Optimising Oncolytic Virotherapy and Immunotherapy for the treatment of Disseminated Colorectal Cancer

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Colorectal cancer (CRC) is one of the most prevalent malignancies in the Western world with a 5 year survival rate of patients with metastatic disease of less than 10%. As such, there remains a pressing need for novel treatment strategies and modalities. Established treatments including anti-EGFR antibodies, for example cetuximab, have improved survival although disappointingly only 10-20% of patients obtain an objective clinical response. Advancing treatment modalities include oncolytic viruses (Reovirus, Vaccinia Virus (JX-594)), which preferentially replicate in cancer cells causing cell death and stimulate anti-tumour immunity, and BH3-mimetic inhibitors (ABT-263), which antagonise the BCL-2 family of pro-survival proteins, may enhance CRC patient survival. Harnessing the potential immune and anti-cancer effects of these treatment modalities, alone and in combination, at primary tumour and sites of metastatic and micrometastatic (for example lymph nodes) disease could form the basis of successful clinical adjuvant strategies.

This MD thesis aims to investigate the efficacy of two OV, Reovirus and JX-594 vaccinia virus against CRC. In particular, the work outlined in this thesis has examined whether i) Reovirus directly kills CRC cell lines with differential mutational status, alone or in combination with BH3-mimetics and ii) can activate immune effector cells to enhance killing of EGFR targeted cells in order to optimise the use of anti-EGFR therapy; iii) delineate the mechanism of cell death induced by JX-594 treated CRC cells, and iv) test the ability of JX-594 to activate and induce an innate immune response in the blood and lymph nodes.

Studies investigating Reovirus in combination with ABT-263 and Cetuximab were performed. ABT-263 in combination with Reovirus demonstrated that this strategy did not yield enhanced killing over either agent alone, with only additive effects observed in a single cell line, SW620. However, initial studies combining Cetuximab with OV did reveal the potential of Reovirus to increase EGFR-mediated ADCC against a KRAS mutant cell line, SW480,

demonstrating the importance of OV immune activation in combination therapeutic approaches. To date, the immune potential of reovirus is well recognised however, less is known about the immune potential of JX-594, an OV currently in clinical testing at Leeds Teaching Hospitals NHS Trust. To test the ability of JX594 to activate immune cell populations, healthy donor blood, along with blood and lymph nodes from CRC patients, were collected and treated with JX-594. JX-594 treated NK cells from patient blood and lymph nodes demonstrated CD69 activation, enhanced degranulation and increased cytotoxicity against CRC cell line targets. In blood, NK cell activation was dependent on IFN production and the presence of $CD14^{+ve}$ monocytes however in lymph node mononuclear cells this was IFN independent and the mechanism remains to be elucidated. Importantly, OV activation of immune effector cells known to reside in LN is encouraging for targeting distant micrometastatic disease.

OV hold promise as a novel treatment modality. Direct tumour-specific lysis, transgene expression and the induction of tumour specific innate immunity in isolation, or in combination with adjunct antitumour treatment modalities, means that they may provide a two-pronged attack against the tumour at different disease sites.

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List of Abbreviations

3-MA	3-Methyladenine
µL	Microlitre
µM	Micromole
°C	Degrees Celsius
^{51}Cr	^{51}Chromium
ADCC	Antibody-dependent cell cytotoxicity
ADCP	Antibody-dependent cell phagocytosis
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
BCL-2	B cell lymphoma-2
BCL_{-XL}	B-cell lymphoma-extra large
B-GAL	β-galactosidase
BH3	Bcl-2 homology domain 3
BIM	Bcl-2 interacting mediator of cell death
BNIP-1	Bcl-2 interacting protein - 1
CEA	Carcinoembryonic Antigen
CMC	Carboxymethylcellulose
cpm	Counts per minute
CRC	Colorectal Cancer
CLL	Chronic lymphocytic leukaemia
CTL	Cytotoxic T-lymphocyte
DAMPS	Damage associated molecular patterns
DC	Dendritic Cell

ddVV	Double deleted vaccinia virus
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
Ds-RNA	Double-stranded Ribonucleic acid
DSMZ	Deutsche Sammiung von Mikroorganismen und Zellkulturen Database
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EEV	Extracellular enveloped viruses
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicine Agency
E:T Ratio	Effector:Target Ratio
ETOH	Ethanol
EV	Extracellular virus
FACS	Fluorescence-activated cell sorting (FACS)
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GAG	Glucosoaminoglycans
GFP	Green Fluorescent Protein
GMCSF	Granulocyte macrophage colony stimulating factor (GMCSF)
H&E	Hematoxylin and Eosin
HBSS	Hanks Buffered Salt Solution
HCC	Hepatocellular Carcinoma
HMGB1	High-mobility group protein B1
HNC	Head and Neck Cancer

HNSCC	Head and neck squamous cell
HRP	Horseradish peroxidase
HSV-1	Herpes Simplex Virus – Type 1
IFN	Interferons
Ig	Immunoglobulin
IHC	Immunohistochemistry
IMV	Intracellular enveloped viruses
i.p.	intraperitoneal
ISG	IFN stimulated genes
JAK	Janus Kinase
KRAS	Kirsten rat sarcoma viral oncogene
LMC	Liver Mononuclear Cells
LNMNC	Lymph Node Mononuclear Cells
MA	Methyladenine
MACS	Magnetic-activated cell sorting
MCL-1	Induced myeloid leukaemia cell differentiation protein
MEK1	Mitogen-activated protein kinase/ERK kinase
MHC	Major Histocompatability complex
mL	Millilitre
MLKL	Mixed lineage kinase domain-like protein
MOI	Multiplicity of Infection
mRNA	messenger RNA
MTT	Methylthiazoyldiphenyl-tetrazolium bromide
MV	Mature virus
NCR	Natural cytotoxicity receptors
NHS	National Health Service
NK	Natural Killer

nm	nanometer
OV	Oncolytic Virus
PAMPS	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	PBS + 0.1% (v/v) Tween
PD-1	Programmed Cell Death Protein-1
PDL-1	Programmed Cell Death Ligand-1
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein Complex
PFA	Paraformalderhyde
PFU	Plaque forming units
pg	Picograms
PI3K	Phosphoinositide 3-kinase
PKR	protein kinase R
PRR	Pattern recognition receptor
RIG-1	Retinoic Acid inducible gene 1
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute 1640
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
S.D	standard deviation
S.E.M	Standard Error of the Mean
SMN	Survival motor neuron
STAT	signal transducer and activator of transcription
T1L	Type 1 Lang Reovirus serotype
T2J	Type 2 Jones Reovirus serotype

T3D	Type 3 Dearing Reovirus serotype
TAA	Tumour associated antigens
TAM	Tumour associated macrophages
TAN	Tumour associated neutrophils
Th1	T-helper 1 cell
TK	Thymidine Kinase
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis induced ligand
T-Vec	Talimogene Laherparepvec
VACV	Vaccinia Virus
VARV	Variola Virus
VEGF	Vascular endothelial growth factor
VGF	Vaccinia Growth Factor
VSV	Vesicular Stomatitis Virus
v/v	Volume/Volume concentration
WBC	White blood cells
WHO	World Health Organisation
WR	Western Reserve
w/v	Weight/Volume Concentration
YFP	Yellow fluorescent protein
ZVAD-FMK	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

Chapter 1
Introduction

1.1 Colorectal Cancer

1.1.1 Epidemiology

After breast and lung cancer, colorectal cancer is the third most prevalent cancer in the UK. However despite this it remains the second leading cause of cancer death[1,2]. Globally there were 1.36 million new cases diagnosed in 2012 with incidence rates being reported to have increased by up to 29% since 1975. By 2030, the burden of colorectal cancer is expected to increase to more than 2.2 million new cases and 1.1 million deaths.[3] Bowel cancer is more prevalent in males than females with a lifetime risk of developing bowel cancer in the UK quoted as 1 in 14 in men and 1 in 19 in women[4]. Increasing age is a prominent risk factor with approximately 75% of bowel cancer cases occurring in patients over 65 years of age[5].

1.1.2 Staging

In 1932, Cuthert Dukes (1890-1977) published his seminal paper describing a staging system for rectal cancer. With the addition of some small modern additions, his work continues to this day to provide a simple and reproducible method of staging colorectal malignancy providing the bedrock for prognostic and therapeutic strategies in the field. The four stages, are summarised in Table 1.1.

Dukes A	Confined to the mucosa and submucosa
Dukes B	Invasion through the muscularis without lymph node involvement
Dukes C	Invasion through the muscularis with regional node metastasis
Dukes D	Tumour metastasis to distant sites

Table 1.1 Dukes staging of Colorectal Cancer. [6]

As Dukes originally observed, there continues to be a strong association between disease staging and patients expected five-year survival rate. The overall five-year relative survival of colorectal cancer patients in England is 50.7%, however, 93.2% of patients diagnosed with Dukes A disease survived five-years from diagnosis compared to only 6.6% of those with advanced disease (Dukes D). Whilst Dukes A cancer therefore represents a good prognostic outcome, currently only approximately 13% of those patients diagnosed with a colorectal cancer are Dukes A. With the implementation of national bowel screening in December 2009 this figure is set to increase however there remains a pressing need for novel treatment modalities to increase the therapeutic armamentarium to treat late stage metastatic colorectal cancer [5].

1.1.3 Metastatic Colorectal Cancer

The word ‘metastasis’ has its derivation in the Greek words ‘meta’ meaning next to and ‘stasis’ meaning placement, which translates together into change of place or displacement. Simplistically, the process of metastasis can be summarised as ‘a complex process in which a specific population of cancer cells within the primary can adapt to selective pressure which allow those cells to spread, invade and flourish in hostile, non-native environments’ [7].

1.1.4 Metastatic Dissemination

In 1889, a British Surgeon by the name of Stephen Paget proposed one of the early theories of metastatic dissemination. His theory was based on the observation and analysis of 735-autopsy records of women with metastatic breast cancer. Paget observed that there was a non-random involvement of organs by associated metastatic disease that did not occur by chance and a result hypothesised the seed and soil theory. In this model, certain favoured tumour cells with metastatic activity ('seeds') will have a special affinity for the growth-enhancing milieau within specific organs ('soil') and therefore the site of metastasis will depend on the affinity of the tumour for the microenvironment [8].

There has been a great body of work since then focussing on the mechanistic aspects of tumour spread involving tumour biology, lymphangiogenesis, immunology and simply the anatomical proximity of primary tumours to drainage systems, a good example being colon cancer metastasising to the liver via the portal system. It is beyond the scope of this work to review all the mechanistic literature, however, Ribatti and colleagues[8] provide a useful summary of the principle components required for tumour spread. These are:

- Neoplasms are biologically heterogeneous and contain subpopulations of cells with different angiogenic, invasive and metastatic properties.
- The process of metastasis is selective for cells that succeed in invasion, embolisation, survival in the circulation, arrest in distant capillary beds and extravasation into and multiplication within distant organ parenchyma.
- Metastatic outcome depends on multiple interactions of the metastastic cells within host homestatic mechanisms of which the tumour cells can escape.

1.1.5 Lymph Nodes

Cancer related mortality associated with colorectal neoplasia is inextricably linked to the stage of disease at diagnosis with those patients with lymph node metastasis found at the time of surgery demonstrating an adverse prognosis. Accurate radiological and histological lymph node staging is therefore crucial

not only for risk stratification but also to guide targeted therapeutic strategies. Other factors conferring a worse prognosis include peritoneal invasion, perforation, extramural venous invasion, incomplete resection and extensive extramural spread.[9,10]

Of note, there is a 25% recurrence rate in patients reported to have tumour negative lymph nodes suggesting that nodal staging may often be inadequate by traditional hematoxylin and eosin (H&E) staining or alternatively other factors are at play.[11] Focussing on lymph nodes, surgical resection undoubtedly plays a pivotal role on patient outcomes with a study in 2006 of 80,000 colorectal cancer patients in the United States demonstrating that resection of at least 15 lymph nodes during colectomies was associated with a 54-month increase in the median survival of patients with node negative disease.[12]

Treatment strategies are discussed in more detail later in this chapter. Typically node positive colon cancer patients (Dukes C) will receive adjuvant chemotherapy, the evidence for which is largely undisputed with adjuvant treatment in this patient cohort proving to be cost effective, while demonstrating a reduction in recurrence and mortality and increasing disease free survival.[13] The role of adjuvant therapy in those patients with node negative disease (Dukes B) remains less clear and is based on individuals risk stratification. Factors that are considered may include surgical and histo-pathological lymph node yields, levels of primary tumour extramural spread and most relevant to this work, the detection of occult tumour cells in the surrounding lymph nodes, commonly referred to as micrometastasis.[14,15]

Micrometastasis are defined by the American Joint Committee on Cancer (AJCC) as cohesive deposits of tumour cells of 2mm or less but larger than 0.2mm. Since the first reports regarding micrometastasis were published there has been fierce debate as to the prognostic impact of these cells on patient outcomes. Indeed, it is estimated that only 0.05% of circulating tumour cells may survive to initiate a metastatic focus [16]. However, a recent meta-analysis by Sloothak and colleagues [15] concluded that the detection of micrometastasis was associated with an increased risk of cancer recurrence in patients with

stage II colorectal cancer and recommended adjuvant therapies be initiated in these patients.

There is an increasing drive to use enhanced methods to increase the rate of detection of these cells. Serial sectioning, immunhistochemistry (IHC) using cytokeratin markers, reverse transcriptase polymerase chain reaction (RT-PCR) and more recently sentinel lymph node mapping have all increased diagnostic yields. Combined data from 16 studies reviewed by Sirop et al. (2011)[17] revealed an overall micrometastasis prevalence rate of 26.5% (24.7% with IHC, 36.6% with RT-PCR) with most studies in the analysis suggesting that the presence of micrometastasis carried a worse prognostic outcome when compared with node negative disease. These finding are supported by Ueda et al. (2013) who analysed thirteen clinicopathological variable and 5 biological markers in stage II colorectal cancer and identified lymph node micrometastasis and lymphatic invasion to be independent prognostic factors with respect to 5 year survival in patients diagnosed with stage II colorectal cancer.[18]

Limited data exist with regards to the benefit of treating patients with proven micrometastatic disease with regards to survival benefit. Interestingly, a pilot study of 106 patients who underwent sentinel lymph node mapping at the time of surgery concluded that patients diagnosed with micrometastasis, and treated with chemotherapy as a result of being considered as having 'high-risk' disease showed improved outcomes.[17] Conversely, a recent study of 2000 colorectal cancer patients in the United States demonstrated no benefits of adjuvant chemotherapy in patients with stage II disease regardless of the presence or absence of poor prognostic features.[19]

Despite mixed reports in the literature, it seems clear that traditional staging procedures provide insufficient information to identify those high-risk patients who may or may not benefit from adjuvant therapy. Minimal residual disease certainly acts as a strong prognostic predictor of patient survival and could be a used to guide and stratify individualised treatment strategies for these patients. Further clinical trials are certainly needed, however it is clear that the presence of occult disease in the form of micrometastasis in lymph nodes or

simply circulating tumour cells could provide exciting new targets for novel treatment strategies.[20]

1.2 The Immune System

1.2.1 General Principles

The immune system is an organisms primary defence system and comprises of an array of biological processes and structures that allow an organism to protect itself against disease. Fundamentally the immune system works by distinguishing 'self' from 'non-self'. The immune system consists of two basic arms, the innate and adaptive immune response. Functioning as 'the first line of defence', the innate immune response is non-specific with no immune memory and responds to foreign stimuli in a generic fashion. Adaptive (acquired) immunity creates immunological memory following an initial response to a specific pathogen. Accordingly, subsequent encounters with the same pathogen are met with an enhanced or primed host response. Adaptive immunity is the basis of vaccination, whereby a low volume pathogenic trigger primes immunological memory and lasting immunity.[21]

Broadly speaking the innate immune system initiates an acute primary inflammatory response stimulated and regulated by a range of cytokines, prostaglandins and complement proteins. Inflammation acts as a physical barrier against the spread of infection and promotes healing of damaged tissues following pathogenic clearance. Antigen presenting cells including dendritic cells (DC's) and macrophages, express pattern recognition receptors (PRR's) such as Toll-like Receptors (TLR) or RIG-1 like receptors (RLR) on their cell surface, in endosomes and within their cytoplasm. PRR's recognise specific small molecular motifs expressed by foreign pathogens known as pathogen associated molecular patterns (PAMPS) and/or biomolecules (nuclear or cytosolic proteins, tumour DNA, HMGB-1) released outside cells or exposed on the surface of cells following tissue damage, known as damage-associated molecular patterns (DAMPS). Receptor activation by a PAMP/DAMP triggers a plethora of signalling cascades leading to gene transcription, release of inflammatory mediators and immune cell recruitment and activation.[22]

The adaptive immune response is highly pathogen specific, creates immunological memory and provides long lasting cell protection. The adaptive system uses specialised leukocytes derived from multipotent haematopoetic stem cells of the bone marrow called lymphocytes. Two broad classes are responsible for the antibody (humoral) response and the cell-mediated response, B cells and T cells, respectively. Adaptive immunity relies on the capacity of immune cells to distinguish between so called 'self' and foreign antigens. Antigens, are recognised by major histocompatibility complex (MHC) and professional APC's such as DC, B-cells and Macrophages. MHC class I and II molecules found on the surface of APCs mediate the process of antigen presentation. MHC class I and class II are similar in function and act to deliver short peptides to the cell surface for recognition by cytotoxic $CD8^+$ and helper $CD4^+$ T cells respectively. Endogenous (intracellular) peptides bind to MHC-I and exogenous (extracellular) peptides bind to MHC-II. APC's express antigen MHC complexes, along with co-stimulatory ligands on their cell surface which are recognised by specific T-cell receptors (TCR) to activate specialist T-cell subgroups as described above.

Virus infection triggers an endogenous antigen response secondary to intracellular viral replication. After virus destruction, viral antigens are presented to naïve cytotoxic T cells by coupling of their TCR with a peptide bound MHC class I leading to activation of cytotoxic $CD8^+$ T lymphocytes (CTL). Effector CTL release perforin and granzyme leading to apoptosis and cell death.

Exogenous antigens (bacteria and toxins) are engulfed by DC which migrate via chemotactic signals to lymph nodes rich in T cells. Following a process of maturation and pathogenic destruction, DC's display the non-self antigen on their surface and form an MHC:antigen complex (MHC-class II for exogenous antigens) which activates a $CD4^+$ T Helper 1 (TH1) or T Helper 2 (Th2) cell response depending on the antigen (Figure 1.1). Helper T cells release interleukins amongst other cytokines, which stimulate B cells to produce antibodies.

Humoral, adaptive immunity is governed by B cells which are the major cell type involved in the creation of immunoglobulins. B cells express a unique B

cell receptor (BCR) which recognise antigens in their native form and trigger a series of events leading to antibody production by terminally differentiated plasma cells. Antibodies recognise unique antigens and form antigen-antibody complexes which coordinate the induction of several cell death mechanisms. Mechanisms include agglutination, activation of the complement cascade causing inflammation and cell lysis, opsonisation which enhances phagocytosis, ADCC (see section 1.2.3.2) and neutralisation.[23]

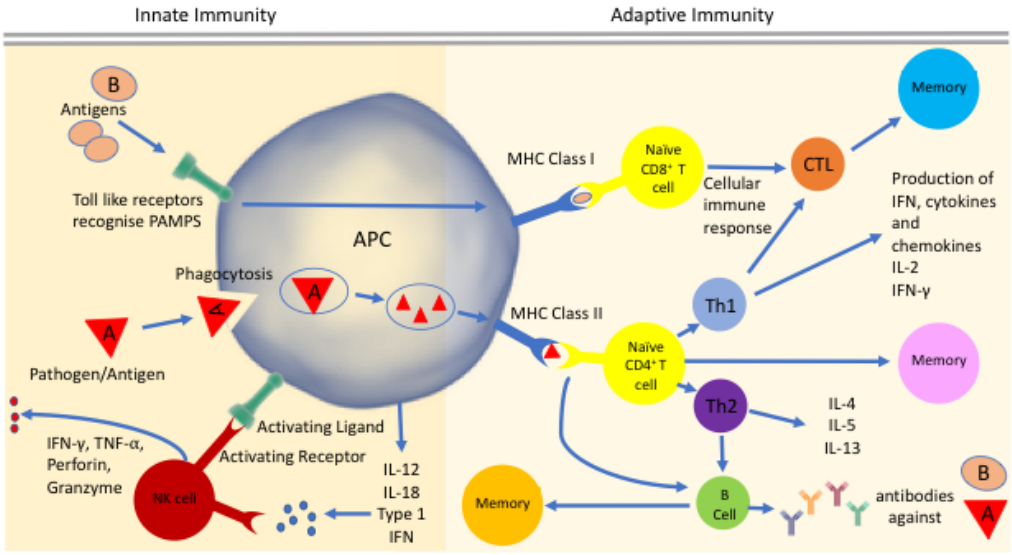


Figure 1.1 The Innate and Adaptive Immune Response; An Overview.

Innate immune mechanisms include the production of cytokines and the activation of antigen-presenting cells (macrophages and dendritic cells) and natural killer (NK) cells. Antigens are degraded into smaller peptides in endosomes/lysosomes in the APCs and are subsequently expressed on the cell surface in MHC class II peptide complexes, which can be recognized by CD4+ T helper lymphocyte cells. T helpers assist B cells to proliferate and mature into antibody-producing plasma cells. DCs can also present epitopes to CD8+ T cells. This is also known as cross-presentation.

1.2.2 Cancer and the Immune System

The role of the immune system in cancer has been the subject of debate for many years after it was first highlighted by Virchow et al. over 150 year ago. In his studies, Virchow made the crucial observation that certain cancers resulted in inflammation that were inherently associated with white blood cells. Thereafter, numerous reports of spontaneous tumour regression relating to acute concomitant infections (influenza, smallpox, tuberculosis, hepatitis and gonorrhoea) were published.[24,25] Following the observation of tumour regression in a patient with streptococcal infection in an ulcerated tumour, William Coley, the 'Father of Immunotherapy' embraced the potential relationship between cancer and the immune system. His experiments involved directly injecting tumours or metastatic deposits with a so called 'vaccine' containing a mixture of Gram negative (*Serratia marcescens*) and Gram-positive (*Streptococcus pyogenes*) bacteria. Coley's Toxin was reported to cause spontaneous regression in several tumours including lymphoma and sarcoma, however despite some positive results, treatment related deaths, reporting inconsistencies, and poorly controlled experimental design led to general criticism and disbelief from the wider scientific community.[25,26] In the last 30 years rapid advances in the understanding of immunooncology has validated many of Coley's initial observations with respect to the interplay between cancer pathogenesis and immune activation.

With regard to cancer, for the immune system to discriminate between self and non-self and mount an appropriate response it must be able to recognise differences in the antigenic make-up between transformed cells such as tumours, and normal cells. As such the underlying relationship between cancer and immunity, is for the immune system to survey for the development of malignancy and eliminate tumour cells when they arise. The three principals involved include detection of non-self-antigens from malignant cells, effector functions to target and destroy the malignant cell whilst protecting the host, and the development of immunological memory via the adaptive immune response. However, cancer cells arise from normal host cells and as such are recognised by the immune system as self or altered self-antigens.

As a direct result, the immune system is now thought to play a dual role in cancer, the paradigm known as immunoediting, which provides a positive and negative balance of immune surveillance, encompassing the recognition of altered self and tumour elimination with cancer progression.[27] The multi-faceted mechanism consists of three phases, elimination, equilibrium and escape where the immune system can suppress tumour growth by destroying or inhibiting tumour cell development, and promote tumour progression either by selecting for tumour cells with acquired resistance or genetic modification, or enhancing conditions within the tumour microenvironment that facilitate tumour growth.[28]

One of the hallmarks of cancer is the ability of cancer to evade or escape immune response embodied by both host and tumour related mechanisms. Failure to mount an effective anti-tumour immune response has obvious sequelae for the patient in the form of tumour progression but also, significantly presents one of the key factors which limits the efficacy of anti-cancer immunotherapies. Several important mechanisms exist by which tumours evade an immune response and are summarised below.

1.2.2.1 Reduction in expression of cell surface antigens

Loss of antigen expression and resultant reduction in overall immunogenicity results from tumour cell genome instability. Genetic mutations lead to a passive loss of MHC-1 expression resulting in tumours avoiding recognition by specific TCR and reduced sensitivity to cytotoxic T cell mediated lysis. Interestingly, Rees et al. (1999)[29] described the loss of all MHC-1 molecules in some colorectal and prostate malignancies, render tumour cells completely unrecognisable by CTL's, but instead make the tumour more susceptible to NK-cell mediated killing. Complete loss of MHC-1 expression is rare and more commonly human tumours downregulate their expression to approximately 50% conferring an overall tumour cell survival benefit.[30]

1.2.2.2 Modulation of immune cell killing mechanisms

Three distinct processes ultimately induce immune cell killing of tumour cells which include i) formation of death-inducing signalling complexes leading to apoptosis (eg binding of 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 perforin and granzyme from cytotoxic granules.[31]

Metastatic CRC cells amongst others have been reported to show decreased and/or mutated levels of FasR and TRAIL-R on their cell surface resulting in tumour cell protection.[31] Interestingly, recent data has demonstrated upregulation of FasR in various tumour types which whilst making tumours more susceptible to Fas-mediated apoptosis is conversely thought to upregulate cell proliferation and differentiation, chemokine production and inflammatory responses through a series of non-cytotoxic, non-apoptotic signalling cascades leading to the promotion of tumour growth.[31]

Furthermore, with respect to Fas-mediated tumour immune protection, O'Connell et al. (1997) first described the so call 'Fas Counterattack' mechanism of immune escape in colon, melanoma, and liver cancer cells which relates to the active killing of Fas-sensitive tumour infiltrating lymphocytes (TIL) by tumour derived FasL thereby imparting an 'immune privilege' on the tumour.[32] Several findings support this theory including i) various cancer cell lines have been shown to express FasL and kill lymphocytes through Fas-mediated apoptosis *in vitro;* ii) human cancers are known to express variable amounts of Fas-L, and iii) inducing FasR-mediated apoptosis of activated leukocytes results in immune tolerance and gives immunological protection to the tumour as demonstrated in animal allograft transplantation models.[32–34]

1.2.2.3 Modification of the tumour microenvironment

Tumour can evade NK cell-mediated innate immune cell death by shedding NK ligands (e.g. NKG2D ligands; MICA, MICB and RAET/ULBP proteins) into the tumour microenvironment and as such avoiding NK cell recognition pathways. Salih et al. (2008)[35] reported that in a variety of tumours, release of

NKG2DL had been observed to interfere with NKG2D-mediated tumour immunity. Mechanisms of NK cell escape utilised by tumours include a reduction of ligand density on the tumour cell surface, decreased expression of NK receptors (described below) and blocking of the NKG2D-binding site.[36]

1.2.3 Components of the immune system

There are multiple vital components of the immune system which fall beyond the scope of this study. However, to understand this work it is important to explore the function of natural killer cells and immunoglobulins in more detail.

1.2.3.1 Natural Killer Cells

Natural killer cells are a specialized population of granular lymphocytes that are highly cytotoxic to both tumour cells and virus-infected cells. NK cells act as the first line of defense against viral infections preceding the development of an antibody response and produce significant levels of cytokines and chemokines (IFN-α, IFN-β, IL-12, IL-18).[37] Activation is dependent on cytokine production with IFN-α and β acting as potent inducers of NK-cell mediated cytotoxicity, and IL-12 resulting in production IFN-γ which induces an antiviral state. The response is regulated by a selection of activating (CD16, NKG2D, NKp30, NKp44, NKp46) and inhibitory receptors, which recognise MHC class I and MHC-like molecules on the surface of target cells. Tumour cells, and virus infected cells therefore become a natural target for NK cells as a result of them down-regulating MHC in order to evade the host adaptive immune response.[38–40]

Given the early activation of NK cells in response to a virus challenge, one would expect NK cell inhibition or depletion to enhance intratumoural spread of an oncolytic virus and enhance tumour killing though lytic pathways. Several studies have indeed validated this theory. Replication, tumour necrosis and animal survival was significantly enhanced following VSV infection in an *in vivo* model of rats bearing intrahepatic multifocal hepatocellular carcinoma (HCC) after antibody mediated depletion of NK cells.[41] A second study showed that HSV activated NK cells preferentially

lysed HSV-infected glioblastoma cells, a process that was dependent on NK cell natural cytotoxicity receptors (NCRs) such as NKp30 and NKp46. In the absence of these receptors (NCR(-/-) knockout mice) HSV titres and efficacy increased.[42]

Conversely, other studies have reported an anti-tumoural effect of NK cells after oncolytic viral treatment. Prestwich et al. (2009) compared the direct oncolytic effect of Reovirus in the resistant murine melanoma cell line, B16ova, compared with the parental cell line, B16. Resistance of B16ova was thought to be due to the absence of the Reovirus receptor, JAM-1, and unsurprisingly Reovirus replication was not detectable after subcutaneous injection of B16ova tumours in mice. Despite this, B16ova tumours regressed *in vivo,* in an NK cell dependent fashion. Further *in vitro* studies by the same group, loaded dendritic cells (DC) with Reovirus infected Mel888 cells known to secrete a broad range of chemokines, causing NK cell migration. The resultant secretion of IFN-β stimulated NK cell-mediated cytotoxicity of the Mel888 cells and concurrent NK cell recruitment caused DC maturation. Both studies together reinforce the role of NK cells in an innate anti-tumoural response.[43,44]

The complex interplay of NK cells in response to viruses serves to adequately highlight just some of the challenges faced in optimising potential oncolytic therapies. On one hand the innate immune response is seen as inhibitory to therapeutic efficacy acting to quash desired viral replication and cell lysis. However counter-intuitively an ever-increasing body of data exists highlighting the important role of oncolytic viruses to induce innate and adaptive immune responses against the tumour thereby increasing their overall therapeutic effect.

1.2.3.2 Antibody-Dependent Cell Cytotoxicity (ADCC)

ADCC is a mechanism of cell-mediated immune defence in which immune effector cells (NK cells, monocytes/macrophages, neutrophils, dendritic cells) actively lyse target cells (tumour cells, cells infected with intracellular pathogen) whose membrane antigens have been bound by specific antibodies (belonging to IgG, IgA or IgE classes). ADCC contributes to the clearance of

infected or foreign cells via the innate and acquired immune system.[45] ADCC mechanisms vary depending on the effector cells that are recruited by antibodies but broadly follow a similar path. Initially, the Fab region of an antibody recognises and binds to a specific antigen found on the surface of a target cell and the Fc region of the antibody binds to Fcγ receptors (FcγR) on effector cells. The target cell-antibody-effector cell complex activates effector cells and results in the release of preformed cytotoxic factors, such as perforin and granzyme, which destroy target cells. Synchronous immunoregulatory cytokine and chemokine release from the activated effector cell further mediates the development of an immune response.

The majority of ADCC research has involved the use of peripheral blood mononuclear cells (PBMC's) in which NK cells are the main effector. The most common FcγR expressed by NK cells is CD16 (FcγRIII) which binds to IgG bound to the surface of target cells (infected with pathogen or Ab-targeted cancer cells). Antibody-dependent NK-mediated killing occurs via several complimentary pathways: 1) exocytosis of cytotoxic granules; 2) TNF family death receptor signalling leading to apoptosis and 3) pro-inflammatory cytokine release, for example IFN-γ. IFN-γ release is reported to inhibit cell proliferation, promotes antigen presentation, increase angiogenesis, increase MHC surface expression and contribute to the upregulation of TRAIL expression on NK cells which work synergistically to promote tumour/infected cell death.[46,47] Less is known about ADCC by non NK-cell populations in peripheral blood and mucosal compartments. However, antibody-dependent cell phagocytosis (ADCP) has been described for phagocytic immune cell populations such as DC, monocytes and macrophages.[48,49]

ADCC is a rapid effector pathway whose efficacy is strongly dependent on a number of variables including the density and stability of the antigen and both antibody affinity and FcR-binding affinity. For example, ADCC involving human IgG1 (the most utilised class by therapeutic antibodies) is highly dependent on the glycosylation profile of the antibody Fc portion and efficacy is effected by FcγRIIa and FcγRIIIa polymorphisms. This variant ADCC activity allows scientists to modulate, enhance and mimic the effects of ADCC in order to develop potential novel therapeutics. Recombinant antibody engineering of the Fc region of IgG can optimise IgG1 antibodies for NK

binding and enhance ADCC. Similarly the use of bispecific antibodies that are able to bind activating molecules expressed by both tumour cells and cytotoxic cells can mimic classical ADCC in experimental studies.[50] Also see section 1.3. for antibody-induced ADCC.

1.2.3.3 Interferons (IFNs)

Interferons (IFNs) are secreted glycoproteins discovered by Isaacs et al. in 1957,[51] so called because they 'interfere' with viral replication within cells. They are a species-specific class of cytokine and have a potent antiviral effect, by allowing communication between cells to trigger the immune system to attack tumour and pathogens. There are three classes of IFN described; Type I, Type II and Type III summarised in Table 1.2. For the purposes of this thesis, this summary focusses largely on type I IFNs as they provide a pivotal component of the innate anti-viral immune response.

Type	Interferon
Type I	IFN-α, IFN-β, IFN-δ IFN-ω IFN-κ
Type II	IFN-γ
Type III	IFN-λ

Table 1.2 The three classes of IFNs.

Type I and III interferons are secreted in response to virus infection and exert their effects via Type I IFN and Type III IFN receptors respectively. Type I IFN receptor is expressed ubiquitously whereas Type III IFN receptor has a more limited distribution within tissues with receptor expression limited to cells of epithelial origin. Type II IFN's are secreted by activated immune cells for example natural killer cells (NK) and/or T-cells. Type II IFN's role is to activate

macrophages and promote a cell-mediated adaptive immune response via T-helper 1 (Th1) cells. [52]

The type I IFN response is initiated after host cell PRR such as membrane-associated TLR and RIG-1-like receptors identify viral PAMPs, for example foreign cytoplasmic RNA. Activation of the PRR stimulate a signalling cascade that initially activates transcription factors such as activator protein-1, NF-κB and IFN-regulatory factor 7 before these proteins are translocated into the nucleus and trigger the transcription of genes encoding type I IFNs, cytokines and chemokines. Initial responses are driven mainly by IFN-β but switch to IFN-α during a subsequent amplification phase of the overall response.[53]

Following secretion of the Type I IFNs, they bind to their respective receptors either on the same cell or neighbouring cells and trigger a Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling cascade leading to the production of IFN-stimulated genes (ISG) which act to confer an antiviral state. ISGs produced include protein kinase R (PKR) which inhibits protein synthesis, 2'-5'-oligoadenylates synthase (OAS) which works to degrade cellular and viral mRNA and ISG15 which conjugates a range of proteins involved in the IFN response stabilising them against degradation. Ultimately the expression of hundreds of ISGs therefore results in a coordinated antiviral response leading to an antiviral state within the cell. [54] A schematic diagram of the IFN response is shown in Figure 1.2.

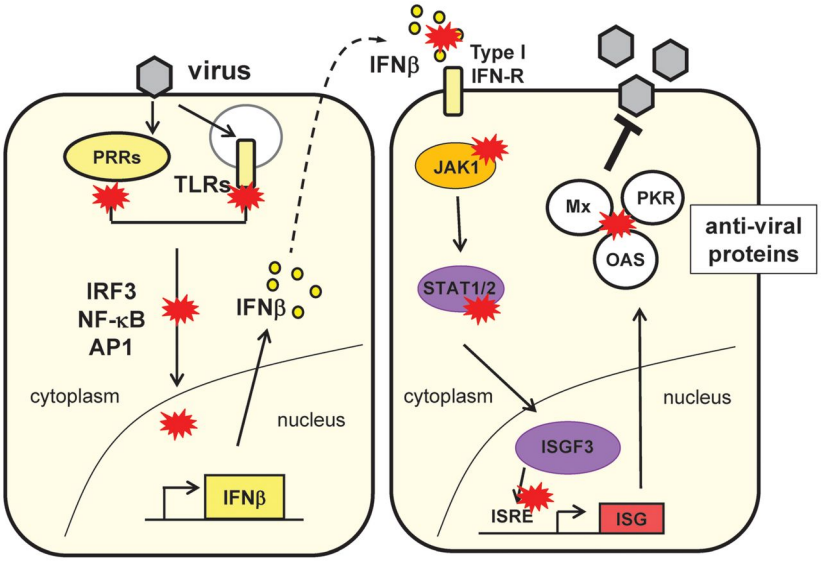


Figure 1.2 Schematic diagram to illustrate antiviral cell IFN response signalling pathway and areas in which oncolytic virus can antagonise the pathway.

Virus infection is sensed by PRRs which trigger signalling cascades leading to activation of transcription factors NF-κB, IRF3 and AP-1. On entering the nucleus they stimulate transcription of the IFN-β gene. IFN-β is secreted from the cell where it binds to the type I IFNR on the same or adjacent cells triggering activation of the JAK/STAT pathway. This leads to the assembly of the ISGF3 complex in the nucleus and the transcription of hundreds of ISGs. The positions where viral proteins can inhibit the production and/or action of IFN are denoted by red stars. This figure is adapted from a review by Haller et al. (2006).[54]

Whilst it is clear viruses stimulate a potent host cell innate immune response via the IFN pathway for successful infection to take place viruses must multiply extensively. Therefore, it is now evident that most viruses have developed means to down-regulate the host IFN response. Using Vaccinia Virus (VV) to illustrate this for the purposes of this report, inhibition of the IFN response is not only multi-mechanistic but also occurs at multiple levels with each individual VV protein potentially antagonising multiple pathways. Some mechanisms utilised by VV to evade immune clearance and allow replication are summarised below.

1. Reduced production and recognition of PAMPs

VV acts to minimise production of PAMPS primarily by minimising the production of dsRNA as a result of structural arrangements within the terminal regions of the genome. Similarly by blocking host cell protein synthesis there is a reduction and loss of IFN transcription. An example of this is the numerous intracellular proteins (A49, B14, C4, K7, M2, N1) that inhibit NF-κB activation. Finally the virus actively expresses proteins such as E3, which sequesters dsRNA via a C-terminal dsRNA binding domain thereby preventing activation of the dsRNA-binding PRR-induced protein signalling cascade.[55,56]

2. Secretion of IFN capture proteins (Decoy receptors).

B18R is an IFN-binding protein that acts both in solution and via glycosaminoglycans (GAG) at the cell surface to sequester and bind type I IFNs, preventing them from activating IFNR in both infected and uninfected cells. B18R targets IFN-α and not IFN-β which relies on more intracellular mechanisms. IFN-α is produced in large quantities by dendritic cells, which are recruited to the site of infection or inflammation and therefore requires neutralisation outside the infected cell.[57]

3. Signal transduction inhibition

VV blocks and antagonises signalling cascades induced by the binding of type I IFNs to their respective receptor complexes. An example of this is the protein VH1, which rapidly dephosphorylates STAT1 and STAT2 after concordant entry into the cell along with infecting virion.[58]

4. Blocks action of intracellular ISGs

Within the infected cell, VV encoded proteins inhibit the actions of protein transcribed ISGs. Examples include the E3 protein which binds ISG15 preventing its antiviral activity and similarly the E3 binds dsRNA to prevent the activation of PKR and OAS.[59]

The relationship between an oncolytic virus and the innate immune response is complex given scientists desire for the innate immune system to mediate part of the viruses therapeutic efficacy. Inhibition of this innate response could theoretically impair potential treatment function. Indeed in VV genetically engineered to express IFN-β (JX-795), Kirn et al. (2007) reported that the generation of anti-tumour immunity resulted in improved tumour selectivity and efficacy when compared with controls. Similarly, engineering IFN-β into Vesicular Stomatitis Virus (VSV) is reported to enhance NK cell activation and increase inflammatory cytokine production.[60,61]

Importantly, there is an active and intact antiviral innate immune response within normal cells but not tumour which prevents toxicity of virus administration. Indeed evidence suggests that severe toxicity is seen from both Reovirus and VSV in normal tissues when administered to IFN-α and IFN-β knockout mice.[62] Balancing the generation of anti-tumour efficacy with potentially unacceptable toxicity is therefore vital to the generation of successful oncolytic viral agents.

1.3 Epidermal Growth Factor Receptor (EGFR)

EGFR is a cell membrane growth factor receptor tyrosine kinase which plays a critical role in the control of key cellular transduction pathways including promoting cell proliferation and opposing apoptosis in both normal and cancerous cells. EGFR is commonly upregulated in various cancers including metastatic colorectal cancer.[63] Overexpression of EGFR is linked to the promotion of cancer progression with EGFR levels being shown to predict tumour grade, relapse rates and patient prognosis.[64,65]

EGFR mediated signal transduction is complex and beyond the scope of this work to detail in full, however Figure 1.3 shows a summary of EGFR induced and KRAS mediated signalling pathways.

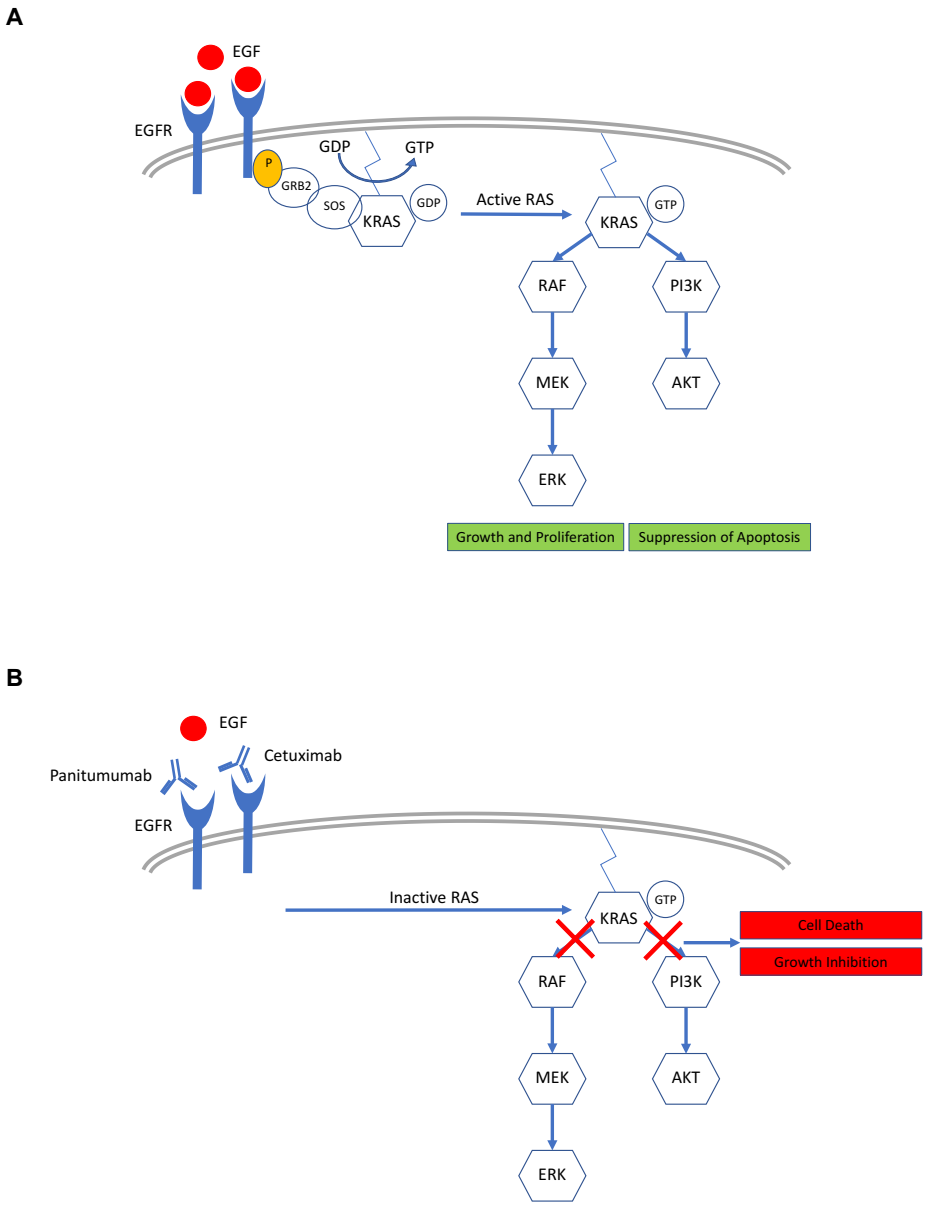


Figure 1.3 EGFR-induced and KRAS-mediated signalling pathways.

A) Activation of EGFR by ligand binding and receptor auto-phosphorylation create a docking site for the SOS/GRB2 complex, resulting in KRAS activation. KRAS then signals through the RAF/MEK/ERK and PI3K/AKT cascades to promote cell growth and suppress apoptosis. B) Cetuximab and Panitumumab, bind to EGFR preventing ligand binding and KRAS activation leading to growth suppression and cell death by inhibition of RAF/MEK/ERK and PI3K/AKT pathways. Mutant KRAS and BRAF cause constitutive activation of the pathway, leading to anti-EGFR antibodies resistance, cell growth and survival. (*Adapted from Knickelbein et al. (2015)*[66])

EGFR downstream signalling pathway are relevant given their interaction with anti-EGFR receptor inhibitors (discussed in section 1.3.1) used for the treatment of CRC and the constitutional activation of the pathway in various tumour cell types, making them an obvious target for cancer-related research. Following EGFR activation, RAS stimulation is triggered. Activated RAS promotes membrane recruitment and activation of its downstream effectors. Over 18 effectors have been reported to be controlled by activated RAS, which provides the common upstream molecule of several pathways. These include RAF/Mek/ERK and PI3k/Akt. Four RAS proteins have been reported (NRAS, HRAS, KRASa and KRASb) which show varying potencies to activate the different cascade pathways. KRAS is considered the stronger inducer of the RAF/MEK/ERK pathway while HRAS induces the PI3k/Akt pathway preferentially.[67] Importantly in human cancer, RAS demonstrates different mutational frequencies with KRAS reported as the most frequently mutated RAS isoform.[68] These mutations result in constitutive activation of the proteins and therefore no longer require ligand binding to EGFR for activation.

Downstream in the RAS-RAF-MEK pathway is the serine/threonine (S/T) kinase Raf family. Activation occurs in a complex series of events mediated by its interaction with RAS. As with RAS, mutation rates in RAF are high in certain cancers for example the BRAF mutation rates are documented as 30% in ovarian cancer, 27-70% in melanoma and 5-22% in colorectal cancer.[69–72] Mutations can occur at different locations in the BRAF protein but the most commonly reported mutation (V600E) occurs at residue number 600 and replaces valine with glutamic acid. The V600E mutation is present in almost 60% of all melanoma patients[73] and interestingly has recently been reported to be responsible for the pre-malignant transformation of benign colorectal polyps into serrated adenomas defining it as a marker and risk factor for the progression of colorectal cancer.[74]

RAF phosphorylation ultimately regulates the activity of mitogen-activated protein kinase/ERK kinase (MEK1). Once activated, the cascade is reported to play a critical role in apoptosis by phosphorylating various apoptotic regulatory factors such as Bas, Bim, Mcl-1, caspase 9 and BCL-2. Furthermore the pathway also influences cell cycle regulation, cell differentiation, migration, metabolism and proliferation.[75]

PI3K can also be activated as a result of ligand binding to EGFR. The signalling cascade induced ultimately recruits Akt, the primary mediator of PI3K-initiated signalling. Akt acts to mediate both the activation of certain substrates (CREB) or the inactivation of others (BRAF, Bim, BAD and Procaspase 9). Interestingly, the pathway also includes several phosphatases which inhibit the growth-promoting effect of PI3k by removing phosphates from PIP2 and PIP3 as such the genes encoding them (e.g. *PTEN*) are referred to as tumour suppressor genes.

1.3.1 Anti- EGFR Receptor Inhibitors

Two pharmacological approaches have been delineated to target and inhibit EGFR functions for cancer treatment, which include anti-EGFR monoclonal antibodies and small molecule tyrosine kinase inhibitors (not discussed further). Anti-EGFR antibodies are competitive antagonists against EGFR which act by occluding the ligand binding region of the extracellular binding domain. Anti-EGFR monoclonal antibodies are highly selective, recognising EGFR exclusively, however resistance to these agents is well documented and is thought to relate to constitutive activation of downstream mediators as a result of mutational variations. In an unselected patient cohort only 10% will be responsive with mutations in the KRAS gene, causing constitutive pathway activation and treatment failure accounting for up to 40% of resistant patients.[76]

To date two anti-EGFR monoclonal antibodies, cetuximab and panitumumab are in mainstream use for metastatic CRC therapy.

1.3.1.1 Cetuximab

Cetuximab (Erbitux™) is a chimeric monoclonal IgG1 antibody composed of variable regions (Fv) of a murine anti-EGFR antibody, which displays high affinity for EGFR, and a constant region (Fc) of a human IgG1 kappa immunoglobulin. Cetuximab is now in widespread clinical use with evidence supporting the first and second line use of Cetuximab as an option for previously untreated epidermal growth factor receptor (EGFR)-expressing,

RAS wild-type, metastatic colorectal cancer in adults in combination with either FOLFOX (5-fluorouracil, folinic acid and oxaliplatin) or FOLFIRI (5 fluorouracil, folinic acid and irinotecan) and third-line as a monotherapy.[77]

Importantly in this patient cohort, retrospective biomarker analysis confirmed KRAS mutation status as an effective predictive biomarker although disappointingly the authors were unable to draw any definitive conclusions with respect to BRAF mutation status due to the small numbers of BRAF mutant genotypes found in the cohort (11 of 309 patients).[78,79] However, analysis of an alternative treated patient cohort comparing objective tumour responses, progression free survival and overall survival following Cetuximab or Panitumumab treatment by Nicolantonio et al. (2009) showed that in the presence of BRAF V600E mutations there was no response. The authors hypothesised that combination strategies to target both EGFR and BRAF may yield interesting outcomes.[80]

Cetuximab's principle mode of action is to inhibit EGFR signalling and by doing so reduce cell proliferation, angiogenesis and cell survival, however in addition Cetuximab is reported to induce ADCC (see section 1.2.3.2) by recruiting immune effector cells and also acts to induce apoptosis by increasing the expression of pro-apoptotic proteins such as Bax and caspase 3 or by inactivating anti-apoptotic proteins for example BCL-2.[81]

ADCC is triggered by the binding of the Fc region of Cetuximab, which is already bound via Fv regions to the target cell, to any of the Fcγ receptors expressed by cells of the innate immune system (for example CD16 on NK cells). The role of specific immune cell types to the anti-tumour ADCC response exerted by anti-EGFR monoclonal antibodies remains unclear. General consensus points to NK cells being the primary immune effector with *in vitro* experiments demonstrating that Cetuximab is able to mediate NK-dependent ADCC with the magnitude of response directly related to CD16 (FcγRIII) polymorphism.[82,83] Conflicting evidence however exists with respect to whether high affinity or low affinity FcγR polymorphisms are required to improve clinical outcomes. Studies by Bibeau et al. (2009)[84] and Lopez-Albaitero et al. (2009)[83] reported longer progression free survival in patients with higher affinity FcγR polymorphisms in metastatic colorectal patients

treated with cetuximab and irinotecan. Conversely, the opposite association was found by Zhang et al. (2007)[85] and Dahan et al. (2011)[86] with better outcomes reported in patients with low affinity polymorphisms. Direct comparison, however, is limited given significant study variation in the underlying number of patients expressing KRAS mutations and other studies incorporating concurrent alternative antibody treatments alongside cetuximab. The impact of these variations is unknown but could arguably account for some of the differences seen in evaluating the impact of FcγR polymorphisms on Cetuximab efficacy.[87]

1.3.1.2 Panitumumab

Panitumumab (Vectibix™) is a humanized IgG2 kappa monoclonal antibody. Like Cetuximab, in the United Kingdom its use is recommended by NICE and the European Medicines agency for use in untreated, RAS wild-type metastatic colorectal cancer in adults as a monotherapy (third-line) or in combination with FOLFOX or FOLFIRI. There is also good evidence that the response to Panitmumab is also limited to only those patients with wild-type KRAS tumours.[88]

Like Cetuximub, Panitumumab works prinicipally via competitive inhibition of the EGFR thus blocking downstream signalling cascades, however it also has been shown to activate an immune response. As an IgG2 anti EGFR antibody, ADCC is induced via cells of myeloid lineage (neutrophil and monocytes). Panitumumab is less effective at inducing NK-dependent ADCC which is likely related to reduced avidity/preference of IgG2 immunoglobulins for CD16 when compared with IgG1.[89,90]

1.3.1.3 GA201

GA201 is a novel anti-EGFR monoclonal antibody designed to have enhanced ADCC properties. GA201 was developed from the humanisation of the rat ICR62 antibody with the Fc region glycoengineered to contain afuscosylated carbohydrates to encourage enhanced binding to FcγRIIIA. Initial *in vitro* data

demonstrated that GA201 had a similar efficacy to Cetuximab, inhibiting EGF ligand binding, downstream signalling and cell proliferation.

Interestingly, GA201 exhibited superior binding to both high and low affinity variants of FcγRIIIA, which when compared with Cetuximab, resulted in enhanced induction of ADCC against both wild-type KRAS and mutant KRAS tumour cell lines. *In vivo* experiments using a variety of immunocompetent mouse xenograft models (pancreas, colorectal and breast) also showed a significant therapeutic benefit of GA201 compared with Cetuximab in both KRAS wild-type and KRAS mutant tumours with variable EGFR expression. Furthermore the efficacy of GA201 was potentiated further when combined with Irinotecan chemotherapy.[91]

With clinical responses of approximately 15% across all sub classes of anti-EGFR monocloncal antibodies, only modest overall survival benefits when compared with best supportive care, and increasingly robust cost-benefit analysis of novel therapies being undertaken by many countries, the challenges are clear. The future of monoclonal antibody therapy is to identify biomarkers and individualise treatment strategies to allow clinicians to predict and target only eligible patients. Strategies should incorporate not only the mechanistic effects of targeting EGFR and downstream signalling pathways but also the potential benefit of exploiting antibody immune potential.[92]

1.4 Oncolytic Viruses

1.4.1 Background

In the late nineteenth century and into the early twentieth century cancer therapy as a field of medicine was wholly reliant on surgical excision with a clear push towards alternative treatments. Viruses as an option for the treatment of cancer therefore developed in a somewhat innocuous fashion under the observational premise that in some cases cancer patients who contracted an infectious disease went into brief periods of clinical remission. Particular examples included cases of leukaemia where it became well

recognised that the contraction of influenza virus could in some cases demonstrate beneficial effects[93].

Thereafter, throughout the 20th century viruses have been the focus of fluctuating attention in the quest to find novel experimental agents for the treatment of cancer. Interest initially peaked in the 1950's and 60's with the evaluation of the oncolytic properties of numerous viruses including Bunyamera, Ilheus, West Nile Virus, Semliki Forest Virus and adenovirus to name a few being explored in human tumour cell lines and then immunosuppressed rats. Proof of concept of the 'oncolytic virus' was driven by Moore et al.[94] who using a novel *in vivo* tumour model in mice demonstrated that the virus, Russian Far East Encephalitis Virus, could selectively target and kill cancer cells (Sarcoma 180), and in some cases completely destroy it. Unsurprisingly, however, infection did eventually cause fatal encephalitis in the treated mice[94,95].

Over the next two decades little to no research progress was made, however in the last fifteen years developments in virology, genetic manipulation and molecular biology have enabled there to be a resurgence in the field. Recombinant technology has allowed researchers to genetically engineer viruses to enhance their safety profile by selectively targeting tumours cells, an approach first published by Martuza et al. (1991) using a herpes simplex virus type one (HSV-1) in an experimental glioma model[96]. Thereafter, a huge wave of interest has driven both pre-clinical and clinical research. Investigation of viruses for oncolytic and/or anti-tumour properties, and the potential interactions between viruses and the immune system, continues unabated with clinical grade viruses now reaching phase III trials [97].

1.4.2 Vaccinia Virus

1.4.2.1 History

Variola Virus (Variola major) is a member of the poxvirus family and for thousands of years was responsible for devastating epidemic outbreaks of smallpox and millions of deaths worldwide. The origin of smallpox as a natural disease has been lost in history but is believed to date back to 10,000BC.

Colloquially, known as ‘speckled monster’ the disease was non selective, affecting all levels of society and in Europe alone in the 18th century was responsible for 400,000 deaths annually and a fatality rate of up to 60% in adults and 98% in children.[98,99]

Throughout time it was well established that survivors of smallpox became immune to the disease and the practice of variolation or inoculation, by which a small of amount of fresh matter from an infected pustule was injected subcutaneously into an uninfected individual to impart immunity, became widely accepted practice.[100] In the late 18-hundreds Edward Jenner discovered the protective effects of cowpox to variola virus (VARV) after overhearing tales of dairymaids being protected from smallpox naturally after having suffered from cowpox and established a vaccination against the disease. Based on his work, and following a worldwide vaccination campaign the World Health Organisation (WHO) declared smallpox to be eradicated in 1980.[101,102] The agent used for the vaccination programme was not the same and its exact origins are unclear. It was called vaccinia virus (VV) and demonstrates a close relationship to the cowpox virus suggesting that it may be a hybrid of cowpox and VARV.[103,104]

By the 1980’s, VV had emerged as an increasingly useful adjunct for use in alternative biological and medical applications. Several factors enabled the virus to develop in this way including VV’s large double-stranded DNA genome, which enables insertion of up to 25 kb of foreign DNA without any loss of infectivity. As a direct result of its historical importance as a smallpox vaccine, the biology and pathogenesis of VV has been investigated extensively and there is a large and established safety profile for the virus. Finally, and importantly, VV continues to demonstrate high infection efficiency making them important in the field of oncolytic virotherapy.[105]

A variety of recombinant viruses now exist derived from various vaccinia strains and continue to be heavily investigated in the laboratory, these include Modified Vaccinia Ankara (MVA), Western Reserve (WR), Lister (LIVP), New York Vaccinia Virus (NYVAC) and Copenhagen (COP). There use in a wide variety of solid tumours continues to show promise in a pre-clinical setting with several forms progressing into clinical trials.

1.4.2.2 Structure, Replication and Life Cycle of VV

1.4.2.2.1 Structure

Vaccinia virus remains one of the largest viruses to replicate within humans and as a result of its success as a live viral vaccine it is also one of the most extensively studied members of the poxviridae family.

The vaccinia virion is a single membrane barrel-like structure measuring 320-380nm in the long axis by 260-340nm in the short.[106,107] The virion has a complex internal structure featuring a bioconcave core flanked by lateral bodies (Figure 1.4).[108] The core is made up of a proteinaceous wall with pore-like structures throughout enclosing a nucleocapsid, while the lateral bodies surrounding this structure are comprised of amorphous protein structures that to date have no known function (Figure 1.5).[109,110]

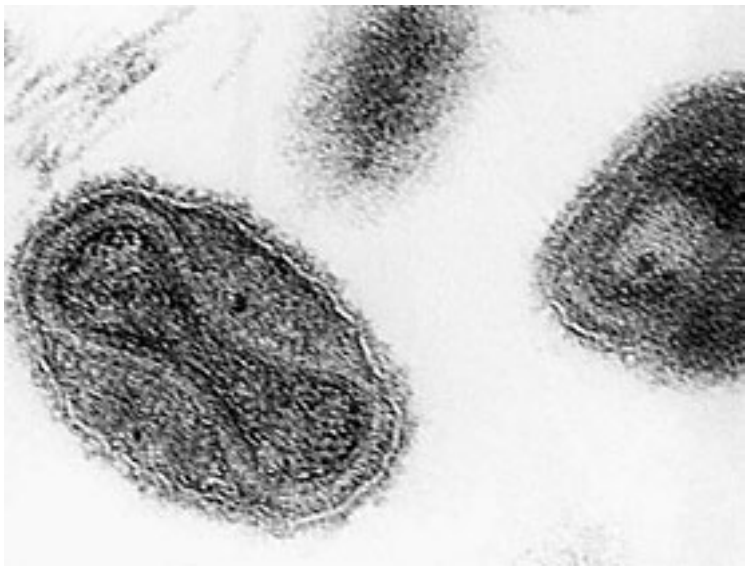
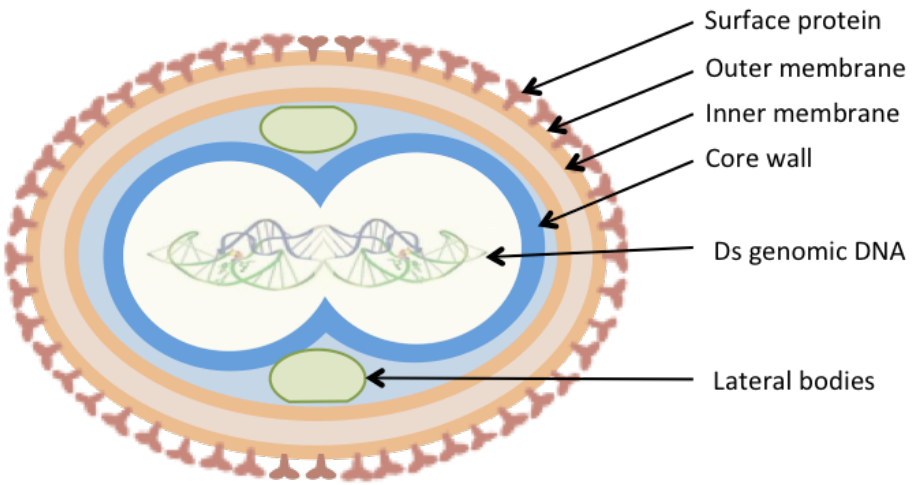


Figure 1.4 Electron micrograph of thin-sectioned vaccinia virus particles demonstrating an outer membrane surrounding a bioconcave core.

(reproduced from swissinfo.ch)

DNA sequencing has confirmed that the genome consists of 191,636 base pairs with a basic composition of 66.6% A & T within which there are 198 ‘major’ protein-coding regions and 65 overlapping minor regions encoding a potential 263 genes.[111] Mass spectrometry has analysed the protein composition of the mature virions and identified at least 70 virus proteins although the localization of many of these virion proteins within the genome remains unknown.

a



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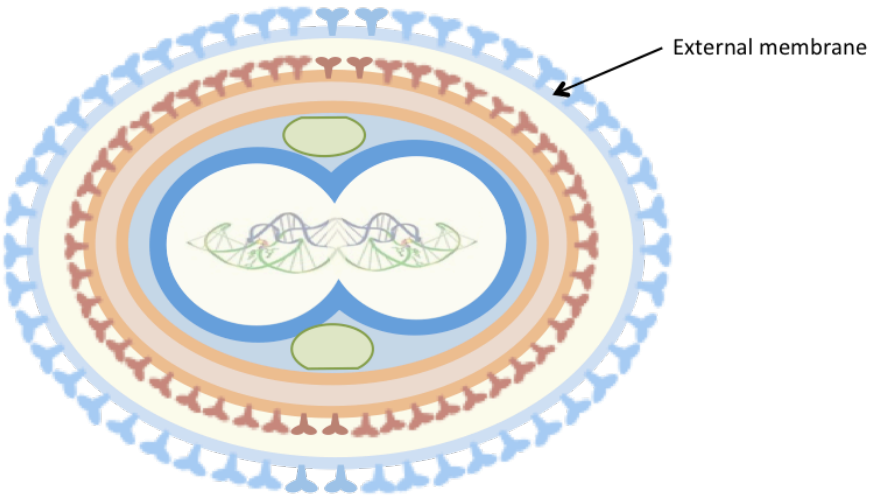


Figure 1.5 Illustrative representation of two vaccinia virion structures, IMV (a) and EEV (b).

The Intracellular mature virion (a) is the most abundant form and is the first virus particle transcribed in the host cell. The single membrane structure has a bioconcave core flanked by lateral bodies. IMV's are responsible for local cell-cell spread. The extracellular enveloped virion (b), develops from the IMV form and gains a second membrane as a result of a wrapping process undertaken in the golgi apparatus. The EEV's are responsible for distant virus dissemination and the extra membrane covering allows the EEV's to play a role in evasion of host innate immunity (ds; double stranded).

1.4.2.2.2 Morphogenesis and Replication

Virion morphogenesis is a complex process ultimately leading to the production of two major classes of mature virion differentiated from one another by their number of membranes. Intracellular mature virus (IMV) has a single membrane and remains within the cytoplasm of the infected cell until cell lysis. The IMV's are thought to be responsible for the horizontal spread of poxvirus and remain largely intracellular until cell lysis. The second set of mature virions acquire an additional wrapping from the host trans-golgi network, resulting in a second membrane. The so-called extracellular enveloped viruses, (EEV) are exported from the cell, prior to cell death by fusing with the plasma membrane before being released via exocytosis. The EEV's are thought to be responsible for the distant spread of the virus within the host.[112,113] Importantly, in addition to their role in dissemination, and as a direct result of being wrapped in a host derived membrane they play a pivotal role in enabling VV to evade host antibody and complement responses.[114]

Unique to the poxvirus family, the entire life cycle including transcription, replication and virus assembly occurs within the host cell cytoplasm. Virus replication is initiated by the binding and entry of virions into susceptible cells. After direct fusion of cellular and viral membranes, the inner viral core is delivered by microtubules into the cytoplasmic compartment where it remains intact for several hours. Fusion of both types of virion is facilitated by the interaction of a group of virion entry/fusion proteins (A21, A28, H2 and L5)[115,116] within the virus membrane with either glucosoaminoglycans (GAGS) or laminins on the cell surface.[117,118] These proteins enable fusion of the virus to the host cell but also facilitate cell to cell spread.

The core contains structural proteins, a tightly compacted viral DNA genome and various transcriptional enzymes necessary to initiate replication. Within several minutes of infection, the virus genome is transcribed into early messenger RNA (mRNA) by the virus associated DNA-dependent RNA polymerase. Translated proteins from these mRNA's allow replication of virus DNA, and modification of the cell microenvironment to the advantage of the virus, enabling the virus to escape from the host innate immune response. Later gene transcription follows, with genes encoding for new virus particles and enzymes packaged into virions that initiate transcription in the next

infected cell.[119] The immature virion (IV) is released upon cell lysis and a minority of virions are transported for ‘wrapping’, the addition of a second cellular membrane to form the EEV population.[114] Host cells undergo lysis 7-24 hours after initial infection releasing both infectious EEV and IMV forms with each individual infected cell yielding approximately 10,000 new viral particles. A summary of the life cycle is shown in Figure 1.6.

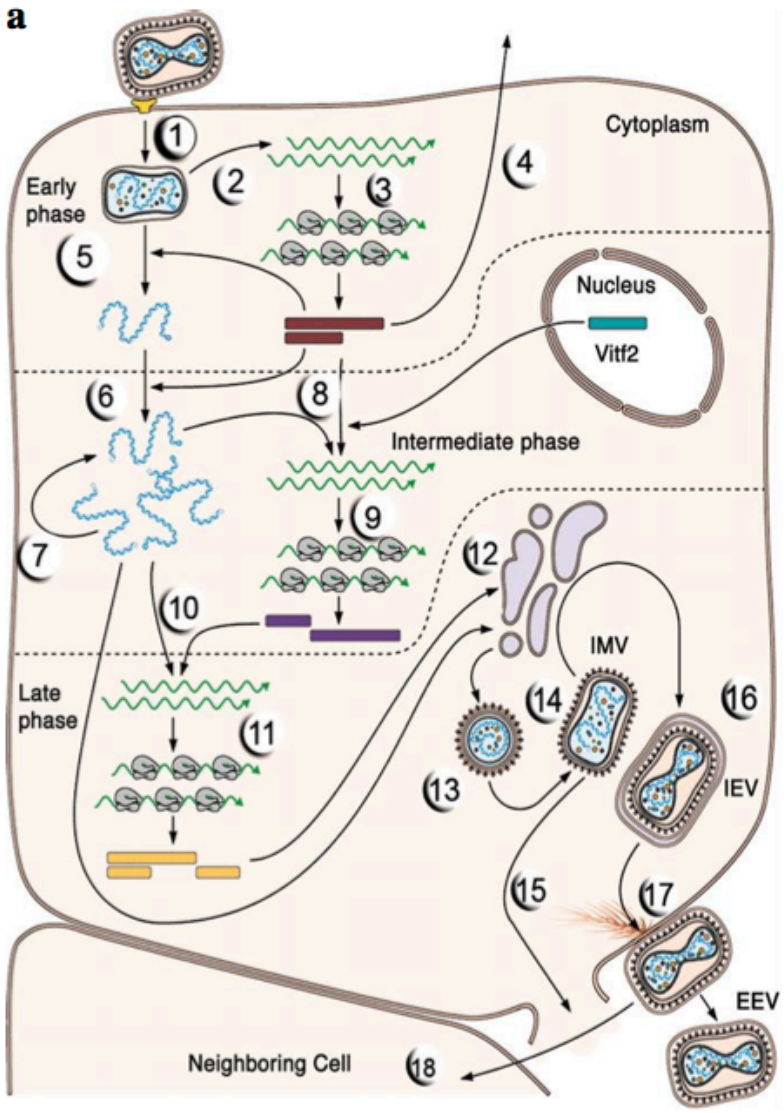


Figure 1.6 Illustrative overview of Vaccinia virus replication and lifecycle.

There are 18 steps in the VV lifestyle which are described below: (1) Entry of EEV's. (2) Core released and early mRNA synthesis. (3) Protein synthesis. (4) Secretion of early proteins including proliferation stimulators and immune evasion factors. (5) Viral DNA genome release. (6,7) Viral DNA synthesis. (8,9)Intermediate phase gene transcription. (10,11) Late phase gene transcription. (12,13) Formation and assembly of the immature virion. (14) Maturation into IMV. (15) Cell lysis and release of IMV. (16) Proportion of IMV's undergo wrapping with second membrane to form intracellular enveloped virion (IEV). (17) IEV transported to the cell surface by microtubules and released via exocytosis in the form of mature EEV's. (18) Membrane fusion and infection of neighbouring cells. (*Figure adapted from Boyle et al. 2009*).[120]

1.4.2.3 Vaccinia as an Oncolytic Virus

As a result of vaccinia's role as a vaccine in the treatment of smallpox, it has become the most extensively researched and understood virus for use in humans. A wealth of literature existed examining virus biology and pathogenesis in both pre-clinical and clinical settings, making it a very attractive proposition for developing into a potential biotherapeutic cancer agent.

Vaccinia virus has several unique features that make it an ideal candidate for use as a potential oncolytic or immunotherapeutic agent and consequently there are a range of recombinant VV viruses being investigated with promising results. Some of these features are discussed below:

1. VV rapidly infects, replicates and lyses target cells when compared with other virus species with the first viral particles reportedly released before 8 hours of infection and cell death seen at between 48 and 72 hours.[121]

2. VV possesses a broad tumour tropism which inherently targets tumour cells in a multi-mechanistic fashion. Primarily this occurs as a result of VV utilization of membrane fusion pathways to infect target cells.[122] In addition tumour cell targeting is also increased by the infrastructure of the cancer cell mimicking that of a virally infected host cells with deregulated cell cycles, defective apoptotic pathways and immune evasion pathways.[123] Finally, cancer cells typically demonstrate activation of the EGFR/RAS/MAPK signal transduction pathway and defective anti-viral type I IFN responses, which support VV replication and spread. In fact, evidence suggests that VV targets these pathways by secretion of an EGF homologue, vaccinia growth factor (VGF), to activate the EGFR/RAS/MAPK pathway and secrete B18R to inhibit type I IFN's.[124]

3. VACV possesses an intrinsic ability to evade host cell defenses by utilising the EEV antigenic virion, therefore making it incredibly attractive for potential intravenous delivery. EEV cloaks itself in a host derived cell membrane containing various host complement proteins. This allows

efficient and protected virus dissemination to distant sites and also allows the virus to be transported unharmed in the bloodstream.[125]

4. As a result of the size of the genome, VACV is easily amenable to genetic manipulation and can be armed with multiple large transgenes to enhance various functional aspects of the chosen therapeutic design.

5. Due to the extensive historical clinical utilization of the poxviridae family they are considered safe in a clinical setting and do not integrate into host DNA due to their replication pathway. Importantly, in the event of an adverse outcome several antiviral agents already exist that could be utilised to treat patients.[126]

1.4.2.4 Virus modification strategies to enhance tumour specificity

While Vaccinia virus has a high natural tropism for tumour cells, research has continued to explore different strategies to enhance tumour specific replication in bioengineered viruses. In JX-594, utilised in this study and discussed later, the genetic modifications are specifically designed to augment its natural intrinsic targeting, enhance tumour specific replication and potentiate the oncolytic potential of vaccinia virus, whilst enhancing anti-tumour immunity through the expression of transgenes.[127] Some of the genetic modifications are discussed in more detail in this section.

1.4.2.4.1 Deletion of Thymidine Kinase

Thymidine kinase (TK) is an E2F-responsive gene involved in deoxyribonucleotide synthesis in dividing cells. Normal cells intrinsically have low nucleotide concentrations and therefore require TK to allow replication, however cancer cells constitutively express high levels of nucleotides rendering TK redundant in cancer cell proliferation cycle. By deleting the TK gene in JX-594, the virus is forced to preferentially replicate in cancer cells, relying on adequate concentrations of nucleotides present in host tumour cells. The TK-deleted virus is quickly cleared from healthy tissue which acts

as an additional protective mechanism against viral toxicity.[128] Various models have validated tumour selective replication as a result of TK deletion, and demonstrated anti-tumour efficacy (positive results) in various animal models (including mouse and rabbit) including melanoma, colon cancer, sarcoma and liver metastasis.[129–132]

1.4.2.4.2 Deletion of Vaccinia Growth Factor (double deleted vaccinia virus).

Vaccinia growth factor (VGF) is a secreted 77 amino acid glycoprotein, which is expressed as an early phase protein in the vaccinia virus lifecycle. It is an epidermal growth factor (EGF) homologue that stimulates cell proliferation by binding to the EGF receptor (EGFR) found on both infected and surrounding non-infected cells. Vaccinia virus relies on the proliferation of cells for efficient virus production and therefore stimulates normal cells to divide using VGF. In contrast, cancer cells naturally proliferate making expression of VGF, to enhance this process, superfluous in this setting. Deletion of VGF from the vaccinia genome therefore enhances tumour selectivity.[133] Studies of a double deleted vaccinia virus (ddVV), involving the combination of TK deletion with VGF deletion have confirmed that this is a promising tumour selective gene therapy vector. *In vitro*, resting and dividing NIH3T3 cells were infected with ddVV-GFP. In resting cells (those requiring VGF to divide) a reduced viral yield was seen when compared with control virus. In contrast, in the freely dividing cells (mimicking cancer cells) virus recovery was equivalent to wild type or single deletion variants. *In vivo* similar results were gained with improved survival and tumour specific replication observed in nude mice following intraperitoneal (i.p.) injection.

1.4.2.4.3 Arming Vaccinia Virus with immune-stimulatory molecules

The fact that oncolytic viruses can amplify in tumour tissue with accompanying transgene expression makes ‘arming’ viruses with therapeutic proteins or immunomodulatory molecules a potential way of enhancing anti-tumour efficacy and/or immunity. Various strategies have been investigated to enhance virus oncolytic activity. For example, secreted transgene products

such as the cytosine deaminase/5-fluorocytosine (CD/5-FC) have been used to stimulate a cytotoxic effect on neighboring non-infected cells and is described as so-called suicide gene system.[134] An alternative strategy utilised is to inhibit tumour angiogenesis; in renal cell cancer models enhanced splenic extramedullary hamatopoieis was seen after systemic treatment with a ddVV armed with vascular endothelial growth factor (VEGF) receptor 1 to sequester VEGF and promote an antiangiogenic effect.[135] Similarly, VACV encoding GLAF-1, a single chain antibody targeting human and murine VEGF, exhibited significantly enhanced therapeutic efficacy *in vitro* and in human xenograft models in nude mice.[136]

1.4.2.5 JX-594

There are a wide variety of recombinant viruses derived from various vaccinia backbones in pre-clinical and clinical testing, however for the remainder of this report we will focus on the experimental virus utilised, JX-594. JX-594, commercially known as Pexa-Vec, was initially designed and manufactured by Jennerex & Partners, however in 2014 the company and product was taken over by Sillajen Biotherapeutics Inc.

JX-594 is an engineered targeted poxvirus with transgene 'arming' developed from the Wyeth vaccine strain (Dryvax; Wyeth Laboratories, Madison, NJ). The virus backbone is genetically modified to disrupt TK to enhance tumour selectivity and attenuation. Immune stimulation is driven by insertion of human granulocyte-macrophage colony-stimulating factor (GMCSF) under the control of an engineered early-late promoter. Finally, a lacZ transgene encoding β-Galactosidase (β-Gal) controlled by the p7.5 early/late promoter aids virus tracking.[127,137] (Figure 1.7)

To date, JX-594 is the most advanced vaccinia based oncolytic virus in the clinical setting, entering phase III trials with promising results. The virus has been given safely to over 250 patients in a selection of advanced tumour types and was the first virus to induce partial and complete tumour responses, be recovered from tumours after systemic intravenous delivery and demonstrate improved survival rates in randomized trials.[138–140]

JX-594 is highly cancer selective, although the mechanisms behind this still remain poorly understood. It is clear however, that it is multi-mechanistic in nature involving EGFR/RAS pathway signaling, cellular TK levels and cancer cell resistance to type I interferons.[124]

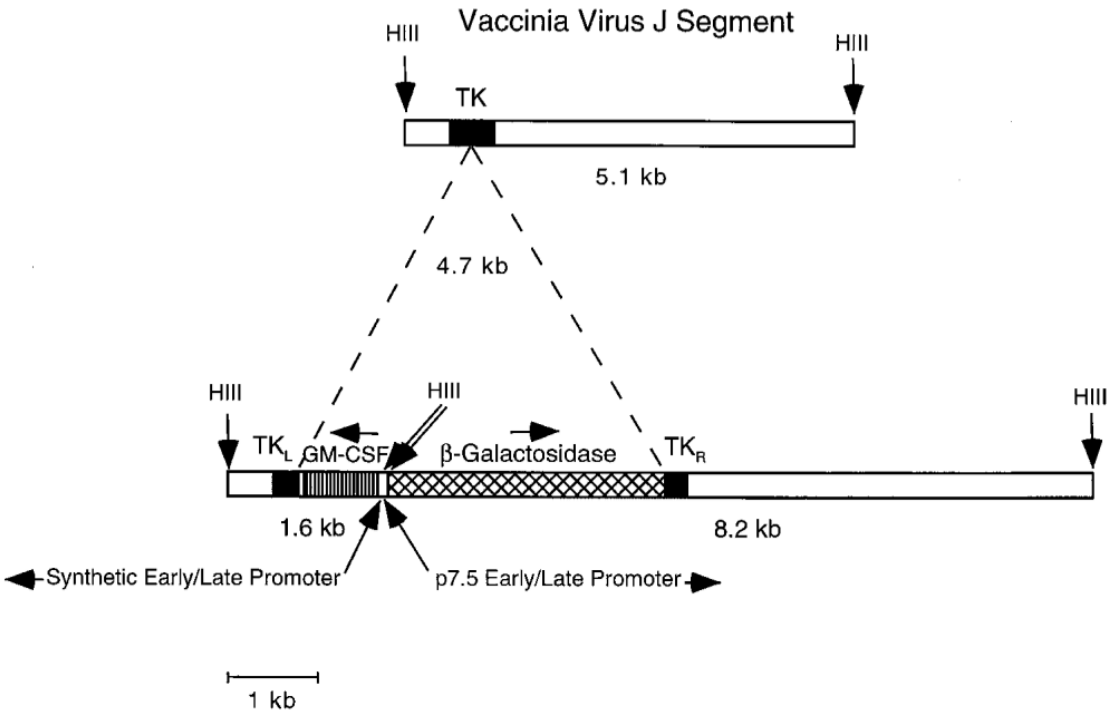


Figure 1.7 Schematic diagram to show modification of Wyeth strain vaccinia virus to construct JX-594.

The top fragment shown represents the virus J segment, a 5.1kb section in which the TK gene is found. A pSC65 vector with a GM-CSF insert is inserted by homologous recombination into the TK gene area and results in TK gene disruption. In addition, the LacZ gene is inserted encoding for β-galactosidase. For experimental JX-594 subtypes this area can be replaced with green fluorescent protein (GFP) or Luciferase (fLUC) to aid virus recognition in experimental models *(Adapted from Mastrangelo et al. (1998)).*[137]

1.4.3 Reovirus

1.4.3.1 Background

Reovirus (Respiratory Enteric Orphan Virus) is a member of the Reoviridae family. The virus was first isolated in 1953 from a rectal swab taken from a healthy human child and thereafter three serotypes were identified and designated into the Reoviridae family. The name Reovirus was coined later in 1959 by Sabin et al. (1959)[141] as an acronym reflecting the fact that the viruses in this group had been found in both the **R**espiratory and **E**nteric tracts with no associated disease, and as such were described as **O**rphan in nature.

As mentioned, three serotypes of Reovirus have been reported based on their activity and are designated Type 1 Lang, Type 2 Jones, Type 3 Abney and Type 3 Dearing. The serotypes are all ubiquitous in both their ability to infect nearly every known mammalian species and their geographical distribution, being largely found in stagnant water and sewerage. Exposure is common, with anywhere between 70-100% of those investigated demonstrating seropositivity and 50% of adults aged 20-30 years demonstrating exposure by carrying antibodies against the virus.[142,143] Despite these high exposure levels, symptoms remain very mild, with reported cases largely limited to the very young and are often subclinical in nature.[144] Interestingly, the non-pathogenic nature of the virus was demonstrated by Rosen et al. (1963)[145] who inoculated (intranasally) 27 volunteers in a correctional institution with serotypes 1,2 and 3; only 30% of those patients developed minor symptoms.

1.4.3.2 Structure, Function and Lifecycle

Reovirus is a non-enveloped virus composed of a genome of 10 segments of double stranded-DNA enclosed in two inner and outer concentric isosahedral protein capsid shells.[146] The virus replicates within the cell cytoplasm and the 10 segments of dsRNA range in length from 1.2 to 3.9 kilobases and are named accordingly (3 x lambda (long), 3 x mu (medium) and 4 x sigma (small) segments). The ten segments encode twelve viral proteins in total, eight structural and four non-structural (Figure 1.8).[147]

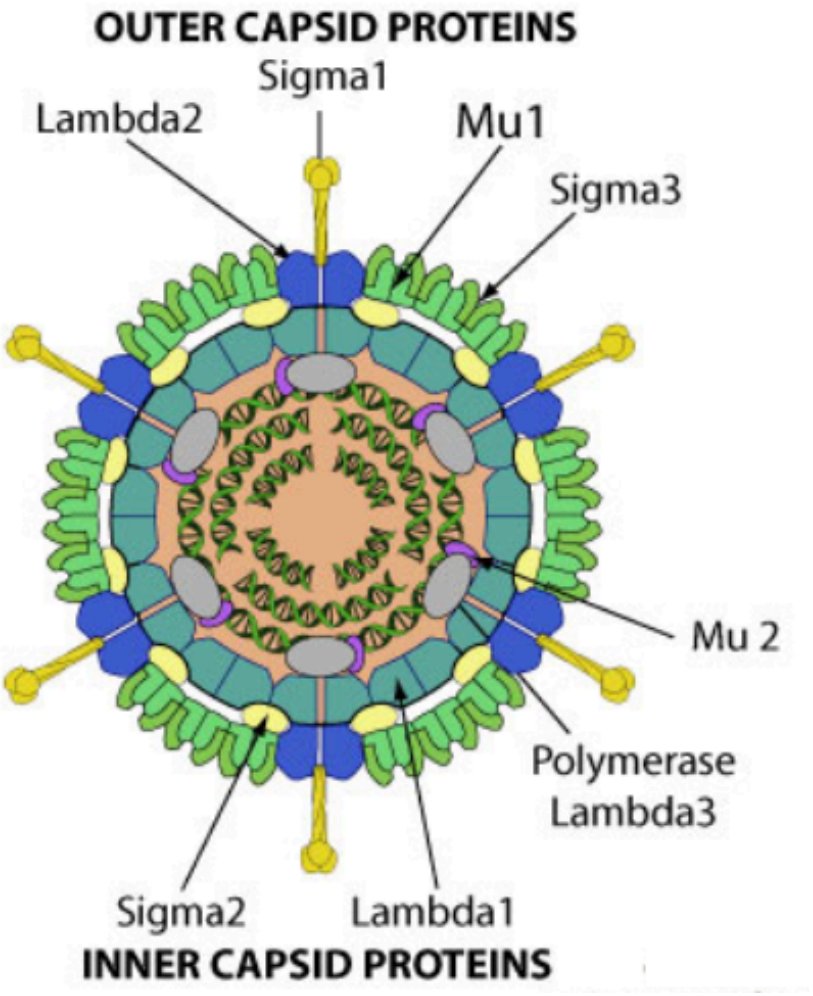


Figure 1.8 Reovirus structure

Reovirus is a non-enveloped dsRNA virus approximately 80nm in diameter. The viral genome consists of 10 segments of dsRNA contained within an outer and inner capsid which encode for structural and non-structural proteins. Sigma-1 has been identified as the viral attachment protein and alongside Sigma-3 is reported to play a role in virulence. Mu-1, Mu-2 and Lambda-3 have roles in viral replication. Figure adapted from Gong and Mita (2014)[148]

Reovirus infection is initiated by the binding of the viral protein sigma 1 (σ1), a filamentous attachment protein, to junctional adhesion molecule-A (JAM-A), a serotype-independent, cell surface receptor.[149] σ1 also binds to cell-surface carbohydrate in a serotype dependent fashion, for example, sialic acid in serotype 3 strains. Following attachment to the cell surface, Reovirus internalisation is initiated via β1 integrins which promote a clathrin-dependent endocytotic process.[147] Within the endosome, the virus undergoes proteolytic disassembly to form sub virion particles (ISVP) in the host cytoplasm and replication proceeds. In addition, cleavage fragment particles escape into the cytoplasm where they activate NF-κB and modulate apoptotic cell death pathways. Interestingly, inhibition of these pathways using either pharmacological agents or via expression of transdominant forms of IκB result in complete cessation of Reovirus-induced apoptosis suggesting a critical role for NF-κB activation in Reovirus cell death.[150]

1.4.3.3 Reovirus as an Oncolytic Agent

Initial experimentation using Reovirus focused on its relatively non-pathogenic nature and therefore it represented a useful model to investigate viral replication and pathogenesis. However, in 1977 Hashiro et al.[151] described the susceptibility of certain tumour cells and spontaneously transformed cell lines of human and murine origin to 'cytotoxic Reovirus induction'. Furthermore, the authors also found that 'normal' cells were resistant to the virus and as such the potential for Reovirus to be used as an oncolytic agent was ignited. These early findings were further validated by Duncan et al (1978)[152] who reported a differential sensitivity to infection with type 3 Reovirus in normal and SV40-tranformed WI-38 cells. The authors reported complete lysis of transformed cells at 96 hours post infection compared with no detectable cell death in normal cells.

1.4.3.4 Reovirus and Clinical Trials

The first reported clinical use of Reovirus was in 1963 in a study to ascertain the clinical manifestations of the virus. All 3 serotypes were given as an

intranasal inoculation to healthy adult volunteers after which they were followed up for 23 days. There was some evidence of minor upper respiratory tract illness and diarrhoea but no significant clinical manifestations.[145]

Following the development of Reolysin by Oncolytics Biotech Inc. (Calgary, Canada) a clinical grade product developed from the type-3 Dearing strain, Reovirus has demonstrated activity in clinical trials across a range of cancer types, including but not limited to breast, prostate malignant glioma, pancreatic, head and neck and ovarian cancers.[153] Various administrative strategies have been explored and similarly trials have examined the virus when given alone and in combination with other cancer therapies.[154]

The first phase 1 cancer related study (REO-001) undertaken was an open-label study to determine the safety and tolerability of Reolysin. 19 patients with clinically accessible and evaluable disease of varying cancer types (head and neck, sarcoma, breast), who had failed to improve on standard treatment regimens, were given escalating doses of intratumoural virus. At the end of six weeks, there was a 37% local target tumour response with one patient exhibiting complete remission. Significantly, it was also noted that three patients had responses recorded in synchronous lesions distant from the site of primary treatment.[155]

Thereafter, Reovirus has been the subject of intense investigation in the clinical setting with 32 clinical trials completed or ongoing. Reovirus is safe to use via both intralesional/intratumoural and systemic routes of administration in Phase I trials. In Phase II trials it has successfully reduced tumour size and burden and promoted tissue necrosis, validating pre-clinical data. The first phase III trial continues to explore the overall survival and progression free survival benefit of intravenous administration of Reolysin in combination with paclitaxel and carboplatin versus chemotherapy alone, in patients with metastatic or recurrent squamous cell carcinoma of the head and neck; initial reports are keenly awaited.

1.5 Oncolytic Virus and the immune system, friend or foe?

Oncolytic viruses have emerged as attractive tools in the development of novel anti-cancer therapies. Historical research has focused on the direct cytotoxic effects of these agents and only recently has the potential role of the immune system in viral therapy been highlighted and become the focus of attention. Understandably, initial hypothesis revolved around the immune system being detrimental and inhibitory to the success of virotherapy with the host immune system lacking the sophistication to identify the difference between malevolent pathogens and therapeutic agents, ultimately leading to viral clearance, neutralization and the negation of direct tumour cell killing. However, it is now a widely accepted and increasingly understood phenomenon that the efficacy of these therapies relies on a three-way interaction between the oncolytic viruses, the immune system and the tumour microenvironment. Acting as an immunotherapy, viruses can prime anti-tumour immunity, generating both innate and adaptive immune responses, and ultimately resulting in tumour regression. [60]

1.5.1 Vaccinia, JX-594 and the immune system

The historical use of vaccinia virus during the eradication of smallpox, has meant that its immune activating potential is well understood and documented. Furthermore, the clinical progress of clinical grade vaccinia virus, such as GMCSF expressing JX-594, has allowed the potential for this virus to target the immune response and enhance virotherapy to be well explored.

The first significant report of vaccinia eliciting an anti-tumour immune response was by Mastrangelo et al. (1999)[137] who conducted a phase 1 trial in seven immunocompetent, revaccinated patients with surgically incurable cutaneous melanoma. For six weeks, the group administered a twice weekly intra-tumoural injection of JX-594 in an escalating dose protocol. An anti-vaccinia humoral response was identified in all seven treated patients with injected tumours becoming inflamed and infiltrated with a number of immune cell types. The clinical outcomes were mixed, however, importantly five of the

seven patients demonstrated tumour regression at both treatment site and distant untreated disease loci, indicative a systemic anti-tumour response.

Regardless of the immune potential elicited by an oncolytic virus, if the virus is not delivered directly into tumour it is important to establish that viral infection is targeted and tumour cell specific. Studies using systemic delivery methods have demonstrated that vaccinia virus can be delivered to tumour in mouse models, however insufficient tumour cell killing and infection of normal tissues remained an issue. Hung et al. (2007),[156] for example, used a non-invasive luminescence imaging system to monitor ovarian tumours treated with intraperitoneal vaccinia in a mouse model. They reported that vaccinia virus was able to selectively target, control and illicit a potent anti-tumour immune response when delivered intraperitoneally but intravenous delivery methods did not yield successful therapy.

One strategy considered to improve tumour selectivity, undertaken by Kirn et al. (2007)[61], was to clone IFN-β gene into a B18R deletion vaccinia mutant. IFN-β in normal tissues is known to inhibit viral replication, however tumour cells are often resistant to the antiviral effects of type I IFNs and as such viral replication should not be inhibited in tumour cells. Using this virus the authors were able to show a complete tumour response following systemic delivery; moreover the response was associated with protection from tumour re-challenge in a CMT93 murine tumour model indicating a systemic and continued anti-tumour immune response.

1.5.2 Reovirus and the immune system

Despite Reovirus progressing to clinical trials for the treatment of various localized and disseminated malignancies, the first study to examine the potential immune contribution to Reovirus efficacy was performed by Errington et al. (2008).[157] The authors examined the ability of Reovirus to activate human dendritic cells (DC), important regulators in both the innate and adaptive immune response, and reported that Reovirus could induce DC maturation and stimulate the production of various inflammatory cytokines including IFN-α, TNF-α, IL-12 and IL-6. Furthermore, Reovirus-activated DC upregulated IFN-γ production and increased NK cell cytolytic activity when co-

cultured with autologous NK cells. Similarly, when Reovirus-activated DC were co-cultured with autologous T cells Reovirus augmented T cell cytokine production and the induction of CTL-mediated tumour cell killing.

In a separate study examining the use of Reovirus in Melanoma, Errington et al. (2008)[158] showed that Reovirus could replicate in and lyse human melanoma cell lines and freshly resected melanoma samples. The study demonstrated that virus-induced cell death was associated with the release of a range of pro-inflammatory cytokines and chemokines along with abrogation of the immunosuppressive cytokine, IL-10. The authors reported that Reovirus-induced cell death may act to reverse the immunosuppressive milieu of the tumour microenvironment, actively recruiting immune effector cells and leading to the generation of an innate anti-tumour immune response.

These data were further supported by Prestwich et al. (2009)[44] who demonstrated that DC's loaded with Reovirus infected melanoma cells and co-cultured with PBMC induced IFN-γ production within the NK cell population in a cell to cell contact dependent manner. Importantly, they also showed that DC loaded with infected melanoma cells induced NK cell cytotoxicity towards tumour cells via an IFN-dependent mechanism. Murine melanoma cell lines (B16ova) were resistant to direct Reovirus oncolysis *in vitro*, however C57BL/6 mice bearing B16ova lymph node and splenic metastasis were cleared of their disease burden when treated intravenously (*i.v.*) with Reovirus loaded T-cells. In the same *in vivo* model using immuno-deficient mice Reovirus had no effect. These results clearly demonstrated the importance of the immune system for the efficacy of Reovirus.[43]

1.6 Apoptosis, Necrosis and Autophagy

1.6.1 Cell death mechanisms and OV

Oncolytic virus mediated cell death traditionally does not follow the classic pathways of apoptosis, necrosis or autophagy but instead typically will display features of all three modalities with some variation seen between different virus types. In an effort to explain the mixed cell death pathways alternative

definitions have emerged including programmed apoptosis, necroptosis and necrosis-like programmed cell death.

In Vaccinia infection the classical pathways have all been implicated to varying degrees with cell lysis considered the common endpoint of infection. Apoptosis has been reported in some cancer cell lines and immune cells, autophagy is disrupted in fibroblasts following infection and necroptosis has also been shown to have a role in the death of vaccinia infected T cells.[159] Reovirus-induced cell death is widely reported to primarily occur by an apoptotic mechanism, however it has also been to shown to activate a caspase independent cell death pathway, inducing features consistent with necroptosis.[160] However, understanding the mechanism of virus-induced cell death will enable complimentary combination strategies to be defined.

1.6.2 Apoptosis

Apoptosis is an essential process to maintain normal development and tissue homeostasis. It is characterised by defined morphological changes such as the formation of apoptotic bodies, caspase activation and loss of integrity from mitochondrial membranes. Apoptotic bodies are removed by phagocytosis after a process of flipping of phosphatidylserines to the outer membrane surface and at the same time anti-inflammatory cytokines are released to minimise immune activation. The process is considered non-immunogenic.[161]

Vaccinia virus is known to encode various inhibitors of apoptosis including F1L, N1L and SPI-2 which combine to prevent premature cell death and may inhibit viral replication and spread. However, apoptosis has been observed following infection with VV. In response to infection with VV, Greiner et al. (2006)[162] demonstrated a time dependent increase in apoptosis but no evidence of necrosis in Mel526 cells as discriminated by annexin-V and 7-AAD staining. Similarly, Humlova et al (2002)[163] suggested VV stimulated apoptosis in a murine macrophage line (J774.G8) using various techniques including mitochondrial membrane potential and annexin-V positivity. Interestingly the study reported that VV-induced apoptosis was dependent on early gene expression and was associated with reduced levels of Bcl-x(L), an anti-apoptotic member of the Bcl-2 family.

Reovirus is reported to induce apoptosis in a wide variety of *in vitro* and *in vivo* experimental models including cancer cells and tumours. Reovirus-induced apoptosis involves the release of TRAIL from infected cells and the activation of TRAIL-associated death receptors (DR), DR4 and DR5. A subsequent activation cascade results in caspase 8 activation, cleavage of Bid and the release of pro-apoptotic mitochondrial factors.[164,165] The combined activation of death receptor and mitochondrial pathways leads to downstream activation of effector caspases, such as caspase-3, the role of which is considered critical to Reovirus-induced apoptosis.[166]

1.6.2.1 The Bcl-2 Family; Proapoptotic versus Prosurvival

One of the hallmarks of cancer is the evasion of apoptosis.[167] Cancer cells demonstrate attrition against a plethora of cellular stresses that would almost certainly result in normal cells undergoing apoptosis. Examples include unfavourable microenvironments, aberrant cell cycle progression and oncogene activation. However, cancer cells have evolved multiple strategies in order to evade these normal cell cycling processes, one of which being the upregulation of the pro-survival BCL-2 family proteins. BCL-2 family members have either pro-apoptotic (BH3-only proteins; Bax, Bak, Bad, Bim, Noxa) or anti-apoptotic (BCL-2, Bcl-XL, Bcl-w, Mcl-1) functions. The so called BH3-only proteins are pro-apoptotic in nature and principally act to control mitochondrial death signalling via the release of cytochrome C, a potent catalyst of apoptosis. On activation by stimuli such as intracellular damage, Bim, Bad and Noxa sequester anti-apoptotic targets and initiate apoptosis by permeabilising the mitochondrial outer membrane and inducing the release of pro-apoptotic enzymes, such as cytochrome C. Cytochrome C then activates the caspase pathway ultimately leading to cell death. Various danger signals can induce the pathway to initiate cell death, for example following DNA damage, the p53 tumour suppressor gene upregulates the expression of Bax, which in turn stimulates mitochondrial release of cytochrome C.[168] In many tumours, apoptosis is dysregulated by upregulation of pro-survival family members or mutations in the p53 pathway which abrogate the normal activation pathways apoptosis triggers.

A summary of the intrinsic and extrinsic apoptotic pathways along with the interaction of BCL-2 family proteins, is presented is in Figure 1.9.

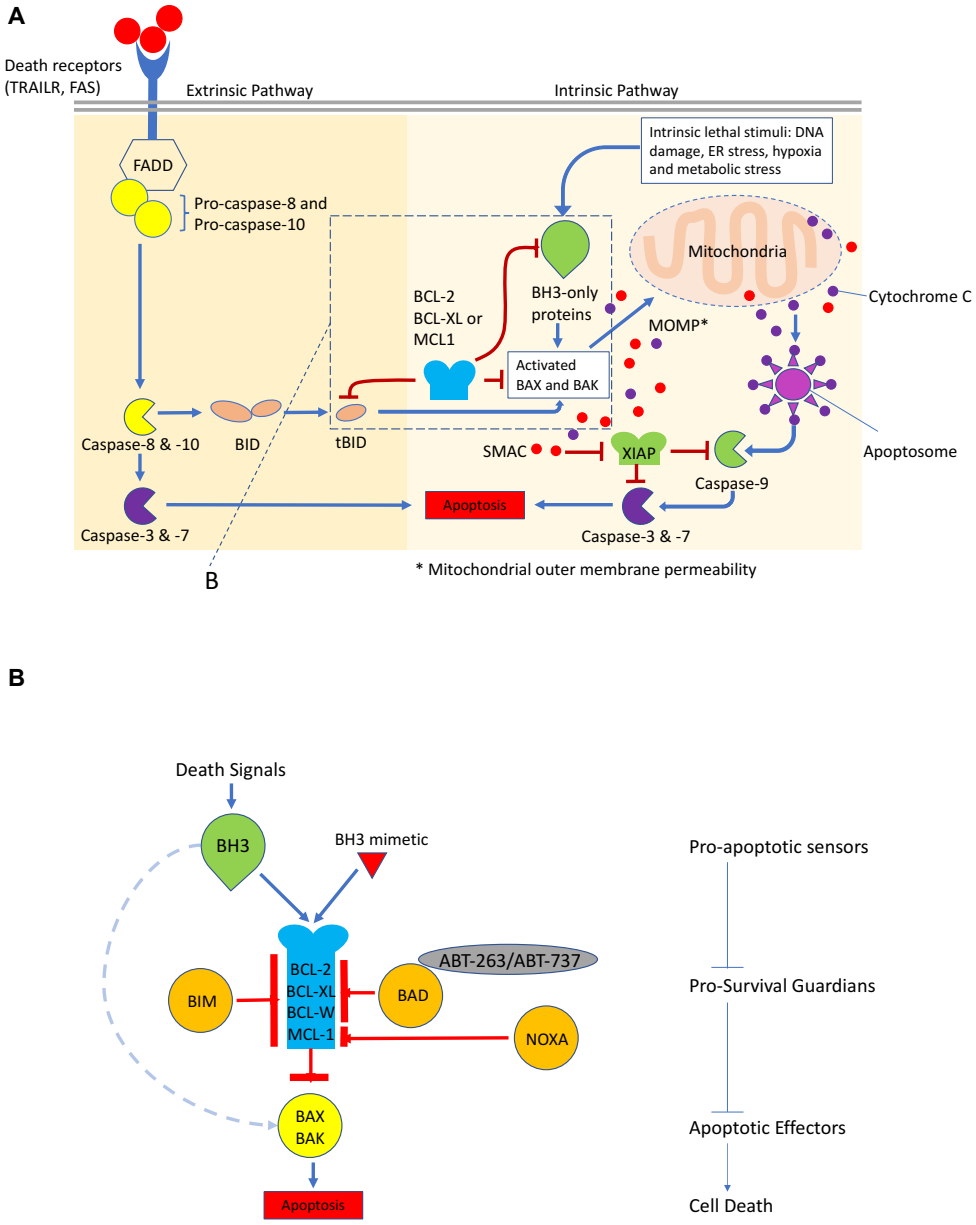


Figure 1.9 Apoptosis and BCL-2 family protein.

A) A summary of the extrinsic and intrinsic signalling cascade leading to activation of caspase 3/7, caspase 8/10 and cell death. B) BCL-2 inhibits apoptosis by interacting with BAK and BAX. BH3-only proteins (BAD, BIM, NOXA) activate apoptosis by inhibiting BCL-2. Specific BH3-only proteins (BID, PUMA) can also induce apoptosis by directly activating BAK and BAX. ABT-263 is a 'BAD-like' mimetic that can bind BCL-2, BCL-XL and BCL-W selectively inhibiting their action.

1.6.3 Necrosis

Necrosis is a less coordinated, studied and defined method of cell death. Cells undergoing necrosis characteristically demonstrate swelling of the cytoplasm and organelles, which is followed by rupture of the plasma membrane and release of the cytoplasmic contents. Necrosis is thought to be immunogenic in nature and is accompanied by the release of various pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α).[169] Necrosis was traditionally thought to be unregulated in nature, with no set pathways or cellular regulation. However, more recent evidence has shown that necrosis can be a controlled mechanism with defined pathways and molecular regulation and as such has been aptly called programmed necrosis or necroptosis.[170]

Necroptosis has been reported in response to vaccinia virus (Lister-dTK strain) in ovarian cancer cell lines although features more consistent with apoptosis and autophagy, such as LC3 lipidation and phosphatidylserine exposure, were also seen. In response to Lister dTK swollen nuclei, ruptured membranes, cytoplasmic vacuolation and HMGB-1 release were observed all of which are associated with necrosis. In addition, the authors reported a significant increase in glycolysis and mitochondrial respiration 24 hours post infection consistent with active necrosis and confirmed this modality as the principle causative process for the cell death observed.[159]

Similarly, Berger et al. (2013)[160] described Reovirus-induced necroptosis cell death in L929 cells treated with Type 3 Dearing Reovirus (T3D). T3D was shown to activate NF-κB and initiate caspases consistent with virus-induced apoptosis. However, interestingly blockade of NF-κB and/or caspases (using ZVAD-FMK) did not affect T3D-induced cell death. The alternative cell death pathway was further defined to be RIP-1 kinase dependent, reduce cellular ATP levels and was associated with cell membrane destruction consistent with necroptosis.

1.6.4 Autophagy

Autophagy is described as a catabolic process designed to protect and ensure an organisms well-being. Autophagy involves the sequestering of a wide array of undesired intracellular constituents into double-membrane vesicles known as autophagosomes which then undergo lysosomal degradation.[171] Autophagy is a reversible process particularly in the context of nutrient starvation. It is not completely clear therefore if autophagy represents a complete cell death mechanism or a trigger to alternative cell death phenomena. Autophagy also represents an important innate immune response against viral infection by enhancing viral antigen presentation on MHC-I, although it is also known that some viruses utilise host cell autophagy to enhance survival and replication.

Vaccinia virus actively disrupts cellular autophagy through a poorly understood novel mechanism involving abnormal LC3 lipidation and direct conjugation of ATG12 and ATG3. Despite this it is clear that there is no general activation of autophagic processes and cell death is not thought to be dependent upon the induction of autophagy. [159,172]

Evidence for Reovirus-induced autophagy remains limited with no conclusive experimental evidence in the literature. Thirukkumaran et al. (2012)[173] studied Reovirus-induced cell death mechanisms in human multiple myeloma cell lines. Apoptosis was the principle mechanism of cell death, however, in addition at 24-48hours post-infection there was formation of autophagosome puncta containing LC3-II, consistent with the activation of autophagy. Interestingly, formation of the autophagosome, was abrogated by the autophagy inhibitor, 3-methyladenine (3-MA). It remains unclear, however to what extent autophagy plays a role in Reovirus-induced cell death with conflicting reports suggesting that the mechanisms behind Reovirus induced-apoptosis might in fact shift the cell away from autophagy like death.[174]

1.7 Agents which may potentiate OV direct killing

1.7.1 BH3 Mimetics

As discussed previously, the antiapoptotic proteins Bcl-2 and Bcl-XL play a critical role in the maintenance of normal cell homeostasis. These proteins are however often overexpressed in solid tumours, and have been implicated in therapy resistance making these proteins attractive targets for the development of novel anticancer drugs.[175] Selective inhibition of these anti-apoptotic proteins was therefore designed to normalise cell death machinery and overcome chemoresistance and may prove useful in potentiating OV therapy.[168]

1.7.1.1 ABT-737

ABT-737 was developed in the mid-2000's by Abbott Laboratories as a novel Bcl-2, Bcl-XL and Bcl-W but not Mcl-1 mimetic. ABT-737 demonstrated higher affinity for its targets than previous molecules and enhanced intrinsic apoptotic signals rather than directly initiating apoptosis.

Oltersdorf et al. (2005)[176] reported 'single agent mechanism based killing' in a variety of cancer cell lines and primary patient derived cells including small-cell lung carcinoma (SCLC) and lymphoma. Hann et al. (2008) also examined the therapeutic efficacy of ABT-737 in *de novo* human primary SCLC tumour xenograft models and standard cell line xenograft models. In the cell line models, ABT-737 induced dramatic tumour regression, however the authors described more mixed results in the primary xenograft models with only one of three SCLC showing a significant response. Variable BCL-2 family protein expression in the primary tumours, in particular increased Mcl-1 (not targeted by ABT-737) and decreased Noxa were suggested as the most likely explanation for ABT-737 resistance. The hypothesis was further supported by multiple *in vitro* experiments demonstrating a reduction in Mcl-1 could overcome ABT-737 resistance.[168,177–179] The findings opened the door to potential combination strategies using alternative agents such as Sorafenib and Cisplatin which have been shown to downregulate Mcl-1 in renal tubular epithelial cells and acute myeloid leukaemia (AML).[180,181]

1.7.1.2 ABT-263 (Navitoclax)

A major limitation of ABT-737 is that it is not orally bioavailable and as such provides a limited ability to translate from the laboratory to the clinic given the inability to perform dose escalation trials. To resolve this issue, ABT-263 a structurally related but orally bioavailable Bad-like BH3 mimetic was designed.

Initial studies performed by Tse et al. (2008)[182] demonstrated ABT-263 could induce Bax translocation, cytochrome C release and apoptosis in *in vitro* models. Faber et al. (2015)[183] examined the efficacy of ABT-263 across a panel of greater than 500 cancer cell lines and using gene expression data were able to demonstrate that high expression of BIM and low MCL-1 predicted ABT-263 sensitivity. Further *in vivo* studies of oral administration of ABT-263 alone induced almost complete tumour cell regression in mouse xenograft models of acute lymphoblastic leukaemia and SCLC.[184,185] In xenograft models of multiple myeloma and B-cell lymphoma only a modest response was seen, however in combination with standard of care treatment regimes, ABT-263 was able to significantly enhance treatment efficacy.[182]

ABT-263 has now been the subject of various Phase I and Phase II clinical trials. The latest phase I trials have explored combination strategies in advanced solid tumours with erlotinib[186], irinotecan[187] and gemcitabine[188] and in refractory lymphoid malignancies with rituximab[189]. Mixed response rates have been reported however the combinations do offer a favourable safety profile. Two recent phase 2 studies have examined the use of navitoclax as a single agent in patients with relapsed SCLC and in combination with or without rituximab in treatment naïve B cell chronic lymphocytic leukaemia (CLL). Limited single agent activity in advanced and recurrent SCLC was reported by Rudin et al. (2012)[190], however its use in combination with rituximab in B-cell CLL resulted in higher response rates and prolonged progression free survival.[191]

Aims of the Study

This thesis covers a broad range of oncolytic virus activity including mechanistic and immunologic function. Significant volumes of work in the field of oncolytic virotherapy exist in the literature and as such this study aimed to focus on some outstanding areas of interest and gaps in knowledge in order to investigate strategies that may increase the therapeutic efficacy of two distinct viruses, Reovirus and Vaccinia Virus.

While the immune potential of Reovirus is well recognised, less is known about JX-594, an OV currently in clinical testing at Leeds Teaching Hospitals NHS Trust. Access to fresh human tissue samples from CRC patients allowed this study to explore the immune potential of JX-594 and furthermore examine for the potential immune effects it may have at distant disease harbouring sites, such as lymph nodes.

The thesis is divided into 3 results chapters with separate stratified aims and include the following:

Chapter 3 – Combination strategies with Reovirus

- Examines Reovirus cytotoxicity in a range of colorectal cell lines with varying EGFR expression and KRAS/BRAF/PI3K mutational variations.
- Explores the ability of ABT-263 (Navitoclax), an orally administered BH3 mimetic, to act synergistically with Reovirus and enhance OV direct cytotoxicity.
- Investigates the ability of Reovirus to enhance the effects of anti-EGFR monoclonal antibody therapy by promoting ADCC/ADCP.

Chapter 4 – Vaccinia Virus (JX-594) mechanism of action.

- Evaluates the mechanism of JX-594 direct oncolysis and cell death mechanisms including apoptosis, necroptosis and autophagy.

Chapter 5 - JX-594-induced innate immune response

- Phenotypes fresh human tissue *ex-vivo* lymph node samples.
- Examines the effects of JX-594 treatment on the innate immune system in blood and lymph nodes, with particular focus on NK cell activation as a marker for innate immune activity.

Chapter 2
Materials and Methods

2.1 General Tissue Culture

Tissue culture was all performed under aseptic conditions in laminar flow NuAire Class II Microbiological Safety Cabinets (NuAire Inc, Plymouth, USA). Cabinets were cleaned routinely pre and post procedures with 2% (w/v) Virkon (Scientific Laboratory Supplies, (SLS), Nottingham, UK) followed by 70% (v/v) ethanol (Sigma-Aldrich Ltd., Dorset, UK) to maintain sterility throughout. Plastic ware used was bought in pre-sterilised sealed packages from Corning Costar (High Wycombe, UK) or BD Biosciences (Oxford, UK). All pipettes used were produced by Gilson Inc. (supplied by Anachem Ltd., Bedfordshire, UK). All centrifugation was carried out for 5 minutes at 400g at room temperature (RT), using an Eppendorf 5810R centrifuge (Eppendorf, Leicestershire, UK) unless otherwise stated. Routine cell observation and cell counts were undertaken with an Olympus CKX41 light microscope (Olympus UK Ltd., South-End-On-Sea, UK) using 0.2% (v/v) Tryphan Blue (Sigma-Aldrich) and a haemocytometer (Weber Scientific International, West Sussex, UK).

2.2 Cell Lines

All cell lines were purchased from American Type Culture Collection (ATCC), the European Collection of Cell Cultures (ECACC) or acquired from collaborative working groups. All cell lines were cryopreserved in liquid nitrogen in 90% foetal calf serum (FCS) supplemented with 10% *(v/v)* dimethyl sulphoxide (DMSO) (Sigma-Aldrich Company Ltd, Dorset, UK) in 1.2ml cryotubes (Nunc, NY, USA). A summary of cell lines used and there derivation is shown in Table 2.1.

Prior to testing all cell lines were authenticated using short tandem repeat (STR) profiling and direct comparison with Leibniz-Institut Deutsche Sammiung von Mikroorganismen und Zellkulturen (DSMZ) databases.

Cell lines were recovered from frozen by rapid thawing in a water bath at 37°C followed by further dilution in fresh media (x10) and centrifugation to remove any residual, potentially toxic, DMSO. Thereafter cells were re-suspended in fresh media, either Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) or Roswell Park Memorial Institute (RPMI) 1640 (Sigma) (Table 2.1) supplemented with 10% (v/v) heat inactivated FCS (56°C, 30 minutes) and maintained in either a 75cm^3 or 150 cm^3 vented plastic tissue culture flasks (Corning Costar, Amsterdam, The Netherlands) in a Sanyo CO_2 incubator at 37°C. Adherent cell lines were routinely passaged or harvested at or near confluence by washing with phosphate buffered saline (PBS) (Sigma, Dorset, UK), with or without the additional supplementation of Ethylenediaminetetraacetic acid (EDTA) where required (Table 2.1), followed by Trypsin-EDTA (10x stock solution diluted in Hanks Buffered Salt Solution (HBSS) (Sigma)). Non-adherent cell lines were washed in PBS and centrifuged before being re-suspended in fresh media.

Cell Lines	Culture Properties	Culture Medium	Wash	Purchased
Colorectal cell Lines				
SW480	Adherent	DMEM + 10% FCS	PBS + EDTA	ATCC
SW620	Adherent	DMEM + 10% FCS	PBS + EDTA	ATCC
HCT116	Adherent	DMEM + 10% FCS	PBS + EDTA	ATCC
Colo320	Suspension, multi cell aggregates	DMEM + 10% FCS	PBS	ATCC
HT29	Adherent	DMEM + 10% FCS	PBS + EDTA	ATCC
LoVo	Adherent	DMEM + 10% FCS	PBS + EDTA	ATCC
LIM 1215	Adherent	RPMI + 10% FCS	PBS + EDTA	Donated
LIM1899	Adherent	RPMI + 10% FCS	PBS + EDTA	Donated
LIM 2408	Adherent	RPMI + 10% FCS	PBS + EDTA	Donated
Normal Kidney Cell Lines				
VERO	Adherent	DMEM + 10% FCS	PBS + EDTA	ATCC
Osteosarcoma Cell lines				
U20S	Adherent	DMEM + 10% FCS	PBS + EDTA	Donated
Miscellaneous				
PBMC	Suspension	RPMI + 10% FCS	PBS	Fresh
LNMNC	Suspension	RPMI + 10% FCS	PBS	Fresh

Table 2.1 Cell Lines and growing conditions

2.3 Measurement of viral titre by Plaque Assay

2.3.1 Reovirus

Reovirus Type 3 Dearing Strain was provided by Oncolytics Biotech Inc. Stock virus titres were determined using a plaque assay technique. Titred aliquots were stored at 4°C for up to 1 month to avoid loss of efficacy through repeated freeze-thaw, or at -80°C for longer storage.

The mouse C3H/An connective tissue cell line, L929, is known to be sensitive to Reovirus and was therefore used to determine the plaque forming activity of Reovirus stock virus titres and Reovirus treated samples. L929 cells were seeded into 6 well plates at a density of $1x10^6$ cells/well in 2mL of DMEM containing 10% FCS, and incubated for 4 hours, at 37°C, to allow for cell adherence. Stock virus and sample dilutions were prepared by serial dilutions in DMEM to give a logarithmic dilution ranging from 10^{-2}-10^{-11}.

At 4 hours, culture media was removed carefully from the adherent cells with special care taken not disturb the monolayer and replaced in either duplicate or triplicate with 500µL of the prepared virus samples. Plates were incubated for a further 4 hours at 37°C. The virus dilutions were then removed from the wells and replaced with 2mL of overlay medium (2:1 ratio of DMEM containing 10% FCS and 1.6% (w/v) of carboxymethylcellulose (CMC)). Cells were then incubated for a further 3 days at 37°C. After 3 days the media:CMC solution was removed and the wells washed with PBS before being fixed with 0.5mL 1% glutaradehyde for 10 minutes at RT. Glutaradehyde was then removed prior to the addition of 0.5mL 1% methylene blue (in 50% ETOH) for 3 minutes in order to stain the cells and visualise the plaques. Areas that were clear as a result of Reovirus-induced lysis (plaques) were counted using a lightbox to enhance visualization and the following formula used to calculate viral titre.

$$\text{Plaque forming units/mL} = \frac{\text{average no. of plaques} \times 2}{\text{dilution}}$$

2.3.2 JX-594

JX-594 was provided by John Bell and Colleagues (Ottawa Hospital Research Institute, Ottawa, Canada) on behalf of Jennerex Biotherapeutics Inc (recently acquired by Sillajen Inc, Korea). Several genetically modified strains were provided for use in different experimental models with the principle form used in these studies being JX-594-GMCSF-eGFP (referred to as JX-594 if not further clarified from this point forward). The insertion of the genes encoding for granulocyte macrophage colony stimulating factor (GMCSF) and green fluorescent protein (GFP) under an early/late promoter is useful not only due to its clinical relevance but because they can also be used as surrogate markers for the assessment of viral replication in experimental models. Other versions included in these studies were JX-594-GMCSF-fLuc (Luciferase) and JX-594-YFP (Yellow Fluorescent Protein) which does not express GMCSF.

Titred aliquots were stored at -80°C and thawed when required. The JX-594 viral backbone is understood to tolerate repeated freeze-thawing with regards to maintaining viral titre levels. However where possible this was minimized and when in use virus was stored at 4°C for no longer than 72 hours.

The human bone osteosarcoma epithelial cell line U2OS (originally known as 2T) were seeded into 6-well plates at a density of approximately $8.4x10^5$ cells/well in 2mLs DMEM containing 10% FCS, and incubated overnight at 37°C.

Stock virus and virus-treated samples for viral quantification underwent serial dilutions in serum free DMEM with a dilution factor range of 10^{-1} to 10^{-8} in a 96-well plate. Media was then carefully removed from the cells and 1mL of virus dilution added to appropriate wells, virus was incubated for a further 2hr incubation at 37°C. After 2 hours, the media was replaced with 2mL of over lay medium (1:1 ratio CMC mix with 3% CMC and 2 x DMEM + 20% FBS) and plates incubated for 96 hours at 37°C. Thereafter the overlay medium was aspirated and a single PBS wash of the cell monolayer undertaken. Plaques were fixed and stained with 0.5mL 1% glutaradehyde for 10 minutes followed by 0.5mL 1% methylene blue (in 50% ETOH) for 3 minutes. Plaque counting and calculations were undertaken as previously described to attain virus titres.

2.4 Flow Cytometry – Fluorescence-Activated Cell Sorter (FACS)

Flow cytometry was used to analyse the physical and chemical characteristics of all experimental cell types. Multiple fluorochoromes were utilised to allow for the identification of different subset populations within the mixed-cell sample population. Flow cytometry antibodies are outlined in Table 2.2. All flow cytometry outlined in the materials and methods was performed using the Attune® NxT Acoustic Focusing Cytometer (Life Technologies, Paisley, UK; Thermo Fisher Scientific, Loughborough, UK) and data analysed using the Attune® Cytometric Software. (v2.1.0) (Life Technologies, Paisley, UK; Thermo Fisher Scientific, Loughborough, UK)

TARGET MOLECULE	FLUOROCHROME	VOLUME ADDED	MANUFACTURER	INDICATION
CONTROLS				
Triple Isotype	FITC/PE/PErCP	2 µL	Dako Cytomation	Control Antibody
Isotype IgG1	FITC/ PE	10 µL 5 µL	Dako Cytomation	Control Antibody
Isotype IgG2B	PerCP	5 µL	Dako Cytomation	Control Antibody
IMMUNE CHARACTERISATION				
CD3	PerCP	10 µL	BD Biosciences	T-Cell Marker
CD4	FITC	5 µL	BD Biosciences	T-Helper Cell marker
CD8	FITC	5 µL	BD Biosciences	Cytotoxic T-Cell marker
CD11B	PERcP	10 µL	BD Biosciences	Pan-Macrophage marker
CD11C	PE	5 µL	BD Biosciences	Dendritic Cell (DC) Marker
CD14	PE	5 µL	BD Biosciences	Monocytes/ Macrophage marker
CD19	PE	5 µL	BD Biosciences	B-Cell Marker
CD56	PE FITC	2 µL 10 µL	BD Biosciences	NK Cell and activated T cell (NKT) marker
CD68	FITC	Intra-cellular staining	BD Biosciences	Pan-macrophage marker
CD69	FITC	10 µL	BD Biosciences	Early activation marker
CD80	PE	2 µL	BD Bioscience	Activation/ Maturation marker of Mature DC's
CD86	PE	2 µL	BD Biosciences	Early activation/ maturation marker of DC's

CD107a	FITC	5 µL	BD Biosciences	NK Cell degranulation marker
CD107b	FITC	5 µL	BD Biosciences	NK Cell degranulation marker
CD274	PE	5 µL	Sigma-Aldrich	Marker for Programmed Death Ligand -1 (PDL-1)
CD279	PE	5 µL	Sigma-Aldrich	Marker of programmed cell death protein 1 (PD-1)
Class II	PE/FITC	2 µL	BD Biosciences	Cell Surface expression on antigen presenting cells (APC's)
CELL LINE CHARACTERISATION				
CEA	FITC	5 µL	BD Biosciences	Marker of Colorectal carcinoma
EGFR	PE	5 µL	BD Biosciences	Epidermal growth factor receptor (EGFR) – stimulates cell growth and differentiation
BerEp4 (Anti-EpCAM)	FITC	5 µL	AbCam	Epithelial specific antigen. Tumour cell marker in lymph nodes, highly conserved expression in carcinoma

Table 2.2 Flow Cytometry Antibodies

2.5 Viability Assays

2.5.1 Measurement of Cell Viability using Live/Dead™

Cell viability was assessed using the Live/Dead™ reactive dye (Life Technologies). Following experimental conditions, cells were harvested into FACS tubes (BD Falcon) and washed in 1mL PBS by centrifugation for 5 minutes at 400g at RT. The supernatant was discarded and cell pellets were re-suspended in 1mL of PBS containing 1µL of PE-conjugated Live/Dead™ fluorescent dye (Invitrogen, Paisley, UK). The cell suspension was incubated in the dark for 30 minutes at RT. A final PBS wash was performed before the cells were fixed in 300µL of 1% Paraformaldehyde (PFA). FACS analysis (Section 2.4) was undertaken immediately where possible or alternatively cells were stored at 4°C and acquisition was performed within one week of sample preparation.

2.5.2 Methylthiazoyldiphenyl-tetrazolium bromide (MTT) metabolic activity.

Cells were seeded in triplicate at a density of 8 x 10^3 cells/well (in 200µL) into 96-cell well plates (Costar) and incubated overnight at 37°C. After 24 hours cells were treated with required virus doses and/or inhibitors and incubated at 37°C until assay end points. At the designated time points 20µL MTT (5mg/mL diluted in PBS) was added to the existing medium in each well and incubated for a further 4 hours at 37°C. After 4 hours, all media was carefully aspirated from the wells, taking particular care not to disrupt the adherent cell monolayer, and replaced with 150µL DMSO and left for 5 minutes to allow cells to solubilize. Optical density was measured using a multi-well scanning spectrophotometer (Multiskan EX plate reader (Thermo Fisher Scientific, Northumberland, UK)) at a wavelength of 550nm and viability calculated by direct comparison and normalization against an untreated control.

2.6 EGFR expression, Cetuximab and GA201 Binding studies

Cell lines were harvested as per section 2.2 and divided into FACS tubes at a density of $5x10^5$ cells/mL. Cells were washed in 2mL of FACS buffer (PBS + 1% (v/v) FCS and 0.1% (w/v) sodium azide) and pelleted by centrifugation for 5 minutes at 400g (RT), with the supernatant discarded. EGFR expression was examined using 5µL of stated antibody (Table 2.2) and incubated at 4°C for 30mins. To assess efficiency of anti-EGFR antibody drug binding, cell pellets were re-suspended in 1mL of media (DMEM, 10% FCS) and 0, 1.25, 2.5, 5, 10µg/mL of Cetuximab, Panitumumab and GA201 added to the cell suspension. The antibodies were incubated for 30 minutes at 37°C before cell were washed in 2mL of FACS buffer. Following this, 10µL FITC Mouse anti-human IgG (Table 2.2) was added to the cell pellets and cells were incubated for a further 30 minutes at 4°C. After a final wash in 2mL FACS buffer cells were pelleted by centrifugation at 400g for 5 minutes, supernatants discarded and cells fixed by the addition of 300µL of 1% PFA. Binding of Cetuximab, Panitumumab and GA201 was determined by flow cytometry (section 2.4) immediately where possible, or cells were stored at 4°C in the dark for a maximum of one week before data acquisition was undertaken.

2.7 ^{51}Chromium (^{51}Cr) Cytotoxicity Release Assay

Tumour target cells (SW480 and SW620) and effector cells (PBMC ± overnight pre-treatment with virus) were harvested, washed and centrifuged to form a cell pellet as described in section 2.2. Tumour target cells were labelled with 50µCi of ^{51}Chromium (Perkin Elmer, Cambridgeshire, UK) per one million cells and incubated for 1 hour at 37°C. Radioactive substance handling precautions were used at all times and any excess unbound ^{51}Cr was removed by three further wash steps using 50mL Hanks Buffered Salt Solution (HBSS) (Sigma-Aldrich). Target cells were finally re-suspended in RPMI containing 10% FCS at $1x10^5$ cells/mL. Following the harvest of effector cells, pellets were also re-suspended in RPMI/10% FCS at a specific cell density to enable a known target to effector ratio to be set up. Serial dilutions of the effector cell suspension was undertaken to achieve a final volume of 100µl

per well, seeded out in triplicate in 96-well round bottomed plates (NUNC, Thermo Scientific, Roskilde, Denmark). 50µL of the previously ^{51}Cr-labelled target cells were added to each well where required. To control for spontaneous release and quantify maximum ^{51}Cr release binding from cell targets, two simultaneous control plates were set up containing either target cells alone in media or target cells treated with 1% (v/v) Triton-X (Sigma) in RPMI (10% FCS) respectively. All plates were incubated for 4 hours at 37°C before being centrifuged (5 minutes, 400g, RT) and harvesting of 50µL of the supernatant from each well onto Luma scintillation plates (Perkin Elmer, Warrington, Cheshire). Luma plates were allowed to dry overnight at RT and levels of ^{51}Cr release determined using a 1450 MicrobetaJet Scintillation Counter (Wallac EG & G Ltd., Milton Keynes, UK). The ^{51}Cr release was measured in counts per minute (cpm). Results were converted to and expressed as 'percentage tumour cell lysis' using the following formula:

$$\% \text{ Lysis} = \frac{(\text{sample cpm} - \text{spontaneous cpm})}{(\text{max cpm} - \text{spontaneous cpm})} \times 100$$

2.8 Human primary tissue and blood

For human tissue and blood experiments written, informed consent was obtained from all patients in accordance with local institutional ethics review and approval guidelines. (Leeds (East) Research Ethics Committee, 06/Q1206/106). For healthy donor volunteers, verbal consent was obtained and samples collected in accordance with University of Leeds Institute of Cancer and Pathology guidelines.

2.8.1 Isolation of peripheral blood mononuclear cells (PBMC) from fresh blood using density gradient separation.

Whole peripheral blood was collected using standard local precautions from patients (Leeds Teaching Hospitals NHS Trust) with colorectal cancer undergoing colonic surgical resection and/or patients with metastatic

colorectal liver cancer undergoing liver resection surgery. Similarly, whole peripheral blood was also collected from healthy donor controls. Blood was first diluted 1:1 with HBSS and thereafter carefully layered onto Lymphoprep© (Axis Shield, Cambridgeshire, UK) at a ratio of two parts blood, one part lymphoprep in a 50mL Falcon Tube. Tubes were centrifuged at RT for 25 minutes at 800g with no brake. Excess plasma was removed and the white cell layer collected using a wide-tipped Pasteur pipette (Alpha laboratories Ltd. Hampshire, UK). The collected interface PBMC layer was washed in 50mLs HBSS and cells pelleted by further 10 minute centrigugation at 400g at RT. Cells were washed a second time in 50mLs HBSS and centrifuged at 300g for 5 minutes at RT. PBMC's were counted and resuspended in fresh RPMI containing 10% FCS culture medium at $2x10^6$ cells/mL.

2.8.2 Primary Tissue Collection

Fresh tissue samples were acquired from patients undergoing elective, planned resection for primary colorectal cancers of any type. The operations from which tissue was acquired were wide ranging and tumour location and staging at time of operation was variable. Written, informed consent was taken from all donors in accordance with institutional ethics review and approval (Leeds (East) Research Ethics Committee, 06/Q1206/106). Whole resection specimens were collected under sterile conditions as per the operating surgeons preferences and taken fresh, in the absence of formalin, immediately to a Consultant Histopathologist for processing. Samples were collected at the discretion of the histopathologist to avoid inadequate clinical processing, however, tissue considered of primary interest and priority was normal and/or tumour draining lymph nodes taken from the specimens mesenteric fat. Where possible additional tissue collected included healthy full thickness bowel (mucosa to serosa), primary colorectal tumour and in the case of concurrent operations to remove liver metastasis, liver tumour and hepatic parenchymal samples. As a result of the nature of collection, and understandable different disease profiles within donors there was some variability to the samples available from each specimen. Samples were transported to the laboratory in sterile 150mL screw-topped pots in RPMI

containing 10% FCS with the addition of 1% antibiotic, antimycotic solution (A combination of 10,000 units Penicillin, 10mg Streptomycin and 25µg Amphotericin B (Sigma)), and processed immediately.

2.8.3 Isolation of lymph node mononuclear cells (LNMNC) from fresh lymph node specimens using density gradient separation

Fresh lymph node samples were placed in a Petri dish containing 10mL, RPMI, 10% FCS and 1% antibiotic and carefully dissected to remove any excess fat, necrotic debris or surrounding non-lymph node tissue. Clean lymph nodes were then repeatedly injected with media to disaggregate them using a 21 gauge, 40mm green needle and 10mL syringe. The remaining cell suspension was then passed through a 70µm cell strainer (BD Biosciences) and any remaining debris removed using a 50mL large volume wash and centrifugation for 5 minutes at 400g, supernatant was discarded leaving a single cell suspension of LNMNC's.

The remaining single cell suspension of lymph node cells was diluted to a total volume of 30mLs in HBSS and layered carefully onto 15mLs of Lymphoprep© in a 50mL falcon tube before being centrifuged at 800g for 25 minutes at RT with no brake. The plasma containing supernatant was slowly removed with a pipette before the remaining LNMNC layer was collected using a wide-tipped Pasteur pipette. Cell were washed in 50mLs of HBSS and centrifuged for 10 minutes at 400g at RT before a second wash again in 50mLs of HBSS with centrifugation for 5 minutes at 300g at RT. Cells were counted and resuspended in RPMI at $1x10^6$ cells/mL or $2x10^6$ cells/mL depending on cell population yields which were variable between samples.

2.8.4 Characterisation of LNMNC's

To identify cell populations and the phenotypic appearance cells, LNMNC's cells were harvested and aliquoted into FACS tubes at a density of $5x10^5$-$1x10^6$ cells/tube. Cells were washed in 2mL of FACS buffer, centrifuged (Sorvall RT6000B refrigerated centrifuge, Kendro Lab Products,

Hertfordshire, UK) at 400g for 5 minutes at 4°C before discarding the supernatants. To identify cell populations, a targeted selection of fluorescently-conjugated antibodies were added (see Table 2.2) and cells incubated for 30 minutes in the dark at 4°C. After 30 minutes cells were washed in 2mL of FACS buffer and centrifuged again. The supernatant was discarded and the stained cells were fixed in 300µL of 1% PFA. FACS analysis (Section 2.4) was undertaken immediately where possible or alternatively cells were stored at 4°C and analysis performed within one week of acquisition.

2.8.5 CD14 Positive cell separation of PBMC's/LNMNC's using magnetic-activated cell sorting (MACS) bead selection.

Fresh PBMC's/LNMNC's were prepared as described in sections 2.8.1 and 2.8.3, respectively. Cell separation of the CD14 positive and negative fractions was then undertaken. The harvested mononuclear cells were washed in 5mL of MACS buffer (1% (v/v) FCS + 2mM EDTA in PBS), centrifuged and the cell pellet re-suspended in 80µL of MACS buffer per $1x10^7$ total cells in a 15mL Falcon tube. 20µL per $1x10^7$ cells of MACS CD14 Microbeads (Miltenyi Biotec Ltd., Surrey, UK) were added to the falcon tubes and incubated for 15 minutes at 4°C prior to being washed in 2mL of MACS buffer and centrifuged at 300g for 5 minutes at RT. The supernatant was again discarded and the remaining cell pellet re-suspended in 500 µL/$1x10^8$ cells of MACS buffer. A MACS LS magnetic separation column was mounted on a magnetic board (both Mltenyi Biotec Ltd., Surrey, UK) and washed with 3mLs of MACS buffer prior to use. The cell suspension was then loaded and allowed to pass through the column. The $CD14^{-ve}$ cell population remained unbound and was collected as the run-off after 3 further 3mL MACS buffer washes. To collect the bound $CD14^{+ve}$ cell population retained in the column, the column was immediately removed from the magnet and flushed using a supplied column plunger. Thereafter 5mL of MACS buffer was added to the column and the resultant cell suspension was collected into a 50mL Falcon tube. The separated cell populations were then counted and utilised as required.

2.9 Virus Treatment of Cell Lines, PBMC'S and LNMNC's

2.9.1 Cell lines

Harvested cell lines were seeded into 6 well plates at a density of $2x10^5$ cells/mL in 3mls of culture medium. Cells were incubated for 4 hours in order to allow adherence prior to virus treatment at a range of doses (0, 0.1, 1, 10pfu/cell). Plates were further incubated thereafter at 37°C for the required experimental time under investigation.

2.9.2 PBMC's and LNMNC's

PBMC's and LNMNC's were cultured at $2x10^6$ cells/mL in 24-well plates. Cell lines were allowed to settle as required and thereafter were able to be used immediately if required. Virus was added at a range of doses (0, 0.1, 1, 10pfu/cell) and incubated at 37°C for the required experimental time under investigation.

Cell lines, LNMNC and PBMC's were then utilised where required in further experiments described in this chapter: NK cell activation studies such as CD69 and CD107 degranulation (section 2.11.2 and 2.11.3 respectively); chromium release assays (section 2.7); cell specific phenotyping studies (section 2.4) and culture supernatants were collected throughout and analysed by ELISA (section 2.12).

2.10 Inhibition of Apoptosis, Necroptosis and Autophagy Death Pathways

SW480 and SW620 cell lines were seeded into a 24-well plate at a density of $7.5x10^4$ cells in 1mL of culture medium and incubated for a minimum of 4 hours at 37°C to allow adherence. The apoptotic, necroptosis and autophagy inhibitors were then added to the wells at the doses listed in Table 2.3 as single agents or in combination and incubated for 1 hour at 37°C. Cells were treated with 0 – 10pfu/cell Reovirus or JX-594 for up to 96 hours. At specific

time points cells were harvested and cell viability was analysed using Live/Dead™ Cell Viability Assay as described in Section 2.5.1.

Inhibitor	Inhibits	Company	Concentration Used
Z-VAD-FMK	Apoptosis	Calbiochem, Nottingham, UK	50μM
Necrostatin-1	Necroptosis	Tocris Bioscience, Bristol, UK	50μM
Necrosulphonamide	Necroptosis	Tocris Bioscience, Brsitol, UK	10μmol/l
3-Methyladenine (3-MA)	Autophagy	Sigma-Aldrich, St Louis, MO	50μM

Table 2.3 Cell death mechanistic inhibitors

2.11 Immune Activation

2.11.1 NK Cell Activation

Natural Killer Cells (NK) within both a PBMC and LNMNC population ('effector cells') were assessed utilising the cell surface expression of CD69 (Section 2.11.2) and degranulation (section 2.11.3) assessed using CD107a/b. Treated effector cell populations were cultured overnight in RPMI containing 10% FCS in the presence and absence of virus. Target cell populations (SW480 and SW620 cell lines) were harvested as previously described (section 2.2), washed in PBS and pelleted by centrifugation. Where appropriate supernatants were saved and stored at -80°C for further analysis by ELISA (section 2.12).

2.11.2 CD69 Expression Assay

$5x10^5$ effector cells in 200µL of RPMI (10% FCS) were added to a FACS tube, washed with 2mL of FACS buffer and centrifuged at RT for 5 minutes at 400g. The pellet was then stained with the fluorescently-conjugated antibodies CD3 PerCP, CD56 PE and CD69 FITC (as per Table 2.2) and incubated in the dark at RT for 30 minutes. Cells were finally washed in a further 2mL of FACS buffer and fixed in 1% PFA before being analysed by flow cytometry (section 2.4).

2.11.3 CD107 Degranulation Assay

$5x10^5$ effector cells in 200µL of RPMI (10% FCS) were added to FACS tubes and $5x10^4$ target cells in 200µL of RPMI (10% FCS) added to give a final 10:1 effector:target ratio in a total volume of 400µL of media. Cells were co-cultured in the FACS tubes for 1 hour at 37°C. After 1 hour an antibody mix containing 10µL CD3 PerCP, 2µL CD56 PE (for identification of NK cells), 5µL anti-CD107a FITC, 5µL anti-CD107b FITC and 0.5mL Brefeldin A (Sigma) giving a working concentration of 1µL/mL (see Table 2.2) made up to 100µL volume with media was added to each tube. Tubes were then incubated at 37°C for a further 4 hours before being washed in 2mL of FACS buffer, and fixed in 300µL of 1% (w/v) PFA. Analysis was undertaken immediately if possible or cells were stored in the dark at 4°C for a maximum of a week prior to data acquisition by flow cytometry (section 2.4).

2.11.4 Interferon Blocking of Natural Killer (NK) cell mediated virus activation.

To assess the pathways involved in virus-induced NK cell activation, type I Interferon α/β was blocked and CD69 activation and CD107 degranulation assays repeated as described above (sections 2.11.2 and 2.11.3 respectively). PBMC's or LNMNC's cells were seeded at $1x10^6$ cells/mL in a 12-well plate and incubated for 1 hour at 37°C to allow cells to settle. 30 minutes prior to the addition of virus 15µL of medium was added to each of

the control wells, a combination of 7.5µL anti-IFN α sheep serum (sigma), 7.5µL anti-IFN β sheep serum and 12.5µL anti-α/β receptor antibodies were added to the block interferon and finally 15µL sheep serum and 12.5µL IgG2a isotype antibody were added to treatment control wells. Thereafter, cells were infected in triplicate (control, interferon block and isotype control) with 0, 0.1 and 1pfu JX-594 and incubated for 24 hours at 37°C. Cells were harvested as detailed in (section 2.8.1 and 2.8.3) and $5x10^5$ effector cells resuspended in 200µl RPMI/10% FCS before continuing with assays to assess CD69 expression and CD107 a/b degranulation (section 2.11.2 and 2.11.3 respectively).

2.12 Enzyme-linked Immunosorbent Assay (ELISA)

2.12.1 ELISA for IFN-α, IFN-γ, IP-10, GMCSF and TNF- α

Maxisorp© (96 well, flat-bottomed plates) (Nunc) were coated with pre-optimised dilutions of capture antibodies (see Table 2.4) diluted in either PBS or coating buffer (100mM $NAHCO_3$ in ddH_2O) as indicated. Plates were wrapped in cling film and stored at 4°C overnight. The following morning plates were washed three times with PBS-Tween (0.05% Tween (Sigma) in PBS) using a plate washer (Skan Washer 300, Molecular devices, Berkshire, UK) and blocked with 200µL of blocking solution (PBS containing 10% FCS) for 2 hours at RT. Plates then underwent a further 3 washes with PBS-Tween. 200µL of recombinant protein standards (of known concentration) were added in triplicate to the top wells and halving serial dilutions into 100µL of media to create a standard curve with halving concentration of recombinant standards; the lower most channel only containing media to act as control for background optical density readings. Thereafter plates were loaded with sample supernatants in triplicate at 100µL/well, wrapped in cling film and stored overnight at 4°C. Following the incubation period, plates were washed six times in PBS-Tween and 100µL of biotinylated detection antibody diluted in blocking solution was added for a further 2 hours at RT. Plates were washed again six times in PBS-Tween before 100µL of Extravidin-alkaline phosphatase conjugate (Sigma), diluted 1:5000 times with PBS-Tween, was

added and incubated for 1 hour at room temperature. Plates were washed three times with PBS-Tween and three times with ddH_2O. Finally substrate solution (p-nitrophenyl phosphate alkaline phosphatase, prepared to manufacturers guidelines (Sigma)) was added at 100µL/well and plates left to develop in the dark for 15-60 minutes.

Plates were read using a Multiskan EX plate reader (Thermo Fisher Scientific) at a wavelength of 450nM to record optical densities. Using the standard curve optical density readings were then used to reveal sample protein quantities (pg/mL).

Antibody	Capture	Detection	Standard (Top Concentration)	Type	Manufacturer
IFN-α	1:250	1:500	5000pg/ml	Mouse Anti-Human	Mabtech
IFN-γ	1:250	1:500	10000pg/ml	Mouse Anti-Human	BD Biosciences
IP-10	1:500	1:500	5000pg/ml	Mouse Anti-Human	BD Biosciences
CXCL-10	1:180	1:180	2000pg/ml	Goat Anti-Human	R&D Systems, Bio-techne.
GMCSF	1:500	1:1000	2000pg/ml	Mouse Anti-human	Mabtech
TNF- α	1:1000	1:1000	2000pg/ml	Mouse Anti-human	BD Biosciences

Table 2.4 ELISA antibodies and protein standards

2.12.2 ELISA for IFN-β

ELISA for IFNβ production was undertaken utilizing a Verikine™ Human IFN Beta ELISA kit (PBL Interferon Source, NJ, USA). Pre-coated 96-well plates provided with the kit were loaded in duplicate with serial dilutions of recombinant protein standards as per manufacturer's instructions before 50µL of sample supernatants were added and incubated for 1 hour at RT. Plates were washed three times utilizing a supplied wash buffer. 100µL of detection antibody, appropriately diluted in a concentrate diluent was then added before a further 1 hour incubation at RT. Plates were washed once more 3 times in wash buffer prior to addition of 100µL of appropriately diluted Horseradish peroxidase (HRP) for a further 1 hour incubation at RT. A final single wash in wash buffer was then undertaken before 100µL of TMB substrate (provided with kit) was added to each well and allowed to develop for 15 minutes in the dark. Following the addition of 100µL of stop solution, optical densities were determined using a Multiskan plate reader at a wavelength of 405nm. Optical densities were converted to protein quantities (pg/mL) using the standard curve generated.

2.13 Western Blots for HMGB1

Cell free supernatants were harvested from pre-cultured SW480 and SW620 cell lines infected with an multiplicity of infection (MOI) of 0, 0.1 or 1 for 24, 48 and 72 hours. Supernatants were then stored at -80°C until required. Alongside this, cell pellets were prepared from harvested DLD-1 cell lines cultured in T75 flasks to act as a positive control.

Running gels and stacking gels were prepared using: ddH_2O, 30% acrylamide mix; 10% (w/v) SDS, 1.5M Tris pH 8.8 (running gel), 1M Tris pH6.8 (stacking gel), 10% (w/v) ammonium persulphate and Tetramethylethylenediamine (TEMED) according to previously described protocols and quantities described by Sambrook et al. (1989)[192]. A 10% running gel was poured into prefabricated cassettes (Invitrogen Life Technologies) and allowed to set. The cassette was then rinsed with ddH_2O, and the stacking gel and comb added. 20µL of each of the cell free supernatants diluted 1:1 with Laemmli buffer (4%

SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl at pH 6.8) were loaded into the sample lanes alongside 2µL of protein molecular marker (Odyssey, LI-COR Biosystems, Lincoln, USA) added to the control lane in order to identify the size of the detectable proteins.

Gels were run at 160-180V for approximately 60 minutes (until dye had run to the bottom of the gel) using a XCell SureLock™ Mini Cell system (Invitrogen) and a Bio-Rad laboratories PowerPac™ HC. Protein transfer was then performed at 25V for 120 minutes onto a Hybond™ C Super nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was washed three times in PBS containing 0.1% (v/v) Tween (PBST) and blocked overnight in Odysssey blocking buffer (LI-COR Biosciences).

Staining for HMGB1 was undertaken using a monoclonal mouse anti-human HMGB1 antibody (R&D systems, Abingdon, UK) at a 1:500 dilution in a 1:1 mix of blocking buffer and PBST. The membrane was placed carefully in a 50mL Falcon tube and 5mL of the antibody mix added and placed on roller apparatus for 1 hour at room temperature. The membrane was then washed four times for 5 minutes each in PBST. The secondary antibody used was goat anti-mouse conjugated with AlexaFluor 680 (Molecular Probes, Invitrogen), this was used at a 1:5000 dilution in 5mL of 1:1 ratio mix of blocking buffer and PBST. Incubation for 1 hour at RT in the dark was again achieved by placing the membrane in a 50mL Falcon and continuously rotating on a roller to ensure consistent antibody/membrane coverage. The membrane was finally washed four further times for 5 minutes, each in PBST before Supersignal™ West Pico Chemiluminescent substrate (Thermo Fisher Scientific, Loughborough, UK) was added to the membranes for 5 minutes. Excess liquid was removed and the membrane was taken to the dark room and exposed to Amersham Hyperfilm™ECL™ film (GE Healthcare Life Sciences, Buckinghamshire, UK) and developed using a Kodak X-OMAT 3000RA Processor.

2.14 Statistical Analysis

Data was represented graphically as mean ± standard error of the mean (S.E.M). Statistical analysis was performed using Graphpad Prism for Mac (v6.0, Graphpad Software, Inc). p values were calculated by using the one-way or two-way analysis of variants (ANOVA) with Bonferroni *post-hoc* test or a paired student t-test with two-tailed distribution. p values were considered significant at $p<0.05$.

Chapter 3
Combination Strategies

3.1 Introduction

The development of novel targeted therapies for CRC, such as cetuximab and Panitumumab, has improved colorectal cancer patient overall survival although anti-epidermal growth factor receptor (EGFR) immunotherapy is not suitable for all patients, with only 10-20% achieving clinical responses. KRAS, BRAF and PI3KCA mutations (causing constitutively activated EGFR-signalling) have been implicated in cellular resistance to anti-EGFR therapy.

Cetuximab (an IgG1 chimeric anti-EGFR antibody) and Panitumumab (an IgG2 fully-humanised anti-EGFR antibody) induce their anti-cancer effects by two distinct mechanisms; blockade of EGF-receptor binding preventing pro-survival/proliferation signals, and ADCC/ADCP. A role for monocytes and NK cells has been described for cetuximab-ADCC/ADCP, whereas Panitumumab utilises monocytes and neutrophils for its immune-mediated effects; these targeted interactions are regulated by Fcγ receptor expression on different immune effector cells.[193] GA201, a humanised-IgG1 glyco-engineered anti-EGFR antibody is reported to have increased Fc-gamma binding affinity and induction of ADCC in KRAS wild-type and mutant cells, and is also in pre-clinical development.[194]

ADCC should occur irrespective of EGFR-signalling mutations and one would expect patients with *KRAS*-mutations to demonstrate some clinical response if ADCC played an important role *in vivo*. Disappointingly, large clinical studies have shown that *KRAS*-mutant patients do not benefit from anti-EGFR therapy (regardless of antibody mechanism of action) and it is currently only offered to patients with wild-type *KRAS* cancers.[195] Immune cell populations within the tumour microenvironment can either support tumour growth or contribute to anti-tumour activity and as such can be used as diagnostic and prognostic biomarkers. High lymphocyte counts typically are reported to correlate with good prognosis and high tumour-associated macrophages (TAM)/neutrophils (TAN) are associated with poor prognosis.[196] Interestingly, a controversial role for TAM has been described for CRC with both pro- and anti-tumour activities

being reported. Acting as 'pro-tumour macrophages' TAMS promote initiation and metastatic progression of tumour cells, stimulate tumour angiogenesis and via the modulation of T-cell activity inhibit anti-tumour immune responses.[196] Furthermore, TAMS are reported to protect colorectal cancer cells from TNF-related apoptosis induced ligand (TRAIL)-mediated apoptosis.[197]

Conversely, strong evidence also exists for the anti-tumour activity of TAMS with several authors reporting an association between increased macrophage infiltration and improved outcomes, the mechanisms behind which remain less clear.[198–201] Some suggested mechanisms include macrophages along the tumour margin inducing apoptosis in a Fas ligand-dependent process, as reported by Sugita et al. (2002)[199] or alternatively peritumoral macrophages differentiating into a tumoricidal phenotype as a result of reduced tumour derived cytokine exposure and being located in a less hypoxic area.[202,203] Clearly, harnessing the immune cell repertoire within the tumour is likely to be important for generating objective clinical responses and it is possible that immune cell activation could increase the efficacy of anti-EGFR immunotherapy for both wild-type and mutant-*KRAS* CRC.

Oncolytic viruses (OV) preferentially replicate in cancer cells causing cell death. Oncolytic Reovirus, a clinically advanced OV, exerts its cytotoxic effects by direct oncolysis (reported to be caspase-dependent and therefore apoptotic in nature) and/or activation of anti-tumour immunity.[204] Reovirus sensitivity has been reported to be dependent on *KRAS*-mutations, or downstream activating signalling mutations (such as PI3K) (Figure 1.3) hence *KRAS*-mutant patients that are resistant to cetuximab should be susceptible to Reovirus-induced oncolysis. As such we hypothesise, that immune activation of monocytes, neutrophils and NK cells by Reovirus may potentiate cetuximab/panitumumab mediated-ADCC/ADCP. In support of this, published research has demonstrated that Reovirus can; activate monocytes within white blood cells (WBCs)[205]; activate NK cells both *in vitro* and *in vivo*[206]; be isolated from CRC liver metastases after systemic delivery[206]; and enhance rituximab-mediated ADCC of autologous CLL cell targets.[205]

Recently, detailed analysis of CRC liver metastases demonstrated that B-Cell Lymphoma Extra Large (BCL-$_{XL}$), an anti-apoptotic, pro-survival protein associated with advanced disease and resistance to chemotherapy, is upregulated in CRC and associated with *KRAS*-mutations.[207] Interestingly, inhibition of BCL-$_{XL}$, using the pro-apoptotic BH3 mimetic (ABT-737) which mechanistically antagonizes the BCL-2 family of anti-apoptotic proteins, was shown to sensitise HRAS^{G12V}-expressing colorectal cancer cells to cetuximab cytotoxicity.[207] As such, it has been proposed that combination strategies incorporating ABT-263/Navitoclax (a clinical grade BH3-mimetic) could have the potential to increase OV direct cytotoxicity.[208]

These hypotheses remain untested and could provide strong evidence to support the use of OV, in appropriate combinations with either cetuximab/Panitumumab and/or BCL-2 family antagonists for all CRC patients, including those with EGFR-signalling mutations or specifically to target those patients resistant to anti-EGFR monotherapy.

3.2 Results

3.2.1 Colorectal cell lines are variably susceptible to Reovirus killing

Previous members of the group have demonstrated that a number of CRC cells are susceptible to Reovirus-induced oncolysis[204] and replication-competent Reovirus can be recovered from CRC liver metastases after intravenous injection prior to surgical resection.[206] Previous work has however not been contextualised with regard to genetic mutations and has utilised a selective and limited panel of colorectal cell lines. This study therefore initially aimed to broaden the range of cell lines investigated, examining their susceptibility to OV killing using a Live/Dead™ flow cytometry cell viability assay.

Nine colorectal cell lines in total were available for experimentation from either collaborative groups or from within the local cell bank library. A summary is shown in Table 3.1.

Cell Line	Age	Ethnicity	Gender	Organ	Disease	Stage	Derivation	Morphology
SW480	50	Caucasion	Male	Colon	Colorectal Adenocarcinoma	Dukes B	Primary Tumour	Epithelial
SW620	51	Caucasion	Male	Lymph Node Metastasis	Colorectal Adenocarcinoma	Dukes C	Metastasis	Epithelial
HCT116	48	Caucasion	Male	Ascending Colon	Colorectal Adenocarcinoma	Dukes D	Primary Tumour	Epithelial
Colo320	55	Caucasion	Female	Sigmoid Colon	Colorectal Adenocarcinoma	Dukes C	Primary Tumour	Rounded & Refractile
HT29	44	Caucasion	Female	Colon	Colorectal Adenocarcinma	Dukes C	Primary Tumour	Epithelial
LoVo	56	Caucasion	Male	Left Supraclavicular Lymph Node	Colorectal Adenocarcinma	Dukes C	Metastasis	Epithelial
LIM1215	34	Caucasion	Male	Omental Metastasis	Heridatory nonpolyposis colorectal cancer	Duke D	Metastatis	Epithelial
LIM1899	UN	Unknown	Unknown	Colon	Colorectal Adenocarcinoma	Dukes C	Primary Tumour	Epithelial, Rounded & clustered
LIM2408	UN	Unknown	Unknown	Splenic Flexure	Moderately differentiated adenocarcinoma	Dukes C	Primary Tumour	Epithelial

Table 3.1 CRC cell line library

Summary of original patient demographics, cancer site and disease profile. *(un = unknown)*

A broad range of mutational profiles exist within this panel of cell lines (Table 3.2) and we therefore planned to utilise this library of CRC cell lines to test susceptibility of various *KRAS*/*BRAF*/*PI3KCA* mutations to Reovirus cytotoxicity using cell viability (Live/Dead™ and MTT (*data not shown*)) assays. This is important given that we know Reovirus can access CRC in particular, liver metastasis, following systemic delivery to cancer patients. Levels of cell death were examined in the context of *KRAS*/*BRAF*/*PI3KCA* mutations to determine whether a specific genetic mutation, or combinations thereof, could be used to predict Reovirus sensitivity.

	SW480	SW620	HCT116	Colo320	HT29	LOVO	LIM1215	LIM1899	LIM2408
EGFR expression	Yes	Low	Yes	No	Yes	Yes	Yes	Yes	Yes
KRAS	G12V	G12V	G13D	WT	WT	G13D	WT	G12A	WT
BRAF	WT	WT	WT	WT	V600E	WT	WT	WT	V600E
P13K	WT	WT	H1047R	WT	WT	WT	WT	WT	WT

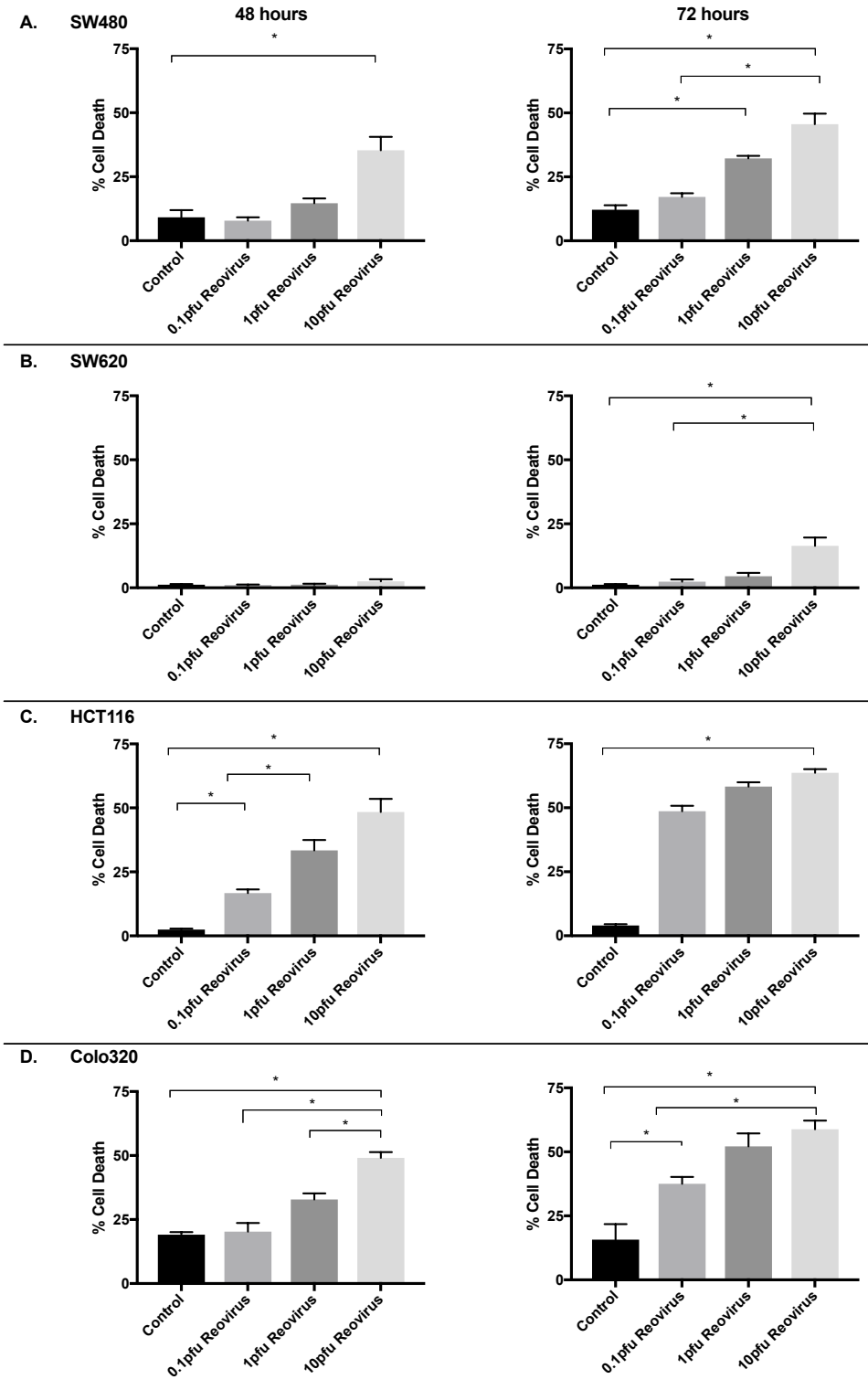
Table 3.2 CRC cell line panel.

Table highlighting reported EGFR expression and KRAS/BRAF/PI3KCA mutational status within the experimental CRC cell line panel.

3.2.2 Reovirus demonstrates variable levels of cytotoxicity against CRC cells with different mutational characteristics.

Initial experimentation therefore involved the direct cytotoxic potential of Reovirus on our panel of CRC cell lines. All nine available cell lines were initially studied, however, for the purposes of this report two of the lines were excluded. Both LOVO and LIM1215 cell lines showed wide variability in results and high levels of cell culture death in the absence of treatment. Further examination demonstrated mycoplasma infection and despite attempts to decontaminate the stored samples a decision was made to exclude these lines from further experimentation.

SW480, SW620, HCT116, Colo320, HT29, LIM1899 and LIM2408 cell lines were directly infected with 0, 0.1, 1 and 10pfu/cell Reovirus for 24-96 hours before cell viability was determined using Live/Dead™ cell viability assay and flow cytometry. Figure 3.1 shows data for the 48 and 72 hour time points.


A.
SW480
48 hours
72 hours
B.
SW620
C.
HCT116
D.
Colo320
% Cell Death
0
25
50
75
Control
0.1pfu Reovirus
1pfu Reovirus
10pfu Reovirus
*

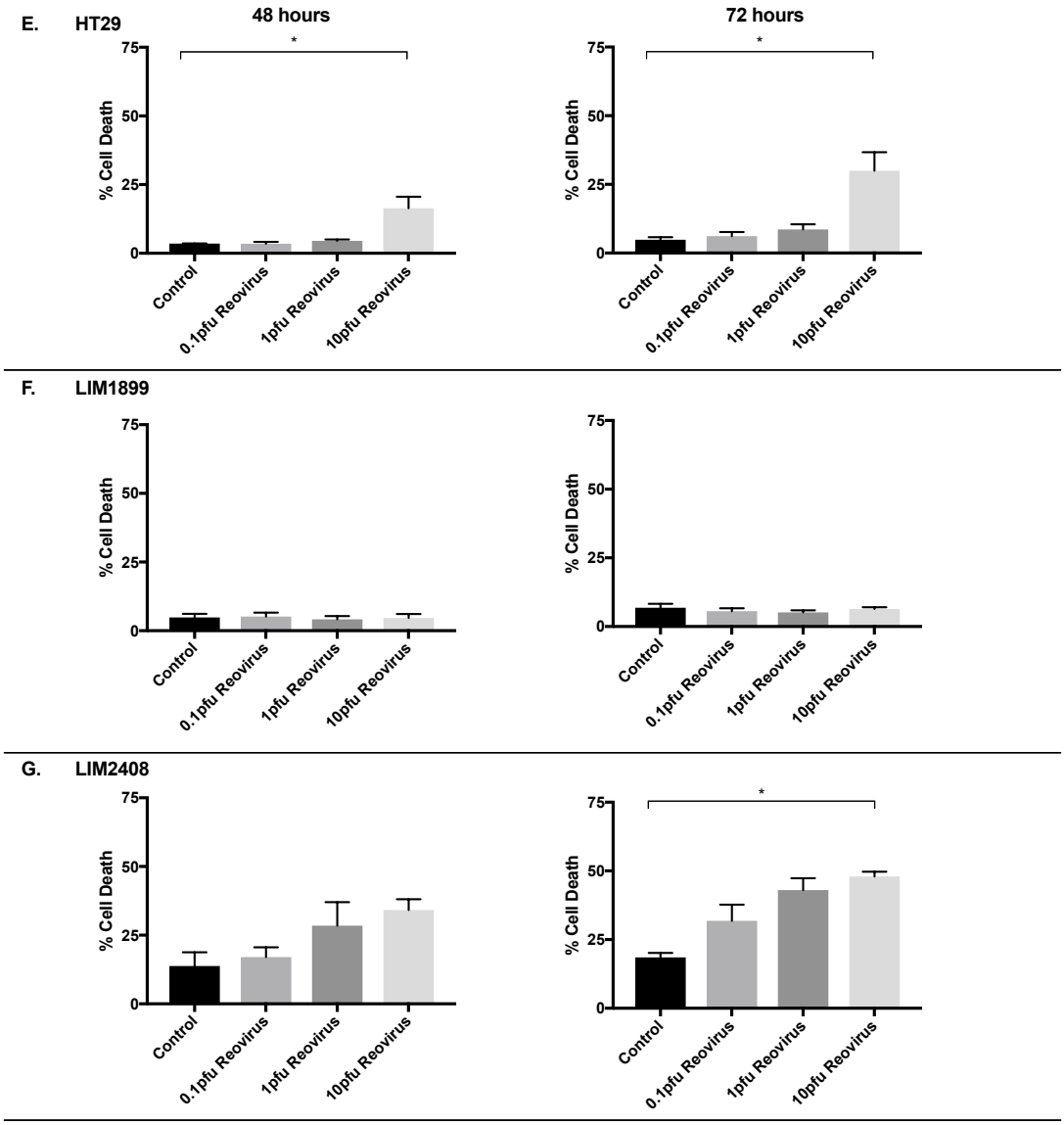


Figure 3.1 Reovirus-induced cell death in CRC Cell Lines.

Cell lines A-G (A-SW480, B-SW620, C-HCT116, D-Colo320, E-HT29, F-LIM1899 and G-LIM2408) were treated with 0 (control), 0.1, 1 and 10pfu/cell Reovirus for 48 and 72 hours. At each time point cells were harvested and stained with Live/Dead™ for assessment by flow cytometry. Graphs show mean percentage cell death of at least 3 independent experiments + SEM. Statistical significance is denoted by * $p<0.05$ (one-way ANOVA)

Our panel of cell lines responded with mixed sensitivity to infection with Reovirus. Consistent with previous data from our laboratory, SW480 cells showed a repeatable dose-dependent and time dependent response with maximal death seen at 72 hours with 10pfu/cell Reovirus. SW620 cells, the metastatic line relating to the SW480 primary are known to be more resistant to virotherapy and as such no death was seen at 48 hours but thereafter Reovirus killed in a dose and time dependent fashion. The suggestion of a continuing effect and further death was seen at 96 hours (*data not shown*) but for the purposes of this study we did not extend this time point further. Similar reduced sensitivity to Reovirus was observed in HT29 cell lines with only low level cell death seen following high dose, 10pfu Reovirus treatment. Increased cytotoxicity was seen in a time dependent fashion (16.3% to 29.9% for 10pfu at 48 and 72 hours respectively) but remained low when compared with other cell lines.

As with SW480, Colo320 and LIM2408 cells were sensitive to Reovirus-induced oncolysis even at lower doses of Reovirus. Maximal percentage cell death (58.3% and 47.9% respectively) was observed after 72 hours and treatment with 10pfu Reovirus. Moreover, HCT 116 cells demonstrated the most sensitivity to Reovirus cytotoxicity with cell death observed at early time points and low concentrations of virus (16.7% cell death at 48hrs and 0.1pfu/cell Reovirus; 48.6% cell death at 72hrs and 0.1pfu/cell Reovirus) when compared to other cell lines.

There was no cytotoxicity seen in LIM1899 across the time points implying that this cell line is completely resistant to Reovirus infection. Across our panel of cell lines there was no evidence that Reovirus induced death demonstrated any correlation to mutational status, however of note HCT116 were the most sensitive and contain mutational changes in the RAS pathway. Interestingly, Colo320 which are wild type and harbour no mutations in the KRAS/BRAF/PI3K pathway, and therefore maybe expected to be resistant are in fact sensitive to Reovirus-induced cell death. It remains unclear as to the mechanism behind this but hypothetically this could be due to mutations in alternative uncharacterised genes in the RAS pathway or alternatively that Reovirus sensitivity is not ubiquitously controlled by the RAS signalling pathway.

3.3 EGFR Binding studies

Given that the mutational status of CRC cell lines did not correlate with Reovirus sensitivity, we wanted to confirm that the cell profiling reported in the literature was consistent with our in house cell populations. Particular focus was placed on EGFR binding, given its relevance to work discussed later in this chapter regarding anti-EGFR monoclonal antibody combination strategies. Cell surface expression of EGFR was determined using an anti-human EGFR-PE monoclonal antibody by flow cytometry. All cell lines were tested and representative results are shown for 4 cell lines (Figure 3.2). SW480 cells demonstrated the most significant EGFR cell surface expression, whilst moderate expression was seen in HT29 and HCT116. As reported Colo320 CRC cells showed no evidence of EGFR expression. For the remaining cell lines tested, EGFR expression correlated with published reports (See Table 3.2).

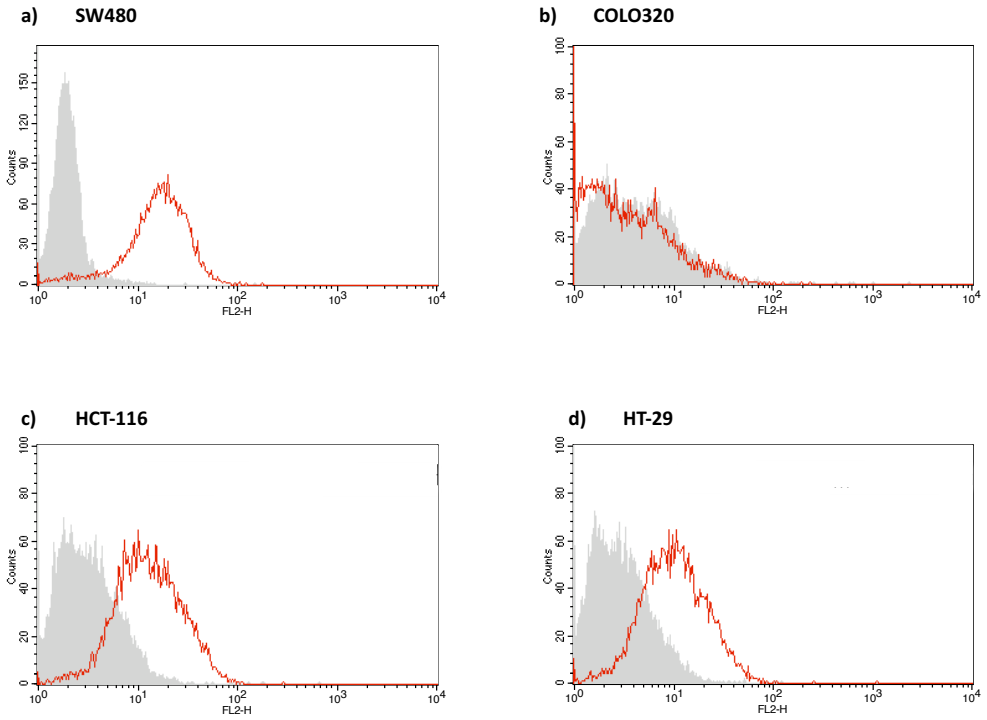


Figure 3.2 Cell surface EGFR expression for four CRC cell lines.

Freshly harvested cell lines were stained with an anti-human-EGFR-PE antibody or IgG isotype control. Cell surface expression was determined by flow cytometry (Red line: EGFR expression; shaded area: Isotype control). Data shown is representative of 3 independent experiments.

3.4 Combination strategies with BCL-2 antagonists.

The PI3K-AKT and RAS-ERK pathways are two of the most important signaling pathways that regulate cell proliferation and survival. Functional mutations in genes for these two signaling pathways, such as RAF, RAS and PI3K are frequently observed in malignant tumours and result in abnormal activation causing primary drug resistance and reduced efficacy of chemotherapeutic agents.[168,209,210] These pathways therefore present promising therapeutic targets however the targeting of individual molecules often results in only limited anti-tumour efficacy and as such combination strategies to target various aspects of the pathway could provide a more promising therapeutic effect. Interestingly, Leverson et al. (2015) [210] showed that inhibition of the PI3K-AKT or RAS-ERK pathway (by for example anti-EGFR antibodies) upregulates the pro-apoptotic proteins BIM or BAD and downregulates the pro-survival proteins BCL-2 and MCL-1. These effects were significantly enhanced by co-treatment with the BH3 mimetic, ABT-263, and resulted in 'on-target cancer cell killing activity'.

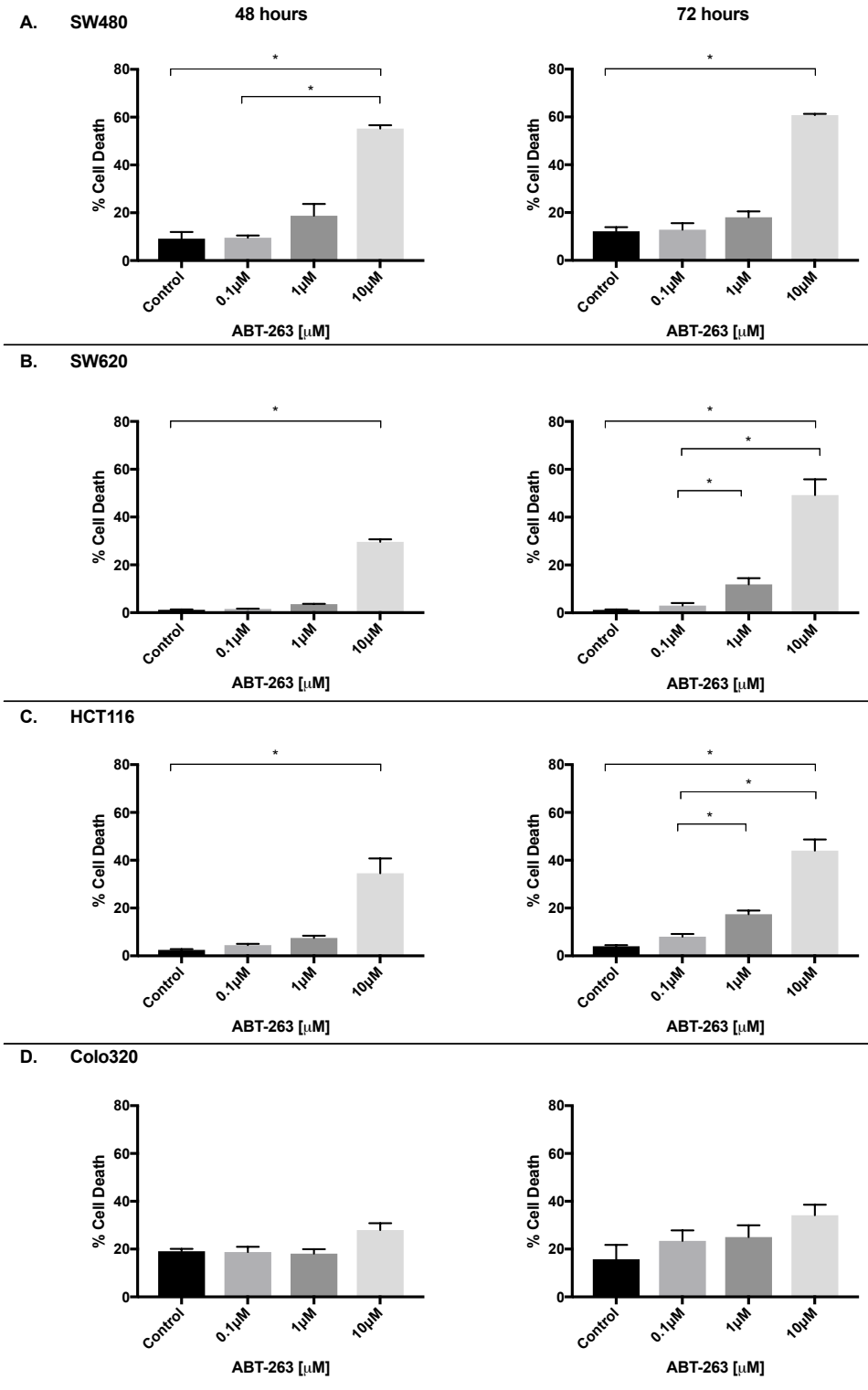
ABT-263 (Navitoclax) is an orally administered active analogue of ABT-737, a BH3 mimetic designed to induce apoptosis by blocking the functions of the pro-survival BCL-2 family proteins. Combination strategies using ABT-263 with various FDA approved drugs have reached clinical trials for the treatment of both solid tumours and haematological malignancies, however tumour cells expressing high levels of MCL-1 have shown resistance to both ABT-263 and ABT-737.[178,211] Furthermore after long exposure to the BH3 mimetic inhibitors, resistance is acquired as a result of upregulation of MCL-1.[212]

3.4.1 ABT-263 induced cell death in Colorectal Cell lines

Given the mechanism of action of ABT-263 (i.e. modulation of the pro vs anti apoptotic pathway), we hypothesised that combination with OV (which can kill via apoptotic mechanisms) may increase the efficacy of oncolytic virotherapy producing a synergistic effect. ABT-263 activity and cell death is thought to rely on releasing BIM to promote apoptosis[213]. High levels of BIM are reported to predict an increased sensitivity to ABT-263 while resistance is correlated

with high MCL-1 levels. Various studies have documented that almost 50% of cancer cell lines are resistant to ABT-263 as a direct result of the variation in the levels of these pro vs anti-apoptotic molecules.[183,214,215] Therefore, before exploring combination strategies we initially examined whether our library of CRC cell lines were sensitive to ABT-263 monotherapy.

SW480, SW620, HCT116, Colo320, HT29, LIM1899 and LIM2408 cell lines were treated with 0, 0.1, 1 and 10μM ABT-263 for 24-96 hours before cell viability was determined using Live/Dead™ cell viability assay and flow cytometry. Results for 48 and 72 hours are shown in Figure 3.3.


A. SW480
48 hours
72 hours
% Cell Death
ABT-263 [μM]
Control
0.1μM
1μM
10μM
B. SW620
C. HCT116
D. Colo320

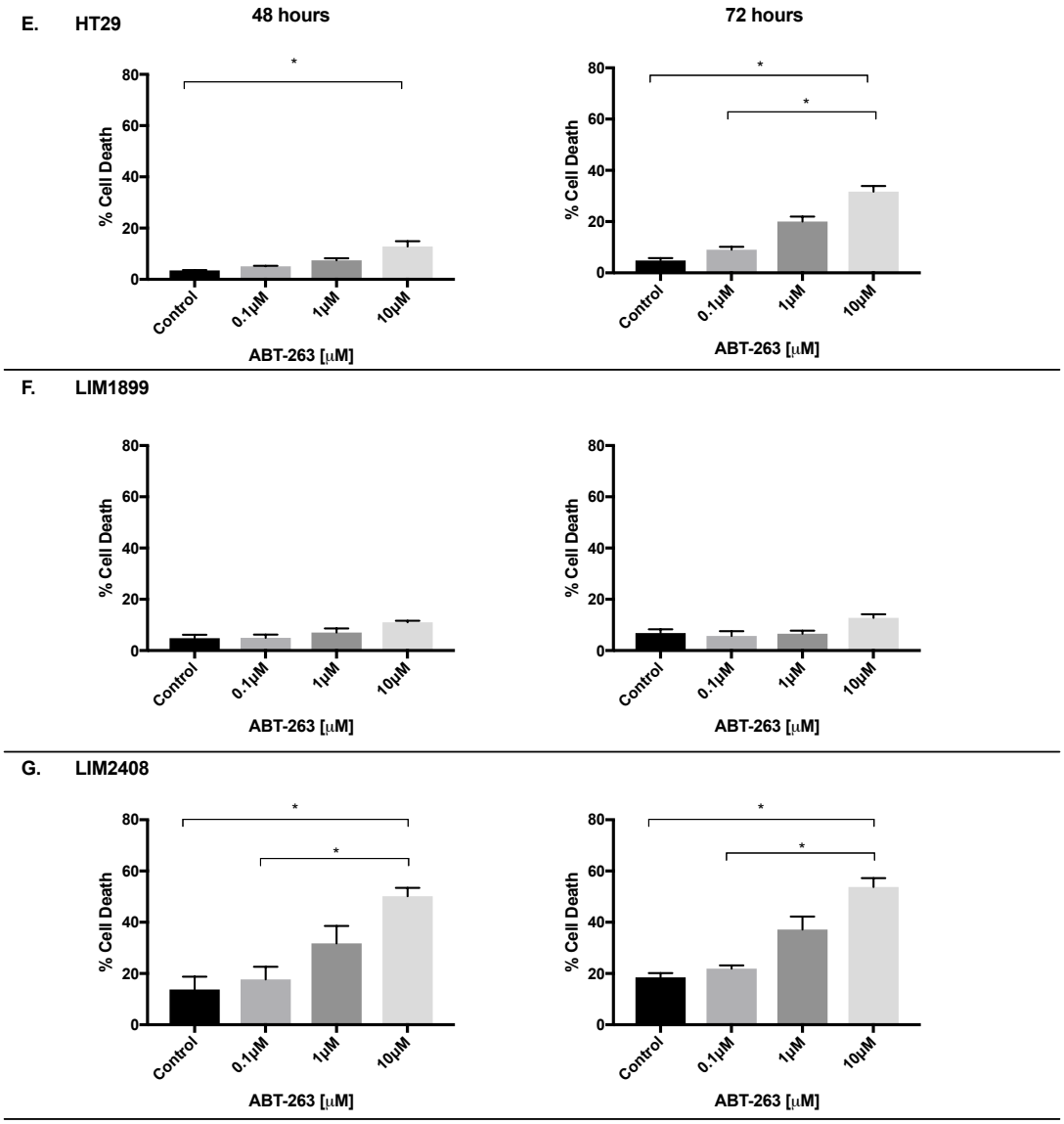


Figure 3.3 ABT-263 induced cell death in CRC Cancer Cell Lines.

Cell lines A-G (A-SW480, B-SW620, C-HCT116, D-Colo320, E-HT29, F-LIM1899 and G-LIM2408) were treated with 0, 0.1, 1 and 10μM ABT-263 for 48 and 72 hours. At each time point cells were harvested and stained with Live/Dead™ for assessment of cell viability using flow cytometry. Graphs show mean percentage cell death of at least 3 independent experiments + SEM. Statistical significance is denoted by * $p<0.05$ (one-way ANOVA).

Treatment of our cell lines with ABT-263 resulted in a mixed response. Following treatment, two cell lines LIM1899 and Colo320 showed no significant change in cell viability, either in a concentration or time dependent manner. SW480 and LIM2408 cells were killed in the presence of ABT-263 in a concentration dependent manner, with no significant change in effect seen at different time points. For example, 10μM ABT-263 treatment of LIM2408 resulted in 50.1% and 53.7% cell death at 48 and 72 hours, respectively, and similarly in SW480 cells treatment resulted in 55.7% and 60.6% cell death at the same time points. The remaining cell lines tested, SW620, HT29 and HCT116 were less sensitive to ABT-263 treatment at the 48 hour time point (29.4%, 12.8% and 34.1% cell death for 10μM ABT-263 at 48hrs, respectively) but responded to treatment in both a dose and time dependent fashion with increased death at 72 hours for SW620 (49.26%). HT29 (31.8%) and HCT116 (43.9%).

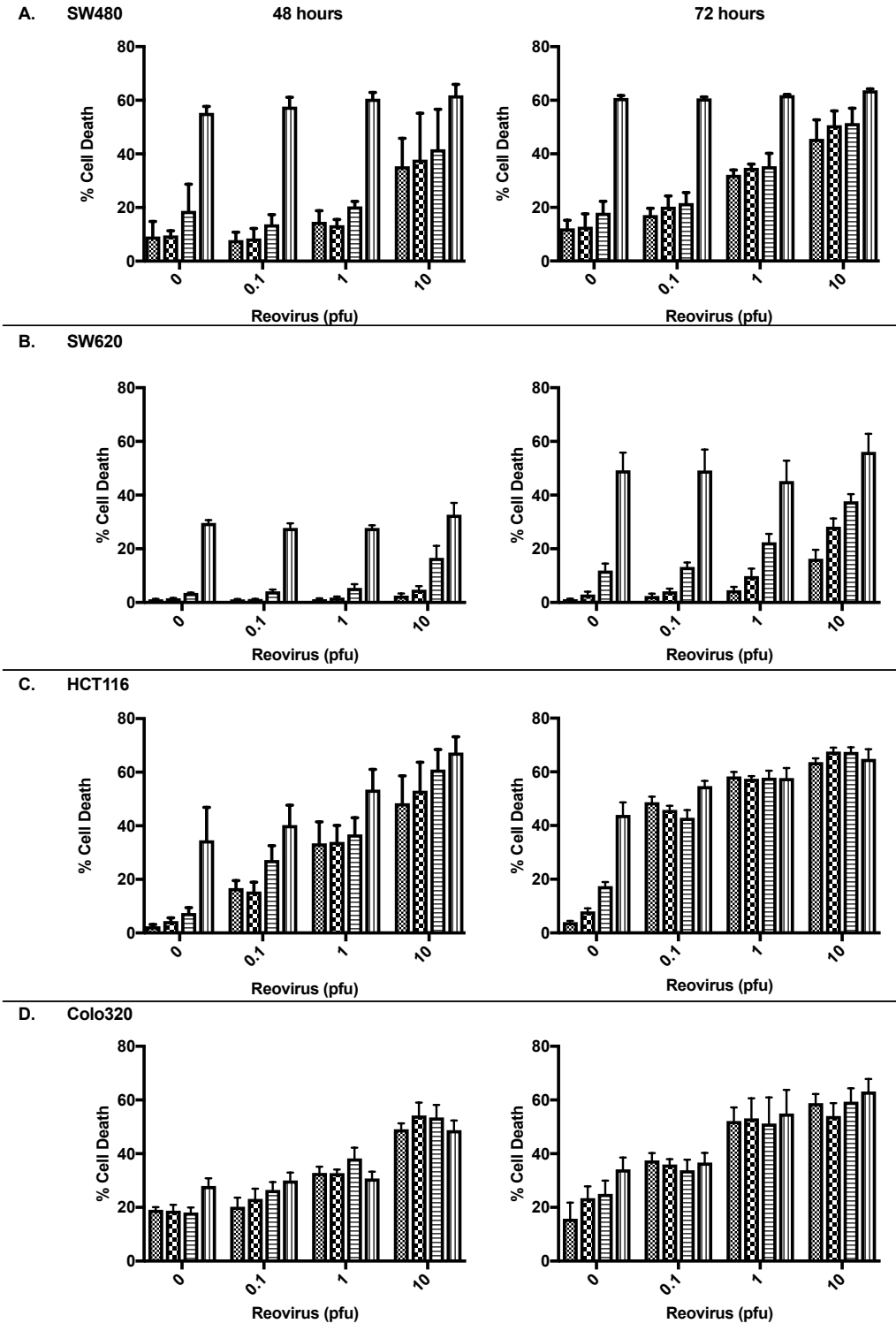
As with Reovirus, across our panel of cell lines there was no evidence that ABT-263 induced cell death demonstrated any correlation to cell line mutational status. Of note, however is that Colo320, which is wild-type in KRAS/BRAF/PI3K genotype was the least sensitive to ABT-263 killing. Whilst this may indicate a requirement for a positive mutational status to be present to allow independent ABT-263 killing a similar lack of response was also seen in the LIM1899 cell line which is KRAS mutant. The relevance of this remains unclear, but could result from differential downstream signalling pathways being modulated in response to the different codon mutations. To support this hypothesis, and whilst it remains controversial, some authors have suggested that sensitivity to certain therapeutic agents may be effected by the exact codon effected by the mutation.[216,217] For example, Kumar et al (2014)[217] suggested that whilst mutational constitutive activation of the RAS/RAF/MAPK signalling pathway is generally accepted to result in drug resistance a proportion of patients with the KRAS G13D mutation did in fact respond to anti-EGFR antibody therapy. The complex interaction of cell signalling processes is beyond the scope of this study but it is clear that the interplay between EGFR signalling and expression of pro vs anti-apoptotic BCL-2 protein family members is important to understanding how we might utilise,

and optimise, potential combination strategies both in the lab and translationally into the clinic.

3.4.2 Efficacy of combining ABT-263 and Reovirus *in vitro*

Given the variable response of our cell lines to both Reovirus, which kills by inducing apoptosis and ABT-263, a pro-apoptotic molecule, we then went on to investigate whether using both agents in combination may induce synergistic killing, enhancing the oncolytic potential of Reovirus.

The panel of CRC cell lines were treated with various combinations of high-dose and low-dose ABT-263 and high dose and low dose Reovirus alone, or in combination, and cell viability at various time points was assessed. Graphical representation of the data is presented in Figure 3.4.


A. SW480
48 hours
72 hours
% Cell Death
Reovirus (pfu)
0
0.1
1
10
B. SW620
C. HCT116
D. Colo320

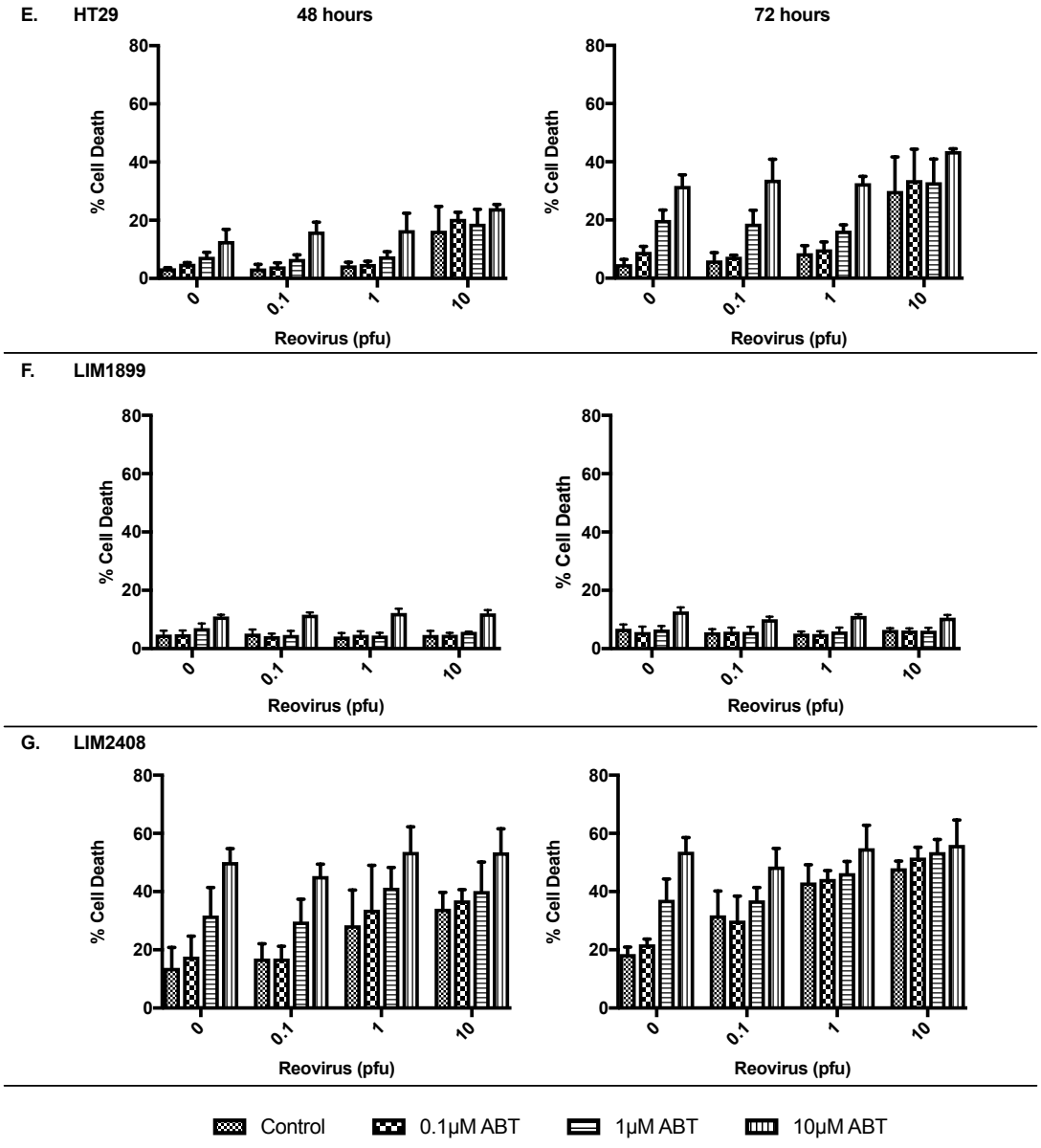


Figure 3.4 Reovirus and ABT-263 combination induced cell death in CRC cell lines.

Cell lines A-G (A-SW480, B-SW620, C-HCT116, D-Colo320, E-HT29, F-LIM1899 and G-LIM2408) were treated with 0, 0.1, 1 and 10μM ABT-263 and/or 0, 0.1, 1 and 10pfu/cell Reovirus for 48-72 hours. At each time point cells were harvested and stained with Live/Dead™ for assessment of cell viability using flow cytometry. Graphs show mean percentage cell death of at least 3 independent experiments + SEM. Statistical significance is denoted by * $p<0.05$ (one-way ANOVA).

Combinations of Reovirus and ABT-263 demonstrated a mixed but largely limited effect of the combination therapy. SW620 CRC cells were the only cell line which showed a significant increase in cell death when compared with single agent therapy in a dose dependent and time dependent manner. Notably, however in LIM2408, SW480 and HT29 cell lines, maximal death was achieved with high dose ABT-263 alone and Reovirus demonstrated no additional effect over and above this treatment. To further analyse these data, and ascertain if there was either a synergistic or additive effect present, further analysis of the data was undertaken using a single chosen dose of both ABT-263 (1μM) and Reovirus (10pfu/cell), at a single time point (72hrs) as a representative sample of response across the cell lines. Data is shown in Figure 3.5.

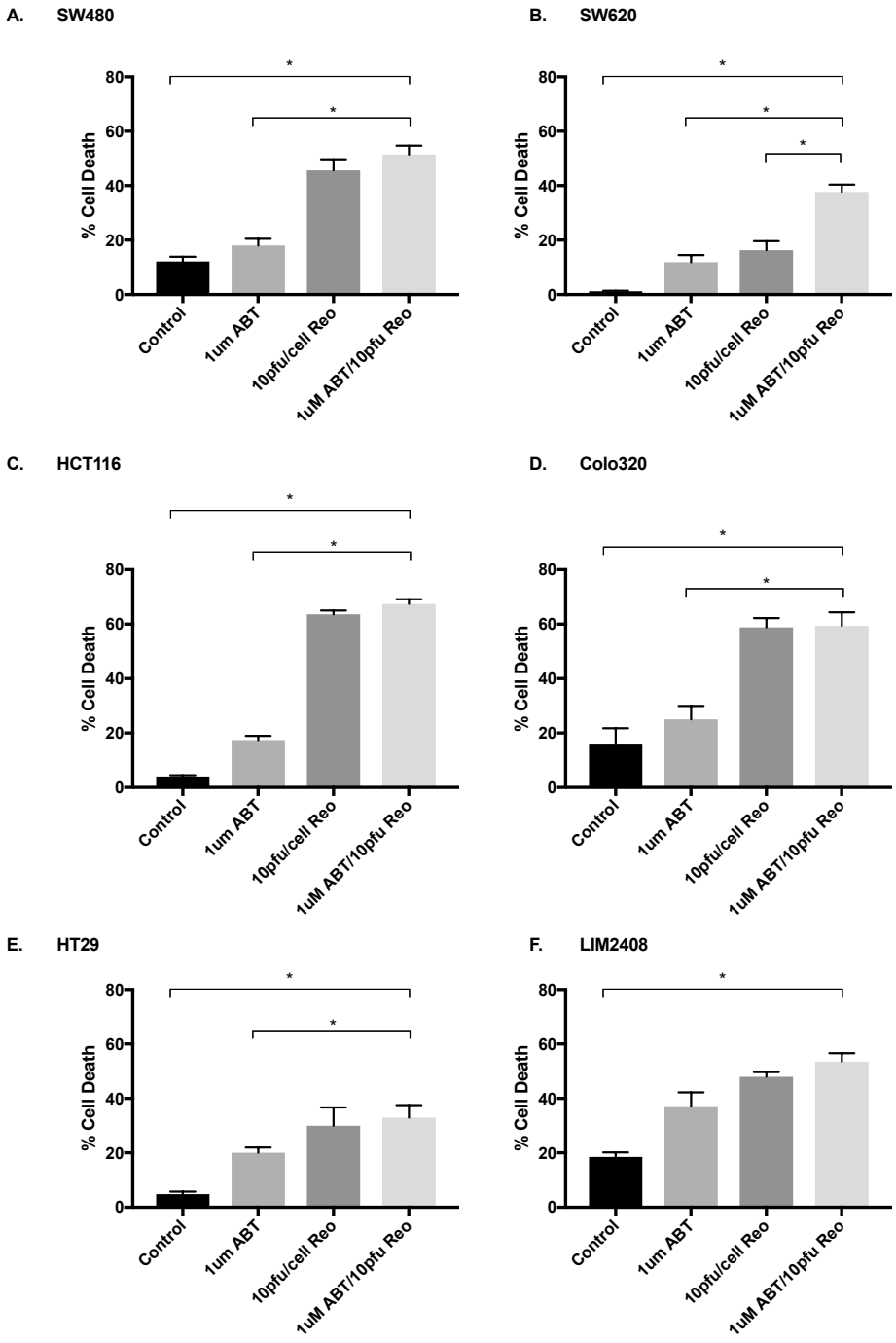


Figure 3.5 The effect of combining 10pfu Reovirus and 1μM ABT-263 on CRC cell death.

Cell lines A-F (A-SW480, B-SW620, C-HCT116, D-Colo320, E-HT29 and F-LIM2408) were treated with 1μM ABT-263 and 10pfu/cell Reovirus for 72 hours. Cells were harvested and stained with Live/Dead™ for assessment of cell viability using flow cytometry. Graphs show mean percentage cell death of at least 3 independent experiments + SEM. Statistical significance is denoted by * $p<0.05$ (paired student t-test).

Disappointingly, increased death was only seen as a direct result of combination therapy in the SW620 cell line. Coefficient regression analysis, however, demonstrated no synergistic effect suggesting that the increased death seen was additive and the result of both agents working in mechanistically different ways rather than acting to enhance the effect of each other.

3.5 Combination strategies to optimise Anti-EGF Receptor monoclonal antibody (Cetuximab/GA201) therapy.

As discussed previously, alternative therapies that may act in combination or hypothetically be modulated by oncolytic Reovirus, are the anti-EGFR receptor monoclonal antibodies. Clinically, Cetuximab and GA201 are directly cytotoxic against *KRAS* wild-type cells but exert limited toxicity against *KRAS*-mutant cells. Hypothetically, Reovirus could enhance killing by promoting the ADCC/ADCP mechanisms of the anti-EGFR monoclonal antibodies. However, of note, it is possible that this effect may be countered, certainly in wild type cell lines, by antibodies blocking EGFR signalling and directly impeding the RAS signalling mechanisms used by Reovirus for direct killing, given that Reovirus oncolyis can be facilitated by constitutively activated EGFR-signalling pathways.[218]

Utilising our panel of CRC cell lines with varying EGFR expression/genetic susceptibility mutations (Table 3.2) we first aimed to test the direct cytopathic effects of cetuximab and GA201 (data no shown) before investigating the ability of potential reciprocal interaction with Reovirus treatment. Having confirmed EGFR cell-surface expression in our CRC cell line was in accordance with reported literature (Figure 3.2), we then sought to confirm EGFR-antibody binding to CRC cell lines using FACS (Figure 3.6).

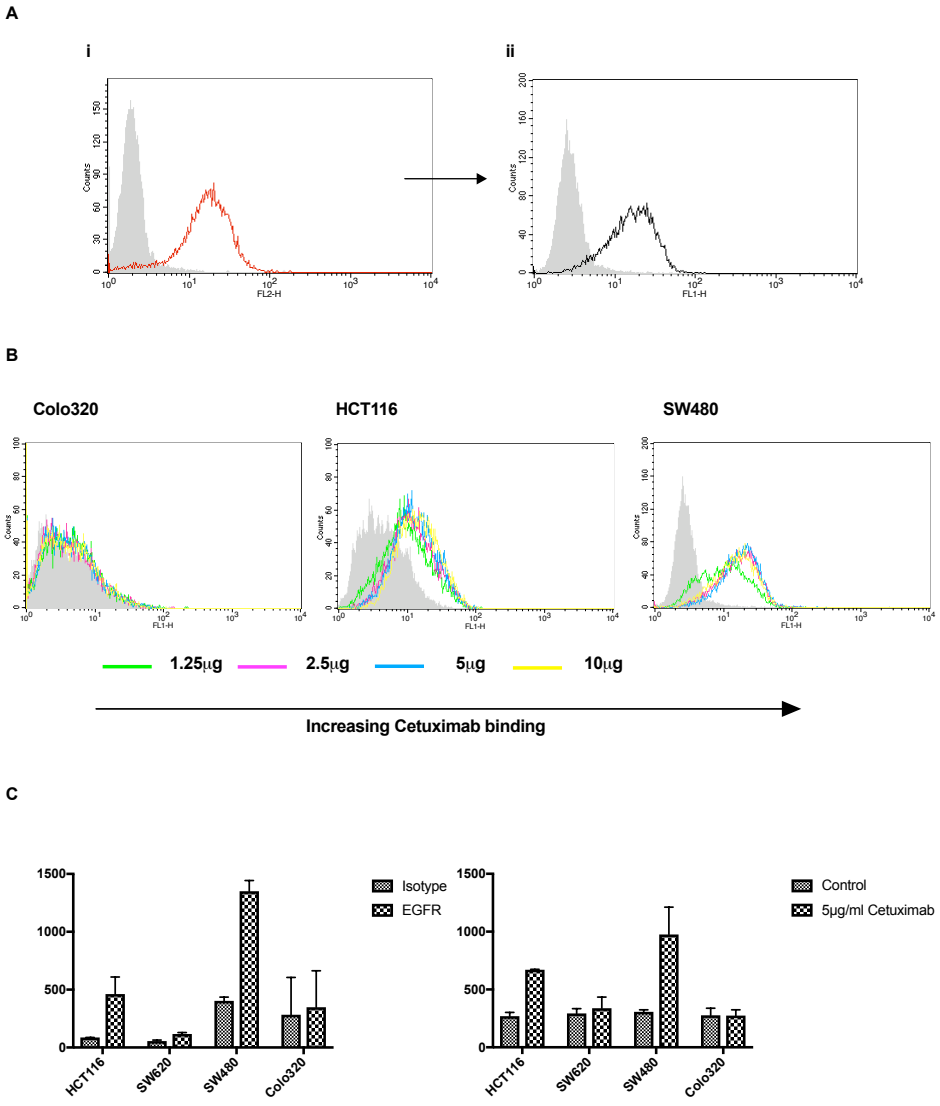


Figure 3.6 Cetuximab binding studies in CRC cell lines with varying EGFR expression.

Four cell lines (HCT116, SW620, SW480 and Colo320) were selected for their variable EGFR expression status. EGFR expression and Cetuximab binding were compared. A.(i) shows a FACS plot of EGFR expression on SW480 CRC cell lines by flow cytometry compared with A(ii) showing SW480 cells labelled with 5µg/mL cetuximab for 30mins at 37°C; Binding of Cetuximab was determined using a FITC-conjugated anti-human IgG1 antibody and flow cytometry. B. Cell lines were labelled with increasing doses of cetuximab (0, 1.25, 2.5, 5 and 10µg/mL) for 30mins at 37°C and binding was determined using FITC-conjugated anti-human IgG1 antibody and flow cytometry; Representative FACS plots are shown. C. FACS data was quantified as mean fluorescent intensity. Bar charts represent EGFR (left) and cetuximab binding (5µg/mL) (right) for each cell line investigated. Graphs show mean + SEM of 2 independent experiments.

Four cell lines were selected for their variability in EGFR expression and KRAS mutant status. SW480 and HCT116 both express EGFR on their cell surface, SW620 has low EGFR cell surface expression and Colo320 has no EGFR cell surface expression as confirmed in earlier experimentation (Figure 3.2) and consistent with reported literature. As expected the data demonstrates binding of cetuximab in the cell lines with high EGFR expression, limited cetuximab binding in SW620 cell lines, consistent with the low EGFR expression and no evidence of cetuximab binding on Colo320 cells. Importantly, of note, both SW480 and HCT116 cell lines are KRAS genotype mutants and additionally HCT116 also has a PI3K mutation. As such cetuximab is likely to demonstrate less efficacy as a direct agent but may act via ADCC triggered by FCγR engagement with NK cells. In support of this there is good evidence that cetuximab-mediated ADCC activity is correlated, with the cell surface expression level of EGFR, regardless of the mutational status of the CRC cell line.[219]

3.5.1 Cetuximab induced cell death in Colorectal Cell lines

Clinically cetuximab only achieves a less than ten percent objective response rate when utilised as a monotherapy in an unselected patient population and only 25% clinical response in those patients genotyped for wild-type KRAS and BRAF genes.[220,221] Before investigating combination strategies we aimed to assess whether our panel of colorectal cell lines would respond in a similar fashion to cetuximab monotherapy *in vitro.* SW480, HCT116, HT29, SW620, Colo320 and LIM1899 cell lines were directly treated with 0, 0.1, 1 and 10µg/mL Cetuximab for 48 (data not shown) and 72 hours before cell viability was determined using Live/Dead™ cell viability assay and flow cytometry. Similarly, to confirm methodological accuracy treated cell lines were assessed by MTT assay to determine any anti-proliferative and cytotoxic effects. Figure 3.7 shows data at 72 hour time point for both MTT and Live/Dead™ viability assays with increasing doses of cetuximab.

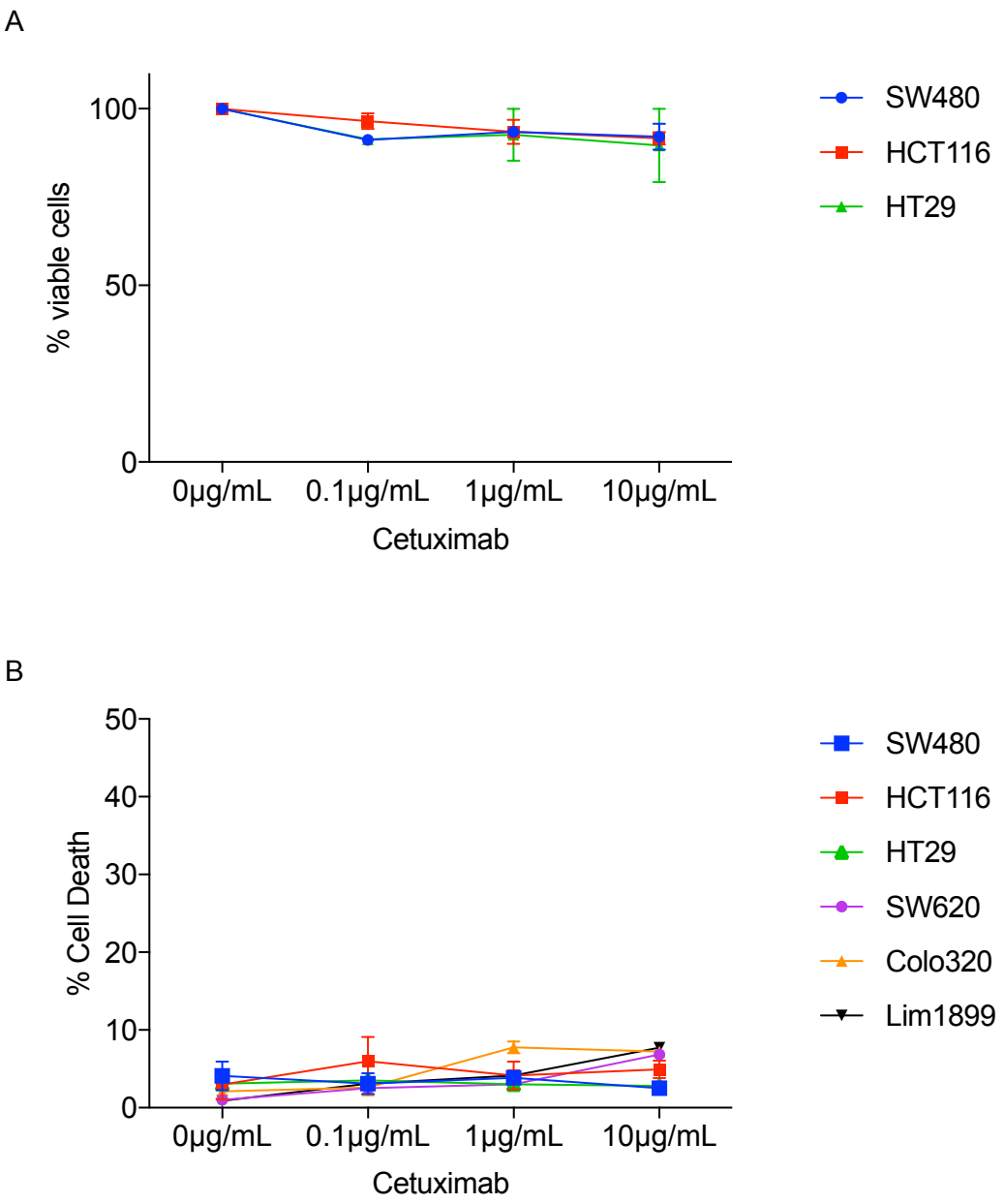


Figure 3.7 Cetuximab monotherapy was not cytotoxic against CRC cell lines despite KRAS/BRAF status.

A) CRC cell lines SW480, HCT116 and HT29 were seeded at 8 x 10^3 cells per well in a 96-well plate and treated with cetuximab at concentrations of 0, 0.1, 1 and 10 µg/mL. 72 hours post treatment, MTT was added for 4 hours and DMSO used to solubilise cells. Percentage viability was calculated as absorbance measured relative to untreated cells. Graphs show mean ± SEM for 3 independent experiments. B) Colorectal cell lines were treated with 0, 0.1, 1 and 10µg/mL Cetuximab for 72 hours before being harvested and stained with Live/Dead™ to assess cell viability by flow cytometry. Graphs show mean percentage cell death of 3 independent experiments ± SEM.

In our cohort of cell lines tested, we were unable to illicit or demonstrate any cetuximab cytoxicity using either MTT or Live/Dead™ cell viability assays. No cell death was seen even at high cetuximab doses and previously reported significant time points. These findings are consistent with recently published data which suggests that single agent cetuximab-induced cytotoxicity is only seen in colorectal cell lines which are EGFR positive and RAS wild type in their genotype. The only such cell line in our panel which would meet this criteria is LIM1215 which was excluded from our study based on mycoplasma contamination issues mentioned previously. The other RAS wild type cell line included Colo320, does not express EGFR and would therefore preclude the action of an anti-EGFR binding antibody; Similarly HT29 which whilst RAS wild type, has a BRAF mutation and as such acts to constitutively activate the RAS-BRAF-PI3K cascade, downstream of EGFR. It is therefore perhaps not surprising that in the tested cell line panel we were unable to illicit any demonstrable single agent cetuximab-induced cytotoxicity. However, the absence of direct cetuximab killing enabled us to assess the efficacy of cetuximab-mediated immune based killing mechanisms such as NK-mediated ADCC.

3.5.2 The combination of cetuximab and Reovirus increases ADCC–mediated killing.

Strategies to overcome the inhibitory effect of RAS-RAF-MAPK mutations have been widely explored and tested in order to enhance the efficacy of anti-EGFR antibody therapy, these include consideration of cell-mediated ADCC/ADCP.

After a monoclonal antibody binds to the tumour antigen, the Fc portion of the monoclonal antibody will interact via the FcγR on the surface of an immune effector cell (e.g. NK cells). Responses to this can be stimulatory or inhibitory depending on both the FcγR triggered and the effector cell involved. Several immune effector cells (NK cells, T cells and macrophages) have the ability to recognise target cell populations through FcR-mediated antibody binding mechanisms, for example EGFR on the surface of colorectal cells will bind Cetuximab and can lead to potent anti-tumour immunity. NK cells can be

activated by binding monoclonal antibodies (such as cetuximab) through engagement of FcγR on their surface and death of the antibody opsonised cancer cell target. Various ADCC mechanisms have been reported including pro-inflammatory cytokine release (IFNγ), cytotoxic granule exocytosis and TNF family death receptor signalling which ultimately result in target cell apoptosis.[222]

As previously discussed, it is well understood that monocytes and NK cells play a crucial role in cetuximab-mediated ADCC and our laboratory has previously demonstrated that NK cells isolated from both healthy donor and colorectal cancer patient PBMCs can be activated by Reovirus, both *in vitro* and *in vivo*.[204] We therefore planned to investigate if Reovirus activation of PBMCs and resultant NK cell activation could increase cetuximab mediated-ADCC in a mutant KRAS colorectal cell line population (SW480 cells) using a ^{51}Chromium release assay. Results are shown in Figure 3.8.

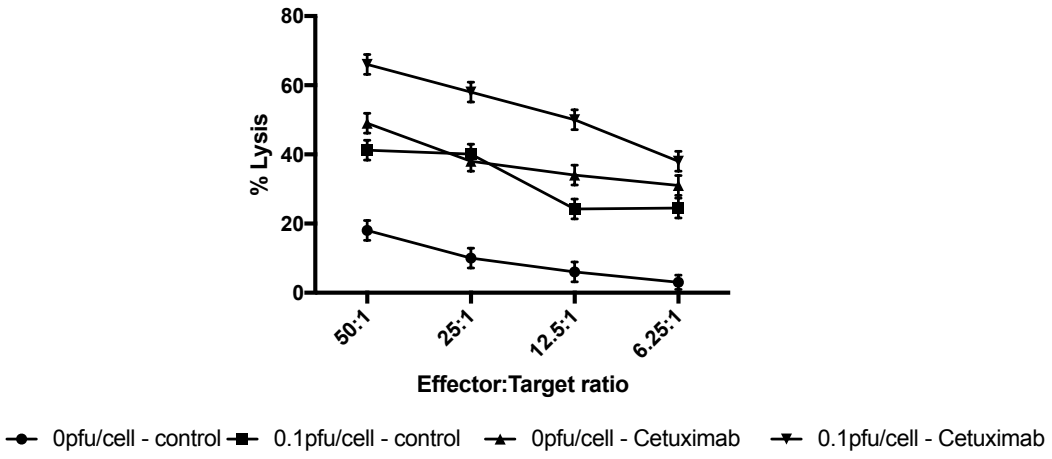


Figure 3.8 Combination of cetuximab and Reovirus increases ADCC-mediated killing.

Healthy donor PBMCs were harvested from whole blood and treated with 0pfu/cell or 0.1pfu/cell Reovirus overnight. Treated PBMCs were then co-cultured with ^{51}Cr-labelled SW480 cells ± cetuximab-binding at different effector:target ratios for 4hrs; % lysis was determined by ^{51}Cr-release using a Microbetajet scintillation counter. Data is presented from a single experiment.

At all effector:target ratios examined Reovirus activated PBMC's demonstrated a clear increase in cetuximab-induced ADCC. Of note, dose dependent cell lysis was seen in both independently treated cell populations. Reovirus activated PBMCs in the absence of cetuximab resulted in 40% lysis at the 50:1 E:T ratio, consistent with activated NK cells acting as the main cytolytic agent. Interestingly, despite SW480 demonstrating a mutant KRAS genotype, cetuximab treatment alone also resulted in 45% lysis at the 50:1 E:T ratio. This is consistent with reported literature demonstrating that cetuximab has the potential to act via two independent pathways; as used in the clinical setting, in WT cell lines through direct ligand binding and disruption of the RAS-RAF-PI3K pathway and in this case, in a constitutively activated mutant cell line, SW480, by recruitment and activation of NK cells via FcR binding and induction of ADCC. Despite the fact that low level cell lysis was seen within those cell populations treated with Reovirus and cetuximab independently, a combination strategy produced improved killing in the experimental setting with Cetuximab induced-ADCC being enhanced by Reovirus activation with a resultant 67% lysis at the 50:1 E:T ratio, 17% and 12% greater than Reovirus and cetuximab treatment alone, respectively.

3.6 Discussion

This chapter aimed to examine the use of oncolytic Reovirus as a potential combination partner with existing and developing colorectal cancer therapies. Anti-EGFR antibody treatment has proved highly effective for those patients who are susceptible, however they remain ineffective as a monotherapy for the large majority of colorectal cancer patients seen in the clinical setting. Hypothetically the use of these agents in combination with oncolytic virus should complement, enhance and improve their anti-tumour efficacy. Likewise ABT-263 has been lauded as an agent with great promise for the treatment of various solid tumour types. Clinically Navitoclax has reached phase II trials for the treatment of both SCLC and haematological malignancies,[190,223,224] however, disappointingly despite positive early data has demonstrated limited single agent activity in the clinical setting. Despite limited tumour responses,

correlation with several putative biomarkers measured at the time suggest that Navitoclax is acting with a positive benefit in patients and may enhance the sensitivity of solid tumours to traditional cytotoxic agents.[190] The focus, therefore shifted to treatment strategies involving its potential use in combination with other agents. Previous data reported from our lab has demonstrated that Reovirus when combined with ABT-263 shows increased efficacy against CLL targets and therefore the combined activity against a panel of colorectal targets was of interest.[225]

With regard to ABT-263 and despite previous promising early data generated in *ex-vivo* CLL samples which showed enhanced cytotoxicity at low doses of virus and ABT-263, initial experimentation using our cell line panel was disappointing. The results of combining ABT-263 with Reovirus only showed evidence of increased killing in one cell line, SW620. Maximal percentage cell death across the other cell lines tested was seen with single Reovirus treatment, and the addition of ABT-263 offered no propensity to demonstrate an increase in recorded cell death levels. In SW620 CRC cells, where an objective response to combination treatment was seen, statistical analysis showed no evidence of synergistic activity between the two agents. It is presumed therefore that the increased oncolysis witnessed was therefore a result of an additive effect rather than as hypothesised, ABT-263 acting to alter the sensitivity of the CRC cells.

Given the lack of synergistic efficacy witnessed in these preliminary screening experiments further mechanistic exploration was not pursued, however it would be interesting to further examine the expression of anti-apoptotic proteins such as BCL-2/BCL-$_{XL}$/BCL-w by intracellular-FACS or western blot. Hypothetically it might be that Reovirus is acting to modulate the pro vs anti-apoptotic profile of the cancer cell and by doing so in CRC cells is rendering ABT-263 activity ineffectual. Moreover, it is possible that the CRC cells tested in this study do not have dysregulated or overexpressed anti-apoptotic proteins (ie. BCL-2/BCL-$_{XL}$/BCL-w)

Further consideration might also go to whether the 'scheduling' of agents may effect potential synergistic efficacy. Reovirus infection is known to alter the expression of genes associated with apoptosis and pathogenesis and as such can act to modulate the pro vs anti-apoptotic profile of cancer cells. Specifically relevant to ABT-263 combination strategies, Clarke et al. (2005)[226] report a detailed analysis of the apoptotic mechanisms involved in Reovirus infection. The authors demonstrated induction of MCL-1, BNIP-1 (a BCL-2 interacting protein) and additionally the upregulation of a survival motor neuron (SMN) gene which encodes for SMN proteins. The SMN protein interacts with BCL-2 conferring protective effects against both BAX-induced and FAS-mediated apoptosis. It is feasible, therefore, that to see maximal efficacy in this combination strategy we need to initially 'prime' CRC cell lines with Reovirus to upregulate anti-apoptotic proteins and induce a resistant phenotype before treatment with ABT-263 to modulate this Reovirus-induced phenotype and thereby enhance killing. However, interestingly previous studies reporting positive synergistic outcomes with BH3-mimetics, in combination with other small molecule inhibitors in a variety of haematological and solid tumours have not reported the need for treatment scheduling.[183,208,210]

To date, there is no published literature reporting the use of Reovirus with BH3-mimetics, however Samuel et al. 2010[227] examined the use of the BCL-2 inhibitor Obatoclax (GX15-070), which acts via a similar mechanism to ABT-263, and vesicular stomatitis virus (VSV) in chronic lymphocytic leukaemia (CLL). In CLL patients, high levels of BCL-2 correlates with chemoresistance and a reduced overall survival rate as a result of enhancing leukemogenesis by interfering with apoptosis. Additionally, BCL-2 expression has also been shown to interfere with VSV-oncolysis *in vitro* in primary CLL cell lines. The authors therefore rationalised that combining VSV with Obatoclax could potentially act synergistically to enhance anti-tumour activity. In both *in vitro* and *in vivo* models, an enhanced effect was seen in response to combination treatment. Further examination of the mechanistic process driving this enhanced killing revealed stimulation of the apoptotic pathway, with increased caspase-3 and -9 cleavage, NOXA up-regulation and the release of BAX and

MCL-1. Thereafter the same group used a similar strategy combining VSV with ABT-737 in primary *ex vivo* CLL patient samples. As before, they reported that the combination resulted in an enhanced therapeutic effect by inducing apoptosis to mediate the cytolytic effect of VSV.[228] Alternative tumour types and/or oncolytic viruses have not been explored further in the literature and as such would certainly provide an interesting direction of investigation for future studies.

With this in mind, ABT-263 monotherapy also had a variable cytotoxic effect on our cell line panel. There is no published data available with respect to the 'LIM' cell lines however our findings with respect to ABT-263 sensitivity in colorectal cell lines are generally consistent with other groups.[211,229] As discussed, cell death occurs via an apoptotic mechanism and variation in the BCL-$_{XL}$ to BAX ratio plays a key role in cell sensitivity and level of ABT-263-death seen. Cell lines with high expression of BCL-$_{XL}$ tested include HT29 and SW480 which both showed increased sensitivity to direct ABT-263 cytotoxicity.[230] No significant cell-death was observed in response to ABT-263 treatment in Colo320 cells however, these cells are reported to have no BCL-$_{XL}$ expression but similar levels of BAX to the other cell lines tested. The result of this is a much lower BCL-$_{XL}$ to BAX ratio and reduced sensitivity to ABT-263.[231] Indeed this may only be the case for ABT-263 as you might generally expect that cells with lower levels of anti-apoptotic proteins (such as BCL-$_{XL}$) may have an increased sensitivity to drugs that kill by apoptotic mechanisms.

There is very limited use of LIM cell lines within the literature which may reflect some of the difficulties we encountered in this study. However consideration should be given to the ABT-263 and Reovirus induced cytotoxic resistance, demonstrated by LIM1899 cells, which may be caused hypothetically by high expression of an alternative anti-apoptotic protein, MCL-1. In a range of colorectal cell lines tested, Shao et al. (2013)[211] highlighted high levels of MCL-1 expression on tumour cells to be directly associated with resistance to ABT-263. Similarly, Kelly et al. (2012)[232] and Knowlton et al. (2012)[233] showed that Reovirus mediated apoptosis induces the pro-apoptotic BCL-2 homology 3 (BH3)-only protein, NOXA and knockdown models confirm a significant

reduction in Reovirus-induced apoptosis in those cells lacking this pro-death protein. The NOXA/MCL-1 ratio is widely reported to be critical to apoptosis with those cells expressing NOXA and MCL-1 at different ratios correlating well with the extent of apoptosis seen.[234,235] Given this, it is likely that over expression of MCL-1, may also impede Reovirus-toxicity. No reported literature exists for LIM1899 cell lines with respect to the levels of NOXA and MCL-1 protein expression however, detailed characterisation would be valuable to examine the potential role of MCL-1 in Reovirus and ABT-263 resistance observed in this cell line.

Apoptotic mechanisms with respect to anti-EGFR antibody therapy demonstrate, more predictably perhaps, that high levels and not low levels of BCL-$_{XL}$ are associated with drug resistance and interestingly inhibition of these apoptotic proteins can enhance cetuximab cytotoxicity in KRAS-mutant cell populations.[207] Kasper et al. (2012)[207] defined the mechanism by suggesting that mutant RAS confers anti-EGFR antibody resistance not purely by constitutively activating the RAS/RAF/PI3K cascade but also by blocking apoptotic caspase activation through a pathway regulated by upregulation of BCL-$_{XL}$. As a result, the authors were successfully able to reverse RAS-mediated resistance to cetuximab using ABT-737, a closely related pre-cursor to ABT-263. By antagonising the anti-apoptotic activities of BCL-2, BCL-$_{XL}$ and BCL-w but not MCL-1 in RAS mutant HRAS^{G12V}-Difi cells, cetuximab-induced apoptosis was also restored. Unfortunately our study did not investigate the ability of ABT-263 to enhance Cetuximab killing of mutated CRC cells but this would be of interest for future studies.

Cetuximab resistance remains complex and clinically its use remains limited to the treatment of wild-type KRAS colorectal cancer (CRC). Combination strategies continue to be explored to overcome these therapeutic challenges and include combinations with various chemotherapeutic agents and immunomodulators. To date there has been limited success at translating promising pre-clinical data into the clinical setting.

Reovirus also exerts its cytotoxic effects by two mechanisms; direct oncolysis via an apoptotic pathway or alternatively, activation of anti-tumour immunity.

Interestingly, Maitra et al.[236] demonstrated that Reovirus preferentially exerts its apoptotic effect in KRAS mutant CRC cell lines and not wild-type cells, the opposite to cetuximab direct killing. They showed, for example that induction of apoptosis in the KRAS mutant cell line, HCT116, was more significant when compared to its isogenic KRAS WT derivative. In our cell line cohort, Colo320 represents the only wild type cell line for the KRAS/BRAF/PI3K pathway tested, and we would therefore expect this to be more resistant to Reovirus killing, however contrary to this, our data showed that this cell line was in fact one of the more sensitive CRC cell lines to direct Reovirus killing. Predicting biomarkers for Reovirus response is complex and multi-factorial in nature but our data supports that of Twigger et al. (2012)[237] who also failed to identify any correlation between Reovirus sensitivity and the EGFR/RAS/MAPK pathway in a panel of 15 Head and Neck cancer cell lines.

Similarly HT29 cells showed the least sensitivity to Reovirus treatment with the exception of LIM1899 which showed no evidence of cell death at all. HT29 cells have a ^{V600E}BRAF mutation and would therefore, based on previous reported literature be expected to respond well to Reovirus therapy. Roulstone et al. (2015) [238], investigated the use of BRAF inhibitors (PLX4720) to enhance Reovirus cytotoxicity and found that against expectation, BRAF inhibition paradoxically led to enhanced cell killing in BRAF mutated melanoma cell lines again suggesting that the mechanism of Reovirus sensitivity remains incompletely understood. As a result attempts to study a single element independently within the complex interplay of mechanisms involved is likely to have significant limitations in translational endpoints making the study of combination therapies for the treatment of solid tumours a challenging area.

Interestingly whilst not continued in the context of this research, preliminary results did demonstrate the ability of Reovirus to increase EGFR-mediated ADCC in a KRAS mutant cell line, SW480, as demonstrated by ^{51}Cr release-assay. The initial data is certainly promising and should form the basis of further work looking at a spectrum of CRC cell lines and experimental duplication to validate these results. Additionally, whilst beyond the scope of this thesis, this preliminary work does provoke many questions that would

certainly be of interest. Examples include exploring whether OV-induced changes in NK cells, macrophages, or levels of cell surface EGFR expression on target cells may influence the efficacy of anti-EGFR antibodies such as cetuximab; potentially reversing the suppressive cytokine milieu and increasing the anti-tumour properties seen.

Indeed, various work has previously been done confirming the role of both NK cells and monocytes in mediating antibody-dependent lysis in CRC cells but NK depletion studies could be used to further clarify if cetuximab-induced ADCC is NK cell dependent across our panel of cell lines, regardless of mutational status. Abdullah et al. (1999)[239] isolated NK cell populations from normal and colorectal cancer patients and compared their ability to mediate ADCC against HT29 CRC cells, before using depletion experiments to confirm their findings. They found NK cells to be the most important effectors mediating ADCC *in vitro* and furthermore confirmed an impairment in NK cell function in CRC patients. Similarly Kurai et al. (2007)[240] studied cetuximab-mediated ADCC in lung cancer and compared the effector cells; PBMCs, purified T cells, monocytes and NK cells from healthy and patient donors. They concluded that $CD3^{-}CD56^{+}$ NK cells were primarily responsible for cetuximab-induced ADCC and interestingly this could be augmented by IL-2, raising the question of using cytokines as additional combination immunotherapy.

With respect to Reovirus, Adair et al (2013)[204], demonstrated that patient PBMC-NK cells from CRC patients were activated by Reovirus to a similar level, and by the same mechanism, as healthy donor PBMC-NK cells. Furthermore, liver mononuclear cells (LMC)- NK cells isolated from patients with metastatic colorectal cancer were also activated by Reovirus to target CRC tumour cells. An inflammatory milieu was generated in response to Reovirus activation which would likely negate the potential need for additional cytokines as suggested above.

Blockade of Fcγ receptors (CD16/CD32/CD64) could identify which FcR are important in the mechanism involved. A number of studies have shown that

anti-tumour activity for various tumour specific monoclonal antibodies is associated with high affinity FcRs, based on their genotype. Broadly speaking FCγR IIa and IIIa polymorphisms have been shown to play an important role in clinical outcomes.[82,84] For example, further specific analysis by Zhang et al. (2007) suggested that the FCγR IIIa FF genotype invoked an increased survival and treatment benefit with Cetuximab over the FCγR IIIa VV genotype,[85] this has however since been disputed by Meller et al. (2013) who concluded that differences in FCγR IIIa genotype inferred no ability to predict cetuximab sensitivity.[241]

EGFR is overexpressed in various solid tumours and as such targeting it using monoclonal antibodies is attractive. Similarly, identifying reliable biomarkers of clinical efficacy, such as EGFR expression remain a priority in order to personalise targeted therapies towards individual patients in the clinical setting. However, controversy remains with regard to how EGFR surface expression may relate to cetuximab efficacy with conflicting evidence presented in various tumour types. Pirker et al. (2012)[242] demonstrated that high EGFR expression can be used as a positive predictive biomarker of efficacy in patients with advanced non-small cell lung cancer (NSCLC) after analysing data generated from a phase III First-Line ErbituX in lung cancer (FLEX)[243] study. Conversely, Licitra et al. (2013)[244] prospectively collected tumour immunohistochemistry data from patients in two further phase III trials examining the effect of cetuximab in both recurrent/metastatic squamous cell carcinoma of the head and neck (SCCHN) and KRAS wild-type metastatic colorectal cancer (EXTREME[245] and CRYSTAL,[79] respectively). The authors showed that the addition of cetuximab to chemotherapy in these tumours improved survival regardless of tumour EGFR expression.

Whilst clinical trial data is useful translationally in the clinic, the mechanism of action behind this variation remains unclear from these studies. A systematic review by Derer et al. (2013)[246] therefore set out to examine the mode of action of EGFR antibodies against EGFR cell surface expression. The authors concluded from their findings that the different mechanisms of cetuximab action discussed earlier may be more effective in different settings

depending on EGFR tumour expression. The study suggests that cetuximab-ADCC may predominate in tumours expressing high levels of EGFR, whereas effects resulting from direct ligand binding inhibition may be more important in those patients with tumours expressing low EGFR levels. Given these findings exploring further the effects seen in our *in vitro* studies (Figure 3.7; Figure 3.8) with respect to both direct and immune-mediated cetuximab mechanism of action against the variable cell surface EGFR expression of our cell line panel (Figure 3.2) may have yielded interesting correlating data.

Finally, of note, problems encountered with the LIM cell lines, and particularly LIM1215 certainly resulted in a less complete data interpretation given the loss of our EGFR positive, KRAS/BRAF/PIK3 wild-type cell line. Useful analysis would have been generated from comparable results against this control cell line, however disappointingly this proved not to be possible. There are indeed limited reported cell line options available that are EGFR positive and KRAS/BRAF/PIK3 wild-type in their genotypic/phenotypic makeup but a suitable alternative for future work could be Caco-2 cells used in similar studies by Shigeta et al. (2013).[247] In this study, the authors used Caco-2 as a wild-type control to evaluate the correlation between EGFR levels detected by Cetuximab and drug sensitivities of SW480 and HCT116 CRC cell lines. They conclude that in their study, high EGFR expression correlates with strong Cetuximab response. Acquisition of this cell line may allow direct comparison with our panel of mutant cell lines and may lead to further conclusions being drawn.

Chapter 4
JX-594 Mediated Direct Oncolysis and Cell Death Mechanisms

4.1 Introduction

Infection of tumour cells by JX-594 triggers a cascade of events which ultimately leads to tumour cell death. JX-594 is known to kill target cells by direct oncolysis but also induces a pro-inflammatory tumour microenvironment which promotes immune cell recruitment stimulating innate and adaptive anti-tumour immunity.[248]

Various modes of administration have been tested and shown to be safe in early clinical studies for a wide variety of oncolytic agents. Delivery methods tested have included intratumoural (IT), intraperitoneal (IP), intrapleural (IPl) and intravenous (IV), however it is clear for the widespread translational application of oncolytic viruses, and to harness their full potential to target disseminated malignant disease, systemic delivery remains the most practical approach.[249]

In order to allow both direct oncolysis, activate the innate immune response and prime adaptive anti-tumour immunity, it is considered vital that the virus can primarily access tumour. Previous members of the Melcher group, using oncolytic Reovirus as an OV, have demonstrated that not only can Reovirus be recovered from tumour (colorectal liver metastases) after systemic delivery in humans, moreover, there was no evidence of toxicity as a result of widespread infection, dissemination and replication.[206] Likewise, JX-594 (Pexa-Vec) has been evaluated in 12 completed and ongoing clinical trials including greater than 1000 individual Pexa-vec treatments in total (IV or IT).[250] Systemic delivery was safe and well tolerated, associated with dose-dependent delivery to multiple solid tumour types (including colorectal cancer, lung cancer, pancreatic cancer and mesothelioma) and demonstrates antitumour activity at high doses.[138,251]

There remains limited published data, however with regard to the effects of JX-594 once it has reached its primary tumour target and similarly, little is

known about the potential viral effects in other tissues not directly related to the primary tumour. Clearly this may be of significant therapeutic importance. With most adjuvant therapy focussing on the clearance of micro-metastatic disease to prevent recurrence or non-resectable disseminated malignancy, organs and tissues that may harbour micrometastases, for example lymph nodes or blood where circulating tumour cells reside (CTC's) are of particular interest. Conceptually, OV could eradicate this low volume disease by direct oncolysis (Chapter 4) or activation of an appropriate immune response (Chapter 5).

Vaccinia virus has been described as being able to utilise various cytolytic mechanisms to implement tumour cell death, however these mechanisms remain poorly understood. Evidence of apoptosis, necrosis, programmed necrosis (also known as necroptosis) and autophagy have all been reported at least in part in response to vaccinia virus in a variety of cell types and in some reports various components of each pathway interacting with one another have been described.[159] However there remains limited work characterising these mechanisms of cell death in malignant tumour cells

Understanding the mechanism surrounding direct tumour oncolysis is a rapidly evolving field and it is increasingly becoming clear that signalling pathways regulating the mechanisms of cell death and cell survival are tightly regulated via complex molecular interplay. Indeed it is likely that all the cell death modalities (apoptosis, necrosis, programmed necrosis and autophagy) triggered by death receptor activation in cells are unlikely to be mutually exclusive and as such interact with each other via a complex cascade of intracellular signalling and crosstalk.[252]

There are no previous reports in the literature studying death mechanisms of JX-594 in colorectal cancers cells specifically and as such this chapter aims to explore some of the direct oncolytic mechanisms used by the virus as part of its therapeutic armamentarium against cancer targets. Identifying these mechanisms may allow complimentary combination approaches to be identified.

Of note, some of the preliminary work referred to in this thesis was completed in part by my predecessors, Rajiv Dave. This work is as yet unpublished but

has been submitted and accepted as his MD thesis to the University of Leeds (2014).[253]

4.2 Colorectal cell lines are susceptible to JX594 killing, viral replication and infection.

Initial work completed by the Melcher group had demonstrated that the colorectal cell lines, SW480, SW620, LoVo and HCT116 were susceptible to killing by JX-594, as assessed by Live/Dead™ and MTT assays.[253] Furthermore, cell lines were infected by JX-594 as demonstrated using a green fluorescent protein (GFP) expressing virus (JX594-GFP) and visualisation of GFP using a confocal laser scanning microscopy. Plaque assays, thereafter, demonstrated that JX-594 replicated in all cell lines tested (data not shown).

For the purposes of this work, we focussed on the cell lines SW480 and SW620. These cells lines were derived from the same 50 year old patient prior to any adjuvant therapy and represent a primary (SW480) and metastatic (SW620) colorectal tumour, respectively. SW480 cells were established from a resected Dukes B primary large bowel cancer specimen and the SW620 cells from an intraabdominal lymph node metastasis resected six-months after primary surgery.

Figure 4.1 shows images of both cells lines taken during *in vitro* cell culture. SW480 cells (Figure 4.1A) were larger in size and displayed an epithelial-like morphology. SW620 cells (Figure 4.1B) demonstrate rounder, more aggregated characteristics and notably grow much faster than the primary cell line, SW480.

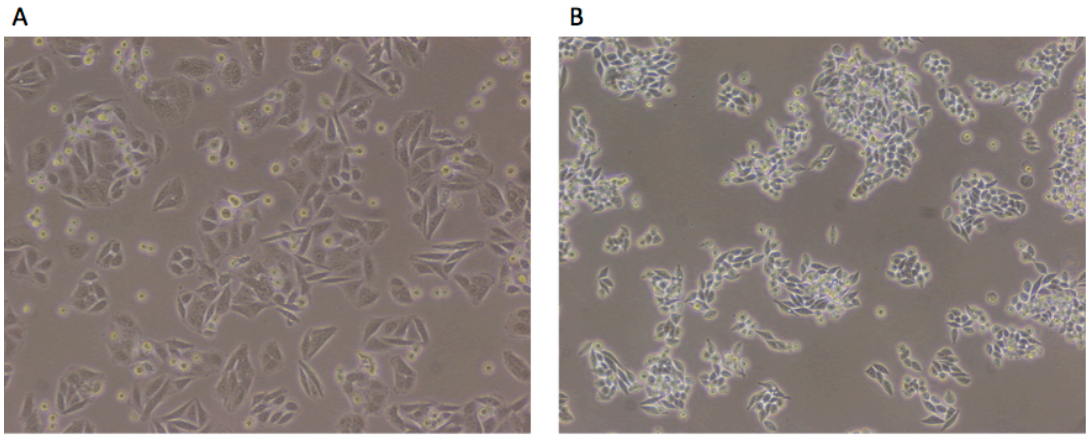


Figure 4.1 In vitro images of SW480 and SW620 human colorectal adenocarcinoma cell lines.

SW480 (A) and SW620 (B) were cultured as described in section 2.2. DMEM supplemented with 10% FCS and passaged regularly at sub confluent levels. Original magnification 10x.

To corroborate the groups previous findings and standardise data, Live/Dead™ assays were repeated with new JX-594 virus stock on SW480 and SW620 cell lines. With increasing doses of virus, and increasing time (48-96hours), JX-594 demonstrates a statistically significant dose-dependent and time-dependent cytotoxic effect, with maximal death observed at 10pfu/cell and 96 hours post-infection for both cell lines (Figure 4.2). Given the related nature of the cell lines, direct comparison is interesting and whilst not statistically significant the SW620 metastatic cell line appeared, particularly at earlier time points to be more resistant to the JX-594 oncolysis and required higher viral titres to produce comparable levels of killing. (Figure 4.3)

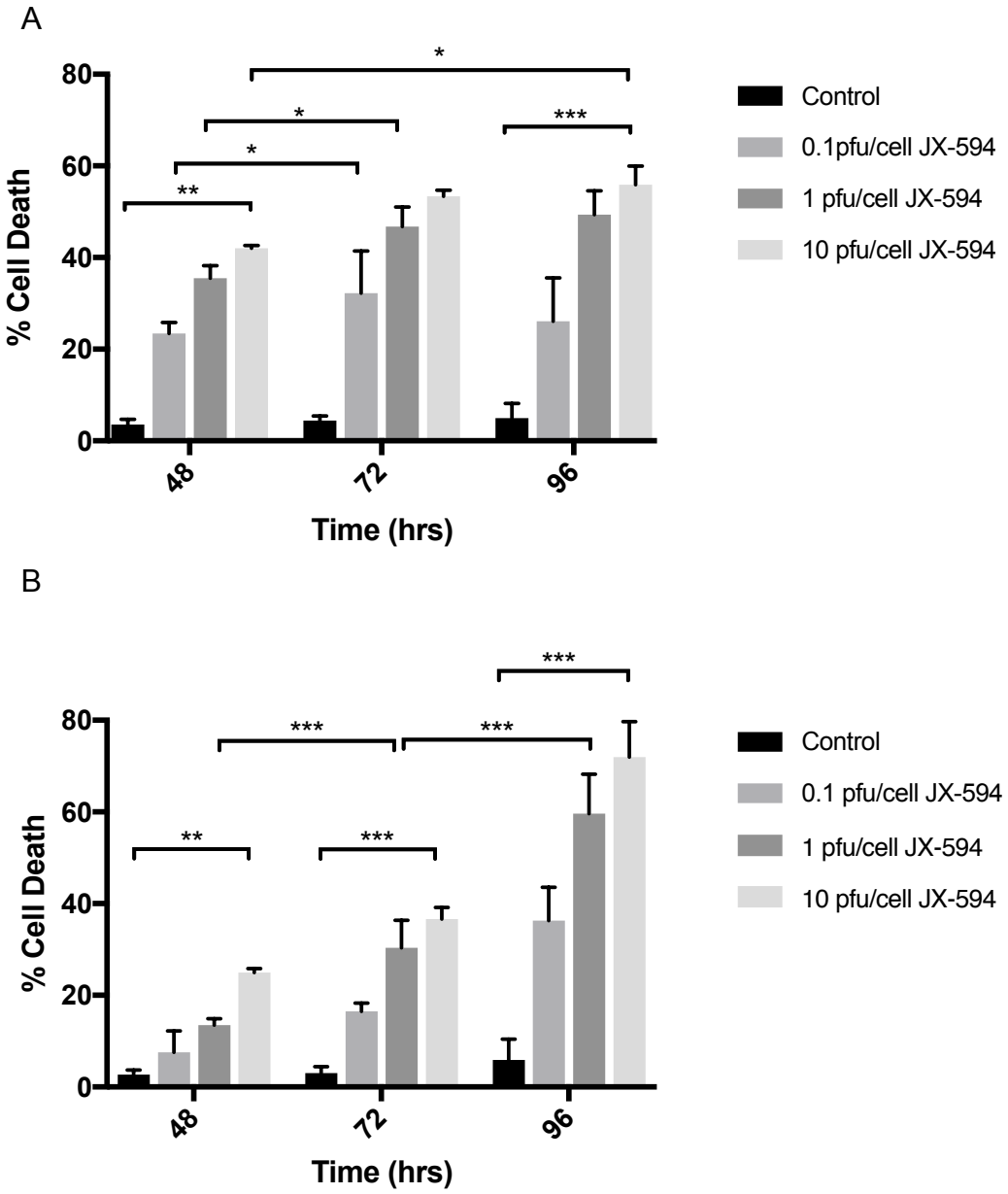


Figure 4.2 Colorectal cell lines are susceptible to JX-594-induced oncolysis.

Colorectal cell lines (a) SW480 (b) SW620 were seeded at 2 x 10^5 cells/well in a 6-well plate and infected with JX-594 at concentrations of 0, 0.1, 1 and 10 pfu/cell for 48-96 hours. At each time point cells were harvested and stained with Live/Dead™ for assessment by flow cytometry. Graphs show mean + SEM of three (N=3) independent experiments. Statistical significance is denoted by *p<0.05, ** p<0.005, ***p<0.001 (one-way ANOVA).

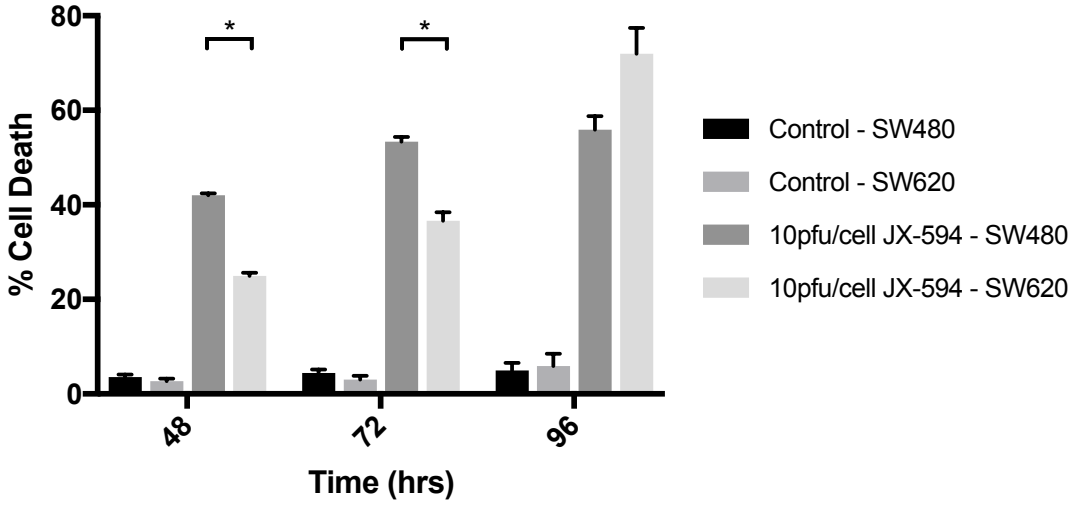


Figure 4.3 SW620 metastatic CRC lines are more resistant to JX-594 oncolysis than SW480 lines.

SW480 and SW620 were seeded at 2 x 10^5 cells/well in a 6-well plate and infected with JX-594 at concentrations of 0 and 10 pfu/cell for 48-96 hours. At each time point cells were harvested and stained with Live/Dead™ for assessment by flow cytometry. Graphs show mean + SEM of 3 (N=3) independent experiments.

4.3 Evaluating mechanisms by which JX-594 kills CRC tumour cells

4.3.1 Caspase-dependent apoptosis

Having demonstrated that colorectal cell lines were susceptible to JX-594 killing, the mode of tumour cell death was then explored given that understanding the molecular mechanism involved in cell death could allow us to develop combination strategies with oncolytic viruses

Previous work has demonstrated increased expression of active Caspase-3, a member of the Cysteine-aspartic acid protease (Caspase) family, important for the execution phase of apoptosis, following infection with JX-594. Using an active caspase-3 apoptosis kit, Dave (2015) (etheses.whoterose.ac.uk/6887) showed approximately 60-65% of SW480 cells and 35-40% of SW620 cells expressed active caspase-3, 96 hours following JX-594 infection (data not shown). Hence, it was therefore hypothesized that JX-594 may induce death in a caspase-dependent manner, classically associated with programmed apoptosis.

Building on these initial observations, this study aimed to further elucidate the role of caspase-mediated apoptosis in SW480 and SW620 cell lines using Z-VAD-FMK, a pan-caspase inhibitor which inhibits caspase-dependent apoptosis by binding irreversibly to the catalytic site of caspase proteases. Mel888 cells, a human melanoma cell line, have previously been shown to undergo oncolysis in response to Reovirus in an apoptotic fashion and therefore acted as a positive control. Z-VAD-FMK was added to the cell culture medium 1 hour prior to infection with JX-594 and cell death was measured 72 and 96 hours following infection using a Live/Dead™ flow cytometry assay. Results are shown in Figure 4.4.

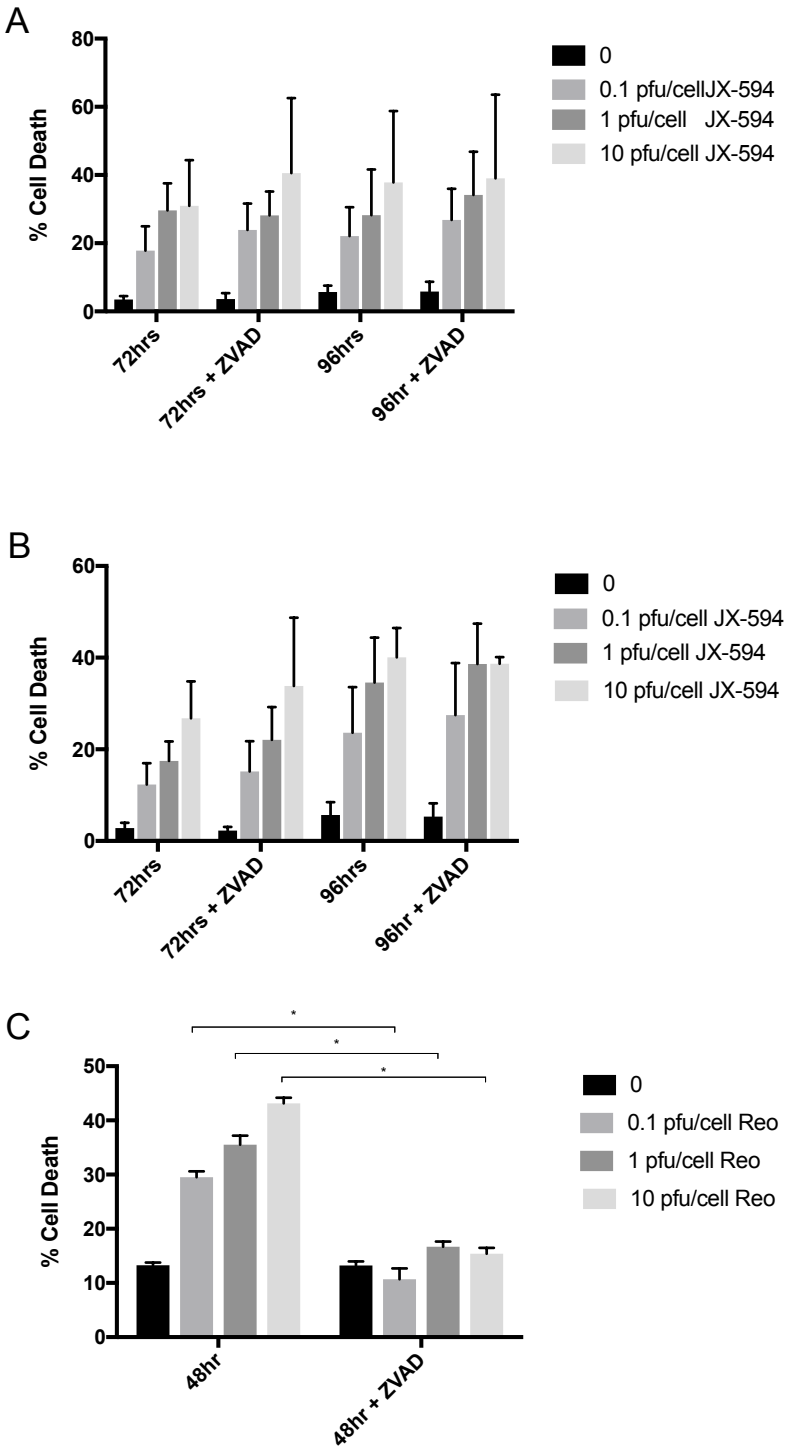


Figure 4.4 Effect of Z-VAD-FMK on JX-594 induced cell death.

SW480 (a), SW620 (b) and Mel888 (c) cell lines were treated with ± 50μM Z-VAD-FMK for 1 hour prior to infection with 0, 0.1, 1 and 10 pfu/cell JX-594 (A & B) or Reovirus (C). Cells were harvested 72 and 96 hours post JX-594-infection or 48 hours after Reovirus treatment. Cell death was assessed using Live/Dead™ cell viability assay. Graphs show mean percentage cell death (n=3) + S.E.M. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA).

The addition of Z-VAD-FMK prior to infection significantly decreased Reovirus-induced cell death in the Mel888 control cell line (44% to 16% at 10pfu/cell) demonstrating that the inhibitor used was functional. In contrast, Z-VAD-FMK did not inhibit JX-594-induced cell death at 72 or 96 hours in either the SW480 or SW620 cell lines. These results were consistent between the two cell lines.

Taken together the results suggest that Reovirus induces death in a caspase dependent apoptotic manner, however JX-594, despite induction of caspase-3 does not induce caspase-dependent apoptosis. These findings are consistent with previous literature studying VV mechanism of death in ovarian cancer cells. Whilding et al. (2013) [159] reported upregulation of various markers of apoptosis following VV infection, including caspase-3, however inhibition of apoptosis by Z-VAD-FMK failed to attenuate the cytotoxicity of VV. This study also highlighted that VV utilises multiple mechanisms to induce cell death and there is therefore a need for the use of a broad spectrum of assays to evaluate OV-induced cell death.

4.3.2 Necrosis/Necroptosis

Given the lack of evidence for caspase-dependent apoptotic cell death pathway by JX-594, alternative death pathways were investigated. As discussed previously, necrosis and its regulated form necroptosis are cellular mechanisms of necrotic cell death induced by apoptotic stimuli under conditions where apoptotic execution is prevented.[254] Using Necrostatin-1 which inhibits the kinase activity of RIP-1, and Necrosulfonamide which targets mixed lineage kinase domain like protein (MLKL), we investigated whether JX-594 kills colorectal cell lines by necroptosis, as previously described by Whilding et al. (2013)[159] in ovarian cancer cell lines. Interestingly the authors reported a dose dependent inhibition of VV activity by both RIP-1 and MLKL antagonism, both alone and in combination with Z-VAD-FMK.

As such Necrostatin-1 ± Z-VAD-FMK and Necrosulphonamide, respectively were added to the cell culture medium 1 hour prior treatment with JX-594 for 96 hours and subsequent levels of cell death measured using Live/Dead™ and flow cytometry. Results are shown in Figure 4.5.

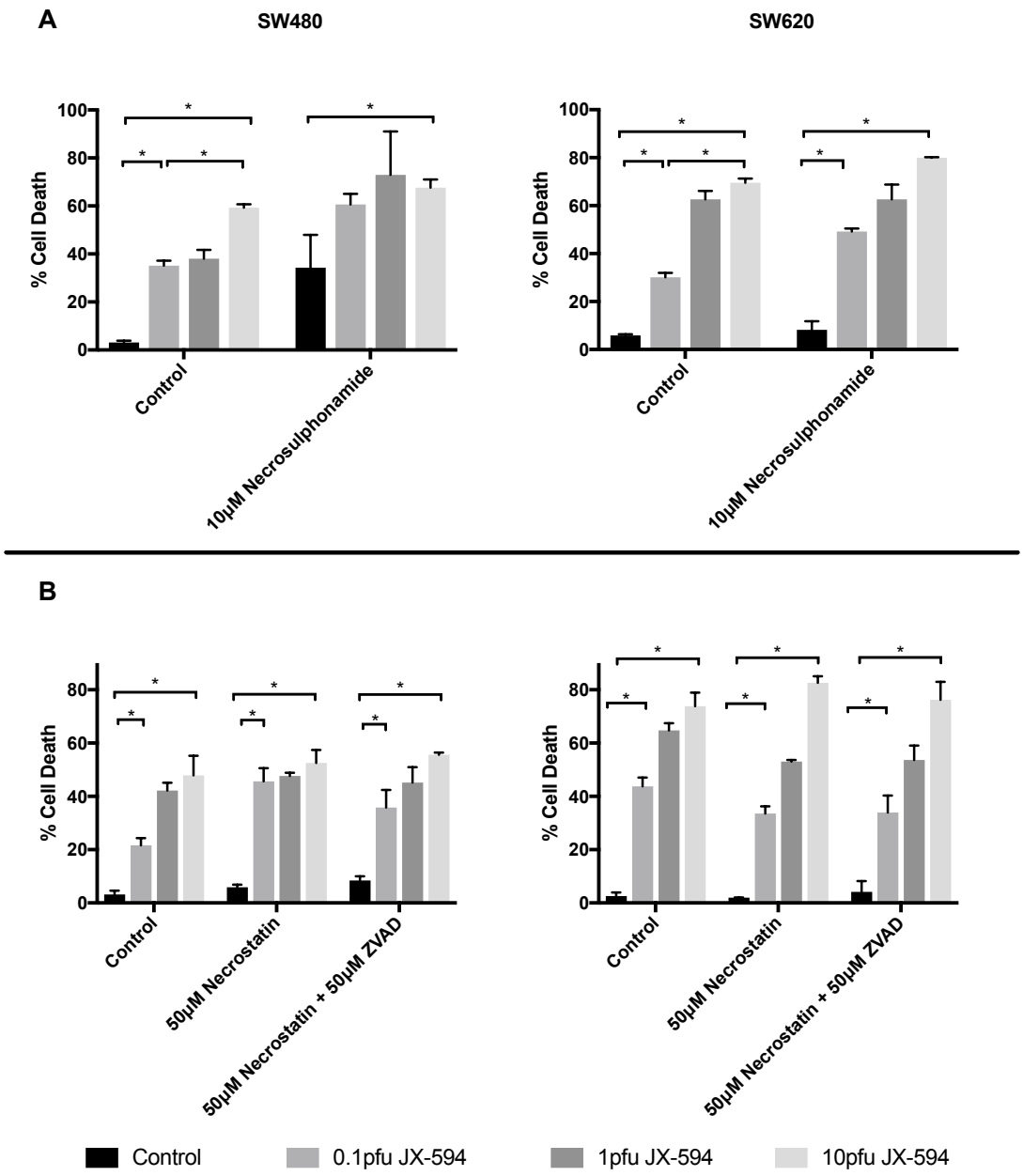


Figure 4.5 Role of Necrosis/Necroptosis in JX-594-induced cell death.

SW480 (Left) and SW620 (Right) cell lines were treated with JX-594±10μM Necrosulphonamide (A), or 50 μM Necrostatin-1 in the presence or absence of 50μM Z-VAD-FMK (B), 1 hour prior to infection with 0, 0.1, 1 and 10 pfu/cell JX-594. Cell were harvested at 96 hours and level of cell death assessed using Live/Dead™ cell viability assay. Graphs show mean percentage cell death (n=2) + S.E.M. Statistical significance is denoted by *$p<0.05$ (Student t-test).

In a similar fashion to the apoptotic inhibitor Z-VAD-FMK, both necrostatin-1 and necrosulphonamide had no effect on the levels of cell death induced by JX-594 at 96 hours suggesting the virus is not acting via a necroptotic pathway. Similarly, the combination of Z-VAD-FMK and necrostatin-1 had no discernible effect on percentage cell death. Interestingly, necrostatin-1 appeared to enhance the virus induced cytotoxicity in SW480 cell lines at lower doses of virus; Percentage cell death increased from 21.6% to 45.6% after treatment with 0.1pfu JX-594 for 96 hours in the presence of 50μM necrostatin-1. This effect was not seen in the metastatic SW620 cells. The mechanism behind this remains unclear with limited data in the literature investigating the mechanistic process behind VV-induced cell death. Interestingly though both Whilding et al. (2013) and Baird et al. (2008) also observed an increase in cell death in response to cell death inhibitors. In IGROV1 cells, an ovarian cancer cell line, treatment with combinations of Z-VAD-FMK and VV and 3-MA and Adenovirus resulted in increased cell cytoxiticity.[159,255] Clearly VV-induced cell death remains a complex process with multiple positive and negative feedback pathways, with the potential for some pathways to be acting to promote cell survival.

4.3.3 High Mobility Group Box Protein B1 (HMGB1) is absent from JX-594-mediated cell death

HMGB1 is a non-histone nuclear protein involved in the structural organization of DNA and is released following cell activation or alternatively as a result of cell necrosis. After its release from the cell, HMGB1 acts as a potent danger signal and mediates inflammation and immune activation.[256] Free HMGB1 release is not usually a feature of apoptotic cell death and instead is translocated into membranous vesicles generated by apoptosing cells.[256] In contrast, cells undergoing necrosis are reported to release HMGB1 into the extracellular space and vaccinia virus specifically has been shown to induce a necrosis-like morphology with resulting, and demonstrable, HMGB1 release in ovarian cancer cell lines.[159]

To further delineate the cellular death mechanisms at play we examined HMGB1 release in cell free supernatants harvested from JX-594 infected SW480 and SW620 cell lines.

Treatment with 1pfu/cell JX-594 for 72 and 96 hours results in significant cell death in both SW480 and SW620 cell lines, as shown in Figure 4.2. Western blots of cell-free supernatants taken at these time points however, showed no evidence of detectable levels of HMGB1, despite strong and proportionate bands with the positive controls (DLD-1 cell lysates; Figure 4.6). These data would fit with a more apoptotic cell death morphology given the previously observed induction of caspase-3, lack of effect observed in combination with necrostatin-1 and necrosulphonamide and the expectation of necrotic cell death to produce high levels of extracellular HMGB1.

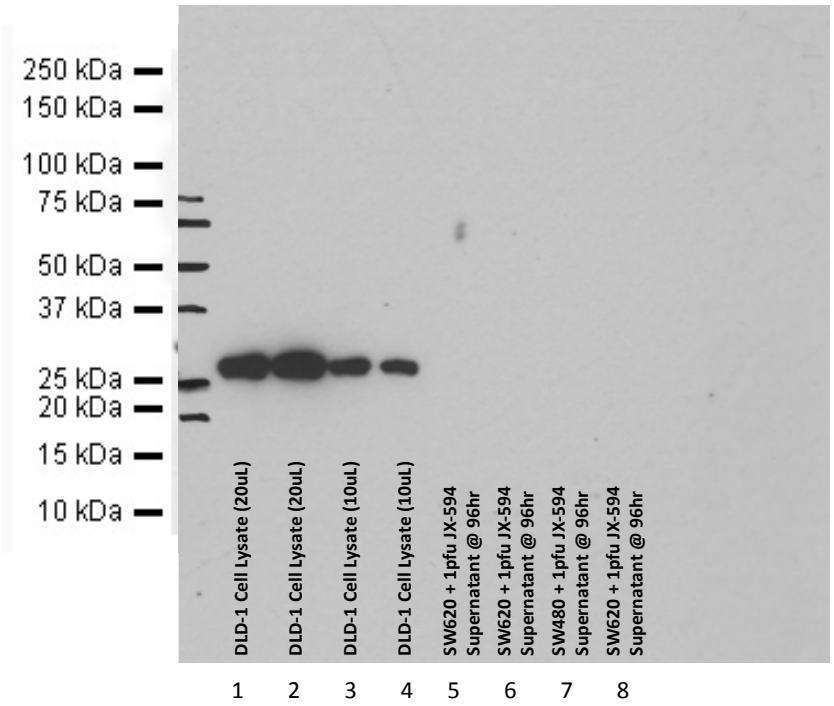


Figure 4.6 Detection of HMGB1 by Western Blot.

Cell-free supernatants were collected from SW480 and SW620 cells, 72 (data not shown) and 96 hours after treatment with JX-594 at 1pfu/cell. Supernatants were prepared for western blot by mixing 1:1 in Laemmli buffer prior to loading onto a 12% SDS-PAGE gel in duplicate (channel 5-8). Molecular weight markers and control DLD-1 cell lysates were also mixed 1:1 in Laemmli buffer and loaded in duplicate at 20µl (channel 1 and 2) and 10µl (channel 3 and 4) respectively. Following protein transfer to nitrocellulose membranes, membranes were blocked and stained with an anti-human HMGB1 antibody followed by a goat anti-mouse secondary antibody. The result shown is a representative example of n=3 independent experiments.

4.3.4 Autophagy

Autophagy is a conserved homeostatic mechanism of lysosomal degradation. Accumulating evidence suggests that OVs interact with the autophagy machinery in infected tumor cells, and may play a role in virus-mediated cancer cell death. A number of studies have reported that modulating autophagy with autophagy inducers, such as rapamycin or RAD001, can augment the anti-tumour effect of adenovirus in glioma cells and Newcastle disease virus in lung cancer cells.[257–259] With respect to vaccinia virus, Whilding et al.[159] suggested that VV may interfere with the autophagic process but does not rely on autophagy to induce cell death. Given these previous findings it was therefore of interest to examine the effect of autophagic inhibition on JX-594-induced oncolysis in the colorectal cell lines, SW480 and SW620.

3-Methyladenine (3-MA) inhibits PI3K which is known to control the activation of mTOR, a key regulator of autophagy. By inhibiting PI3K, 3-MA serves to block autophagosome formation and thus inhibits autophagy.[260] 3-MA was added to the cell culture medium 1 hour prior to infection with JX-594 and cell death was measured using Live/Dead™ flow cytometry. Results are shown in Figure 4.7.

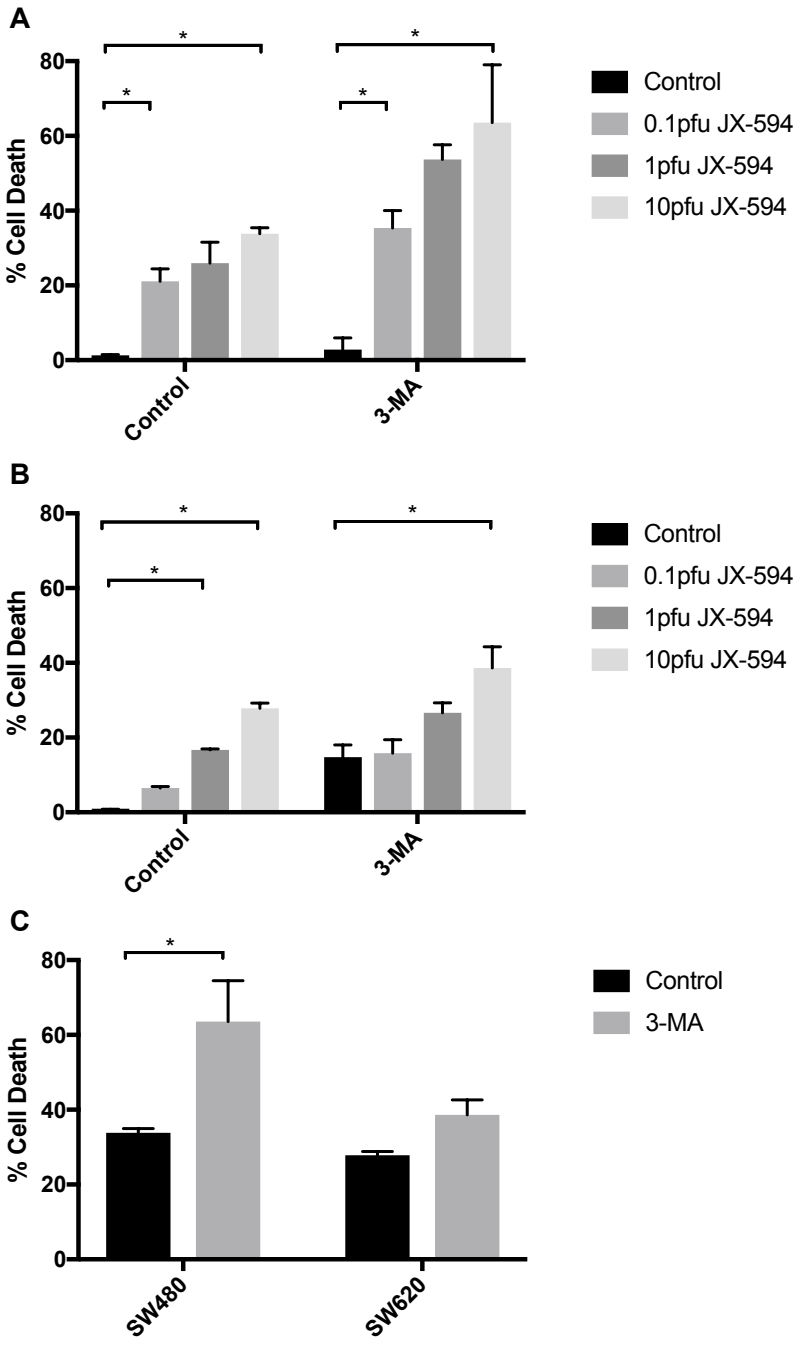


Figure 4.7 Effect of 3-MA, an autophagy inhibitor on JX-594-induced SW480 and SW620 cell death.

SW480 (A) and SW620 (B) cell lines were treated with ± 50μM 3-MA, 1 hour prior to infection with 0, 0.1, 1 and 10 pfu/cell JX-594. Cell were harvested at 96 hours and level of cell death assessed using a Live/Dead™ cell viability assay. Graphs show mean percentage cell death (n=2) + S.E.M. Graph (C) represents the same data directly comparing the effect of 3-MA on SW480 and SW620 cell lines after infection with 10pfu/cell JX-594. Statistical significance is denoted by *$p<0.05$ (Student t test).

As with apoptosis and necrosis/necroptosis inhibition, 3-MA did not inhibit JX-594-mediated oncolysis in either the SW480 or SW620 cell lines suggesting the mechanism of cell death is not autophagy related, as previously reported in ovarian cancer models. Interestingly, as seen in Figure 4.5 as a direct result of necrostatin-1, JX-594-induced cell death of SW480 cells was in fact significantly augmented by 3-MA with the percentage cell death increasing from 25.95% to 53.75% and 33.85% to 63.55% for 1pfu and 10pfu/cell JX-594 (Figure 4.7C), respectively. The mechanism behind this effect remains unclear but may simply be the result of autophagy eradicating virus from the cell.

4.4 Discussion

Taken together these data show that JX-594 triggers significant cell oncolysis associated with up-regulation of caspase-3 and the absence of HMGB1 release which is consistent with apoptosis being the primary cell death pathway. Activation of the full apoptotic effector pathway however is not induced given the inability of Z-VAD-FMK to block cell death. This suggests death may not be caspase mediated or apoptosis alternatively apoptosis can be bypassed. Similarly virus oncolysis does not occur via necroptosis or autophagy given the inability of necrostatin-1, necrosulphonamide or 3-MA to block JX-594 induced cell death.

Studies available in the literature examining the mechanisms by which oncolytic JX-594 induces tumour death are limited, however, those available both support and refute our findings. Probably the most comparable study in the literature was conducted by Whilding et al. (2013)[159] who evaluated apoptosis, necroptosis and autophagy in ovarian cancer cell lines following infection with a lister-strain VV. They demonstrated as with our study that despite some features of apoptosis being present, (the appearance of sub-G1 DNA populations, phosphatidylserine externalisation and PARP cleavage), VV did not induce death via an apoptotic mechanism.

Conversely, however, VV-induced apoptotic death has been reported in various malignant cell lines including both melanoma and cervical cancer.[162,261] In these studies, both authors utilised a wild type VV strain

Western Reserve (WR), and these data may therefore represent strain specific variation. Furthermore, further analysis reveals the conclusions drawn by Liskova et al. (2011)[261] in HeLa and BSC-40 cell lines were solely based on the upregulation and activation of caspase 2, 3 and 4 using the WR strain. As with both our study, and Whilding and colleagues, the authors failed to demonstrate any significant effect using both pan-caspase inhibitors (Z-VAD-FMK) and inhibitors of calpain and cathepsin D and E.

Examining apoptosis in isolation is clearly complex with no single assay able to adequately demonstrate the significance of the role it plays. As such, there is a need for multiple assays to explore the various aspects of these pathways in combination, however, this remains challenging. To illustrate the challenges, examples in the literature include identifying features classically reported as consistent with apoptosis, including Bax-mediated release of apoptosis-inducing factor, which have subsequently been described as a critical step in programmed necrosis.[262] It is now generally accepted that the pathways involved in apoptosis, necrosis and autophagy share a large numbers of similarities and it is therefore unsurprising that delineating a single pathway in this study was unsuccessful.

Further challenges are highlighted by the role of alternative pathways that can be bypassed by traditional inhibitor molecules. Lafont et al. (2010)[263] studied the function of caspase-10 in response to FasL (a member of the TNF receptor super family) to mediate cell death. They reported that in the presence of Z-VAD-FMK, FasL activation initiated by RIP-1 could trigger an alternative, non-apoptotic caspase-dependent form of cell death dependent on caspase-10. The study highlights that whilst Z-VAD is often utilised as a pan-caspase inhibitor, it may be that cell death pathways exist utilising alternative caspases not effectively targeted by Z-VAD. Similarly, a study by Lieberman et al. (2010)[264] demonstrated that granzyme A, a serine protease found in killer cell cytotoxic granules, could activate caspase-independent programmed cell death that morphologically resembled apoptosis but had unique substrates and mediators. Indeed whilst Z-VAD-FMK has been used in many studies to block apoptotic cell death, the literature also reports that it may sensitise cells to necrosis and actively induce autophagy.[265]

Given the mixed evidence for an independent apoptotic pathway being utilised by JX-594, we went on to explore alternative mechanisms, such as necroptosis and autophagy. Whilding et al. (2013) demonstrated that vaccinia virus was capable of 'inducing a regulated and programmed necrotic cell death' in ovarian cancer cell lines. Their data demonstrated that VV induced a reduction in intracellular ATP, modified mitochondrial metabolism and released HMGB1, consistent with necroptosis. Furthermore using the necroptosis inhibitors, necrostatin-1 and necrosulphonamide, they demonstrated a dose-dependent abrogation of virus induced cell death. Similarly, Heinrich et al. (2017)[266] explored the type of cell death induced by VV in melanoma cell lines using FACS staining for phosphatidylserine (expressed by early apoptotic cells) and DNA of necrotic cells stained with PI. Treated cells demonstrated a more necrotic type cell death.

In our study in colorectal cell lines, despite good levels of JX-594-induced cell death we were unable to demonstrate any effect of either necrosulphonamide or necrostatin-1 and no evidence of HMGB1 release in the cell free supernatants. These data are at odds with Whilding's study and would support the suggestion of a classical apoptotic mechanism. Whilst it is possible that results are variable between different cancer models tested, interestingly, this is also the opposite to findings by Guo et al. (2005)[267], using the CRC cell line HT-29. The authors reported a time-dependent increase in HMGB1 detection by Western Blots following infection with a WR VV. The same paper also reported features consistent with apoptotic death reconfirming the complex nature of cell death pathways. In addition, differences between virus strains as well as cancer cell population variability may also account for the conflicting results observed. Indeed, the various VV strains commonly used have been reported to encode for different apoptotic and necroptosis inhibitors, and inducers of immunogenic cell death which makes like for like comparison across a spectrum of viruses studied difficult. Examples of molecules encoded by VV include the direct caspase/serine protease inhibitor, cytokine response modifier A (CrmA) and F1L which is a direct inhibitor of Bax and Bak.[268] Interestingly, in a recent study by Schock et al. (2017)[269] examining the prevalence of virus-induced necroptosis across a broad range of virus strains,

only two of the seven viruses investigated resulted in significant necroptotic cell death demonstrating the diversity of these agents.

Very limited data exist with regard to the role of autophagy in VV-induced cell death, however interestingly there is an increasing body of evidence to suggest that various OV may interact with intrinsic autophagic mechanism to exert anti-tumour effects. Our study did not identify any evidence of autophagy as the underlying pathway responsible for cell death but did suggest that modulating autophagy could be used to enhance direct lytic killing by VV. Autophagy was first recognised as a virus-induced cell death mechanism by Ito et al. (2006)[257] using a glioma cell model infected with adenovirus. Autophagy was assessed by examining cell morphology, the formation of acidic vesicular organelles, signalling via mammalian target of rapamycin (mTOR) and Western Blot analysis to monitor 'an autophagy associated molecule'. However, in a recent review of the current literature by Hu et al. (2017)[270] the authors concluded that the underlying mechanisms by which autophagy exerts both direct and anti-tumour effects remains largely unknown. However, interestingly they identified four distinct OV's that had been reported in the literature to modulate autophagic pathways to induce both an innate and adaptive immune response (Herpes simplex, Adenovirus, Newcastle disease virus and Measles) indicating autophagy may also contribute to OV-mediated immunotherapy.

As described, the pathways involved in JX-594 are complex, multifaceted and remain to be completely elucidated. Our data is consistent with JX-594 inducing significant cell oncolysis which has previously been reported by colleagues to be associated with the upregulation of caspase-3. The additional absence of HMGB1 release further supports apoptosis being the primary cell death pathway. However, it is possible that the full apoptotic effector pathway is not induced given the inability of Z-VAD-FMK to inhibit JX-594-induced oncolysis. Whilst definitive conclusions are difficult it is possible that death is not solely caspase mediated, or that tumour cells utilise alternative caspase pathways, not inhibited by Z-VAD-FMK, for example caspase 10 not thought to be inhibited. It remains unclear how JX-594 induces tumour cell death however, it is likely that a mixed non-linear morphology

probably exists, incorporating complex feedback systems and a programmed series of events.

Understanding the complex interplay of OV's with cell death mechanisms remains an area of significant research and as such continues to provide potential therapeutic targets and areas of interest for future studies. For example, recent advances in our understanding of the potential for autophagy modulators to regulate OV-induced antitumour immune responses (as reviewed by Hu and colleagues (2017)[270]) could provide potential therapeutic combination strategies and would certainly provide interesting continuation work for this thesis. Furthermore, delineating further the mechanisms behind our observation of necrostatin-1 potentiating VV-induced cytotoxicity in primary cancer SW480 cell lines would significantly enhance our understanding and may provide options to explore novel targeted strategies.

Chapter 5
JX-594-induced innate immune response

5.1 Innate immune activation in the blood

It is increasingly evident that for oncolytic viruses to be effective as anti-cancer therapies, irrespective of their direct oncolytic effect or cytotoxic pathways, they must be highly immunogenic and stimulate an innate and/or adaptive immune response. Likewise, as more work has been undertaken to evaluate the mechanisms underpinning cancer immunotherapy, the importance of immune activation has been recognised as increasingly important. The interaction between innate and adaptive immune effectors such as cross-activation between DC and NK cells and evidence of improved clinical outcomes in colorectal tumour infiltrated with both innate NK cell and adaptive T cells, highlights the importance of harnessing both arms of the immune response.[38,271–273]

As previously discussed, the immune response triggered by oncolytic viruses, such as JX-594 or Reovirus is of critical benefit to the clinical efficacy of these treatments. Importantly, these anti-tumour immune effects have been enhanced in virus platforms by the use of genetic modification and the insertion of various immune genes. Examples include, Kottke et al. (2009)[274] who showed that the addition of IL-2 enhances the anti-tumour immune effects of Reovirus and is associated with an adaptive response against tumour-associated antigens (TAA) in tumour draining lymph nodes. Similarly, the insertion of GMCSF into VV generates a systemic anti-tumour response *in vivo* associated with the recruitment and activation of DC into the tumour microenvironment.[275]

Furthermore, the trafficking of immune cells to lymph nodes is well established to play a pivotal role in immunity and as such, clearance and detection of lymph node micrometastasis is the focus of multiple pre-clinical and clinical trials particularly in the field of colorectal and breast cancer.[276–278] The combination of OV-immunogenicity and the prevalence of both micrometastasis and circulating tumour cells therefore makes the study of both lymph nodes and blood an obvious target for OV research. Indeed,

unpublished data from Breitbach et al., a collaborative group in Canada demonstrated that, as seen with Reovirus, JX-594 can be recovered from blood, lymph nodes, normal liver, primary colon and metastatic colorectal liver metastasis after i.v. delivery in patients with metastatic colorectal cancer.

Therefore, to address the potential wider immune response to systemic delivery of JX-594, and assess the viruses ability to generate anti-tumour effects both in blood and at distant sites harbouring non-resected microscopic disease, we investigated the innate effects of JX-594. Initial studies focussed on the blood of healthy volunteers and colorectal cancer patients and thereafter we examined the same immune responses in freshly resected lymph node tissue from colorectal cancer patients.

5.2 Natural Killer Cells in JX-594 infection

Following intravenous administration of JX-594, high-doses of virus will be present in the circulating blood. Not only is this relevant to virus carriage but the presence of circulating tumour cells (CTC) within the blood has been reported to be associated with significantly increased recurrence, mortality rates and poor prognostic outcomes.[279] In colorectal cancer the stimulation of an anti-tumour innate immune response in this setting, and indeed in other micrometastatic sites, may be sufficient to provide a therapeutic effect and clear tumour even if there is minimal virus access and yield.

Within the group, NK cells have been the focus of attention as a marker of innate immune activation. Colleagues have previously demonstrated NK cell activation and immune-mediated killing in PBMCs following treatment with Reovirus.[206]

Defined as $CD3^{-ve}CD56^{+ve}$ lymphocytes, NK cells constitute between 10-20% of the PBMC population. They can be sub-classified into two distinct forms based on their discrete functions, properties and phenotypes. $CD56^{Dim}$ cells are highly cytotoxic and are predominantly found in the blood and at sites of inflammation whereas, $CD56^{Bright}$ cells are reported to have little cytotoxic activity, and play an important role in immunoregulation, rapidly inducing

cytokine production. This phenotype makes up the majority of NK cells found in lymph nodes.[280]

Figure 5.1 denotes the typical phenotypic appearances of NK cell populations in healthy donor PBMC as seen following flow cytometry.

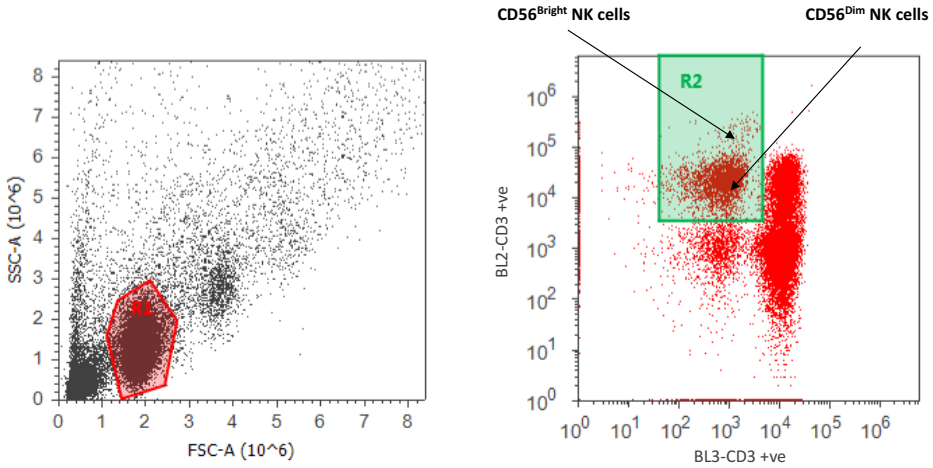


Figure 5.1 Phenotypic appearances of $CD3^{-}CD56^{+}$ lymphocytes (NK cells) and their discrete subpopulations $CD56^{Dim}$ and $CD56^{Bright}$ in PBMCs as demonstrated by Flow Cytometry.

PBMCs were prepared from whole blood of healthy volunteers and stained using CD3-PerCP and CD56-PE anti-human antibody. Cell were analysed using flow cytometry. (A) denotes a forward scatter (FSC) *vs* side scatter (SSC) dot plot with the R1 gate incorporating the lymphocyte cell population. (B) shows a BL3 fluorescence channel (CD3- PerCP) *vs* BL2 (CD56-PE) of the R1 selected cell population. The R2 gates surrounds the total NK cell population ($CD3^{-}CD56^{+}$), 10-15% NK cells within total PBMCs. Bright and dim arrows delineate the sub-populations within this gate.

5.3 NK Cell Activation and CD107 Degranulation

CD69 is a membrane receptor glycoprotein which is transiently expressed after activation of haematopoietic derived cells with the exception of erythrocytes[281]. In resting lymphocytes it is not normally detectable, however selective expression occurs as a result of active immune responses and in chronic inflammatory infiltrates. Activation of NK cells leads to rapid CD69 expression associated with NK-cell derived cytotoxicity, regulation of NK cell proliferation and down regulation of cell auto-immunity.[281] Furthermore, CD69 acts to regulate the expression of other functional activation molecules such as TNF-α, CD25 and intracellular adhesion molecule-1 (ICAM-1) and mobilizes calcium in pre-activated NK cells.[282,283] CD69 can therefore be utilised as an important early marker of NK cell activation. A typical histogram plot showing CD69 expression (FITC) of a resting NK cell population is shown in Figure 5.2.

A second marker that can be utilised for the investigation of NK cell functional activity is a highly glycosylated lysosomal membrane protein, CD107 (also known as lysosomal-associated membrane protein (LAMP-1). NK cells contain high concentrations of preformed cytolytic granules within their cytoplasm containing cytolytic proteins, such as perforin and granzyme. These granules are specifically designed to induce death in target cells following their release. Perforin and granzyme work synergistically to induce target cell death, with perforin being responsible for pore formation in cell membranes allowing delivery of granzymes (serine proteases), where they activate distinct cell death pathways.[264,284] Activation of NK cells results in rapid release of these granules triggering cell lysis and death. CD107 is found lining the surface of these granules and has been described as a good marker of NK cell functional activity.[285] As such, the transient expression of CD107 on the cell surface in concordance with loss of perforin correlates well with NK cell mediated lysis of target cells. Brefeldin A is utilised in the assay to prevent CD107 recycling into the Golgi complex, resulting in accumulation of proteins in the endoplasmic reticulum and enhanced detection of molecules of interest at the cell surface. Expression of CD107 is observed within 2 hours of cell stimulation making CD107a and CD107b ideal markers of NK cell activity.[285–

[288] A representative flow cytometry picture of NK cell degranulation is shown in Figure 5.2.. Increased CD107a/b expression is consistent with enhanced NK cell degranulation.

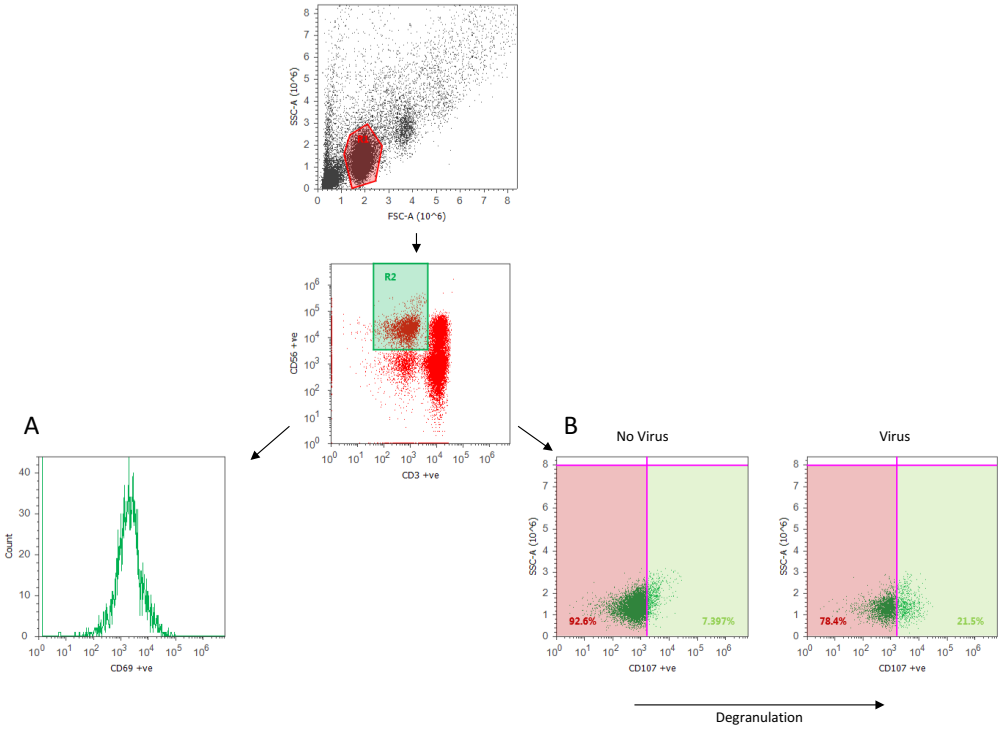


Figure 5.2 Flow Cytometry analysis of NK cells ($CD3^{-}CD56^{+}$ lymphocytes), CD69 (FITC) expression and degranulation within PBMCs.

PBMCs were prepared from whole blood of healthy volunteers and stained using CD3-PerCP and CD56-PE anti-human antibodies. FSC *vs* SSC dot plot with the R1 gate incorporating the lymphocyte cell population and a BL3 fluorescence channel (CD3-PerCP) *vs* BL2 (CD56-PE) of the R1 selected cell population, as shown in figure 5.1 to identify NK cells. (A) delineates a typical histogram plot showing CD69 expression (FITC) on NK cells. (B) shows a representative flow cytometry picture of NK cell degranulation following co-culture with a target cell population (SW480) ± VV; PBMCs were stained with antiCD107a-FITC and anti-CD107b-FITC in the presence Brefeldin A.

5.3.1 JX-594 activates NK cells in healthy donor PBMCs

Utilising CD69 and CD107 as markers of NK cell activation we investigated whether JX-594 could activate NK cells within healthy donor PBMCs. PBMCs were isolated from whole blood venesection of healthy donor volunteers by density gradient centrifugation. Isolated PBMCs were treated with 0, 0.1 and 1pfu/cell of JX-594 for 24 hours before being harvested for flow cytometry; CD69 expression was quantified using mean fluorescence intensity after staining with a CD69-FITC anti-human antibody. Treatment of PBMCs with JX-594 activated NK cells, increasing CD69 expression in a dose-dependent manner (Figure 5.3A).

Similarly PBMCs from the same donor blood were treated with 0, 0.1 and 1 pfu/cell JX-594 overnight before being co-cultured with SW480 and SW620 target cells at a ratio of 10:1 (PBMCs:Target cells) for 4 hours. Cells were stained with CD107a and CD107b and expression was examined using flow cytometry. The presentation of target cells to JX-594-treated PBMCs resulted in a dose dependent increase in NK cell degranulation with similar enhancement of expression observed in response to both SW480 (18% control, 28% 0.1pfu/cell and 36% 1pfu/cell JX-594) and the metastatic cell line SW620 (17% control, 27% 0.1pfu/cell and 32% 1pfu/cell JX-594). Results are shown in Figure 5.3B.

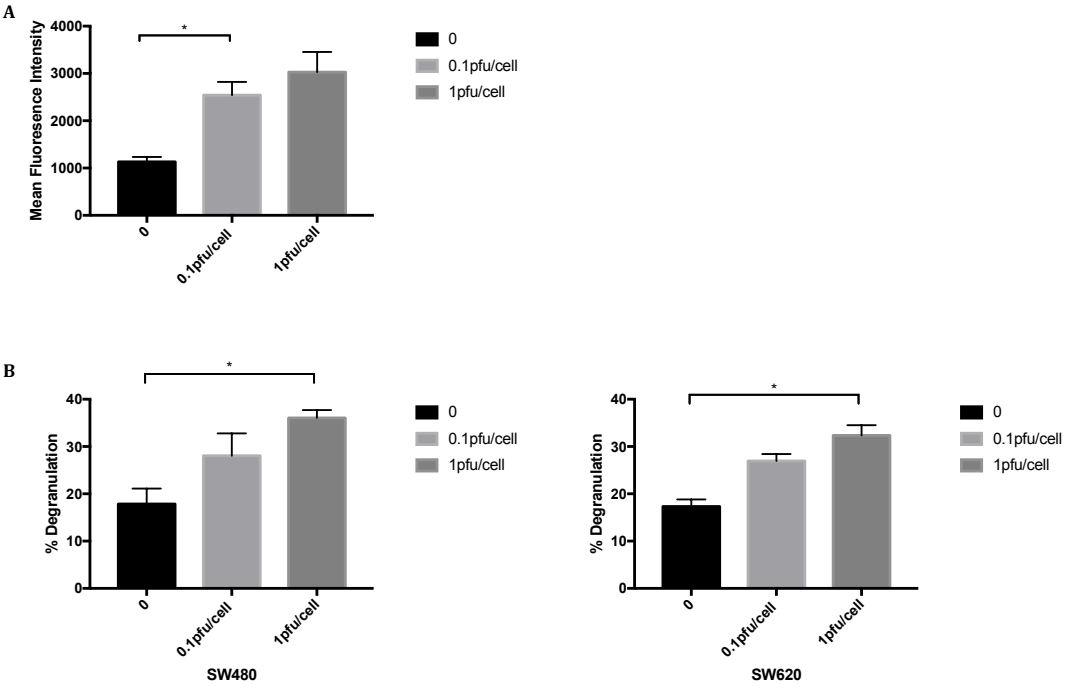


Figure 5.3 JX-594 activates NK cells in healthy donor PBMCs.

PBMCs were isolated from whole blood of healthy donor volunteers by density gradient centrifugation. Isolated PBMCs were treated with 0, 0.1 and 1pfu/cell JX-594 for 24 hours before being harvested and stained with CD3/CD56/CD69-FITC antibody or co-cultured with SW480 and SW620 colorectal cell lines prior to staining with CD3/CD56/CD107a/b-FITC antibodies. Mean CD69 expression (A) and the percentage of NK cells expressing CD107a/b (B) measured using flow cytometry; graphs show mean + SEM for 4 individual healthy donors.

5.4 JX-594 induces type I IFN and GMCSF release in healthy donor PBMCs

To further delineate the mechanism by which NK cells were activated in response to JX-594, we analysed the cell free supernatants from PBMC cell cultures after infection with JX-594. The innate immune response relies on cell-cell cross talk, with NK cells responding to interaction with other immune cells, such as dendritic cells and macrophages, or the secretion of soluble factors (cytokines). Cytokines known to activate NK cells include IFN-α, IFN-β (Type I IFNs), IL-2, IL-10, IL-12, IL-15 and IL-18, whilst cytokines released by activated NK cells include IFNγ, TNF-α, IL-13 and GMCSF.[289]

To examine the secretion of selected cytokines, cell-free supernatants were collected from PBMCs which had been treated with 0, 0.1, 1pfu/cell JX-594, or 0.1 and 1pfu/cell Reovirus for comparison and analysed by ELISA. There was no evidence of IL-2, IL-6, IL-8, IL-10, TNF-α or IFNγ (data not shown). However, JX-594 did lead to the production of IFN-α and GMCSF, but no IFN-β (Figure 5.4). Our group has previously demonstrated the secretion of IFN-α and IFN-β in response to Reovirus treatment and a direct virus comparison was of therefore of interest. As demonstrated in Figure 5.4, JX-594 infected PBMCs produce a relatively low level of IFN-α (A) compared to Reovirus and no IFN-β (B), however there is a significant dose related increase in GMCSF (C) which was not seen after Reovirus treatment. It remains unclear if this increase in GMCSF is as a result of immune activation and cytokine release from activated NK/PBMC cells, or secondary to the early replication of JX-594, which has been genetically engineered to encode GMCSF. To examine this further, GMCSF release was examined following treatment of PBMC with a non-GMCSF encoded virus (VV-LUC) using independent healthy donors. Despite the absence of the virus encoded GMCSF virus modification, significant levels of GMCSF secretion were still seen following infection with VV-LUC (1549pg/ml, 1pfu/cell VV-LUC). Graphical representation is shown in Figure 5.5. Whilst this was less than that seen with JX-594-GMCSF (3874.8pg/ml, 1pfu/cell JX-594-GMCSF-GFP) it is strongly suggestive that GMCSF release is in fact, at least in part, as a result of immune cell activation rather than simply the result of viral replication.

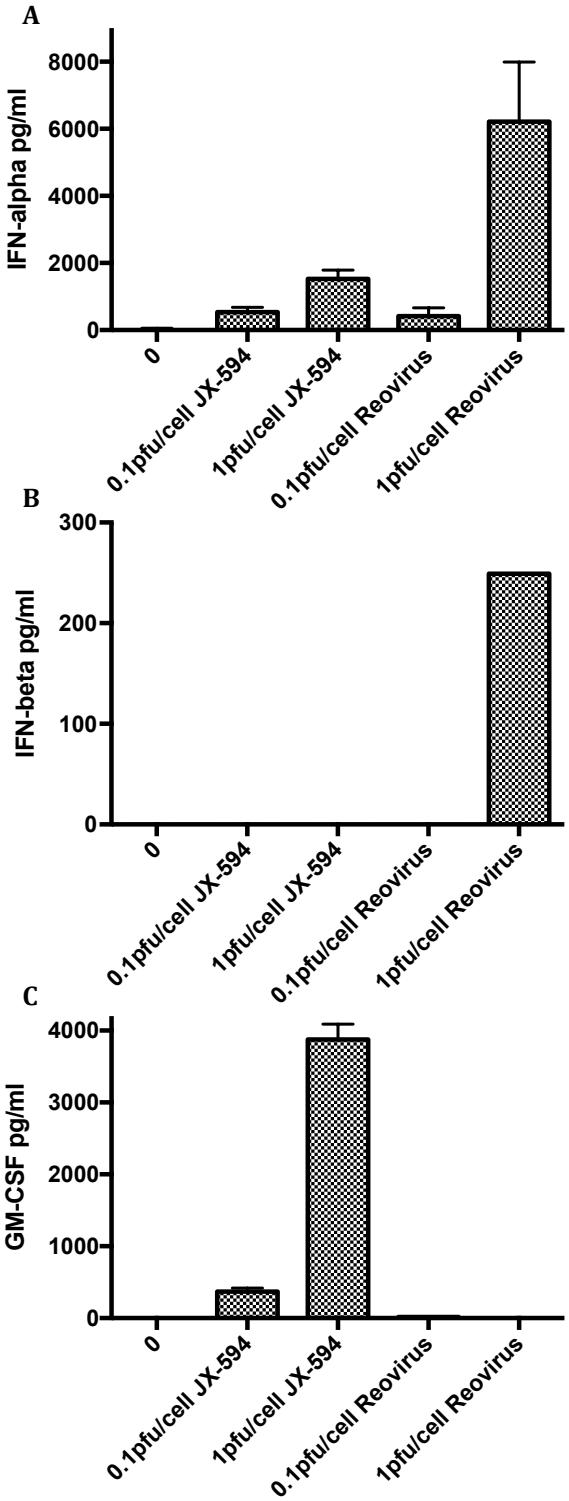


Figure 5.4 IFN and GMCSF production from JX-594 treated PBMCs.

PBMCs were isolated from peripheral blood of healthy donors. Cells were treated overnight with 0, 0.1 and 1pfu/cell JX-594 and/or 0.1 and 1pfu/cell reovirus. Cell-free supernatants were collected and analysed by ELISA for the presence of IFN- α (A), IFN- β (B) and GMCSF (C). Graphs show mean + S.E.M for 3 individual donors.

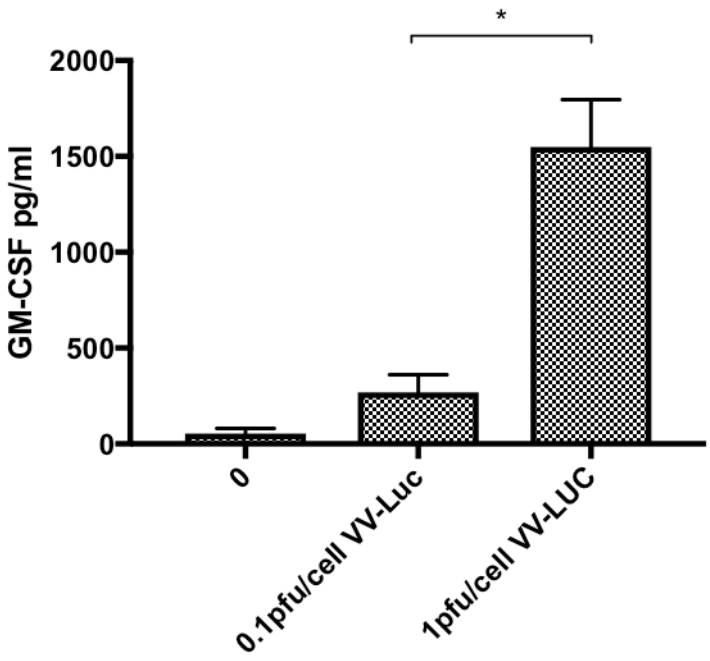


Figure 5.5 GMCSF production from VV-Luciferase treated PBMC.

PBMCs were isolated from peripheral blood of healthy donors. Cells were treated overnight with 0, 0.1 and 1pfu/cell VV-luciferase. Cell-free supernatants were collected and analysed by ELISA for the presence of GMCSF. Graphs show mean + SEM for 4 individual donors. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA).

5.5 JX-594 mediated NK cell activation is dependent on type I IFN in healthy donor PBMCs

The role of the type I IFNs is of particular interest with several studies reporting them to have a decisive role in the activation of NK cells. Despite relatively low levels of Type I IFNs identified in PBMC supernatants as a result of JX-594 treatment we wanted to confirm whether the NK cell activation demonstrated in Figure 5.3 was dependent on type I IFN production. CD69 activation and CD107 degranulation assays were therefore repeated in the presence or absence of IFN blocking antibodies in three independent healthy donor volunteers. Assays were carried out in a directly comparable fashion to previous experiments with the modification that 30 minutes prior to the addition of JX-594 to PBMCs, combinations of soluble IFN blocking serum and IFN receptor blocking antibodies were added alongside appropriate serum/isotype antibody controls.

Figure 5.6A demonstrates increased CD69 expression following infection with JX-594 in a dose dependent fashion. Significantly this response is completely removed in the presence of IFN blocking serum antibodies and restored to normal levels in the presence of serum/isotype controls. Similarly, Figure 5.6B shows that JX-594 activated NK cells degranulate to a significant level against both SW480 and SW620 target cells. In addition, degranulation was also IFN-dependent and was inhibited to baseline levels when IFN blocking antibodies were used. Importantly, isotype controls did not significantly impede NK cell degranulation and results were consistent between the primary and metastatic cell line.

Therefore, JX594 treatment of donor PBMCs activates NK cells to degranulate against colorectal cancer target cells. Despite the low levels of IFN production in response to JX-594, NK cell activation is an IFN-dependent process with both NK cell phenotypic activation and degranulation being prevented in the absence of type I IFN signalling.

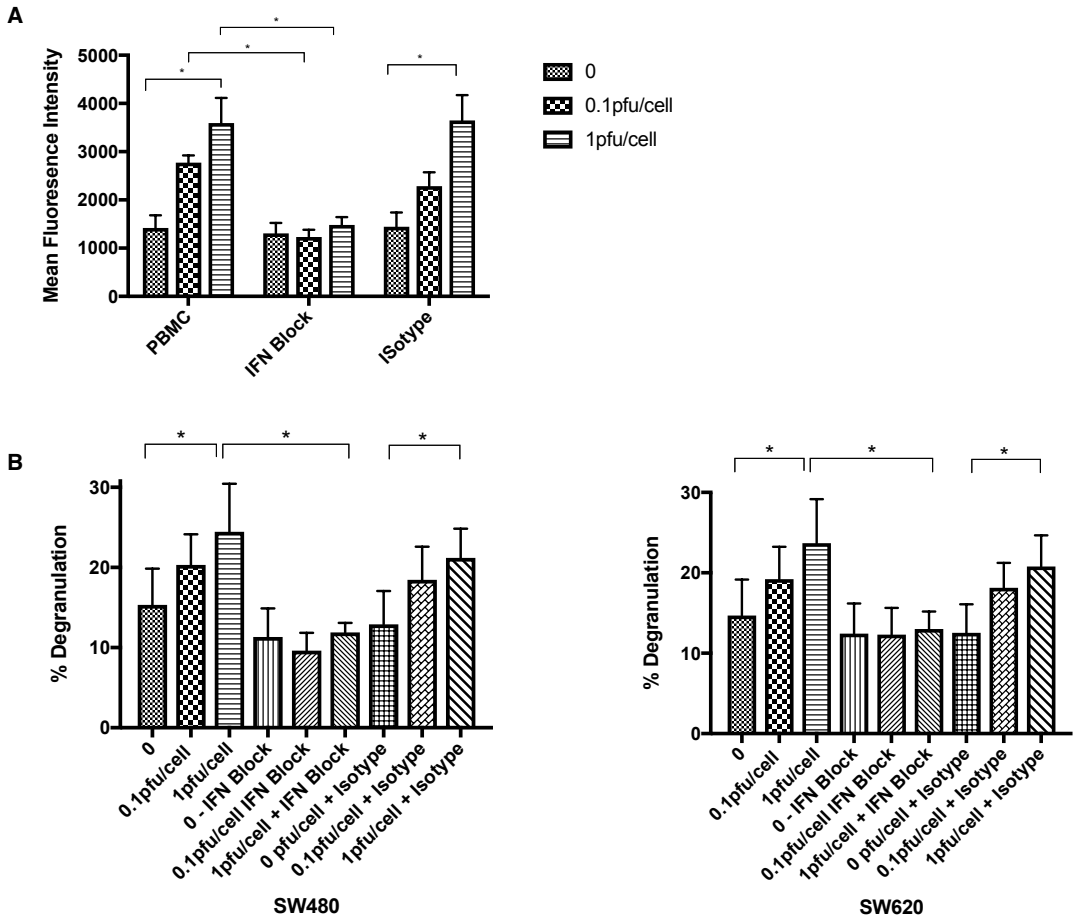


Figure 5.6 JX-594 activation of NK cells is dependent on type 1 interferons in PBMCs.

PBMCs were isolated from whole blood of healthy donor volunteers prior to JX-594 treatment, PBMCs were treated with control media, IFN-α/IFN-β blocking antibody serum and IFNα/β receptor blocking antibodies or IgG2a isotype and control sheep serum. Following this pre-incubation, PBMCs were treated with 0, 0.1 and 1pfu/cell of JX-594 for 24 hours before being stained with (A) CD3/CD56/CD69-FITC antibodies for 30 mins or (B) co-cultured and incubated with SW480 and SW620 colorectal cell lines (10:1 ratio) for 4 hours prior to assessment of CD107a/b expression using flow cytometry. Graphs show mean + SEM for three individual donors. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA).

5.6 JX-594 mediated NK cell activation is dependent on monocytes in healthy donor PBMCs

It should be noted that these data examine only an element of the innate immune response to oncolytic virus therapy. Hou et al. (2012)[290] for example suggested VV infection induces monocytes to differentiate into mature dendritic cells with enhanced capacity to activate T cells, and activate innate and acquired antiviral immunity.

Interestingly therefore, previous work by the group investigated the primary immune cell target for JX-594 and using a GFP-expressing vaccinia virus demonstrated that $CD14^+$ monocytes were the predominant cells within PBMCs to become infected with JX-594 and hence express the GFP transgene (data not shown). Of interest to this work, previous studies have demonstrated a role of $CD14^+$ monocytes in the virus-induced activation of NK cells[291] and as a result we therefore wished to further explore these concepts for JX-594.

PBMCs from 3 independent healthy donors were collected and using magnetic beads CD14+ monocytes were removed. Whole PBMCs and CD14- PBMC cell populations from the same donors were treated with 0, 0.1 and 1pfu/cell of JX-594 for 24 hours before being harvested and NK cell activation investigated using CD69 and CD107 degranulation assays. These data demonstrates that; 1) in the CD14- population there was no upregulation in CD69 expression on NK cells following JX-594 treatment, and 2) that degranulation against the two CRC cell targets, SW480 and SW620 was significantly reduced (Figure 5.7). Hence, $CD14^+$ monocytes clearly play a role in the activation of NK cells within PBMCs in response to JX-594 infection with CD69 expression being completely abrogated and degranulation significantly reduced.

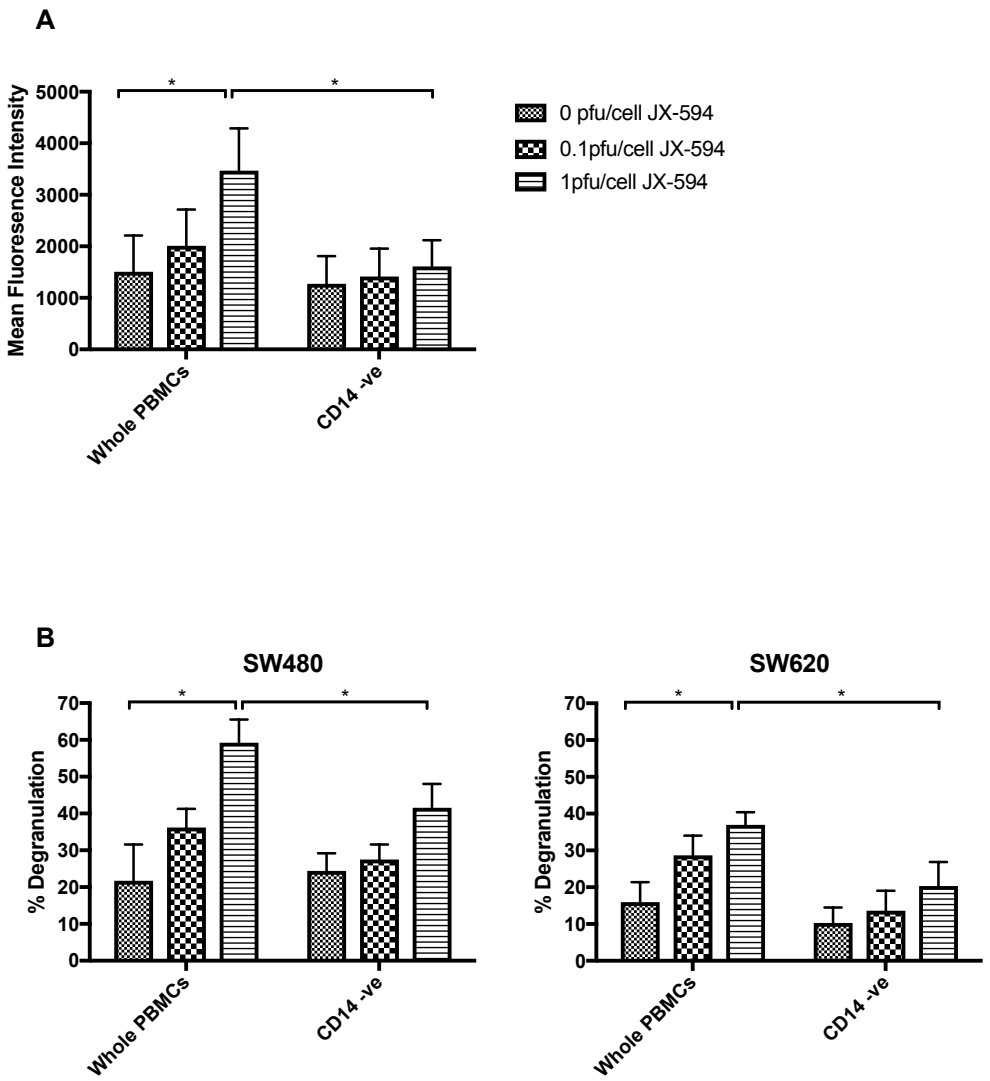


Figure 5.7 JX-594 activation of NK cells is dependent on monocytes in PBMC.

PBMC were isolated from whole blood of healthy donor volunteers by density gradient centrifugation and $CD14^-$ cells depleted using magnetic beads. $CD14^+$ and $CD14^-$ PBMCs were treated with 0, 0.1 and 1pfu/cell JX-594 for 24 hours before being harvested and (A) stained with CD3/CD56/CD69-FITC for 30 mins or (B) co-cultured and incubated with SW480 and SW620 cell lines prior to staining with CD3/CD56/CD107a/b-FITC. CD69 and CD107a/b expression, respectively, was assessed using flow cytometry. Graphs show mean + SEM for 3 separate healthy donors. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA).

As previously demonstrated NK cell activation was dependent on IFN and as a direct result of removing $CD14^+$ monocytes from PBMC cell populations, NK cell activation was also inhibited. Importantly there was no evidence of interferon release in the supernatants depleted of $CD14^+$ monocytes (Figure 5.8).

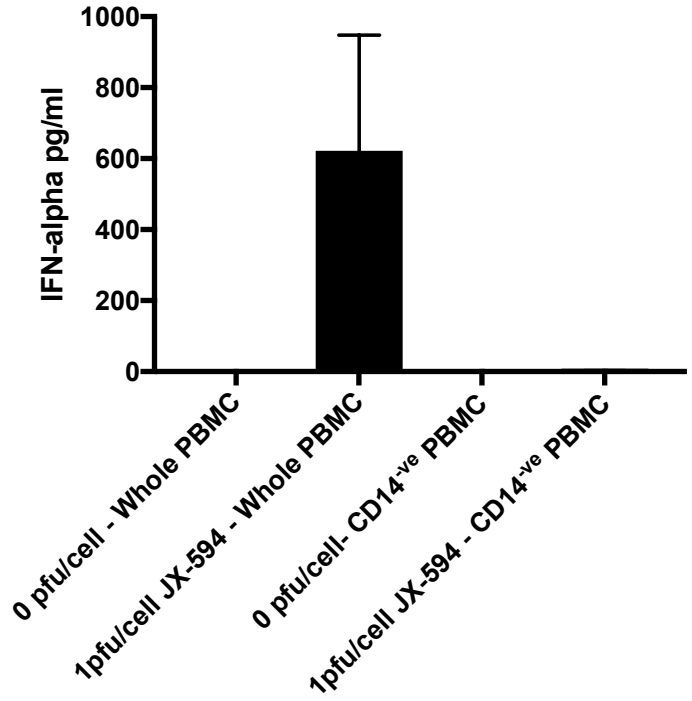


Figure 5.8 IFN-α production in JX-594 treated whole PBMCs and CD14$^-$ PBMC population.

Cell-free supernatants were collected during the experiment described in Figure 5.7. Supernatants were analysed by ELISA for the presence of IFN- α. Graphs show mean + SEM for 3 individual donors. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA). No statistically significant values were observed.

5.7 Innate immune characterisation and NK cell activation in *ex-vivo* freshly resected human lymph node tissue

Having confirmed in PBMCs that JX-594 can activate NK cell in blood we next addressed the potential for JX-594 to demonstrate a similar effect in lymph nodes. As discussed in Chapter 1, cancer related mortality is intimately linked to the progression of disease at diagnosis with the presence of visible lymph node disease providing a clear marker of poor prognosis. Occult disease is more difficult to assess but with a reported 25% recurrence rate in patients with initial node negative staging at the time of diagnosis there is a clear desire for novel therapies to target these areas.

Several studies have described effective transit of virus to tumour draining lymph nodes in *in vivo* models following *i.t.* injection using various OV's.[292,293] Indeed using an orthotopic breast cancer mouse model, Gholami and colleagues (2012)[294] showed that a Lister strain, GFP-expressing VV (GLV-1h153) could not only be tracked in metastatic lymph nodes 48 hours after a single dose *i.t.* injection but 5 weeks after treatment, the authors were unable to find any evidence of metastatic cells within the harvested lymph nodes within the treatment group. Untreated controls, however, continued to demonstrate detectable metastatic cells. Clearly, translationally this could provide a plethora of exciting new treatment options and the ability of JX-594 to act by both direct oncolysis and activate an innate immune response at these distant tumour locations, rich in immune cells, is of significant interest.

5.7.1 Examination of immune cell components in lymph nodes.

Access to fresh tissue samples, particularly lymph nodes holds significant challenges and to date little is reported in the literature about the phenotypic appearance of fresh human lymph nodes samples. Initial work focussed, therefore on characterising the cell populations we were able to identify within these lymph nodes with a particular interest in NK cells. Lymph Node Mononuclear cells (LNMNC) isolated from CRC donor patients were prepared by density centrifugation and cell populations identified by flow cytometry (Figure 5.9).

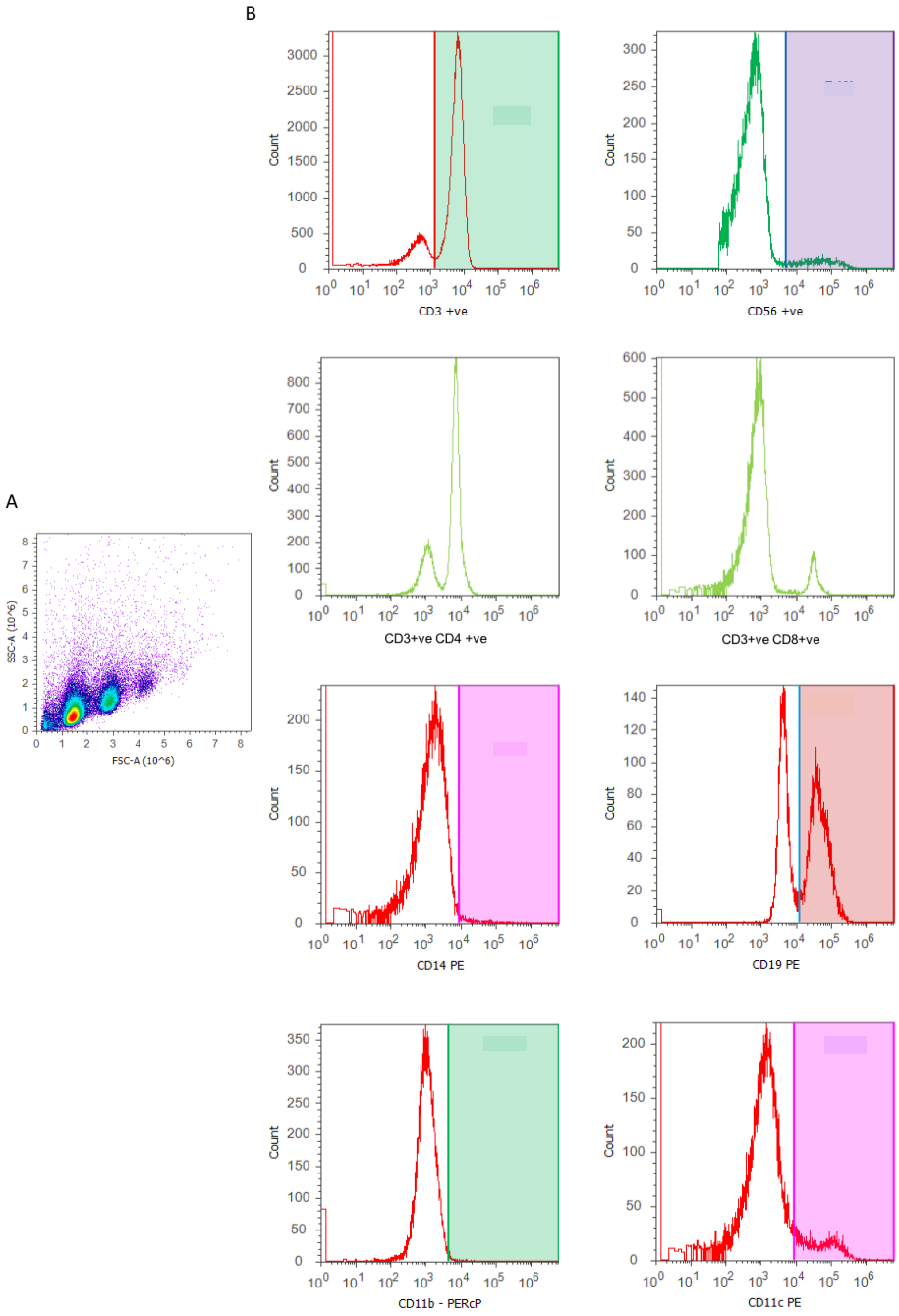


Figure 5.9 Immune cell populations in lymph nodes.

Freshly resected lymph node tissue retrieved from the mesentery of patients undergoing colorectal resection for malignancy were disaggregated into single cell suspensions and cell population assessed by staining with CD3-PERCP, CD3/CD4-FITC, CD3/CD8-FITC, CD11b-PERCP, CD11c-PE, CD14-PE, CD19-PE, CD3/CD56-FITC and CD69-FITC for 30 minutes prior to analysis using flow cytometry. Data is representative of at least 3 separate lymph nodes. A) shows a representative FSC *vs* SSC for lymph node biopsies. B) shows representative histogram plots for each of the antibody panels used.

Given our previous work in PBMCs, we wanted to see what cell populations/markers we could detect in lymph nodes in order to define what might be utilised to respond to virus therapy and furthermore identify cell populations responsible for possible immune activation. Phenotyping experiments identified, as expected, non-tumour LNMNCs did not contain monocytes. Perhaps surprisingly CD11b cells were also not detectible; CD11b is globally expressed on the surface of neutrophils, monocytes, macrophages and NK cells and mediates the inflammatory response by regulating leukocyte adhesion and migration. Previous published data describe human lymph nodes as locations for immature NK cell populations that express low levels of CD11b intergrin would also support this observation.[295] The lymph node cell population did however, contain $CD11c^+$ and $CD19^+$ cells typically found on the surface of DC and B-cells, respectively. CD11c is a type 1 transmembrane protein found in high levels on most human DC but also expressed on monocytes, macrophages and B cells. CD11c DC are required for lymphocytes to traffic towards lymph nodes *in vivo* and have been implicated as likely carriers of pathogens to lymph nodes.[296,297] Clearly with respect to OVs this may have direct implications with regards virus carriage and access to distant disease harbouring sites.

5.8 JX-594 activates NK cells in human, non-tumour, lymph nodes

Studies on human lymph node tissue are scarce, but mouse data suggests that NK cells are 'either normally excluded or at low levels in resting naïve lymph nodes but can be rapidly recruited to lymph node draining sites in response to immune signalling'.[298] It is thought that the primary innate immune response causes activated NK cells to kill target cells and/or produce inflammatory cytokines such as IFN-γ.[299] Thereafter, IFN-γ production plays an important role in T cell priming, inducing $CD4^+$ T cell Th1 differentiation and a CD8 cytotoxic response. Of note, NK cell recruitment to lymph nodes is tightly regulated by activation status and requires endogenous IFN-γ, although

the mechanism by which this controls NK cell recruitment to lymph nodes remains unclear.[298]

In human tissue, one study identified the presence $CD56^{Bright}$ NK cells in human lymph nodes consistent with our data presented below (Figure 5.11). The authors demonstrated that the lymph node residing NK cells could be stimulated by T-cell derived IL-2, acting through constitutively expressed high affinity IL-2 receptors to secrete IFN-γ, clearly illustrating the cross talk between the innate and adaptive immune response.[300] Induction of an innate immune response and NK cell activation in the lymph node tissue following OV therapy could translate into potential targeted therapeutic effects in micrometastases, initiating a focal anti-tumour immune response..

Having identified cell types present in the LNMNC and demonstrated the presence of NK cells ($CD3^-/CD56^+$) (Figure 5.10), we further investigated whether JX-594 could activate NK cells and trigger an innate immune response within the lymph nodes, similar to that seen previously in the blood.

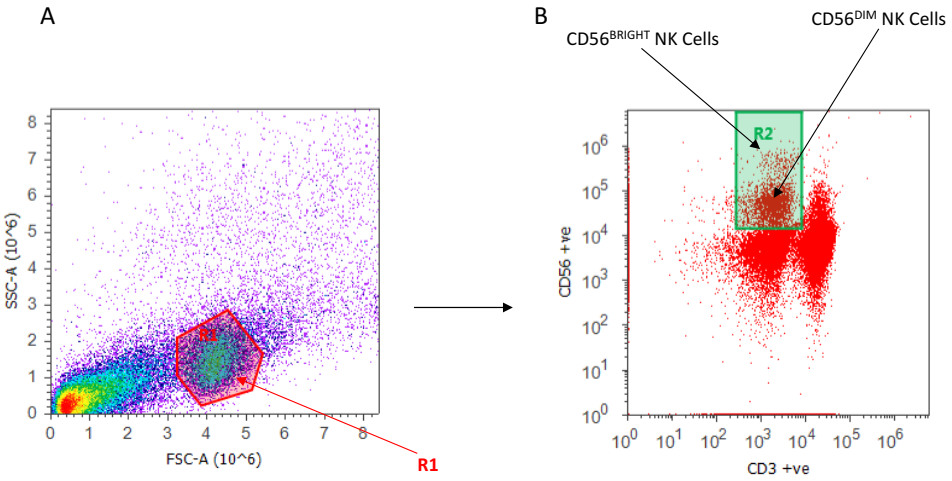


Figure 5.10 The Phenotypic appearances of $CD3^{-}CD56^{+}$ lymphocytes (NK cells) and their discrete NK cell subpopulations ($CD56^{Dim}$ and $CD56^{Bright}$) within LNMNCs.

LNMNCs were prepared from disaggregated fresh lymph node tissue and stained with CD3 PerCP and CD56 PE anti-human antibodies. Cells were analysed using flow cytometry. (A) denotes a forward scatter (FSC) *vs* side scatter (SSC) dot plot, with the R1 gate incorporating the lymphocyte cell population. (B) Shows CD3 PerCP *vs* CD56 PE expression within the R1 selected cell population. The R2 gates surrounds the total NK cell population ($CD3^{-}CD56^{+}$) with $CD56^{Dim}$ and $CD56^{Bright}$ NK Cell populations highlighted.

As previously described, CD69 and CD107 staining were utilised as representative markers of NK cell activation. Prepared LNMNC cells from lymph nodes acquired from the mesentery of colorectal cancer specimens were treated with JX-594 (Figure 5.11).

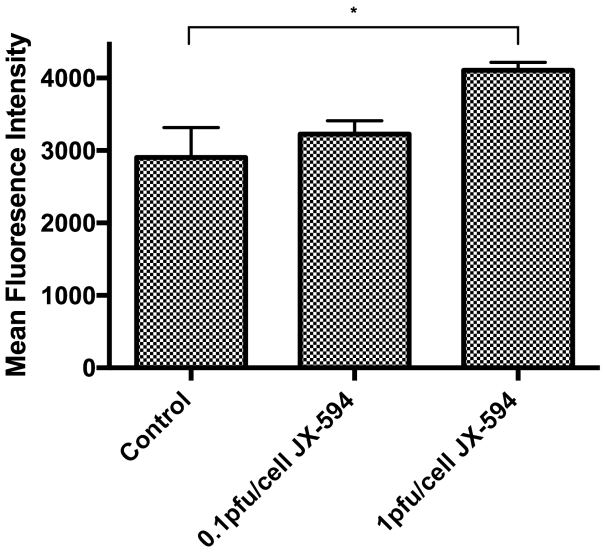


Figure 5.11 JX-594 activates NK cells in LNMNCs.

LNMNCs were prepared from disaggregated fresh lymph node tissue as previously described. Isolated LNMNCs were treated with 0, 0.1 and 1pfu/cell of JX-594 for 24 hours before being harvested and stained with CD69-FITC antibody for 30 mins and then washed and fixed with 1% PFA prior to analysis using flow cytometry. Graphs show mean + SEM for 3 individual lymph nodes. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA).

As a direct result of virus challenge, NK cell activation was observed in non-tumour bearing lymph nodes, as demonstrated by a significant increase in CD69 expression after treatment with 1pfu/cell JX-594. Whilst still significant this was less pronounced than that previously demonstrated within PBMCs. (Figure 5.3A)

Similarly, to assess NK cell degranulation, LNMNCs were treated with increasing doses of JX-594 and co-cultured with SW480 and SW620 colorectal cell line targets. Figure 5.13 demonstrates that JX-594-activated LNMNC NK cells degranulate against both SW480 and SW620 cell line targets which was significant compared to untreated controls. No significant degranulation was observed in the absence of SW480 or SW620 cell targets.

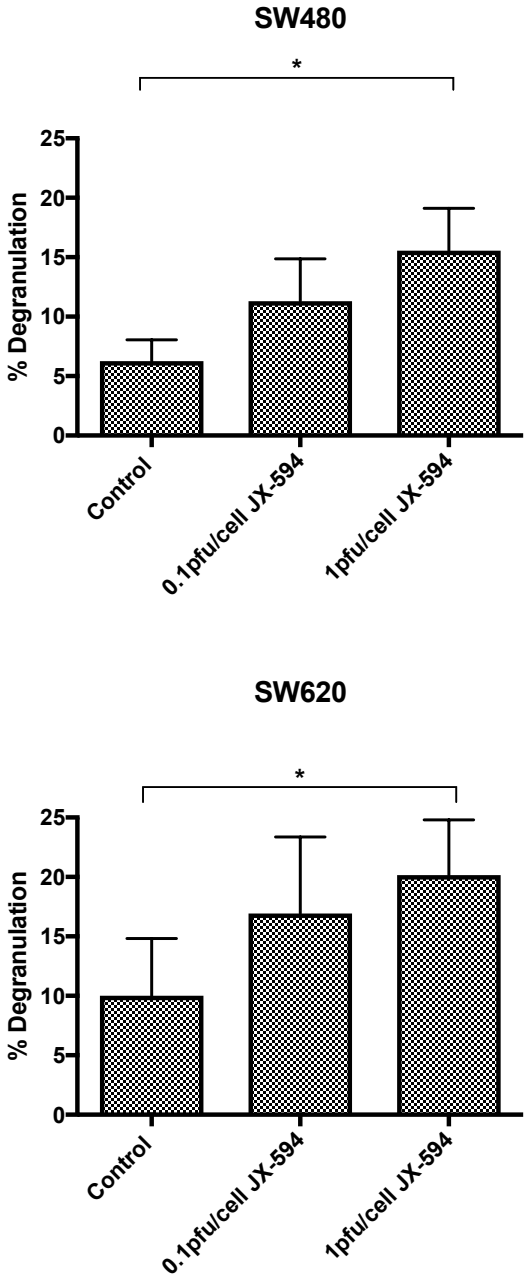


Figure 5.12 JX-594-activated LNMNC NK cells degranulate against colorectal cell line targets.

LNMNCs were isolated from disaggregated fresh lymph node tissue obtained from CRC patients undergoing surgical resection and treated with 0, 0.1 and 1pfu/cell of JX-594 for 24 hours before being harvested. LNMNCs were then co-cultured with SW480 and SW620 colorectal cell lines for 4 hours prior to staining with CD107a/b-FITC antibodies. The percentage of NK cells expressing CD107a/b was quantified using flow cytometry and recorded as percentage degranulation. Graphs show mean + SEM for 3 separate lymph node donors. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA).

Of note, as with healthy donor PBMC (Figure 5.3) higher levels of degranulation were seen against the metastatic colorectal target cell line SW620 cells compared with SW480 cells. No statistically significant increase was observed in the absence of targets (data not shown). Whilst we acknowledge small differences and low levels of degranulation involved, purely hypothetically this could present significant translational potential for targeting of micrometastasis.

5.9 Cytokines involved in JX-594 activation of NK cells in LNMNC.

To explore the mechanistic process behind the NK cell activation observed we analysed the cell free supernatants after infection with JX-594 to quantify specific cytokines that may be involved in NK cell activation. In PBMCs, type I IFN's appeared to play a key role in NK cell activation, as demonstrated by the production of IFN-α in response to JX-594 infection, and the cessation of NK cell activation when assays were performed in the presence of IFN blocking (Figure 5.6).

Unlike in PBMCs, however, LNMNCs did not appear to stimulate the production of type 1 IFNs (α/β). At 1pfu/cell JX-594, IFN-α was just detectable at very low levels (less than 100pg/ml; Figure 5.13A) and IFN-β was not present (data not shown) after treatment with JX-594. GMCSF was secreted in a dose-dependent manner, with increased expression associated with increasing virus dose; levels were comparable to that seen with PBMCs (Figure 5.13C).

Given previous literature regarding lymph nodes which suggests an important role for IFN-Interferon-γ in both NK cell recruitment and activation we also looked for interferon gamma-induced protein 10 (IP-10). IP-10 (also known as C-X-C motif chemokine 10 (CXCL10)) is a small cytokine belonging to the alpha chemokine family. It is constitutively expressed at low levels in lymph node stroma[301] and secreted by various cell types including monocytes, fibroblasts and endothelial cells. IP-10 plays an important role in T-cell

trafficking and mediates the IFN-γ response. Interestingly, given the absence of significant IFN-α or IFN-β, IP-10 was also detected at low levels, although levels detected demonstrated wide variability across the repeat experiments (Figure 5.13B).

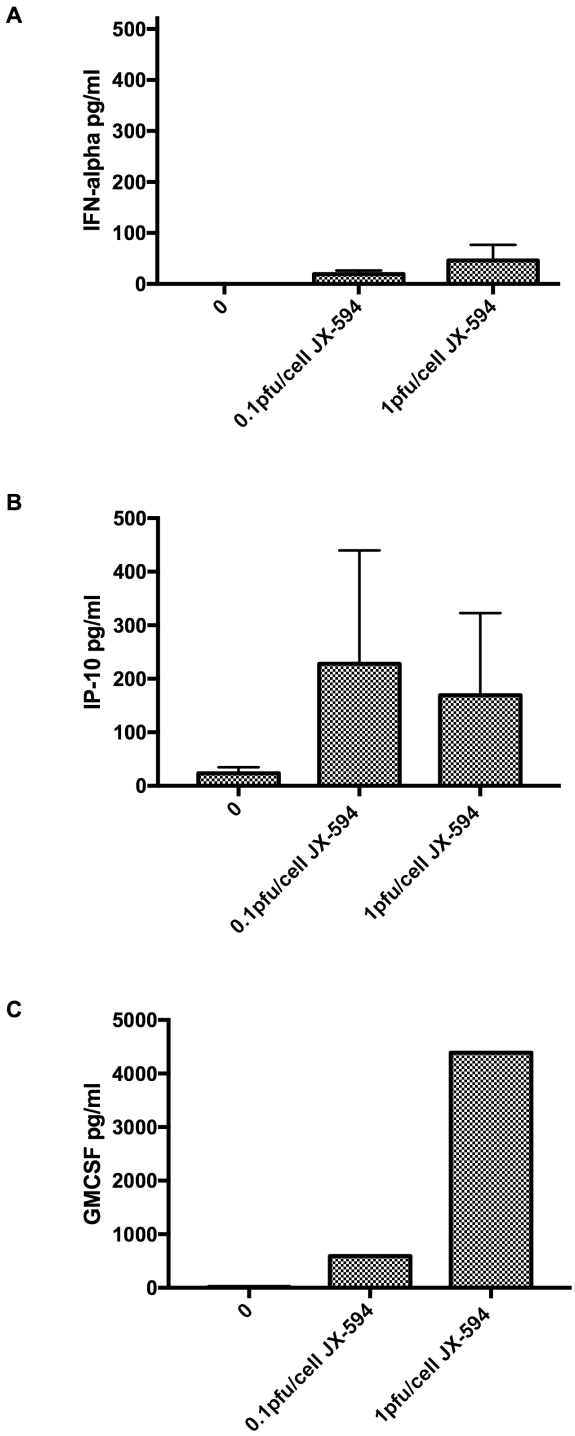


Figure 5.13 IFN- α, IP-10 and GMCSF production from JX-594 treated LNMNCs.

Cell-free supernatants were collected from treated LNMNCs and analysed by ELISA for the presence of IFN- α, IP-10 and GMCSF. Graphs A & B show mean + SEM for 3 separate donors. Graph C is data from a single donor. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA). No statistically significant values were observed.

5.10 JX-594 activation of NK cells in donor LNMNCs is independent of type 1 IFNs

Data from PBMCs demonstrated that despite relatively low levels of Type I IFNs identified as a result of JX-594 infection, NK cell activation was indeed dependent on type I IFN production. For example, CD69 expression and NK cell CD107 degranulation were both inhibited in the presence of IFN blocking.

Hypothetically it was anticipated that the same should be true for LNMNC response to JX-594 treatment. However, given the unexpectedly low, almost undetectable levels of Type I IFN found in the supernatants of infected LNMNCs we postulated that IFN may not be the primary mechanism of NK cell activation within lymph nodes. To elucidate the importance of IFN for NK cell activation in lymph node samples we used interferon blocking experiments as previously described to allow direct comparison with data acquired for PBMCs. The CD69 and CD107 degranulation assays on LNMNCs in the presence and absence of IFN blocking serum antibodies yielded no significant reduction in CD69 activation or degranulation.

Similar levels of CD69 activation (3.6% fold increase from control to 1pfu JX-594) were observed in response to JX-594 treatment, however, the addition of IFN blockade or isotype controls did not inhibit CD69 upregulation in response to JX-594, suggesting a mechanism different to that seen in blood, and not type I IFN dependent (Figure 5.14A). Likewise, NK cell degranulation, was also independent of type 1 IFN with no demonstrable difference in NK degranulation seen in the presence or absence of IFN blockade. Of note, this was consistent between the SW480 and SW620 target cells with no difference seen between the primary and metastatic cell lines (Figure 5.14B).

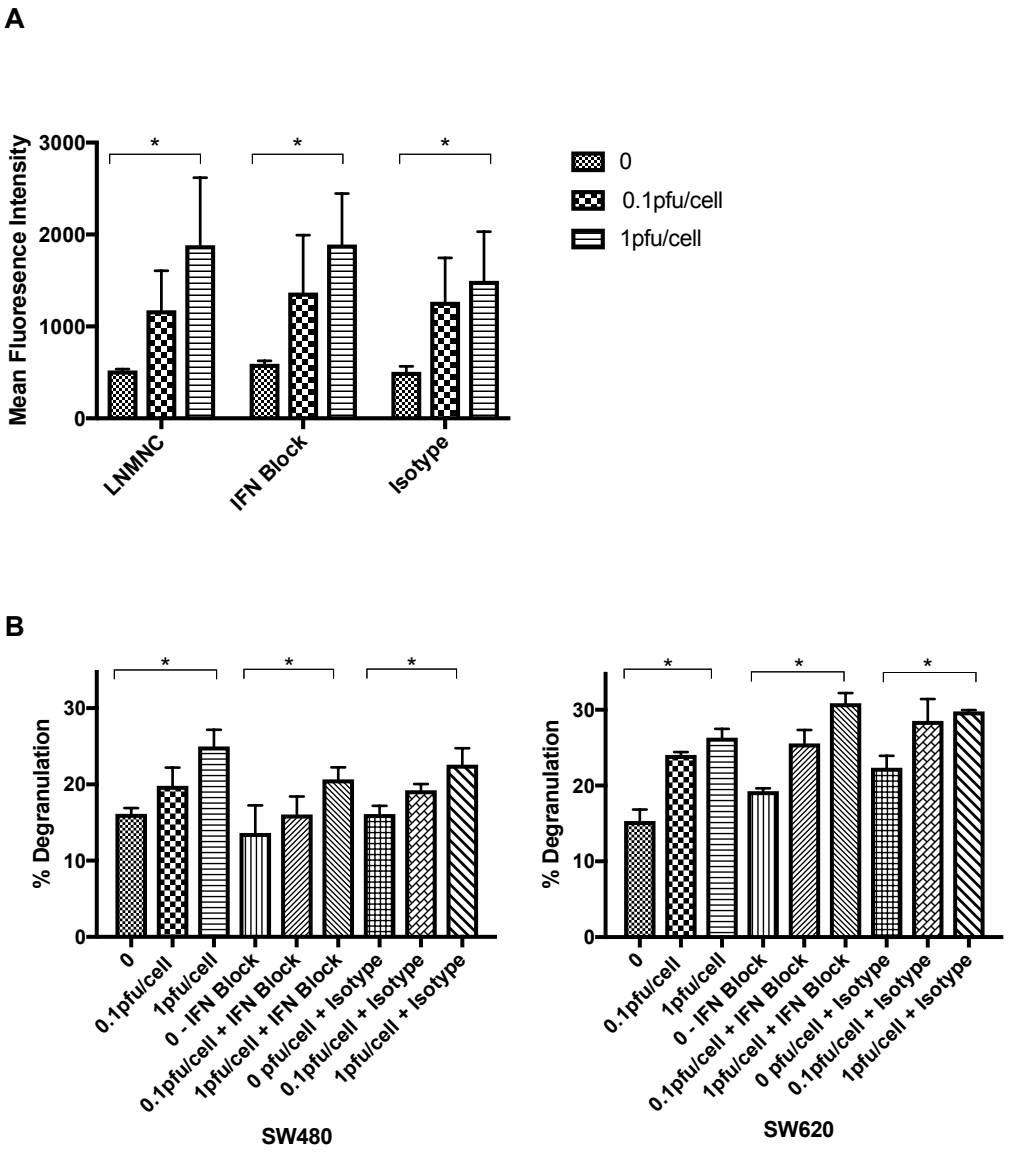


Figure 5.14 JX-594 activation of NK cells is independent on type 1 interferons in patient LNMNCs.

LNMNCs were isolated from lymph node samples retrieved from patients undergoing colorectal cancer resection prior to JX-594 treatment. LNMNCs were treated with control media, IFN-α, IFN-β blocking serum and an IFNα/β receptor blocking antibody or IgG2a isotype and control sheep serum. Following this pre-incubation, LNMNCs were treated with 0, 0.1 and 1pfu/cell of JX-594 for 24 hours before being stained with (A) CD3/CD56/CD69-FITC antibodies or (B) co-culture with SW480 and SW620 colorectal cell lines (10:1 ratio) for 4 hours prior to assessment of CD107a/b on NK cells. Graphs show mean + SEM for 3 separate patient samples. Statistical significance is denoted by *$p<0.05$ (One-way ANOVA).

5.11 Reovirus induced NK cell activation in LNMNCs

As discussed earlier in this chapter, previous work by colleagues in the laboratory have largely focused on an alternative virus, Reovirus. Given this previous body of work, it was of interest to see if NK cell activation could be initiated by Reovirus in lymph nodes in a similar fashion to JX-594.

As with JX-594, Reovirus resulted in a significant increase in CD69 with results largely consistent with those observed for JX-594. Significant NK cell activation was observed at lower doses of Reovirus (0.1pfu/cell) resulting in a significant increase in CD69 expression (2903.7 - 4422.4mfi; Figure 5.15).

Similarly, we also examined the ability of Reovirus to promote NK cell degranulation within our LN samples. As with JX-594, Reovirus-activated LNMNC NK cells resulted in degranulation against colorectal targets in a dose dependant fashion. Percentage degranulation against both colorectal cancer cell targets were however almost 3-fold greater than those seen with JX-594, with similar enhanced levels of degranulation seen in response to both the primary and metastatic target cell population (Figure 5.16).

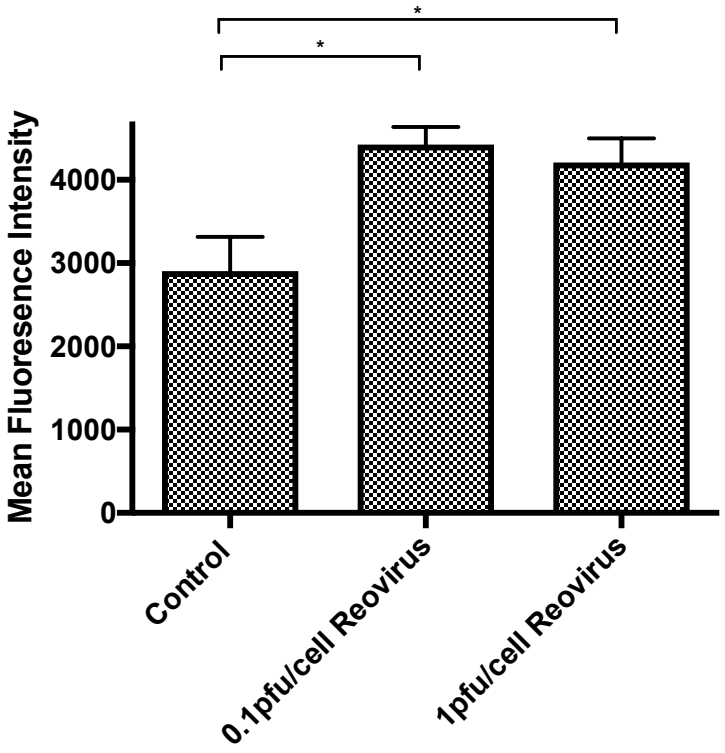


Figure 5.15 Reovirus activates NK cells in LNMNCs.

LNMNCs were prepared from disaggregated fresh lymph node tissue as previously described. Isolated LNMNCs were treated with 0, 0.1 and 1pfu/cell of Reovirus for 24 hours before being harvested and stained with CD69-FITC antibody for 30 mins prior to analysis using flow cytometry. Graphs show mean + SEM for 3 individual lymph nodes. Statistical significance is denoted by *$p<0.05$.

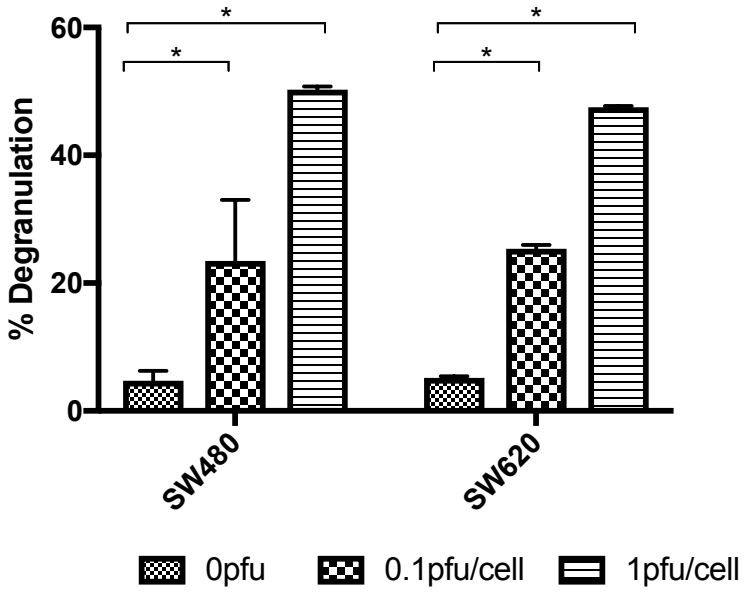


Figure 5.16 Reovirus-activated LNMNC NK cells degranulate against colorectal cell line targets.

LNMNCs were isolated from disaggregated fresh lymph node tissue from CRC patients undergoing surgical resection and treated with 0, 0.1 and 1 pfu/cell of Reovirus for 24 hours before being harvested. LNMNCs were then co-cultured with SW480 and SW620 colorectal cell lines (10:1 ratio) for 1 hour prior to staining with CD107a and b - FITC antibodies in the presence of Brefeldin A for a further 4 hours. Percentage of cells expressing CD107a/b was measured using flow cytometry and recorded as percentage degranulation. Graphs show mean + SEM for 3 separate lymph node donors. Statistical significance is denoted by *$p<0.05$.

Given the levels of NK cell degranulation seen in response to Reovirus it was important to also further define the mechanism of Reovirus-induced NK degranulation in lymph nodes given the mechanism of action adopted by JX-594 does not appear to utilise type-1 IFN. In response to Reovirus, significant levels of IFN-α were produced (Figure 5.17) consistent with a type 1 IFN response. IP-10 was also produced at low levels (Figure 5.17), however no GMCSF production (data not shown).

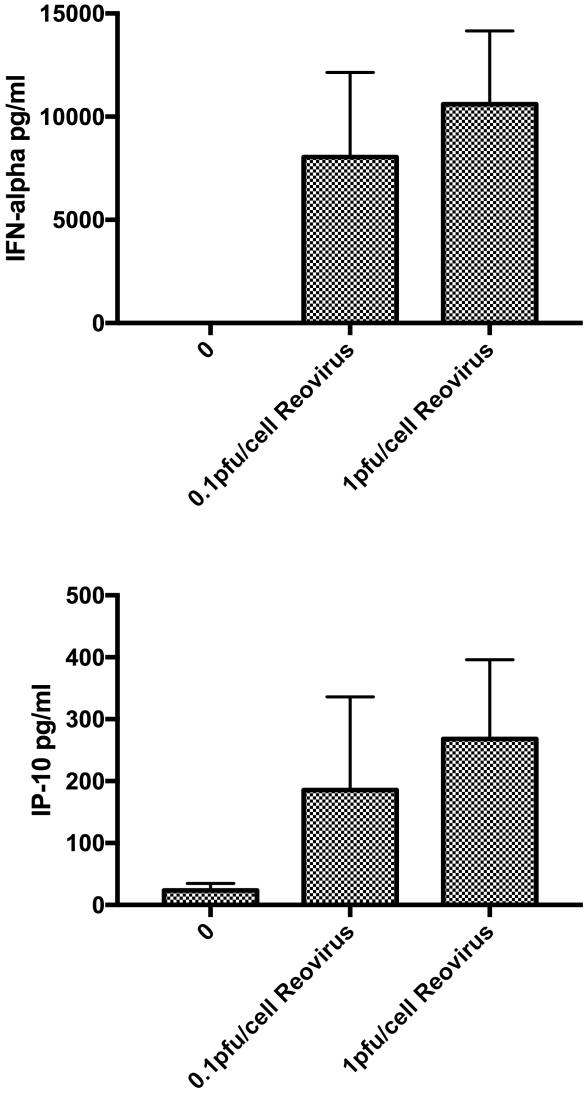


Figure 5.17 IFN-α and IP-10 production from Reovirus treated LNMNCs.

Cell-free supernatants were collected from Reovirus treated LNMNCs and analysed by ELISA for the presence of IFN- α, and IP-10. Graphs show mean + SEM for 3 separate donors.

Hypothetically, the high level of IFN-α produced in response to Reovirus in LNMNC, in contrast to JX-594, would suggest the mechanism of NK cell activation is likely to be type I IFN dependent as observed in PBMC. Whilst beyond the scope of this work, and as a direct result of limited access to further lymph node samples, a single preliminary experiment using 10pfu Reovirus, was carried out in the presence of interferon block. Surprisingly, in the experiment undertaken, IFN blocking yielded no significant reduction in NK cell activation or degranulation (Figure 5.18).

Drawing firm conclusions from single experiments is difficult and further repeats would be necessary to corroborate these initial findings, but it seems possible that two different mechanisms of action are involved in OV-induced NK cell activation in PBMCs and LNMNCs, regardless of the virus chosen. OV-induced NK cell activation in PBMCs is type-1 IFN dependent and likely controlled by IFN-α, albeit at low levels. In LNMNCs, despite variable IFN production depending on the OV chosen, there appears to be no correlation between the presence of type 1 IFNs and NK cell activation, in fact, the mechanism appears to be independent of type-1 IFNs.

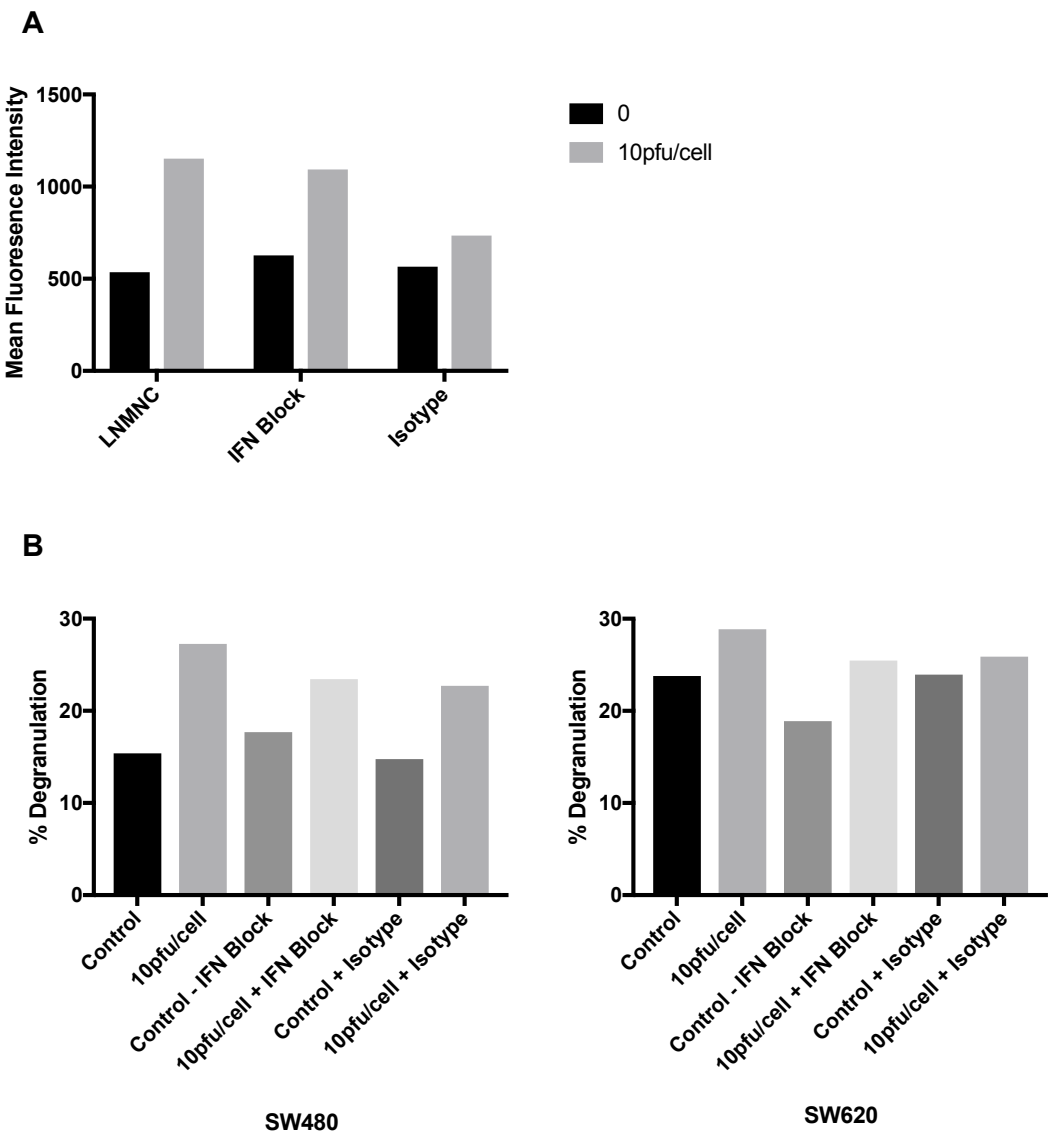


Figure 5.18 Reovirus activation of NK cells may be independent of type 1 interferons in patient LNMNC.

LNMNC were isolated from lymph node samples retrieved from patients undergoing colorectal cancer resection prior to Reovirus treatment. LNMNCs were treated with control media, IFN-α, IFN-β blocking serum and an IFNα/β receptor blocking antibody or IgG2a isotype and control sheep serum. Following this pre-incubation, LNMNCs were treated with 0, 10 pfu/cell Reovirus for 24 hours before being stained with (A) CD3/CD56/CD69-FITC antibodies or (B) co-cultured with SW480 and SW620 colorectal cell lines (10:1 ratio) for 4 hours prior to assessment of CD107a/b on NK cells. Graphs show data from a single patient sample.

5.12 Discussion

The initial benefits of OV therapy focussed on their direct oncolytic effects, however, more recently modulating OV immune response has been the focus of various groups around the world. OV's have a natural tropism for tumour cells, which can be enhanced by genetic modifications to the virus backbone resulting in improved cell specificity and supporting anti-tumour immunity. Tumour cells also have an impaired antiviral response, demonstrating both a diminished IFN response and an increased biological tolerance of viral infection making them attractive cancer therapy agents.[52] This chapter aimed therefore to further define the mechanisms by which JX-594 acts as an immunotherapeutic agent.

The focus of the chapter relates to NK cell activation which has been used by our group extensively in previous similar studies as a direct marker of innate immune activation and thereafter examined the consequences of associated cytokine release. Specific focus was placed on type 1 IFNs, the role of which have been reviewed by Biron et al. (1999)[40] and Le Bon et al. (2002)[302] but act to govern a number of immune-regulatory functions that control both the innate and adaptive immune response, for example regulating phenotypic and functional changes in DC. The production of IFN-α was initially demonstrated by Gerosa et al. (1991)[303] to induce the upregulation of CD69 expression on resting human NK cells and more recent data demonstrated that Reovirus infection could induce CD69 expression on NK cells in whole PBMCs in an IFN dependent fashion.[204] With respect to this, our group has also previously demonstrated that RNA viruses, such as Reovirus, oncolytic VSV and measles virus activate a generalised inflammatory response following tumour cell infection, characterized by the secretion of a range of molecules, in addition to the type I IFNs (α and β), including, RANTES, IL-6, IL-8, MIP-1a and MIP-1b, which have been shown to be able to regulate innate tumour killing.[43,206,304,305]

For JX-594, unpublished data reported by my predecessor analysed the profile of chemokines and cytokines secreted following JX-594 treatment. JX-594 infection of CRC cell lines did not result in the secretion of significant levels of any of the inflammatory molecules listed above with the exception of

IL-6 and GMCSF, where GMCSF is encoded by the virus. However, a dose-dependent reduction in the classically immunosuppressive cytokines, VEGF and IL-10 was observed suggesting that JX-594 can promote a pro-inflammatory, anti-angiogenic tumour microenvironment (Data not shown; see also R Dave unpublished thesis).[253]

To progress these data we have demonstrated that JX-594 treatment of healthy donor PBMCs activates NK cells to degranulate against colorectal cancer target cells. NK cell activation was an IFN-dependent process with both activation and degranulation being prevented when IFN signalling was abrogated by IFN blocking serum. Interestingly, this occurs despite the relatively low levels of IFN production reported in response to JX-594 when compared with alternative OV reported in the literature.[157,304,306]

Interestingly, previous studies examining the effect of OV (Reovirus) in isolated NK cells *in vitro,* have shown an inability of Reovirus to activate NK cells in isolation. Rather it acts to induce phenotypic maturation of DC/monocytes and the production of inflammatory cytokines leading to the activation of NK cells upon co-culture.[157] The effect of JX-594 therefore in whole PBMCs is interesting and broadly mimics the response observed by both Adair et al. (2013)[204] and Parrish et al. (2015)[205] seen after Reovirus infection of whole PBMCs albeit, with lower IFN production levels. The findings further highlight the complex interaction at play in response to virus infection of whole PBMCs, with DC, a plethora of inflammatory cytokines and NK cells involved in cell-cell cross-talk driving an inflammatory response. Within this pathway, IFN-α, secreted from DC, drives an anti-viral response by activating other effector cells and propagating the immune response against target cells.[307] However, various authors have demonstrated, including my predecessor for JX-594 (*data unpublished)* that this anti-viral IFN-α response does not completely abrogate direct oncolysis or viral replication.[308,309] Indeed, these mechanisms underly JX-594 inherent tumour selectivity. As a result of cellular infection by JX-594, the virus secretes viral proteins from the infected cell in order to manipulate pathways that allow it to replicate, spread and evade IFN-mediated anti-viral clearance. Two distinct proteins are released, vaccinia growth factor (VGF) which activates the EGFR/RAS/MAPK

signal transduction pathway driving virus replication and spread and B18R which inactivates type 1 IFNs.[124]

The other cytokine produced in significant quantities was GMCSF. Initially it remained unclear if this increase in GMCSF was as a result of cytokine release from activated NK cells or simply as a result of replication of JX-594, which has been genetically engineered to encode for GMCSF within PBMCs. VV-Luciferase treatment of PBMCs, a virus not engineered to encode GMCSF, also resulted in a significant, albeit reduced increase in GMCSF. It would seem likely therefore that JX-594 stimulates GMCSF production directly from immune cells in response to virus challenge, although additional production from early phase replication of the virus in PBMCs cannot be discounted.

Currently, GMCSF is the immune gene that has been inserted most successfully into a range of clinical grade OV. Interest stems from its underlying ability to generate a potent systemic adaptive anti-tumour immune response following expression in tumour cells, this is associated with differentiation, maturation and recruitment of DC within the tumour microenvironment.[271] Numerous clinical trials have now shown clinical benefit of this strategy with large scale accumulation of DC seen at injection sites, resulting in the processing and presentation of tumour antigens to activate tumour-specific T cells.[310,311] The findings in this study of both induced and encoded GMCSF expression in response to JX-594 infection further supports these translational strategies.

A further interesting observation in this study is that it is evident that $CD14^+$ monocytes play a crucial role in the activation of NK cells within PBMCs in response to JX-594. As previously mentioned prior work by the group demonstrated that $CD14^+$ monocytes were the predominant cell population within PBMCs to become infected with JX-594, an observation supported by Hou et al. (2012)[290] who demonstrated a role of $CD14^+$ monocytes in the virus-induced activation (VSV, vaccinia, influenza A virus, circulating swine-origin virus) of NK cells. Likewise, Sanchez-Puig et al. (2004)[312] and Byrd et al (2013)[313] both conclude that VV infection of peripheral blood leukocytes show a strong bias towards the infection of monocytes over alternative immune cell populations.

In a similar vein to our data, the important relationship between monocytes, NK cells and type 1 IFNs in response to virus challenge is consistent with recent data published in knock out mice experiments carried out by Lee et al. (2017)[314]. In response to Herpes Simplex Virus Type-2 (HSV-2) infection in an *in vivo* murine model, the authors concluded that type 1 IFN receptor signalling in monocytes, not DC's or NK cells, is essential for NK cell activation. In their study, knock-out mice deficient in type 1 IFN signalling resulted in 'significantly lower levels of inflammatory monocytes, deficient IL-18 production and lacked NK cell-derived IFN-γ' following treatment with HSV-2. Furthermore, depletion of monocytes (as with our study) resulted in complete loss of NK cell activation.[314]

Interestingly, $CD14^+$ monocyte dependent NK cell activation in virus treated PBMCs has also been reported with Reovirus. Parrish and colleagues (2015)[205] abrogated CD69 expression and NK cell degranulation in $CD14^+$ depleted Chronic Lymphocytic Leukaemia (CLL) patient PBMC following Reovirus treatment suggesting monocytes are critical for activation to occur. Hypothetically, both our data and previous studies within the group suggest that within PBMCs, monocytes take up JX-594 causing a phenotypic shift (data not shown) which results in a cascade of events that ultimately leads to IFN-α production and NK cell activation. Given the immune response demonstrated in PBMCs, one might expect to see similar results in LNMNCs. Indeed, JX-594 infection of donor LNMNCs did activate NK cells to degranulate against CRC targets, as expected. However, our data does not support a role for an IFN dependent system, as with PBMCs, and the mechanism of virus activation in lymph nodes remains to be fully elucidated and is discussed further below.

Notable and important differences exist in the phenotyping of LNMNCs when compared with PBMCs, namely the lack of monocytes ($CD14^+$ cells) which appear critical to the virus response of PBMCs. This is not unexpected for lymph nodes in their resting state as typically during infection, monocytes or monocyte/derived cells are recruited from the blood into lymph nodes to mediate, and promote, an adaptive immune response.[315] Vaccinia virus has been reported to induce type 1 IFN expression by inflammatory monocytes, and recruitment to lymph nodes requires toll-like receptor 2 (TLR2) stimulation

by viral ligands in endosomes of virus infected cells. Following recruitment to the lymph nodes, monocytes differentiate along distinct pathways based on distinct pathogen-derived ligands.[316] Indeed only eighteen hours after VV infection, different subsets of DC can be identified characterised in part by increased cell surface expression of the DC maturation marker CD83, and is associated with rapid disappearance of $CD14^+$ cells.[290]

Within the lymph nodes, the main initiators of a cellular immune response are thought to be antigen presenting cells (APC) which act to present processed viral antigens to naïve T cells in the draining lymph nodes. Of these APC, DC are reported to be the most important in stimulating T-cell proliferation. In murine lymph nodes several phenotypically distinct DC populations exist but consistent with our data all are $CD11c^+$.[317] Interestingly a review by Colonna et al. (2002) demonstrated that $CD11c^+$ DC's secreted large amounts of type 1 IFN *in vitro* following infection with HSV-1 in both humans and mice.[318] This is at odds to the minimal IFN production seen in response to JX-594 but is consistent with the IFN production observed following Reovirus treatment. Hypothetically, this may reflect different virus acting on different DC subsets, i.e. plasmayctoid DC or conventional DC both of which express a different array of TLR.[316]

To examine potential reasons for the differences observed between both the JX-594 and Reovirus and PBMC and LN responses, it is important to explore human NK cell biology in more detail. Human NK cells, as previously discussed are identified by CD56 expression and the absence of the T cell marker CD3. CD56 NK cells can be sub-classified further into $CD56^{Dim}$ and $CD56^{Bright}$ which can be found in different quantities depending on tissue type examined. The $CD56^{Dim}$ subset are largely found in the blood and are characterised by high cytotoxicity and low cytokine production, in keeping with our PBMC findings which demonstrate low levels of $CD56^{Bright}$ in phenotyping studies (Figure 5.1) and low levels of cytokine production in response to NK cell activation. Conversely, $CD56^{Bright}$ NK cells are largely found in lymph node tissues, and are reported to show low cytotoxicity, and produce large amounts of cytokines, specifically IFN-γ,[319] not examined in this study. Our phenotyping of lymph nodes certainly demonstrated an increased proportion of $CD56^{Bright}$ when compared with PBMC data and perhaps suggests OV-induced NK cell

activation is exerting its effect via two different subpopulations of NK cell in the two different tissue types, as reviewed by Zwirner et al. (2017)[320].

Different cell types and cytokine release is supported by a previous study examining NK cells in mice and human lymph nodes by Watt et al. (2008)[298]. The authors identified mature NK cells to be recruited preferentially into draining lymph nodes following DC challenge in a tightly regulated IFN-γ - dependent mechanism. Thereafter, DC induced NK cell activation was reported to produce IFN-γ which played an important role in inducing Th1 responses. Several reports suggest a tightly coordinated network involving DC, T cell and NK cells which act within the local LN microenvironment to determine an immune response.[321–323] Interestingly, consistent with these findings, Rajani et al. (2015) [324] also demonstrated an adaptive T cell response in LN following Reovirus treatment. In mice treated with *i.t*. Reovirus, an IFN$-\gamma$ memory recall response to B16 tumour lysates was detected in pooled splenocytes and lymph nodes suggestive of Reovirus priming anti-tumour T-cell responses.

Furthermore, Hayakawa et al. (2008)[321] examined the effect of prior NK cell activation with two molecules, poly(I:C) and α-GalCer on NK cell recruitment to draining LN. Poly(I:C) and α-GalCer are known to be type I and type II IFN dependent in their mechanism of action, however acted to diminish NK cell recruitment to draining LN whilst downregulating expression of the activated NK cell chemokine receptor, CXCR3. The authors hypothesise that reduced CXCR3 expression may be driven by the cell microenvironment to avoid any undesired contribution that pre-activated NK cells may play during T cell priming. It is clear from these studies, as with our data that NK cell biology in the lymph nodes may act through different mechanism to that utilised by PBMCs. In this study, we did not look at IFN$-\gamma$, however the literature suggests that IFN$-\gamma$ plays a pivotal role in coordinating both NK cell recruitment and activation in lymph nodes and this would therefore be an interesting continuation of this work. As previously stated, confirming a different mechanism by which OV-induced immune priming can act between PBMCs and LNMNCs could open the window to the targeting of micrometastatic disease in lymph nodes in addition to the blood.

Whilst much work remains examining the wider immune response to systemic virus and the potential anti-tumour effects, this could hold the key to treating non-resectable microscopic disease. It is likely that multiple other effectors, not examined here also play a role in driving innate immune responses, an example being neutrophils which are reported to contribute to the innate anti-tumour efficacy of other oncolytic viruses (Reviewed by Choi et al. (2016))[325].

In addition, our preliminary data with Reovirus showed that Reovirus is also capable of activating LNMNC NK cells and as observed for PBMC, was more efficient than JX-594 at inducing IFN-α and activating NK cell populations. Reovirus anti-tumour efficacy is widely reported to depend upon a potent anti-tumour immune response mediated through the activation of DC to stimulate NK cells and T cell mediated cytotoxicity.[43,157,324,326] Our preliminary findings, suggest improved type 1 IFN release from LNMNC as a result of Reovirus treatment with significantly higher levels of IFN-α recorded compared with JX-594. However, as observed for JX-594 NK cell activation did not appear to be type-1 IFN dependent in LNMNCs in the pilot experiment performed.

This study may also provide the basis for exploring further combination strategies to treat micrometastatic disease, for example, the use of OV with immune checkpoint inhibitors. Rajani et al. (2015)[324] reported a significantly improved survival benefit in mice treated with a combination of Reovirus and anti-PD-1 antibody compared with either PBS controls or single agent treatment. Preliminary phenotyping experiments in our LNMNC population (*data not shown)* showed significant levels of PD-1 and PDL-1 and as such the potential for translational combination strategies in the clinical setting must be considered promising and provides an area of interest to explore further in more detail in future experiments.[324]

It is clear from these studies that JX-594 is able to alter both phenotypic and functional aspects of NK cell activity acting via different mechanisms in the blood and lymph nodes. These different mechanistic processes may allow translational opportunities with JX-594 targeting tumour cells in both blood and lymph node tissue. Indeed, if low levels of virus can access any non-resected tissue, activation of an innate immune response at specific tissue sites may provide sufficient therapy to treat this micrometastatic disease and our data

suggests that JX-594 holds promise as a novel treatment modality. Moreover, direct tumour-specific lysis, transgene expression and the induction of tumour specific innate immunity means that it may provide a multi-pronged, and selective attack, against the tumour at various disease sites.

Chapter 6
Conclusion

As discussed colorectal cancer remains a significant clinical problem. By 2030 CRC globally is estimated to be diagnosed in 2.2 million new cases per year and account for 1.1 million deaths, an increase of 60%.[3] Nonetheless bowel cancer survival has undoubtedly improved. In the UK alone survival has more than doubled in the last 40 years (22%-57%), however these figures remain dependent on stage of disease at diagnosis, patient demographics, comorbid state and tumour biology.[327]

In part, improved survival has transpired as a result of now established treatments such as anti-EGFR antibodies (Cetuximab, Panitumumab, and GA201). Unfortunately they are currently only offered to about 50% of CRC patients, based on identification of appropriate genetic susceptibility; disappointingly only 10-20% of patients obtain objective clinical responses with KRAS, BRAF and PI3KCA mutations (causing constitutively activated EGFR signalling) implicated in this cellular resistance. Newer classes of anticancer agents include oncolytic viruses (T-VEC and HSV-1) which have been approved by regulatory bodies for mainstream use in the treatment of inoperable melanoma, and BH3-mimetic (Navitoclax) which continue to develop as experimental agents in phase II clinical trials.

The laboratory has a broad breath of experience with respect to delineating the cytotoxic and immune potential of a variety of oncolytic viruses in a range of tumour types and as such this study set out to explore the potential for OV involvement in combination with alternative immune-mediated therapies. Furthermore, viral effects in areas other than the tumour itself and its microenvironment may also be therapeutically important. This is particularly the case in organs which can harbour micrometastases such as lymph nodes, which may potentially be targeted and cleared by appropriate activation of an innate and adaptive immune response. An alternative OV, JX-594 was therefore chosen to investigate if it could preferentially replicate, and kill, CRC at micrometastastic sites, and activate and induce an innate immune response in both the blood and lymph nodes.

This thesis examined the ability of Reovirus and JX-594 to kill a range of CRC cell lines regardless of their EGFR/KRAS/BRAF/PI3K mutational phenotype. Variable levels of cytotoxicity were demonstrated against cells with different mutational profiles with some cell lines demonstrating consistent dose-dependent, time dependent killing (SW480, SW620) and other cell lines completely resistant to OV treatment (LIM1899). However, no correlation between mutational status and OV-induced death was elicited.

Before exploring treatments in combination, the cytotoxic potential of ABT-263 and Cetuximab in isolation was examined. Consistent with data that reports up to 50% of cancer cell lines are resistant to ABT-263 because of variable expression of pro vs anti-apoptotic molecules, our data also elicited a mixed response. Some cell lines tested were completely resistant whilst others showed time-dependent, dose-dependent increases in cytotoxicity. Once again, no correlation was seen between killing and cell line mutational status although the WT cell line, Colo320, was least sensitive suggesting a potential requirement for positive mutational status to allow ABT-263 cytotoxic death. Despite positive results with monotherapies, the effect of combining Reovirus and ABT-263 was disappointing. A single cell line (SW620) elicited increased cell death as a direct result of using both treatments in combination however, statistical analysis revealed only additive effects.

Cetuximab induces its anti-cancer effect by two distinct mechanisms; 1) antagonism of EGFR binding preventing pro-survival and proliferation signals, and 2) immune-mediated ADCC. Irrespective of EGFR signalling mutations, ADCC should occur and Reovirus immune activation of monocytes, neutrophils and NK cells, as demonstrated previously by the group might potentiate this antibody-induced ADCC.[328] Interestingly, regardless of EGFR cell surface expression or KRAS/BRAF/PI3K mutational status cetuximab induced no direct cytotoxicity against the selected CRC cell lines, although we were unable to test this model specifically against an EGFR positive, RAS wild type cell line, which has been reported by other groups to be sensitive to direct killing by cetuximab. Despite the lack of direct killing we did demonstrate preliminary evidence that pre-treatment of PBMCs with Reovirus, and resultant NK cell activation, did increase cetuximab-mediated ADCC in a

KRAS mutant cell line (SW480). Further work is needed to validate this data fully but certainly provides promise and direction for future experimentation.

JX-594 like Reovirus can kill target cells by direct oncolyis, replicate within CRC cell lines, and induce a pro-inflammatory, anti-angiogenic tumour microenvironment by promoting immune cell recruitment to stimulate an innate and adaptive immune response. However, VV-induced death mechanisms in CRC remain poorly understood and similarly the effects of VV on distant tissues, not related to the primary tumour, remain to be elucidated. These areas of study are however important given that targeting micrometastatic disease, in for example the blood and lymph nodes, with novel treatment strategies such as combinations of anti-EGFR antibodies and OV might prevent recurrence and the development of non-resectable disseminated disease.

Significantly, JX-594 infection has been shown to be associated with increase caspase-3 expression in previous work by the group. Caspase-3 is an important protein used in apoptosis and as such JX-594 was expected to, at least in part, kill by this mechanism. Despite the upregulation of these markers, and having confirmed JX-594 could induce direct killing in our panel of cell lines, we were unable to elicit any attenuation of VV-induced cytotoxicity using a pan-caspase inhibitor. Similarly, necroptosis inhibitors had no effect on JX-594-mediated death, which along with the absence of HMGB1 in post-treatment cell free supernatants was suggestive that necrosis and necroptosis were also not the primary mechanisms of action. The final mechanism of cell death investigated was autophagy, a process which VV has been shown to interfere with in ovarian cancer cell lines. In the CRC cell lines used in this study, no evidence of autophagy was demonstrated. Therefore, whilst it is clear that JX-594 induces significant cell oncolysis, the exact mechanism remains to be identified. It would seem likely that cell death is a coordinated, multi-faceted process involving parts of each pathway investigated and as such beyond the scope of this work to delineate further.

It is now well understood that for a virus to be an effective therapeutic agent, it must be highly immunogenic and stimulate an innate and adaptive immune response. Indeed, even if systemic delivery yields relatively low levels of virus

to non-tumour tissue, such as blood and lymph nodes, activation of an innate immune response may be sufficient to provide adequate therapy against CTCs and micrometastatic disease. We therefore first examined the innate effects of JX-594 on blood, which would be readily accessed at high dose following intravenous administration and which can harbour circulating tumour cells, a feature of CRC associated with poor prognosis. Virus treated NK cells from donor blood demonstrated CD69 activation, NK cell degranulation and increased cytotoxicity against CRC cell line targets. This was dependent on IFN production and the presence of $CD14^+$ monocytes. The relatively low levels of IFN-α and absence of IFN-β contrasts with Reovirus which produced relatively high levels of type-I IFNs. It is likely that this may be due to proteins such as B18R, encoded by VV, whose role is to blunt the infected cells IFN response. High levels of GMCSF were also seen following JX-594 treatment. Taken together the findings demonstrate the ability of JX-594 to infect monocytes within PBMC, leading to IFN-α mediated NK cell activation for potential killing of CTC's.

The study then addressed whether JX-594 mediated innate immune activation may have therapeutic effects in LN tissue. Data pertaining to the examination of fresh human lymph nodes using *ex vivo* models is relatively scarce in the literature, presumably because of the challenge of accessing samples. Initial work therefore determined the phenotypic appearance of lymph node tissue and identifiable cell populations. Not surprisingly, LNMNCs did not contain monocytes and CD14 was absent. NK cells were however identified, along with the presence of CD11c, which is implicated as a likely carrier of pathogens to lymph nodes, which as discussed raises the potential for virus carriage to distant disease harbouring sites.

Having identified NK cells in LNMNCs, we repeated PBMC experimental protocols. Again virus treated LNMNC demonstrated CD69 activation, degranulation and increased cytotoxicity against CRC targets. Interestingly however this was a type-I IFN independent process with CD69 expression and NK cell CD107 degranulation not abrogated by the presence if IFN blocking. In addition, there was almost undetectable levels of type 1 IFN found in the cell free supernatant. Interestingly, similar results were observed in a

preliminary experiment using Reovirus treated LNMNC despite the production of high levels of IFN-α.

This work opens the door to several areas of interest for continuing work. Defining the mechanism behind LNMNC viral induced NK cell activation, and validating the preliminary finding for Reovirus induced activation, will help delineate treatment strategies further. Exploration of IFN-γ would also provide an important extension of this work.

Furthermore, this study may also provide the basis for examining alternative OV combination strategies for example with immune checkpoint inhibitors given the significant levels of PD-1 and PDL-1 found in our LNMNC population. Anti-PD-1 antibody inhibitors are the focus of promising recent research and have been shown to improve survival in *in vivo* mouse models when combined with Reovirus.[324] Given the findings from our LNMNC populations examining this combination would certainly be a promising experimental avenue to explore.

Indeed the arrival of the first two clinical grade PD-1 checkpoint inhibitors, Nivolumab and Pembrolizumab for the treatment of advanced melanoma and non-small cell lung cancer have shown significant promise in the field of immuno-oncology and as such are beginning to change the landscape of available treatment strategies for advanced disease.[329] As a result of these novel molecules, exploring combination strategies holds understandable appeal and as such the field of immuno-oncology continues to pursue the ideal combination to enhance patient outcomes. It is however, becoming apparent that this process is complicated with potential significant safety concerns associated with certain tested strategies. Pre-clinical testing of combined immune checkpoint blockade with a PD-1 inhibitor and CTLA-4 inhibitor for the treatment of various cancer types has demonstrated unprecedented efficacy, however is associated with a considerable toxic profile and high incidence of adverse drug reaction raising significant concerns about safety.[330]As such the challenges remain clear, and balancing improved treatment efficacy with toxicity is vital. Novel combination strategies clearly hold great promise, and as such the potential for immune checkpoint inhibitors

in combination with oncolytic virus provides an exciting avenue of further research.

Whilst there continues to be significant focus on immune checkpoints other areas of development and research include small molecule agents that modulate the tumour immune microenvironment such as CSF1R, IDO1, A2AR and KIR, cell therapies that engineer immune cells such as T cells to directly attack cancer cells (e.g. anti-CD19 CAR-T) and cancer vaccines that induce antigen specific antitumour immunity (e.g. sipuleucel-T).[331] Indeed the field is broad with currently 940 clinical-stage immuno-oncology agents including oncolytic viruses registered modulating 271 different targets.[329]

Immunomodulatory targets continue to be approved as the standard of care for many advanced tumour types, however with such a crowded and diverse molecular profile available some authors have recently questioned the efficiency of resources available to test all these agents effectively. Indeed the therapeutic milieu available to scientists and clinicians has been reported to be overwhelming to even the most experienced investigators, with limited availability of patients to register for clinical trials and as such may be delaying future innovation and clinical development.[331] Without doubt, progress continues to be rapid however the challenges remain multifaceted moving forward. Exploring combination strategies, controlling adverse effects and toxicity and targeting tumours that don't respond as yet to immunotherapy all hold potential for future research. As an aside, there is increasing calls for the development of collaborative working practices within academic institutions, biotech companies and pharmaceutical organisations as a way of bringing promising cancer immunotherapies to patients, sooner rather than later which must be the goal of any research study within this field.

In summary, it is evident from this work that both JX-594 and Reovirus hold promise as novel treatment modalities both as a monotherapy and in combination with adjuvant therapies. Both viruses tested can alter the phenotypic and functional aspects of NK cell activity via different mechanisms in the blood and lymph nodes. Moreover, direct tumour-specific lysis, transgene expression, the induction of tumour specific innate immunity and the potential to modulate other immunotherapies, means that these viruses

may be able to provide multi-faceted and selective targeted therapy against tumour at both primary and distant locations.

References

1. National Institute for Health and Care Excellence. *Colorectal cancer: the diagnosis and management of colorectal cancer*. *Nice Guideline* **CG131,** (2011).
2. Ait Ouakrim, D. *et al.* Trends in colorectal cancer mortality in Europe: retrospective analysis of the WHO mortality database. *BMJ* **351,** 4970 (2015).
3. Arnold, M. *et al.* Global patterns and trends in colorectal cancer incidence and mortality. *Gut* **66,** 683–691 (2017).
4. Ferlay, J. *et al.* Cancer incidence and mortality patterns in Europe: Estimates for 40 countries in 2012. *Eur. J. Cancer* **49,** 1374–1403 (2013).
5. Network, N. C. I. *Colorectal cancer survival by stage*. (2009).
6. Haq, A. I., Schneeweiss, J., Kalsi, V. & Arya, M. The Dukes staging system: a cornerstone in the clinical management of colorectal cancer. *Lancet Oncol.* **10,** 1128 (2009).
7. Leong, L. *Clinical Relevance of Targeting Cancer Metastases*. *Madame Curie Bioscience Database (Internet)* (2013).
8. Ribatti, D., Mangialardi, G. & Vacca, a. Stephen Paget and the 'seed and soil' theory of metastatic dissemination. *Clin. Exp. Med.* **6,** 145–149 (2006).
9. Petersen, V. C., Baxter, K. J., Love, S. B. & Shepherd, N. a. Identification of objective pathological prognostic determinants and models of prognosis in Dukes' B colon cancer. *Gut* **51,** 65–69 (2002).
10. Märkl, B. *et al.* The clinical significance of lymph node size in colon cancer. *Mod. Pathol.* **25,** 1413–1422 (2012).
11. Bilchik, A. J. *et al.* Prognostic impact of micrometastases in colon cancer: interim results of a prospective multicenter trial. *Ann. Surg.* **246,** 568-575-577 (2007).
12. Chen, S. L. & Bilchik, A. J. More extensive nodal dissection improves

survival for stages I to III of colon cancer: a population-based study. *Ann. Surg.* **244,** 602–610 (2006).

13. Dukes, CE, B. H. The Spread of Rectal Cancer and its effect on Prognosis. *Br. J. Cancer* **12,** 309–320 (1932).

14. Quirke, P. & Morris, E. Reporting colorectal cancer. *Histopathology* **50,** 103–112 (2007).

15. Sloothaak, D. a M. *et al.* The prognostic value of micrometastases and isolated tumour cells in histologically negative lymph nodes of patients with colorectal cancer: a systematic review and meta-analysis. *Eur. J. Surg. Oncol.* **40,** 263–9 (2014).

16. Talbott, J. a. Looking forward. *J. Nerv. Ment. Dis.* **199,** 65 (2011).

17. Sirop, S. *et al.* Detection and prognostic impact of micrometastasis in colorectal cancer. *J. Surg. Oncol.* **103,** 534–537 (2011).

18. Ueda, Y. *et al.* Biological predictors of survival in stage II colorectal cancer. *Mol. Clin. Oncol.* **1,** 643–648 (2013).

19. O'Connor, E. S. *et al.* Adjuvant Chemotherapy for Stage II Colon Cancer With Poor Prognostic Features. *J. Clin. Oncol.* **29,** 3381–3388 (2011).

20. Bork, U. *et al.* Prognostic relevance of minimal residual disease in colorectal cancer. *World J. Gastroenterol.* **20,** 10296–10304 (2014).

21. Janeway, C. *Immunobiology 5 : the immune system in health and disease*. (Garland Pub, 2001).

22. Mahla, R. S., Reddy, C. M., Prasad, D. & Kumar, H. Sweeten PAMPs: role of sugar complexed PAMPs in innate immunity and vaccine biology. *Front. Immunol.* **4,** 248 (2013).

23. LeBien, T. W. & Tedder, T. F. B lymphocytes: how they develop and function. *Blood* **112,** 1570–80 (2008).

24. Balkwill, F. *et al.* Inflammation and cancer: back to Virchow? *Lancet* **357,** 539–45 (2001).

25. Nauts, H. C. & McLaren, J. R. Coley toxins--the first century. *Adv. Exp. Med. Biol.* **267,** 483–500 (1990).

26. McCarthy, E. F. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop. J.* **26,** 154–8 (2006).

27. Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science (80-.).* **331,** 1565–1570 (2011).

28. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat. Immunol.* **3,** 991–998 (2002).

29. Rees, R. C. & Mian, S. Selective MHC expression in tumours modulates adaptive and innate antitumour responses. *Cancer Immunol. Immunother.* **48,** 374–381 (1999).

30. Bubeník, J. MHC class I down-regulation: tumour escape from immune surveillance? (review). *Int. J. Oncol.* **25,** 487–91 (2004).

31. Guicciardi, M. E. & Gores, G. J. Life and death by death receptors. *FEBS J.* **23,** 1625–37 (2009).

32. O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K. & Shanahan, F. The Fas counterattack: a molecular mechanism of tumor immune privilege. *Mol. Med.* **3,** 294–300 (1997).

33. Bennett, M. W. *et al.* The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma. *J. Immunol.* **160,** 5669–75 (1998).

34. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R. & Ferguson, T. A. Fas Ligand-Induced Apoptosis as a Mechanism of Immune Privilege. *Science (80-.).* **270,** 1189–92 (1995).

35. Salih, H. R., Holdenrieder, S. & Steinle, A. Soluble NKG2D ligands: prevalence, release, and functional impact. *Front. Biosci.* **13,** 3448–56 (2008).

36. Nausch, N. & Cerwenka, A. NKG2D ligands in tumor immunity. *Oncogene* **27,** 5944–5958 (2008).

37. Souza-Fonseca-Guimaraes, F., Adib-Conquy, M. & Cavaillon, J.-M. Natural killer (NK) cells in antibacterial innate immunity: angels or

devils? *Mol. Med.* **18,** 270–85 (2012).

38. Coca, S. *et al.* The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* **79,** 2320–8 (1997).

39. Biron, C. A. Activation and function of natural killer cell responses during viral infections. *Curr. Opin. Immunol.* **9,** 24–34 (1997).

40. Biron, C. A., Nguyen, K. B., Pien, G. C., Cousens, L. P. & Salazar-Mather, T. P. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* **17,** 189–220 (1999).

41. Altomonte, J. *et al.* Exponential enhancement of oncolytic vesicular stomatitis virus potency by vector-mediated suppression of inflammatory responses in vivo. *Mol. Ther.* **16,** 146–53 (2008).

42. Alvarez-Breckenridge, C. A. *et al.* NK cells impede glioblastoma virotherapy through NKp30 and NKp46 natural cytotoxicity receptors. *Nat. Med.* **18,** 1827–34 (2012).

43. Prestwich, R. J. *et al.* Immune-mediated antitumor activity of reovirus is required for therapy and is independent of direct viral oncolysis and replication. *Clin. cancer Res.* **15,** 4374–81 (2009).

44. Prestwich, R. J. *et al.* Reciprocal human dendritic cell-natural killer cell interactions induce antitumor activity following tumor cell infection by oncolytic reovirus. *J. Immunol.* **183,** 4312–21 (2009).

45. Hashimoto, G., Wright, P. F. & Karzon, D. T. Antibody-dependent cell-mediated cytotoxicity against influenza virus-infected cells. *J. Infect. Dis.* **148,** 785–94 (1983).

46. Schroder, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. Interferon- : an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* **75,** 163–189 (2003).

47. Wang, R., Jaw, J. J., Stutzman, N. C., Zou, Z. & Sun, P. D. Natural killer cell-produced IFN- and TNF- induce target cell cytolysis through up-regulation of ICAM-1. *J. Leukoc. Biol.* **91,** 299–309 (2012).

48. Gómez Román, V. R., Murray, J. C. & Weiner, L. M. in *Antibody Fc* 1–

27 (2014). doi:10.1016/B978-0-12-394802-1.00001-7

49. Gül, N. & van Egmond, M. Antibody-Dependent Phagocytosis of Tumor Cells by Macrophages: A Potent Effector Mechanism of Monoclonal Antibody Therapy of Cancer. *Cancer Res.* **75,** (2015).

50. Pollara, J. *et al.* High-throughput quantitative analysis of HIV-1 and SIV-specific ADCC-mediating antibody responses. *Cytometry* **79,** 603–12 (2011).

51. Isaacs, A., Lindenmann, J. & Valentine, R. C. Virus Interference. II. Some Properties of Interferon. *Proc. R. Soc. B Biol. Sci.* **147,** 268–273 (1957).

52. Smith, G. L. *et al.* Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *J. Gen. Virol.* **94,** 2367–2392 (2013).

53. Marié, I., Durbin, J. E. & Levy, D. E. Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *EMBO J.* **17,** 6660–9 (1998).

54. Haller, O., Kochs, G. & Weber, F. The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* **344,** 119–30 (2006).

55. Smith, G. Poxviruses: Interfering with Interferon. *Semin. Virol.* **8,** 409–418 (1998).

56. Chang, H. W., Watson, J. C. & Jacobs, B. L. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4825–9 (1992).

57. Waibler, Z. *et al.* Vaccinia virus-mediated inhibition of type I interferon responses is a multifactorial process involving the soluble type I interferon receptor B18 and intracellular components. *J. Virol.* **83,** 1563–71 (2009).

58. Najarro, P., Traktman, P. & Lewis, J. A. Vaccinia virus blocks gamma interferon signal transduction: viral VH1 phosphatase reverses Stat1 activation. *J. Virol.* **75,** 3185–96 (2001).

59. Guerra, S., Cáceres, A., Knobeloch, K.-P., Horak, I. & Esteban, M. Vaccinia virus E3 protein prevents the antiviral action of ISG15. *PLoS Pathog.* **4,** e1000096 (2008).

60. Prestwich, R. J. *et al.* The case of oncolytic viruses versus the immune system: waiting on the judgment of Solomon. *Hum. Gene Ther.* **20,** 1119–32 (2009).

61. Kirn, D. H., Wang, Y., Le Boeuf, F., Bell, J. & Thorne, S. H. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. *PLoS Med.* **4,** e353 (2007).

62. Qiao, J. *et al.* Cyclophosphamide facilitates antitumor efficacy against subcutaneous tumors following intravenous delivery of reovirus. *Clin. cancer Res.* **14,** 259–69 (2008).

63. Ushiro, H. & Cohen, S. Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. *J. Biol. Chem.* **255,** 8363–5 (1980).

64. Cowley, G. P., Smith, J. A. & Gusterson, B. A. Increased EGF receptors on human squamous carcinoma cell lines. *Br. J. Cancer* **53,** 223–9 (1986).

65. Sainsbury, J. R., Farndon, J. R., Needham, G. K., Malcolm, A. J. & Harris, A. L. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet* **1,** 1398–402 (1987).

66. Knickelbein, K. & Zhang, L. Mutant KRAS as a critical determinant of the therapeutic response of colorectal cancer. *Genes Dis.* **2,** 4–12 (2015).

67. Yan, J., Roy, S., Apolloni, A., Lane, A. & Hancock, J. F. Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *J. Biol. Chem.* **273,** 24052–6 (1998).

68. Steelman, L. S. *et al.* JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* **18,** 189–218 (2004).

69. Garnett, M. J. & Marais, R. Guilty as charged. *Cancer Cell* **6,** 313–319 (2004).

70. Davies, H. *et al.* Mutations of the BRAF gene in human cancer. *Nature* **417,** 949–954 (2002).

71. Fransen, K. *et al.* Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas. *Carcinogenesis* **25,** 527–533 (2003).

72. Libra, M. *et al.* Analysis of BRAF Mutation in Primary and Metastatic Melanoma. *Cell Cycle* **4,** 1382–1384 (2005).

73. Barras, D. BRAF Mutation in Colorectal Cancer: An Update. *Biomark. Cancer* **7,** 9–12 (2015).

74. Noffsinger, A. E. Serrated Polyps and Colorectal Cancer: New Pathway to Malignancy. *Annu. Rev. Pathol. Mech. Dis.* **4,** 343–364 (2009).

75. Wee, P. & Wang, Z. Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers (Basel).* **9,** 52 (2017).

76. Meriggi, F., Di Biasi, B., Abeni, C. & Zaniboni, A. Anti-Egfr Therapy in Colorectal Cancer: How to Choose The Right Patient. *Curr. Drug Targets* **10,** 1033–1040 (2009).

77. Karapetis, C. S. *et al. K-ras* Mutations and Benefit from Cetuximab in Advanced Colorectal Cancer. *N. Engl. J. Med.* **359,** 1757–1765 (2008).

78. Bokemeyer, C. *et al.* Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. *Ann. Oncol.* **22,** 1535–1546 (2011).

79. Van Cutsem, E. *et al.* Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N. Engl. J. Med.* **360,** 1408–17 (2009).

80. Di Nicolantonio, F. *et al.* Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J. Clin. Oncol.* **26,** 5705–12 (2008).

81. Galizia, G. *et al.* Cetuximab, a chimeric human mouse anti-epidermal growth factor receptor monoclonal antibody, in the treatment of human colorectal cancer. *Oncogene* **26,** 3654–3660 (2007).

82. Taylor, R. J. *et al.* FcγRIIIa polymorphisms and cetuximab induced cytotoxicity in squamous cell carcinoma of the head and neck. *Cancer Immunol. Immunother.* **58,** 997–1006 (2009).

83. López-Albaitero, A. *et al.* Role of polymorphic Fc gamma receptor IIIa and EGFR expression level in cetuximab mediated, NK cell dependent in vitro cytotoxicity of head and neck squamous cell carcinoma cells. *Cancer Immunol. Immunother.* **58,** 1853–1862 (2009).

84. Bibeau, F. *et al.* Impact of FcγRIIa-FcγRIIIa Polymorphisms and KRAS Mutations on the Clinical Outcome of Patients With Metastatic Colorectal Cancer Treated With Cetuximab Plus Irinotecan. *J. Clin. Oncol.* **27,** 1122–1129 (2009).

85. Zhang, W. *et al.* FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. *J. Clin. Oncol.* **25,** 3712–8 (2007).

86. Dahan, L. *et al.* Pharmacogenetic profiling and cetuximab outcome in patients with advanced colorectal cancer. *BMC Cancer* **11,** 496 (2011).

87. Lièvre, A. *et al.* KRAS Mutation Status Is Predictive of Response to Cetuximab Therapy in Colorectal Cancer. *Cancer Res.* **66,** 3992–5 (2006).

88. Amado, R. G. *et al.* Wild-Type *KRAS* Is Required for Panitumumab Efficacy in Patients With Metastatic Colorectal Cancer. *J. Clin. Oncol.* **26,** 1626–1634 (2008).

89. López-Albaitero, A. & Ferris, R. L. Immune Activation by Epidermal Growth Factor Receptor–Specific Monoclonal Antibody Therapy for Head and Neck Cancer. *Arch. Otolaryngol. Neck Surg.* **133,** 1277 (2007).

90. Schneider-Merck, T. *et al.* Human IgG2 Antibodies against Epidermal Growth Factor Receptor Effectively Trigger Antibody-Dependent Cellular Cytotoxicity but, in Contrast to IgG1, Only by Cells of Myeloid Lineage. *J. Immunol.* **184,** 512–520 (2010).

91. Gerdes, C. a *et al.* GA201 (RG7160): A Novel, Humanized,

Glycoengineered Anti-EGFR Antibody with Enhanced ADCC and Superior In Vivo Efficacy Compared with Cetuximab. *Clin. Cancer Res.* **19,** 1126–38 (2013).

92. Martinelli, E., De Palma, R., Orditura, M., De Vita, F. & Ciardiello, F. Anti-epidermal growth factor receptor monoclonal antibodies in cancer therapy. *Clin. Exp. Immunol.* **158,** 1–9 (2009).

93. Kelly, E. & Russell, S. J. History of oncolytic viruses: genesis to genetic engineering. *Mol. Ther.* **15,** 651–659 (2007).

94. Moore, A. E. The destructive effect of the virus of Russian Far East encephalitis on the transplantable mouse sarcoma 180. *Cancer* **2,** 525–34 (1949).

95. Moore, A. E. & O'Conner, S. Further studies on the destructive effect of the virus of Russian Far East encephalitis on the transplantable mouse sarcoma 180. *Cancer* **3,** 886–90 (1950).

96. Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L. & Coen, D. M. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science (80-.).* **252,** 854–6 (1991).

97. Prestwich, R. J. *et al.* Oncolytic viruses: a novel form of immunotherapy. *Expert Rev. Anticancer Ther.* **8,** 1581–8 (2008).

98. Barquet, N. & Domingo, P. Smallpox: the triumph over the most terrible of the ministers of death. *Ann. Intern. Med.* **127,** 635–42 (1997).

99. Riedel, S. Edward Jenner and the history of smallpox and vaccination. *Proc. (Bayl. Univ. Med. Cent).* **18,** 21–5 (2005).

100. Stearns, R. P. & Pasti, G. Remarks upon the introduction of inoculation for smallpox in England. *Bull. Hist. Med.* **24,** 103–22

101. Fenner, F. A successful eradication campaign. Global eradication of smallpox. *Rev. Infect. Dis.* **4,** 916–30 (1982).

102. Breman, J. G. & Arita, I. The confirmation and maintenance of smallpox eradication. *N. Engl. J. Med.* **303,** 1263–73 (1980).

103. Baxby, D. Should smallpox virus be destroyed? The relevance of the origins of vaccinia virus. *Soc. Hist. Med.* **9,** 117–9 (1996).

104. Baxby, D. The origins of vaccinia virus. *J. Infect. Dis.* **136,** 453–5 (1977).

105. Smith, G. L. & Moss, B. Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene* **25,** 21–8 (1983).

106. Malkin, A. J., McPherson, A. & Gershon, P. D. Structure of Intracellular Mature Vaccinia Virus Visualized by In Situ Atomic Force Microscopy. *J. Virol.* **77,** 6332–6340 (2003).

107. Griffiths, G. *et al.* Structure and assembly of intracellular mature vaccinia virus: isolated-particle analysis. *J. Virol.* **75,** 11034–55 (2001).

108. Moussatche, N. & Condit, R. C. Fine structure of the vaccinia virion determined by controlled degradation and immunolocalization. *Virology* **475,** 204–18 (2015).

109. Condit, R. C., Moussatche, N. & Traktman, P. In a nutshell: structure and assembly of the vaccinia virion. *Adv. Virus Res.* **66,** 31–124 (2006).

110. Cyrklaff, M. *et al.* Cryo-electron tomography of vaccinia virus. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 2772–7 (2005).

111. Goebel, S. J. *et al.* The complete DNA sequence of vaccinia virus. *Virology* **179,** 247–266 (1990).

112. Traktman, P. in *DNA Replication in Eukaryotic Cells* 775–798 (1996).

113. Moss, B. Poxvirus entry and membrane fusion. *Virology* **344,** 48–54 (2006).

114. Roberts, K. L. & Smith, G. L. Vaccinia virus morphogenesis and dissemination. *Trends Microbiol.* **16,** 472–9 (2008).

115. Senkevich, T. G., Ward, B. M. & Moss, B. Vaccinia Virus A28L Gene Encodes an Essential Protein Component of the Virion Membrane with Intramolecular Disulfide Bonds Formed by the Viral Cytoplasmic Redox Pathway. *J. Virol.* **78,** 2348–2356 (2004).

116. Townsley, A. C., Senkevich, T. G. & Moss, B. The Product of the Vaccinia Virus L5R Gene Is a Fourth Membrane Protein Encoded by All Poxviruses That Is Required for Cell Entry and Cell-Cell Fusion. *J. Virol.* **79,** 10988–10998 (2005).

117. Chiu, W.-L., Lin, C.-L., Yang, M.-H., Tzou, D.-L. M. & Chang, W. Vaccinia virus 4c (A26L) protein on intracellular mature virus binds to the extracellular cellular matrix laminin. *J. Virol.* **81,** 2149–57 (2007).

118. Lin, C.-L., Chung, C.-S., Heine, H. G. & Chang, W. Vaccinia Virus Envelope H3L Protein Binds to Cell Surface Heparan Sulfate and Is Important for Intracellular Mature Virion Morphogenesis and Virus Infection In Vitro and In Vivo. *J. Virol.* **74,** 3353–3365 (2000).

119. Broyles, S. S. Vaccinia virus transcription. *J. Gen. Virol.* **84,** 2293–2303 (2003).

120. Boyle, K. & Traktman, P. *Viral Genome Replication*. (Springer US, 2009). doi:10.1007/b135974

121. Wein, L. M., Wu, J. T. & Kirn, D. H. Validation and analysis of a mathematical model of a replication-competent oncolytic virus for cancer treatment: implications for virus design and delivery. *Cancer Res.* **63,** 1317–24 (2003).

122. Thorne, S. H. *et al.* Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. *J. Clin. Invest.* **117,** 3350–8 (2007).

123. Everts, B. & van der Poel, H. G. Replication-selective oncolytic viruses in the treatment of cancer. *Cancer Gene Ther.* **12,** 141–61 (2005).

124. Parato, K. a *et al.* The Oncolytic Poxvirus JX-594 Selectively Replicates in and Destroys Cancer Cells Driven by Genetic Pathways Commonly Activated in Cancers. *Mol. Ther.* **20,** 749–758 (2012).

125. Kirn, D. H., Wang, Y., Liang, W., Contag, C. H. & Thorne, S. H. Enhancing Poxvirus Oncolytic Effects through Increased Spread and Immune Evasion. *Cancer Res.* **68,** 2071–2075 (2008).

126. Kirn, D. H. & Thorne, S. H. Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat. Rev. Cancer* **9,** 64–71 (2009).

127. Kim, J. *et al.* Systemic Armed Oncolytic and Immunologic Therapy for Cancer with JX-594, a Targeted Poxvirus Expressing GM-CSF. *Mol.*

Ther. **14,** 361–370 (2006).

128. Shen, Y. & Nemunaitis, J. Fighting cancer with vaccinia virus: teaching new tricks to an old dog. *Mol. Ther.* **11,** 180–95 (2005).

129. Gnant, M. F. *et al.* Tumor-specific gene delivery using recombinant vaccinia virus in a rabbit model of liver metastases. *J. Natl. Cancer Inst.* **91,** 1744–50 (1999).

130. Puhlmann, M. *et al.* Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. *Cancer Gene Ther.* **7,** 66–73 (2000).

131. Gnant, M. F., Puhlmann, M., Bartlett, D. L. & Alexander, H. R. Regional versus systemic delivery of recombinant vaccinia virus as suicide gene therapy for murine liver metastases. *Ann. Surg.* **230,** 352-60–1 (1999).

132. Buller, R. M., Smith, G. L., Cremer, K., Notkins, A. L. & Moss, B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* **317,** 813–5 (1985).

133. Guse, K., Cerullo, V. & Hemminki, A. Oncolytic vaccinia virus for the treatment of cancer. *Expert Opin. Biol. Ther.* **11,** 595–608 (2011).

134. Foloppe, J. *et al.* Targeted delivery of a suicide gene to human colorectal tumors by a conditionally replicating vaccinia virus. *Gene Ther.* **15,** 1361–71 (2008).

135. Guse, K. *et al.* Antiangiogenic arming of an oncolytic vaccinia virus enhances antitumor efficacy in renal cell cancer models. *J. Virol.* **84,** 856–66 (2010).

136. Frentzen, A. *et al.* Anti-VEGF single-chain antibody GLAF-1 encoded by oncolytic vaccinia virus significantly enhances antitumor therapy. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 12915–20 (2009).

137. Mastrangelo, M. J. *et al.* Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther.* **6,** 409–22 (1999).

138. Breitbach, C. J. *et al.* Intravenous delivery of a multi-mechanistic

cancer-targeted oncolytic poxvirus in humans. *Nature* **477,** 99–102 (2011).

139. Kim, M. K. *et al.* Oncolytic and immunotherapeutic vaccinia induces antibody-mediated complement-dependent cancer cell lysis in humans. *Sci. Transl. Med.* **5,** 185ra63 (2013).

140. Israeli, E. *et al.* Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nat. Med.* **19,** 329–36 (2013).

141. Sabin, A. B. Reoviruses. A new group of respiratory and enteric viruses formerly classified as ECHO type 10 is described. *Science (80-.).* **130,** 1387–9 (1959).

142. Jackson, G. G. & Muldoon, R. L. Viruses causing common respiratory infection in man. IV. Reoviruses and Adenoviruses. *J. Infect. Dis.* **128,** 811–66 (1973).

143. Selb, B. & Weber, B. A study of human reovirus IgG and IgA antibodies by ELISA and western blot. *J. Virol. Methods* **47,** 15–25 (1994).

144. Norman, K. L. & Lee, P. W. K. Not all viruses are bad guys: The case for reovirus in cancer therapy. *Drug Discov. Today* **10,** 847–855 (2005).

145. Rosen, L., Evans, H. E. & Spickard, A. Reovirus infections in human volunteers. *Am. J. Hyg.* **77,** 29–37 (1963).

146. Coombs, K. M. Reovirus structure and morphogenesis. *Curr. Top. Microbiol. Immunol.* **309,** 117–67 (2006).

147. Danthi, P., Guglielmi, K. M., Kirchner, E., Mainou, B. & Dermody, T. S. From Touchdown to Transcription: The Reovirus Cell Entry Pathway HHS Public Access. *Curr Top Microbiol Immunol* **343,** 91–119 (2010).

148. Gong, J. & Mita, M. M. Activated ras signaling pathways and reovirus oncolysis: an update on the mechanism of preferential reovirus replication in cancer cells. *Front. Oncol.* **4,** 167 (2014).

149. Campbell, J. A. *et al.* Junctional adhesion molecule a serves as a receptor for prototype and field-isolate strains of mammalian reovirus. *J. Virol.* **79,** 7967–78 (2005).

150. Danthi, P., Holm, G. H., Stehle, T. & Dermody, T. S. in *Advances in experimental medicine and biology* **790,** 42–71 (2013).

151. Hashiro, G., Loh, P. C. & Yau, J. T. The preferential cytotoxicity of reovirus for certain transformed cell lines. *Arch. Virol.* **54,** 307–15 (1977).

152. Duncan, M. R., Stanish, S. M. & Cox, D. C. Differential sensitivity of normal and transformed human cells to reovirus infection. *J. Virol.* **28,** 444–9 (1978).

153. Clements, D., Helson, E., Gujar, S. & Lee, P. Reovirus in cancer therapy: an evidence-based review. *Oncolytic Virotherapy* **3,** 69–82 (2014).

154. Bilsland, A. E., Spiliopoulou, P. & Evans, T. R. J. Virotherapy: cancer gene therapy at last? *F1000Research* **5,** (2016).

155. Morris, D. G. *et al.* REO-001: A phase I trial of percutaneous intralesional administration of reovirus type 3 dearing (Reolysin®) in patients with advanced solid tumors. *Invest. New Drugs* **31,** 696–706 (2013).

156. Hung, C.-F. *et al.* Vaccinia virus preferentially infects and controls human and murine ovarian tumors in mice. *Gene Ther.* **14,** 20–29 (2007).

157. Errington, F. *et al.* Reovirus Activates Human Dendritic Cells to Promote Innate Antitumor Immunity. *J. Immunol.* **180,** 6018–26 (2008).

158. Errington, F. *et al.* Inflammatory tumour cell killing by oncolytic reovirus for the treatment of melanoma. *Gene Ther.* **15,** 1257–1270 (2008).

159. Whilding, L. M. *et al.* Vaccinia virus induces programmed necrosis in ovarian cancer cells. *Mol. Ther.* **21,** 2074–86 (2013).

160. Berger, A. K. & Danthi, P. Reovirus activates a caspase-independent cell death pathway. *MBio* **4,** e00178-13 (2013).

161. Woller, N., Gurlevik, E., Ureche, C.-I., Schumacher, A. & KÃ¼hnel, F. Oncolytic Viruses as Anticancer Vaccines. *Front. Oncol.* **4,** 188 (2014).

162. Greiner, S. *et al.* The highly attenuated vaccinia virus strain modified

virus Ankara induces apoptosis in melanoma cells and allows bystander dendritic cells to generate a potent anti-tumoral immunity. *Clin. Exp. Immunol.* **146,** 344–53 (2006).

163. Humlová, Z., Vokurka, M., Esteban, M. & Melková, Z. Vaccinia virus induces apoptosis of infected macrophages. *J. Gen. Virol.* **83,** 2821–32 (2002).

164. Clarke, P. *et al.* Mechanisms of Reovirus-Induced Cell Death and Tissue Injury: Role of Apoptosis and Virus-Induced Perturbation of Host-Cell Signaling and Transcription Factor Activation. *Viral Immunol.* **18,** 89–115 (2005).

165. Clarke, P. & Tyler, K. L. Reovirus-induced apoptosis: A minireview. *Apoptosis* **8,** 141–50 (2003).

166. Kominsky, D. J., Bickel, R. J. & Tyler, K. L. Reovirus-induced apoptosis requires both death receptor- and mitochondrial-mediated caspase-dependent pathways of cell death. *Cell Death Differ.* **9,** 926–933 (2002).

167. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100,** 57–70 (2000).

168. van Delft, M. F. *et al.* The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10,** 389–399 (2006).

169. Fadok, V. A., Bratton, D. L., Guthrie, L. & Henson, P. M. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J. Immunol.* **166,** 6847–54 (2001).

170. Feoktistova, M., Leverkus, M. & Leverkus, C. M. Programmed necrosis and necroptosis signalling. *FEBS J.* **282,** 19–31 (2015).

171. Kim, H. J., Lee, S. & Jung, J. U. When autophagy meets viruses: a double-edged sword with functions in defense and offense. *Semin. Immunopathol.* **32,** 323–341 (2010).

172. Moloughney, J. G. *et al.* Vaccinia virus leads to ATG12–ATG3 conjugation and deficiency in autophagosome formation. *Autophagy* **7,** 1434–47 (2011).

173. Thirukkumaran, C. M. *et al.* Reovirus as a Viable Therapeutic Option for the Treatment of Multiple Myeloma. *Clin. Cancer Res.* **18,** 4962–72 (2012).

174. Chiu, H. C., Richart, S., Lin, F. Y., Hsu, W. L. & Liu, H. J. The interplay of reovirus with autophagy. *Biomed Res. Int.* **2014,** 1–8 (2014).

175. Petros, A. M. *et al.* Discovery of a Potent Inhibitor of the Antiapoptotic Protein Bcl-x $_{L}$ from NMR and Parallel Synthesis. *J. Med. Chem.* **49,** 656–663 (2006).

176. Oltersdorf, T. *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435,** 677–681 (2005).

177. Hann, C. L. *et al.* Therapeutic Efficacy of ABT-737, a Selective Inhibitor of BCL-2, in Small Cell Lung Cancer. *Cancer Res.* **68,** (2008).

178. Lin, X. *et al.* 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-XL inhibitor ABT-737. *Oncogene* **26,** 3972–3979 (2007).

179. Tahir, S. K. *et al.* Influence of Bcl-2 Family Members on the Cellular Response of Small-Cell Lung Cancer Cell Lines to ABT-737. *Cancer Res.* **67,** 1176–83 (2007).

180. Yu, C. *et al.* The role of Mcl-1 downregulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. *Oncogene* **24,** 6861–6869 (2005).

181. Yang, C., Kaushal, V., Shah, S. V. & Kaushal, G. P. Mcl-1 is downregulated in cisplatin-induced apoptosis, and proteasome inhibitors restore Mcl-1 and promote survival in renal tubular epithelial cells. *Am. J. Physiol. - Ren. Physiol.* **292,** 1710–7 (2007).

182. Tse, C. *et al.* ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. *Cancer Res.* **68,** 3421–3428 (2008).

183. Faber, A. C. *et al.* Assessment of ABT-263 activity across a cancer cell line collection leads to a potent combination therapy for small-cell lung cancer. *Proc. Natl. Acad. Sci. U. S. A.* **112,** E1288-96 (2015).

184. Suryani, S. *et al.* Cell and Molecular Determinants of In Vivo Efficacy of

the BH3 Mimetic ABT-263 against Pediatric Acute Lymphoblastic Leukemia Xenografts. *Clin. Cancer Res.* **20,** 4520–4531 (2014).

185. Shoemaker, A. R. *et al.* Activity of the Bcl-2 Family Inhibitor ABT-263 in a Panel of Small Cell Lung Cancer Xenograft Models. *Clin. Cancer Res.* **14,** 3268–3277 (2008).

186. Tolcher, A. W. *et al.* Safety, efficacy, and pharmacokinetics of navitoclax (ABT-263) in combination with erlotinib in patients with advanced solid tumors. *Cancer Chemother. Pharmacol.* **76,** 1025–1032 (2015).

187. Tolcher, A. W. *et al.* Safety, efficacy, and pharmacokinetics of navitoclax (ABT-263) in combination with irinotecan: results of an open-label, phase 1 study. *Cancer Chemother. Pharmacol.* **76,** 1041–1049 (2015).

188. Cleary, J. M. *et al.* A phase I clinical trial of navitoclax, a targeted high-affinity Bcl-2 family inhibitor, in combination with gemcitabine in patients with solid tumors. *Invest. New Drugs* **32,** 937–945 (2014).

189. Roberts, A. W. *et al.* Phase 1 study of the safety, pharmacokinetics, and antitumour activity of the BCL2 inhibitor navitoclax in combination with rituximab in patients with relapsed or refractory CD20 $^{+}$ lymphoid malignancies. *Br. J. Haematol.* **170,** 669–678 (2015).

190. Rudin, C. M. *et al.* Phase II Study of Single-Agent Navitoclax (ABT-263) and Biomarker Correlates in Patients with Relapsed Small Cell Lung Cancer. *Clin. Cancer Res.* **18,** 3163–3169 (2012).

191. Kipps, T. J. *et al.* A phase 2 study of the BH3 mimetic BCL2 inhibitor navitoclax (ABT-263) with or without rituximab, in previously untreated B-cell chronic lymphocytic leukemia. *Leuk. Lymphoma* **56,** 2826–2833 (2015).

192. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular cloning: a laboratory manual*. *Cold Spring Harbor Laboratory Press* (Cold Spring Harbor Laboratory Press, 1989).

193. Wilson, N. S. *et al.* An Fcγ receptor-dependent mechanism drives antibody-mediated target-receptor signaling in cancer cells. *Cancer Cell* **19,** 101–113 (2011).

194. Iannello, A. & Ahmad, A. Role of antibody-dependent cell-mediated cytotoxicity in the efficacy of therapeutic anti-cancer monoclonal antibodies. *Cancer Metastasis Rev.* **24,** 487–499 (2005).

195. Jimeno, A., Messersmith, W. A., Hirsch, F. R., Franklin, W. A. & Eckhardt, S. G. KRAS Mutations and Susceptibility to Cetuximab and Panitumumab in Colorectal Cancer. *Cancer J.* **15,** 110–113 (2009).

196. Erreni, M., Mantovani, A. & Allavena, P. Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer. *Cancer Microenviron.* **4,** 141–54 (2011).

197. Kaler, P., Galea, V., Augenlicht, L., Klampfer, L. & Cammareri, P. Tumor Associated Macrophages Protect Colon Cancer Cells from TRAIL-Induced Apoptosis through IL-1β- Dependent Stabilization of Snail in Tumor Cells. *PLoS One* **5,** e11700 (2010).

198. Hara, J. *et al.* Expression of costimulatory molecules B7-1 and B7-2 in macrophages and granulomas of Crohn's disease: demonstration of cell-to-cell contact with T lymphocytes. *Lab. Investig.* **77,** 175–84 (1997).

199. Sugita, J. *et al.* Close association between Fas ligand (FasL; CD95L)-positive tumor-associated macrophages and apoptotic cancer cells along invasive margin of colorectal carcinoma: a proposal on tumor-host interactions. *Japanese J. cancer Res.* **93,** 320–8 (2002).

200. Zhou, Q. *et al.* The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. *J. Transl. Med.* **8,** 13 (2010).

201. Khorana, A. A., Ryan, C. K., Cox, C., Eberly, S. & Sahasrabudhe, D. M. Vascular endothelial growth factor, CD68, and epidermal growth factor receptor expression and survival in patients with Stage II and Stage III colon carcinoma. *Cancer* **97,** 960–968 (2003).

202. Noy, R. & Pollard, J. W. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* **41,** 49–61 (2014).

203. Mantovani, A. & Allavena, P. The interaction of anticancer therapies with tumor-associated macrophages. *J. Exp. Med.* **212,** 435–45 (2015).

204. Adair, R. A. *et al.* Cytotoxic and immune-mediated killing of human colorectal cancer by reovirus-loaded blood and liver mononuclear cells. *Int. J. Cancer* **132,** 2327–2338 (2013).

205. Parrish, C. *et al.* Oncolytic reovirus enhances rituximab-mediated antibody-dependent cellular cytotoxicity against chronic lymphocytic leukaemia. *Leukemia* **29,** 1799–1810 (2015).

206. Adair, R. a *et al.* Cell carriage, delivery, and selective replication of an oncolytic virus in tumor in patients. *Sci. Transl. Med.* **4,** 138ra77 (2012).

207. Kasper, S. *et al.* Oncogenic RAS simultaneously protects against anti-EGFR antibody-dependent cellular cytotoxicity and EGFR signaling blockade. *Oncogene* 1–9 (2012). doi:10.1038/onc.2012.302

208. Cragg, M. S., Harris, C., Strasser, A. & Scott, C. L. Unleashing the power of inhibitors of oncogenic kinases through BH3 mimetics. *Nat. Rev. Cancer* **9,** 321–326 (2009).

209. Tahir, S. K. *et al.* Identification of Expression Signatures Predictive of Sensitivity to the Bcl-2 Family Member Inhibitor ABT-263 in Small Cell Lung Carcinoma and Leukemia/Lymphoma Cell Lines. *Mol. Cancer Ther.* **9,** 545–557 (2010).

210. Leverson, J. D. *et al.* Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). *Cell Death Dis.* **6,** e1590 (2015).

211. Shao, H. *et al.* Apigenin sensitizes colon cancer cells to antitumor activity of ABT-263. *Mol. Cancer Ther.* **12,** 2640–50 (2013).

212. Stamelos, V. A., Redman, C. W. & Richardson, A. Understanding sensitivity to BH3 mimetics: ABT-737 as a case study to foresee the complexities of personalized medicine. *J. Mol. Signal.* **7,** 1–15 (2012).

213. Del, V. *et al.* Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J. Clin. Invest.* **117,** 112–121 (2007).

214. Besbes, S., Pocard, M., Mirshahi, M. & Billard, C. The first MCL-1-

selective BH3 mimetics have therapeutic potential for chronic lymphocytic leukemia. *Crit. Rev. Oncol. Hematol.* **100,** 32–36 (2016).

215. Inoue-Yamauchi, A. *et al.* Targeting the differential addiction to anti-apoptotic BCL-2 family for cancer therapy. *Nat. Commun.* **8,** 16078 (2017).

216. De Roock, W. *et al.* Association of KRAS p.G13D Mutation With Outcome in Patients With Chemotherapy-Refractory Metastatic Colorectal Cancer Treated With Cetuximab. *JAMA* **304,** 1812 (2010).

217. Kumar, S. S. *et al.* KRAS G13D Mutation and Sensitivity to Cetuximab or Panitumumab in a Colorectal Cancer Cell Line Model. *Gastrointest. cancer Res.* **7,** 23–6 (2014).

218. Strong, J. E. & Lee, P. W. The v-erbB oncogene confers enhanced cellular susceptibility to reovirus infection. *J. Virol.* **70,** 612–6 (1996).

219. Seo, Y. *et al.* Cetuximab-mediated ADCC activity is correlated with the cell surface expression level of EGFR but not with the KRAS/BRAF mutational status in colorectal cancer. *Oncol. Rep.* **31,** 2115–22 (2014).

220. Medico, E. *et al.* The molecular landscape of colorectal cancer cell lines unveils clinically actionable kinase targets. *Nat. Commun.* **6,** 7002 (2015).

221. Cunningham, D. *et al.* Cetuximab Monotherapy and Cetuximab plus Irinotecan in Irinotecan-Refractory Metastatic Colorectal Cancer. *N. Engl. J. Med.* **351,** 337–345 (2004).

222. Wang, W., Erbe, A. K., Hank, J. A., Morris, Z. S. & Sondel, P. M. NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Cancer Immunotherapy. *Front. Immunol.* **6,** 368 (2015).

223. Wilson, W. H. *et al.* Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. *Lancet Oncol.* **11,** 1149–1159 (2010).

224. Roberts, A. W. *et al.* Substantial Susceptibility of Chronic Lymphocytic Leukemia to BCL2 Inhibition: Results of a Phase I Study of Navitoclax

in Patients With Relapsed or Refractory Disease. *J. Clin. Oncol.* **30,** 488–496 (2012).

225. Scott, G., Parrish, C., Hillmen, P., Melcher, A. & Errington-Mais, F. Increased efficacy of Reolysin when combined with ABT263 against CLL. *Hum. Gene Ther.* A24–A24 (2014).

226. Clarke, P., Richardson-Burns, S. M., DeBiasi, R. L. & Tyler, K. L. Mechanisms of apoptosis during reovirus infection. *Curr. Top. Microbiol. Immunol.* **289,** 1–24 (2005).

227. Samuel, S. *et al.* VSV Oncolysis in Combination With the BCL-2 Inhibitor Obatoclax Overcomes Apoptosis Resistance in Chronic Lymphocytic Leukemia. *Mol. Ther.* **18,** 2094–2103 (2010).

228. Samuel, S. *et al.* BCL-2 Inhibitors Sensitize Therapy-resistant Chronic Lymphocytic Leukemia Cells to VSV Oncolysis. *Mol. Ther.* **21,** 1413–1423 (2013).

229. Yang, L. *et al.* BH3 mimetic ABT-737 sensitizes colorectal cancer cells to ixazomib through MCL-1 downregulation and autophagy inhibition. *Am. J. Cancer Res.* **6,** 1345–57 (2016).

230. Koehler, B. C. *et al.* Beyond Cell Death – Antiapoptotic Bcl-2 Proteins Regulate Migration and Invasion of Colorectal Cancer Cells In Vitro. *PLoS One* **8,** e76446 (2013).

231. Nita, M. E. *et al.* 5-Fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. *Br. J. Cancer* **78,** 986–92 (1998).

232. Kelly, K. R. *et al.* Reovirus therapy stimulates endoplasmic reticular stress, NOXA induction, and augments bortezomib-mediated apoptosis in multiple myeloma. *Oncogene* **31,** 3023–3038 (2012).

233. Knowlton, J. J., Dermody, T. S. & Holm, G. H. Apoptosis Induced by Mammalian Reovirus Is Beta Interferon (IFN) Independent and Enhanced by IFN Regulatory Factor 3- and NF- B-Dependent Expression of Noxa. *J. Virol.* **86,** 1650–1660 (2012).

234. Mei, Y. *et al.* Noxa/Mcl-1 balance regulates susceptibility of cells to

camptothecin-induced apoptosis. *Neoplasia* **9,** 871–81 (2007).

235. Haschka, M. D. *et al.* The NOXA–MCL1–BIM axis defines lifespan on extended mitotic arrest. *Nat. Commun.* **6,** 6891 (2015).

236. Maitra, R. *et al.* Oncolytic reovirus preferentially induces apoptosis in KRAS mutant colorectal cancer cells, and synergizes with irinotecan. *Oncotarget* **5,** 2807–19 (2014).

237. Twigger, K. *et al.* Reovirus exerts potent oncolytic effects in head and neck cancer cell lines that are independent of signalling in the EGFR pathway. *BMC Cancer* **12,** 368 (2012).

238. Roulstone, V. *et al.* BRAF- and MEK-Targeted Small Molecule Inhibitors Exert Enhanced Antimelanoma Effects in Combination With Oncolytic Reovirus Through ER Stress. *Mol. Ther.* **23,** 931–42 (2015).

239. Abdullah, N., Greenman, J., Pimenidou, A., Topping, K. P. & Monson, J. R. The role of monocytes and natural killer cells in mediating antibody-dependent lysis of colorectal tumour cells. *Cancer Immunol. Immunother.* **48,** 517–24 (1999).

240. Kurai, J. *et al.* Antibody-Dependent Cellular Cytotoxicity Mediated by Cetuximab against Lung Cancer Cell Lines. *Clin. Cancer Res.* **13,** 1552–61 (2007).

241. Mellor, J. D., Brown, M. P., Irving, H. R., Zalcberg, J. R. & Dobrovic, A. A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. *J. Hematol. Oncol.* **6,** 1 (2013).

242. Pirker, R. *et al.* EGFR expression as a predictor of survival for first-line chemotherapy plus cetuximab in patients with advanced non-small-cell lung cancer: analysis of data from the phase 3 FLEX study. *Lancet. Oncol.* **13,** 33–42 (2012).

243. Pirker, R. *et al.* Cetuximab plus chemotherapy in patients with advanced non-small-cell lung cancer (FLEX): an open-label randomised phase III trial. *Lancet* **373,** 1525–1531 (2009).

244. Licitra, L. *et al.* Predictive value of epidermal growth factor receptor

expression for first-line chemotherapy plus cetuximab in patients with head and neck and colorectal cancer: Analysis of data from the EXTREME and CRYSTAL studies. *Eur. J. Cancer* **49,** 1161–1168 (2013).

245. Vermorken, J. B. *et al.* Platinum-Based Chemotherapy plus Cetuximab in Head and Neck Cancer. *N. Engl. J. Med.* **359,** 1116–1127 (2008).

246. Derer, S., Lohse, S. & Valerius, T. EGFR expression levels affect the mode of action of EGFR-targeting monoclonal antibodies. *Oncoimmunology* **2,** e24052 (2013).

247. Shigeta, K. *et al.* Expression of Epidermal Growth Factor Receptor Detected by Cetuximab Indicates Its Efficacy to Inhibit In Vitro and In Vivo Proliferation of Colorectal Cancer Cells. *PLoS One* **8,** e66302 (2013).

248. Lichty, B. D., Breitbach, C. J., Stojdl, D. F. & Bell, J. C. Going viral with cancer immunotherapy. *Nat. Rev. Cancer* **14,** 559–67 (2014).

249. Russell, S. J., Peng, K.-W. & Bell, J. C. Oncolytic virotherapy. *Nat. Biotechnol.* **30,** 658–70 (2012).

250. Breitbach, C., Bell, J. C., Hwang, T.-H., Kirn, D. & Burke, J. The emerging therapeutic potential of the oncolytic immunotherapeutic Pexa-Vec (JX-594). *Oncolytic Virotherapy* **Volume 4,** 25 (2015).

251. Park, B.-H. *et al.* Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet. Oncol.* **9,** 533–42 (2008).

252. Vanden Berghe, T., Kaiser, W. J., Bertrand, M. J. & Vandenabeele, P. Molecular crosstalk between apoptosis, necroptosis, and survival signaling. *Mol. Cell. Oncol.* **2,** e975093 (2015).

253. Dave, R. Oncolytic vaccinia virus for the treatment of liver cancer. (University of Leeds, 2014).

254. Degterev, A. *et al.* Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat. Chem. Biol.* **4,** 313–21 (2008).

255. Baird, S. K. *et al.* Oncolytic adenoviral mutants induce a novel mode of

programmed cell death in ovarian cancer. *Oncogene* **27,** 3081–90 (2008).

256. Schiller, M. *et al.* During apoptosis HMGB1 is translocated into apoptotic cell-derived membranous vesicles. *Autoimmunity* **46,** 342–6 (2013).

257. Ito, H. *et al.* Autophagic Cell Death of Malignant Glioma Cells Induced by a Conditionally Replicating Adenovirus. *J. Natl. Cancer Inst.* **98,** 625–636 (2006).

258. Meng, S., Xu, J., Wu, Y. & Ding, C. Targeting autophagy to enhance oncolytic virus-based cancer therapy. *Expert Opin. Biol. Ther.* **13,** 863–73 (2013).

259. Ke Jiang, Yingchun Li, Qiumin Zhu, Jiansheng Xu, Yupeng Wang, Wuguo Deng, Quentin Liu, Guirong Zhang, S. M. Pharmacological modulation of autophagy enhances Newcastle disease virus-mediated oncolysis in drug-resistant lung cancer cells. *BMC Cancer* **14,** 1471–2407 (2014).

260. Li, J. *et al.* Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. *Ann. Surg. Oncol.* **16,** 761–71 (2009).

261. Liskova, J., Knitlova, J., Honner, R. & Melkova, Z. Apoptosis and necrosis in vaccinia virus-infected HeLa G and BSC-40 cells. *Virus Res.* **160,** 40–50 (2011).

262. Boujrad, H., Gubkina, O., Robert, N., Krantic, S. & Susin, S. A. AIF-Mediated Programmed Necrosis: A Highly Orchestrated Way to Die. *Cell Cycle* **6,** 2612–2619 (2007).

263. Lafont, E. *et al.* Caspase-10-Dependent Cell Death in Fas/CD95 Signalling Is Not Abrogated by Caspase Inhibitor zVAD-fmk. *PLoS One* **5,** e13638 (2010).

264. Lieberman, J. Granzyme A activates another way to die. *Immunol. Rev.* **235,** 93–104 (2010).

265. Vandenabeele, P., Vanden Berghe, T. & Festjens, N. Caspase Inhibitors Promote Alternative Cell Death Pathways. *Sci. STKE* **2006,**

pe44-pe44 (2006).

266. Heinrich, B. *et al.* Immunogenicity of oncolytic vaccinia viruses JX-GFP and TG6002 in a human melanoma in vitro model: studying immunogenic cell death, dendritic cell maturation and interaction with cytotoxic T lymphocytes. *Onco. Targets. Ther.* **10,** 2389–2401 (2017).

267. Guo, Z. S. *et al.* The Enhanced Tumor Selectivity of an Oncolytic Vaccinia Lacking the Host Range and Antiapoptosis Genes SPI-1 and SPI-2. *Cancer Res.* **65,** 9991–9998 (2005).

268. Galluzzi, L. *et al.* Viral strategies for the evasion of immunogenic cell death. *J. Intern. Med.* **267,** 526–542 (2010).

269. Schock, S. N. *et al.* Induction of necroptotic cell death by viral activation of the RIG-I or STING pathway. *Cell Death Differ.* **24,** 615–625 (2017).

270. Hu, L., Jiang, K., Ding, C. & Meng, S. Targeting Autophagy for Oncolytic Immunotherapy. *Biomedicines* **5,** (2017).

271. Melcher, A., Parato, K., Rooney, C. M. & Bell, J. C. Thunder and lightning: immunotherapy and oncolytic viruses collide. *Mol. Ther.* **19,** 1008–1016 (2011).

272. Andrews, D. M. *et al.* Cross-talk between dendritic cells and natural killer cells in viral infection. *Mol. Immunol.* **42,** 547–555 (2005).

273. Galon, J. *et al.* Type, Density, and Location of Immune Cells Within Human Colorectal Tumors Predict Clinical Outcome. *Science (80-.).* **313,** 1960–1964 (2006).

274. Kottke, T. *et al.* Improved Systemic Delivery of Oncolytic Reovirus to Established Tumors Using Preconditioning with Cyclophosphamide-Mediated Treg Modulation and Interleukin-2. *Clin. Cancer Res.* **15,** 561–569 (2009).

275. Dranoff, G. *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 3539–43 (1993).

276. Sambi, M. *et al.* Alternative therapies for metastatic breast cancer:

multimodal approach targeting tumor cell heterogeneity. *Breast cancer* **9,** 85–93 (2017).

277. Ong, M. L. H. & Schofield, J. B. Assessment of lymph node involvement in colorectal cancer. *World J. Gastrointest. Surg.* **8,** 179–92 (2016).

278. Nicastri, D. G., Doucette, J. T., Godfrey, T. E. & Hughes, S. J. Is occult lymph node disease in colorectal cancer patients clinically significant? A review of the relevant literature. *J. Mol. diagnostics* **9,** 563–71 (2007).

279. Akagi, Y., Kinugasa, T., Adachi, Y. & Shirouzu, K. Prognostic significance of isolated tumor cells in patients with colorectal cancer in recent 10-year studies. *Mol. Clin. Oncol.* **1,** 582–592 (2013).

280. Cooper, M. A. & Caligiuri, M. A. Isolation and characterization of human natural killer cell subsets. *Curr. Protoc. Immunol.* **Chapter 7,** Unit 7.34 (2004).

281. Testi, R., D'Ambrosio, D., De Maria, R. & Santoni, A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunol. Today* **15,** 479–83 (1994).

282. Borrego, F., Peña, J. & Solana, R. Regulation of CD69 expression on human natural killer cells: differential involvement of protein kinase C and protein tyrosine kinases. *Eur. J. Immunol.* **23,** 1039–43 (1993).

283. Borrego, F., Robertson, M. J., Ritz, J., Peña, J. & Solana, R. CD69 is a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD94 inhibitory receptor. *Immunology* **97,** 159–65 (1999).

284. Voskoboinik, I., Whisstock, J. C. & Trapani, J. A. Perforin and granzymes: function, dysfunction and human pathology. *Nat. Rev. Immunol.* **15,** 388–400 (2015).

285. Alter, G., Malenfant, J. M. & Altfeld, M. CD107a as a functional marker for the identification of natural killer cell activity. *J. Immunol. Methods* **294,** 15–22 (2004).

286. Betts, M. R. *et al.* Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* **281,** 65–78 (2003).

287. Aktas, E., Kucuksezer, U. C., Bilgic, S., Erten, G. & Deniz, G. Relationship between CD107a expression and cytotoxic activity. *Cell. Immunol.* **254,** 149–54 (2009).

288. Moretta, L. *et al.* Human NK cells and their receptors. *Microbes Infect.* **4,** 1539–44 (2002).

289. Lee, S. & Margolin, K. Cytokines in cancer immunotherapy. *Cancers (Basel).* **3,** 3856–93 (2011).

290. Hou, W. *et al.* Viral infection triggers rapid differentiation of human blood monocytes into dendritic cells. *Blood* **119,** 3128–31 (2012).

291. Newman, K. C. & Riley, E. M. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. *Nat. Rev. Immunol.* **7,** 279–291 (2007).

292. Brader, P. *et al.* Imaging of lymph node micrometastases using an oncolytic herpes virus and [F]FEAU PET. *PLoS One* **4,** e4789 (2009).

293. Eisenberg, D. P. *et al.* Real-time intraoperative detection of breast cancer axillary lymph node metastases using a green fluorescent protein-expressing herpes virus. *Ann. Surg.* **243,** 824-30–2 (2006).

294. Gholami, S. *et al.* Vaccinia virus GLV-1h153 is effective in treating and preventing metastatic triple-negative breast cancer. *Ann. Surg.* **256,** 437–45 (2012).

295. Freud, A. G. *et al.* Evidence for discrete stages of human natural killer cell differentiation in vivo. *J. Exp. Med.* **203,** 1033–43 (2006).

296. Shetron-Rama, L. M., Herring-Palmer, A. C., Huffnagle, G. B. & Hanna, P. Transport of Bacillus anthracis from the lungs to the draining lymph nodes is a rapid process facilitated by CD11c+ cells. *Microb. Pathog.* **49,** 38–46 (2010).

297. Moussion, C. & Girard, J.-P. Dendritic cells control lymphocyte entry to lymph nodes through high endothelial venules. *Nature* **479,** 542–546 (2011).

298. Watt, S. V., Andrews, D. M., Takeda, K., Smyth, M. J. & Hayakawa, Y. IFN-γ-Dependent Recruitment of Mature CD27high NK Cells to Lymph

Nodes Primed by Dendritic Cells. *J. Immunol.* **181,** 5323–5330 (2008).

299. Martín-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat. Immunol.* **5,** 1260–5 (2004).

300. Fehniger, T. A. *et al.* CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood* **101,** 3052–7 (2003).

301. Gattass, C. R., King, L. B., Luster, A. D. & Ashwell, J. D. Constitutive expression of interferon gamma-inducible protein 10 in lymphoid organs and inducible expression in T cells and thymocytes. *J. Exp. Med.* **179,** 1373–8 (1994).

302. Le Bon, A. & Tough, D. F. Links between innate and adaptive immunity via type I interferon. *Curr. Opin. Immunol.* **14,** 432–6 (2002).

303. Gerosa, F. *et al.* Interferon alpha induces expression of the CD69 activation antigen in human resting NK cells, while interferon gamma and tumor necrosis factor alpha are ineffective. *Int. J. cancer* **48,** 473–5 (1991).

304. Donnelly, O. G. *et al.* Measles virus causes immunogenic cell death in human melanoma. *Gene Ther.* **20,** 1–14 (2011).

305. Lillard, J. W. *et al.* MIP-1␣ and MIP-1␥ differentially mediate mucosal and systemic adaptive immunity. doi:10.1182/blood-2002

306. Samson, A. *et al.* Oncolytic reovirus as a combined antiviral and anti-tumour agent for the treatment of liver cancer. *Gut* **10,** 1136 (2016).

307. Fitzgerald-Bocarsly, P. & Feng, D. The role of type I interferon production by dendritic cells in host defense. *Biochimie* **89,** 843–55 (2007).

308. Steele, L. *et al.* Pro-inflammatory cytokine/chemokine production by reovirus treated melanoma cells is PKR/NF-kappaB mediated and supports innate and adaptive anti-tumour immune priming. *Mol. Cancer* **10,** 20 (2011).

309. Hall, K. *et al.* Reovirus-mediated cytotoxicity and enhancement of innate

immune responses against acute myeloid leukemia. *Biores. Open Access* **1,** 3–15 (2012).

310. Müller-Hübenthal, B. *et al.* Tumour Biology: tumour-associated inflammation versus antitumor immunity. *Anticancer Res.* **29,** 4795–805 (2009).

311. Gupta, R. & Emens, L. A. GM-CSF-secreting vaccines for solid tumors: moving forward. *Discov. Med.* **10,** 52–60 (2010).

312. Sánchez-Puig, J. M., Sánchez, L., Roy, G. & Blasco, R. Susceptibility of different leukocyte cell types to Vaccinia virus infection. *Virol. J.* **1,** 10 (2004).

313. Byrd, D. *et al.* Primary Human Leukocyte Subsets Differentially Express Vaccinia Virus Receptors Enriched in Lipid Rafts. *J. Virol.* **87,** 9301–9312 (2013).

314. Lee, A. J. *et al.* Inflammatory monocytes require type I interferon receptor signaling to activate NK cells via IL-18 during a mucosal viral infection. *J. Exp. Med.* **214,** 1153–1167 (2017).

315. Shi, C. & Pamer, E. G. Monocyte recruitment during infection and inflammation. *Nat. Rev. Immunol.* **11,** 762–774 (2011).

316. Barbalat, R., Lau, L., Locksley, R. M. & Barton, G. M. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nat. Immunol.* **10,** 1200–7 (2009).

317. Kassim, S. H., Rajasagi, N. K., Zhao, X., Chervenak, R. & Jennings, S. R. In vivo ablation of CD11c-positive dendritic cells increases susceptibility to herpes simplex virus type 1 infection and diminishes NK and T-cell responses. *J. Virol.* **80,** 3985–93 (2006).

318. Colonna, M., Krug, A. & Cella, M. Interferon-producing cells: on the front line in immune responses against pathogens. *Curr. Opin. Immunol.* **14,** 373–9 (2002).

319. Bhat, R. & Rommelaere, J. Emerging role of Natural killer cells in oncolytic virotherapy. *ImmunoTargets Ther.* **4,** 65–77 (2015).

320. Zwirner, N. W. & Ziblat, A. Regulation of NK Cell Activation and Effector

Functions by the IL-12 Family of Cytokines: The Case of IL-27. *Front. Immunol.* **8,** 25 (2017).

321. Hayakawa, Y., Watt, S. V, Takeda, K. & Smyth, M. J. Distinct receptor repertoire formation in mouse NK cell subsets regulated by MHC class I expression. *J. Leukoc. Biol.* **83,** 106–11 (2008).

322. Bajénoff, M. *et al.* Natural killer cell behavior in lymph nodes revealed by static and real-time imaging. *J. Exp. Med.* **203,** 619 (2006).

323. Martín-Fontecha, A. *et al.* Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH1 priming. *Nat. Immunol.* **5,** 1260–1265 (2004).

324. Rajani, K. *et al.* Combination Therapy With Reovirus and Anti-PD-1 Blockade Controls Tumor Growth Through Innate and Adaptive Immune Responses. *Mol. Ther.* **24,** 166–74 (2016).

325. Choi, A. H., O'Leary, M. P., Fong, Y. & Chen, N. G. From Benchtop to Bedside: A Review of Oncolytic Virotherapy. *Biomedicines* **4,** (2016).

326. Prestwich, R. J. *et al.* Tumor Infection by Oncolytic Reovirus Primes Adaptive Antitumor Immunity. *Clin. Cancer Res.* **14,** 7358–7366 (2008).

327. Bannister, N. & Broggio. *Cancer survival by stage at diagnosis for England (experimental statistics): Adults diagnosed 2012 , 2013 and 2014 and followed up to 2015*. *Office of National Statistics* (2016).

328. West, E. J., Scott, K. J., Jennings, V. A. & Melcher, A. A. Immune activation by combination human lymphokine-activated killer and dendritic cell therapy. *Br. J. Cancer* **105,** 787–95 (2011).

329. Emens, L. A. *et al.* Cancer immunotherapy: Opportunities and challenges in the rapidly evolving clinical landscape. *Eur. J. Cancer* **81,** 116–129 (2017).

330. Hassel, J. C. *et al.* Combined immune checkpoint blockade (anti-PD-1/anti-CTLA-4): Evaluation and management of adverse drug reactions. *Cancer Treat. Rev.* **57,** 36–49 (2017).

331. Tang, J., Shalabi, A. & Hubbard-Lucey, V. M. Comprehensive analysis of the clinical immuno-oncology landscape. *Ann. Oncol.* 84–91 (2017).

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