Neuroblastoma Immunotherapy Using A Novel Vector System

KONSTANTINA ELENA SIAPATI

A thesis submitted for the degree of Doctor of Philosophy

2001

Molecular Immunology Unit
Institute of Child Health
University College London

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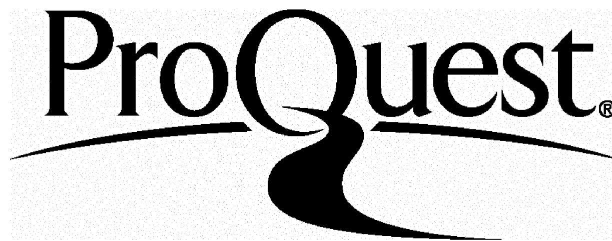

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ABSTRACT

Neuroblastoma is one of the commonest paediatric solid tumours and its treatment using conventional therapy has so far been of limited success. Immunotherapy in the form of tumour vaccination is an alternative therapeutic approach which aims at enhancing the immunogenicity of the tumour and involves the *ex vivo* manipulation of tumour cells to express certain immunomodulatory molecules. The aim of this project was to develop a cell-based neuroblastoma vaccine expressing Interleukin-2 and/or Interleukin-12, and examine its effect in a murine model for the disease. A non-viral vector system (LID) consisting of an integrin-targeting peptide and Lipofectin was used to deliver the cytokine genes into neuroblastoma cells. Optimisation of the vector components resulted in transfection of neuroblastoma cells at high efficiency (20-60%) utilising an $\alpha_5\beta_1$ integrin-targeting peptide. Examination of the intracellular trafficking of LID complexes in these cells suggested that the LID vector may employ a non-coated pit endocytic or phagocytic pathway to mediate cell entry. Despite the transient nature of transfection, expression of biologically active cytokines persisted above potentially therapeutic levels for at least 10 days, suggesting a sufficient time window for an immune response to be elicited. Indeed, transfection of the mouse Neuro-2A cell line with IL-2 and/or IL-12 completely abrogated its tumourigenicity in a syngeneic mouse model for neuroblastoma. Pre-vaccination with irradiated Neuro-2A cells conferred protective immunity against subsequent challenge with parental cells, which was, however, irrespective of the cytokine expression by the cells. There was a clear effect of IL-12 with reduced tumour growth in the presence of IL-2. *In vivo* immunodepletion experiments suggest that $CD8^+$ T cells may be responsible for the initial rejection of Neuro-2A cells expressing both IL-2 and IL-12, while tumour regression seems to be mediated by leukocyte infiltration and necrosis. These results indicate that the complex nature of anti-tumour immune responses may require multi-modality treatments, such combination of cytokines, for efficient tumour eradication and generation of systemic immunity. The improved anti-tumour immunity of co-transfection of IL-2 and IL-12, therefore, provides a promising immunotherapeutic approach for the treatment of neuroblastoma.

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ABBREVIATIONS

APC	Antigen presenting cell
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BM	Bone marrow
bp	Base pair(s)
BSA	Bovine serum albumin
CDA	Cytosine deaminase
cDNA	Complementary DNA
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
dH_2O	Distilled water
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOPE	dioleoyl phosphatidylethanolamine
DOTMA	N-[1(-2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluoroscein isothiocyanate
GM-CSF	Granulocyte macrophage cell stimulating factor
hIL-2	Human Interleukin-2

HRP	Horseradish peroxidase
HSV-tk	Herpes Simplex Virus thymidine kinase
ICE	Interleukin-1β converting enzyme
kb	Kilobase pair(s)
kDa	Kilo Daltons
LB	Luria-Bertani (media)
LD	Lipid-DNA
LID	Lipid-integrin targeting peptide-DNA
LOH	Loss of heterozygosity
MDR	Multi-drug resistance
MHC	Major histocompatibility complex
mIL-12	Murine IL-12
MRP	Multi-drug resistance associated protein
NCAM	Neural cell adhesion molecule
NK	Natural killer cell
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PD	Peptide-DNA
PFA	Paraformaldehyde
PHA-L	*Phaseolus* leucoagglutinin
PI3K	Phosphatidylinositol 3-kinase
PLL	Polylysine
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TAP	Transporters associated with antigen processing
TCR	T cell receptor
TE	Tris/EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine

Tris	2-amino-2-[hydroxymethyl]-1,3 propandiol
Tween 20	Polyethylene-sorbitan monolaurate
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
w/v	Weight per volume

AMINO ACIDS

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

PUBLICATIONS

This thesis has contributed to the following publications:

K.E.Siapati, C.Kinnon, A.Michalski, R.Anderson, A.Thrasher and S.L.Hart. *Improved anti-tumour immunity in murine neuroblastoma with a combination of IL-2 and IL-12.* Manuscript in process

Published abstracts:

K.E.Siapati, P.Brickell, C.Kinnon, A.Michalski, A.Thrasher and S.Hart (2000). Neuroblastoma Immunotherapy Using a Novel Vector System. *Molecular Therapy* **1**: 270

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisors Dr Steve Hart and Dr Adrian Thrasher for their help and support throughout this project. Special thanks also to Prof Christine Kinnon for her encouragement and invaluable advice on the preparation of this thesis. I am grateful to Mike Blundell, Kate Parseley and Susie Barker for their great help with the animal work and the long trips to the Royal Veterinary College! I would like to acknowledge Dr Anthony Michalski and Dr John Anderson for the patient neuroblastoma samples and Caroline Marshall for her assistance and helpful practical tips on the preparation and histology of tumour specimens. I would also like to thank Dr Richard Parkes for critical review and heated discussions on certain parts of this thesis.

Last but not least I'm indebted to my family for the enormous psychological support and encouragement throughout these four years.

1

INTRODUCTION

1.1 NEUROBLASTOMA

1.1.1 Features of the disease

Neuroblastoma is an extracranial solid tumour that accounts for approximately 10% of the solid malignancies occurring in children. It arises from precursor cells of the sympathetic nervous system and primary sites include the adrenal gland and the abdomen. Localisation of the tumour in the thorax and the neck are less common (reviewed in Ninane and Pearson, 1997). Recently, a number of studies have reported spread of the disease at CNS sites (Kramer *et al.*, 2001). Neuroblastoma has the highest rate of spontaneous regression but one of the poorest outcomes when it occurs as disseminated disease. Stage 4S neuroblastoma is a special form of the malignancy characterised by localised primary tumour with metastases that exclude involvement of the bone (**Table 1.1**). The prognosis for stage 4S, even in the absence of any therapy, is very good, with the majority of tumours undergoing spontaneous regression. Due to its high incidence, neuroblastoma has been extensively studied and various therapeutic approaches have been tested.

Neuroblastoma is a genetically heterogeneous disease with the majority of tumours demonstrating deletions or rearrangements of several genomic regions. These include loss of heterozygosity at 1p36 (Takeda *et al.*, 1996) where a number of tumour suppressor genes such as p73 have been identified (Jost *et al.*, 1997; Kaghad *et al.*, 1997). In addition, LOH for chromosome 14q and 11q, and chromosome 17 aberrations have been reported (Takayama *et al.*, 1992; Lastowska *et al.*, 1997; Hoshi *et al.*, 2000). LOH at chromosome 19q13 is associated with aggressive disease (Mora *et al.*, 2001). Another very common genetic abnormality in neuroblastoma is amplification of the N-*myc* oncogene, which is located at chromosome 2p23-24. The family of myc genes encodes transcription factors that control cell proliferation (Bouchard *et al.*, 1998). Transgenic mice over-expressing N-*myc* in neural crest cells develop neuroblastoma tumours a few months after birth strongly implicating N-*myc* in tumourigenesis (Weiss *et al.*, 1997). N-*myc* amplification is also associated with high telomerase activity and poor prognosis in 94% of neuroblastomas. This

phenomenon could be due to a failure to repress telomerase during development or because of reactivation of telomerase activity at a later stage accompanied by other genetic abberations leading to neuroblastoma development (Hiyama *et al.*, 1995).

A tumour-associated marker has not been identified for neuroblastoma. Many studies have used the disialoganglioside GD_2 as a target antigen either for the generation of specific cytotoxic T lymphocytes (CTL) (Zhao and Cheung, 1995) or for the direction of certain immunostimulatory molecules such as Interleukin-2 fused to monoclonal antibodies (Lode *et al.*, 1998a). In addition, monoclonal antibodies targeted to GD_2 display antibody-dependent cytotoxicity against neuroblastoma cells when used together with lymphokine activated killer cells *in vitro* (Honsik *et al.*, 1986). This suggests that GD_2 may be an effective immunotherapeutic target for neuroblastoma.

Table 1.1 International Neuroblastoma Staging System (INSS)[1]

Stage 1	Localised tumour with complete surgical resection; distant lymph nodes negative for tumour (microscopically)
Stage 2A	Localised tumour with incomplete resection; lymph nodes negative for tumour
Stage 2B	As in Stage2A but ipsilateral lymph nodes positive for tumour while contralateral lymph nodes must be negative for tumour
Stage 3	Unresectable tumour with or without regional lymph node involvement <u>or</u> localised tumour with contralateral lymph node involvement <u>or</u> tumour crossing the vertebral column by infiltration or by lymph node involvement
Stage 4	Primary tumour with metastases in bone, bone marrow, lymph nodes, liver and/or other organs
Stage 4S	Localised primary tumour with metastases limited to skin, liver or bone marrow. Limited to children under 1 year of age

[1] Adapted from (Castel *et al.*, 1999).

GD_2 is highly expressed in many human tumours including neuroblastoma and is believed to play a role in the clinical phenotype of neuroblastoma. Gangliosides are membrane-bound glycosphingolipids that are prominent in neuronal tissue. Biosynthesis of gangliosides from ceramide occurs in two distinct pathways, a and b, with b being associated with early brain development. Comparison between neuroblastoma patients younger than 1 year of age with a group of patients older than 1 year, revealed that b pathway gangliosides were prominent in 92% of patients in the former group compared to just 40% of the latter group (Kaucic *et al.*, 2001). This suggests that the pathway of ganglioside synthesis is important for the clinical differences observed among different stages of neuroblastoma biology and that the b pathway is probably associated with better prognosis.

1.1.2 Conventional therapy

The type of therapy chosen to treat patients with neuroblastoma depends on the stage of the disease but has mainly consisted of chemotherapy followed by surgical resection of the tumour or radiotherapy (Pinkerton *et al.*, 2000). Although the disease is responsive to therapy, survival of high-risk group patients at 2 years is 50% and survival at 5 years is 33% (Philip *et al.*, 1997). The main drawback of such approaches is the acquired drug resistance after exposure to chemotherapeutic agents. Patient cell lines exhibit higher than 1000-fold resistance after etoposide and doxorubicin treatment (Keshelava *et al.*, 1997). Multi-drug resistance depends on the expression of two genes, multi-drug resistance (MDR) (Haber *et al.*, 1997) or multi-drug resistance-associated protein (MRP) genes that encode ATP-dependent membrane transport proteins. There is a strong correlation between the expression patterns of these genes in neuroblastoma tumours lacking N-*myc* amplification (Haber *et al.*, 1997). Although expression of the MDR1gene is associated with poor outcome, it is found to inversely correlate with amplification of N-*myc*. Another drawback of chemotherapy is its non-specific nature, which does not target the minimal residual disease and causes patients to relapse. Strategies to address that problem have included high-dose induction chemotherapy followed by stem cell rescue. Patients who survive for 5 years

following initial surgical resection or chemotherapy have an 80% chance of becoming long-term survivors if they receive bone marrow transplantation (Philip *et al.*, 1997).

In order to increase the specificity of the treatment, the use of anti-neuroblastoma IgM antibodies found in healthy individuals has been investigated. Administration of such antibodies in a metastatic neuroblastoma model in nude rats completely abrogated the tumourigenicity of human LAN-1 neuroblastoma cells. In addition, there was a 90% reduction in the initial volume of established adrenal gland tumours following treatment. The main anti-tumour functions of these antibodies are achieved through complement activation and apoptosis (Engler *et al.*, 2001).

1.2 GENE THERAPY FOR CANCER

The concept of gene therapy for treating cancer arose with the recognition that all tumours arise as a result of genetic lesions. It has been proposed that anti-tumour therapy can be achieved by genetic intervention, a process that can induce selective killing of tumour cells and eliminate cytotoxic side effects caused by chemotherapeutic agents and radiotherapy. Although numerous approaches have been devised, the common aim is to specifically eradicate tumour cells. Limitations of the available gene transfer vectors make it impossible to achieve 100% transduction efficiency of the tumour cells. However, due to a process known as the 'bystander effect', whereby the cytotoxic effect of a treatment is diffused to neighbouring cells, 100% transduction efficiency is not always required. This effect can be local, by release of cytotoxic agents to neighbouring cells, immune, where tumour-specific immune responses are generated, or angiostatic.

1.2.1 Drug sensitivity

Transduction of tumour cells with a gene whose product can convert a non-toxic administered prodrug to a toxic metabolite is a very efficient approach of inducing cell

death. Herpes Simplex Virus thymidine kinase (HSV-tk) is a well-studied suicide gene that converts ganciclovir (GV) to the toxic metabolite ganciclovir-triphosphate once inside a cell expressing HSV-tk. Cell death is caused by incorporation of the toxic metabolite into the DNA chain and termination of DNA synthesis. The advantage of this system is that adjacent untransduced cells are rendered sensitive to ganciclovir. This effect occurs only when cells are in direct contact with HSV-tk-transduced cells and has been attributed to the transfer of the toxic metabolite through gap junctions (Bi *et al.*, 1993). Retroviral transfer of HSV-tk into murine neuroblastoma tumours and subsequent administration of ganciclovir results in tumour regression and extensive necrosis as indicated by TUNEL staining (Cho *et al.*, 1999). Another well-studied suicide gene is cytosine deaminase (CDA) that converts 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). This is then converted to 5-FU monophosphate and 5-FU triphosphate that interact with DNA and RNA synthesis respectively and lead to cell death. The 'bystander effect' occurs with CDA as well but probably by a different mechanism where 5-FU freely diffuses through the cell membrane. Adenoviral transfer of the CDA gene to colon carcinoma cells increases expression of CD95 and leads to apoptotic cell death upon treatment with 5-FC (Ju *et al.*, 2000). Combination of other treatments such as administration of lymphotactin, results in greater inhibition of tumour growth, which is mediated by $CD4^+/CD8^+$ T cells and natural killer (NK) cells.

1.2.2 Oncogenes and tumour suppressor genes

Transformation of a normal to a malignant phenotype is the result of genetic mutations causing loss of tumour suppressor function or acquisition of oncogenic phenotype. As a result of this observation, gene therapy strategies have been devised that involve the introduction or restoration of tumour-suppressor function by correcting for the mutated allele. The tumour suppressor gene p53 is crucial in induction of apoptosis and its absence is believed to render cells resistant to mutations caused by irradiation and chemotherapeutic compounds. Loss of p53 is associated with resistance of tumour cells to certain anticancer therapies (Lowe *et al.*, 1993).

50% of human cancers either express a mutant p53 or a non-functional protein emphasising the need for effective therapies for tumours that lack p53 function. In a human non-small cell lung cancer study, tumour biopsies following retroviral administration of p53 gene show apoptotic death of tumour cells and tumour regression in 33% of patients (Roth *et al.*, 1996). The majority of neuroblastoma cells lack p53 function or its closely related p73 tumour suppressor gene, implicating it in the process of malignant transformation. Expression of the human polyomavirus BK (BKV) large T antigen by neuroblastoma interferes with p53 function. Antisense oligonucleotides against BKV large T antigen restore p53 expression and induce apoptosis (Jorgensen *et al.*, 2000). In addition, p53 inactivation may be a consequence of antagonistic function of the glucocorticoid receptor (GR) on p53-downstream targets such as p21 and bcl2 (Sengupta *et al.*, 2000).

Lack of p53 expression in many tumour cells has been exploited as a target for tumour therapy. A number of viral proteins bind to and inactivate p53 including the protein encoded by E1B gene of adenovirus. A virus lacking E1B should, in theory, be able to infect and lyse p53-deficient tumour cells. Administration of ONYX-15, an E1B gene-attenuated adenovirus, in nude mice engrafted with p53 mutant tumours (C33A cervical cancer) or tumours expressing defective form of p53 (HLaC laryngeal cancer) resulted in tumour regression. No cytolysis was observed in tumours expressing a fully functional p53 gene (Heise *et al.*, 1997). Phase I-II clinical trials with ONYX-15 have been initiated in a number of cancers. However, administration of this replication-selective adenovirus alone failed to exhibit any anti-tumour effects unless it was combined with chemotherapy. The virus was well tolerated in all doses and routes administered, but further modifications are required to enhance its efficacy in certain tumours (Kirn, 2001).

1.2.3 Anti-angiogenesis

Generation of new blood vessels is a prerequisite for tumour growth and metastasis formation. Human neuroblastoma cell lines secrete metalloproteinases, MMP-2 and

MMP-9 that degrade the extracellular matrix, induce proliferation of human vascular endothelial cells and promote angiogenesis *in vivo* (Ribatti *et al.*, 1998). Activin A, a member of the TGF-β superfamily, is present during embryonic development and suppresses angiogenesis *in vivo*. An inverse correlation of activin A with N-*myc* expression has been demonstrated in human neuroblastoma cell lines. The down-regulation involves interaction of the N-*myc* with the activin A promoter implicating the former in the angiogenesis process (Breit *et al.*, 2000).

In a clinical context, high vascular density correlates with N-*myc* amplification and poor prognosis in neuroblastoma patients (Meitar *et al.*, 1996). Expression of angiogenic factors such as vascular endothelial growth factor (VEGF), VEGF-B, VEGF-C, transforming growth factor (TGF-α), angiopoietin-1 and –2, and platelet-derived growth factor (PDGF) is higher in patients with more advanced stages of the disease (stages 3 and 4S) (Eggert *et al.*, 2000). The fact that several angiogenic factors are co-expressed in most patients, suggests that they act in synergy to create highly vascularised neuroblastoma tumours. Therefore, multi-modality treatments may be required that target a number of different factors. Treatment of experimental neuroblastoma tumours with the angiogenesis inhibitor TNP-470 [*O*-(*N*-chloroacetyl-carbamoyl)-fumagillol significantly reduces the tumour growth rate but has no effect in animals with large tumour burdens (Katzenstein *et al.*, 1999). Although this angiostatic agent acts on endothelial proliferation independently of angiogenic factor expression, reducing the vascularisation of large tumours has not been possible.

Retroviral transduction of murine fibroblasts with the gene for the soluble VEGFR-2 (flk-1) and co-administration with neuroblastoma cells into SCID mice significantly decreased murine tumour growth after 25 days (Davidoff *et al.*, 2000). In a similar way, VEGFR-2 transduction of neuroblastoma cells reduced their tumourigenicity in SCID mice so that the resulting tumours were about one third the volume of those of controls (Davidoff *et al.*, 2001). The flk-1 produced by transduced neuroblastoma cells in that study showed *in vitro* bioactivity by inhibiting human umbilical vein endothelial cell (HUVEC) proliferation and VEGF-mediated migration.

Expression of certain integrins such as $\alpha_v\beta_3$ by endothelial cells is implicated in the formation of blood vessels (Brooks *et al.*, 1994). Cytokines such as TNF and IFN-γ do not only induce an immune response but also demonstrate angiostatic properties. Treatment with either of these cytokines inhibits $\alpha_v\beta_3$–mediated endothelial cell adhesion and survival *in vitro*. Their administration in melanoma patients disrupt tumour vascularisation by causing the detachment of $\alpha_v\beta_3$ –positive endothelial cells (Ruegg *et al.*, 1998).

1.2.4 Immunotherapy

Cancer immunotherapy depends on the proposition that most tumours possess specific antigens that are either of embryonic origin and are absent from 'mature' tissues, or are expressed as a result of mutations, or other genetic aberrations, and are thus attractive targets for immunotherapy. However, malignant transformation may be accompanied by a number of phenotypic changes in the cancer cells that allow tumours to evade the immune system. Although those changes depend largely on the individual neoplasm, certain common mechanisms have been identified.

1.2.4.1 Tumour immune evasion

1.2.4.1.1 Down-regulation of MHC molecules

Down-regulation of MHC molecules is a frequent observation in malignant cells and over-expression of MHC class I reverses the oncogenic effects (Tanaka *et al.*, 1986). Cytotoxic T cells (CTLs) require antigen presentation through MHC class I molecules while NK cells recognise MHC class I via their inhibitory receptors (KIRs). Down-regulation of MHC class I molecules may cause tumour evasion of immune attack either by insufficient antigen presentation to CTLs or inhibition of NK cell function. In neuroblastoma, down-regulation of MHC class I molecule expression is associated with N-*myc* amplification. This occurs through suppression of the p50 subunit of the

transcription factor NF-κB, which is involved in up-regulation of IL-2 expression by T cells and their subsequent proliferation. Transfection of neuroblastoma cells with p50 restores surface expression of MHC class I molecules (van't Veer *et al.*, 1993).

Neuroblastoma immunogenicity is related to MHC class II expression. Retroviral transfer of syngeneic, allogeneic or xenogeneic MHC class II molecules, causes murine neuroblastoma cells to lose their tumourigenicity. However, cells transduced with human DR (xenogeneic) or I-A^b (allogeneic) induce a more rapid immune response that correlates with enhanced stimulation of naive splenocytes compared to the syngeneic (I-A^k) MHC expressing vaccines (Hock *et al.*, 1996). The speed of the observed immune response may be due to the foreign nature of the transduced antigen that may result in an initial alloreactive response.

Defects in the assembly pathway of MHC molecules, such as the TAP (transporters associated with antigen processing) complex have been reported in cervical carcinomas. TAP consists of TAP1, TAP2 and tapasin and mediates the transportation of the antigenic peptides from the proteosome to the endoplasmic reticulum. In neuroblastoma cell lines with N-*myc* amplification and deletion at 1p, low expression of HLA class I molecules correlates with absence of TAP1. Expression of all three members of the TAP complex as well as surface HLA class I molecule expression can be restored by treatment with IFN-γ (Corrias *et al.*, 2001). The importance of TAP in tumour immunogenicity has been demonstrated by incorporation of a functional TAP gene in a recombinant vaccinia virus in a small-cell lung carcinoma model. TAP-transduced cells exhibited improved immunogenicity and enhanced antigen presentation (Alimonti *et al.*, 2000).

1.2.4.1.2 Role of Fas/FasL

Cytotoxic T lymphocytes and NK cells are among the most effective mediators of anti-tumour immunity. Killing of target tumour cells is initiated by cell contact followed by release of perforin and granzymes that penetrate the tumour cells and

cause apoptotic death. Apoptosis can also be induced by interaction of CD95L (FasL) on the effector cell with the 'death receptor' CD95 (Fas) on the target cell. Ligation of the receptor triggers a cascade of caspase proteins such as caspase 1 or interleukin-1β-converting enzyme (ICE). The latter cleaves interleukin-1β to its active form or activates IL-18. Both of these cytokines have pro-inflammatory effects and can trigger an anti-tumour immune response. Expression of ICE mRNA has been detected in 69% of stage 1, 2 and 4S neuroblastoma tumours while only 19% of patients with poorer prognosis (stage 3 and 4) express ICE. In addition, ICE expression inversely correlates with N-*myc* amplification (Ikeda *et al.*, 1997).

Reports on Fas/FasL interaction and its importance in tumour immune evasion are quite controversial. Although most neuroblastoma cell lines express very low levels of Fas and FasL (Bernassola *et al.*, 1999), expression of FasL induces T cell death *in vitro* indicating a potential mechanism of T cell elimination at the tumour environment (Shurin *et al.*, 1998). Indeed, melanoma specific T cells after MHC-class-I recognition undergo apoptosis, a process that is inhibited with anti-Fas antibodies. FasL expression, in that particular study, could not be detected on tumour cells but rather on T lymphocytes following activation (Zaks *et al.*, 1999). The observed T cell apoptosis could be due to Fas/FasL interaction among T cells after prolonged antigen activation. Alternatively, Fas upregulation can occur in tumours *in vivo*, suggesting that Fas/FasL interaction plays an important role in immune escape especially at the early stages of tumour growth (Rose *et al.*, 2000). On the other hand, vaccination with FasL expressing neuroblastoma tumour cells showed efficacy in neuroblastoma immunotherapy. FasL-expressing cells induced a strong inflammatory response consisting mainly of neutrophils and provided a $CD8^+$ T cell tumour-specific protective immunity in a murine model. This vaccine also proved very effective in eradicating established tumours (Shimizu *et al.*, 1999).

In a similar way, other tumour-associated molecules may act as a protection by tumours against immune surveillance. For example, RCAS1 (receptor-binding cancer antigen expressed in SiSo cells) expression has been observed in uterine, ovarian, colon and lung cancers and recently its receptor has been identified on T, B and NK

cells. Upon binding with RCAS1, receptor-expressing cells cease to proliferate and undergo apoptotic death (Nakashima *et al.*, 1999).

1.2.4.1.3 Inhibition of apoptosis

Resistance to apoptosis can be an outcome of inactivation or functional loss of certain members of the apoptotic pathway. Caspase 8 inactivation has been reported in neuroblastomas with N-*myc* oncogene amplification (Teitz *et al.*, 2000). Recently, a number of viral proteins that inhibit transmission of apoptotic signals through Fas or other death receptors have been identified (FLIPs). These inhibitors are produced by herpes viruses and inhibit interaction of the cytoplasmic death domain of Fas receptor (FADD) with caspase proteins. Transduction of A20 lymphoma cells with Kaposi's sarcoma herepesvirus FLIP protein enhances their tumourigenicity *in vivo* and protects them from Fas-induced apoptosis, demonstrating the tumourigenic effect of such inhibitors (Djerbi *et al.*, 1999). However, T cell-mediated lysis of CD95/FLIP-transfected lymphoma cells can still take place *in vitro* through the perforin pathway. The importance of this pathway is demonstrated in perforin knockout mice, where administration of cells expressing high levels of FLIP results in progressive tumour growth. In contrast, cells that express low levels of FLIP are cleared. Therefore, as CD95/FLIP expression in both wild-type and perforin-knockout mice results in tumour growth, CD95-dependent apoptosis seems to be the most prominent mechanism of tumour eradication *in vivo* (Medema *et al.*, 1999).

1.2.4.1.4 T cell anergy

Alternative explanations of failure of immune surveillance include the phenomenon of T cell anergy. This unresponsive state of T cells is characterised by lack of particular responses despite optimal stimulation through their T cell receptor (TCR). T cell unresponsiveness may also be a consequence of lack of co-stimulatory molecules such as B7.1 or B7.2 from tumour cells. T cells require secondary confirmatory signals via

their CD28 receptor in order to become activated and absence of a simultaneous co-stimulatory signal leads to T cell anergy. Secretion of immunomodulatory molecules, such as IL-10, TGF-β or other cytokines, from the tumour cells or their surrounding environment may also contribute to T cell anergy. Anergic T cells exhibit reduced production of certain cytokines such as IL-2 and GM-CSF whereas IFN-γ levels remain unchanged. The decreased IL-2 levels and reduced cellular proliferation can be caused by the production of certain inhibitors such as Nil-2a that block cytokine transcription or p21 activation. These effects can be reversed in the presence of co-stimulatory signals during antigen presentation or by signalling through the IL-2 receptor (reviewed in Schwartz, 1996). Also, *in vivo* administration of activating antibody against CD40, which activates antigen-presenting cells, is capable of reversing the peptide-induced T cell unresponsiveness in a renal cell carcinoma model expressing influenza haemaglutinnin (Sotomayor *et al.*, 1999).

Groux *et al*, (1996) have reported that human $CD4^+$ T cells fail to proliferate or secrete any cytokines in a mixed lymphocyte reaction culture in the presence of IL-10. The induced anergic state cannot be reversed with anti-CD3 or anti-CD28 antibodies suggesting that IL-10 impairs signalling through the TCR/CD3 complex. However, after treatment with PMA these T cells proliferate normally indicating that a direct activation of protein C kinase is required (Groux *et al.*, 1996). Secretion of IL-10 and TGF-β may also explain why animals with big tumour burdens fail to respond to immunotherapeutic treatments. Hsieh *et al*, (2000) show that the expression of these two cytokines at the tumour site correlates with its progression stage. Addition of IL-10 and TGF-β during *in vitro* T cell function assays from vaccinated animals inhibits the proliferative and cytolytic activity of these T cells (Hsieh *et al.*, 2000). IFN-γ has been widely used in many cancer animal models to enhance the immunogenicity of the cells. A recent report, however, demonstrates its involvement in promoting immune escape in a CT26 murine colon carcinoma model. The decreased immunogenicity of the tumour cells is due to infiltrating $CD8^+$ cells secreting IFN-γ and down-regulating the immunodominant antigen gp70 (Beatty and Paterson, 2000).

1.2.4.1.5 Loss of immunodominant antigens

Tumours can also escape immune surveillance by shutting down genes expressing immunodominant antigens. MAGE-1, -3 and -6 are genes encoding melanoma rejection antigens and are presented to CTLs in an HLA-I context. 59% of clinical neuroblastoma samples and 43% of cell lines express those antigens and in clinical tumours expression of MAGE antigens inversely correlated with clinical stage (Ishida *et al.*, 1996). This may suggest that during neuroblastoma progression, silencing of MAGE genes comprises a mechanism of immune escape that provides neuroblastoma with a growth advantage. Furthermore, shedding of gangliosides that have an immunosuppressive function by neuroblastoma tumours may be an alternative way of destroying the host immune response. Tumour derived gangliosides inhibit IL-2 mediated proliferation of T cell lines and inhibit production of TNF-α by monocytes (Lu and Sharom, 1995). In addition, generation of DCs from murine bone marrow progenitors incubated with neuroblastoma cell lines is significantly impaired. Furthermore, when DCs are cultured in the presence of neuroblastoma cells, their antigen presenting capacity is inhibited in an allogeneic mixed leukocyte reaction (MLR) (Shurin *et al.*, 2001). Treatment of neuroblastoma cell lines with D-PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), an inhibitor of glycosphingolipid synthesis, significantly reduces the production and shedding of gangliosides (Li and Ladisch, 1996). This could potentially restore immune suppression and increase the immunogenicity of the tumour.

In conclusion, a number of potential mechanisms that allow tumours to escape immune surveillance have been identified. The precise mechanism varies for each type of cancer and is probably a combination of more than one. In order to increase the immunogenicity of many cancers and by-pass any immune suppressive effects, a number of studies have been performed using immunomodulatory molecules such as cytokines or chemokines systemically or in the form of a vaccine. In the latter case, patient tumour cells or established cell lines have been genetically modified to secrete these immunomodulatory molecules. The main advantages of this approach are the generation of specific anti-tumour immunity and the localised production of cytokines

at the tumour site thereby avoiding toxicity caused by systemic administration of cytokines.

1.2.4.2 Cytokine vaccines

Certain cytokines such as interferons have direct anti-tumour effects through localized tumour killing while others work by augmenting the T cell-mediated immunity against the tumour as a result of localised expression at the tumour site. For example, IL-4 and TNF-α induce inflammatory responses by attracting eosinophils and macrophages at the tumour site and by regulating the expression of certain adhesion molecules on the surface of endothelial cells, so that the transmigration of inflammatory cells is enabled (reviewed in Tepper and Mule, 1994). GM-CSF augments the immunity for a number of tumours and its mechanism is thought to be $CD4^+$ and $CD8^+$ T-cell dependent. An alternative mechanism of action could be through the activation and differentiation of DCs (reviewed in Pardoll, 1995). Other cytokines demonstrated to augment T cell-mediated tumour immunity are IL-6 and IL-7. IL-6 enhances tumour-specific cytotoxic T-lymphocytes and NK cell activity, while the actions of IL-7 are less clear.

1.2.4.2.1 Interleukin-2

Interleukin-2 (IL-2) is a 15.5 kDa protein, which is produced mainly by activated T cells and in certain cases by B cells. Immature human T cells express low affinity IL-2 receptors consisting of IL2Rα chain alone or dimeric interactions among the α, β and γ chains. Upon antigenic activation, the three chains form a complex that comprises the high-affinity IL-2 receptor. The main function of IL-2 on T cells is their clonal expansion following antigenic stimulation (**Fig 1.1**). IL-2 production by $CD4^+$ T cells (T_{H1}) also stimulates the growth of B cells and increases the cytotoxic activity of NK cells. Myeloid cells such as macrophages and peripheral blood monocytes also express

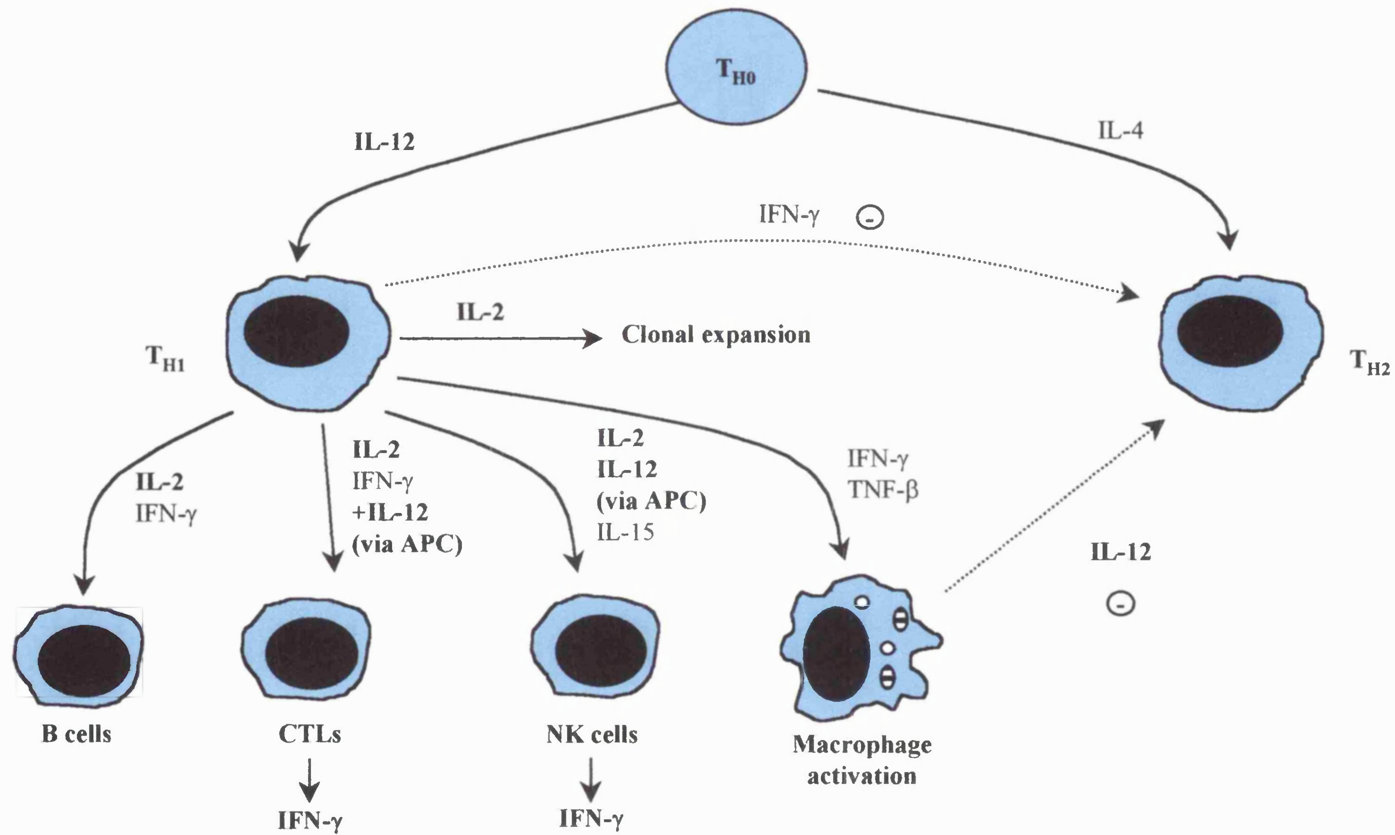


Figure 1.1 Effects of IL-2 and IL-12 on the immune system. The anti-tumour activity of IL-2 has been demonstrated in a number of studies mainly due to its ability to activate and enhance the cytotoxic activity of NK cells and CTLs (Fearon et al, 1990; Gansbacher et al, 1990; Arienti et al, 1994). IL-12 is a potent activator of natural killer and T cells and promotes the development of $CD4^+$ T_{H1} inducing anti-tumour immunity (Trinchieri, 1998). Many of its functions are mediated through the actions of IFN-γ but it was initially identified as an NK cell stimulatory factor (Gately et al, 1991).

IL2-Rβ and respond to IL-2. Also, proliferation and differentiation of $CD8^+$ T cells may be induced by direct ligation of IL-2 secreted by $CD4^+$ T cells (reviewed in Gaffen *et al.*, 1998).

IL-2 was among the first cytokines to be used in immunotherapeutic studies. Initially, it was used to expand tumour-infiltrating lymphocytes (TIL) *ex vivo* followed by adoptive transfer to melanoma patients. Detection was enabled after TIL retroviral transduction with the neomycin resistance gene and circulation of TILs in treated patients persisted up to 2 months (Rosenberg *et al.*, 1990). The introduction of IL-2 into tumour cells has been based on the speculation that the failure of the immune system to eliminate tumours is due to defective T cell help. At first, IL-2 was administered systemically a route that has, however, been associated with numerous side effects such as TNF and nitric oxide (NO) production (Fraker *et al.*, 1989; Kasid *et al.*, 1989). Co-administration of lymphokine-activated killer (LAK) cells together with IL-2 enhanced survival of immunodeficient mice compared to recombinant IL-2 alone and inhibited liver metastases formation (Sabzevari *et al.*, 1994). Studies utilising IL-2 have shown infiltration of $CD8^+$ and NK cells but not $CD4^+$ T cells, suggesting that the need for helper T cells can be bypassed (Fearon *et al.*, 1990). It is important to note that each experimental model exhibits distinct patterns of anti-tumour immunity with different effector cells playing key role in tumour eradication. Alternative strategies to achieve specific delivery of the cytokine to the tumour site include fusion of IL-2 to an anti-GD_2 antibody. The GD_2-IL-2 fusion protein exhibits an NK cell-mediated immunity in a murine model of neuroblastoma metastasis and treatment of murine splenocytes with anti-MHC I antibody enhance their cytolytic activity against NXS2 target cells (Lode *et al.*, 1998a). In the same study, recombinant IFN-γ administration failed to activate T cells and resulted in tumour formation when treated animals were challenged subcutaneously. This could be attributed to T cell anergy induced by IL-10 and TGF-β1 secreted by NXS2 cells, which have an activating effect on NK cells.

One of the first studies to utilise IL-2-transduced tumour cells was in a murine fibrosarcoma model. Vaccination of animals inhibited tumour formation and tumour-

specific cytolytic activity could be detected. Subsequent investigations, however, showed that initial rejection of IL-2-secreting cells was not T cell-mediated. Although a strong T cell response was induced upon re-challenge with parental cells, T cells could not be detected at the site of injection (Gansbacher *et al.*, 1990). However, in a murine melanoma model, pre-vaccination of immunocompetent animals not only offered protective immunity upon challenge with wild-type melanoma cells, but had no effect in athymic mice. This suggests that the immune response to IL-2-transduced tumour cells is T cell-mediated (Zatloukal *et al.*, 1995). Moreover, transfer experiments of the T lymphocyte-enriched population from immunised mice, indicates that IL-2 needs both $CD4^{+}$ and $CD8^{+}$ T cells for an effective anti-tumour response. However, different tumours may elicit distinct immune responses. In a murine model for neuroblastoma the predominant infiltrating cells were $CD11b^{+}$ and $CD90^{+}$ upon vaccination with IL-2- or GMCSF-expressing C1300 cells (Yoshida *et al.*, 1999).

Failure to identify a neuroblastoma-specific antigen has directed immunotherapeutic strategies to using whole-tumour cell vaccines engineered to express certain immunomodulatory molecules. The main cytokine used to transduce neuroblastoma cells has been IL-2 (Leimig *et al.*, 1994; Corrias *et al.*, 1998). Neuroblasts producing IL-2 have been used in patient clinical trials (Brenner, 1992). Bowman *et al.*, (1998) have used an adenoviral vector to deliver the IL-2 gene in autologous tumour cells and a retroviral vector in allogeneic patient cell lines. These IL-2-transduced neuroblasts have been used to vaccinate 10 and 12 children, respectively with neuroblastoma. In the autologous study, five patients showed tumour responses with anti-tumour antibody production and enhanced MHC-restricted CTL activity against autologous tumour cells. The results from the allogeneic study are, however, less striking. No direct CTL killing against the immunising cell line could be detected, but 3 of the 5 patients studied exhibited an increased frequency of tumour reactive CTL precursors. Only one child showed response and 7 had stable disease. These data suggest that the IL-2 tumour vaccination approach generates immunomodulatory responses consisting of cytotoxic T cell activity that correlate with tumour responses (Bowman *et al.*, 1998a, b). However, such responses were more evident when autologous cells were used although an allogeneic vaccine would offer certain advantages. In another study,

immunisation of neuroblastoma patients with IL-2 transduced autologous tumour cells induced a tumour-specific B cell response. This was revealed by isolating patient B cells and cloning anti-tumour antibody fragments from IgG scFv phage display libraries (Rossig *et al.*, 2000). A high percentage of the isolated clones (13%) were specific for neuroblastoma cells and although the target antigens were not identified, the antibodies exhibited a different pattern of reactivity compared to known neuroblastoma antigens such as GD_2 and N-CAM.

It is becoming apparent, that due to the complexity of the immune response against a parental tumour and the number of steps required to achieve long-lasting immunity, a combination of therapies may be necessary. In order to achieve therapeutic efficacy, treatments that target different compartments of tumour biology can be combined. Lode *et al.*, (1999) report the synergistic effect of targeted immunotherapy with an anti-GD_2 antibody-IL2 fusion protein and anti-angiogenic therapy using an α_v integrin antagonist. While each therapy alone results in a delay in tumour growth, simultaneous application of both treatments induces regression of experimental tumours of neuroblastoma, colon carcinoma and melanoma. Furthermore, there is significant reduction in tumour vasculature and enhancement of infiltrating cells (Lode *et al.*, 1999a).

1.2.4.2.2 Interleukin-12

There has been an increasing interest in Interleukin-12 (IL-12) as a potent anti-tumour agent. IL-12 was originally identified as an NK cell stimulatory factor and cytotoxic T cell maturation factor (Perussia *et al.*, 1992) (**Fig 1.1**). In addition, it induces $CD4^+$ T cell-specific immune responses by shifting the differentiation of T_{H0} cells towards the T_{H1} pathway (Manetti *et al.*, 1993; Tsung *et al.*, 1997; Trinchieri, 1998). IL-12 is an inflammatory cytokine secreted by macrophages at the site of infection. It is also involved in the proliferation of activated T cells. Upon antigenic stimulation, expression of IL-12 receptor on the surface of T cells is up-regulated enabling them to proliferate in response to this cytokine independently of IL-2 (Desai *et al.*, 1992).

Administration of IL-12 in mice with pulmonary metastases of melanoma results in prolonged survival by augmenting the NK and lymphokine activated killer cell (LAK) cytotoxicity and inducing IFN-γ production. $CD8^+$ T cells are the cellular infiltrate and the anti-tumour response is sensitive to $CD8^+$ and $CD4^+$ T cell depletion, suggesting that $CD4^+$ T cell-help is important for the anti-tumour function of IL-12 (Brunda *et al.*, 1993; Nastala *et al.*, 1994). $CD8^+$ T cells were also the dominant effector cell type in a murine model for neuroblastoma metastasis as revealed by *in vivo* immunodepletion experiments (Lode *et al.*, 1998b). Furthermore, vaccination with IL-12-secreting cells induced protective immunity and resulted in complete eradication of established liver metastases. Direct intra-tumoural administration of a vector carrying the cytokine transgene is an alternative approach of inducing a local immune response. Adenoviral *in situ* transduction of murine tumours with IL-12 results in 75% partial or complete tumour regression. The immune response consists of $CD4^+$ and $CD8^+$ T cells and protective immunity can be generated (Davidoff *et al.*, 1999). However, in a clinical context the drawback of this approach would be its limited use on patients with localised tumour.

IFN-γ is thought to be one of the most important mediators of the IL-12 anti-tumour effects. Although IFN-γ is a potent stimulator of macrophages and can therefore result in direct tumour killing, it has been shown to up-regulate tumour cell expression of MHC class I and MHC class II molecules. This is believed to enhance their ability to present tumour antigens. In addition, it has angiostatic effects mediated by chemokines such as interferon IP-10 (inducible protein 10). Indeed, the anti-tumour effect of murine IL-12 was completely abrogated when antibodies against IP-10 were used during the immunisation phase (Pertl *et al.*, 2001). In addition, generation of tumour specific $CD8^+$ T cells was inhibited.

Many tumours are thought to express tumour-specific antigens that are, however, not efficiently presented to T cells since tumour cells do not express co-stimulatory molecules. Co-expression of B7-1 and IL-12 by adenocarcinoma cells is very effective in a murine model resulting in complete tumour regression in 70% of the animals (Putzer *et al.*, 1997).

1.2.4.2.3 Lymphotactin

Lymphotactin belongs to the family of chemokines whose biological function is to primarily chemoattract lymphocytes at sites of inflammation. To date, there are four chemokine groups, CC, C, CXC and CX_3C, depending on the number and positioning of the four conserved cysteine residues found in their amino termini. However, lymphotactin lacks two of those four cysteines and, as a result, maintains only one disulfide bridge within the molecule.

Lymphotactin was initially discovered and cloned from a mouse progenitor T cell cDNA library but was shown to be expressed by activated $CD8^+$ T cells, and also mouse intraepithelial $\gamma\delta$ T cells (Kelner *et al.*, 1994; Boismenu *et al.*, 1996). Parallel studies have resulted in the identification of two molecules designated ATAC (activation-induced, T cell-derived, and chemokine-related) and single cysteine motif-1, which are in essence the same as lymphotactin.

Lymphotactin has proven to be an attractive candidate for immunotherapy due to its role in lymphocyte trafficking and inflammatory responses. On its own, lymphotactin does not have any anti-tumour effects although it induces infiltration of $CD4^+$ and $CD8^+$ T cells. When combined with IL-2 or IL-12, however, it results in tumour regression of experimental breast cancer tumours (Emtage *et al.*, 1999). Transduction of DCs with lymphotactin induces protective and therapeutic anti-tumour immunity against murine lung carcinoma tumours. Administration of lymphotactin-transduced peptide-pulsed DCs as a vaccine gives rise to more potent cytotoxic T cells compared to peptide-pulsed untransduced DCs. The immune response depends largely on $CD8^+$ and NK cells while depletion of $CD4^+$ T cells at the immunization stage abrogates any anti-tumour effects. This demonstrates the requirement of $CD4^+$ T cells for cross-priming of $CD8^+$ T cells (Cao *et al.*, 1998).

1.2.4.3 Dendritic cell vaccines

Initially it was speculated that the tumour cells were capable of presenting tumour-associated antigens to T cells via MHC class I and/or MHC class II molecules (**Fig 1.2 A**). However, since most tumours are MHC class II negative and in the case of neuroblastoma MHC class I low, it was not clear how T cells could be primed. Recent studies on the immune system function have provided a better appreciation of the mechanism of antigen presentation and the role of antigen-presenting cells (APC) in the initiation of cell-mediated immune responses. Increasing evidence that MHC class I-negative tumour cells are not capable of acting as APCs but priming of naïve $CD8^+$ cells is actually carried out by dendritic cells (DCs) favours the second model of antigen presentation (**Fig 1.2B**). This hypothesises that DCs are actually responsible for presenting tumour specific antigens to $CD4^+$ or $CD8^+$ T cells in the context of MHC class II and MHC class I complexes (Albert *et al.*, 1998). These cells are thought to circulate in the tissues, phagocytose antigens from apoptotic cells and travel to the draining lymph nodes where they cross-present antigen to $CD4^+$ and/or $CD8^+$ T cells (Bannerji *et al.*, 1994). The current view is, therefore, that cytokines produced by modified tumour cells in vaccine studies attract DCs or other inflammatory cells at the vaccination site and enhance the uptake of apoptotic material by DCs. Furthermore, generation of CTLs by DCs does not require $CD4^+$ T cell help if signalling through the CD40 molecule on DCs occurs (Ridge *et al.*, 1998).

A number of subsequent investigations have focused on the use of DCs in novel therapies for the treatment of cancer. Since in most cancers identification of tumour-specific antigens has not been possible, DCs are pulsed with tumour peptides acid-eluted from MHC class I molecules on the surface of tumour cells. Vaccination results in the eradication of established murine tumours generating a T cell-dependent anti-tumour response that requires the delivery of co-stimulatory signals (Zitvogel *et al.*, 1996). An alternative approach exploits DCs fused with carcinoma cells providing DCs with the full repertoire of tumour cell antigens. The fused cells exhibit DC morphology with expression of MHC class I, class II and co-stimulatory molecules.

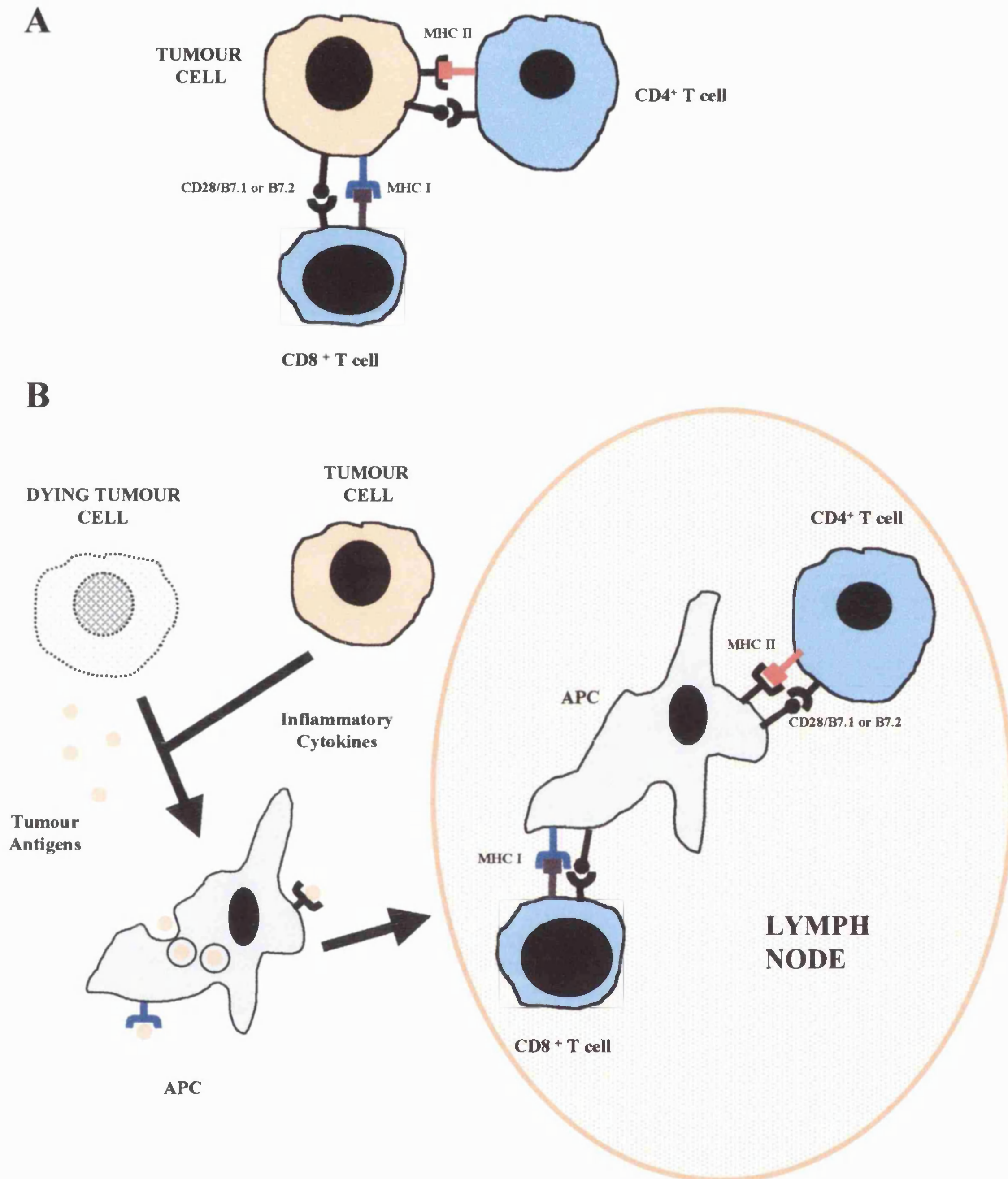


Figure 1.2 Potential mechanisms of tumour antigen presentation. A. The tumour cell itself may act as an antigen presenting cell, directly activating CD4$^+$ and CD8$^+$ T cells through MHC and co-stimulatory molecules. **B.** Inflammatory cytokines produced by the tumour cells attract a number of effector cells such as neutrophils, NK cells or DCs at the vaccination site. Tumour antigens become available for antigen presentation due to the irradiated nature of the vaccine or direct attack by infiltrating cells. Professional APCs then travel to the draining lymph nodes where they present tumour antigens to T cells in the context of MHC molecules.

The interesting aspect is that the *in vivo* tumourigenicity of the fused cells is completely abrogated (Gong *et al.*, 1997). Administration of these cells can also protect animals against subsequent challenge with parental tumour cells and generate tumour-specific CTLs.

Transduction of DCs with CD40L is believed to overcome the need for $CD4^+$ T cells and directly activate DCs. CD40L-transduced DCs suppress the growth of established melanoma and colon adenocarcinoma tumours (Kikuchi *et al.*, 2000). In addition, DCs traffic to the spleen and induce T cell-mediated anti-tumour immunity, which is transferable to naive mice by spleen cells.

An obvious candidate for DC therapy is melanoma since a number of melanoma specific antigens (gp100, MART-1, MAGE-1, MAGE-3, tyrosinase) have been identified. DCs obtained from patient peripheral blood monocytes after culturing with GM-CSF and IL-4 were pulsed with a cocktail of peptides derived from the above antigens depending on the patients' HLA haplotype. Although there was some delayed type hypersensitivity (DTH) reaction, regression of tumours was observed in 5 out of 16 patients and peptide-specific CTLs could be recovered from skin biopsies (Nestle *et al.*, 1998). Despite evidence of autoimmunity in DC-vaccinated animals with antigens that are shared with normal cells (Ludewig *et al.*, 2000) and the early stages of clinical evaluation of DC vaccination, there is substantial evidence that such a therapeutic approach is effective against a number of human cancers.

1.3 GENE THERAPY VECTORS

The development of vectors for gene therapy applications has tried to mimic the nature of viruses and the way they deliver their nucleic acid into infected cells. Crucial to vector construction is the delivery of the transgene specifically to the cells of interest especially if they reside amongst a heterogeneous population. Specific cell targeting also avoids transduction of dendritic cells and elicitation of an immune response against the vector. The host immune response comprises one of the main

obstacles to efficient gene delivery. Vector components should in theory be immunologically inert both at the cellular and humoral level. Failure to do so will result in elimination of the transduced cells by cellular immunity or make vector re-application ineffective due to circulating antibodies. The safety of gene therapy vectors with respect to viral reproduction is also very important as well as their ability to transduce a large number of cells. Furthermore, vectors should not present any limitations to the size and type of DNA to be delivered. Depending on their nature, gene therapy vectors have been divided into synthetic and viral whose development is based on recombinant viruses.

1.3.1 Retrovirus vectors

Vectors based on recombinant retroviruses were the first to be developed for gene transfer applications. Their RNA contains three essential genes *gag*, which encodes the viral structural proteins, *pol* that encodes the reverse transcriptase and integrase enzymes, and *env* that codes for the viral envelope glycoprotein. One of the main potential problems with initial retroviral vectors was the generation of replication-competent virus particles. New vectors have been developed, where the retroviral genes have been replaced with a therapeutic gene and provided *in trans* in a packaging cell line (reviewed in Somia and Verma, 2000). This allows production of virus particles capable of infecting the target cells. Further advances include the design of self-inactivating (SIN) vectors with deletions in the 3' LTR that allow non-viral regulation of the transduced gene upon proviral integration into the host genome (Yu *et al.*, 1986). The use of alternative envelope proteins has extended the range of target cells to amphotropic (both rodent and human cells) and xenotropic (most cells except rodent) (Danos and Mulligan, 1988). Retroviral vectors, however, require mitotic activity for infection, which limits their use to tissues containing actively dividing cells. Another potential disadvantage of retroviral vectors is that transcription of the transgene may gradually shut off (Palmer *et al.*, 1991).

Retroviral vectors have been extensively used to transduce many cell types, mostly tumour cells and haematopoietic stem cells. The first gene therapy studies utilising TILs in melanoma patients exploited retroviral marking of the cells with the neomycin resistance gene (Rosenberg *et al.*, 1990). Since then a number of clinical trials have incorporated retroviral vectors in their protocols. Both direct vector administration *in vivo* or transduction of tumour cells for the development of cancer vaccines have been exploited (Roth *et al.*, 1996; Bowman *et al.*, 1998b).

Correction of severe combined immunodeficiency-X1 (SCID-X1) in RAG2/γ_c deficient mice can be achieved using retroviral transfer of the γ_c gene into bone marrow haematopoietic stem cells. Treated animals exhibit normal lymphocyte development and immunoglobulin production (Soudais *et al.*, 2000). A gene therapy clinical trial for SCID-X1 has also been initiated. A Moloney retroviral vector carrying the human gene for γ_c was used to transduce CD34$^+$ cells from two patients. T and NK cells expressing γ_c were detected 10 months post-therapy and patients had developed functional lymphoid cells capable of mounting antigen-specific responses (Cavazzana-Calvo *et al.*, 2000).

1.3.2 Lentivirus vectors

Lentiviruses are complex retroviruses that are capable of shuttling their genome into the nucleus without the requirement of mitotic division. First-generation lentiviral vectors consist of a three-construct system. A packaging construct, where expression of Env and Vpu, which assists in the assembly of the virus particles, is blocked, a plasmid that encodes different envelope proteins (VSV-G or amphotropic MLV envelope) to broaden the host range of the vector, and a transfer vector, which provides the Gag and Rev responsive element (Naldini *et al.*, 1996). However, due to the possibility of generating pathogenic virus, vector systems have been devised where four out of the nine genes are deleted but the ability of the vector to transduce non-dividing cells *in vitro* is retained (Zufferey *et al.*, 1999). Further improvements of lentiviral vectors include the incorporation of the central poly-purine tract (cPPT),

which is required for the second-strand DNA synthesis of lentiviruses and is believed to aid in the transport of the provirus to the nucleus (Zennou *et al.*, 2000). Also inclusion of the woodchuck post-transcriptional regulatory element (WPRE) enhances the transduction efficiency of lentiviral vectors (Zufferey *et al.*, 1999).

Lentiviral transfer of the cytotoxic gene for gibbon ape leukaemia virus envelope fusogenic membrane protein (GALV-FMG) into human fibrosarcoma xenografts has demonstrated the potential of lentiviral vectors for cancer gene therapy (Diaz *et al.*, 2000). In addition, lentiviral vectors efficiently transduce human acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) cell lines and primary cells. Gene delivery of GM-CSF and CD80 into primary leukaemia cells has illustrated the feasibility of these vectors to transduce poor proliferating cells and induce anti-leukaemic immune responses (Stripecke *et al.*, 2000). However, due to the relatively recent development of these vectors as well as the concerns regarding the generation of replication-competent viral particles, there are no clinical trials to date for cancer gene therapy.

1.3.3 Adenovirus vectors

Adenoviruses are DNA viruses that were identified by their ability to cause tumours in rodents. Wild-type adenovirus can only package 2 kb of insert DNA and therefore adenoviral vectors have been constructed by deleting various regions of the viral genome. First generation adenoviral vectors consisted of deletion of E1 and E3 regions, which are involved in viral transformation and the host response to adenovirus, respectively. This offered a transgene capacity of 8.2 kb. Since those vectors elicited significant immune responses, further inactivation of viral genes was necessary resulting in second-generation adenoviral vectors. Deletion of E2A and E2B that encode the DNA binding and DNA polymerase activities respectively, as well as parts of the E4 viral genome has increased the insert capacity of the vectors to 14 kb (reviewed in Danthinne and Imperiale, 2000). Removal of all viral sequences in the

so-called 'helper-dependent' adenoviral vectors can facilitate a packaging capacity of 37 kb (Kochanek *et al.*, 1996).

Due to their large size adenoviral vectors are produced by homologous recombination between the viral DNA and a shuttle vector containing the viral sequences to be deleted and the gene of interest. This is performed in helper cells such as 293 cells expressing E1 proteins. Purification of recombinant adenoviral vectors occurs by two or three rounds of plaque assay (Danthinne and Imperiale, 2000).

Any targeting limitations of adenoviral vectors due to inefficient expression of receptors on certain cell types such as human airway epithelial cells can be circumvented by complexing the virus with cationic liposomes. In this case, binding occurs via ionic interactions with the negatively charged cell membrane but the ability to escape endosomes and deliver the DNA efficiently, is retained (Fasbender *et al.*, 1997). Alternatively, to redirect adenoviral binding or increase specificity of gene delivery, adenoviral vectors can be incubated with fusion proteins composed of an anti-fibre single-chain antibody linked to EGF (Watkins *et al.*, 1997). This has proven valuable in altering the host range of adenoviral vectors by replacing EGF with an alternative ligand.

One of the main disadvantages of replication-deficient adenoviral vectors is the elicitation of host immune responses. Adenovirus-infected cells are believed to present antigens to CTLs via an MHC class I–restricted mechanism that does not require viral replication or protein synthesis (Kafri *et al.*, 1998). Although this problem hampers re-administration of adenoviral vectors, elicitation of immune response may prove advantageous for tumour immunotherapy. Adenoviral vectors have been used in number of gene therapy clinical trials to treat breast cancer, melanoma as well as brain tumours (Stewart *et al.*, 1999; Trask *et al.*, 2000). An adenoviral transduced autologous vaccine expressing IL-2 was also used in a neuroblastoma clinical trial (Bowman *et al.*, 1998a). Patient responses were variable and elevation of adenovirus antibodies could be detected in some cases. However,

these studies confirmed the feasibility and safe administration of adenoviral vectors for cancer gene therapy.

1.3.4 Adeno-Associated Virus (AAV) vectors

AAV is a single-stranded DNA non-enveloped parvovirus that requires the help of adenovirus or herpes simplex virus (HSV) to replicate. Its genome consists of two genes, *cap* that encodes the structural proteins of the virus and *rep* that codes for the replication and integration functions of AAV. The two genes are located within two inverted terminal repeats (ITRs) that are required for packaging the viral genome into capsids. A number of helper proteins are provided *in trans* by adenovirus, which enables exit of AAV due to its lytic function. AAV vectors are constructed by replacing the rep and cap genes with the therapeutic gene and supplying the Rep/Cap construct and the adenoviral helper proteins *in trans*. Normally AAV can integrate in a specific site at chromosome 19 through Rep protein but AAV vectors have lost this ability and integration is random (Somia and Verma, 2000).

The main limitations of AAV vectors are the difficulty to obtain really high titers and the restricted 4.5 kb packaging capacity (reviewed in Rabinowitz and Samulski, 2000). The latter problem has, however, been addressed after the finding that AAV genomes undergo intermolecular concatamerisation following transduction. Dividing the rAAV cassette into two vectors results in similar efficiency to a single vector and will potentially enhance the packaging capacity of AAV vectors (Nakai *et al.*, 2000). Trans-splicing events between two AAV vectors delivering 5' and 3' parts of erythropoietin into muscle result in production of functional protein that protects mice from renal-failure induced anaemia (Yan *et al.*, 2000).

An AAV vector has been used to treat retinal degeneration in mice with successful restoration of the structure and function of photoreceptors demonstrating potential of these vectors for gene therapy applications (Ali *et al.*, 2000). The host range of AAV vectors can also be expanded to specifically target haematopoietic cells by

construction of a chimeric capsid protein containing the variable region of an antibody against CD34 (Yang *et al.*, 1998). The potential of AAV vectors for cancer gene therapy has been illustrated in experimental glioma tumours by delivery of the HSV-tk and IL-2 genes followed by ganciclovir administration (Okada *et al.*, 1996). Although *in vivo* delivery of these genes resulted in a significant anti-tumour response, there are no clinical trials on cancer patients to date employing AAV vectors. Nonetheless, the feasibility of utilising these vectors in clinical trials has been demonstrated by intramuscular delivery of factor IX in haemophilia B patients (Kay *et al.*, 2000).

1.3.5 Herpes simplex virus (HSV) vectors

Herpes simplex virus 1 (HSV) is an enveloped virus with a 152 kb double-stranded DNA genome. HSV infection is quite common in humans, causing cold sores or more severely encephalitis. Upon cell infection, expression of immediate early (IE) genes, which are involved in regulating the viral replication and encapsidation functions, is induced by VP16 (Robbins *et al.*, 1998).

There are two types of HSV vectors, recombinant viral vectors and amplicon vectors. Recombinant viral vectors result from deletion of one or more viral genes, which reduces the toxicity and enable incorporation of transgenes. Such vectors have a great transgene capacity and can accommodate up to 30-50 kb of foreign DNA. Further advantages of HSV vectors include their ability to infect neuronal cells and replicate episomally. HSV amplicon vectors consist of a plasmid containing the HSV origin of replication and packaging sequence. This is co-transfected in cells with cosmids containing the essential viral sequences but no packaging signal (Wang *et al.*, 1997). The disadvantage of this approach, however, is the production of low titre virus.

HSV vectors have been used for gene therapy in experimental models of multiple sclerosis (MS) (Furlan *et al.*, 2001) and Parkinson's disease (Yamada *et al.*, 1999). The oncolytic property of HSV has been exploited for cancer gene therapy. Development of conditionally replicating HSV vectors that can replicate and lyse

rapidly dividing cells, such as those found within tumours, reduces the neurovirulence of HSV. Such vectors contain mutations in the *U_L39* and/or *$\gamma_1 34.5$* genes that encode the ribonucleotide reductase function and promote replication in non-dividing cells, respectively (reviewed in Martuza, 2000). Preliminary data from a Phase I clinical trial of glioma using a conditionally replicating HSV vector demonstrates the efficacy and safety of such vectors for the treatment of brain tumours (Markert *et al.*, 2000). Furthermore, replication-competent attenuated HSV vectors have been used in combination with other anti-tumour therapies, such as the *in situ* tumour delivery of IL-12 in experimental intracranial neuroblastoma (Parker *et al.*, 2000).

1.3.6 Non-viral vectors

1.3.6.1 Naked DNA

Studies utilizing naked DNA have focused on the direct *in vivo* injection into muscle or skin. Although the transfection efficiency is rather low, expression has been shown to persist for a long time (Davis *et al.*, 1993a, b). Expression of reporter genes using DNA-coated microprojectiles is detectable up to 14 days in mouse liver and skin cells (Williams *et al.*, 1991). *In vivo* injection of plasmid DNA into muscle results in transgene expression for up to 19 months (Wolff *et al.*, 1992). Studies in *mdx* mice with delivery of the human dystrophin gene, have suggested that the susceptibility of skeletal muscle to naked DNA transfection is due to the architectural structure of myofibrils (Acsadi *et al.*, 1991).

1.3.6.2 Lipoplexes

Lipoplexes are formed by the electrostatic association of DNA with a cationic lipid or the encapsulation of DNA inside a liposomal carrier. Lipoplexes interact and fuse with the cellular membrane avoiding the endosomal lysosomal route and achieve higher transfection efficiency than naked DNA. Dioleoyloxypropyl-trimethylammonium

Table 1.2 **Viral vectors for gene therapy applications**

VECTOR	INSERT SIZE	VIRAL SEQUENCES	ADVANTAGES	DISADVANTAGES
RETROVIRUS	8 kb	Deletions in *gag*, *pol* and *env*	Integration of the transgene into the host genome	Transduction limited to proliferating cells Risk of insertional mutagenesis
ADENOVIRUS	2 kb	Deletions in E1B, E1A, E3	Broad host range Transduces dividing and non-dividing cells Episomal replication so no risk of insertional mutagenesis	Induction of inflammatory responses Transient expression of transgene
	4 kb	Further deletions in E2A, E2B and E4		
	37 kb	Deletion of all viral sequences		
AAV	4.5 kb	Inverted terminal repeats	Broad host range	Laborious purification from helper virus Risk of insertional mutagenesis
LENTIVIRUS		Deletions in 4 out of 9 viral genes Gag and Rev provided *in trans*	Transduction of quiescent cells Integration into host genome	Possibility of generating pathogenic virus
HSV	30 kb	Deleted IE genes HSV ori and packaging signal	Very effective on neuronal cells No immunogenicity Episomal replication	No integration Potential cytotoxicity

chloride (DOTMA) has been used in many studies alone or in combination with dioleoylphosphatidylethanolamine (DOPE), which is a neutral lipid. This combination is available commercially as Lipofectin.

Various liposomes such as DC-chol-DOPE (Caplen *et al.*, 1995a) and N -(1-(2,3-dioleoyl-oxy) propyl)-N, N, N -trimethyl-ammonium methylsulphate (DOTAP) (McLachlan *et al.*, 1995) have been used for cystic fibrosis gene therapy. Expression of the gene under the control of a CMV promoter in mouse lung epithelium persists up to 17 days (McLachlan *et al.*, 1995). The lipoamine DC-chol in combination with DOPE has been used to deliver the *CFTR* gene in a clinical trial for cystic fibrosis. Though transient, partial restoration of the electrophysiological defect was observed in patients receiving the *CFTR* cDNA as measured by the potential difference across the nasal epithelium (Caplen *et al.*, 1995b). *In vitro* culturing of airway epithelial cells show that poorly differentiated cells are less transfectable with liposome-DNA complexes due to reduced negative surface charge and decreased phagocytic activity (Matsui *et al.*, 1997). Intratumoural administration of plasmid DNA complexed with DC-chol was capable of *in situ* transduction and generation of effective anti-tumour responses (Egilmez *et al.*, 1996). The main disadvantage of lipoplex formulations is that their systemic administration is of limited use due to the binding and destabilization by blood serum proteins. This could be overcome by coating cationic liposomes with human serum albumin (HSA) (Simoes *et al.*, 2000). Alternatively, combination of dioctadecylamidoglycylspermidine and dioleoyl phosphatidylethanolamine (DLS liposomes) with an episomally replicating DNA plasmid retains expression *in vivo* up to 3 months (Thierry *et al.*, 1995).

1.3.6.3 Polyplexes

Polyplex vectors utilise DNA-binding compounds such as polyethylenimine (PEI), poly-L-lysine (PLL) alone or together with a lipid to deliver DNA into the cell. DNA condensation has also been demonstrated with cationic α-helical peptides of an amphiphilic nature that contain repeated amino acid sequences (Niidome *et al.*, 1997).

The incorporation of targeting components into these vectors that allow specific binding and transfection of cells has raised considerable interest. A number of ligands have been used to target such synthetic vectors including transferrin (Wagner *et al.*, 1990; Zenke *et al.*, 1990; Wagner *et al.*, 1991)), epidermal growth factor (EGF) (Xu *et al.*, 1998), histone H1 and antibody fragments. Receptor-mediated gene transfer to hepatocytes *in vitro* and *in vivo* was demonstrated with galactosylated PLL (Perales *et al.*, 1997). Conjugation of transferrin to PEI increases the transfection efficiency up to 1000-fold in a number of tumour cell lines and results in higher expression of murine IL-2 (Kircheis *et al.*, 1997). PEI-transferrin polyplexes mediate cell entry by receptor-mediated endocytosis since addition of soluble transferrin significantly inhibits transfection efficiency. Short synthetic peptides that bind to integrin receptors and are fused to an oligolysine chain have also been used for DNA delivery (Hart *et al.*, 1997; Harbottle *et al.*, 1998).

Specific delivery of non-viral complexes to neuroblastoma cells has been achieved with antibodies directed against certain molecules with a neuronal-restricted expression pattern. A monoclonal antibody (chCE7) that recognises a 190 kDa neuroblastoma surface molecule was linked to PLL and the resulting DNA complexes showed neuroblastoma-specific transfection *in vitro* (Coll *et al.*, 1997). Selective gene transfer was also achieved with a biotinylated antibody (NBL-1) to RET, a receptor tyrosine kinase specifically expressed on cells originating from the neural crest. This antibody was used in combination with avidin-conjugated PLL-DNA complexes. Internalisation of the complexes occurred via endocytosis but transfection efficiency *in vitro* only reached 1% (Yano *et al.*, 2000).

1.4 BARRIERS TO NON-VIRAL GENE DELIVERY

Gene transfer into the cell using non-viral vectors, has to overcome a number of cell barriers. Initially, binding to the cell surface either specifically, via receptor-ligand interaction or non-specifically, via ionic interaction with the negatively charged cell membrane, must occur. There are three pathways by which synthetic complexes can

Table 1.3 **Synthetic vector systems for gene therapy applications**

VECTOR	COMPOSITION	ADVANTAGES	DISADVANTAGES
Naked DNA	Plasmid DNA	High levels of sustained expression when delivered *in vivo* to muscle	*In vivo* application limited to muscle
LIPOPLEXES	Cationic liposomes	No DNA size limitations No immunogenicity	Transient transgene expression No specificity Limited *in vivo* application due to rapid clearance by serum proteins
CATIONIC POLYMERS	Poly-L-Lysine (PLL) Polyethylenimine (PEI)	Condensation of DNA May have lysosomotropic effects PEI may allow nuclear targeting No immunogenicity	Low efficiency compared to viral vectors
POLYPLEXES	Liposomes or cationic polymers in conjuction with receptor targeting peptides	Targeting specificity	

enter the cell: receptor-mediated endocytosis, non-coated pit endocytosis or phagocytosis. Endocytosis of DNA complexes by the classical receptor-mediated endocytic pathway is a clathrin-dependent process. This mechanism normally operates for the uptake of extracellular fluid and receptor-bound ligands. Binding of synthetic vector complexes to their respective receptors triggers a process that involves concentration of clathrin molecules in the receptor-ligand region and formation of vesicles after invagination of the cell membrane (**Fig 1.3**). These vesicles are approximately <0.2μm in diameter and therefore, condensation of DNA is required for complexes to be internalised by this pathway. Internalised molecules in clathrin-coated vesicles are then transported to endosomes (Mellman, 1996).

Endocytosis can still occur when formation of clathrin-coated pits is blocked. Non-coated pit endocytosis may involve cytoplasmic regions characterised by concentration of the protein caveolin or through pinocytosis, a mechanism prominent in macrophages, DCs and fibroblasts. Pinocytosis reflects the passive uptake of extracellular fluid into vesicles 1-5μm in diameter. Formation of caveolae in endothelium correlates with transport of solutes across the cell rather than fusion with endosomes. Molecules inside pinosomes can be delivered to lysosomes or recycled. Phagocytosis on the other hand, involves the internalisation of large particles (>0.5 μm). It represents an integral mechanism of mammalian leukocytes against parasitic infection and also allows the processing and presentation of foreign antigens for adaptive immunity. Binding to specific receptors that can trigger an intracellular phagocytic signal results in polymerisation of actin and pseudopod extension surrounding the bound particles. Subsequent engulfment of the particles gives rise to phagosomes that eventually release their contents to endosomes and/or lysosomes. Therefore, the size of synthetic vector complexes is a crucial parameter for their internalisation mechanism and intracellular fate. Receptor targeted vectors will get endocytosed via clathrin-mediated endocytosis provided they have a size of ~ 100 nm. Synthetic complexes of larger size, on the other hand, or those that bind non-specifically to the cell membrane will mediate cell entry via non-coated pit endocytosis or phagocytosis.

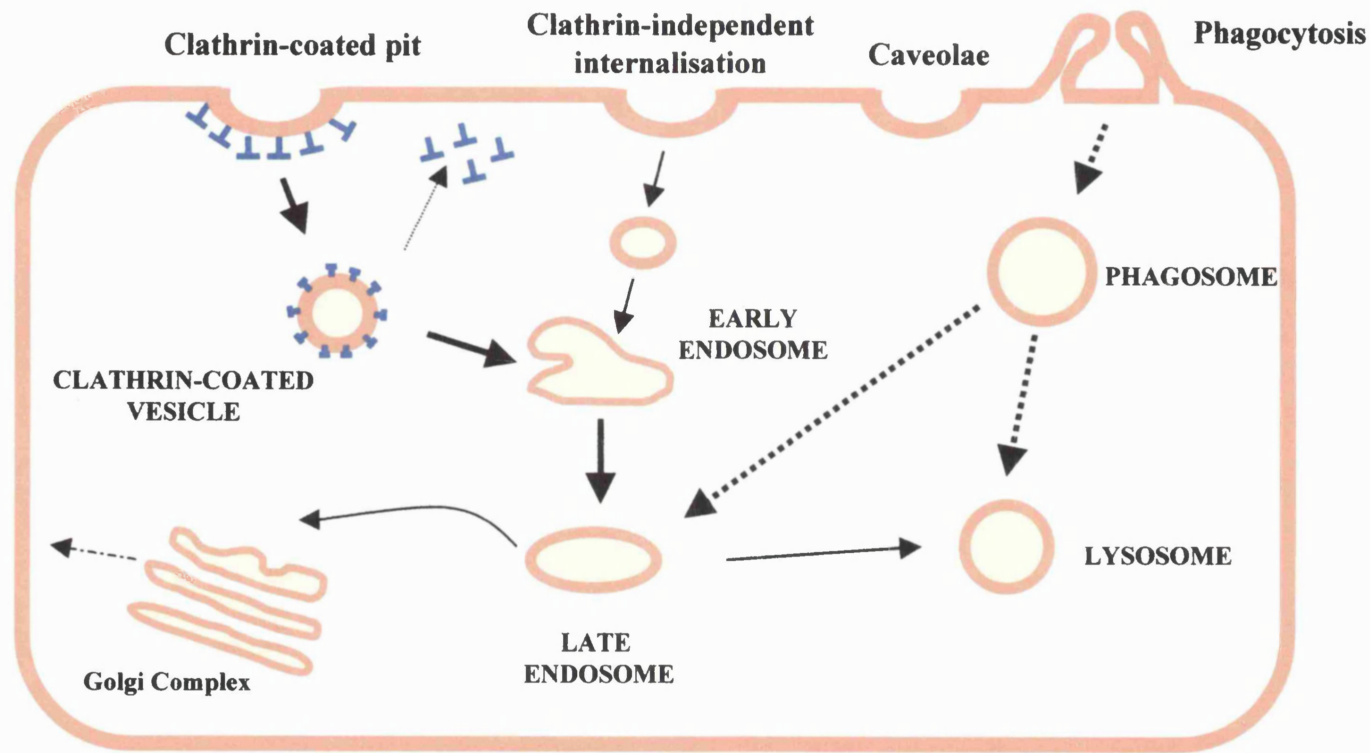


Figure 1.3 **Endocytosis in mammalian cells.** Endocytosis is the cellular uptake of molecules and involves the formation of different membrane vesicles. Large particles are engulfed by an actin-dependent process known as phagocytosis. Clathrin-dependent endocytosis or non-coated pit endocytosis are responsible for the absorption of molecules <0.2 µm in size. The former mechanism is triggered by ligand-receptor interactions that lead to the concentration of clathrin molecules in the immediate region of the membrane. Non-coated pit endocytosis delivers molecules that bind non-specifically to the cell membrane and may involve the formation of caveolae. The main role of each of these vesicles is to deliver their contents to endosomes/lysosomes where molecular sorting and degradation takes place. (Figure adapted from Mellman, 1996)

Early endosomes are the first group of intracellular vesicles encountered after internalisation of particles with an internal pH 6.3-6.8. Their main function is sorting of endocytosed material and may facilitate dissociation of receptor-ligand complexes. Concentration of endocytosed particles then occurs by vacuolar detachment from early endosomes and fusion with late endosomes after travelling on the intracellular microtubule network. Late endosomes contain lysosomal enzymes and may initiate the degradation process. The final step in the endocytic process involves fusion with lysosomes where the main degradation takes place.

This comprises one of the major obstacles to efficient gene transfer and a number of approaches have been employed to avoid destruction of the complexes. These include buffering agents such as chloroquine that raise the lysosomal pH, incorporation of inactivated adenoviral particles or viral fusogenic peptides such as influenza haemagglutinin (Wagner *et al.*, 1992) or *Pseudomonas* exotoxin A (Fominaya and Wels, 1996) to promote endosomal lysis. PLL in the presence of glycerol is capable of rupturing vesicles of neutral pH such as early endosomes (Zauner *et al.*, 1997). Furthermore, condensing the DNA with synthetic polycations such as PEI or cationic lipids, which possess an endosomalytic function and interact with the anionic lipids of lysosomes, has been shown to increase the transfection efficiency of non-viral vectors allowing cytoplasmic escape of the complexes. PEI, due to its composition of amines that are ionisable at different pHs, can alter the vesicle pH and induce endosomal disruption (Boussif *et al.*, 1995). Alternatively, approaches that bypass the endosomal-lysosomal endocytic pathway have been devised. Incorporation of a synthetic peptide based on a subunit of Diphtheria toxin into synthetic vectors and delivery of the complexes occurs via the endoplasmic reticulum (Uherek *et al.*, 1998).

A further barrier to efficient gene transfer is transportation to the nucleus. The exact mechanism by which DNA enters the nucleus is not known. It is not clear whether complexes bind to nuclear pore peptides and aid the uptake of DNA. Nuclear transport through the nuclear pores of molecules greater than 9nm is an energy-requiring process that involves shuttle molecules. Most non-viral systems seem to require mitotic activity of the target cells, when breakdown of the nuclear membrane occurs

and nuclear localisation is enabled (Mortimer *et al.*, 1999). Alternatively, incorporation of a peptide with a nuclear localisation signal such as the basic peptide from SV40 large tumour antigen enhances transfection efficiency (Zanta *et al.*, 1999).

1.5 INTEGRINS

Integrins are a large group of heterodimeric molecules that are involved in cell shape and movement via interactions with the extracellular matrix (ECM) (reviewed in Hughes and Pfaff, 1998). They are also responsible for cell-cell interactions and mediate a number of downstream signalling cascades that modulate cell survival, proliferation and migration (reviewed in Hynes, 1992). They are composed of one of 16 alpha and one of 8 beta chains, both of which are transmembrane glycoproteins. Currently 16α and 8β subunits have been identified in humans and combinations of the two chains have given rise to 22 known integrin receptors (reviewed in Humphries and Newham, 1998). The ligand specificity of these receptors varies extensively and the ligand-binding pocket lies near the interface of the two subunits. Their distribution may be ubiquitous such as $\alpha_5\beta$ and $\alpha_v\beta_3$, or restricted to specific cell types such as $\alpha_M\beta_2$ integrin on macrophages, which is involved in receptor-mediated phagocytosis of complement-opsonised pathogens. Binding to integrin receptors for gaining cell entry has been exploited by a number of pathogens such as *Yersinia pseudotuberculosis*, adenovirus and Adeno-associated virus type 2 (AAV-2) (Haas and Plow, 1994).

1.5.1 Integrins as receptors for cell entry

Yersinia pseudotuberculosis is a gut pathogen that binds to host integrins by a single protein called invasin. Invasin binds to $\alpha_5\beta_1$ integrin receptors on the surface of the cell and uptake of the bacterium follows a "zipper-up" process that resembles phagocytosis (Isberg and Van Nhieu, 1995). This process differs from the classical receptor-mediated endocytosis in that there is no size constraint in the particles to be

internalised. Adenovirus binds with its fibre protein to a cell-surface receptor known as CAR (Coxsackie and Adenovirus Receptor) and facilitates internalisation by binding with its penton base to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins or $\alpha_M\beta_2$ in haematopoietic cells (Wickham *et al.*, 1993; Huang *et al.*, 1996). This is mediated by RGD sequence motifs found in five identical domains of the penton base. AAV-2 interacts with heparan sulphate proteoglycan receptors using $\alpha_v\beta_5$ integrins and fibroblast growth factor receptor 1 as co-receptors (Summerford *et al.*, 1999). However, AAV-2 lacks an RGD sequence suggesting that binding takes place in an integrin-independent way. Other viruses such as Foot and Mouth Disease Virus, Coxsackie A9 and echovirus also bind to cell-surface integrins (Bergelson *et al.*, 1992; Jackson *et al.*, 1997). Such investigations have, therefore, proved that integrin-mediated entry into the cell is a conserved process that could be potentially exploited as a non-viral gene delivery system. Obvious advantages of such an approach include the fact that integrin expression occurs on the surface of most cells and secondly, large particles can be delivered.

1.5.2 Integrin-targeting peptides

The original identification of RGDS as the minimal amino acid sequence to bind fibronectin led to the discovery that most integrins bind to the RGD sequence (Pierschbacher and Ruoslahti, 1984). This sequence has very high specificity and a single amino acid change of aspartic acid (RGD) to glutamic acid (RGE) reduces binding affinity up to 100-fold (Hautanen *et al.*, 1989). Identification and isolation of peptide sequences specific for integrin receptors has been facilitated by the development of phage display libraries. These are formed by fusion of DNA sequences with the phage coat proteins pIII or pVIII and displayed on the surface of filamentous phage. For example, the peptide CWLDVC was found to strongly bind to $\alpha_4\beta_1$ integrin while RRETAWA was identified to associate exclusively with $\alpha_5\beta_1$ (Koivunen *et al.*, 1994).

1.5.3 Integrin-targeted vector systems

Synthetic peptides containing the RGD motif have been widely used to target polyplex and lipopolyplex vectors to cell-surface integrin receptors, thereby mimicking the integrin-dependent internalisation process of bacteria and viruses. Invasin, the protein required for internalisation of *Yersinia pseudotuberculosis* has been expressed as a fusion protein with the yeast transcription factor GAL-4. When used in conjunction with poly-L-lysine, this resulted in the formation of complexes capable of transfecting a number of target cells in an integrin-specific manner (Paul *et al.*, 1997). In an attempt to mimic adenovirus cell binding, the integrin-binding peptide CYGGRGDTP was conjugated to PEI via a disulfide bond. Internalisation of these complexes was again integrin-mediated since the control peptide CYGGRGETP had no effect. Also, transfection efficiency of integrin expressing cell lines was enhanced up to 100-fold compared to PEI alone (Erbacher *et al.*, 1999).

Conjugation of integrin-targeting peptides to an oligo-lysine chain results in bifunctional peptides that can target integrin receptors but also have the ability of condensing DNA. The integrin-binding moiety of these peptides may be short, consisting of just the RGD motif, synthetic such as GGCRGDMFGC or isolated from a phage display library (GACRRETAWACG). Incorporation of Lipofectin (Hart *et al.*, 1998) or Lipofectamine, which is a 3:1 mixture of DOSPA and DOPE, in these systems significantly enhances transfection efficiency possibly by mediating endosomal escape (Colin *et al.*, 2000). Integrin-targeted vectors can transfect a wide range of cell types and although transfection is transient, efficiency has exceeded 50% (Hart *et al.*, 1998).

Advantages of such vector systems for gene transfer compared to viral vectors, include the fact that they are non-pathogenic, they are safe and easy to use, and do not have any packaging-related size constraints that limit the delivery capacity of viral vectors. Delivery of large 110 kb PAC constructs into mammalian cells is possible with an integrin-targeted vector system and expression has been shown to persist for up to 35 weeks (Compton *et al.*, 2000). This is very important for gene transfer of

large genes that require their natural genomic promoters and maintain tissue-specificity. In addition, the integrin-targeting peptides have potentially low immunogenicity and the target gene does not integrate into the host genome so there is no risk of insertional mutagenesis. Any problems of immunogenicity could be overcome by using alternative peptides for gene targeting so that repeated administration could be possible. Integrin-targeted vectors have many potential applications since the cell distribution of integrins can be widespread or more restricted depending on the molecule. Also some integrins can be activated or their tissue expression can be altered during progression of the disease state giving rise to greater variability which can be exploited for selective integrin targeting and gene transfer.

The exploitation of integrins as targeting receptors for synthetic vector systems requires maximal expression and function on the target cells. In a range of cell types, integrins may be present in an inactive conformational form that can be altered by treatment with cytokines or PMA. For example, transduction of human monocytes and T cells with adenoviral vectors is improved by expression of α_v integrins upon treatment with MCSF and GMCSF. In a similar fashion, exposure of Caco-2 intestinal epithelial cells to Interleukin-1β enhances adenoviral transduction due to upregulation of integrin expression (Croyle *et al.*, 1998).

1.5.4 Integrins and neuroblastoma

Integrins together with other cell adhesion molecules play a very important role in tumour formation. Angiogenesis and tumour growth in animal models is dependent on endothelial cell anchorage, which is mediated by RGD-binding integrins, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. *In vitro* blocking of these integrins with RGD cyclic peptides results in apoptosis of brain endothelial cells and 50% increase in endogenous ceramide, a lipid second messenger that is implicated in apoptotic pathways (Erdreich-Epstein *et al.*, 2000). The same study showed that expression of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was elevated in the microvascular endothelium of high-risk neuroblastomas (stage IV S

and N-myc-amplified stage III) compared to low-risk groups (stages I, II and non-N-myc-amplified stage III). In melanoma, tumour progression and mortality is also associated with increased expression of $\alpha_v\beta_3$ integrin.

Integrin expression on cultured neuroblastoma cells depends on their morphological features. Substrate-adherent cells show higher expression of integrin fibronectin receptors and especially $\alpha_5\beta_1$ integrins, compared to neuroblastic cells that mainly express $\alpha_v\beta_1$ and $\alpha_4\beta_1$ integrins (Yoshihara *et al.*, 1992). In neuroblastoma cell lines, expression of integrins that bind fibronectin or collagen inversely correlates with N-*myc* amplification (Flickinger *et al.*, 1994). SKNSH neuroblastoma cells normally express little or no N-*myc* but do express β_1, α_2 and α_3 integrins. Transfection of N-*myc* results in down-regulation of β_1 expression (Judware and Culp, 1995). Identification of N-*myc* binding sites on the β_1 integrin promoter has suggested that its product affects transcription from certain integrin genes and exerts a negatively regulatory effect on the integrin mRNA levels (Judware and Culp, 1997).

In neuroblastoma, the stage of cell differentiation may also play a vital role in the levels and type of integrin receptors expressed. Treatment of neuroblastoma cell lines with differentiating agents such as IFN-γ and TNF-α or retinoic acid enhances their $\alpha_1\beta_1$, $\alpha_2\beta_2$ and $\alpha_3\beta_1$ integrin expression (Rozzo *et al.*, 1993). In a similar way, the levels of α_v integrin receptors in SHSY5Y neuroblastoma cells are increased after neuronal differentiation with TPA and retinoic acid treatment (Linnala *et al.*, 1997).

In conclusion, integrin expression depends on the type of tumour and its progression state. Loss of integrins would result in detachment of cancer cells from the original tumour mass while expression of new adhesion molecules would enable those cells to migrate and colonise new tissues. A number of other factors must of course participate in the complex process of metastasis formation and it is not the result of just integrin expression. However, the unique integrin expression pattern of individual tumours could be exploited for targeted gene delivery.

1.6 AIMS AND OBJECTIVES

The aim of this project was to develop an immunotherapeutic strategy for the treatment of neuroblastoma. We have employed a novel non-viral vector system to transfect neuroblastoma cells with IL-2 and IL-12 and used these as a tumour vaccine. The rationale behind using combination of IL-2 and IL-12 lies in their synergistic effect in the generation of LAK cell activity against tumour targets, induction of IFN-γ production and activation of antigen-specific CTLs (Storkus *et al.*, 1998; Trinchieri, 1998). Peripheral blood mononuclear cells from neuroblastoma patients showed increased cytotoxic activity when activated *in vitro* with both cytokines than with IL-2 or IL-12 alone (Rossi *et al.*, 1994). Furthermore, intratumoural injection of vectors expressing both cytokines, resulted in better response with distal tumours undergoing regression and exhibiting enhanced IFN-γ production in 65% of the animals (Addison *et al.*, 1998). Anti-tumour therapies should not only eradicate parental tumour by inducing cytotoxic activity but also have to maintain and amplify anti-tumour immunity. To achieve this, a combination of an IL-12 whole tumour cell vaccine and a mAb-IL-2 fusion protein were used in a mouse model of neuroblastoma (Lode *et al.*, 1999b).

Initially the transfection conditions with the LID vector were optimised on neuroblastoma cell lines, determining the appropriate combination of integrin-targeting peptide and lipid component. After verifying cytokine expression by transfected neuroblastoma cells, a mouse model for the disease was used in order to test the vaccine *in vivo* and assess whether an enhanced immune response could be elicited. The ability of this vaccine to eradicate established murine tumours was determined as well as its prophylactic application. *In vivo* immunodepletion experiments were performed to delineate the immune effector cells responsible for the observed immune response. Furthermore, the effect of vaccination on the histology of tumours and the presence of infiltrating effector cells was determined.

2

MATERIALS AND METHODS

2.1 Reagents

All chemical reagents were purchased from Sigma unless otherwise stated. ^{3}H-thymidine [51 Ci/mmol] was obtained from Amersham. The PI3K inhibitors wortmannin and LY29400 were purchased from Calbiochem and were resuspended in dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 1 mg/ml and 5 mg/ml, respectively. Chloroquine (Sigma) was resuspended in OptiMEM (Life Technologies) at a concentration of 1 mM while cytochalasin-B (Sigma) was resuspended in DMSO at 5 mg/ml final concentration. Recipes for buffer solutions are given in 2.10.

2.2 Cell culture

2.2.1 Primary cells

Primary neuroblastoma cells were obtained from bone marrow samples of patients at Great Ormond Street Hospital NHS Trust, London, UK. Samples were subjected to density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia) to isolate the monocyte fraction and plated on EHS Natrix cellware (Becton Dickinson). Cells were allowed to adhere for 16 h and the non-adherent fraction was replaced with RPMI growth medium (Life Technologies) supplemented with 10% FCS (Sigma) heat-inactivated for 1h at 56 °C, 2mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all by Life Technologies). Primary cells were maintained in a 37 °C incubator supplied with 5% CO_2 and were retained in culture for 2-3 months.

2.2.2 Cell lines

The human IMR-32 and the mouse Neuro-2A neuroblastoma cell lines were obtained from the American Tissue Culture Collection (ATCC) while the SHSY-5Y human neuroblastoma cell line was purchased from the European Collection of Cell Cultures (ECACC). Cell lines were cultured in Dulbecco's MEM- Glutamax-1 (Life

Technologies) supplemented with 10% FCS (Sigma) heat-inactivated for 1hr at 56 °C, non-essential amino acids (100x), 1mM sodium pyruvate (100x), 100 IU/ml penicillin and 100 μg/ml streptomycin (all by Life Technologies). The cultures were maintained in a 37 °C incubator supplied with 5% CO_2 and split every three or four days at a 1 in 5 density. Trypsin-EDTA (Life Technologies) was used for 5-10 min at 37 °C to detach cells from tissue culture flasks.

The hybridoma cell lines YTS191.1 and YTS169 were a kind gift from Dr Ali (Institute of Ophthalmology, London) and produce rat (IgG_{2b}) anti-mouse CD4 and CD8 antibodies, respectively. Cells were cultured in RPMI containing 10% FCS, 2mM glutamine and 10 μg/ml gentamicin until confluent. The medium was then replaced with Hybridoma-SFM medium (Life Technologies) containing 10 μg/ml gentamicin for 48 h. The cell culture supernatant was collected, centrifuged to remove cell debris and frozen at –80 °C for subsequent antibody purification.

2.2.3 Antibody purification

Rat (IgG_{2b}) anti-mouse CD4 and CD8 antibodies were purified on 1 ml HiTrap Protein G columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Briefly, the hybridoma cell culture supernatant was centrifuged at 12,000 rpm for 30 min at 4 °C, its composition adjusted to 20 mM sodium phosphate pH 7.0 by addition of the appropriate volume of 100 mM sodium phosphate buffer pH 7.0 and filtered through a 0.22 μm Stericup filter (Nync). A peristaltic pump P-1 (Pharmacia Fine Chemicals) was used to control the flow rate through the protein G column. The column was initially equilibrated with 10 volumes of 20 mM phosphate buffer at a flow rate of 1 ml/min and then the antibody sample was passed through at the same rate. After washing the column with 5-10 volumes of 20 mM phosphate buffer, the antibody was eluted with 5 volumes of 0.1 M glycine-HCl, pH 2.7. 100 μl of 1 M Tris-HCl pH 9 was added per ml of fraction to adjust the pH. After extensive dialysis in PBS, the antibody concentration was determined using Protein Assay Reagent (Biorad) according to the manufacturer's recommendations. BSA standards were used to generate a standard curve.

2.3 LID transfection

2.3.1 Preparation of LID complexes

Cells were seeded on 48-well plates at $2.5x10^4$ cells per well and incubated overnight in a 37 °C incubator. LID transfection complexes were formed using Lipofectin (Life Technologies), Integrin-targeting peptide (Zinsser Analytic) (See Table 2.1 for sequences and integrin specificity) and plasmid DNA at a ratio 0.75 μg Lipofectin per μg of plasmid DNA. Peptides were used at a 7:1 charge ratio to DNA (see Table 2.1 for weight ratios to plasmid DNA). Lipofectin and plasmid DNA were diluted to a concentration of 0.75μg/100 μl OptiMEM (Life Technologies) and 1μg/100 μl OptiMEM, respectively, mixed with the integrin-targeting peptide (0.1 mg/ml in OptiMEM) and complexes were allowed to form for 1.5 h at RT. Lipofectin-DNA (LD) complexes were prepared by mixing Lipofectin and plasmid DNA at a weight ratio of 5:1. After the incubation period, the transfection complexes were diluted in OptiMEM to a concentration of 1 μg DNA per 0.25 ml of complexes per well. Transfection was performed at 37 °C for 4 h and reporter gene activity was performed 48 h afterwards. Transfection using other lipids (Table 2.2) was carried out in a similar way by first mixing the lipid with the integrin-targeting peptide and finally adding the DNA.

Primary cells were detached from plates as described above and seeded on 24-well plates at $5x10^4$ cells per well. LID, LD and PD complexes were prepared as described above in OptiMEM and 0.5 ml of complexes were added per well. Transfection was performed for 4 h and reporter gene expression assays were carried out 48 h post-transfection.

Transfection of neuroblastoma cells with the plasmids carrying the cytokine genes was performed on 24-well plates using peptide 6. Cells were seeded at $5x10^4$ cells/well the night before in culture medium and transfected the next day. 24 h after transfection and at certain time points, the cells were fed with 1 ml medium, incubated for 24 h

and the supernatant was collected. This was then centrifuged for 5 min at 12,000 rpm to eliminate any particulate residues and stored at –70 °C.

Table 2.1 Peptides used in LID transfection experiments

PEPTIDE	SEQUENCE	TARGETING INTEGRIN(S)	μg of peptide for 6.8:1 charge ratio (per μg of plasmid DNA)
Pep1	$[K]_{16}$GACRGDMFGCA	α_v, α_5	3.5
Pep6	$[K]_{16}$GACRRETAWACG	$\alpha_5\beta_1$	4
Pep6J	$[K]_{16}$GACATRWARECG	-	4
Pep10	$[K]_{16}$GACWLDVCA	α_4	3.8
Pep11	$[K]_{16}$GACRGEMFGCA	-	3.5
Pep12	$[K]_{16}$XSXGACRRETAWACG	$\alpha_5\beta_1$	4.5
K_{16}	$[K]_{16}$	-	2.7
Soluble RGD	GRGDSP	α_v, α_5	
Soluble RGE	GRGESP	-	

Table 2.2 Lipids used in LID transfection experiments

LIPID	FORMULATION	MANUFACTURER
Lipofectin	1:1 (w/w) formulation of DOTMA [N-[1(-2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride] and DOPE (dioleoyl phosphatidylethanolamine)	Life Technologies
TransIT Polyamine Transfection Reagents	polyamine	Mirus, UK

2.3.2 Calculation of charge of transfection complexes

The charge ratios of LID complexes were deduced from the molecular weight and charge parameters of each of the peptide and DNA components. In the peptides, lysine (K) and arginine (R) residues contribute a charge of +1 whereas each aspartic acid (D) and glutamic acid (E) contributes a charge of –1. The negative charge of plasmid DNA was estimated on the basis that each phospholipid per base of DNA contributes one negative charge. Therefore, 1μg of pCI-luciferase plasmid DNA (5,693 bp, $M=3.76x10^6$) contains 0.27 pmol of DNA and [0.27 pmol x 5,693 x 2]= 3.1 nmol negative charge.

2.3.3 Luciferase assay

Cells transfected with the pGL3-CMV850 or pCI-luc vectors were assayed for luciferase activity 48 h after transfection. The cells were washed twice with PBS and 100 μl of 1x Reporter Lysis Buffer (Promega) in sterile water was added. The cells were incubated at 4 °C for 15 min and subsequently scraped off the plates using a pipette tip. After mixing, the cell suspension was frozen for a minimum of 30 min at -70 °C. After allowing for the suspension to thaw, followed by a 5 min centrifugation to remove cell debris, 20 μl of the supernatant was assayed for Luciferase activity using a Luciferase Assay kit (Promega). The relative light units emitted in each reaction were measured on an Anthos Lucy 1 Luminometer (Labtech) and were then corrected for the protein concentration of the samples determined with Protein Assay Reagent (Biorad) according to the manufacturer's instructions. Luciferase enzyme activity was expressed as RLU/mg.

2.3.4 Cytokine ELISA

In order to quantify the hIL-2 or murine IL-12 secreted by transfected neuroblastoma cells in the supernatant, a DuoSet ELISA Development System was used (R&D Systems, Oxford, UK) according to the manufacturer's instructions. Nunc-Immuno™

MaxiSorp 96-well plates (Life Technologies) were coated with cytokine capture antibody reconstituted and diluted to 4 µg/ml working concentration in PBS according to the manufacturer's instructions. Detection was carried out using a biotinylated secondary antibody followed by streptavidin conjugated to horseradish-peroxidase (HRP) and an equimolar mixture of hydrogen peroxide (H_2O_2) and tetramethylbenzidine (R&D Systems). The reaction was stopped with 2M H_2SO_4 and the optical density determined at 450 nm with wavelength correction at 570 nm, which corrects any optical imperfections in the plate. A standard curve was constructed using the readings for the cytokine standards and the concentration in the supernatant was extrapolated taking into consideration the appropriate dilution of the samples. For human IL-12, an IL-12 p70 ELISA system (Amersham Pharmacia) was used according to the manufacturer's instructions.

2.3.5 Cytokine Bioassay

Mononuclear cells from the peripheral blood of healthy donors were obtained by density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia). These cells were monocyte-depleted by plastic adherence and then resuspended at 10^7 cells/ml in RPMI 1640 medium (Life Technologies) containing 2.5 µg/ml PHA-L (Sigma) and cultured for 3 days. Cells were then diluted 1:1 and 50 IU/ml rhIL-2 (R&D Systems) was added for 24 h. Complete medium was used to wash the cells prior to adjusting their concentration to $4x10^5$ cells/ml. For the cytokine bioassay, 50 µl of cell suspension was incubated for 24 h with 50 µl of either a dilution of the reference standard or the supernatant of IL-2-, IL-12- or dual-transfected neuroblastoma cells. Cell proliferation was measured by addition of ^{3}H-thymidine (0.5 µCi/well) for 16 h and samples were analysed for incorporated tritium.

2.4 Nucleic acids

2.4.1 Preparation of competent cells

E.Coli DH5α cells were used to transform plasmid DNA. A 1 ml overnight inoculum was used to propagate a 200 ml LB bacterial culture for 2-3 h at 37 °C with agitation or until the spectrophotometric density of the culture reached an OD_{600nm}=0.4. The bacterial cell pellet was then resuspended in 80 ml of 100 mM $CaCl_2$ solution and incubated on ice for 30 min. After centrifugation, 8 ml of $CaCl_2$ and 2 ml of glycerol were added and the cells snap-frozen in liquid nitrogen.

2.4.2 Transformation of plasmid DNA

For plasmid DNA transformation, 200 μl of competent cell suspension were used for 100 ng of DNA by initially incubating the cells on ice and subsequently heat-shocking at 42 °C for 90 sec. Cells were then incubated at 37 °C with shaking for 1 h and finally plated on LB agar plates containing the appropriate antibiotic.

2.4.3 Plasmid DNA

The plasmids pGL3-850CMV and pCI-luciferase (gifts from O.Schwickerath, Institute of Child Health, London) carry the luciferase reporter gene under the control of a cytomegalovirus immediate early promoter-enhancer (CMV). The pEGFP-N1 plasmid, which carries the gene for the green fluorescence protein, was obtained from Clontech (California, USA). The plasmid pCMV-Flexi12 was a gift from Dr Anderson (Royal Free Hospital, London) and the retroviral constructs MFGS-IL2, MFGS-IL12p35 and MFGS-IL12p40 were kindly donated by Prof Collins (University College London).

2.4.4 Plasmid DNA purification

Plasmid DNA was purified by alkaline lysis on Hybaid resin columns (Hybaid) and precipitated with isopropanol according to the manufacturer's instructions. The plasmid DNA pellets were washed in 70% ethanol and then resuspended in pyrogenic-free water. For small-scale preparations, plasmid DNA was extracted from alkaline-lysed bacteria using Hybaid Recovery Quick Flow miniprep kit. The concentration of DNA was quantitated by spectrophotometric analysis at OD_{260nm} using GeneQuant RNA/DNA Calculator (Pharmacia Biotech) and gel electrophoresis by comparison with lambda HindIII ladder (Life Technologies) or Hyperladder I (Bioline).

2.4.5 Enzyme digestion of plasmid DNA

Restriction enzyme (Promega) digestion of bacterial plasmid DNA was carried out in 1xRestriction enzyme buffer using an excess of enzyme 5-10U/μg DNA according to the manufacturer's instructions.

2.4.6 Agarose gel electrophoresis

0.8-1% (w/v) agarose gels (Life Technologies) were prepared in 1xTAE containing 400 ng/ml Ethidium Bromide and DNA samples were loaded after addition of 6xDNA loading buffer. 1kb Plus Ladder (Life Technologies) or HyperLadder (Bioline) were loaded alongside plasmid DNA for sizing and the gels were run in 1x TAE buffer at 70-120 mV depending on size. Agarose gels were observed under a UV MultiImage™ Light Cabinet, images taken using AlphaImager™ 1200 (Flowgen) and processed in Adobe™ Photoshop (Microsoft).

2.4.7 Gel purification of DNA fragments

DNA fragments were separated by agarose gel electrophoresis and the DNA was purified using the Qiagen Gel Extraction kit (Qiagen) according to the manufacturer's instructions.

2.4.8 DNA Ligation

Ligation of DNA fragments with complementary ends was carried out at 14 °C overnight using T4 DNA ligase (Promega) and 1x ligase buffer according to the manufacturer's recommendations. In order to prevent self-ligation of the plasmid vector, it was pretreated with shrimp alkaline phosphatase (Amersham) at 0.01U/pmol of ends to remove the 5' phosphate groups. The enzyme was heat-inactivated at 65 °C for 15 min and removed using a Nucleotide removal kit (Qiagen) according to the manufacturer's instructions.

2.5 Flow cytometry

2.5.1 EGFP expression

The percentage of transfection efficiency of cells with pEGFP-N1 plasmid vector was estimated through FACS analysis. The cells were transfected as described in 2.3.1 in 48-well plates using the pEGFP-N1 vector. 48 h post-transfection, the cells were washed twice with PBS, and they were detached from the plate by addition of 200 μl of Trypsin-EDTA (Life Technologies). The trypsin solution was subsequently removed, the cells washed and resuspended in 1xPBS-0.01% Azide and either put directly through the FACS sorter (FACSCalibur, Becton Dickinson) using CellQuest software or fixed in 4% paraformaldehyde in PBS and stored at 4 °C.

2.5.2 FACS antibody staining

Human neuroblastoma cell lines were stained for integrin receptors using one of the primary antibodies (Serotec) listed in Table 2.3. A mouse IgG antibody (Chemicon International) was used as the isotype control. All antibodies were used at a concentration of 1μg/10^6 cells in a 50 μl reaction in staining buffer and incubation was performed for 1 h on ice. After three washes in staining buffer, a secondary goat anti-mouse FITC-labelled antibody (DAKO) was applied for 40 min. The cells were finally fixed in 1%PFA (w/v) in PBS and expression was analysed by flow cytometry in a FACS sorter (Beckman Coulter). Staining for mouse α_5 integrin expression on Neuro-2A cells was performed using an RPE-conjugated rat anti-mouse CD49e antibody (Pharmingen) or an RPE-conjugated rat IgG_{2b} isotype control (Pharmingen) as described above.

Table 2.3 Antibodies used for integrin expression

ANTIBODY	SOURCE	ISOTYPE	WORKING DILUTION
Human anti-CD49e	Serotec	Mouse	1:50
Human anti-CD49d	Serotec	Mouse	1:50
Human anti-CD51/61	Serotec	Mouse	1:50
Mouse IgG	Chemicon	Mouse	1:50
Mouse anti-CD49e-RPE	Pharmingen	Rat	1:50
Rat IgG_{2b}-RPE	Pharmingen	Rat	1:50

CD4 and CD8 staining of mouse splenocytes was performed by homogenising whole spleens through a cell strainer using a syringe barrel. The single cell suspension obtained was subjected to red blood cell (RBC) cell lysis by addition of 1 ml RBC Lysis solution (Sigma) for 5 min on ice. After addition of 20 ml RPMI, the cells were centrifuged at 15,000 rpm at 4 °C and washed twice in antibody staining buffer. Blocking of $F_{c\gamma}$ receptors was performed initially by 10 min incubation in Fc antibody (1:100) which was maintained during CD4 or CD8 staining. All reactions (50 μl) were performed in antibody staining buffer for 40 min on ice followed by three washes in

antibody staining buffer. Positive cells were detected by 20 min incubation in streptavidin-CY (Pharmingen) and FACS analysis.

Table 2.4 Antibodies used for FACS staining of mouse splenocytes

ANTIBODY	SOURCE	ISOTYPE	WORKING DILUTION
Mouse anti-CD4-biotin	Pharmingen	Rat	1:20
Mouse anti-CD8a-biotin	Pharmingen	Rat	1:20
Rat IgG_{2a}-biotin	Pharmingen	Rat	1:20
Mouse CD16/CD32 ($F_{c\gamma}$ III/II)	Pharmingen	Rat	1:100

Primary neuroblastoma cells were stained for GD_2 and NCAM surface expression in a similar way to that described above. Both mouse anti-human NCAM antibody (DAKO) and mouse anti-human GD_2 (clone 14.G2a) (Chemicon International Inc) were used at 1:50 dilution in antibody staining buffer for 1 h on ice. A secondary RPE-conjugate ant-mouse antibody was applied for 40 min and detection was again performed by FACS analysis.

2.5.3 Annexin-V/PI staining

Detection of apoptosis after transfection or irradiation of cells was performed with Annexin V-FITC and propidium iodide staining (Pharmingen). Annexin V binds to the phospholipid phosphatidylserine, which on apoptotic cells is exposed onto the outer membrane. Cells were resuspended in 1x binding buffer at a concentration of 10^6 cells/ml and 100 μl were used for staining. 5 μl of annexin V-FITC and 10 μl of a 50μg/ml propidium iodide stock solution in PBS was prepared which stains the nuclei of necrotic or dead cells. Cells were incubated at RT for 15 min and then analysed by flow cytometry after addition of 400 μl 1x binding buffer. Flow cytometry compensation and quadrants were set up using unstained cells, and cells stained only with annexin V-FITC or propidium iodide.

2.6 Western analysis

2.6.1 Preparation of cell lysates

Transfected cells ($5x10^5$-$2x10^6$) were trypsinised, washed twice in cold PBS and lysed on ice by addition of 100 µl NP-40 lysis buffer for 10 min. The cell debris was removed by centrifugation and an equal volume of SDS protein sample buffer was added to the supernatant. The samples were subsequently boiled at 100 °C for 5 min.

2.6.2 SDS-PAGE analysis

A 4% stacking gel [1.3ml 30% acrylamide (Protogel™, National Diagnostics), 2.5 ml stacking gel buffer, 6.1 ml H_20] was poured onto a 20% resolving gel [12 ml 30% acrylamide, 5 ml main gel buffer, 3 ml H_20]. For polymerisation of the gels, 0.1% (v/v) TEMED and 0.1% (w/v) ammonium persulphate were used. The protein concentration of samples was estimated by spectrophotometric analysis at OD_{550nm} using BioRad Protein Assay Reagent (BioRad) according to the manufacturer's recommendations. An equal amount of protein (~200 µg) from each sample was added per well and they were electrophoresed across a voltage of 100-180 V. 10 µl of Rainbow markers (Amersham Pharmacia) were loaded alongside the samples for sizing. Recombinant mouse lymphotactin (200ng) (R&D Systems) was also loaded.

2.6.3 Transfer to nitrocellulose membranes

Gels were immersed in protein transfer buffer for 5 min and then the protein was transferred onto nitrocellulose membranes (Micron Separations Inc) using a semi-dry transfer cell (BioRad) for 20-30 min across a voltage of 12 V.

2.6.4 Immunoblotting

The protein membrane was blocked overnight in PBS-T containing 5% (w/v) milk and (1:200) rabbit IgG at 4 °C. The primary antibody of choice was applied for the appropriate incubation time [goat anti-mouse lymphotactin antibody (0.3 μg/ml for 1 h at RT) and rabbit anti-mouse β-actin (Sigma) (1/1000 dilution for 2 h at RT)]. The membrane was washed x5 in PBS-T and a secondary rabbit anti-goat HRP-conjugated (Sigma) (lymphotactin) or anti-rabbit (β-actin) HRP-conjugated antibody (Sigma) was used for 30 min. After repeating the washes with PBS-T, reactive species were detected using enhanced chemiluminescence plus system (Amersham Pharmacia) and autoradiographed for 5 sec-10 min.

Table 2.5 Antibodies used in Western blotting

PRIMARY ANTIBODY	SOURCE	ISOTYPE	WORKING DILUTION
Anti-mouse lymphotactin	R&D	Goat	3:1000
Anti-β actin	Sigma	Rabbit	1:1000

2.7 Animal studies

2.7.1 Transfection of Neuro-2A cells

Neuro-2A cells were detached from plastic by incubation with Trypsin-EDTA, counted by trypan blue (Life Technologies) exclusion and seeded onto 150 cm^2 tissue culture flasks at $4x10^6$ cells/flask. LID transfection was performed the following day using 40 μg of DNA per 150 cm^2 flask for 4 h. The cells were trypsinised after ~17 h, washed in RPMI-1640 medium and resuspended at the appropriate dilution for inoculation into A/J mice.

2.7.2 Animal inoculation

A/J mice are syngeneic with Neuro-2A mouse neuroblastoma cell line and were obtained from Harlan Laboratories (UK). All experiments were conducted in accordance with guidelines for animal use and care established by the Animals Scientific Procedures Act (1986) at the Royal Veterinary College (London, UK) or at the Institute of Child Health. Mice (n=6 per group, unless otherwise stated) were subcutaneously inoculated in the posterior flank with 10^6 Neuro-2A (total volume=100 μl) cells transfected with cytokine genes, empty vector or untransfected as described in 2.7.1. The two perpendicular diameters of subcutaneous tumours were measured every 2-3 days using calipers. Mice were sacrificed when tumours reached a diameter of >17mm. The tumour volume was calculated by the formula:

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

2.8 Immunohistochemistry

2.8.1 Tissue embedding and sectioning

Subcutaneous tumours were isolated from culled A/J mice, dissected appropriately and fixed in 10% PFA by overnight incubation at 4 °C. Tissue specimens were dehydrated through serial 1 h alcohol washes to 100% ethanol. Paraffin embedding was performed by two 40 min incubations in HistoClear (National Diagnostics) followed by three 40 min incubations in an equimolar mixture of paraffin wax (Raymond Lamb) and HistoClear. Finally tumours were embedded by 40 min incubations in paraffin wax and allowed to set overnight at 4°C. Tumour tissue was sectioned (6-8μm) on a Microm HM325 Rotary Microtome (Raymond Lamb), mounted onto poly-L-lysine coated glass slides (BDH) and allowed to air-dry overnight at 37 °C.

2.8.2 Immunohistochemical staining

Sections were de-waxed twice in HistoClear and re-hydrated through serial 3 min alcohol washes to 50% ethanol. After a final wash in dH_2O, sections were pre-treated to unmask antigen by microwaving in antigen unmasking solution (Vector Laboratories) (3.75 ml in 400 ml dH_2O) for 10 min and cooling rapidly in water. They were then washed twice in dH_2O prior to blocking endogenous peroxidase activity in 0.3% (v/v) H_2O_2-0.1% (w/v) NaN_3 in PBS for 15 min. The sections were washed twice in PBS and non-specific antibody binding was blocked by incubation in PBS containing 10% (w/v) rabbit serum (Sigma) for 30 min at RT. The antibody of interest was applied onto the sections after draining off the excess blocker. Rabbit anti-mouse CD45 or CD34 (Pharmingen), were used at 1:20 dilution in PBS 1% BSA and the sections incubated overnight at 4 ^{o}C in a humid chamber. Control staining consisted of overnight incubations with 10% rabbit serum in PBS. The sections were subsequently washed three times in PBS and a rabbit anti-rat biotinylated antibody (Vector Laboratories) was used at a 1:250 dilution in PBS 1% BSA for 30 min at RT followed by a streptavidin-biotinylated peroxidase complex (ABC Reagent) (VectaStain). Peroxidase activity was visualised using the chromogen 3,3'-Diaminobenzidine (SIGMA FAST DAB) (Sigma). After stopping the reaction by three washes in PBS, the sections were counterstained with methyl green (Vector Laboratories) for 10 min and rinsed twice by immersing 10 times in dH_2O followed by a 30 sec wash in fresh dH_2O. They were then dehydrated by immersing ten times in butanol-1 (BDH) for a total of two washes, incubated in fresh butanol-1 for 30 sec and then in HistoClear for 10 min. The slides were mounted with VectaMount (Vector Laboratories) permanent mount using a glass coverslip (BDH). Slides were viewed using an AxioPhot 2 (Zeiss) microscope attached to a ProgRes 3012 (Kontron Electonic) digital camera and images were processed in Adobe^{TM} Photoshop (Microsoft).

2.8.3 Histology

Examination of the morphology of tumour tissue was performed by conventional histological staining using Ehrlich's haematoxylin (BDH) and 1% aqueous solution of eosin (Raymond Lamb). Sections were immersed in HistoClear for 5 min, dehydrated by sequential washes in 100%, 95% and 70% ethanol and rinsed briefly in dH_2O. Staining with haematoxylin was performed for 10 min at RT. Excess dye was removed by rinsing sections in dH_2O for 5 min and quickly dipping them in acid alcohol [1% (v/v) conc.HCl in 70% ethanol]. The sections were then placed under running tap water, stained in eosin for 10 min and washed again in tap water. Dehydration of sections was performed by briefly immersing them into subsequent solutions of 95% and 100% ethanol. They were finally placed in HistoClear solution for 5 min and mounted as described in 2.8.2.

2.9 Statistical analysis

The non-parametric Mann-Whitney test was used to assess the significance between different experimental animal groups. For cytokine production and bioassays, comparison among different groups was performed at each time point. Comparison of the cell proliferation among the transfected neuroblastoma cells was performed using the Student *t* test.

2.10 Buffers

All buffers were prepared in double distilled water (ddH_2O) autoclaved at 121 °C for 15 min.

Ampicillin	25 mg/tablet (Stratagene) diluted in H_2O; working concentration 100 μg/ml
Antibody Staining Buffer	PBS. 0.01% (w/v) NaN_3, 1% BSA
Kanamycin	Stock solution 50 mg/ml, working dilution 50μg/ml
LB broth	1% (w/v) tryptone peptone (Becton Dickinson), 0.5% (w/v) yeast extract (Becton Dickinson), 171 mM NaCl (BDH), pH 7.0. For solid media 15 g/lt LB agarose was added.
Main Gel Buffer	1.5M Tris-HCl, 0.1% (v/v) SDS, pH 8.8
NP-40 Lysis Buffer	1% NP40, 20 mM Tris-HCl (pH 8.0), 1300mM NaCl, 10 mM NaF, 1 mM PMSF, 100 μM Na_3VO_4, 1 mM DTT, 20 μM leupeptin, 1% aprotinin
PBS	Phosphate buffered saline tablets (Oxoid) were dissolved in water at 1tablet/100ml
PBS-T	PBS, 0.1% Tween 20

Phosphate Buffer (0.1 M)	57.7 ml of 1 M Na_2HPO_4 and 42.3 ml of 1M NaH_2PO_4
Protein Transfer Buffer	48 mM Tris-HCl, 39 mM glycine (Calbiochem), 20%(v/v) methanol (BDH), pH9.2
Stacking Gel Buffer	125 mM Tris-HCl, pH 6.8, 0.4% SDS
TAE (x50)	0.2 M Tris, 1 M glacial acetic acid (BDH), 50 mM EDTA, pH8.0
SDS-PAGE Running Buffer	25 mM Tris, 192 mM glycine, 0.1% SDS
SDS Protein Sample Buffer	125 mM Tris-HCl, 0.01% (w/v) bromophenol blue (BDH), 2% (v/v) SDS, 0.4% water, pH 6.8, 0.02% (v/v) β-mercaptoethanol

3

LID Vector Optimisation

3.1 INTRODUCTION

The development of a gene therapy application necessitates the optimisation of certain parameters that will ensure maximum transfection efficiency and expression of the desired transgene. In the case of *ex vivo* transfection of tumour cells for vaccine development, the number of transfected cells directly affects the expression levels of cytokines or other immunomodulatory molecules that will effectively induce an immune response.

Variation in the total amount of DNA to be delivered, the number of cells and their mitotic state contribute to the transfection efficiency of a particular vector. In addition, the incubation time of the transfection complexes plays an important role. Optimisation of a viral vector protocol on a specific cell type requires titration of the multiplicity of infection (MOI) of the virus as well as the number of rounds of infection necessary to achieve maximum transduction efficiency. For non-viral vector systems where a combination of molecules is used to deliver the DNA into the cells, the concentrations of each of the components must be carefully titrated. In addition, non-viral complexes directed to a particular cell surface receptor have to express maximal number of ligands at high avidity to ensure strong interaction with the receptor. The surface charge density, also known as zeta potential (z), of such complexes also plays an important role in the efficiency of the vector because it determines the non-specific interaction with the cell membrane. Potential obstacles to efficient transfection such as inefficient endosomal escape can be overcome using lysomotropic agents. Low expression may be enhanced by utilising a stronger promoter that will drive higher expression of the transgene.

The aim of the following experiments was to obtain the LID complex formulation by combining the appropriate lipid and integrin-targeting peptide. The weight ratio among the vector components as well as the maturation of complexes were titrated in order to achieve maximum transfection efficiency of neuroblastoma cells with the LID vector.

3.2 RESULTS

3.2.1 Enhanced transfection efficiency by incorporation of a lipid component

Previous studies using the LID vector showed that the integrin-targeting peptide could condense DNA and mediate delivery into a variety of cell types. However, efficient transfection could only be achieved in the presence of Lipofectin (Hart *et al.*, 1998). Neuroblastoma cells were transfected as described in 2.3.1 with LID, LD (Lipofectin-DNA at a weight ratio of 5:1) or PD (peptide 6- DNA) complexes and assayed for luciferase gene expression 48 h later. In all cell lines transfection efficiency using the full LID formulation gave higher RLU/mg values than LD or PD complexes ($p<0.009$) (**Fig 3.1**). This increase in reporter gene activity achieved using LID complexes correlated with an increase in the percentage of IMR-32-transfected cells as revealed by pEGFP-N1 transfections and subsequent flow-cytometric analysis. LD transfection in these cells resulted in just 10% transfection efficiency. In SHSY5Y and Neuro-2A cells, however, LD complexes were equally efficient with LID complexes – 30% and 60% respectively.

3.2.2 Titration of the LID vector components

Charge ratios were calculated as described in the Materials and Methods (2.3.2). The overall charge of plasmid DNA is attributed to its phospholipid backbone. The charge of integrin-targeting peptides was based on their amino acid sequence and the charge properties of each amino acid. A net positive charge was attributed to lysine or arginine amino acids while glutamic acid and aspartic acid have a negative charge. Lipofectin is a commercially available lipid consisting of an equimolar ratio of the cationic lipid *N*-[1-(2,3,-dioleyloxy) propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and the neutral lipid dioleoyl posphatidylethanolamine (DOPE). DOTMA has a single net positive charge while DOPE is neutral. The overall cationic charge of Lipofectin was therefore taken as +1 per molecule of DOTMA.

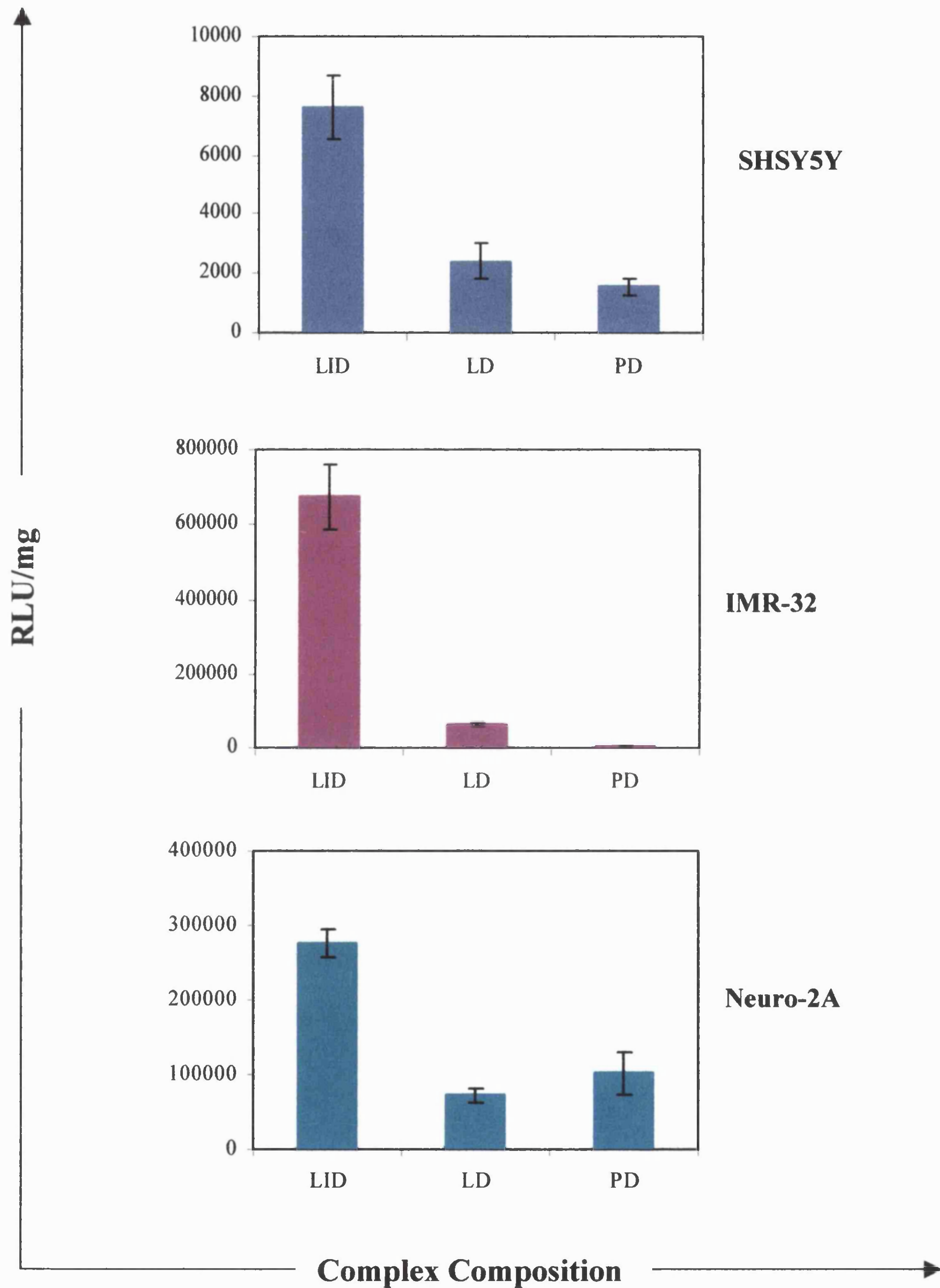


Figure 3.1 Effect of Lipofectin on transfection efficiency of the LID vector. Enhanced luciferase gene expression in neuroblastoma cell lines by transfection with the full LID formulation compared to LD (Lipofectin/DNA) or PD (Pep6/DNA) complexes. Luciferase expression was assayed after 48 h and the error bars represent standard deviations from triplicate transfections.

3.2.2.1 DNA

Initially, the LID formulation was optimised by titration of the Lipofectin and DNA components. Neuroblastoma cell lines were transfected with increasing amounts of DNA (1-5 μg) per 2.5×10^4 cells whilst maintaining the weight ratio of Lipofectin to DNA (0.75:1) and peptide 6 to DNA (4:1) constant. In IMR32 cells, luciferase gene expression decreased with increasing DNA concentration while in the SHSY5Y cell line 2 μg of DNA gave rise to significantly higher RLU/mg values than any other DNA concentrations ($p<0.05$) (**Fig 3.2**). In Neuro-2A cells, 5 μg of DNA resulted in a 5-fold increase in luciferase gene expression compared to 2 μg of DNA. However, this may be due to a toxic effect of the transfection process. This was evident by a reduction in the total amount of cellular protein in the samples used in the luciferase assay, which affected the total RLU/mg value. There was no improvement in the luciferase expression in Neuro-2A cells when 2 μg of DNA were used compared to 1 μg (**Fig 3.2**).

3.2.2.2 Lipofectin

In these experiments the total amount of DNA (1μg) and the peptide/DNA weight ratio (4:1) were retained whilst varying the amount of Lipofectin per μg of DNA. In transfections of SHSY5Y cells there was a 1.5-fold increase in luciferase expression when 1 μg of Lipofectin per μg of DNA was used compared to 0.75 μg Lipofectin per μg of DNA ($p<0.05$) (**Fig 3.3**). Further increase of Lipofectin concentration (1.5-3 μg per μg of DNA) decreased expression levels similar to those achieved with 0.75 μg of Lipofectin. In IMR-32 cells, on the other hand, 0.75 μg of Lipofectin proved to be the most efficient concentration. Increasing the amount of Lipofectin above 0.75 μg per μg of DNA resulted in a dose-dependent decrease in luciferase expression. In the mouse Neuro-2A cell line, 0.75 and 1 μg of Lipofectin resulted in similar RLU/mg levels of 10^6 and 1.1×10^6 respectively. At concentrations of 1.5 or 3 μg of Lipofectin

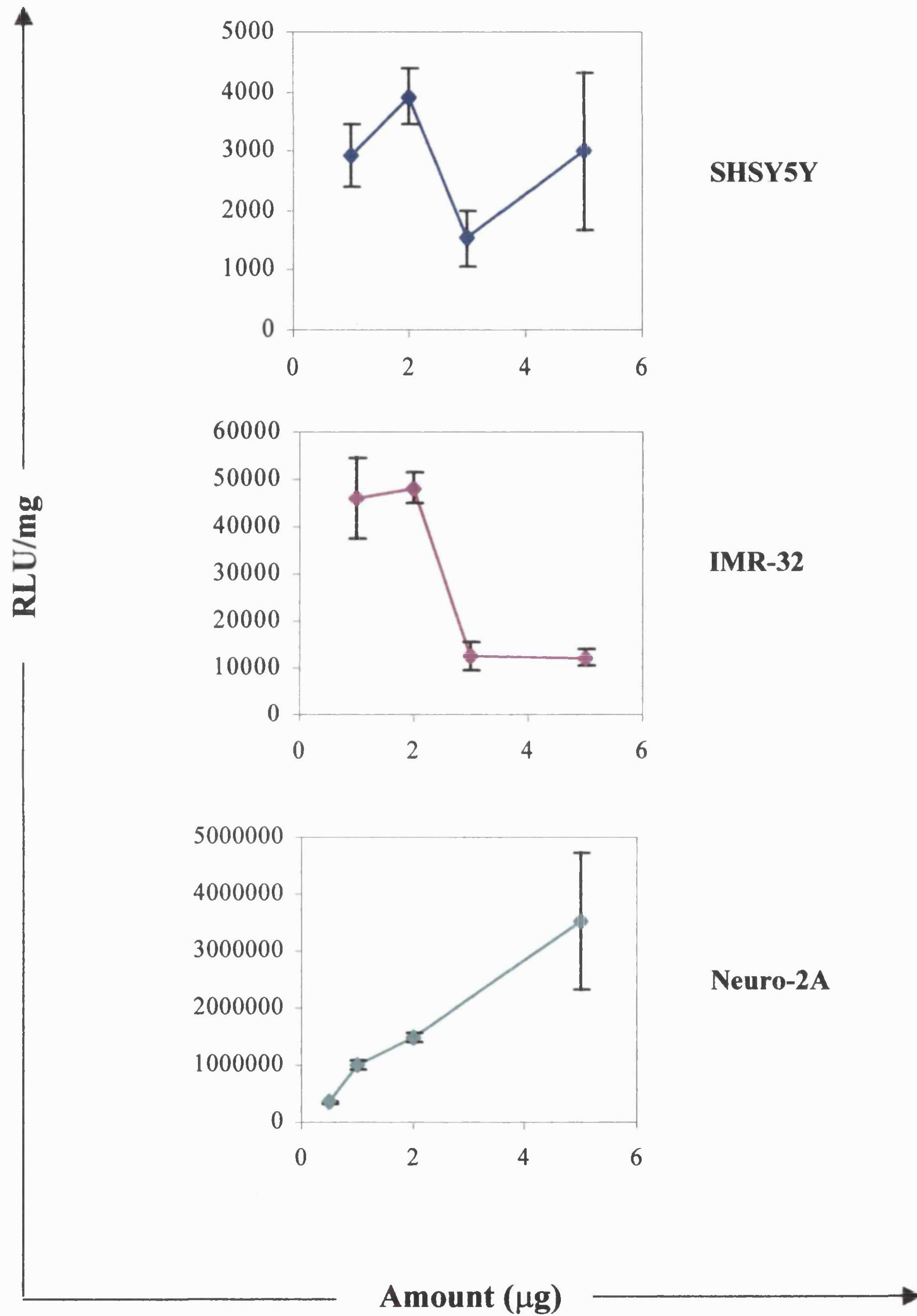


Figure 3.2 Titration of the DNA component of the LID vector on neuroblastoma cells. The weight ratio of peptide 6 to DNA and Lipofectin to DNA were retained while changing the total amount of DNA that was used to transfect the cells. Transfections were performed in triplicate wells and one representative experiment is shown.

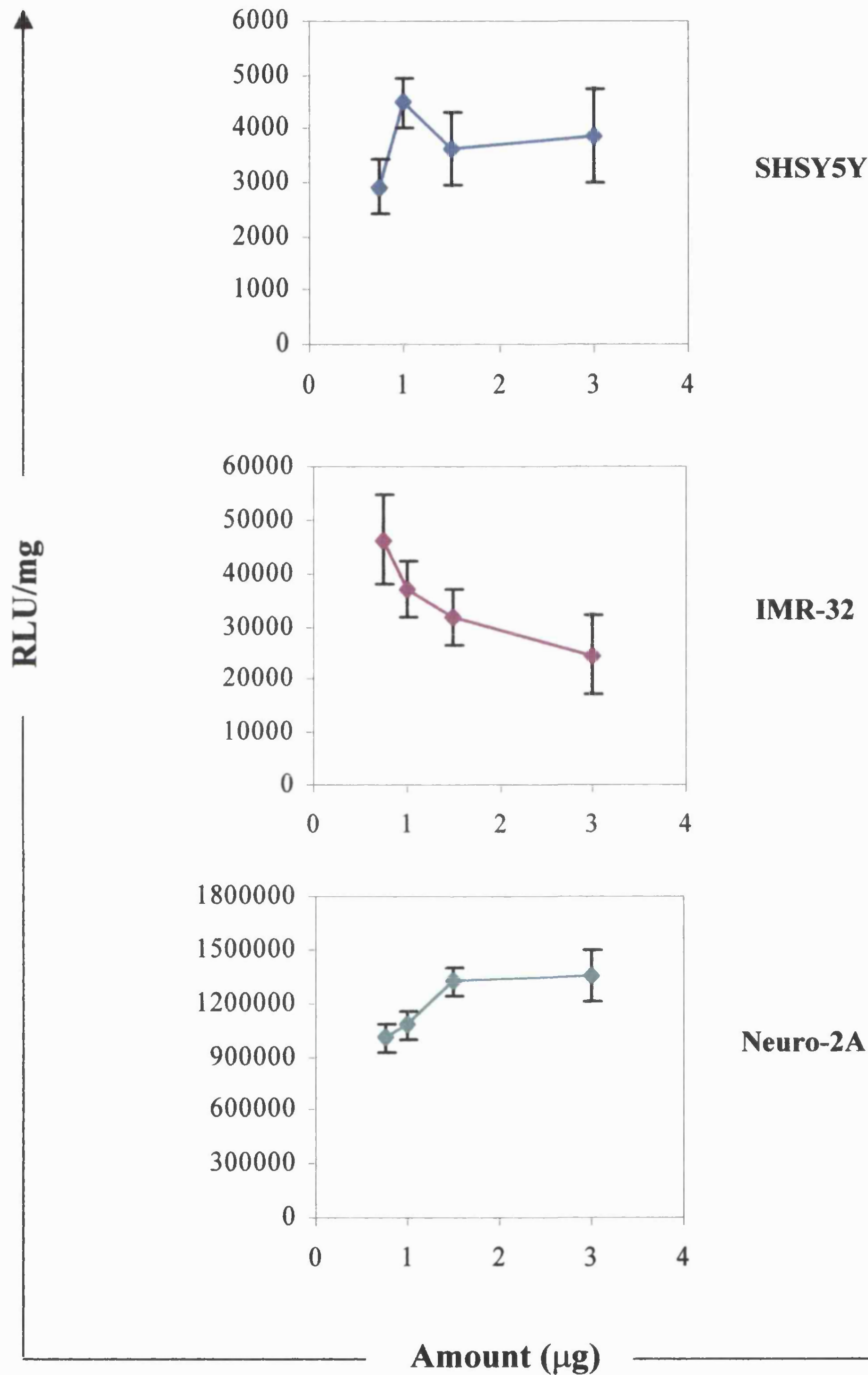


Figure 3.3 Titration of Lipofectin in neuroblastoma cell lines. Luciferase gene activity in neuroblastoma cells as a function of Lipofectin concentration. Cells were transfected with the same total amount of DNA per well but by varying the amount of Lipofectin per μg of DNA. The peptide 6/DNA weight ratio was retained constant and complexes were formed for 1 h.

per μg of DNA the observed RLU/mg values were 1.3-fold higher than those seen with 0.75 μg of Lipofectin.

Since a common optimal LID vector formulation was required for all cell lines, 1 μg of DNA per $2.5x10^4$ cells and a weight ratio of 0.75 μg Lipofectin per μg of DNA were chosen as the most efficient transfection ratio.

3.2.2.3 Integrin-targeting peptide

Different integrin-targeting peptides (see Table 2.1 in Materials and Methods) were used to transfect neuroblastoma cells. All peptides contain a cyclic integrin-targeting domain, which is formed by a disulfide bond between two cysteine residues flanking the integrin-binding amino acid sequence. Binding of LID complexes to cells is dependent on peptide cyclisation (Hart *et al.*, 1997). Peptide 6 contains the targeting sequence RRETAWA, which is specific for α5β1 integrin and was isolated from a phage display library (Koivunen *et al.*, 1994). Neuroblastoma cells were transfected using pCI-luc plasmid and peptides at a charge ratio of 1:6.8 (see Table 2.1). Peptides 9 and 12 have the same targeting domain as peptide 6. However, peptide 9 has two domains attached to a sixteen- lysine chain and peptide 12 contains extra amino acids between the targeting domain and the lysine chain. This is speculated to increase the flexibility of the molecule and prevent steric hindrance of the targeting domain. Transfection of IMR-32 and Neuro-2A cells with peptides 9 and 12 resulted in similar transfection efficiency to peptide 6 (**Fig 3.4**). Transfection of human cell lines with peptide 8, which targets the α_4 integrin chain, resulted in equally high luciferase expression as with peptide 6 (see chapter 4 for integrin expression on these cells). In conclusion, none of the peptides tested produced higher transfection levels than that achieved with peptide 6 and hence the latter was incorporated in the optimal transfection protocol.

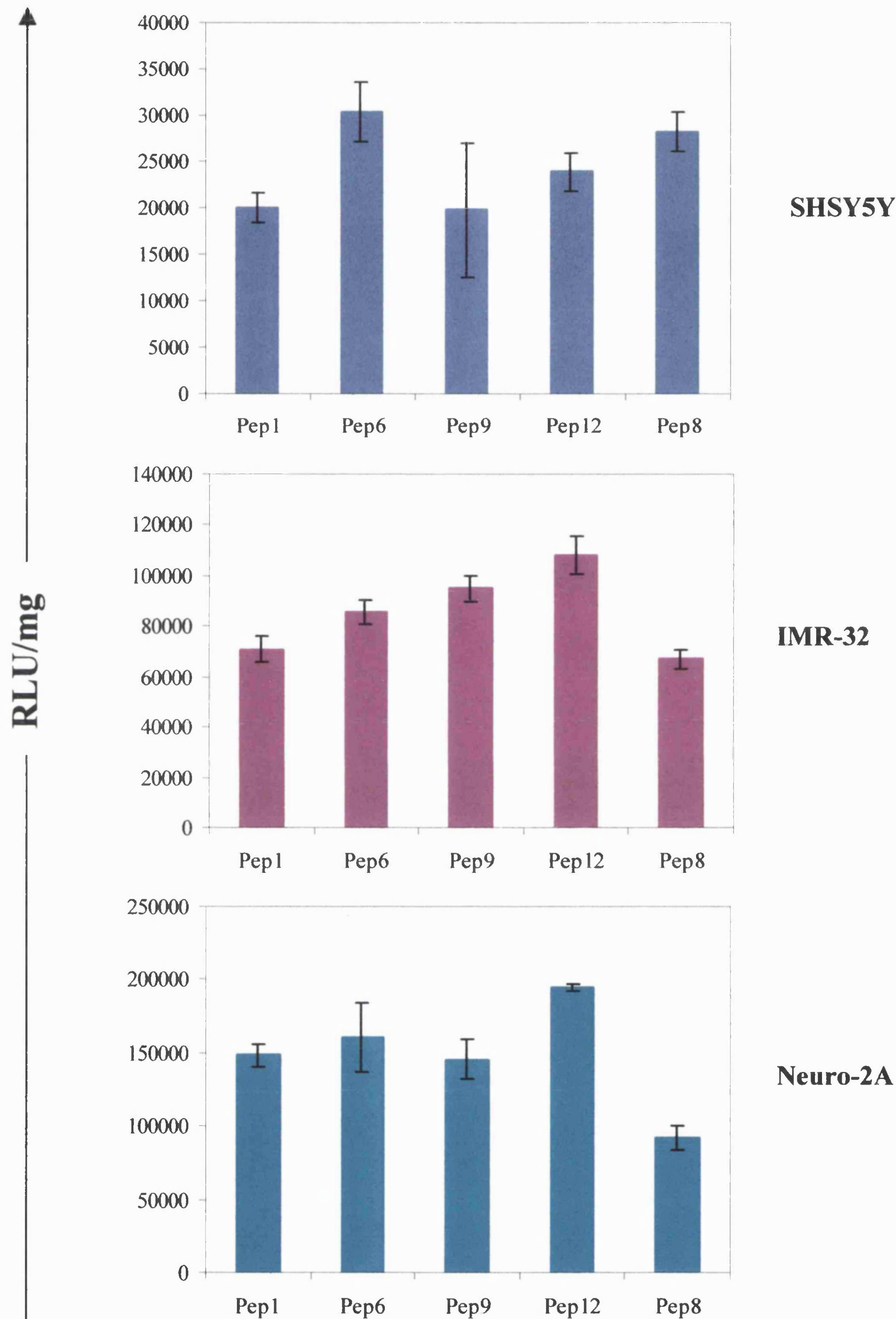
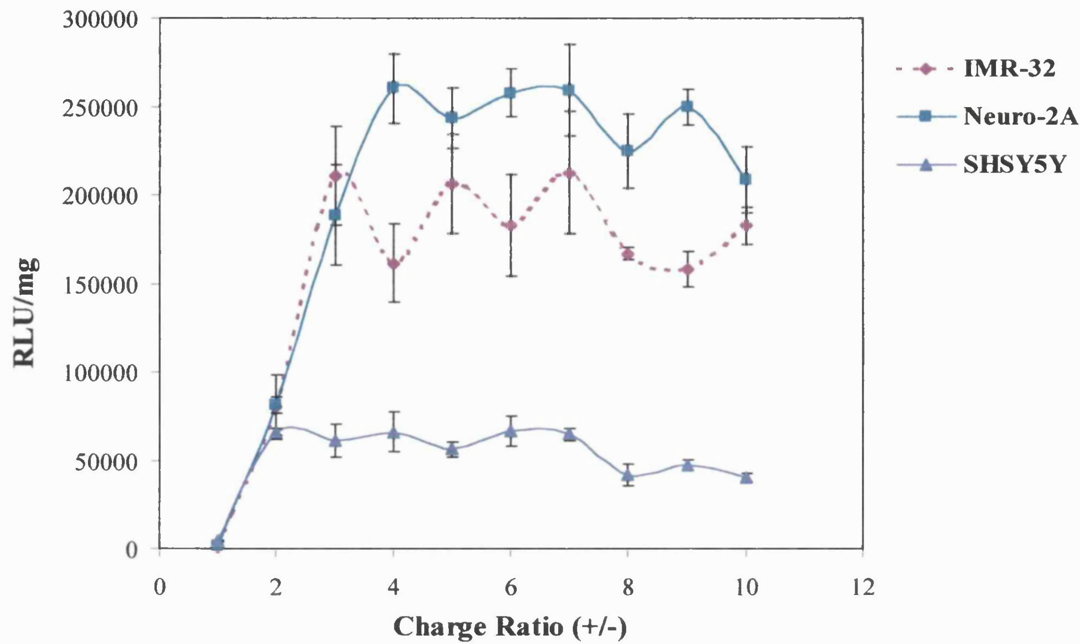


Figure 3.4 Optimisation of the integrin-targeting peptide component of the LID vector. Different integrin-peptides were used to formulate LID complexes and transfect neuroblastoma cell lines with pCI-luc plasmid. Error bars represent the standard deviations from triplicate transfections.

Titration of the integrin-targeting component of the LID vector was performed by varying the amount of peptide 6, whilst maintaining the total amount of DNA and the DNA/Lipofectin weight ratio. In most lipopolyplex systems, the ionic interaction among the components of the system determines the surface charge density of the complexes and their ability to bind to the negatively charged cell membrane. At peptide/DNA ratios close to neutrality the transfection efficiency was below 4,500 RLU/mg in all cell lines (**Fig 3.5 A**). However, reporter gene expression increased 100% at a ratio of 2:1 (Peptide 6/DNA) in all cell lines as revealed by the RLU/mg values. At a peptide/DNA ratio of 4 there was a further 70% increase in the luciferase expression in Neuro-2A cells while complexes formulated at a charge ratio 2:1 raised reporter gene expression in IMR-32 cells by 100%. At higher ratios, transfection efficiency of all neuroblastoma cell lines was constant. The most reproducibly efficient charge ratio of peptide/DNA in all three cell lines was 6.8:1.

Gel retardation assays showed that addition of an integrin-targeting peptide at a 1:1 charge ratio to DNA is sufficient to condense DNA and retard it on an agarose gel (Hart *et al.*, 1995). Further addition of peptide is thought to increase the surface charge density of the complexes. Zeta potential (z) measurements provide an indication of the surface charge of the polycationic complexes. Cationic particles have been reported to be efficient gene delivery vehicles due to their association with the negatively charged cell-surface proteoglycans and subsequent endocytosis (Mounkes *et al.*, 1998). The transfection efficiency of DOPE-cholesterol cationic derivatives correlated with their zeta potential (Takeuchi *et al.*, 1996). The zeta potential of LID particles formulated in PBS at various charge ratios was measured using a zeta sizer (Becton and Dickinson). LID particles at low charge ratios (0.5:1 to 2:1 peptide/DNA) had a negative (-40 mV to –25 mV) zeta potential (**Fig 3.5 B**). At a charge ratio of 2.5:1 there was a shift of the zeta potential value to +4 mV. A further increase to +10 mV was observed with particles at 3:1 or higher charge ratios. Therefore, the increased reporter gene expression achieved with LID complexes formulated at a 2:1 charge ratio of peptide 6/DNA correlates with an increase in the zeta potential of the complexes from –40 mV to +4 mV. A similar shift in zeta potential from negative to positive values with increase in charge ratio was observed with other cationic polymers such as PLL and

A



B

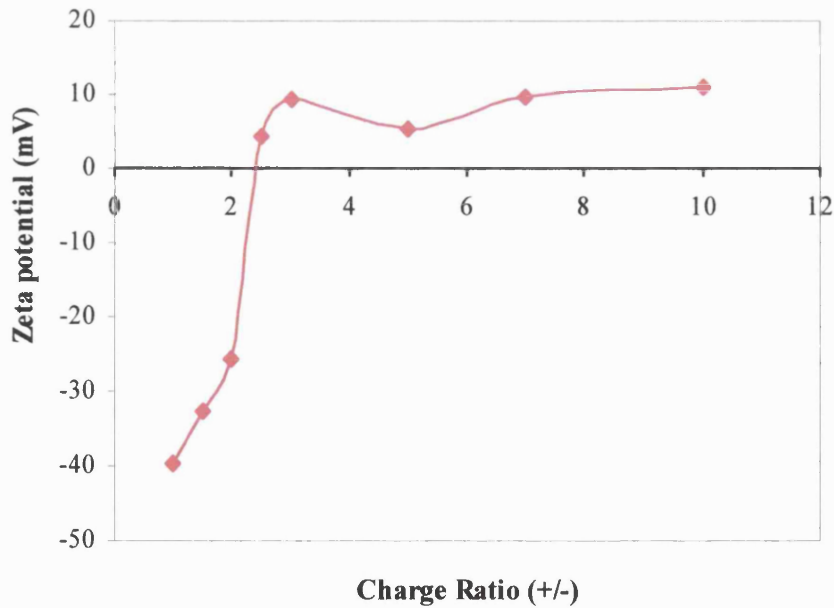


Figure 3.5 Influence of the peptide/DNA charge ratio on the transfection efficiency of LID complexes. A. LID complexes were constructed by increasing the amount of the integrin-targeting peptide (Pep 6) per μg of DNA whilst retaining the total amount of DNA constant. Analysis for luciferase expression was performed 48 h after transfection and one representative experiment is shown. Error bars represent standard deviations from triplicate transfections. **B.** Surface charge density of LID complexes at various charge ratios formulated in PBS.

PEI (Tang and Szoka, 1997). This would suggest that an increase in the charge ratio of peptide/DNA enables a greater number of LID complexes to gain entry into the cell by a charge interaction with the cell membrane.

3.2.2.4 Alternative Lipids

Alternative lipids were evaluated to investigate potential enhancement in transfection efficiency. An amphipathic polyamine (Tfx Reagent 1, Mirus) was used as a substitute for Lipofectin. Its effect on neuroblastoma cell lines was dose-dependent and interestingly, transfection with LID complexes incorporating Tfx resulted in a 2-fold enhancement of luciferase gene expression in IMR-32 cells, compared to Lipofectin, and a 3-fold enhancement of expression in Neuro-2A cells (**Fig 3.6**). The most striking effect was observed with SHSY5Y cell line where Tfx Reagent resulted in a 5-fold increase in luciferase expression. This improvement was only observed in combination with peptide 6 and was abolished when the lipid was used alone with DNA. However, Tfx Reagent 1 proved to be toxic to the cells. There was an increase in the number of dead floating cells as observed by light microscopy and also the protein concentration of the samples was reduced by 2-3-fold. This was observed only when the lipid was complexed with an integrin-targeting peptide. Therefore, Tfx Reagent was considered unsuitable for an application where cell viability is crucial and Lipofectin was chosen as the most suitable lipid component for the LID vector.

3.2.3 Maturation of LID complexes

The following experiments investigate the effect that LID complex maturation has on the transfection efficiency and reporter gene activity of neuroblastoma cells. LID complexes consisting of peptide 6 and pCI-luc or pEGFP-N1 plasmids were incubated at RT for various periods of time and subsequently used to transfect neuroblastoma

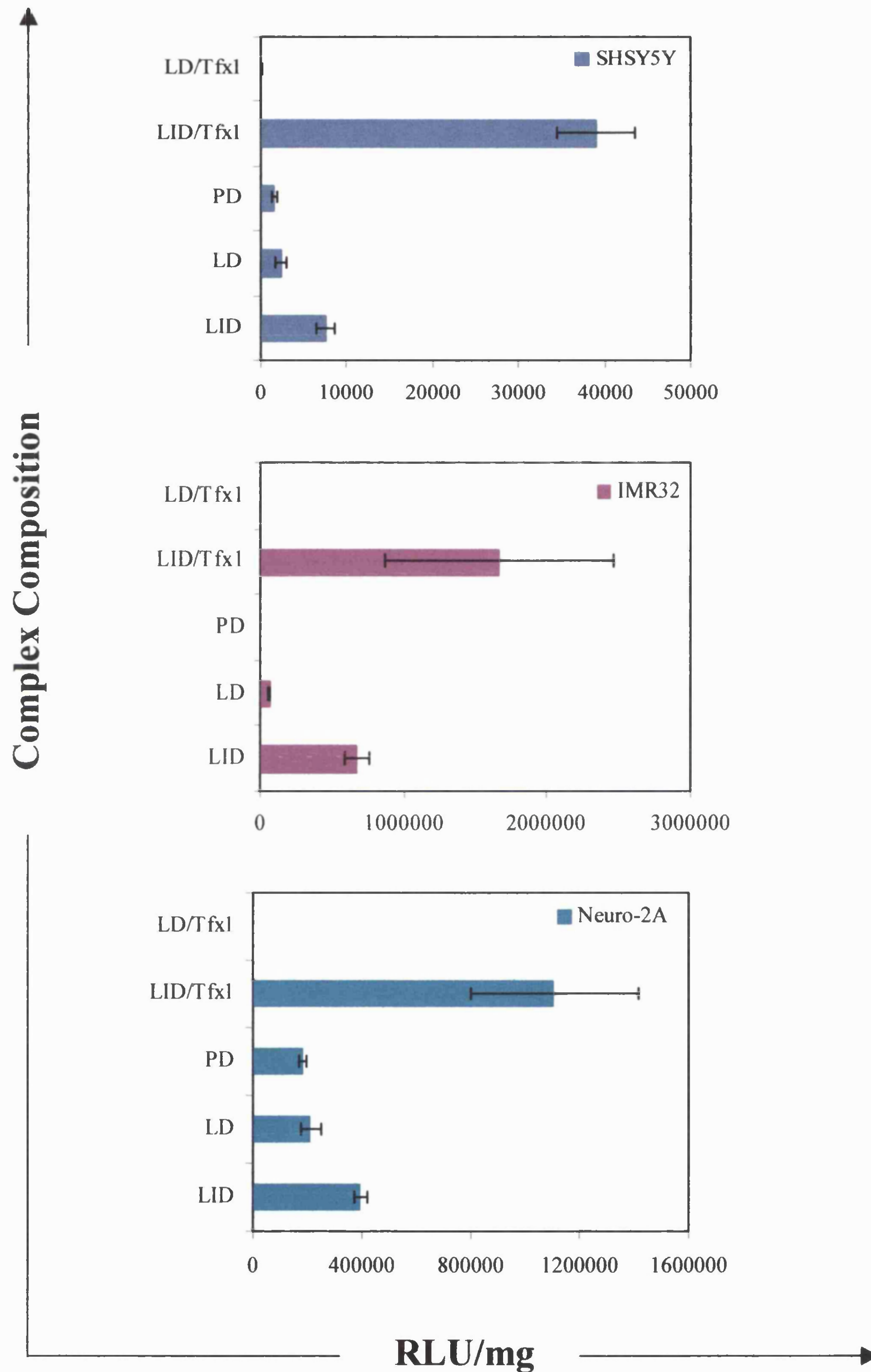


Figure 3.6 Transfection of neuroblastoma cell lines using Tfx Reagent 1. LID, PD and LD complexes were formulated as described previously while Tfx1 Reagent was used at a weight ratio of 0.5:1 to DNA in LID/Tfx1 complexes. Peptide 6 was used as the integrin-targeting component and the complexes were pre-incubated for 1.5 h prior to a 4 h-transfection. Luciferase expression is presented as RLU/mg and error bars are plotted from triplicate transfections.

cell lines for 4 h. In general, prolonged incubation of complexes correlated with an increase in transfection efficiency in all cells tested (**Fig 3.7**). In SHSY5Y cells, maximum transfection efficiency was achieved with complexes pre-incubated for 30 min. Further incubation of complexes did not induce any significant enhancement in transfection. However, the overall RLU/mg values were reduced when complexes pre-incubated for 180 min were used. Flow cytometric analysis of pEGFP-N1-transfected SHSY5Y cells revealed that most of the transfected cells were expressing the green fluorescent protein weakly ($>10^3$ on the fluorescence log scale as revealed by FACS). Transfection with complexes formulated with the luciferase construct (pCI-luc) resulted in greater RLU/mg values when complexes had been allowed to mature for 30 min. Luciferase expression remained stable when the cells were transfected with particles pre-incubated for longer.

IMR-32 cells were most efficiently transfected (64%) with LID complexes pre-incubated for 90 min (**Fig 3.7**). Again there was a direct correlation between transfection efficiency and maturation of complexes up to 90 min. At that time point, as shown by flow-cytometric analysis, IMR-32 cells exhibited highest expression of EGFP compared to transfection with complexes matured for 0-60 min. Transfection efficiency dropped to 35% when complexes were allowed to stand for 180 min prior to transfection. Reporter gene activity, as measured by luciferase expression peaked when the cells were transfected with complexes pre-incubated for 60 min and reached a plateau thereafter.

A similar pattern to that of IMR32 cells was observed in the Neuro-2A cell line. Both luciferase reporter gene expression and the percentage of transfected cells increased with pre-incubation of LID complexes (**Fig 3.7**). The highest RLU/mg values were obtained at 90 min, while maximum transfection efficiency (64%) was achieved with complexes matured for 60 min. The efficiency of the LID vector was not significantly lower at 30 min or 90 min but since luciferase expression peaked at 90 min that time point was chosen as the optimal incubation period. For this particular application, a combination of high transfection efficiency and transgene expression were desirable

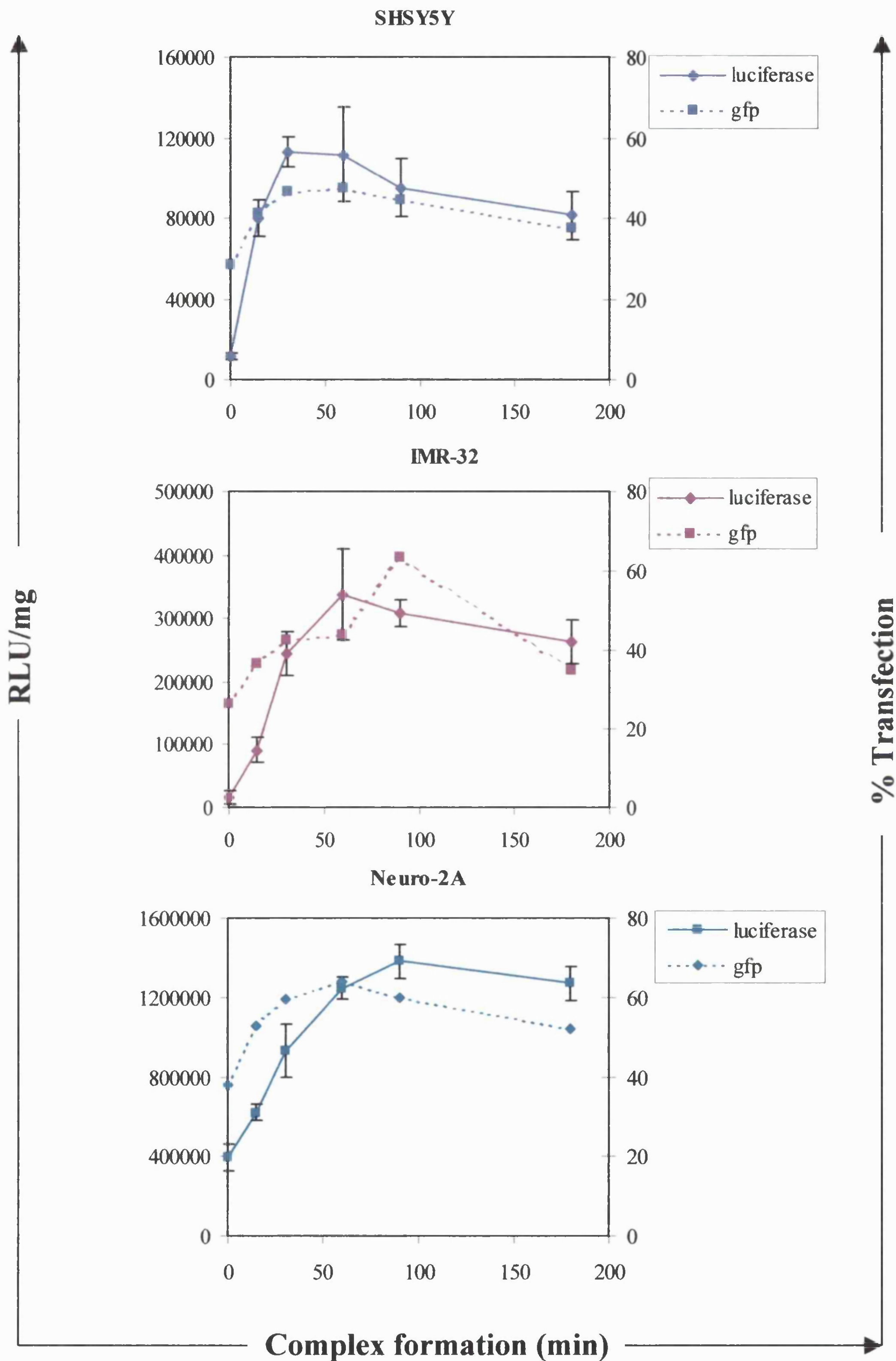


Figure 3.7 Correlation between luciferase gene activity and efficiency of transfection as a function of duration of complex formation. Neuroblastoma cells were transfected with LID complexes consisting of either pCI-luc or pEGFP-N1 plasmids, after incubating the complexes for different periods of time. Analysis by luciferase assay or flow cytometry was conducted 48 h after transfection.

so 90 min was selected as the optimal time-point that offered the highest RLU/mg values and percentage of transfected cells. Transfection of neuroblastoma cell lines with the LID vector proved to be very efficient resulting in a median efficiency of 22% (n=4) for SHSY5Y, 59% for IMR-32 (n=7) and 60% for Neuro-2A cell line (n=7) (**Fig 3.8; 3.9**). There was variation in the number of transfected cells among experiments but this is an expected outcome considering the nature of the transfection process. Transfection efficiency depends on a number of variables such as the condition and passage number of the cells, the DNA and peptide preparations as well as the transfection or culturing medium. All these factors may vary from experiment to experiment resulting in the observed variability in transfection efficiency.

3.3 DISCUSSION

Optimisation of the LID vector transfection protocol for neuroblastoma cells consisted of determining the most efficient combination of lipid and integrin-targeting peptide, as well as their respective weight ratio to DNA. A number of neuroblastoma cell lines were used in order to determine the potential transfection efficiency of the LID vector. SHSY5Y and IMR32 cells are clones of patient neuroblastoma cells while the Neuro-2A cell line originates from C1300 cells, a clone derived from a spontaneous neuroblastoma in an albino strain A mouse. Although these cells are expected to have distinct patterns of cellular division and integrin expression, critical factors for integrin-targeted non-viral gene delivery, further optimisation of the LID transfection protocol would be required for transfection of human primary cells. *Ex vivo* culturing of primary neuroblastoma cells is difficult due to very low mitotic division and optimisation of all the previously described parameters would be required in order to obtain maximum levels of transfection.

In this study it is important that the cells used in the vaccine exhibit maximum expression of cytokines rather than highest levels of transfection efficiency. In that respect the LID formulation offered superior transgene expression than LD complexes in all cell lines tested.

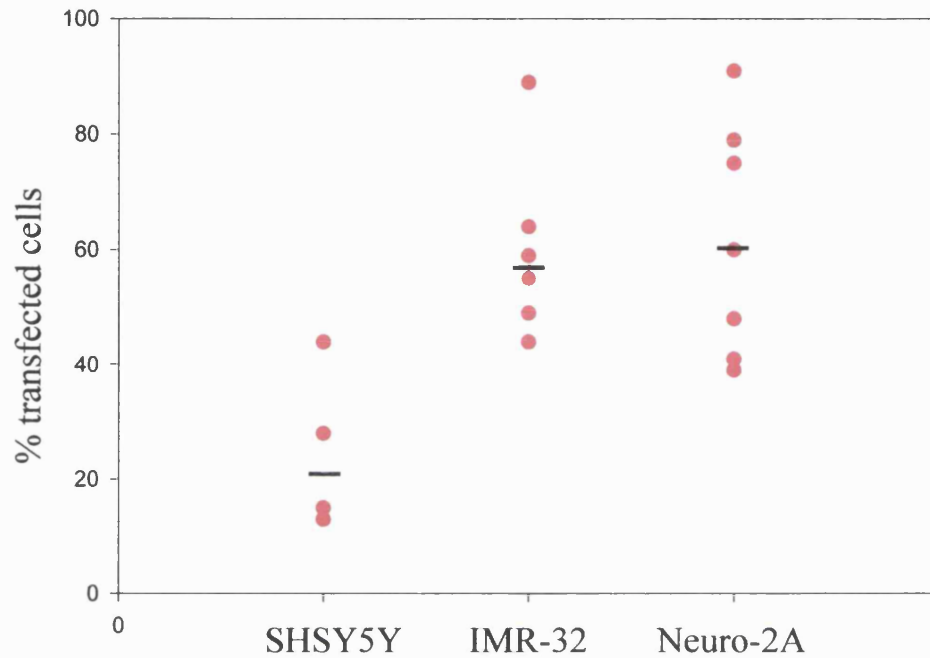
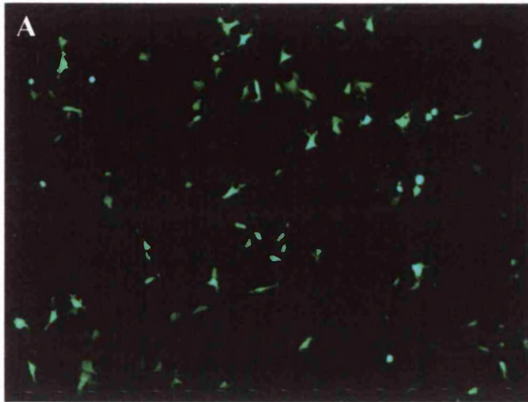
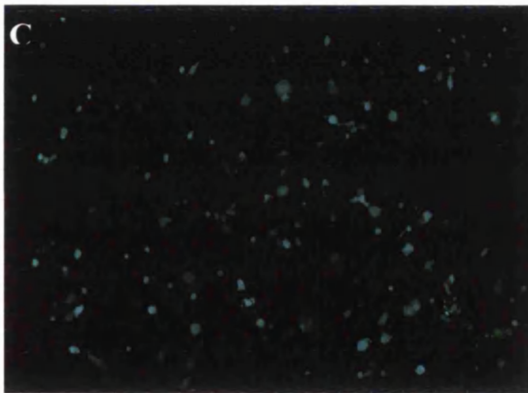


Figure 3.8 Transfection efficiency of neuroblastoma cells using the LID vector. Cells were transfected using peptide 6 and pEGFP-N1 plasmid and analysed by flow cytometry for GFP-positive cells 48 h later. The results of a number of experiments are depicted [SHSY5Y (n=4), IMR-32 and Neuro-2A (n=7)] and the median % of transfected cells is represented by a horizontal line.

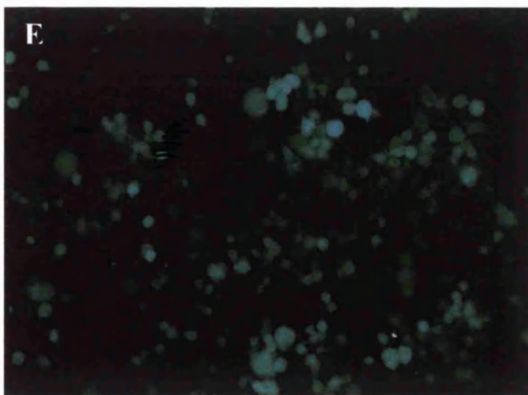
SHSY5Y



IMR-32



Neuro-2A



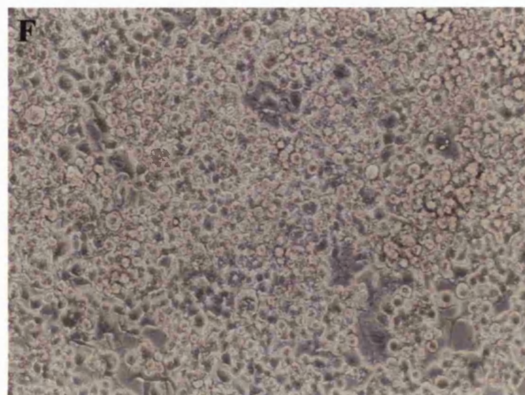


Figure 3.9 Photographs of LID-transfected neuroblastoma cell lines. LID complexes consisted of peptide 6, Lipofectin and pEGFP-N1 plasmid. Transfection efficiency was estimated by flow cytometric analysis to be 25% (A), 55% (C) and 40% (E). B,D and F are phase-contrast photos of the transfected cells.

Enhancement of transfection activity by LID complexes over PD complexes is not believed to be a charge-mediated effect. Lipofectin contributes minimally to the overall charge of the complexes compared to the integrin-targeting peptide (Hart *et al.*, 1998). It is, therefore, speculated that Lipofectin has an effect at releasing the internalised complexes from the endosomal compartment.

The neutral lipid DOPE has been shown to destabilise the membrane of endosomes and its presence in the same endosome is required for efficient release of the complexes in the cytoplasm (Zhou and Huang, 1994); (Farhood *et al.*, 1995). Furthermore, cationic liposomes are believed to interact with and neutralise the anionic lipids of the endosomal membrane resulting in release of the DNA into the cytoplasm. This is thought to occur after the DNA complexes induce destabilisation of the endosomal membrane and exposure of the anionic lipids to the inner part of the vesicle (Xu and Szoka-FC, 1996). Therefore, the cationic lipid DOTMA, which is present in Lipofectin, could be aiding in the release of LID complexes in the cytoplasm by binding to anionic lipids of the endosomal membrane and displacing the DNA.

LID complexes give rise to significantly greater transgene expression than LD complexes suggesting that the integrin-targeting peptide facilitates cellular binding and entry. This would result in more complexes gaining entry into the cell thus achieving high levels of reporter gene expression. Alternatively, after release of the complexes from the endosomal compartment, the peptide may remain associated with the DNA molecule and facilitate its transport to the nuclear membrane (Hart *et al.*, 1998). This would allow more DNA molecules to reach the transcriptional machinery and result in greater reporter gene expression. In conclusion, it is speculated that the integrin-targeting peptide helps to condense the plasmid DNA through its sixteen-lysine tail and also aids binding of the complexes to the cell. The Lipofectin component is probably responsible for efficient release of the complexes from the endosomal compartment.

An $\alpha_5\beta_1$ integrin-specific peptide was found to work most efficiently in all the cells tested. Increasing the avidity of the LID vector for this integrin or enhancing the spacer between the integrin-targeting domain and the sixteen-lysine chain did not improve transfection efficiency.

Formation of LID complexes commences immediately after mixing the DNA, Lipid and peptide components (Hart *et al.*, 1998). During formation of lipoplexes, displacement of ethidium bromide from DNA by addition the cationic lipid DOTAP is completed within 100 s indicating rapid association and complete encapsidation of the DNA by lipid (Xu and Szoka-FC, 1996). However, further incubation of lipoplexes may result in aggregation and formation of polydisperse particles (Ross and Hui, 1999). The precise effect of complex size on transfection efficiency is still under investigation. Factors that influence particle size include liposome/DNA charge ratio, the liposome charge and molecular weight, as well as the buffer composition. The presence of serum in the medium has also been reported to play an important role in transfection with small size complexes. This effect is absent when using lipoplexes of large size (750nm) (Turek *et al.*, 2000). Ross and Hui, (1999) have attributed this property of serum to a possible size growth arrest of the particles, which is abolished by using large multilamellar vesicles.

LID complex maturation resulted in improved transfection efficiency in the neuroblastoma cell lines. Analysis of LID particles in water by atomic force microscopy (AFM) suggested a spherical structure of 44 nm in diameter (Hart *et al.*, 1998). Preliminary photon correlation spectroscopy (PCS) data indicate that increased incubation of LID complexes formed in serum-free media (Opti-MEM) leads to aggregation and formation of large particles with a diameter greater than 1000nm (Li Kim Lee, personal communication). This phenomenon has been observed elsewhere and is due to the presence of salts in the medium (Ogris *et al.*, 1998). Although the optimal lipoplex size for efficient transfection is still debatable, the endocytic process sets a cutoff point for uptake of molecules due to size limitations of endosomes. This may vary on the cell type and its endocytic activity, and can extend up to 2μm for CHO cells (Ross and Hui, 1999). Neuroblastoma cell lines exhibit enhanced

transfection efficiency with particles pre-incubated for 90 min. Ogris *et al.*, (1998) report that transfection efficiency of Neuro-2A cells was greater with transferrin-PEI aggregates formed in HBS than with small particles in salt-free conditions. These data are therefore, in agreement with observations that large particles result in enhanced transfection efficiency and greater reporter gene activity. This could be attributed to sedimentation of large aggregates onto the cell surface during transfection that makes complexes more accessible for internalisation (Ogris *et al.*, 1998).

Optimisation of transfection parameters in the presence of serum was not undertaken since the vaccination protocol requires *ex vivo* transfection of neuroblastoma cells. Destabilisation of LID complexes by serum proteins would be studied if the vector were to be used intravenously or directly at the tumour site. In addition, very efficient transfection was achieved with large LID particles. Therefore, methods to prevent particle growth, such as addition of serum, were not explored. Optimisation of parameters affecting LID complex transfection and particle size including charge ratio, particle size, lipid component and the specificity of the integrin-targeting domain has been undertaken. As a result, LID transfection of Neuro-2A cells resulted in much higher efficiency (60%) than that reported with other non-viral systems (12-28%) (Ogris *et al.*, 1998).

4

MECHANISM OF THE LID VECTOR

4.1 INTRODUCTION

The main objective of these experiments was to study the integrin specificity of the LID vector and delineate its uptake mechanism in neuroblastoma cells. The importance of the integrin-targeting domain in the peptide component of the LID vector has been demonstrated in umbilical vein endothelial ECV304 cells by transfecting the cells with a combination of peptide 6 and K_{16} (Hart *et al.*, 1998). The transfection efficiency in these cells increases with increasing amounts of peptide 6. In addition, competition with integrin-blocking antibodies inhibits LID transfection by 35% indicating an integrin affinity of the vector. This partial blocking with integrin antibodies also stresses the importance of the charge property of the vector. However, since different cell types possess distinct integrin expression patterns, it was necessary to investigate the mechanism of internalisation of the LID vector in neuroblastoma cells.

Most studies on cellular uptake of polyplexes by receptor-mediated endocytosis have utilised free ligands such as transferrin (Zenke *et al.*, 1990). Cell-surface receptors were blocked with free ligand and the subsequent effect on transfection efficiency was examined (Carpenter and Minchin, 1998). Although peptide motifs such as RGDMFG (O'Neil *et al.*, 1992) and RRETAWA (Koivunen *et al.*, 1994) isolated from phage display libraries are known to bind integrin receptors, coupling to a lysine tail may alter their affinity for the natural receptors. Previous investigations on binding of complexes containing these peptides to integrin receptors performed competitive cell-binding studies on fibronectin or vitronectin coated wells (Harbottle *et al.*, 1998). Alternatively, addition of soluble RGD or RGE peptides was performed to investigate the effect of blocking the cellular integrin receptors on transfection efficiency with such peptides (Hart *et al.*, 1995).

The intracellular trafficking of non-viral complexes once they have entered the cell has been studied using a number of pharmacological drugs in order to differentiate between endocytosis, phagocytosis or pinocytosis. Wortmannin and LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] are two widely used inhibitors of

phosphatidylinositol 3-kinase (PI3K). Wortmannin acts irreversibly on the catalytic subunit p110 of PI3K while LY294002 acts on the ATP-binding site of the enzyme. They have been used to delineate the function of PI3K and its precise involvement in intracellular membrane trafficking. PI3K plays a role in the endosome-endosome and endosome-lysosome fusion (Li *et al.*, 1995). Its function in fluid-phase pinocytosis and phagocytosis is well established (Araki *et al.*, 1996). Wortmannin blocks Fc receptor-mediated phagocytosis in macrophages by preventing the formation of macropinosomes and phagosomes. PI3K has an alternative role in integrin downstream signalling. Binding of adenovirus to α_v integrin induces activation of PI3K and disruption of the enzyme using wortmannin completely abrogates virus-mediated gene transfer (Li *et al.*, 1998).

Endosomal degradation is one of the major barriers to efficient transfection by lipoplexes. Chloroquine is a basic molecule that neutralises the acidic pH of endosomes inhibiting the function of many lysosomal enzymes. It has been used to enhance endosomal release of non-viral complexes and, therefore, its effect on reporter gene expression should give an indication of endosomal involvement in the transfection process. Investigation of the uptake mechanism of many targeted non-viral systems has focused on blocking the surface receptors with free ligand. However, receptor-mediated endocytosis can also be studied by disrupting the formation of clathrin-coated pits using hypertonic media (Heuser and Anderson, 1989). Transfection in media containing 0.45 mM sucrose was used to study the importance of clathrin molecules in the endocytic pathway of a non-viral integrin-targeting vector (Colin *et al.*, 1998). Phagocytosis is a cellular mechanism responsible for the uptake of large (> 0.5μm in diameter) molecules that is largely dependent on the cytoskeleton. The drug cytochalasin-B disrupts formation of F-actin microfilaments thereby blocking phagocytosis and has been used to study the mechanism of internalisation of certain synthetic vectors.

4.2 RESULTS

4.2.1 Integrin expression

In order to determine whether the LID vector employs an integrin-mediated internalisation pathway, we first investigated the integrin expression on the surface of human neuroblastoma cell lines. Cells were stained with antibodies against CD49e (α_5), CD49d (α_4) or CD51/61 ($\alpha_v\beta_3$) molecules and analysed by flow cytometry on a FACS sorter. Expression of the integrin α_5 chain on SHSY5Y cells line was greater (48%) compared to the IMR-32 cell line which exhibited only a marginal staining with CD49e antibody (5.4%) (**Fig 4.1**). A similar expression pattern to that for α_5 was observed for $\alpha_v\beta_3$ integrin. Approximately 4% of IMR-32 cells were positive for CD51/61 antibody while SHSY5Y showed higher expression of α_v integrin (37%). Interestingly, α_4 integrin was equally expressed on both IMR-32 (86%) and SHSY5Y (78%) cell lines. Mouse IgG1 was used as the isotype control in all experiments.

4.2.2 Competitive inhibition of LID transfection

In the following experiments the binding of LID complexes to integrin receptors on neuroblastoma cells was investigated by blocking studies using either integrin-binding antibodies or soluble integrin-binding peptides.

4.2.2.1 Integrin-binding antibodies

The aim of these experiments was to block transfection by antibodies to specific integrins targeted by LID complexes. Since both IMR-32 and SHSY5Y were shown to express α_4 integrin, antibodies against the α_4 chain were used to block binding of peptide 8 LID complexes. 2 μg of either CD49d or mouse IgG1 antibody were applied per $5x10^4$ cells for 30 min at 4 °C. LID complexes composed of peptide 8, which

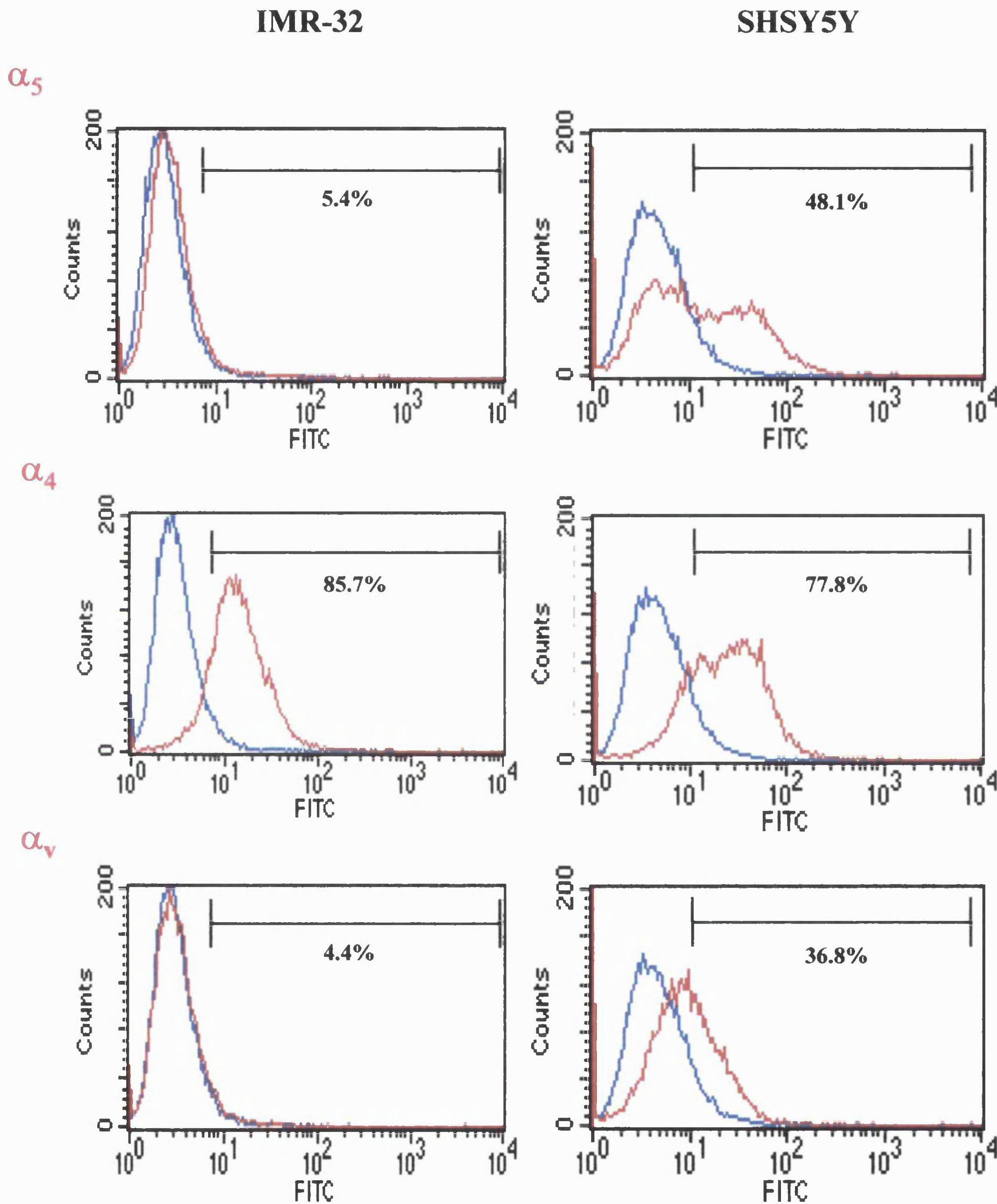


Figure 4.1 Integrin expression on human neuroblastoma cell lines. Cells were stained with mouse anti-human CD49e (α_5), CD49d (α_4)or CD51/61 (α_v) antibodies (red line) and a mouse IgG1 antibody was used as the isotype control (blue line). Flowcytometric analysis by FACS was performed after using a secondary goat anti-mouse FITC antibody. Representative data from 10,000 captured events are shown and gating on live cells was performed.

targets the α_4 integrin, or K_{16} that has no integrin-targeting domain were applied onto the cells and transfection incubations performed for 1hr at 4 °C in the presence of the antibodies. Cells were then returned to 37 °C, and luciferase gene expression was assessed 48hr later. As expected, there was no difference in transfection efficiency with K_{16} complexes between cells pre-treated with CD49d or control antibody (**Fig 4.2**). Transfection with K_{16} peptide is mainly charge-mediated so internalisation of K_{16} LID particles should not be affected by blocking of the integrin receptors. When peptide 8 LID complexes were used, however, pre-treatment with an anti-α_4 antibody did not inhibit transfection in either cell line. Surprisingly, in SHSY5Y cells, incubation with CD49d antibody significantly increased the reporter gene expression by 20% compared to transfection in the presence of an isotype control antibody ($p<0.04$).

In a similar way, pre-incubation of neuroblastoma cells with antibodies against α_5 integrin receptors did not block transfection with peptide 6 LID complexes (**Fig 4.3**). SHSY5Y cells express α_5 integrins while IMR-32 cell line does not. IMR-32 cells were, therefore, used as a control for competitive inhibition of α_5 integrin-mediated transfection by the LID vector. Pre-pulsing of the cells with integrin antibodies (anti-α_5, anti-α_4 or isotype control) was performed as described above. Subsequently, LID complexes consisting of peptide 6, which specifically targets the $\alpha_5\beta_1$ integrin receptor, or K_{16} were added to the cells and competition with the integrin antibodies was allowed for a further 1hr. Again cells were returned to 37 °C and analysed for reporter gene expression 48hr later. In the SHSY5Y cell line no inhibition could be observed when anti-α_5 antibody was used. In IMR-32 cells, as expected, there was no inhibition of LID transfection with peptide 6 complexes after treatment with integrin antibodies. The transfection levels with K_{16} complexes in these cells were the same regardless of antibody competition. These results indicate that internalisation of LID complexes cannot be blocked by antibodies against integrin receptors.

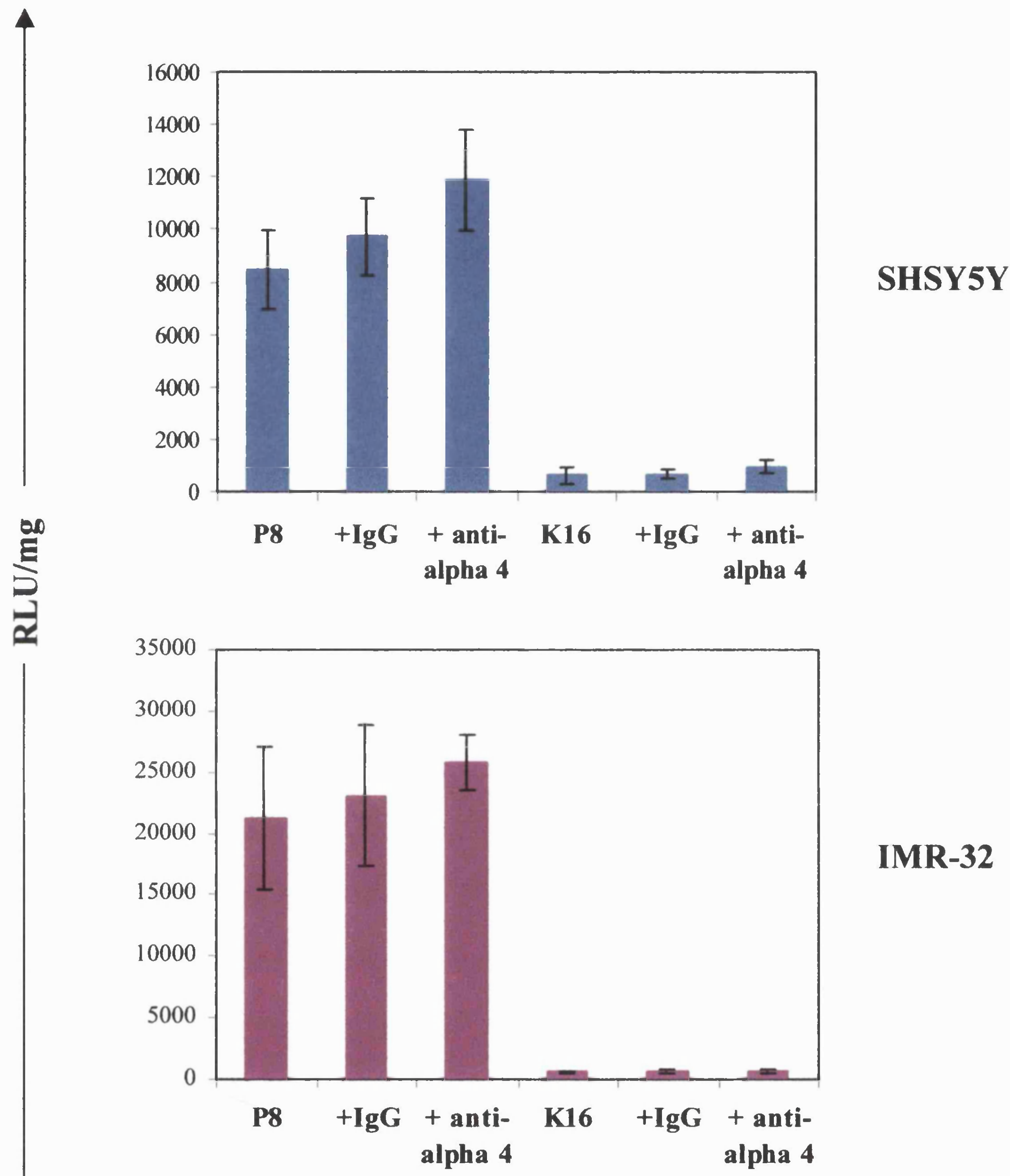


Figure 4.2 **Blocking of LID transfection with α_4 integrin antibodies.** Pre-incubation of neuroblastoma cells with anti-integrin antibodies fails to block LID transfection. Human neuroblastoma cell lines were incubated with anti-human CD49d or isotype control antibodies (1µg/2.5x10^4 cells) for 30 min at 4 °C. LID complexes consisting of peptide 8 or K_{16} formed at 1:3 charge ratio were added for a further 1 h at 4°C. The transfection complexes were then removed and the cells assayed for luciferase expression 48 h later. Error bars are standard deviations from six wells and one representative experiment is shown.

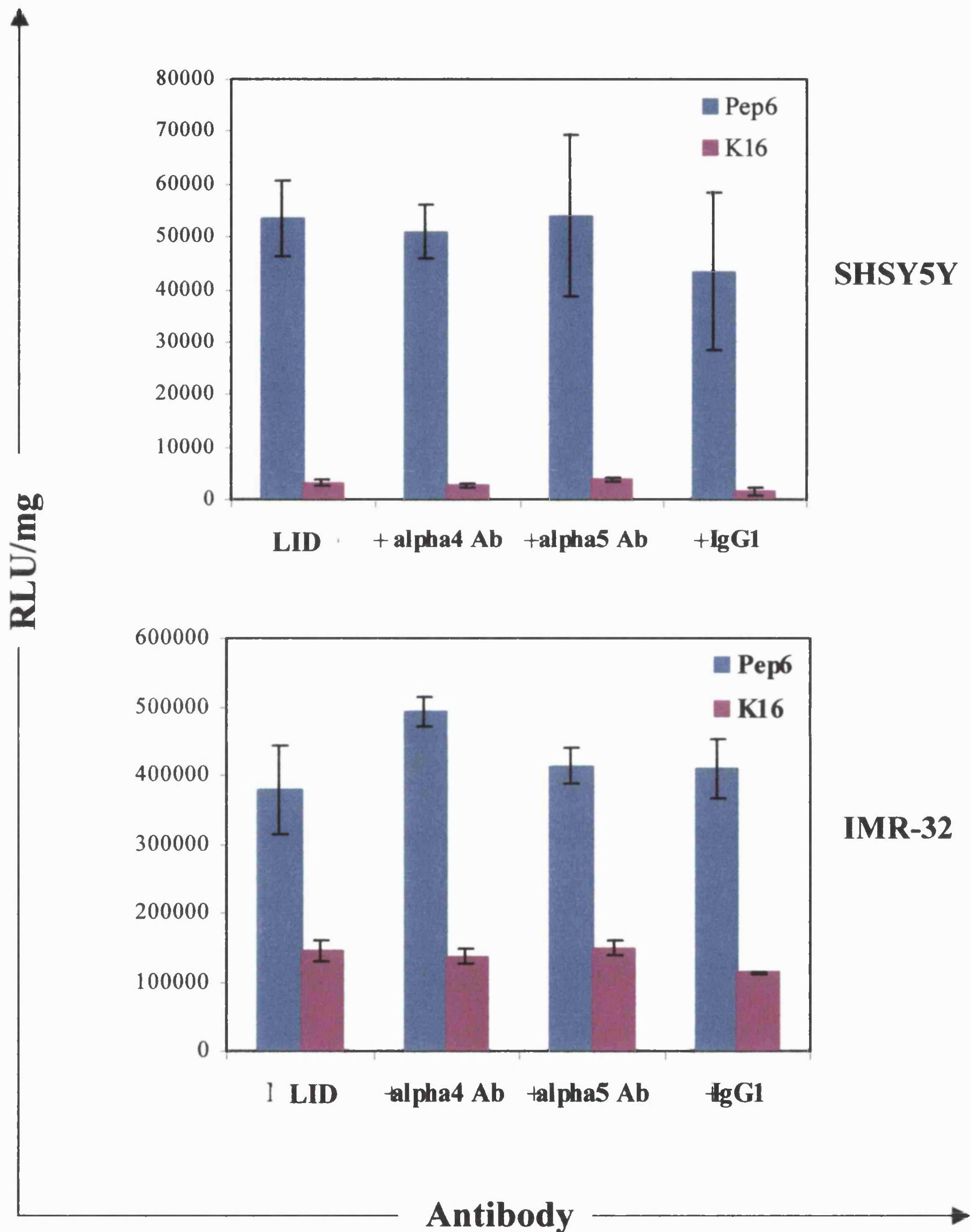


Figure 4.3 Blocking of LID transfection with α_5 integrin antibodies. Competitive inhibition of transfection using integrin antibodies. Human neuroblastoma cell lines were transfected using LID complexes consisting of peptide 6 or K_{16} in the presence of anti-alpha4, alpha 5 or isotype control antibodies. Error bars are standard deviations from triplicate transfections and one representative experiment is shown.

4.2.2.2 Soluble RGD/RGE peptides

Blocking of LID transfection using soluble GRGDSP or GRGESP peptides was investigated next. RGD is the minimal amino acid sequence required for fibronectin binding (Pierschbacher and Ruoslahti, 1984). It is a highly specific sequence as a single amino acid change of aspartic acid (RGD) to glutamic acid (RGE) abolishes binding affinity (Hautanen *et al.*, 1989). A peptide containing the RRETAWA sequence acts as a direct competitive inhibitor of RGD binding to $\alpha_5\beta_1$ integrin, suggesting that the binding sites of these two sequences overlap (Mould *et al.*, 1998). Therefore, the GRGDSP peptide should, in theory, compete with peptide 6 for binding to $\alpha_5\beta_1$ integrins on SHSY5Y cells.

SHSY5Y cells (2.5×10^4) were pre-incubated with 1mg of either peptide in OptiMEM for 30 min at 4 °C. Control transfections consisted of pre-incubation with OptiMEM. Subsequently, LID complexes consisting of peptide 6 and pCI-luc plasmid formulated at 3:1 charge ratio were added to the cells and transfection was performed for 1 h. Luciferase assays revealed that there was no difference in reporter gene expression among control-transfected cells and those that soluble peptides had been added to (**Fig 4.4A**). This indicates that LID transfection on SHSY5Y cells cannot be blocked by soluble RGD peptides.

4.2.2.3 Integrin-targeting peptides transfect neuroblastoma cells more efficiently than K_{16}

An alternative way of examining whether LID vector internalisation is mediated via integrin receptors was to directly compare the transfection efficiency of integrin-targeting peptides with their controls. Cells were transfected with LID complexes consisting of either peptide 6 ($\alpha 5\beta 1$), K_{16}, peptide 1 (RGD) or peptide 11 (RGE) (see Table 2.1 for peptide sequences). All LID complexes were formulated at 7:1 (peptide:DNA) charge ratio.

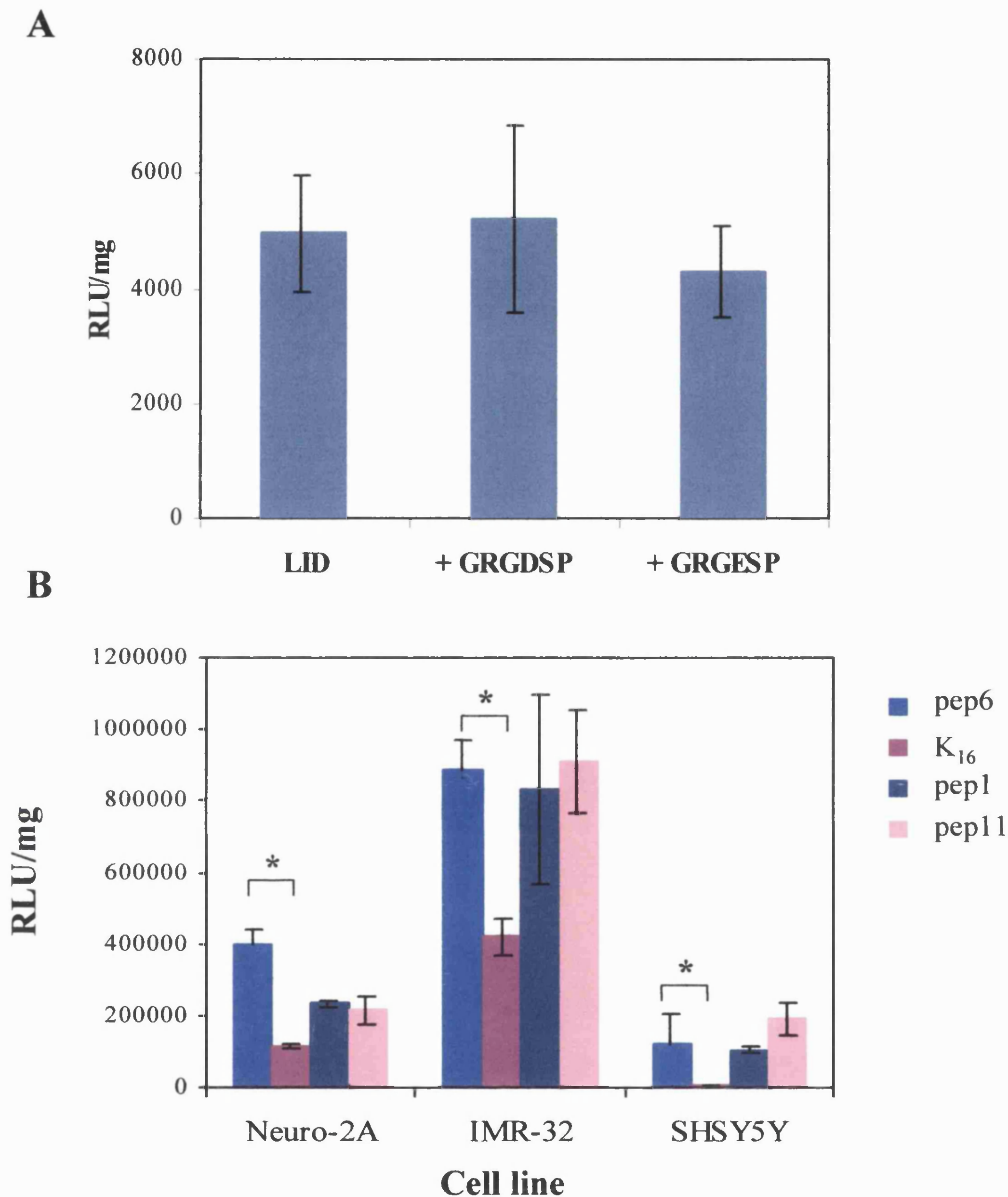


Figure 4.4 Competitive inhibition of LID transfection. A. SHSY5Y neuroblastoma cells were pre-incubated with 1mg (per 2.5×10^4 cells) of GRGDSP or GRGESP peptides in OptiMEM for 30 min at 4 °C. LID complexes consisting of peptide 6 formulated at 3:1 charge ratio were then added and allowed to compete with the peptides for a further 1 h. Luciferase reporter gene assays was performed 48 h post-transfection. **B.** Transfection of neuroblastoma cell lines with LID complexes consisting of either an integrin-specific targeting peptide or its control. Luciferase gene expression using peptide 6 was compared with that of K_{16}, which lacks an integrin-targeting domain. Peptide 1 has an RGD sequence motif and was also used alongside its control in which aspartic acid (D) has been substituted for glutamic acid (E) [RGE sequence] and integrin binding is abolished.

Transfection of all neuroblastoma cell lines with K_{16} LID complexes resulted in significantly lower reporter gene activity than LID complexes consisting of peptide 6 formulated at the same charge ratio ($p<0.05$) (**Fig 4.4B**). In the SHSY5Y cell line there was a 15-fold decrease in luciferase gene expression when cells were transfected with K_{16} compared to peptide 6. Luciferase expression was reduced 2-fold in IMR-32 cell line when the cells were transfected with K_{16} complexes compared to peptide 6. In Neuro-2A cells, the difference in transfection efficiency between LID complexes containing K_{16} and those consisting of peptide 6 was 3.5-fold. Transfection with the sixteen lysine reflects the extent to which transfection with the LID vector is charge-mediated and suggests that any difference in transfection efficiency between K_{16} and peptide 6 could be attributed to the extra integrin-targeting domain present in peptide 6. This is reinforced by the transfection efficiency of peptides 1 and 11, that contain an RGD and an RGE motif respectively, attached to a sixteen-lysine chain. Transfection with LID complexes containing either of these peptides significantly improves reporter gene expression compared to K_{16} LID complexes in all three cell lines. Although peptide 11 should not bind to any integrin receptors, the transfection efficiency with that peptide confirms that the extra amino acid sequence attached to the sixteen-lysine chain is responsible for that enhanced efficiency.

4.2.3 Inhibition of endocytosis

4.2.3.1 Phosphatidylinositol 3-kinase Inhibitors

In order to further delineate the internalisation mechanism of LID particles in neuroblastoma cells, a number of inhibitors that impair vesicle trafficking were used to study their effect on transgene expression. The PI3K inhibitors wortmannin and LY294002 were used at various concentrations to pre-treat the neuroblastoma cells for 30 min at 37 °C prior to transfection. LID complexes consisting of peptide 6, pCI-luc plasmid and Lipofectin at the optimised ratio were then applied and transfection was performed in the presence of the inhibitors. In the SHSY5Y cell line, neither wortmannin nor LY294002 inhibited LID transfection. Even at concentrations of

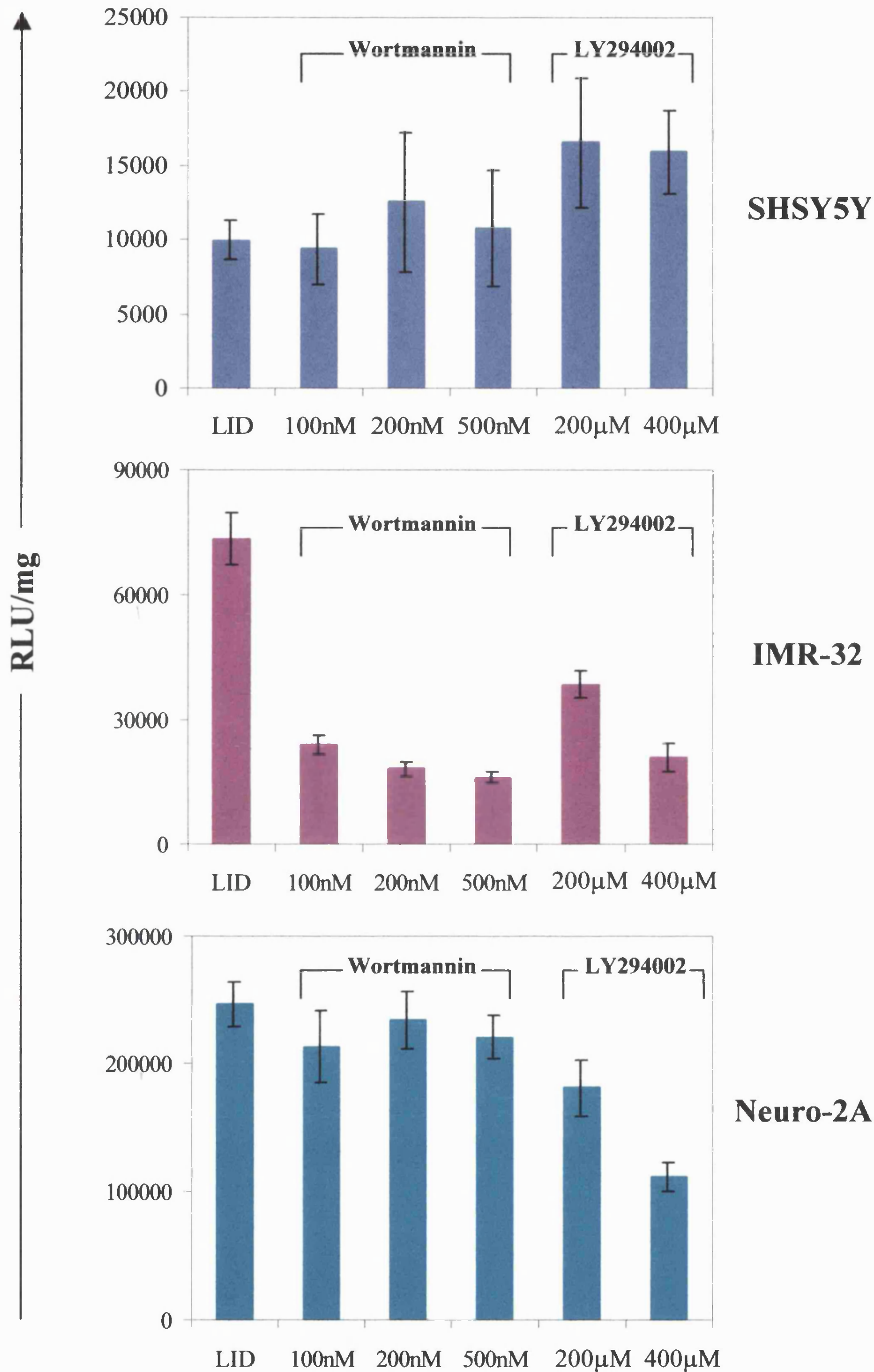


Figure 4.5 Involvement of endosomes in the intracellular trafficking of LID complexes. LID transfection is dependent on the endocytic function. Neuroblastoma cells were pretreated with the PI3K inhibitors wortmannin or LY294002 for 30 min at 37 °C. LID complexes consisting of peptide 6 and pCI-luc plasmid were applied onto the cells in the presence of the endocytic inhibitors for a further 4 h. Expression of luciferase reporter gene was examined 48 h post-transfection.

500 nM (wortmannin) and 400 μM (LY294002) the reporter gene expression levels – 10,743 RLU/mg for wortmannin and 15,887 RLU/mg for LY294002 - were similar to those achieved with the control LID transfection (9,964 RLU/mg) ($p>0.2$) (**Fig 4.5**).

However, transfection of IMR-32 cells in the presence of either wortmannin or LY294002 resulted in significant inhibition of LID transfection ($p<0.007$). There was a 70% and 50% reduction in the total RLU/mg values compared to control LID transfection when wortmannin or LY294002 (200 μM) were used, respectively. Saturation of the inhibitory effect of wortmannin was achieved above a concentration of 100 nM. In contrast, doubling the concentration of LY294002 (400μM) resulted in a further 20% reduction in reporter gene expression in comparison to control transfection. This suggests that the full inhibitory effect of LY294002 on IMR-32 occurred at a concentration of 400 μM.

In Neuro-2A cells, wortmannin at a concentration of 100 nM inhibited LID transfection by 15% ($p<0.05$). LY294002 exhibited a superior inhibitory effect on these cells than wortmannin. At 200 μM there was a 25% reduction in RLU/mg values whereas at 400 μM reporter gene expression was suppressed by 55% compared to LID transfection in the absence of any inhibitors ($p<0.004$). Above 400 μM LY294002 proved toxic to all cell lines tested as observed by cell death and reduced protein concentration.

These results show that PI3K is implicated in the transfection pathway of the LID vector in IMR-32 and Neuro-2A cells. In the SHSY5Y cell line, on the other hand, inhibition of PI3K did not affect LID transfection.

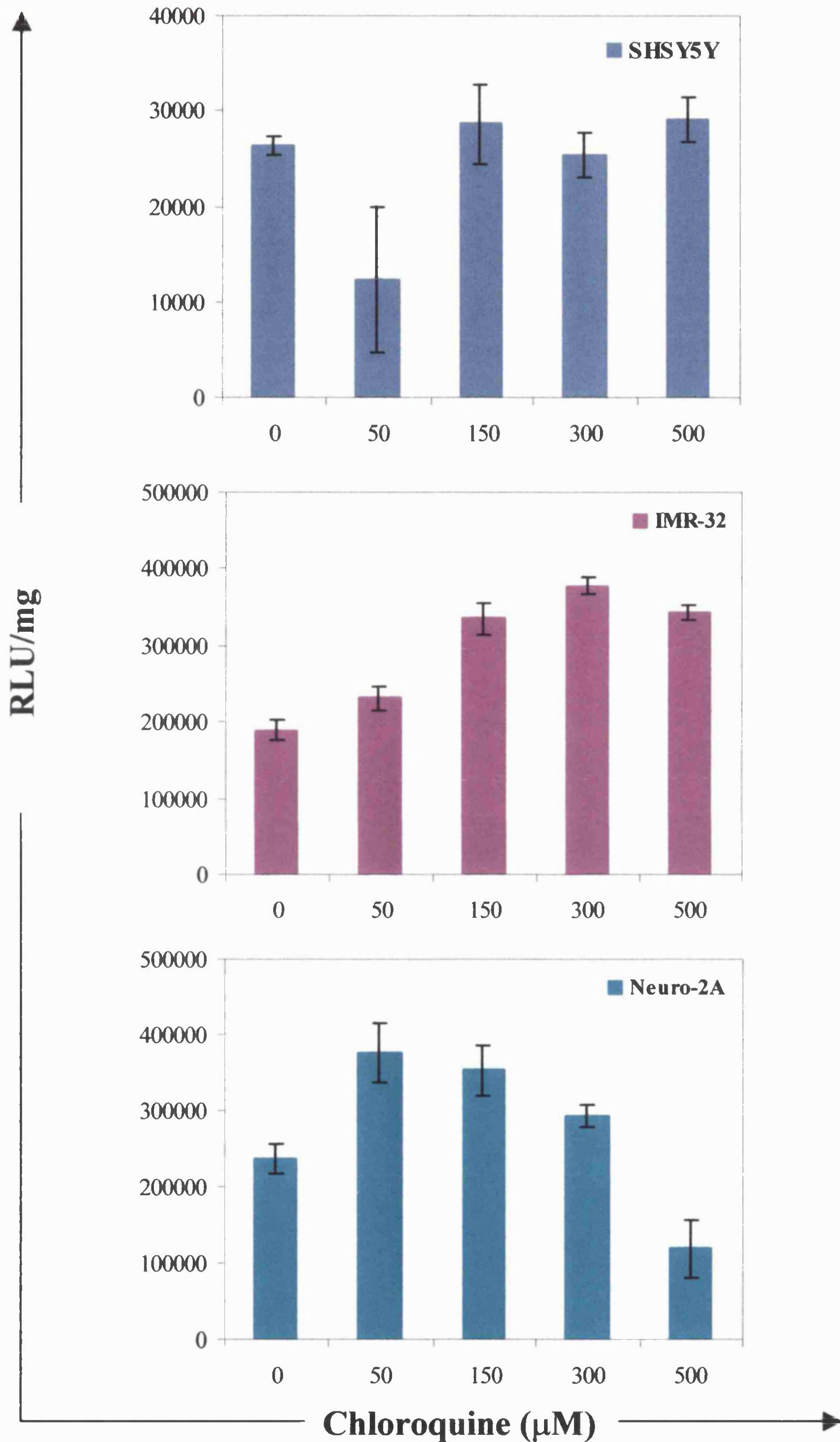


Figure 4.6 Effect of the endosomal pH on LID transfection. Neuroblastoma cell lines were transfected withLID complexes consisting of peptide 6 and pCI-luc plasmid in the presence of the indicated concentrations of chloroquine. Expression of luciferase reporter gene was examined 48 h post-transfection.

4.2.3.2 Chloroquine

The effect of chloroquine on transfection efficiency of the LID vector on neuroblastoma cells was investigated by adding the drug at the indicated concentrations and LID transfections were performed in the presence of chloroquine.

Luciferase expression in transfected SHSY5Y cells remained stable despite the addition of chloroquine (**Fig 4.6**). In IMR-32 cells, on the other hand, chloroquine resulted in a dose-dependent increase in RLU/mg values, which peaked at 300 μM. At that chloroquine concentration, the total RLU/mg values for that cell line were nearly double compared to those achieved by LID alone in the absence of chloroquine. Similarly, the luciferase expression in Neuro-2A cells was enhanced in the presence of chloroquine. At 50 μM, there was a 40% enhancement in the RLU/mg values compared to control, from 237,379 to 376,310. Surprisingly, at higher concentrations, transfection efficiency decreased again to control levels when 300 μM of chloroquine was used. This could be due to toxicity effects of the drug. At 500 μM, the observed 50% reduction in transfection efficiency was due to toxicity as seen by increased cell death.

The results from these experiments indicate that there is endosomal involvement during intracellular trafficking of the LID vector in IMR-32 and Neuro-2A cells. SHSY5Y cells again displayed a different response compared to the other two cell lines. Treatment with chloroquine had no effect on the transfection efficiency of SHSY5Y cells suggesting that gene expression is not affected by the endosomal pH.

4.2.3.3 Disruption of clathrin molecules

In order to test whether LID particles were internalised by receptor-mediated endocytosis in neuroblastoma cells, clathrin molecules were disrupted by treatment with hypertonic media as described previously (Colin *et al.*, 2000).

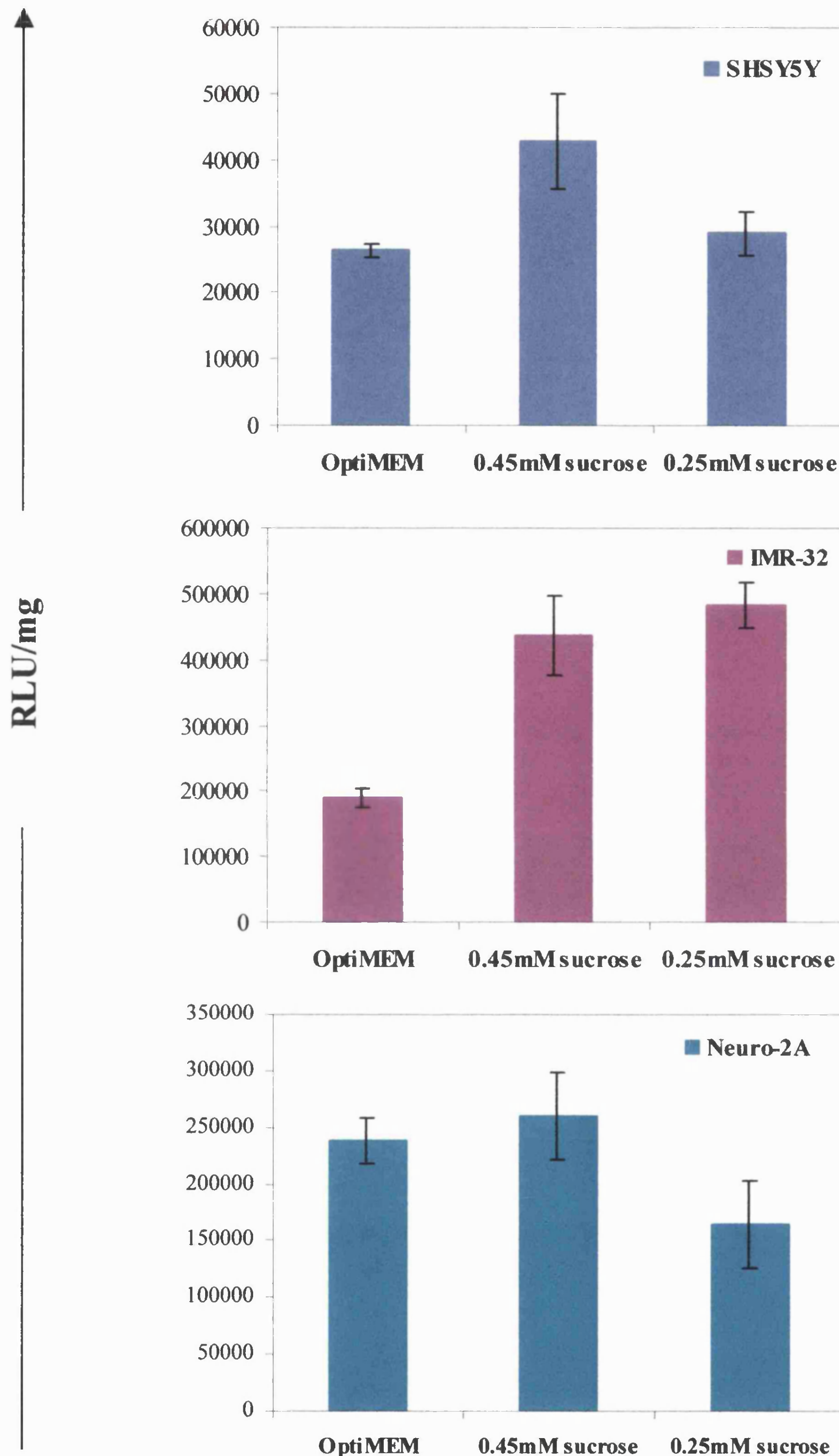


Figure 4.7 The importance of clathrin-coated pits in LID internalisation. Blocking of clathrin-coated pit formation does not inhibit LID transfection. Hypertonic (0.45mM sucrose) or isotonic (0.25mM sucrose) media were applied onto neuroblastoma cell lines for 45 min and subsequently transfections was performed with LID complexes formed in hypertonic, isotonic media or OptiMEM. Graphs represent mean RLU/mg values ± standard deviation of triplicate transfections.

Neuroblastoma cells were pretreated with OptiMEM containing 0.45 mM (hypertonic) or 0.25 mM (isotonic) sucrose for 45 min prior to adding LID complexes formulated in hypertonic or isotonic medium respectively. Transfections performed in isotonic medium or OptiMEM served as the controls.

The use of hypertonic media did not reduce transfection efficiency of neuroblastoma cell lines compared to OptiMEM with 0.25 mM sucrose (**Fig 4.7**). Surprisingly, it proved to be a better transfection medium than OptiMEM alone in two cell lines. In SHSY5Y cells, there was a 40% increase in the RLU/mg values when cells were treated with hypertonic media compared to transfections performed in OptiMEM or isotonic media. In IMR-32 cells, hypertonic media resulted in over 100% enhancement of transfections performed in OptiMEM while no effect was observed in Neuro-2A cells.

Blocking of receptor-mediated endocytosis by disruption of clathrin molecules did not alter the LID transfection efficiency in any of the cell lines tested, suggesting that LID particles must mediate internalisation in neuroblastoma cells by an alternative pathway. These results do not exclude the possibility that LID particles may bind to other cell-surface receptors. They indicate that clathrin molecules are not involved in the endocytic process of LID uptake by these cells.

4.2.4 Inhibition of phagocytosis

The effect of cytochalasin-B on LID transfection was studied by pre-incubating neuroblastoma cells with various concentrations of the drug for 40 min at 37 °C. LID complexes were then added to the cells and transfection was performed for 4 h at 37°C.

Reporter gene expression assays showed that in SHSY5Y cells cytochalasin-B consistently enhanced luciferase expression especially at a concentration of 1 μg/ml (**Fig 4.8**). This increase in reporter gene expression possibly reflects an increase in

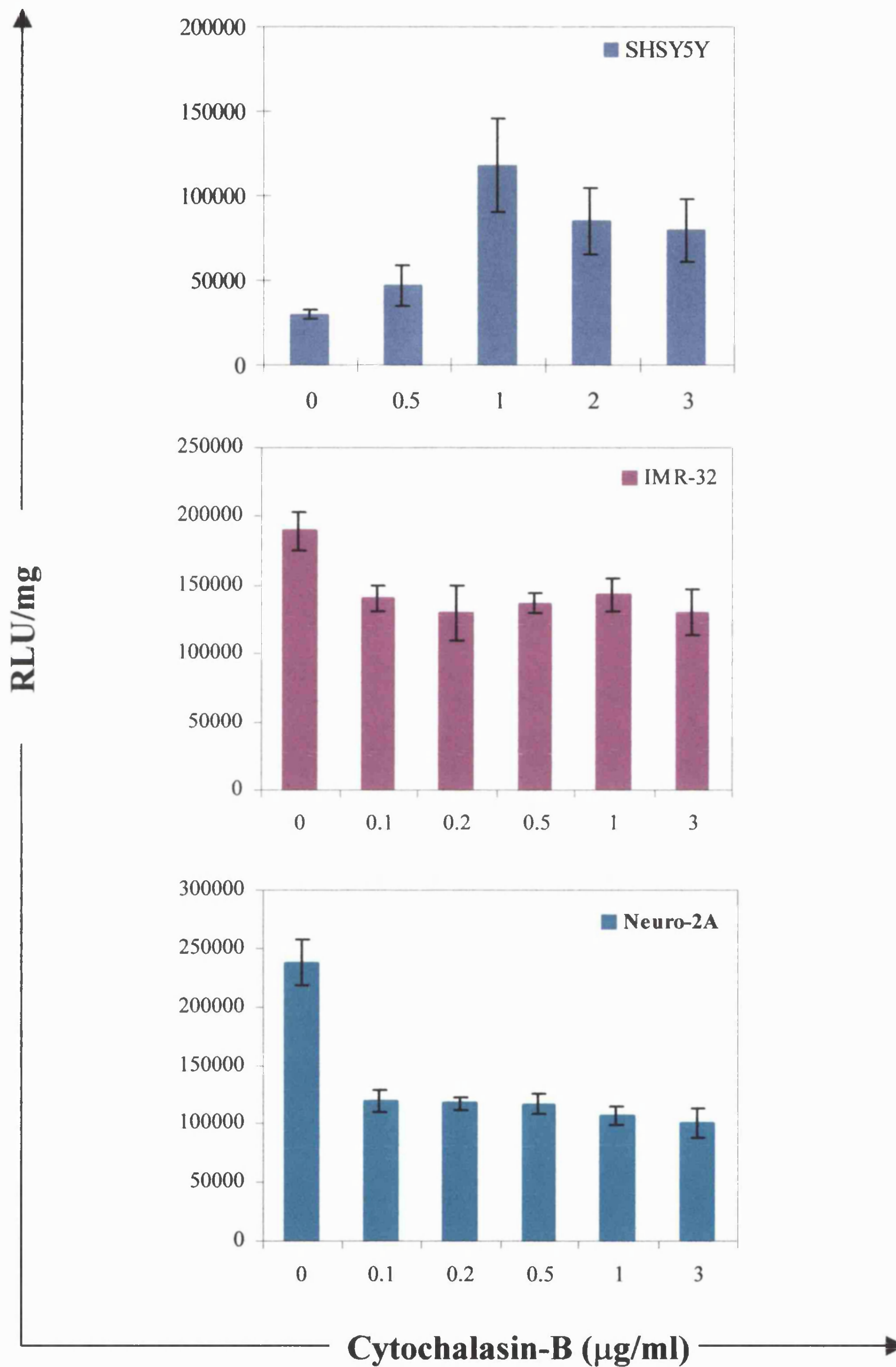


Figure 4.8 Cytochalasin-B inhibits transfection with the LID vector. Neuroblastoma cell lines were treated with cytochalasin-B prior to transfection with LID particles. The effect of the drug on transfection efficiency was estimated by measuring the luciferase expression 48 h later. Error bars represent standard deviations from 4 wells and data from one representative experiment is shown.

uptake of the LID vector in these cells. In contrast, internalisation of the LID vector in IMR-32 and Neuro-2A cell lines was significantly inhibited at as low concentrations of the drug as 0.1 μg/ml. Increasing the concentration of cytochalasin-B up to 3 μg/ml did not correlate with further inhibition of luciferase expression. These results suggest that phagocytosis is a potential uptake mechanism of LID particles in IMR-32 and Neuro-2A cells. In the SHSY5Y cell line, however, LID internalisation does not seem to be mediated by a phagocytic process.

4.3 DISCUSSION

In this chapter the mechanism of internalisation of the LID vector in three different neuroblastoma cell lines was investigated. The integrin specificity of the vector was initially examined by blocking of surface integrin receptors by antibodies or soluble peptides. Inhibition of LID transfection using antibodies against integrins failed possibly because the antibody is directed against a different epitope than the integrin ligand-binding pocket, and therefore no inhibition of transfection could be observed. The integrin-receptors are involved in binding of the LID complexes but the high charge property of these complexes also plays an important role in transfection (Hart *et al.*, 1997). Although the SHSY5Y cell line exhibited 48% expression of the alpha 5 integrin, incubation with soluble GRGDSP peptide failed to block internalisation of LID particles. This suggests that SHSY5Y neuroblastoma cells do not take up LID particles by integrin-mediated endocytosis. However, higher concentrations of the soluble peptides may be required to block LID transfection in these cells.

Differences in transfection efficiency between K_{16} and peptides containing a targeting sequence (peptides 6, 1 and 11) indicate that the extra targeting motif attached to the sixteen-lysine chain might enhance transfection efficiency. Peptide 6 was most efficient in all human neuroblastoma cell lines despite their low α_5 integrin expression. Also, the RRETAWA sequence is specific for human $\alpha_5\beta_1$ integrin but worked efficiently in the mouse Neuro-2A cell line. Furthermore, transfection using

an RGE- containing peptide (peptide 11) resulted in higher transfection efficiency than K_{16} in all cell lines despite the fact that peptide 11 does not target any integrins. These results indicate that LID complexes consisting of peptides with extra amino acid sequences attached to the sixteen-lysine chain confer higher transfection efficiency than K_{16} LID complexes. The superior transfection efficiency of these peptides may be mediated by increased charge interactions of the LID complexes with the cell membrane or via cross binding to other cell surface receptors.

Alternative internalisation mechanisms were investigated by observing the effects on transfection using a number of inhibitors. Uptake of the LID vector and transfection of IMR-32 and Neuro-2A neuroblastoma cells occurs in a wortmannin-sensitive manner. Transfection in the presence of wortmannin or LY294002 significantly reduced reporter gene expression. Both of these inhibitors act specifically on PI3K. Since PI3K is involved in endosome and lysosome fusion and integrin downstream signalling, one or both of these steps must be important in the intracellular trafficking of the LID vector. However, since none of these two cell lines express $\alpha_5\beta_1$ integrins, it is unlikely that involvement of PI3K affects internalisation of the LID vector in these cells via an integrin downstream signalling pathway. These results implicate PI3K in trafficking of the LID vector through endosomes in IMR-32 and Neuro-2A cells.

Table 4 Effects of various drugs on transfection efficiency of the LID vector[2]

	SHSY5Y	IMR-32	Neuro-2A
Expression of α_5 integrin	+	-	Mouse
Inhibition with integrin antibodies	-	-	ND
Inhibition with soluble RGD peptides	-	ND	ND
Inhibition with PI3K inhibitors	-	+	+
Enhancement with chloroquine	-	+	+
Hypertonic medium	-	-	-
Inhibition with cytochalasin-B	-	+	+

[2] EFFECT ON TRANSFECTION: - no effect; + significant effect; ND not determined

The involvement of endosomes in the uptake of LID particles by IMR-32 and Neuro-2A cells is supported by the use of chloroquine since luciferase expression in these cells consistently increased in the presence of chloroquine. This also suggests that the action of Lipofectin in releasing particles from endosomes may be enhanced by raising the acidic pH using chloroquine.

In order to further clarify whether clathrin-coated pits were involved in the internalisation mechanism of LID complexes, hypertonic media were used that disrupt formation of clathrin-coated pits. Treatment and transfection of neuroblastoma cells in hypertonic media did not inhibit LID-mediated luciferase expression, strongly refuting the involvement of clathrin-coated pits in the internalisation mechanism of the vector. Cytochalasin-B, on the other hand, which inhibits phagocytosis by disrupting the F-actin network, significantly reduced reporter gene expression in IMR-32 and Neuro-2A cells. This result taken together with that of hypertonic media indicates that internalisation of LID complexes probably occurs via non-coated pit endocytosis or phagocytosis.

Interestingly, the SHSY5Y cell line responded differently to treatment with most drugs compared to the other two neuroblastoma cell lines (**Table 4**). The transfection efficiency of SHSY5Y cells remained unaffected by addition of chloroquine or pre-treatment with PI3K inhibitors. A possible explanation for this result is that efficient release of the internalised LID complexes occurs and additional inhibition of lysosomal enzymes is not required. Alternatively, the LID vector may mediate entry in these cells via an alternative pathway such as phagocytosis. However, cytochalasin-B did not inhibit luciferase expression in SHSY5Y cells, disproving the possibility of phagocytosis as the main mechanism of internalisation of LID complexes in that cell line. It has to be noted, though, that SHSY5Y is the only cell line that expresses α_5 integrins. Although the precise internalisation mechanism of the LID vector in these cells remains unclear, their different response to the pharmacological inhibitors studied possibly indicates a distinct uptake mechanism to that of IMR-32 and Neuro-2A cells.

Although none of the experiments mentioned above studied the uptake of LID particles by neuroblastoma cells directly, luciferase expression reflected to some extent the intracellular fate of these particles and their ability to be expressed. Although invaluable in studying the intracellular trafficking of non-viral complexes, drugs such as chloroquine and the PI3K inhibitors have certain limitations. Their effects on endosomal trafficking do not distinguish between different endocytic pathways. Therefore, more detailed studies are required to determine the precise internalisation mechanism of LID complexes in neuroblastoma cells. Fluorescent labelling of DNA, integrin receptors or endosomal markers and subsequent analysis by confocal microscopy would illustrate the interaction of the complexes with different components of the membrane and intracellular trafficking vesicles.

Integrin targeting is not important when non-specific delivery of a transgene is required as in *ex vivo* manipulation of cells for cancer vaccines, since the vector is not limited by the integrin receptor availability on the cells. On the contrary, it may prove advantageous in cancer models where integrin expression is variable. Expression of various integrins is elevated in the tumour microenvironment with progression of the disease (Erdreich-Epstein *et al.*, 2000). As seen in chapter 3, the LID vector transfects neuroblastoma cells with high efficiency so it is suitable for such an *ex vivo* strategy. Improvement of the peptide ligand for targeting would offer greater specificity especially for *in vivo* applications.

5

In vitro Cytokine Expression

5.1 INTRODUCTION

Following optimisation of the LID vector for use in neuroblastoma cells it was essential to determine the *in vitro* expression of the cytokine genes to be introduced in these cells. *In vitro* culturing of transfected cells and their protein expression may not always reflect the exact *in vivo* situation but it comprises the most immediate step prior to proceeding to an *in vivo* model for the studied disease.

5.1.1 Cytokine bioactivity assays

In vitro protein expression of transfected/transduced cells is usually detected using techniques such as Western analysis and enzyme immunoassay. Both of these techniques rely on the binding of an antibody that recognises certain epitopes on the protein in question but do not verify that the detected protein levels coincide with biologically active protein. Therefore, assays that will provide an indication of the function of the protein in an *in vitro* setting are required before progressing to the *in vivo* model. Such assays usually look into special properties of the proteins in question, and in the case of IL-2 and IL-12, their ability to enhance proliferation of PHA-stimulated lymphocytes. T cells derived from the non-adherent fraction of peripheral blood mononuclear cells can be activated in a number of ways, including the use of monoclonal antibodies that can directly interact with the CD3/TCR complex or lectins such as PHA or concavalin-A (ConA). The precise mechanism of such agents is unknown, but they are thought to indirectly cross-link the TCR receptor. When activated, T cells respond to and produce IL-2 and any differences in their proliferative responses should give an indication of the extent of the T cell activation.

Immature human T cells express low affinity IL-2 receptors consisting of IL2Rα chain alone or dimeric interactions among the α, β and γ chains. Upon antigenic activation, the three chains form a complex that comprises the high-affinity IL-2 receptor. Activated T cells also up-regulate expression of IL-12 receptor that enables them to proliferate in response to this cytokine independently of IL-2 (Desai *et al.*, 1992).

Therefore, by incubating mitogen-activated T cells in cytokine-transfected cell culture supernatant, it should be possible to directly compare the ability or levels of these cytokines to trigger T cell proliferation. However, the effect of IL-2 or IL-12 on PHA-activated lymphocytes cannot be distinguished as they both induce cellular proliferation.

Functional studies of lymphotactin, which is a chemoattractant of NK and T cells, have focused on its ability to cause infiltration of cells after injection into the peritoneal lavage of animals or the induction of chemotaxis *in vitro* across a chemokine gradient (Hedrick *et al.*, 1997).

5.1.2 γ-Irradiation

For a vaccine application, it is vital that certain parameters are optimised before looking at the *in vivo* immune response elicited against the vaccine. For example, the number of transfected cells used for vaccination determines the amount of tumour antigens as well as the cytokine levels secreted by the cells. These are critical factors for antigen presentation and direction of a specific T cell response. The input levels of cytokines such as IL-2 and IL-12, whose function is to attract and activate T and NK cells, is important for the extent of T cell-mediated or non T cell-mediated responses that they induce. Furthermore, when developing a cell-based vaccine for clinical use in cancer patients, it is vital that dead or dying cells are used in order to avoid increasing the patients' tumour burden. Therefore, cells are required to express the immunostimulatory molecules that they have been modified with but to have ceased proliferating. Many studies have reported the use of γ-irradiated cells as a vaccine, which retain their ability to express certain proteins.

Gamma and ultraviolet irradiation induce DNA damage and can trigger cell death by apoptosis by signalling through a number of protein kinases such as ataxia-telangiectasia mutated protein (ATM) and DNA-dependent protein kinase (DNA-PK). Upon exposure to gamma rays, ATM leads a signal-transduction pathway with

tumour-suppressor p53 being one of the concluding targets (reviewed in Westphal, 1997). Activation of p53 leads to either cell cycle arrest at G_1 and G_2 through the cyclin-dependent kinase inhibitor p21, or initiation of apoptosis depending on a number of factors such as the cell type, stage of cell cycle or extent of DNA damage. The p53-dependent apoptotic pathway is more complex, implicating proteins such as Bax, the insulin-growth factor I (IGF-1) receptor and the IGF binding protein-3 (reviewed in Evan and Littlewood, 1998).

5.1.3 Apoptosis

There has been an ongoing controversy regarding the immune response towards apoptotic and necrotic cells and which type of cells should be used to develop vaccines. For vaccine development, an antigen-specific T cell response is required, but enhancement of inflammation by using apoptotic or necrotic cells as an adjuvant, may be a desirable outcome. Apoptosis is a physiological process contrary to the pathological death of necrosis that is supposed to provide 'danger signals' and lead to inflammatory responses. The activation of dendritic cells, the immune system's professional antigen presenting cells, is believed to depend on the nature of death of the cell to be phagocytosed. Induction of DC maturation was shown to occur only by exposure to necrotic cells or their soluble derivatives (Sauter *et al.*, 2000). However, a contradicting study suggests that neither apoptotic nor necrotic cells are capable of activating DCs but the presence of mycoplasma infection will stimulate DC maturation (Salio *et al.*, 2000).

Apoptosis or 'programmed cell death' is a physiological mode of cell death upon reaction to certain stimuli that involves a progression from cell cycle arrest to cell death. The early stages of apoptosis are followed by a number of morphological changes in the cell shape and size, due to dehydration of the cytoplasm, but the cell membrane remains intact. Other very characteristic features include condensation of chromatin, hyperchromasia of DNA and formation of apoptotic bodies after disintegration of the nuclear envelope. There are many experimental methods for

detecting apoptosis, which rely on changes in cell morphology, membrane permeability to certain dyes or altered mitochondrial or lysosomal membrane function. An alternative approach utilises the property of annexin-V, a Ca^{+2}-dependent protein that binds to negatively charged phospholipids such as phosphatidylserine. The latter resides in the inner part of the cell membrane of live cells but during apoptosis it is translocated and exposed to the outer leaflet of the membrane, so that it can be detected by binding to annexin-V. Annexin-V conjugated to fluorescein isothiocyanate (annexin-V-FITC) used in conjunction with propidium iodide, that stains the cell nucleus, allows for differentiation between necrotic and apoptotic cells by flow-cytometric analysis. Early apoptotic cells will stain positively for annexin-V but negatively for propidium iodide due to the integrity of the plasma membrane that will exclude the dye. As apoptosis progresses and the plasma membrane disintegrates the cells will be stained with propidium iodide and so will necrotic cells.

5.2 RESULTS

5.2.1 Cloning of cytokine genes

The human IL-2 cDNA was kindly provided by Prof M.Collins (UCL) in a retroviral construct, MFGS-IL2, and expression of the cytokine was driven by Moloney leukaemia virus LTRs. However, transfection of neuroblastoma cells with this construct resulted in very low expression and an alternative vector had to be sought (data not shown). The pCI plasmid was chosen as the vector for cloning the human IL-2 and murine lymphotactin cDNAs. It is a mammalian expression vector where expression of the cDNA is regulated by an immediate CMV enhancer/promoter. The additional feature of this vector is a chimeric intron that is located 5'of the cDNA, and is claimed to increase expression of the cDNA.

The hIL-2 cDNA was isolated by digesting MFGS-IL2 plasmid with *Bam*HI, and then was separated and purified by gel electrophoresis. Subsequently, it was ligated into *Bam*HI-digested and dephosphorylated pBluescript plasmid. Plasmid DNA was extracted from transformed bacterial clones and digested with *Bsm*AI to confirm the

fragment orientation. pBluescript contains two *Bsm*AI sites and there is also one *Bsm*AI site at the 3' end of the IL-2 cDNA approximately 110 bp from the *Bam*HI site. Digestion of pBluescript-IL2 (anti-sense orientation) with *Bsm*AI would give rise to three fragments of the following sizes: 776 bp, 1216 bp, 1496 bp compared to bacterial clones containing the plasmid with the IL2 cDNA in the sense orientation, which would lead to a 776 bp, a 902 bp and a 1810 bp band. Clones with the fragment being in the anti-sense orientation were isolated and pBluescript-IL2 was subsequently digested with *Eco*RI and *Not*I (Life Technologies) in a single digestion reaction. The hIL-2 cDNA was purified by gel electrophoresis and ligated into *Eco*RI/*Not*I-digested pCI vector. The pCI plasmid had been dephosphorylated to avoid any self-ligation of molecules that had been digested only by a single enzyme.

Mouse lymphotactin cDNA was isolated from pBluescript-Ltn plasmid by digestion with *Xba*I and *EcoR*I enzymes, and in a similar way to that described for hIL-2 cDNA, it was subcloned into pCDNA3.1(-). pDNA3.1-Ltn clones were digested with *Eco*RI and *Nhe*I to excise the lymphotactin fragment that was next inserted into pCI. One of the pCI-IL2 and pCI-lymphotactin clones after digestion with *Eco*RI/*Not*I and *Eco*RI/*Nhe*I, respectively are shown in **Fig 5.1**. The original pCI plasmid was also shown for comparison. Excision of a ~600 bp fragment from pCI-Ltn and a ~550 bp fragment from pCI-IL2 were detected which correspond to the murine lymphotactin and hIL-2 fragments, respectively.

Human IL-12 p35 and p40 subunits were carried on pCMV-Flexi12 plasmid joined by a linker sequence. This results in the production of a single full-length IL-12 molecule that retains its biological activity when tested on PHA-activated lymphocytes (Anderson *et al.*, 1997). pCDNA3.1scIL12 plasmid was used for delivery of the murine IL-12 gene into Neuro-2A cells since human IL-12 does not activate mouse lymphocytes. This construct carries a single chain IL-12 fusion protein, prepared by linking the p35 and p40 subunits of mIL-12 by a synthetic linker (Lode *et al.*, 1998). The plasmid maps are summarised in Appendix A.

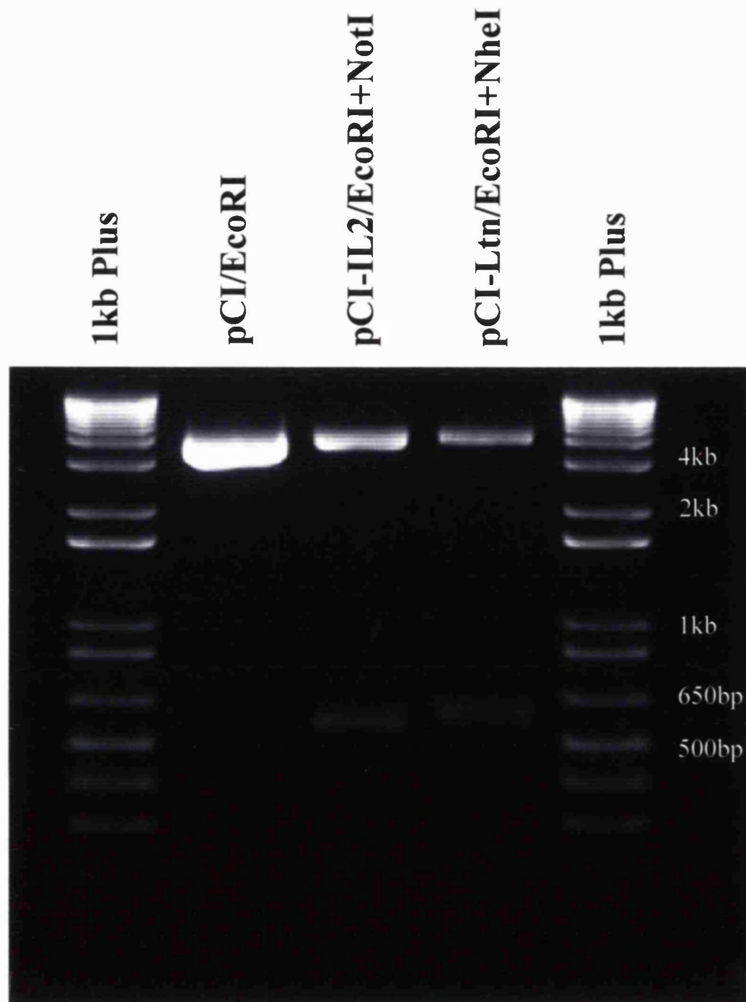


Fig 5.1 Cloning of cytokine genes. Agarose gel electrophoresis of pCI-IL2 and pCI-Ltn clones digested with EcoRI/NotI and EcoRI/NheI enzymes respectively so that the cytokine inserts were excised. The parental pCI plasmid digested with EcoRI was also shown for comparison. Restriction enzyme digests were electrophoresed on a 1.5% agarose gel and 1 kb Plus DNA ladder (Life Technologies) was used for confirming the size of the DNA fragments.

5.2.2 Cytokine expression

Delivery of cytokine genes into neuroblastoma cells was performed using the LID vector as described in section 2.3.1. Peptide 6 ($\alpha5\beta1$) and pCI-IL2, pCMV-Flexi12 or a combination of the two plasmids were employed to transfect human neuroblastoma cell lines, while for mouse Neuro-2A cells, pCI-IL2 and pCDNA3.1scIL12 were used. Cytokine levels were measured by ELISA analysis from the cell culture supernatant collected at 24 h intervals from $5x10^4$ cultured cells and the values expressed as ng/ml/24h/10^6 cells.

5.2.2.1 Interleukin-2

Human IL-2 secretion by human neuroblastoma cell lines reached a peak 24-48 h post-transfection in both cell lines with IMR-32 cells expressing an average of 350±5.5 ng/ml/24h/10^6 cells and SHSY5Y cell line 11 ng/ml/24h/10^6 cells on day 1 **(Fig 5.2 A,B)**. By day 10, IL-2 expression by IMR-32 cells had decreased to 29 ng/ml/24h/10^6 cells and for SHSY5Y cells to 6 ng/ml/24h/10^6 cells. Co-transfections with hIL-12 resulted in slightly lower levels of IL-2 expression than single transfections. IMR-32 cells expressed an average of 232±7 ng/ml/24h/10^6 cells of IL-2 for the first two days of culture and there was a reduction to 15 ng/ml/24h/10^6 cells by day 10. In SHSY5Y cells co-transfection experiments resulted in similar IL-2 levels to single transfections starting at 12 ng/ml/24h/10^6 cells on day 1 and declining to 5 ng/ml/24h/10^6 cells by day 10.

The levels of hIL-2 secreted by transfected Neuro-2A cells were higher than mIL-12, starting at 385 ng/ml/24h/10^6 cells and dropping to 170 ng/ml/24h/10^6 cells by day 7 of *in vitro* culture **(Fig 5.2 C)**. There was no significant difference in the IL-2 expression levels by Neuro2A cells between single and co-transfection experiments. In all experiments there was no detectable production of IL-2 by pEGFP-N1 transfected cells ($p<0.04$).

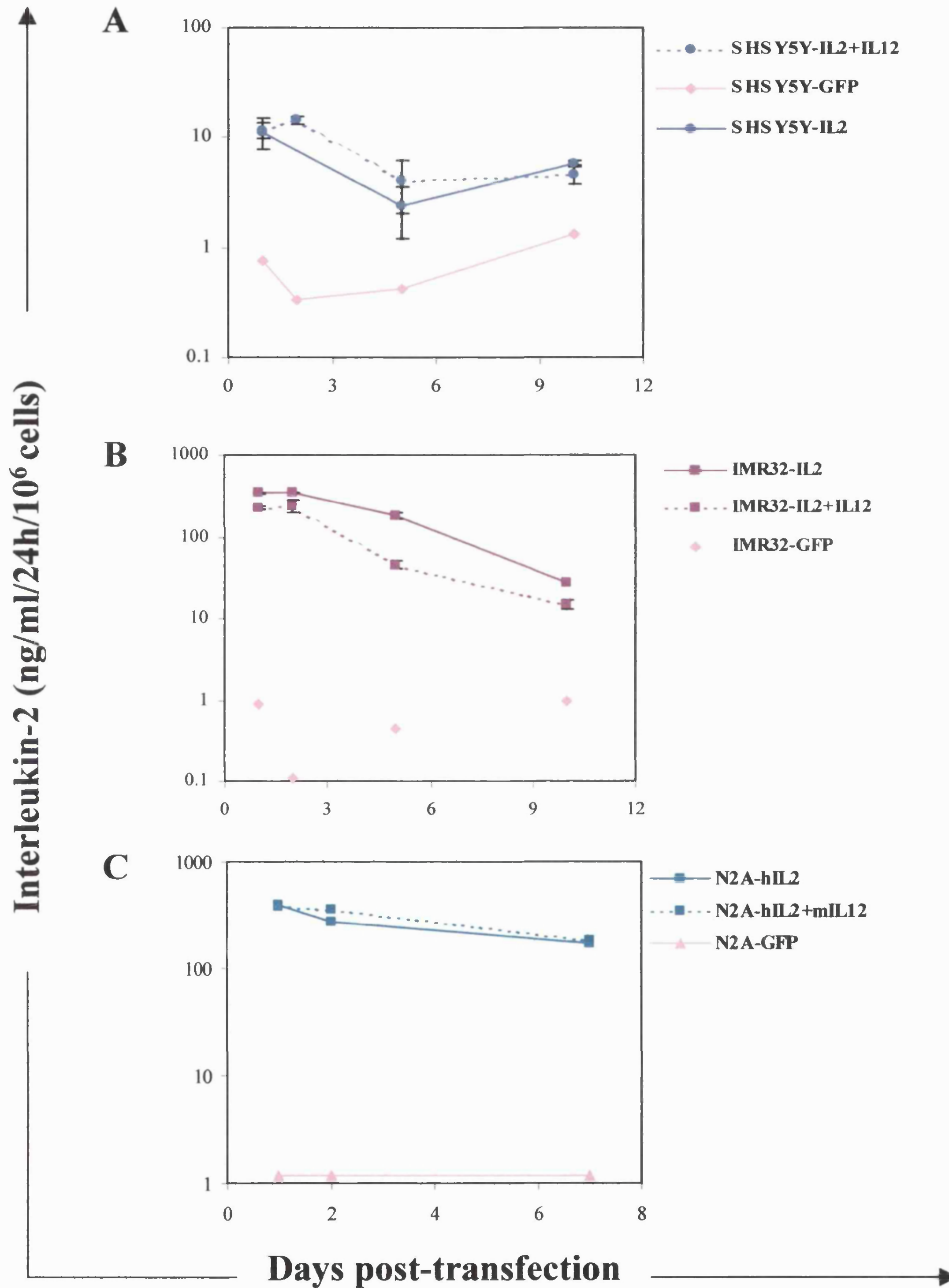


Fig 5.2 Interleukin-2 expression by neuroblastoma cell lines. *In vitro* expression of human IL-2 is depicted as a function of time. Cells were transfected with pCI-IL2 alone or in combination with pCMV-Flexi12 (human cells) or pCDNA3.1scIL12 (mouse). Secretion of IL-2 was analysed at 24 h intervals by ELISA and pEGFP-N1 transfected cells were used as a negative control. Results from one representative experiment are shown.

5.2.2.2 Interleukin-12

In both IMR-32 and SHSY5Y cell lines, hIL-12 expression followed a rather stable expression pattern throughout a 10-day culture period. IMR-32 expressed an average 185±20 ng/ml/24h/10^6 cells of IL-12 for 10 days while IL-12 production by SHSY5Y was maintained at an average of 27±7 ng/ml/24h/10^6 cells for the same period of time **(Fig 5.3 A,B)**. Single cytokine transfection experiments resulted in significantly higher production of cytokines than co-transfecting IL-2 and IL-12 genes ($p<0.05$) despite the fact that an equal amount of cytokine cDNA was used to transfect these cells. Co-transfection of IMR-32 cells with IL-2 and IL-12 resulted in 200 ng/ml/24h/10^6 cells of IL-12 production on day 1 with a decline to 46 ng/ml/24h/10^6 cells by day 10. Co-transfection experiments of SHSY5Y cells resulted in an average 12±4 ng/ml/24h/10^6 cells IL-12 expression for 10 days.

Murine IL-12 expression by mouse Neuro-2A cells retained similar expression levels throughout the seven days of analysis. It reached 115 ng/ml/24h/10^6 cells at day 1 declining slightly to 90 ng/ml/24h/10^6 cells seven days after transfection. No significant difference in expression was observed between single and co-transfection experiments where both cytokine genes were introduced into Neuro-2A cells ($p>0.05$) **(Fig 5.3 C)**. No detectable IL-12 levels were secreted by pEGFP-N1 transfected cells ($p<0.04$).

5.2.3 Cytokine Bioassay

Undiluted supernatant from cytokine-transfected neuroblastoma cells over a 7-10 day culture period was applied onto PHA-activated lymphocytes for 24 h. The effect of the cytokines on the proliferation of PHA-activated lymphocytes was examined by addition of ^{3}H-thymidine for 16 h.

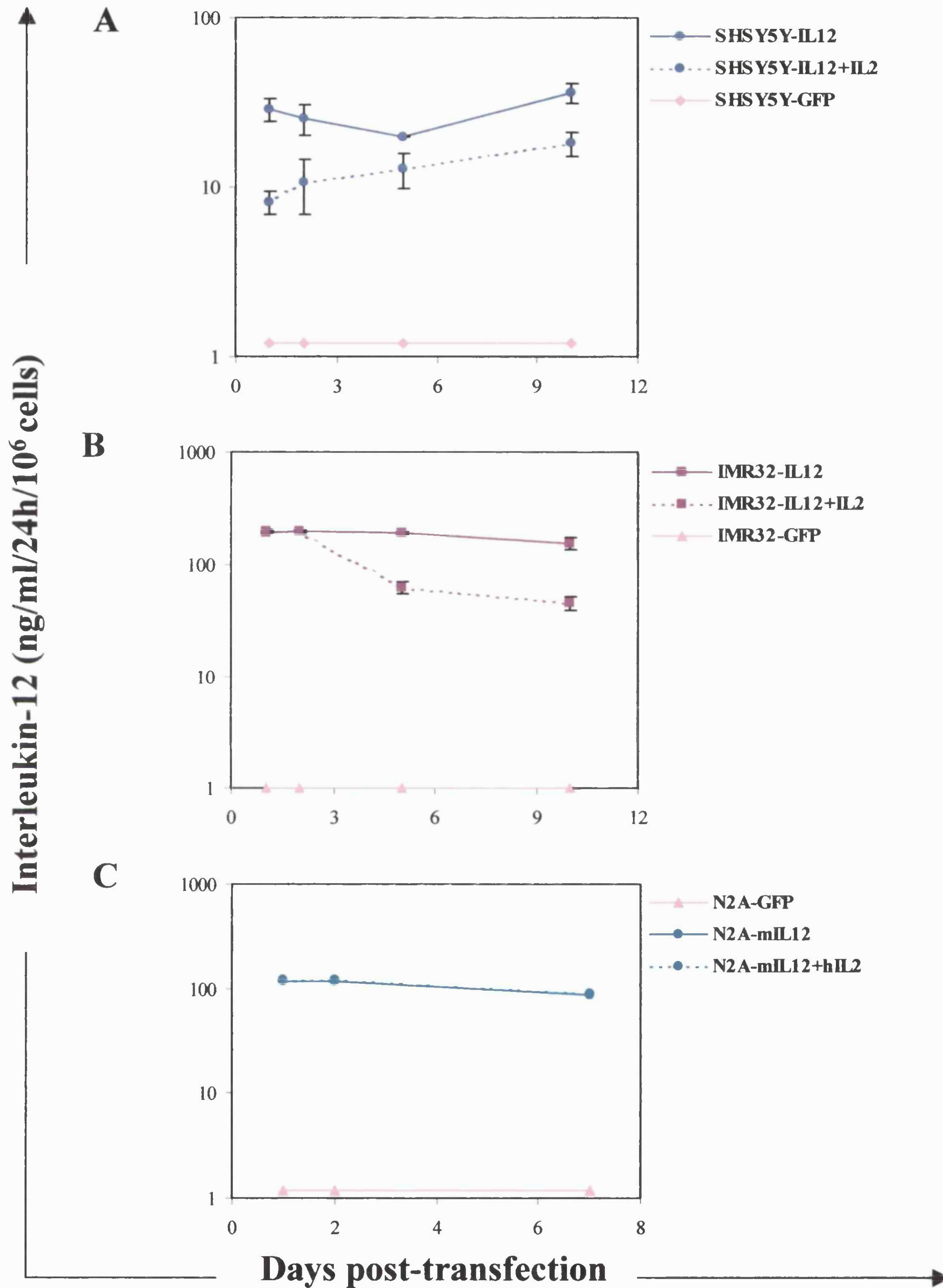


Fig 5.3 ***In vitro* expression of Interleukin-12 by neuroblastoma cell lines.** Supernatant from transfected cells in culture was collected every 24 h and analysed by cytokine immunoassay. Cytokine levels from one representative experiment are plotted on a log scale and error bars represent the standard deviation of triplicate transfections.

The cell culture supernatant of hIL-12-transfected SHSY5Y and IMR-32 cell lines over a period of ten days caused stable proliferation suggesting that its biological activity was retained *in vitro* for that period of time (**Fig 5.4 A,B**). The biological activity of hIL-2 secreted in the cell culture supernatant of single or co-transfected cells, however, dropped after day 2 as indicated by the reduction in ^{3}H-thymidine incorporation in PHA-activated lymphocytes. In both cell lines, hIL-2 produced after ten days in culture, had no significant mitogenic effect compared to supernatant from pEGFP-N1-transfected cells. This result, however, contradicts the immunoassay data where IL-2 concentration at day ten was estimated to be 45 ng/ml/24h/10^6 cells for IMR-32 and 10 ng/ml/24h/10^6 cells for SHSY5Y. However, the difference in IL-2 production by the two cell lines obtained by ELISA analysis, was confirmed by the poorer proliferative potential of supernatant from SHSY5Y cells on PHA-activated lymphocytes.

The cytokines expressed by transfected mouse Neuro-2A neuroblastoma cells exhibited a comparable effect on PHA-activated lymphocytes to those expressed by SHSY5Y and IMR-32. The cell culture supernatant of IL-2-transfected Neuro-2A cells demonstrated analogous proliferation of PHA-activated lymphocytes to that of co-transfected cells. Murine IL-12 had a significantly higher mitogenic effect than supernatant from pEGFP-N1-transfected cells, but compared to IL-2 it did not result in equally high incorporation of ^{3}H-thymidine in PHA-blasts. In addition, the presence of both cytokines did not have a cumulative effect on proliferation of these cells but the degree of proliferation was retained at the same levels when supernatant from different time points was tested (**Fig 5.4 C**). This was indicative of stable cytokine concentrations throughout the seven-day culture period and is in agreement with the ELISA data obtained for both hIL-2 and mIL-12.

Although Neuro-2A cells produced similar concentrations of hIL-2 to IMR-32, the scintillation counts of PHA-activated lymphocytes were lower when the latter were incubated with supernatant from IL-2-transfected Neuro-2A cells. This could suggest lower biological activity of IL-2 or more likely it could be due to experimental variation, which is expected in such a procedure where the donor and preparation of

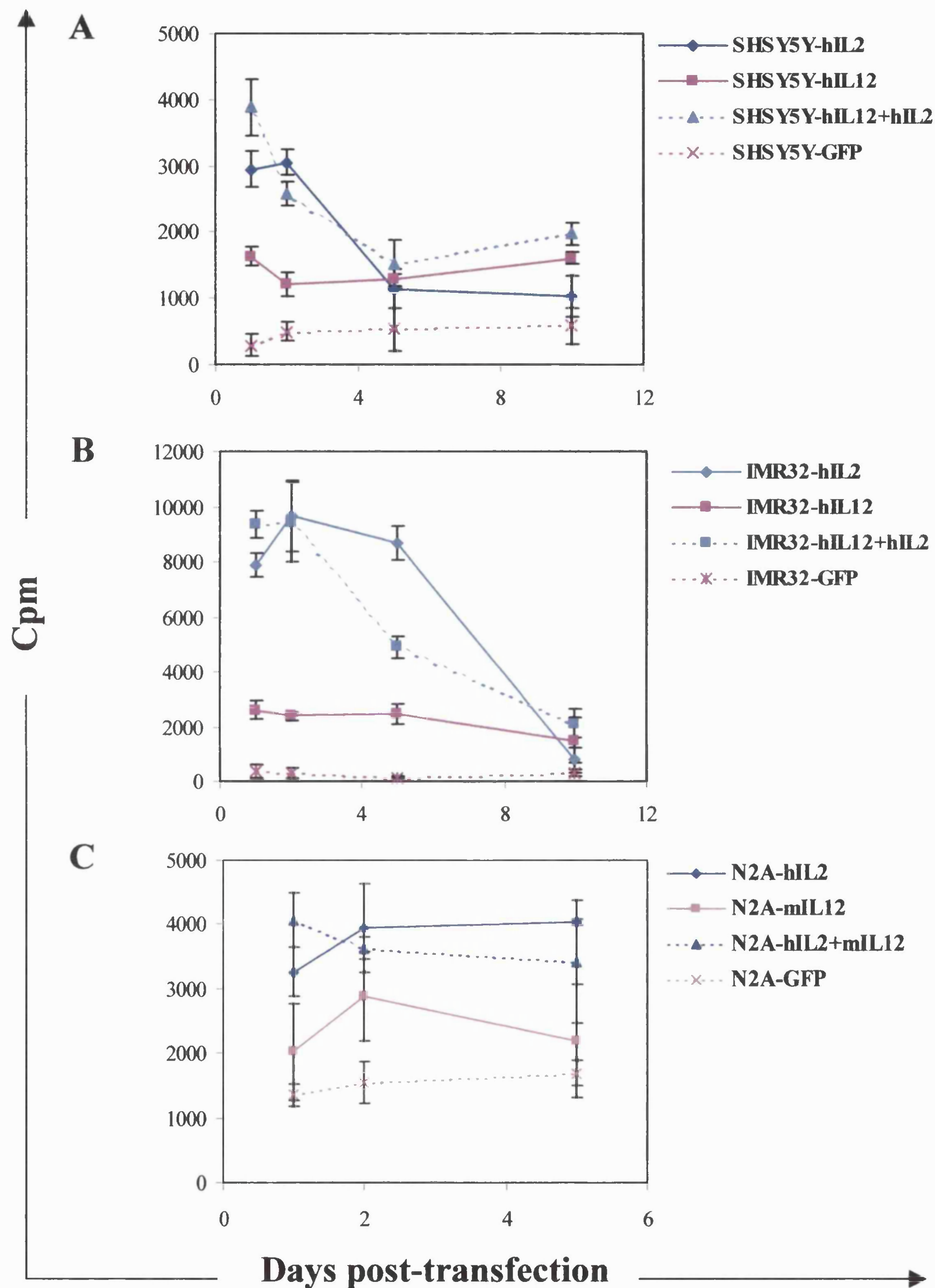


Fig 5.4 Biological activity of IL-2 and IL-12 expressed by transfected neuroblastoma cell lines. The supernatant from transfected neuroblastoma cell lines was tested on human lymphocytes after stimulation with PHA and rhIL-2. Cell proliferation was assessed by ^{3}H-thymidine incorporation over a 16 h incubation time. Results are expressed as scintillation counts and one representative experiment is shown.

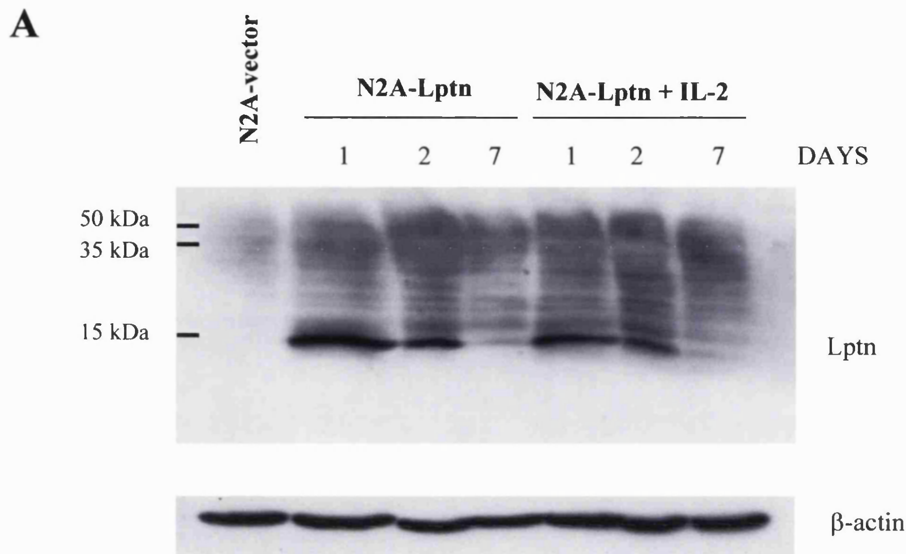
the T cells is of vital importance. The background proliferation of the PHA-activated lymphocytes was stable in all experiments.

5.2.4 Lymphotactin expression by mouse Neuro-2A cells

Initial studies on lymphotactin had mainly detected expression by investigating the presence of a copy of the lymphotactin gene by Northern hybridisation of RNA isolated from thymic and spleen cells (Kelner *et al.*, 1994). Recombinant murine lymphotactin expressed in *E.coli*, was electrophoresed on Tris-glycine gradient gel to reveal two species running below 14 kDa (Hedrick *et al.*, 1997).

Murine lymphotactin expression in neuroblastoma cells was analysed by Western blotting of intracellular protein levels from transfected cells. Neuro-2A cells placed on 6-well plates at a density of $3x10^5$ cells/well were transfected with LID complexes consisting of lipofectin, peptide 6 and either pCI-Ltn or pCI-IL2 vectors. At certain time points after transfection, cells were removed from culture, detached from the tissue culture plate and subsequently analysed by Western blotting.

Lymphotactin expression is restricted to the T cell lineage and NK cells, so parental mouse Neuro-2A cells transfected with an empty vector (pCI plasmid) were used as a negative control. The expected size of the protein, as determined by its cDNA sequence, is approximately 18 kDa. Recombinant murine lymphotactin is a single protein species of 10 kDa and runs just below the 15 kDa protein marker on SDS polyacrylamide gel (**Fig 5.5 B**). Antibody detection of lymphotactin in transfected Neuro-2A cells, revealed one prominent band, running next to the 15 kDa protein marker. The expression levels were slightly lower in cells that had been co-transfected with hIL-2 as indicated by less intense bands on the immunoblot. Lymphotactin expression had faded by day seven after transfection as can be seen by the disappearance of the corresponding band on the immunoblot (**Fig 5.5 A**). β-actin expression, indicated by the lower bands running at 42 kDa, confirmed that



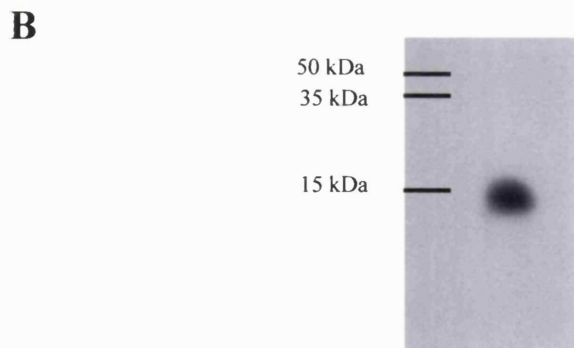


Figure 5.5 Expression of murine lymphotactin by Neuro-2A cells. A. Murine lymphotactin alone or in conjunction with human IL-2 were used to transfect Neuro-2A cells and analysis was performed at various time points by SDS-PAGE for the presence of lymphotactin. A protein species could be detected as a single band running at approximately 12-15 kDa on a polyacrylamide gel. Equal protein loading was confirmed by β-actin expression which is shown in the bottom part of the figure. **B.** Recombinant murine lymphotactin (200 ng) can be detected as a single protein species at ~10 kDa as seen by SDS-PAGE.

comparable protein levels were loaded in each lane of the gel and any difference in intensity of the lymphotactin bands was due to altered expression patterns.

It is not clear whether the 15 kDa protein species appearing after SDS-polyacrylamide gel electrophoresis corresponds to unmodified or post-transcriptionally glycosylated form of murine lymphotactin. Approximately 60% of human ATAC (activation-induced, T cell-derived and chemokine-related), which is the same molecule as lymphotactin but was independently identified at the same time as lymphotactin, is being secreted by activated T cells as an unmodified 10.3k Da protein running in SDS-PAGE at 12 kDa, while a substantial percentage is subjected to *O*-glycosylation. These glycosylated protein species appear as 15 kDa and 17-19 kDa proteins in SDS-PAGE and are secreted within 30-40 min after synthesis (Dorner *et al.*, 1997). Murine lymphotactin could follow a similar pathway and be subjected to post-transcriptional glycosylation.

5.2.5 γ-Irradiation

5.2.5.1 Annexin-V/ PI staining for detection of apoptosis

Mouse neuroblastoma Neuro-2A cells were subjected to 2500 rad (25 Gy) γ-irradiation, counted with trypan blue exclusion and plated at $3x10^5$ cells/well in a 6-well plate. At certain time points thereafter, cells were detached and stained with Annexin-V-FITC and propidium iodide to detect apoptosis. Non-irradiated cells were used as a negative control. No visible change in the cell morphology or binding to annexin-V could be detected up to 48 h after irradiation. At that stage only 4.6% of Neuro-2A cells were positive for apoptosis whereas nearly 60% remained intact. This was calculated by subtracting the physiological apoptotic levels exhibited by normal Neuro-2A cells. The percentage of early apoptotic cells increased with time to 8.5% at day 3, 27% at day 5 and finally 40% at day 7. By that point, there was a shift of the entire cell population from early to late apoptosis (**Fig 5.6**). Non-irradiated Neuro-2A cells plated at the same time and at density as the irradiated ones, were used as the

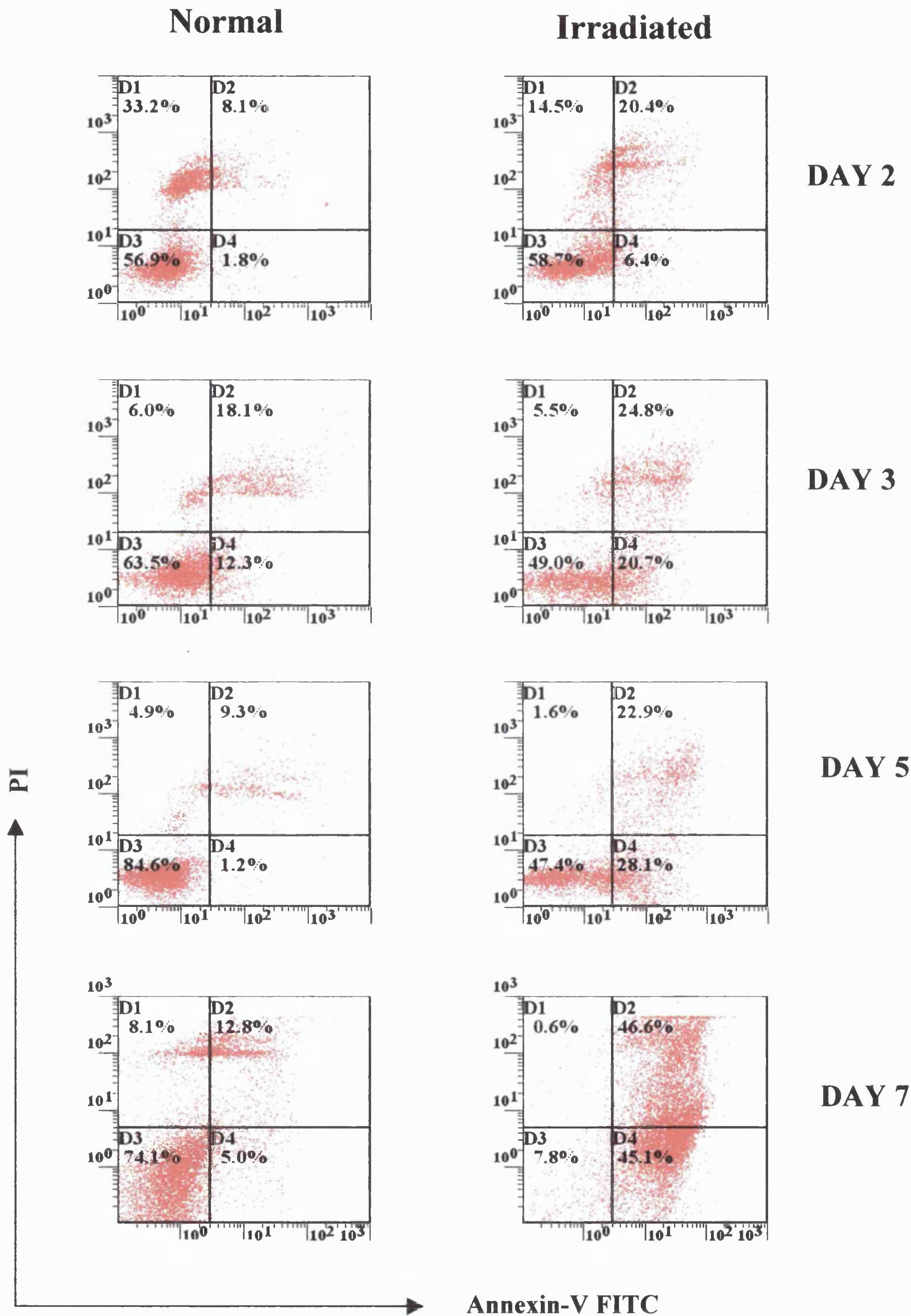


Figure 5.6 Apoptosis of γ-irradiated Neuro-2A cells. Neuro-2A cells following γ-irradiation (25Gy) were incubated with Annexin-V FITC and propidium iodide and analysed by flow cytometry. Setting up of the FACS sorter was performed with parental Neuro-2A cells stained with either annexin-V or PI. Normal cell death was estimated by non-irradiated cells.

control to determine physiological cell death. The interesting feature of apoptosis of Neuro-2A cells was the change in cell morphology. Irradiated cells by day 5 were enlarged with neurite outgrowths as indicated by their forward and side scatter distribution on the FACS sorter.

5.2.5.2 *In vitro* cell proliferation

Neuro-2A cells following γ-irradiation were cultured on 96-well plates and their *in vitro* proliferation measured by ^{3}H-thymidine incorporation over seven days. Comparison with non-irradiated cells, one and two days post-irradiation, showed a significant reduction in scintillation counts, suggesting marked inhibition of proliferation ($p<0.05$) **(Fig 5.7)**. Untransfected or cytokine-transfected cells followed an identical pattern of proliferation after irradiation. Following the proliferation of these cells seven days after irradiation revealed that mitotic division had ceased altogether as indicated by the extremely low incorporation of ^{3}H-thymidine (data not shown). Examination by light microscopy confirmed that result, since most cells had detached from the tissue culture plate or had formed neurite projections, an indication of differentiation (data not shown).

5.2.5.3 IL-2 and IL-12 Expression

Before determining whether apoptotic or necrotic cells induce the highest immune response *in vivo*, their *in vitro* production of cytokines was analysed. Neuro-2A cells were subjected to freeze-thawing, γ-irradiation or left untreated. They were cultured for seven days, their supernatant collected and the cytokine levels estimated by ELISA. Both mIL-12 and hIL-2 reached comparable concentrations in both untreated and irradiated cells with a decline after the second day. The expression pattern was almost identical suggesting that within the seven-day analysis period, γ-irradiated cells were still able to transcribe and translate the cytokine transgene. Necrotic cells on the other hand, expressed very low quantities of either cytokines that did not surpass

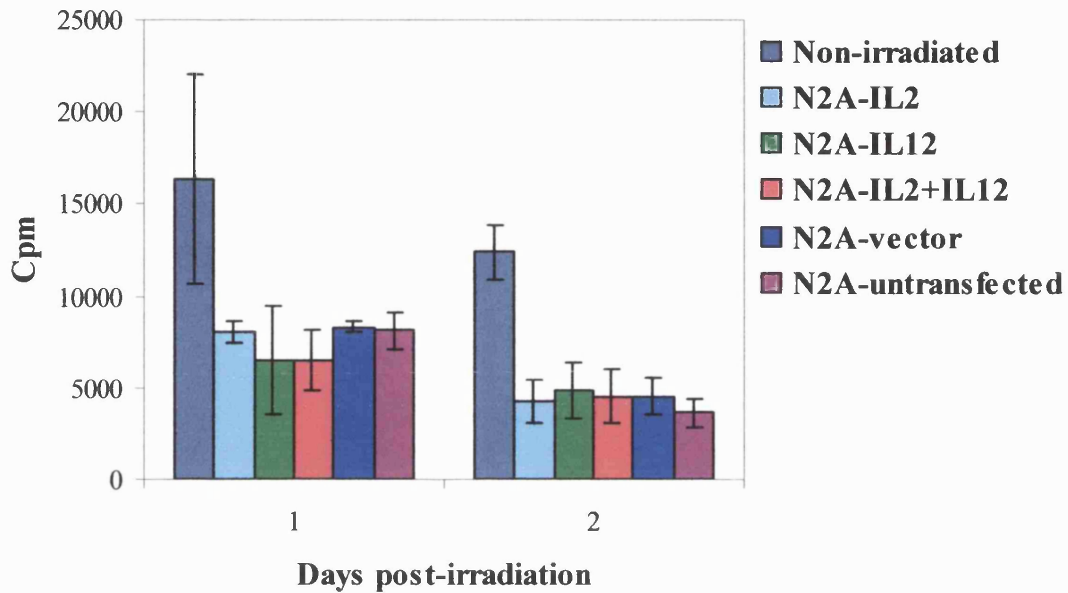


Fig 5.7 Effect of γ-irradiation on proliferation of LID-transfected Neuro-2A cells. Cells were transfected using the LID vector and 24 h later subjected to 2500 RAD (25 Gy). Their proliferation was estimated at various time points by incorporation of ^{3}H-thymidine over a 16 h incubation period. No difference in proliferation could be observed among the differently transfected groups but there was significant reduction compared to non-irradiated parental Neuro-2A cells.

1 ng/ml at day 1 (**Fig 5.8**). These cytokine levels could be attributed to few live or dying cells that may have survived the freeze-thawing cycle. This result was in accordance with the morphological cell changes following freezing which is disruption of the cell membrane and immediate death. This was confirmed by staining with annexin-V FITC and propidium iodide, which revealed a >90% necrotic cell population (data not shown).

5.2.5.4 Lymphotactin Expression

In order to determine expression of murine lymphotactin in irradiated Neuro-2A cells, transfection was carried out as mentioned in 5.5 and the cells were γ-irradiated (25 Gy) 18-20 h afterwards. Cell lysates were obtained from cells at different time points of *in vitro* culture and lymphotactin expression analysed by SDS-PAGE. Interestingly, two protein species were observed, approximately 15 kDa and ~17 kDa and their expression was persistent for at least seven days after irradiation. The higher band was more visible in cells that had been co-transfected with hIL-2 and its intensity faded with time while the reverse seemed to occur in single lymphotactin transfectants. Indeed, cell lysates from lymphotactin transfectants after seven days in culture revealed higher levels of the 17 kDa protein species than cells at earlier time points. In general, lymphotactin expression declined with time. The bands appearing in day 7 lysates from both lymphotactin and lymphotactin and hIL-2 transfectants were less sharp than those from days 1 and 2 but nonetheless, still visible. Interestingly, lymphotactin levels appeared to be higher in irradiated than non-irradiated cells and *in vitro* expression of the chemokine persisted longer (**Fig 5.9**). No lymphotactin expression was detected in irradiated cells transfected with an empty vector (pCI). In order to exclude the possibility that γ-irradiation may be inducing secretion of endogenous lymphotactin, pCI-transfected cells were analysed seven days after irradiation, at which time point, the entire population was undergoing apoptosis.

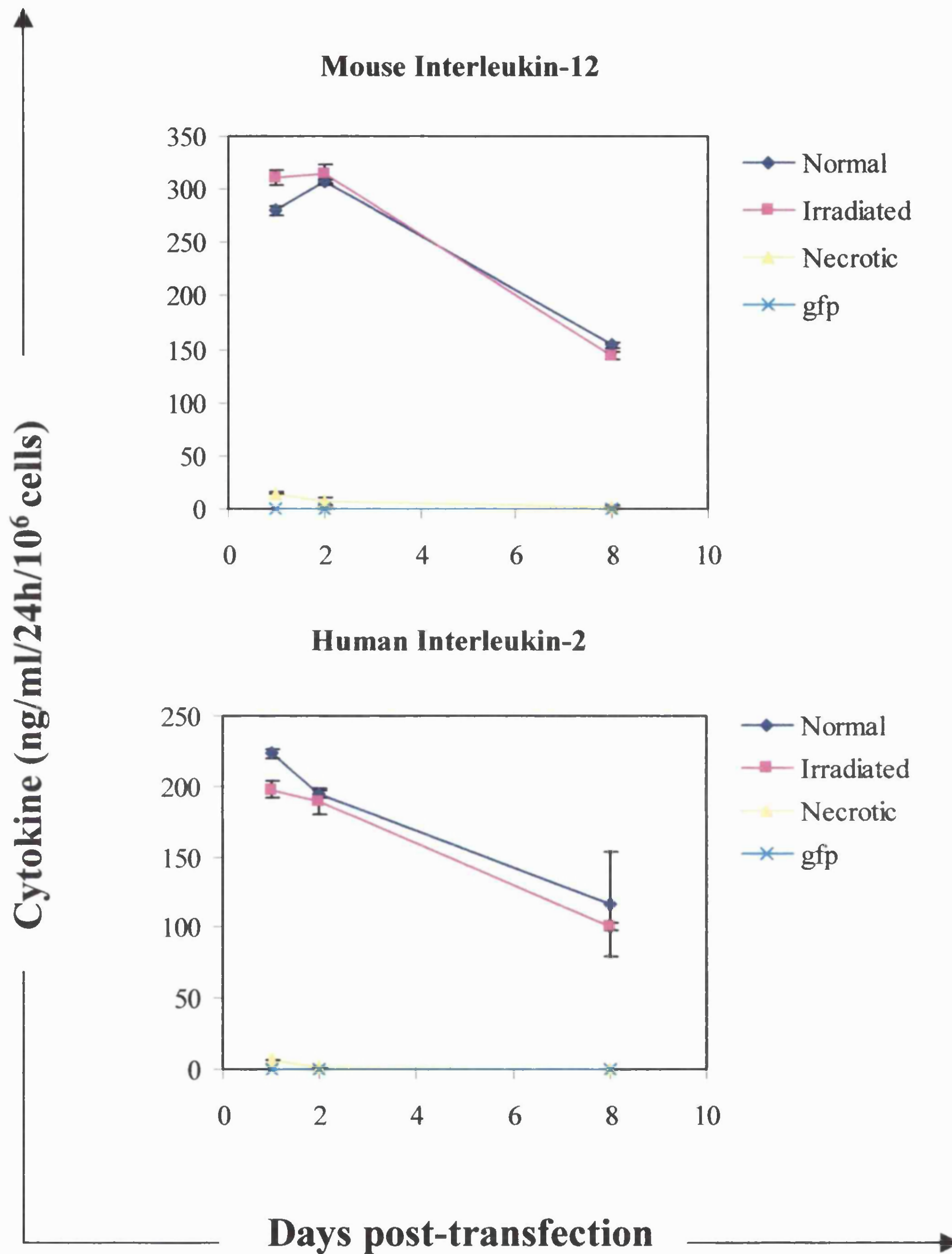


Fig 5.8 Comparison of cytokine expression by irradiated, necrotic and normal transfected Neuro-2A cells. Cells were transfected with cytokine genes and 18 h later subjected to γ-irradiation, freeze-thawing at –70 °C or no treatment, respectively. Supernatants were collected as described before and analysis was performed by ELISA.

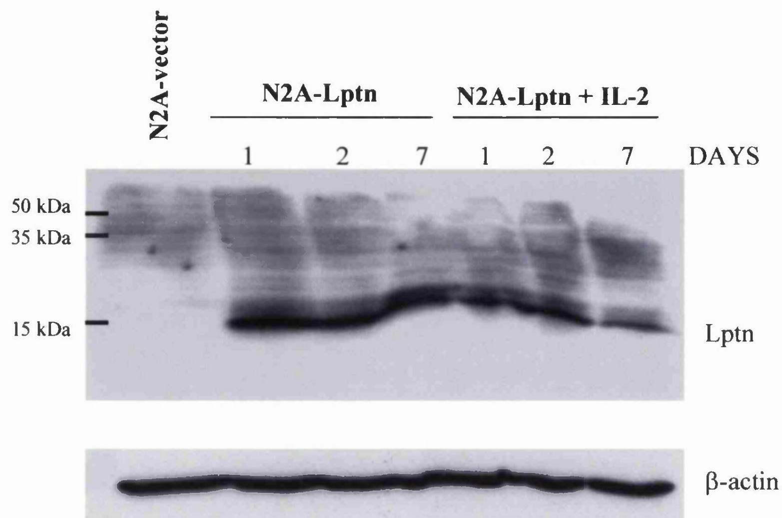


Figure 5.9 Expression of murine lymphotactin by γ-irradiated transfected Neuro-2A cells. Following transfection with murine lymphotactin alone or in conjunction with human IL-2 , Neuro-2A cells were subjected to γ-irradiation (25 Gy) 18 hrs after transfection. Murine lymphotactin could be detected as a protein species of 12-15 kDa size while a secondary species (~17 kDa) was visible which corresponds to a glycosylated form of the protein. Equal protein loading was confirmed by β-actin expression.

5.3 DISCUSSION

Transfection of two human and one mouse neuroblastoma cell lines using the LID vector resulted in the secretion of significant concentrations of IL-12 and IL-2 that are above potentially therapeutic levels (150 pg/ml/24h/10^6cells) required to elicit an immune response (Brenner *et al.*, 2000, clinical protocol). Despite the fact that transfection is transient and no integration of the transgene will occur unless the transfectants are placed under selection, *in vitro* expression of the cytokine genes persisted for at least ten days for the human cells and seven days for the mouse cell line. The levels of cytokines were comparable to reported systems, where delivery of the cytokine cDNAs is carried out using a viral construct (Leimig *et al.*, 1996; Emtage *et al.*, 1999).

The interesting aspect of the LID vector was that delivery of both IL-2 and IL-12 genes into the cells was performed by an easy transfection process with the cytokine cDNAs being carried on bacterial plasmids. Although the cytokine concentrations were significantly lower than that of single transfections in human cells, the cytokine levels were sustained above the therapeutic levels for at least ten days. In the mouse Neuro-2A cell line, co-transfection of both cytokines, produced identical levels of both IL-2 and IL-12 to those of single transfection experiments. A similar effect was observed when lymphotactin and IL-2 were introduced in these cells as determined by Western analysis although lymphotactin levels appeared to decline seven days after transfection. Quantitative analysis of lymphotactin levels was not performed and the aim was to directly test its biological activity *in vivo* as described previously (Emtage *et al.*, 1999).

The cytokines produced by transfected neuroblastoma cell lines induced the proliferation of PHA-stimulated lymphocytes compared to supernatant from control-transfectants. Lymphocytes activated with PHA or ConA and rhIL-2 are known to constitutively express high levels of the IL12Rβ1 and IL12Rβ2 receptors that comprise the IL-12 receptor complex (Nagayama *et al.*, 2000). Binding of IL-12 to these cells triggers a number of functions such as cell proliferation and secretion of

certain cytokines such as IL-12, IFN-γ, TNF-α and IL-6. IL-2 has a superior mitogenic effect on PHA-activated lymphocytes than IL-12, although it requires much higher concentrations compared to IL-12, which exerts its mitogenic effect at nanomolar concentrations (Gately *et al.*, 1991). When recombinant human IL-2 and IL-12 were used at varying concentrations, proliferation of PHA-activated lymphocytes reached a plateau above a concentration of 0.5 ng/ml for both cytokines, but the effect of IL-2 was much higher than IL-12. Diluting the samples up to 50-fold did not seem to alter the proliferative response of the PHA-stimulated lymphocytes (data not shown).

Furthermore, PHA-activated lymphocytes respond to a combination of IL-2 and IL-12 only when sub-optimal concentrations are used. The proliferative effect in such an instance does not surpass the maximum of that induced by IL-2 alone (Gately *et al.*, 1991). Therefore, the fact that supernatant from IL-2 and IL-12 co-transfected cells did not have an additive proliferative effect compared to IL-2 itself could be due to a saturation effect in proliferation. This would mean that although both IL-2 and IL-12 receptors are occupied and proliferation of PHA-activated lymphocytes is induced independently by each cytokine, there is a maximum mitogenic effect that can take place. In the case of murine IL-12, it enhances proliferation of human T cells since it can bind to the human form of IL12R (Schoenhaut *et al.*, 1992). The bioactivity of mIL-12 expressed by the pDNA3.1scIL12 construct was tested on ConA-stimulated murine splenocytes (Lode *et al.*, 1998). When secreted by transfected mouse Neuro-2A cells, mIL-12 was effective on human lymphocytes at levels equivalent to hIL-12. The low ^{3}H-thymidine incorporation observed in PHA-blasts compared to reported values, could be attributed to the mitogenic stimulation period of the cells or even due to the fact that in the constructs used both subunits of IL-12 are co-expressed using a linker. The end molecule is still bioactive, but may have reduced proliferative efficacy on T cells compared to recombinant IL-12. Alternatively, it could be due to the lymphocyte preparation as the number of IL-12R sites and their binding and dissociation constants depend on the individual preparations of lymphocytes (Gately *et al.*, 1991). In general, the cytokines secreted by LID-transfected neuroblastoma cell

lines exhibited biological efficacy by inducing a significant proliferation of PHA-stimulated lymphocytes, which provides an indication for their function *in vivo*.

Subjecting tumour cells to γ-irradiation is a widely used technique for inhibiting their growth. Irradiation should be performed to a degree that will allow continued cytokine expression but will prevent cells from dividing, thus avoiding the risk of adding to the patients' tumour burden. Following the growth pattern of irradiated Neuro-2A cells, their proliferation was reduced compared to parental non-irradiated cells immediately after irradiation. By day 7, they had ceased proliferating and were progressing from early to late apoptosis as revealed by staining with annexin-V FITC and propidium iodide. The expression pattern of cytokines, however, was not altered. IL-2 and IL-12 levels secreted by transfected irradiated Neuro-2A cells were identical to those of non-irradiated cells as was detected by ELISA. Lymphotactin expression was similar if not higher in irradiated cells. Interestingly, irradiated cells seemed to express higher levels of a ~17 kDa protein species which was very faintly visible in non-irradiated cells.

In conclusion, cytokine transfection with the LID vector was determined in neuroblastoma cell lines and certain parameters optimised prior to applying the vaccine to a mouse model for the malignancy. The cytokine levels produced by transfected Neuro-2A cells should be adequate for immunomodulation despite the limited duration of expression. The *in vitro* cytokine expression persisting *in vivo* for ten days should provide a sufficient time window for an immune response to be elicited.

6

In vivo Studies

6.1 INTRODUCTION

Study of a number of diseases has been enabled by the use of animal models. Such models facilitate understanding of the biology of disease and, in many cases, including some cancer models, allow the investigation of potential therapies. A murine model for neuroblastoma has been developed based on clones of a spontaneous neuroblastoma in strain A mice. The C1300 cell line or its clone, Neuro-2A, have been extensively used to characterise murine neuroblastoma and define its effectiveness as a model of human disease.

Numerous similarities in tumour growth and metastatic behaviour have been reported between human and murine neuroblastoma. Human neuroblastoma grows progressively at sites of neural crest cell origin and can invade surrounding tissues. In a similar way, murine neuroblastoma exhibits progressive growth with invasion of neighbouring sites until the death of the animal. Although both human and murine neuroblastoma consist of cells that are mainly in the resting stage, histological examination of murine disease has failed to reveal rosette formation of neuroblasts, which is a characteristic of human disease. Metastases formation of human neuroblastoma is frequent and involves sites such as lymph nodes, liver, bone marrow and bone cortex. The metastatic behaviour of murine neuroblastoma is limited to very late stages of the disease and it usually involves regional lymph nodes but rarely any other organs. However, spontaneous regression has rarely been observed in the murine model although it is a well-documented phenomenon in patients with clinical stage 4S neuroblastoma especially those under 1 year of age. Human and murine neuroblastoma also share expression of certain surface molecules, such as neural cell adhesion molecule (NCAM) and low levels of MHC class I molecules (Ziegler *et al.*, 1997).

Furthermore, there are indications of similar host immunoregulation mechanisms operating in human and murine neuroblastoma. Patients demonstrate natural NK cytotoxic activity to neuroblastoma and lack of specific CTL function (Main *et al.*,

1985). In a similar way, the C1300 murine neuroblastoma cell line exhibits NK cell sensitivity suggesting that augmentation of this effector cell population may comprise a potential anti-tumour immunotherapeutic protocol (Choi *et al.*, 1989). These similarities between human and murine disease have rendered the mouse model for neuroblastoma an invaluable research tool and have prompted numerous studies especially on the investigation of therapeutic regimes. However, it is vital that the efficacy and safety of such protocols is tested on human subjects in phase I clinical trials.

The experiments described in this chapter are based on testing cytokine-transfected Neuro-2A cells as a vaccine in the A/J mouse model for neuroblastoma. Various aspects of this vaccine have been addressed such as the effect of cytokine transfection on the immunogenicity of the cells and the potential for prophylactic immunisation. In addition, the effect of vaccination on established experimental tumours as well as the nature of the anti-tumour immune response have been examined.

6.2 RESULTS

6.2.1 *In vivo* tumourigenicity of cytokine-transfected neuroblastoma cells

To establish whether the cytokines produced by neuroblastoma cells were biologically active *in vivo* and to test the cytokine-transfected cells in an animal neuroblastoma model, 6-8 week-old A/J mice were subcutaneously inoculated with 10^6 unmodified or cytokine-transfected Neuro-2A cells. Transfection was performed one day prior to inoculation into syngeneic mice (**Fig 6.1A**). Animals receiving cells transfected with IL-12 or a combination of IL-2 and IL-12 remained tumour-free for at least 75 days. IL-2 secreting Neuro-2A cells also failed to grow tumours in 83% of the animals. All mice inoculated with wild-type Neuro-2A cells developed tumours within 12 days after injection (**Fig 6.1B**). The tumourigenicity of cytokine-transfected cells (N2A-IL-2, N2A-IL-12 and N2A-IL-2+IL-12) was significantly reduced compared to vector-transfected cells that induced tumour formation in 70% of the animals ($p<0.05$).

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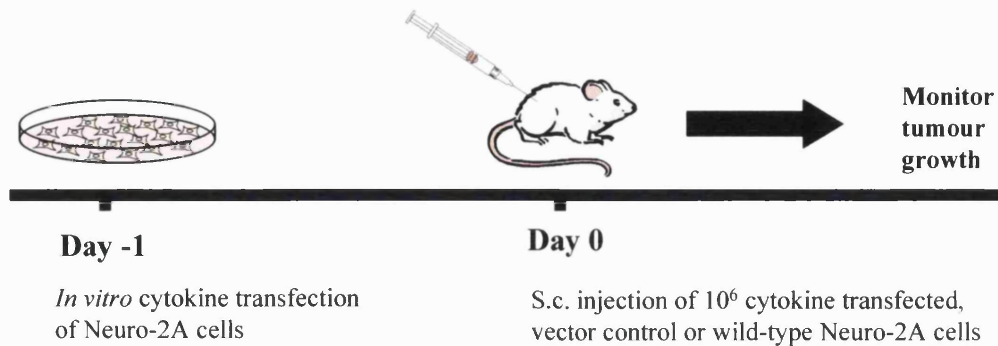

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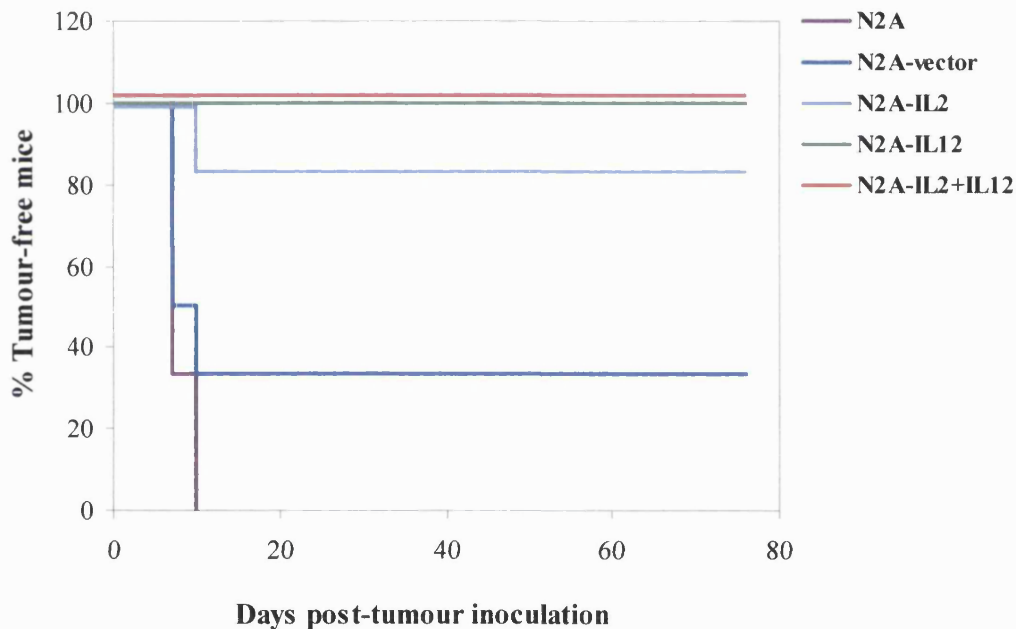

Figure 6.1 *In vivo* tumourigenicity of cytokine-transfected Neuro-2A cells in syngeneic mice. A. Overview of experimental procedure. Cells were transfected one day prior to inoculation into A/J mice. 10^6 live untransfected, vector control, IL-2, IL-12 or IL-2 and IL-12- producing cells were administered subcutaneously into n=6 mice per group. **B.** Reduced in *vivo* tumourigenicity of cytokine-transfected Neuro-2A cells compared to vector-transfected cells. Tumour growth was monitored over 75 days and a survival curve was constructed. Representative results from one out of two experiments are shown.

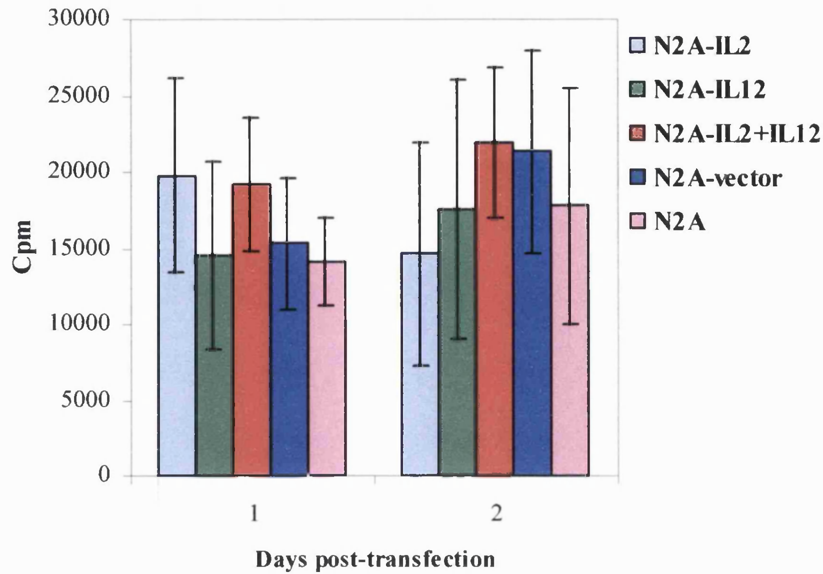

Figure 6.2 ***In vitro*** **proliferation of cytokine-transfected Neuro-2A cells used in syngeneic mice.** Proliferation of transfected Neuro-2A cells was examined one and two days after transfection by ^{3}H-thymidine incorporation over a 16 h period. No difference in the cpm values between the different cell types could be observed.

Cells were tested for their *in vitro* proliferation by ^{3}H-thymidine incorporation to ensure that any differences in tumourigenicity were not a result of altered cell proliferation (**Fig 6.2**). No difference in cell proliferation could be observed between cytokine-transfected and vector-transfected cells, suggesting that their different tumourigenicity *in vivo* was a result of cytokine expression. Also, the proliferation of all LID-transfected cells was similar to that of unmodified parental Neuro-2A cells. For cytokine expression of the cells used in this experiment see Appendix C1.

6.2.2 Prophylactic application of the vaccine

In order to determine whether protective immunity could be generated after immunisation with cytokine-transfected Neuro-2A cells, animals were subcutaneously vaccinated with 10^6 γ-irradiated (25 Gy) cytokine- or vector-transfected cells. Transfection was again performed one day prior to vaccination. Control vaccinations consisted of PBS or untransfected irradiated Neuro-2A inoculations. Seven days later, animals were challenged with 10^6 parental Neuro-2A cells by subcutaneous inoculation on the opposite flank and tumour growth was monitored every two days (**Fig 6.3A**). The cytokine expression of transfected Neuro-2A cells was monitored *in vitro* and is shown in Appendix C2a.

Animals that had been inoculated with PBS rapidly grew tumours upon challenge with wild type Neuro-2A cells (**Fig 6.3B**). Vaccination with N2A-IL-12 or N2A-IL2+IL12 cells offered 80% protection against challenge with parental cells and vaccinated animals remained tumour-free for the entire course of the experiment. A similar effect to that of cytokine-transfected Neuro-2A cells was observed with control-transfected Neuro-2A cells. Indeed, 100% of mice receiving N2A-vector cells exhibited protective immunity upon challenge with parental Neuro-2A cells. Administration of either N2A-IL-2 or unmodified irradiated Neuro-2A cells exhibited protective immunity in 60% of the animals. There was no significant survival difference between the different vaccination groups suggesting that irradiated Neuro-2A cells alone are immunogenic. This was true in a number of experiments where animals were pre-

vaccinated with 10^6 N2A cells (**Table 6**). Although 91% of the animals vaccinated with PBS developed tumours, 88% of mice receiving N2A-vector cells were protected against challenge with parental Neuro-2A cells.

In order to investigate whether the amount of tumour antigens provided by the irradiated cell vaccine masked the effect of cytokines expressed by these cells, a titration experiment was performed. N2A-IL-12 and N2A-IL-2+IL-12 vaccines offered similar protective immunity to animals upon challenge with parental cells (**Fig 6.3B**). Therefore, vaccination with various concentrations of N2A-IL-12 cells was compared against the same number of N2A-vector cells. Cytokine expression of these cells is depicted in Appendix C2b. The number of cells used to pre-vaccinate animals (n=6 per group) ranged from 10^3-10^6. The cells were again transfected one day before immunisation and γ-irradiated at 25 Gy.. Upon challenge with 10^6 wild-type Neuro-2A cells a week later, both groups of N2A-IL-12 and N2A-vector vaccinated animals exhibited systemic immunity when they had been immunised with either 10^6 or $2x10^5$ cells. There was no actual survival difference between the groups of animals receiving 10^6 or $2x10^5$ cells. 70-100% of the immunised mice remained tumour-free for over 70 days (**Fig 6.4A**). When the number of cells used in the pre-vaccination step decreased to 10^5 cells per animal, there was a steep reduction in survival. Protective immunity was evident in only 20-40% of the immunised mice when 10^5 cells were used. Again there was no significant survival difference between N2A-IL-12 and N2A-vector pre-vaccinated animals (**Fig 6.4B**). Reducing the number of vaccinating cells even further to 10^4 or 10^3 per animal totally abrogated any protective immunity against parental Neuro-2A cells. All animals immunised with 10^4 or 10^3 cells (N2A-IL-12 or N2A-vector) developed tumours within 7 days after challenge with wild-type cells. These results verify the immunogenicity of Neuro-2A cells when provided at a concentration of up to $2x10^5$ irradiated cells per animal. Pre-vaccination of animals with irradiated cells alone seems to induce sufficient protective immunity against challenge with wild-type cells.

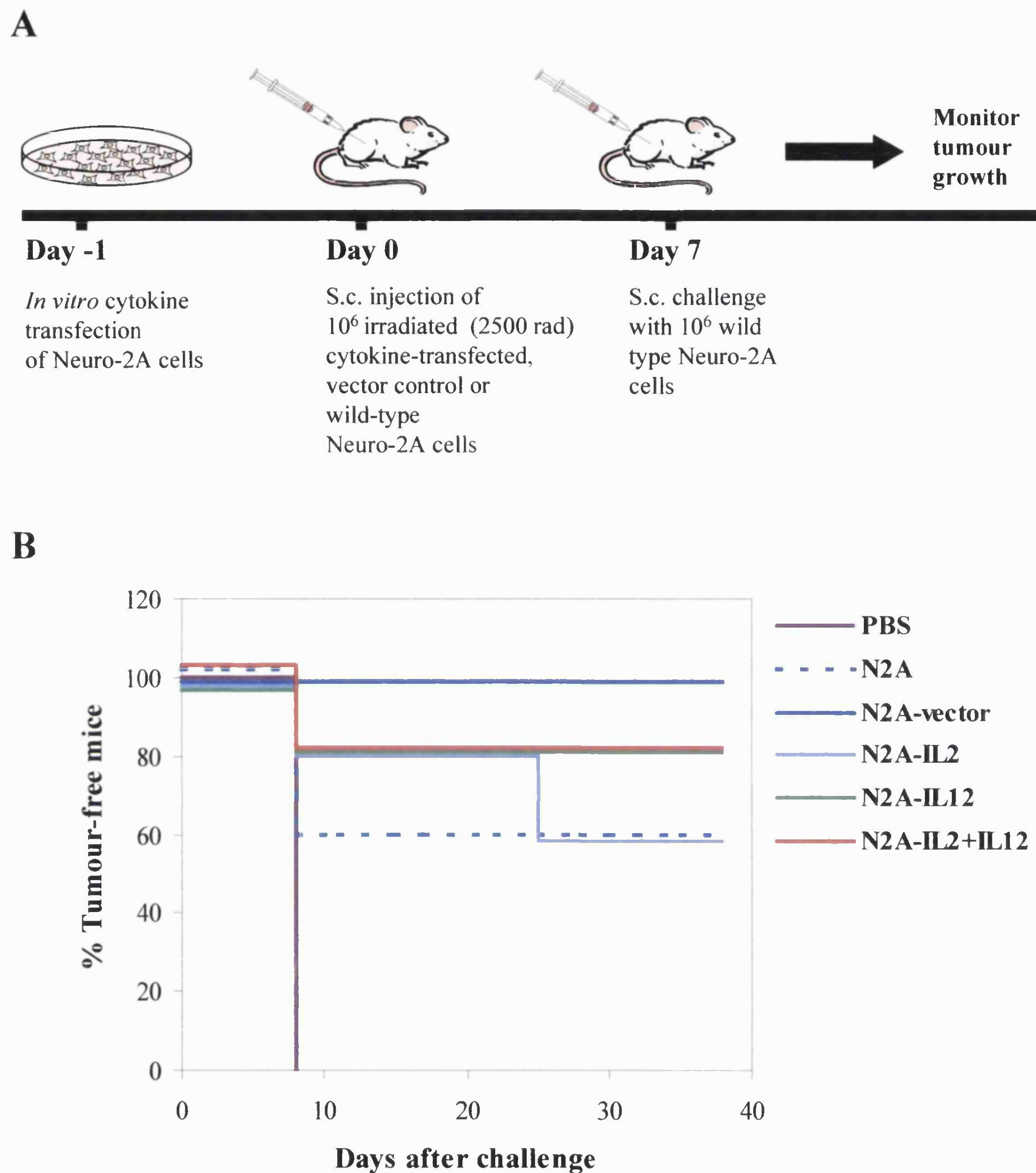

Figure 6.3 Effect of vaccine application on systemic anti-tumour immunity. A. Overview of experimental procedure. Cells were transfected with vector control, IL-2, IL-12 or IL-2 and IL-12 and γ-irradiated (25Gy). On day 0 animals were pre-vaccinated with 10^6 irradiated Neuro-2A cells and challenged one week later on the opposite flank with the same number of parental Neuro-2A cells. **B.** Survival of pre-vaccinated mice over a period of 38 days. Pre-vaccination with irradiated cells alone generated protective immunity irrespective of cytokine production.

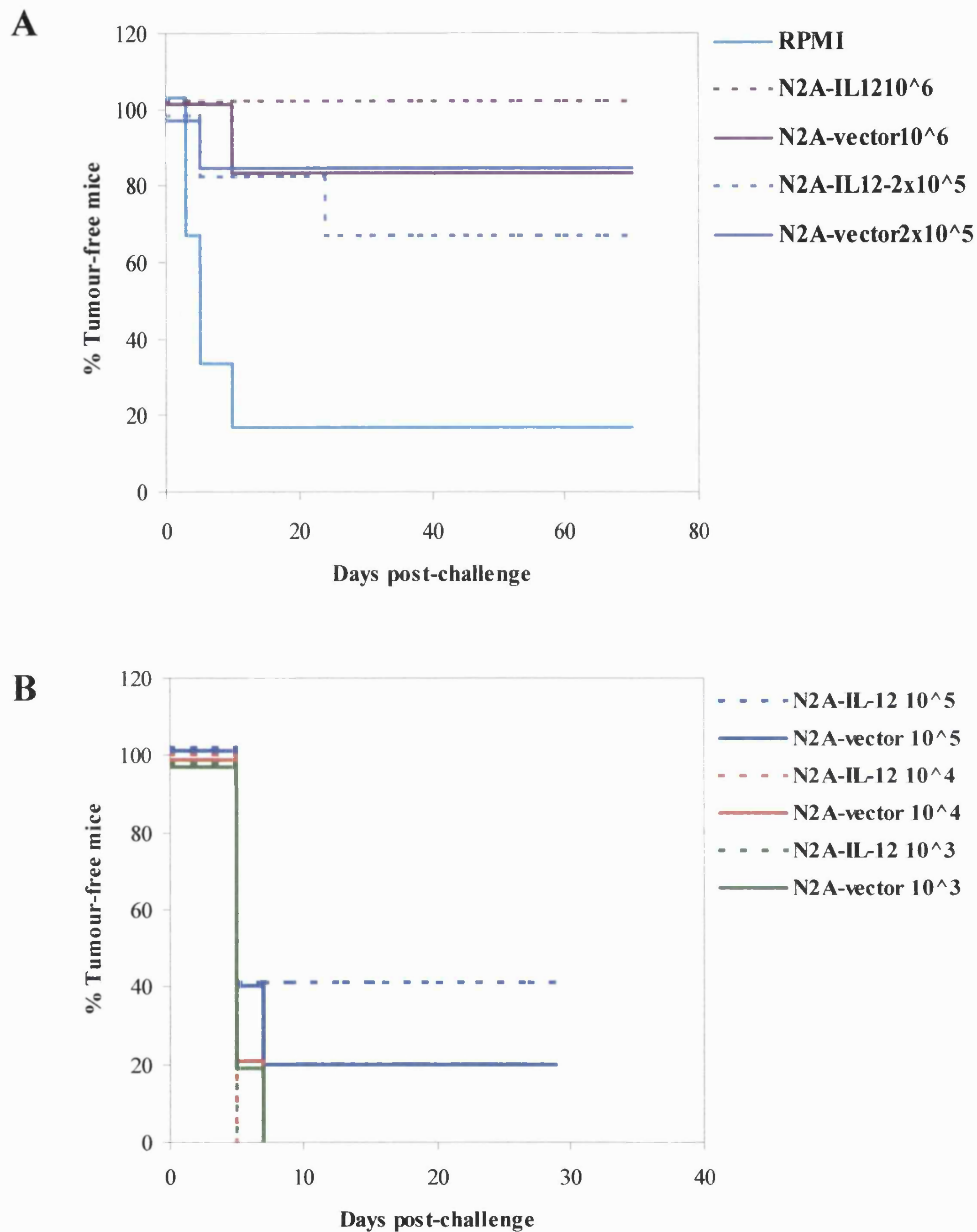

Figure 6.4 Titration of the number of Neuro-2A cells used in pre-vaccination experiments. Pre-vaccination of syngeneic A/J mice was performed by varying the number of cells present in the vaccine (10^3-10^6). The experimental strategy was similar to that described in Fig 6.2 and vaccinated animals were challenged with 10^6 parental Neuro-2A cells 7 days post-vaccination.

Table 6 Summary of results of pre-vaccination experiments

Vaccine	Animals with tumours	Percentage
PBS	10/11	91%
N2A	12/21	57%
N2A-vector	3/26	12%
N2A-IL-2	6/10	60%
N2A-IL-12	4/16	25%
N2A-IL-2+IL-12	8/22	36%

6.2.3 Eradication of established tumours

Most cancer vaccines will be used on patients with primary tumours or on those who after initial treatment have relapsed to show metastases formation in other tissues. Therefore, the most clinically relevant aspect of a cancer vaccine is its efficacy on established disease. In order to address this issue, we induced subcutaneous tumours in A/J mice by inoculation in the right flank with 10^6 wild-type Neuro-2A cells. When a tumour mass was palpable and the tumour had reached a diameter of approximately 5 mm, animals received an intratumoural vaccination of 10^6 vector- or cytokine-transfected Neuro-2A cells that had been γ-irradiated (25 Gy) (**Fig 6.5A**). There was no significant difference among the tumour sizes of animals in each experimental group on the day of vaccination ($p>0.2$). Cytokine expression of the cells used in this vaccination experiment is shown in Appendix C3a.

Tumour growth in vaccinated animals was monitored every two days. Mice vaccinated with vector-transfected or IL-2-transfected Neuro-2A cells showed rapid tumour progression and were sacrificed 7 days after vaccination because of their tumour burden. The interesting aspect of combining IL-2 and IL-12 in the vaccine strategy can be observed by looking at the tumour growth pattern of individual mice. 50% (3/6) of mice receiving the N2A-IL-2+IL-12 vaccine showed delayed tumour

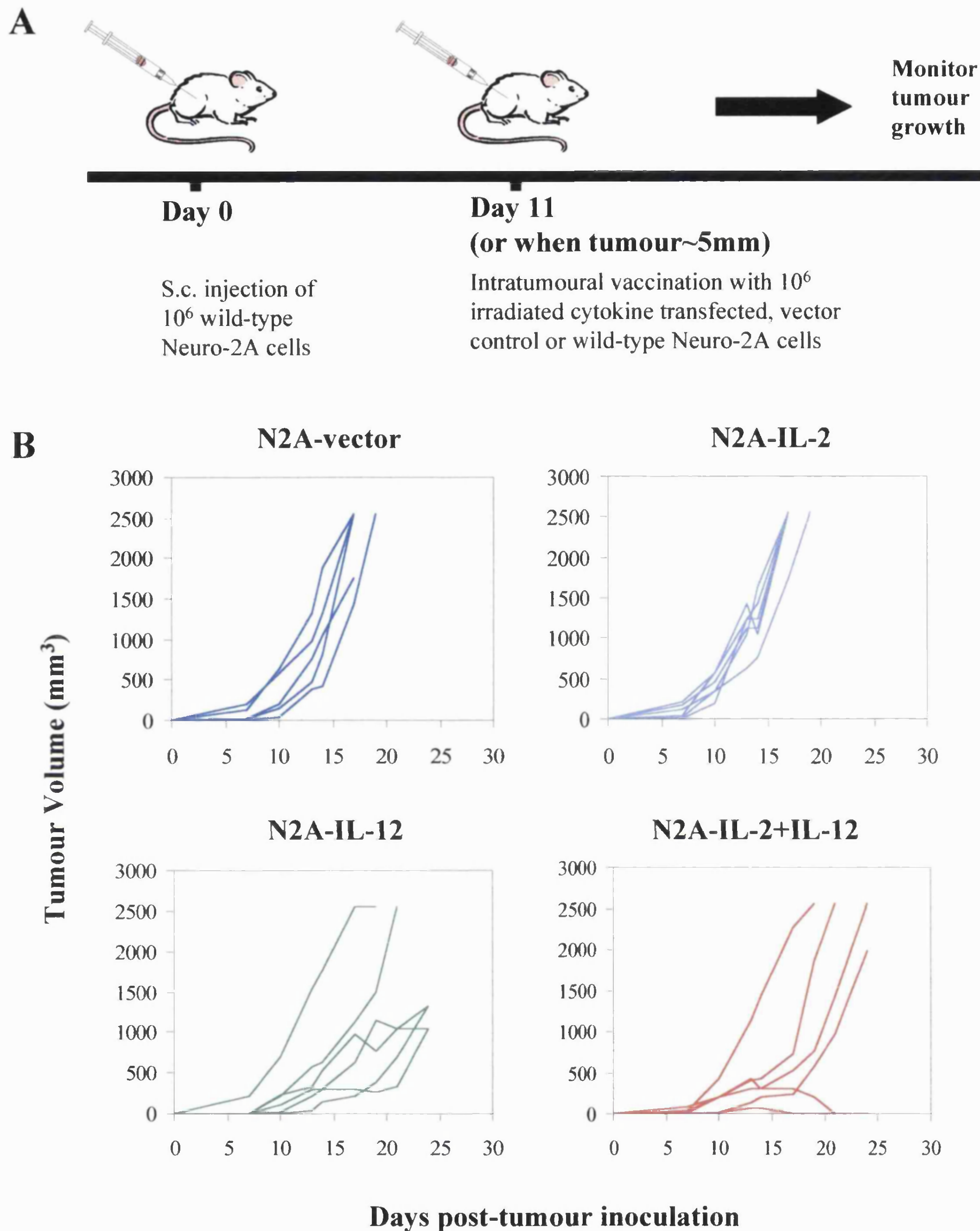

Figure 6.5 Effect of vaccination on established murine tumours. A. A/J mice (n=6 per group) were subcutaneously inoculated with 10^6 parental Neuro-2A cells and 11 days later vaccinated intratumourally with an equal number of irradiated (25 Gy) vector- or cytokine-transfected Neuro-2A cells. The tumour growth in each animal was monitored and is represented by a single line. **B.** Tumour growth of individual mice following intratumoural vaccination. The tumour size of individual mice in mm^3 is plotted over time. Each line represents the tumour growth pattern of one animal.

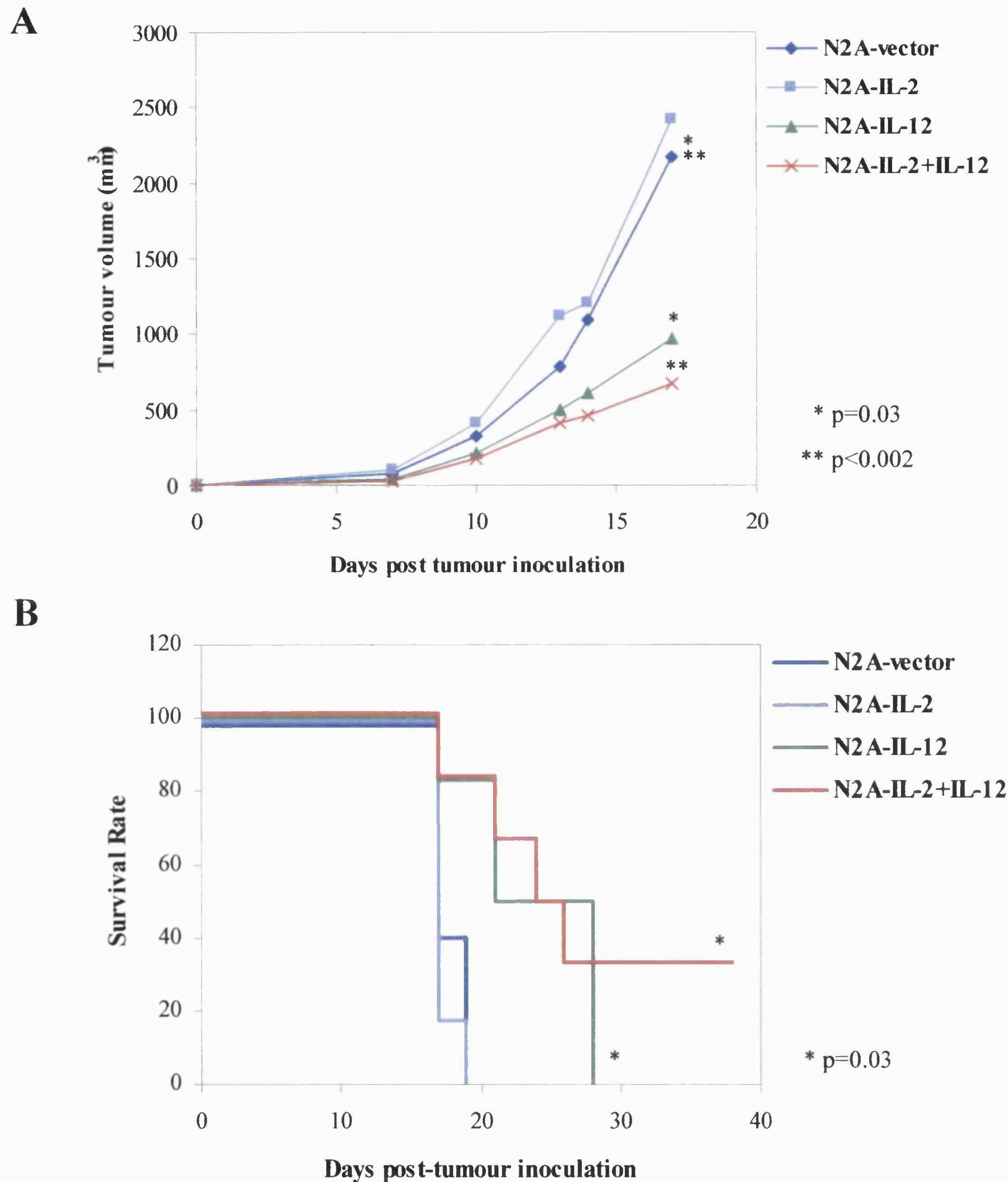

Figure 6.6 Effect of intratumoural vaccination on the growth and survival of animals with established tumours. A. Vaccination of animals with cells expressing IL-12 alone or in combination with IL-2 resulted in significant delay (p=0.03 and 0.002 respectively) in tumour growth compared to control-transfected cells. **B.** Enhanced survival of animals receiving N2A-IL12 or N2A-IL2+IL12 vaccine. Mice receiving N2A cells expressing IL-12 alone or in combination with IL-2 survived longer than those receiving the control vaccine (p=0.03). Representative results from one out of two experiments are shown.

growth compared to animals receiving N2A-vector cells. Complete tumour regression was evident only in this group in 33% mice, which remained tumour-free for the entire course of the experiment (>40 days) (**Fig 6.5B**). Expression of IL-12 by the immunising cells delayed tumour growth in 4/6 mice compared to the group receiving the N2A-vector vaccine. Comparison of tumour size 6 days after vaccination (day 17 post-tumour inoculation) among groups receiving N2A-vector cells and those immunised with the N2A-IL-12 or N2A-IL-2+IL-12 vaccines, revealed that the tumours of the latter two groups of animals were smaller ($p<0.04$ and $p<0.002$ respectively) (**Fig 6.6A**). No difference in tumour growth was observed after vaccination with N2A-IL2 or N2A-vector cells ($p>0.3$).

In addition, the survival of animals immunised with N2A-IL-12 or N2A-IL-2+IL-12 cells was significantly enhanced compared to animals vaccinated with N2A-vector cells ($p<0.03$) (**Fig 6.6B**). Mice receiving the N2A-IL-2+IL-12 vaccine survived for 27±9 days compared to only 18±1 days for those vaccinated with vector-transfected Neuro-2A cells and 17±1 days for animals receiving N2A-IL-2 cells. The N2A-IL-12 vaccine prolonged survival at an average of 24±5 days.

In a second eradication experiment, vaccination with N2A-IL-2 or N2A-vector had again no effect in tumour growth of immunised animals (**Fig 6.7**). Examination of the tumour growth pattern in individual mice revealed that 50% of animals receiving the N2A-IL-2+IL-12 vaccine showed delayed tumour growth while another 50% exhibited tumour regression. However, these regressing tumours grew back 8-11 days after vaccination. Interestingly, 60% (3/5) animals that received the N2A-IL-12 vaccine completely regressed while the tumours in the remaining 40% mice grew aggressively, resembling the tumour growth pattern of animals vaccinated with N2A-vector or N2A-IL-2 cells.

Comparison of the mouse tumour volumes among groups on day 14 post-tumour inoculation revealed that only N2A-IL-2+IL-12 significantly inhibited tumour growth this time compared to vaccination with vector-transfected Neuro-2A cells ($p<0.002$) (**Fig 6.8A**). Animals treated with N2A-IL-12 cells developed tumours that were not

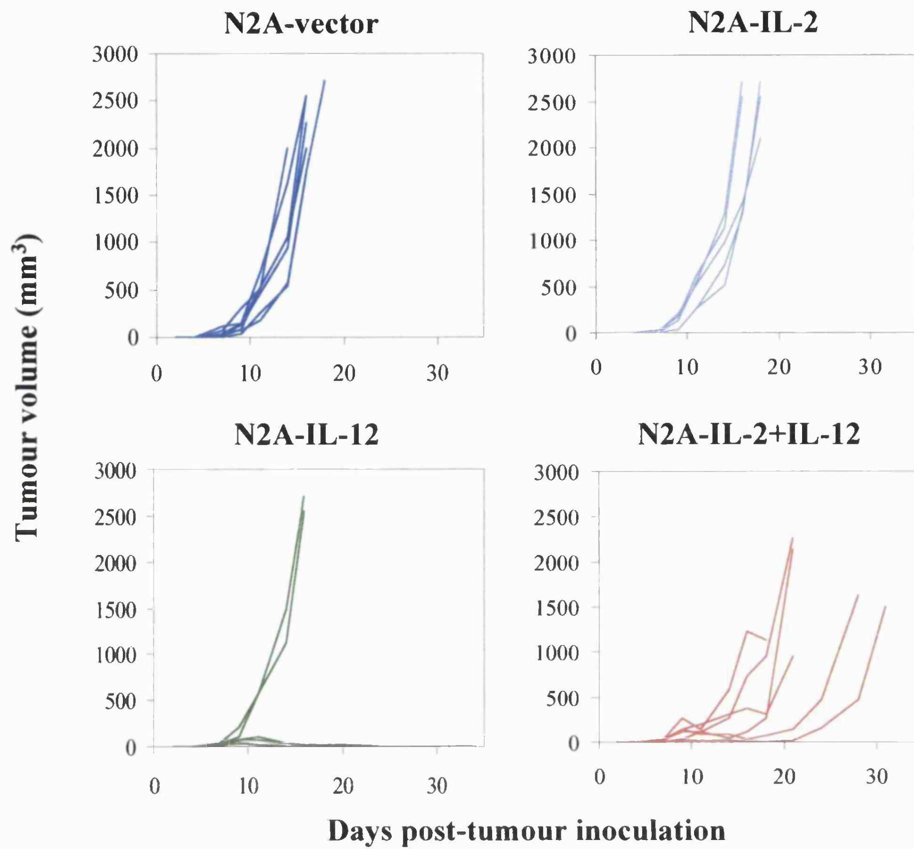

Figure 6.7 Tumour growth of individual animals used in a second eradication experiment. Each graph represents a different experimental group. The tumour growth pattern of each mouse in the different experimental groups is depicted as a single line and plotted over time.

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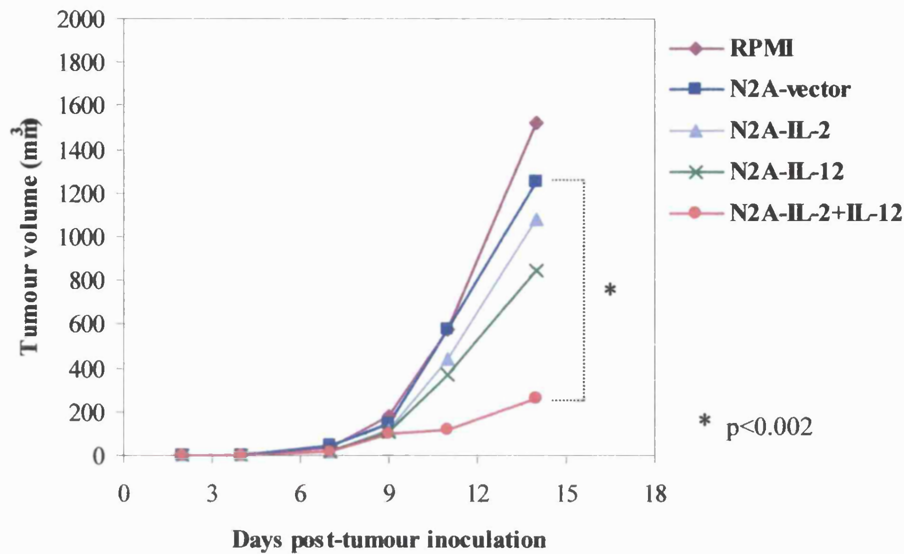

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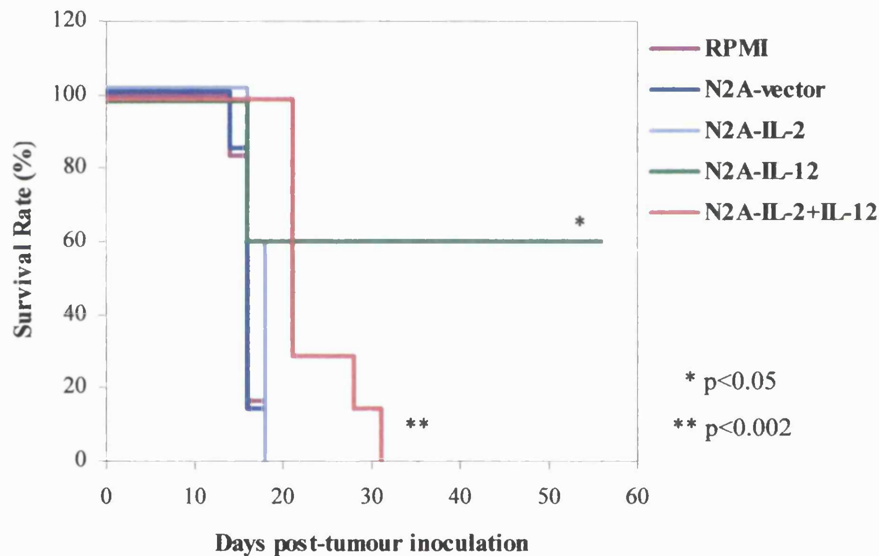

Figure 6.8 Average tumour growth and survival of animals from eradication experiment 2. A. Tumour inoculation was performed as described in Fig 6.5A and intratumoural vaccination was carried out on day 9 post-inoculation. The average tumour size in each experimental group is plotted over time. **B.** Survival rate of vaccinated animals in different experimental groups. Vaccination with N2A-IL-12 or N2A-IL-2+IL-12 cells significantly prolonged survival compared to vaccination with N2A-vector cells.

significantly smaller on day 14 than those of N2A-vector vaccinated animals ($p>0.3$). Vaccination with either N2A-IL-12 or N2A-IL-2+IL-12 cells significantly prolonged survival compared to vaccination with N2A-vector cells ($p<0.05$ and $p<0.002$ respectively) (**Fig 6.8B**). Animals receiving the N2A-vector or N2A-IL-2 vaccine survived for 16±1 d and 17±1 d, respectively, while the survival of those vaccinated with N2A-IL-12 or N2A-IL-2+IL-12 was extended to 40±22 d and 23±5, respectively.

The cytokine levels produced by transfected Neuro-2A cells used in the eradication experiment 2 are shown in Appendix C3b.

6.2.4 Inoculation into γ_c/RAG2 knockout mice

As discussed in 6.2.1, cytokine-transfected Neuro-2A cells completely lose their tumourigenicity *in vivo*. In order to show that the cytokines expressed by these cells are effectively generating an immune response, γ_c/RAG2 knockout mice were subcutaneously inoculated with N2A-vector or N2A-IL2+IL12 cells. γ_c/RAG2 knockout mice lack any T, B and NK cells as they have a non-functional immunoglobulin γ chain and mutations in the recombination-activating gene 2 (RAG2) (Goldman *et al.*, 1998). The experimental procedure was similar to that described in 6.2.1. 10^6 live Neuro-2A cells that had been transfected the day before, were inoculated into γ_c/RAG knockout mice (n=3 per group) and tumour growth was monitored over a period of 20 days (**Fig 6.9A**). The mean tumour growth of animals in each experimental group is depicted in **Fig 6.9B**. Both groups of mice developed tumours within 8 days post-inoculation and their mean survival was 18 days. The *in vitro* proliferation of N2A-vector and N2A-IL-2+IL-12 was verified by ^{3}H-thymidine incorporation. No significant difference in proliferation could be observed between N2A-vector and N2A-IL-2+IL-12 cells indicating that their *in vivo* growth was not due to altered proliferation (**Fig 6.10**). Also the cytokine levels produced by the cells inoculated into gc/RAG2 knockout mice were monitored *in vitro* and are shown in Appendix C4. This result indicates that the reduced *in vivo* tumourigenicity of

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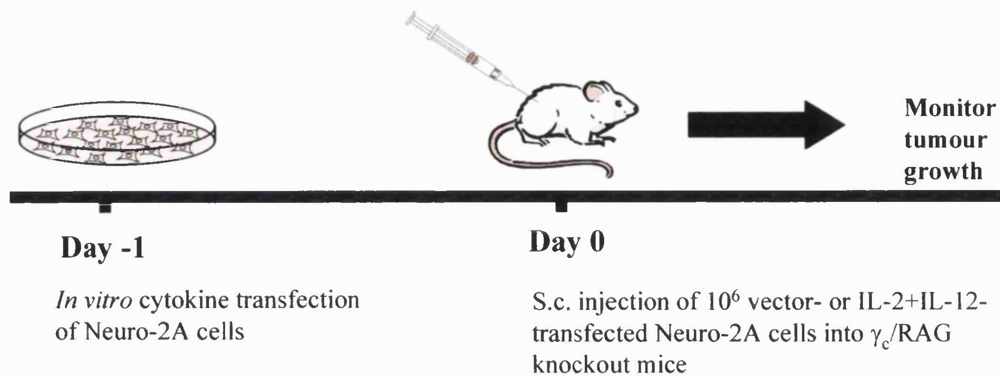

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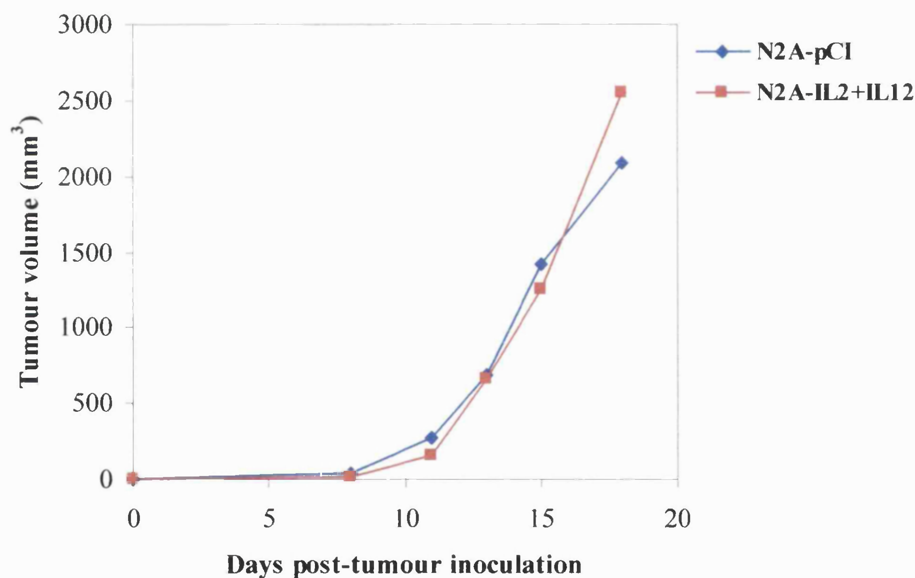

Figure 6.9 ***In vivo*** **tumourigenicity of N2A-IL-2+IL-12 cells in γ_c/RAG2 knockout mice. A.** Overview of experimental procedure. Cells were transfected one day prior to subcutaneous inoculation into γ_c/RAG2 knockout mice (n=3 per group) and the tumour growth monitored. **B.** Neuroblastoma tumour growth following s.c. inoculation. In the absence of immune effector cells, the *in vivo* tumourigenicity of cytokine-transfected Neuro-2A cells is restored. Mean tumour volumes of n=3 mice per group expressed in mm^3 are plotted over a period of 20 days.

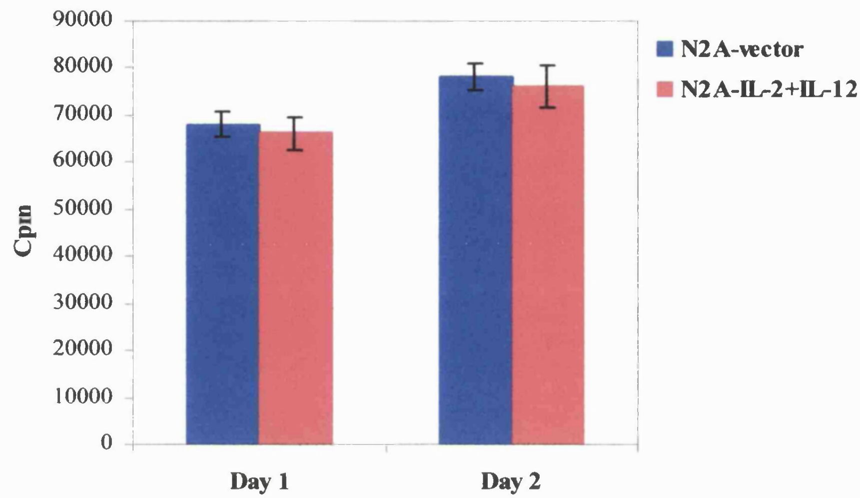

Figure 6.10 ***In vitro*** **proliferation of Neuro-2A cells used to inoculate γ_c/RAG2 knockout mice.** Transfection of Neuro-2A cells with an empty vector or combination of IL-2 and IL-12 did not alter their *in vitro* proliferation. This was estimated by incorporation of ^{3}H-thymidine one and two days following transfection.

cytokine-transfected Neuro-2A cells is an immunological phenomenon depending on T, B or NK cells.

6.2.5 *In vivo* immunodepletion

Studies in γ_c/RAG2 knockout mice indicated the involvement of immune cells in the abrogation of tumourigenicity of N2A-IL-2+IL-12 cells. However, more precise delineation of the effector cell group was necessary. Therefore, animals were immunodepleted *in vivo* by intraperitoneal administration of anti-CD4 or anti-CD8 antibodies. Immunodepletion commenced on days –6 and –2 with intraperitoneal inoculation of 100 μg anti-CD4, 180 μg anti-CD8 or PBS. The concentrations of administered antibodies were titrated *in vivo* (data not shown). Following subcutaneous injection of 10^6 N2A-IL-2+IL-12 cells, depletion of CD4 and CD8 cells was maintained by weekly antibody intraperitoneal administration (**Fig 6.11A**). In order to verify the state of immunodepletion, one mouse per group was sacrificed prior to N2A-IL-2+IL-12 inoculation and their splenocytes stained for CD4 or CD8. 21.4% and 16.9% of splenocytes from immunocompetent mice stained positive for CD4 and CD8, respectively (**Fig 6.12**). CD4-depletion resulted in disappearance of the distinct population in quadrant C1 and CD4 staining was only evident in 1.4% of splenocytes. After subtraction of the isotype control background, animals treated with anti-CD4 antibody had been depleted of 99% of their $CD4^+$ cells. Similarly, administration of anti-CD8 antibody, gave only 2.2% positive staining for CD8 which correlated to 93% CD8 depletion compared to immunocompetent mice. The CD8 and CD4 levels of CD4- and CD8-depleted animals respectively remained unaffected.

Tumour growth of N2A-IL-2+IL-12 cells in CD4- and CD8-depleted animals was compared to that in untreated immunocompetent mice. In addition, animals inoculated with control-transfected or untransfected Neuro-2A cells were used as controls. Wild-type Neuro-2A cells and control-transfected cells gave rise to subcutaneous tumours as shown in **Fig 6.11B**. Although there was a delay in tumour development in animals receiving N2A-vector cells compared to parental untransfected Neuro-2A cells, all

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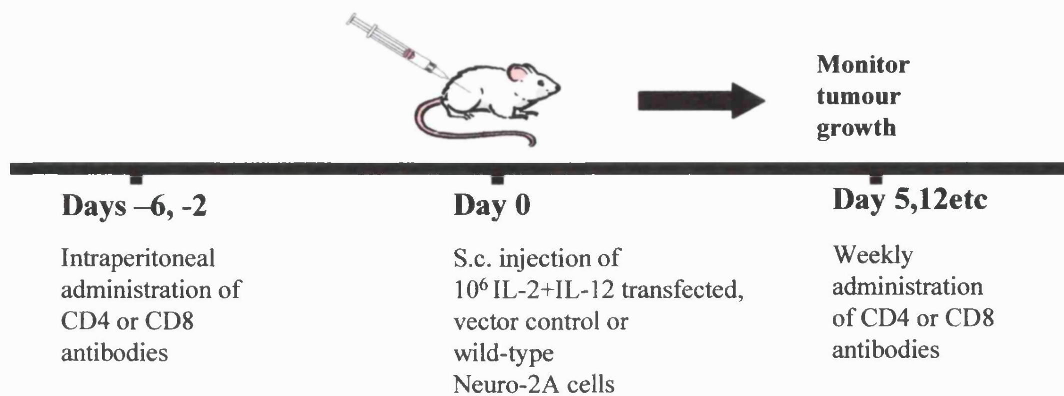

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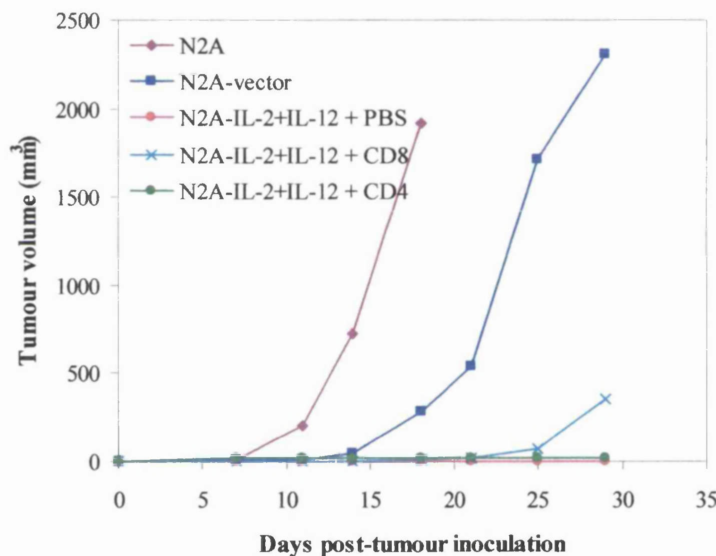


Figure 6.11 ***In vivo*** **CD4 and CD8 immunodepletion.** **A.** Overview of experimental procedure. Intraperitoneal administration of antibodies was carried out on days –6, -2 and weekly thereafter. Subcutaneous inoculation of 10^6 live untransfected, control- or IL-2+IL-12-transfected Neuro-2A cells was performed on day 0. **B.** Tumour growth of N2A-IL-2+IL-12 cells in CD4-, CD8-depleted or immunocompetent mice. The mean tumour volume of n=6 mice per group is plotted over time.

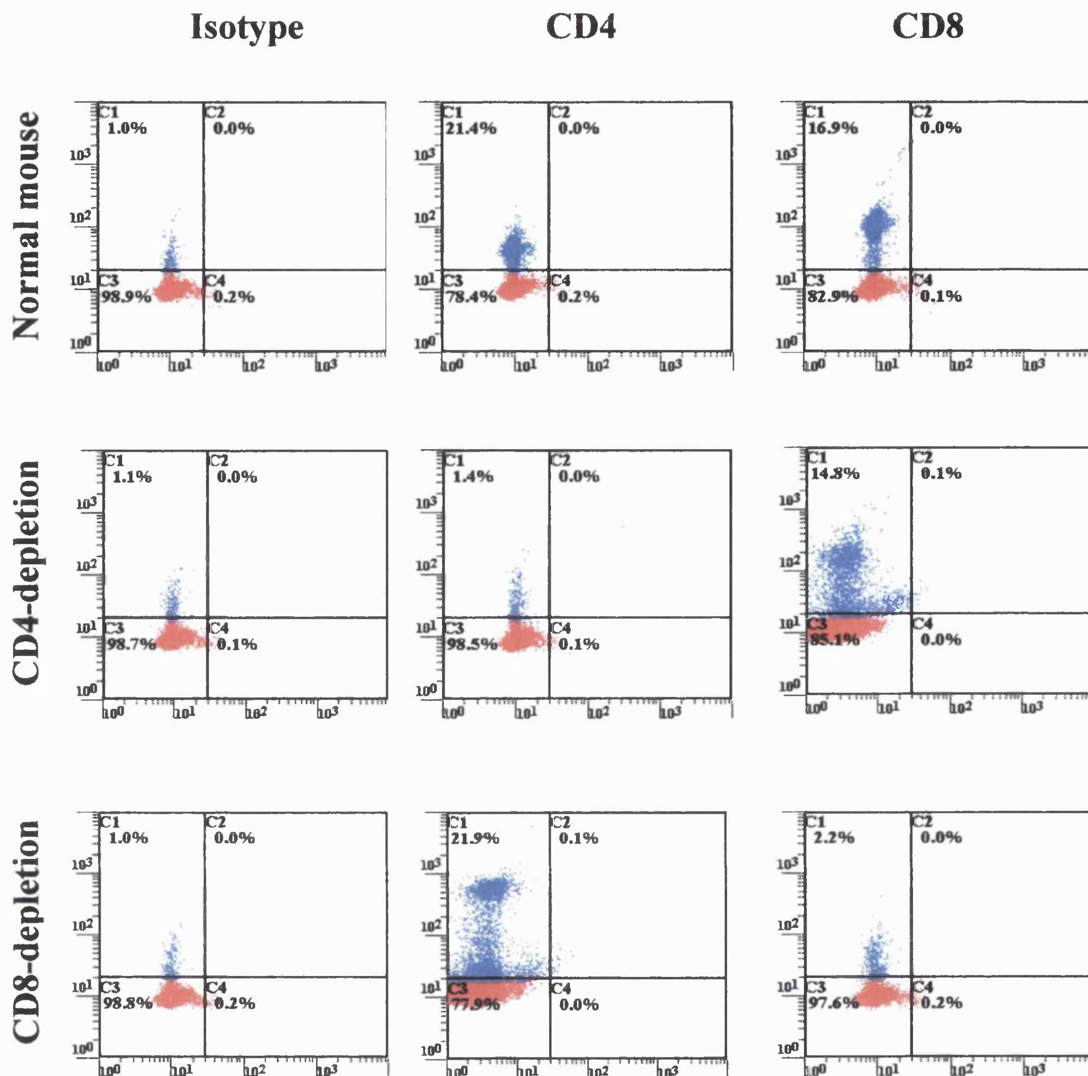


Figure 6.12 CD4 and CD8 staining of splenocytes from immunodepleted animals. Following two intraperitoneal administrations of anti-CD4 and anti-CD8 antibodies, one animal per group was sacrificed and their splenocytes analysed by FACS for CD4 and CD8 expression. Comparison with the CD4 and CD8 values of splenocytes from an immunocompetent mouse revealed that there was 99% CD4 and 93% CD8 depletion in animals treated with anti-CD4 and anti-CD8 antibodies, respectively.

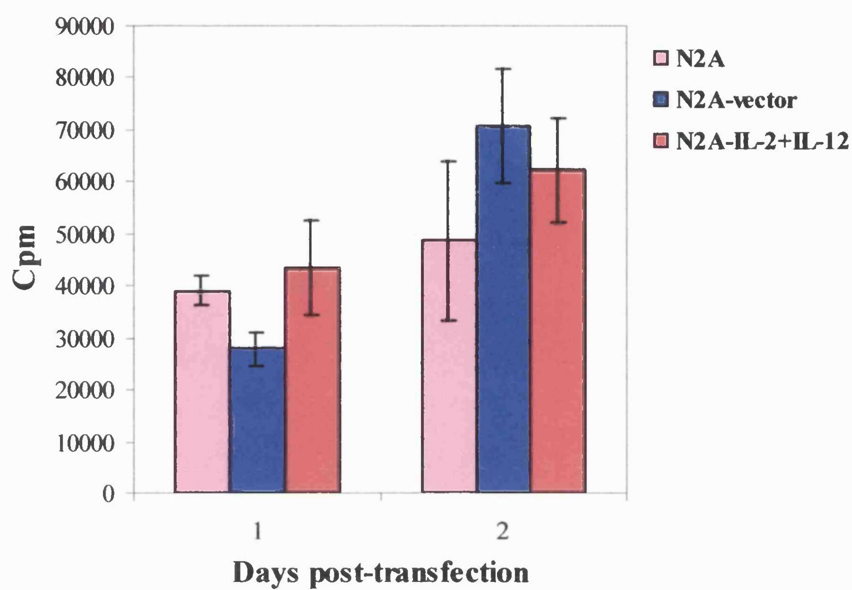


Figure 6.13 ***In vitro* proliferation of Neuro-2A cells used in the *in vivo* immunodepletion experiment.** The proliferation of N2A-vector and N2A-IL-2+IL-12 cells was determined by incorporation of ^{3}H-thymidine.

mice in both groups developed tumours within 14 days after inoculation. Neuro-2A cells transfected with a combination of IL-2 and IL-12 were immunogenic in mice injected with PBS and no animals developed tumours. This result is in agreement with that seen in Fig 6.1. No tumour growth was observed in any of the animals depleted of $CD4^+$ cells. However, 2/6 (33%) animals depleted of $CD8^+$ cells developed tumours. These results indicate that $CD4^+$ T cells are not responsible for the abrogation of tumourigenicity of N2A-IL-2+IL-12 cells while $CD8^+$ T cells may be involved in the rejection of N2A-IL-2+IL-12 cells.

The cells used in the immunodepletion experiment were monitored *in vitro* for their cytokine expression and proliferation. ^{3}H-thymidine incorporation one and two days after transfection revealed that there was no significant difference in the proliferative ability of N2A-vector and N2A-IL-2+IL-12 (**Fig 6.13**). Therefore, the lack of tumour growth in animals receiving N2A-IL-2+IL-12 was not due to altered proliferation of the cells after transfection. For cytokine expression of the cells used in this experiment see Appendix C5.

6.2.6 Histology of murine neuroblastoma tumours

Histological examination of wild-type murine neuroblastoma was performed as described in 2.8.3 on tumours of 5-7 mm in diameter. Paraffin sections stained with haematoxylin and eosin revealed extensive tissue vascularisation (**Fig 6.14A,B**). As described before, no rosette formation could be detected and the majority of the cells were in resting stage as indicated by the uniform nuclei staining. The tissue vasculature was verified by staining of blood vessel endothelial cells with antibody against CD34. Positive CD34 staining was evident only around blood vessels indicating the presence of endothelial cells (**Fig 6.14C**). Indeed, positive cells appeared flat and elongated characteristic of endothelial cells compared to the round appearance of neuroblasts. No background staining was observed using just the secondary antibody (**Fig 6.14D**).

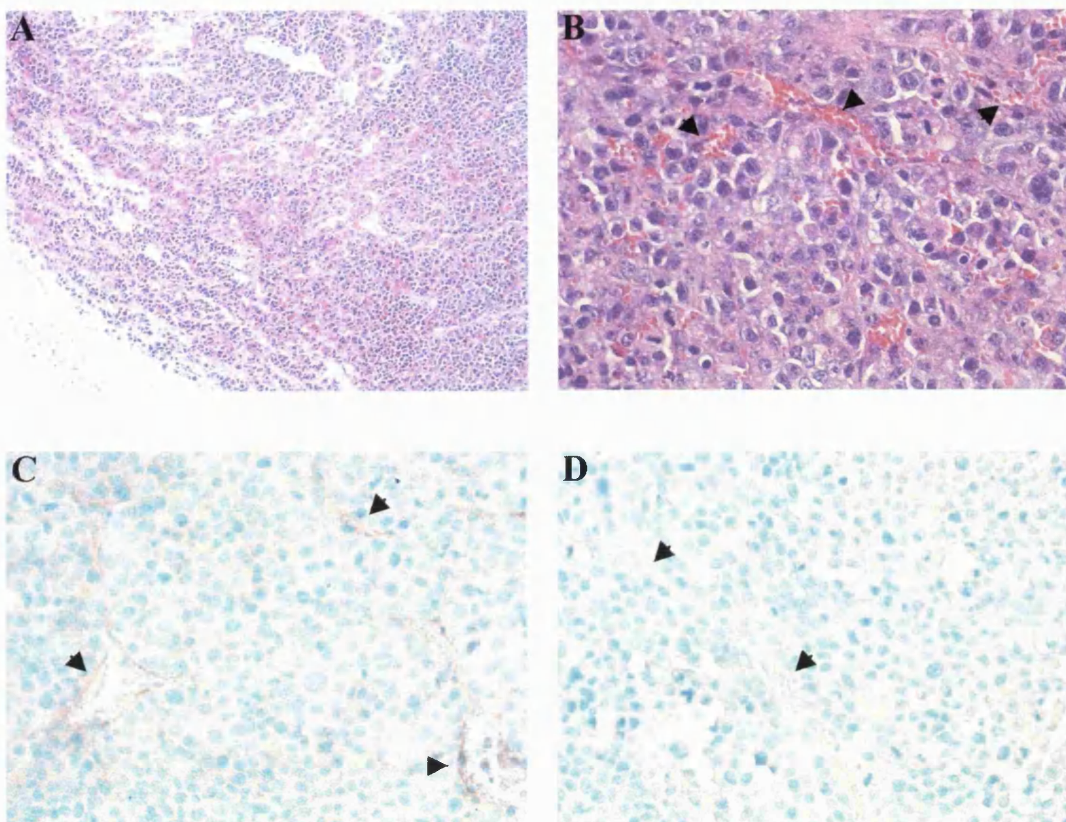


Fig 6.14 Histology of wild-type murine neuroblastoma tumours. Haematoxylin and eosin stained wild-type tumours of ~5-7mm in diameter (A-B). Immunohistochemical analysis of paraffin-embedded wild-type tumour sections for CD34 expression (C) or isotype control (D). Magnification x40 (B-D) or x10 (A). Arrowheads indicate blood vessels.

After determining the superior anti-tumour effect of the N2A-IL-2+IL-12 vaccine, tumours of immunised animals were histologically examined. Tumours that were in regression phase due to vaccination with N2A-IL-2+IL-12 were isolated 5 days post-immunisation and stained with haematoxylin and eosin. Paraffin-embedded sections revealed large areas of necrosis as indicated by punctate staining of nuclei (**Fig 6.15A,B**). The majority of blood vessels in these tumours were located mainly in the periphery as indicated by the arrows in **Fig 6.15A**. Immunohistochemical staining with anti-CD34 antibody was performed to confirm the presence of blood vessels (**Fig 16.5C**). In order to examine whether tumours were infiltrated with leukocytes, immunohistochemical analysis with anti-CD45 antibody was performed. Leukocyte infiltration was evident in certain parts of the tumour sections especially closer to the periphery (**Fig 6.15E**). Images at higher magnification revealed the presence of cells with clear surface staining (**Fig 6.15F**). No background staining could be detected when only the secondary antibody was used (**Fig 6.15D**). Also such infiltration was absent from wild-type tumours of similar size (data not shown).

6.3 DISCUSSION

As seen in chapter 5, the cytokine levels secreted by transfected Neuro-2A cells did not only surpass potentially therapeutic levels but also exhibited bioactivity *in vitro* by inducing the proliferation of PHA-activated lymphocytes. Following inoculation into syngeneic A/J mice, Neuro-2A cells transfected with cytokines (mIL-12, hIL-2 or combination of two) exhibited significantly reduced tumourigenicity compared to untransfected or vector control cells. This reduced tumourigenicity was not due to altered cell proliferation as shown by *in vitro* ^{3}H-thymidine incorporation but was rather a specific effect of cytokine expression.

In many animal model studies vaccination is performed with either virally transduced cells or stable transfectants. Although there are numerous advantages to such an approach, associated with the simplicity and reproducibility of experimental procedures, it does not reflect the clinical situation. In a clinical trial, cytokine

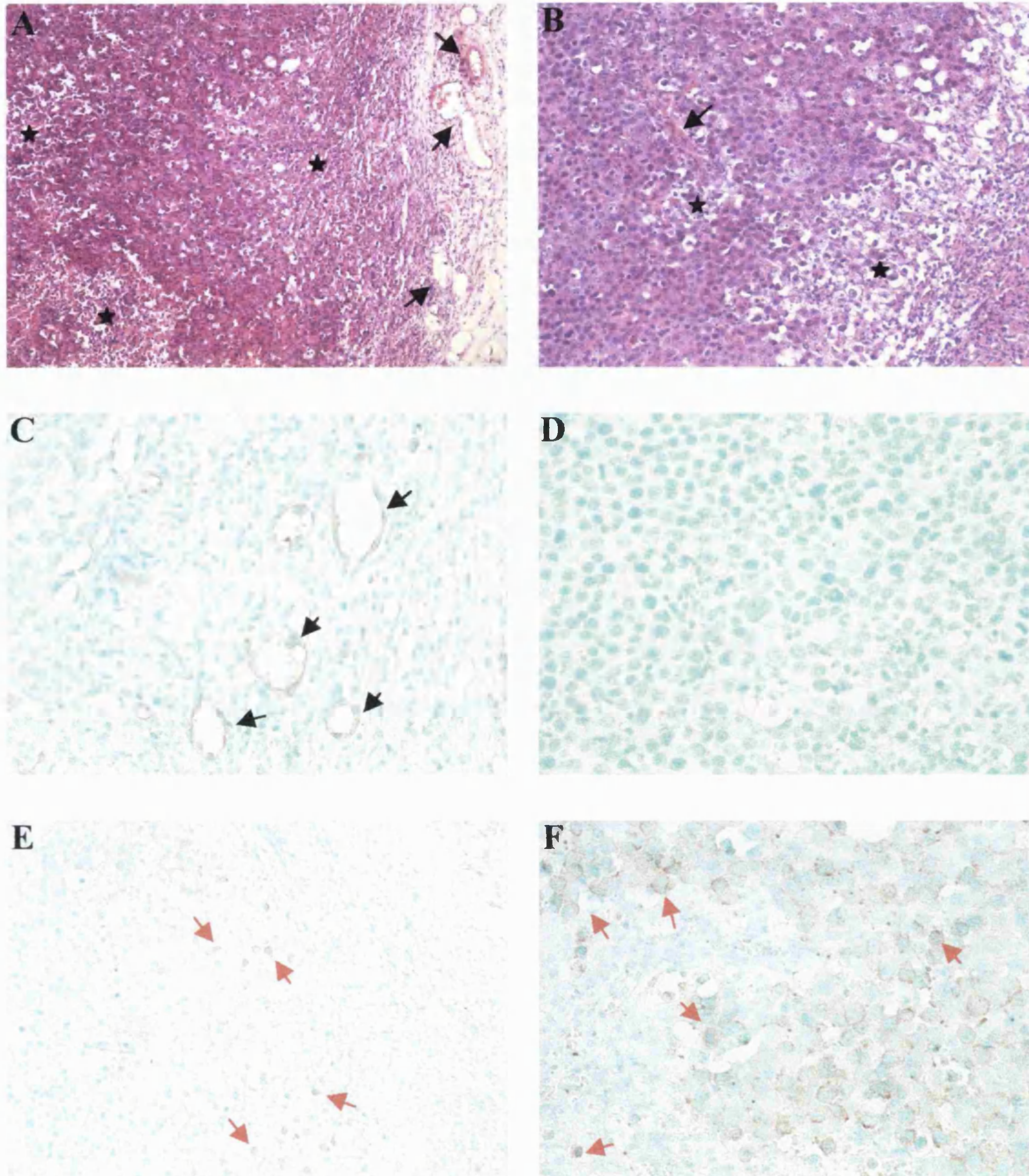


Fig 6.15 Histology of N2A-IL-2+IL-12-vaccinated neuroblastoma tumours. Paraformaldehyde-fixed, paraffin-embedded murine tumours vaccinated with N2A-IL-2+IL-12 cells were stained with haematoxylin and eosin (A, B), CD34 (C), CD45 antibody (E,F) or isotype control (D). Black arrowheads indicate blood vessels and black stars necrotic areas. Infiltrating leukocytes are denoted with red arrowheads. Magnification x10 (A), x20 (B,E) and x40 (C,D,F).

expression by primary neuroblasts is likely to vary regardless of the vector system used to deliver the cytokine genes due to variations in cell culture and transduction efficiency. Therefore, in this study separate transfections were performed for each experiment. Although the cytokine levels varied, they were sufficient to examine the effect of the N2A-IL-2+IL-12 vaccination on neuroblastoma tumours.

Pre-vaccination of animals with irradiated Neuro-2A cells induced immunity independently of cytokine expression. Contrary to previous reports, where mice vaccinated with IL-2-expressing Neuro-2A induced enhanced tumour protection in comparison to untransduced cells upon challenge with parental cells (Katsanis *et al.*, 1994), in this study pre-vaccination with cells alone or control-transfected cells resulted in protective immunity in 43% and 88% of the animals, respectively. Although this seems to indicate that the transfection process itself may render the cells more immunogenic, pre-vaccination with N2A-IL-2 cells resulted in similar tumour development (60%) to that of untransfected N2A cells, indicating that protective immunity was irrespective of transfection or cytokine expression. In addition, no IL-2 or IL-12 could be detected by N2A-vector cells thus excluding the possibility that transfection may be stimulating endogenous expression of either of these two cytokines. These pre-vaccination results suggest that Neuro-2A cells, when provided in an irradiated form, are potentially immunogenic. Reducing the number of vaccinating cells to less than $2x10^5$ abrogated this protective effect emphasising the importance of the amount of antigen present in the vaccine. However, reduction of the number of cells by tenfold equated to a tenfold reduction in the levels of cytokine expressed by the vaccine cells. This makes differentiation between the protective effect of the amount of antigen provided and levels of cytokine expression difficult to interpret.

Irradiated Neuro-2A cells expressing IL-12 or a combination of IL-2 and IL-12 exhibited an immunological effect against parental tumours. Indeed, in one experiment 33% of animals receiving the N2A-IL-2+IL-12 vaccine showed complete tumour regression, while delay in tumour growth was observed in another 33%. In a second eradication experiment, 50% of the immunised animals exhibited tumour

growth inhibition while partial tumour regression was observed in the remaining 50%. However, tumour growth resumed in the latter animals a few days later indicating that tumour regression was not due simply to alteration of cell growth. This emphasises the need for repeated vaccination. The variation in individual responses to N2A-IL2+IL-12 vaccination in the two eradication experiments may be due to cytokine expression. Comparison of the IL-2 expression levels by N2A-IL-2+IL-12 cells in the two eradication experiments, suggests that sustained expression of high levels of IL-2 may be required in order to achieve tumour regression.

Vaccination of established tumours with N2A-IL-12 cells resulted in a delay in tumour growth in 66% of the animals in one experiment, while 60% of N2A-IL-12 vaccinated animals exhibited complete tumour regression in a second experiment. Both N2A-IL-12 and N2A-IL-2+IL-12 vaccines prolonged survival of immunised animals compared to vaccination with N2A-vector cells. Interestingly, IL-2 alone did not show any anti-tumour efficacy although it totally abrogated the *in vivo* tumourigenicity of IL-2 transfected Neuro-2A cells. Despite the variability in response of individual animals to vaccination with N2A-IL-12 or N2A-IL-2+IL-12, it is evident from both experiments that overall vaccination with N2A-IL-2+IL-12 cells exhibited some anti-tumour effect, either complete regression or growth inhibition, in nearly 100% of the treated animals.

Experiments in γ_c/RAG2 knockout mice suggest that abrogation of tumourigenicity of IL-2+IL-12-transfected Neuro-2A cells is an immunological phenomenon, depending on T, B or/and NK cells. However, $CD4^+$ T cells are excluded as the important effector cell population since depletion of that cell type in A/J mice did not result in tumour formation by N2A-IL-2+IL-12 cells. In contrast, CD8-depletion resulted in tumour formation in 33% of the animals indicating a potential role of $CD8^+$ T cells in the initial rejection of N2A-IL-2+IL-12 cells. These results suggest that direct killing of N2A-IL-2+IL-12 by $CD8^+$ cells may be taking place. However, experiments using larger numbers of animals are needed to confirm this result. In previous studies $CD8^+$ T cells alone or in combination with $CD4^+$ were involved in primary tumour rejection of IL-12-expressing neuroblastoma cells (Balicki *et al.*, 2000). Activation of NK cells

may be an alternative immune mechanism induced through local secretion of IL-2 and IL-12. However, immunodepletion studies using anti-asialo GM antibody are required to confirm this hypothesis. Identification of NK cells as the important effector cell population agrees with a synergistic mechanism of IL-2 and IL-12 on these cells. This may be due to IL-2 up-regulation of IL-12 receptors and STAT4 on NK cells, which increases their activation response to IL-12 (Wang *et al.*, 2000).

In a retroperitoneal model of neuroblastoma, local IL-2 production by retrovirally transduced Neuro-2A cells induced activation of T cells. T cell depletion prior to vaccination indicated that $CD8^+$ cells mediated the priming phase of the immune response (Katsanis *et al.*, 1994). $CD8^+$ T cells were also responsible for reducing the tumourigenicity of B7-1-transduced or B7-1+IFN-transduced Neuro-2A cells (Katsanis *et al.*, 1995;Katsanis *et al.*, 1996). In a similar way, the systemic response against NXS2 neuroblastoma cells expressing IL-12 in an experimental neuroblastoma model metastasis was dependent on $CD8^+$ cells (Lode *et al.*, 1998a). In the same model, on the other hand, GD_2-targeted IL-2 suppressed tumour growth and eradicated liver metastases in an NK-cell-mediated way (Lode *et al.*, 1998b). Therefore, it seems that the immune effector cell population responsible for anti-tumour immunity in each case depends on the cells used as well as the cytokine expressed by the vaccine. When IL-2 and IL-12 were used concomitantly in a murine model of neuroblastoma metastasis tumour-targeted IL-2 amplified $CD8^+$ T cells after IL-12 vaccination and enhanced MHC class I-mediated killing (Lode *et al.*, 1999).

The N2A-IL-2+IL-12 vaccine induced leukocyte infiltration in treated tumours as seen by CD45 staining. Unfortunately it was not possible to precisely determine the nature of the infiltrating cells, as there are no CD4, CD8 or NK mouse antibodies available that work on paraformaldehyde-fixed paraffin-embedded sections. Most commercially available antibodies work on frozen specimens. However, cryopreservation did not maintain the integrity of the tumour tissue and fixing in 10% PFA had to be applied.

The improved anti-tumour effect shown by the combination of IL-2 and IL-12 suggests that an inflammatory cytokine such as IL-12 functions to attract effector cells

such as NK cells at the vaccination site and also to activate DCs. Its anti-tumour immunity has also been shown to include non-T cell mediated events (Nastala *et al.*, 1994) and angiostatic effects (Voest *et al.*, 1995). There was some indication of reduced vascularisation in N2A-IL-2+IL-12-vaccinated tumours compared to wild-type tumours of similar size and the majority of blood vessels were localised at the periphery. However, studies on a greater number of animals are required with actual quantification of the number of blood vessels in order to confirm an angiostatic effect of the N2A-IL-2+IL-12 vaccine. A possible synergy between IL-2 and IL-12 may lie in the up-regulation of T cell CD25 expression by IL-12 so that T cell proliferation in response to IL-2 is enhanced (Nguyen *et al.*, 2000). A second cytokine, such as IL-2, that exhibits a stimulatory effect on a broad range of immune cell types and is also capable of clonally expanding activated T cells may be vital for strengthening the immune response against the tumour and achieving long-term immunity. Although the dominant immune effector cell population responsible for the initial rejection of N2A-IL-2+IL-12 seems to be $CD8^+$ T cells, NK cells may participate in the eradication of established neuroblastoma tumours. However, immunodepletion experiments prior to vaccinating established tumours are required in order to determine the precise immune mechanism of tumour regression.

In conclusion, we have shown that co-expression of IL-2 and IL-12 results in improved anti-tumour immunity with enhanced eradication of established murine neuroblastoma tumours. This vaccine provides a potential therapeutic strategy for the treatment of neuroblastoma.

7

Discussion

Cancer vaccination, unlike traditional prophylactic immunisation against infectious agents, aims at the therapeutic treatment and elimination of established tumours. A number of mechanisms have been proposed to explain the failure of the immune system to recognise tumour as 'foreign' and elicit an immune response. These include down-regulation or absence of tumour MHC molecules, co-stimulatory molecules or other antigen processing machinery, secretion of inhibitory cytokines or loss of immunodominant antigens. Whatever the mechanism of tumour immune evasion, cancer vaccination aims to mount an immune response sufficient to elicit clinical benefit. Studies on murine models have demonstrated the importance of $CD8^+$ T cells as the primary anti-tumour effector population. In addition, down-regulation of MHC class I molecules correlates with clinical disease progression suggesting that MHC-I-dependent antigen presentation is important in anti-tumour immunity. Therefore, antigens recognised by CTLs would comprise the most effective tumour rejection antigens. However, the significance of $CD4^+$ T cells in priming CTLs *in vivo* has been clearly demonstrated so antigens capable of eliciting $CD4^+$ T cell responses should be equally important in generating anti-tumour immunity. The advantage of whole cell tumour vaccination is that a large repertoire of tumour antigens is provided. Immunogenicity would be further enhanced by concurrent local expression of immunomodulatory molecules. The principal behind this approach is that low avidity auto-reactive T cells that have attained functional anergy due to the acquisition of peripheral tolerance will be activated and will eradicate tumours. Although with such an approach there is the potential risk of autoimmunity, murine studies generating CTLs against self-epitopes show that it may not be a serious problem (Morgan *et al.*, 1998).

Neuroblastoma is a common childhood tumour with very poor prognosis when it occurs as disseminated disease. Although it is quite responsive to surgical resection and treatment with radiotherapy or/and chemotherapy, the majority of patients relapse within 3 years. In order to eradicate minimal residual disease a number of groups have attempted to activate immune responses against the parental tumour. This thesis describes the development of an immunotherapeutic strategy for the treatment of

neuroblastoma based on the *ex vivo* modification of tumour cells to secrete a combination of Interleukin-2 and Interleukin-12.

7.1 LID transfection

A non-viral vector consisting of an integrin-targeting peptide and Lipofectin (LID) (Hart *et al.*, 1998) was utilised to transfect neuroblastoma cells. Optimisation of the transfection parameters is necessary in order to achieve maximum transfection efficiency and cytokine delivery. The effectiveness of the LID vector in *ex vivo* transfection experiments was assessed on three neuroblastoma cell lines. The peptide component of the vector is synthesised and consists of a sixteen-lysine chain coupled to an integrin-targeting motif. The oligolysine chain resembles poly-L-lysine in its ability to interact and condense the negatively charged DNA. The integrin-targeting domain of the peptide contributes to the overall charge of the LID complexes and targets integrin receptors (Hart *et al.*, 1998). Several peptides with different integrin-targeting specificities were tested on neuroblastoma cells. An $\alpha_5\beta_1$ integrin specific peptide (peptide 6) exhibited the highest transfection efficiency. Peptide 6 contains the RRETAWA sequence, which was isolated from a phage display library for its high affinity for $\alpha_5\beta_1$ integrin (Koivunen *et al.*, 1994).

Incorporation of the commercially available lipid Lipofectin was shown previously to enhance the transfection efficiency of integrin-targeting peptide-DNA complexes (Hart *et al.*, 1998). Indeed, all neuroblastoma cell lines exhibited significantly higher reporter gene expression when Lipofectin was included in the transfection protocol. The mechanism whereby Lipofectin enhances efficiency of LID transfection is not known, however, it is speculated to aid the release of complexes from the endosomal compartment. Both DOTMA and DOPE, which are present in an equimolar mixture in Lipofectin, have been described independently to participate in the endosomal release of complexes (Farhood *et al.*, 1995; Xu and Szoka-FC, 1996). The importance of the surface charge density of LID particles was also demonstrated. Transfection of neuroblastoma cells significantly increased at charge ratios of 3:1 (peptide:DNA) or

higher, emphasising the significance of a cationic zeta potential of LID complexes. Transfection efficiency with cationic liposomes consisting of DOPE and various cationic derivatives of cholesterol was consistent with their zeta potential values (Takeuchi *et al.*, 1996).

Application of the LID vector to neuroblastoma cell lines resulted in high efficiency transfection (20-60%). Transfection efficiency of Neuro-2A cells with the LID vector was 2-fold higher than that reported with other non-viral systems (Ogris *et al.*, 1998). Cytokine gene transfer resulted in production of high levels of IL-2 and IL-12 similar to those reported using an adenoviral vector (Leimig *et al.*, 1996). IL-12 and IL-2 expression by mouse Neuro-2A cells was maintained for at least 7 days at similar levels in both single and co-transfection experiments. In the IMR-32 cell line co-transfection experiments resulted initially in the expression of similar levels of IL-12 to those of single transfections but this declined over time. IL-2 expression in these cells reached a peak at 48 h post-transfection but decreased over time. In SHSY5Y cells, both single and co-transfection experiments resulted in a rather stable cytokine expression pattern although the levels in the latter experiments were significantly lower than those of single transfections. Nonetheless, the cytokine levels were maintained above the potentially therapeutic levels (150 pg/ml; (Brenner, 1992) clinical protocol) for at least up to 10 days.

The biological activity of IL-2 and IL-12 produced by transfected neuroblastoma cells was tested on PHA-stimulated peripheral blood lymphocytes. The *in vitro* biological activity of the secreted cytokines was examined by assaying their proliferative effect on PHA-lymphocytes via ^{3}H-thymidine incorporation. IL-2 and IL-12, whether transfected alone or co-transfected, exhibited a mitogenic effect on PHA-activated lymphocytes, which was significantly higher than the proliferation induced by the supernatant of pEGFP-N1-transfected cells. Therefore, cytokine transfection of neuroblastoma cells resulted in the production of IL-2 and IL-12 that were not only significantly higher than the required therapeutic levels, but exhibited bioactivity *in vitro*.

7.2 Internalisation pathway of the LID vector

The integrin-targeting property of the LID vector has been demonstrated on human umbilical vein endothelial cells (ECV304) by competitive inhibition with integrin antibodies. The integrin-targeting domain of the peptide component plays a vital role in transfection efficiency by the LID vector as shown by gradual substitution of K_{16} by peptide 6 in LID complexes. Transfection efficiency with LID particles containing peptide 6 in ECV304 cells is higher compared to K_{16} complexes, suggesting that this difference is due to the integrin targeting ability of the former peptide (Hart *et al.*, 1998). The importance of the extra integrin-targeting motif was also demonstrated in neuroblastoma cells. Transfection using peptide 6, peptide 1 or control peptide 11 complexes resulted in significantly higher gene expression than K_{16} complexes. Interestingly, the efficiency of the former peptides was irrespective of their integrin targeting.

Investigation of the endocytic mechanism of the LID vector not only provides information on the intracellular trafficking of LID complexes, but also suggests possible ways of improving transfection efficiency. In addition, further development of the targeting property of the vector may also be possible. The intracellular trafficking of LID particles in neuroblastoma cells was studied using several drugs that disrupt certain endocytic pathways. Chloroquine enhanced gene expression in two cell lines suggesting that LID particles are shuttled through an endocytic pathway in these cells. Wortmannin and LY294002 supported the involvement of PI3K in the uptake mechanism of the vector. Although receptor-mediated endocytosis was excluded as a potential internalisation mechanism, as disruption of clathrin molecules did not affect LID transfection, there is some indication that LID complexes may be internalised by phagocytosis or non-coated pit endocytosis. Blocking of integrin receptors in SHSY5Y human neuroblastoma cell line using integrin antibodies or soluble RGD peptides did not inhibit LID transfection, despite the fact that these cells expressed α_5 integrins. Examination of the ligand-binding domain of $\alpha_5\beta_1$ integrin revealed that a peptide containing the RRETAWA sequence directly inhibited binding of only one anti-α_5 antibody (mAb16) to $\alpha_5\beta_1$ (Mould *et al.*, 1998). If the binding

domain of RRETAWA on α_5 overlaps with only that antibody, that may explain why blocking of the integrin receptors on SHSY5Y cells with commercially available antibodies did not have any inhibiting effect on transfection with the LID vector. Furthermore, SHSY5Y cells did not require PI3K for efficient gene expression, indicating that binding of LID complexes did not trigger an integrin downstream signalling pathway. Integrin signalling requires clustering of receptors upon ligand binding and conformational change in order to trigger a downstream cascade. If the integrins on SHSY5Y cells are inactive, such events will not take place. Interestingly, the SHSY5Y cell line exhibited a different response to treatment with pharmacological drugs compared to IMR-32 and Neuro-2A cells. This implies that uptake of the LID vector in these cells may occur via an alternative pathway to that of IMR-32 and Neuro-2A cells.

The experiments described in chapter 4 have not precisely defined the mechanism of internalisation of the LID vector in neuroblastoma cells. Studies of the zeta potential of the LID complexes show that cationic surface charge is required to mediate high transfection efficiency. In addition, neuroblastoma cells transfect more efficiently with particles matured for 1.5 h. Photon correlation spectroscopy data indicates that such complexes are polydisperse and form aggregates of ~1000nm in diameter (Li Kim Lee personal communication). Therefore, it seems that uptake of LID complexes by neuroblastoma cells may be mediated by more than one endocytic processes. Large aggregates may be phagocytosed while smaller particles may mediate cell entry via non-coated pit endocytosis after association with integrin receptors or charge-mediated interaction with the cell membrane. Although these findings cannot exclude integrin-specific mechanisms of uptake, alternative pathways are clearly involved in all or some cell types.

These results are in agreement with most reports on endocytosis of polyplex and lipopolyplex systems. Endocytosis is the main internalisation route of such complexes. However, a similar non-viral vector consisting of an RGD-K_{16} peptide in combination with Lipofectamine was shown to mediate cell entry via receptor-mediated endocytosis (Colin *et al.*, 2000). Despite the similarity of the peptide component of

this system with that of the LID vector, the latter study was performed on human tracheal epithelial cells. These cells may differ from neuroblastoma cells in a number of ways including their integrin expression pattern and rate of mitotic division. Uptake of complexes may occur via alternative routes in these cells.

The experiments described here provide some insight into the route of intracellular uptake of the LID vector in neuroblastoma cells. Future studies to delineate the exact endocytic pathway of the LID vector in these cells could include complex labelling and confocal microscopy. Nonetheless, these experiments have provided useful insight into ways of enhancing transgene expression in neuroblastoma cells. Utilisation of OptiMEM containing sucrose has proved to be a better transfection medium than OptiMEM alone. Also transfection efficiency may be further improved by performing transfections in the presence of chloroquine.

High-risk neuroblastomas (stage IV S and N-myc-amplified stage III) exhibit increased expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins compared to low-risk groups (stages I, II and non-N-myc-amplified stage III). Integrin expression on cultured neuroblastoma cells depends on their morphological features (Yoshihara *et al.*, 1992) and is inversely proportional to N-*myc* amplification (Flickinger *et al.*, 1994). Indeed, the neuroblastoma cell lines used in this thesis exhibited variations in integrin expression. In addition, the levels and type of integrin receptors by neuroblastoma cells may depend on the cell differentiation state. Up-regulation of integrin expression may be possible with differentiating agents such as IFN-γ and TNF-α or retinoic acid (Rozzo *et al.*, 1993). This could be used to increase specific targeting of gene delivery.

For *ex vivo* modification of tumour cells, the LID vector has demonstrated high transfection efficiency and expression of the delivered cytokine genes. The integrin availability on the cell lines used in this thesis does not seem to limit the application of the vector. Further development on the targeting properties of the vector would be required, however, for *in vivo* applications. For this to be possible novel peptides, that specifically target neuroblastoma cells, could be isolated by phage display libraries after *in vivo* panning on established neuroblastoma tumours. Alternatively, antibodies

directed against a neuroblastoma marker such as GD_2 could be incorporated into the LID vector to enhance its targeting specificity.

7.3 *In vivo* anti-tumour effect of vaccination with IL-2 and IL-12 transfected neuroblastoma cells

Investigating the therapeutic effect of the vaccine in a mouse model for neuroblastoma was the obvious next step after verifying the *in vitro* bioactivity of the cytokines produced by transfected Neuro-2A cells. Although human responses to such vaccination may vary considerably compared to that observed in mice and toxicity issues may require careful consideration, using cytokine-transfected Neuro-2A cells in a mouse model provides important insight into both the effect of vaccination *in vivo* and into the potential mechanism of anti-tumour immunity.

IL-2 and IL-12 have both distinct and overlapping effects on a wide range of immune cell types. IL-2 induces the proliferation of activated T cells and is also capable of enhancing the cytolytic activity of NK cells (Gaffen *et al.*, 1998). IL-12 is a potent stimulator of NK and T cells and promotes the differentiation of $CD4^+$ T cells into T_{H1} cells (Trinchieri, 1998). It also up-regulates T cell CD40L expression which is crucial for activation of dendritic cells and macrophages (Peng *et al.*, 1998). Independently, both IL-2 and IL-12 have demonstrated anti-tumour efficacy in a number of experimental models. Vaccination with IL-2-expressing cells prolonged survival of animals with experimental neuroblastoma tumours, generating anti-tumour specific immunity (Katsanis *et al.*, 1994). In addition, systemic administration of GD_2-targeted IL-2 through fusion with an anti-GD_2 antibody efficiently eradicated established liver and bone marrow metastases (Lode *et al.*, 1998b). This was also achieved by vaccination with IL-12-transduced neuroblastoma cells (Lode *et al.*, 1998a). Moreover, the anti-tumour effect of IL-12 has been shown by direct *in situ* transduction of neuroblastoma tumours (Davidoff *et al.*, 1999a).

The synergy between these two cytokines has been demonstrated in their proliferative effect on T cells. IL-12 induces CD25 expression thereby enhancing the proliferation of T cells in response to IL-2 (Nguyen *et al.*, 2000). In addition, IL-2 enhances the functional responses of NK cells to IL-12 by up-regulating IL-12 receptor expression and maintaining levels of STAT4, which is critical for IL-12 signalling (Wang *et al.*, 2000). This effect on NK cells is also observed *in vivo* after administration of IL-2. These observations suggest that a combination of these two cytokines may prove advantageous for the treatment of cancer. Indeed, IL-2 and IL-12-treated NK cells exhibit enhanced *in vitro* cytolytic activity and lyse neuroblastoma cells (Rossi *et al.*, 1994) and human osteosarcoma cells more efficiently (Mariani *et al.*, 2000).

In this thesis, we examined the novel simultaneous gene transfer of both IL-2 and IL-12 in neuroblastoma cells and their synergistic effect in anti-tumour immunity. Initially, the effect of cytokine expression on the tumourigenicity of the cells was investigated. After subcutaneous inoculation of untransfected, control- or cytokine-transfected Neuro-2A cells into syngeneic A/J mice it was evident that the tumourigenicity of IL-2 and/or IL-12 transfected cells was completely abrogated. Such abrogation of tumourigenicity upon cytokine transfection of tumour cells has been illustrated in a number of studies. IL-12-transduced neuroblastoma cells failed to induce liver and bone marrow metastasis (Lode *et al.*, 1998a), while the tumourigenicity of IL-2-transduced Neuro-2A cells was reduced in a retroperitoneal neuroblastoma model (Katsanis *et al.*, 1994).

Pre-vaccination with transfected or untransfected irradiated cells generated protective immunity in 88% (N2A-pCI) and 43% (N2A) of the animals, respectively. Immunised animals receiving untransfected, control- or cytokine-transfected cells failed to form tumour upon challenge with parental Neuro-2A cells 7 days later. These results indicate that, contrary to previous reports, Neuro-2A cells are immunogenic without the requirement for cytokine expression (Katsanis *et al.*, 1994). Nonetheless, pre-vaccination experiments do not reflect the actual clinical situation since most candidates for such therapy would already be presented with established disease.

Vaccination of animals with established subcutaneous tumours, gave a very interesting outcome. Intratumoural inoculation of animals with Neuro-2A cells expressing a combination of IL-2 and IL-12 resulted in complete tumour regression in 33% and inhibition of tumour growth in 50% of the animals. In a second experiment, there was a delay in tumour growth in 50% of the animals and partial tumour regression in the remainder 50%. Individually, IL-2 expression exhibited no anti-tumour activity on established experimental tumours in any of the experiments performed. This result contradicts previous studies where animals vaccinated with neuroblastoma cells expressing IL-2 exhibited prolonged survival (Katsanis *et al.*, 1994) and eradication of established tumours (Yoshida *et al.*, 1999). The N2A-IL-12 vaccine, however, did exhibit some anti-tumour effect. It induced a delay in tumour growth in 66% of the animals in one experiment, and tumour regression in 60% of the animals in a second experiment. Although both N2A-IL-12 and N2A-IL-2+IL-12 prolonged survival of the immunised mice, the N2A-IL-2+IL-12 vaccine exhibited an overall superior anti-tumour effect than N2A-IL-12. In both eradication experiments 83-100% of N2A-IL-2+IL-12-immunised animals responded to vaccination by tumour growth inhibition, partial or complete tumour regression. This suggests that those animals with a delayed tumour growth or partial regression may benefit from multiple vaccinations to achieve complete tumour eradication. A similar synergistic combination between IL-2 and IL-12 was observed in a Lewis lung carcinoma model where only vaccination with dual-gene transduced cells inhibited the growth of established tumours (Tanaka *et al.*, 2000).

The improved anti-tumour effect of IL-2 and IL-12 when co-transfected in tumour cells suggests that IL-2 may be amplifying the IL-12-induced immune response for eradication of established tumours. Systemic administration of IL-2 following vaccination with IL-12-transfected cells has been shown to amplify T cell-mediated immune responses in a neuroblastoma metastasis model (Lode *et al.*, 1999a). Immunodepletion experiments and inoculation in γ_c/RAG2 knockout mice indicated that the abrogation of tumourigenicity of N2A-IL-2+IL-12 occurs probably through activation of $CD8^+$ T cells. However, further studies are required to precisely determine the nature of the immune response during regression of established

tumours. Preliminary data indicate that the N2A-IL-2+IL-12 vaccine induces necrosis, leukocyte infiltration and possibly a reduction in the tumour vasculature in the vaccinated animals. A tumour angiostatic effect may be possible since IL-12 is known to mediate anti-angiogenesis by chemokines such as IP-10 (Interferon inducible protein 10) (Pertl *et al.*, 2001).

Anti-tumour responses following vaccination seem to depend on the cytokine expressed by the vaccine as well as the cell type and route of vaccine administration. Transduced Neuro-2A cells with IL-2, B7-1 or B7-1+IFN induced activation of $CD8^+$ T cells during the priming phase of the immune response in a retroperitoneal model of neuroblastoma (Katsanis *et al.*, 1994, 1996). $CD8^+$ T cells also generated a systemic response against IL-12-expressing NXS2 neuroblastoma cells in an experimental model for neuroblastoma metastasis (Lode *et al.*, 1998a) whereas in the same model, systemic administration of IL-2 targeted to GD_2 receptors induced an NK cell-mediated anti-tumour response (Lode *et al.*, 1998b). Concomitant application of IL-2 and IL-12 seems to generate a $CD8^+$ T cell-dependent effect (Lode *et al.*, 1999a) while an oral squamous cell carcinoma study has demonstrated that expression of CD80 and IFN-γ are essential for tumour regression by these two cytokines (Thomas *et al.*, 2000).

In order to further elucidate the mechanism of the immune response involved in tumour eradication following vaccination with N2A-IL-2+IL-12 cells, a number of further experiments could be carried out. *In vivo* immunodepletion prior to vaccination of established tumours would provide an indication of the immune cell type that is responsible for tumour regression. In addition, although vaccination with N2A-IL-2+IL-12 cells resulted in tumour regression, further investigation is required to confirm that prolonged protective immunity is generated. This could be performed by challenging animals exhibiting complete tumour eradication with parental Neuro-2A cells, and also by examining the cytotoxicity of splenocytes against tumour cell targets. This would provide some insight into the potential of using this vaccine to induce sustained tumour-specific immunity.

The route of vaccine administration is another parameter that requires careful examination. Intratumoural vaccination may be effective in murine models but its application is limited only to patients with primary accessible tumours. Also, treatment of minimal residual disease requires alternative vaccination routes such as subcutaneous vaccine administration. Furthermore, in most clinical studies, patient vaccination is performed subcutaneously (Bowman *et al.*, 1998a; Sun *et al.*, 1998). Therefore, the efficacy of subcutaneous application of the N2A-IL-2+IL-12 vaccine should be examined in the A/J neuroblastoma model. In a Lewis lung carcinoma model, IL-12 gene transfer to a site distant from the tumour exhibited a less effective anti-tumour response compared to local delivery. However, after surgical excision of the primary tumour, both local and distant IL-12 treatment showed similar inhibition of lung metastases (Oshikawa *et al.*, 2001). This indicates that subcutaneous vaccination may not be effective for eradication of primary tumours but is probably sufficient to treat minimal residual disease.

Several factors need to be considered for the development of a cell-based tumour vaccine including the type and concentration of cytokine expressed by the tumour cells. The concentration and rate of cytokine production are crucial for the generation of an inflammatory response at the tumour site. The effectiveness of the vaccine depends on the kinetics of inflammation compared to that of tumour growth. In addition, the tumour-associated antigen(s) should be provided in an immunogenic form. The results obtained with the combination of IL-2 and IL-12 suggest that the concentrations of the cytokines produced by transfected Neuro-2A cells are sufficient to generate an anti-tumour response. Although cytokine expression was variable among experiments it persisted above at least 30 ng/ml/24h/10^6 cells. Previous vaccination studies on experimental neuroblastoma metastases have utilised IL-12-transduced tumour cells expressing 6.5 ng/ml/24h/10^6 cells (Lode *et al.*, 1998a). Therefore, the cytokine levels expressed by transfected Neuro-2A cells were sufficient for the development of a tumour vaccine. Furthermore, despite the transient nature of transfection using a non-viral vector, expression of IL-2 and IL-12 seems to persist sufficiently to abrogate the tumourigenicity of Neuro-2A cells and induce tumour regression in vaccinated animals. Delivery of the IL-2 or IL-12 genes to a

neuroblastoma cell line using a histone H2A transfection system also induced anti-tumour effects in a syngeneic neuroblastoma model, thus validating the use of transient transfection in the development of a cancer vaccine (Balicki *et al.*, 2000).

The Neuro-2A murine model for neuroblastoma shares numerous similarities with human disease. Expression of certain molecules, such as NCAM and low levels of MHC class I occurs in both murine and human neuroblastoma (Ziegler *et al.*, 1997). In addition, neuroblastoma patients show a natural NK cytolytic activity against neuroblastoma while the C1300 neuroblastoma cell line exhibits NK cell sensitivity. This model resembles early stage clinical neuroblastoma with favourable prognosis since metastasis of murine neuroblastoma is limited to regional lymph nodes and occurs at very late stages of the disease. However, metastases formation is a frequent event of human neuroblastoma. Therefore, in order to study the effect of vaccination on more advanced stage neuroblastoma, it would be useful to use the TBJ neuroblastoma clone. This clone arose from C1300 neuroblastoma cells and induces metastases in A/J mice as it grows more aggressively than C1300 or Neuro-2A cells (Ziegler *et al.*, 1997).

7.4 Potential problems

A Phase I Clinical trial initiated at St Jude's Hospital for Children, used autologous patient cells transduced with Interleukin-2 as a vaccine (Bowman *et al.*, 1998a). In this particular study, autologous patient cells were isolated from bone marrow or tumour biopsy specimens. The cells were stained with a panel of five monoclonal antibodies that exhibited >95% reactivity with neuroblastoma cells and separated by magnetic beads or cell sorting. Although the results from that study showed clinical benefit, the difficulties experienced in culturing primary cells and obtaining sufficient numbers for repeated vaccination has prompted the development of an allogeneic vaccine (Bowman *et al.*, 1998b). Standardisation of cytokine levels produced by transduced primary cells was also hindered by the great variability among individual tumours. In

the allogeneic vaccine, a cell line derived from patient primary neuroblasts transduced with IL-2 was used to vaccinate HLA-A2 positive patients with neuroblastoma.

Personal attempts to maintain primary neuroblasts in culture were not satisfactory. However, previous studies indicate that the anti-tumour effect of vaccination with IL-2-transduced allogeneic tumour cells was inferior to that elicited by vaccination with autologous neuroblastoma cells, suggesting that autologous cells provide superior immunogenicity (Bowman *et al.*, 1998a, b). Furthermore, autologous vaccination studies emphasise the importance of a neuroblastoma specific antibody that can reliably detect neuroblasts with minimal cross-reactivity to other tissues.

A major limitation of our ability to culture primary neuroblasts during this thesis was lack of a reliable method of identifying them. Ganglioside GD_2 is strongly expressed on the surface of neuroblastoma cells (Kaucic *et al.*, 2001). Antibodies against GD_2 have been extensively used to treat neuroblastoma patients alone (Yu *et al.*, 1998), or in combination with IL-2 (Frost *et al.*, 1997) or GMCSF (Ozkaynak *et al.*, 2000) with some anti-tumour efficacy. In addition, GD_2 antibodies have been used for diagnostic purposes to detect neuroblastoma infiltration either by bone marrow immunofluorescence or tumour immunohistochemistry (Kramer *et al.*, 1998). In this thesis, failure to reliably detect neuroblastoma infiltration in the bone marrow using a GD_2 antibody may be solely due to primary cell death at the time of analysis. A monoclonal neuroblastoma-specific antibody (chCE7) was used in combination with PLL for gene delivery in human neuroblastoma cells (Coll *et al.*, 1997). This antibody has been used for imaging in neuroblastoma and a study in renal carcinoma cells revealed that it binds to L1-CAM (Meli *et al.*, 1999). Furthermore, an antibody (NBL-1) to RET, a tyrosine kinase receptor expressed on neuroblastoma cells and cells of the substantia nigra has been used as a gene delivery method to neuroblastoma cells (Yano *et al.*, 2000). This antibody may also be exploited as a diagnostic tool for the detection and isolation of neuroblastoma cells.

Although a neuroblastoma antibody that doesn't cross-react with other tissues would alleviate the problem of detecting infiltrating cells in the bone marrow allowing

positive selection of neuroblasts by cell sorting, it is highly unlikely that sufficient numbers of primary cells from bone marrow samples could be generated. As a result, alternative vaccination approaches must be investigated. In order to overcome the limitation of obtaining enough primary material to develop an autologous vaccine, a stably cytokine-transfected cell line could be generated similar to that reported by Bowman *et al*, (1998b). If tumour biopsy is available, tumour lysate could be administered as an adjuvant during vaccination.

This thesis has demonstrated the potential of the LID vector to efficiently transfect neuroblastoma cell lines. Due to difficulties in growing primary neuroblasts, optimisation of the transfection parameters for these cells was not possible. In addition, failure to detect neuroblasts after *in vitro* culture of patient bone marrow samples indicates that the actual cells transfected by the LID vector may not be neuroblasts. Upon improvement of the technique for maintaining primary neuroblastoma cells in culture, further development of the LID vector for transfection of these cells will possible.

7.5 Future directions

The process of tumour development and eradication are complex and still poorly understood. In order to reach therapeutic efficacy, a number of steps from tumour-specific immune activation to development of long-lasting protective immunity have to be attained. The different effects of cytokines on the immune system could be exploited for cancer gene therapy. In many cases, combinations of cytokines or co-expression of MHC or co-stimulatory molecules have proved more efficient in recruiting inflammatory cells to the tumour site, resulting in eradication of established animal tumours. (Putzer *et al.*, 1997; Addison *et al.*, 1998; Chong *et al.*, 1998). The effective combination of IL-2 and IL-12 described in this study supports the practice of multi-modality treatments for cancer therapy. In the same notion, alternative combinations of cytokines may be used.

Lymphotactin is a strong T cell chemoattractant that would be ideal for enhancing infiltration of T cells at the tumour site. Its anti-tumour effect has been demonstrated in a breast cancer model when combined with IL-2 or IL-12 (Emtage *et al.*, 1999). Also, transduction of peptide-pulsed DCs with lymphotactin enhances the cytotoxicity of T cells compared to vaccination with untransduced DCs (Cao *et al.*, 1998). Its use is currently being investigated in combination with IL-2 in an autologous vaccination study of neuroblastoma (Brenner *et al.*, 2000). Although, lymphotactin expression by transfected Neuro-2A cells was verified in this study, unfortunately time did not permit the elucidation of its effect in the mouse neuroblastoma model.

Other attractive candidates include GM-SCF, a potent attractor of DCs, whose efficient use in cancer vaccination has been established in many animal models (Chong *et al.*, 1998; Yoshida *et al.*, 1999). IL-18, which induces IFN-γ production by T and NK cells, has also demonstrated anti-tumour efficacy. When the inactive precursor of IL-18 (pro-IL-18) was delivered into the skin of animals bearing murine mammary adenocarcinoma tumours together with IL-12 and ICE, the enzyme required for generation of active IL-18, tumour regression was evident (Oshikawa *et al.*, 1999).

In order to overcome the technical difficulties of expanding primary autologous tumour cells in a clinical setting, gene transfer into cutaneous fibroblasts isolated from skin biopsy could be exploited. Vaccination of experimental melanoma tumours with IL-2 and IL-12-secreting syngeneic fibroblasts prolonged survival of treated animals and resulted in complete tumour eradication (Govaerts *et al.*, 1999). This study illustrated the potential of using alternative autologous cells to locally deliver cytokine genes for cancer therapy.

The exploitation of the antigen presenting capacity of DCs for gene therapy of cancer offers certain advantages. These cells can be easily generated from patient peripheral blood and can be pulsed with peptides isolated from MHC class I molecules on the surface of tumour cells (Zitvogel *et al.*, 1996), or fused to tumour cells resulting in the formation of a hybrid cell type that retains DC morphology while expressing the full repertoire of tumour cell antigens (Gong *et al.*, 1997). Both of these approaches

exhibit anti-tumour efficacy and could potentially be exploited for the treatment of neuroblastoma. Using tumour lysate to pulse DCs provides a large repertoire of tumour antigens and overcomes the problem of identification of tumour-specific antigens. Furthermore, priming of $CD8^+$ T cells by DCs is possible even when irradiated allogeneic cells are used to pulse the DCs (Berard *et al.*, 2000). In that study, DCs loaded with allogeneic melanoma cells were capable of priming T cells directed against the four shared melanoma antigens (MAGE-3, gp100, tyrosinase and MART-1). On the other hand, vaccination with peptide-pulsed DCs may result in autoimmunity if antigen expression is shared with normal tissues (Ludewig *et al.*, 2000).

The potential of vaccination with DCs to generate anti-tumour immunity has been illustrated in clinical trials. Treatment of melanoma patients with autologous DCs pulsed with a cocktail of peptides from gp100, MART-1, MAGE-1, MAGE-3 and tyrosinase, exhibited tumour regression in 30% of the patients (Nestle *et al.*, 1998). This would also overcome the problem of culturing primary cells and obtaining sufficient numbers to generate an autologous tumour vaccine. Furthermore, DCs trigger NK cell-mediated anti-tumour immunity through cell-to-cell contact that enhances the cytolytic activity of NK cells and their IFN-γ production (Fernandez *et al.*, 1999). This may prove advantageous for tumours with down-regulated MHC class I expression, such as neuroblastoma, which may escape NK cell immune attack. It may also enhance the natural host NK cell cytotoxicity to neuroblastoma (Main *et al.*, 1985). Furthermore, direct comparison between vaccinations with genetically modified DCs and GMCSF-secreting tumour cells in a melanoma model revealed the superior efficacy of the former. MAGE-1-transduced DCs exhibited a superior anti-metastatic effect while further modification to express GMCSF or CD40L enhanced their therapeutic effect in a subcutaneous melanoma model (Klein *et al.*, 2000). This result not only confirmed that DCs may comprise a better vaccination approach for certain tumours, but also reinforced the concept of applying multi-modality treatments for cancer gene therapy.

The treatment of neuroblastoma is an area of considerable unmet medical need. Current treatment protocols fail to adequately control the disease and mortality rates are high. The development of targeted therapeutic strategies to control tumour growth is a prerequisite for providing therapeutic options for patients that fail current treatment regimes. Tumour immunotherapy provides a rational and potentially viable approach to this problem especially for targeting minimal residual disease. Immunisation strategies, using both autologous or allogeneic tumour cells in various tumour types, have previously shown potential in reducing or eliminating tumour growth in pre-clinical models. The work described in this thesis suggests that utilisation of vaccine tumour cells expressing IL-2 and IL-12 may offer a potential therapeutic approach for the treatment of neuroblastoma.

REFERENCES

Acsadi, G., Dickson, G., Love, D. R., Jani, A., Walsh, F. S., Gurusinghe, A., Wolff, J. A., and Davies, K. E. (1991). Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. *Nature* **352**:815-818.

Addison, C. L., Bramson, J. L., Hitt, M. M., Muller, W. J., Gauldie, J., and Graham, F. L. (1998). Intratumoral coinjection of adenoviral vectors expressing IL-2 and IL-12 results in enhanced frequency of regression of injected and untreated distal tumors. *Gene Ther* **5**:1400-1409.

Albert, M. L., Pearce, S. F., Francisco, L. M., Sauter, B., Roy, P., Silverstein, R. L., and Bhardwaj, N. (1998). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* **188**:1359-1368.

Albert, M. L., Sauter, B., and Bhardwaj, N. (1998). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**:86-89.

Ali, R. R., Sarra, G. M., Stephens, C., Alwis, M. D., Bainbridge, J. W., Munro, P. M., Fauser, S., Reichel, M. B., Kinnon, C., Hunt, D. M., Bhattacharya, S. S., and Thrasher, A. J. (2000). Restoration of photoreceptor ultrastructure and function in retinal degeneration slow mice by gene therapy. *Nat Genet* **25**:306-310.

Alimonti, J., Zhang, Q. J., Gabathuler, R., Reid, G., Chen, S. S., and Jefferies, W. A. (2000). TAP expression provides a general method for improving the recognition of malignant cells in vivo. *Nat Biotechnol* **18**:515-520.

Anderson, R., Macdonald, I., Corbett, T., Hacking, G., Lowdell, M. W., and Prentice, H. G. (1997). Construction and biological characterization of an interleukin-12 fusion protein (Flexi-12): delivery to acute myeloid leukemic blasts using adeno-associated virus. *Hum Gene Ther* **8**:1125-1135.

Araki, N., Johnson, M. T., and Swanson, J. A. (1996). A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* **135**:1249-1260.

Arienti, F., Sule-Suso, J., Melani, C., Maccalli, C., Belli, F., Illeni, M. T., Anichini, A., Cascinelli, N., Colombo, M. P., and Parmiani, G. (1994). Interleukin-2 gene-transduced human melanoma cells efficiently stimulate MHC-unrestricted and MHC-restricted autologous lymphocytes. *Hum Gene Ther* **5**:1139-1150.

Balicki, D., Reisfeld, R. A., Pertl, U., Beutler, E., and Lode, H. N. (2000). Histone H2A-mediated transient cytokine gene delivery induces efficient antitumor responses in murine neuroblastoma. *Proc Natl Acad Sci U S A* **97**:11500-11504.

Bannerji, R., Arroyo, C. D., Cordon, C. C., and Gilboa, E. (1994). The role of IL-2 secreted from genetically modified tumor cells in the establishment of antitumor immunity. *J Immunol* **152**:2324-2332.

Beatty, G. L. and Paterson, Y. (2000). IFN-gamma can promote tumor evasion of the immune system in vivo by down-regulating cellular levels of an endogenous tumor antigen. *J Immunol* **165**:5502-5508.

Berard, F., Blanco, P., Davoust, J., Neidhart-Berard, E. M., Nouri-Shirazi, M., Taquet, N., Rimoldi, D., Cerottini, J. C., Banchereau, J., and Palucka, A. K. (2000). Cross-priming of naive CD8 T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells. *J Exp Med* **192**:1535-1544.

Bergelson, J. M., Shepley, M. P., Chan, B. M., Hemler, M. E., and Finberg, R. W. (1992). Identification of the integrin VLA-2 as a receptor for echovirus 1 [see comments]. *Science* **255**:1718-1720.

Bernassola, F., Scheuerpflug, C., Herr, I., Krammer, P. H., Debatin, K. M., and Melino, G. (1999). Induction of apoptosis by IFNgamma in human neuroblastoma cell lines through the CD95/CD95L autocrine circuit. *Cell Death Differ* **6**:652-660.

Bi, W. L., Parysek, L. M., Warnick, R., and Stambrook, P. J. (1993). In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSV tk retroviral gene therapy. *Hum Gene Ther* **4**:725-731.

Boismenu, R., Feng, L., Xia, Y. Y., Chang, J. C., and Havran, W. L. (1996). Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. *J Immunol* **157**:985-992.

Bouchard, C., Staller, P., and Eilers, M. (1998). Control of cell proliferation by Myc. *Trends Cell Biol* **8**:202-206.

Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* **92**:7297-7301.

Bowman, L., Grossmann, M., Rill, D., Brown, M., Zhong, W. Y., Alexander, B., Leimig, T., Coustan, S. E., Campana, D., Jenkins, J., Woods, D., Kitchingman, G., Vanin, E., and Brenner, M. (1998a). IL-2 adenovector-transduced autologous tumor cells induce antitumor immune responses in patients with neuroblastoma. *Blood* **92**:1941-1949.

Bowman, L. C., Grossmann, M., Rill, D., Brown, M., Zhong, W. Y., Alexander, B., Leimig, T., Coustan, S. E., Campana, D., Jenkins, J., Woods, D., and Brenner, M. (1998b). Interleukin-2 gene-modified allogeneic tumor cells for treatment of relapsed neuroblastoma. *Hum Gene Ther* **9**:1303-1311.

Breit, S., Ashman, K., Wilting, J., Rossler, J., Hatzi, E., Fotsis, T., and Schweigerer, L. (2000). The N-myc oncogene in human neuroblastoma cells: down-regulation of an angiogenesis inhibitor identified as activin A. *Cancer Res* **60**:4596-4601.

Brenner, M. K. (1992). Clinical protocol: Phase I Study of Cytokine-Gene Modified Autologous Neuroblastoma Cells for Treatment of Relapsed/Refractory Neuroblastoma. *Hum Gene Ther* **3**:655-676.

Brenner, M. K., Heslop, H., Krance, R., Horowitz, M., Strother, D., Nuchtern, J., Grilley, B., Martingano, E., and Cooper, K. (2000). Phase I study of chemokine and cytokine gene-modified autologous neuroblastoma cells for treatment of relapsed/refractory neuroblastoma using an adenoviral vector. *Hum Gene Ther* **11**:1477-1488.

Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. (1994). Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **79**:1157-1164.

Brunda, M. J., Luistro, L., Warrier, R. R., Wright, R. B., Hubbard, B. R., Murphy, M., Wolf, S. F., and Gately, M. K. (1993). Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med* **178**:1223-1230.

Cao, X., Zhang, W., He, L., Xie, Z., Ma, S., Tao, Q., Yu, Y., Hamada, H., and Wang, J. (1998). Lymphotactin gene-modified bone marrow dendritic cells act as more potent adjuvants for peptide delivery to induce specific antitumor immunity. *J Immunol* **161**:6238-6244.

Caplen, N. J., Kinrade, E., Sorgi, F., Gao, X., Gruenert, D., Geddes, D., Coutelle, C., Huang, L., Alton, E. W., and Williamson, R. (1995a). In vitro liposome-mediated DNA transfection of epithelial cell lines using the cationic liposome DC-Chol/DOPE. *Gene Ther* **2**:603-613.

Caplen, N. J., Alton, E. W., Middleton, P. G., Dorin, J. R., Stevenson, B. J., Gao, X., Durham, S. R., Jeffery, P. K., Hodson, M. E., and Coutelle, C. (1995b). Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat Med* **1**:39-46.

Carpenter, D. S. and Minchin, R. F. (1998). Targeting of a cholecystokinin-DNA complex to pancreatic cells in vitro and in vivo. *Gene Ther* **5**:848-854.

Castel, V., Garcia-Miguel, P., Canete, A., Melero, C., Navajas, A., Ruiz-Jimenez, J. I., Navarro, S., and Badal, M. D. (1999). Prospective evaluation of the International Neuroblastoma Staging System (INSS) and the International Neuroblastoma Response Criteria (INRC) in a multicentre setting. *Eur J Cancer* **35**:606-611.

Cavallo, F., Giovarelli, M., Gulino, A., Vacca, A., Stoppacciaro, A., Modesti, A., and Forni, G. (1992). Role of neutrophils and CD4+ T lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene. *J Immunol* **149**:3627-3635.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint, B. G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Deist, F. L., and Fischer, A. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**:669-672.

Cho, H. S., Park, Y. N., Lyu, C. J., Yang, C. H., Ryoo, K. Y., Kim, B. S., Kim, K. Y., and Kim, Y. S. (1999). Effects of retroviral-mediated herpes simplex virus thymidine kinase gene transfer to murine neuroblastoma cell lines in vitro and in vivo. *Acta Oncol* **38**:1093-1097.

Choi, S. H., Reynolds, J. V., and Ziegler, M. M. (1989). Systematic analysis of the immunoregulation of murine neuroblastoma. *J Pediatr Surg* **24**:15-19.

Chong, H., Todryk, S., Hutchinson, G., Hart, I. R., and Vile, R. G. (1998). Tumour cell expression of B7 costimulatory molecules and interleukin-12 or granulocyte-macrophage colony-stimulating factor induces a local antitumour response and may generate systemic protective immunity. *Gene Ther* **5**:223-232.

Colin, M., Harbottle, R., Knight, A. M., Kornprobst, M., Cooper, R. G., Miller, A. D., Trugnan, G., Capeau, J., Coutelle, C., and Brahimi-Horn, M. C. (1998). Liposomes enhance delivery and expression of an RGD-oligolysine gene transfer vector in human tracheal cells. *Gene Ther* **5**:1488-1498.

Colin, M., Maurice, M., Trugnan, G., Kornprobst, M., Harbottle, R. P., Knight, A., Cooper, R. G., Miller, A. D., Capeau, J., Coutelle, C., and Brahimi-Horn, M. C. (2000). Cell delivery, intracellular trafficking and expression of an integrin- mediated gene transfer vector in tracheal epithelial cells. *Gene Ther* **7**:139-152.

Coll, J. L., Wagner, E., Combaret, V., Metchler, K., Amstutz, H., Iacono-Di-Cacito, I., Simon, N., and Favrot, M. C. (1997). In vitro targeting and specific transfection of human neuroblastoma cells by chCE7 antibody-mediated gene transfer. *Gene Ther* **4**:156-161.

Compton, S. H., Mecklenbeck, S., Mejia, J. E., Hart, S. L., Rice, M., Cervini, R., Barrandon, Y., Larin, Z., Levy, E. R., Bruckner-Tuderman, L., and Hovnanian, A. (2000). Stable integration of large (>100 kb) PAC constructs in HaCaT keratinocytes using an integrin-targeting peptide delivery system. *Gene Ther* **7**:1600-1605.

Corrias, M. V., Basso, S., Meazza, R., Musiani, P., Santi, L., Bocca, P., Occhino, M., Ferrini, S., and Pistoia, V. (1998). Characterization and tumorigenicity of human neuroblastoma cells transfected with the IL-2 gene. *Cancer Gene Ther* **5**:38-44.

Corrias, M. V., Occhino, M., Croce, M., De Ambrosis, A., Pistillo, M. P., Bocca, P., Pistoia, V., and Ferrini, S. (2001). Lack of HLA-class I antigens in human neuroblastoma cells: analysis of its relationship to TAP and tapasin expression. *Tissue Antigens* **57**:110-117.

Croyle, M. A., Walter, E., Janich, S., Roessler, B. J., and Amidon, G. L. (1998). Role of integrin expression in adenovirus-mediated gene delivery to the intestinal epithelium. *Hum Gene Ther* **9**:561-573.

Danos, O. and Mulligan, R. C. (1988). Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci U S A* **85**:6460-6464.

Danthinne, X. and Imperiale, M. J. (2000). Production of first generation adenovirus vectors: a review. *Gene Ther* **7**:1707-1714.

Davidoff, A. M., Kimbrough, S. A., Ng, C. Y., Shochat, S. J., and Vanin, E. F. (1999a). Neuroblastoma regression and immunity induced by transgenic expression of interleukin-12. *J Pediatr Surg* **34**:902-906.

Davidoff, A. M., Stevenson, S. C., McClelland, A., Shochat, S. J., and Vanin, E. F. (1999b). Enhanced neuroblastoma transduction for an improved antitumor vaccine. *J Surg Res* **83**:95-99.

Davidoff, A. M., Leary, M. A., Ng, C. Y., and Vanin, E. F. (2000). Retroviral vector-producer cell mediated angiogenesis inhibition restricts neuroblastoma growth in vivo. *Med Pediatr Oncol* **35**:638-640.

Davidoff, A. M., Leary, M. A., Ng, C. Y., and Vanin, E. F. (2001). Gene therapy-mediated expression by tumor cells of the angiogenesis inhibitor flk-1 results in inhibition of neuroblastoma growth in vivo. *J Pediatr Surg* **36**:30-36.

Davis, H. L., Whalen, R. G., and Demeneix, B. A. (1993a). Direct gene transfer into skeletal muscle in vivo: factors affecting efficiency of transfer and stability of expression. *Hum Gene Ther* **4**:151-159.

Davis, H. L., Demeneix, B. A., Quantin, B., Coulombe, J., and Whalen, R. G. (1993b). Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum Gene Ther* **4**:733-740.

Desai, B. B., Quinn, P. M., Wolitzky, A. G., Mongini, P. K., Chizzonite, R., and Gately, M. K. (1992). IL-12 receptor. II. Distribution and regulation of receptor expression. *J Immunol* **148**:3125-3132.

Diaz, R. M., Bateman, A., Emiliusen, L., Fielding, A., Trono, D., Russell, S. J., and Vile, R. G. (2000). A lentiviral vector expressing a fusogenic glycoprotein for cancer gene therapy. *Gene Ther* **7**:1656-1663.

Djerbi, M., Screpanti, V., Catrina, A. I., Bogen, B., Biberfeld, P., and Grandien, A. (1999). The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J Exp Med* **190**:1025-1032.

Dorner, B., Muller, S., Entschladen, F., Schroder, J. M., Franke, P., Kraft, R., Friedl, P., Clark-Lewis, I., and Kroczek, R. A. (1997). Purification, structural analysis, and

function of natural ATAC, a cytokine secreted by CD8(+) T cells. *J Biol Chem* **272**:8817-8823.

Eggert, A., Ikegaki, N., Kwiatkowski, J., Zhao, H., Brodeur, G. M., and Himelstein, B. P. (2000). High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. *Clin Cancer Res* **6**:1900-1908.

Egilmez, N. K., Cuenca, R., Yokota, S. J., Sorgi, F., and Bankert, R. B. (1996). In vivo cytokine gene therapy of human tumor xenografts in SCID mice by liposome-mediated DNA delivery. *Gene Ther* **3**:607-614.

Emtage, P. C., Wan, Y., Hitt, M., Graham, F. L., Muller, W. J., Zlotnik, A., and Gauldie, J. (1999). Adenoviral vectors expressing lymphotactin and interleukin 2 or lymphotactin and interleukin 12 synergize to facilitate tumor regression in murine breast cancer models. *Hum Gene Ther* **10**:697-709.

Engler, S., Thiel, C., Forster, K., David, K., Bredehorst, R., and Juhl, H. (2001). A novel metastatic animal model reflecting the clinical appearance of human neuroblastoma: growth arrest of orthotopic tumors by natural, cytotoxic human immunoglobulin M antibodies. *Cancer Res* **61**:2968-2973.

Erbacher, P., Remy, J. S., and Behr, J. P. (1999). Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. *Gene Ther* **6**:138-145.

Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L. S., Kim, K. S., Stins, M. F., Seeger, R. C., and Durden, D. L. (2000). Integrins alpha(v)beta3 and alpha(v)beta5 are expressed by endothelium of high-risk neuroblastoma and their inhibition is associated with increased endogenous ceramide. *Cancer Res* **60**:712-721.

Evan, G. and Littlewood, T. (1998). A matter of life and cell death. *Science* **281**:1317-1322.

Farhood, H., Serbina, N., and Huang, L. (1995). The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta* **1235**:289-295.

Fasbender, A., Zabner, J., Chillon, M., Moninger, T. O., Puga, A. P., Davidson, B. L., and Welsh, M. J. (1997). Complexes of adenovirus with polycationic polymers and cationic lipids increase the efficiency of gene transfer in vitro and in vivo. *J Biol Chem* **272**:6479-6489.

Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., Karasuyama, H., Vogelstein, B., and Frost, P. (1990). Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell* **60**:397-403.

Fenczik, C. A., Sethi, T., Ramos, J. W., Hughes, P. E., and Ginsberg, M. H. (1997). Complementation of dominant suppression implicates CD98 in integrin activation [see comments]. *Nature* **390**:81-85.

Fernandez, N. C., Lozier, A., Flament, C., Ricciardi-Castagnoli, P., Bellet, D., Suter, M., Perricaudet, M., Tursz, T., Maraskovsky, E., and Zitvogel, L. (1999). Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. *Nat Med* **5**:405-411.

Flickinger, K. S., Judware, R., Lechner, R., Carter, W. G., and Culp, L. A. (1994). Integrin expression in human neuroblastoma cells with or without N-myc amplification and in ectopic/orthotopic nude mouse tumors. *Exp Cell Res* **213**:156-163.

Fominaya, J. and Wels, W. (1996). Target cell-specific DNA transfer mediated by a chimeric multidomain protein. Novel non-viral gene delivery system. *J Biol Chem* **271**:10560-10568.

Fraker, D. L., Langstein, H. N., and Norton, J. A. (1989). Passive immunization against tumor necrosis factor partially abrogates interleukin 2 toxicity. *J Exp Med* **170**:1015-1020.

Frost, J. D., Hank, J. A., Reaman, G. H., Frierdich, S., Seeger, R. C., Gan, J., Anderson, P. M., Ettinger, L. J., Cairo, M. S., Blazar, B. R., Krailo, M. D., Matthay, K. K., Reisfeld, R. A., and Sondel, P. M. (1997). A phase I/IB trial of murine monoclonal anti-GD2 antibody 14.G2a plus interleukin-2 in children with refractory neuroblastoma: a report of the Children's Cancer Group. *Cancer* **80**:317-333.

Furlan, R., Poliani, P. L., Marconi, P. C., Bergami, A., Ruffini, F., Adorini, L., Glorioso, J. C., Comi, G., and Martino, G. (2001). Central nervous system gene therapy with interleukin-4 inhibits progression of ongoing relapsing-remitting autoimmune encephalomyelitis in Biozzi AB/H mice. *Gene Ther* **8**:13-19.

Gaffen, S. L., Goldsmith, M. A., and Greene, W. C. (1998). Interleukin-2 and the Interleukin-2 Receptor. *In* "The Cytokine Handbook" pp. 73-103, Academic Press Limited.

Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R., and Gilboa, E. (1990). Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *J Exp Med* **172**:1217-1224.

Gately, M. K., Desai, B. B., Wolitzky, A. G., Quinn, P. M., Dwyer, C. M., Podlaski, F. J., Familletti, P. C., Sinigaglia, F., Chizonnite, R., Gubler, U., and et, a. (1991). Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J Immunol* **147**:874-882.

Ginsberg, M. H., Du, X., and Plow, E. F. (1992). Inside-out integrin signalling. *Curr Opin Cell Biol* **4**:766-771.

Goldman, J. P., Blundell, M. P., Lopes, L., Kinnon, C., Di Santo, J. P., and Thrasher, A. J. (1998). Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. *Br J Haematol* **103**:335-342.

Gong, J., Chen, L., Chen, D., Kashiwaba, M., Manome, Y., Tanaka, T., and Kufe, D. (1997). Induction of antigen-specific antitumor immunity with adenovirus-transduced dendritic cells. *Gene Ther* **4**:1023-1028.

Govaerts, A. S., Guillaume, T., Andre, M., Bayat, B., Feyens, A. M., Hawley, T. S., Fong, A. Z., Hawley, R. G., and Symann, M. (1999). Retroviral-mediated transfer of genes encoding interleukin-2 and interleukin-12 into fibroblasts increases host antitumor responsiveness. *Cancer Gene Ther* **6**:447-455.

Groux, H., Bigler, M., de Vries, J. E., and Roncarolo, M. G. (1996). Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* **184**:19-29.

Haas, T. A. and Plow, E. F. (1994). Integrin-ligand interactions: a year in review. *Curr Opin Cell Biol* **6**:656-662.

Haber, M., Bordow, S. B., Haber, P. S., Marshall, G. M., Stewart, B. W., and Norris, M. D. (1997). The prognostic value of MDR1 gene expression in primary untreated neuroblastoma. *Eur J Cancer* **33**:2031-2036.

Harbottle, R. P., Cooper, R. G., Hart, S. L., Ladhoff, A., McKay, T., Knight, A. M., Wagner, E., Miller, A. D., and Coutelle, C. (1998). An RGD-oligolysine peptide: a prototype construct for integrin-mediated gene delivery [see comments]. *Hum Gene Ther* **9**:1037-1047.

Hart, S. L., Knight, A. M., Harbottle, R. P., Mistry, A., Hunger, H. D., Cutler, D. F., Williamson, R., and Coutelle, C. (1994). Cell binding and internalization by filamentous phage displaying a cyclic Arg-Gly-Asp-containing peptide. *J Biol Chem* **269**:12468-12474.

Hart, S. L., Harbottle, R. P., Cooper, R., Miller, A., Williamson, R., and Coutelle, C. (1995). Gene delivery and expression mediated by an integrin-binding peptide [published erratum appears in Gene Ther 1996 Nov;3(11):1032-3]. *Gene Ther* **2**:552-554.

Hart, S. L., Collins, L., Gustafsson, K., and Fabre, J. W. (1997). Integrin-mediated transfection with peptides containing arginine-glycine-aspartic acid domains. *Gene Ther* **4**:1225-1230.

Hart, S. L., Arancibia, C. C., Wolfert, M. A., Mailhos, C., O'Reilly, N. J., Ali, R. R., Coutelle, C., George, A. J., Harbottle, R. P., Knight, A. M., Larkin, D. F., Levinsky, R. J., Seymour, L. W., Thrasher, A. J., and Kinnon, C. (1998). Lipid-mediated enhancement of transfection by a nonviral integrin-targeting vector. *Hum Gene Ther* **9** :575-585.

Hautanen, A., Gailit, J., Mann, D. M., and Ruoslahti, E. (1989). Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor. *J Biol Chem* **264**:1437-1442.

Hedrick, J. A., Saylor, V., Figueroa, D., Mizoue, L., Xu, Y., Menon, S., Abrams, J., Handel, T., and Zlotnik, A. (1997). Lymphotactin is produced by NK cells and attracts both NK cells and T cells in vivo. *J Immunol* **158**:1533-1540.

Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D. D., and Kirn, D. H. (1997). ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med* **3**:639-645.

Heuer, J. G., Tucker, M. C., Gonin, R., and Hock, R. A. (1996). Retrovirus-mediated gene transfer of B7-1 and MHC class II converts a poorly immunogenic neuroblastoma into a highly immunogenic one. *Hum Gene Ther* **7**:2059-2068.

Heuser, J. E. and Anderson, R. G. (1989). Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* **108**:389-400.

Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M. A., and Shay, J. W. (1995). Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* **1**:249-255.

Hock, R. A., Reynolds, B. D., Tucker-McClung, C. L., and Heuer, J. G. (1996). Murine neuroblastoma vaccines produced by retroviral transfer of MHC class II genes. *Cancer Gene Ther* **3**:314-320.

Honsik, C. J., Jung, G., and Reisfeld, R. A. (1986). Lymphokine-activated killer cells targeted by monoclonal antibodies to the disialogangliosides GD2 and GD3 specifically lyse human tumor cells of neuroectodermal origin. *Proc Natl Acad Sci U S A* **83**:7893-7897.

Hoshi, M., Otagiri, N., Shiwaku, H. O., Asakawa, S., Shimizu, N., Kaneko, Y., Ohi, R., Hayashi, Y., and Horii, A. (2000). Detailed deletion mapping of chromosome band 14q32 in human neuroblastoma defines a 1.1-Mb region of common allelic loss. *Br J Cancer* **82** :1801-1807.

Hsieh, C. L., Chen, D. S., and Hwang, L. H. (2000). Tumor-induced immunosuppression: a barrier to immunotherapy of large tumors by cytokine-secreting tumor vaccine. *Hum Gene Ther* **11**:681-692.

Huang, S., Kamata, T., Takada, Y., Ruggeri, Z. M., and Nemerow, G. R. (1996). Adenovirus interaction with distinct integrins mediates separate events in cell entry and gene delivery to hematopoietic cells. *J Virol* **70**:4502-4508.

Hughes, P. E. and Pfaff, M. (1998). Integrin affinity modulation. *Trends Cell Biol* **8**:359-364.

Humphries, M. J. and Newham, P. (1998). The structure of cell-adhesion molecules. *Trends Cell Biol* **8**:78-83.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**:11-25.

Ikeda, H., Nakamura, Y., Hiwasa, T., Sakiyama, S., Kuida, K., Su, M. S., and Nakagawara, A. (1997). Interleukin-1 beta converting enzyme (ICE) is preferentially expressed in neuroblastomas with favourable prognosis. *Eur J Cancer* **33**:2081-2083.

Isberg, R. and Van Nhieu, G. (1995). The mechanism of phagocytic uptake promoted by invasin-integrin interaction. *Trends CellBiol* **5**:120-124.

Ishida, H., Matsumura, T., Salgaller, M. L., Ohmizono, Y., Kadono, Y., and Sawada, T. (1996). MAGE-1 and MAGE-3 or -6 expression in neuroblastoma-related pediatric solid tumors. *Int J Cancer* **69**:375-380.

Jackson, T., Sharma, A., Ghazaleh, R. A., Blakemore, W. E., Ellard, F. M., Simmons, D. L., Newman, J. W., Stuart, D. I., and King, A. M. (1997). Arginine-glycine-aspartic acid-specific binding by foot-and-mouth disease viruses to the purified integrin alpha(v)beta3 in vitro. *J Virol* **71**:8357-8361.

Jorgensen, G. E., Johnsen, J. I., Ponthan, F., Kogner, P., Flaegstad, T., and Traavik, T. (2000). Human polyomavirus BK (BKV) and neuroblastoma: mechanisms of oncogenic action and possible strategy for novel treatment. *Med Pediatr Oncol* **35**:593-596.

Jost, C. A., Marin, M. C., and Kaelin-WG, J. (1997). p73 is a human p53-related protein that can induce apoptosis [see comments]. *Nature* **389**:191-194.

Ju, D. W., Tao, Q., Cheng, D. S., Zhang, W., Zhang, M., Hamada, H., and Cao, X. (2000). Adenovirus-mediated lymphotactin gene transfer improves therapeutic efficacy of cytosine deaminase suicide gene therapy in established murine colon carcinoma. *Gene Ther* **7** :329-338.

Judware, R. and Culp, L. A. (1995). Over-expression of transfected N-myc oncogene in human SKNSH neuroblastoma cells down-regulates expression of beta 1 integrin subunit. *Oncogene* **11**:2599-2607.

Judware, R. and Culp, L. A. (1997). Concomitant down-regulation of expression of integrin subunits by N-myc in human neuroblastoma cells: differential regulation of alpha2, alpha3 and beta1. *Oncogene* **14**:1341-1350.

Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat Genet* **17**:314-317.

Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L., and Verma, I. (1998). Cellular immune response to adenoviral vector infected cells does not require de novo viral gene expression: implications for gene therapy. *Proc Natl Acad Sci U S A* **95**:11377-11382.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997).

Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90**:809-819.

Kasid, A., Director, E. P., and Rosenberg, S. A. (1989). Induction of endogenous cytokine-mRNA in circulating peripheral blood mononuclear cells by IL-2 administration to cancer patients. *J Immunol* **143**:736-739.

Katsanis, E., Orchard, P. J., Bausero, M. A., Gorden, K. B., McIvor, R. S., and Blazar, B. R. (1994). Interleukin-2 gene transfer into murine neuroblastoma decreases tumorigenicity and enhances systemic immunity causing regression of preestablished retroperitoneal tumors. *J Immunother Emphasis Tumor Immunol* **15**:81-90.

Katsanis, E., Xu, Z., Bausero, M. A., Dancisak, B. B., Gorden, K. B., Davis, G., Gray, G. S., Orchard, P. J., and Blazar, B. R. (1995). B7-1 expression decreases tumorigenicity and induces partial systemic immunity to murine neuroblastoma deficient in major histocompatibility complex and costimulatory molecules. *Cancer Gene Ther* **2**:39-46.

Katsanis, E., Bausero, M. A., Panoskaltsis-Mortari, A., Dancisak, B. B., Xu, Z., Orchard, P. J., Davis, C. G., and Blazar, B. R. (1996). Irradiation of singly and doubly transduced murine neuroblastoma cells expressing B7-1 and producing interferon-gamma reduces their capacity to induce systemic immunity. *Cancer Gene Ther* **3**:75-82.

Katzenstein, H. M., Rademaker, A. W., Senger, C., Salwen, H. R., Nguyen, N. N., Thorner, P. S., Litsas, L., and Cohn, S. L. (1999). Effectiveness of the angiogenesis inhibitor TNP-470 in reducing the growth of human neuroblastoma in nude mice inversely correlates with tumor burden. *Clin Cancer Res* **5**:4273-4278.

Kaucic, K., Etue, N., LaFleur, B., Woods, W., and Ladisch, S. (2001). Neuroblastomas of infancy exhibit a characteristic ganglioside pattern. *Cancer* **91**:785-793.

Kay, M. A., Manno, C. S., Ragni, M. V., Larson, P. J., Couto, L. B., McClelland, A., Glader, B., Chew, A. J., Tai, S. J., Herzog, R. W., Arruda, V., Johnson, F., Scallan, C., Skarsgard, E., Flake, A. W., and High, K. A. (2000). Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* **24**:257-261.

Kelner, G. S., Kennedy, J., Bacon, K. B., Kleyensteuber, S., Largaespada, D. A., Jenkins, N. A., Copeland, N. G., Bazan, J. F., Moore, K. W., Schall, T. J., and . (1994). Lymphotactin: a cytokine that represents a new class of chemokine. *Science* **266**:1395-1399.

Keshelava, N., Seeger, R. C., and Reynolds, C. P. (1997). Drug resistance in human neuroblastoma cell lines correlates with clinical therapy. *Eur J Cancer* **33**:2002-2006.

Kikuchi, T., Moore, M. A., and Crystal, R. G. (2000). Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. *Blood* **96**:91-99.

Kikuchi, T., Miyazawa, N., Moore, M. A., and Crystal, R. G. (2000). Tumor regression induced by intratumor administration of adenovirus vector expressing CD40 ligand and naive dendritic cells. *Cancer Res* **60**:6391-6395.

Kircheis, R., Kichler, A., Wallner, G., Kursa, M., Ogris, M., Felzmann, T., Buchberger, M., and Wagner, E. (1997). Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Ther* **4**:409-418.

Kirn, D. (2001). Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? *Gene Ther* **8**:89-98.

Klein, C., Bueler, H., and Mulligan, R. C. (2000). Comparative analysis of genetically modified dendritic cells and tumor cells as therapeutic cancer vaccines. *J Exp Med* **191**:1699-1708.

Kochanek, S., Clemens, P. R., Mitani, K., Chen, H. H., Chan, S., and Caskey, C. T. (1996). A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc Natl Acad Sci U S A* **93**:5731-5736.

Koivunen, E., Wang, B., and Ruoslahti, E. (1994). Isolation of a highly specific ligand for the alpha 5 beta 1 integrin from a phage display library. *J Cell Biol* **124**:373-380.

Kramer, K., Gerald, W. L., Kushner, B. H., Larson, S. M., Hameed, M., and Cheung, N. K. (1998). Disialoganglioside G(D2) loss following monoclonal antibody therapy is rare in neuroblastoma. *Clin Cancer Res* **4**:2135-2139.

Kramer, K., Kushner, B., Heller, G., and Cheung, N. K. (2001). Neuroblastoma metastatic to the central nervous system. The Memorial Sloan-kettering Cancer Center Experience and A Literature Review. *Cancer* **91**:1510-1519.

Lastowska, M., Roberts, P., Pearson, A. D., Lewis, I., Wolstenholme, J., and Bown, N. (1997). Promiscuous translocations of chromosome arm 17q in human neuroblastomas. *Genes Chromosomes Cancer* **19**:143-149.

Leimig, T., Foreman, N., Rill, D., Coze, C., Holladay, M., and Brenner, M. (1994). Immunomodulatory effects of human neuroblastoma cells transduced with a retroviral vector encoding interleukin-2. *Cancer Gene Ther* **1**:253-258.

Leimig, T., Brenner, M., Ramsey, J., Vanin, E., Blaese, M., and Dilloo, D. (1996). High-efficiency transduction of freshly isolated human tumor cells using adenoviral interleukin-2 vectors. *Hum Gene Ther* 7:1233-1239.

Li, E., Stupack, D., Klemke, R., Cheresh, D. A., and Nemerow, G. R. (1998). Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide-3-OH kinase. *J Virol* **72**:2055-2061.

Li, G., D'Souza-Schorey, C., Barbieri, M. A., Roberts, R. L., Klippel, A., Williams, L. T., and Stahl, P. D. (1995). Evidence for phosphatidylinositol 3-kinase as a regulator of endocytosis via activation of Rab5. *Proc Natl Acad Sci U S A* **92**:10207-10211.

Li, R. and Ladisch, S. (1996). Abrogation of shedding of immunosuppressive neuroblastoma gangliosides. *Cancer Res* **56**:4602-4605.

Linnala, A., Lehto, V. P., and Virtanen, I. (1997). Neuronal differentiation in SH-SY5Y human neuroblastoma cells induces synthesis and secretion of tenascin and upregulation of alpha(v) integrin receptors. *J Neurosci Res* **49**:53-63.

Lode, H. N., Dreier, T., Xiang, R., Varki, N. M., Kang, A. S., and Reisfeld, R. A. (1998). Gene therapy with a single chain interleukin 12 fusion protein induces T cell-dependent protective immunity in a syngeneic model of murine neuroblastoma. *Proc Natl Acad Sci U S A* **95**:2475-2480.

Lode, H. N., Xiang, R., Dreier, T., Varki, N. M., Gillies, S. D., and Reisfeld, R. A. (1998). Natural killer cell-mediated eradication of neuroblastoma metastases to bone marrow by targeted interleukin-2 therapy. *Blood* **91**:1706-1715.

Lode, H. N., Xiang, R., Duncan, S. R., Theofilopoulos, A. N., Gillies, S. D., and Reisfeld, R. A. (1999). Tumor-targeted IL-2 amplifies T cell-mediated immune response induced by gene therapy with single-chain IL-12. *Proc Natl Acad Sci U S A* **96**:8591-8596.

Lode, H. N., Moehler, T., Xiang, R., Jonczyk, A., Gillies, S. D., Cheresh, D. A., and Reisfeld, R. A. (1999). Synergy between an antiangiogenic integrin alphav antagonist and an antibody-cytokine fusion protein eradicates spontaneous tumor metastases. *Proc Natl Acad Sci U S A* **96**:1591-1596.

Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**:957-967.

Lu, P. and Sharom, F. J. (1995). Gangliosides are potent immunosuppressors of IL-2-mediated T-cell proliferation in a low protein environment. *Immunology* **86**:356-363.

Ludewig, B., Ochsenbein, A. F., Odermatt, B., Paulin, D., Hengartner, H., and Zinkernagel, R. M. (2000). Immunotherapy with dendritic cells directed against tumor antigens shared with normal host cells results in severe autoimmune disease. *J Exp Med* **191**:795-804.

Main, E. K., Lampson, L. A., Hart, M. K., Kornbluth, J., and Wilson, D. B. (1985). Human neuroblastoma cell lines are susceptible to lysis by natural killer cells but not by cytotoxic T lymphocytes. *J Immunol* **135**:242-246.

Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M. P., Maggi, E., Trinchieri, G., and Romagnani, S. (1993). Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med* **177**:1199-1204.

Mariani, E., Meneghetti, A., Tarozzi, A., Cattini, L., and Facchini, A. (2000). Interleukin-12 induces efficient lysis of natural killer-sensitive and natural killer-resistant human osteosarcoma cells: the synergistic effect of interleukin-2. *Scand J Immunol* **51**:618-625.

Markert, J. M., Medlock, M. D., Rabkin, S. D., Gillespie, G. Y., Todo, T., Hunter, W. D., Palmer, C. A., Feigenbaum, F., Tornatore, C., Tufaro, F., and Martuza, R. L. (2000). Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a phase I trial. *Gene Ther* **7**:867-874.

Martuza, R. L. (2000). Conditionally replicating herpes vectors for cancer therapy. *J Clin Invest* **105**:841-846.

Matsui, H., Johnson, L. G., Randell, S. H., and Boucher, R. C. (1997). Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J Biol Chem* **272**:1117-1126.

McLachlan, G., Davidson, D. J., Stevenson, B. J., Dickinson, P., Davidson-Smith, H., Dorin, J. R., and Porteous, D. J. (1995). Evaluation in vitro and in vivo of cationic liposome-expression construct complexes for cystic fibrosis gene therapy. *Gene Ther* **2**:614-622.

Meddeb, M., Danglot, G., Chudoba, I., Venuat, A. M., Benard, J., Avet-Loiseau, H., Vasseur, B., Le Paslier, D., Terrier-Lacombe, M. J., Hartmann, O., and Bernheim, A. (1996). Additional copies of a 25 Mb chromosomal region originating from 17q23.1-17qter are present in 90% of high-grade neuroblastomas. *Genes Chromosomes Cancer* **17**:156-165.

Medema, J. P., de Jong, J., van Hall, T., Melief, C. J., and Offringa, R. (1999). Immune escape of tumors in vivo by expression of cellular FLICE-inhibitory protein. *J Exp Med* **190**:1033-1038.

Meitar, D., Crawford, S. E., Rademaker, A. W., and Cohn, S. L. (1996). Tumor angiogenesis correlates with metastatic disease, N-myc amplification, and poor outcome in human neuroblastoma. *J Clin Oncol* **14**:405-414.

Meli, M. L., Carrel, F., Waibel, R., Amstutz, H., Crompton, N., Jaussi, R., Moch, H., Schubiger, P. A., and Novak-Hofer, I. (1999). Anti-neuroblastoma antibody chCE7 binds to an isoform of L1-CAM present in renal carcinoma cells. *Int J Cancer* **83**:401-408.

Mellman, I. (1996). Endocytosis and molecular sorting. *Annu Rev Cell Dev Biol* **12**:575-625.

Mora, J., Cheung, N. K., Chen, L., Qin, J., and Gerald, W. (2001). Loss of heterozygosity at 19q13.3 is associated with locally aggressive neuroblastoma. *Clin Cancer Res* **7**:1358-1361.

Morgan, D. J., Kreuwel, H. T., Fleck, S., Levitsky, H. I., Pardoll, D. M., and Sherman, L. A. (1998). Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J Immunol* **160**:643-651.

Mortimer, I., Tam, P., MacLachlan, I., Graham, R. W., Saravolac, E. G., and Joshi, P. B. (1999). Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Ther* **6**:403-411.

Mould, A. P., Askari, J. A., Aota, S., Yamada, K. M., Irie, A., Takada, Y., Mardon, H. J., and Humphries, M. J. (1997). Defining the topology of integrin alpha5beta1-fibronectin interactions using inhibitory anti-alpha5 and anti-beta1 monoclonal antibodies. Evidence that the synergy sequence of fibronectin is recognized by the amino-terminal repeats of the alpha5 subunit. *J Biol Chem* **272**:17283-17292.

Mould, A. P., Burrows, L., and Humphries, M. J. (1998). Identification of amino acid residues that form part of the ligand-binding pocket of integrin alpha5 beta1. *J Biol Chem* **273**:25664-25672.

Mould, A. P., Askari, J. A., and Humphries, M. J. (2000). Molecular basis of ligand recognition by integrin alpha 5beta 1. I. Specificity of ligand binding is determined by amino acid sequences in the second and third NH2-terminal repeats of the alpha subunit. *J Biol Chem* **275**:20324-20336.

Mounkes, L. C., Zhong, W., Cipres-Palacin, G., Heath, T. D., and Debs, R. J. (1998). Proteoglycans mediate cationic liposome-DNA complex-based gene delivery in vitro and in vivo. *J Biol Chem* **273**:26164-26170.

Nagayama, H., Sato, K., Kawasaki, H., Enomoto, M., Morimoto, C., Tadokoro, K., Juji, T., Asano, S., and Takahashi, T. A. (2000). IL-12 responsiveness and expression of IL-12 receptor in human peripheral blood monocyte-derived dendritic cells. *J Immunol* **165**:59-66.

Nakai, H., Storm, T. A., and Kay, M. A. (2000). Increasing the size of rAAV-mediated expression cassettes in vivo by intermolecular joining of two complementary vectors. *Nat Biotechnol* **18**:527-532.

Nakashima, M., Sonoda, K., and Watanabe, T. (1999). Inhibition of cell growth and induction of apoptotic cell death by the human tumor-associated antigen RCAS1. *Nat Med* **5**:938-942.

Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996). Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A* **93**:11382-11388.

Nastala, C. L., Edington, H. D., McKinney, T. G., Tahara, H., Nalesnik, M. A., Brunda, M. J., Gately, M. K., Wolf, S. F., Schreiber, R. D., Storkus, W. J., and et, a. (1994). Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. *J Immunol* **153**:1697-1706.

Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. (1998). Vaccination of melanoma patients with pep. *Nat Med* **4**:328-332.

Nguyen, T., Wang, R., and Russell, J. H. (2000). IL-12 enhances IL-2 function by inducing CD25 expression through a p38 mitogen-activated protein kinase pathway. *Eur J Immunol* **30** :1445-1452.

Nickerson, H. J., Matthay, K. K., Seeger, R. C., Brodeur, G. M., Shimada, H., Perez, C., Atkinson, J. B., Selch, M., Gerbing, R. B., Stram, D. O., and Lukens, J. (2000). Favorable biology and outcome of stage IV-S neuroblastoma with supportive care or minimal therapy: a Children's Cancer Group study. *J Clin Oncol* **18**:477-486.

Niidome, T., Ohmori, N., Ichinose, A., Wada, A., Mihara, H., Hirayama, T., and Aoyagi, H. (1997). Binding of cationic alpha-helical peptides to plasmid DNA and their gene transfer abilities into cells. *J Biol Chem* **272**:15307-15312.

Ninane, J. and Pearson, A. D. J. (1997). Neuroblastoma. *In* "Paediatric Oncology" (C. R. Pinkerton and P. N. Plowman, Eds.), pp. 442-483, Chapman & Hall, London.

O'Neil, K. T., Hoess, R. H., Jackson, S. A., Ramachandran, N. S., Mousa, S. A., and DeGrado, W. F. (1992). Identification of novel peptide antagonists for GPIIb/IIIa from a conformationally constrained phage peptide library. *Proteins* **14**:509-515.

Ogris, M., Steinlein, P., Kursa, M., Mechtler, K., Kircheis, R., and Wagner, E. (1998). The size of DNA/transferrin-PEI complexes is an important factor for gene expression in cultured cells. *Gene Ther* **5**:1425-1433.

Ogris, M., Brunner, S., Schuller, S., Kircheis, R., and Wagner, E. (1999). PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther* **6**:595-605.

Okada, H., Miyamura, K., Itoh, T., Hagiwara, M., Wakabayashi, T., Mizuno, M., Colosi, P., Kurtzman, G., and Yoshida, J. (1996). Gene therapy against an experimental glioma using adeno-associated virus vectors. *Gene Ther* **3**:957-964.

Oshikawa, K., Shi, F., Rakhmilevich, A. L., Sondel, P. M., Mahvi, D. M., and Yang, N. S. (1999). Synergistic inhibition of tumor growth in a murine mammary adenocarcinoma model by combinational gene therapy using IL-12, pro-IL-18, and IL-1beta converting enzyme cDNA. *Proc Natl Acad Sci U S A* **96**:13351-13356.

Oshikawa, K., Rakhmilevich, A. L., Shi, F., Sondel, P. M., Yang, N., and Mahvi, D. M. (2001). Interleukin 12 gene transfer into skin distant from the tumor site elicits antimetastatic effects equivalent to local gene transfer. *Hum Gene Ther* **12**:149-160.

Ozkaynak, M. F., Sondel, P. M., Krailo, M. D., Gan, J., Javorsky, B., Reisfeld, R. A., Matthay, K. K., Reaman, G. H., and Seeger, R. C. (2000). Phase I study of chimeric human/murine anti-ganglioside G(D2) monoclonal antibody (ch14.18) with

granulocyte-macrophage colony-stimulating factor in children with neuroblastoma immediately after hematopoietic stem-cell transplantation: a Children's Cancer Group Study. *J Clin Oncol* **18**:4077-4085.

Palmer, T. D., Rosman, G. J., Osborne, W. R., and Miller, A. D. (1991). Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc Natl Acad Sci U S A* **88**:1330-1334.

Pardoll, D. M. (1995). Paracrine cytokine adjuvants in cancer immunotherapy. *Annu Rev Immunol* **13**:399-415.

Pardoll, D. M. and Topalian, S. L. (1998). The role of CD4+ T cell responses in antitumor immunity. *Curr Opin Immunol* **10**:588-594.

Parker, J. N., Gillespie, G. Y., Love, C. E., Randall, S., Whitley, R. J., and Markert, J. M. (2000). Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors. *Proc Natl Acad Sci U S A* **97**:2208-2213.

Paul, R. W., Weisser, K. E., Loomis, A., Sloane, D. L., LaFoe, D., Atkinson, E. M., and Overell, R. W. (1997). Gene transfer using a novel fusion protein, GAL4/invasin. *Hum Gene Ther* **8**:1253-1262.

Peng, X., Remacle, J. E., Kasran, A., Huylebroeck, D., and Ceuppens, J. L. (1998). IL-12 up-regulates CD40 ligand (CD154) expression on human T cells. *J Immunol* **160**:1166-1172.

Perales, J. C., Grossmann, G. A., Molas, M., Liu, G., Ferkol, T., Harpst, J., Oda, H., and Hanson, R. W. (1997). Biochemical and functional characterization of DNA complexes capable of targeting genes to hepatocytes via the asialoglycoprotein receptor. *J Biol Chem* **272**:7398-7407.

Pertl, U., Luster, A. D., Varki, N. M., Homann, D., Gaedicke, G., Reisfeld, R. A., and Lode, H. N. (2001). IFN-gamma-inducible protein-10 is essential for the generation of a protective tumor-specific CD8 T cell response induced by single-chain IL-12 gene therapy. *J Immunol* **166**:6944-6951.

Perussia, B., Chan, S. H., D'Andrea, A., Tsuji, K., Santoli, D., Pospisil, M., Young, D., Wolf, S. F., and Trinchieri, G. (1992). Natural killer (NK) cell stimulatory factor or IL-12 has differential effects on the proliferation of TCR-alpha beta+, TCR-gamma delta+ T lymphocytes, and NK cells. *J Immunol* **149**:3495-3502.

Philip, T., Ladenstein, R., Lasset, C., Hartmann, O., Zucker, J. M., Pinkerton, R., Pearson, A. D., Klingebiel, T., Garaventa, A., Kremens, B., Bernard, J. L., Rosti, G., and Chauvin, F. (1997). 1070 myeloablative megatherapy procedures followed by stem cell rescue for neuroblastoma: 17 years of European experience and conclusions. European Group for Blood and Marrow Transplant Registry Solid Tumour Working Party. *Eur J Cancer* **33**:2130-2135.

Pierschbacher, M. D. and Ruoslahti, E. (1984). Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc Natl Acad Sci U S A* **81**:5985-5988.

Pinkerton, C. R., Blanc Vincent, M. P., Bergeron, C., Fervers, B., and Philip, T. (2000). Induction chemotherapy in metastatic neuroblastoma--does dose influence response? A critical review of published data standards, options and recommendations (SOR) project of the National Federation of French Cancer Centres (FNCLCC). *Eur J Cancer* **36** :1808-1815.

Putzer, B. M., Hitt, M., Muller, W. J., Emtage, P., Gauldie, J., and Graham, F. L. (1997). Interleukin 12 and B7-1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. *Proc Natl Acad Sci U S A* **94**:10889-10894.

Rabinowitz, J. E. and Samulski, R. J. (2000). Building a better vector: the manipulation of AAV virions. *Virology* **278**:301-308.

Ribatti, D., Alessandri, G., Vacca, A., Iurlaro, M., and Ponzoni, M. (1998). Human neuroblastoma cells produce extracellular matrix-degrading enzymes, induce endothelial cell proliferation and are angiogenic in vivo. *Int J Cancer* **77**:449-454.

Ridge, J. P., Di Rosa, F., and Matzinger, P. (1998). A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* **393**:474-478.

Robbins, P. D., Tahara, H., and Ghivizzani, S. C. (1998). Viral vectors for gene therapy. *Trends Biotechnol* **16**:35-40.

Rose, D. M., Cardarelli, P. M., Cobb, R. R., and Ginsberg, M. H. (2000). Soluble VCAM-1 binding to alpha4 integrins is cell-type specific and activation dependent and is disrupted during apoptosis in T cells. *Blood* **95**:602-609.

Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L., and . (1990). Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* **323**:570-578.

Ross, P. C. and Hui, S. W. (1999). Lipoplex size is a major determinant of in vitro lipofection efficiency. *Gene Ther* **6**:651-659.

Rossi, A. R., Pericle, F., Rashleigh, S., Janiec, J., and Djeu, J. Y. (1994). Lysis of neuroblastoma cell lines by human natural killer cells activated by interleukin-2 and interleukin-12. *Blood* **83**:1323-1328.

Rossig, C., Nuchtern, J. G., and Brenner, M. K. (2000). Selection of human antitumor single-chain Fv antibodies from the B-cell repertoire of patients immunized against autologous neuroblastoma. *Med Pediatr Oncol* **35**:692-695.

Roth, J. A., Nguyen, D., Lawrence, D. D., Kemp, B. L., Carrasco, C. H., Ferson, D. Z., Hong, W. K., Komaki, R., Lee, J. J., Nesbitt, J. C., Pisters, K. M., Putnam, J. B., Schea, R., Shin, D. M., Walsh, G. L., Dolormente, M. M., Han, C. I., Martin, F. D., Yen, N., Xu, K., Stephens, L. C., McDonnell, T. J., Mukhopadhyay, T., and Cai, D. (1996). Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer. *Nat Med* **2**:985-991.

Rozzo, C., Ratti, P., Ponzoni, M., and Cornaglia-Ferraris, P. (1993). Modulation of alpha 1 beta 1, alpha 2 beta 1 and alpha 3 beta 1 integrin heterodimers during human neuroblastoma cell differentiation. *FEBS Lett* **332**:263-267.

Ruegg, C., Yilmaz, A., Bieler, G., Bamat, J., Chaubert, P., and Lejeune, F. J. (1998). Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med* **4**:408-414.

Sabzevari, H., Gillies, S. D., Mueller, B. M., Pancook, J. D., and Reisfeld, R. A. (1994). A recombinant antibody-interleukin 2 fusion protein suppresses growth of hepatic human neuroblastoma metastases in severe combined immunodeficiency mice. *Proc Natl Acad Sci U S A* **91**:9626-9630.

Salio, M., Cerundolo, V., and Lanzavecchia, A. (2000). Dendritic cell maturation is induced by mycoplasma infection but not by necrotic cells. *Eur J Immunol* **30**:705-708.

Salmon, P., Kindler, V., Ducrey, O., Chapuis, B., Zubler, R. H., and Trono, D. (2000). High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. *Blood* **96**:3392-3398.

Sauter, B., Albert, M. L., Francisco, L., Larsson, M., Somersan, S., and Bhardwaj, N. (2000). Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med* **191**:423-434.

Schmidt, W., Schweighoffer, T., Herbst, E., Maass, G., Berger, M., Schilcher, F., Schaffner, G., and Birnstiel, M. L. (1995). Cancer vaccines: the interleukin 2 dosage effect. *Proc Natl Acad Sci U S A* **92**:4711-4714.

Schoenhaut, D. S., Chua, A. O., Wolitzky, A. G., Quinn, P. M., Dwyer, C. M., McComas, W., Familletti, P. C., Gately, M. K., and Gubler, U. (1992). Cloning and expression of murine IL-12. *J Immunol* **148**:3433-3440.

Schwartz, R. H. (1996). Models of T cell anergy: is there a common molecular mechanism? *J Exp Med* **184**:1-8.

Sengupta, S., Vonesch, J. L., Waltzinger, C., Zheng, H., and Wasylyk, B. (2000). Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells. *EMBO J* **19**:6051-6064.

Shimizu, M., Fontana, A., Takeda, Y., Yagita, H., Yoshimoto, T., and Matsuzawa, A. (1999). Induction of antitumor immunity with Fas/APO-1 ligand (CD95L)-transfected neuroblastoma neuro-2a cells. *J Immunol* **162**:7350-7357.

Shurin, G. V., Gerein, V., Lotze, M. T., and Barksdale, E. M., Jr. (1998). Apoptosis induced in T cells by human neuroblastoma cells: role of Fas ligand. *Nat Immun* **16**:263-274.

Shurin, G. V., Shurin, M. R., Bykovskaia, S., Shogan, J., Lotze, M. T., and Barksdale, E. M., Jr. (2001). Neuroblastoma-derived gangliosides inhibit dendritic cell generation and function. *Cancer Res* **61**:363-369.

Simoes, S., Slepushkin, V., Pires, P., Gaspar, R., Pedroso de Lima, M. C., and Duzgunes, N. (2000). Human serum albumin enhances DNA transfection by lipoplexes and confers resistance to inhibition by serum. *Biochim Biophys Acta* **1463**:459-469.

Somia, N. and Verma, I. M. (2000). Gene therapy: trials and tribulations. *Nat Rev Genet* **1**:91-99.

Sotomayor, E. M., Borrello, I., Tubb, E., Allison, J. P., and Levitsky, H. I. (1999). In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. *Proc Natl Acad Sci U S A* **96**:11476-11481.

Soudais, C., Shiho, T., Sharara, L. I., Guy-Grand, D., Taniguchi, T., Fischer, A., and Di Santo, J. P. (2000). Stable and functional lymphoid reconstitution of common cytokine receptor gamma chain deficient mice by retroviral-mediated gene transfer. *Blood* **95** :3071-3077.

Staveley, O. K., Sotomayor, E., Montgomery, J., Borrello, I., Hwang, L., Fein, S., Pardoll, D., and Levitsky, H. (1998). Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A* **95**:1178-1183.

Stewart, A. K., Lassam, N. J., Quirt, I. C., Bailey, D. J., Rotstein, L. E., Krajden, M., Dessureault, S., Gallinger, S., Cappe, D., Wan, Y., Addison, C. L., Moen, R. C., Gauldie, J., and Graham, F. L. (1999). Adenovector-mediated gene delivery of interleukin-2 in metastatic breast cancer and melanoma: results of a phase 1 clinical trial. *Gene Ther* **6**:350-363.

Storkus, W. J., Tahara, H., and Lotze, M. T. (1998). Interleukin-12. *In* "The Cytokine Handbook" pp. 391-425, Academic Press Limited.

Stripecke, R., Cardoso, A. A., Pepper, K. A., Skelton, D. C., Yu, X. J., Mascarenhas, L., Weinberg, K. I., Nadler, L. M., and Kohn, D. B. (2000). Lentiviral vectors for efficient delivery of CD80 and granulocyte-macrop. *Blood* **96**:1317-1326.

Summerford, C., Bartlett, J. S., and Samulski, R. J. (1999). AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med* **5**:78-82.

Sun, Y., Jurgovsky, K., Moller, P., Alijagic, S., Dorbic, T., Georgieva, J., Wittig, B., and Schadendorf, D. (1998). Vaccination with IL-12 gene-modified autologous melanoma cells: preclinical results and a first clinical phase I study. *Gene Ther* **5**:481-490.

Takayama, H., Suzuki, T., Mugishima, H., Fujisawa, T., Ookuni, M., Schwab, M., Gehring, M., Nakamura, Y., Sugimura, T., Terada, M., and . (1992). Deletion mapping of chromosomes 14q and 1p in human neuroblastoma. *Oncogene* **7**:1185-1189.

Takeda, O., Handa, M., Uehara, T., Maseki, N., Sakashita, A., Sakurai, M., Kanda, N., Arai, Y., and Kaneko, Y. (1996). An increased NM23H1 copy number may be a poor prognostic factor independent of LOH on 1p in neuroblastomas. *Br J Cancer* **74**:1620-1626.

Takeuchi, K., Ishihara, M., Kawaura, C., Noji, M., Furuno, T., and Nakanishi, M. (1996). Effect of zeta potential of cationic liposomes containing cationic cholesterol derivatives on gene transfection. *FEBS Lett* **397**:207-209.

Tanaka, K., Hayashi, H., Hamada, C., Khoury, G., and Jay, G. (1986). Expression of major histocompatibility complex class I antigens as a strategy for the potentiation of immune recognition of tumor cells. *Proc Natl Acad Sci U S A* **83**:8723-8727.

Tanaka, M., Saijo, Y., Sato, G., Suzuki, T., Tazawa, R., Satoh, K., and Nukiwa, T. (2000). Induction of antitumor immunity by combined immunogene therapy using IL-2 and IL-12 in low antigenic Lewis lung carcinoma. *Cancer Gene Ther* **7**:1481-1490.

Tang, M. X. and Szoka, F. C. (1997). The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther* **4**:823-832.

Teitz, T., Wei, T., Valentine, M. B., Vanin, E. F., Grenet, J., Valentine, V. A., Behm, F. G., Look, A. T., Lahti, J. M., and Kidd, V. J. (2000). Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nat Med* **6**:529-535.

Tepper, R. I. and Mule, J. J. (1994). Experimental and clinical studies of cytokine gene-modified tumor cells. *Hum Gene Ther* **5**:153-164.

Thierry, A. R., Lunardi-Iskandar, Y., Bryant, J. L., Rabinovich, P., Gallo, R. C., and Mahan, L. C. (1995). Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci U S A* **92**:9742-9746.

Thomas, G. R., Chen, Z., Enamorado, I., Bancroft, C., and Van Waes, C. (2000). IL-12- and IL-2-induced tumor regression in a new murine model of oral squamous-cell carcinoma is promoted by expression of the CD80 co-stimulatory molecule and interferon-gamma. *Int J Cancer* **86**:368-374.

Topalian, S. L. (1994). MHC class II restricted tumor antigens and the role of CD4+ T cells in cancer immunotherapy. *Curr Opin Immunol* **6**:741-745.

Trask, T. W., Trask, R. P., Aguilar-Cordova, E., Shine, H. D., Wyde, P. R., Goodman, J. C., Hamilton, W. J., Rojas-Martinez, A., Chen, S. H., Woo, S. L., and Grossman, R. G. (2000). Phase I study of adenoviral delivery of the HSV-tk gene and ganciclovir administration in patients with current malignant brain tumors. *Mol Ther* **1**:195-203.

Trinchieri, G. (1998). Proinflammatory and immunoregulatory functions of interleukin-12. *Int Rev Immunol* **16**:365-396.

Tsung, K., Meko, J. B., Peplinski, G. R., Tsung, Y. L., and Norton, J. A. (1997). IL-12 induces T helper 1-directed antitumor response. *J Immunol* **158**:3359-3365.

Turek, J., Dubertret, C., Jaslin, G., Antonakis, K., Scherman, D., and Pitard, B. (2000). Formulations which increase the size of lipoplexes prevent serum-associated inhibition of transfection. *J Gene Med* **2**:32-40.

Uherek, C., Fominaya, J., and Wels, W. (1998). A modular DNA carrier protein based on the structure of diphtheria toxin mediates target cell-specific gene delivery. *J Biol Chem* **273**:8835-8841.

van't Veer, L. J., Beijersbergen, R. L., and Bernards, R. (1993). N-myc suppresses major histocompatibility complex class I gene expression through down-regulation of the p50 subunit of NF-kappa B. *EMBO J* **12**:195-200.

Voest, E. E., Kenyon, B. M., O'Reilly, M. S., Truitt, G., D'Amato, R. J., and Folkman, J. (1995). Inhibition of angiogenesis in vivo by interleukin 12. *J Natl Cancer Inst* **87**:581-586.

Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M. L. (1990). Transferrin-polycation conjugates as carriers for DNA uptake into cells. *Proc Natl Acad Sci U S A* **87**:3410-3414.

Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M. L. (1991). Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc Natl Acad Sci U S A* **88**:4255-4259.

Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M. L. (1992). Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci U S A* **89**:7934-7938.

Wang, K. S., Frank, D. A., and Ritz, J. (2000). Interleukin-2 enhances the response of natural killer cells to interleukin-12 through up-regulation of the interleukin-12 receptor and STAT4. *Blood* **95**:3183-3190.

Wang, S., Di, S., Young, W. B., Jacobson, C., and Link, C. J., Jr. (1997). A novel herpesvirus amplicon system for in vivo gene delivery. *Gene Ther* **4**:1132-1141.

Watkins, S. J., Mesyanzhinov, V. V., Kurochkina, L. P., and Hawkins, R. E. (1997). The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery. *Gene Ther* **4**:1004-1012.

Weiss, W. A., Aldape, K., Mohapatra, G., Feuerstein, B. G., and Bishop, J. M. (1997). Targeted expression of MYCN causes neuroblastoma in transgenic mice. *EMBO J* **16**:2985-2995.

Westphal, C. H. (1997). Cell-cycle signaling: Atm displays its many talents. *Curr Biol* **7**:R789-R792.

Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell* **73**:309-319.

Williams, R. S., Johnston, S. A., Riedy, M., DeVit, M. J., McElligott, S. G., and Sanford, J. C. (1991). Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. *Proc Natl Acad Sci U S A* **88**:2726-2730.

Wolff, J. A., Ludtke, J. J., Acsadi, G., Williams, P., and Jani, A. (1992). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum Mol Genet* **1**:363-369.

Xu, B., Wiehle, S., Roth, J. A., and Cristiano, R. J. (1998). The contribution of poly-L-lysine, epidermal growth factor and streptavidin to EGF/PLL/DNA polyplex formation. *Gene Ther* **5**:1235-1243.

Xu, Y. and Szoka-FC, J. (1996). Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* **35**:5616-5623.

Yamada, M., Oligino, T., Mata, M., Goss, J. R., Glorioso, J. C., and Fink, D. J. (1999). Herpes simplex virus vector-mediated expression of Bcl-2 prevents 6-hydroxydopamine-induced degeneration of neurons in the substantia nigra in vivo. *Proc Natl Acad Sci U S A* **96**:4078-4083.

Yan, Z., Zhang, Y., Duan, D., and Engelhardt, J. F. (2000). Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. *Proc Natl Acad Sci U S A* **97**:6716-6721.

Yang, Q., Mamounas, M., Yu, G., Kennedy, S., Leaker, B., Merson, J., Wong-Staal, F., Yu, M., and Barber, J. R. (1998). Development of novel cell surface CD34-targeted recombinant adenoassociated virus vectors for gene therapy. *Hum Gene Ther* **9**:1929-1937.

Yano, L., Shimura, M., Taniguchi, M., Hayashi, Y., Suzuki, T., Hatake, K., Takaku, F., and Ishizaka, Y. (2000). Improved gene transfer to neuroblastoma cells by a monoclonal antibody targeting RET, a receptor tyrosine kinase. *Hum Gene Ther* **11**:995-1004.

Yoshida, H., Enomoto, H., Tagawa, M., Takenaga, K., Tasaki, K., Nakagawara, A., Ohnuma, N., Takahashi, H., and Sakiyama, S. (1998). Impaired tumorigenicity and decreased liver metastasis of murine neuroblastoma cells engineered to secrete interleukin-2 or granulocyte macrophage colony-stimulating factor. *Cancer Gene Ther* **5**:67-73.

Yoshida, H., Tanabe, M., Miyauchi, M., Kawamura, K., Takenaga, K., Ohnuma, N., Sakiyama, S., and Tagawa, M. (1999). Induced immunity by expression of interleukin-2 or GM-CSF gene in murine neuroblastoma cells can generate antitumor response to established tumors. *Cancer Gene Ther* **6**:395-401.

Yoshihara, T., Esumi, N., Humphries, M. J., and Imashuku, S. (1992). Unique expression of integrin fibronectin receptors in human neuroblastoma cell lines. *Int J Cancer* **51**:620-626.

Yu, A. L., Uttenreuther-Fischer, M. M., Huang, C. S., Tsui, C. C., Gillies, S. D., Reisfeld, R. A., and Kung, F. H. (1998). Phase I trial of a human-mouse chimeric anti-disialoganglioside monoclonal antibody ch14.18 in patients with refractory neuroblastoma and osteosarcoma. *J Clin Oncol* **16**:2169-2180.

Yu, S. F., von Ruden, T., Kantoff, P. W., Garber, C., Seiberg, M., Ruther, U., Anderson, W. F., Wagner, E. F., and Gilboa, E. (1986). Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc Natl Acad Sci U S A* **83**:3194-3198.

Zaks, T. Z., Chappell, D. B., Rosenberg, S. A., and Restifo, N. P. (1999). Fas-mediated suicide of tumor-reactive T cells following activation by specific tumor: selective rescue by caspase inhibition. *J Immunol* **162**:3273-3279.

Zanta, M. A., Belguise-Valladier, P., and Behr, J. P. (1999). Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci U S A* **96**:91-96.

Zatloukal, K., Schneeberger, A., Berger, M., Schmidt, W., Koszik, F., Kutil, R., Cotten, M., Wagner, E., Buschle, M., Maass, G., and et, a. (1995). Elicitation of a systemic and protective anti-melanoma immune response by an IL-2-based vaccine. Assessment of critical cellular and molecular parameters. *J Immunol* **154**:3406-3419.

Zauner, W., Kichler, A., Schmidt, W., Mechtler, K., and Wagner, E. (1997). Glycerol and polylysine synergize in their ability to rupture vesicular membranes: a mechanism for increased transferrin-polylysine-mediated gene transfer. *Exp Cell Res* **232**:137-145.

Zenke, M., Steinlein, P., Wagner, E., Cotten, M., Beug, H., and Birnstiel, M. L. (1990). Receptor-mediated endocytosis of transferrin-polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. *Proc Natl Acad Sci U S A* **87**:3655-3659.

Zennou, V., Petit, C., Guetard, D., Nerhbass, U., Montagnier, L., and Charneau, P. (2000). HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101**:173-185.

Zhao, X. J. and Cheung, N. K. (1995). GD2 oligosaccharide: target for cytotoxic T lymphocytes. *J Exp Med* **182**:67-74.

Zhou, X. and Huang, L. (1994). DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochim Biophys Acta* **1189**:195-203.

Ziegler-Heitbrock, H. W., Kafferlein, E., Haas, J. G., Meyer, N., Strobel, M., Weber, C., and Flieger, D. (1992). Gangliosides suppress tumor necrosis factor production in human monocytes. *J Immunol* **148**:1753-1758.

Ziegler, M. M., Ishizu, H., Nagabuchi, E., Takada, N., and Arya, G. (1997). A comparative review of the immunobiology of murine neuroblastoma and human neuroblastoma. *Cancer* **79**:1757-1766.

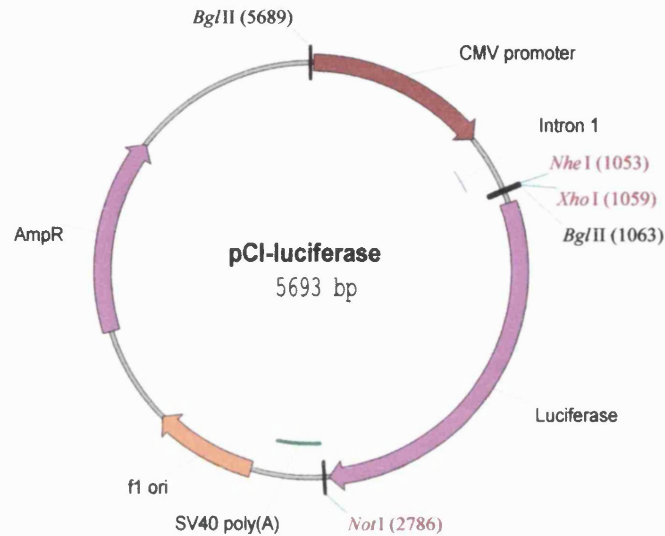
Zitvogel, L., Couderc, B., Mayordomo, J. I., Robbins, P. D., Lotze, M. T., and Storkus, W. J. (1996). IL-12-engineered dendritic cells serve as effective tumor vaccine adjuvants in vivo. *Ann N Y Acad Sci* **795**:284-293.

Zufferey, R., Donello, J. E., Trono, D., and Hope, T. J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* **73**:2886-2892.

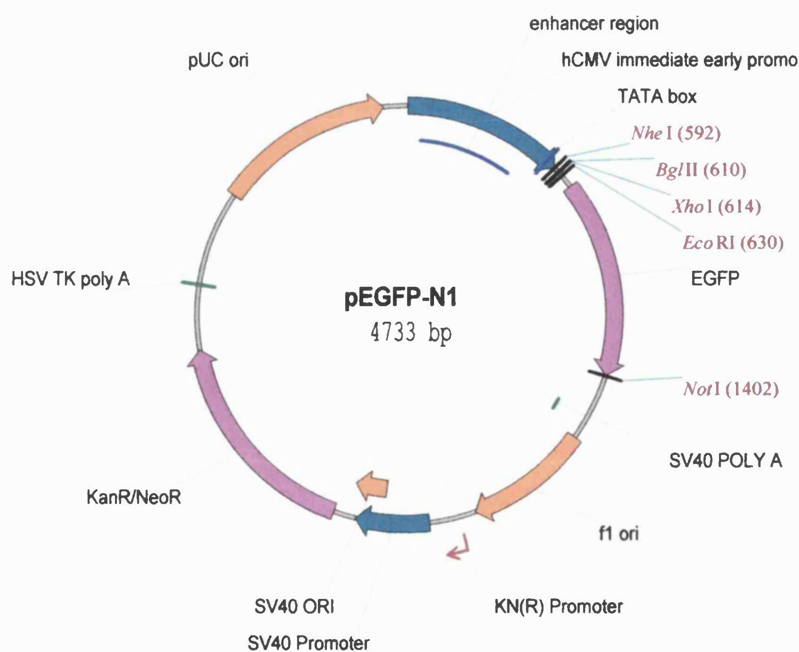
APPENDICES

A. Bacterial Plasmids Used In LID Transfection Experiments

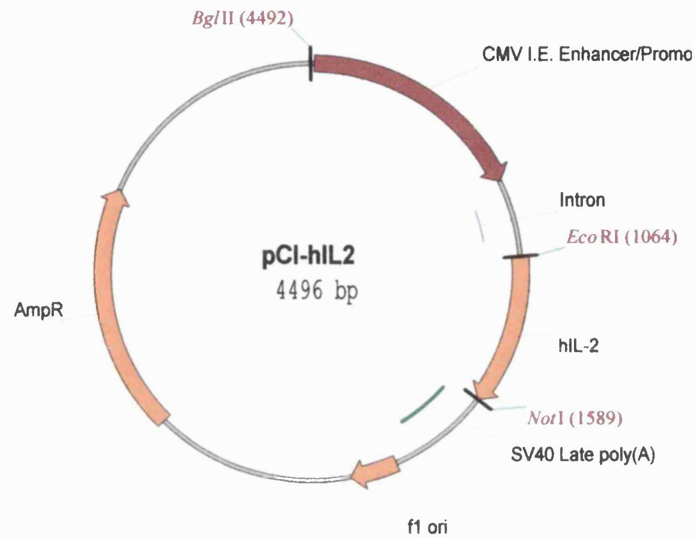
A1. Luciferase plasmid



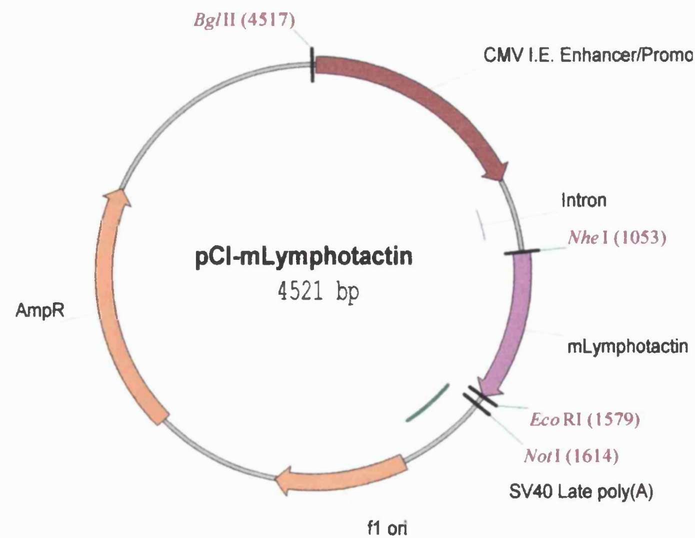
A2. Green fluorescent protein plasmid



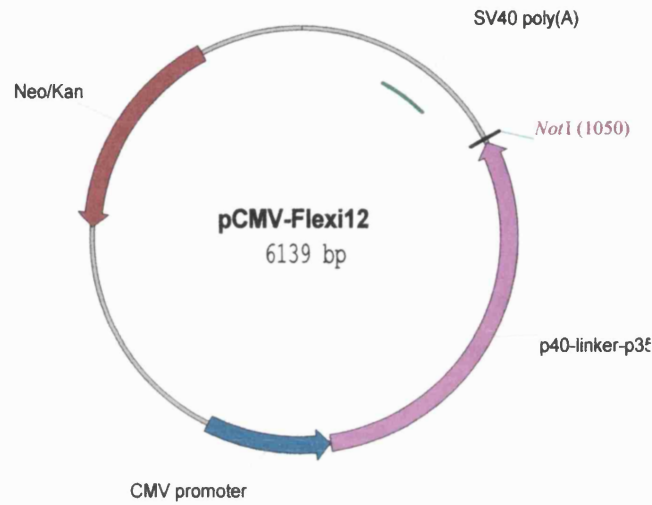
A3. Human IL-2 plasmid



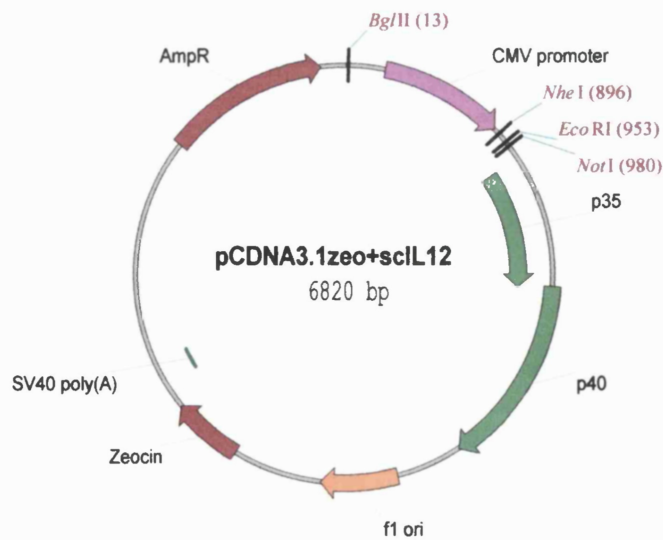
A4. Murine Lymphotactin plasmid



A5. Human IL-12 plasmid



A6. Murine IL-12 plasmid



B. Culturing Of Primary Neuroblastoma Cells

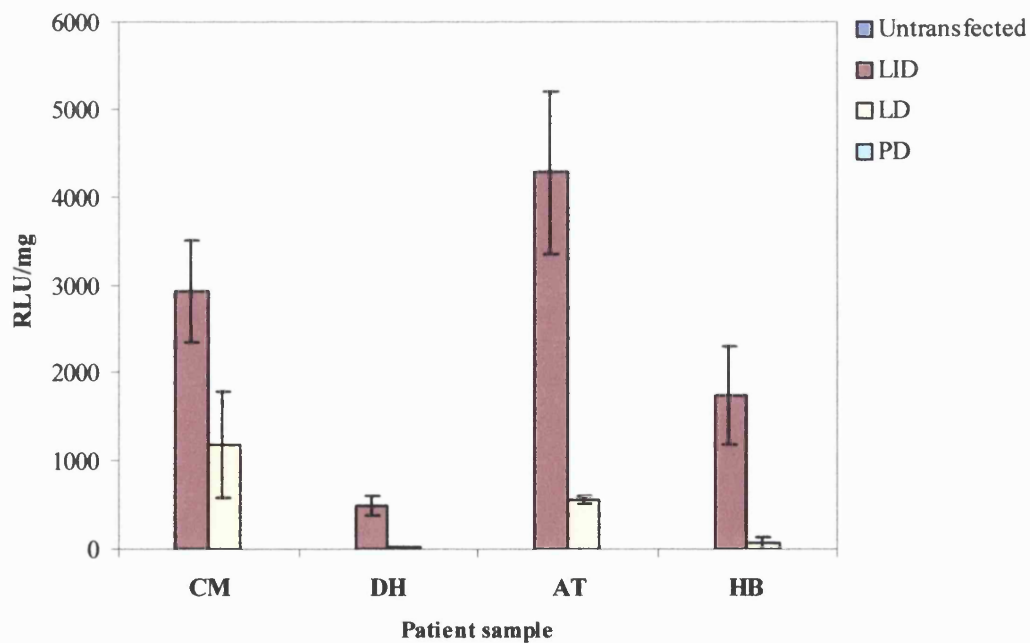
Development of an autologous tumour cell vaccine requires optimisation of the culture conditions of primary cells. We, therefore, wanted to determine the ease of growing primary neuroblasts from patient bone marrow samples obtained from Great Ormond Street Hospital for Children NHS Trust, London. The samples were subjected to Ficoll gradient separation followed by plastic adherence of monocytes on EHS Natrix cellware specially coated with a natural extracellular matrix that was synthesized by an Engelbreth-Holm-Swalm tumour-derived cell line. Adherence of neuroblastoma cells or other cells of neuronal origin to this substrate is believed to promote cell growth and differentiation. Primary neuroblasts were retained in culture for up to 2-3 months and prior to LID transfection they were stained with anti-GD_2 and anti-NCAM I antibodies to confirm presence of neuroblastoma cells and to ensure that overgrowth of stroma cells did not inhibit that of neuroblasts. Normal BM stroma cells were used as a negative control.

Three out of four patient samples were positive for GD_2 staining ranging from 14.9-22.8%. The antibody against NCAM-I did not exhibit such strong binding with only two patient samples showing a weak 1-1.5% staining. Histology performed at the Cytogenetics Department at Great Ormond Street Hospital for Sick Children NHS Trust, confirmed infiltration of neuroblasts to the bone marrow in three out of four patients (**Table B**). However, A.T. who was negative of neuroblastoma infiltration in the bone marrow exhibited 14.9% cross-reactivity with anti-GD_2 antibody. The reverse occurred with patient C.M., whose histology was positive but no staining could be detected with either anti-GD_2 or anti-NCAM-I antibodies. The antibody results for the other two patients, D.H. and H.B., were in agreement with the histological observations performed at GOS Hospital. However, these controversial results obviate the need of alternative antibodies that will be specific for neuroblastoma cells. Neuroblastoma infiltration in the BM of relapsed patients does not normally exceed 10%, and, therefore, it may not be possible to culture enough cells for vaccination especially if the administration is performed in successive

Table B. Antibody staining of patient bone marrow samples

Patient	HISTOLOGY	Anti-GD2 Ab	Anti-NCAMI Ab
Normal BM	-	1.5%	0.1%
A.T.	Negative	14.9%	0.1%
C.M.	Positive	0	0.1%
D.H.	Positive (10%)	22.8%	1.1%
H.B.	Positive	20.7%	1.5%

Fig B. LID transfection of primary neuroblasts



inoculations. A potential solution would be to stain the cells with specific antibodies and subsequently sort positive cells by FACS. By enriching for primary neuroblasts, it may prove easier to culture without the risk of growing BM stroma cells as well.

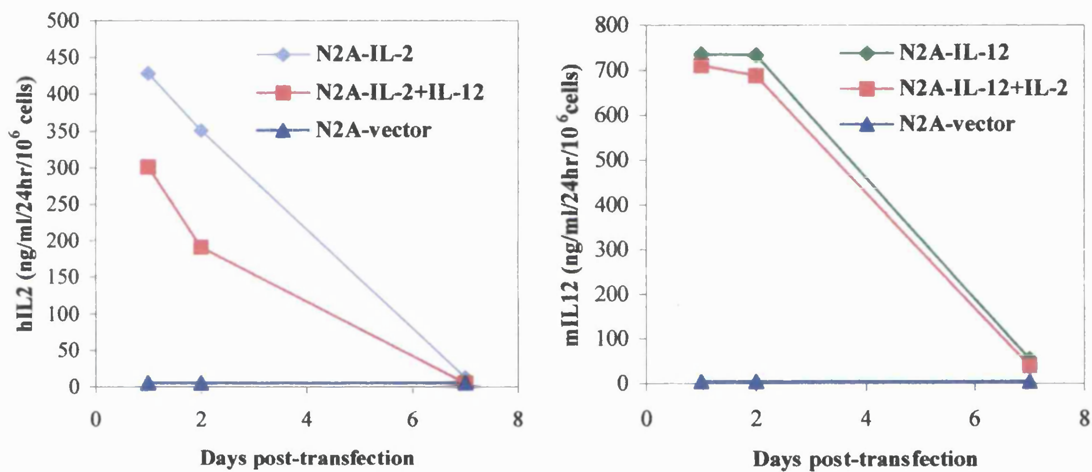
Transfection of primary neuroblastoma cells was performed as described in the Materials and Methods (2.3.1) using the optimal transfection conditions determined by transfection of cell lines. Primary cells were seeded on 24-well plates at a density of $5x10^4$ cells per well and transfected using either LID, LD or PD complexes for 4 h. Luciferase expression was analysed 48 h later and revealed that transfection with the full LID formulation resulted in higher reporter gene expression compared to either LD or PD complexes (**Fig.B**). Although the levels of luciferase expression varied among different patient samples, there was at least 2-fold enhancement in the RLU/mg levels when LID complexes were used compared to the LD formulation. No detectable luciferase expression was observed in transfections with PD complexes.

Primary neuroblasts were also transfected with the pEGFP-N1 vector using the full LID formulation in order to assess the efficiency of transfection. FACS analysis of transfected cells revealed an average of 2% EGFP-positive cells 48 h post-transfection indicating the difficulty of transfecting these primary cells (data not shown).

C. Cytokine Expression By Transfected Neuro-2A Cells Used In Mouse Experiments

C1. *In vivo* tumourigenicity of cytokine-transfected Neuro-2A cells

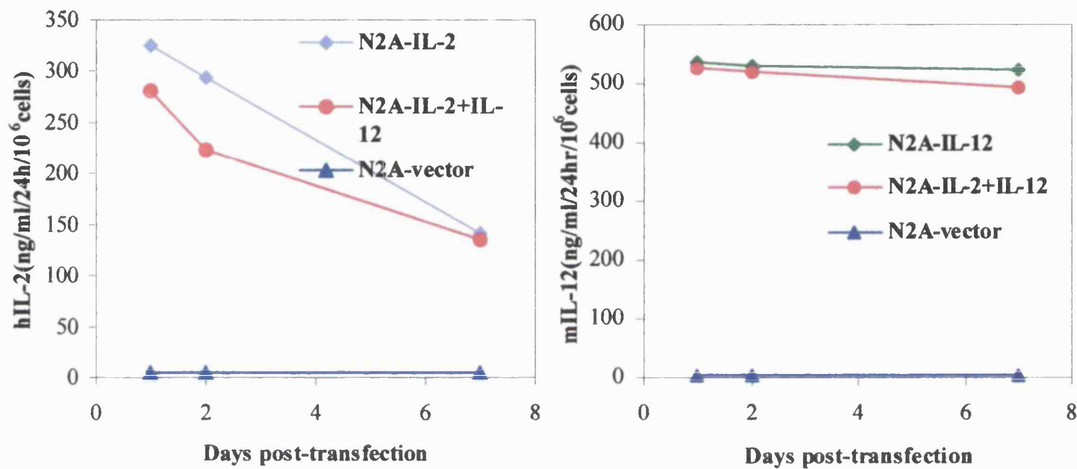
(Experiment described in 6.2.1)



The levels of IL-2 and IL-12 production by transfected Neuro-2A cells were monitored *in vitro*. Transfected cells were seeded on 24-well plates, their supernatant collected at 24 h intervals and subsequently analysed by ELISA. IL-12 expression by either N2A-IL-12 or N2A-IL-2+IL-12 was retained at ~700 ng/ml/24h/10^6 cells for days 1 and 2 and decreased to 40-50 ng/ml/24h/10^6 cells by day 7. Expression of IL-2 by N2A-IL-2 cells ranged from 430 ng/ml/24h/10^6 cells on day 1 to 350 ng/ml/24h/10^6 cells on day 2 and was reduced to just 1 ng/ml/24h/10^6 cells by day 7. The IL-2 levels produced by N2A-IL-2+IL-12 cells were lower than those of N2A-IL-2 cells. Expression commenced at 300 ng/ml/24h/10^6 cells on day 1 and decreased to 190 ng/ml/24h/10^6 cells on day 2 and to 1 ng/ml/24h/10^6 cells by day 7.

C2. Pre-vaccination experiments (described in 6.2.2)

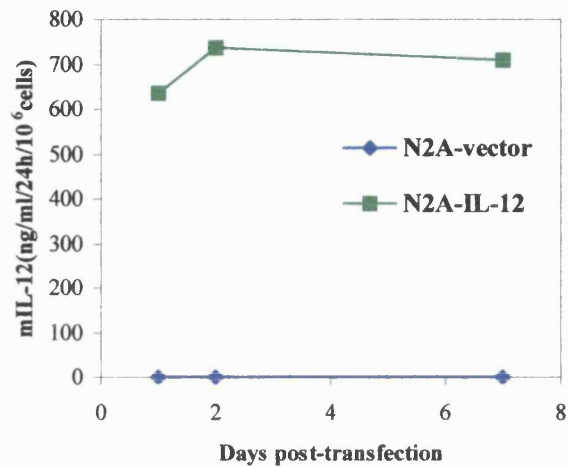
C2a (corresponding to Fig 6.3)



In this experiment, IL-12 expression by either N2A-IL-12 or N2A-IL-2+IL-12 was sustained at 520±15 ng/ml/24h/10^6 cells during 7 days in culture. The average IL-2 levels produced by N2A-IL-2 or N2A-IL-2+IL-12 were 235±80 ng/ml/24h/10^6 cells up to at least 7 days post-transfection.

C2b. Titration of the number of cells used in pre-vaccination experiments

(Corresponding to Fig 6.4)

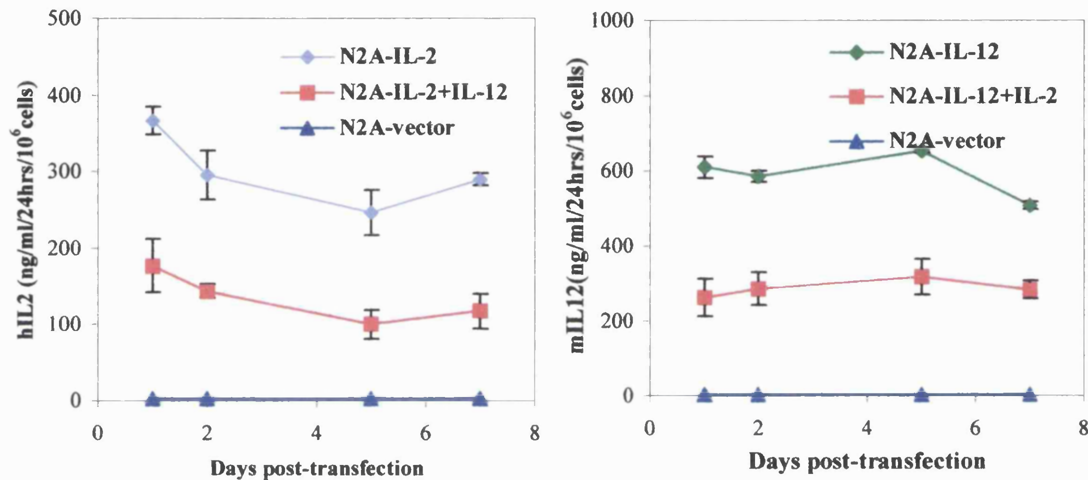


In vitro expression of IL-12 by N2A-IL-12 cells was retained at 694±52 ng/ml/24h/10^6 cells for at least 7 days after transfection as determined by ELISA analysis.

C3. Eradication of established tumours (described in 6.2.3)

C3a. 1ST EXPERIMENT

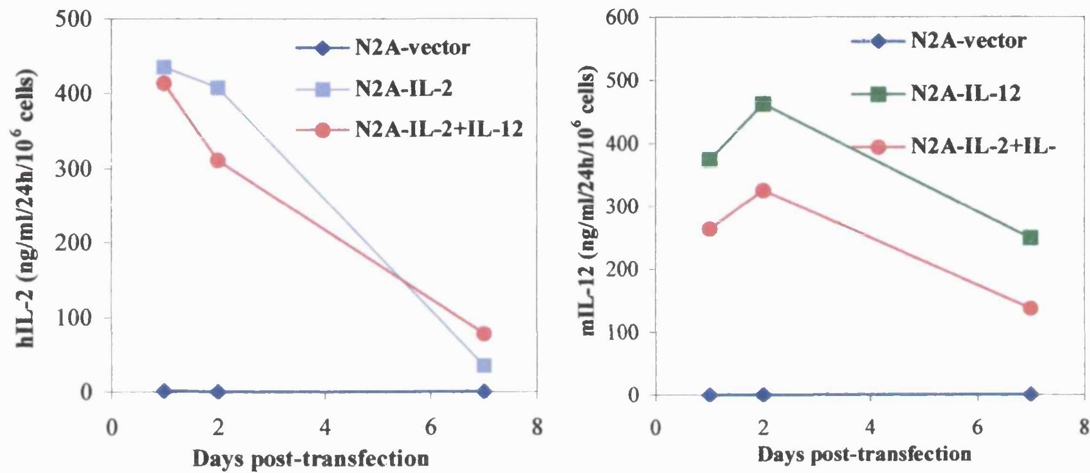
(Corresponding to Fig 6.5 & Fig 6.6)



As indicated by *in vitro* culture, IL-2 expression by N2A-IL2 cells in that particular experiment was maintained at 300±50 ng/ml/24h/10^6 cells throughout a 7-day period. N2A-IL2+IL-12 cells sustained *in vitro* IL-2 expression at 135±35 ng/ml/24h/10^6 cells for 7 days. IL-12 expression was higher than that of IL-2. IL-12-transfected Neuro-2A cells retained IL-12 levels at 590±60 ng/ml/24h/10^6 cells over 7 days in culture while IL-12 expression by N2A-IL-2+IL-12 cells reached 285±20 ng/ml/24h/10^6 cells for the same period of time.

C3b. 2ND EXPERIMENT

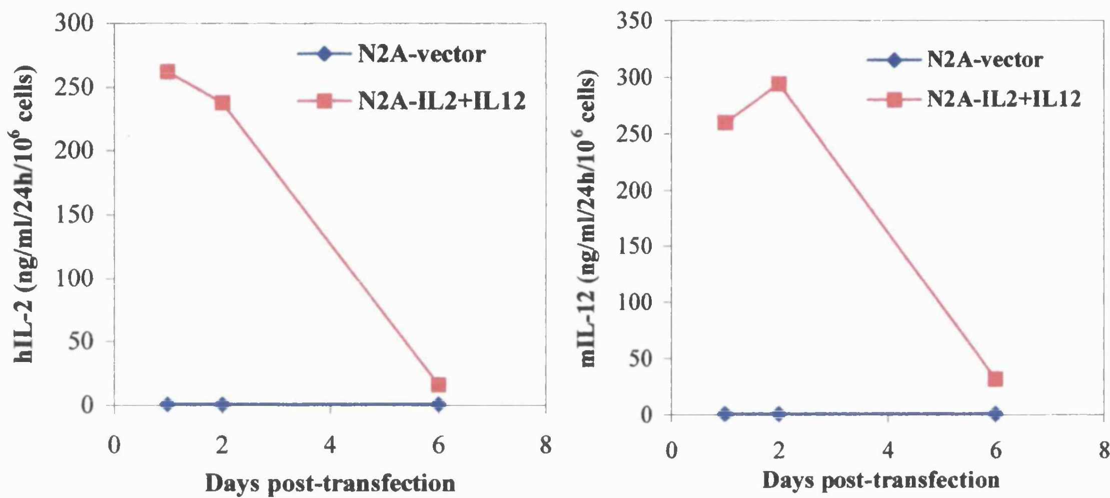
(Corresponding to Fig 6.7 & Fig 6.8)



N2A-IL-12 cells retained IL-12 expression levels at 362±107 ng/ml/24h/10^6 cells for at least up to 7 days while co-transfection with IL-2 resulted in 242±95 ng/ml/24h/10^6 cells of IL-12 for the same period of time. IL-2 expression by either N2A-IL-2 or N2A-IL-2+IL-12 cells was maintained at an average of 392±46 ng/ml/24h/10^6 cells up to 2 days post-transfection. However, IL-2 levels significantly decreased by day 7 to 36 ng/ml/24h/10^6 cells for N2A-IL-2 and 78 ng/ml/24h/10^6 cells for N2A-IL-2+IL-12.

C4. Inoculation into γ_c/RAG2 knockout mice

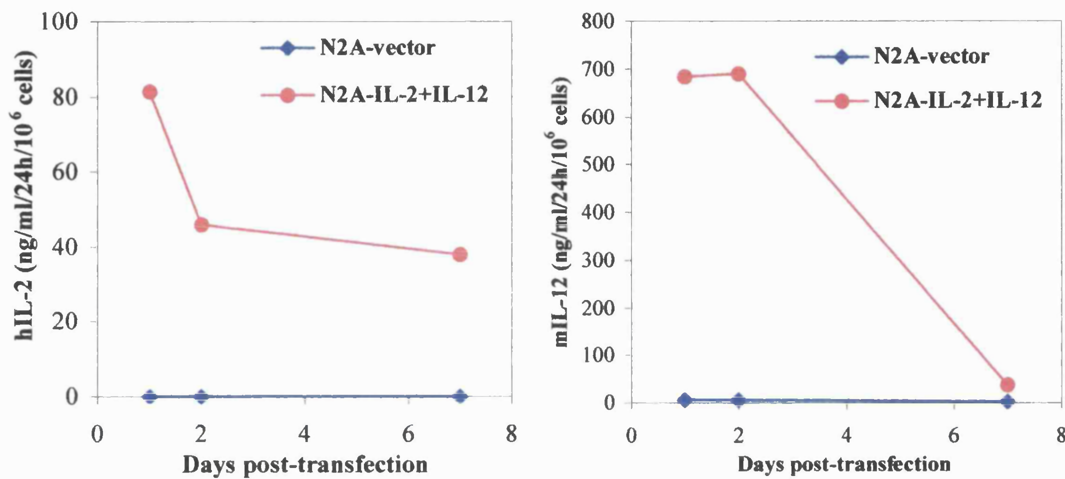
(Described in 6.2.4)



IL-2 and IL-12-transfected Neuro-2A cells used to inoculate γ_c/RAG2 knockout mice maintained IL-12 expression at 250±17 ng/ml/24h/10^6 cells up to 48 h post-transfection. By day 7, the IL-12 levels had decreased to 17 ng/ml/24h/10^6 cells. IL-2 expression persisted at an average 277±17 ng/ml/24h/10^6 cells for 48 h and declined to 32 ng/ml/24h/10^6 cells by day 7.

C5. *In vivo* immunodepletion experiment

(Described in 6.2.5)



IL-12 expression by N2A-IL-2+IL-12 cells used in the *in vivo* immunodepletion experiment was maintained at 687±3 ng/ml/24h/10^6 cells for two days but decreased below 37 ng/ml/24h/10^6 cells by day 7. IL-2 expression was relatively low compared to previous transfection experiments. It commenced at 82 ng/ml/24h/10^6 cells on day 1 while only 46 ng/ml/24h/10^6 cells could be detected two days following transfection. By day 7 the IL-2 levels had decreased to <38 ng/ml/24h/10^6 cells. Control-transfected Neuro-2A cells produced no detectable cytokine levels.