IMMUNE MEDIATED AND DIRECT KILLING OF COLORECTAL CANCER BY REOVIRUS.

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Abstract

Colorectal cancer remains the second most prevalent cancer in the world. Novel treatment strategies are necessary, especially for the treatment of metastatic disease. Reovirus is a naturally occurring oncolytic virus which acts by both direct and immune-mediated mechanisms. Having shown promise in early clinical trials, its therapeutic potential may be limited by inactivation following systemic delivery. This study addressed whether reovirus can be shielded from neutralising antibodies by cell carriage, and whether virus-loaded blood or hepatic innate immune effector cells are activated to kill colorectal cancer cells metastatic to the liver in human systems. Reovirus induced oncolysis of SW480, SW620, LoVo and LS174T tumour cells and the mode of cell death was apoptosis. Direct tumour cell killing by neat virus was significantly reduced in the presence of neutralising serum. Reovirus was protected from neutralisation when loaded onto peripheral blood mononuclear cells and could be handed off to tumour targets for direct oncolytic killing. Moreover, NK cells within reovirus-loaded patient blood mononuclear cells were stimulated to kill tumor targets but not normal hepatocytes; similarly, NK cells within liver mononuclear cells became selectively cytotoxic towards tumour cells when activated by reovirus. This blood cell carriage has the potential to instigate both direct and innate immune-mediated therapy against human colorectal cancer which has metastasised to the liver.

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Abbreviations and Definitions

°C	Degrees Celsius
µm	Micrometre
µM	Micromole
^{51}Cr	^{51}Chromium
ADCC	Antibody-dependent Cell-mediated Cytotoxicity
Ad-HSV-tk	Adenovirus Encoding HSV Thymidine Kinase
ANOVA	Analysis of Variance
APC	Antigen Presenting Cells
BID	BH3 Interacting-domain Death Agonist
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CD	Cluster of Differentiation
CEA	Carcinoembryonic Antigen
cm	Centimetre
CO_2	Carbon Dioxide
COX2	Cyclooxygenase-2
cpm	Counts per Minute
CRLM	Colorectal Liver Metastases
CT	Computerised Tomography
CTL	Cytotoxic T Lymphocyte
CTLA-4	CTL-associated Antigen-4
CTX	Cortical Thymocyte Marker of Xenopus Protein
DC	Dendritic Cells
DISC	Death-inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid

DNAM	DNAX Accessory Molecule
DR	Death Receptor
dsRNA	Double-stranded Ribonucleic Acid
E:T	Effector to Target
EBNA	Epstein Bar Virus Nuclear Antigen
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
eIF2α	Eukaryotic Initiation Factor 2α
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial–mesenchymal Transition
FACS	Fluorescence Activated Cell Sorting
FasL	Fas Ligand
FasR	Fas Receptor
FcR	Fc Receptor
FCS	Foetal Calf Serum
Fig	Figure
FITC	Fluorescein Isothiocyanate
FOLFIRI	Folinic Acid, Fluorouracil, Irinotecan
FOLFOX	Folinic Acid, Fluorouracil, Oxaliplatin
Foxp3	Forkhead Box P3 Transcription Factor
g	Gram
G	Gravitational
GITR	Glucocorticoid-induced TNF Receptor
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
GMP	Good Manufacturing Practice
GTP	Guanosine Triphosphate
HBSS	Hank’s Balanced Salt Solution
HBV	Hepatitis B virus

HCC	Hepatocellular Cancer
HIV	Human Immuno-deficiency Virus
HLA	Human Leucocyte Antigen
HMBG-1	High Mobility Group Box-1
HPV	Human Papilloma Virus
Hr	Hours
HS	Human AB Serum
HSP	Heatshock Protein
HSV	Herpes Simplex Virus
i.p.	Intra-peritoneal
i.t.	Intra-tumoural
i.v.	Intra-venous
ICAM-1	Intercellular Adhesion Molecule 1
iDC	Immature Dendritic Cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Inc.	Incorporated
ISVP	Infectious Sub-viral Particles
JAM-1	Junctional Adhesional Molecule-1
JAM-A	Junctional Adhesional Molecule- A
JNK	c-Jun N-terminal Kinase
kDa	Kilodalton
KIR	Killer-cell Immunoglobulin-like Receptors
Kras	Kirsten rat sarcoma viral oncogene homlogue
LAK	Lymphokine Activated Killer
L-Arg	Levorotary-arginine
L-glutamine	Levorotary-glutamine

LIR	Leucocyte Inhibitory Receptor
LMNC	Liver-derived Mononuclear Cells
LNK	Liver-derived Natural Killer Cells
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MACS	Magnetic Activated Cell Sorting
MART-1	Melanoma-associated Antigen Recognised by T cells
mDC	Mature Dendritic Cell
MDSC	Myeloid-derived Suppressor Cells
mg	Milligram
MHC	Major Histocompatability Complex
MICA/B	Major Histocompatibility Complex Class I Chain-related Gene A/B
Mins	Minutes
MIP	Macrophage Inflammatory Protein
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of Infection
MTD	Maximum Tolerated Dose
MUC-1	Mucin-1
MV	Measles Virus
NAB	Neutralising Antibody (anti-reovirus)
NCR	Natural Cytotoxicity Receptor
NDV	Newcastle Disease Virus
NFκB	Nuclear Factor Kappa B
NHSBT	National Health Service Blood and Transplant Service
NICE	National Institute of Health and Clinical Excellence
NK	Natural Killer Cells
NKT	Natural Killer T cells

nM	Nanomolar
OS	Overall Survival
PBMC	Peripheral Blood Mononuclear Cells
PBNK	Peripheral Blood Natural Killer Cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline- Tween
PCR	Polymerase Chain Reaction
PDZ	Postsynaptic Density Protein, Disc-large, zo-1
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein Complex
PFA	Paraformaldehyde
PFS	Progression Free Survival
pfu	Plaque Forming Units
pg	Picogram
PGE_2	Prostaglandin E2
PI	Propiodium Iodide
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PKR	dsRNA Dependent Protein Kinase
PRR	Pattern Recognition Receptors
PSA	Prostate Specific Anitgen
PTEN	Phosphatase and tensin homologue
RAG	Recombination Activating Genes
RalGEF	Ral Guanine Nucleotide Exchange Factor
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
rpm	Revolutions per Minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
rtPCR	Real Time Polymerase Chain Reaction

SEM	Standard Error of the Mean
sFasL	Soluble Fas Ligand
SJUH	St James's University Hospital
STAT-1	Signal Transducer and Activator of Transcription-1
TAA	Tumour Associated Antigens
TCID	Tissue Culture Infectious Dose
TCR	T cell Receptor
TGF	Transforming Growth Factor
Th	T helper
TIL	Tumour-infiltrating Lymphocytes
tk	Thymidine Kinase
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TRAIL	T Apoptosis-inducing Ligand
TRAIL-R	T Apoptosis-inducing Ligand Receptor
T-reg	Regulatory T Cells
TRP-2	T cell Response Towards Tyrosinase-related Protein 2
UK	United Kingdom
USA	United States of America
UV	Ultra-violet
v/v	Volume/Volume Concentration
VEGF	Vascular Endothelial Growth Factor
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus
w/v	Weight/Volume Concentration
ZVAD-FMK	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone
µCi	Microcurie
µg	Microgram

µl	Microlitre

1 Introduction

1.1 Colorectal Cancer

1.1.1 The Clinical Problem

Colorectal cancer is the third most prevalent cancer in the world. In 2008, an estimated 1.24 million new cases were diagnosed world-wide and it is now the fourth most common cancer in men and the third in women (Ferlay *et al*., 2010). Survival estimates at 5 years currently stand at 65% in North America, 54% in Western Europe, 34% in Eastern Europe and 30% in India. The global distribution of the disease is similar, although greater variations between countries have been noted for colon cancer than for rectal cancer (Parkin, 2005). The incidence of the disease appears to be increasing across Europe, although survival rates have doubled over the past 30 years, with the overall 5 year survival for colorectal cancer now standing at approximately 50% in the United Kingdom (UK) (www.nice.org.uk). Despite this, 16,000 people die from colorectal cancer in the UK annually (www.cancerresearchuk.org), with around 20% of patients still presenting with advanced disease. Of those who undergo complete surgical resection of all macroscopic disease, approximately 50% will relapse (Fong *et al.,* 1999).

Around 50% of all patients with colorectal cancer will develop metastatic disease in the liver over the course of their illness (Scheele *et al.,* 1991). Left untreated, the prognosis for these patients is extremely poor, with a median overall survival of approximately 12 months (Altendorf-Hofmann *et al.,* 2003). No surgical randomised control trial data exists to direct therapeutic strategy and to date surgical resection of hepatic metastases remains the only possibility of cure. Currently, 5 year overall survival rates of between 36% and 58% have been reported in several large retrospective case series (Fong *et al.,* 1999; Abdalla *et al.,* 2004; Scheele *et al.,* 1990; Choti *et al.,* 2002; Adson *et al.,* 1984; Schlag *et al.,* 1990). However, relapse rates of up to 70% have been described and there is little data available on the patterns or rates of recurrence following curative intent surgery (de Jong *et al.,*

2009). Novel techniques such as metastasectomy, non-anatomical resections and portal vein embolisation have increased the number of patients suitable for such surgery (Abdalla *et al.,* 2006; Berri *et al.,* 2009) and there are an increasing number of reports where favourable outcomes have been achieved after more than one liver resection (Adair *et al*., 2012a). In the UK, fluoropyrimidines such as fluorouracil and its pro-drug, capecitabine, as well as the topoisomerase inhibitor, irinotecan, and the DNA synthesis inhibitor, oxaliplatin, are the current standard cytotoxic drugs used in treating metastatic colorectal cancer (Seymour, 2007). In the United States, bevacizumab, a humanised monoclonal antibody (mAb) against vascular endothelial growth factor (VEGF), combined with fluoropyrimidine-based chemotherapy is now the standard first-line treatment for metastatic colorectal cancer. Combination chemotherapy may further improve survival by rendering patients with inoperable metastatic disease operable. Such patients have achieved similar survival to their operable counterparts in recent studies (Nordlinger *et al.,* 2008a); however long term toxicity remains problematic.

Cetuximab is a chimeric immunoglobulin (Ig) G1 mAb which blocks epidermal growth factor receptor (EGFR) and therefore abrogates EGFR-dependent cell proliferation. It has efficacy in Kirsten rat sarcoma (K*ras*) wild type metastatic colorectal cancer as a monotherapy and in combination with irinotecan in irinotecan-resistant patients (Cunningham *et al.,* 2004). The main evidence base for the use of cetuximab stems from two randomised control trials. CRYSTAL, a phase III, multicentre, open-label randomised controlled trial, comparing cetuximab in combination with folinic acid and irinotecan (FOLFIRI) with FOLFIRI alone, examined progression-free survival as the primary outcome. OPUS (n = 336), a phase II, multicentre, open-label randomised controlled trial, compared cetuximab in combination with folinic acid, fluorouracil and oxaliplatin (FOLFOX) with FOLFOX alone, and examined response rate as the primary outcome. The National Institute for Health and Clinical Excellence (NICE) now recommends the use of cetuximab as first line treatment in patients

who present with a resectable primary colorectal cancer and unresectable colorectal liver metastases (CRLM).

The CRYSTAL trial demonstrated a statistically significant increase in progression-free survival in the K*ras* wild-type subgroup, with a median progression-free survival of 9.9 months. The OPUS trial showed a statistically significant increase in progression-free survival, with a median survival of 7.7 months in the K*ras* wild-type subgroup. Cetuximab in combination with FOLFOX was also associated with a statistically significant increase in response rate compared with FOLFOX alone (Van Custem *et al.,* 2009).

The absence of mutation in the proto-oncogene K*ras* appears to be a reliable marker for predicting cetuximab efficacy. Other factors, such as EGFR amplification (Moroni *et al.,* 2005; Personeni *et al.,* 2008), tumour expression of nuclear factor-kB (NF-kB) (Scartozzi *et al.,* 2007), PTEN (Frattini *et al.,* 2007), BRAF (Di Nicolantonio *et al.,* 2008) or PIK3CA (Loupakis *et al.,* 2009) may also predict response to cetuximab but these have not been validated and have not therefore been fully incorporated into clinical practice. Cetuximab's effect is thought to be due to the direct anti-proliferative and apoptotic capabilities of the antibody; however, a further mechanism of action may be through antibody-dependent cell-mediated cytotoxicity (ADCC) by Natural Killer (NK) cells, macrophages and polymorphonuclear leukocytes.

Despite significant therapeutic advances and improvements in overall survival for colorectal cancer from 22% to 50% in the last 10 years in the UK (www.cancerresearchuk.org), the high proportion of patients presenting with advanced disease means that novel, systemic, therapeutic options are warranted. Oncolytic viral therapy has shown promising results in various types of tumour. One particular type of oncolytic virus currently under investigation in clinical trials is reovirus. Reovirus therapy has been targeted towards tumours with K*ras* mutations and as approximately 50% of colorectal tumours have K*ras* oncogene mutations

(Forrester *et al.,* 1987), colorectal cancer seems a suitable target for reovirus-based oncolytic therapy.

1.2 Cancer and the Immune System

1.2.1 Background

The role of the immune system in cancer has been the subject of debate for many years. Numerous accounts of spontaneous tumour regression have been reported and almost all of these have coincided with acute, concomitant infections, including gonorrhoea, hepatitis, influenza, malaria, measles, smallpox, syphilis and tuberculosis, as well as various other pyogenic and non-pyogenic infections (Nauts *et al.,* 1990). These reports, in turn, led to the practice of deliberately encouraging sepsis following surgical procedures in the latter part of the 19th century, resulting in various early accounts of delayed tumour recurrence (Thiery, 1909). Consequently, by the early 1900s, active immunotherapies were being readily pursued.

William Coley, an American surgeon, was the first to undertake a systematic approach to the concept of immunotherapy. He observed tumour regression in a patient with a streptococcal infection in an ulcerated tumour (Coley, 1893). Based on this, he developed a "vaccine" containing Gram-positive *Streptococcus pyogenes* and Gram-negative *Serratia marcescens*, which later became known as "Coley's Toxins". Injected directly into tumours or metastatic deposits, these produced the local and systemic effects of bacterial infection without the pathogenicity. As a result, spontaneous regression was noted in several different tumours, including lymphoma and sarcoma (Coley, 1928). However, with the advent of widespread antibiotic use, surgical asepsis, as well as chemotherapy and radiotherapy, interest in the use of Coley's Toxins faded in the 1950s.

A more contemporary appraisal of the importance of the role of the immune system in preventing cancer can also be demonstrated in numerous series of solid organ transplant

patients where follow-up studies have consistently demonstrated a higher incidence of a variety of cancers (London, 1995). In a large study of Norwegian kidney transplant patients, a 2- to 5-fold increase in cancers of the colon, larynx, lung and bladder were seen in both sexes, as well as an increase in cancers of the prostate and testes in men. Up to a 30-fold increase in non-melanomatous skin cancers was noted in both sexes. Moreover, a higher incidence of non-Hodgkin's lymphoma was also reported, as were cancers of the cervix and vulva in women (Birkeland *et al*., 1995). Further evidence of the involvement of the immune system in cancer can be seen in the higher incidence of Kaposi's sarcoma in immune-compromised patients with Human Immuno-deficiency Virus (HIV) (Moss *et al.,* 1989).

1.2.2 Immune Recognition of Tumours

Historically, it has been argued that tumour cells are not sufficiently "foreign" for successful immunotherapy. The concept of the immune system being able to discriminate "self" from "non-self" was first postulated in 1959 (Burnet *et al.,* 1959). It followed, therefore, that differences in the antigenic make-up of tumours and normal tissue were seen as a crucial element in the development of effective immune responses against cancer.

1.2.2.1 The Danger Theory

This model proposes that immune activation is dependent upon the release of "danger signals" from stressed or dying cells (Matzinger *et al.,* 1994). These immunogenic danger signals include heat shock proteins (HSP), interferon (IFN)-α, uric acid, hyaluronic acid and high mobility group box-1 (HMBG-1), which can stimulate antigen presenting cells (APC). These activated or mature APC such as dendritic cells (DC), macrophages or B cells, subsequently produce the required co-stimulation to invoke a naive T cell response. Based on this concept, cancer cells do not appear "dangerous" to the immune system and therefore an effective T cell response cannot be generated.

1.2.2.2 The Immune Surveillance Theory

According to the immune surveillance theory, tumours expressing antigens are regarded as "non-self" by the immune system. A major function of the immune system is surveillance for the development of malignancy with the elimination of tumour cells as they arise. The initial immune surveillance theory relied upon the "activation only" state of mature lymphocytes and their ability to recognise tumour-associated antigens (TAA) as foreign and much of our understanding of immune surveillance has come about by removing specific components of the innate and adaptive immune systems in mouse models. For example, mice lacking perforin have been shown to develop disseminated lymphoma (Smyth *et al.,* 2000). Those lacking recombination activating genes (RAG) have deficiencies in Natural Killer T (NKT), T cells and B cells and have been shown to develop sarcomas (reviewed by Dunn *et al.,* 2004). Mice lacking signal transducer and activator of transcription-1 (STAT-1), with resultant deficiencies in IFN-γ production, have a preponderence towards developing mammary carcinomas (Chan *et al.,* 2012).

1.2.2.3 Immunoediting

Cancer immunoediting describes a process whereby highly immunogenic tumour cells can be removed by the immune system. The concept has evolved from the immune surveillance theory to incorporate the tumour prevention functions of the immune system as well as its ability to modify developing tumours (Dunn *et al.,* 2002). Cancer immunoediting encompasses three processes: elimination, equilibrium and escape. Elimination equates to the historical concept of immune surveillance and can result in the complete removal of tumour cells without progression to the other phases of the editing process. Equilibrium occurs when the immune system and any tumour cell variant which may have survived the elimination process enter into a state of dynamic balance and tumour growth is controlled. Existing tumour variants may be destroyed and lymphocytes and IFN-γ can exert a restrictive effect upon, but not fully clear, existing tumour cells. It is thought that equilibrium may persist for anything up to three years (Dunn *et al.,* 2002). In the final stage, escape, un-eliminated

tumour cell variants that have undergone genetic changes and acquired resistance to immunogenic detection, proliferate and expand in an uncontrolled manner and lead to increased tumour burden and eventually host death if left unchecked.

1.2.3 Tumour Immune Evasion Strategies

Dysfunction in the host immune response represents one of the most fundamental ways in which tumours evade immune detection. However, both host and tumour-related mechanisms can lead to a failure to mount an effective anti-tumour immune response and these are frequently the key factors limiting the efficacy of anti-cancer immunotherapy.

1.2.3.1 Low Immunogenicity

The instability seen in the genome of tumour cells can enable the loss of antigen expression leading to a reduction in their overall immunogenicity. As well as this, genetic mutations may lead to a passive loss of Major Histocompatability Complex (MHC) -I expression. Loss of all MHC-I molecules, seen in some colorectal and prostate adenocarcinomas, renders tumour cells unrecognisable to cytotoxic T cells (CTL) but make the tumour more susceptible to NK cell-mediated killing (Browning *et al.,* 1992; Rees *et al.,* 1999). However, the loss of all MHC-I is rare. More commonly, a reduction in the level of Human Leucocyte Antigen (HLA) protein expression by up to 50% can occur, which may in turn confer a survival advantage for the tumour (Rees *et al.,* 1999). In losing only some MHC-I expression, tumours avoid recognition by specific CTL, whilst at the same time remaining relatively resistant to NK-mediated killing.

1.2.3.2 Antigenic Modulation

Antigenic modulation is a dynamic process where antibody-induced internalisation and degradation of antigens or variation in the expression of antigens on the tumour cell-surface can lead to a selective advantage to the tumour when recognised by lymphocytes specific for an antigen. For example, binding of Type I mAb to CD20 on the surface of B cell lymphomas is 5 times less effective than the Type II mAb, rituximab, despite both operating exclusively

via activatory Fc Receptor (FcR)–expressing macrophages. Type I mAb induces a profound modulation of CD20, resulting in a reduction in effector cell-mediated clearance of B cells by the internalisation of CD20 and the consumption of CD20/mAb complexes (Beers *et al.,* 2010). Whilst the process of antigenic modulation is more common in haemopoetic malignancies, the modulation of antigens which act as growth factors for solid tumours, such as VEGF, represents a further potential strategy for therapy.

1.2.3.3 Resistance to Immune Cell Killing

Immune cell killing of tumour cells occurs by three main processes: i) ligation of death receptors (e.g. Fas Ligand (FasL) to Fas Receptor (FasR)); ii) binding of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) to tumour necrosis factor-related apoptosis-inducing ligand receptor (TRAIL-R) and iii) the release of granules containing perforin and granzymes. However, some tumours develop resistance to these mechanisms, enabling tumours cells to escape immune detection.

A variety of tumours, including hepatocellular cancer (HCC), colon cancer and melanoma show decreased or mutated expression of FasR, TRAIL-1 and TRAIL-2 on their cell-surface (Strand *et al.,* 1996; Higaki *et al.,* 1996; Möller *et al.,* 1994; Das *et al.,* 2000; Lee *et al.,* 1999; Sjostrom-Mattson *et al.,* 2009; Chatzitolios *et al.,* 2010; Lee *et al.,* 2001). Whilst transcriptional mechanisms are likely to account for the majority of this down-regulation, oncogenic K*ras* has also been implicated (Peli *et al.,* 1999). However, the complete loss of FasR is rarely seen in human cancers (Peter *et al.,* 2005). In fact, many tumours actually express large quantities of FasR and are highly susceptible to Fas-mediated apoptosis (Elnemr *et al*., 2001). It now seems that FasR may actually promote tumour growth through its non-apoptotic activities and that inhibition of its activity, rather than enhancement, may prove more beneficial in cancer therapy (Chen *et al.,* 2010).

A further method by which tumour cells can avoid immune-mediated cell death is by shedding NK ligands into the tumour microenvironment. This enables them to avoid NK cell

recognition. Soluble NKG2D ligands have been demonstrated in tumour samples from neuroblastoma, colorectal, prostate, melanoma and ovarian cancer patients (Raffaghello *et al.,* 2004; Doubrovina *et al.,* 2003; Wu *et al.,* 2004; Paschen *et al.,* 2009; Wang *et al.,* 2008).

1.2.3.4 Tumour Counter-attack

Tumour counter-attack relates to the active killing of Fas-sensitive tumour-infiltrating lymphocytes (TIL) by tumour-derived FasL. FasL is a type II trans-membrane protein which belongs to the tumour necrosis factor (TNF) superfamily. It signals through the tri-merisation of FasR, which spans the membrane of the target cell and can trigger apoptosis upon binding with it. FasL has been associated with sites of immune privilege. High levels are seen in the eye, testes and in neurons and Fas/FasL interactions are thought to explain the high percentage of corneal transplants which are accepted without tissue typing or immunosuppression (Niederkorn, 2003). A variety of tumour cells have been shown to express FasL and can induce apoptosis in Fas-sensitive TIL (Hahne *et al*., 1996; Strand *et al*., 1996; Niehans *et al*., 1998; Bennett *et al.,* 1998). Inhibition of FasL on colon cancer cells has been shown to improve anti-tumour immunity and reduce tumour growth *in vivo* (Ryan *et al.,* 2005). Higher levels of FasL expression have also been demonstrated on CRLM when compared with their matched primary tumours (Mann *et al.,* 1999). It seems apparent, therefore, that FasL-expressing tumour cells counter-attack TIL and establish sites of immune privilege around the tumour.

In addition to this, membrane-bound FasL may be cleaved from the surface of cells by matrix metalloproteinase-like enzymes and presented in a soluble form (sFasL), which retains an extracellular region for binding to FasR (Tanaka *et al.,* 1996). Serum sFasL has been shown to be significantly elevated in colorectal cancer patients and this, in turn, corresponded to higher levels of apoptotic tumour-infiltrating and peripheral blood lymphocytes (Song *et al.,* 2001).

1.2.4 Tumour-induced Immune Tolerance

The tumour micro-environment promotes a variety of inhibitory networks that normally function to prevent auto-immunity. These inhibitory networks include the promotion of conditions in which APC are unable to generate an appropriate immune response and therefore enable tolerance to TAA. One method of achieving this is through the release of inhibitory soluble factors such as VEGF. These inhibitory networks also include regulatory T cells (T-reg) and other inhibitory cells such as myeloid-derived suppressor cells (MDSC) (Murdoch *et al.,* 2008).

1.2.4.1 Soluble Suppressive Factors

Tumour cells and surrounding stromal cells can produce a variety of immunosuppressive cytokines and soluble factors, including transforming growth factor (TGF)-β, VEGF, interleukin (IL)-10, and prostaglandin E2 (PGE_2). Mutations in TGF-β signalling pathways in tumour cells can overcome the inhibitory effects of TGF-β on cell growth. Moreover, TGF-β can convert naïve T cells into T-reg, having the overall effect of suppressing effective T cell responses (Chen *et al.,* 2003).

VEGF has been shown to promote angiogenesis (reviewed by Ferrara, 2002) and is produced by almost all tumours. It has been shown to prevent haemopoietic progenitor cells from differentiating into functional DC (Oyama *et al.,* 1998). As well as this, it can recruit immature myeloid cells into the tumour microenvironment, enriching it with tolerogenic immature DC (iDC), macrophages and B cells (Gabrilovich *et al.,* 1998). Further, VEGF production by stromal macrophages can further suppress DC differentiation and function, resulting in an ineffective immune response.

PGE_2 is a pro-inflammatory bioactive lipid produced by a variety of human solid tumours, including colorectal cancer, stomach, and breast cancers (Rigas *et al.,* 1993; Uefuji *et al.,* 2000, Rolland *et al.;* 1980). PGE_2 has been shown to down-regulate T helper (Th) 1 cytokines (Harris *et al.,* 2000) and up-regulate Th2 cytokines such as IL-4, IL-10, and IL-6

(Shreedhar *et al.,* 1998; Huang *et al.,* 1998; Della Bella *et al.,* 1997). PGE_2 has also been shown to modulate immune function by inhibiting DC differentiation (Yang *et al.,* 2003) and T cell proliferation (Goodwin *et al.,* 1983). In other instances, cytokines may also act on stromal cells to instigate structural changes within peri-tumoural tissue which act as a physical barrier to immune cell entry, thereby blocking any potential anti-tumour immune response (Mantovani *et al.,* 2008).

1.2.4.2 Regulatory T cells

T-reg are essential for sustaining self-tolerance and immune homeostasis. Whilst they suppress a wide variety of physiological and pathological immune responses against self and non-self-antigens, the exact mechanism for T-reg-induced anti-tumour immune suppression is not fully understood. Two functionally distinct subsets are thought to exist: i) natural T-reg which develop in the thymus gland and express CD4 and CD25 and suppress in a cell to cell, contact-dependent manner and ii) adaptive T-reg which are dependent upon IL-10 and/or TGF-β for their suppressive functions (Tang *et al.,* 2004).

The most physiologically relevant T-reg population are $CD25^+$ $CD4^+$ T-reg, which make up approximately 10% of all $CD4^+$ T cells and express the forkhead box P3 transcription factor (Foxp3). Foxp3 controls expression of several genes for cell-surface molecules including the α chain of the IL-2 receptor, CD25 and other co-stimulatory molecules such as glucocorticoid-induced TNF receptor (GITR) family regulated gene and CTL-associated antigen-4 (CTLA-4) (Shevach, 2009). It also inhibits T cell receptor (TCR) activation-dependent production of effector cytokines such as IL-2 and IFN-γ. Moreover, $Foxp3^+$ T-regs are also able to secrete immunosuppressive cytokines such as IL-10, TGF-β and IL-35 (reviewed by Tran, 2011). Several tumour types have been shown to actively recruit $Foxp3^+$ T-reg into the tumour micro-environment and in some cases to the draining lymph node via chemokine receptor (CCR) 6 (Curiel *et al.,* 2004; Chen *et al.,* 2011). Moreover, the presence of T-reg within human tumours is associated with poor prognosis (Leffers *et al.,* 2008; Bates

et al., 2006) and systemic depletion of T-reg and their removal from the tumour micro-environment has been shown to enhance anti-tumour responses that have led to the inhibition of tumour growth in murine models (Attia *et al.,* 2005; Dannull *et al.,* 2005).

1.2.4.3 Myeloid-derived Suppressor Cells

MDSC consist of myeloid progenitor cells and immature myeloid cells which have the ability to suppress immune responses by depleting L-arginine (L-Arg) from the tumour microenvironment. L-Arg is required for T cell proliferation, ζ-chain peptide and TCR complex expression as well as the development of T cell memory (Bronte *et al.,* 2003; Ochoa *et al.,* 2001).

1.2.4.4 Tumour-associated Macrophages

Tumour–associated macrophages represent approximately 50% of solid tumour mass (reviewed by Solinas *et al.,* 2009). They promote tumour progression and invasion as well as tumour cell migration, angiogenesis and tumour-associated immunosuppression. Two main phenotypes exist: the M1 polarised (IL-12^{high}/IL-10^{low} high APC function) and the M2 polarised (IL-12^{low}/IL-10^{high} low APC function). Those expressing the M2 phentotype are the most predominant within the tumour microenvironment. Here, they produce a variety of immunosuppressive factors including IL-10, TGF-β and PGE_2 (Sica *et al.,* 2006). As well as this, T-regs have been shown to induce B7-H4 expression on tumour-associated macrophages which negatively regulates T cell proliferation and cytokine production (Galani *et al.,* 2010).

1.2.5 Promotion of Cancer Growth

Despite the evidence supporting the role of the immune system in the prevention of cancer, it has been shown that it can, in fact, alternatively promote the development of cancer. Chronic inflammation has been shown to promote carcinogenesis; the risk of developing HCC is related to the duration of the inflammatory response generated by Hepatitis B and C infection (Karin, 2006). Inflammation as a result of *Helicobacter pylori* infection is now

recognised as a major cause of gastric cancer (de Martel *et al.,* 2009). The release of pro-inflammatory cytokines, including VEGF and TNF-α, can promote tumour development and mast cell infiltration in lung tumours is associated with poorer outcomes (Imada *et al.,* 2000). Evidence for the role of chronic inflammation in cancer development can also be seen in epidemiological studies where inhibiting chronic inflammation in patients with pre-malignant disease had a preventative effect (Giardiello *et al.,* 1993; Phillips *et al.,* 2002). These studies demonstrated that long-term usage of anti-inflammatory drugs, such as aspirin and selective cyclooxygenase-2 (COX2) inhibitors, significantly reduced cancer risk, indicating that COX2 or other key molecules that are involved in prostaglandin biosynthesis might be effective anti-cancer targets.

1.3 Cellular Components of Anti-tumour Immunity

Innate immunity is the host first line of defence against pathogens and transformed cells. It does not require prior antigen stimulation and its main cellular effector populations are: neutrophils, macrophages, NK cells, NKT cells, gamma delta (γδ) T-cells and DC. The adaptive immune response consists of antigen-specific reactions of both the humoral (B cell) and cellular (T cell) type. These result in the development of immunological memory. Both systems are intimately linked and play integral roles in the recognition and destruction of primary tumour and metastatic cells.

1.3.1 Natural Killer Cells

First isolated in mice in 1975 (Kiessling *et al.,* 1975), NK cells are a distinct subset of lymphocytes of the innate immune system which monitor the cell surfaces of autologous cells for aberrant expression of MHC-I molecules and cell stress markers. In humans, NK cells are defined as $CD3^-/CD56^+$ lymphocytes and constitute approximately 5-20% of the peripheral blood lymphocytes. They can be divided into two broad categories: $CD56^{bright}$ and $CD56^{dim}$. $CD56^{dim}$ NK cells predominate in the blood and at sites of inflammation and make up approximately 95% of total blood NK cells (Cooper *et al.,* 2004). They are highly cytotoxic and express MHC-I inhibitory receptors such as killer-cell immunoglobulin-like receptors

(KIR) and leukocyte inhibitory receptors (LIR). The $CD56^{bright}$ subset makes up approximately 75% of total lymph node NK cells. They display limited cytotoxicity but produce a variety of immuno-regulatory cytokines upon activation, such as IFN-γ, TNF-β, GMCSF, and IL-13 (Cooper *et al.,* 2001). They also express high levels of the low-affinity FcR for IgG (FcγRIII or CD16) which enables them for ADCC (Ljunggren *et al.,* 2007). NKT cells are a heterogeneous population of T cells which exhibit properties of both T and NK cells. Initially thought to have a protective role in cancer, they are now known to also exhibit inhibitory effects on tumour immune-surveillance and cancer immunotherapy. This is due to different sub-sets of NKT cells which impact on different immune cells including DC, MDSC and NK cells (reviewed by Terabe *et al.,* 2008)

1.3.1.1 Activation and Effector Function of NK Cells

In addition to recognising lack of MHC-I molecules, NK cells must be stimulated by target cell ligands which bind to specific activating receptors on the NK cell-surface. Healthy cells express low levels of NK activatory ligands on their cell-surface, whereas some malignant cells can up-regulate NK activatory ligands and express low levels of MHC-I making them susceptible to NK-mediated killing. NK cell target recognition is a closely regulated balance between activatory and inhibitory signalling. It is also apparent that NK cells do not possess one dominant activating receptor. Instead, they rely on the integration of signals from various activating and inhibitory receptors and ligands (Table 1).

It appears that a critical threshold of signalling which exceeds the influence of the inhibitory receptors must be achieved before NK activation occurs. With the exception of CD16, no activating receptor can activate an NK cell in isolation. Instead, different pairs or combinations of receptors must be cross-linked before effector functions can occur. The recognition of this led to the phrase “co-activating receptors” to be used in the description of the interplay between different activatory NK cell receptors (Bryceson *et al.,* 2006).

NK cells can also be stimulated by several types of exogenous cytokines, including IL-12, IL-15, IL-18 and IL-2 (Fehniger *et al.,* 1999). In humans, $CD56^{bright}$ NK cells in particular, once activated by these cytokines, proliferate and secrete IFN-γ, TNF-α and GM-CSF, thus stimulating inflammatory responses. Upon contact with an appropriate target and suitable receptor ligation, cytotoxic granules move into the synapse between effector and target cells and fuse with the plasma membrane of the target cell. Effector molecules, such as perforin, a membrane-disrupting protein, as well as proteolytic serine proteases known as granzymes are subsequently released into the synaptic cleft by a process of polarized delivery (Tschopp *et al.,* 1987). This ensures delivery of the effector molecules only via the immune synapse and in turn leads to target cell membrane perforation and subsequent induction of apoptotic cell death by granzymes. $CD56^{bright}$ NK cells express lower levels of perforin than their more mature, $CD56^{dim}$ counterparts and are therefore less cytotoxic (Bryceson *et al.,* 2011).

Table 1 Human NK Cell Receptors and Ligands

Activating Receptor(s)	**Ligand(s)**
NKp30, 44, 46	Viral haemagglutins
NKG2D	MICA/B and ULBP 1-5
DNAM-1	CD112 and CD155
CD16	IgG
NKG2DC	HLA-C (low affinity)
Inhibitory receptor(s)	**Ligand(s)**
KIR	MHC-I (HLA-A,B,C)
NKG2A/CD94	HLA-E

As well as this, cytokine secretion by activated NK cells influences adaptive immune responses by promoting growth and differentiation of DC, macrophages and monocytes (Raulet *et al.,* 2004). For example, NK-derived IFN-γ has been shown to stimulate DC, resulting in the production of IL-12 which promoted a CD8 anti-tumour response (Adam *et al.,* 2005). NK cells which reach terminal differentiation upon activation by cytokines i.e. $CD56^{dim}$ $CD62L^{+}$ $CD57^{+}$ NK cells, exhibit high levels of perforin, efficient cytotoxicity, and the ability to produce ample amounts of cytokines in response to target cell recognition (Cooper *et al.,* 2001).

1.3.1.2 NK Cell-mediated Immuno-surveillance of Cancer

NK cell anti-tumour activities may directly lead to tumour eradication by means of cytolysis or IFN-γ secretion but may also indirectly contribute to tumour control by inducing an efficient T cell-mediated anti-tumour response. In humans, low NK-like cytotoxicity of peripheral blood lymphocytes correlates with an increased lifetime risk of developing cancer and several studies report improved outcomes in tumours with high levels of NK cell infiltration (Takeuchi *et al*., 2001; Imai *et al.,* 2000; Sconnochia *et al*., 2011;). Perhaps the most striking evidence of the importance of NK cells in the control of human malignancies comes from studies where allo-reactive NK cells were given to leukaemia patients during haemopoetic stem cell transplantation and significant improvements in survival and relapse rates were seen in patients lacking MHC-I ligands for donor-inhibitory KIR (Hsu *et al.,* 2005; Ruggeri *et al.,* 2007).

1.3.2 Dendritic Cells

DC are professional APC that play a vital role in co-ordinating the innate and adaptive immune systems. Derived from bone marrow progenitor cells, they can prime naïve T and B cells and are also known to recruit and reciprocally interact with NK cells. The classic model of DC maturation states that they exist in two functional forms: immature and mature (Steinman, 1991). iDC are characterised by their high endocytic activity and low T cell activation potential. They reside mainly in peripheral tissues and constantly sample their

surrounding environment for antigen and danger signals, utilising pattern recognition receptors (PRR) such as toll-like receptors (TLR).

iDC are in a constant state of migration between the peripheral tissues and secondary lymphoid tissue (Huang *et al.,* 2000a). They capture antigen using a variety of methods, including phagocytosis and receptor-mediated endocytosis. Antigen uptake with co-stimulation results in DC activation and maturation, characterised by up-regulation of DC cell-surface markers such as CD40, CD86 and CD80. Mature DC (mDC) then present antigen in the context of MHC molecules to T cells to initiate an adaptive immune response.

DCs have been shown to play a key role in the induction of tumour-specific immune responses by the presentation of TAA to T cells (Armstrong *et al.,* 1998). However, in trials utilising DC in patients, clinically relevant responses have been disappointing (Engell-Noerregaard *et al.,* 2009; Rosenberg *et al.,* 2005; Robson *et al.,* 2010). Despite these disappointing clinical outcomes, around 200 DC-based clinical trials have been conducted in cancer patients. The lack of efficacy seen in early trials can be explained by well-defined tumour escape mechanisms and more recent trials have attempted to address these by combining DC vaccination with other approaches, such as CTLA-4 (Ribas *et al.,* 2009), CD25 blockade (Dannull *et al.,* 2005) or loading DC with autologous tumour lysates in combination with agents such as GM-CSF, pegylated IFN, and cyclophosphamide (Alfaro *et al.,* 2011).

Further limitations of the numerous DC trials which have been conducted have been the heterogeneity of trial protocols, the use of non-standardised cellular products and the lack of an established method of measuring clinical and immune responses; these have made it impossible to draw valid conclusions from individual clinical trials. A recent meta-analysis of 29 trials involving 906 prostate and renal cancer patients has shown, as proof of principle, a statistically significant association between cellular immune response and clinical benefit (Draube *et al.,* 2011). It seems likely therefore, that Phase III trials utilising DC-based

immunotherapies warrant further exploration; however, standardised vaccines, trial protocols and criteria for the assessment of immunological and clinical responses must be implemented in a similar manner to those for other drugs.

1.3.3 T Cells

T cells play a central role in cell-mediated immunity. They possess a TCR on their cell-surface and require presentation of antigen in the context of MHC molecules. Abnormal or foreign proteins can be presented to the TCR of $CD8^+$ cytotoxic T cells bound to MHC-I molecules. If appropriate co-stimulation is provided, once activated, antigen specific, $CD8^+$ cytotoxic T cells undergo clonal expansion, mediated by IL-2. In contrast, $CD4^+$ T cells recognise MHC-II molecules on APC. The genetic instability associated with tumour cells can result in the expression of particular peptides and their subsequent presentation on MHC molecules. These TAA can be broadly classified into six main categories:

i. Tumour specific antigens: these are generally a result of point mutations during the process of oncogenesis. These point mutations may allow binding of the resulting peptide to MHC-I de novo or augment MHC-I binding to an existing peptide. An example is Carcinoembryonic Antigen (CEA) in colorectal cancer.
ii. Germ cell antigens: these are expressed on germ cells which do not express MHC-I. The antigens are found on melanoma, breast and glioma tumours and because germ cells cannot present peptides to T cells, they are effectively tumour specific.
iii. Differentiation antigens: these are expressed only in particular types of tissue and best demonstrated by the differentiation antigens expressed in normal melanocytes and malignant melanoma.
iv. Abnormal gene expression: these antigens are overexpressed in tumours compared to their normal counterparts. An example is HER2/neu which is homologous to EGFR.

v. Abnormal post translational modifications: an example of these is cosylated mucin (MUC-1) expressed on several tumours including breast and pancreas.

vi. Oncoviral proteins: expressed on tumours with a known viral aetiology. Examples include Epstein Bar Virus Nuclear Antigen (EBNA) 1, 2 and 3 antigens on Burkitt's and Hodgkin's lymphomas, the Hepatitis B virus (HBV) antigens on hepatomas and the Human Papilloma virus (HPV) E6 and E7 antigens presented on cervical cancers.

Whilst each category of TAA may provoke an anti-tumour immune response *in vitro* or *in vivo*, the spontaneous regression of established tumours remains extremely rare and it is this apparent failure of the immune system to recognise tumours that poses an immense challenge for contemporary tumour immunologists. Vast numbers of TAA are now recognised prompting the National Cancer Institute to define criteria for prioritising which molecules should be trialled for vaccine-based therapy (Cheever *et al.,* 2009).

Despite several early clinical trials using *ex vivo* expanded TIL in combination with IL-2 resulting in tumour regression (Topalian *et al.,* 1988; Rosenberg *et al.,* 1988), lack of specificity of many TAA has prompted concern as to the therapeutic benefits. Many immunotherapy regimes have shown early promise but have failed to confer long-lasting anti-tumour immunity. As well as this, antigen-targeted therapy has the potential to trigger the autoimmune destruction of normal tissues. The fact that a wide range of tumours can develop in immune-competent hosts despite a huge array of TAA has led to the formation of several models of immune recognition in an attempt to gain some understanding of why the immune system fails to clear cancer cells.

1.4 Oncolytic Viruses

1.4.1 Background

Viruses with cytotoxic anti-cancer properties are known as oncolytic viruses. They replicate selectively in, and lyse, cancer cells and this property can be inherent or genetically engineered. The association between viral infection and human cancer therapy dates back to the early part of the 20th century where early anecdotal accounts of spontaneous tumour regression in association with concomitant viral infection with an unrelated virus were described (Dock, 1904). Based on these accounts, an interest in the treatment of human cancers with naturally occurring viruses developed in the 1950s and several clinical trials were undertaken. Out of technological necessity, these early studies focused on naturally occurring viruses such as poliomyelitis virus, coxsackie virus and adenovirus (Pond *et al.,* 1964; reviewed by Kunin 1964). Outcomes were poor, however, with limited anti-tumour efficacy demonstrated. Overwhelming infection caused significant morbidity and mortality and it was quickly apparent that not all tumours were responsive to viral therapy. In those that did respond, the effects were not sustained, with the recipient's anti-viral immune response often abrogating any therapeutic benefit (Huebner *et al.,* 1955; Huebner *et al.,* 1956). It was for these reasons that oncolytic viral therapy was all but abandoned until the late 1970s.

The advent of genetic engineering brought about the ability to modify existing viruses or create novel ones and sparked renewed interest in viral therapy for cancer. Genetic modification enables manipulation of the viral strategies which control host cell response and govern viral replication. By altering these, better tumour selectivity can be achieved or replication in normal cells prevented. As well as this, the addition of tumour-specific promoters may also enable selective tumour replication. Several viruses, including adenoviruses, influenza viruses, herpes viruses (HSV) and vaccinia viruses (VV) have all been genetically modified to enhance tumour selectivity or to promote an anti-tumour

immune response (Suzuki et al., 2001; Bergmann *et al.,* 2001; Martuza *et al.,* 1991; McCart *et al.,* 2001).

1.4.2 Naturally Occurring Oncolytic Viruses

Inherently tumour-selective, wild-type viruses, such as reovirus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV) or autonomous parvovirus depend upon the host for replication. They can specifically target cancer by exploiting cellular aberrations that occur in tumour cells, such as surface attachment receptors, activated *ras* and Akt, or via defective IFN pathways. As well as this, cellular changes which occur upon infection, including increased cell cycling, oncogene activation and signalling pathway alterations, have been shown to enhance the ability of some viruses to replicate in malignant cells (O'Shea *et al*., 2005; Shmulevitz *et al*., 2005). They are capable of destroying tumours and even lymph node metastases in immuno-deficient animals (Prestwich *et al*., 2009c). However, in immuno-competent animals, the anti-viral immune response has been shown to inhibit viral dissemination (Fulci *et al.,* 2003; Altomonte *et al.,* 2008). Currently, one of the main aims of viral therapy is to improve this ratio between the anti-tumour and the anti-viral immune response.

1.4.3 Engineered Tumour-selective Viruses

Engineering viruses to become more tumour-selective involves modification of cellular tropism at the viral replication level. This makes the virus dependent upon specific characteristics of the tumour cell for replication. Various viruses have also been engineered to express immuno-stimulatory molecules including cytokines, HSP and other co-stimulatory molecules. One of the earliest examples of genetic engineering is the adenovirus, Onyx-015, which was modified by removing two DNA elements. It was initally thought that removing the E1B 55□kDa fragment would facilitate replication of Onyx-015 in cells with a defective *p53* pathway. It became apparent, however, that the virus was not specific for *p53*-null cells (Lechner *et al.,* 1992). Recently, a follow-up agent, H103, an adenovirus over-expressing HSP_{70}, has been tested as an intra-tumoral vaccination in a phase I clinical trial in patients

with advanced solid tumours. Distant, un-injected tumours demonstrated regression in three patients (Li *et al.,* 2009).

A further technique for improving the anti-tumour immunogenicity of viruses includes expressing GM-CSF in viruses with the aim of improving antigen presentation by DC. OncoVEXGM-CSF, an HSV virus expressing GM-CSF, showed anti-tumour effect when delivered *i.t.* to a range of cutaneous tumour deposits from breast, gastrointestinal and head and neck primaries as well as nodules of malignant melanoma (Hu *et al.,* 2006); this virus has now been tested in a complete Phase III trial. JX594, a VV expressing GM-CSF with a deletion of the thymidine kinase (tk) gene, has also demonstrated anti-tumour efficacy and an alleged statistically significant benefit in overall survival in a recently completed Phase II trial in patients with advanced liver cancer (www.jenerex.com). In an earlier Phase I trial utilising *i.t.* injection, tumour responses were seen in both injected and un-injected tumours (Park *et al.,* 2008).

Engineering viruses to express other cytokines for improved immunogenicity has also been carried out. IFN-β, expressed by Measles virus (MV) (Li *et al.,* 2010) and VSV (Wang *et al.,* 2010) caused an influx of immune cells into the tumour microenvironment and in the case of VSV, reduced tumour angiogenesis in murine models. IL-12 and IL-18 have also been used to promote anti-tumour activity by inducing NK and T cell proliferation in the context of an oncolytic adenovirus expressing these cytokines (RdB/IL-12/IL-18) (Choi *et al.,* 2011).

Despite initial safety concerns associated with the use of genetically modified viruses in the clinical setting, Phase I/II studies have been undertaken and have shown favourable results with good safety profiles (Park *et al.,* 2008; Xu *et al.,* 2003). All have been manufactured according to Good Manufacturing Practice (GMP) and unlike most Phase I drug trials, the majority of virus studies have never reached a maximum tolerated dose (MTD). Whilst this may be due to technical restriction on the quantity of virus produced, it is also the case that replication competent viruses have unclear dosing regimens, with no clear correlation

between dose and efficacy or toxicity. For this reason, many concurrent trials are ongoing with the same agent and the primary outcome measure remains safety and MTD. The rationale for this is that the interactions between replication competent viruses, host and environment are difficult to predict.

It is now abundantly clear however, that virus-based therapies have shown anti-tumour efficacy and it is likely that their acceptance into mainstream clinical use will occur in combination with other current treatment modalities. The resurgence of oncolytic viral therapy is reflected by the huge increase in publications- around 200 per month- on the topic. In August 2012, a search of the U.S National Institutes of Health- www.clinicaltrials.gov revealed 36 ongoing or completed Phase I and II clinical trials using thirteen different oncolytic viruses in the United States. Some agents have reached the Phase III testing, such as Reolysin®, a clinical grade reovirus which is currently under investigation for the treatment of platinum-refractory head and neck cancers in combination with paclitaxel and carboplatin.

1.4.4 Obstacles to Effective Viral Therapy

Systemic delivery of oncolytic viruses represents the most clinically pragmatic method of administration. However, several host strategies are utilised to clear viral particles from the circulation in immuno-competent animals and can eliminate viral particles before they reach the site of the tumour. These include the uptake of virus by non-blood cells, binding to erythrocytes and viral neutralisation by complement (Lyons *et al*., 2001; Shashkova *et al.,* 2008). Prior exposure to virus can prompt the release of neutralising IgM, preventing viral binding to the cell-surface receptors necessary for infection (White *et al.,* 2008). Whilst several mechanisms to overcome the humoral anti-viral immune response are currently the focus of clinical investigation, some of these strategies may have immunotherapeutic consequences.

The tumour itself can also represent a significant barrier to effective viral therapy. The structure of tumour tissue is not conducive to viral spread. The vasculature is disordered and

the basement membranes of tumours are irregular (Jain *et al.,* 2007). Areas of necrosis also pose problems for viral delivery and the relatively hypoxic and acidotic environment within many tumours may have deleterious effects on viral entry, transcription and the induction of apoptosis. As well as this, fibrillar collagen in the extra-cellular matrix of many tumours differs from normal tissue and has been shown to hamper the spread of HSV within human xenograft models (McKee *et al.,* 2006).

1.4.4.1 Enhancing Viral Delivery

Numerous mechanisms for enhancing viral delivery and anti-tumour efficacy have been investigated. Immuno-suppressive drugs, such as cyclophosphamide and cyclosporine A reduce the ability of the immune system to mount effective anti-viral responses and clear the virus. Pre-conditioning with the alkylating agent, cyclophosphamide and IL-2 prior to *i.v.* reovirus infusion has been shown to enhance reovirus localisation and therapy in C57Bl/6 mice bearing subcutaneous B16 tumours (Kottke *et al.,* 2008). This may, in part, be due to a reduction in circulating neutralising antibodies, a reduction in T-reg numbers (Di Paolo *et al.,* 2006), reduced vascular tumour permeability (Kurozumi *et al.,* 2007) or by causing a reduction in innate immune cell infiltration (Fulci *et al.,* 2006).

It seems reasonable to assume, therefore, that suppression of the anti-viral immune response will enhance oncolytic virus efficacy. However, cyclophosphamide also exhibits immuno-stimulatory effects by the induction of cytokines and has been shown to promote homeostatic proliferation of lymphocyte populations (Bracci *et al.,* 2007). Therefore, the immunosuppressive effects of cyclophosphamide may in fact represent only one component of the multiple pleiotropic effects attributable to cytokine induction within the tumour microenvironment.

Cyclosporine A, a calcineurin inhibitor used following solid organ transplantation, has been shown to enhance reovirus therapy for CRLM in murine models (Smakman *et al.,* 2006a). In

other pre-clinical studies its use led to reduced tumour burden and improved outcomes when used in combination with anti-CD4 and anti-CD8 antibodies (Hirasawa *et al.,* 2003).

Cell carriage of virus particles may represent a further mechanism by which oncolytic viruses can be delivered to tumours in hosts with prior immunity. It has recently been demonstrated that reovirus-loaded mDC can deliver the virus to lymph node B16tk melanoma metastases in immune C57Bl/6 mice (Ilett *et al.,* 2009). Monocytes (Iankov *et al.,* 2007) and tumour cells (Power *et al.,* 2007) have also been shown to prevent viral elimination by carriage. Cells used in this process may simply deliver their viral cargo at the site of the tumour - a process known as “hitch-hiking” - or, in some cases, they promote viral replication during carriage, thus amplifying delivery. Other strategies involve the use of hitch-hiking cells which are themselves active against tumour such as lymphokine activated killer (LAK) cells. The exact mechanism of viral “hand-off” once these cells reach the tumour remains unclear but may include enzymes such as heparinases in the tumour micro-environment (Cole *et al.,* 2005). Whilst many of these observations may be virus specific, it is likely that future clinical trials will have to focus on the equilibration of the humoral anti-viral immune response and anti-tumour immunity.

Several attempts have been made to enhance the delivery of virus to tumours using agents such as proteases which target the extracellular matrix (Kuriyama *et al.,* 2001). In this human glioblastoma multiforme-derived, xenograft model, significantly higher levels of tumour regression were noted on pre-treatment with a mixture of collagenase and dispase prior to the administration of a recombinant adenovirus encoding HSV thymidine kinase (Ad-HSV-tk).

As well as this, attempting to modify the extra-cellular matrix of the tumour by inducing apoptosis has been shown to improve viral delivery. The creation of “void spaces” within the tumour architecture using cytotoxic agents such as doxycyline, paclitaxel and TRAIL has been shown to induce heterogeneous areas of apoptosis and the formation of channel-like

structures, which enhance the initial viral penetration in human breast carcinoma xenograft models (Nagano *et al.,* 2008). It seems likely, therefore, that combination therapies within clinical trials may show promise in overcoming some of the mechanical barriers within the tumour to viral dissemination and efficacy.

1.5 Reovirus

1.5.1 Background

Reovirus (Respiratory Enteric Orphan virus) is a member of the Reoviridae family and was first isolated from the gastrointestinal and respiratory tracts of humans (Sabin, 1959). Found predominantly in stagnant water and untreated sewage, reovirus is ubiquitous in the natural environment. Reovirus exposure is common, with up to 100% of the population demonstrating sero-positivity (Selb *et al.,* 1994). Virtually all mammals, including humans, serve as hosts, with infection normally occurring before the age of 5. Reovirus-induced disease in humans is limited to the very young; however, its pathogenicity in new born mice has established its use as an experimental model for viral infection and replication (Forrest *et al.,* 2003). Little is known about the natural history of reovirus infection in humans and this is possibly as a result of its lack of pathogenicity. A few longitudinal studies have tried to address the issue of virulence and pathogenicity and have found several peaks of sero-prevalence at various ages, suggesting that re-infection occurs from late childhood through to old age (Douville *et al.,* 2008).

1.5.2 Structure of Reovirus

Reovirus has a non-enveloped, icosahedral capsid consisting of an inner and outer protein shell. This stable structure is partly responsible for the ubiquity of the virus. It replicates within the cytoplasm of cells and its genome consists of 10 segments of dsRNA, arranged in 3 distinct size classes, designated Lambda (λ), Mu (μ) and Sigma (σ). These 10 segments encode 12 viral proteins; 8 structural and 4 non-structural (Chandran *et al.,* 2001).

Three serotypes of reovirus have been defined, based on their antibody neutralisation and haemagglutination inhibitory activities: Type 1 Lang, Type 2 Jones and Type 3 Abney, as well as Type 3 Dearing (Rosen *et al.,* 1960). All three serotypes utilise junctional adhesion molecule-A (JAM-A) as a serotype-independent, cell-surface receptor and infection is initiated with the attachment of the σ1 protein (Campbell *et al.,* 2005). Sigma 1 is a homo-trimer consisting of a fibrous tail and an externally facing, globular head at the C terminus. The σ1 proteins of the T1 Lang strain and the T3 Dearing contain an independent receptor domain which binds JAM-A.The fibrous tail also contains further receptor domains which bind α-linked sialic acid (Chappell *et al.,* 2000).

1.5.3 Replication of Reovirus

Following host infection, reovirus cellular binding occurs via a multi-step process. Initially, the tail portion of σ1 on the virion binds with low affinity to the target cell via surface sialic acid (Chandran *et al.,* 2001; Barton *et al.,* 2001; Chappell *et al.,* 1997). Subsequently, the head of the virus binds to JAM-A and virions are internalised by a process of receptor-mediated endocytosis (Barton *et al.,* 2001). The virus then undergoes proteolytic disassembly within the endosome, resulting in the loss of the outermost capsid protein, σ3 (Alain *et al.,* 2007). The σ1 portion is then shed with the underlying viral proteins μ1/μ1c undergoing structural rearrangement. As well as this, changes in the conformation of the outer tail take place. This process culminates in the production of infectious sub-virion particles (ISVP; Figure 1-2) (reviewed by Lemay, 1988).

It is believed that ISVP are intermediates, capable of penetrating endosomes, lysosomes or plasma membranes (Alain *et al.,* 2007). They have the ability to deliver cores capable of transcription into the cytoplasm of the infected cell, where replication of reovirus can take place. Inhibiting proteolytic disassembly by blocking endosomal acidification or the endosomal proteases has been shown to prevent apoptosis in both *in vivo* and *in vitro* reovirus-infected systems, highlighting that viral un-coating and disassembly are crucial for

apoptosis to occur (Marcato *et al.,* 2007). Furthermore, the addition of an RNA synthesis blocker to cells infected with reovirus does not prevent apoptosis occurring. In addition to this, viral particles lacking dsRNA are also capable of inducing apoptosis indicating that the initiating event in apoptosis precedes and is independent of, viral replication (Connolly *et al.,* 2002).

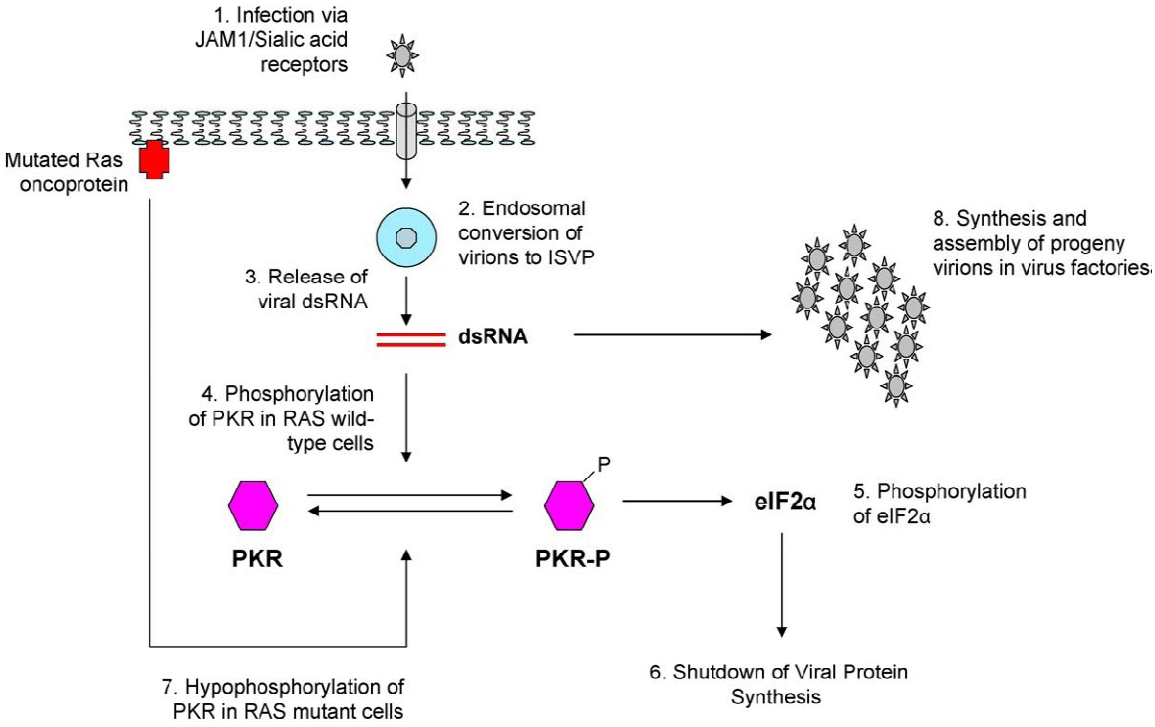


Figure 1-1 Schematic of the Reovirus Infective Cycle

(1) Cellular entry occurs through interaction between the virion, JAM-A and sialic acid residues on the cell membrane. (2) The virus is internalised in an endosomal compartment to form the ISVP. (3) dsRNA escapes from the endosome. (4) In *ras* wildtype cells, the presence of dsRNA leads to phosphorylation of PKR (5) and subsequent activation of eukaryotic initiation factor 2α (eIF2a). (6) This leads to shutdown of viral protein synthesis, thus aborting a productive infection. (7) In cells with mutant *ras* or an activated *ras* pathway, PKR remains in a hypo-phosphorylated form and viral RNA species are able to direct synthesis and assembly of new virions (8) (Adapted from Harrington *et al.,* 2010).

1.5.4 Junctional Adhesion Molecule-A

Junctional adhesion molecule-A, formerly known as JAM-1, was first recognised as the main reovirus receptor as recently as 2001 (Tyler *et al.,* 2001). It is a broadly expressed, trans-

membrane protein which belongs to the cortical thymocyte marker of Xenopus protein (CTX) family of the Ig superfamily of adhesion molecules (Forrest *et al.,* 2003). The protein consists of two Ig-like domains: a trans-membrane section and a short cytoplasmic tail (Kostrewa *et al.,* 2001; Prota *et al.,* 2003). In humans, it is localised to epithelial and endothelial tight junctions and is found on the surface of platelets and leucocytes.

Homophilic JAM-A interactions play a role in peri-cellular permeability and leucocyte transmigration in response to inflammation. During an inflammatory response, JAM-A redistributes from the tight junctions to the apical surface and engages leucocytes in preparation for diapedesis through the endothelium. Neutralisation of JAM-A has been shown to reduce both monocyte and neutrophil transmigration across brain endothelium in a mouse meningitis model (Del Maschio *et al.,* 1999) and more recently the receptor has been reported to play a role in breast cancer invasion (Naik *et al.,* 2008). Whilst JAM-A-independent cellular entry by reovirus can occur, it has been shown that both JAM-A and sialic acid binding, in combination with proteolytic disassembly of the viral capsid, are required for the induction of cellular apoptosis (Alain *et al.,* 2007).

1.5.5 The Activated *ras* Pathway

ras proteins are low-molecular-weight GTP-binding proteins and make up a large superfamily. Three members of the *ras* family, H*ras*, K*ras* and N*ras* are closely related, with 85% of their amino acid sequence being identical (reviewed by Downward, 2003). K*ras* is expressed in almost all cell types and has been shown to be essential for normal cell development in mice (Johnson *et al.,* 1997). Human tumours frequently express *ras* proteins that have been activated by point mutation. It is estimated that approximately 30% of all tumours have undergone at least one activating mutation in one of the *ras* genes. They occur most frequently in pancreatic cancer (90%), colorectal cancer (50%), lung cancer (40%) and myeloid leukaemia (30%) (reviewed by Bos,1989). Constitutive changes in *ras* signalling, brought about by oncogenic changes in the signalling pathway upstream and downstream of *ras*, are even more prevalent in human cancers (reviewed by Downward, 2003). In these

tumours, the activated *ras* protein contributes to several aspects of the malignant phenotype, including tumour-cell growth, inhibition of apoptosis, the promotion of invasiveness, and angiogenesis (Shields *et al.,* 2000).

In normal cells, viral infection induces activation and phosphorylation of dsRNA-dependent protein kinase (PKR) (Strong *et al.,* 1998). Activated PKR inhibits translation of viral transcripts via phosphorylation of eIF2α which halts protein synthesis and therefore abrogates viral replication. The addition of a chemical inhibitor of PKR phosphorylation to normal cells induces susceptibility to reovirus infection. In *ras*-transformed cells, PKR is not phosphorylated in response to reovirus infection, allowing viral transcription to occur, leading to replication and accumulation of newly synthesised virus and eventual release of the progeny through cell lysis. This "activated *ras* pathway" has been proposed as the mechanism underlying the selective oncolysis exhibited by reovirus and it has been demonstrated that reovirus killing is enhanced in cells which over-express H*ras* (Coffey *et al.,* 1998; Strong *et al.,* 1998).

Exactly how activation of *ras* signalling leads to the inhibition of PKR phosphorylation remains unclear, however the ral guanine nucleotide exchange factor (RalGEF) signalling pathway appears to be important for allowing selective reovirus replication (Norman *et al.,* 2004). Downstream of RalGEF, *p38*, a signalling element, has been shown to regulate viral protein synthesis in *ras* transformed cells (reviewed by Shmulevitz *et al.,* 2005). Despite this, the precise mechanisms by which RalGEF and *ras* promote reovirus replication remain unclear.

The impairment of the PKR-mediated inhibition of viral protein translation by *ras* transformation was previously thought to fully explain the selective tumour oncolysis by reovirus. More recent studies, however, have found factors other than the absence of PKR phosphoylation which may account for the susceptibility to reovirus infection seen in cells

with an activated *ras* pathway. It is now known that *ras* transformation promotes three steps in viral replication, which results in substantial amplification of virion yield after one cycle of viral replication (Marcato *et al.,* 2007). Firstly, *ras* transformation enhances viral un-coating which is thought to be due to up-regulation of lysosomal cathepsins in *ras* transformed tumours. Secondly, transformed cells facilitate the production of new virions which are highly infectious. Whilst the reasons for this are not fully understood, it is has been proposed that *ras* transformation may confer subtle structural changes during virion assembly which increase their infectivity. Finally, *ras* activated tumour cells are more sensitive to reovirus-mediated apoptosis and deletion of K*ras* in colorectal cell lines has been shown to block apoptosis but not replication (Smakman *et al.,* 2005).

Murine and human tumour models have also demonstrated that the presence of an activated *ras* pathway may facilitate the induction of reovirus-induced apoptosis (Smakman *et al.,* 2005, Smakman *et al.,* 2006b). In these studies, neither reovirus protein synthesis nor yield of virions were reduced by the knockdown of *ras*, however reovirus-induced tumour cell apoptosis was abrogated. This suggests that the mechanism underlying reovirus induced oncolysis may involve the sensitization of tumour cells by a *ras*-dependent pathway and that reovirus replication is not *ras*-dependent *per se*. For example, reovirus is known to induce apoptosis via the activation of the cellular stress kinase, c-Jun N-terminal kinase (JNK), and NF-κB (Clarke *et al.,* 2004; Connolly *et al.,* 2000; Pruitt *et al.,* 2002). Since *ras* transformation results in JNK and NF-κB activation, these pathways may play a role in the enhanced sensitivity of *ras* transformed cells to reovirus-induced apoptosis.

The inhibition of PKR phosphorylation, whilst important, is only one of many functional consequences of activated *ras* signalling utilised by reovirus and may not be as critical as once thought in determining susceptibility of tumour cells to reovirus infection. Moreover, a recent study suggested that reovirus infection of cancer cells is not dependent upon *ras* status at all. No clear correlation between levels of activated PKR, *ras* status or reovirus-

induced apoptosis was found across multiple cell lines (Song *et al.,* 2009). Instead, it has been suggested that tumour cell susceptibility to reovirus infection is dependent upon its expression of JAM-A. This was demonstrated by transducing glioma cells which do not express JAM-A which were resistant to infection with lentiviral vectors encoding JAM-A. This in turn led to reovirus infection and cell death (Song *et al.,* 2009).

1.5.6 Reovirus Activation of Nuclear Factor κ B

The Nuclear Factor κB family of transcription factors plays a key role in cell growth and survival. In quiescent cells, NF-κB is prevented from migrating and binding to DNA by the IkB family of inhibitory proteins. Several stimuli, including TNF-α, IL-1, and lipopolysaccharide (LPS) activate NK- kB via a mechanism of site-specific phosphorylation and proteasomal degradation of IkB (Brown *et al.,* 1995). Reovirus infection has been shown to activate NF-kB in a variety of cell lines by electrophoretic mobility shift assays. It appears that the activation is as a result of targeted degradation of IkB and the accumulation of nuclear *p65* (Steele *et al.,* 2011). The inhibition of NF-kB by the addition of protease inhibitors has been shown to abrogate reovirus-induced apoptosis, as have targeted disruptions in the genes encoding several subunits of NF-kB (Connolly *et al.,* 2000). It now seems likely that the detection of dsRNA by PKR induces the activation of NF-κB-dependent chemokines and cytokines in tumour cells rather than reovirus transcription or translation being a prerequisite for activation, as was previously thought.

Reovirus activation of NF-kB, like apoptosis induction, requires both sialic acid and JAM-A binding, in addition to viral disassembly (Connolly *et al.,* 2000). Exactly how receptor binding and viral disassembly activate NF-kB remains elusive, however several theories have been put forward. It has been postulated that the conformational change in σ1 that occurs during disassembly may increase the affinity of it for the receptors, or that proteolytic processing of μ-1/μ-1C during disassembly influences virus-receptor interactions, causing receptor aggregation and the stimulation of intra-cellular signalling. A further theory is that JAM-A acts as an intra-cellular binding partner of the *ras* target postsynaptic density protein, disc-

large, zo-1 (PDZ) domain protein, AF-6, and that NF-kB activation occurs via a *ras*-mediated signalling pathway (reviewed by Clarke *et al.,* 2003).

1.5.7 Apoptosis is Independent of Reovirus Replication

The addition of ribavirin, a RNA synthesis inhibitor to cultures of HeLa cells infected with reovirus inhibits reovirus replication but not apoptosis (Connolly *et al.,* 2002). Ultra-violet (UV) light- inactivated virions, which contain σ1 but not σ1s and are transcriptionally-inactive, have been shown to induce cell death, but higher numbers of virions- up to 100 fold - are required. This suggests that it is the S1 gene product, σ1, which is responsible for apoptosis induction. The necessity for higher multiplicity of infection (MOI) of UV-inactivated virions to induce apoptosis, suggests that at low MOI, replication may be necessary to raise the viral inoculum to a threshold level before apoptosis is induced. The higher MOI required for UV-inactivated induction of apoptosis appear to trigger a faster kinetic of apoptosis as initial replication is not necessary for viral amplification (Tyler *et al.,* 1995).

1.5.8 Reovirus and the Immune System

Despite vigorous investigation of the direct cytotoxic effects of oncolytic viruses, including reovirus, it is only recently that the role of the immune system in viral therapy has been studied. The majority of animal experiments investigating oncolytic viruses have been performed using xenografts and immuno-deficient mouse models, precluding assessment of the role of the immune system in therapy. Whilst initially it was thought that the immune system must inevitably be detrimental to oncolytic viral therapy by the neutralisation of virus and abrogating direct tumour cell killing, it has become clear that viral therapy may alternatively support priming of anti-tumour immune responses, which can contribute significantly to tumour regression (Prestwich *et al.,* 2009a).

Reovirus replicates in and lyses several human melanoma cell lines as well as freshly resected melanoma samples (Errington *et al.,* 2008a). This study also demonstrated that reovirus-induced cell death was associated with the release of a number of chemokines and

pro-inflammatory cytokines. Macrophage inflammatory protein (MIP) -1α, MIP-1β, IL-8 and Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES) were all found, as well as the pro-inflammatory cytokine, IL-6. Moreover, reovirus-nduced phenotypic maturation of DC in the absence of tumour cells and these activated DC secreted a number of cytokines, including IL-6, IL12p70, IFN-α and TNF-α. Additionally, reovirus-infected melanoma cell lines loaded onto purified DC induced NK-mediated cytotoxicity against melanoma targets (Prestwich *et al.,* 2009b). This process was modulated by IFN-β. These findings raise the possibility of utilising reovirus infection to generate a pro-inflammatory cytokine response with the induction of innate anti-tumour immunity.

Reovirus also has the ability to generate adaptive anti-tumour immune responses. In a murine model, intravenous delivery of the virus resulted in the generation of a melanoma-specific, T cell response towards tyrosinase-related protein (TRP) -2 and B16 antigens (Prestwich *et al.,* 2009b). As well as this, reovirus-infected melanoma cell lines not only matured DC in a reovirus dose-dependent manner, but when cultured with autologous peripheral blood mononuclear cells (PBMC), DC loaded with reovirus-infected human Mel888 melanoma cell antigens cross-primed CD8+ T cells specific against the human tumour-associated antigen Melanoma-associated antigen recognised by T cells (MART-1) (Prestwich *et al.,* 2009b). Moreover, reovirus loaded T cells purged murine melanoma, B16ova lymph node and splenic metastases in C57BL/6 mice. B16ova is resistant to direct reovirus oncolysis, highlighting, for the first time that the immune system was vital for reovirus therapeutic efficacy and that viral replication was not essential. As well as this, reovirus-infected tumour cells have been shown to induce a chemotactic response resulting in the recruitment of CTL in the absence of live virus (Steele *et al.,* 2011).

In the several clinical trials which have been completed using reovirus, an increase in neutralising antibodies in recipients has been a consistent finding (White *et al.,* 2008;

Harrington *et al.,* 2010). Suppressing this anti-viral immune response to avoid rapid viral clearance is likely to be crucial in maximising the efficacy of reovirus *in vitro*. One possible way in which viral elimination may be abrogated, is the use of cell carriers such as T cells, DC or monocytes (Qiao *et al.,* 2008a; Iankov *et al.,* 2007; Power *et al.,* 2007; Ilett *et al.,* 2009).

1.5.9 Reovirus in Clinical Trials

In order to ascertain the clinical features of reovirus infection, one study was undertaken in 1963 during which each of the 3 serotypes of reovirus were inoculated into adult volunteers (Rosen *et al.,* 1963). In this study, male participants were given intra-nasal inoculations and followed up for 23 days for signs of symptomatic illness. This study confirmed that reovirus may cause minor upper respiratory tract illnesses and diarrhoeal symptoms, as had been previously suggested (Jackson *et al.,* 1961; Jarudi *et al.,* 1973). The wild type, Dearing strain of Reovirus has been manufactured to clinical grade (Reolysin®) by Oncolytics Biotech Inc. (Calgary, Canada). Thirty clinical trials have been completed or are ongoing as of May 2012. The first 3 studies were conducted at the University of Calgary, Alberta, Canada with others being undertaken at several centres internationally.

In the first Phase I cancer related study, 19 patients with clinically evident metastatic or recurrent non-lymphoma solid tumours that were accessible for injection and measurement by direct observation or palpation were enrolled. Intra-tumoural injection of the virus was undertaken at escalating dose levels ranging from 10^7 plaque forming units (pfu) to 10^{10} pfu. The patients had a variety of end-stage tumour types, including head and neck cancer, melanoma, breast adenocarcinoma and sarcoma. Only minor illnesses such as flu-like and diarrhoeal symptoms were noted. The best responses at 10 or 14 weeks included 3 partial responses and two with stable disease in the primary tumour. One patient had a complete response, 1 a partial response and 8 more patients had no measurable progression in their disease. Perhaps more significantly, 3 patients had responses noted in synchronous

lesions distant from the site of the primary lesion (www.oncolyticsbiotech.com/clinical-trials/27).

Having shown promise via direct *i.t.* delivery and that the virus was well tolerated, further investigations of reovirus therapy using differing routes of administration, either alone or in combination with other agents, were undertaken. Pre-clinical studies have shown that reovirus in combination with ionising radiation acts synergistically to promote apoptosis in cell lines (Twigger *et al.,* 2008). On this basis, a Phase Ia/Ib study was undertaken where radiotherapy was given in conjunction with *i.t.* injection of the virus (Harrington *et al.,* 2010). Partial responses were again seen in several different tumour types including colorectal and oesophageal adenocarcinomas. REO 008, a phase II study, evaluating radiation with *i.t.* Reolysin® injection in 16 heavily pre-treated patients with a variety of malignancies, demonstrated a total disease control rate of 93% in the treated lesions (http://www.oncolyticsbiotech.com/clinical-trials/20).

REO 05, a phase I study of *i.v.*-delivered Reolysin® in patients with advanced cancer, found evidence of anti-tumour activity (Vidal *et al.,* 2008). All patients had evidence of progressive disease prior to entry. At least 4 of 18 patients in the completed cohorts had stable disease with varying degrees of tumour regression. One patient with metastatic prostate cancer treated in the $3x10^9$ Tissue Culture Infectious Dose- 50 ($TCID_{50}$) cohort had a 50% decrease in Prostate Specific Antigen (PSA) levels with evidence of reovirus replication in tumour and tumour necrosis on Computerised Tomography (CT) scanning. Two patients with metastatic colorectal cancer in the $1x10^9$ and $3x10^9$ $TCID_{50}$ cohorts had 60% and 27% reduction in CEA levels, having received 6 and 3 courses of treatment respectively. All patients demonstrated reovirus sero-positivity prior to receiving the infusion. Titres increased approximately 1 week after treatment, eventually reaching a plateau.

REO 013, a biological endpoint trial which recently completed, studied the effects of *i.v*. reovirus administered to patients undergoing curative intent surgery for CRLM (Adair *et al.,* 2012b). Ten patients received 1 cycle of $1x10^{10}$ $TCID_{50}$ reovirus, 6-28 days prior to elective, curative-intent hepatic resection. Despite the presence of neutralising antibody (NAB) before viral infusion, replication-competent reovirus was recovered from PBMC. Moreover, recovered virus retained cytotoxicity towards target cells and assessment of the surgical specimens demonstrated greater expression of reovirus σ3 in tumour cells than in surrounding normal liver parenchyma. As well as this, replicating virus was isolated from tumour tissue only. These findings suggest for the first time, that reovirus may be protected from NAB after systemic administration by immune cell carriage of the virus and that these cells could in turn deliver reovirus to intra-hepatic tumour sites. Although not the direct subject of this thesis, as I was the lead author on this trial and its findings relate to the preclinical data presented here, the publication from this study is included in the Appendix (Adair *et al*., 2012b).

Reo 018, the first randomised, two-arm, double-blind, multicentre, two-stage, adaptive Phase III trial of carboplatin/paclitaxel, plus reovirus or placebo, in patients with relapsed or metastatic, platinum-refractory, squamous cell cancer of the head and neck is on-going. All patients receive 3 weekly (21 day cycles) of paclitaxel and carboplatin and either *i.v.* placebo or intravenous Reolysin®. All dosing takes place in the first 5 days of each cycle, with patients receiving standard *i.v.* doses of paclitaxel and carboplatin on day 1 only. On days 1 to 5, they receive either *i.v.* placebo or Reolysin® at a dose of 3×10^{10} $TCID_{50}$. Patients continue to receive the trial combination therapy for up to 8, 21-day cycles and, thereafter, blinded placebo or Reolysin® until the patient either develops progressive disease or meets other criteria for removal from the trial. The primary endpoint for the trial is overall survival (OS) with secondary endpoints including progression free survival (PFS), objective response rate, duration of response, and safety and tolerability of Reolysin®, when administered in combination with paclitaxel and carboplatin. The first non-adaptive stage of the trial is

designed to enrol 80 patients, with the second adaptive stage designed to enrol between 100 and 400 patients. Results are eagerly awaited.

2 Aims of the Study

The aim of this study was to examine the therapeutic potential of reovirus for the treatment of colorectal cancer. The direct cytoxic effects of the virus, as well as well its ability to generate an innate anti-tumour immune response were investigated. In particular, this study focused on:

1. The cell suface expression of JAM-A on colorectal cancer cell lines and human PBMC.
2. The cytotoxicity of reovirus against colorectal cancer *in vitro*.
3. The mode of reovirus-induced cell death.
4. Examining the deleterious effect of NAB on direct reovirus cytotoxicity.
5. Investigating the potential of human PBMC to act as viral carriers.
6. The effect of reovirus infection on NK cells within whole PBMC.
7. Reovirus-activated NK cell-mediated cytoxicity towards colorectal cancer.
8. The isolation of liver mononuclear cells from fresh human resection specimens.
9. The effect of reovirus infection on hepatic NK cells.

3 Materials and Methods

3.1 General

Tissue culture was performed under aseptic conditions using NuAire Class II Microbiological Safety Cabinets (NuAire Inc., Plymouth, UK). The cabinets were routinely cleaned before and after each use with 2% (w/v) Virkon (Scientific Laboratory Supplies (SLS), Nottingham, UK), then 70% (v/v) ethanol (Sigma-Aldrich Ltd., Dorset, UK). All plasticware used was purchased as pre-sterilised sealed packages from Corning Costar (High Wycombe, UK) or BD Biosciences (Oxford, UK). Cells were incubated in vented flasks (25 cm^2 – 150 cm^2 sizes) or 6-well tissue culture plates in Sanyo incubators (Sanyo Inc., Loughborough, UK) at 37^oC, in a humidified atmosphere of 5% CO_2 in air. All pipettes used were produced by Gilson Inc. (supplied by Anachem Ltd., Bedfordshire, UK). Centrifugations were carried out at room temperature (RT), using an Eppendorf 5810R centrifuge (Eppendorf, Leicestershire, UK). All centrifugations were done at 400g for 5 mins at RT, unless stated otherwise. An Olympus CKX41 light microscope (Olympus UK Ltd., South-end-on-Sea, UK) was used for routine cell observations and cell counts were performed using 0.2% (v/v) Trypan Blue (Sigma) and a standard haemocytometer (Weber Scientific Int., West Sussex, UK).

3.2 Cell Culture and Primary Cell Isolation

Cell lines and primary cells were maintained in either Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute (RPMI) -1640 (both Sigma) and supplemented as indicated in Table 2. Foetal calf serum (FCS) and pooled human AB serum (HS) (both Biosera, Ringmer, UK) were heat-inactivated at 56 ^oC for 30 mins prior to use. L-glutamine, sodium pyruvate, HEPES, non-essential amino acids and 2β-mercaptoethanol were all purchased from Sigma. All cell lines were routinely passaged or harvested at or near confluence using trypsin-EDTA (Sigma).

Table 2: Cell Lines, Primary Cells and Culture Media

Cell Line / Primary Cell	Culture Medium
LoVo Human colorectal adenocarcinoma	DMEM + 10% (v/v) FCS + 2 mM L-glutamine (10% DMEM)
LS174T Human colorectal adenocarcinoma	10% DMEM
SW480 Human colorectal adenocarcinoma	10% DMEM
SW620 Human colorectal adenocarcinoma	10% DMEM
L929 Murine fibrosarcoma	10% DMEM
Primary colorectal metastatic cells	10% DMEM
Primary hepatocytes	10% DMEM
PBMC	RPMI-1640 + 7.5% HS + 2 mM L-glutamine + 1% (v/v) sodium pyruvate + 1% (v/v) HEPES +1% (v/v) non-essential amino acids + 20 µM 2β-mercaptoethanol (lymphocyte culture medium)
Liver-derived mononuclear cells (LMNC)	Lymphocyte culture medium

3.2.1 Isolation of PBMC by Density Gradient Separation

Peripheral blood was collected from healthy donors and patients with colorectal cancer metastatic to the liver. Written, informed consent was obtained from all patients in accordance with local institutional ethics review and approval. Samples were diluted 1:1 with Hank's Balanced Salt Solution (HBSS) (Sigma) and then slowly layered at a 2:1 ratio onto Lymphoprep© (Axis Shield, Cambridgeshire, UK). Tubes were centrifuged at RT for 25 mins

at 800g with no brake, before excess plasma was pipetted off and discarded. The white cell layer was collected using a wide-tipped Pasteur pipette (SLS). 50 ml HBSS was added and cells were pelleted by centrifugation at 400g for 10 mins at RT. Cells were washed again in 50 ml HBSS and centrifuged at 300g for 5 mins at RT. PBMC were cultured at 2 x 10^6 / ml.

3.2.2 Primary Tissue Collection

Tumour and normal hepatic parenchyma samples were taken from patients undergoing routine, planned resection of colorectal cancer metastatic to the liver. Written, informed consent was obtained from all donors in accordance with institutional ethics review and approval. Samples were collected under sterile conditions (Geoffrey Giles Theatre Suites, SJUH, Leeds, UK), preserving margins for histological diagnosis and confirmation of the adequacy of resection. Immediately following resection, the tissue was transported back to the laboratory in sterile, 150 ml screw-topped pots (SLS) containing either cold physiological saline or HBSS and processed at once.

3.2.3 Isolation of Primary Colorectal Metastatic Cells

Tumour samples were placed in a Petri dish containing 10 ml 10% DMEM and dissected into 5mm cubes, removing any fatty or necrotic tissue. Cubes were then disaggregated using a 60 µm Cell Dissociation Sieve & Tissue Grinder Kit (Sigma). This cell suspension was passed through a 70 µm cell strainer (BD Biosciences) and any debris removed by 2 large volume washes and centrifugations. The resulting CRLM single cell suspension was cultured at 2 x 10^6 / ml.

3.2.4 Isolation of Liver-derived Mononuclear Cells and Primary Hepatocytes

Freshly resected normal liver tissue was collected and a single cell suspension was prepared as described above. Cells were then diluted in 30 ml HBSS, layered slowly over 15 ml Lymphoprep© and centrifuged at RT for 25 mins at 800g with no brake. Supernatant was pipetted off and discarded before LMNC were collected from the resulting layer using a wide-tipped Pasteur pipette. Hepatocytes were isolated from directly above the red blood cell

pellet, again using a wide-tipped Pasteur pipette. 50 ml HBSS was added and cells were pelleted by centrifugation at 400g for 10 mins at RT. Cells were washed again in 50 ml HBSS and centrifuged at 300g for 5 mins at RT. Both cell populations were cultured at 2 x 10^6 / ml.

3.3 Reovirus

Reovirus Type 3 Dearing Strain was provided by Oncolytics Biotech Inc. Stock virus titre was determined using a standard plaque assay protocol on L929 cells as described in section 3.3.6. Aliquots of $1x10^9$pfu/ml were stored in the dark at 4°C for up to 1 month or -80°C for longer term storage.

3.3.1 Assessment of Direct Reovirus-induced Cytotoxicity in Colorectal Cancer Cell Lines

LoVo, LS174T, SW480 and SW620 cells were seeded into 6-well plates at a density of $2x10^5$ cells in 3 ml culture medium. Cells were incubated for 4 hr to allow them to adhere and then treated with 0, 1 or 10pfu/cell reovirus. Following incubation for 24-72 hr, cells were harvested and their viability was determined using the Live/Dead® Cell Viability Assay (Life Technologies, Paisley, UK) (section 3.4.5) and flow cytometry. Where the effect of NARA was to be determined, cells were also cultured in DMEM supplemented with 1-2% (v/v) HS and 1% (v/v) L-glutamine.

3.3.2 Assessment of Reovirus Replication in Colorectal Cancer Cell Lines

SW480 and SW620 cells were seeded into 6-well plates at a density of $2x10^5$ cells in 3 ml culture medium. Cells were incubated for 4 hr to allow them to adhere and then treated with 0, 1 or 10pfu/cell reovirus. Following incubation for 24-72 hr, cells/supernatants were harvested using a wide-tipped Pasteur pipette and stored at -80 °C prior to use. Lysates were then prepared by means of 3 cycles of freezing/thawing (10 mins in methanol/dry ice followed by 10 mins in a water bath at 37ºC). The reovirus titre in each sample was determined by plaque assay (section 3.3.6).

3.3.3 Assessment of Apoptotic Intracellular Active Caspase-3 Production

SW480 and SW620 cells were seeded into 6-well plates at a density of $2x10^5$ cells in 3 ml culture medium. Cells were incubated for 4 hr to allow them to adhere and then treated with 0 or 10pfu/cell reovirus. After 72 hr, cells were harvested and active caspase-3 production was measured using the PE-conjugated Active Caspase-3 Apoptosis Kit (BD Pharmingen, Oxford, UK) (Section 3.4.6) and flow cytometry.

3.3.4 Assessment of Inhibition of Apoptosis

SW480 and SW620 cells were seeded into 6-well plates at a density of $2x10^5$ cells in 3 ml culture medium. Cells were incubated for 4 hr to allow them to adhere and then treated ± the irreversible pan-caspase inhibitor, Z-VAD-FMK (Calbiochem, Nottingham, UK) at a concentration of 50 µM. Cells were incubated for 1 hr prior to the addition of 0 or 10pfu/cell reovirus. After 72hr, cells were harvested and viability was determined using the Live/Dead® Cell Viability Assay (section 3.4.5) and flow cytometry.

3.3.5 Assessement of Reovirus Replication in PBMC

PBMC were cultured at 2×10^5 cells/ml and treated with 1pfu/cell reovirus. Following 24-72 hr of incubation, cell/supernatants were harvested and stored at -80 ^oC prior to use. Lysates were then prepared (section 3.3.2) and reovirus titre in each sample was determined by plaque assay (3.3.6).

3.3.6 Measurement of Reovirus Titre by Plaque Assay

The reovirus-sensitive, L929 cell line was used to determine the plaque forming activity of reovirus-treated samples. L929 cells were seeded in 6-well plates at a density of 1×10^6 cells in 2 ml culture medium and incubated for 4 hr to allow cells to adhere. Serial dilutions between 2×10^{-2} and 2×10^{-9} of sample lysates and reference virus stock were prepared in DMEM + 2mM L-glutamine (virus dilution media). Culture medium was removed from L929 cells and replaced with 500 µl of diluted viral samples in triplicate, taking care not to disturb the cell monolayer. Cells were then incubated for 4 hr, rocking occasionally to ensure even

coverage of the viral sample. Melted, 2% (w/v) agar (Sigma) and overlay medium (2×DMEM (Gibco), sterile filtered and supplemented with 10% (v/v) FCS + 2mM L-glutamine) were prepared and pre-incubated in a waterbath at 46°C for 30 mins prior to use. Virus samples were gently removed from the wells, before the agar and overlay medium were mixed at a 1:1 ratio and 3 ml was added immediately to each well, pipetting slowly to avoid the formation of bubbles. After solidification, plates were incubated at 37°C for 96 hr. 500 µl 0.02% (v/v) Neutral Red solution (Sigma) was added to each well and plates were incubated for 1 hr before excess dye was removed. Plates were then placed back in the incubator for a further 3 hr. A light box was used to visualise and count the clear plaques where reovirus-induced L929 cell lysis had occurred. Virus concentration in each sample was calculated using the following formula:

$$\text{pfu/ml} = \frac{\text{average number of plaques} \times 2}{\text{dilution}}$$

and fold increase in viral titre was determined by comparison with levels of input virus.

3.3.7 Reovirus Activation of Cells

PBMC, LMNC, SW480 or SW620 cells were cultured at 2 x 10^6 cells/ml. 0 or 1pfu/cell reovirus was added and cells were incubated overnight at 37ºC. PBMC and LMNC were then used in either CD107 degranulation assays (section 3.4.7), chromium release assays (section 3.5), analysed for phenotypic changes (section 3.4.2) or culture supernatants were collected and analysed by ELISA (section 3.6 and section 3.7). Reovirus-treated colorectal cancer cell lines were analysed for phenotypic changes (section 3.4.1).

3.3.8 Reovirus Binding on PBMC

PBMC were cultured at 2 x 10^6 cells/ml and 0, 1 or 10pfu/cell reovirus was added before cells were incubated for 4 hr. Cells were then harvested and 20 ml phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK) added before cells were pelleted by centrifugation. After supernatant was discarded, this washing procedure was carried out twice more to remove

any unbound reovirus. Cells were stained for cell-surface expression of reovirus as described in section 3.4.3.

3.3.9 Assessment of Reovirus Hitch-hiking on PBMC and Hand off to Colorectal Cancer Cell Lines

SW480 and SW620 cells were seeded in 6-well plates at a density of $1x10^5$ cells in 2 ml 10% DMEM and incubated for 4 hrs to allow cells to adhere. PBMC were isolated before 5-$10x10^6$ were placed in Universals tubes (Sterilin Ltd., Caerphilly, UK). Cells were centrifuged, the resulting pellets resuspended in 1 ml of lymphocyte culture medium and treated with 0 or 1pfu/cell reovirus. After 4 hr incubation at 4^{o}C, any unbound virus was removed by 3 washes and centrifugations in 20 ml PBS. Cell pellets were resuspended in lymphocyte culture medium at $1x10^5$/ml and, once 10% DMEM had been removed from tumour targets, 1 ml of each PBMC condition was added to duplicate wells. Plates were incubated for 4 hr, rocking occasionally to ensure good coverage of PBMC over tumour targets. PBMC were then removed and wells were gently washed down with 1 ml PBS. 3 ml lymphocyte culture medium was added to each well and cells were incubated for 48-120 hr. Separate SW480 and SW620 targets were directly infected with 0.005pfu/cell reovirus (the dose equivalent to that with is hitch-hiked on PBMC) and cultured in 3 ml lymphocyte culture medium for 120 hr. After incubation, SW480 and SW620 cells were harvested for assessment of their viability by propidium iodide (PI) staining (section 3.4.4) or cells/supernatants were collected and stored at -80^{o}C until plaque assays were performed to determine their viral titre (section 3.3.6).

3.4 Flow Cytometry- Fluorescence-activated Cell Sorter (FACS)

All flow cytometry was performed using a FACSCalibur machine and data analysed using the CellQuest©Pro Software package (v4.0.1) (both Becton Dickinson, Oxford, UK).

3.4.1 Phenotypic Analysis of Colorectal Cancer Cell Lines, Primary Metastatic Tumour Cells and Primary Hepatocytes

Cells (see sections 3.3.7 and 3.2.3-4) were harvested and aliquoted into FACS tubes (BD Biosciences) at a density of 1×10^5-1×10^6 per tube. 2 ml FACS buffer (PBS + 1% (v/v) FCS and 0.1% (w/v) sodium azide) was added and cells were centrifuged at 400g for 5 mins at 4^{o}C in a Sorvall RT6000B refrigerated centrifuge (Kendro Lab Products, Hertfordshire, UK). After supernatants were discarded, fluorescently-conjugated antibodies were added (Table 3) and cells incubated for 30 mins at 4^{o}C in the dark. 2 ml FACS buffer was then added to each tube and cells pelleted by centrifugation. Cells were resuspended in 100-200 µl 1% (w/v) paraformaldehyde (PFA) (Sigma) and stored at 4^{o}C for up to 1 week prior to acquisition.

Table 3 FACS Antibodies Used for Colorectal Cancer Cell Lines, Primary Metastatic Tumour Cells and Primary Hepatocytes

Target Molecule	Fluorochrome	Volume Added per $5x10^5$ Cells	Species of Origin	Clone	Manufacturer
JAM-A	PE	3 µl	Mouse	43	Santa Cruz (Calne, UK)
MHC-I	PE	3 µl	Mouse	G46-2.6	BD Biosciences
ULBP-1	PE	3 µl	Mouse	170818	R & D Systems (Abingdon, UK)
ULBP-2	PE	3 µl	Mouse	165903	R & D Systems
ICAM-1	PE	3 µl	Mouse	3E2	BD Pharmingen
CD112	PE	3 µl	Mouse	R2.525	BD Pharmingen
MIC A/B	PE	3 µl	Mouse	6D4	BD Pharmingen
BerEp4	FITC	5 µl	Mouse	MCF7	Dako Cytomation (Stockport, UK)
CEA	FITC	5 µl	Mouse	B1.1	BD Pharmingen
IgG1 Isotype Mix	FITC, PE, PerCP	5 µl	Mouse	DAK-G01	Dako Cytomation

3.4.2 Phenotypic Analysis of PBMC/LMNC

The phenotype of NK cells within PBMC/LMNC and the JAM-A expression of PBMC sub-populations was determined using 3-colour flow cytometry. PBMC/LDMC (section 3.3.7) were harvested, stained and stored as described in the above section, using the antibodies detailed in Table 4.

Table 4 FACS Antibodies Used for PBMC/LMNC

Target Molecule	Fluorochrome	Volume Added per $5x10^5$ Cells	Species of Origin	Clone	Manufacturer
CD3	FITC	5 µl	Mouse	SK7	BD Biosciences
CD3	PerCP	3 µl	Mouse	SK7	BD Biosciences
CD4	PerCP	3 µl	Mouse	SK3	BD Biosciences
CD8	PerCP	3 µl	Mouse	SK1	BD Biosciences
CD8	PerCP	3 µl	Mouse	SK1	BD Biosciences
CD14	PerCP	3 µl	Mouse	MΦP9	BD Biosciences
CD16	FITC	3 µl	Mouse	3G8	BD Pharmingen
CD19	FITC	5µl	Mouse	4G7	BD Biosciences
CD56	FITC	5 µl	Mouse	NCAM 16.2	BD Biosciences
CD56	PE	2 µl	Mouse	C5.9	Serotec (Kidlington, UK)

CD69	FITC	10 µl	Mouse	FN50	BD Pharmingen
CCR7	PE	10 µl	Mouse	150503	R & D Systems
DNAM-1	PE	3 µl	Mouse	DX11	BD Pharmingen
JAM-A	PE	3 µl	Mouse	43	Santa Cruz
NKG2D	PE	3 µl	Mouse	ID11	BD Pharmingen
NKp30	PE	3 µl	Mouse	P30-15	BD Pharmingen
NKp44	PE	3 µl	Mouse	P44-8.1	BD Pharmingen
NKp46	PE	3 µl	Mouse	9E2	Miltenyi Biotec (Gladbach, Germany)
IgG2a, κ	PE	3 µl	Mouse	G155-178	BD Pharmingen
IgG2b, κ	PE	3 µl	Mouse	27-35	BD Pharmingen
IgG1 Isotype Mix	FITC, PE, PerCP	5 µl	Mouse	DAK-G01	Dako Cytomation

3.4.3 Cell-surface Binding of Reovirus on PBMC

Cell-surface binding of reovirus σ3 capsid protein on sub-populations of PBMC was determined by 3-colour flow cytometry, using both directly-conjugated and un-conjugated antibodies (Table 5). PBMC (see section 3.3.8) were harvested, washed in 2 ml FACS buffer

and pelleted by centrifugation. After supernatants were discarded, anti-reovirus σ3 antibody was added and cells incubated for 20 mins at 4ºC in the dark. Cells were then washed in 2 ml FACS buffer and pelleted by centrifugation. Supernatants were discarded and FITC-labelled secondary antibody was added for 20 mins at 4ºC in the dark. After a further wash and centrifugation in FACS buffer, supernatant was poured off and cells were subjected to a stain with directly conjugated antibodies (as described in section 3.4.1) to allow for identification of PBMC sub-populations. Cells were fixed in 1% (w/v) PFA and stored for up to 1 week before acquisition.

Table 5 FACS Antibodies Used to Determine Reovirus Binding on PBMC Sub-populations

Target Molecule	Fluorochrome	Volume Added per $5x10^5$ Cells	Species of Origin	Clone	Manufacturer
CD3	PE	3 µl	Mouse	SK7	BD Biosciences
CD3	PerCP	3 µl	Mouse	SK7	BD Biosciences
CD4	PerCP	3 µl	Mouse	SK3	BD Biosciences
CD8	PerCP	3 µl	Mouse	SK1	BD Biosciences
CD14	PerCP	3 µl	Mouse	MΦP9	BD Biosciences
CD19	PE	3 µl	Mouse	HIB19	BD Pharmingen
CD56	PE	2 µl	Mouse	C5.9	Serotec
Reovirus σ3	Un-conjugated	1 µl	Mouse	4F2	DSHB (University of Iowa, USA)
Ig (2^o Ab)	FITC	100 µl of 1:50 dilution	Goat	Polyclonal	BD Pharmingen
IgG1 Isotype Mix	FITC, PE, PerCP	5 µl	Mouse	DAK-G01	Dako Cytomation

3.4.4 Measurement of Cell Viability by PI Staining

Cells were harvested into FACS tubes, washed in 2 ml FACS buffer and pelleted by centrifugation. Supernatant was discarded and 0.05 mg/ml PI (Sigma) was added per tube. Cells were incubated at RT for 15 mins in the dark and analysed by flow cytometry immediately.

3.4.5 Measurement of Cell Viability Using the Live/Dead® Cell Viability Assay

Cells were harvested into FACS tubes, washed with 1 ml PBS and pelleted by centrifugation. Supernatant was discarded and cells were resuspended in 1 ml PBS containing 1µl of supplied PE-conjugated, fluorescently-activated dye. Cells were incubated for 30 mins at RT in the dark, before being washed with 1 ml PBS and pelleted by centrifugation. A further wash with 1 ml PBS/1% (v/v) FCS and centrifugation was performed before cells were fixed in 100-200 µl 1% (w/v) PFA. Cells were stored at 4°C for up to 1 week prior to acquisition.

3.4.6 Measurement of Apoptosis Using the Intracellular Active Caspase-3 Apoptosis Kit

Cells were harvested into FACS tubes, washed in 2 ml cold PBS and pelleted by centrifugation. Cells were resuspended in 500 µl supplied Cytofix/Cytoperm buffer™ and incubated on ice in the dark for 20 mins. After centrifugation and the removal of excess buffer, cells were subjected to a further 2 centrifugations in 500 µl supplied BD Perm/Wash™ buffer. 20 µl of anti-caspase-3 antibody was then added per tube and cells incubated at RT for 30 mins in the dark. 1 ml of supplied BD Perm/wash™ buffer was added and cells were pelleted by centrifugation. Supernatant was discarded before cells were resuspended in 500 µl of BD Perm/wash buffer™ and stored at 4°C for up to 1 week prior to acquisition.

3.4.7 CD107 Degranulation Assay

SW480 and SW620 tumour target cells and effector cells (PBMC/LMNC ±reovirus treatment; see section 3.3.7) were harvested, washed in PBS and pelleted by centrifugation. Cells were

then resuspended in lymphocyte culture medium before effector cells were added to FACS tubes either alone or with each tumour target at a 1:1 ratio, in a final volume of 400 µl. After 1 hr incubation, 5 µl each of anti-CD107a FITC and anti-CD107b FITC antibodies (both BD Biosciences) as well as 10 µg/ml Brefeldin A (Sigma) was added to each tube, before a further 4 hr of incubation. Cells were then washed with 2 ml FACS buffer and pelleted by centrifugation before being stained with anti-CD3 PerCp and anti-CD56 PE (section 3.4.1) for 30 mins at 4°C (to allow identification of NK cell populations). Cells were washed in 2 ml FACS buffer, centrifuged and fixed in 200 µl 1% (w/v) PFA. Cells were stored at 4^{o}C for a maximum of 1 week prior to acquisition.

3.5 ^{51}Chromium (^{51}Cr) Cytotoxicity Assay

SW480 and SW620 tumour target cells and effector cells (PBMC ± reovirus treatment; see section 3.3.7) were harvested, washed and pelleted by centrifugation. Target cells were labelled with 50 µCi ^{51}Cr (Perkin Elmer, Cambridgeshire, UK) per million cells and incubated for 1 hr. Excess ^{51}Cr was then removed by 3 centrifugations in 50 ml PBS. Cells were resuspended in lymphocyte culture medium at 1x10^{5} cells/ml. Effector cells were resuspended in lymphocyte culture medium at a concentration that would provide known effector to target (E:T) cell ratios. Serial dilutions of effectors cells were seeded out in triplicate into round-bottomed 96-well plates (Nunc Nalgene, Loughborough, UK) in a final volume of 100 µl per well, before 50 µl of ^{51}Cr-labelled target cells were added to each appropriate well. Maximum and spontaneous target release plates were set up as controls, consisting of either target cells alone or treated with 1% (v/v) Triton-X (Sigma). Following 4 hr of incubation, plates were centrifuged and 50 µl of supernatant from each well was transferred onto Luma scintillation plates (Perkin Elmer) before being left to dry at RT overnight. ^{51}Cr release in cell supernatants was measured in counts per minute (cpm), using a 1450 MicrobetaJet Scintillation Counter (Wallac EG & G Ltd., Milton Keynes, UK). Results were expressed as percent tumour cell lysis, using the formula:

% lysis = 100 x (sample cpm – spontaneous cpm) / (maximum cpm -spontaneous cpm).

For NK depletion experiments, effector PBMC were harvested from culture, washed in 50 ml MACS buffer (PBS + 2% (v/v) FCS + 2 mM EDTA) and pelleted by centrifugation. Cells were then treated with CD56 microbeads (Miltenyi) as per the manufacturer's instructions. Briefly, microbeads were added at 10 µl per million PBMC and incubated at 4°C for 15 mins. 50 ml of MACS buffer was then added and, following a centrifugation at 300g for 10 mins, cells were resuspended in MACS buffer and passed through a MACS LS separation column (Miltenyi) attached to a magnetic stand. CD56-depleted cells (those that passed freely through the column) were collected and used as effectors as described above.

3.6 Measurement of IL-28 and IFN-α Production by Enzyme-linked Immunosorbant Assay (ELISA)

Flat-bottomed 96-well Maxisorp® plates (Nunc) were coated with 100 µl optimised concentrations of capture antibodies, diluted in PBS. Plates were wrapped in foil and stored at 4°C overnight. Plates were then washed 3 times with PBS-T (PBS + 0.05% (v/v) Tween20 (Sigma)), using the SkanWasher300 (Molecular Devices, Berkshire, UK). 200 µl of blocking solution (PBS + 10% (v/v) FCS) was added to each well and plates left, wrapped in foil, for 2 hr at RT. Following a further 3 washes in PBS-T, 100 µl of recombinant protein standard (in serial dilution) and sample supernatants were added to the plates in triplicate. Plates were then wrapped in foil and stored at 4°C overnight, before being washed 6 times with PBS-T. Optimised concentrations of biotinylated detection antibodies, diluted in blocking solution, were added at 100 µl/well and plates were left, wrapped in foil, at RT for 2 hr. Plates were then washed 6 times with PBS-T before 100 µl Extravidin®-alkaline phosphatase conjugate (Sigma), diluted 1:5000 in PBS-T, was added per well. After 1 hr incubation at RT, the plates were washed 3 times with PBS-T and 3 times with double-distilled water. Sigmafast™ pNPP alkaline phosphatase substrate (Sigma) was prepared according to the manufacturer's instructions and added at 100 µl per well. Plates were allowed to develop in the dark for 10-30 mins. A Multiscan EX plate reader (Thermo Fisher Scientific, Surrey, UK) was used to

measure optical density at a wavelength of 405nm. Details of all ELISA antibodies and standards are given in Table 6.

Table 6 ELISA Antibodies and Protein Standards

Target Molecule	Role	Dilution / top standard concentration	Species of Origin	Manufacturer
IFN-α	Capture	1:180	Mouse	Mabtech AB (Buro, Germany)
IFN-α	Detection	1:180	Goat	Mabtech AB
IFN-α	Standard	5000 pg/ml	Human	Mabtech AB
IL-28	Capture	1:250	Mouse	R & D Systems
IL-28	Detection	1:1000	Mouse	R & D Systems
IL-28	Standard	8000 pg/ml	Human	R & D Systems

3.7 Measurement of IFN-β Production by ELISA

IFN-β production was measured using the *Verikine*™ Human IFN-β ELISA Kit (PBL InterferonSource, New Jersey, USA), following the manufacturer's instructions. All reagents were supplied with the kit and all incubations were performed at RT. Briefly, 50 µl of sample supernatants and serial dilutions of protein standards (at a top concentration of 4000 pg/ml) were added in triplicate to pre-coated 96 well plates. Plates were incubated for 1 hr then washed 3 times with Wash Buffer. Antibody Concentrate was diluted in Concentrate Diluent and 100 µl added per well before 1 hr incubation, followed by 3 washes in Wash Buffer. 100 µl Horseradish peroxidise (HRP), diluted in Concentrate Diluent, was added per well before

plates were incubated for 1 hr then washed a further 3 times with Wash Buffer. 100 µl TMB substrate was added per well and plates were allowed to develop in the dark. After 15 mins, 100 µl Stop Solution was added to each well and optical densities read at a wavelength of 405nm, using a Multiscan EX plate reader.

3.8 Statistical Analysis

p values were calculated using paired student's *t* test with 2-tailed distribution or 2-way ANOVA with Bonferroni *post hoc* testing. Statistical significance was taken as $p<0.05$.

4 Results

4.1 The Cytotoxic Effect of Direct Reovirus Infection on Human Colorectal Cancer Cell Lines

4.2 Aims

1. To assess the cell-surface expression of the reovirus receptor, JAM-A, on human colorectal cancer cell lines.

2. To examine the cytotoxic effect of direct reovirus infection on human colorectal cell lines *in vitro*.

3. To elucidate the mechanism of reovirus-induced cell death.

4. To determine the level of reovirus replication in human colorectal cell lines *in vitro*.

5. To demonstrate the effect of NAB on reovirus-induced cell death.

4.3 Introduction

As previously discussed, reovirus has proven cytotoxic effect against a wide range of tumour types and has been shown to have an acceptable safety profile in a number of Phase I/II studies. Whilst the initial focus on the importance of *ras* status has shifted in recent years, there is no doubt that in colorectal cancer mutant *ras* status promotes apoptosis in the context of reovirus oncolysis (Smakman *et al.,* 2006b). The finding that approximately 50% of human colorectal cancers display mutant *ras* status therefore makes reovirus a viable therapeutic option in the treatment of colorectal cancer. This chapter examines the direct, oncolytic potential of reovirus, using a number of human colorectal cell lines *in vitro*.

4.3.1 Cell-surface expression of JAM-A on human colorectal adenocarcinoma cell lines

Reovirus utilises JAM-A as a serotype-independent, cellular receptor and infection is initiated with the attachment of the σ1 protein. The membrane-distal Ig-like D1 domain of JAM-A is required for homodimerization and binding to reovirus attachment protein σ1. In order to assess the potential susceptibility of human colorectal cancer cells lines to reovirus infection, a panel of *ras* mutant human colorectal cancer cell lines was screened for cell-surface expression of JAM-A using flow cytometry.

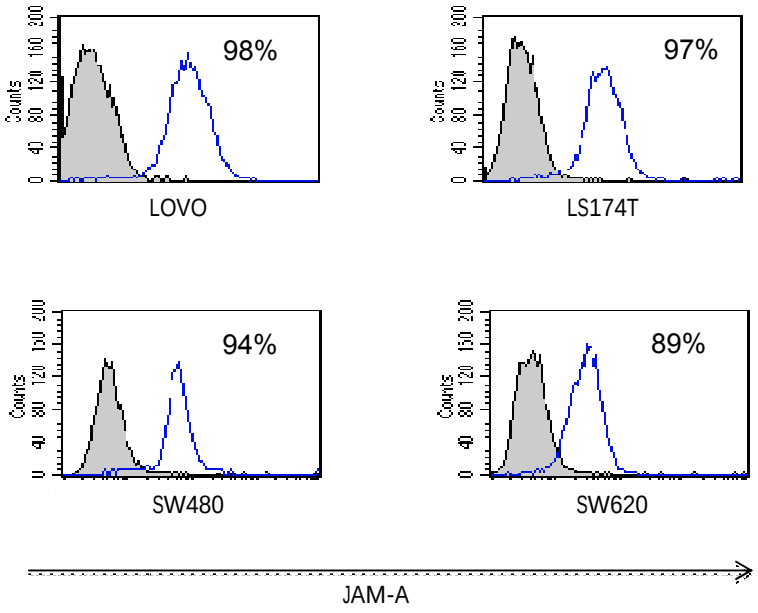


Figure 4-1 Expression of Cell-surface JAM-A on Human Colorectal Adenocarcinoma Cell Lines

LoVo, LS174T, SW480 and SW620 cells were harvested and stained with anti-human JAM-A PE-conjugated antibody. Cell-surface expression of JAM-A was determined by flow cytometry (blue line: JAM-A; shaded grey: isotype-matched control; numbers represent percentage of gated cells positive for JAM-A expression). Data is representative of 3 independent experiments.

Figure 4-1 shows representative FACS histogram plots showing the expression of cell-surface JAM-A on LoVo, LS174T, SW480 and SW620 human colorectal adenocarcinoma cell lines. All cell lines exhibited high levels of surface JAM-A expression. LoVo, LS174T and SW480 cells exhibited the greatest expression at 98%, 97% and 94% of cells, respectively.

4.3.2 Reovirus-induced Cytotoxicity in Human Colorectal Cancer Cell Lines

Having established that human colorectal cancer cell lines express JAM-A, the direct cytotoxic potential of reovirus was next assessed. LoVo, LS174T, SW480 and SW620 cell lines were directly infected with 0, 1 and 10pfu/cell reovirus for 24-72 hr before cell viability was determined using a Live/Dead® cell viability assay and flow cytometry.

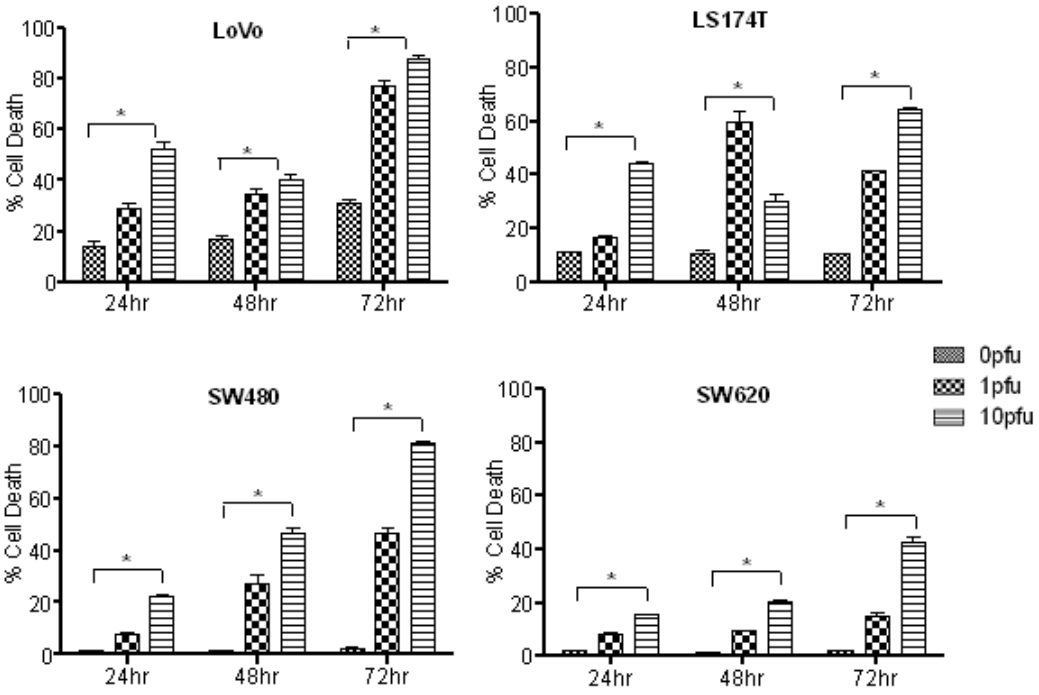


Figure 4-2 Reovirus-induced Cell Death in Colorectal Adenocarcinoma Cancer Cell Lines

LoVo, LS174T, SW480 and SW620 cells were treated with 0, 1 or 10pfu/cell for 24-72 hr. At each time point, cells were harvested and stained using the Live/Dead® cell viability assay. The percentage of cell death was then determined by flow cytometry. Graphs show mean percentage cell death + SEM of 3 separate experiments. Statistical significance is denoted by *$p<0.05$ (two-way ANOVA, Bonferroni *post hoc* test).

Figure 4-2 demonstrates that LoVo, LS174T, SW480 and SW620 colorectal cell lines were all susceptible to direct reovirus-mediated cell death. The level of death seen in each cell line was both time and dose dependent and reached statistically significant levels in all cases. LoVo and SW480 cells exhibited the greatest sensitivity, with the mean level of cell death reaching 87% and 81% respectively at the latest time points and highest dose of reovirus. The SW620 cell line exhibited the lowest level of sensitivity across all time points, with the mean level of death only reaching 43% at the latest time point and highest dose of virus.

Having established the direct cytotoxic potential of reovirus in colorectal cancer cell lines *in vitro,* the focus of further work centred upon the SW480 and SW620 lines. These cells

represent an *in vitro* model of both primary (SW480) and metastatic (SW620) colorectal cancer and were derived from the same patient, prior to any adjuvant therapy. SW480 cells were established from a resected primary large bowel cancer specimen and the SW620 line from an intra-abdominal lymph node metastasis resected six months later.

Figure 4-3 shows photographs taken of both cell lines during *in vitro* culture. The SW480 cells (Fig 4-3A) displayed an epithelial-like morphology and were larger in size than the SW620 cells (Fig 4-3B) which exhibited rounder, more spindle-like characteristics. The metastatic SW620 cells were also observed to grow at a much faster rate than the SW480 cells.

A **B**

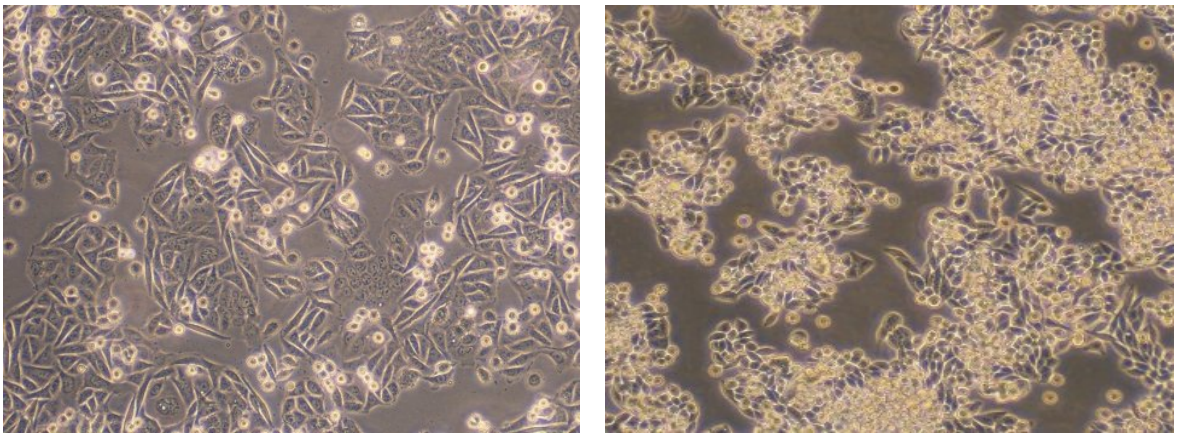


Figure 4-3 Photographs of SW480 and SW620 Human Colorectal Adenocarcinoma Cell Lines *in vitro*

SW480 (A) and SW620 (B) cell lines were cultured in DMEM supplemented with 10% FCS and routinely passaged at or near confluence. Cells were visualised using a C7070 Canon camera and light microscope at x10 magnification.

4.3.3 Reovirus Activates Caspase-3 in SW480 and SW620 Cell Lines

Reovirus has been shown to induce apoptotic cell death in a variety of human tumour cell types *in vitro* (reviewed by Clarke *et al.,* 2000). Reovirus-induced apoptosis involves the release of TRAIL from infected cells and the activation of TRAIL-associated death receptors (DR) 4 and 5. DR activation is followed by activation of caspase-8, cleavage of the Bcl-family protein Bid, and the subsequent release of pro-apoptotic mitochondrial factors (reviewed by Clarke, 2003). Caspases-8 and -9 also contribute to the activation of the effector caspases such as caspase-3. Caspase-3 acts on intracellular substrates and is essential for dismantling cellular structures and the formation of apoptotic bodies, the hallmark of apoptosis.

In order to confirm that apoptosis was the main mechanism of reovirus-induced cell death in colorectal cancer cells, SW480 and SW620 cells were cultured in the presence of 0 or 10pfu/cell reovirus for 72 hr. Cells were then harvested and the level of intracellular, active caspase-3 was measured using flow cytometry.

Fig 4-4 shows that reovirus infection induced significant levels of active caspase-3 production in SW480 and SW620 cell lines. A higher percentage of SW480 cells expressed intracellular caspase-3 after reovirus infection when compared to SW620 cells (60% vs 43% respectively). This is in keeping with the level of cell death observed in these cell lines upon infection with reovirus (Fig 4-2) and suggests that SW480 cells are more susceptible to reovirus-induced apoptosis than the metastatic SW620 line.

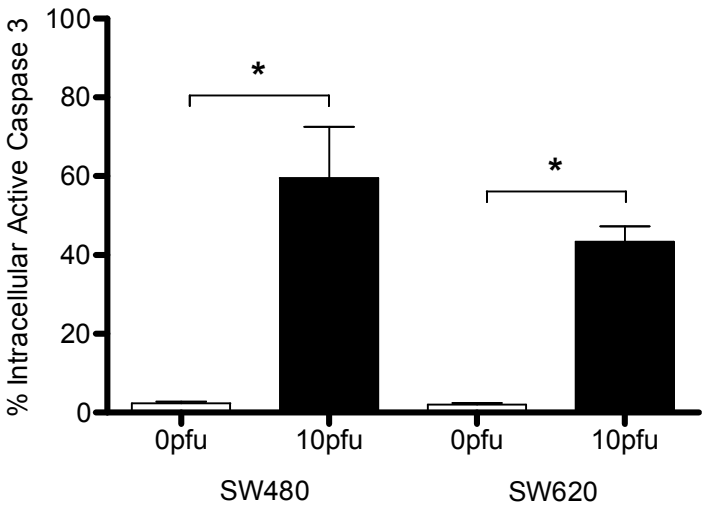


Figure 4-4 Intracellular Active Caspase-3 Production Following Direct Reovirus Infection of SW480 and SW620 Cells Lines

SW480 and SW620 cells were treated with 0 or 10pfu/cell for 72 hours. Cells were then harvested and stained using the PE-conjugated Active Caspse-3 Apoptosis Kit. The percentage of cells expressing intracellular caspase-3 was then determined using flow cytometry. Graph shows mean + SEM of 3 independent experiments. Statistical significance is denoted by *p<0.05 (two-way ANOVA, Bonferroni *post hoc* test).

To confirm the role of apoptosis in the reovirus-induced oncolysis of SW480 and SW620 cells, Z-VAD-FMK, a cell permeant, pan-caspase inhibitor which binds irreversibly to the catalytic site of caspase proteases and inhibits apoptosis induction, was added to the cell cultures prior to the addition of reovirus. The subsequent level of cell death was then determined using the Live/Dead® cell viability assay and flow cytometry. The results are shown in Fig 4-5.

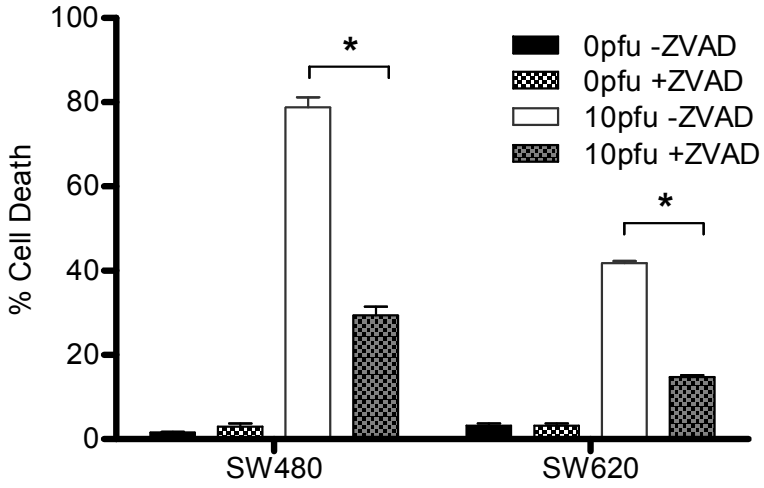


Figure 4-5 Effect of Z-VAD-FMK on Reovirus-induced SW480 and SW620 Cell Death

SW480 and SW620 cells were treated ± 50μM Z-VAD-FMK 1 hour prior to the addition 0 or 10pfu/cell reovirus. Cells were harvested at 72 hr and the level of cell death was assessed using the Live/Dead® cell viability assay and flow cytometry. Graph shows mean percentage cell death + SEM of 3 independent experiments. Statistical significance is denoted by *p<0.05 (two-way ANOVA, Bonferroni *post hoc* test).

The addition of ZVAD-FMK prior to infection with reovirus significantly decreased reovirus-induced cell death in SW480 and SW620 cultures and confirms the role of apoptosis in this process. The level of reduction in cell death induced by the addition of ZVAD-FMK to the cultures was more marked in the SW480 cell line (79% vs 29%) than in the SW620 cells (42% vs 15%).

4.3.4 SW480 and SW620 Cell Lines Support Reovirus Replication

Several *in vitro* studies have demonstrated reovirus' predilection for replication in *ras* activated cells. NIH3T3 cells are naturally resistant to reovirus infection; however, when transformed with the v-erb oncogene, a truncated, EGFR-lacking, ligand-binding, extracellular domain, containing a constitutively active tyrosine kinase cytoplasmic domain which activates the *ras* signalling pathway, they become highly permissive to reovirus infection (Strong *et al.,* 1998).

The mutation status of K*ras,* however, has been shown not to predict sensitivity to reovirus oncolysis in a panel of colorectal cell lines (Smakman et. al., 2006b). In that study, viral replication was observed in all cell lines tested regardless of K*ras* status and was not affected by the deletion of endogenous mutant K*ras*. Deletion did, however, affect reovirus-induced apoptosis, suggesting that oncolysis is not dependent upon viral replication but on tumour cells' susceptibility to apoptosis. *Ras*-transformed cells demonstrate increased susceptibility to Type 3 Dearing Strain reovirus via the inactivation of the dsRNA-activated protein kinase phosphorylation pathway (Coffey *et al.,* 1998; Strong *et al.,* 1998). Importantly in the context of this study, SW480 and SW620 were shown to express JAM-A (Fig 4-1) and are known to express a *ras* codon 12 mutation (Bos, 1989).

Having demonstrated that SW480 and SW620 cells expressed cell-surface JAM-A and were permissive to reovirus-induced cell death, their ability to support viral replication upon infection was investigated. SW480 and SW620 cells were infected with 1pfu/cell reovirus for 24-72 hr and standard plaque assays were performed using L929 cells.

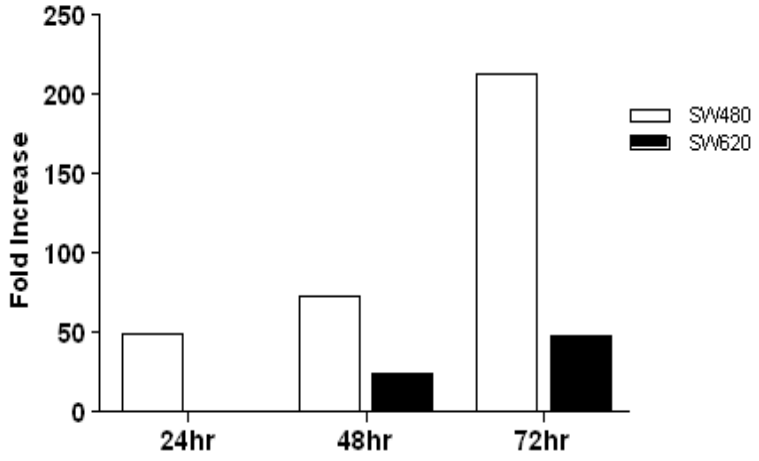


Figure 4-6 Replication of Reovirus in SW480 and SW620 Cells

SW480 and SW620 cells were treated with 1pfu/cell reovirus for 24-72 hr. At each time point, cells and supernatants were harvested, before lysates were prepared and the viral titre determined by plaque assay. The graph shows fold increase in reovirus titre compared to input level and is representative of 3 separate experiments.

Figure 4-6 demonstrates the fold increase in reovirus titre compared to the initial viral input dose for each cell line. SW480 and SW620 cells supported reovirus replication in a time dependent manner. Whereas SW480 cells permitted replication of the virus as early as 24 hr after infection, SW620 cells did not begin to promote replication until 48 hr. Moreover, the fold increase in viral titre was much greater in the SW480 cell line than the SW620 cells at each time point examined (213- fold increase vs 42-fold increase at 72 hr, respectively). This result is consistent with the levels of both cell death (Fig 4-2) and intracellular active caspase-3 production (Fig 4-3) in each cell line following direct infection with reovirus, with SW480 being more susceptible than SW620 cells.

4.3.5 Effect of Human Serum on Direct Reovirus-induced Cytotoxicity

Several studies have demonstrated the inhibitory effect of neutralising anti-reovirus antibody on oncolysis (White *et al.,* 2008). Blunting the effect of NAB using immunomodulatory agents such as cyclophosphamide has been shown to improve the efficacy of systemically delivered oncolytic viruses (Kottke *et al.,* 2009, Lun *et al.,* 2009). Whilst NAB certainly antagonises the oncolytic activity of reovirus, it has also been shown in murine models that complete abrogation of NAB may lead to significant toxicities, with viral replication occurring in normal tissues (Qiao *et al.,* 2008a).

To investigate the effect of NAB on the direct oncolytic potential of reovirus, SW480 and SW620 cells were treated with 0 or 10pfu/cell reovirus in the presence (human serum, HS) or absence (FCS) of NAB. Cell viability was then measured using the Live/Dead® cell viability assay and flow cytometry. The results are shown in Fig 4-7.

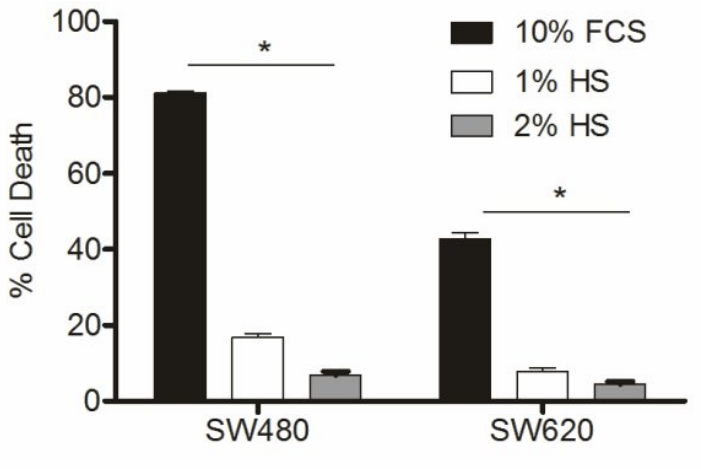


Figure 4-7 Effect of NAB on Reovirus-induced Cytotoxicity

SW480 and SW620 cell lines were cultured in the presence of either 10% FCS or 1- 2% HS. Reovirus was added at 0 or 10pfu/cell for 72 hours. Cells were then harvested and the level of cell death was measured using the Live/Dead® cell viability assay and flow cytometry. The graph shows the mean percentage of cell death + SEM of 3 independent experiments. Statistical significance is denoted by *p<0.05 (paired Student's t test).

The addition of NAB to the cultures significantly reduced the level of reovirus-induced cytotoxicity in both cell lines. In the SW480 cell line, the level of reovirus-induced cell death in the absence of NAB was 81%, whereas in the presence of 1% NAB-containing HS, the level of cell death reached only 17%. In the SW620 cell line, the presence of NAB in the cultures reduced the level of cell death from 43% to 8%. These findings highlight the deleterious effect of NAB on the cytotoxic potential of direct treatment with reovirus.

4.4 Discussion

This chapter examined the direct effects of clinical grade reovirus on the human colorectal cell lines, LoVo, LS174T, SW480 and SW620. These cell lines were all found to express JAM-A, the main reovirus receptor, on their cell-surface (Fig 4-1). In addition, all cell lines demonstrated susceptibility to infection and the levels of cell death observed were both time and dose dependent (Fig 4-2). The differential susceptibility of SW480 and SW620 to direct reovirus killing, as well as the differences in JAM-A expression between the two cell lines,

demonstrates not only the heterogeneity of the cell lines themselves, but also the differing tumour biology between primary and metastatic tumour cells.

It is well known that JAM-A plays an essential role in tight junction integrity (Mandell *et al.,* 2005). Disruption of tight junction integrity has been implicated in cancer cell metastasis by inducing epithelial mesenchymal transition (EMT) (Gumbiner *et al.,* 2000), and down-regulation of JAM-A in breast cancer has been shown to serve as a key negative regulator in cellular invasion and migration (Naik *et al.,* 2008). It is therefore possible that the lower level of JAM-A expression seen on the metastatic SW620 cell line (Fig 4-1) could have a deleterious effect on the level of reovirus binding and subsequent cellular entry.

Aberrant expression of cell-surface JAM-A by tumour cells is likely to represent a significant barrier to meaningful direct oncolysis. Whilst low level cellular entry can occur via sialic acid binding and there are reports of JAM-A-independent cellular entry by reovirus (Danthi *et al.,* 2006), it is the reovirus-JAM-A interaction which has proven the most efficient method of cellular entry. A further confounding factor is the mislocalisation of JAM-A on the surface of CRLM. It has been shown that both primary colorectal cancers and, to a greater extent CRLM, express JAM-A throughout the cell cytoplasm and not on the cell-surface. Moreover, the addition of reovirus to single cell suspensions and to small fragments of colorectal metastasis tissue did not result in any reduction in cell viability, suggesting a lack of oncolytic efficacy (van Houdt *et al.,* 2008).

The finding that reovirus infection of SW480 and SW620 cells promoted the activation of caspase-3 confirmed that the predominant mode of cellular death following infection was apoptosis (Fig 4-6). This correlates with previous reports that apoptosis is a critical mechanism for inducing cell death upon host infection (reviewed by Clarke *et al.,* 2003). More recently, it has been shown that caspase-3 gene deletion in mice not only resulted in reduced central nervous system toxicity but also enhanced survival rates in mice that had been delivered a potentially lethal intra-cerebral dose of reovirus (Beckham *et al.,* 2010).

Further evidence for the role of apoptosis in colorectal cancer cell line death following reovirus infection was seen by addition of the pan caspase blocker, ZVAD-FMK to *in vitro* cultures (Fig 4-7). Here, a significant reduction in the level of reovirus-induced cell death was observed. However, the addition of ZVAD-FMK did not completely abrogate cell death at 72 hr post-infection (Fig 4-7), suggesting that a longer time course is needed to see complete cell death in the cultures. This is in-keeping with previous findings (Smakman *et al.,* 2005).

It is well documented that activated *ras* signalling significantly enhances reovirus replication and spread. Both SW480 and SW620 cell lines express a *ras* mutation on codon 12 (Bos *et al.,* 1988). Whilst the levels of reovirus-induced cell death in the SW480 and SW620 cell lines correlated with their levels of reovirus replication, this cannot be explained by activated *ras* status alone. The differential level of replication between the two cell lines may in part be explained by the higher level of JAM-A expression seen on SW480 cells (Fig 4-1). It is likely, however, that the differing susceptibility is due to a number of complex molecular changes which have occurred as part of the process of developing metastatic capability.

As Fig 4-7 demonstrates, the presence of very low levels of NAB-containing HS led to significant blockade of direct reovirus-induced cytotoxicity. This deleterious effect of NAB on direct oncolysis by reovirus has led to concerns over the clinical efficacy of systemically administered reovirus. Given that the level of HS in these cultures was significantly less than the phyisiological level (~30%), concerns regarding the lack of anti-tumour effect due to viral neutralisation would seem valid. In contrast, however, several studies have demonstrated the presence of virus in tumour tissue following *i.v.* administration (Vidal *et al.,* 2008; Comins *et al.,* 2010). Furthermore, in the REO 013 Phase I clinical trial of patients undergoing *i.v.* infusion of Reolysin® prior to curative resection of CRLM, viral factories within tumour were identified and replicating virus was recovered from tumour tissue despite pre-existing anti-viral immunity (Adair *et al.,* 2012b).

These findings raise interesting questions about how the virus might evade NAB in the circulation after systemic delivery. Recently, purified populations of *ex-vivo*-loaded, human DC and T cells have been shown to be capable of delivering reovirus to Mel-888 melanoma targets for effective killing in the absence of human serum (Ilett *et al.,* 2009). Only DC, however, acted as efficient cell carriers in the presence of NAB but with lower efficacy than in the absence of HS. Viral carriage on a clinically relevant population of immune cells provides one interesting mechanism by which reovirus may evade the deleterious effects of NAB in the systemic circulation, to be delivered to tumour targets *in vivo* and forms the basis of the work shown in the following chapter.

5 Results

5.1 Cellular Carriage of Reovirus by Immune Cells, Protection from NAB and 'Hand-off' to Colorectal Tumour Cell Targets

5.2 Aims

1. To assess cell-surface expression of the reovirus receptor, JAM-A, on human PBMC.

2. To examine the level of reovirus cell-surface binding on human PBMC in the presence of NAB.

3. To determine the ability of human PBMC to support reovirus replication.

4. To demonstrate the ability of PBMC to 'hitch-hike' and hand-off reovirus to SW480 and SW620 cell line targets.

5. To assess the replication competence of reovirus which has been hitch-hiked on PBMC and handed off to SW480 and SW620 cell line targets.

5.3 Introduction

The use of cell carriers with inherent tumour-trafficking properties appears an attractive strategy in enhancing viral delivery. Immune cells such as antigen-specific T cells and cytokine-induced killer cells have been used for this purpose (Cole *et al.,* 2005; Thorne *et al.,* 2006). As well as this, reovirus-loaded mDC and T cells can be used to deliver reovirus to melanoma in murine models (Ilett *et al.,* 2009). Furthermore, DC internalise reovirus in the presence of NAB and can subsequently deliver the virus to melanoma targets *in vitro*. Moreover, these reovirus-loaded DC remain capable of both phagocytosis and T cell priming (Ilett *et al.,* 2011).

Whilst the most clinically pragmatic mode of delivery of reovirus to patients remains the systemic, *i.v.* route, factors such as complement proteins, antibodies and immune scavenger cells have been shown to eliminate viral particles almost instantly upon systemic administration of virus. Within two minutes of *i.v.* injection into an immuno-competent mouse, VSV has been shown to be almost entirely cell-associated (Willmon *et al.,* 2009a). Whilst much of this cellular uptake is likely to be by cells equipped for viral clearance, opportunistic adhesion to the surface of cells may occur with subsequent viral dissociation at distant sites (Cole *et al.,* 2005).

5.3.1 Cell-surface Expression of JAM-A on Human PBMC

To establish the potential of immune cells within whole PBMC to bind and carry reovirus, PBMC were isolated from the peripheral blood of healthy donors and the level of cell-surface JAM-A expression in the constituent cell populations was measured by flow cytometry.

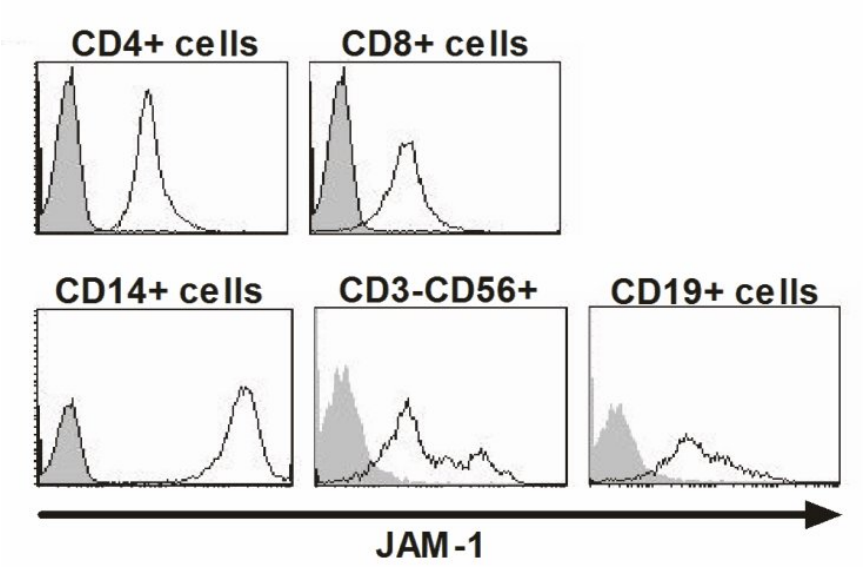


Figure 5-1 Cell-surface Expression of JAM-A on Human PBMC

PBMC were isolated from healthy donor peripheral blood. Cells were stained with anti-human: CD3, CD8, CD4, CD56, CD14 and CD19 antibodies in the presence of JAM-A antibody. The cell-surface expression of JAM-A within each sub-population was then measured using flow cytometry (black line: JAM-A; shaded grey: isotype-matched control). Data is representative of 3 independent experiments.

Figure 5-1 demonstrates that the main subpopulations which make up human PBMC express cell-surface JAM-A. High levels of expression (95-100%) were seen on the cell-surface of CD8 and CD4+ T cells, B cells as well as NK cells. Monocytes ($CD14^+$) demonstrated 100% cell-surface expression of JAM-A. This highlights the potential for human PBMC to bind reovirus to their cell-surface.

5.3.2 Cell-surface Binding of Reovirus on PBMC

Having demonstrated high levels of JAM-A expression on the surface of several PBMC sub-populations, suggesting a potential pathway for viral binding and carriage, the ability of these cells to bind reovirus to their surface was then assessed. An antibody specific to the reovirus capsid protein σ3 was used to detect reovirus binding to the surface of PBMC sub-populations *in vitro* using flow cytometry. Cells were cultured in the presence of reovirus and HS (to ensure the presence of NAB and mimic the situation *in vivo* in patients), for 4 hr.

Having washed off unbound virus, anti σ3 antibody was then added. Reovirus binding to each PBMC sub-population was then determined using flow cytometry.

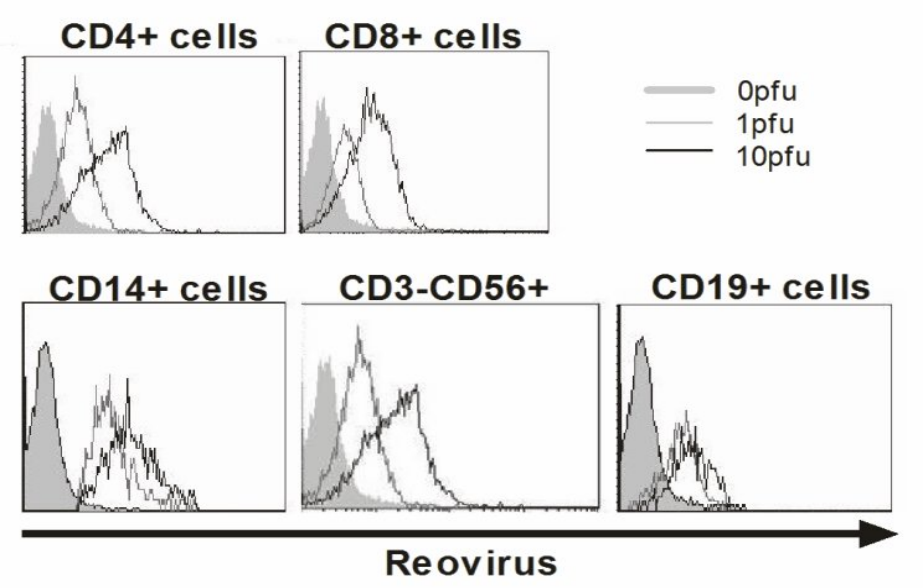


Figure 5-2 Cell-surface Binding of Reovirus on Human PBMC

PBMC were isolated from healthy donor blood and treated with 0, 1 or 10pfu/cell reovirus for 4 hr in the presence of HS. Excess virus was then washed off and the cells were stained for cell-surface binding of reovirus σ3 within the $CD4^+$, $CD8^+$, $CD3^-CD56^+$, $CD14^+$ and $CD19^+$ PBMC sub-populations. σ3 positive cells within each sub-population were then determined using flow cytometry (black line: 10pfu/cell; grey line: 1pfu/cell; shaded grey: 0pfu/cell). Data is representative of 3 independent experiments.

Figure 5-2 demonstrates that the major cellular constituents of human PBMC can all bind reovirus to their cell-surface in the presence of NAB and that virus is detectable by flow cytometry after 4 hr of culture. With the exception of B cells, all other sub-populations exhibited increased levels of surface binding with the higher dose of virus.

5.3.3 Replication of Reovirus in Human PBMC

Having established that PBMC not only expressed cell-surface JAM-A, but also that reovirus can bind to their cell-surface, the potential of PBMC to support reovirus replication and therefore act as viral amplification factories, was investigated. PBMC were isolated from healthy volunteers, treated with reovirus and cultured in presence of NAB for 24-72 hr. Plaque assays using L929 cells were then performed to quantify viral titre.

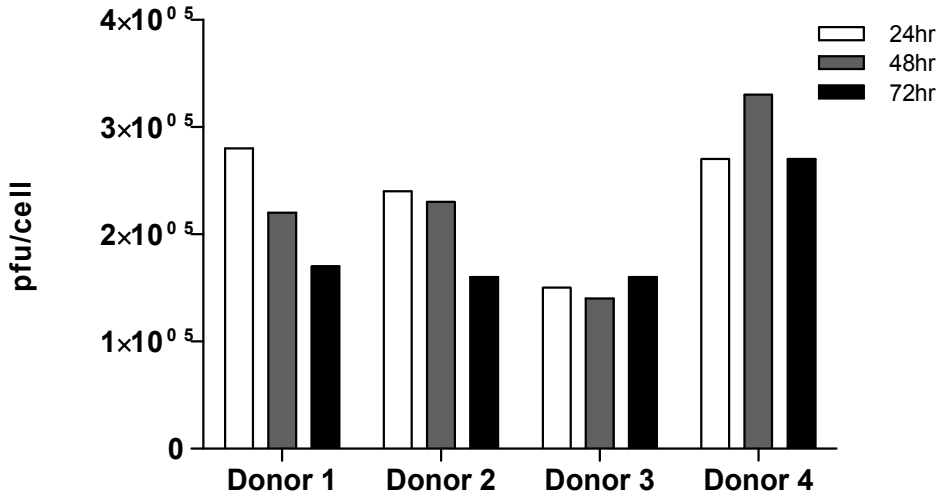


Figure 5-3 Replication of Reovirus in Human PBMC

PBMC were isolated from the peripheral blood of healthy donors. $2x10^5$ cells were treated with 1pfu/cell reovirus for a total of 72 hr in the presence of HS. At each time point, cells and supernatants were harvested and lysates were made. Viral titre was determined using standard plaque assay.

As $2x10^5$ PBMC were treated with 1pfu/cell reovirus, Figure 5-3 demonstrates that whole PBMC do not support reovirus replication. A reduction in the overall viral yield was demonstrated in 2 of the 4 donors and no net increase in yield was demonstrated in the remaining 2. Although the levels of virus yielded from donor 4 were slightly higher than the initial treatment dose, it is likely that this represents a margin of error in the initial stock virus titre or the plaque assay, rather than true replication. It is also important to note that reovirus did not exhibit cytotoxicity towards PBMC at a dose of 5pfu/cell (data not shown).

5.3.4 Human PBMC can Hitch-hike and Hand-off Reovirus to SW480 and SW620 Cell Targets in the Presence of NAB

Three critical, sequential phases are required for successful carrier cell-based delivery of oncolytic viruses: *ex vivo* loading, stealth delivery and virus production at the tumour site (reviewed by Power *et al.,* 2008). In the first of these phases, carrier cells are exposed to virus for a sufficient time to allow viral binding to occur. Following this, the carrier cells must pass through the systemic circulation avoiding destruction by complement proteins, phagocytes or cytotoxic cells. The third phase involves viral delivery to the tumour and the exact mechanism of how this process occurs is dependent upon the type of carrier cells used. By using carriers which permit viral replication, delivery of high numbers of virions due to viral amplification may be possible. Having established that PBMC expressed the main reovirus receptor, JAM-A and that reovirus could bind to the cell-surface of the sub-populations making up human PBMC in the presence of HS, the potential for PBMC to carry reovirus and deliver it to tumour cell targets *in vitro* was investigated.

To this end, PBMC were loaded with 0 or 1pfu/cell reovirus for 4 hr in the presence of HS (i.e. NAB). Following this, the cells were washed in PBS to remove any unbound virus and then co-cultured with adherent SW480 and SW620 cell targets. After 4 hr, PBMC were removed and the targets cultured for 48-120 hr. Concomitantly, separate targets cells were pulsed directly with 0.005pfu/cell reovirus (this dose represented the equivalent 'direct' virus dose to that retained and hitch-hiked on PBMC, as determined by plaque assay (data not shown). All cultures were performed in the presence of neutralising HS. The level of tumour target cell death was then measured using PI staining and flow cytometry.

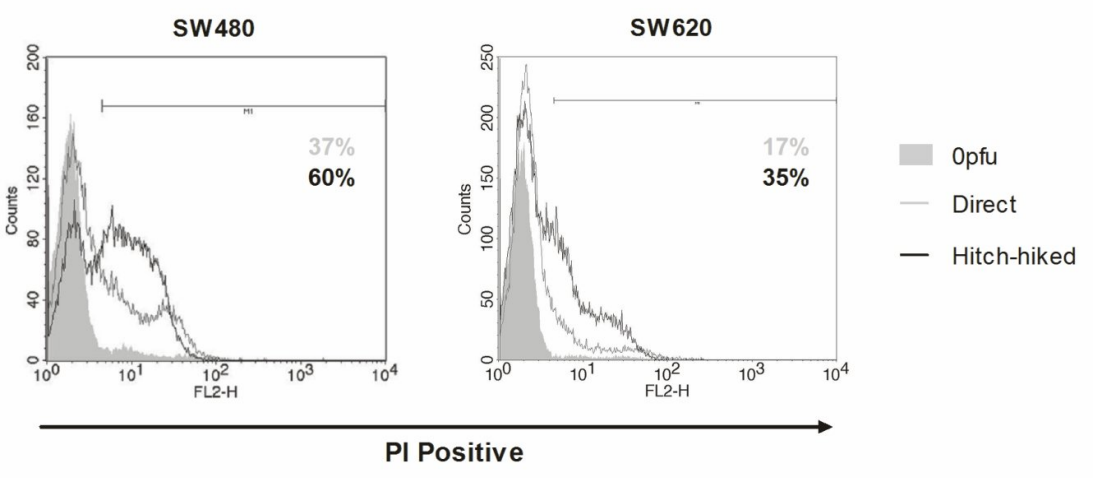


Figure 5-4 Death of SW480 and SW620 Cells After Virus Hitch-hiking and Hand-off by PBMC

PBMC were isolated from healthy donor peripheral blood. Cells were treated with 0, or 1 pfu/cellreovirus for 4 hr in the presence of HS. Cells were then washed to remove unbound virus and co-cultured with SW480 or SW620 targets for a further 4 hr. PBMC were then removed from the cultures before targets were incubated for 120 hr. All cultures were performed in the presence of NAB-containing HS.Killing by an equivalent dose of 'direct' virus under these conditions was also tested. The percentage of target cell death was measured by PI staining and flow cytometry. Histograms are representative of 5 donors.

Figure 5-4 demonstrates the ability of human PBMC to hitch-hike reovirus on their cell-surface in the presence of NAB. Furthermore, these cells were able to hand-off the virus to both SW480 and SW620 targets. Moreover, reovirus that had been handed-off to tumour target cells remained cytotoxic, as determined by PI positivity of SW480 and SW620 cells after culture. Interestingly, the level of PI positive tumour targets after hitch-hiking was found to be greater in both cell lines than the level seen upon direct infection with an equivalent direct dose of reovirus in the presence of NAB (60% vs 37% in SW480 and 35% vs 17% in SW620 cells).

5.3.5 Hitch-hiked Virus Remains Replication-competent

This study has demonstrated that PBMC can bind reovirus to their cell-surface in the presence of neutralising anti-reovirus antibodies. As well as this, they have been shown to deliver cytotoxic virus to SW480 and SW620 colorectal cancer cell line targets. Moreover,

viral carriage by PBMC resulted in higher levels of anti-tumour efficacy than the equivalent direct dose of the virus.

In order for reovirus-induced apoptosis to occur, binding of σ1 to both JAM-A and sialic acid must occur (Connolly *et al*., 2002) and reovirus oncolysis is dependent upon proteolytic disassembly of the outer capsid to create ISVP (Alain *et al.,* 2007). In order to confirm that viral carriage on PBMC had not altered the ability of reovirus to undergo disassembly or to bind to tumour cell-surface JAM-A, we aimed to assess the replication competence of reovirus which had been handed off to tumour cell targets. Plaque assays were therefore undertaken to determine viral replication in SW480 and SW620 tumour cell line targets which had received virus via the hitch-hiking process.

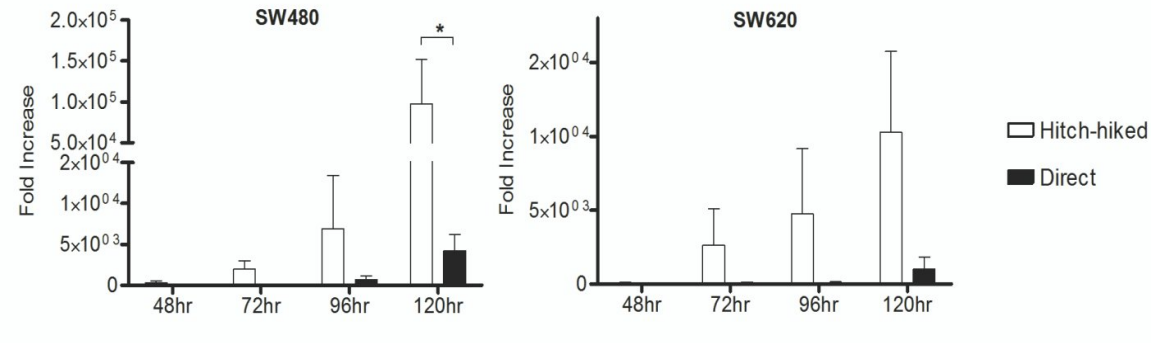


Figure 5-5 Reovirus Replication in SW480 and SW620 Cells Following Hitch-hiking and Direct Reovirus Infection

Cells and supernatants from SW480 and SW620 culutres treated as in Fig 5-4 were collected at 48-120 hr post viral hand-off and stored at -80^{o}C until required. Lysates were then prepared, before the viral titre in each sample was assessed by standard plaque assay. Graphs show mean fold increase in virus titre compared to input dose + SEM of 3 separate experiments. Statistical significance is denoted by *$p<0.05$ (paired Student's t test).

Reovirus which has been hitch-hiked by PBMC in the presence of HS and has been handed off to colorectal cell line targets retains the ability to replicate, as demonstrated in Fig 5-5. Higher levels of replication were seen in SW480 cells than in SW620 cells following hand-off, which correlates with the pattern of replication seen in Fig 4-6. Moreover, viral hand-off by

PBMC facilitated significantly higher levels of replication in SW480 cells than 'direct' virus at 120 hr, with a maximum fold increase of 100,000, compared to just 5000 in the directly infected cells. Whilst statistical significance was not reached in the SW620 samples, a maximum fold increase of 10,000 was demonstrated in the hitch-hiked conditions, compared to a 1000-fold increase in the directly infected cultures.

5.4 Discussion

One of the first descriptions of cell-based carriage for oncolytic viral therapy utilised human teratocarcinoma PA-1 as carrier cells for the *i.p.* delivery of a replication competent HSV in a murine, xenograft, ovarian cancer model (Coukos *et al.,* 1999). Here, carrier cells successfully delivered the virus and resulted in a reduction in tumour burden with larger areas of tumour infection evident on immunohistochemistry than those treated with direct virus. Further evidence for the use of immune cells as carriers to deliver virus to tumour targets was demonstrated using syngeneic C26 murine colorectal cancer cells infected with VSV. In this study, anti-tumour efficacy against established lung metastases was observed despite the presence of NAB (Power *et al.,* 2007).

Several differing approaches, aimed at protecting viral particles from neutralisation by IgG within the systemic circulation and ensuring tumour delivery, have been investigated. These have included techniques such as viral coat modification with lipid encapsulation and polymer coating (Kangasniemi *et al.,* 2011; Fisher *et al.,* 2007; Engelmann *et al.,* 1999). Other approaches have included serotype switching to evade specific antibody neutralisation (reviewed by Bangari *et al.,* 2006). Pre-clinical studies have shown that carriage by purified T cells, (Qiao *et al.,* 2008a) monocytes, and endothelial cells (Iankov *et al.,* 2007) can prevent viral elimination. These cells may simply act as carriers and deliver virus to the target tumour in a process known as hitch-hiking, or the virus may actively replicate within the cell, facilitating amplified viral delivery. Cellular migration within the tumour environment can also improve micro-distribution after direct delivery, thereby improving bio-distribution (Power *et*

al., 2007). Utilising clinically relevant, circulating immune cells as viral carriers or "Trojan horses" in the context of systemic delivery, however, has not been examined in detail.

The main constituents of human PBMC were found to express JAM-A (Fig 5-1), highlighting their potential to bind reovirus on their cell-surface. Indeed, when these PBMC sub-populations were stained for expression of the reovirus capsid protein σ3, they were all found to be capable of binding reovirus, even in the presence of NAB (Fig 5-2). Although PBMC did not support reovirus replication (Fig 5-3) and could not therefore act as viral replication factories, there was no evidence of reovirus-induced cytotoxicity towards PBMC (data not shown).

As demonstrated in Fig 5-4, PBMC were capable of hitch-hiking and handing-off reovirus to SW480 and SW620 cell targets in the presence of NAB. Furthermore, the level of tumour target death following this process was greater than that seen in the equivalent to hitch-hiked dose of directly added virus. This implies that the process of viral hitch-hiking on, and hand-off by, PBMC affords a protective effect against NAB in human serum. This finding has important clinical implications. As mentioned previously, several studies have demonstrated reduced oncolytic efficacy upon systemic administration of virus because of circulating NAB. Whilst techniques to abrogate this anti-viral immune response have been reported (Kottke *et al.,* 2009), this is the first account of a physiologically relevant population of immune cells acting as potential chaperones for reovirus to evade the anti-viral immune response in the context of systemic delivery.

The substantially higher levels of reovirus replication in both tumour cell targets observed following hitch-hiking (Fig 5-5) compared with an equivalent directly infected dose, highlights a further potentially useful clinical characteristic of immune cell carriage of reovirus. Although the MTD has not been reached in any of the clinical trials using Reolysin® to date, this finding implies that giving even low systemic doses may result in substantial anti-tumour efficacy following hand-off by autologous immune cells. In the clinical setting, data from the

recently completed REO 013 trial have shown that PBMC, granulocytes and platelets, though not red blood cells, collected from treated, NAB-positive patients, effectively hitch-hiked and protected reovirus for hand-off and killing of target cells *ex vivo*. Furthermore, in 4 of 4 patients tested, replication-competent reovirus was recovered from tumour samples (but not normal liver) and evidence of viral factories was observed in the metastases, implying that, even in the presence of NAB, virus can be delivered to tumour sites in patients (Adair *et al.,* 2012b).

6 Results

6.1 The Effects of Reovirus on Innate Immune Cells within PBMC in the Presence of Neutralising Human Serum

6.2 Aims

1. To assess the cytokine/chemokine response of reovirus-treated PBMC.

2. To examine the effects of reovirus on the activation status of NK cells within whole PBMC.

3. To investigate the level of NK ligand expression on SW480 and SW620 colorectal cell lines.

6.3 Introduction

It has been shown that reovirus infection activates isolated DC with the production of IFN-α, TNF-α, IL-12p70, and IL-6 (Errington *et al.,* 2008b). Interactions between purified reovirus-activated DC and autologous NK cells have been shown to induce the production of IFN-γ upon co-culture with PBMC in a cell-to-cell contact-dependent manner (Prestwich *et al.,* 2009b). Furthermore, melanoma-loaded DC, cultured in reovirus-infected tumour cell-conditioned media, primed an adaptive anti-tumour immune response (Steele *et al.,* 2011). These findings suggest that reovirus-activated DC can trigger innate immune cell activation and that the pro-inflammatory cytokines and chemokines produced in response can recruit adaptive immune effectors to the tumour microenvironment.

NK cells are an important cell population in the development of an anti-tumour immune response. The ability of isolated NK cells to produce IFN-γ upon contact with reovirus-activated DC demonstrates the importance of cellular cross-talk. Moreover, the ability to activate NK cells by the administration of reovirus to provoke innate immune cell killing of tumour cells may have significant clinical benefits. For this reason, the focus of further study centres on the NK cell response to reovirus treatment of whole PBMC in the presence of HS.

NK cells express an array of receptors that modulate their cytotoxicity against tumours and infected cells. These include NK-specific receptors known as natural cytotoxicity receptors (NCRs). NKp30, NKp44 and NKp46 make up the NCRs and these are complemented by other receptors, including NKG2D and the cell-surface receptor DNAM-1, for inducing NK cell activation. DNAM-1 is expressed on the surface of healthy cells and has been implicated in the recognition of target cells by cytotoxic cells (Shibuya *et al.,* 1996). Two ligands for DNAM-1 are known: nectin 2 (CD112) and nectin-like molecule 5 (CD155), which is also the receptor for poliovirus (Bottino *et al.,* 2003; reviewed by Fuchs *et al.,* 2006). DNAM-1 has been shown to regulate NK cell-mediated killing of a number of tumour types, including myeloid and lymphoblastic leukaemia (Pende *et al.,* 2005), ovarian carcinoma (Carlsten *et al.,* 2007) and myeloma (El-Sherbiny *et al.,* 2007).

NKG2D is expressed on virtually all NK cells and binds to a number of cell-surface ligands, including MICA/B, ULBP 1-5 and isoform proteins (Bauer *et al.,* 1999; Cosman *et al.,* 2001). As the most prominent activating receptor, it mediates immune responses that are important in NK-mediated surveillance against cancer and infection (reviewed by Raulet *et al.,* 2003).

CD69 is a glycoprotein, membrane receptor which is transiently expressed on the activation of all bone marrow derived cells with the exception of erythrocytes (reviewed by Testi *et al.,* 1994). It is not usually detected on resting lymphocytes, and is selectively expressed in chronic inflammatory infiltrates and at the sites of active immune responses *in vivo* (Sancho *et al.,* 2005). Once activated, NK cells rapidly express CD69 (Borrego *et al.,* 1993) and its importance in NK cell-mediated cytotoxicity has been demonstrated (Moretta *et al.,* 1991). As well as this, CD69 regulates NK cell proliferation, TNF-α production and the expression of other, functionally relevant, activation molecules, including CD25 and intracellular adhesion molecule-1 (ICAM-1) (Borrego *et al.,* 1999). More recenlty, CD69 has also been shown to mediate TGF-β production and to down-regulate auto-immunity (Sancho *et al.,* 2003).

A small subset of NK cells- $CD56^{bright}$ $CD16^{-}$ KIR^{-} $NKG2A^{+}$- express CCR7, which plays a pivotal role in the migration of NK cells to lymph nodes and participates in their proliferation and activation (Robertson *et al.,* 2000). NK cells are largely excluded from lymph nodes unless those nodes are undergoing an immune response. They may, however, acquire CCR7 from other CCR7-expressing cells via cell to cell contact and subsequent CCR7 receptor uptake from them. This process is regulated by the interaction of MHC-I and NK inhibitory ligands and occurs prior to cytolysis (Marcenaro *et al.,* 2009).

Having demonstrated that reovirus can be carried on immune cells in the presence of HS and that those carrier cells could deliver the virus to colorectal cancer cell line targets which support replication *in vitro*, the effect of reovirus infection on the innate immune cells within whole PBMC was next examined.

6.3.1 Cytokine and Chemokine Release by Reovirus-infected PBMC

Several studies have implicated a role for Type I IFNs in the activation of NK cells; however the exact mechanism for this remains unclear (Martinez *et al.,* 2008; Zhu *et al.,* 2008). To examine the secretion of IFN-α and IFN-β, supernatants from PBMC cultured with or without 1pfu/cell reovirus in the presence of HS were analysed by ELISA and Luminex®.

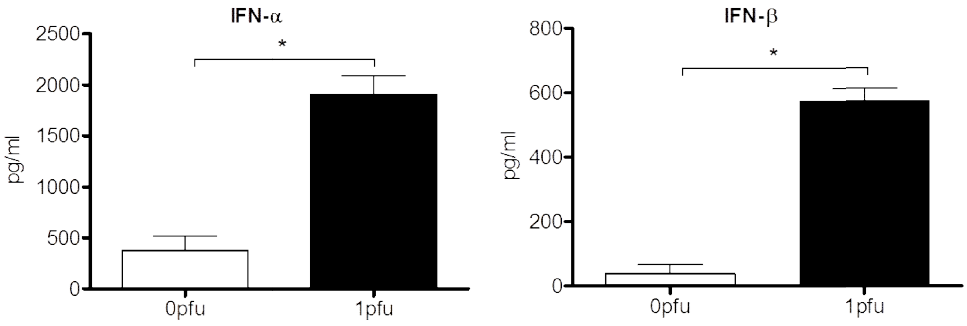


Figure 6-1 Type I Interferon Release from Reovirus-treated PBMC

PBMC were isolated from healthy donor peripheral blood. Cells were treated with 0 or 1 pfu/cell reovirus and cultured overnight in the presence of HS. Supernatants were collected and analysed by ELISA for the presence of IFN-α and IFN-β. Graphs show mean cytokine concentration + SEM of 3 separate donors. Statistical significance is denoted by *$p<0.05$ (paired Student's t test).

Figure 6-1 demonstrates the production of IFN-α and IFN-β by PBMC upon overnight culture with 1pfu/cell reovirus. Significant levels of both IFN-α (375pg/ml vs 1905pg/ml) and IFN-β (38pg/ml *vs* 574pg/ml) production were seen in response to reovirus treatment in the presence of NAB.

The Type III IFN, IL-28, plays a key role in the immune response to viral infection by inducing the production of proteins essential for innate anti-viral immunity (Kempuraj *et al.,* 2004). However, IL-28 is also associated with NK cell expansion and when given in combination with IL-12, significantly increased the production of IFN-γ and the overall anti-tumour effect in a murine fibrosarcoma model (Numasaki *et al.,* 2007). Moreover, IL-28 induction by

oncolytic VSV sensitised B16ova melanoma cells to NK killing *in vivo* (Wongthida *et al.,* 2010).

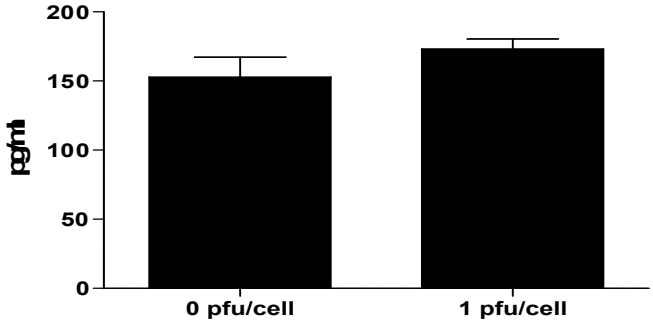


Figure 6-2 IL-28 Release from Reovirus-treated PBMC

PBMC were isolated from the peripheral blood of healthy donors. Cells were teated with 0 or 1pfu/cell reovirus and cultured overnight in the presence of HS. Supernatants were collected and analysed by ELISA for the presence of IL- 28. Graph shows mean concentration of cytokine + SEM for 3 separate donors.

Figure 6-2 demonstrates that IL-28 was not induced from PBMC in response to overnight treatment with 1pfu/cell reovirus. Moreover, the type II IFN, IFN-γ, was also not produced by reovirus-treated PBMC, in keeping with previous reports (Prestwich *et al.,* 2009b). As well as this, no significant increase was seen in the levels of the pro-inflammatory cytokines/chemokines MIP-1α, MIP-1β, RANTES or TNF-α (data not shown).

6.3.2 Activation of NK Cells within PBMC by Reovirus

In order to quantify the effect of reovirus on NK cell activation receptors and molecules, PBMC were infected with 0 or 1pfu/cell reovirus overnight and the cell-surface expression of each molecule on CD3- CD56+ NK cells was assessed by flow cytometry.

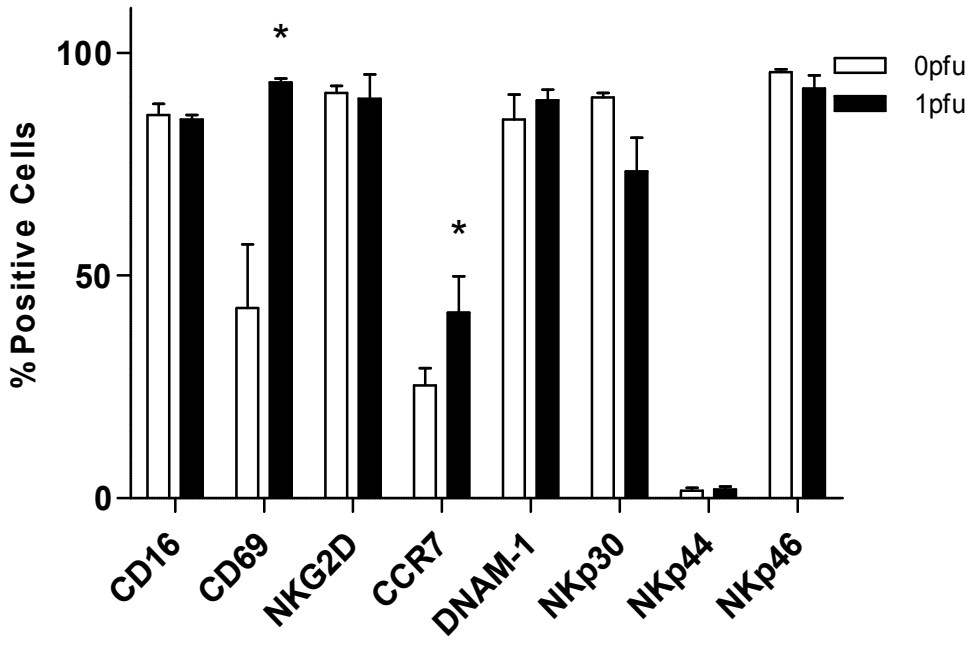


Figure 6-3 Expression of NK Cell-surface Activation Markers on Reovirus-activated PBMC

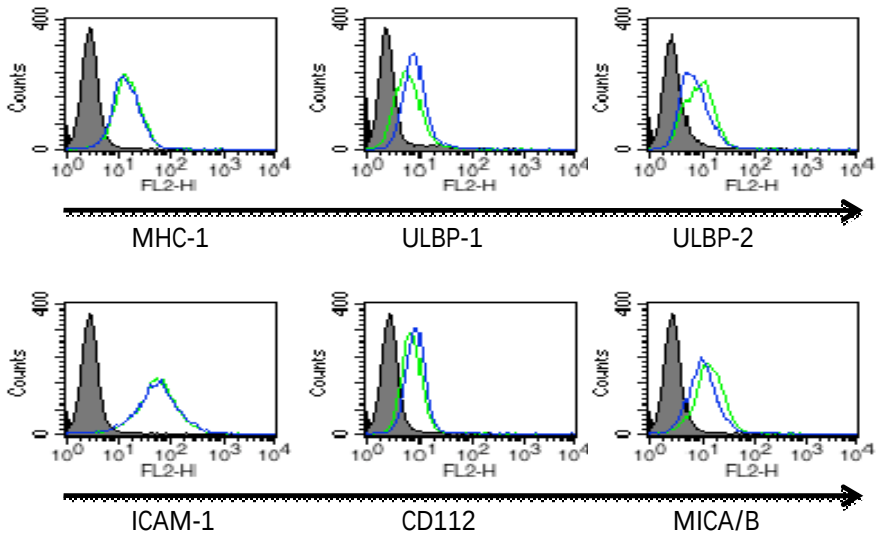
PBMC were isolated from healthy donor peripheral blood. Cells were treated with 0 or 1pfu/cell reovirus and cultured overnight in the presence of HS. Cells were then stained with anti-human CD3 and CD56 (to identify NK cells), alongside anti-human: CD16, CD69. NKG2D, CCR7, DNAM-1, NKp30, NKp44 and NKp46 antibodies. The cell-surface expression of each activation marker on $CD3^{-}CD56^{+}$ NK cells was then determined by flow cytometry. Graph shows percentage of cells positive for each marker + SEM of 5 separate experiments. Statistical significance is denoted by *$p<0.05$ (paired Student's t test).

Figure 6-3 demonstrates that reovirus infection of whole PBMC induced up-regulation of the NK cell-surface glycoprotein, CD69, and that the increase in mean percentage expression (43% vs 96%) seen in 5 separate, healthy, donors reached statistical significance. Reovirus treatment of PBMC also induced significant up-regulation of the migratory receptor, CCR7, on NK cells (22% vs 42%). No significant change was noted, however, in the levels of the Fc receptor, CD16, or in the NCRs, NKp30 and NKp46. Likewise, the NK cell surface expression of DNAM-1 and NKG2D did not alter after overnight treatment with reovirus. This may be the result of the background levels of expression being very high. Cell-surface expression of NKp44 was negligible both before and after virus treatment.

6.3.3 Cell-surface Expression of NK Ligands on SW480 and SW620 Tumour Targets

Having demonstrated the ability of reovirus to cause activation of NK cells by the up-regulation of CD69 and CCR7 within a mixed population of PBMC, the cell-surface expression of NK ligands on SW480 and SW620 cells was examined, to explore their potential as targets for killing by reovirus-activated NK cells. SW480 and SW620 cells were cultured in the presence or absence of 1pfu/cell reovirus to determine any changes in the expression of the NKG2D ligands, ULBP-1, ULBP-2 and MICA/B and the DNAM ligand, CD112. Changes in the level of cell-surface MHC-I expression were also examined (Fig 6-4).

A



B

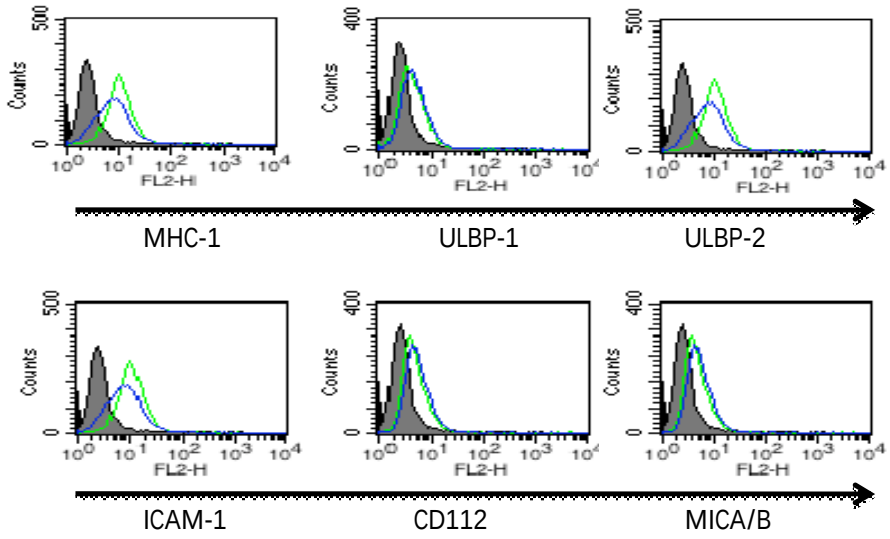


Figure 6-4 Cell-surface Expression of NK Ligands on SW480 and SW620 Cells

SW480 (A) and SW620 (B) cells were treated with 0 or 1pfu/cell reovirus overnight. Cells were then stained with anti-human: MHC-I, ULBP-1, ULBP-2, ICAM-1, CD112 or MICA/B antibodies and the percentage cell-surface expression was determined by flow cytometry (blue line: 0pfu/cell; green line: 1pfu/cell; shaded grey: isotype control). Data is representative of 3 independent experiments.

Fig 6-4 demonstrates the cell-surface expression of NK ligands on SW480 and SW620 cells in response to 0 or 1pfu/cell reovirus treatment, overnight. Both cell lines were found to

express MHC-I, although lower levels of expression were seen on the metastatic SW620 cell line than on SW480 cells. Moreover, reovirus infection caused a small increase in the level of SW620 MHC-I expression, whereas no increase was noted in the SW480 cell line. Both cell lines also expressed the stress ligands ULBP-1 and ULBP-2; however reovirus infection had no effect on the level of ULBP-1 expression. A minimal increase in the level of ULBP-2 expression was seen on the SW620 cell line following incubation with reovirus.

Whilst SW480 cells were observed to have a higher basal level of cell-surface ICAM-1 than SW620 cells, they did not increase its expression after reovirus treatment, unlike the marginal increase seen on SW620 cells. Similarly, higher levels of expression of the DNAM-1 ligand, CD112, were noted on the SW480 cell line; however reovirus exposure did not result in an increase in CD112 expression on either tumour cell line. Higher baseline levels of MICA/B expression were noted in the SW480 cell line, and reovirus infection resulted in a small increase in its surface expression on this tumour cell line only.

6.4 Discussion

The finding that reovirus infection stimulates significant levels of IFN-α and IFN-β production by PBMC (Fig 6-1) is potentially beneficial for cancer therapy. Type I IFNs are secreted by normal cells in response to viral infection. They govern a number of immune-regulatory functions which alter both innate and adaptive immune responses (reviewed by Biron *et al.,* 1999), including phenotypic and functional changes on DC (reviewed by Le Bon *et al.,* 2002). IFN-α has been shown to have anti-tumour effects. It can cause growth inhibition via Jak-STAT signalling in renal cancer cells (Shang *et al*., 2011) and has been shown to exert an anti-angiogeneic effect via the inhibition of VEGF gene transcription (von Marschall et al., 2003). IFN-β production by reovirus-infected, Mel888-loaded, DC has been shown to activate NK cells and enhance cytotoxicity towards Mel888 cell targets (Prestwich *et al.,* 2009b). As well as this, IFN-β release may prove functionally significant within the immunosuppressive tumour microenvironment. IFN-β has been shown to suppress the growth of pulmonary metastases in murine breast cancer and melanoma models (Studeny *et*

al., 2004) and, when given in combination with ionising radiation, demonstrated synergy for the treatment of alveolar rhabdomyosarcoma (Sims *et al.,* 2010). As well as this, IFN-β appears to be capable of inducing an anti-tumour phenotype in neutrophils (reviewed by Di Carlo *et al.,* 2001). To capitalise on this, IFN-β has been genetically engineered into a number of oncolytic viruses in an attempt to improve their tumour cell specificity and to support priming of anti-tumour immunity (Kirn *et al.,* 2007; Willmon *et al.,* 2009b); moreover the expression of IFN-β by such oncolytic viruses may increase protection of normal cells.

It is worth highlighting that no IFN-γ was produced on overnight infection of PBMC, despite significant increases in the levels of $CD3^-$ $CD56^+$ NK cell surface expression of CD69. This is in-keeping with previous findings, where reovirus infection of PBMC, as well as co-culturing PBMC with reovirus-infected Mel888 cells, failed to induce a significant IFN-γ response (Prestwich *et al.,* 2009b). One implication of this finding is that IFN-γ has been shown to be critical for the generation of tumour-specific T cells (Kelly *et al.,* 2002). It is likely, therefore, that the production of IFN-γ and the subsequent generation of tumour-specific CTL will depend upon complex tumour/DC/NK cell interactions, which will involve the secretion of chemokines and IL-12 (Prestwich *et al.,* 2009b), resulting in the recruitment of NK cells into the tumour microenvironment.

It has already been demonstrated that reovirus infection does not activate isolated NK cells *in vitro*. It does, however, induce phenotypic maturation of isolated DC with the production of inflammatory cytokines and these DC in turn activate autologous NK cells upon co-culture (Errington *et al.,* 2008b). However, the effect of reovirus infection on whole PBMC, a more physiologically relevant cell population, had not previously been explored. The finding that reovirus infection up-regulated CD69 expression on $CD3^-CD56^+$ NK cells in the context of whole PBMC and in the presence of HS is, therefore, an interesting finding. The up-regulation of CD69 expression on NK cells can be brought about by the production of IFN-α (Gerosa *et al.,* 1991) and by the cross-linking of FcγRIII (CD16). Therefore, this finding likely

highlights the complex interactions within the inflammatory milieu of whole PBMC upon reovirus infection between DC, multiple inflammatory cytokines and NK cells.

A further interesting observation is the ability of reovirus to cause up-regulation of CCR7 on NK cells (Fig 6-3). To date, this finding has not been reported previously. CCR7 is expressed by $CD56^{bright}$ NK cells but not on $CD56^{dim}$ NK cells (Berahovich *et al.,* 2006). KIR^{+} NK cell populations can express CCR7 *de novo* upon co-culture with mDC and become capable of lymph node migration in response to the endogenous CCR7-specific ligands, CCL19 and CCL21 (Marcenaro *et al.,* 2009). Therefore, administering an agent which promotes the migration of activated NK cells to lymph nodes containing metastatic tumour has obvious potential clinical benefits. This must be tempered however, by the finding that protein expression for CCL19 and CCL21 does not differ in CRLM tissue when compared to surrounding normal tissue and therefore represents one obstacle to the recruitment of activated NK cells to intra-hepatic CRLM (Rubie *et al.,* 2006).

The lower levels of MHC-I expressed on the surface of SW620 cells represents a further significant finding with regards to NK-mediated killing (Fig 6-4). It follows, therefore, that SW620 cells may be more susceptible to NK-induced cytotoxicity. Given the propensity of metastatic colorectal cancer cells to invade loco-regional lymph nodes, their low MHC-I expression, coupled with high levels of CCR7 expression on reovirus activated NK cells, provides an interesting basis for investigating further the anti-tumour cytotoxic role of reovirus-activated NK cells.

The NKG2D ligands, MICA/B are structurally similar to MHC-I and are expressed by normal gastrointestinal epithelium, endothelial cells, and fibroblasts (Bahram *et al.,*1994; Zwirner *et al.,* 1999). As well as this, MICA/B is expressed on the surface of a range of haematological and solid tumours, including colorectal cancer (Pende *et al.,* 2002). Whilst the processes which regulate the expression of NKG2D ligands on the surface of cancer cells are not widely understood, DNA damage response pathways and BCR/ABL oncogene expression

have been implicated (Boissel *et al.,* 2006). Expression of MICA/B is up-regulated in response to cellular stress such as viral infection (Groh *et al.,* 2001) and the slight increase in MICA/B expression by SW620 cells (Fig 6-4) in response to reovirus infection supports this finding. ULBP-1/2 are distantly related to MHC-I and are expressed on normal human epithelium and show no increased expression on tumour cells (Kubin *et al.,* 2001). The up-regulation of ULBP-2 by both SW480 and SW620 cells (Fig 6-4) in response to reovirus infection may increase their susceptibility to NK cell-mediated cytotoxicity.

As discussed previously, cancer cells may develop ways to evade anti-tumour immunity through immuno-editing. There are now a number of recognised mechanisms which enable tumours cells to evade NKG2D-mediated immune responses. These include processes which lead to persistent expression of NKG2D ligands, which in turn results in the down-regulation of NKG2D expression (Oppenheim *et al.,* 2005; Groh *et al.,* 2002). As well as this, some tumours can shed soluble NKG2D ligands or secrete immunosuppressive cytokines, such as, TGF-β which has been shown to down-regulate NKG2D expression (Crane *et al.,* 2010). *Ras* activation has also been implicated in the disruption of the antigen presenting system, controlling immune recognition in colorectal cancer cells through co-suppression of MHC I, NKG2D ligands and peptide transporter and proteosomal genes (Sers *et al.,* 2009).

NK cell to target cell adhesion is mediated by two integrins, LFA-1 and Mac-1, on NK cells and ICAM-1 on target cells (reviewed by Orange *et al.,* 2008). ICAM-1 mediates cell to cell and cell to matrix interactions and is expressed on a variety of haematopoietic and non-haematopoietic cells (reviewed by van de Stolpe *et al.,* 1996). It is rapidly up-regulated in response to inflammatory stimuli, including viral infection and pro-inflammatory cytokine release (van de Stolpe *et al.,* 1996). In some tumour types, high surface expression of ICAM-1 is associated with increased risk of metastasis (Johnson *et al.,* 1989; Sun *et al.,* 1999). It seems likely, therefore, that ICAM-1 expression on SW480 and SW620 cells should facilitate NK cell-induced cytolysis (Fig 6-4). For instance, ICAM-1 expression by oral neoplastic cell lines demonstrated increased levels of adhesion to PBMC and LAK cell-

mediated cytotoxicity (Huang *et al.,* 2000b). The findings described in this chapter suggest that reovirus pulsing of PBMC can activate NK cells within them, even in the presence of neutralising anti-reovirus antibodies, and that colorectal cancer may be a target for killing by these reovirus-activated innate effectors.

7 Results

7.1 The Cytolytic Activity of NK Cells Following Activation by Reovirus in the Presence of Neutralising Human Serum

7.2 Aims

1. To assess the cytolytic effects of reovirus-activated peripheral blood NK cells (PBNK) against SW480 and SW620 colorectal cancer cell line targets, using ^{51}Cr-release and CD107 degranulation assays.

2. To confirm that NK cells are the main anti-tumour effector cells within reovirus-activated PBMC.

7.3 Introduction

The previous chapter demonstrated that reovirus activated NK cells within a mixed PBMC population, as shown by a significant increase in the cell-surface expression of the early NK activation marker, CD69 (Fig 6-3). NK cells are known to acquire potent cytotoxicity in response to acute viral infection (reviewed by Biron *et al.,* 2001). It is likely that this occurs in response to Type I IFN production, in concert with IL-12 release and the subsequent production of IFN-γ. As well as this, interactions between cytokine receptors, adhesion molecules, and the recognition of ligands by activating receptors, all contribute to NK cell activation.

The exact mechanism by which reovirus activates NK cells has not been fully elucidated; however, it is has been shown that isolated NK cells are not activated by direct reovirus infection (Errington *et al.,* 2008b). DC have previously been reported to activate NK cells through the release of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-15 and IL-12 (Gerosa *et al.,* 2005; Ferlazzo G *et al.,* 2004). It is likely therefore, that reovirus infection leads to the phenotypic and functional maturation of DC and the subsequent activation of NK cells in whole PBMC.

NK cells exert their cytotoxic effects by a range of different mechanisms, including exocytosis of perforin/granzyme and via interactions between the TNF superfamily death receptors and their ligands, such as Fas/FasL. NK cells contain high concentrations of cytolytic granules in their cytoplasm as they circulate in the periphery (Cooper *et al.,* 2001). These vesicles contain a number of cytolytic proteins, such as perforin and granzyme, uniquely designed to induce death in target cells upon release (Cooper *et al.,* 2001; Tschopp *et al.,* 1990). Following activation, NK cells rapidly release these granules at the immunological synapse, inducing apoptotic death of the target cell (Cooper *et al.,* 2001; Moretta *et al.,* 2002).

The lysosomal-associated membrane protein-1 and lysosomal-associated membrane protein-2 (LAMP-1 and LAMP-2, also known as CD107a and CD107b, respectively) line the membrane of cytotoxic granules (Winchester, 2001; Peters *et al.,* 1991). The flow cytometry-based CD107 release assay detects the surface expression of CD107a and CD107b which occurs upon NK cell degranulation. CD107 expression has been shown to correlate well with both cytokine release from, and cell-mediated lysis of target cells, by NK cells (Mittendorf *et al.,* 2005).

7.3.1 Reovirus-activated NK Cells are Cytolytic Against SW480 and SW620 Targets

The previous chapter established that SW480 and SW620 colorectal cancer cells expressed ligands for the NK receptors, DNAM-1 and NKG2D (Fig 6-4), and that reovirus treatment led to the activation of NK cells within PBMC (Fig 6-3). Coupled with the known ability of NK cells to lyse transformed cells, the cytolytic potential of reovirus-activated NK cells within PBMC (PBNK) against SW480 and SW620 targets was next assessed. PBMC were cultured overnight in the presence of HS with or without 1pfu/cell reovirus. These cells were then co-cultured with SW480 and SW620 cells in a CD107 assay and the level of NK cell degranulation in response to target cells was measured by flow cytometry.

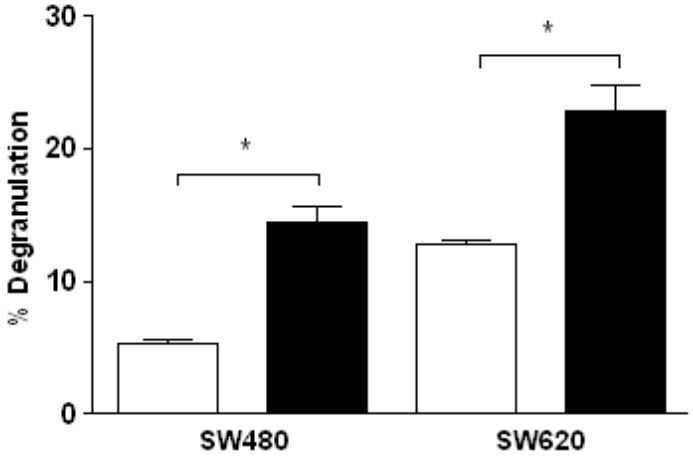


Figure 7-1 Degranulation of PBNK in Response to SW480 and SW620 Targets.

PBMC were isolated from healthy donor peripheral blood and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. PBMC were then co-cultured with SW480 and SW620 targets at a 1:1 ratio for 4 hr,in the presence of anti-human CD107a and CD107b antibodies. After subsequent staining with anti-human CD3 and CD56 antibodies, cell-surface expression of CD107 within NK cells was then determined by flow cytometry. Graph shows mean percentage of CD107 degranulation + SEM of 3 separate experiments. Statistical significance is denoted by *$p<0.05$ (paired Student's t test).

Fig 7-1 demonstrates that reovirus-activated PBNK degranulated against both SW480 and SW620 cell line targets. The level of degranulation in the reovirus–activated PBMC was significantly higher than that seen in non reovirus-treated PBMC (5% vs 15% against the SW480 cell line and 14% vs 23% against the SW620 cell line). The overall level of degranulation seen was higher against the SW620 cells than against the SW480 line (23% vs 15%). No degranulation was seen in response to targets by either NKT cells or CD3+ cells within reovirus-activated PBMC (data not shown).

In order to confirm that CD107 release by reovirus-activated PBNK did in fact correlate with innate immune-mediated cell killing of colorectal target cells, standard 4hr ^{51}Cr release assays were undertaken. Untreated and reovirus-treated effector PBMC were co-cultured with ^{51}Cr-labelled SW480 and SW620 target cells and the level of target cell lysis was measured.

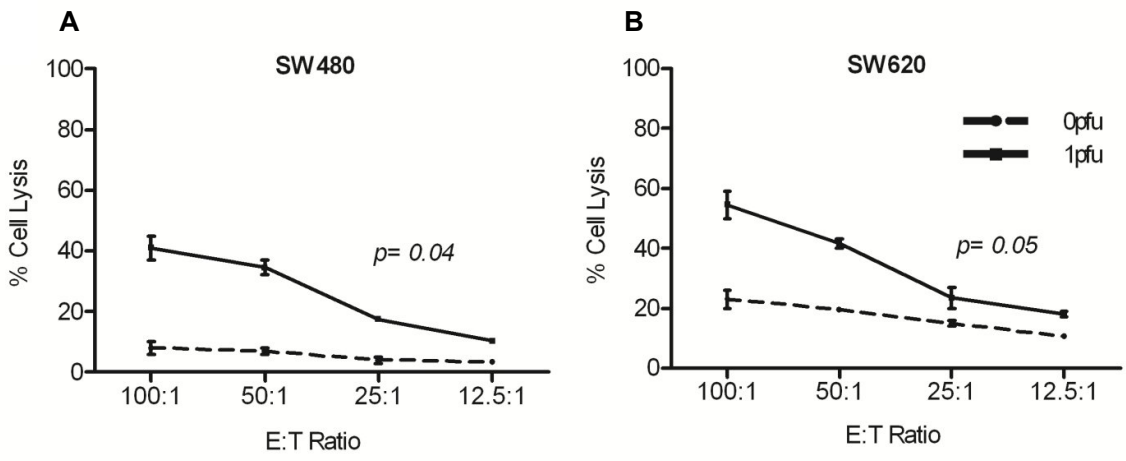


Figure 7-2 Cytolytic Activity of Reovirus-activated PBNK against SW480 and SW620 cells

PBMC were isolated from healthy donor peripheral blood and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. PBMC were then co-cultured with ^{51}Cr-labelled SW480 (A) and SW620 (B) cell targets at different E;T cell ratios for 4 hr. Culture supernatants were then harvested onto luma scintillation plates and the level of tumour cell lysis was determined using a MicrobetaJet scintillation counter. Graphs show mean percentage target cell lysis +/- SEM of 3 separate experiments. Statistical significance is taken as <0.05 (paired Student's t test).

Fig 7-2 demonstrates that significantly higher levels of SW480 (A) and SW620 (B) target cell lysis occurred after co-culture with reovirus-treated PBMC than when compared with untreated controls, even in the presence of neutralising HS. Higher percentages of cell lysis were observed in the SW620 cell line than the SW480 cells (59% vs 37% at an E:T ratio of 100:1). These findings correlate with the higher level of NK cell degranulation seen against the SW620 cell line compared to SW480 cells seen in Fig 7-1; a noteable point as it differs from the pattern of target cell death seen after SW480 and SW620 cells were infected with direct reovirus (Fig 4-2).

7.3.2 NK Cells are the Main Effectors in Reovirus-activated PBMC

In order to confirm that the cytolytic activity against SW480 and SW620 cell targets demonstrated above was due to the activity of NK cells within the whole reovirus-activated

PBMC population, NK depletion experiments were performed. PBMC were treated with 0 or 1pfu/cell reovirus in the presence of HS overnight, before some PBMC were depleted of CD56+ cells and ^{51}Cr-release assays were carried out.

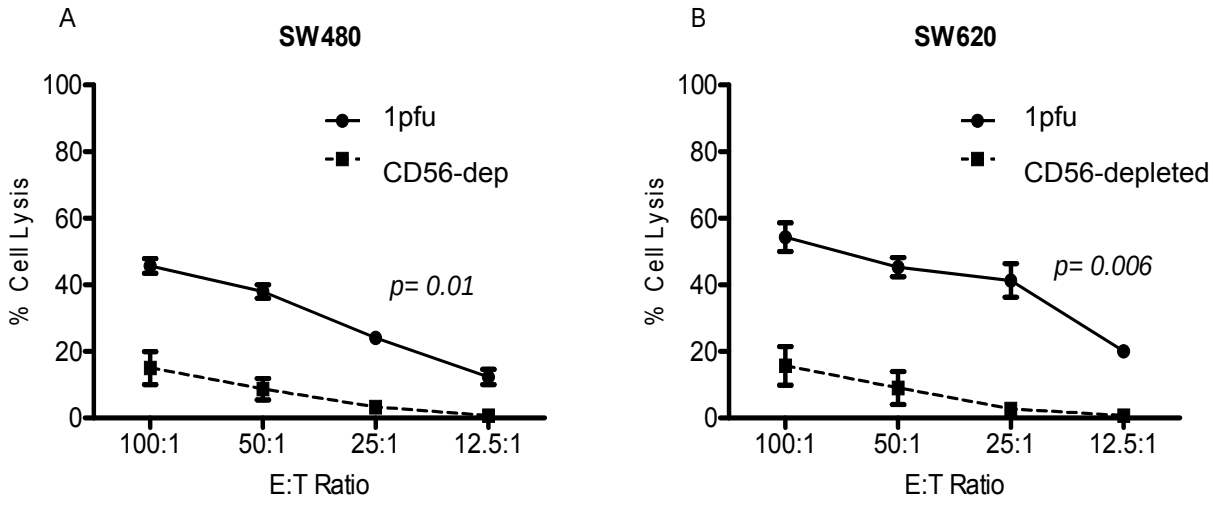


Figure 7-3 Cytolytic Activity of NK-depleted Reovirus-activated PBNK Against SW480 and SW620 cells

PBMC were isolated from healthy donor peripheral blood and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. PBMC were then co-cultured with ^{51}Cr-labelled SW480 (A) and SW620 (B) cell targets at different E:T cell ratios for 4 hr. Where depletions were performed, PBMC were treated with CD56 microbeads before ^{51}Cr assays were set up. Culture supernatants were then harvested onto luma scintillation plates and the level of tumour cell lysis was determined using a MicrobetaJet scintillation counter. Graphs show mean percentage target cell lysis +/- SEM of 3 separate experiments. Statistical significance was taken as $p<0.05$ (paired Student's t test).

Fig 7-3 demonstrates a significant reduction in the levels of cytolytic activity of CD56-depleted reovirus-activated PBMC against both SW480 (A) and SW620 (B) target cells, when compared to whole reovirus-activated PBMC. The mean levels of killing were reduced to 15% from 46% in SW480 cells and 16% from 54% in SW620 cells, at an E:T ratio of 100:1. This confirms that the NK cell population within reovirus-activated whole PBMC are the likely main cytolytic effector cells.

Taken together, the data in this chapter confirm that healthy donor PBMC be activated by reovirus to degranulate in response to, and lyse, colorectal cancer cell line targets, even in the presence of HS containing neutralising anti-reovirus antibodies. Furthermore, it demonstrates that it is the NK cell population within the reovirus-activated PBMC which are the main effector cell population.

7.4 Discussion

Reovirus-activated, healthy donor PBMC were demonstrated in this chapter to have cytolytic activity towards colorectal cancer cell lines even in the presence of human serum, which is known to contain NAB. CD107 is a marker of NK cell degranulation (i.e. release of cytotoxic granules) in response to target cells. Significant levels of CD107 was observed on the surface of NK cells within reovirus-treated PBMC when co-cultured with SW480 and SW620 cells, compared with untreated controls (Fig 7-1). Moreover, reovirus-activated PBMC also demonstrated significant levels of cytolytic activity against the tumour cell targets when measured via ^{51}Cr release (Fig 7-2). The confirmation that NK cells represented the main cytolytic component of reovirus-activated whole PBMC (Fig 7-3) provides an interesting focus for further investigation, as much of the work examining reovirus-activation of immune cells has been conducted using purified or isolated cell populations (Errington *et al*., 2008b).

The discovery that reovirus-treated PBMC led to more NK cell degranulation in response to, and cell lysis of, SW620 cells than SW480 cells was interesting. When infected directly with virus, SW480 cells consistently displayed higher percentages of reovirus-induced cell death than SW620 cells (Fig 4-2). Although both cell lines express MHC-I (a negative regulator of NK-mediated cytotoxicity), its level was demonstrated to be lower on SW620 cells than SW480 cells (Fig 6-4). This would be consistent with a potential role for MHC-I in the sensitivity of these cell lines to NK-mediated cytotoxicity. In the clinical setting, metastatic colorectal cancers have been shown to express less MHC-I than primary tumour (Lopez-

Nevot *et al.*, 1989). Hence, class I-low tumour cells, if resistant to direct oncolysis, may alternatively be killed by reovirus activation of innate immune effectors.

The finding that reovirus can activate innate immune cells within whole PBMC to lyse cancer cell targets has potentially important clinical implications. As discussed previously, the direct oncolytic effects of reovirus can be abrogated in the presence of neutralising antibodies, which the majority of patients will possess after exposure to the ubiquitous virus. Using PBMC to "carry" and protect reovirus from neutralisation for delivery at the site of the tumour (Fig 5-4) represents one method of circumventing the deleterious effects of NAB and enabling direct oncolysis. However, concomitant reovirus-activation of NK cells which become cytotoxic to tumour targets (i.e the generation of an innate anti-tumour immune response), represents an additional bystander mechanism for enhancing the overall efficacy of reovirus therapy via cell delivery. The following chapter investigates the potential for reovirus to activate and promote degranulation of NK cells within the whole PBMC, as well as liver, of patients with metastatic colorectal cancer.

8 Results

8.1 The Effect of Reovirus on Colorectal Cancer Patient PBNK and Liver-derived NK Cells (LNK) and their Cytolyic Potential Against Autologous Metastatic Cells

8.2 Aims

1. To assess the effects of reovirus on colorectal cancer patients' PBMC.

2. To examine the functionality of reovirus-treated patient PBNK against SW480 and SW620 cell line targets using CD107 degranulation assays.

3. To isolate and characterise single cell suspensions of CRLM and hepatocytes from freshly resected surgical specimens.

4. To examine the functionality of reovirus-treated patient PBNK against autologous CRLM and hepatocytes using CD107 degranulation assays.

5. To isolate and characterise liver mononuclear cells (LMNC) from freshly resected liver tissue

6. To examine the functionality of reovirus-treated patient LNK against SW480, SW620 cell line targets as well as autologous CRLM and hepatocytes, using CD107 degranulation assays.

8.3 Introduction

The NK cells of cancer patients have been shown to differ in phenotype to those of healthy donors and it seems likely that the production of soluble NK ligands by tumours may, in part, account for some of the reduced cytolytic activity seen (Fuertes *et al.,* 2008). As well as this, lower levels of the activating receptors CD16, NKp46 and NKp30 have been demonstrated in the peripheral NK cells of human melanoma patients (Konjević *et al.,* 2009). Furthermore, in one study examining the NK cells from the ascites of ovarian cancer patients, reduced levels of DNAM-1 and CD16 were observed, with lower levels of ADCC exhibited when compared with autologous peripheral blood NK cells (Carlsten *et al.,* 2009).

NK cells are known to be scarce in colorectal cancer specimens, despite MICA/B expression on the tumour cells' surface and the production of a variety of chemokines and cytokines (Halama *et al.,* 2011). Moreover, reduced NK cell gene expression has been highlighted as one of the major immune characteristics in the peripheral blood of colorectal cancer patients (Xu *et al.,* 2011). This scarcity of tumour-infiltrating NK cells, coupled with the technical difficulties in isolating them effectively, has hampered *ex-vivo* expansion and functional analysis; however it is reasonable to assume that the altered functionality of NK cells in cancer patients may pose a potential barrier to effective activation and cytolytic activity by reovirus.

Having established that reovirus could activate the NK cells within PBMC of healthy donors and that those cells demonstrated cytotoxicity towards SW480 and SW60 cell targets, even in the presence of NAB, the functionality of NK cells from patients with CRLM was investigated.

8.3.1 The Effect of Reovirus Treatment on PBNK Isolated from CRLM Patients

The effects of reovirus on the PBNK cells of patients undergoing liver resection for CRLM was next assessed. PBMC were isolated from three patients prior to resection and treated with 0 or 1 pfu/cell reovirus overnight in the presence of HS. The cells were then stained for

cell-surface expression of the same activation markers and surface molecules as healthy donors (Fig 6-3) and the percentage expression of each marker on NK cells within the PBMC population was determined by flow cytometry.

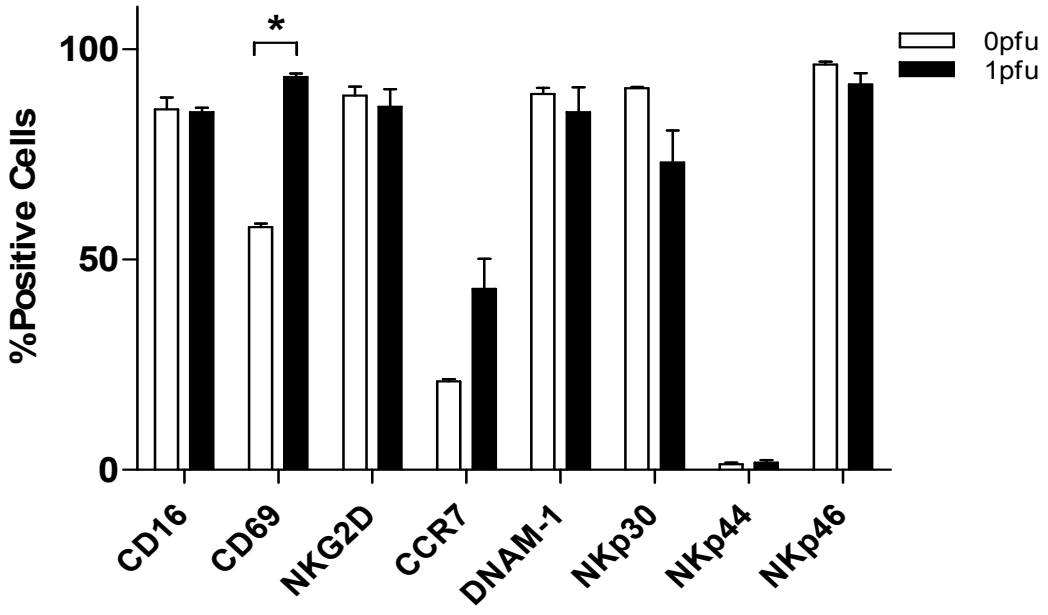


Figure 8-1 Effect of Reovirus Treatment on CRLM Patient PBNK

PBMC were isolated from patients with CRLM the night before hepatic resection and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. Cells were then stained with anti-human CD3 and CD56 (to identify NK cells), alongside anti-human: CD16, CD69. NKG2D, CCR7, DNAM-1, NKp30, NKp44 and NKp46 antibodies. The cell-surface expression of each activation marker on $CD3^{-}CD56^{+}$ NK cells was then determined by flow cytometry. Graph shows percentage of cells positive for each marker + SEM of 3 separate experiments. Statistical significance is denoted by *$p<0.05$ (paired Student's t test).

Fig 8-1 demonstrates that NK cells isolated from patients with CRLM were activated by reovirus in the presence of HS. Significantly higher levels of surface CD69 were seen on the surface of NK cells in the reovirus-treated PBMC (93% vs 58%). As with the healthy donor group (Fig 6-3), no significant increase was noted in the levels of NKG2D, NKp30, NKp44, NKp46 or DNAM-1 expression in response to reovirus infection. Similarly to healthy donors,

an increase in the level of cell-surface CCR7 (21% vs 46%) was observed on patient PBNK in response to reovirus treatment, although the levels did not reach statistical significance.

8.3.2 Cytolytic Activity of Reovirus-activated Patient NK cells

Having demonstrated the ability of reovirus to activate patient PBNK by up-regulation of CD69, the cytolytic capability of reovirus-activated patient PBNK was assessed using CD107 release assays. The level of $CD3^{-}CD56^{+}$ NK cell degranulation in reovirus-treated and untreated PBMC against SW480 and SW620 cell targets was assessed.

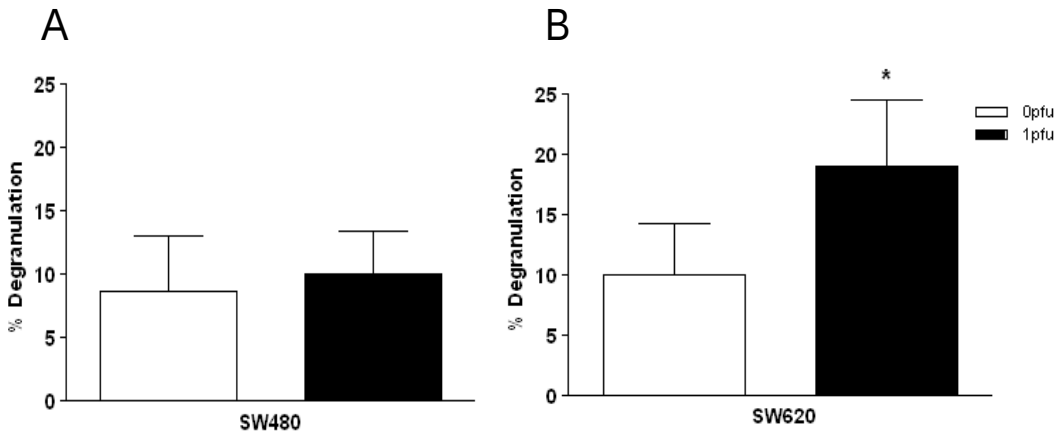


Figure 8-2 Degranulation of Patient PBMC Against Colorectal Cell Line Targets

PBMC were isolated from the peripheral blood of patients with CRLM the night before hepatic resection and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. PBMC were then co-cultured with SW480 (A) and SW620 (B) targets at a 1:1 ratio for 4 hr,in the presence of anti-human CD107a and CD107b antibodies. After subsequent staining with anti-human CD3 and CD56 antibodies, cell-surface expression of CD107 within NK cells was then determined by flow cytometry. Graph shows mean percentage of CD107 degranulation + SEM of 3 separate experiments. Statistical significance is denoted by *p<0.05 (paired Student's t test).

Fig 8-2 confirms that reovirus-activated, patient PBNK did not degranulate to a significant level against SW480 cells (8% vs 9%) in the presence of HS. This may be due to immunosuppression in patients with CRLM. Degranulation against SW620 cells did occur, however, and the level seen was statistically significant (9% vs 19%).

8.3.3 Isolation of Primary CRLM Cells and Hepatocytes

Obtaining representative cells from freshly resected tissue is technically difficult. Numerous studies have documented the difficulty in establishing colorectal cancer cell lines from primary bowel cancers (Namba *et al.,* 1983; Oh *et al.,* 1999). Well characterised, low passage, cell lines are rare and their generation from resected specimens remains important for the study of oncogenic mechanisms in colorectal cancer, as well as new therapeutic strategies. Recognising these challenges, having utilised an *in vitro* primary and metastatic colorectal cancer cell line model for the investigation of the anti-tumour and immune effects of reovirus, we nevertheless next tried to test if the findings correlated with cells derived from freshly resected tissue taken from patients with colorectal liver metastases. First, single cell suspensions of primary metastatic cells and hepatocytes were isolated from liver tumour and normal tissue, respectively. Fig 8-3 shows the microscopic appearance of isolated hepatocytes (A) and colorectal liver metastasic cells (B) at x20 magnification. Flow cytometry plots demonstrating the size, granularity and gating strategies of hepatocytes (C) and tumour cells (D) are also shown. Whilst hepatocytes were observed to remain viable in culture for up to 96 hr, death of primary CRLM was seen to occur after approximately 24-48 hr (data not shown).

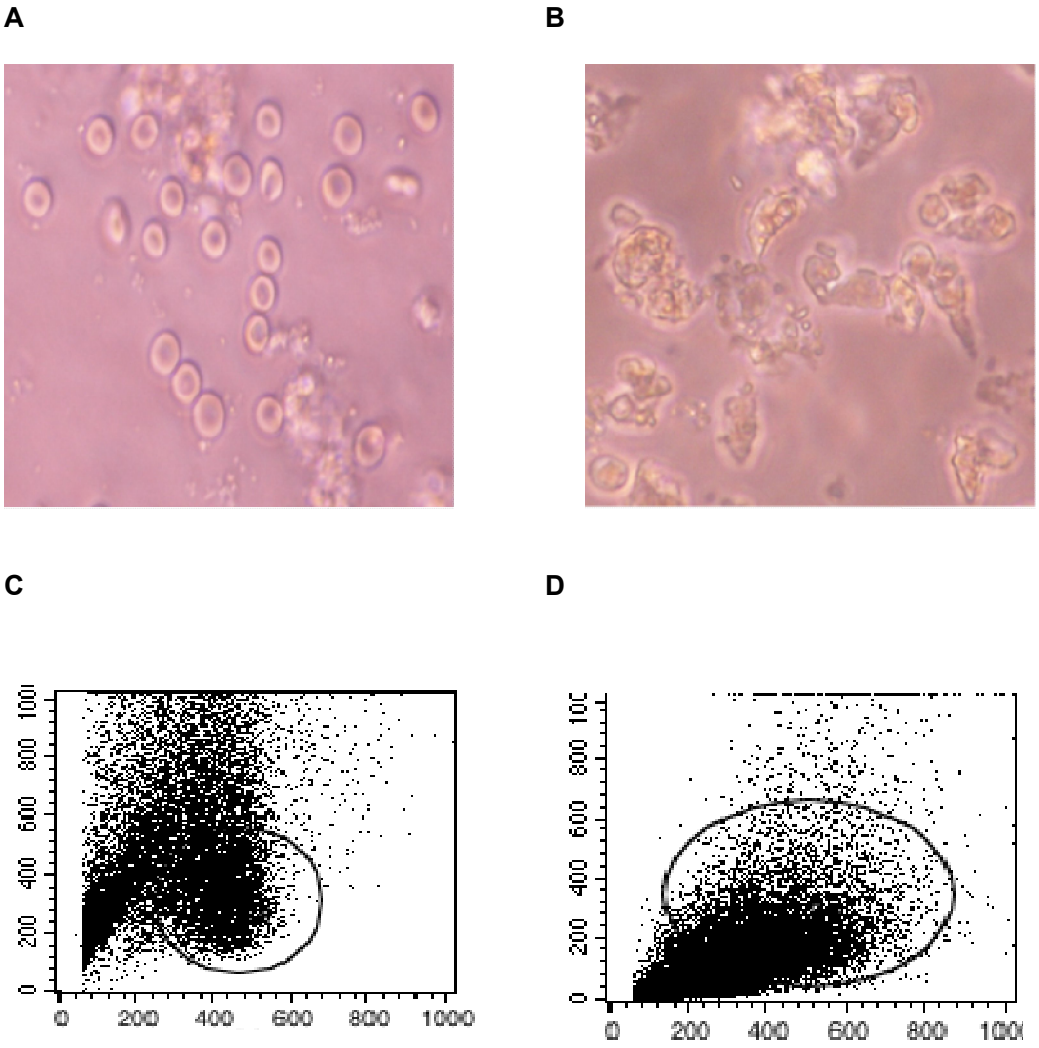


Figure 8-3 Microscopic and Flow Cytometric Appearances of Primary Hepatocytes and CRLM

Single cell suspensions of hepatocytes (A) and tumour cells (B) were isolated from freshly resected normal liver parenchyma and colorectal liver metastases, respectively. Cells were photographed using an Olympus C-7070 camera and light microscope at x20 magnification. Representative flow cytometry dot plots, showing size, granularity and gating strategies of hepatocytes (C) and tumour cells (D) are also shown.

8.3.4 Phenotypic Characterisation of Primary Hepatocytes and CRLM

Having successfully isolated both tumour cells and hepatocytes from freshly resected specimens, phenotypic characterisation was carried out. CEA, a glycoprotein involved in cell adhesion and expressed on a variety of epithelial tumour cells but not on the surface of

normal hepatocytes was used. The monoclonal antibody, BerEp4, directed against an epitope on human epithelial cells was also used to discriminate epithelial cells from hepatocytes. BerEp4 is not expressed on the surface of adult hepatocytes and exhibits highly conserved expression on carcinomas (Latza *et al.,* 1990). Cells were also assessed for the presence of the reovirus receptor, JAM-A, on their cell-surface and the results are shown in Fig 8-4.

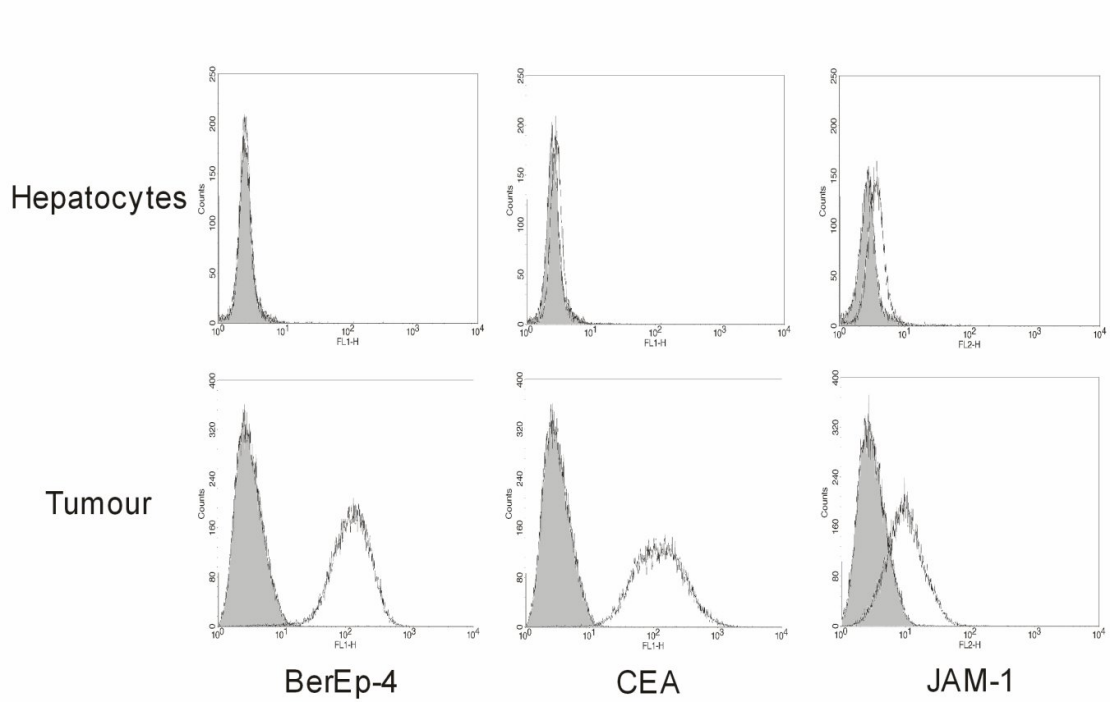


Figure 8-4 Cell-surface Expression of BerEp4, CEA and JAM-A on Primary CRLM and Hepatocytes.

Freshly resected tumour and liver tissue were disaggregated into single suspensions. Cells were then stained with anti-humanBer-Ep4, CEA or JAM-A antibodies and the level of cell-surface expression was determined by flow cytometry (black line: surface marker expression, shaded grey: isotype control). Data is representative of 3 independent experiments.

Fig 8-4 demonstrates the cell-surface expression of Ber-Ep4, CEA and JAM-A on the surface of freshly resected CRLM and hepatocytes. A clear distinction can be made on the basis of this analysis between the two cell populations, with tumour cells shown to express high

levels of the epithelial marker Ber-Ep4 and the TAA, CEA. In contrast, hepatocytes did not express either cell-surface Ber-Ep4 or CEA. Futhermore, whilst primary CRLM were shown to express high levels of the reovirus receptor, JAM-A, hepatocytes were observed to express it at only relatively low levels on their cell-surface.

8.3.5 Cytolytic Activity of Patient PBNK against Autologous CRLM cells and Hepatocytes

Having demonstrated that discrete populations of tumour cells and hepatocytes could be isolated from freshly resected surgical specimens, the cytotoxic potential of reovirus-activated patient PBNK against autologous freshly isolated CRLM cells and hepatocytes was next examined.

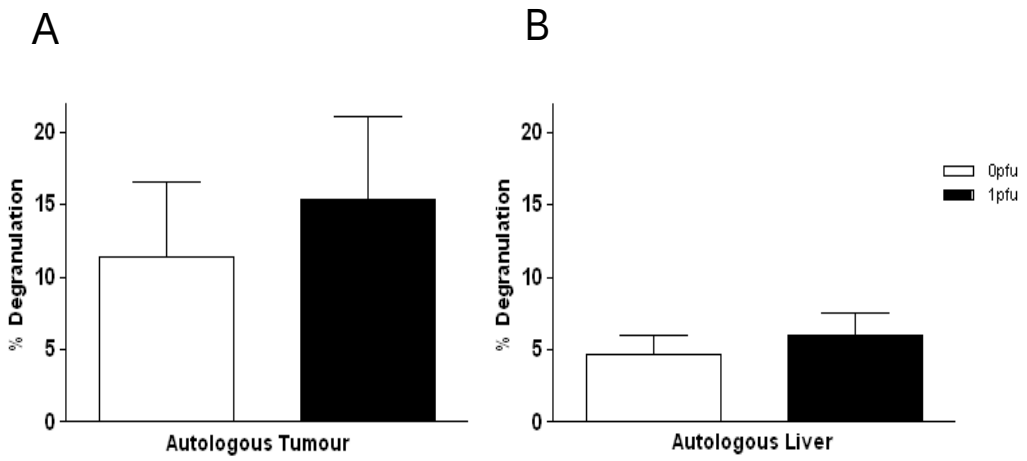


Figure 8-5 Degranulation of Patient PBNK against Autologus CRLM cells and Hepatocytes

PBMC were isolated from the peripheral blood of patients with CRLM the night before hepatic resection and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. Single cell suspensions of hepatocytes and CRLM cells were isolated from freshly resected normal liver parenchyma and colorectal liver metastases, respectively. PBMC were then co-cultured with CRLM (A) or hepatocytes (B) at a 1:1 ratio for 4 hr in the presence of anti-human CD107a and CD107b antibodies. After subsequent staining with anti-human CD3 and CD56 antibodies, cell-surface expression of CD107 within patient PBNK was then determined by flow cytometry. Graph shows mean percentage of CD107 degranulation + SEM of 3 separate experiments.

Fig 8-5 highlights a general trend of increased cytolytic activity of reovirus-activated patient PBNK against autologous tumour cells, however the difference in the level of activity did not reach statistical significance. There was background PBNK degranulation in response to primary hepatocytes (approximately 5%) but reovirus treatment had no effect on the level seen. Chromium release cytotoxicity experiments were not possible using these samples as primary tumour cells did not label with ^{51}Cr despite repeated attempts. This is likely to be a reflection of their low metabolic activity.

8.3.6 Isolation of Liver Mononuclear Cells

NK cells account for around one third of the total lymphocyte population of the liver, making them crucial in the innate immune defence against microbial infection. As well as this, they have been shown to act as adaptive immune response regulators involved in cross talk between DC and T cells (Ferlazzo *et al.,* 2002; Zingoni *et al.,* 2004). They therefore play not only a central role in innate immunity, but also shape the adaptive immune response within the liver microenvironment.

In order to assess the effect of reovirus on innate cells within the liver, LMNC were isolated from freshly resected liver tissue. Autologous peripheral blood was also collected from the patients at the time of resection and PBMC prepared for comparison. Cells were then stained with anti-human CD3 and CD56 antibodies and the relative NK cell populations of both LMNC (LNK) and PBMC (PBNK) from a representative patient are shown in Fig 8-5.

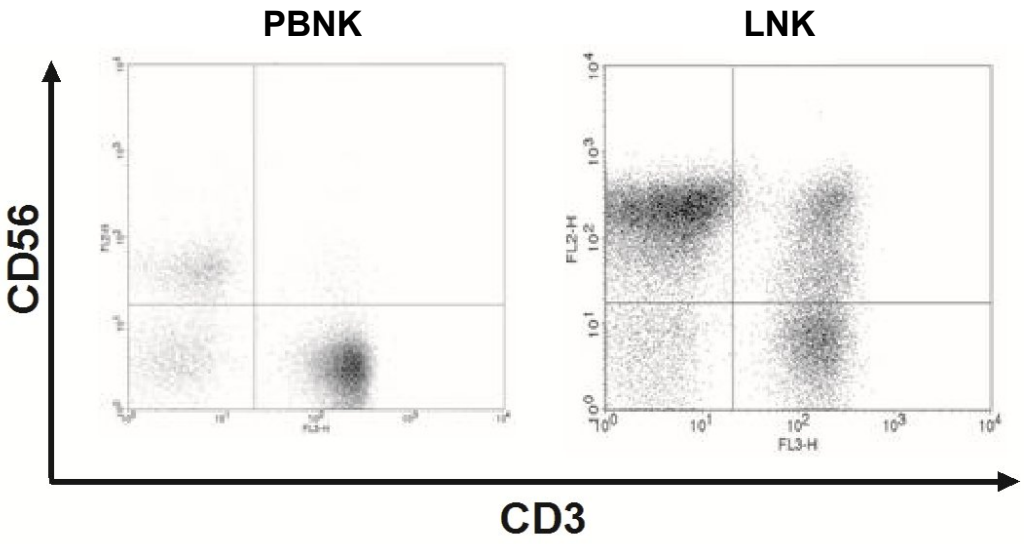


Figure 8-6 Characterisation of NK Cell Populations within PBMC and LMNC

PBMC and LMNC were isolated from the peripheral blood and liver parenchyma of patients with CRLM, respectively. Cells were stained with anti-human CD56 and CD3 antibodies and cell-surface expression of each marker was determined byflow cytometry. Dot plots are representative of 3 separate donors.

Fig 8-6 demonstrates the distinct populations of $CD3^{-}CD56^{+}$ NK cells within matched PBMC and LMNC. Higher numbers of both NK and NKT ($CD3^{+}/CD56^{+}$) cells were seen in LMNC compared to peripheral blood, whereas the number of $CD3^{+}$/ $CD56^{-}$ cells was observed to be higher in PBMC.

8.3.7 Reovirus Activation of LNK

Having isolated LMNC from patients and identified the NK cells within them (LNK), the effect of reovirus treatment on LNK phenotype in the presence of human serum was next assessed.

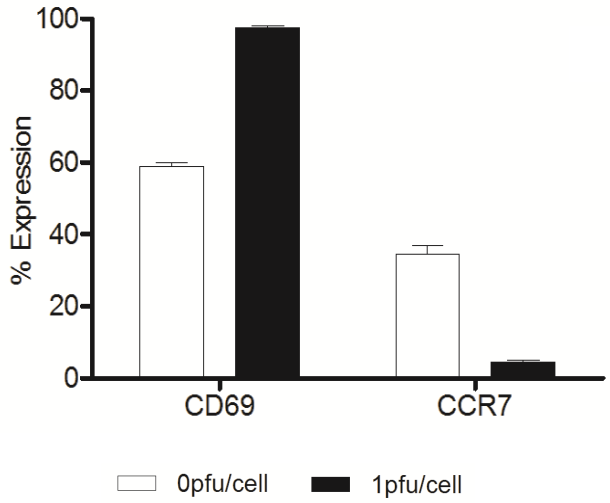


Figure 8-7 Effect of Reovirus on CRLM Patient LNK Cells

LMNC were isolated from the liver parenchyma of patients and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. Cells were then stained with anti-human CD56 and CD3 antibodies (to identify LNK cells), alongside anti-human CD69 and CCR7 antibodies. Percentage cell-surface expression of each marker was determined by flow cytometry. Graph shows mean percentage expression + SEM from 2 independent experiments.

Fig 8-7 demonstrates up-regulation of CD69 on the surface of LNK after activation with reovirus when compared to controls (59% vs 98%). In contrast to levels seen in healthy donor and patient PBMC, overnight infection with reovirus resulted in a decrease in the expression of CCR7 on the cell-surface of LNK within LMNC (36% vs 7%).

8.3.8 Cytolytic Activity of LNK

Having shown that the NK population of LMNC could be isolated and that they demonstrated up-regulation of the early NK activation marker, CD69, upon treatment with reovirus, the cytolytic activity of patient LNK cells against SW480 and SW620 colorectal cancer cell targets was examined. Cell-surface expression of CD107 from LNK in response to SW480 and SW620 cell line targets was determined by flow cytometry.

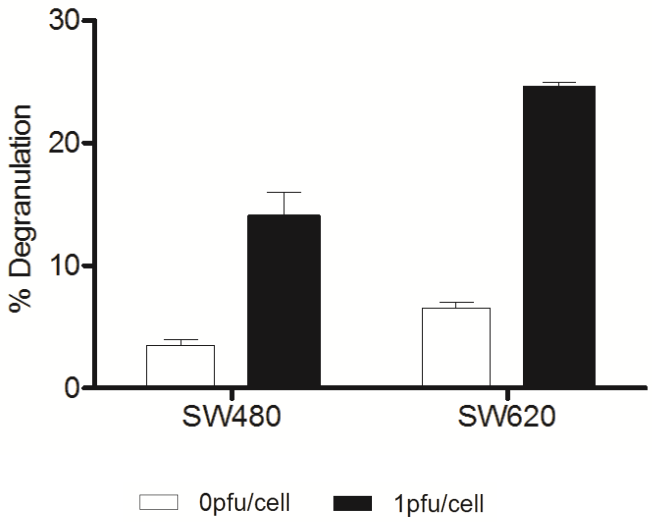


Figure 8-8 Degranulation of Patient LNK Against Colorectal Cell Line Targets

LMNC were isolated from the liver parenchyma of patients with CRLM and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. LMNC were then co-cultured with SW480 and SW620 target cells at a 1:1 ratio for 4 hr in the presence of anti-human CD107a and CD107b antibodies. After subsequent staining with anti-human CD3 and CD56 antibodies, cell-surface expression of CD107 within patient LNK was then determined by flow cytometry. Graph shows mean percentage of CD107 degranulation + SEM of 2 separate experiments.

Fig 8-8 demonstrates that reovirus-activated LNK cells are cytolytic towards SW480 and SW620 cell line targets in the presence of HS. Similar to healthy donor PBMC (Fig 7-1), higher levels of degranulation were again seen against the SW620 cells compared with SW480 cell line (15% vs 25% at 1pfu/cell).

Having demonstrated that reovirus-activated patient PBNK (Fig 8-5) and LNK (Fig 8-8) cells exhibited cytolytic activity against SW480 and SW620 cell targets, their cytotoxic potential against autologous tumour was next assessed.

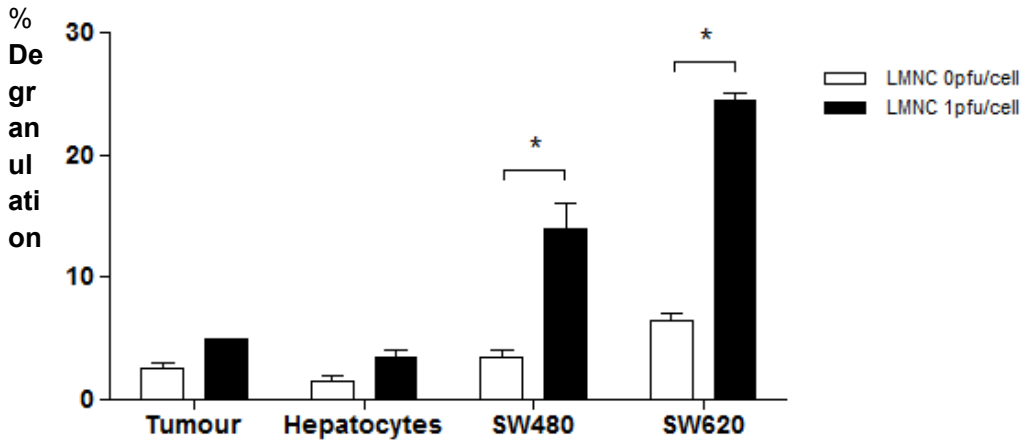


Figure 8-9 Cytolytic Activity of Reovirus-activated Patient LNK against Autologous CRLM and Hepatocytes

LMNC were isolated from the liver parenchyma of patients with CRLM and cultured overnight with 0 or 1pfu/cell reovirus in the presence of HS. Single cell suspensions of hepatocytes and CRLM cells were isolated from autologous freshly resected normal liver parenchyma and colorectal liver metastases, respectively. LMNC were then co-cultured with CRLM or hepatocyte at a 1:1 ratio for 4 hr in the presence of anti-human CD107a and CD107b antibodies. After subsequent staining with anti-human CD3 and CD56 antibodies, cell-surface expression of CD107 within patient LNK was then determined by flow cytometry. Graph shows mean percentage of CD107 degranulation + SEM of 2 separate experiments. Statistical significance is denoted by *$p<0.05$ (paired Student's t test).

Fig 8-9 demonstrates the cytolytic activity of LNK cells against autologous CRLM cells and hepatocytes. Whilst low levels of background degranulation were seen against autologous tumour, a possible trend towards higher levels of degranulation against tumour by reovirus-activated LNK was seen (2.5% vs 5%). It is worth noting, however, that the levels of LNK degranulation in response to autologous tumour were lower than those seen in patient PBNK (Fig 8-5) Very low levels of cytolytic activity were demonstrated against hepatocytes (1.5% vs 3.5%). This graph also includes degranulation against SW480 and SW620 (as shown in Fig 8-8) for comparison; again further chromium release assays were not possible due to inefficient labelling of primary tumour and hepatocyte samples.

8.4 Discussion

Colorectal cancer has been shown to be immunosuppressive in its own right, with tumour tolerance beginning in the local peri-tumoural environment. As the disease progresses, this suppression gradually involves the entire immune system (reviewed by Zou, 2005). A reduction in the rate of PBMC proliferation and reduced production of Th1 cytokines, such as IFN-γ, IL-2 and TNF-α seen in patients with Stage III/IV disease, represents a systemic suppression of cell-mediated immunity (Evans *et al.,* 2010). The reduced levels of cytolytic activity seen in the NK cells isolated from patients with CRLM when compared to healthy donors (Figs 8-2 and 7-1) may be a reflection of gene variations and alterations in NK cell signalling described in cancer patients. It has been demonstrated that NK cell activity against K562 and DLD-1 colorectal cancer cells is reduced in patients with metastatic colorectal cancer (Nüssler *et al.,* 2007).

Reovirus treatment of PBNK isolated from CRLM patients induced significant up-regulation of CD69 in the presence of HS, albeit at lower levels that those seen in healthy donor PBNK (Fig 8-1). As with healthy donors, no significant increase in the level of the Fc receptor, CD16 or in the NCRs NKp30, NKp44 and NKp46, was demonstrated after reovirus treatment. Likewise, the level of DNAM-1 expression following overnight co-culture with reovirus did not significantly increase. The finding that CCR7 did not significantly increase after culture with reovirus differs from the healthy donor group. This serves as further potential evidence of the heterogeneity of genes associated with NK cell signalling and cytotoxicity in patients with advanced colorectal cancer (Xu *et al*., 2011).

The cytolytic activity demonstrated by patient PBNK cells after activation with reovirus does, however, have important potential clinical implications, particularly in the context of metastatic disease. Up to 50% of those undergoing curative intent resection for CRLM develop intra-hepatic recurrence (Nordlinger *et al*., 1996; Kin *et al.,* 1998). Hepatic micro-metastatic disease, defined as discrete microscopic tumour cells or clusters of cells within the hepatic parenchyma or portal tracts surrounding the dominant macroscopic hepatic

tumour, have been implicated as one of the main causes of recurrence. As well as this, numerous studies have focused on the detection of circulating epithelial tumour cells in the peripheral blood of patients diagnosed with colorectal cancer (Wyld *et al.,* 1998; Zhang *et al.,* 2005). More recently, circulating tumour cells have been shown to predict progression-free and overall survival for both ovarian and colorectal cancer (Poveda *et al.,* 2011; Cohen *et al.,* 2009). The potential, therefore, to activate PBNK cells with *i.v.* reovirus in patients with metastatic colorectal cancer to kill circulating tumour cells represents a further interesting, potential, therapeutic strategy.

The successful disaggregation of tumour and liver tissue to provide *ex-vivo* CRLM and hepatocytes enabled phenotypic evaluation of these cell populations (Fig 8-3). It is noteworthy that freshly resected tumour cells expressed lower levels of cell-surface JAM-A than the SW480 and SW620 cell lines (Figs 8-4 and 4-1, respectively). This reduced surface expression correlates with a previous report where primary colorectal tumours and CRLM displayed aberrant distribution of JAM-A (van Houdt *et al.,* 2008). This poses an obvious problem; lower cell-surface expression of JAM-A will potentially reduce the ability of reovirus to bind to the cell surface, thereby lowering any direct oncolytic potential. Therefore, the immune-mediated effects of reovirus infection, particularly in the context of NK cells, represent a further strategy for increasing anti-tumour efficacy.

In the liver, JAM-A has been shown to modulate E-cadherin in hepatocytes, playing a critical role in tight junction integrity and hepatocyte adhesion (Konopka *et al.,* 2007). One area of concern regarding the use of reovirus as an oncolytic agent has been hepatotoxcitiy. Tissues which can undergo de-differentiation and replication, such as the liver, have been shown to be more susceptible to reovirus infection (Piccoli *et al.,* 1990). In murine models, reovirus replication within hepatocytes and Kuppfer cells is enhanced with concomitant hepatic insults such as surgical trauma or the use of hepatotoxins but the pattern of replication seen has been shown to be dependent on the timing of the inoculum (Rubin *et al.,* 1990). Hepatocytes were shown to express very low levels of JAM-A (Fig 8-4); however

reovirus-induced cell death was not demonstrated upon infection (data not shown). Moreover, none of the early clinical trials utilising reovirus have demonstrated significant hepatotoxicity, although the combination of surgical insult associated with hepatic resection, coupled with the hepatoxic effects of chemotherapy agents such as oxaliplatin, will need to be considered carefully if reovirus is to be administered concomitantly with cytotoxic agents in any future clinical trials prior to liver resection.

In keeping with previous reports, LNK cells were demonstrated to differ in comparison to PBNK. They are more numerous and the proportion of NKT cells was also shown to be higher (Fig 8-6). NKT cells have previously been described as featuring in the millieu of immune cells within the liver. This group of heterogeneous T lymphocytes recognise the lipid antigens presented by the non-classical MHC class I-like molecule, CD1, and express αβ or γδ TCR as well as various NK receptors, such as CD16, CD69 and CD161 (Gao *et al.,* 2009). In human liver, NKT cell numbers vary greatly, accounting for approximately 5-25% of hepatic lymphocytes (Doherty *et al.,* 2000). They play an important role in regulating both innate and adaptive immunity by the production of cytokines such as IFN-γ. It is the close interaction between these immune cells as well as the enrichment and activation of NK and NKT cells within normal healthy liver that are likely to play a key role in immune surveillance and tumour cell clearance within the liver.

It has also been shown that LNK cells show marked phenotypic and functional differences to PBNK cells, possessing higher cytotoxicity against tumour cells, higher numbers of vesicles and granules, along with higher levels of TRAIL, perforin and granzyme B (Vanderkerken *et al.,* 1990; Tu *et al.,* 2011; Vermijlen *et al.,* 2002). A large population of LNK cells also express DC markers such as CD11c and these cells display increased cytotoxicity against tumour cells and a greater IFN-γ response, compared with $CD11c^-$ NK cells (Burt *et al.,* 2008; Taieb *et al.,* 2006). The finding that LNK cells demonstrated reduced surface expression of CCR7 upon reovirus infection may be a reflection of their role as *in situ* innate immune cells (Figure 8-7), whereas PBNK cells migrate to lymph nodes to exert their

immune effects. Further evidence of the migratory capacity of peripheral NK cells is seen in the ability of bone marrow-derived NK cells to migrate to the liver where they can undergo further differentiation into liver specific NK cells (Vanderkerken *et al.,* 1993).

The ability of reovirus to activate LNK cells in patients with CRLM (Fig 8-7) has not been described previously. Indeed, little is known about how LNK cells respond to viral infection. Much of the work examining LNK cells has focused around hepatitis infection and in this context, activated LNK cells have been shown to inhibit hepatic fibrosis by their cytotoxic effect against hepatic stellate cells, the major cell type implicated in the development of hepatic fibrosis (reviewed by Gao *et al.,* 2007). It has also been shown that Fas/Fas ligand and NKG2D/NKG2D ligand-mediated killing of LNK cells contributes to hepatocyte necrosis in virus-induced liver failure (Zou *et al.,* 2010).

The finding that reovirus can activate LNK cells (Fig 8-7) without seemingly causing significant hepatotoxicity as demonstrated in patients undergoing hepatic resection following reovirus infusion (Adair *et al.,* 2012b), has important clinical applications. Increasing the cytolytic potential of LNK cells using reovirus may prove beneficial for the treatment of micrometastatic disease which is not detectable using contemporary radiological imaging. It is important to note that neither patient reovirus-activated LNK nor PBNK degranulated significantly against autologous hepatocytes (Figs 8-5 and 8-9, respectively), suggesting that there may be a useful therapeutic index between reovirus-mediated innate immune stimulation against tumour and surrounding normal cells within the liver. It is also noteworthy that primary hepatocytes were observed to express very low levels of the reovirus receptor, JAM-A, whereas almost 100% of primary metastatic tumour cells expressed the receptor on their cell surface (Fig 8-4).

Taken together, these data highlight that, despite the acknowledged immunosuppression associated with cancer (reviewed by Kim *et al.,* 2006), both circulating and hepatic innate

effector cells from patients with CRLM can be activated by reovirus, even in the presence of neutralising anti-reovirus antibodies, to become potentially cytotoxic against tumour cells.

9 Conclusion

Colorectal cancer remains a significant clinical problem, with approximately 600,000 people dying from the disease worldwide in 2008 (www.who.int/en/). Whilst survival rates have undoubtedly improved in recent decades, the morbidity associated with surgery, radiotherapy and contemporary cytoxic agents remains a significant problem (reviewed by Ooi *et al*., 1999). The development of biological response modifiers for the treatment of colorectal cancer has been the focus of recent investigation. Cetuximab is now recommended as first line treatment for resectable primary colorectal cancer and inoperable CRLM where the primary tumour is resectable and the metastatic disease is confined to the liver (www.nice.org). Given the finding that overall survival following resection for CRLM is not influenced by chemotherapy (Nordlinger *et al*., 2008), the development of new agents for the treatment of systemic disease is crucial.

Oncolytic viruses such as reovirus represent a promising new class of anti-cancer agents, which have undergone extensive testing in Phase I and II clinical trials (reviewed by Donnelly *et al*., 2011). These early results have been encouraging enough for reovirus to enter into Phase III testing in combination with carboplatin and paclitaxel chemotherapy in platinum-resistant head and neck cancer. The high incidence of *ras* mutations makes colorectal cancer a promising target for reovirus, and the recently reported REO 013 trial of *i.v.* reovirus prior to liver resection for CRLM has demonstrated repilciation competent virus in resected tumour specimens (Adair *et al*., 2012b). On the basis of these findings, a Phase I, dose escalation study combining *i.v*. Reolysin® with FOLFIRI chemotherapy in patients with advanced metastatic colorectal cancer is ongoing. (http://oncolyticsbiotech.com/clinical.html).

The current study initially focused on the direct cytopathic effect of reovirus infection upon colorectal tumour cells *in vitro* and demonstrated a dose-dependent cytotoxic effect (Fig 4-2) which was mediated by apoptosis (Fig 4-4 and Fig 4-5). The presence of NAB in human serum has been a consistent, confounding factor in the efficacy of many oncolytic virus trials including reovirus-based studies; however several studies also report anti-tumour effect

following systemic delivery (Vidal *et al*., 2008; Comins *et al*., 2010). Having demonstrated significant levels of reduction in reovirus-induced cell death against SW480 and SW620 cells when cultured in the presence of NAB (Fig 4-7), we examined peripheral blood samples from the REO 013 trial. These suggested that a reovirus signal could be detected using rtPCR from the PBMC of patients having had a Reolysin® infusion 1 hour previously. This prompted the investigation of the potential of immune cells within human PBMC to act as viral carriers which could theoretically shield the virus from the deleterious effects of NAB.

Although several blood components (e.g. red cells, platelets and granulocytes) may transport viruses, we focused on the potential of mononuclear cells to act as carriers as this fraction contained both the recognised carriers (DC, T cells) and innate immune effector cells (DC, NK cells). Innate effector cells have been shown previously to be involved in the efficacy of reovirus treatment (Prestwich *et al*., 2009b; Qiao *et al*., 2008b). PBMC components were found to express JAM-A (Fig 5-1) and more importantly could bind reovirus to their cell-surface (Fig 5-2). PBMC did not, however, support viral replication (Fig 5-3).

Significantly, reovirus-loaded PBMC could 'hitch-hike' reovirus to SW480 and SW620 target cells (Fig 5-4) and virus which had been "handed off" to these target cells remained capable of replication and tumor cell killing even in the presence of HS (Fig 5-5). This suggests that reovirus carried by immune cells in patients following *i.v.* injection may be protected from neutralisation by NAB and can be successfully delivered to tumours in patients (Vidal *et al*., 2008; Comins *et al*., 2010). This theory is further supported by the REO 013 trial, where successful reovirus delivery to CRLM was demonstrated following *i.v.* administration (Adair *et al*., 2012b).

The current study also demonstrated that reovirus-loading of whole PBMC can exert immune-mediated anti-tumour effects. Reovirus treatment induced the production of pro-inflammatory cytokines and chemokines (Fig 6-1 and Fig 6.2) and led to an activated NK cell phenotype (Fig 6-3). Moreover, NK cells within whole PBMC were capable of degranulation

against (Fig 7-1), and causing lysis of (Fig 7-2 and Fig 7-3), SW480 and SW620 cell line targets in the presence of NAB. These findings were also demonstrated in PBMC isolated from patients with CRLM (Fig 8.2).

In addition to activated peripheral blood innate effector cells accessing the tumour as carrier cells, the liver itself contains virus-sensitive cells which have the potential to be activated *in situ* by reovirus, with the subsequent stimulation of anti-tumour immune effector function. The potential of this therapeutic mechanism is supported by clinical data showing a mild, transient, elevation of liver transaminases in patients treated with *i.v.* reovirus (Gollamudi *et al*., 2009; Vidal *et al*., 2008). The activation of resident innate immune effector cells may be particularly effective for targeting micro-metastatic tumours within the liver which are undetectable using conventional radiological imaging.

This study highlights that LNK from patients with CRLM, similar to patient and normal donor PBNK, are activated by reovirus to target SW480 and SW620 tumour cells and that again, the metastatic SW620 cells were more susceptible than the primary SW480 cell line (Fig 8-8). Importantly, neither patient reovirus-activated LNK nor PBNK degranulated significantly against autologous hepatocytes, suggesting that there may be a useful therapeutic index between OV-mediated innate immune stimulation against tumour and surrounding normal cells within the liver. These data also show that, despite the acknowledged general immunosuppression associated with cancer (Kim *et al*., 2006) both circulating and hepatic innate effectors from these patients with CRLM can be activated by reovirus to become cytotoxic.

In summary, this work confirms that colorectal cancer is a viable target for reovirus therapy mediated both by direct cytoxic and innate immune cell killing. Although serum can neutralise reovirus, this data demonstrates a model in which blood cells can protect and transport reovirus for delivery to target tumour cells following *i.v.* injection. These cell carriers, as well as hepatic immune cells, are activated in response to reovirus and

demonstrate an innate anti-tumour effect with no detriment to normal hepatic parenchyma. As well as supporting the further development of reovirus as a systemic treatment for CRLM, this study suggests that the rapid clearance of reovirus from the circulation which has restricted therapy in murine models, may not inevitably apply in patients treated with *i.v.* reovirus, where immune cells may act both not only as protective cell carriers but also as peripheral therapeutic effectors against tumour cells.

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