if therapy of one tumour could also mediate a therapeutic effect on a tumour at a distant site. One group of mice received syngeneic DC in addition to AJ3.1-IL2-IL12 to investigate whether the presence of APCs would increase the immune response against the tumour cells. The group treated with AJ3.1-IL2-IL12 + irradiated N2a cells on the opposite flank from the tumour was a model for treating metastatic lesions, with the irradiated N2a cells included as a pool of tumour antigen at the vaccine

site. Control mice received 100µl RPMI i.t. Each group received three vaccines, two days apart (i.e. days 5, 7 and 9) (**Figure 5.13A**). Cytokine expression of transfected cells is shown in appendix 5E. Two mice from each group were culled 13 days after initial engraftment for *in vitro* analysis of splenocyte responses and tumour histology.

By day 12 post engraftment, average tumour volume of the mice vaccinated with N2a-IL2-IL12 i.t. was significantly lower ($354 \pm 172\text{mm}^3$; $p < 0.05$) than tumours in control mice receiving RPMI ($1085 \pm 695\text{mm}^3$) (**Figure 5.13B**). Twelve tumours were vaccinated intratumourally with AJ3.1-IL2-IL12, which significantly reduced tumour volume after 12 days ($520 \pm 434\text{mm}^3$; $p < 0.05$) compared to controls. Of these 12 mice, 6 were engrafted with a single tumour that was vaccinated with AJ3.1-IL2-IL12 i.t. and a further 6 were engrafted with tumours on each flank, of which one was vaccinated with AJ3.1-IL2-IL12 i.t. Intratumoural vaccination of AJ3.1-IL2-IL12 + DC also reduced tumour growth compared to controls, but this was not significant ($802 \pm 550\text{mm}^3$; $p > 0.2$). The tumours engrafted in animals receiving transfected AJ3.1 cells in the opposite flank from the tumour grew at a similar rate to controls, with an average tumour volume of $965 \pm 612\text{mm}^3$ on day 12. However, the tumour growth in individual mice in each group shows more clearly the therapeutic effect of intratumoural vaccination.

Tumour growth was effectively retarded by the intratumoural vaccine of cytokine-transfected N2a cells, with two of six mice undergoing complete tumour regression. The remaining four tumours in this group grew at a reduced rate compared to controls, slowing disease progression, thus this vaccine had an effect on 100% of tumour bearing mice (**Figure 5.14**). Two of these four mice were culled for *in vitro* analysis at 13 days post- engraftment, but when the mice were culled, these tumours also were growing more slowly

A



Day 0: inject 10^6 wild type N2a cells s.c. in left flank

Days 4, 6, 8: Thaw out appropriate numbers of AJ3.1 and N2a cells transfected with pCI-IL2 and pCI-IL12

Days 5, 7, 9: Inject cytokine-transfected AJ3.1 or N2a cells intratumourally **OR** cytokine-transfected AJ3.1 cells plus irradiated N2a cells in the opposite (right) flank

B

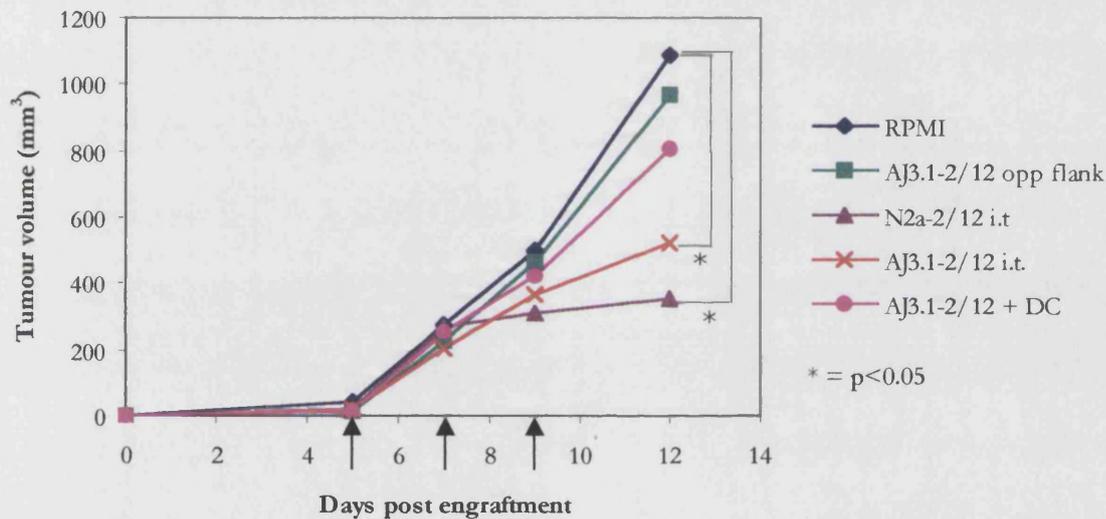


Figure 5.13 Average tumour growth of different experimental groups in eradication experiment. (A) Schematic representation of experimental protocol. Mice ($n=6$ per group) were engrafted with 10^6 wild-type N2a cells in the left flank plus another group ($n=6$) were engrafted with a tumour on each flank. Three vaccines were administered, each two days apart starting on day 5 after engraftment when tumour masses were $\sim 3\text{--}4$ mm in diameter. **(B)** Mice vaccinated with RPMI or those vaccinated with IL-2 and IL-12 expressing AJ3.1 fibroblasts in the opposite flank rapidly developed aggressive tumours. Data of the mice engrafted with two tumours, of which one was treated i.t., and the mice with a single tumour treated with AJ3.1-IL2-IL12 administered i.t. alone were pooled to give a group of $n=12$. Intratumoural vaccination of N2a-IL2-IL12 cells or AJ3.1-IL2-IL12 cell mediated a significant delay in tumour growth ($p < 0.05$, calculated by t-test). Arrows indicated vaccine administration.

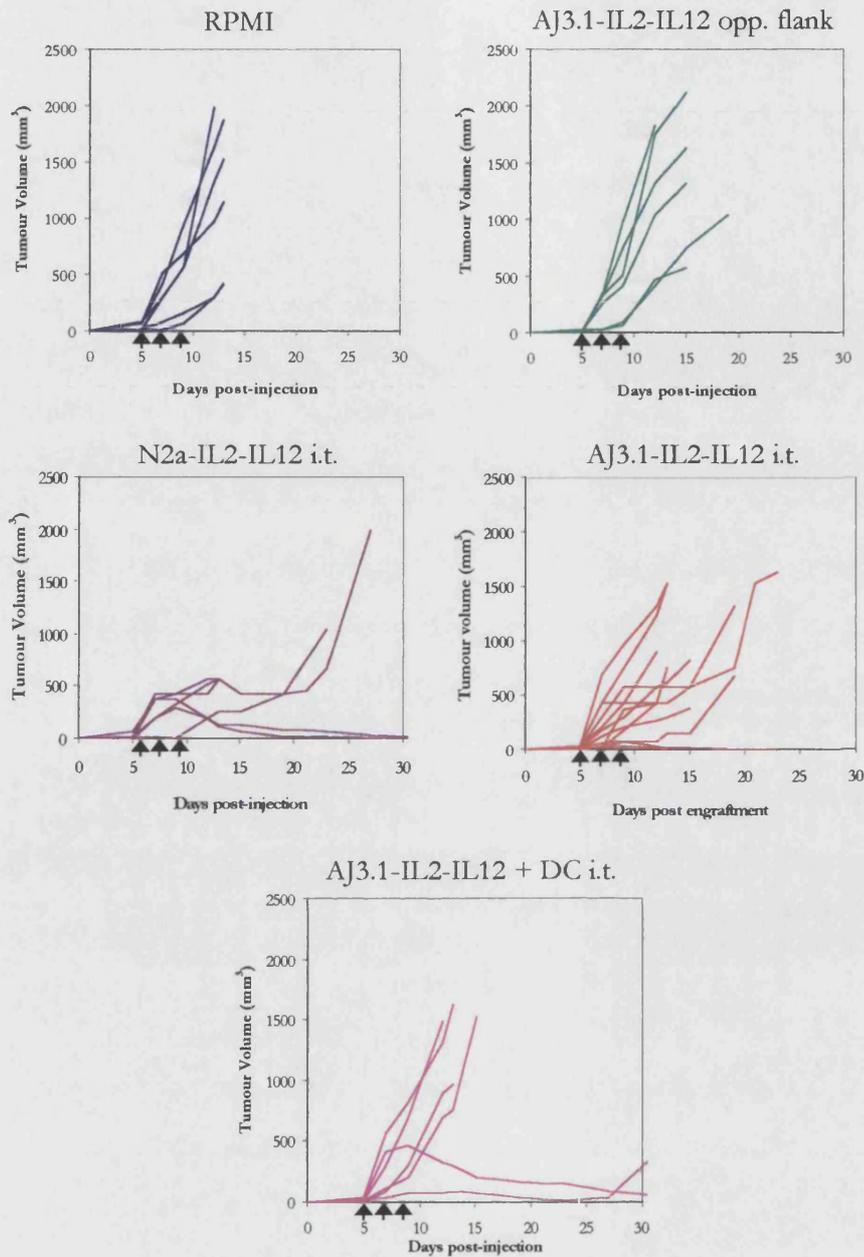


Figure 5.14 Effect of vaccination with transfected AJ3.1 fibroblasts on established N2a-derived tumours. Tumour growth curves for each experimental group. Each line represents one mouse. Tumours were measured every 2-3 days with calipers and tumour volumes calculated from two perpendicular axes. AJ3.1-IL2-IL12 graph shows pooled data from mice engrafted with one tumour that was treated, and data from mice with two tumours, of which one was treated. Two mice from each group were culled on day 13 for use in *in vitro* assays. Arrows indicate vaccine administration.

than controls. In the remaining two mice, tumours developed progressively but this was delayed by up to three weeks compared with controls.

Intratumoural cytokine-transfected AJ3.1 fibroblasts retarded tumour growth in eight out of twelve mice. Tumour growth was slowed for up to two weeks compared to controls in six tumours and a further two tumours were eradicated completely. The two tumours that were eradicated were in mice that were also engrafted with untreated tumours on the opposite flank. The progressive growth of the contralateral untreated tumour necessitated these mice to be culled. The mice receiving DC in addition to cytokine-expressing fibroblasts showed severely retarded tumour growth in two of six mice, although by day 27 post-engraftment a tumour redeveloped at the original vaccination site in one of these mice. However, the tumours of the other four mice in the group grew rapidly, so did not appear to be responding to the vaccine. The mice receiving transfected AJ3.1 + irradiated N2a on the opposite flank did not show a therapeutic anti-tumour effect and tumours grew at the same rate as controls (**Figure 5.14**).

The group of mice with tumour cells engrafted on each flank demonstrated a significant difference ($p < 0.01$) between the average growth of the treated tumour ($342 \pm 326 \text{mm}^3$) and the untreated tumour ($1119 \pm 552 \text{mm}^3$), clearly showing the efficacy of the AJ3.1-IL2-IL12 vaccine (**Figure 5.15A**). Examination of the tumour growth in individual mice in this group shows an interesting feature: in some mice showing a response to the treated tumour, there appears to be some level of response in the untreated tumour, although this is not significant (**Figure 5.15B**). The mice represented by the dark blue and burgundy lines show a substantial decrease in the growth of the treated tumour, and the untreated tumour also appears to develop more slowly than the tumours in control mice. This association was not seen in all mice as the mouse represented by the green line shows

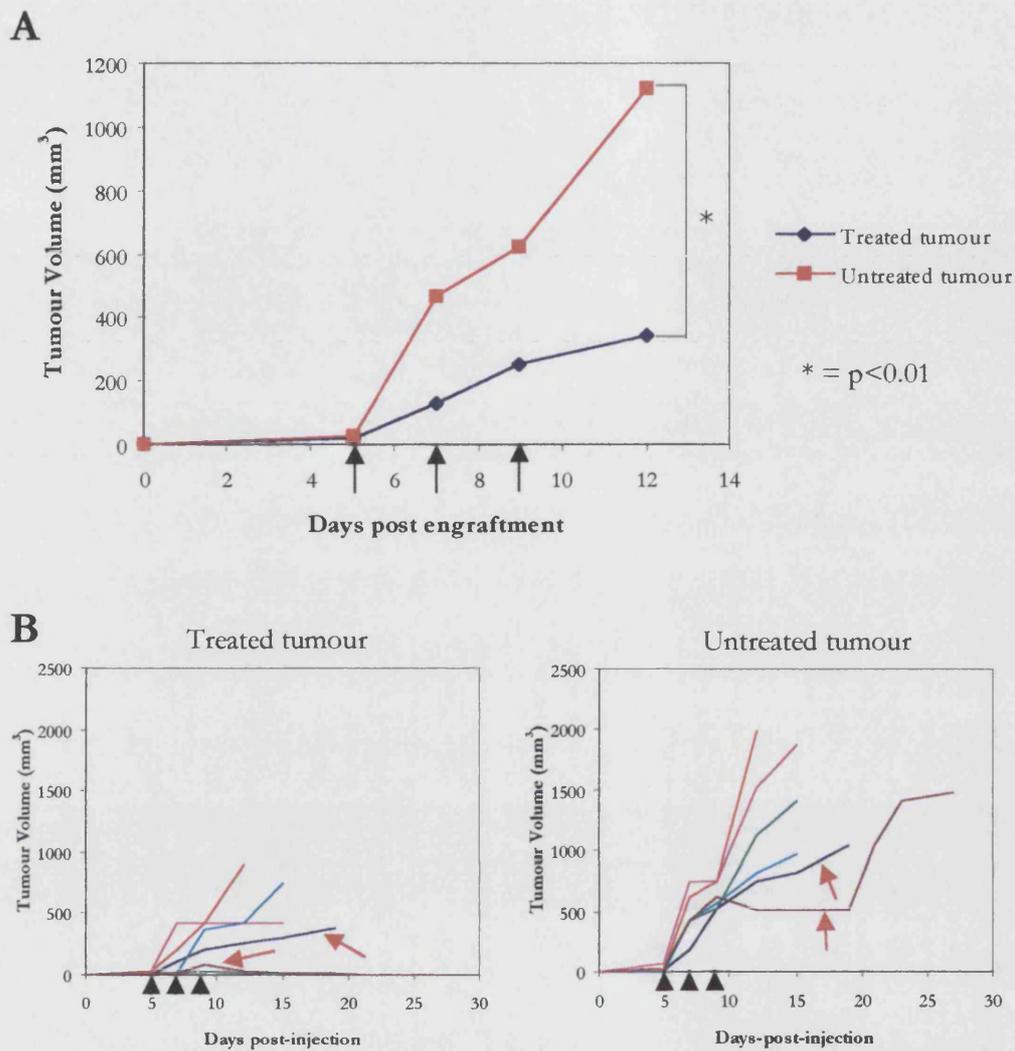


Figure 5.15 Effect of vaccination on tumours at a distant site. Mice ($n=6$) were engrafted with 10^6 wild type N2a cells on each flank. Tumours were palpable after 5 days, and mice received three vaccinations, each two days apart, of AJ3.1-IL2-IL12 cells into the tumour on the left flank. **(A)** Comparison of average growth of treated and untreated tumours. Tumour volumes on each flank were equivalent at the beginning of the vaccination course and tumour mass on day 12 was significantly lower ($p < 0.01$, calculated by paired t-test) in the treated tumour compared to the contralateral untreated tumour. Black arrows indicate vaccine administration. **(B)** Tumour growth of individual mice. Lines of the same colour between graphs indicate the same mouse. Tumours were measured every 2-3 days and volumes calculated from two perpendicular axes. Two mice were culled on day 13 for use in *in vitro* assays. Black arrows indicate vaccine administration, and the red arrows highlight the difference between treated and untreated tumours in the same mouse.

eradication of the treated tumour, but aggressive growth of the untreated tumour. Nevertheless, this suggests some evidence of antitumour activity at a site distant from the vaccination, but the response is too weak to mediate substantial anti-tumour effect.

All the groups of mice treated with the AJ3.1-IL2-IL12 vaccine showed some reduction of tumour growth, but it is difficult to interpret the effect of the vaccine on survival due to the sacrifice of mice for *in vitro* assays (**Figure 5.16**). Larger groups would be required to study the effect of the fibroblast vaccine on survival. Nonetheless, one mouse in the group receiving AJ3.1-IL2-IL12 + DC and two mice receiving N2a-IL2-IL12 showed complete eradication of tumour and remained tumour free for over 70 days.

5.2.3.6 *In vitro* evidence of anti-tumour activity

Despite some mice in each therapeutic group developing tumours, it was important to determine whether immune responses were being generated against N2a cells. Therefore, 13 days into this experiment, two mice from each group were culled and their splenocytes used for *in vitro* assays. Mice with regressing or slow-growing tumours were kept alive to determine whether tumours could be eradicated. Consequently, the mice used in the ELISpot and LDH release assays had progressing tumours but it was important to determine if these mice were raising immune responses against wild-type N2a cells.

5.2.3.6.1 ELISpot

Splenocytes were isolated from two mice per group 13 days into the experiment. ELISpot assays were performed to determine if splenocytes produced IL-2 and IFN- γ in response to co-culture with wild-type N2a cells. Splenocytes were co-cultured with 5×10^3 irradiated N2a cells in 96-well U-bottomed TC plates at effector:target ratios of 100:1, 50:1, 20:1 and 10:1. Positive control wells contained splenocytes cultured with 5 μ g/ml ConA, negative

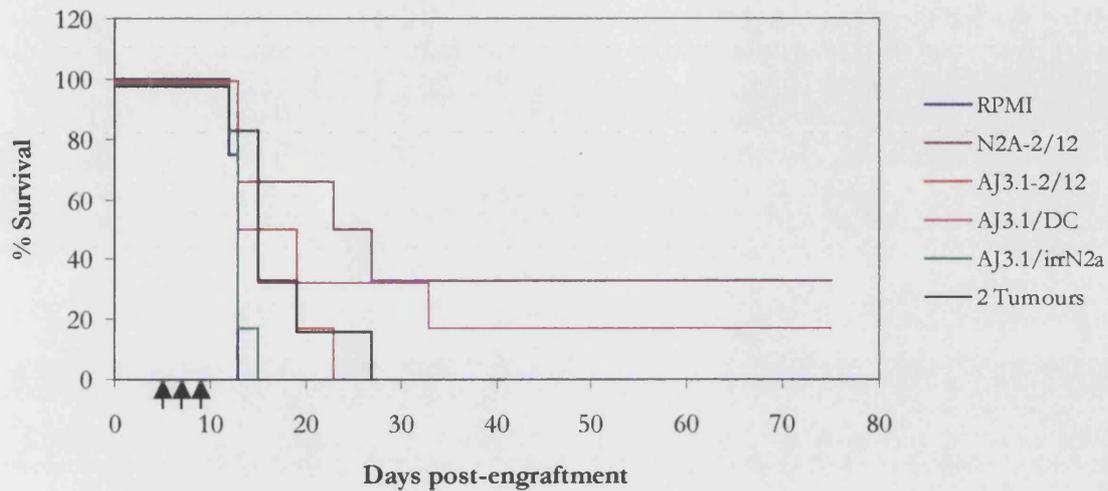


Figure 5.16 Survival of mice in eradication experiment. Mice (n=6 per group) received three vaccinations, as indicated by arrow heads, and tumour growth was monitored. Two mice per group were sacrificed on day 13 for *in vitro* assays, and the tumour progression of the remaining four was followed. Two of six mice vaccinated with N2a-IL2-IL12 and one mouse vaccinated with AJ3.1-IL2-IL12 + DC remained tumour free for over 70 days.

wells contained splenocytes only. Cells were incubated overnight at 37°C before being transferred to ELISpot plates pre-coated with capture antibody and incubated for a further 24h at 37°C. Plates were developed and analysed.

Very few spots were present in negative control wells, indicating there was little non-specific splenocyte activation, while the positive control wells showed very dense spots, confirming the splenocytes were functional. As in the rechallenge ELISpot assay, the 100:1 E:T ratio gave the clearest result for every animal tested, so all data discussed refers to this E:T ratio and representative wells are shown in **Figure 5.17A**.

Splenocytes from mice receiving AJ3.1-IL2-IL12 secreted high amounts of IL-2 (123 ± 11.7 spots) and IFN- γ (114 ± 15.3 spots) following co-culture with wild-type N2a cells (**Figure 5.17B**). Importantly, this level of cytokine production by splenocytes was equivalent to that observed from mice receiving N2a-IL2-IL12, with 128 ± 15.2 IL-2 spots and 120 ± 9.5 IFN- γ spots. Mice with two tumours receiving AJ3.1-IL2-IL12 in one tumour only and mice receiving AJ3.1-IL2-IL12 + DC also showed high IL-2 (136 ± 9.2 spots and 122 ± 9.2 spots respectively) and IFN- γ production (117 ± 11.8 spots and 123 ± 8.3 spots respectively). Splenocytes from mice receiving AJ3.1-IL2-IL12 in the opposite flank showed reduced cytokine production compared to mice which were vaccinated intratumourally, with 84 ± 12.3 IL-2 spots per well and 83 ± 9.9 IFN- γ spots per well.

Control mice receiving RPMI i.t. secreted IL-2 (85 ± 7.1 spots) and IFN- γ (83 ± 9.6) in response to co-culture with N2a cells at equivalent levels to the mice vaccinated in the opposite flank with AJ3.1-IL2-IL12. All mice vaccinated intratumourally produced significantly more IL-2 and IFN- γ than RPMI-treated controls ($p < 0.01$ for all groups

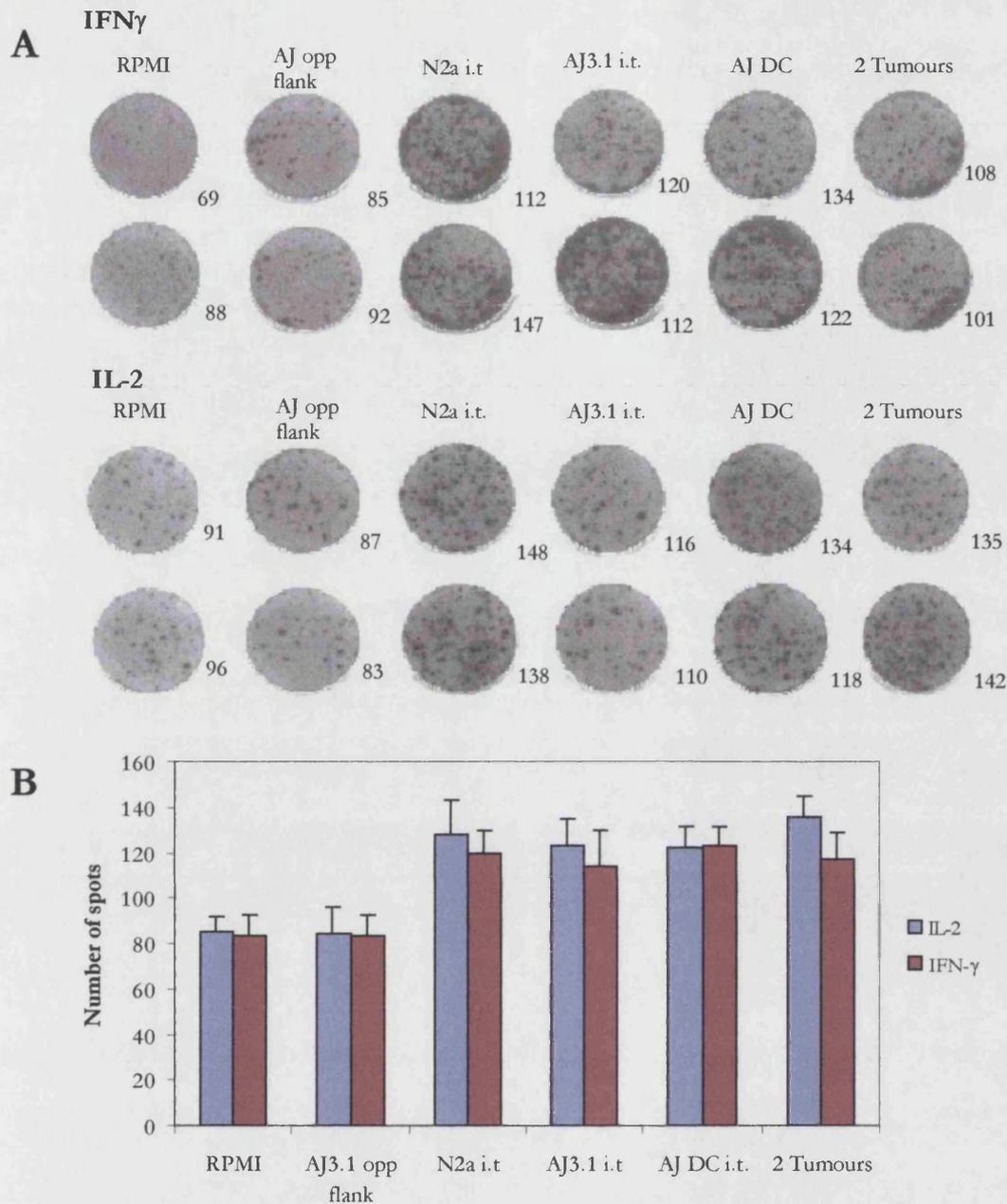


Figure 5.17 *In vitro* evidence of immune response against wild-type N2a cells in vaccinated mice by ELISpot. Splenocytes were isolated from two mice in each group and co-cultured with N2a cells. Numbers of spots representing IL-2 and IFN- γ secreting splenocytes were quantified using a plate reader. **(A)** Wells shown are of an effector:target ratio of 100:1 and are representative of each experimental group. **(B)** Average number of spots at the 100:1 E:T ratio from each experimental group. Error bars represent the standard deviation from six experimental wells. All groups of mice vaccinated intratumourally with cytokine-transfected cells showed significantly increased cytokine expression compared to RPMI controls ($p < 0.01$) or mice vaccinated with AJ3.1-IL2-IL12 in the opposite flank ($p < 0.01$). Significance was calculated using a t-test.

compared to controls) and mice vaccinated in the opposite flank ($p < 0.01$ for all intratumoural groups compared with AJ3.1-IL2-IL12 + irradiated N2a).

5.2.3.6.2 LDH release cytotoxicity assay

Splenocytes were co-cultured with irradiated N2a cells at an effector:target ratio of 50:1 in the presence of 60IU rhIL-2 for 6 days. CTL effectors were harvested and co-incubated with live N2a at ratios of 27:1, 9:1, 3:1 and 1:1 for 5 hours and LDH release from N2a target cells quantified and used to calculate % cytotoxicity.

As with the rechallenge LDH assay, results from the 27:1 and 9:1 E:T ratios gave the clearest and most consistent results and are shown in **Figure 5.18**. RPMI treated tumours showed cytotoxicity of $3 \pm 5\%$ and $6 \pm 3\%$ at E:T ratios of 9:1 and 27:1 respectively. Mice receiving AJ3.1-IL2-IL12 together with irradiated N2a cells in the opposite flank from the tumour showed marginally increased cytotoxicity against N2a targets with $6 \pm 17\%$ and $13 \pm 12\%$ lysis at 9:1 and 27:1 E:T ratios respectively, although this was not significantly greater than RPMI-treated controls at either E:T ratio ($p > 0.2$). Mice vaccinated i.t. with N2a-IL2-IL12 showed significantly greater cytotoxicity at both 9:1 ($14 \pm 7\%$; $p < 0.05$) and 27:1 E:T ratios ($24 \pm 9\%$; $p < 0.01$) compared to RPMI-treated controls. Mice vaccinated i.t. with AJ3.1-IL2-IL12 showed significantly greater cytotoxicity against N2a targets compared to RPMI-treated controls at a 9:1 E:T ratio ($19 \pm 11\%$; $p < 0.05$) but this was not significant at the 27:1 E:T ratio due to the large standard deviation ($22 \pm 14\%$; $p > 0.05$). Mice receiving AJ3.1-IL2-IL12 + DC showed increased cytotoxicity against N2a cells compared to RPMI-treated controls, but this was not significant at either E:T ratio ($8 \pm 3\%$; $p > 0.1$ at 9:1 and $10 \pm 6\%$; $p > 0.2$ at 27:1). The mice engrafted with two tumours of

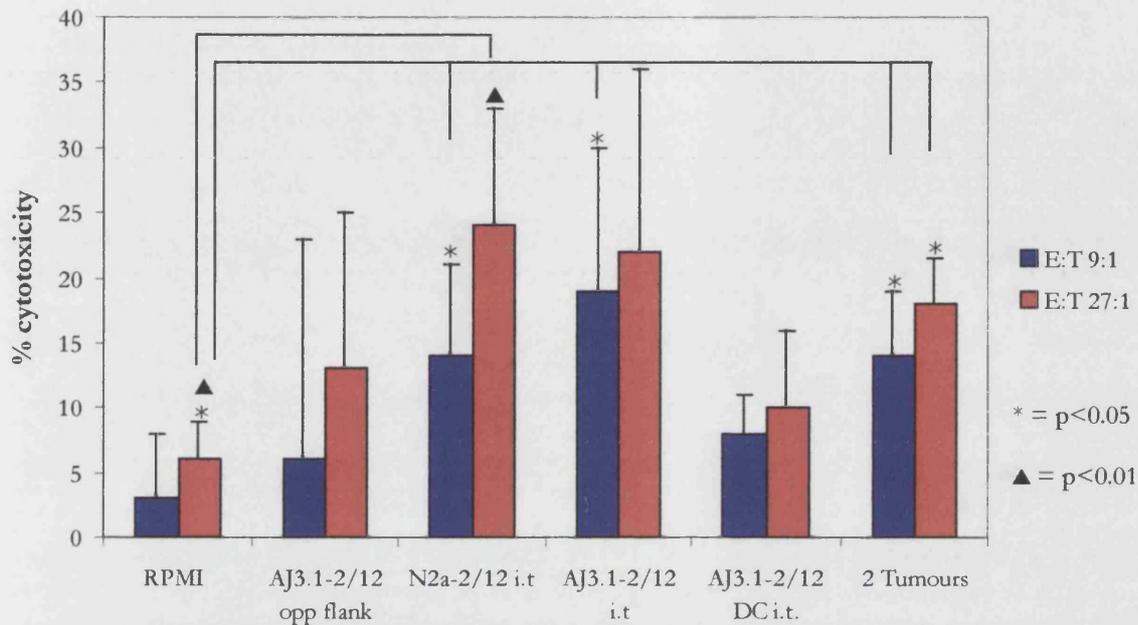


Figure 5.18 *In vitro* evidence of cytotoxicity against wild-type N2a cells in vaccinated mice. Splenocytes were isolated from two mice in each group of the eradication experiment and splenocyte-mediated cytotoxicity against N2a targets was determined by LDH assay. The mice used in this assay had developed tumours, but tumour burden was lower than the RPMI control group. The data shows average cytotoxicity values at two different effector:target ratios and is representative of each experimental group. Error bars represent standard deviation from quadruplicate samples. Significance was calculated using a t-test. Where significance is indicated, this refers to experimental vaccinated test groups compared with the RPMI control group at the 27:1 E:T ratio.

which one was treated showed significantly enhanced cytotoxicity compared with RPMI-treated controls of $14 \pm 5\%$ at 9:1 and $18 \pm 3.5\%$ at 27:1 (both $p < 0.05$) (Figure 5.18).

5.2.3.7 Histology of N2a-derived tumours

Tumours were excised from the same mice that were used for the LDH and ELISpot assays, fixed in 4% PFA, embedded in paraffin wax and $7\mu\text{m}$ sections mounted on polylysine coated slides. Sections from RPMI-treated control tumours were stained with hemotoxylin and eosin, revealing highly vascularised tumours with dense capillary networks. Larger vessels were also present around the tumour periphery. As is typical for murine NB tumours, no rosettes of neuroblasts could be detected and the majority of cells were in the resting stage, with only a few dividing cells observed (Figure 5.19 A&B). Staining with an anti-CD45 antibody showed negligible leukocyte infiltration of control tumours (Figure 5.19C). Control staining with the secondary layer only showed no background staining (Figure 5.19D).

Tumours from mice vaccinated intratumourally with N2a-IL2-IL12 (Figure 5.20 A&B) or AJ3.1-IL2-IL12 (Figure 5.20 C&D) were also stained with haemotoxylin and eosin. These treated tumours showed decreased presence of capillaries within the tumour compared to RPMI-treated controls, with most vasculature confined to the tumour periphery. Necrotic areas could be seen in the treated tumours, defined by punctate nuclear staining. In some areas the cells also appeared to be aggregating together more loosely than in viable tumour tissue, with a complete lack of nuclear staining, suggesting areas of dead cells.

Staining of sections with CD45 revealed infiltrating leukocytes (Figure 5.21). Tumours vaccinated intratumourally with N2a-IL2-IL12 (Figure 5.21 A&B) or AJ3.1-IL2-IL12

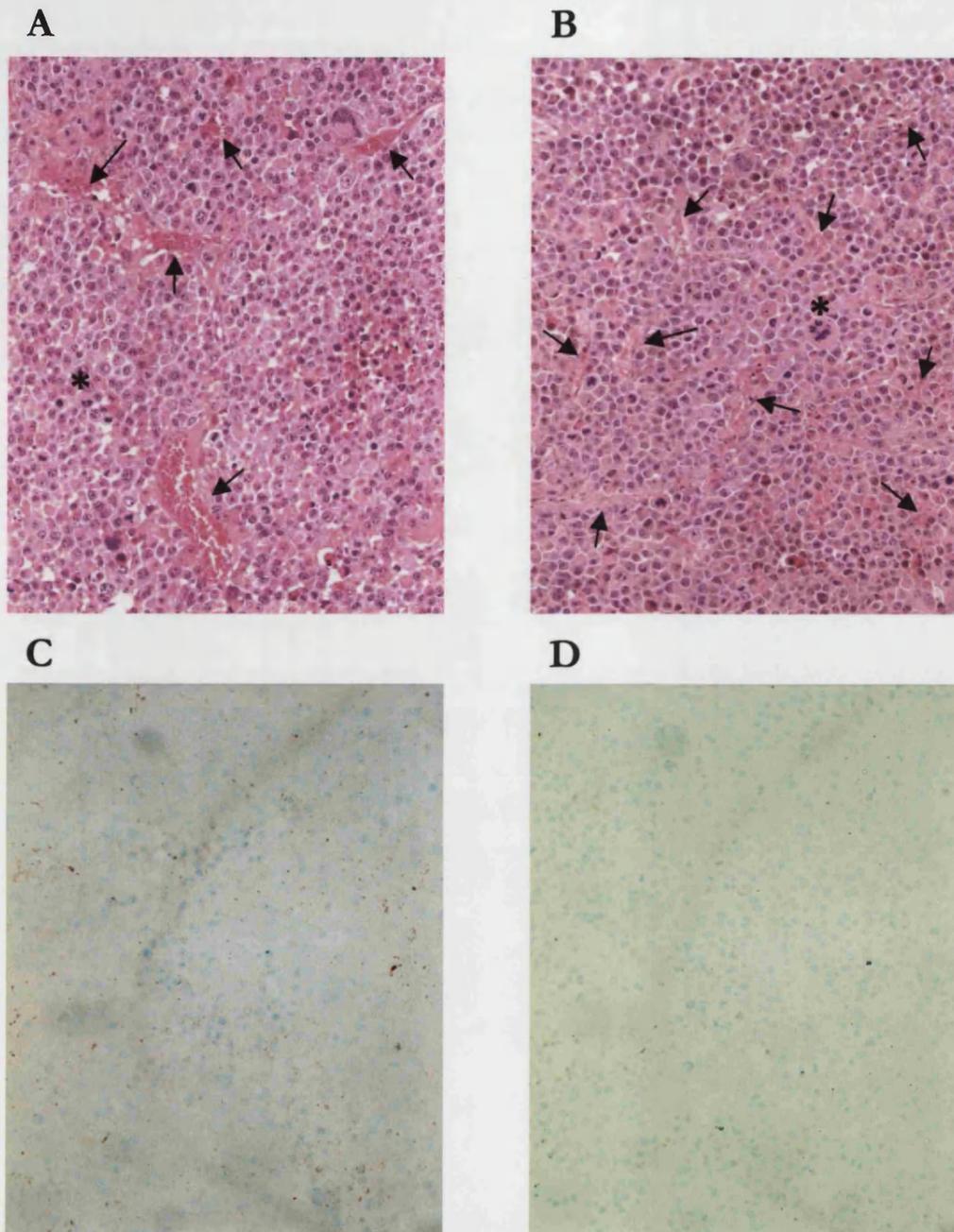


Figure 5.19 Histology and immunohistochemistry of tumours from RPMI-treated control mice. **(A & B)** Haematoxylin and eosin staining, showing highly vascularised tumours. Arrows denote blood vessels and stars denote dividing cells. **(C)** Staining for CD45, showing minimal infiltration of leukocytes. **(D)** Control staining with secondary layer only. Magnification x 40 (A-D).

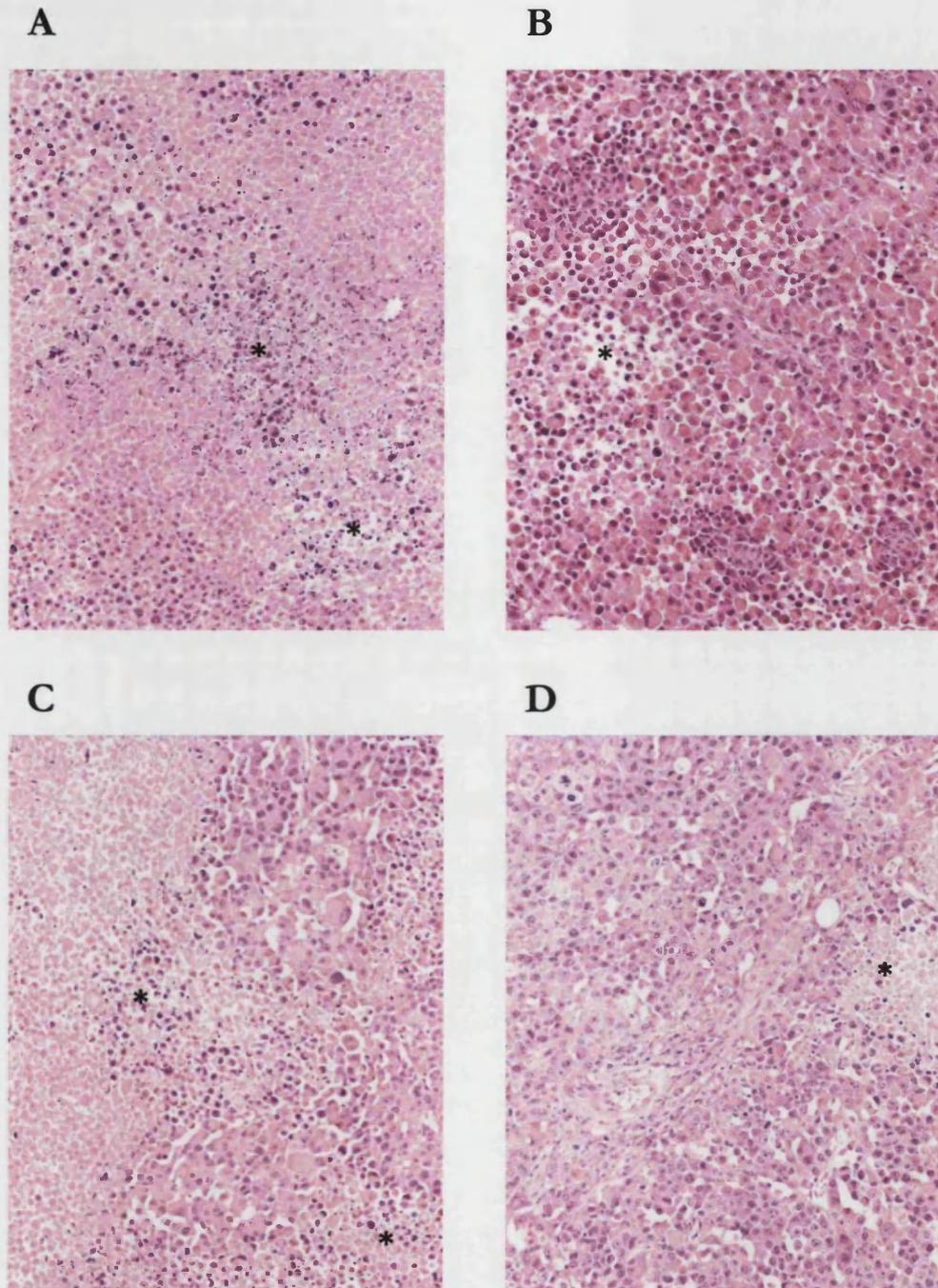


Figure 5.20 Histology of treated tumours. (A&B) Haemotoxylin and eosin staining of paraffin-embedded tumours from mice vaccinated intratumourally with N2a-IL2-IL12. **(C&D)** Paraffin-embedded tumours stained with haemotoxylin and eosin from mice vaccinated intratumourally with AJ3.1-IL2-IL12. Stars indicate areas of necrosis. Magnification x 40.

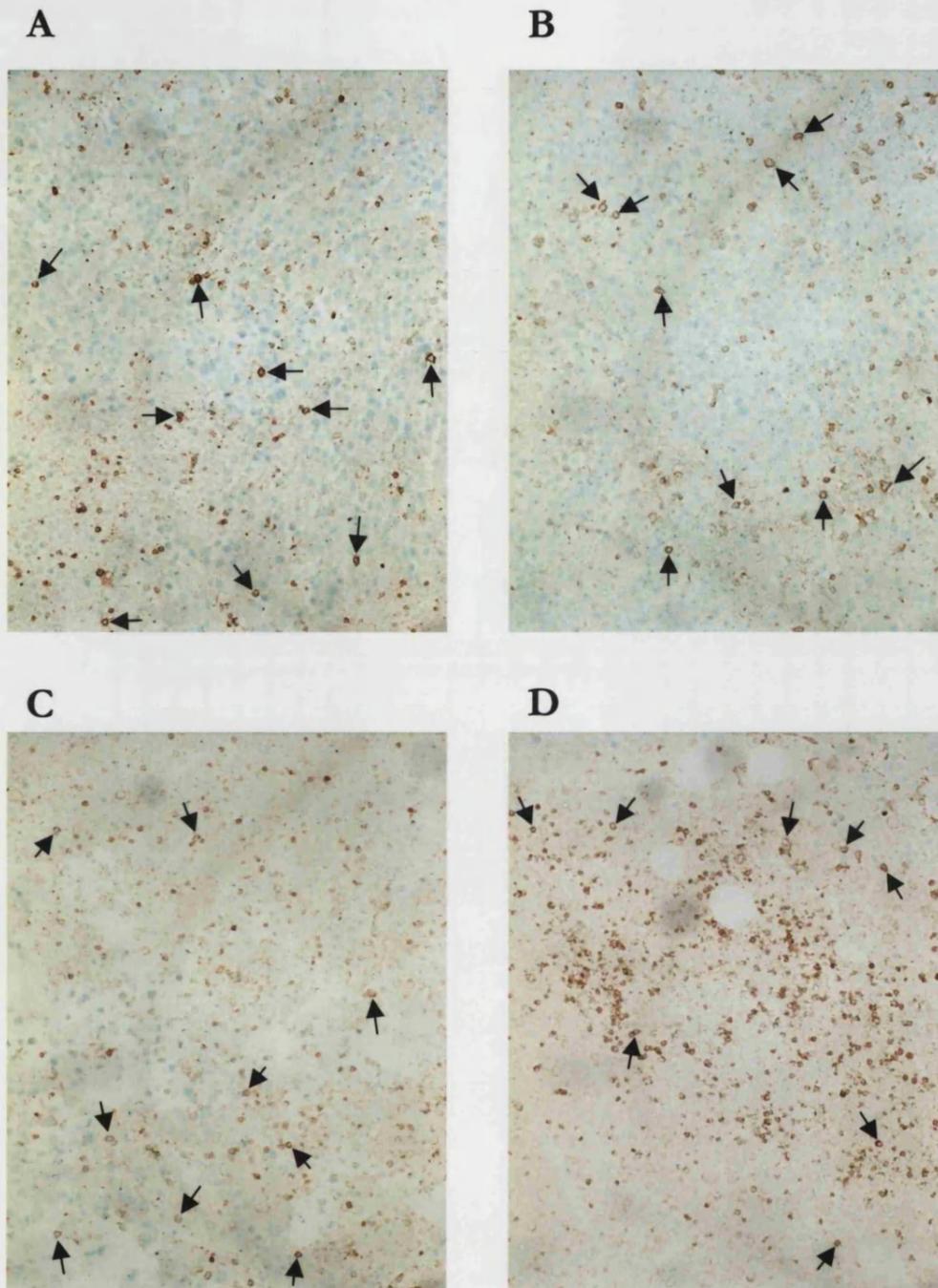


Figure 5.21 Immunohistochemistry of treated tumours. (A&B) CD45 staining of paraffin-embedded tumours from mice vaccinated intratumourally with N2a-IL2-IL12. **(C&D)** Paraffin-embedded tumours stained for CD45 from mice vaccinated intratumourally with AJ3.1-IL2-IL12. Arrows denote examples of CD45⁺-infiltrating leukocytes. Magnification x 40.

(Figure 5.21 C&D) demonstrated extensive leukocyte infiltration, particularly around the tumour periphery, indicated by strong CD45 surface staining of positive cells. This contrasts with the absence of CD45 staining in RPMI-treated tumours (Figure 5.19C).

5.2.3.8 Rechallenge of tumour-eradicated mice with wild-type N2a cells

Three mice in the eradication experiment displayed tumour regression after vaccination and remained tumour free for over 70 days (two treated with the N2a-IL2-IL12 vaccine, one treated with AJ3.1-IL2-IL12 + DC). To determine if these mice had developed systemic immunity, they were rechallenged with 10^6 wild-type N2a cells in the opposite flank from the original tumour/vaccination after 11 weeks. While the number of mice is too small to provide statistical data, it was an interesting observation that all three mice showed no evidence of tumour development at the site of rechallenge or the original injection site for over 90 days, suggesting systemic anti-tumour immune memory.

5.3 DISCUSSION

Fibroblasts transfected to express cytokines are an attractive potential alternative to autologous tumour vaccines due to the problems of culturing and manipulating primary tumour cells. Transfected fibroblasts provide local sustained cytokine expression in an analogous manner to transfected tumour cells, and autologous fibroblasts expressing IL-2 or IL-12 have already demonstrated therapeutic effects in the treatment of solid tumours in phase I trials (Sobel *et al.*, 1999; Kang *et al.*, 2001).

Preparing patient-tailored vaccines may take some time, so cytokine-transfected allogeneic fibroblast lines that could be rapidly reconstituted from frozen stocks may be advantageous. When vaccinated in the tumour locale, the allogeneic cells may mediate

enhanced inflammatory responses, stimulating antitumour immunity. Therefore, the aim of this chapter was to compare the *in vivo* therapeutic efficacy of syngeneic and allogeneic cytokine-expressing fibroblasts with cytokine-expressing N2a cells, which are known to have potent antitumour properties in a murine NB model (Siapati *et al.*, 2003).

The threshold level of cytokine expression required for antitumour responses in this model had not been established, so was titrated *in vivo*. Tumour engraftment was prevented in 83% of mice receiving N2a cells expressing 235ng IL-12 + 40ng IL-2. The level of cytokine produced by cytokine-transfected AJ3.1 and NIH-3T3 murine fibroblasts, as determined in chapter 4, is between the levels administered to the mice in the two highest dose groups (between 2350ng IL-12 + 400ng IL-2/24h and 235ng IL-12 + 40ng IL-2/24h). This suggests the IL-2 and IL-12 levels expressed by transfected fibroblasts exceed the therapeutic threshold required to prevent engraftment and is also within the range previously shown to stimulate antitumour responses against established NB tumours (Siapati *et al.*, 2003). Therefore, the therapeutic efficacy of AJ3.1 and NIH-3T3 cells expressing IL-2 and IL-12 was compared with cytokine-expressing N2a cells *in vivo*.

Tumourigenicity experiments compared cytokine-transfected allogeneic and syngeneic fibroblasts with cytokine-transfected N2a cells for their ability to prevent engraftment of co-injected wild-type N2a cells. Allogeneic NIH-3T3 cells inhibited N2a tumour engraftment in virtually all mice, whether the cells were transfected with cytokine genes, empty plasmid or untransfected, showing that NIH-3T3 cells alone mediated antitumour effects. Cytokine-transfected syngeneic AJ3.1 prevented N2a-derived tumour engraftment, while mice receiving untransfected AJ3.1 cells with wild-type N2a cells developed tumours at the same rate as N2a-only control mice, showing that unmodified AJ3.1 had no inhibitory effect on tumour growth. The cytokine-expressing AJ3.1 were as

effective as cytokine-expressing N2a cells at inhibiting N2a tumour engraftment with 83% of mice in each group remaining tumour free for over 40 days. This compares well with results from a colorectal carcinoma model where live tumour cells mixed with IL-2 expressing fibroblasts significantly inhibited tumour engraftment compared to wild-type fibroblasts (Fakhrai *et al.*, 1995), while IL-12-expressing syngeneic fibroblasts significantly inhibited the development of tumours by co-vaccinated tumour cells in a murine ovarian cancer model (Sanches *et al.*, 2000).

No IL-2 or IL-12 was expressed by untransfected cells or cells transfected with empty vector. This suggests inhibition of engraftment by allogeneic fibroblasts was independent of cytokine expression, while syngeneic fibroblasts inhibited tumour engraftment in a cytokine-dependent manner. However, fibroblasts were being investigated here as an alternative to autologous tumour cells to mediate cytokine-dependent tumour regression. Therefore, allogeneic NIH-3T3 cells were unsuitable due to their non-specific antitumour effect. Previous work has also suggested that autologous cells are more effective than allogeneic cells as cancer vaccines (Aruga *et al.*, 1997; Bowman *et al.*, 1998a, b). However, this contrasts with other reports that indicate that cytokine-expressing allogeneic cell vaccines are superior to cytokine-expressing syngeneic cells, and this improved tumour regression is cytokine-dependent (Kayaga *et al.*, 1999).

Many clinical procedures require irradiated cells, so the tumourigenicity experiment was repeated with irradiated AJ3.1-IL2-IL12. However, the irradiated cells mediated reduced protection against tumour engraftment compared to the live vaccine. Preliminary assays had shown irradiation was not detrimental to cytokine production, yet IL-2 and IL-12 expression by the irradiated AJ3.1 cells used in these vaccinations decreased rapidly compared with live AJ3.1 cells. Irradiated AJ3.1 maintained *in vitro* became apoptotic

sooner than N2a cells receiving the same radiation dose, indicated by the AJ3.1 becoming very granular and non-adherent. This could explain why the higher cell dose did not increase the therapeutic effect, as the duration of cytokine expression was reduced. Comparing the rate of apoptosis of irradiated AJ3.1 with irradiated N2a cells by staining with annexin-V and propidium iodide would allow quantitative measurements of apoptosis. Optimisation of the radiation dose for AJ3.1 cells may prolong cytokine expression while ensuring the cells are growth-arrested.

The most clinically relevant aspect of any cancer vaccine is to inhibit the progression of tumours as most patients present with established disease. Therefore, cytokine-transfected AJ3.1 fibroblasts were compared with cytokine-transfected N2a cells in the treatment of solid tumours. Intratumoural administration of N2a-IL2-IL12 cells or AJ3.1-IL2-IL12 fibroblasts mediated a significant reduction in average tumour volume 12 days after engraftment compared with RPMI-treated control mice.

N2a-IL2-IL12 cells mediated antitumour responses in all treated mice. Four tumours grew more slowly compared to controls, and two tumours regressed completely. The AJ3.1-IL2-IL12 i.t. vaccine mediated antitumour effects in 67% (eight of twelve) of treated mice. The tumour progression of six mice was slowed by up to two weeks compared with controls, and two tumours regressed completely. Six of the twelve mice were engrafted with a tumour on each flank but received AJ3.1-IL2-IL12 in only one tumour to investigate whether the vaccine would mediate regression of the contralateral tumour. The average volume of the treated tumour was significantly reduced compared to the untreated tumour, indicating the vaccine is most effective when administered in the tumour locale. However, the mice with eradicated tumours were from the group engrafted with tumours on both flanks, so it was not possible to follow their progress due to the aggressive growth

of the untreated tumours. Interestingly, one mouse with an eradicated tumour had slower growth and a transient reduction in mass of the contralateral untreated tumour. Other work suggests that further vaccinations may be necessary to increase the proportion of eradicated tumours. Fibroblasts expressing IL-12 could mediate regression of established sarcoma when administered for three weeks (Zitvogel *et al.*, 1995).

As the most potent APC, it was hypothesised that the addition of DC may enhance tumour antigen presentation in the pro-inflammatory environment, increasing antitumour responses and augmenting tumour regression. However, the addition of DC to the AJ3.1-IL2-IL12 i.t. vaccine did not enhance tumour rejection. Previous work has shown that addition of DC in cytokine tumour vaccines has a more marked effect in preventing tumour growth after rechallenge, and does not significantly enhance primary tumour eradication (Redlinger *et al.*, 2003).

Cytokine-transfected AJ3.1 fibroblasts induced systemic antitumour immunity as effectively as cytokine-transfected N2a cells in the tumourigenicity experiment. Tumour free mice initially vaccinated with live AJ3.1-IL2-IL12 or N2a-IL2-IL12 cells were rechallenged after 7 weeks with wild type N2a cells on the opposite flank and two of five mice in each group (40%) remained tumour free for over 25 days. This is consistent with a murine colorectal carcinoma model, which showed that mice injected with cytokine-expressing syngeneic fibroblasts or cytokine-expressing tumour cells showed similar levels of induced antitumour immunity against rechallenge (Shawler *et al.*, 1995). Vaccination of established tumours mediated tumour eradication in two mice receiving N2a-IL2-IL12 i.t. and one receiving AJ3.1-IL2-IL12 + DC i.t. Rechallenge with wild-type N2a cells in these mice 11 weeks later revealed evidence of systemic immunity, as they remained tumour free

for 90 days. Larger groups of animals are required to determine if these results are significant and repeated vaccination may increase systemic immunity.

Cellular immune memory responses were investigated *in vitro* by cytokine production from splenocytes and LDH cytotoxicity assay. ELISpot assays with mice from the tumourigenicity and rechallenge experiment showed that all mice previously injected with N2a cells produced significantly higher numbers of IL-2-expressing splenocytes following co-culture with wild-type N2a cells compared with naïve control mice. All mice that had previously been injected with N2a cells also demonstrated increased lysis of N2a targets in the LDH cytotoxicity assay compared with naïve mice, suggesting that even wild-type N2a cells may stimulate some degree of immune response.

Splenocytes from mice in the tumourigenicity and rechallenge experiment showed equivalent levels of IL-2 production ELISpot between mice that had rejected tumour and those which developed tumour. This contrasts with the result from the LDH release assay, which revealed differences in the level of cytotoxicity against N2a target cells between tumour bearing and tumour free mice. Splenocytes from mice that rejected two doses of tumour cells had a substantially greater cytotoxic response against N2a cells than mice that developed tumours. Mice that had rejected one tumour, but succumbed to rechallenge displayed an intermediate level of cytotoxicity against N2a targets.

The initial vaccine of N2a-IL2-IL12 cells or AJ3.1-IL-2-IL12 fibroblasts was administered to the mice 7 weeks before tumour free individuals were rechallenged with wild-type N2a cells and 11 weeks before the ELISpot and LDH assays were performed. Therefore, the responses observed in these assays against wild-type N2a cells suggest evidence of induction of cellular immune memory. Importantly, production of IL-2 and the cytotoxic

response by splenocytes from mice that received AJ3.1-IL-2-IL12 fibroblasts was equivalent to mice that received N2a-IL2-IL12 cells.

ELISpot assays from mice with established tumours showed that all vaccinated mice responded to N2a cells by increased IL-2 and IFN- γ secretion compared with naïve mice. The splenocytes of all groups of mice vaccinated intratumourally with N2a-IL2-IL-12 or AJ3.1-IL2-IL12 produced equivalent numbers of IL-2 and IFN- γ spots, and these were significantly higher than the number of IL-2 and IFN- γ spots from RPMI-vaccinated controls or mice vaccinated with AJ3.1-IL2-IL12 in the opposite flank.

In the LDH release assay, mice vaccinated intratumourally with N2a-IL2-IL12 or AJ3.1-IL2-IL12 showed significantly higher lysis of N2a cells compared with RPMI-vaccinated controls or mice vaccinated with AJ3.1-IL2-IL12 in the opposite flank. Importantly, N2a lysis was equivalent between mice receiving AJ3.1-IL2-IL-12 and N2a-IL2-IL12 and further supports fibroblasts as a suitable alternative to tumour cells. This level of target cell cytolysis also compares well with other reports of IL-12 expressing syngeneic cellular vaccines (Pertl *et al.*, 2001). In another report, mice vaccinated with IL-12-expressing NSX2 NB cells with an IL-2-anti-GD₂ antibody fusion protein mediated significantly higher lysis of wild-type NSX2 targets than mice receiving IL-12-expressing NSX2 cells with recombinant IL-2 (Lode *et al.*, 1999), suggesting that targeting of cytokines to tumour cells may further enhance antitumour responses.

Tumour sections showed extensive leukocyte infiltration in tumours vaccinated with N2a-IL2-IL12 or AJ3.1-IL2-IL12 compared to negligible staining of leukocytes in control tumours, suggesting the fibroblast vaccine recruits immune effector cells to the tumour site as effectively as the tumour cell vaccine. As well as adaptive immune functions, IL-12

is well documented to elicit angiostatic effects (Duda *et al.*, 2000), largely mediated through induction of IP-10 (Coughlin *et al.*, 1998). Histology suggested that the N2a-IL2-IL12 or AJ3.1-IL2-IL12 treated tumours had reduced vascularisation compared with controls. Analysis of several sections from each tumour and a larger number of animals would be required for quantification of an antiangiogenic effect of the cytokine-expressing vaccines. Staining with CD31 or CD34 could also be used to give better definition of blood vessels by staining of vessel endothelia.

Haematoxylin and eosin staining revealed necrotic areas of tumour tissue which were more prevalent in treated tumours, showing that the vaccine could mediate destruction of tumour cells, probably due to a combination of the reduced vascular network, limiting the supply of nutrients and oxygen, and extensive infiltration of immune effector cells. To quantify the level of cell death within tumours, TUNEL staining (terminal deoxynucleotidyl transferase-mediated nick end labelling), which detects apoptotic cells, could be used in future experiments. However, this technique is performed on frozen sections, so could not be implemented on the paraffin embedded sections studied here.

In conclusion, syngeneic fibroblasts expressing IL-2 and IL-12 prevented engraftment of wild-type N2a cells and induced long lasting systemic immune memory that protected mice from rechallenge with wild type N2a cells as effectively as cytokine-transfected N2a cells. A significant therapeutic antitumour effect was also seen when IL-2 and IL-12-expressing syngeneic fibroblasts were vaccinated into established N2a-derived tumours. The fibroblast vaccine induced equivalent levels of splenocyte-mediated IL-2 and IFN- γ release and cytotoxicity against wild type N2a cells as the tumour cell vaccine, and extensive leukocyte infiltration was observed in established tumours vaccinated with cytokine-transfected fibroblasts. Therefore, a cellular vaccine consisting of syngeneic

fibroblasts co-transfected with IL-2 and IL-12 is a potential therapy for the treatment of neuroblastoma.

CHAPTER 6

DISCUSSION

Neuroblastoma is a common paediatric malignancy, with a poor outlook for advanced stage patients. The majority of patients with disseminated disease will relapse within three years, even after initially successful therapy. Conventional chemotherapy and surgery are the main treatments, but do not always eradicate minimal residual disease, so improved treatment is necessary to reduce the relapse rate and increase long-term survival. The work in this thesis describes the development of a cellular immunotherapeutic vaccine using a synthetic gene delivery vector to treat experimental murine NB. Initially DC were considered for the vaccine as these are the most potent APC and may augment tumour antigen presentation to T cells. Cytokine-expressing fibroblasts were also investigated as an alternative means of enhancing pro-inflammatory antitumour immune responses.

The aim of cancer immunotherapy is to educate the immune system to recognise and eliminate cancer cells. Tumours exploit many ways to evade immune detection including down-regulation of MHC I or tumour antigen expression, reduced expression of death receptors, T-cell anergy and secretion of immunosuppressive factors, which all contribute to the immune escape of tumours. Immunotherapeutic strategies to treat such tumours aim to induce inflammatory responses that mediate destruction or inhibit further growth of established tumours. An important goal of immunotherapy is to induce systemic immune responses that protect an individual from disease relapse.

6.1 Transfection using the synthetic LPD vector

The synthetic LPD vector system used in this project was composed of plasmid DNA, lipid and cationic targeting peptide. One advantage of synthetic vectors is the short preparation time. Selection of stable transfectants or retrovirally-transduced clones may take weeks to produce, while LPD transfection takes only 24 hours.

Murine bone marrow-derived DC consistently showed transfection levels of around 5% with the LPD vector and confocal microscopy showed the main barrier to transfection of DC was low vector internalisation. Other reports have suggested that this level of gene transfer into DC is sufficient to prime T-cells (Irvine *et al.*, 2000), but it was not possible here to show antigen-specific immune responses following LPD transfection of a model antigen into murine DC.

An alternative approach is to increase the concentration of pro-inflammatory mediators in the tumour environment. LPD transfection of IL-2 and IL-12 into N2a tumour cells abrogates their tumorigenicity *in vivo* in syngeneic mice and mediates regression of established N2a tumours (Siapati *et al.*, 2003). However, difficulties with culturing and manipulating patient primary NB cells makes this approach difficult to transfer to the clinic. Therefore, cytokine-secreting autologous fibroblasts may provide a suitable alternative method of supplying sustained, local, high-level cytokine expression.

Transfection of IL-2 and IL-12 into murine fibroblast lines using the LPD vector resulted in cytokine expression equivalent to that of LPD-transfected N2a cells which are known to mediate antitumour effects *in vivo* (Siapati *et al.*, 2003). Moreover, the expression of IL-2 and IL-12 from LPD-transfected primary human fibroblasts and murine fibroblast lines was higher than achieved with many other synthetic vectors and at least equivalent to many viral gene transfer systems.

Cytokine-expressing syngeneic fibroblasts prevented the engraftment of N2a-derived tumours and, importantly, transfected fibroblasts were as effective as transfected N2a cells at inducing systemic antitumour immunity. Furthermore, transfected syngeneic fibroblasts

significantly inhibited the growth of established N2a-derived tumours when vaccinated intratumourally compared to RPMI-vaccinated controls.

Splenocytes from mice vaccinated with AJ3.1-IL2-IL12 demonstrated significantly increased cytotoxicity against wild-type N2a target cells and the tumour tissue from these mice revealed extensive leukocyte infiltration compared with RPMI-vaccinated controls, suggesting induction of cellular antitumour immunity even in tumour bearing mice.

Individual lymphocyte subsets were not investigated in this project, but previous work has shown that both CD4⁺ and CD8⁺ T-cells are required for tumour rejection in the A/J model (Siapati *et al.*, 2003). In another model of murine NB, NXS2 murine NB cells expressing IL-12 also induced CD8⁺-mediated immunity in A/J mice (Lode *et al.*, 1999). This IL-12-induced protective immunity was dependent on IP-10 as depletion of IP-10 abrogated the IL-12-mediated antitumour effects and inhibited tumour cell lysis by CD8⁺ T-cells (Pertl *et al.*, 2001). CD8⁺ T-cells are also essential for antitumour immune responses in experimental colon and renal cell carcinoma (Mendiratta *et al.*, 1999).

A combination of IL-2 and IL-12 was used in this project. Previous work with this NB model (Siapati *et al.*, 2003) and a Lewis lung carcinoma model (Tanaka *et al.*, 2000) has shown the efficacy of IL-2 and IL-12 together is superior to either individual cytokine. The combination of IL-12 and tumour-targeted IL-2 in another NB model also enhanced CD8⁺ and memory T-cell responses compared to IL-12 alone (Lode *et al.*, 1999). The enhanced response of administering both cytokines may be due to the IL-2-enhanced expression of the IL-12 receptor and STAT4 in NK cells. STAT4 is critical in IL-12-mediated signalling, leading to increased IFN- γ production and killing of target cells (Wang

et al., 2000). Furthermore, IL-12 can enhance IL-2 function by inducing expression of IL-2R α , the subunit required for the high-affinity IL-2R (Nguyen *et al.*, 2000).

6.2 Fibroblasts as vaccines

Many experimental cancers have been successfully treated with gene-modified syngeneic tumour cells. However, when these approaches are transferred to the clinic the vaccines tend not to fulfil the potential shown in murine models. This may be due to poor gene transfer and viability of the transfected or transduced primary autologous tumour cells, or in some cases, there is insufficient tumour material available from which to engineer such cellular vaccines. A cellular vaccine composed of fibroblasts could circumvent these problems. Fibroblasts are easily cultured from skin explants, so there is no problem with availability of cells. Preliminary work in this project also showed that primary dermal fibroblasts can be efficiently transfected with a synthetic LPD vector and the levels of cytokines expressed are comparable to those achieved following transduction of primary human fibroblasts with retroviral vectors (Sobol *et al.*, 1999; Park *et al.*, 1997).

Syngeneic fibroblasts have also shown promise as gene delivery “vehicles” in a number of other experimental systems, such as delivery IL-4 in collagen-induced rheumatoid arthritis (Bessis *et al.*, 2002) and production of proteolipid protein to induce T-cell anergy in experimental autoimmune encephalomyelitis, the murine model of multiple sclerosis (Weiner *et al.*, 2004). Primary fibroblasts have also been used to deliver BDNF and NT-3 in experimental models of spinal cord injury and this was shown to provide limited regeneration (Tobias *et al.*, 2003). Therefore, there are many potential applications for gene modified fibroblasts, and LPD-transfection provides a safe, easy and quick method of efficiently transferring genes to these cells.

6.3 Clinical strategies

Results from this project suggest that the vaccine is most effective when administered in the tumour locale. Therefore, potential clinical strategies may include intratumoural vaccination or peritumoural vaccine administration at the time of surgical resection. The vaccine would most likely be administered in addition to other antitumour strategies, possibly including antiangiogenic or standard chemotherapeutic treatments. Retrovirally transduced autologous dermal fibroblasts have already been used in phase I clinical trials to treat adult solid tumour (Sobol *et al.*, 1999; Kang *et al.*, 2001), which showed that the treatment was well tolerated and that anti-cancer immune responses were induced in some patients. Therefore, this vaccine should also be tolerated by paediatric patients.

Intratumoural vaccination of cytokine-expressing fibroblasts induced antitumour effects, but vaccination at a distant site did not inhibit the growth of established tumours. In a clinical situation, the tumour may not be accessible for direct treatment, or multiple metastases may be present that cannot be treated individually. Therefore the vaccine efficacy at sites distant from the tumour requires improvement. One possibility is including DC in the vaccine. DC did not enhance the fibroblast vaccine in the mice treated intratumourally, but this may be due to the immature phenotype of the DC as injection of immature antigen-pulsed DC into humans resulted in inhibition of antigen-specific CTL function with reduced IFN- γ production and cytolytic activity (Dhodapkar *et al.*, 2001). Therefore, mature DC could be used in the vaccination as these may show enhanced migration to lymph nodes. Alternatively, pulsing DC with autologous tumour lysate to load the DC with tumour antigens may enhance their antitumour response. Vaccination of DC co-cultured with live tumour cells also mediated protective immunity against tumour rechallenge in murine colon carcinoma (Kim *et al.*, 2001). Furthermore, injecting DC intratumourally may not be optimal due to the immunosuppressive effects of

the tumour environment and NB-derived gangliosides are known to inhibit DC function (Shurin *et al.*, 2001).

6.4 Further work

The work presented in this thesis supports the concept that cytokine-transfected fibroblasts may offer an effective alternative to transfected autologous tumour cells and these initial experiments have demonstrated their antitumour efficacy. However, additional work is required to further characterise these antitumour responses.

Extensive infiltration of leukocytes was observed in tumours treated with cytokine-expressing fibroblasts or tumour cells, but it was not possible to identify the subsets of immune cells present in the tumours. Therefore, an alternative could be to isolate the leukocytes from tumour tissue with anti-CD45 magnetic beads, allowing the frequency of individual immune cell subsets could be determined from the CD45⁺ population. Work with melanoma-infiltrating T-cells has shown that individual CD4⁺ and CD8⁺ cells can be clonally expanded and are capable of lysing autologous tumour cells *in vitro* (Terheyden *et al.*, 2000). Therefore, expansion of infiltrating lymphocytes may provide sufficient cells for *in vitro* analysis of antigen specificity. Furthermore, it may even be possible to adoptively-transfer the infiltrating cells into naïve mice and challenge these hosts with tumour cells.

In vitro assays demonstrated that splenocytes from mice vaccinated with AJ3.1-IL2-IL12 could lyse N2a target cells. A more comprehensive study of acquired antitumour immunity could isolate and investigate tumour-specific CTLs from mice that rejected tumour. Co-culture of splenocytes with tumour cells activates the tumour-specific CTL population to produce IFN- γ , which could then be isolated with magnetic beads. A stringent measure of the antitumour capacity of isolated CTLs would be to adoptively

transfer them into naïve hosts, then challenge the hosts with tumour cells. Alternatively CTLs could be transferred into tumour-bearing hosts.

Only a combination of IL-2 and IL-12 was considered in this project. Now cytokine delivery by transfected syngeneic fibroblasts has demonstrated therapeutic efficacy in this NB model, different cytokine combinations could be compared. GM-CSF is a good candidate as it has shown antitumour responses in several animal models (Sandler *et al.*, 2003; Moret *et al.*, 2001), and has reached clinical trial for a variety of human cancers (Jaffee *et al.*, 2001; Kushner *et al.*, 2001). Lymphotactin is a chemokine that could be investigated in this model. It has been shown to enhance antigen specific immunity in murine melanoma (Xia *et al.*, 2002), and has also been transduced into autologous tumour cells together with IL-2 in a clinical trial for NB (Brenner *et al.*, 2001).

Expression of IL-18 by transduced N2a cells abrogates their tumourigenicity in a T-cell mediated manner and induced immunity against wild-type N2a cells (Heuer *et al.*, 1999), thus IL-18-transfected fibroblasts may be a good therapy for established tumours. NKT cells activated with a combination of IL-12 and IL-18 exhibited antitumour immunity when adoptively transferred into syngeneic hosts (Baxevanis *et al.*, 2003), while peritumoural injection of IL-2 with IL-18 synergistically mediated the regression of established murine NB, although immune memory was not induced (Redlinger *et al.*, 2003c). However, the same route of administration and dose of IL-2 and IL-18 eradicated established murine fibrosarcomas and induced CD4⁺-dependent memory responses (Son *et al.*, 2003), indicating antitumour immune responses are often tumour-specific. Furthermore, IL-18 and IL-2 synergistically increase the proliferation, cytotoxicity and IFN- γ production of human primary lymphocytes (Son *et al.*, 2001).

Alternatively, fibroblasts could be used for intratumoural delivery of a soluble VEGF-R to inhibit tumour angiogenesis by sequestering the pro-angiogenic cytokine VEGF. Such a receptor inhibits angiogenesis in a murine model of retinal neovascularisation (Bainbridge *et al.*, 2002), and combined with IL-12, could be a potent angiostatic therapy.

The cytokines explored in cancer therapeutics are predominantly pro-inflammatory factors or T_H1 cytokines to enhance the immunogenicity of the tumour environment. However, the T_H2 cytokine, IL-4, has also demonstrated potent antitumour properties. IL-4-expressing cancer cell lines lose their tumorigenicity *in vivo*, while T-cell proliferation in glioblastoma patients can be improved by IL-4 (reviewed in Mocellin *et al.*, 2001). Tumours frequently express IL-10, which is generally acknowledged to act in an immunosuppressive manner. However, IL-10 has also demonstrated antitumour immunostimulatory properties; murine colon carcinoma transduced with IL-10 showed a reduction of their malignant phenotype and induced a systemic tumour-specific T_H2 response (Adris *et al.*, 1999). Murine melanoma and mammary adenocarcinoma cell lines also show reduced tumorigenicity when transfected with IL-10, while breast cancer and melanoma metastases can regress following IL-10 administration (reviewed in Mocellin *et al.*, 2001).

Another method of enhancing vaccine efficacy is blocking the function of CTLA-4. Following binding of CD80/86 to CD28, CTLA-4 is expressed on the T-cell, which then binds CD80/86 with higher avidity than CD28. Signalling through CTLA-4 limits T-cell proliferation and reduces cytokine production (Chambers *et al.*, 2001). The importance of CTLA-4 regulation is highlighted by the observation that CTLA-4 knock-out mice develop a fatal lymphoproliferation and multi-organ degeneration (Tivol *et al.*, 1995). However, transient inhibition of CTLA-4 function enhanced the antitumour effects of GM-CSF-

expressing vaccines in mouse models and in patients with melanoma or ovarian carcinoma, although mild autoimmune reactions were observed (reviewed in Dranoff, 2004). Such data demonstrates that carefully controlled, temporary abrogation of CTLA-4 could improve the efficacy of cytokine-expressing vaccines.

Further evidence also supports the notion that a vigorous immune response is required for successful antitumour responses. Perez-Diez *et al.* (2002) used a murine colon carcinoma line expressing a model antigen to show that strength of the immune response directed against the model antigen, rather than its mere presence or absence, strongly correlated with the reduction of tumour burden. A clinical trial treating pancreatic cancer with GM-CSF-secreting tumour cells showed that the patients with the strongest DTH skin reactions against autologous tumour cells also showed the best clinical responses and the longest disease-free survival (Jaffee *et al.*, 2001). This requirement for strong immune responses is particularly important considering the immunosuppressed state of most tumour microenvironments.

One potential major advantage of transfected fibroblasts over transfected tumour cells is that they may be effective against multiple tumour types. Cytokine-expressing autologous tumour cells have generally only been used against the parental tumour, and allogeneic tumour cells are only used to treat tumours of the same type. However, autologous dermal primary fibroblasts expressing IL-2 or IL-12 have already been used in clinical trials to treat many types of tumour (Sobol *et al.*, 1999; Kang *et al.*, 2001), and IL-2 engineered syngeneic fibroblasts have also been injected intracerebrally to treat experimental glioma (Lichter *et al.*, 2002). Therefore, transfected AJ3.1 fibroblasts may be effective against other syngeneic tumours. Firstly, treatment of other syngeneic NB cell lines such as NSX2 or the aggressive metastatic TBJ could be compared with N2a-derived tumours. It is also

important to investigate the effectiveness of transfected AJ3.1 cells against different tumour types, such as the SCK murine mammary carcinoma, which is syngeneic with A/J mice and has previously been shown to be sensitive to IL-12 (Coughlin *et al.*, 1998).

The complexity of the human disease plus the polymorphisms present in the human population compared with the inbred strain of mice used here means that caution is necessary when extrapolating the results obtained in this project to what could potentially be attained clinically. Furthermore, possible toxicity issues related to cytokine dose would also need to be addressed. Nonetheless, cytokine-transfected syngeneic fibroblasts demonstrated therapeutic efficacy in this murine NB model. The syngeneic fibroblast vaccine compared extremely well with the tumour cell vaccine in every experiment, abrogating the tumorigenicity of wild-type N2a cells, inducing systemic protective antitumour immunity and mediating significant regression of established tumours. *In vitro* assays demonstrated cellular responses to wild-type N2a cells, while vaccinated tumours revealed necrosis and extensive leukocyte infiltration. In conclusion, syngeneic fibroblasts expressing IL-2 and IL-12 present a viable alternative to autologous tumour vaccines and warrant further investigation for the treatment of neuroblastoma and other solid neoplasms.

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APPENDIX*

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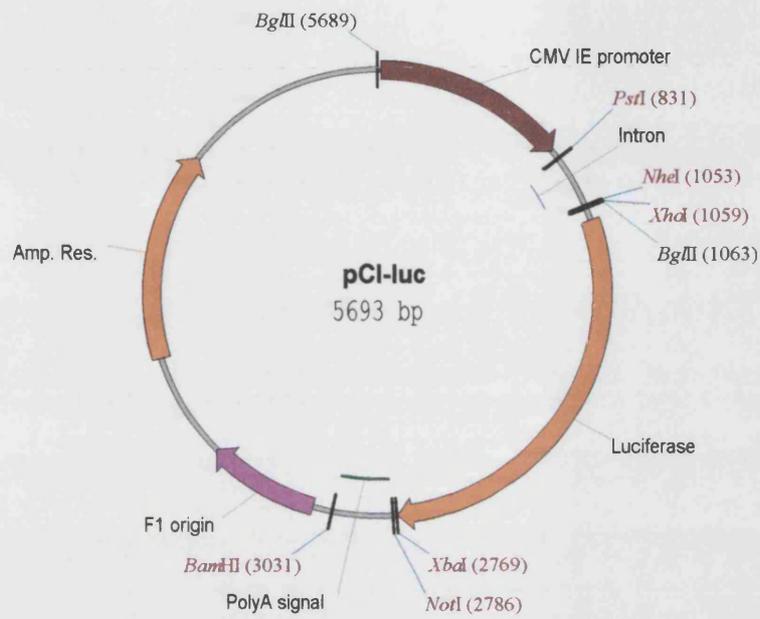
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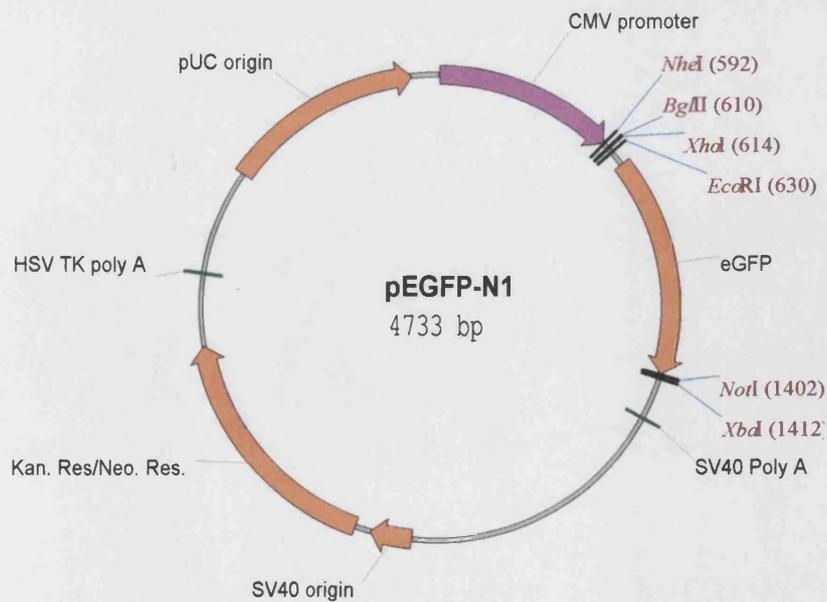
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Appendix 1: Plasmids used for LPD transfections

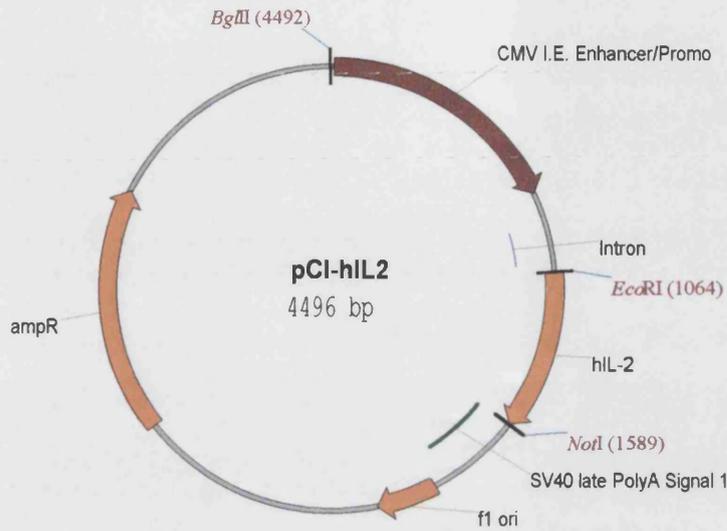
1A: pCI-Luciferase



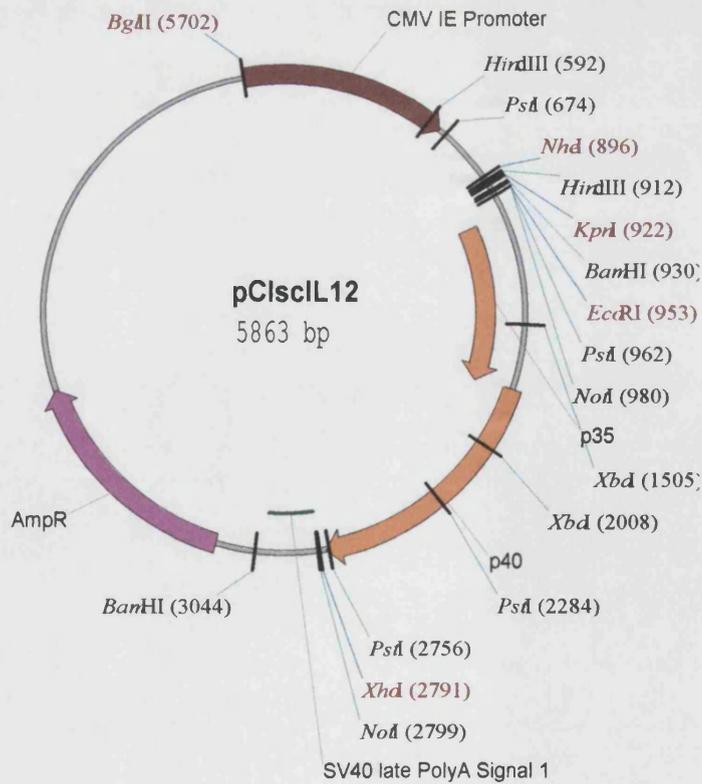
1B: Enhanced Green Fluorescent Protein-N1



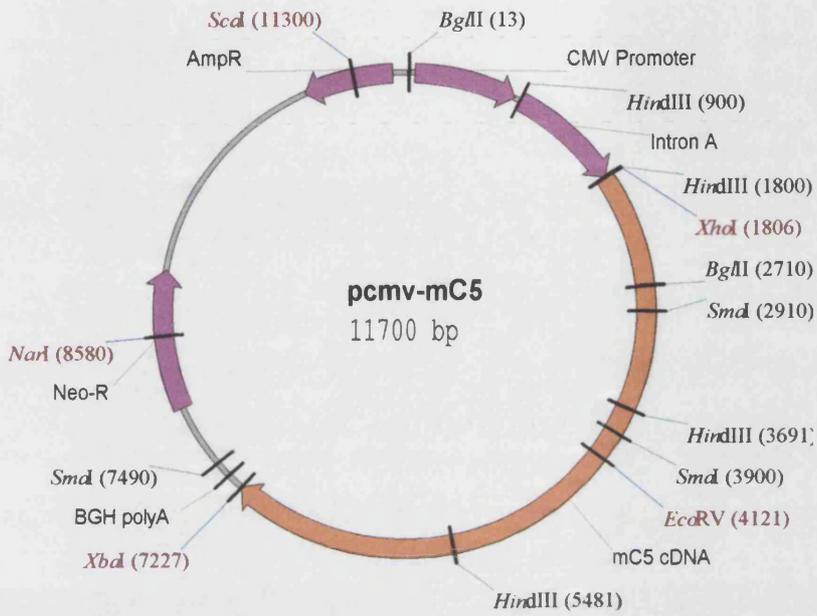
1C: pCI-human Interleukin-2



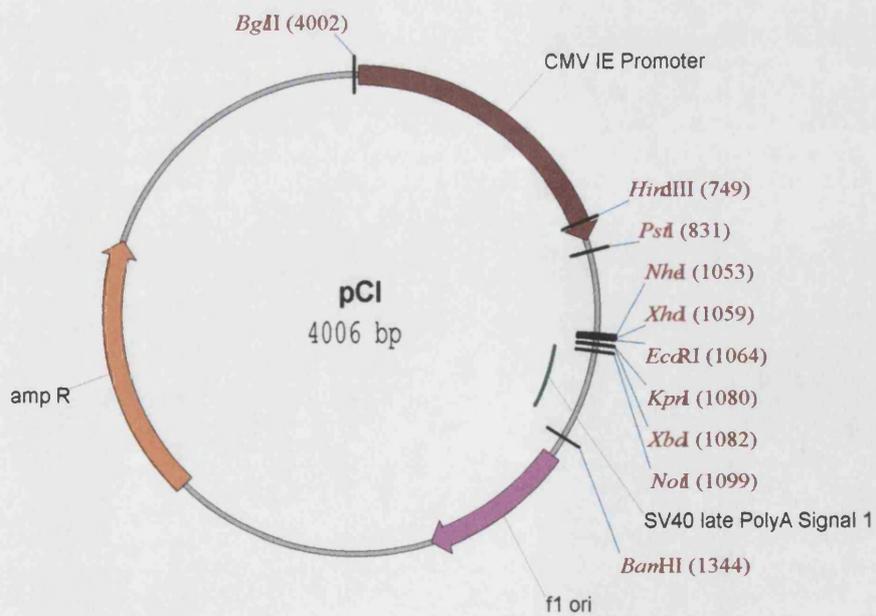
1D: pCI-single chain murine Interleukin-12



1E: pCMV-murine complement protein 5



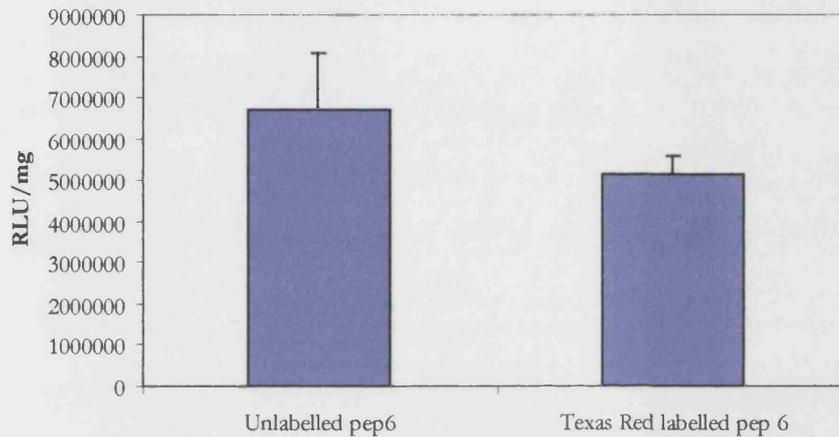
1F: pCI



Appendix 2: Phenotype of MACS separated DC compared with unseparated DC

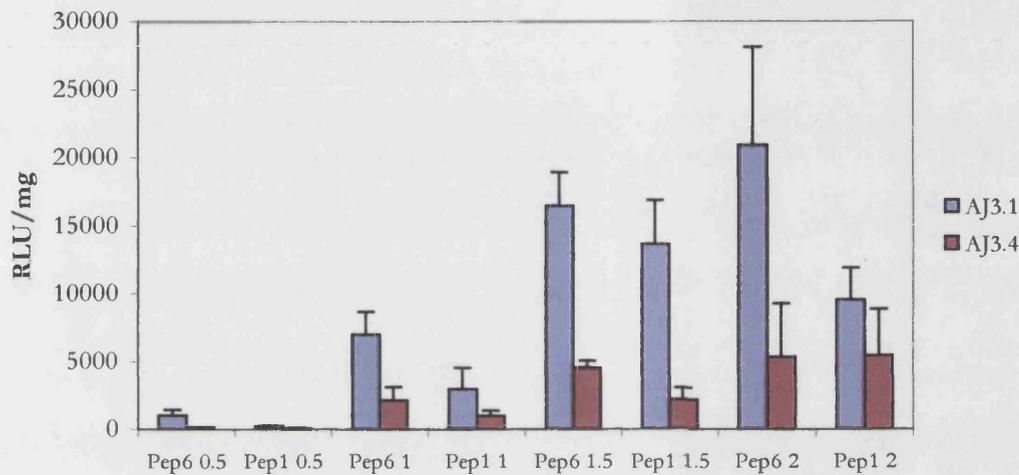
Surface Marker	Unseparated immature DC	Unseparated mature DC	MACS separated immature DC	MACS separated mature DC
CD40	14.3%	72.4%	10.5%	87.1%
CD80	21.2%	31.4%	17.5%	35.8%
CD86	18.1%	41%	17.1%	50.6%
MHC II	24.9%	51%	21.5%	49.0%

Comparison of the phenotype of unseparated DC or DC separated by positive selection on MACS CD11c microbeads. Cells were analysed by flow cytometry in their immature state or after maturation by incubation overnight in the presence of 1µg/ml LPS. Double staining with CD11c-PE and antibodies against CD80-FITC, CD86-FITC, MHC II-FITC or CD40-biotin and streptavidin-CyChrome identified the DC fraction of the unseparated cells. This data shows that the MACS separation procedure itself does not affect the DC phenotype, and neither does it affect the ability of the DC to mature in response to stimulus.

Appendix 3: Effect of fluorescently-labelled peptide on transfection

Transfection with a luciferase reporter gene was performed to investigate whether peptide 6 labelled with Texas Red used for the confocal microscopy experiments would interfere with the transfection process. The representative data shown here shows that transfection with labelled peptide results in a slight reduction in transgene expression, but no substantial detrimental effect is observed. Error bars represent standard deviations from triplicate transfections.

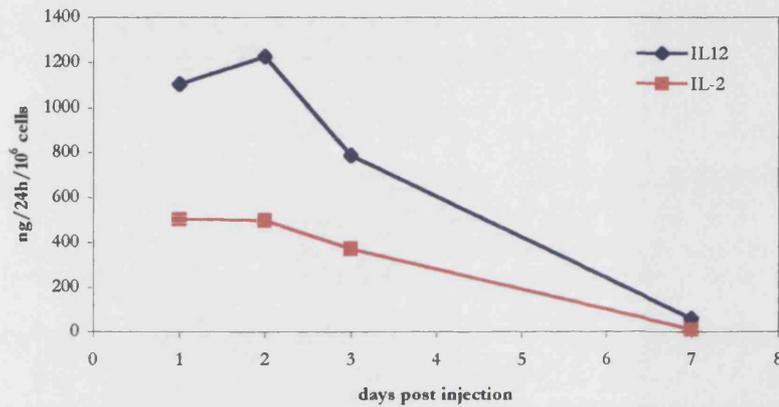
Appendix 4: Preliminary MEF reporter gene transfections



Two MEF cultures were established with the morphology of fibroblasts, AJ3.1 and AJ3.4. Preliminary transfections were performed to determine which cell line would be used for all future experiments. A luciferase reporter gene was used to test peptides 1 and 6 at a charge ratio of +7, and amounts of DNA were varied between 0.5-2 μ g per well. Cells were analysed by luciferase assay 24 hours after transfection. Representative data is shown and error bars indicate standard deviations of triplicate transfections.

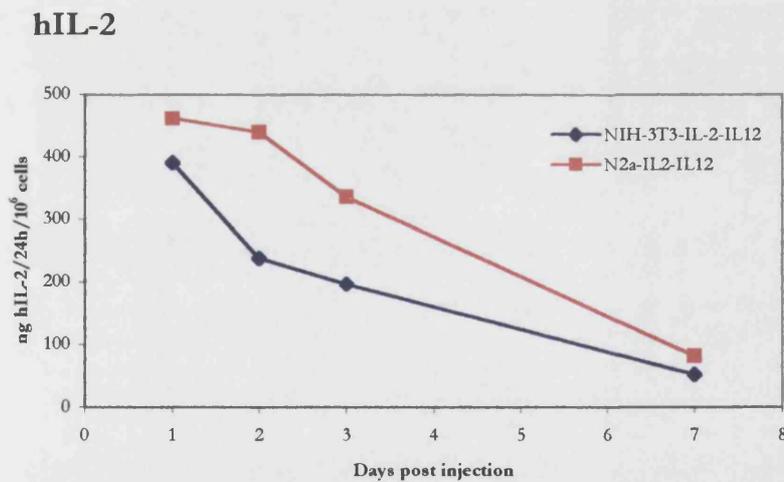
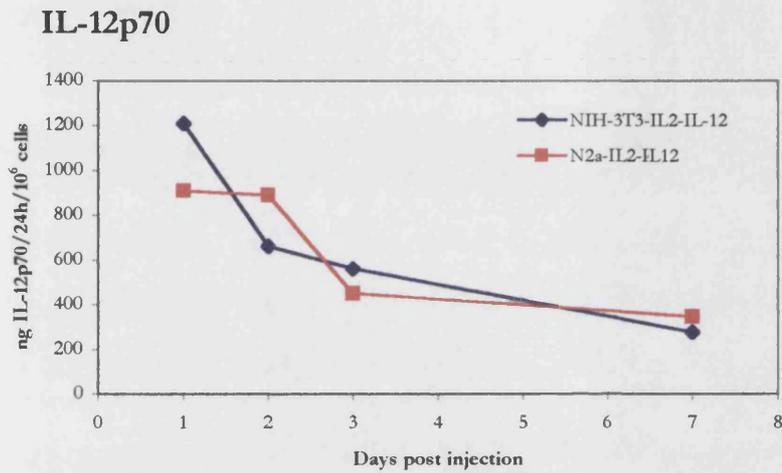
The data clearly shows that AJ3.1 expressed higher levels of transgene than AJ3.4 under every condition tested. Peptide 6 mediated higher luciferase expression than peptide 1 at equal amounts of DNA. Therefore, AJ3.1 was used in all subsequent experiments with syngeneic MEFs, and peptide 6 was used for comparison with other targeting peptides.

Appendix 5A: Cytokine expression of irradiated NIH-3T3 cells used in first tumourigenicity experiment using allogeneic fibroblasts



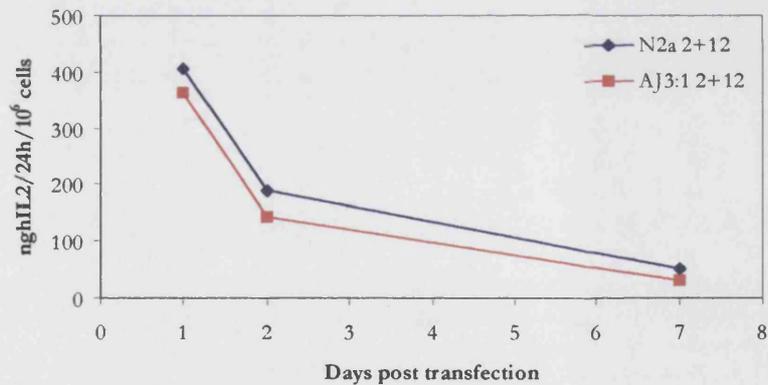
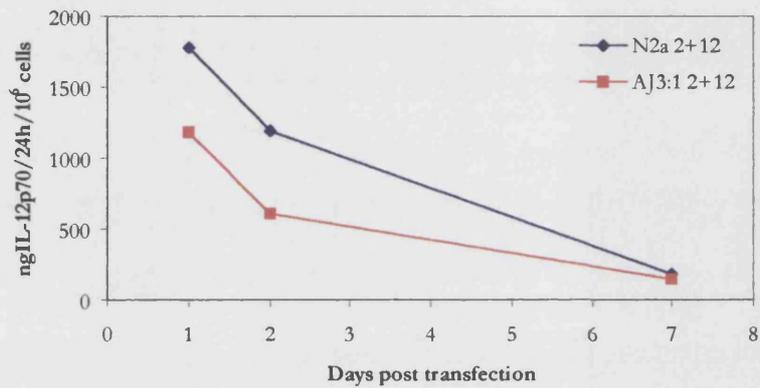
Expression of IL-2 and IL-12 by NIH-3T3 cells used in the first tumourigenicity experiment using allogeneic fibroblasts. These cells were transfected, and irradiated with 2500 rads shortly before s.c. administration with live wild-type N2a cells.

Appendix 5B: Cytokine expression of live transfected NIH-3T3 cells and transfected N2a cells used in second tumourigenicity experiment using allogeneic fibroblasts



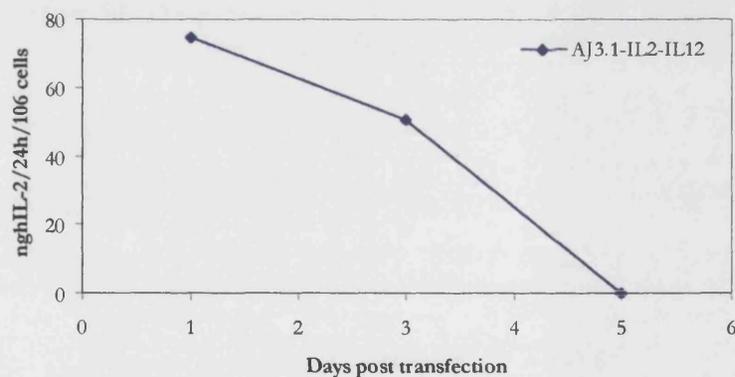
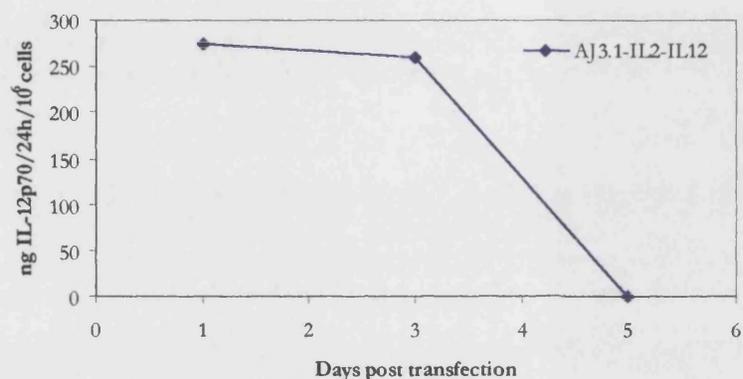
Expression of IL-2 and IL-12 by NIH-3T3 cells and N2a cells used in the second tumourigenicity experiment using allogeneic fibroblasts. These cells were transfected and administered s.c. together with live wild-type N2a cells as a live cell vaccine.

Appendix 5C: Cytokine expression of live transfected AJ3.1 fibroblasts and transfected N2a cells used in the first tumourigenicity experiment using syngeneic fibroblasts



Cytokine expression of transfected AJ3.1 and N2a cells used in first tumourigenicity experiment with syngeneic fibroblasts.

Appendix 5D: Cytokine expression from transfected irradiated AJ3.1 fibroblasts in the second tumourigenicity experiment using syngeneic fibroblasts

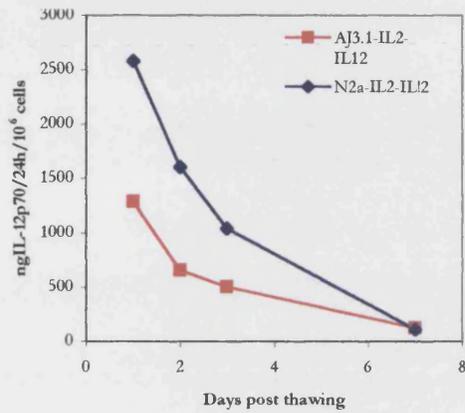


Expression of IL-2 and IL-12 by AJ3.1 cells used in the second tumourigenicity experiment using syngeneic fibroblasts. These cells were transfected, and irradiated with 2500 rads shortly before s.c. administration with live wild-type N2a cells.

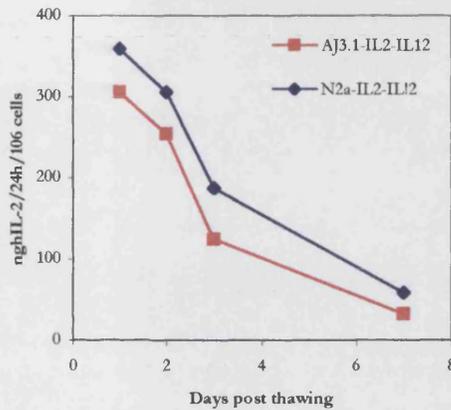
Appendix 5E: Cytokine expression of transfected AJ3.1 fibroblasts and N2a cells used in the eradication experiment

Injection 1

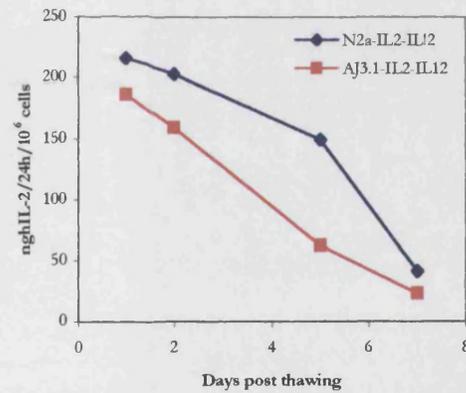
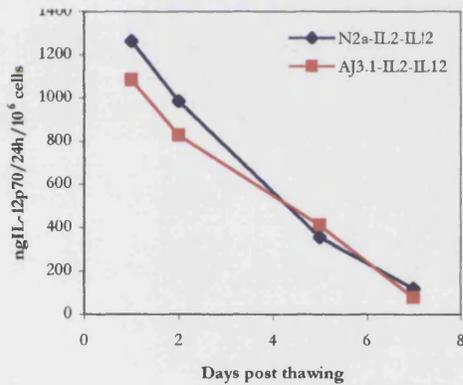
IL-12p70



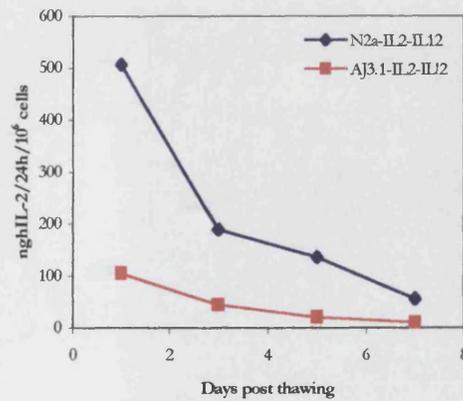
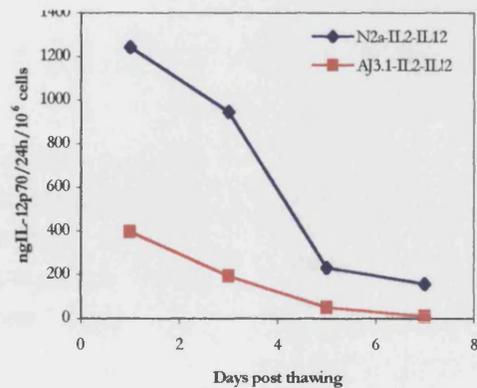
hIL-2



Injection 2

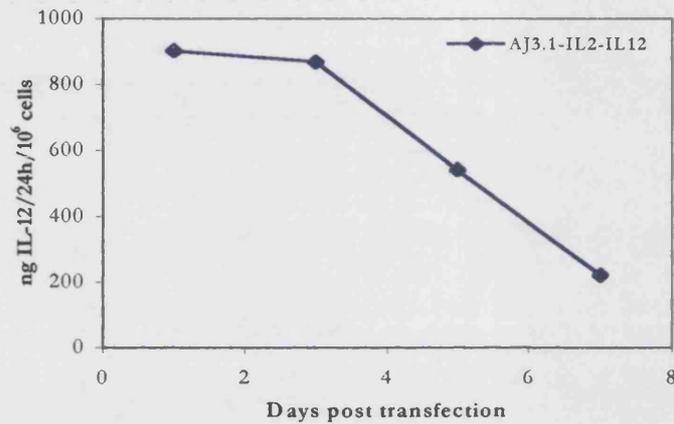


Injection 3

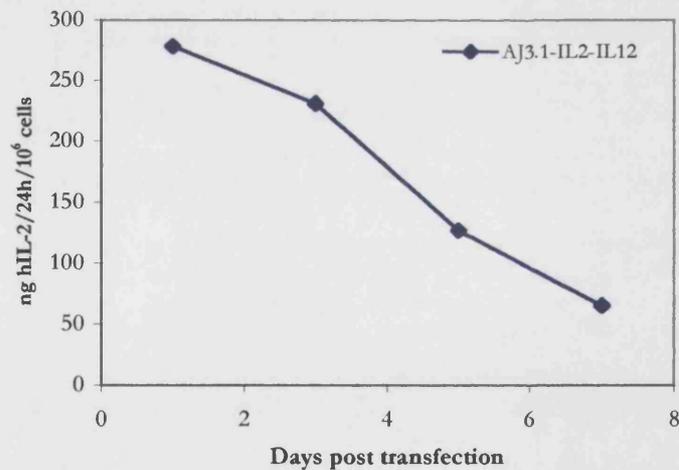


Appendix 6: Cytokine expression of irradiated cytokine-transfected AJ3.1 fibroblasts

IL-12p70



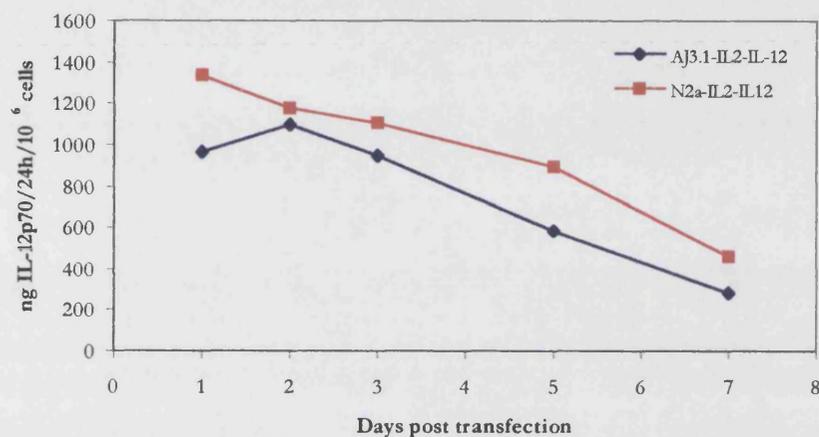
hIL-2



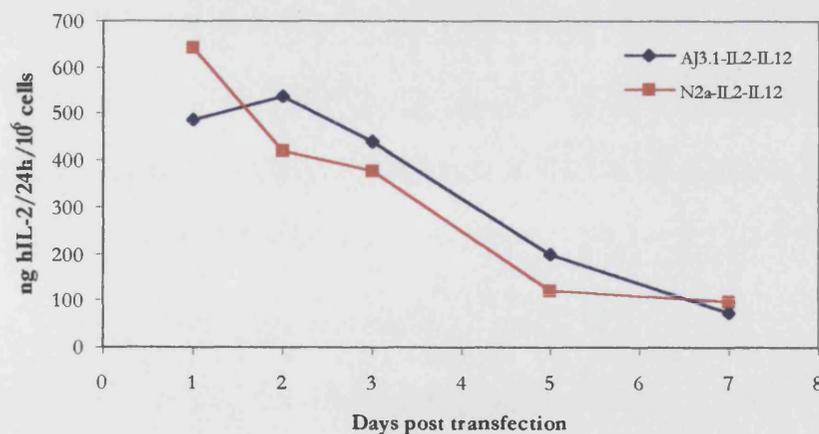
Cytokine-transfected AJ3.1 cells were irradiated with 2500 rads 24h after transfection, then cultured *in vitro* and supernatant harvest over 24h periods and analysed by ELISA for expression of IL-12 and IL-2. Cytokine expression is comparable with that from live cytokine-transfected AJ3.1 and was maintained for over 7 days.

Appendix 7: Cytokine expression from frozen transfected cells

IL-12p70



hIL-2



Cytokine expression from AJ3.1 fibroblasts and N2a cells transfected with IL-2 and IL-12, then frozen down to -80°C 24h after transfection. One week later, cells were thawed, and cultured at 37°C . Supernatant was collected over 24h intervals for 7 days. Cytokine expression is equivalent to that obtained from freshly transfected cells.