The Efficacy of Coxsackievirus A21 in Combination with Radiotherapy for the Treatment of Colorectal Cancer.

Miss Jennifer Ann Kingston

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Chapter 3.2.8 contains work from jointly authored publication 'Differential and Longitudinal Immune Gene Patterns Associated with Reprogrammed Microenvironment and Viral Mimicry in Response to Neoadjuvant Radiotherapy in Rectal Cancer', Wilkins A, Fontana E, Nyamundana G, Ragulan C, Patil Y, Mansfield D, Kingston J, Errington-Mais F, Bottomley D, Harrington K, West N, Melcher A, Sadanandam A, Journal for Immunotherapy of Cancer, 9(3) (2021).

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Abstract

Colorectal cancer (CRC) is a common malignancy which is gradually increasing in incidence. Survival is closely associated with stage of disease, with metastatic colorectal cancer having a five-year survival of less than 10%. Up to 40% of patients with CRC present at a late stage, already with metastatic burden, and up to 40% of patients with CRC who are treated with curative intent will go on to develop either local recurrence or metachronous metastases. There is therefore substantial scope with which to develop novel treatments that can improve outcome and decrease recurrence risk within this common disease.

Oncolytic viruses (OV) are a novel form of cancer treatment with only a few approved treatments thus far. They are viruses with low pathogenicity which preferentially infect and kill cancerous cells due to their altered molecular architecture. In addition to direct lytic killing, OVs have the capacity to induce an anti-tumour immune response, which not only provides a secondary mode of tumour cell death but could potentially provide prolonged protection against tumour recurrence.

The use of OVs in the treatment of CRC is in the early stage of research. Within this study we aimed to investigate the potential use of one such OV, Coxsackievirus A21 (CVA21) against CRC. This is the first time this particular OV has been studied within the context of CRC. Here, we have demonstrated that CVA21 exhibits direct cytotoxic effects on CRC cell lines, the extent of which correlates positively with the level of expression of CVA21 entry receptors. In addition to direct mediated cytotoxicity, we have also demonstrated that CVA21 infection activates NK cells and thereby results in immune-mediated death of CRC *in vitro*. Importantly, within this study we have shown that the concurrent use of radiotherapy results in enhancement of CVA21 cytotoxicity against CRC, both via direct and indirect cytotoxic pathways. This is highlighted by the demonstration of *de novo* viral replication within resistant cell lines, increased reduction in cell viability and enhanced NK cell-mediated death seen in all cell lines following dual treatment. Crucially, this enhanced response is demonstrated in CRC cell lines resistant to either treatment in isolation. In summary, we have demonstrated

that CVA21 not only has significant potential as a treatment for CRC, but as an adjunct alongside standard therapies could further enhance their treatment of CRC.

Abbreviations

Ad	Adenovirus
AJCC	American Joint Committee on Cancer
AML	Acute Myeloid Leukaemia
APC	Adenomatous Polyposis Coli Gene
APCs	Antigen Presenting Cells
ATCC	American Type Culture Collection
CEA	Carcinoembryonic Antigen
CMC	Carboxymethylcellulose
CRC	Colorectal Cancer
CRLM	Colorectal Liver Metastases
CRUK	Cancer Research UK
CTLs	Cytotoxic T Lymphocytes
CVA21	Coxsackievirus A21
CVB3	Coxsackievirus B3
DAF	Decay Accelerating Factor
DAMPs	Damage Associated Molecular Patterns
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EGFR	Epidermal Growth Factor
EMR	Endoscopic Mucosal Resection
ESD	Endoscopic Submucosal Resection
FCS	Foetal Calf Serum
gFOBt	Guaiac Faecal Occult Blood Test
GMCSF	Granulocyte Macrophage Colony-Stimulating Factor
HIPEC	Hyperthermic Intraperitoneal Chemotherapy
HMGB1	High Mobility Group B1
ICAM-1	Intercellular Adhesion Molecule-1

IR	Interventional Radiology
FACS	Fluorescence-Activated Cell Sorting
FAP	Familial Adenomatous Polyposis
FCS	Foetal Calf Serum
FDA	United States Food and Drug Administration
5FU	5-Fluorouracil
HBSS	Hanks Balanced Salt Solution
HSV	Herpes Simplex Virus
ICAM-1	Intercellular Adhesion Molecule-1
IFN	Interferon
IHC	Immunohistochemistry
IMA	Inferior Mesenteric Artery
IM	Intramuscular
IR	Ionizing Radiation
IV	Intravenous
LCC	Left Colonic Cancer
LCCRT	Long Course Chemoradiotherapy
Mab	Monoclonal Antibody
mCRC	Metastatic Colorectal Cancer
MDT	Multidisciplinary Team
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
MMR	Mismatch Repair
MRB	Maraba Virus
MSI	Microsatellite Instability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NABs	Neutralizing Antibodies
NHS	National Health Service
NHS BCSP	NHS Bowel Cancer Screening Program
NHSBT	NHS Blood and Transplant
NICE	The National Institute for Health and Care Excellence
NK	Natural Killer Cell

NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
OV	Oncolytic Virus
PAMPs	Pathogen Associated Molecular Patterns
PE	Plating Efficiency
PBS	Phosphate Buffered Saline
PBMCs	Peripheral Blood Mononuclear Cells
PD-1	Programmed Death-1 Receptor
PD-L1	Programmed Death Ligand-1
PFA	Paraformaldehyde
PFS	Progression Free Survival
PRR	Pattern Recognition Receptors
RC	Rectal Cancer
RCC	Right Colonic Cancer
Reo	Reovirus
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RTX	Radiotherapy
SABR	Stereotactically Ablative Body Radiation
SBRT	Stereotactically Guided Single Dose Radiotherapy
SCID	Severe Combined Immunodeficient Mouse
SCRT	Short Course Radiotherapy
SF	Surviving Fraction
sICAM-1	Soluble ICAM-1
SIRT	Selective Internal Radiation Therapy
SMA	Superior Mesenteric Artery
TAA	Tumour Associated Antigen
T*is*	Carcinoma *in situ*
TLR	Toll-Like Receptor
TME	Tumour Microenvironment
TNF	Tumour Necrosis Factor
TNM	Tumour Node Metastasis

VEGF	Vascular Endothelial Growth Factor
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus

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1 Introduction

1.1 Cancer

1.1.1 The Hallmarks of Cancer

Cancer is the uncontrolled proliferation of abnormal cells within the body, with architecture that has varied from the parent tissue. The six major hallmarks of cancer were described in a seminal review by Hanahan and Weinberg [1] where they summarized the complex, multistep process of tumorigenesis into a common pathway which allows for normal cells to transform into malignancy. They described cancer cells as having uncontrolled growth as a consequence of both self-sufficiency in growth and insensitivity to growth-inhibitory signals, an ability to evade apoptosis, having unlimited replicative potential, sustained angiogenesis and possess the fundamental property of being able to invade adjacent tissue and metastasize to distant sites. Over recent years these original six hallmarks have been expanded to include deregulation of metabolism and the avoidance of immune destruction [2, 3].

1.1.2 The Role of the Immune System in Cancer

The immune system has an important role in the prevention of cancer development, so called 'immune surveillance'. This has been highlighted by the increased incidences of cancer in immunosuppressed populations [4]. A disruption between the intricate relationship between altered or abnormal cells and components of both the innate and adaptive immune system can lead to the establishment of clinical disease (**Figure 1-1**). This process has been described as cancer immunoediting and encompasses three phases, elimination, equilibrium and escape. Elimination occurs as a result of both recognition and killing of cancer cells by components of both the innate and the adaptive immune system, the latter of which mounts a more specific response against the cancerous cells. The equilibrium phase involves continual evolution of the tumour cells that survive the process of elimination, with selection of those with reduced immunogenicity and preferential replication of the cells with greater resistance. The final stage of immunoediting is escape, a state whereby a cancerous cell, as a result of genetic changes, is able to evade the host immune surveillance. The resultant cell is then able to replicate in an uncontrolled manner until it becomes a clinically detectable tumour [5].

Ultimately, cancerous cells are able to shield themselves from immune-mediated clearance by production of immunosuppressive cytokines [6, 7], dampened function of immune effector cells [8], failure of immune cell chemotaxis and infiltration [9], reduced antigen presentation [10] and modulated major histocompatibility complex (MHC) expression [11].

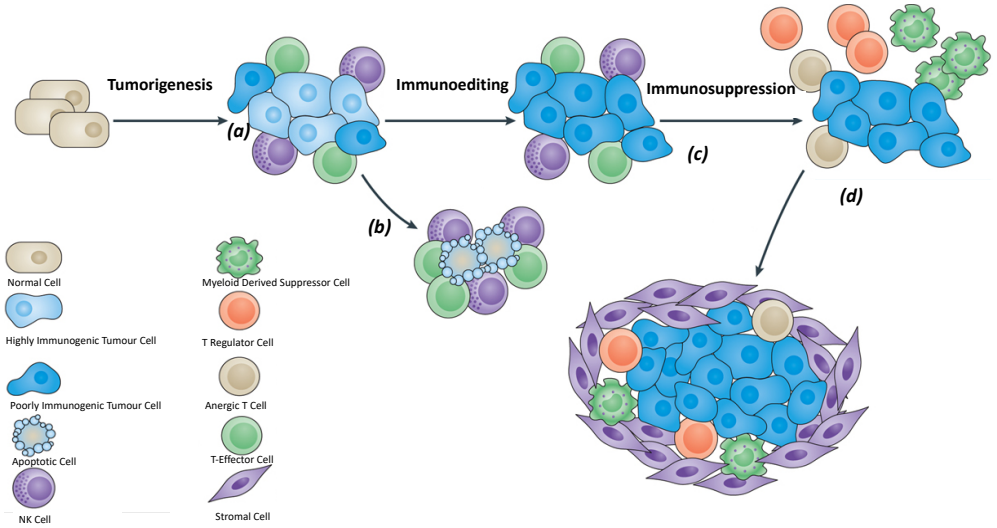


Figure 1-1: Cancer Development and Immune Evasion. The immune system interacts with tumour antigens in three distinct phases before it becomes clinically detectable: elimination, equilibrium and escape. During development, cancers acquire neoantigens (a) which may be recognized and eliminated by cells of both the innate and adaptive immune system (b). If the cancer evolves and survives elimination, it proceeds to an equilibrium phase, natural selection of the tumour cells, wherein there is a down-regulation of antigen processing and presenting and the most immunogenic antigens are removed (c). At this equilibrium phase, cancerous cells are in a stable and still clinically undetectable position. The final stage, escape, is when a tumour becomes clinically detectable. It is established by a combination of downregulation of immune activation pathways, upregulation of immunosuppressive pathways, production of immunosuppressive cytokines and recruitment of immune cells which mediate tolerance, such as T-regulatory cells and myeloid-derived suppressor cells (d). Image adapted from publication in Nature [12].

1.2 Colorectal Cancer

1.2.1 Pathogenesis

Colorectal cancer (CRC) begins anywhere within the large bowel epithelium of the mucosa with the majority, up to 96%, being adenocarcinomas [13]. Anatomically the cancers are either rectal or colonic, often subdivided into right colonic cancers (RCC), left colorectal cancers (LCC), and rectal cancers (RC). In embryological development the right colon, which anatomically extends from the caecum to two-thirds of the length of the transverse colon (Figure 1-2), is derived from the mid gut, and the left colon and rectum originate from the hindgut. Consequentially these areas have differing blood supplies. The right colon is supplied from the superior mesenteric artery (SMA), the left by the inferior mesenteric artery (IMA) and the rectum, although partially supplied by the IMA, also obtains some supply from the internal iliac and pudendal arteries. The incidence of left sided cancers is slightly more frequent, with up to 60% of all CRC occurring within this area [14]. There are some important differences in the pattern of disease presentation and survival depending on location of CRC, with RCC often presenting later and having a worse survival rate [15-17]. These differences in rates of presentation and survival are partly due to embryological development and anatomical considerations already discussed, as well as differing levels of exposure to potential carcinogens [18, 19]. Although small differences occur due to location of CRC, the course of disease progression and their histological derivation mean that they are generally considered as one group of cancers.

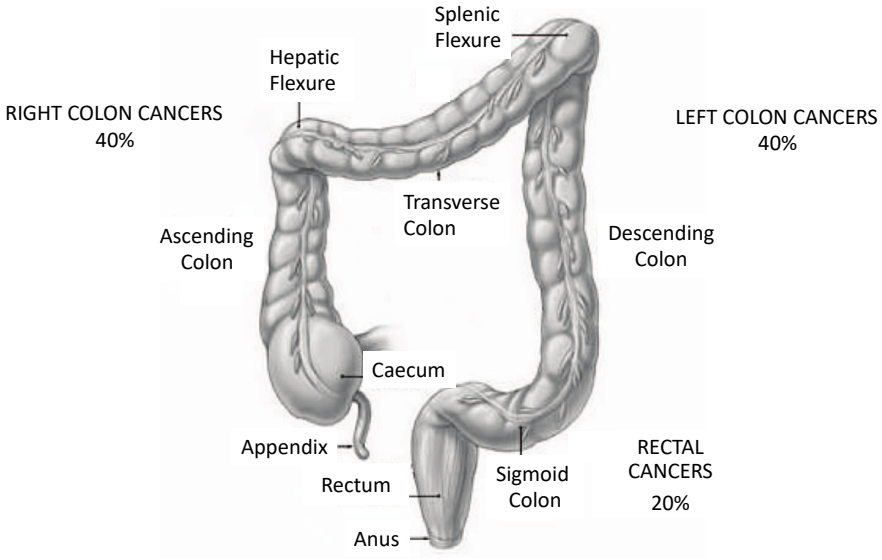


Figure 1-2: Gross Anatomy of The Large Intestine and Distribution of Adenocarcinomas. The right colon arises at the caecum and continues into the proximal two thirds of the transverse colon, the left colon is the area covering the distal third of the transverse colon, descending and sigmoid colon, up to the upper border of the rectum. 96% of all colorectal cancers are adenocarcinomas, with majority occurring within the left colon and rectum. Image adapted from Carvallo et. al. 2008 [20].

Most CRC develop and begin as an adenomatous polyp which can remain pre-cancerous for many years before undergoing cellular changes and developing into malignant lesions. There is an established, well documented pathway for this, known as the adenoma-carcinoma sequence (**Figure 1-3**), whereby specific molecular and genetic events occur resulting in the transformation of normal colonic epithelium into benign tumours, known as adenomatous polyps. These adenomatous polyps can subsequently transform into malignancies [21]. The first stage in the adenocarcinoma sequence is loss of the tumour suppressor gene adenomatous polyposis coli gene (APC), which normally negatively regulates cell growth. This is followed by mutations involving combinations of the genes controlling cell proliferation (K-ras), cell adhesion (E-cadherin), cellular repair (MLH1, MSH2) and apoptosis (TP53) [22, 23]. Adenomas are a common finding at endoscopic investigation and can be present in up to 50% of the population over the age of 70 [24-26]. Risk factors for malignant transformation from adenomatous polyps include location (rectal polyps carry the highest risk of malignancy), dysplasia, increasing size (25mm or greater inferring considerable risk), and the presence of multiple polyps [27]. Malignant transformation may take many years to occur or may not occur at all. It has been estimated, based on data acquired from surveillance imaging prior to the advent of routine colonoscopy, that risk of malignant transformation of adenomatous polyps is 2.5%, 8% and 24% at 5, 10 and 20 years, respectively [28].

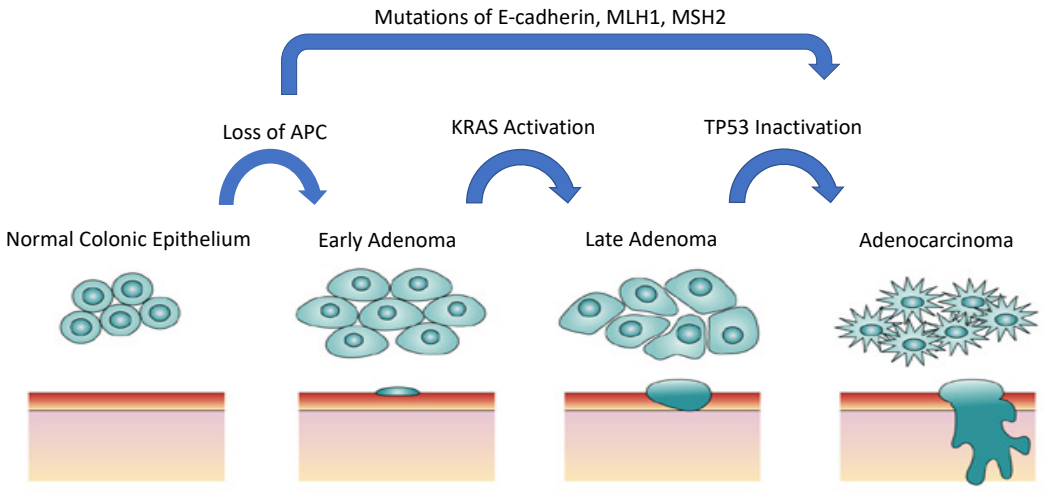


Figure 1-3: The Adenoma-carcinoma Sequence. The molecular events leading to progression of normal colonic epithelium to polyp and subsequent adenocarcinoma (the origin of 85-90% of CRC). The likely first stage is loss of APC gene, a tumour suppressor gene, due to inactivating genetic mutations. This results in altered signalling pathways and dysregulated cell proliferation which results in development of adenoma. Following this, mutations in the oncogene KRAS result in increased cell signalling and further dysregulated cell growth and replication. Finally, loss of the tumour suppressor p53 gene results in a failure of cell apoptosis, the final mutation which will result in transition from adenoma to cancer [29]. Image adapted from publication in Gastrointestinal Cancer Research [30].

1.2.2 Epidemiology

According to statistics compiled by Cancer Research UK (CRUK), CRC is the fourth most common cancer in the UK, but the second most common cause of cancer death, accounting for around 10% of all cancer deaths in this country [31]. In 2016 there were 34,952 new cases reported in England alone (Cancer Registration Statistics, England) [32]. Globally, CRC had an incidence of 23.2 per 100,000 (1.8 million cases) in 2017 [33], a figure which demonstrates that there has been a gradual increase over the last twenty years from a previous incidence of 13.7 per 100,000. This increasing incidence may in part be attributable to an ageing population, and has been predicted to rise by a further 2% in the UK by 2030 [34].

Risk factors for CRC are multifactorial and can be stratified by modifiable and non-modifiable risk factors. Incidence of CRC is more frequent in males than females and is strongly correlated with advancing age [35]. The incidence of CRC rises sharply above the age of 50 and the difference in incidence rate in male to female widens with increasing age [33]. There are known genetic conditions which predispose to CRC, with the most commonly inherited forms being attributable to Lynch syndrome, a disorder of mutations in DNA mismatch repair genes, and Familial Adenomatous Polyposis (FAP), a disorder resulting from mutations of APC. In addition to these, patients with a history of two first degree relatives who have had CRC under the age of 60 years are offered colonoscopy screening from age 50 due to a moderate-high risk of developing CRC [36]. Patients with inflammatory bowel disease, either Crohn's or Ulcerative Colitis also have a higher risk of developing CRC [37]. Modifiable risk factors for CRC include obesity, poor levels of physical activity, dietary factors (high intake of red meat, processed meat, alcohol, animal fats and sugar, low levels of vegetable and fruit consumption) [38] and cigarette smoking [39].

1.2.3 Staging and Grading

CRC progression is often staged using Duke's classification, a system originally developed from a study of the histology of rectal cancers [40], staging them as A, B or C depending on tumour extension through the bowel wall. It was the first system to simply and accurately predict prognosis following surgery for rectal cancer [41]. It was later further developed to the commonly used Modified Dukes Classification (**Table 1-1**) which uses stages A-D inclusive, with subdivision of stages B and C to describe disease progression [42, 43]. Recent data shows that five-year survival correlating to Dukes stage is as follows; Dukes A 93.2%, Dukes B 77%, Dukes C 47.7% and Dukes D only 6.6% [43].

Many other staging systems exist in addition to Dukes. Staging of CRC within the clinical context is more frequently described using the tumour-node-metastasis (TNM) system as it affords more precise prognostic information [44]. It is a descriptive representation based on depth of tumour invasion into the bowel wall, extension into neighbouring structures or organs, number of involved loco-regional lymph nodes, and the presence or absence of distant metastases. This more detailed descriptive system allows prediction of risk to inform clinical decisions to be made regarding the nature of CRC treatment, for example the appropriateness of neoadjuvant, surgical or adjuvant therapy [45]. A simplified modification of the TNM system, based on the 5th edition of American Joint Committee on cancer (AJCC) staging system [46], will be used for the purpose of this report (**Table 1-2**). It comprises a more straightforward grouping from stage 0-4 inclusive.

Modified Dukes Classification		
A		Tumour confined to the mucosa
B	B1	Tumour confined to the muscularis propria
	B2	Tumour growth through muscularis propria and serosa
C	C1	Tumour spread up to 4 loco-regional lymph nodes
	C2	Tumour involving >4 lymph nodes
D		Distant metastases

Table 1-1: Modified Dukes Classification of Colorectal Cancer. The system is an elaboration of the original classification of rectal cancers described by Cuthbert E. Dukes in 1932 [40]. Advancing stage within this system correlates with poorer prognosis, with Dukes A conferring 93% 5-year survival and Dukes D having only 7% 5-year survival.

	Stage Grouping CRC	TNM equivalent
Stage 0 (T*is*)	Carcinoma *in situ*; cancer cells confined to basement membrane or lamina propria.	T*is*
Stage I	Tumour confined to mucosa or muscularis propria No nodal disease or metastasis	T1 or T2 with N0 M0
Stage II	Tumour Invading muscularis or invading adjacent structures. No nodal disease or metastasis	T3 or T4 with N0 MO
Stage III	Any tumour stage with nodal metastasis	Any T with N1 or N2, M0
Stage IV	Any tumour stage, any nodal stage with distant metastasis	Any T or N but M1

Table 1-2: AJCC staging of CRC [46]. A modification of the TNM system; Stage 0 (Tis) is carcinoma in situ, stage I is equivalent to an early T stage in the TNM system, wherein the tumour has not breached the extent of the muscularis propria or serosa, stage II is locally advanced disease, stage III is any level of local disease with lymph node metastases, and stage IV infers the presence of distant organ metastasis.

1.2.4 Prognosis

Despite the rising incidence, survival in patients with CRC has improved dramatically over recent years (**Figure 1-4**), with the overall five-year survival rate increasing from 25% in the 1970s to a little over 50% in the last decade [47-49]. However, more specific prognostic information may be yielded by observing stage specific survival data as 5-year survival rates drop remarkably with increasing stage of disease at diagnosis; the trend down from stage I through to IV is from 92%, 84%, 65% and 10% respectively [31, 49].

The notable improvement in survival of this disease over the last 50 years is due to improved medical therapy, an increase in hepatic resection [50], and the introduction of the national bowel cancer screening program (NHS BCSP) in the UK [51]. The NHS BCSP was introduced in 2006 using a home-based guaiac faecal occult blood test (gFOBt), a method of detecting any blood within the faeces. Any patient, age 60-74, with a positive result on this initial screen is subsequently offered a colonoscopy. The program was subsequently expanded to offer those over the age of 55 a one-off screening flexible sigmoidoscopy; any adenomas detected, or other significant abnormality would result in endoscopic removal of the adenoma and full completion colonoscopy [51], although this one-off endoscopic screening has recently been suspended. Initial results predicted that screening has the potential to reduce CRC mortality rates by a further 16% by 2030 [52].

At the time of disease presentation, around 26% of patients with rectal cancer and 40% of patients with colon cancer will have metastatic, stage IV disease [53]. Common sites of metastases include liver, lung, peritoneum and bone, with colorectal liver metastases (CRLM) being most frequently observed [15, 54] for all CRC. Even for those patients who are treated with curative intent, local disease recurrence will occur in up to 17% [55], and metastatic disease will subsequently develop in around 25% of patients [56] [57]. When disease recurrence transpires, 30-50% of these recurrences will occur within two years [58].

Patients whom present with more advanced, stage IV, metastatic disease, have significantly poorer outcomes with only around 10% surviving to five years post diagnosis [49] (**Figure 1-5**).

In patients with liver-limited metastatic disease, surgical resection is the most effective way of improving disease survival. Recent data analysis has shown that 5-year survival following hepatic resection for CRLM is up to 74.3%, reducing to 57.5% in patients that experience disease recurrence [59]. Even in patients whom undergo liver resection, disease recurrence is common occurring in up to two thirds of patients [60]. Survival in surgically treatable patients is significantly improved compared to patients that are deemed inoperable. In this cohort the 5-year survival is only around 9% [61].

Prognosis of a patient with CRC is not only predicted by stage of disease, but also histological type of cancer, differentiation, serosal involvement and extra mural vascular invasion. Three degrees of differentiation are frequently described in histological examination of CRC, well, moderate and poor, with survival progressively worsening from well to poorly differentiated tumours [62].

Overall, survival of CRC has more than doubled in the last 30 years in the UK (**Figure 1-4**), but despite these significant advances, there remains scope for dramatic improvement in overall survival (OS) and the prevention of disease recurrence, particularly considering that survival rates are heavily stage dependent, and a more advanced stage is associated with significantly worse mortality and morbidity (**Figure 1-5**) as well as a higher risk of disease recurrence [63, 64].

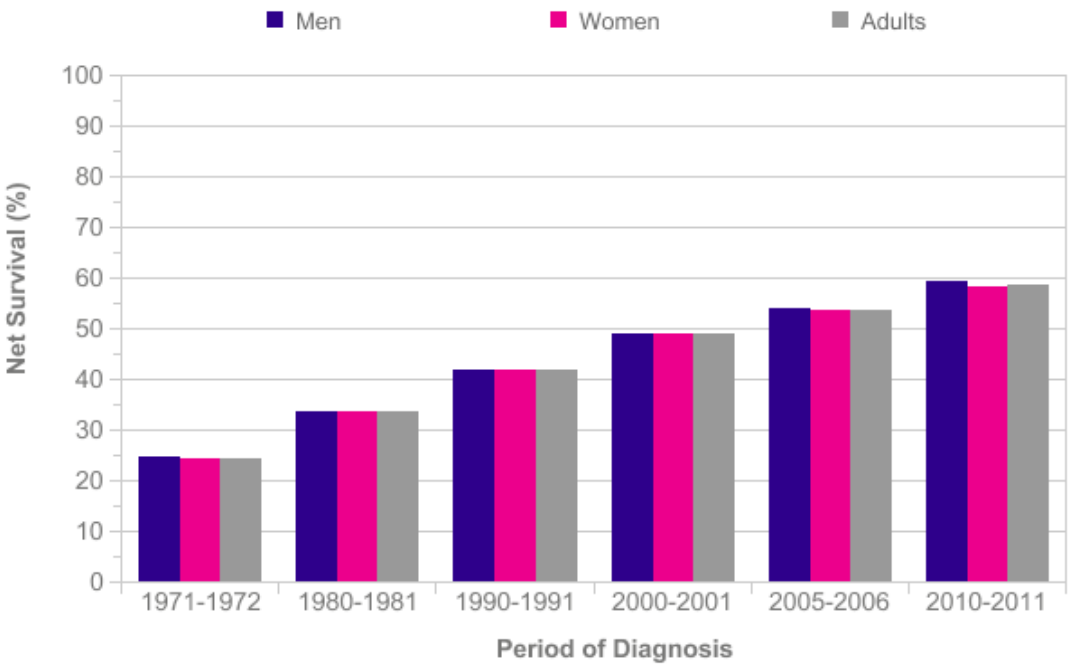


Figure 1-4: Age-Standardized Five-year Net Survival (Adults between age 15-99) in England and Wales. From 1971 until 2011, overall five-year survival (the percentage of people within the group alive five years subsequent to diagnosis) for CRC has increased from 22% to 57%. Data published in 2015 and represents statistics collected from adult patients between age 15-99, graphical image taken from CRUK, 2015 [31].

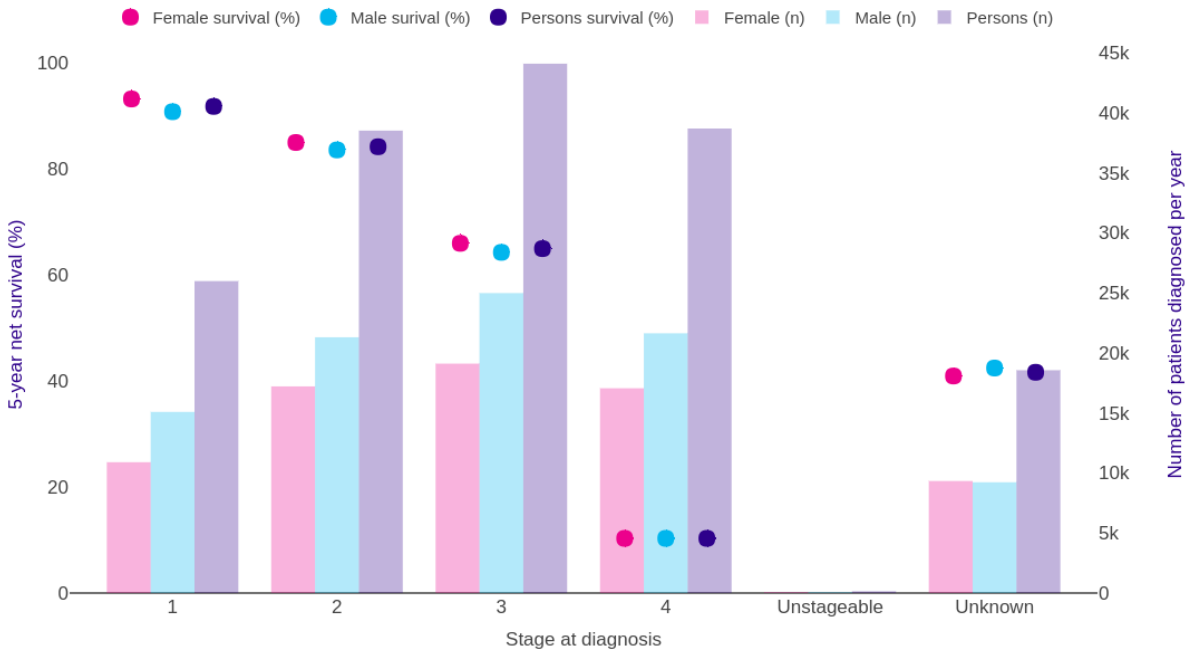


Figure 1-5: Bowel Cancer Incidence and Five-year net Survival by Stage of Disease. Bar charts represent incidence of stage of diagnosis, whereas percentage five-year survival is shown by scatter dots. Data is representative of all adult patients diagnosed between 2013-2017, with follow up data until 2018. Despite the NHS screening program, the majority of diagnoses are still made with a disease stage II or beyond, with the most common being stage III. Five-year net survival for bowel cancer is significantly reduced in patients that have a more advanced stage of disease; five-year net survival ranges from around 90% at Stage 1 to as little as 10% at stage 4. Data taken from Cancer Research UK [65].

1.2.5 Treatment

Following thorough investigation and confirmation of diagnosis of CRC, treatment is dependent on stage of disease and presence or absence of symptoms on presentation, anatomical location, histological findings as well as patient demographics, general fitness and choice, and is subject to discussion at a multi-disciplinary team (MDT) meeting. Treatment options consist of medical, radiological and surgical, in the context of curative intent, disease palliation and management of patients with oligometastatic disease (patients with a limited number of, potentially curable, metastatic deposits). The benefits of one treatment pathway over another are beyond the scope of this thesis, however a generic overview of different available therapies is described.

1.2.5.1 Endoscopic and Surgical Management

Endoscopic techniques can be used for resection of benign polyps as well as resection of early tumours using techniques such as endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD). These techniques are potentially curative in patients with early disease and complete resection spares the patient from undergoing the significant morbidity and mortality risk undertaken with a formal surgical resection [66]. In addition to these techniques, endoscopic colonic stents are available for left sided tumours which present with acute large bowel obstruction or impending obstruction and can be used for palliation or as a bridge to elective surgery with curative intent [67].

Surgical options are available for both primary and metastatic disease and can be undertaken in the elective and emergency setting, depending on the mode of patient presentation. Surgical resection of primary disease, along with its corresponding mesentery and vascular ligation, via open or minimally invasive techniques, is considered the standard treatment for stage I-III CRC [48] and is frequently offered alongside other surgical or medical treatments for patients with stage IV disease. It is generally accepted that surgery offers the greatest chance of disease cure and increased survival [53, 68]. Surgical management of CRC also includes palliative procedures such as de-functioning stomas and tumour debulking. More

aggressive surgical approaches exist for cytoreductive surgery for peritoneal infiltration, with or without hyperthermic intraperitoneal chemotherapy (HIPEC) [69] and resection of pulmonary or liver metastases. As mentioned previously, five-year survival is significantly improved in patients with technically operable metastatic disease, within the liver or lung, when surgical resection for the metastatic burden is undertaken [70-72].

1.2.5.2 Medical Treatment

Systemic medical treatment has advanced considerably over the last 25 years, until the 1980s the only chemotherapeutic agent against colorectal cancer was 5-fluorouracil (5FU) [73]. Over the subsequent decades chemotherapeutic regimes have evolved substantially. Presently, neoadjuvant systemic chemotherapy is offered to patients with stage IV colonic cancer and as part of chemoradiotherapy in patients with rectal cancer with nodal or locally advanced disease [45]. Adjuvant chemotherapy is generally recommended for patients with histological stage II (with high chance of recurrence) or stage III CRC, or in patients with resection margins of less than 1mm following surgery [45, 74], with the aim of preventing relapse and improving survival. Adjuvant chemotherapeutic regimes include capecitabine in combination with oxaliplatin (CAPOX or XELOX), oxaliplatin (FOLFOX) or irinotecan (FOLFIRI) in combination with 5-FU and folinic acid, or single agent fluoropyrimidine (capectitabine or 5-FU), the latter of which tends to be reserved for patients that have poorer performance status and increased co-morbidities. Evidence has shown that these commonly used chemotherapy regimens can increase OS in the region of 20 months [75]. Chemotherapy is also used in the palliative setting to significantly increase the quantity and, possibly, quality of life [76], although its use must be carefully balanced against potential side effects experienced by the patient.

1.2.5.3 Radiotherapy

Radiotherapy (RTX) is a commonly used treatment modality for many cancers. It can either be administered as a monotherapy or as part of a combination treatment regimen alongside chemotherapy, surgical resection, surgical debulking, or any combination of these [77]. Radiotherapy utilizes ionizing radiation (IR) which deposits high energy to tissue cells as it

passes through them, ultimately resulting in a suspension of cellular proliferation and induction of apoptosis. This is achieved by generating DNA damage, either single stranded or double stranded, both directly from ionization and indirectly by the generation of free radicals, with subsequent DNA damage pathways resulting in cell cycle arrest [78]. Four possible sequale to this are (1) DNA damage repair, (2) cell proliferation, (3) senescence or (4) cell death as a result of apoptosis, necrosis, mitotic catastrophe or autophagy [79].

As well as the direct cellular effects of radiation, it is also apparent that the use of ionizing radiation can enhance tumour immunogenicity, as evidenced by the regression of tumours outside of the radiation field, known as an abscopal effect [80-82]. This effect is thought to result from the release of tumour associated-antigens (TAAs) and damage of DNA which induces the release of inflammatory cytokines [83] which promote the recruitment and activation of dendritic cells (DCs) to the radiation damaged tumour cells [84]. DCs then migrate to the lymph nodes where they can prime CD8+ T cells to initiate a cytotoxic T cell response, which can result in systemic tumour regression (i.e., the abscopal effect) [85, 86]. In addition to this adaptive immune response, NK cell-mediated cytotoxicity is an important method of tumour cell death following irradiation as ionizing radiation increases the expression of tumour ligands for NK cell-activating receptors such as NKG2D [87].

Radiotherapy has a firmly established role in the management of rectal cancer as a neo-adjuvant treatment in order to downsize disease (rendering previously inoperable lesions surgically resectable) and reduce risk of local recurrence. Alternatively, it can also be utilized as a palliative treatment providing symptomatic relief to patients with inoperable lesions. It may also be used in the adjuvant setting in the scenario where the resection specimen contains positive margins. There is well established evidence to suggest the use of radiotherapy in rectal cancer reduces the risk of local recurrence and, if patient selection is appropriate, radiotherapy also improves survival [88-90].

External beam radiotherapy is currently offered pre-operatively to patients with locally advanced mid or low rectal tumours and rectal tumours with nodal involvement. Short course radiotherapy (SCRT), used for operable mobile rectal tumours, is the use of 25 Gy administered in 5 Gy doses over 5 days, followed by immediate or delayed surgery. Long

course chemoradiotherapy (LCRT) is the delivery of 45-50 Gy, delivered in 25-28 fractions, with concurrent chemotherapy and surgery approximately 4-8 weeks later. There is no apparent difference in OS or PFS between SCRT and LCRT [91, 92] but factors such time to surgery, systemic and gastrointestinal toxicity, extent and exact location of disease, the potential for sphincter sparing surgery and patient choice are important considerations when selecting the most appropriate treatment.

The use of radiation treatment in CRC is no longer limited to its more traditional SCRT and LCRT therapy. In CRLM not amenable to surgical resection, local ablative therapies using radiation are available. Selective internal radiation (SIRT) is a technique that involves embolising radiolabelled spheres into the arterial supply of the liver and is often used in patients with liver metastases not amenable to surgery [93]. It can be used in isolation or alongside systemic or local chemotherapy, however definitive evidence of a benefit of SIRT for CLRM in respect to OS is yet to be established [94]. Another technique utilizing ionising radiation in the treatment of CRC oligometastatic disease is stereotactically guided single-dose radiotherapy (SBRT), also known as stereotactic ablative body radiation (SABR). This technique is most frequently utilized in hepatic and pulmonary metastases [95] and is a further example of the use of ionising radiation in the management of CRC. It can be used as a treatment for non-resectable lesions, lesions unsuitable for radiofrequency ablation or in patients with co-morbidities that preclude surgery or ablative therapy and can offer a degree of local disease control and improvement in OS [80, 96-98].

1.2.5.4 Biological Agents and Immunotherapy

Over recent years there has been a drive to tailor the treatment of CRC to counteract the limitations of chemotherapy such as systemic toxicity, resistance and poor specificity [99]. With respect to CRC, biological agents, such as monoclonal antibodies cetuximab and panitumumab, epidermal growth factor (EGFR) inhibitors, and bevacizumab, which targets vascular endothelial growth factor (VEGF), are used alongside chemotherapy in advanced, metastatic disease. Evidence suggests that, for RAS wild-type metastatic CRC, panitumumab or cetuximab (in EGFR expressing tumours), in combination with FOLFOX or FOLFIRI, can improve PFS and OS when compared to chemotherapy alone [100, 101]; these regimes are

currently approved as first line treatments by the National Institute for Health and Care Excellence [102].

This use of combination strategies is aimed at improving OS and PFS in patients with advanced, metastatic CRC who would otherwise have limited survival. More recently combinatorial treatments have extended to include immunotherapy with agents such as Pembrolizumab, a treatment option for untreated mCRC with microsatellite instability (MSI) or mismatch repair (MMR). MMR is a process which preserves DNA homeostasis and confers genomic stability. Its purpose is to correct errors occurring during DNA replication; when it is insufficient it fails to correct these errors and as a consequence the mutational rate of the cell increases resulting in MSI [103]. MSI is detected in around 15% of CRC [104]. Programmed death-1 (PD-1) receptor and programmed death ligand-1 (PD-L1) interaction results in an impairment of T-cell mediated anti-tumour response [105]. Research has shown that CRC with MSI highly express PD-L1 and as such Pembrolizumab, a PD-1 inhibitor can improve PFS [106]. Although immunotherapies are showing promise, their use is currently limited to a small subset of patients with CRC. The ongoing investigation to broaden the armoury of immunotherapies to use in combinations with standard therapies is therefore critical to improving outcomes in patients with mCRC.

1.3 Oncolytic Viruses

1.3.1 Mechanism of Action

Oncolytic viruses (OVs) can be either naturally occurring or genetically modified. They preferentially target and kill cancer cells through direct oncolysis and OV-mediated anti-tumour immunity [107]. This is also known as direct and indirect mediated OV killing. The latter can be achieved via a combination of innate and adaptive immune pathways and was originally evidenced by intra-tumoural injection of melanoma resulting in an abscopal effect of regression of distant lesions [108].

For an OV to be considered appropriate for clinical use, it should display a selective affinity towards cancerous cells, preferentially infecting, replicating within and killing them whilst resulting in minimal toxicity to the host organism. It should also persist within the environment for a long enough to induce a clinical effect [109]. The advantage of this selectivity in cancerous cells holds the promise of a self-propagating treatment with immunologic stimulation that could potentially provide long term prevention of disease recurrence via immune memory [110]. Indeed, this effect, that adaptive immune responses can be elicited following treatment, has been demonstrated in both *in vitro* and *in vivo* (human and murine) models using a variety of different OVs [111-116]. Importantly, the induction of adaptive immunity is, potentially, capable of preventing disease recurrence upon tumour rechallenge. One study in which mice were injected with a breast cancer cell line, then treated with either Reovirus (Reo), Vesicular stomatitis virus (VSV), Herpes Simplex Virus (HSV), Maraba virus (MRB) or Adenovirus (Ad), prior to surgical resection, showed such results. For example, not only did OVs show the ability to reduce the size of the primary tumour burden and decreased the incidence of lung metastases but OV treatment also increased survival and controlled tumour growth when the mice were rechallenged with a further injection of the same breast cancer cell line [117]. Another study, evaluating oncolytic Ad in a replication permissive, immune competent, Syrian hamster model, demonstrated that CD3 monoclonal antibody (mAB) deletion of T-cells extended the length of time infectious

virus persisted within tumour cells, but rescinded the anti-tumour efficacy of Ad infection [118].

Cancer cells are fundamentally predisposed to viral infection by OVs compared to healthy host cells for a variety of reasons, including impaired anti-viral response mechanisms within the genetically mutated tumour [110], enhanced virus receptor expression [119], increased cellular proliferation and the availability of required host machinery [109, 120, 121].

Infection of tumour cells with OVs results in activation of both the innate and adaptive immune system. Following tumour cell infection with an OV, pro-inflammatory cytokines are released [116, 122] alongside the generation of pathogen-associated molecular patterns (PAMPs) and damage associated molecular pattern signals (DAMPS) [123], with further cytokine being released from both adjacent cells and recruited immune cells. Pro-inflammatory cytokines within the tumour micro-environment (TME) drive differentiation, proliferation, recruitment and activation of immune effector cells as well as contribute to bystander cytokine killing [124, 125]. PAMPs and DAMPs are recognized by specific pathogen recognition receptors (PRRs) which are expressed by innate immune cells such as Natural Killer (NK) cells and Dendritic Cells (DCs). Interaction between these ligands and receptors act to attract, activate and mature DCs in the TME [126] and activate NK cells to release cytokines, chemokines and cytotoxic granules to induce tumour cell death [127]. Both DCs and NK cells function as a bridge to link innate and adaptive immunological mechanisms. Mature DCs (following PRR interaction with PAMPs and DAMPs) present TAA which subsequently activate antigen specific CD4+ and CD8+ T-Cell responses [111] (**Figure 1-6**). Activated NK cells influence T-cell maturation through pro-inflammatory cytokines and via interactions which improve maturation and effector functions of DCs [128]. NK cells also increase the availability of tumour associated antigens (TAAs) for presentation following NK cell-mediated death of tumour cells [129]. This complex interplay between aspects of innate and adaptive immune responses alongside immunologic cell death of OV-infected tumour cells holds the most exciting promise for the future of OV therapy and the hope of a self-perpetuating treatment with immune memory against cancer.

Despite the potential presence of circulating neutralizing antibodies (NABs) to OVs present in patients, OVs can still persist when given systemically. In a trial using oncolytic Reo, it was established that despite NABs, replicating virus was identified at tumour sites and this was postulated to be due to immune cell carriage (Adair et al., 2012). Indeed, Ilett et. al. demonstrated that oncolytic Reo could be internalized by monocytes and thus protected from inactivation by NABs [130]. Delivering OVs systemically is the preferred choice of delivery as this will allow effective delivery to sites of metastatic disease in order for the OV to have both a direct cytotoxic effect whilst simultaneously contributing to immunological stimulation [131].

OVs are safe in clinical use, with minimal toxicity. There are currently four such viruses licensed for use as anti-cancer therapy. Rigvir (ECHO-7), a picornovirus, was the first OV to be licensed in 2004 in Latvia for use in melanoma via intramuscular (IM) injection [132] and it has been shown to significantly prolong survival in early stage melanoma [133]. Subsequent to this another OV, Oncorine (H101), an attenuated adenovirus, was approved for use in China in 2005 as a therapy against head and neck cancer [134]. This approval was subsequent to a clinical trial which not only demonstrated safety but also showed that Oncorine, when delivered as an intra-tumoural injection alongside chemotherapy resulted in a considerably improved response rate when compared to chemotherapy alone [135].

There are two modified herpes simplex viruses currently approved for clinical use. The first, T-VEC, a modified HSV-1 delivered by intra-tumoural injection, is currently approved by the US Food and Drug Administration (FDA) for use in advanced, inoperable, melanoma following phase III trials that demonstrated a significant improvement in durable response rate and overall patient survival [136]. It is designed to replicate within tumours and secrete granulocyte macrophage colony-stimulating factor (GM-CSF) to enhance systemic anti-tumour immune responses. The other HSV-1 based OV which is most recently approved is DELYTACT. DELYTACT was approved for use in primary brain cancers in Japan in 2021 following data achieved in clinical trials which reflected a remarkable increase in the one year survival of patients with recurrent or residual glioblastoma multiforme [137], an aggressive primary brain tumour with historically poor survival and limited treatment options [138].

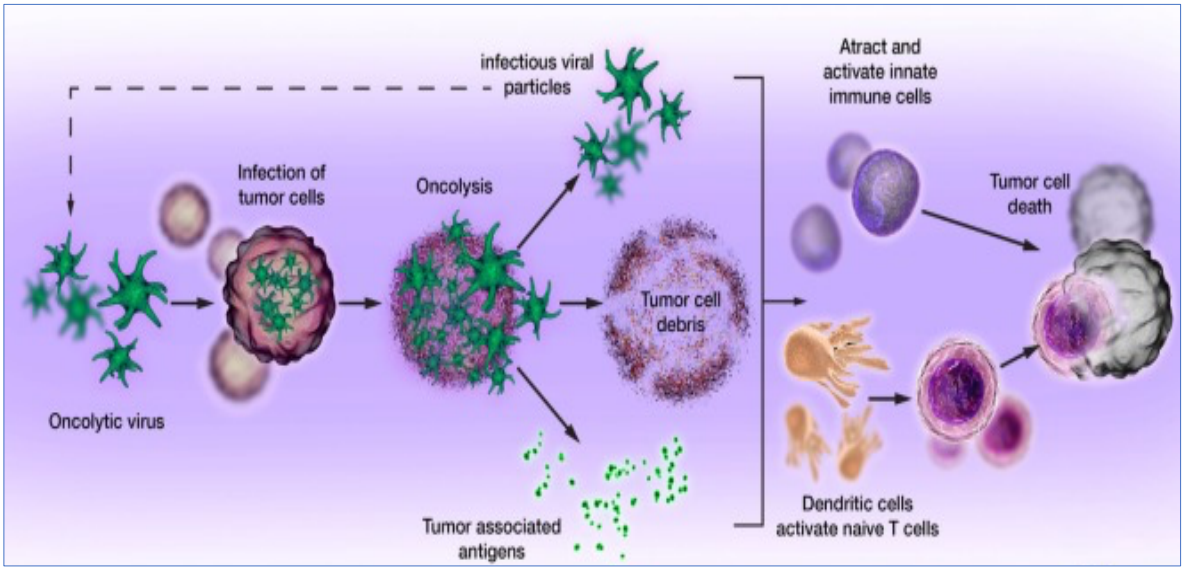


Figure 1-6: Dual Mechanism of Action of Oncolytic Viruses; Direct Lysis and Induction of Host Anti-tumour Immune Response. Cancer cells are more vulnerable to viral infection due to impaired antiviral responses, altered membrane receptor expression and rapid cellular proliferation. This results in direct cytotoxicity of cancer cells as a result of OV infection. As a result of this direct cytotoxicity, pro-inflammatory cytokines and TAAs are released which can stimulate both an innate and adaptive immune response. Image adapted from 'Frontiers in Oncology' [139].

1.3.2 Oncolytic Viruses in Colorectal Cancer

Considerable research has begun into the potential of using OVs as a treatment for CRC, with pre-clinical data and early phase clinical trials investigating a wide range of OVs as single agent [140-143], or as part of a combination therapy [144-146], in both the context of their direct and indirect cytotoxic effects. As yet there remains no published research into the use of Coxsackievirus A21 (CVA21) as a potential treatment for CRC. By contrast, Coxsackievirus B3 (CVB3) has been studied in the context of CRC, however there are some concerns with regard to systemic toxicity of some strains of CVB virus strains, which can cause myocardial or pancreatic inflammation as significant adverse reactions [147]. Coxsackievirus A11 has also shown some *in-vitro* toxicity against CRC cell lines and, in combination with the chemotherapeutic agent oxaliplatin, CRC cells that were resistant to either agent in isolation became susceptible to combination treatment [148]. Other OVs widely researched with respect to CRC include VV, Reo and HSV which are discussed further below.

1.3.2.1 Herpes Simplex Virus (HSV)

A multi-mutated HSV-1 (G207) has been shown to replicate in CRC cell lines [149]. Moreover, in athymic mouse xenografts, direct tumoural injection of G207 resulted in significant suppression of tumour growth ($p < 0.0001$), and portal venous injection reduced the incidence of liver nodules ($p < 0.05$) [149]. In a phase I clinical trial, NV1020, a replication competent HSV-1, was delivered via hepatic artery infusion to patients with metastatic CRC (mCRC) refractory to first line chemotherapy. Subsequent to infusion and regional chemotherapy, patients showed a reduction in the tumour marker carcinoembryonic antigen (CEA), radiological partial response and evidence of HSV infection upon tumour biopsy [140]. A similar early phase clinical trial administered NV1020 to 19 patients with advanced mCRC, which was refractory to treatment, followed by conventional chemotherapy showed stabilisation of liver metastases and a potential for increased OS by re-sensitising tumours to chemotherapy [141].

1.3.2.2 Vaccinia Virus (VV)

Vaccinia Virus (VV) has also been studied extensively as an OV and has shown some promise within the context of CRC. VV demonstrates cytotoxicity against CRC cell lines, with some evidence of synergism when used in combination with chemotherapeutic agents, Oxaliplatin and Irinotecan [146]. Modified strains armed with immunostimulatory cytokines (IL-21 and IL-24) inhibit the growth of CRC, induce oncolysis, apoptosis and stimulate anti-tumour effects [150, 151]. Pexa-Vec (JX594, a Thymidine Kinase-Deactivated VV expressing GMCSF), given intravenously, pre-operatively to patients undergoing liver resection for CRLM resulted in tumour selective histological changes of inflammation and fibrosis. Analysis of peripheral blood mononuclear cells (PBMC) post IV JX594 administration in the same patient demographic identified evidence of activation of both innate and adaptive immune responses [152]. Importantly, there have also been phase I/II studies examining the effects of JX594 in combination with immune checkpoint inhibition in refractory CRC, although no statistically significant difference was seen with PFS, tolerability and safety was established [153].

1.3.2.3 Reovirus (Reo)

Reo is known to infect and replicate in human CRC cell lines and has the ability to result in regression in the size of CRC tumours in animal models [154]. In a translational study undertaken in patients undergoing liver resection for CRLM, Reo was delivered intravenously prior to surgical resection. Reo was able to evade the host immune system and replication competent virus was detected in PBMC and tumour whilst sparing normal liver tissue [155]. Another phase II study has investigated the use of Pelareoprep (clinically formulated Reo) alongside FOLFOX6/Bevacizumab in patients with mCRC, however no difference in OS and an inferior PFS was reported [145].

In summary, many pre-clinical and early phase clinical trials have been commenced to evaluate the safety of OVs in the context of CRC and any possible efficacy they may possess at improving PFS or OS when used alongside standard treatment regimens for advanced disease. However, as mentioned above, there has been no published data investigating the

potential oncolytic efficacy of CVA21 in CRC as a stand-alone treatment, or as part of a combination therapy approach.

1.3.3 Coxsackievirus A21

Coxsackieviruses are un-enveloped, positive-sense RNA enteroviruses and are members of the *Picornaviridae* family and the genus Enterovirus. They are common and produce often self-limiting febrile illness or, more rarely, aseptic meningitis. They are transmitted via the faeco-oral route. The coxsackievirus family is composed of more than twenty viruses, subdivided into one of two groups, Coxsackievirus A (twenty-three serotypes) or Coxsackievirus B (six serotypes). The subdivision originated according to observations of their pathogenicity in mice, with coxsackievirus A producing flaccid paralysis and coxsackievirus B producing spastic paralysis [156]. Coxsackievirus A21 (CVA21) is one such serotype, manufactured commercially as CAVATAK™ (Viralytics Limited, Sydney, New South Wales, Australia). In humans it typically causes mild, self-limiting, upper respiratory tract symptoms [157].

Enterovirus infection, and life cycle within host cells follows a relatively uniform pattern. It is a process initiated by viral attachment to specific cell surface receptors, which allow for receptor-mediated endocytosis. Specifically, with respect to CVA21, two receptors are necessary, Intercellular Adhesion Molecule-1 (ICAM-1) and Delay Accelerating Factor (DAF). ICAM-1 is responsible for internalization of CVA21, whereas DAF functions as a membrane sequestration receptor facilitating the role of ICAM-1. Complete inhibition of CVA21 infection requires dual antibody blocking of both of these receptors [158]. The presence of DAF in isolation is not sufficient to allow internalization of CVA21, as this stage is dependent on ICAM-1. It does however act to accumulate virus at the cell surface, thereby facilitating and optimizing the function of ICAM-1 [159].

Receptor-mediated endocytosis of enteroviruses is followed by virion uncoating, a process which is either mediated by the entry receptor, as is the case for ICAM-1 and CVA21 infection [157, 158], or pH changes within the cytoplasm [160]. This uncoating releases the viral genome from the capsid into the cytoplasm. The viral RNA is subsequently translated into a single, large polyprotein which is proteolytically cleaved into replication and capsid proteins in the cytosol. Replication proteins facilitate genome replication on membranous replication

organelles within the host cell; the resultant positive sense RNA can then undergo further replication or can be packaged into progeny virions. Capsid proteins self-organise into protomers and pentamers, and when combined with genomic viral RNA form pro-virions which are converted to infectious virions subsequent to specific protein cleavage [160] (**Figure 1-7**). Mature virions either exit the cell via release of extra-cellular vesicles, or in latter stages of infection, through cell lysis.

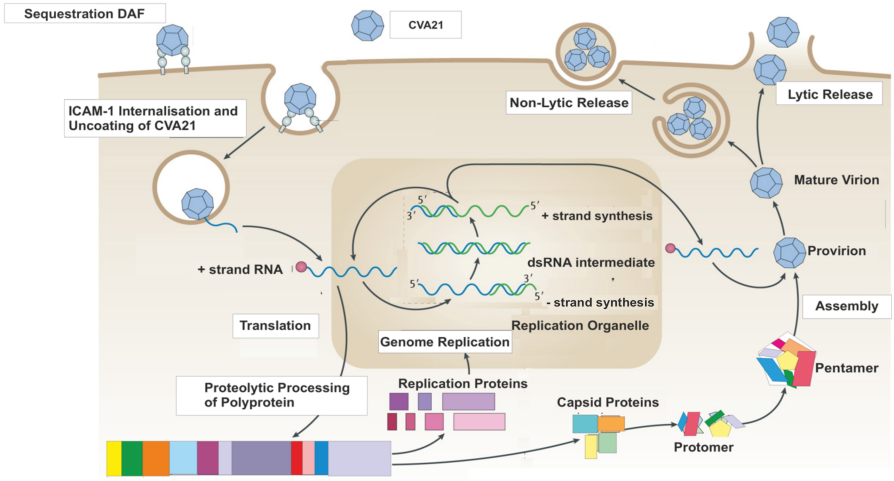


Figure 1-7: Lifecycle of CVA21. CVA21 binds to both DAF and ICAM-1 and undergoes endocytosis and uncoating facilitated via ICAM-1 in order to deliver its positive strand RNA genome into the cytoplasm. Genome translation results in a single polyprotein which is subsequently cleaved into replication and capsid proteins. Genome replication commences on membranous organelles, with a negative strand RNA serving as a template for new positive RNA molecules. These positive RNA sequences can either enter a new round of replication or form constituent parts of progeny virions. The capsid proteins self-organize into protomers and pentamers; in combination with replication machinery and genomic RNA and assemble into provirions. These provirions are converted to mature, infectious virions via protein cleavage, and exit the cell via release of extracellular vesicles or, in late-stage infection, via cell lysis. Image adapted [160].

1.3.3.1 CVA21 Oncolysis

In recent years, CVA21 has been investigated as a promising OV, partly due to the upregulation of ICAM-1 demonstrated on a plethora of cancers relative to comparative normal tissue [161-163]. Specifically, this phenomena of increased ICAM-1 expression in cancerous cells relative to normal tissue has also been demonstrated in CRC [164-166]. Moreover, there is evidence that CVA21 may have potent direct and indirect activity against cancerous cells, locally and in metastatic disease. For example, Shafren *et. al.* showed that human breast cancer cell lines had higher levels of both ICAM-1 and DAF, which rendered them more susceptible to lytic infection compared to normal breast tissue, an effect that was also sustained in 3D spheroid models. Moreover, the use of murine models in this same study resulted in a significant reduction, not only of primary tumour burden, but also of metastatic disease presence following a single dose of IV CVA21 compared to mock-treated animals [163]. A similar study investigating CVA21 as a potential OV in prostate cancer showed comparable findings; *in vitro* cell line models had higher expression of DAF and ICAM-1 relative to normal tissue and there was therefore significant oncolysis following CVA21 treatment reported [167]. Again, in this study, xenograft models demonstrated a significant decrease in tumour burden following systemic delivery of CVA21 [167]. Furthermore, multiple myeloma (MM) cell lines have also shown cytostatic and cytocidal responses to CVA21, an effect that was replicated *ex vivo* in primary tumour samples obtained from bone marrow biopsies [168]. Although ICAM-1 and DAF expressing MM cells were susceptible to CVA21 infection, associated ICAM-1 and DAF expressing peripheral blood lymphocytes remained resistant to infection [169]. In addition to these described studies, CVA21 has also undergone pre-clinical evaluation for the treatment of melanoma, bladder, lung and head and neck cancers [170]. Early phase studies provide valuable information with regard to CVA21 since although CVA21 can cross species, mouse models are limited to severe combined immunodeficient (SCID) mice in order to support xenografts of tumour models. This therefore limits the scientific information which can be obtained with regard to mechanisms of indirect cytotoxicity.

1.3.3.2 CVA21 Immunotherapy

More recently researchers have begun to investigate the possible mechanisms responsible for CVA21 immune-mediated killing. Although the full complexities of this mechanism have not yet been established, there is some important information being published. Damage-associated molecular patterns (DAMPs) is an encompassing term describing immune-stimulatory molecules released from cells following cell stress or death. Bladder tumours infected with CVA21 induce two such DAMPs, ecto-calreticulin and High mobility group B1 (HMGB1) [171]. HGMB1 is a protein found abundantly within cells, but once released into the extracellular milieu activates inflammatory responses [172]. Binding of HMGB1 to Toll-like receptor 4 (TLR4), is important in the activation of DCs and can facilitate antigen presentation to T-cells [173]. Calreticulin is an endoplasmic reticulum-associated chaperone and its presence on the surface of dying tumour cells serves as an "eat me" signal for antigen presenting cells (APCs), such as DCs [174]. Importantly, PMBCs collected from cancer patients following IV administration of CVA21 demonstrate the ability of CVA21 to activate immune effector cells. In the same study, using CVA21-sensitive and resistant models of multiple myeloma (MM) and acute myeloid leukaemia (AML), respectively, CVA21 was shown to induce potent anti-tumour immune responses in the form of cytokine-mediated bystander killing, NK cell-mediated cytotoxicity and priming of cytotoxic T-cells against known TAA [124]. Interestingly, CVA21-induced anti-tumour immune responses were dependent on the presence of ICAM-1 expressing plasmacytoid DC [124]. Previous CVA21 infection is widespread within the population, which could lead to the hypothesis that immune-mediated tumour killing of CVA21 could be abrogated due to the presence of anti-viral immunity and the presence of circulating NABs against CVA21. Interestingly and importantly, one recent study in mice has demonstrated that, despite prior exposure to the virus, intra-tumoural injection of CVA21 not only resulted in influx of neutrophils, increased activation of T-cells and an increase in inflammatory cytokines of tumoural tissue suggesting both an innate and adaptive anti-tumour response. This data suggests that pre-existing host immunity against CVA21 may not impair its activation of immune-mediated killing [175].

1.3.3.3 CVA21 Clinical Trials

Early phase clinical trials to evaluate the safety of CVA21 are currently underway, and between them encompass many different cancers. The main studies to date include CALM, STORM, MITCI, CAPRA and CANON; universally they have all shown that CVA21 is safe and well tolerated, with a minimal side effect profile [136, 170, 176-179]. Each individual study has also added valuable evidence for the mechanisms of CVA21 antitumour effects. The CALM study administered intra-tumoural injections of CVA21 to patients with advanced melanoma and reported responses in both locally injected lesions as well as in sites distal to the primary injection site [180]. The STORM trial is currently investigating intravenous administration of CVA21 in patients with solid tumours (non-small cell lung cancer (NSCLC), melanoma, bladder and prostate cancers) with or without Pembrolizumab (a PD-1 blocking antibody). Early results from this study highlight that CVA21 treatment is safe and well-tolerated even when used in combination with an additional immunotherapeutic agent [178]. The MITCI study [181] also investigated intra-tumoural injection of CVA21 in patients with advanced melanoma, but more specifically in combination with ipilimumab (a CTLA blocking antibody). This study replicated previous evidence and demonstrated good tolerance to combination therapies incorporating both CVA21 and immune checkpoint immunotherapy; minimal side effects were reported along with promising responses in local and distal disease sites. Importantly, tumour regression was also observed in patients who failed to respond to previous lines of immunotherapy [181].

The CANON study was a phase I/II clinical trial of intravesical administration, prior to surgery, of 15 patients with non-invasive bladder cancer, with or without mitomycin-c. In concurrence with the other studies detailed, the CANON study confirmed that CVA21 treatment was well tolerated with minimal side effects [176]. Whilst the study was based on low patient numbers the results were promising, with macroscopic evidence of tumour inflammation in response to CVA21 and one patient experiencing a complete tumour response which was confirmed histologically. The study reported evidence of viral infection in 12 out of 14 resected tumour specimens, with no evidence of replication in normal urothelium or stromal cells; replication competent virus was also detected in patient urinary samples following treatment. These

findings suggest that CVA21 has the ability to specifically target tumour tissue over normal counterparts [176].

Specific information with regard to potential immune mediated killing was established from the CANON study. There was evidence of increased levels of HMGB1 in urine following treatment; in using immunohistochemistry (IHC) it was noted that untreated bladder controls had predominately nuclear localisation of HGMB1 whereas CVA21 treated samples had higher expression in cell cytoplasm [171]. In addition to these findings, nanostring evaluation demonstrated that IFN-inducible genes were increased in CVA21-treated tumour cells. In parallel, there was upregulation of apoptotic genes following CVA21 treatment and more than 50% of the resected tumours showed an increase in the immunogenic cell death marker, Calreticulin [171]. However, there was no evidence of a difference in CD8+ T-cell infiltrates following CVA21/mitomycin-c treatment and there was an increase in immune checkpoint molecules in bladder tumour following CVA21 treatment (PD-L1, LAG3 and IDO) [171]. This finding could suggest that a combination of CVA21 with immune checkpoint inhibition may be of benefit.

In summary, early phase clinical trials in a variety of solid tumours have shown CVA21 to be a safe treatment, regardless of mode of delivery, with evidence of tumour specificity, viral replication, tumour specific cytotoxicity and evidence of immune potentiation. Many of the clinical trials discussed herein have shown that a combination of CVA21 treatment with additional therapeutic modalities have resulted in an increased tumour response. This is in keeping with findings from other, pre-clinical studies; synergism has been shown when using the chemotherapeutic agent Docetaxel in combination with CVA21 treatment on a number of immortal cell lines of NSCLC, and translation of this work into xenografts controlled progression of disease [182]. A similar increase in cytotoxicity has been demonstrated in bladder cancer cell lines in response to a combination of both mitomycin c with CVA21 and radiotherapy with CVA21 [183]. Moreover, combinations of CVA21 and anti-PD-1 mAbs resulted in greater anti-tumour activity than either treatment alone [184].

1.4 Conclusions

OVs take advantage of the TME to preferentially infect, replicate within and ultimately destroy malignant cells. In doing so they create an immunologically hot environment which results in a complex immunological cascade resulting in systemic immunological, target specific death of malignant cells. Since the immune system clearly plays a critical role in the establishment, progression and recurrence of malignant disease, OV treatment provides an exciting avenue for the treatment and long-term control of cancer.

Research into the use of OVs in the context of CRC is in its early stages and has shown some promising results. CVA21 has not yet been investigated as a potential OV for CRC, although it has been shown to result in direct cytotoxicity of other malignancies which express its cell entry receptor, ICAM-1. It has also shown potential to direct immune mediated death of malignant cells. Since ICAM-1 is known to be upregulated in CRC and, given the efficacy of CVA21 against CRC has not been extensively investigated to date, it provides a novel area of research to undertake.

As a further avenue of research, we have briefly discussed the benefits of combining OVs with current standard of care and literature has shown that CVA21 has the ability to work synergistically with chemotherapeutic agents, such as Doxorubicin [185], and radiotherapy in bladder tumours [186]. Given that standard of care of CRC often involves chemotherapy and/or radiotherapy, investigating the potential of CVA21 in combination with these treatments provides a novel approach for this thesis.

1.5 Project Hypothesis and Aims

This study aimed to investigate if CVA21 could be used as an effective treatment for CRC. We designed this research to assess the effects of CVA21 treatment both in the context of direct cellular cytotoxicity and potential immune-mediated killing that could occur. In addition to this, we also sought to investigate how the use of CVA21 may modulate the cellular sequalae which occur as a result of the use of existing standard treatments currently used in the clinical armoury against CRC. In order to achieve this the following specific project aims were devised;

- Establish the potential direct cytotoxicity of CVA21 against CRC through studying the expression of CVA21 entry receptors, resultant infectivity, CVA21 replication and possible cell death.
- Investigate the potential immune mediated killing of CRC following CVA21 treatment through study of activation of immune cell populations.
- Explore possible changes within points 1 and 2 following CRC cell line treatment with radiotherapy.
- Consider translation into human in vivo models by investigating expression of viral entry receptors on human tissue prior to and following radiotherapy treatment.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Cell Culture Methods

Cells were resurrected from cryopreservation by rapid thawing in a 37°C water bath and were promptly washed in 10x excess culture medium before centrifugation (5 minutes at room temperature at 400 *x g* using an Eppendorf 5810 centrifuge) and resuspension in fresh culture medium. Cells were then transferred into vented, plastic tissue culture flasks (Corning® and Costar®) to be maintained at 37°C in humidified atmosphere of 5% CO_2, using an incubator with continuous UV decontamination (Sanyo). Cell cultures were passaged, near confluence (approximately 80%), every 3-4 days under aseptic conditions in a Nuaire Class II laminar flow microbiological safety cabinet. Adherent cells were washed using sterile phosphate buffered saline (PBS, prepared using Dulbecco's A PBS tablets in distilled H_20 [Oxoid™]), then subsequently mobilized using trypsin (10x stock solution diluted to 1:10 with Hanks' Balanced Salt Solution [HBSS], both Sigma-Aldrich) at 37°C. Cells were subsequently passaged following re-suspension in fresh media. Cell counts were undertaken using trypan blue (0.2% in PBS, sigma-Aldrich) and an Improved Neubauer haemocytometer. Cells were replaced regularly to ensure that passage number did not exceed 20. All cells were routinely tested for mycoplasma infection and were free from contamination.

2.1.2 Cell Lines

All CRC cell lines (SW480, SW620, HCT116 and HT29) were purchased from American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified eagles medium (DMEM, Sigma-Aldrich) containing 1% L-glutamine and supplemented with 10% foetal calf serum (FCS, Thermo-Fischer Scientific). Further details regarding the cell lines used are provided in (Table 2-1). CRC cell lines were selected not only to model a primary cancer with subsequent metastasis (SW480 and SW620) and also to provide variation in consensus molecular subtypes (CMS) and MSI [187]. Mel624 (human melanoma) cells (used for plaque assay) were also cultured in DMEM containing 1% L-glutamine and 4500mg/L glucose and 10% FCS.

Cell Line	Origin	Location	Histology	Stage	Derivation
SW480	50-year old male	Descending colon	Moderately differentiated adenocarcinoma	Dukes B	Primary Tumour
SW620	51-year old male	Lymph node metastasis	Moderately differentiated adenocarcinoma	Dukes C	Lymph node metastasis
HCT116	48-year old male	Ascending Colon	Poorly differentiated colon cancer	Dukes D	Primary Tumour
HT29	44-year old female	Colon	Moderately differentiated colon adenocarcinoma	Dukes C	Primary Tumour

Table 2-1: CRC Cell Line Library. A summary of the demographics, histology and site of the CRC cell lines used in experiments.

2.2 Oncolytic Viruses

Wild Type Coxsackievirus A21, *Kuykendall* strain (CVA21, CAVATAK™), was initially provided by Viralytics Ltd (Sydney Australia). Later experiments were conducted using CVA21 propagated from wild-type CVA21 obtained from ATCC® (ATCC® VR-850™) by Dr Matthew Holmes at The University of Leeds.

2.3 Human Blood Sampling and Tissue Specimens

2.3.1 Blood Sampling

Peripheral venous blood samples were collected from healthy volunteers by peripheral vein phlebotomy following informed verbal consent in accordance with University of Leeds Institute of Cancer and Pathology Guidelines. Samples were collected using aseptic, non-touch technique (ANTT) in K3EDTA-coated Vacuette® blood sample tubes (Greiner Bio-one). Additional blood samples were obtained from healthy donor blood in leukocyte apheresis cones and were provided by National Health Service Blood and Transplant (NHSBT).

2.3.2 Isolation of Human PBMCs

For NK cell experiments, whole blood from healthy volunteers was mixed at a 1:1 ratio with Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich). This solution was then carefully layered over the top of 15mL of Lymphoprep™ reagent (Fresenius-Kabi Norge AS). Samples were centrifuged at 800 *x g* for 20 minutes, in the absence of braking. For DC experiments, healthy donor blood was provided by NHSBT in leukocyte apheresis cones. The content of these cones was diluted with 50mL (1:5) HBSS, then layered carefully onto 15mL (2:1) Lymphoprep™, in 2 x 50mL falcon tubes, without allowing the layers to mix. The layered samples were centrifuged at room temperature at 800 *x g*, in the absence of brake. The PBMCs were harvested using a wide-tipped Pasteur pipette (Alpha Laboratories), mixed with 30mL HBSS and further centrifugation at 200 *x g*, room temperature, for 20 minutes with brake set to 3. Two further washes of the PBMC pellets with 30mL HBSS and centrifugation at 300 *x g* for 10 minutes were undertaken. Resultant PBMCs were maintained in Roswell Park Memorial Institute-1640 (RPMI) (Sigma Aldrich) containing 1% L-glutamine and 10% FCS.

2.3.3 Colorectal Cancer Tissues Samples

Colorectal cancer tissue samples were obtained courtesy of Dr Nick West, obtained from archival blocks within the department of Histopathology at Leeds teaching Hospitals NHS Trust (Research Ethics Committee 08/H0903/62).

2.4 Cell Treatments

2.4.1 TNFα Treatment of *in-vitro* Cell Culture

CRC cell cultures were treated with human recombinant TNFα (R&D Systems) at a dose of 0, 10 or 100 U/mL for 24 hours prior to flow cytometric analysis for ICAM-1 expression or treatment with CVA21.

2.4.1 ICAM-1 Supplementation of PBMCs

PBMCs were cultured in complete RPMI supplemented with increasing concentrations of human recombinant soluble ICAM-1 (sICAM-1) (eBioscience). Increasing doses of 0, 200, 300 and 400 ng/mL were used in order to represent sICAM-1 cells associated with healthy controls and those associated with local CRC and widespread disease [188], respectively. PBMCs were incubated in sICAM-1 conditioned media for one hour prior to any subsequent virus treatment.

2.4.2 Irradiation of *In-Vitro* Cell Samples

Cell cultures were washed with PBS, mobilized with trypsin, then further washed with culture media before re-suspension in 3mL of media. Cell suspensions were transported, on ice, in 15mL falcon tubes to be irradiated at 0Gy, 5Gy, 10Gy, 20Gy using either a Metrix NDT X-ray irradiator or RS2000 X-ray irradiator (Rad Source). Following irradiation, cells were counted (as above) and cultured for a period of 24 hours prior to any further treatment being administered. Untreated samples underwent the identical transport procedure.

2.5 Flow Cytometry

All flow cytometric analysis was performed on either 2-laser Attune™ Acoustic Focusing Cytometer (Applied Biosystems®), or, for NK cell studies, on a 4-laser CytoFLEX S (Beckman Coulter).

2.5.1 Phenotypic Analysis

To assess for CRC cell surface expression of ICAM-1 and DAF, CRC cells were washed with PBS and mobilised with trypsin. $1x10^5$ -$1x10^6$ cells were harvested, placed in FACS tubes (BD Falcon™) and washed following centrifugation (400 *x g* for 5 minutes) with 1mL FACS buffer (Phosphate Buffered Saline, 1% Sodium Azide, 1% FCS), then re-suspended in 100µL of the same solution. Respective antibodies for the corresponding assay were added at the volumes detailed in **Table 2-2** and these samples were stained at 4°C in the dark for 30 minutes. Subsequent to this, cells were again washed with FACS buffer and fixed with 300µL of 1% paraformaldehyde (PFA) and stored in the dark at 4°C. All samples were analysed within a 7-day period.

Target Molecule	Protein	Fluorochrome	Volume Added	Species of Origin	Name of Supplier	Catalogue Number
IgG2A		PE	5µL	Mouse	BD Biosciences	3404596
IgG2B		PE	5µL	Mouse	BD Biosciences	555058
IgG1		PE	5µL	Mouse	BD Biosciences	555749
CD54	ICAM-1	PE	5µL	Mouse	BD Biosciences	940072
CD55	DAF	PE	5µL	Mouse	BD Biosciences	555694
CD69	Type II C-lectin receptor (NK activation)	FITC	2µL	Mouse	Biolegend	130-092-166
CD56	Neural Cell Adhesion Molecule	PE	2µL	Mouse	Biolegend	130-090-755
CD3	Cluster of Differentiation 3	PerCP	2µL	Mouse	Biolegend	130-133-131
CD107	Cluster of Differentiation 107a	Viobright	2µL	Mouse	Miltenyi Biotec	130-106-233

Table 2-2: Human Flow Cytometry Antibodies Used for Immunophenotyping.

2.5.2 CellTracker™ Staining

For identification of CRC within PBMC co-cultures, CellTracker™ Green CMFDA (CTG) (Invitrogen) was used from a 5mM stock solution in DMSO. Culture media was removed from cells, and then cells were re-suspended at 10^6/mL in serum free RPMI. Following this, CellTracker™ Green was added at a 1:2000 dilution and incubated for 30 minutes at 37°C. Cells were washed three times with 50mL culture media prior to use in further experiments.

2.5.3 Cell Viability; LIVEDEAD®

Cell viability was examined using LIVE/DEAD® Fixable Red Cell Stain Kit (Invitrogen™). Cell samples were harvested with trypsinisation, placed into FACS tubes and the supernatant washed with 1mL PBS at 400 xg. Specimens were then stained with red fluorescent LIVE/DEAD® stain (1μL/mL in PBS) at 0.5mL/sample and left in the dark at 4°C for a minimum of 30 minutes. Samples were washed with 2mL PBS, fixed with 1% PFA and underwent flow cytometric analysis using 2-laser Attune™ Acoustic Focusing Cytometer (488 nm laser) within 7 days of sample fixation.

For phenotypic analysis following irradiation, samples were double stained to allow ICAM-1 expression to be quantified on viable cells only. $1x10^5$-$1x10^6$ cells were washed with PBS and stained with yellow LIVE/DEAD® fluorescent stain for 30 minutes in the dark, at 4°C, before being washed with FACS buffer prior to staining with relevant phenotype isotype or antibodies described in section 2.51 (**Table 2-2**) and undertaking analysis using 2-laser Attune™ Acoustic Focusing Cytometer (405 nm laser).

2.5.4 NK Cell Activation

CD69 was used as a marker of early activation of NK cells. PBMCs were cultured in 24 well plates in 1mL RPMI +/- sICAM-1 (0, 200 or 400 ng/mL) for 1 hour at 37°C prior to treatment with CVA21 (0, 0.1 or 1 pfu/cell). Samples were left overnight, then centrifuged (5 minutes, 400 xg), and transferred to a 96-well round-bottomed plate (0.5E6 cells in 200μL RPMI). 150μL

of antibody master mix (**Table 2-3**) was added and specimens were incubated in the dark at 4°C for 20 minutes. Samples were washed in FACS, centrifuged (5 minutes, 400 xg) and fixed in 150µL 1%PFA.

Assay	CD3	CD56	CD69	CD107	Breveldin A	FACS solution	RPMI
NK Cell Activation	56µL	56µL	56µL	-	-	4032µL	
NK Cell Degranulation	156	156	-	156	15.6µL	-	3416.4 µL

Table 2-3: Concentrations of Antibody Master-mix for NK Cell Activation and Degranulation Assays.

2.5.5 NK Cell Degranulation

CD107 was used as a functional marker of NK cell activation by way of quantifying degranulation of cytotoxic granules. Assays were undertaken in 96-well, round bottomed plates. PBMCs in RPMI were treated with increasing concentrations of sICAM-1 and incubated at 37 °C for one hour prior to treatment with +/- CVA21. Virus treated samples were incubated overnight, for a minimum of 12 hours. The following day, plates were centrifuged for 5 minutes at 400 xg, and resultant cell pellets were re-suspended in 150µL RPMI. The pre-treated PBMCs were then cultured alone, or co-cultured with target CRC cell lines, at a ratio of 10:1 (5E5 PBMCs, 5E4 CRC), for 1 hour at 37°C in 96-well round-bottomed plates. Following this hour, 50µL of antibody master mix (**Table 2-3**) containing antibody mix and Brefeldin A (1:1000, BioLegend, San Diego, CA) was added to each well and a further incubation period of 4 hours was undertaken. Plates were then centrifuged for 5 minutes, room temperature at 400 xg, then washed with 150µL FACS buffer and fixed in 150µL of 1% PFA.

2.5.6 NK Cell Killing Assay

Target CRC cells were stained with CellTracker™ Green. CVA21 treated PBMCs were added to CRC targets (2.5 x10^4 cells) at a ratio of 20:1in 96-well round-bottom plates in 200μL complete RPMI-1640. Co-cultures were incubated at 37°C for 5 hours then centrifuged at 400 xg for 5 minutes and washed with 250μL/well of PBS before staining with yellow Live/Dead®. Cells were incubated in the dark at 4°C for 30 minutes, washed with PBS and fixed in 150μL 1% PFA.

2.6 Analysis of Cell Viability Using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

MTT was used as a method of assessing metabolic activity, and therefore delineate cell viability following CVA21 infection either alone, or subsequent to TNFα or radiation exposure. CRC cell lines were seeded in triplicate in 96-well flat-bottomed plates at a volume of 200μL and concentration 4x10^4 cells/mL in DMEM + 10% FCS. Cells on 96-well plates were incubated at 37°C for a period of 24 hours prior to CVA21 treatment. Following virus treatment, plates were incubated for 24, 48,72 or 96 hours. Subsequent to culture, 20 μL/well MTT (5mg/mL, Sigma) was added, and cultures further incubated at 37°C for 4 hours. Supernatants were removed and crystals were solubilised in 150μL DMSO before measuring optical density using Thermo Multiskan Ex™ microplate reader, optical filter 405nm.

2.7 Clonogenic Assay

CRC cell lines were seeded in 6 well plates (0.5 x 10^7 cells) and allowed to incubate at 37°C for 24 hours prior to CVA21 treatment. CVA21 was added at a dose of 1pfu/cell to treat samples for 4 hours. Following this, media was removed, samples were washed and CRC cells mobilized using trypsin. Cell lines ± CVA21 treatment at 1 PFU were plated in duplicate at different cell numbers (10, 25, 50 and 100) into 6 well plates and left overnight to become adherent. The following day media was replaced by an overlay of 1:1 20% FCS supplemented

2x DMEM and 3% carboxymethylcellulose (CMC). Cells were left to incubate at 37°C for 8-12 days to enable colony formation.

Plating efficiency (PE) was calculated for each cell line, whereby;

$$\boldsymbol{PE = n/s}$$

$\boldsymbol{n}$ = number of colonies formed, and $\boldsymbol{s}$ = number of cells seeded.

A % surviving fraction (SF) of treated samples could then be calculated as;

$$\%\, \boldsymbol{SF} = \frac{\boldsymbol{nt}}{\boldsymbol{s}} \boldsymbol{x}\, \boldsymbol{PE}$$

Where $\boldsymbol{nt}$ = number of colonies formed after treatment.

2.8 Plaque Assay

2.8.1 Sample Preparation

Plaque assay was used to determine CVA21 concentration in infected CRC cells following +/- radiation treatment. CRC cell lines were cultured in 24 well plates, 7.5 x10^4 cells/well for 24 hours prior to infection with CVA21 at 0 or 1 pfu/cell. Supernatants (1mL) were collected at 30minutes as a control, and 24-hourly intervals thereafter up to a maximum of 72 hours. Samples were centrifuged (5 minutes at room temperature at 400 xg using an Eppendorf 5810 centrifuge) and then supernatants were frozen down immediately to -80°C for storage. Samples were utilised within 30 days of collection.

2.8.2 Sample Dilutions

Using a 96-well plate, ten-fold serial dilutions of thawed sample supernatants and stock CVA21 were prepared in serum free DMEM ranging from 1x10^{-2} – 1 x10^{-13}. For titring of sample supernatant, 25μL of sample supernatant was added to 225μL of serum free media ad 1/10 serial dilutions created across the wells whilst ensuring pipette tips were changed

with each dilution. Titring stock virus followed an identical method, however 2.5μL of stock virus was added to 247.5μL serum free media.

2.8.3 Calculation of CVA21 Concentrations

To quantify PFU/mL in CVA21 treated samples, Mel624 were seeded into 6 well plates at $9x10^5$ cells/well in DMEM supplemented with 10% FCS and placed at 37°C for 24 hours to allow cells to adhere and ensure 95% monolayer confluency. On the subsequent day, media was removed from the Mel624 cultures and replaced with 500μL/well of the 10-fold serial dilutions of sample supernatants and stock CVA21. Samples were added in duplicate for 2 hours to allow for viral cell entry. After 2 hours the supernatants were then removed, taking care not to disturb the monolayer, and 2mL of overlay media consisting of a 2:1 ratio of 20% FCS supplemented DMEM and 3% carboxymethylcellulose (CMC) was added. After 72 hours of incubation at 37°C, the overlay media was removed, cells were then washed with 2mL PBS and fixed in 1% paraformaldehyde solution (PFA) (0.5mL/well) for 20 minutes. PFA was removed and cells were stained with methylene blue for 10 minutes. Subsequently, cells were washed with water and plates were allowed to dry at room temperature. Plaques were counted using a light box and concentration of CVA21 (pfu/mL) was calculated using the following formula (where $\boldsymbol{D}$ = the dilution of which plaques were counted, and $\boldsymbol{V}$= the volume of diluted virus added).

$$\boldsymbol{pfu/mL = Number\ of \frac{Plaques}{DxV}}$$

2.9 Gene Expression Profiling (Nanostring)

Nanostring analysis of CRC samples was facilitated courtesy of Dr Anna Wilkins (Division of Radiotherapy and Imaging, Institute of Cancer Research, London, UK). Archival CRC tissue was macro-dissected from unstained slides and nucleic acids were extracted using the RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fischer Scientific, UK) and quantified using Qubit Fluorometry (Thermo Fischer Scientific, UK). The expression of genes within the NanoString panCancer Immune Panel (NanoString Technologies, Seattle, Washigton) were measured according to manufacturer's instructions and normalized using positive and negative controls and the housekeeping genes included in the panel. The presence of a batch effect in log2 transformed and normalized data was assessed using exploBatch and corrected using *ComBat* from SVA Bioconductor-based R package [189].

2.10 Statistical Analyses

All statistical analyses were performed using Graphpad Prism for MacOS (version 8.4.1, Graphpad Software Inc). Graphical data throughout was represented using mean ± standard error of the mean (SEM). For determination of statistical significance when comparing two groups (results were considered statistically significant if $p<0.05$) *p*-values were calculated using Student's *t*-test with two-tailed distribution. When comparing three or more groups either one-way analysis of variance (ANOVA) or two-way ANOVA were used.

3 Direct Cytotoxicity of CVA21 Against Colorectal Cancer Cell Lines

3.1 Introduction

As discussed in Chapter 1.2, CRC is a common malignancy with increasing numbers of cases and, despite screening programs and significant improvement in treatment options and survival, remains a significant and increasing clinical burden globally [190]. For patients with advanced, or stage IV CRC, predicted 5-year survival is poor, at less than 10% [191], with a median survival of just 30 months [97]. Those patients diagnosed at an earlier stage of CRC may have a significantly better prognosis in terms of 5-year survival, however risk of recurrence, either locally, within the peritoneum, or systemically, is up to 30% [56, 192].

To date, there are currently no approved OVs for use in the treatment of CRC, although there is good evidence from pre-clinical studies [143, 144, 151, 193] and promising results from early phase clinical trials [141, 177, 194-196] to suggest that OVs could provide a novel and safe therapeutic regimen to improve outcomes for CRC patients with advanced or recurrent disease. The plethora of published data demonstrating the potential for effective and safe treatment of CRC with OVs is predominately surrounding HSV, Reo and VV; however, at present there is no research published with respect to CVA21 as a possible treatment option for CRC.

CVA21 is an OV which is not yet licensed for treatment of cancer, although a multitude of studies have investigated its role in melanoma, MM, breast, prostate, bladder, lung and head and neck cancers [124, 170, 171, 183]. Part of the appeal of CVA21 as an OV is that it infects and replicates in cells which express ICAM-1, a membrane receptor which is frequently over expressed in many cancers, including CRC [197]. Given the lack of published work with regards to CVA21 in treatment of CRC, this chapter investigates its direct cytotoxic effects against CRC using *in vitro* cell culture models.

3.2 Expression of CVA21 Receptors on CRC Cell Lines

As detailed in the introduction, CVA21 infection of cells, replication within them and subsequent cell death is dependent on the presence of the surface membrane receptors ICAM-1 and DAF [198]. Therefore, to investigate the potential of CVA21 against CRC, the expression of these viral entry receptors was initially investigated using flow cytometry. **Figure 3-1** demonstrates the level of ICAM-1 (**Figure 3-1a**) and DAF (**Figure 3-1b**) expressed by the selected panel of CRC cell lines. When mean fluorescence intensity (MFI) was used as a quantitative method to assess ICAM-1 expression only one cell line, SW480, expressed high levels of ICAM-1, the remainder expressed low levels in comparison. Overlay plots suggested a complete absence of ICAM-1 expression on SW620 cell line (**Figure 3-2b**). It should also be noted that there was a small degree of heterogeneity demonstrated with staining across individual experiments on all CRC cell lines and this may be explained by small changes in morphology and phenotype resulting from cell passage.

HT-29 and HCT116 expressed the highest levels of DAF, the co-receptor required for CVA21 sequestration. SW620 had markedly low expression of both ICAM-1 and DAF. Given that only one CRC cell line tested expressed high level ICAM-1, it would suggest that CVA21 may not be a particularly successful direct cytotoxic agent against CRC. However, as a counter argument, the relatively high expression of the co-receptor DAF on HT29 and HCT116 (**Figure 3-1c**) could potentially allow for significant virus concentration at the CRC cell surface membranes to facilitate some ICAM-1-mediated CVA21 internalization and subsequent cytotoxicity.

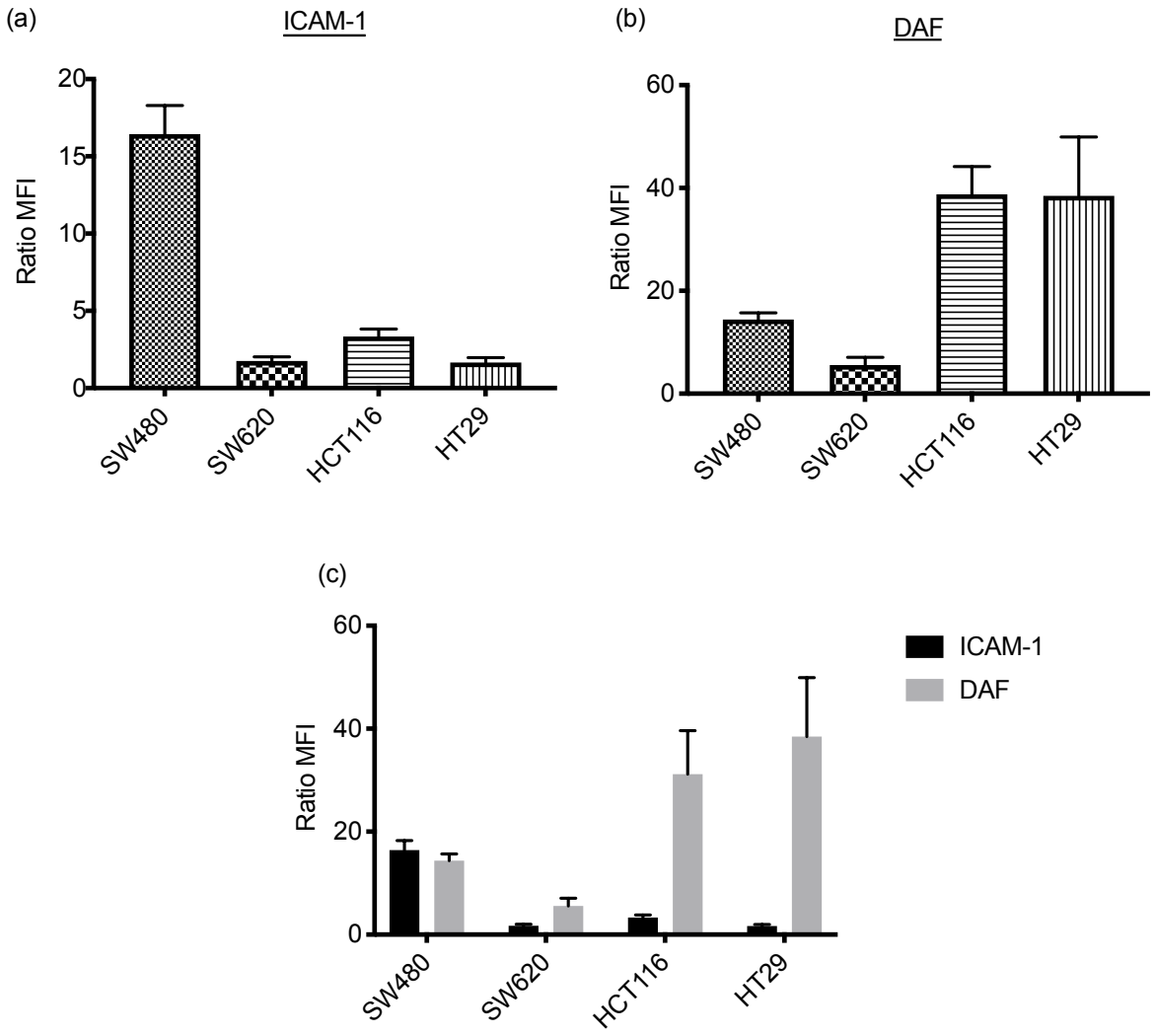


Figure 3-1: Relative Expression of CVA21 Entry Receptors on CRC Cell Lines. Levels of ICAM-1 and DAF expression on a panel of CRC cell lines were quantified using flow cytometry after staining CRC cell lines with either anti-DAF, anti-ICAM-1 antibodies, or an isotype control. Relative levels of receptor expression are represented as a ratio of mean fluorescence intensity (MFI) compared to the relevant isotype. Graphs show mean results of at least n=3 individual experiments with error bars to indicate SEM (standard error of the mean). (a)Illustrates ICAM-1 expression, (b) illustrates DAF expression and (c) compares the proportion of each receptor on each CRC cell line.

3.3 LIVE/DEAD® Analysis of CVA21 Infected CRC Cell Lines.

To assess the susceptibility of CRC cell lines to CVA21 at different time points, LIVE/DEAD® flow cytometry was used, and as expected, cell death correlated positively with expression of ICAM-1 (**Figure 3-2**). Representative overlay plots showing isotype and ICAM-1 staining have also been displayed adjacent to these charts for reference (**Figure 3-2b**). These data confirmed that high levels of ICAM-1 expression correlated positively with significant CVA21-induced cell death, notably demonstrated in the SW480 cell line, which had the highest expression of ICAM-1 (**Figure 3-1a**). CVA21-induced death occurred in a dose and time dependent manner within SW480, with a minimum 48-hour time period required to see significant cell death. At this timepoint cell death was statistically significant at a dose of 5 pfu/cell ($p = 0.0124$, two-way ANOVA). By 72 hours SW480 cell death was significant at both 5 pfu/cell, and also the lower dose of 0.5 pfu/cell ($p = 0.0011$ and $p = 0.0124$, respectively). This statistically significant increase in cell death following CVA21 treatment was maintained at 96 hours. The lowest dose used of 0.05 pfu/cell did not result in any significant cellular death in SW480 at any timepoint. Moreover, no significant cell death at any dose or time was observed in any of the other CRC cell lines tested, confirming that cell death correlated positively with high expression of ICAM-1.

Unfortunately, we were unable to determine whether CVA21-mediated cell death occurred at later timepoints in cell lines that demonstrate lower levels of ICAM-1 expression. This was due to the time frame in which cell cultures needed to be passaged to retain cell viability and health. However, it is possible that CVA21 may result in cell death at later timepoints as a result of low level ICAM-1 and high DAF receptor expression, a pattern exhibited by both HT29 and HCT116 cell lines (**Figure 3-1b**). In support of this hypothesis, it was noted qualitatively, that cell growth as visualised under the microscope following CVA21 treatment, appeared to be impaired in the seemingly CVA21-resistant cell lines when compared to untreated cells. Therefore, it was postulated that CVA21 effects were potentially underestimated using the cell viability LIVE/DEAD® assay.

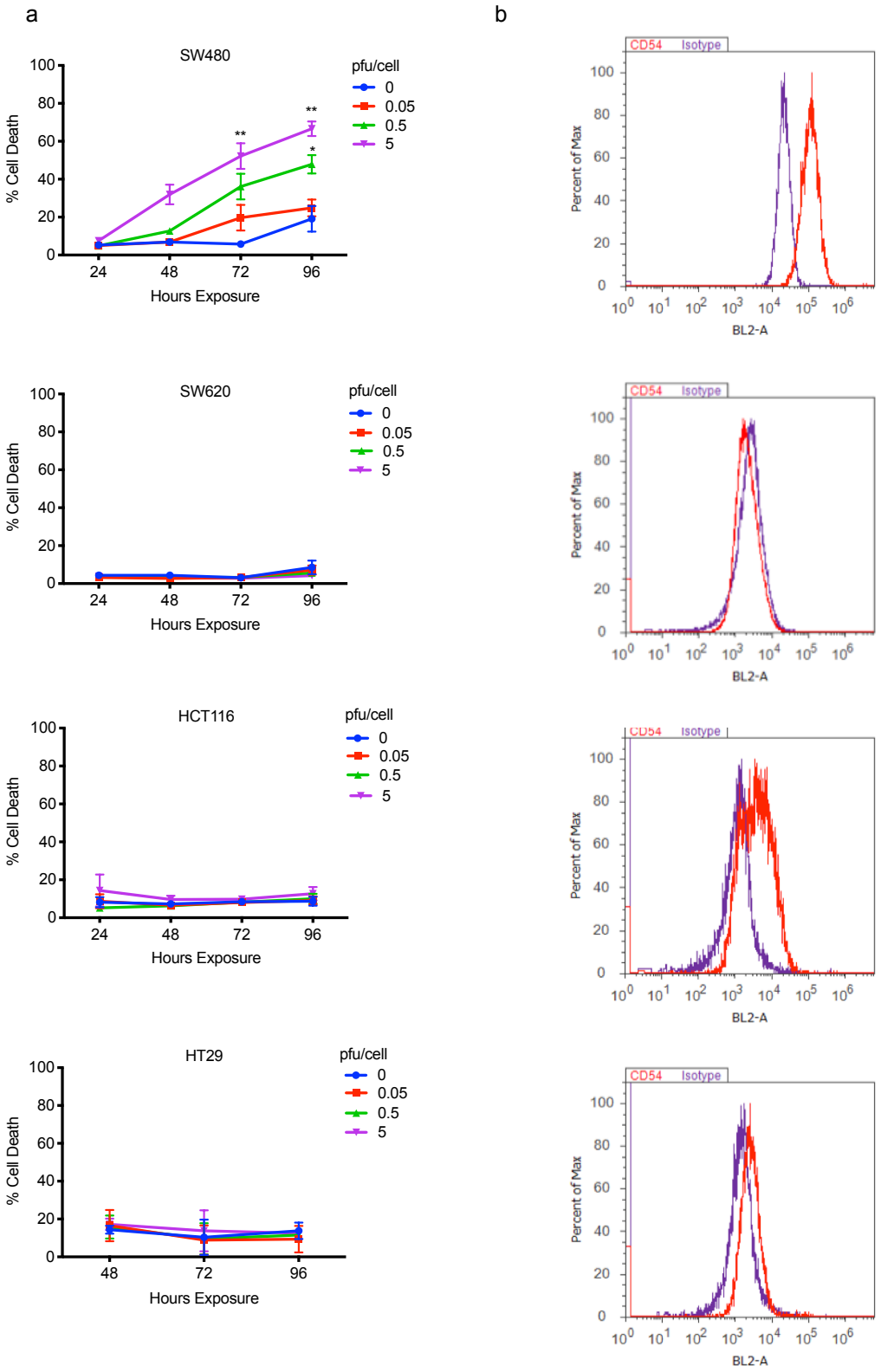


Figure 3-2: Direct Cytotoxicity of CVA21 on CRC Cell Lines. (a) Cell death of SW480, SW620, HCT116 and HT29 following treatment with 0, 0.05, 0.5 or 5 pfu/cell was examined using LIVE/DEAD® staining and flow cytometry. Assessment of cell death was made at 24, 48, 72

and 96 hours following CVA21 treatment. Cell death is expressed as percentage of the parent population, and results presented show the mean percentage of dead cells ±SEM for n= 10 experiments. Statistically significant changes from control experiment at a given timepoint are shown where * represents $p \leq 0.05$ and ** represents $p \leq 0.01$ (calculated using 2-way ANOVA) (b) Flow cytometry overlay plots of ICAM-1 (CD54; red) expression and isotype control (purple) for comparison of degree of viral receptor expression with resultant CVA21-induced cytotoxicity.

3.4 MTT Evaluation of CVA21 Toxicity on CRC Cell Lines

To examine the subjective observation of impaired cellular proliferation, we used MTT as an alternative assay to assess cell viability and growth by investigating the relative metabolism of cell cultures treated with CVA21. Using this method, SW480 cells demonstrated greater than expected susceptibility to CVA21 when compared to the results obtained for LIVE/DEAD® analysis at the same treatment dose (5 pfu/cell) (**Figure 3-3**). For example, statistically significant impairment of relative metabolism was seen at 24 hours (*p = 0.0160*) following treatment of the CVA21-sensitive CRC cell line SW480 with CVA21, however no significant cell death was observed at this timepoint using LIVE/DEAD®. In addition to these significant findings, susceptibility to CVA21 was also evident in the three apparently CVA21-resistant cell lines, HCT 116, HT-29 and SW620 following treatment with CVA21 at a dose of 5pfu/cell (**Figure 3-4**), although effects did not increase in a time-dependent manner. To further confirm this effect, these cell lines were treated with a lower dose of CVA21 (1 pfu/cell), and cell viability/metabolism was again assessed by MTT. At this lower dose of CVA21, a statistically significant decrease in relative cell metabolism/viability was also observed in response to CVA21 treatment in HCT116 and HT29 CRC cell lines, but not the SW620 cell line (**Figure 3-5**). This decreased cell metabolism seen in HCT116 and HT29 was maintained 72 hours following treatment with CVA21 at both the 5pfu/cell and the lower dose of 1pfu/cell (data not shown).

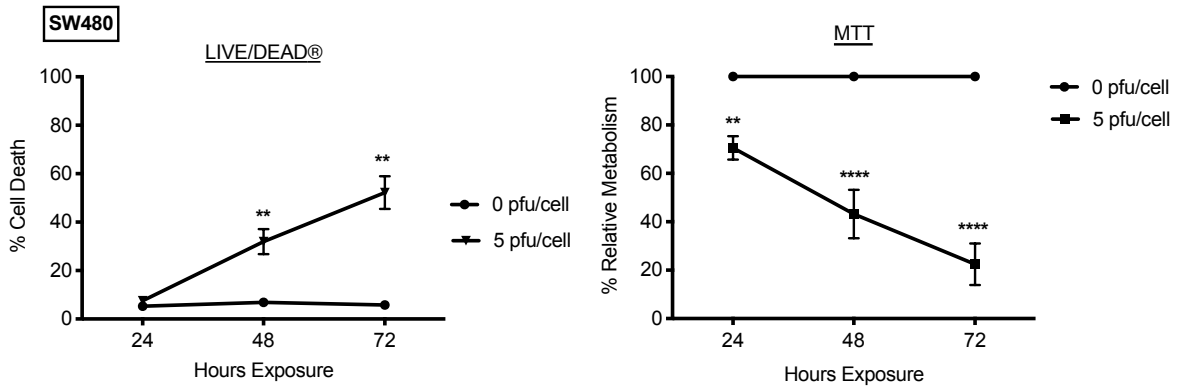


Figure 3-3: SW480 Susceptibility to CVA21; Cellular Death Versus Relative Metabolism. Results show % cell death and % relative metabolism of SW480 for untreated (0 pfu/cell CVA21) versus treated (5 pfu/cell CVA21) SW480 cells at 24, 48 and 72 hours. Data show mean results ±SEM, n =10 for LIVE/DEAD®, n=4 for MTT. Statistically significant differences between 0 and 5 pfu/cell at each timepoint is demonstrated, where ** represents $p \leq 0.01$ and **** represents $p \leq 0.0001$ (calculated using 2-way ANOVA).

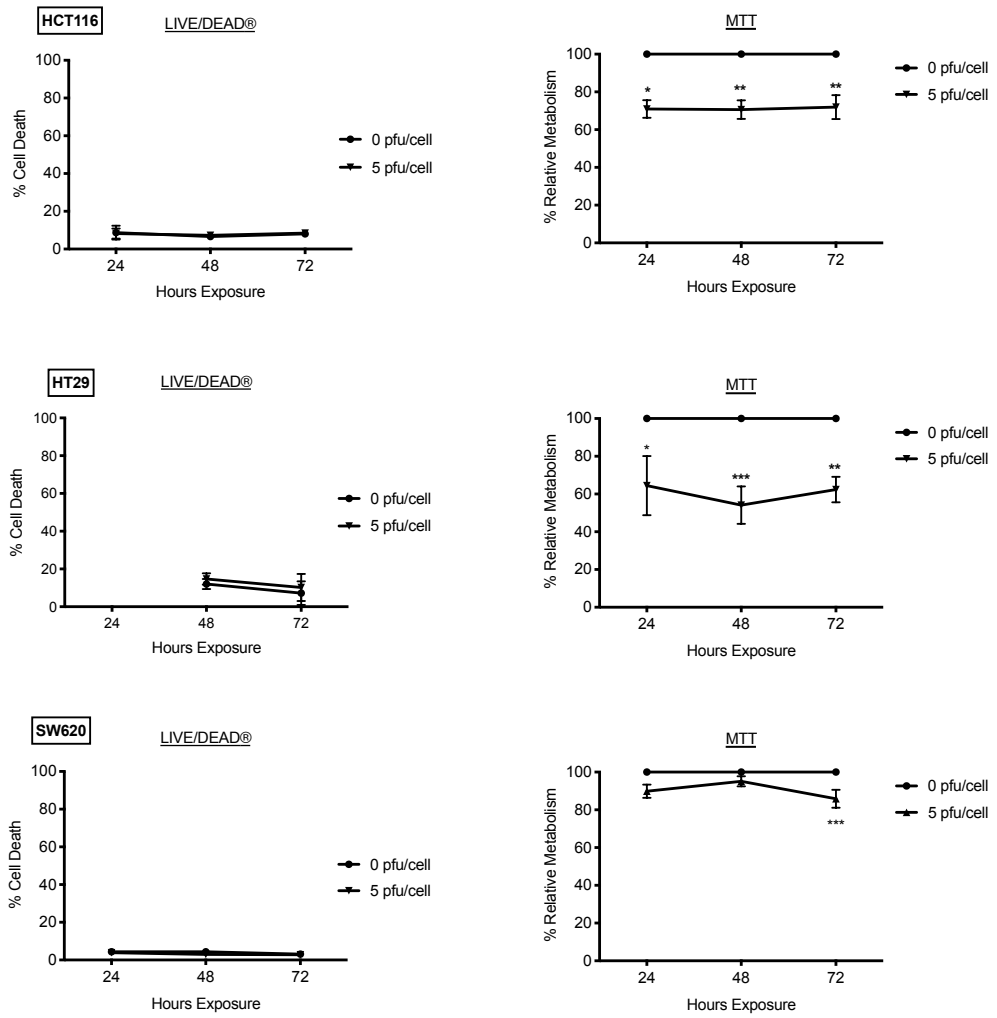


Figure 3-4: HCT116, HT29 and SW620 Susceptibility to CVA21; Cellular Death Versus Relative Metabolism. Results show % cell death and % relative metabolism of HCT116, HT29 and SW620 for untreated (0 pfu/cell CVA21) versus treated (5 pfu/cell CVA21) CRC cell samples at 24, 48 and 72 hours. Data show mean results ±SEM, n =10 for LIVE/DEAD®, n=4 for MTT. Statistical significance between 0 and 5 pfu/cell at individual timepoints is demonstrated, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represents $p \leq 0.001$ (calculated using 2-way ANOVA).

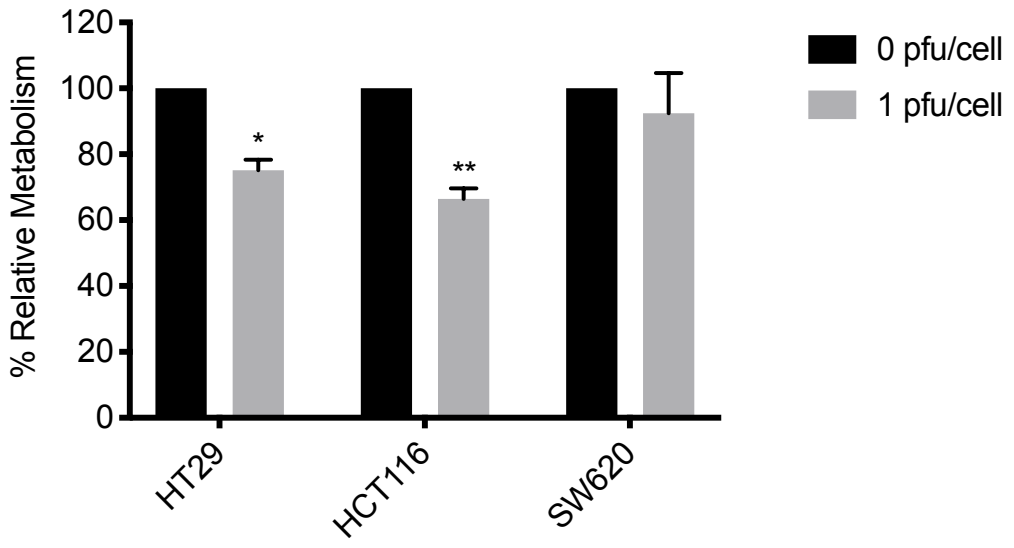


Figure 3-5: MTT Assessment of Cell Viability of CRC Cell Lines Following 48 hours of Low Dose CVA21 Treatment. Graph shows % relative metabolism for CRC cell lines that were resistant to CVA21-mediated cell death using LIVE/DEAD® analysis. Graph depicts MTT data for CRC cell lines treated for 48 hours with CVA21 at a dose of 1pfu/cell. Data shows mean results +SEM, n = 3. Statistical significance compared to 0 pfu/cell is demonstrated, where * represents $p \leq 0.05$ and ** represents $p \leq 0.01$ (calculated using 2-way ANOVA).

Given that MTT evaluation of SW480, SW620, HCT 116 and HT29 demonstrated that relative cell metabolism was significantly impaired following treatment with CVA21, the susceptibility of all four CRC cell lines was re-examined following addition of soluble (sICAM-1) to determine whether sICAM-1, often identified in patients with CRC [199, 200] would impact the sensitivity of CRC cells to the direct cytotoxic/cytostatic effects of CVA21. CRC cell lines were cultured in media, conditioned with increasing doses of recombinant sICAM-1 (0-800ng/mL in 200ng/mL increments) in keeping with serum concentrations of sICAM-1 found in CRC patients [201]. The ICAM-1 enriched CRC cell cultures were treated with CVA21 for 48 hours before cell growth and metabolism was assessed using MTT. No significant change in CRC cell relative metabolism was observed in response to increasing concentrations of sICAM-1. Therefore, this data suggests that the direct cytotoxic/cytostatic effects may not be inhibited by circulating sICAM-1 (**Figure 3-6**).

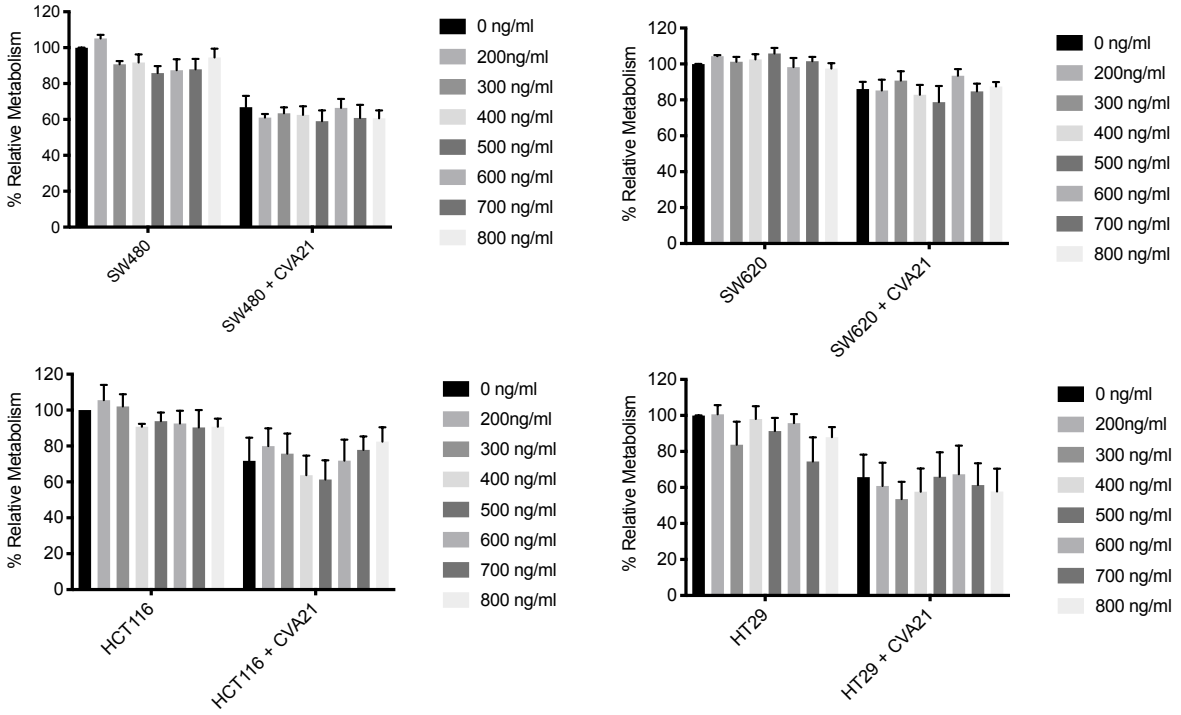


Figure 3-6: Effects of Increasing Concentrations of Recombinant sICAM-1 on CRC Cells +/- CVA21. SW480 cells were treated with 0.1pfu/cell CVA21 due to their marked sensitivity to the direct cytotoxic effects of CVA21, whilst HT29, HCT116 and SW620 were treated with a dose of 1pfu/cell. CRC cells were pre-conditioned for 24 hours with increasing doses of recombinant sICAM-1ranging from 200ng/mL-800ng/mL and then treated with the appropriate doses of CVA21 for a further 48 hours. MTT was then used to quantify changes in relative metabolism. Data show mean results +SEM, n = 3.

3.5 Clonogenic Ability of Colorectal Cancer Cell Lines Following CVA21 Treatment

As a further examination of the hypothesis that proliferation of CRC cells is inhibited by CVA21 treatment, we evaluated the clonogenic capacity of our selected CRC cell lines following a dose of 1 pfu/cell CVA21 (**Figure 3-7**). Unfortunately, due to difficulty in optimising an adequate plating efficiency for the HT29 cell line, consistent results could only be obtained for SW480, SW620 and HCT116. However, results obtained for these three cell lines supported previous findings noted with MTT, that treatment with CVA21 inhibited cell proliferation in the CRC cell lines, SW480 and HCT116. For example, there was a clear and statistically significant decrease in the colony forming ability of SW480 and HCT116 following CVA21 treatment ($p \leq 0.0001$ for each CRC cell line). These results were consistent with the fact that both of these cell lines expressed ICAM-1 (Figure 3-1a) and showed reduced relative metabolism when using the MTT assay (**Figure 3-3, Figure 3-4**). Again, in-keeping with these previous experiments, SW620 cells treated with 1 pfu/cell CVA21 did not show any significant impairment of clonogenic capacity, consistent with its relative lack of ICAM-1 expression (**Figure 3-1a, Figure 3-2b**) and thus, an inability to internalise CVA21 for viral replication.

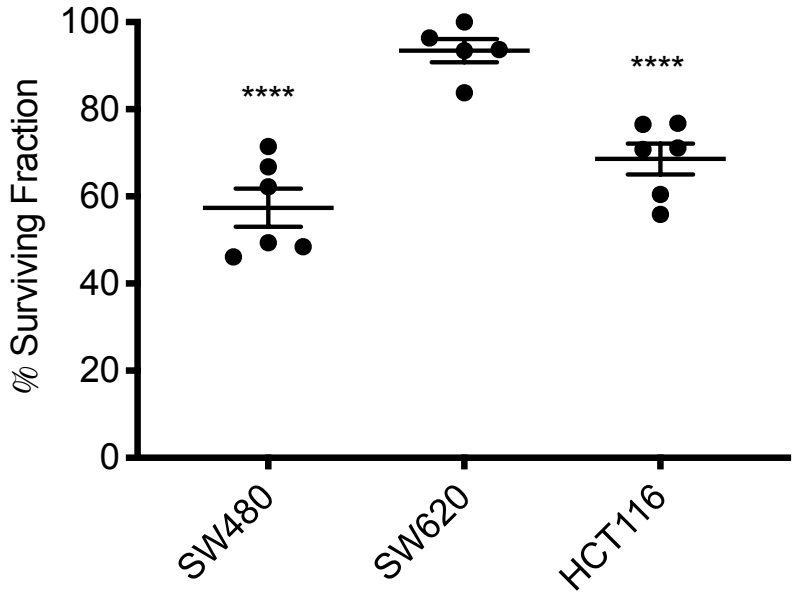


Figure 3-7: Colony Forming Ability Following CVA21 Treatment. CRC cell lines were seeded at a count of $5x10^6$ in 6 well plates 24 hours prior to CVA21 treatment (1pfu/cell) for 4 hours, alongside an untreated control. After CVA21 treatment, supernatants were removed, cells were mobilized and re-seeded at a range of cell densities. After 24 hours, media was replaced with an overlay of 1:1 CMC:2X DMEM and cultures left for a minimum of 7 days. The surviving fraction of cells was calculated as a percentage of surviving colonies proportional to the plating efficiency determined using control, untreated cells. Horizontal bars represent mean percentage surviving fraction ± SEM. Statistical significance is represented where **** demonstrates $p \leq 0.0001$ (2-way ANOVA compared to the untreated control).

3.6 CVA21 Replication Within Colorectal Cancer Cell Lines

To further explore the possible sensitivity of CRC cell lines to CVA21, viral replication in CRC cell lines was assessed by plaque assay (**Figure 3-8**). Data obtained in these experiments shows that CVA21 not only has the ability to infect and replicate in the SW480 cells ($p = 0.0457$ at 24 hours and $p < 0.0001$ at 48 and 72 hours), which were sensitive to its cytotoxic effects (**Figure 3-2a**), but also has the ability to replicate at low levels in both HCT116 and HT29 (**Figure 3-8**). Statistically significant CVA21 replication was demonstrated at both 48 and 72 hours in HT29 ($p = 0.0011$ and $p = 0.0001$) and HCT116 CRC cell lines ($p = 0.0450$ and $p = 0.0013$). The low-level viral replication observed in HCT116 and HT29 cells (**Figure 3-8**) correlates with both the observational finding of impaired CRC culture growth, decreased colony forming ability seen in HCT116 in clonogenic studies (**Figure 3-7**), and the reduced viability of these cell lines as determined using MTT (**Figure 3-4**). There was no evidence of active CVA21 replication in SW620 cells, which is unsurprising given the absence of ICAM-1 expression on this cell line (**Figure 3-2**).

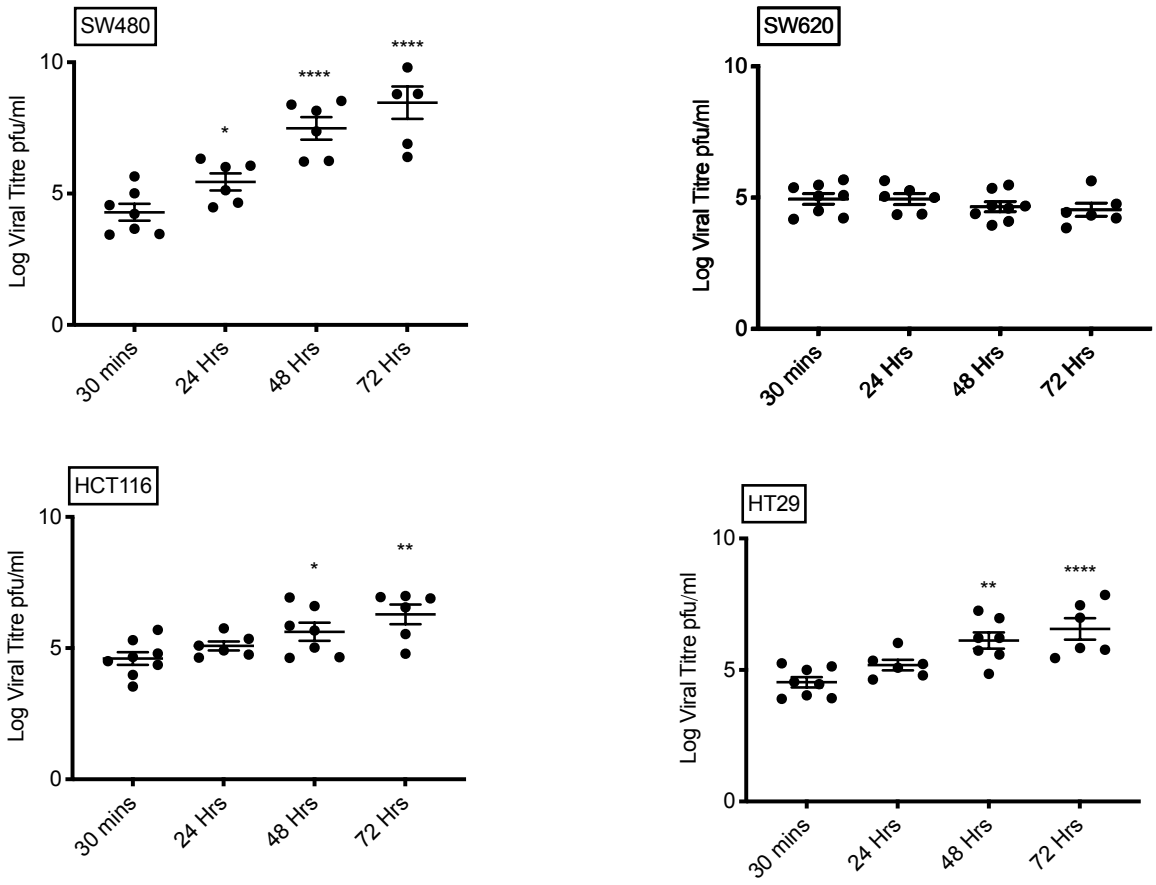


Figure 3-8: CVA21 Replication within CRC Cell Lines. Concentrations of CVA21 (pfu/cell) were determined by plaque assay; 7.5x104 CRC cells from each cell line were treated with 1pfu/cell of CVA21 and the supernatants were harvested at given timepoints of 30 minutes, 24, 48 and 72 hours, frozen at -80 0C and plaque assays were carried out within 1 week. Graphs show log values of mean viral titre (pfu/mL) ±SEM, n = minimum of 5 independent experiments. Statistical significance compared to the 30-minute timepoint is demonstrated, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (calculated using one-way ANOVA).

3.7 Discussion

Although there are currently no licensed OVs for use in CRC, there is good evidence to suggest they may hold therapeutic benefit. For example, patients with CRC liver metastases were included in a Phase I, dose escalation study, of intra-tumoural administration of a modified poxvirus (JX594) study in metastatic and primary liver tumours. The treatment was safe with tolerable, mild, side effects and patients showed a radiological decrease both in tumour size and distant, non-injected lesions despite the presence of circulating antibodies [194]. Subsequent studies have also investigated the use of JX594 in CRC, again showing that OV use results in a tolerable treatment which exhibits anti-tumour activity, detection of replication competent virus in the tumour (despite circulating antibodies) and reduction in size of tumour burden [177, 195]. A genetically engineered HSV (NV1020) has also been shown in early phase clinical studies to stabilise liver metastases in CRC with minimal toxicity [141] and Reolysin has also been investigated in early phase clinical trials in CRC; patients with CRC showed minimal toxicity to treatment and exhibited a marked decrease in tumour markers following treatment [196]. There are currently however, no studies investigating the role of CVA21 use in CRC hence the rationale for this research.

This chapter has herin presented novel investigations regarding potential cytotoxicity of CVA21 in CRC. It describes the presence of membrane bound CVA21 receptors (ICAM-1 and DAF) on CRC cell lines and the subsequent cell death that occurs as a result of CVA21 treatment. In addition to this we have investigated the more subtle effects of CVA21 on CRC cell metabolism and possible cytostatic effects attributable to this OV in the form of MTT and clonogenic studies, in addition to investigating CVA21 replication in CRC cell lines.

Crucially, it is known that ICAM-1 is the receptor responsible for the internalization of CVA21 and DAF is a sequestration co-receptor which can facilitate the function of ICAM-1 [158]. Therefore, in order for CVA21 to be a potential therapy for CRC, receptors need to be displayed at a sufficient level for viral internalization to result in tumour cell death. ICAM-1 has previously been identified within colorectal tumours and adenomas, either bound to cell membranes or found in soluble protein form (sICAM-1) [197, 201] and as such CVA21 may

well have a therapeutic role in the treatment of CRC. Immunohistochemistry of surgically resected CRC tumours has repeatedly demonstrated that ICAM-1 is present in CRC cancer cells, albeit with varying degrees of intensity, but is not evident in adjacent uninvolved, non-cancerous colorectal epithelial cells [197, 202-204]. ICAM-1 has also been observed at high levels in tumour-associated fibroblasts within the stroma of CRC [205].

In addition to high levels of tissue expression of ICAM-1 in CRC, there is a multitude of data to show that the serum of patients with CRC has higher levels of sICAM-1 than a healthy population [188, 201, 206, 207]. In fact, sICAM-1 levels in patient sera have been shown to correlate positively with large tumours and those with lymph node metastasis [188, 201] and liver metastasis [199, 206]. High serum sICAM-1 levels are also associated with poorly differentiated CRC cancer [208] and poor overall survival [188]. Even on the background of normal levels of carcinoembryonic antigen (CEA), a tumour marker for CRC, high sICAM-1 levels are associated with a worse outcome and more advanced disease and it has been suggested that sICAM-1 could therefore be used as a predictor of survival [200]. The paradox exists that, while increasing serum sICAM-1 is associated with increased risk of metastatic disease and worse overall survival, lower levels of membrane bound ICAM-1 are observed in tumours which have metastasized [209], such as in SW620 cell line which we saw no significant ICAM-1 expression, and poorly differentiated tumours seem to have lower levels of ICAM-1 than well differentiated tumours [203]. This again is what we demonstrated in HCT116 and HT29, which originate from poorly differentiated and moderately differentiated tumours respectively. The prognosis of patients with low ICAM-1 expressing CRC cancers is worse [202].

As yet it is unclear how the presence of membrane bound ICAM-1 and sICAM-1 within CRC tumours translate into the levels of sICAM-1 seen in patient sera as there is a paucity of literature relating the two. It may be that there is an underlying mechanism surrounding ICAM-1 for tumour escape. Taglia et al have shown that ICAM-1 mediates tumour cell attachment to the extracellular matrix and therefore potentially prevents cells detaching from the primary tumour mass [203]. Moreover, a downregulation of ICAM-1 in CRC cell lines has been shown to mitigate cell invasion [210], also suggesting that ICAM-1 may be responsible for tumour invasion. In addition, sICAM-1 is documented to be shed from CRC cell

lines [197]. It may be that, once CRC become established that ICAM-1 is shed from the tumour as part of an immune evasion strategy as ICAM-1 is known to have a crucial role in leukocyte-endothelial cell interactions and effector function [211]. The theory of ICAM-1 shedding would be supported by the fact that elevated serum levels of sICAM-1 seen in patients with CRC decrease following curative surgical resection of the tumour [188]. Although high levels of ICAM-1 are expressed in CRC tumours, and high circulating sICAM-1 is also found within patient sera, there are no comprehensive studies that relate the two together and it is currently unknown how the two are related and how this may impact on CVA21 as a potential treatment for CRC. Similar associations have also been documented with regard to elevated sICAM-1 in non-small cell lung cancer [212], gastric [213] and laryngeal cancer [214]. To date, it is currently unknown how levels of sICAM-1 may impact the efficacy of CVA21 as a treatment option for CRC or other cancers.

We have concluded, in keeping with the afore mentioned immunohistochemistry studies, that expression of ICAM-1 on CRC cell lines is present but variable, as displayed in **Figure 3-1** and **Figure 3-2**. High levels of expression of ICAM-1, the receptor required for ICAM-1 internalisation, was only displayed in one (SW480) out of four cell lines investigated and was found to be at low to negligible levels in the remaining 3 cell lines (HCT116, HT29 and SW620). The expression of DAF was ubiquitous across all 4 cell lines investigated (**Figure 3-1, Figure 3-2b**), with the highest level being present in HT29 cells, and the lowest levels in SW620. This is again an unsurprising result given that high DAF expression in CRC has previously been well documented within the literature [215, 216], and although its level of expression in CRC cells does not seem to correlate with stage of disease of prognosis [217, 218], the level of expression within the stroma may correlate with poor outcomes [219].

Of the four cell lines investigated for expression of ICAM-1, three originate from primary CRC tumours (SW480, HCT116 and HT29) and one from a lymph node metastasis and the stage and differentiation of the cell lines are displayed in **Table 2-1**. Even though high levels of membrane bound ICAM-1 are associated with a reduced chance of metastasis, the patient from whom SW480 cell line originated subsequently progressed to develop lymph node metastasis (SW620 cell line). This would imply that, where high ICAM-1 receptor expression is associated with more stable primary CRC, CVA21 could be considered as a treatment

option. By contrast, both HT29 and HCT116 cell lines originated from primary CRC tumours from patients whom had metastatic disease and displayed low levels of ICAM-1 (**Figure 3-1**), discernible from flow cytometry histograms (**Figure 3-2b**), perhaps reflecting their metastatic potential. This could potentially suggest that the efficacy of CVA21 as a treatment in more advanced stages of CRC could be reduced. Given the limited variation of histology on the four CRC cell lines used, it would be interesting to expand the investigation of ICAM-1 and DAF expression to include a much larger panel of CRC cell lines and correlate this with disease stage.

Importantly, and as expected, the level of ICAM-1 but not DAF receptor expression was directly related to the level of CVA21-mediated death seen in LIVEDEAD® studies (**Figure 3-2**) with a maximal percentage cell death of ~67%, 96 hours subsequent to treatment with 5 pfu/cell CVA21. These results are comparable to the amount of cell death achieved in the same cell line with an alternative OV, Reovirus [220], which has been investigated more thoroughly as an OV against CRC. The remaining cell lines, which had poor to no levels of ICAM-1 but all displayed DAF, were resistant to CVA21-mediated cell death at each dose and time-point investigated in our LIVEDEAD® studies. These findings are in keeping with previous research, using melanoma cell lines [169] and prostate cancer [221] which demonstrate that CVA21-DAF interactions in isolation are insufficient to result in CVA21-mediated oncolysis. It is worth noting that, whilst ICAM-1 expression has also been previously documented in HT29 cell line [197, 203, 204], we found only low expression which was not in sufficient levels to allow for CVA21 mediated cell death. However, it is possible that low ICAM-1 expression, alongside high levels of DAF, could induce cytotoxicity at extended timepoints. This hypothesis would be supported by the observation that CVA21 was able to replicate within both HCT116 and HT29 cells.

CVA21 is known to facilitate cell death as a result of a combination of shutdown of host cellular protein synthesis, inhibition of transport of cellular glycoproteins, induction of apoptosis and the proteolytic digestion of transcription factors [222], all of which require cell entry of CVA21 via ICAM-1 in order to occur. As already mentioned, SW480 with high expression of ICAM-1 therefore displayed sensitivity to CVA21 mediated death. The cell lines with low to no expression of ICAM-1 did not show CVA21 mediated cell death in LIVEDEAD ®

studies. Despite this absence of CVA21-mediated cell death observed in the majority of CRC cell lines using LIVEDEAD® studies, it was objectively noted that growth of all the CRC cell cultures subsequent to CVA21 treatment seemed to be impaired or inhibited compared to untreated. We therefore hypothesised that, although CVA21 was not killing HCT116, HT29 or SW620 cells it may still be either successfully infecting them or initiating some aberrant cellular changes to limit cell growth, possibly by way of interaction or binding with DAF. Indeed, the MTT studies we undertook to examine this hypothesis proved to be a more sensitive assay to the subtle cellular effects of CVA21, with three apparently resistant CRC cell lines all displaying significant impairment of cell viability/metabolism in response to treatment CVA21. Importantly, these same MTT studies undertaken in the presence of increasing concentrations of sICAM-1, designed to replicate sICAM-1 levels exhibited by patients with increasing stages of CRC, did not show any inhibition of these effects (**Figure 3-6**).

Clonogenic studies (**Figure 3-7**) confirmed that proliferative capacity was inhibited in SW480 and HCT116 but not in SW620 cells. It would therefore be interesting to see how this cell line would responds in a 3D model of cell culture, such as spheroids in order to ascertain further information with this regard. The results of these findings combined would imply that SW480 shows marked sensitivity to CVA21, HCT 116 and HT29 show intermediate sensitivity and SW620 could be considered to be low to non-responsive.

In analyses of CVA21 titres following plaque assay (**Figure 3-8**), understandably the highest titre achieved of CVA21 was following infection of SW480 cells due to their demonstration of the highest level of ICAM-1. However, CVA21 replication was also evident in the intermediately sensitive cell lines HT29 and HCT116 cells, with a slightly higher level of replication evident in HT29 cells. This is interesting given that HCT116 have marginally higher expression of ICAM-1 than HT29 (**Figure 3-1**), although HT29 have significantly higher levels of DAF, the receptor responsible for CVA21 sequestration at the cell membrane. This finding could potentially highlight a valuable role for DAF in mediating CVA21 responses. Given that SW620 did not express ICAM-1 significantly it is understandable that we did not observe CVA21 replication in this assay, as it is unlikely that the virus could be internalized. However, the fact that we saw a degree of inhibition of metabolism (**Figure 3-4**) despite the lack of

ICAM-1 expression remains of interest. DAF expression was low on this cell line and it raises the question as to whether higher levels of DAF would eventually facilitate CVA21 entry, similar to the findings in HCT116 and HT29.

This is not the first time that such findings have been documented. Skelding et al have also demonstrated that CVA21 replication can occur within breast cancer cell lines with minimal ICAM-1 expression in the presence of high expression of DAF [163]. Shafren et al have also made the observation that pre-treatment of membrane bound DAF with monoclonal antibodies to specific short consensus repeats (SCR) resulted in a dramatic increase in CVA21 infectivity of rhabdomyosarcoma (RD) cells, which are known to be relatively insensitive to CVA21 due to low levels of ICAM-1 [198]. Johansson et al have also reported that that a bioselected variant of CVA21 (CVA21-DAFv) generated by multiple passages in DAF-expressing RD cells acquired the capacity to induce lysis of ICAM-1 deficient cells [223]. In summary, following CVA21 infection of CRC cells, low levels of ICAM-1 are sufficient to result in decreased cellular viability, result in a cytostatic response and sustain viral replication. However, it is possible that all of these responses may be enhanced by the presence of higher levels of DAF expression. By contrast, in the absence of ICAM-1 receptor on CRC, CVA21 does not appear to be internalized or replicate to create viral progeny.

In conclusion, we have demonstrated that that high level of ICAM-1 expression seen within CRC correlates with CVA21 infection, replication, impairment of metabolism and tumour cell death. This is in keeping with findings reported in multiple other studies, with specific reference to melanoma [175], breast [163], bladder [171] and prostate [167] cancers, but is novel work with respect to CRC. We have also demonstrated for the first time that, even in cells with low levels of ICAM-1 expression, CVA21 can replicate and cause cytostatic effects to reduce tumour cell growth. Ultimately, CVA21 displays appreciable direct cytotoxicity within CRC to justify further investigation as an OV in CRC.

4 Combination Treatment of Radiation and CVA21

4.1 Introduction

There is overwhelming evidence to suggest that the combination of an OV with adjunct treatments can enhance treatment efficacy in a variety of cancers, both for haematological [224, 225] and solid tumours [226-229]. Although data is somewhat more limited with respect to combination treatments with OVs in the context of CRC, there is pre-clinical and clinical evidence to support the combination of OVs with other treatments such as chemotherapy, radiotherapy and immune checkpoint inhibition [153, 230-233]. For example, Reo, when used in combination with gemcitabine, delays disease progression and significantly increases survival in a murine model of ovarian cancer, compared to either treatment alone [234]. Reo has also been shown to have therapeutic advantage when used in combination with either radiation or cisplatin for the treatment of paediatric sarcomas implanted in athymic mice [235]. Synergistic effects have also been observed when Reo was used in combination with radiation in a wide range of cancer cell lines, including CRC cell lines [236]. Moreover, phase I clinical trials have shown that Reo, when used in combination treatment with FOLFIRI was safe and well tolerated [237].

There is also a plethora of recent work looking into the treatment of CRC with OV in combination with other treatment modalities. Treatment with an HSV-1 (NV1020) has been shown to sensitise CRC cell lines to the cytotoxic effects of chemotherapeutics, with an additive or synergistically enhanced response [144]. Synergism of combination treatment including Irinotecan and OV in CRC cell lines has also been demonstrated for both Vaccinia virus [146] and Reo [227, 238]. Furthermore, pre-treatment of xenografts with cetuximab prior to Canerpaturev (a naturally occurring HSV) inhibited CRC growth compared to either treatment alone [230].

Radiotherapy has more recently been investigated in combination with OVs with early phase clinical trials in oesophageal cancer [239], breast [240] and lung cancer [241], amongst others. With regard to CVA21, there is evidence suggesting that radiation could improve the efficacy of CVA21 against bladder tumours [183]. Given the current trend to optimise the clinical outcomes of OVs with combination treatments, the encouraging results of pre-existing trials

and early data to suggest that combination treatments may improve outcomes, the aim of this chapter was to investigate whether radiation (and/or chemotherapy) could be used to potentiate the direct cytotoxic effects of CVA21 against CRC.

4.2 TNFα Upregulation of ICAM-1

As ICAM-1 expression correlates with CVA21 toxicity, and only low levels were present on the majority of CRC cell lines examined, the effect of up-regulating ICAM-1 was examined. TNFα is known to up-regulate ICAM-1 surface expression [242-244], therefore cell lines were treated with increasing doses of TNFα for a period of 24 hours and assessed for ICAM-1 expression using flow cytometry (**Figure 4-1**). Doses of TNFα used within this study are known to result in upregulation of ICAM-1 in other studies[242, 245, 246]. Any possible resultant changes in CVA21 cytotoxicity on CRC cell lines following TNFα treatment was subsequently examined using LIVE/DEAD® analysis (**Figure 4-2**), and MTT (**Figure 4-3**). ICAM-1 expression was significantly increased (One-way ANOVA) in both SW480 and HT29 cell lines following treatment with TNFα (**Figure 4-1**). SW480 demonstrated a significant increase of ICAM-1 following doses of both 10 u/mL and 100 u/mL TNFα (an increase in MFI ratio from 16.4 to 42.7 and 50.0, *p = 0.0377 and p = 0.0131,* respectively) and HT29 cell line also demonstrated a significant increase following a dose of 100u/mL (MFI increased from 1.8 to 5.8, *p = 0.0031*). Although there appeared to be an increase in ICAM-1 expression on HCT116 and SW620 cell lines, this was not statistically significant (**Figure 4-1**). It is possible that there could have been a more significant increase if we had investigated ICAM-1 expression at a later time point on these two cell lines.

Since ICAM-1 was upregulated in SW480 and HT29 following treatment with TNFα, LIVE/DEAD® studies were repeated to determine whether this increase would correlate with increased CVA21-induced CRC cell death (**Figure 4-2**). Previous LIVE/DEAD® studies had shown that, out of the panel of four CRC cell lines used, only SW480 showed any sensitivity to CVA21 using this method of analysis (**Figure 3-2**). In this analysis, where cell cultures were treated with TNFα, CVA21-mediated cell death in SW480 was enhanced following pre-treatment with TNFα at doses of both 10 and 100 u/mL (*p = <0.0001*, Two-way ANOVA) (**Figure 4-2a**). However, despite showing an increase in ICAM-1 expression in response to treatment with TNFα, HT29 did not demonstrate any significant increase in cell death when TNFα pre-treated cells were subsequently treated with CVA21 (**Figure 4-2c**), contrary to the result seen for SW480. Cell death was observed in response to TNFα treatment in HT29 CRC

cells at a dose of 100u/mL (*p = 0.0279*) (**Figure 4-2c**) and in HCT116 at doses of both 10u/mL and 100u/mL (*p = <0.0001*) (**Figure 4-2c**). SW620 CRC cells did not show significant death in any of the conditions applied (**Figure 4-2b**).

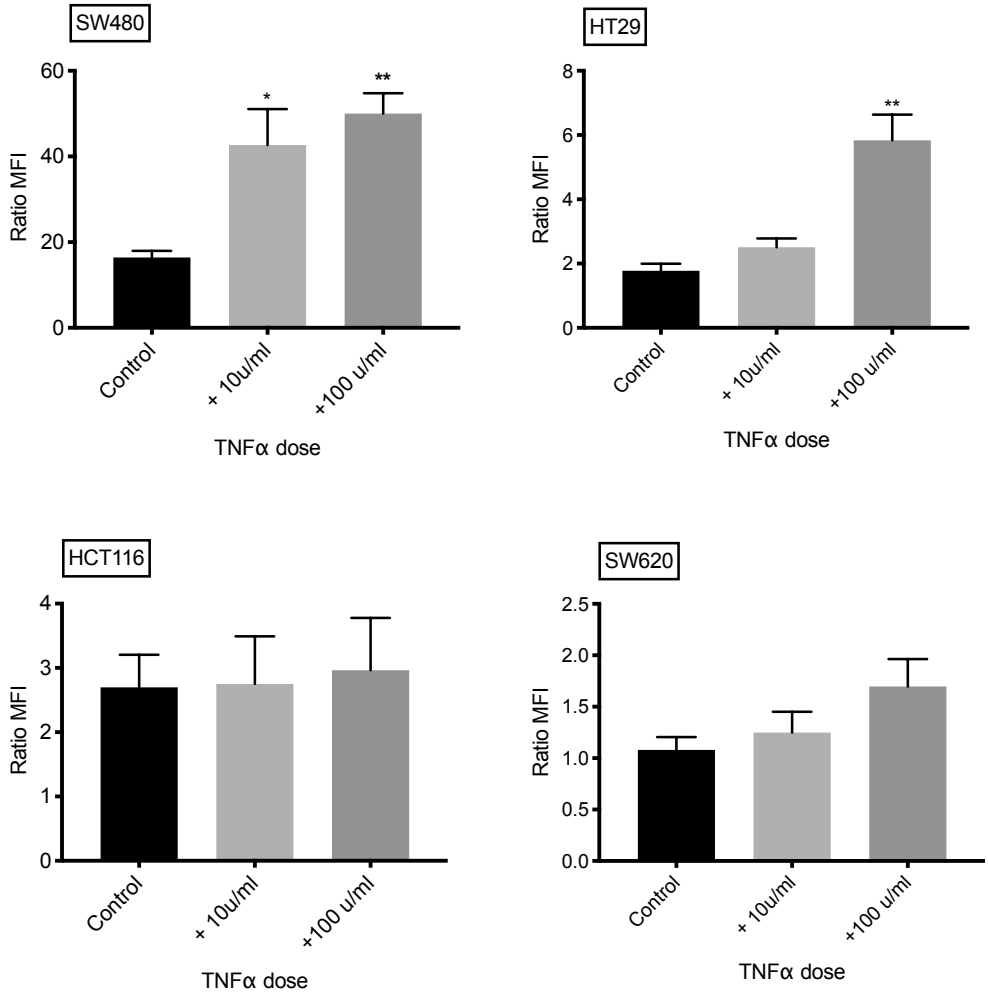


Figure 4-1: Relative Expression of ICAM-1 following CRC Cell Treatment with TNFα. CRC cell lines were cultured in media supplemented with increasing doses of TNFα of 0u/mL, 10u/mL or 100u/mL. Following a 24-hour period of incubation, cells were harvested then dual stained with anti-ICAM-1antibody and LIVE/DEAD® stain. Receptor expression on live cells (LIVEDEAD®) is represented as a ratio of MFI of living cells compared to the relevant isotype control, n=3, with error bars showing SEM. Statistical significance compared to the untreated control is demonstrated, where * represents $p \leq 0.05$ and ** represents $p \leq 0.01$ (calculated using one-way ANOVA).

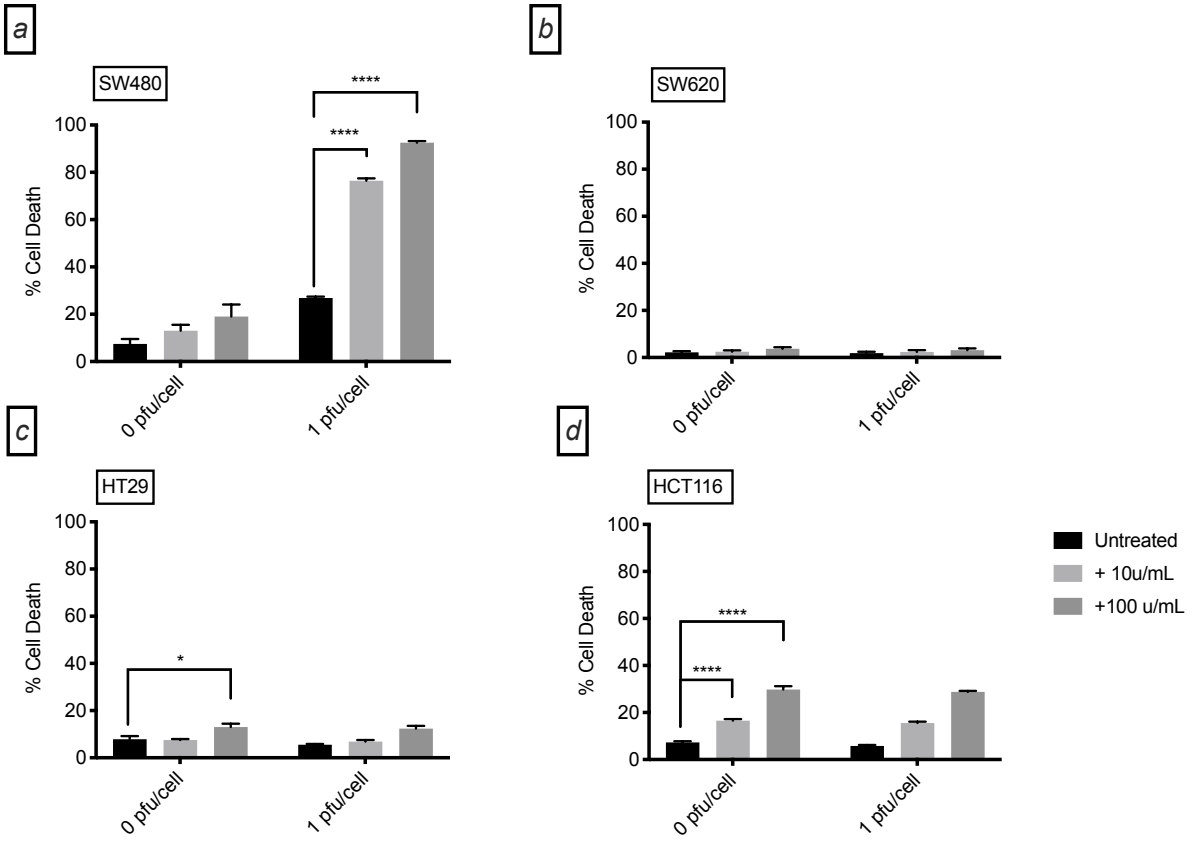


Figure 4-2: Percentage of CRC Cell Death following Conditioning with TNFα and Treatment with CVA21. CRC cells were cultured in media conditioned with doses of 0, 10 or 100u/mL of TNFα. Following a period of 24 hours, cells were treated with a dose of 0 or 1 pfu/cell CVA21 for 48 hours. Cell viability was then examined using LIVE/DEAD® staining and flow cytometry. Cell death is expressed as percentage of the parent population, and results show the mean percentage of dead cells +SEM for n= 3 experiments. Statistically significant results are displayed within the relevant graphs and were calculated using 2-way ANOVA.

Since MTT had previously proved to be a more sensitive method to identify the overall effects of CVA21 on CRC cell lines, we used this technique to further explore whether treatment with TNFα could modulate CVA21 sensitivity (**Figure 4-3**). SW480 relative metabolism/cell viability was significantly reduced in response to CVA21 treatment, but unaffected by dosing with either 10 or 100u/mL of TNFα. When SW480 cells were conditioned for a period of 24 hours with TNFα at a dose of 10u/mL or 100u/mL prior to CVA21 treatment, there was a significant decrease in relative cell metabolism/viability compared to either treatment alone (*p = <0.0006 and p = 0.0003,* respectively) (**Figure 4-3a**).

HT29 cells demonstrated significant decrease in cell viability/metabolism following CVA21 treatment (*p = 0.0067*), but no reduction in relative cell viability/metabolism in response to TNFα. Combination treatment of CVA21 (1 pfu/cell) and TNFα (100 u/mL) on HT29 cells yielded a statistically significant reduction in relative cell viability/metabolism compared to either of these treatments in isolation (*p = 0.0006*) (**Figure 4-3c**). Overall, results of MTT studies using the HT29 cell line confirmed increased sensitivity to CVA21 treatment in accordance with increasing levels of ICAM-1 (**Figure 4-1**) in the presence of TNFα. In conclusion, two CRC cell lines (SW480 and HT29) demonstrated upregulation of ICAM-1 in response to TNFα which increased CVA21-induced cellular toxicity.

Neither SW620 or HCT116 CRC cell lines up-regulated ICAM-1 in the presence of TNFα (**Figure 4-1**). Despite this both SW620 and HCT116 cell lines showed significant reduction in relative metabolism when using a combination of 100 u/mL TNFα and 1 pfu/cell CVA21 (*p = 0.0477* and *p = 0.0294*, respectively). With respect to HCT116, this phenomenon is likely to be due to additive effects of both CVA21 and TNFα-mediated cytotoxicity. SW620 did not show a statistically significant reduction in relative cell metabolism in response to 1 pfu/cell of CVA21 or TNFα at either 10 u/mL or 100 u/mL alone, however it is possible that the significant effect observed in these cells were due to low levels of cumulative toxicity.

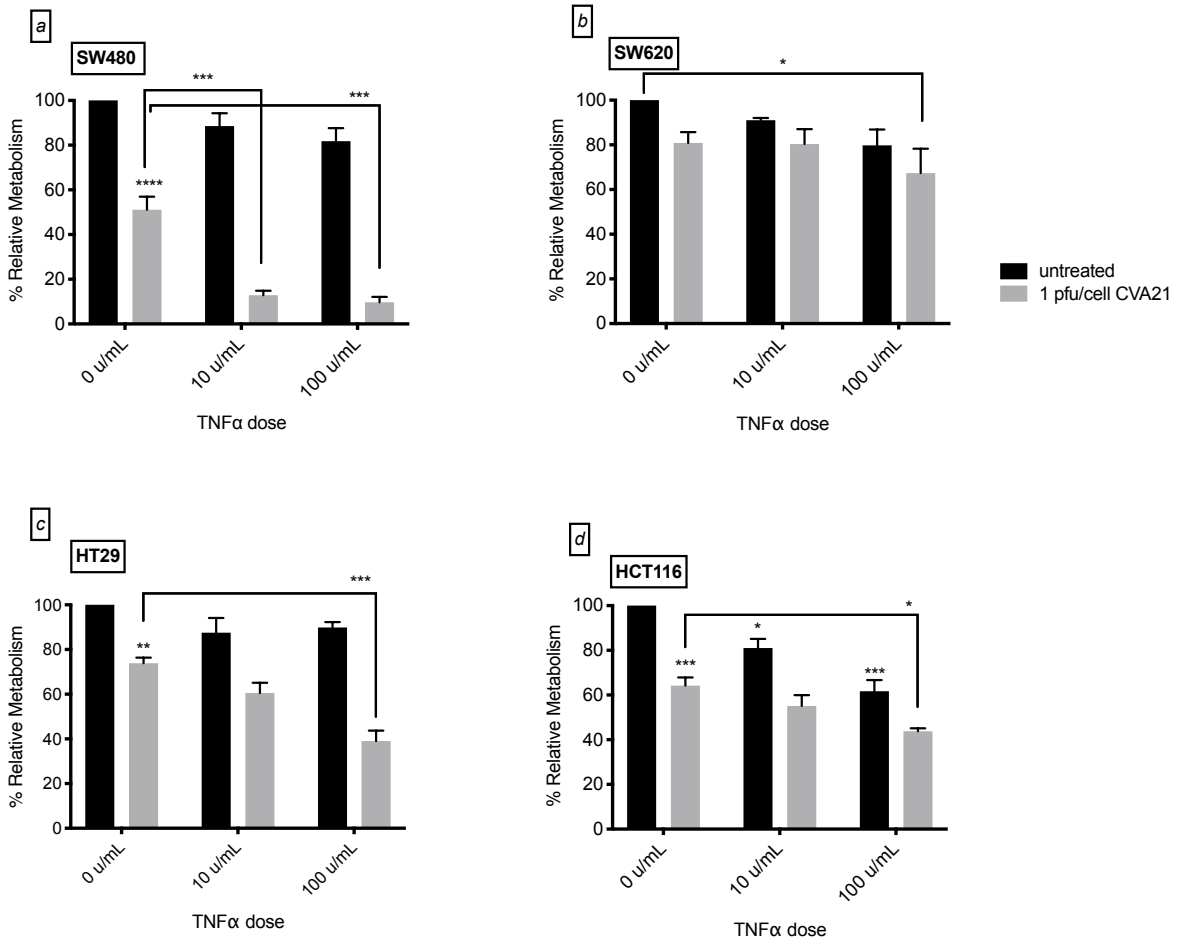


Figure 4-3: MTT of CRC Cell lines Following Treatment with CVA21 +/- TNFα. Changes in CRC cell relative metabolism is represented. CRC cells were cultured for 24 hours in media conditioned with either 0, 10 or 100u/mL of TNFα. Cells were subsequently treated with 0 or 1pfu/mL of CVA21 and analyzed using MTT following a period of 48 hours. Graphs show means with error bars representing ±SEM for n=3 independent experiments. P values are calculated from statistical analysis using Two-way ANOVA, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represents $p \leq 0.001$.

Upon investigating of the effects of TNFα on CRC response to CVA21, as above, the MTT assays appear to be a more sensitive indicator of the cellular effects of CVA21. Overall, these findings have suggested that treatments which can modulate ICAM-1 expression could be used to enhance the susceptibility of CRC cell lines to CVA21 direct cytotoxicity, as demonstrated by SW480 and HT29 cell lines (**Figure 4-1**, **Figure 4-2** and **Figure 4-3**). Therefore, given evidence detailed in the introduction that ICAM-1 can be up-regulated following radiotherapy [164, 165], and the findings that TNFα-mediated increased ICAM-1 correlates positively with increased CVA21 cytotoxicity, it was hypothesized that radiotherapy, a commonly used treatment for CRC, would enhance the sensitivity of CRC cell lines to CVA21-induced direct oncolysis.

4.3 ICAM-1 Upregulation Following Irradiation

Flow cytometry was utilized to test the hypothesis that surface expression of ICAM-1 on the CRC cell lines would be upregulated following irradiation. CRC cells were exposed to a variety of doses of radiation before being cultured for a period of 24, 48 and 72-hours. Cells were subsequently harvested and underwent dual staining with LIVE/DEAD® and anti-ICAM-1 antibody to quantify ICAM-1 expression on viable cells (**Figure 4-4**). There appeared to be an increase of ICAM-1 expression over all cell lines in response to radiation in a dose dependent manner. However, the ICAM-1 increase was most evident 48-hours after treatment with radiation (for clarity this is further illustrated in **Figure 4-5**).

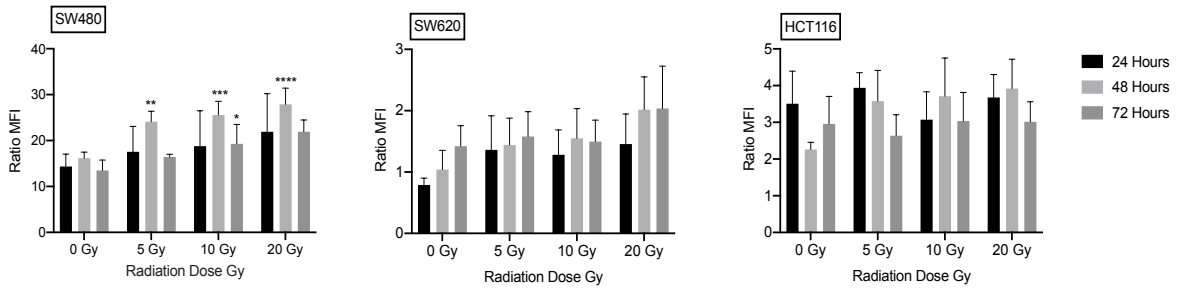


Figure 4-4: Surface Expression of ICAM-1 on CRC Cells Following Radiation Treatment. ICAM-1 expression was quantified following exposure to γ radiation at a range of doses (0-20 Gy) by flow cytometry. CRC cells were irradiated in media and incubated for a period of 24, 48 or 72 hours. CRC cells were then double stained with LIVE/DEAD® (to exclude dead cells from the analysis) and anti-ICAM-1 antibody, or isotype control. Relative levels of receptor expression are represented as a ratio of MFI compared to the relevant isotype, with error bars representing +SEM, n=4. Statistical significance compared to control (0 Gy) is shown where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (2-way ANOVA).

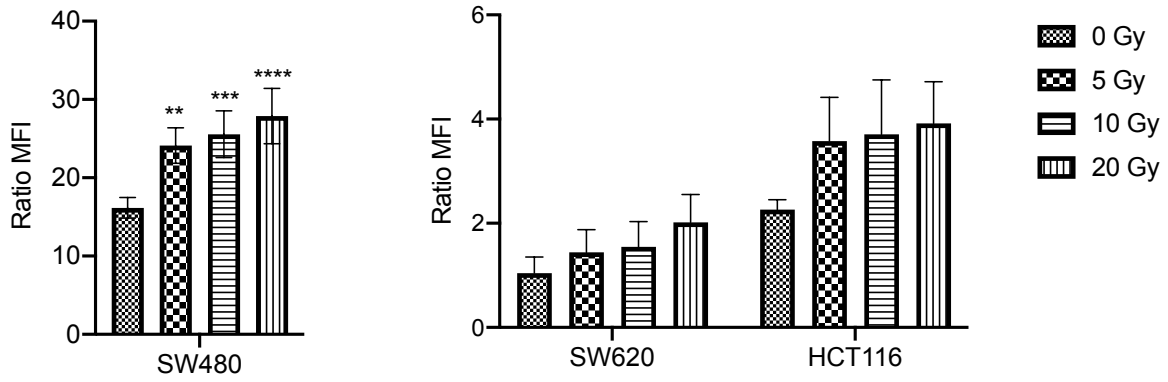


Figure 4-5: Surface Expression of ICAM-1 on CRC Cells 48 Hours Following Radiation Treatment. ICAM-1 expression following exposure to γ radiation at a range of doses (0-20Gy). CRC cell culture samples were double stained with LIVE/DEAD® and anti-ICAM-1 antibody to exclude dead cells from the analysis 48 hours post irradiation. Graphs display MFI of antibody staining/isotype, with error bars representing +SEM, n=4, p values are calculated from statistical analysis using 1 way ANOVA, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$.

4.4 LIVEDEAD® Analysis of Dual Treated CRC Cells

To assess CRC cell line sensitivity to radiotherapy, and whether its use could potentiate the susceptibility to CVA21-mediated death, we proceeded to examine cell viability using LIVE/DEAD® studies. Upon initial assessment of radiation sensitivity, all the cell lines investigated were resistant to radiation-induced cell death 48 hours post treatment at 5Gy, 10Gy or 20Gy. Moreover, CRC cells were resistant to 5 Gy radiation at all timepoints investigated (**Figure 4-6**). The CRC cell line which demonstrated the greatest sensitivity to radiation was SW620 (**Figure 4-6**). There was a significant increase in cell death observed at both 10Gy and 20Gy, 72 hours post-radiation ($p = 0.0346$ and $p = 0.0047$, respectively). This sensitivity to death was also maintained at 96 hours following treatment, with notable significance at both 10Gy and 20Gy ($p = < 0.0001$). SW480 cells displayed marginally less sensitivity to radiation with significant cell death 72 hours following treatment with 20Gy of radiation ($p = 0.00136$) and 96 post treatment with both 10Gy and 20Gy ($p = 0.0150$ and $p = <0.0001$, respectively). HCT116 demonstrated the least sensitivity to radiation, with cell death observed only at 96 hours after a dose of 20Gy ($p = 0.0132$).

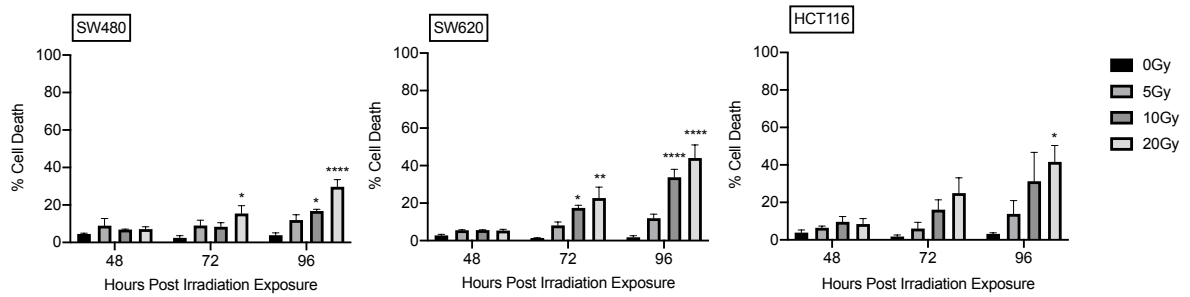


Figure 4-6: Live Dead Analysis of CRC Cells Following Treatment with Radiation. CRC cells were irradiated with a single dose of either 0, 5, 10 or 20Gy. The percentage of subsequent cell death within in each cell line was assessed using LIVE/DEAD® staining and flow cytometry at 24, 48 and 72 hours post treatment. Cell death is expressed as a percentage of the parent population, with error bars representing ±SEM, n=3. Statistically significant cell death compared to control (0 Gy) was calculated using 2-Way ANOVA, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$.

Having defined the sensitivity of CRC cell lines to radiation, we next sought to determine whether radiation could be used to increase sensitivity to CVA21. In order to investigate this, we pre-treated CRC cell lines with a dose of 0, 5, 10 or 20Gy radiation 24 hours prior to treatment with CVA21. This was to ensure that time was allowed for possible ICAM-1 upregulation prior to CVA21 addition. Cell death was then quantified using LIVE/DEAD® analysis 24, 48 and 72 hours post CVA21 treatment. Results showed that, after a period of 24 hours, there was no significant death seen in any cell line in response to CVA21 or radiation as a solitary treatment.

Interestingly, in combining the two treatments of CVA21 and radiation, at specific doses and timepoints, SW480 cell line showed some marginal increases in cell death (**Figure 4-7**), likely as a result of additive effect. At the latter timepoint of 72 hours however, the significance of this effect was negated, likely due to the marked efficacy of each treatment in individual cytotoxicity (data not shown). In addition, HCT116 also showed a relative increased response to dual treatment of CVA21 and radiation using LIVE/DEAD® analysis (**Figure 4-8**, **Figure 4-9**). Following treatment with radiation and CVA21 for 72 hours (not 24 or 48 hours; data not shown), we observed evidence of cell death in HCT116 cell line, suggesting increased sensitivity to CVA21 in this cell line. For example, 72 hours following CVA21 treatment of irradiated HCT116 cells, we observed evidence of additional cell death in using the dual treatment combinations, showing novel sensitivity to CVA21 in this cell line using LIVEDEAD®. 72 hours following CVA21 treatment at a dose of 5 pfu/cell, there was a significant increase in cell death observed in the cells which had been irradiated at 5 Gy; in isolation these two treatments do not result in significant death individually (**Figure 4-9**). This is also demonstrated by the general uptrend of the graphical data and change in *p* values trending towards significance (Fig x). In addition to this, in HCT 116 cells pre-treated with 10 Gy and 20 Gy radiation, there is significant increase in cell death subsequent to addition of 5 pfu/cell CVA21 ($p = 0.0375$ and $p = 0.0412$, respectively) (**Figure 4-8**). Overall, given these findings in HCT116 cell line, which has previously demonstrated resistance to CVA21 mediated death when using LIVE/DEAD® analysis (**Figure 3-2**), it could potentially imply a synergistic response of the two treatments combined.

In summary LIVE/DEAD® studies demonstrate that all the CRC cell lines examined were sensitive to radiation-induced death, but combination treatment with radiation and CVA21 resulted in significantly more death in SW480 and HCT116 than by either radiation or CVA21 treatment alone. The data has suggested an additive benefit of combining treatments with regard to SW480, and a possible synergistic benefit with regard to HCT116. SW620 appeared to maintain resistance to CVA21 treatment and was only sensitive to radiation mediated death after 48 hours (data not shown).

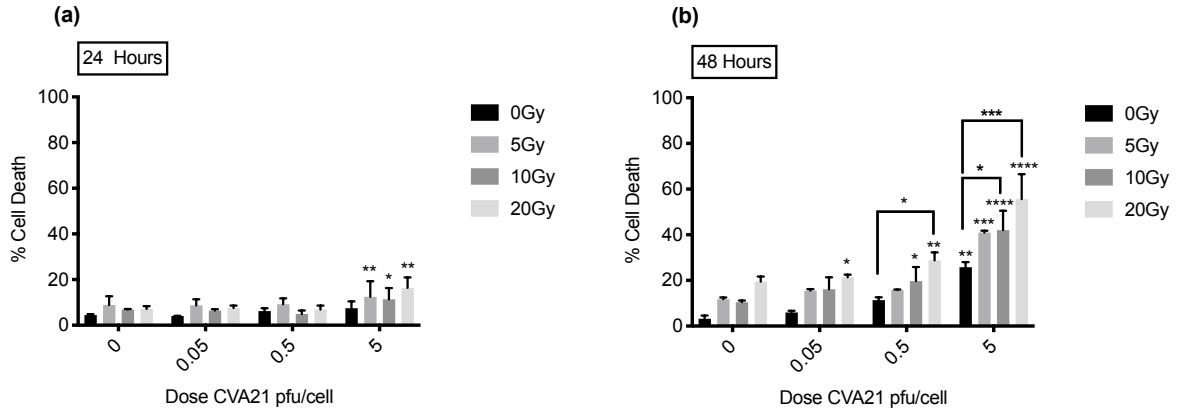
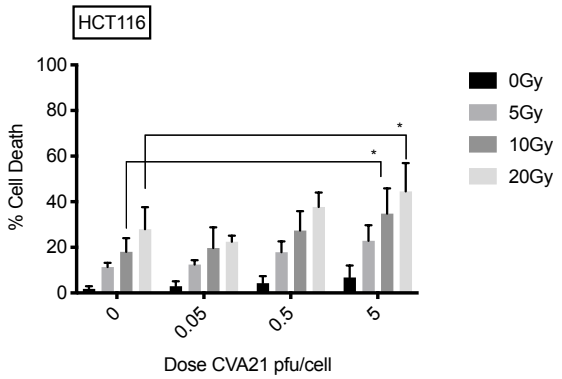


Figure 4-7: LIVE/DEAD® Analysis of SW480 CRC Cells Following Dual Treatment with Radiation and CVA21. SW480 cells were treated with a dose of 0Gy, 5Gy, 10Gy or 20Gy 24 hours prior to treatment with a dose of 0, 0.05, 0.5 or 5 pfu/cell of CVA21. Cell cultures were harvested at (a) 24, (b) 48 and following CVA21 treatment and analyzed using LIVE/DEAD® flow cytometry. Cell death is expressed as percentage of the parent population, and results presented show the mean percentage of dead cells +SEM for n = 3 experiments. Statistically significant results are highlighted on the bar charts highlighted where * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represents $p \leq 0.001$, 2-way ANOVA.



CVA21 Dose (pfu/cell)	Radiation Dose	*p-value*	Sig
0	5 Gy	0.6139	ns
	10 Gy	0.0496	*
	20 Gy	0.0004	***
0.05	0 Gy	>0.9999	ns
	5 Gy	0.4604	ns
	10 Gy	0.0221	*
	20 Gy	0.0059	**
0.5	0 Gy	>0.9999	ns
	5 Gy	0.0532	ns
	10 Gy	0.0006	***
	20 Gy	< 0.0001	****
5	0 Gy	0.9951	ns
	5 Gy	0.0047	**
	10 Gy	< 0.0001	****
	20 Gy	< 0.0001	****

Figure 4-8: LIVE/DEAD® Analysis of HCT116 CRC Cells Following Dual Treatment with Radiation and CVA21, 72 Hour Analysis. HCT116 cells were treated with a dose of 0Gy, 5Gy, 10Gy or 20Gy 24 hours prior to treatment with a dose of 0, 0.05, 0.5 or 5 pfu/cell of CVA21. Cell cultures were harvested following incubation for 72 hours and analysed using LIVE/DEAD® and flow cytometry. Cell death is expressed as percentage of the parent population, and results show the mean percentage of dead cells +SEM for n = 3 experiments. Statistically significant changes in cell death from control untreated cells are charted in the adjacent table. Statistical improvement of radiation mediated cell death with combination of CVA21 + radiation are highlighted on the bar chart where * represents $p \leq 0.05$ and ** represents $p \leq 0.01$ (2-way ANOVA).

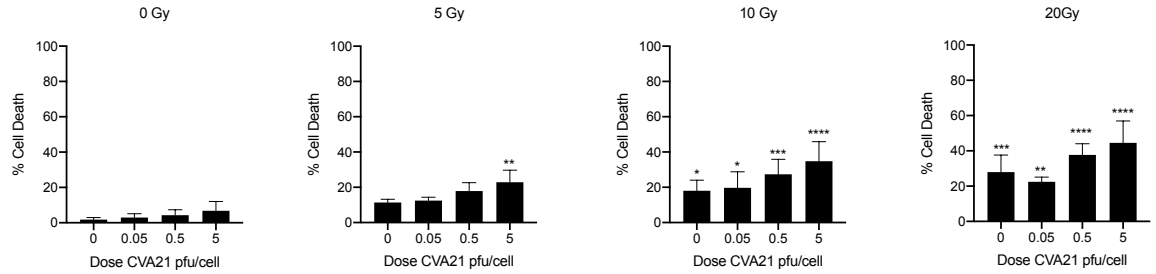


Figure 4-9: LIVE/DEAD® Analysis of HCT116 CRC Cells Following Dual Treatment with Radiation and CVA21, 72 Hour Analysis. HCT116 cells were treated with a dose of 0Gy, 5Gy, 10Gy or 20Gy 24 hours prior to treatment with a dose of 0, 0.05, 0.5 or 5 pfu/cell of CVA21. Cell cultures were harvested following incubation for 72 hours and analysed using LIVE/DEAD® and flow cytometry. Cell death is expressed as percentage of the parent population, and results show the mean percentage of dead cells +SEM for n = 3 experiments. Statistically significant changes in cell death are shown where * represents $p \leq 0.05$ and ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (2-way ANOVA).

4.5 MTT Evaluation of Dual Treatment Toxicity on CRC Cell Lines.

Given that MTT proved to be a superior assay in quantifying the more-subtle effects of CVA21 on CRC cells (3.4, MTT Evaluation of CVA21 Toxicity on CRC Cell Lines), it was again utilized here to investigate any possible additive or synergistic effects of combining CVA21 and radiation. Experiments were carried out using the same timing as those for LIVE/DEAD® studies (4.4, LIVEDEAD® Analysis of Dual Treated CRC Cells); CRC cell lines ± radiation were seeded in triplicate in 96 well flat-bottomed plates at a fixed volume and concentration, then 24-hours later they were treated with an increasing dose of CVA21 (0 – 5 pfu/cell).

Initially the effect of radiation as monotherapy was determined using MTT which suggested that SW620 cells were particularly sensitive to radiation. For example, there was a statistically significant decrease in relative cell metabolism/viability at every dose of radiation used (5, 10 and 20Gy) at all time points investigated (48, 72 and 96 hours) (**Figure 4-10**). The HCT116 cell line also demonstrated significantly impaired metabolism in response to radiation ($p \leq 0.0001$) for 5Gy, 10Gy and 20Gy at both 72-hour and 96-hour timepoints, but no impairment at any dose at the 48-hour timepoint.

Out of the panel of four CRC cells used, SW480 and HT29 cells were the least sensitive to radiation as monotherapy, with SW480 cells only displaying significant changes at the 96-hour timepoint and HT29 cells only showing a decrease in cell metabolism/viability in response to 20 Gy. For SW480 cells, this reduction was evident at both 10 Gy and 20 Gy ($p = 0.0345$ and $p = 0.0008$, respectively) and for HT29 cells the only statistically significant reduction was at 20 Gy at 96 hours post-treatment ($p = 0.0332$). These data support the LIVE/DEAD® analysis (**Figure 4-6**) which suggested that SW620 and HCT116 were the most sensitive to radiation.

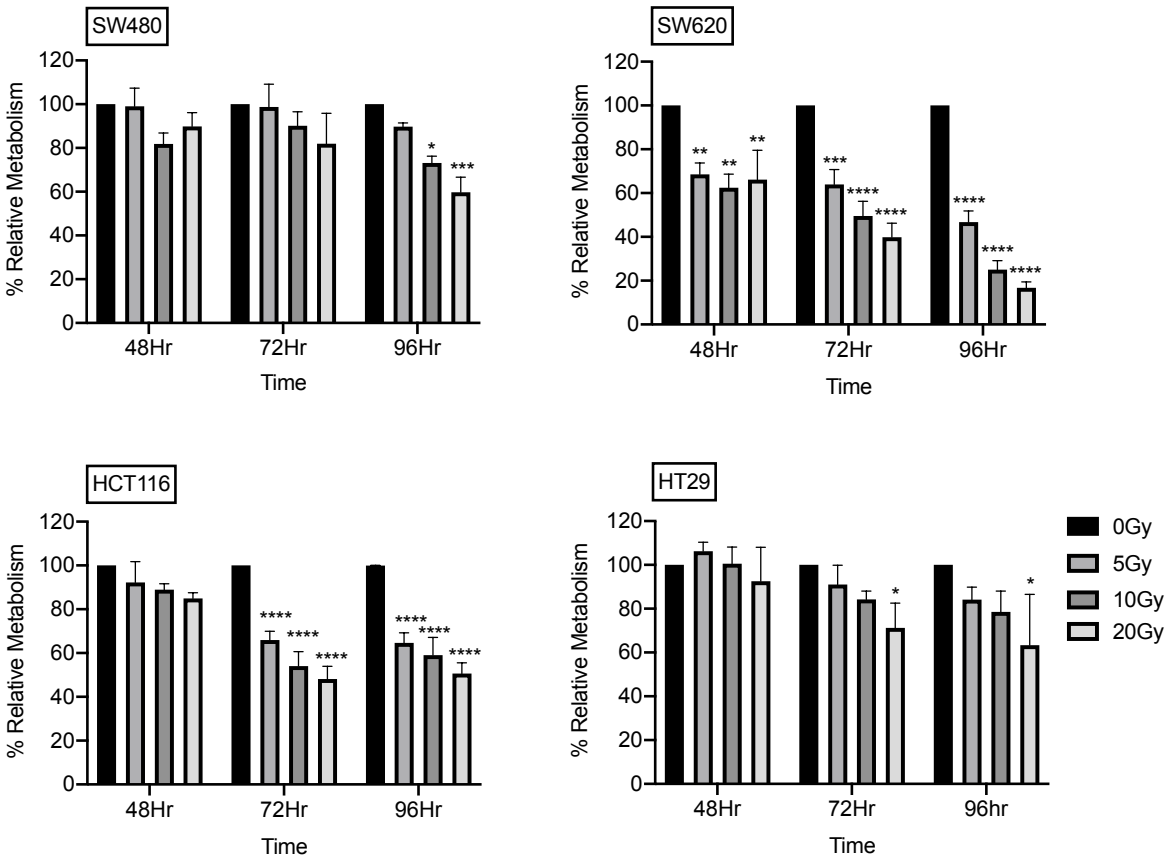


Figure 4-10: MTT Assessment of Relative Metabolism of CRC Cells Following Treatment with Radiation. Cells were irradiated, then seeded at a volume of 200µL at a concentration 4x104 cells/mL. Samples were incubated for a range of time points in 24-hour increments. Results show % relative metabolism compared to untreated samples. Data show mean results +SEM, n = minimum of 3 results. Statistical significance compared to the untreated control is demonstrated, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (calculated using 2-way ANOVA).

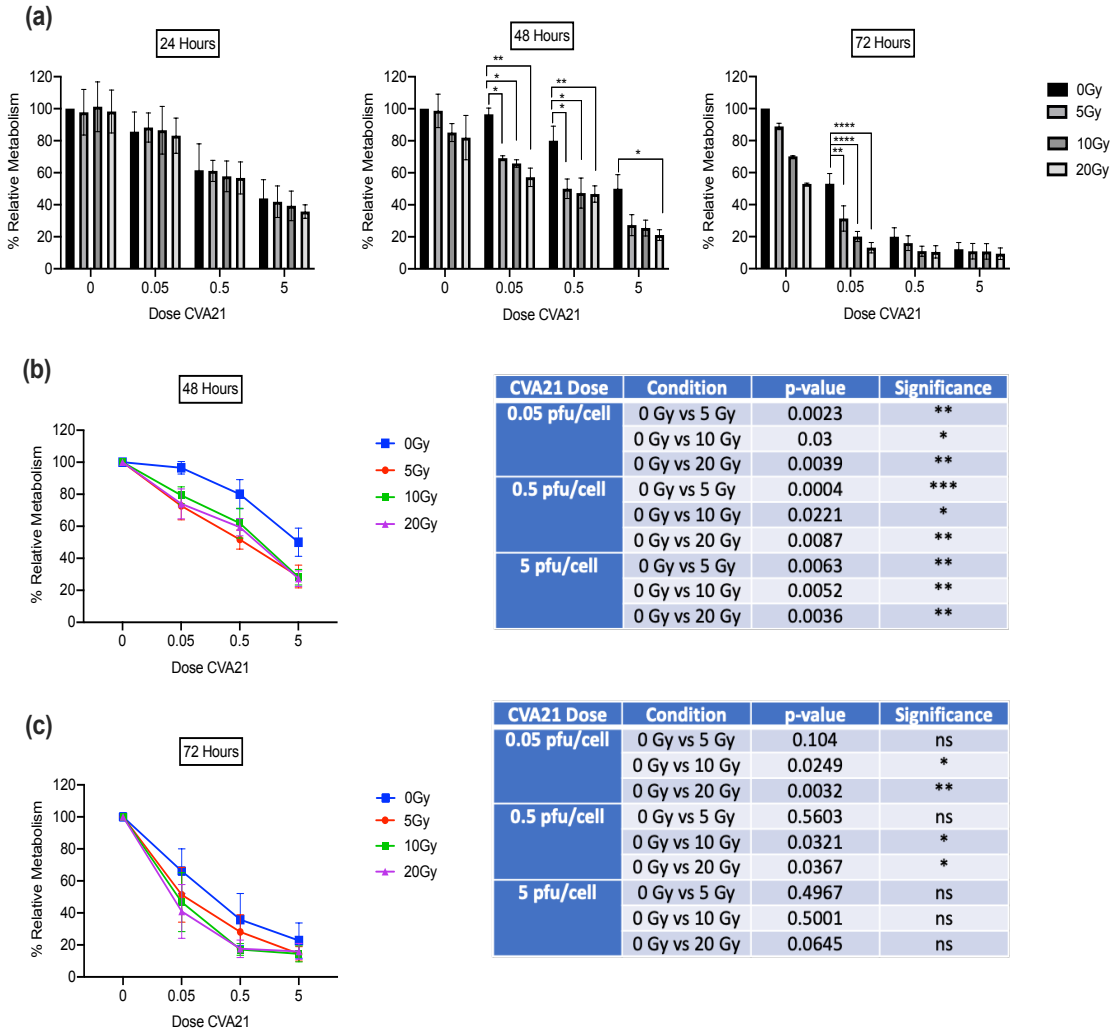
Using MTT to investigate the cytotoxic potential of a combining radiation with CVA21 revealed a significant reduction in cell viability/metabolism compared to either treatment in isolation. For example, SW480 CRC cells that had been pre-treated with radiation, showed significantly reduced cell viability/metabolism in response to CVA21, 48 and 72 hours subsequent to virus treatment (**Figure 4-11**). This was demonstrated most effectively 48 hours following treatment, with CVA21 at a dose of 0.05 pfu/cell and 0.5 pfu/cell. At these two doses of CVA21 in isolation, we do not see any significant impairment of SW480 metabolism/viability. However, it is evident that a combination of CVA21 at 0.05pfu/cell following pre-treatment with radiation at a dose of 5, 10 and 20 Gy elicits a significant decrease in metabolism/viability ($p = 0.0371$, $p = 0.0284$ and $p = 0.0013$, respectively). The same was observed for this timepoint following treatment of SW480 cells with CVA21 at a dose of 0.5 pfu/cell ($p = 0.0193$, $p = 0.0177$ and $p = 0.0077$ with increasing doses of radiation).

Given the relative resistance of SW480 cells to the cytotoxic effects of low dose CVA21 (0.05 and 0.5 pfu/cell) at 48 hours and radiation at 72 hours, the subsequent impairment of metabolism/viability achieved with the combination regimen could suggest a degree of synergism when utilising the two treatments, although this requires further investigation. It is noted, however, that 48 hours following a dose of 5 pfu/cell, the highest dose of CVA21 used, a significant decrease in cell metabolism/viability compared to CVA21 treatment alone was only observed at 20Gy ($p = 0.0251$). At this dose of CVA21, there is a significant impairment of metabolism/viability attributable to CVA21 alone, therefore the more subtle effects of radiation treatment may not be evident.

Following 72 hours of CVA21 of SW480 cells, a significant decrease in metabolism/viability was observed at each dose of virus (0.05, 0.5 and 5 pfu/cell), regardless of whether cells were pre-treated with radiation. However, at a dose of 0.05 pfu/cell, there was a significant reduction in metabolism/viability when cells were pre-treated with increasing doses of radiation ($p = 0.0049$, $p \leq 0.0001$ and $p = \leq 0.0001$ for 5, 10 and 20 Gy, respectively). At higher doses of CVA21 and latter time-points, the statistical benefit of combining treatments is lost, which is likely due to the marked efficacy of individual treatments at later timepoints and higher doses. Notably, CVA21 treatment alone at 0.5 and 5 pfu/cell resulted in a reduction in

cell metabolism/viability of 80 and 88%, respectively; pre-treatment with radiation (10 and 20 Gy) reduces this further to ~90%.

Given the direct cytotoxic effects of radiation on SW480 cells, it was difficult to determine what effect radiation had on CVA21-induced death. To inspect this further, results were normalized to control for the effect of radiation alone (**Figure 4-11c**). These data confirmed that 48 hours post-CVA21 treatment, radiation (5, 10 and 20 Gy) significantly increased susceptibility to CVA21. However, at 72 hours, enhanced CVA21-induced death was only observed after treatment with 10 and 20 Gy and following treatment with lower doses of CVA21 (0.05 and 0.5 pfu/cell). The loss of statistical significance at the highest dose points seen after 72 hours of treatments is likely due to the efficacy of CVA21 direct cytotoxic effects at this timepoint. Collectively, these data suggest that radiation increased the sensitivity of SW480 cells to CVA21 oncolysis.



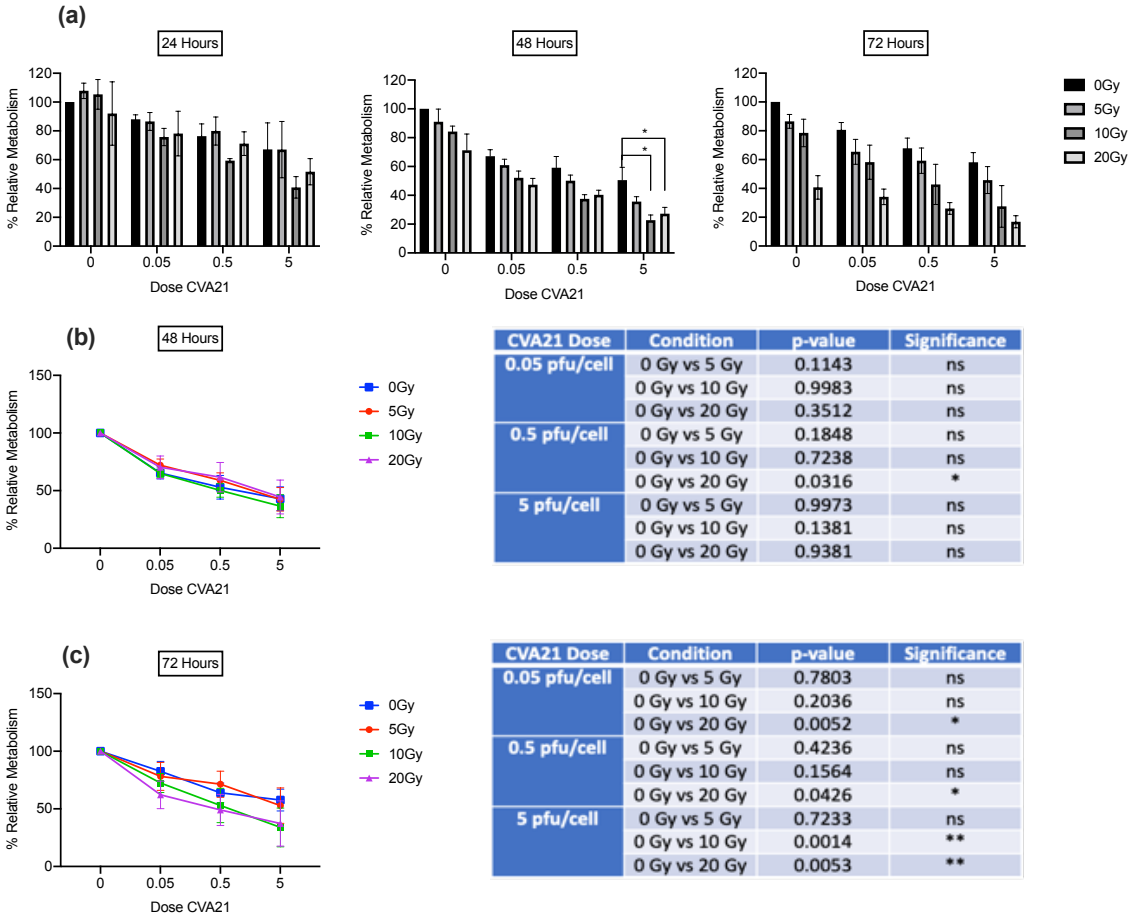
CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.0023	**
	0 Gy vs 10 Gy	0.03	*
	0 Gy vs 20 Gy	0.0039	**
0.5 pfu/cell	0 Gy vs 5 Gy	0.0004	***
	0 Gy vs 10 Gy	0.0221	*
	0 Gy vs 20 Gy	0.0087	**
5 pfu/cell	0 Gy vs 5 Gy	0.0063	**
	0 Gy vs 10 Gy	0.0052	**
	0 Gy vs 20 Gy	0.0036	**

CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.104	ns
	0 Gy vs 10 Gy	0.0249	*
	0 Gy vs 20 Gy	0.0032	**
0.5 pfu/cell	0 Gy vs 5 Gy	0.5603	ns
	0 Gy vs 10 Gy	0.0321	*
	0 Gy vs 20 Gy	0.0367	*
5 pfu/cell	0 Gy vs 5 Gy	0.4967	ns
	0 Gy vs 10 Gy	0.5001	ns
	0 Gy vs 20 Gy	0.0645	ns

Figure 4-11: MTT Assessment of Relative Metabolism of SW480 CRC Cells Following Combination Treatment with Radiation and CVA21. Cells were irradiated and then seeded in triplicate in 96 well plates at a volume of 200µL and a concentration $4x10^4$ cells/mL for 24 hours before being treated with increasing doses of CVA21. Cells were incubated for a range of time points in 24-hour increments. Results show % relative metabolism compared to untreated samples at each timepoint. (a) Mean (+SEM) % relative metabolism of SW480 cells following combination treatment at 24, 48 and 72 hours (b) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 48 hours (mean ± SEM) (c) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 72 hours (mean ± SEM). Data shown is for n = 4 individual experiments; statistically significant differences in cell metabolism/viability are highlighted, where *

represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (calculated using 2-way ANOVA).

Similar to SW480, HT29 CRC cells demonstrate a statistically significant decrease in cell metabolism/viability when combining CVA21 treatment with radiation (**Figure 4-12**). Like SW480 cells, HT29 are relatively resistant to the effects of radiation at the timepoints assessed (**Figure 4-10**), unless treated with the highest dose of radiation, 20 Gy. As we have already shown, they do show a reduction in cell metabolism/viability following CVA21 treatment (**Figure 3-4**, **Figure 3-5**). However, at 48 hours following CVA21 treatment at 5 pfu/cell, cells which were pre-treated with 10 Gy and 20 Gy showed a significant decrease in relative cell viability/metabolism compared to CVA21 alone. The remainder of the data seemed to have an additive benefit of using the two treatments together, with cell metabolism/viability being reduced to ~17%, 72 hours following CVA21 treatment at 5 pfu/cell in cells that had been pre-treated with 20Gy. Again, once the data for this cell line was normalized to control for the effects of radiation, it was evident that sensitivity of HT29 cells to CVA21 oncolysis was enhanced following treatment with ionizing radiation (**Figure 4-12b, Figure 4-12c**). This effect, however, was slightly less pronounced than in SW480 cells, with the greatest enhancement of sensitivity being displayed at the latter timepoint of 72 hours and the higher radiation dose of 20 Gy.

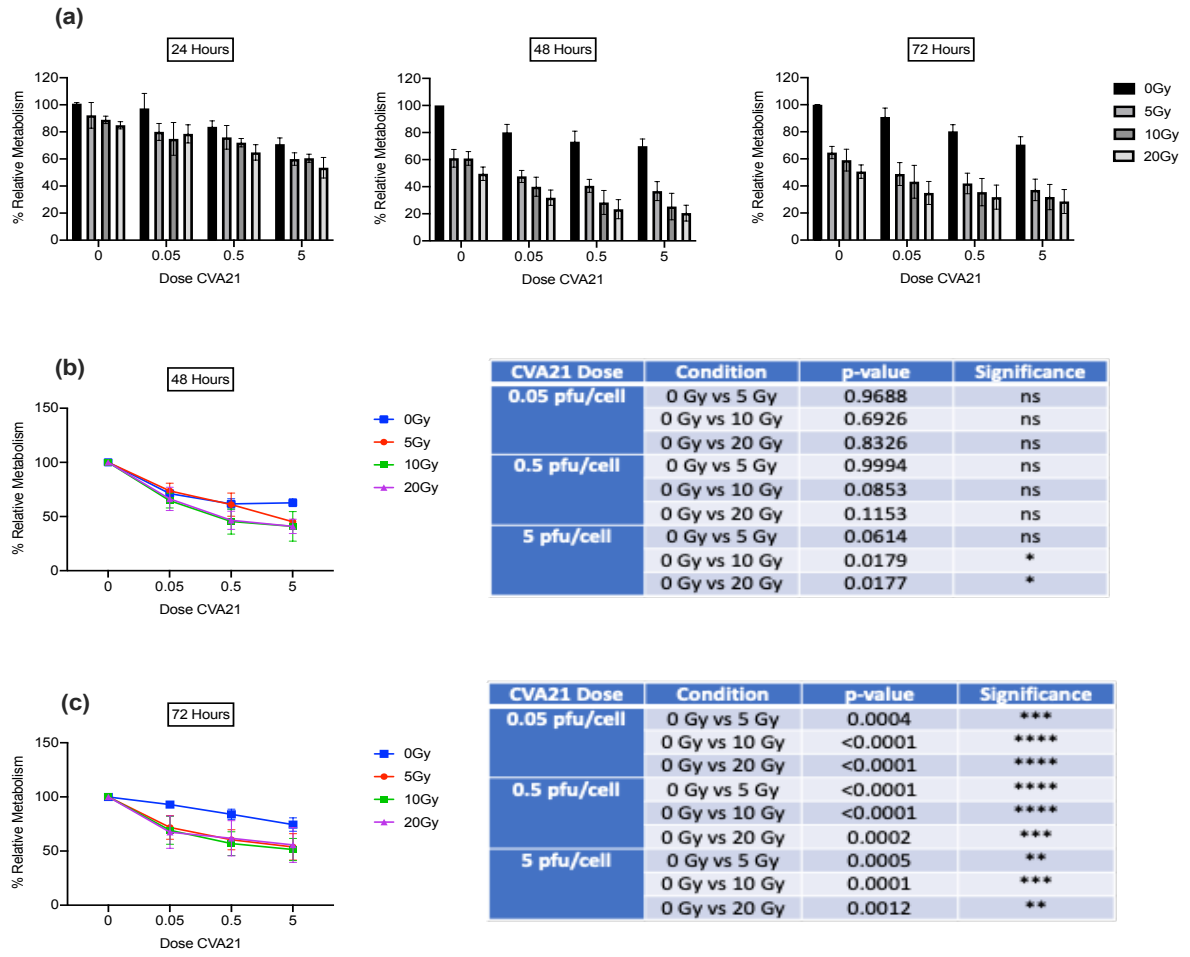


CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.1143	ns
	0 Gy vs 10 Gy	0.9983	ns
	0 Gy vs 20 Gy	0.3512	ns
0.5 pfu/cell	0 Gy vs 5 Gy	0.1848	ns
	0 Gy vs 10 Gy	0.7238	ns
	0 Gy vs 20 Gy	0.0316	*
5 pfu/cell	0 Gy vs 5 Gy	0.9973	ns
	0 Gy vs 10 Gy	0.1381	ns
	0 Gy vs 20 Gy	0.9381	ns

CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.7803	ns
	0 Gy vs 10 Gy	0.2036	ns
	0 Gy vs 20 Gy	0.0052	*
0.5 pfu/cell	0 Gy vs 5 Gy	0.4236	ns
	0 Gy vs 10 Gy	0.1564	ns
	0 Gy vs 20 Gy	0.0426	*
5 pfu/cell	0 Gy vs 5 Gy	0.7233	ns
	0 Gy vs 10 Gy	0.0014	**
	0 Gy vs 20 Gy	0.0053	**

Figure 4-12: MTT Assessment of Relative Metabolism of HT29 CRC Cells Following Combination Treatment with Radiation and CVA21. Cells were irradiated in a uniform manner, then seeded in triplicate in 96 well plates at a volume of 200µL and a concentration $4x10^4$ cells/mL. Samples were incubated for a range of time points in 24-hour increments. (a) % relative metabolism compared to untreated samples at 24, 48 and 72 hours, error bars represent mean +SEM. (b) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 48 hours (mean ± SEM) (c) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 72 hours (mean ± SEM). Data shown is for n = 4 individual experiments; statistically significant differences in cell metabolism/viability are highlighted, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (calculated using 2-way ANOVA).

HCT116 CRC cells show a reduction in relative metabolism/viability to both CVA21 and radiation treatments individually, although they display markedly more sensitivity to the effects of radiation (**Figure 4-10**, **Figure 4-13**). In the case of this cell line, there appeared to be no statistical benefit of combining the two treatments (**Figure 4-13a**) when compared to either radiotherapy or CVA21 alone, albeit a suggestion of an additive reduction in cell viability when combining the two treatments. However, it appears that the relative sensitivity of this particular cell line to both CVA21, and more notably to radiation, makes the data somewhat more difficult to determine without precise dose response relationships. Again, by correcting the data to allow for control of radiation dose shown in **Figure 4-13b** and **Figure 4-13c** it is evident that at 48 hours of CVA21 treatment, 10 and 20 Gy dose of radiation results in significant enhancement of sensitivity to CVA21 at a dose of 5 pfu/cell. 72 hours subsequent to CVA21 treatment this enhancement of sensitivity is demonstrated at every dose of CVA21 and radiation. This is again is in keeping with the findings in SW480 and HT29 cell lines.



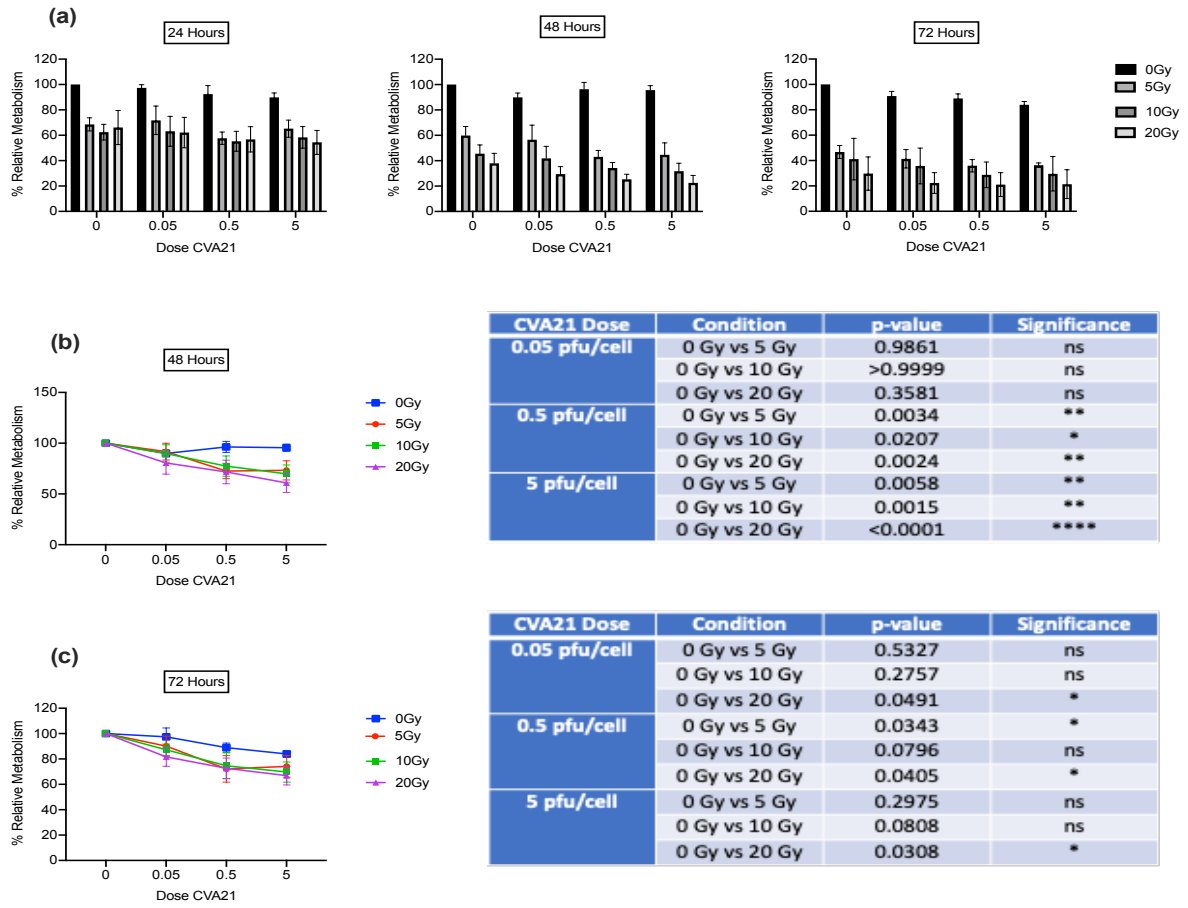
CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.9688	ns
	0 Gy vs 10 Gy	0.6926	ns
	0 Gy vs 20 Gy	0.8326	ns
0.5 pfu/cell	0 Gy vs 5 Gy	0.9994	ns
	0 Gy vs 10 Gy	0.0853	ns
	0 Gy vs 20 Gy	0.1153	ns
5 pfu/cell	0 Gy vs 5 Gy	0.0614	ns
	0 Gy vs 10 Gy	0.0179	*
	0 Gy vs 20 Gy	0.0177	*

CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.0004	***
	0 Gy vs 10 Gy	<0.0001	****
	0 Gy vs 20 Gy	<0.0001	****
0.5 pfu/cell	0 Gy vs 5 Gy	<0.0001	****
	0 Gy vs 10 Gy	<0.0001	****
	0 Gy vs 20 Gy	0.0002	***
5 pfu/cell	0 Gy vs 5 Gy	0.0005	**
	0 Gy vs 10 Gy	0.0001	***
	0 Gy vs 20 Gy	0.0012	**

Figure 4-13: MTT Assessment of Relative Metabolism of HCT116 CRC Cells Following Combination Treatment with Radiation and CVA21. Cells were irradiated in a uniform manner, then seeded in triplicate in 96 well plates at a volume of 200µL and a concentration $4x10^4$ cells/mL. Samples were incubated for a range of time points in 24-hour increments. (a) % relative metabolism compared to untreated samples at 24, 48 and 72 hours, error bars represent mean +SEM. (b) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 48 hours (mean ± SEM) (c) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 72 hours (mean ± SEM). Data shown is for n = 4 individual experiments; statistically significant differences in cell metabolism/viability are highlighted, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (calculated using 2-way ANOVA).

Similar to HCT116 cells, SW620 cells no statistically significant differences in metabolism/viability when adding CVA21 subsequent to radiation (**Figure 4-14a**). Again, this may be attributable to the marked sensitivity the cell line displayed to radiation alone. However, once the results were normalized to control for the effect of radiation alone (**Figure 4-14b**, **Figure 4-14c**) we again observed that sensitivity to CVA21 was improved following radiation treatment. Following 48 hours of CVA21 treatment with 0.5 pfu/cell and 5 pfu/cell, there was significantly enhanced reduction in SW620 cell viability with all doses of radiation investigated. Interestingly, at the 72-hour timepoint, the only dose of radiation which consistently enhanced the cytotoxic effects of CVA21 was 20 Gy, although the reason for this was unclear.

In summary, all of the CRC cell lines seemed to display an apparent benefit to combining CVA21 and radiation, most likely in an additive effect, although this was difficult to interpret when there was sensitivity to both radiation and CVA21. Therefore, in an effort to simplify the data, we decided to normalize the effect of radiation alone across all experiments to further analyse the effect that radiation had on CRC cell susceptibility to CVA21. A further important detail to highlight from all of this data is that pre-treating CRC cells with radiation did not impair the ability of CVA21 to induce cytotoxic/cytostatic effects on CRC cell lines.



CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.9861	ns
	0 Gy vs 10 Gy	>0.9999	ns
	0 Gy vs 20 Gy	0.3581	ns
0.5 pfu/cell	0 Gy vs 5 Gy	0.0034	**
	0 Gy vs 10 Gy	0.0207	*
	0 Gy vs 20 Gy	0.0024	**
5 pfu/cell	0 Gy vs 5 Gy	0.0058	**
	0 Gy vs 10 Gy	0.0015	**
	0 Gy vs 20 Gy	<0.0001	****

CVA21 Dose	Condition	p-value	Significance
0.05 pfu/cell	0 Gy vs 5 Gy	0.5327	ns
	0 Gy vs 10 Gy	0.2757	ns
	0 Gy vs 20 Gy	0.0491	*
0.5 pfu/cell	0 Gy vs 5 Gy	0.0343	*
	0 Gy vs 10 Gy	0.0796	ns
	0 Gy vs 20 Gy	0.0405	*
5 pfu/cell	0 Gy vs 5 Gy	0.2975	ns
	0 Gy vs 10 Gy	0.0808	ns
	0 Gy vs 20 Gy	0.0308	*

Figure 4-14: MTT Assessment of Relative Metabolism of SW620 CRC Cells Following Combination Treatment with Radiation and CVA21. Cells were irradiated in a uniform manner, then seeded in triplicate in 96 well plates at a volume of 200µL and a concentration $4x10^4$ cells/mL. Samples were incubated for a range of time points in 24-hour increments. (a) % relative metabolism compared to untreated samples at 24, 48 and 72 hours, error bars represent mean +SEM. (b) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 48 hours (mean ± SEM) (c) % relative metabolism compared to controls normalized for radiation to investigate any enhancement of CVA21 toxicity mediated by radiation at 72 hours (mean ± SEM). Data shown is for n = 4 individual experiments; statistically significant differences in cell metabolism/viability are highlighted, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$ and **** represents $p \leq 0.0001$ (calculated using 2-way ANOVA).

4.6 CVA21 Replication

Using plaque assays we have previously demonstrated that CVA21 is able to infect and replicate in three of the CRC cell-lines we have investigated; SW480, HCT116 and HT29 (**Figure 3-8**). To investigate the potential impact that radiation treatment may have on the ability of CVA21 to retain infectivity and continue to replicate in CRC cells, we repeated plaque assay, using all four CRC cell lines, after treatment with radiation.

Previously we have shown that CVA21 was able to infect and replicate in SW480 CRC cells with marked efficiency (**Figure 3-8**). This susceptibility was maintained following radiation of this cell line, with CVA21 replication occurring as quickly as 24 hours post-treatment ($p = 0.0023$, $p = 0.0001$, $p = 0.003$ and $p < 0.0001$ for 0 Gy, 5 Gy, 10 Gy and 20 Gy, respectively) (**Figure 4-15**). The effect on CVA21 replication was pronounced, with Log_{10} viral titre reaching a value of 10 at 48 hours following irrespective of pre-treatment with radiation. There was therefore no statistically significant benefit observed with radiation. It is possible that if we repeated this experiment using a lower treatment dose of CVA21 or at an earlier time-point we could possibly observe some differences in CVA21 replication with increasing doses of radiation. However, importantly, there was no impairment of CVA21 replication at any timepoint with radiation pre-treatment.

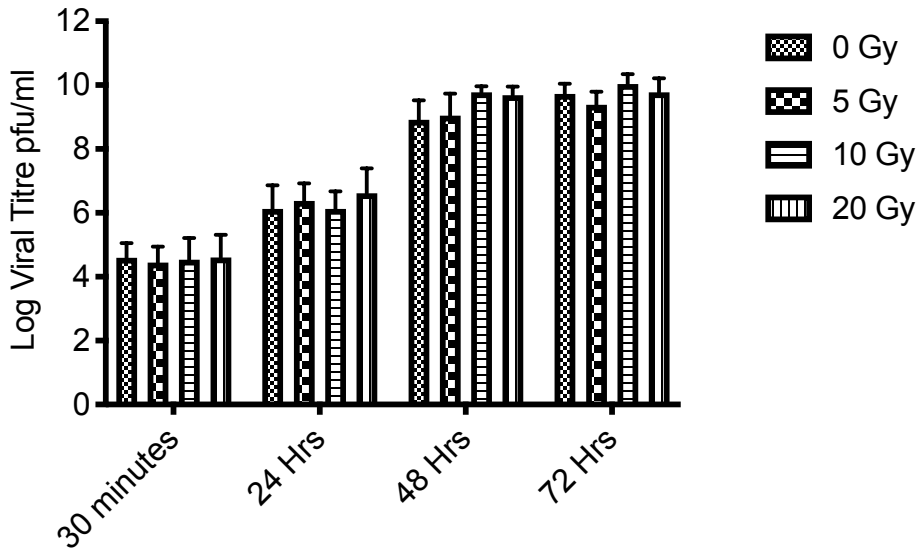


Figure 4-15: CVA21 Replication Within SW480 Cells +/- Radiation. Concentrations of CVA21 (pfu/mL) were determined by plaque assay; SW480 cells were treated with 0, 5, 10 or 20 Gy of radiation and 24 hours later were treated with 1pfu/cell of CVA21. The supernatants were harvested at given timepoints of 30 minutes, 24, 48 and 72 hours, frozen at -80 ^{0}C and plaque assays were carried out within 1 week. Graphs show log values of mean viral titre (pfu/mL) +SEM, n = minimum of 4 results

Despite their relative insensitivity to cell death as a result of CVA21 infection (as measured by LIVEDEAD® **Figure 3-2**), both HT29 and HCT116 CRC cell lines have shown inhibition to cell growth (Figure 3-7), decreased relative metabolism (**Figure 3-4**, **Figure 3-5**) and evidence of CVA21 replication at 48 and 72 hours (**Figure 3-8**). Again, using plaque assay to assess CVA21 replication, both of these cell lines demonstrated an increase in viral titre when cells were irradiated prior to CVA21 treatment. With regard to HCT116 CRC cells, samples that had undergone pre-treatment with 20 Gy displayed a significant increase in CVA21 replication at 24 hours (*p = 0.0221*), an effect which was not evident at 5 Gy or 10 Gy. Moreover, following a time period of 48 and 72 hours, there was statistically greater levels of CVA21 replication seen in cells pre-treated with 10 Gy and 20 Gy than compared to control (**Figure 4-16a**). HT29 cells demonstrated similar results in that there was a significant increase in CVA21 replication, compared with control, seen at 48 hours when cells were pre-treated with 20 Gy radiation (*p = 0.0211*) and at 72 hours if cells were pre-treated with either 10 Gy or 20 Gy (*p = 0.0053* and *p = 0.0139*) (**Figure 4-16b**). This demonstrates that in both HCT116 and HT29 cell lines, CVA21 can infect and replicate within the cells and that radiation can increase CVA21 replication in a dose dependent manner.

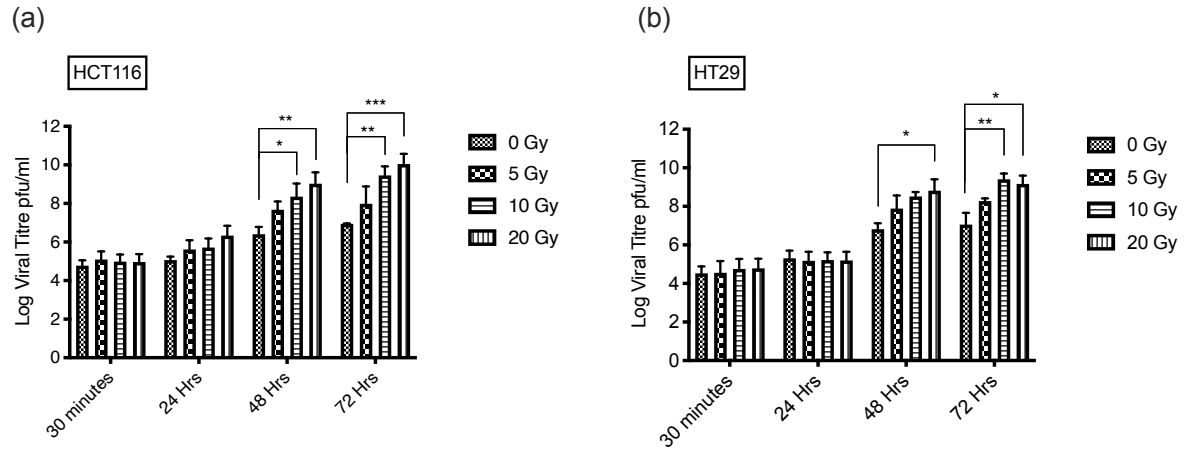


Figure 4-16: CVA21 Replication Within (a) HTC116 and (b) HT29 CRC Cells +/- Radiation. Concentrations of CVA21 (pfu/cell) were determined by plaque assay; CRC cells were treated in a uniform manner with 0, 5, 10 or 20 Gy of radiation. 24 hours subsequent to this $7.5x10^4$ cells, in each condition, were treated with 1pfu/cell of CVA21. The supernatants were harvested at given timepoints of 30 minutes, 24, 48 and 72 hours, frozen at -80 ^{0}C. Plaque assays were carried out within 1 week. Graphs show log values of mean viral titre (pfu/mL) +SEM, n = minimum of 4 results. Statistical significance is demonstrated, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represents $p \leq 0.001$ (calculated using 2-way ANOVA).

Perhaps the most exciting finding was that upon assessment of CVA21 replication in CRC cell lines following radiation was that CVA21 viral replication was detected in the previously resistant cell line, SW620 (**Figure 4-17**). When SW620 cells underwent pre-treatment with 20 Gy radiation, there was significant CVA21 replication after 72 hours (*p < 0.0001);* moreover, there was also a suggestion of viral replication at 24 and 48 hours, although these timepoints lacked statistical significance. This phenomenon was not demonstrated using 5 or 10 Gy of radiation (data not shown).

In summary, over the entire panel of CRC cell lines investigated there was no evidence of inhibition of viral replication following pre-treatment with radiation (**Figure 4-15** - **Figure 4-17**). Interestingly, in three out of four CRC cell lines, CVA21 titre was significantly increased when compared to non-irradiated samples (**Figure 4-16** - **Figure 4-17**). Most notably, in SW620 cells, which had previously not been able to support CVA21 replication, there was statistically significant evidence of CVA21 replication (**Figure 4-17**). Overall, up-regulation of ICAM-1 via radiotherapy on CRC cell lines needs further assessment using alternative methodologies, however collectively, this data suggests that combination of radiotherapy with CVA21 may be used to potentiate CVA21 viral replication and the anti-cancer properties of CVA21 against CRC.

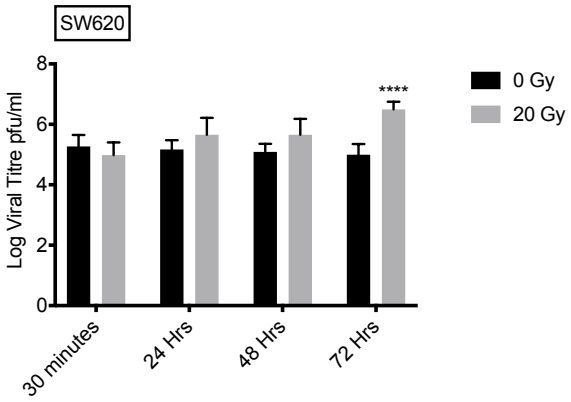


Figure 4-17: CVA21 Replication Within SW620 Cells +/- 20 Gy Radiation. Concentrations of CVA21 (pfu/cell) were determined by plaque assay; SW480 cells were treated with 0, or 20 Gy of radiation. 24 hours subsequent to this $7.5x10^4$ cells at each condition were treated with 1pfu/cell of CVA21. The supernatants were harvested at given timepoints of 30 minutes, 24, 48 and 72 hours, frozen at -80 ^{0}C and plaque assays were carried out within 1 week. Graphs show log values of mean viral titre (pfu/mL) ±SEM, n = 4. Statistical significance is demonstrated, where * represents $p \leq 0.05$ (calculated using 2-way ANOVA).

4.7 CVA21 Compared to 5FU

As a comparison of the potential direct cytotoxic effects of CVA21 combined with radiotherapy, we used MTT to compare this treatment modality against a model of standard chemo-radiotherapy with 5FU, a treatment often utilised in clinical practice. As mentioned in in section 1.2.5, 5FU is the most frequently used chemotherapeutic to be used in the treatment of CRC and is often combined with radiotherapy to potentiate the effect of the latter. Two cell lines, SW480 and its paired lymph node metastatic cell line of SW620 were used. To limit the direct cytotoxic effects of radiotherapy in order to observe the more subtle differences, the dose of radiotherapy was limited to 10Gy and the dose of CVA21 limited to 1 pfu/cell for the purposes of this assay. A range of dose regimens of 5FU used were based on previous published research using CRC cell lines [247-249].

With regard to non-irradiated SW480 cells, we observed a reduction in relative cell viability/metabolism in response to treatment with CVA21 at a dose of 1 pfu/cell as well as a dose dependent reduction in metabolism/viability with increasing doses of 5FU (**Figure 4-18**). However, within the group treated with CVA21 and 5FU, the dose response of 5FU was removed. Pre-treatment of these two experimental groups with 10 Gy of radiation demonstrated further reductions in cell metabolism/viability of both groups, whilst maintaining the dose response of both CVA21 and 5FU. Overall, in SW480, the decreased cell metabolism/viability observed following treatment with CVA21 was more pronounced than that resulting from 5FU at the doses investigated. Following pre-treatment with 10 Gy radiation, the combination of CVA21/radiation remained superior to 5FU/radiation in reducing cell metabolism/viability.

Results from SW620 CRC cells showed, as previously demonstrated (**Figure 3-4**, **Figure 3-5**), the suggestion of a reduction of cell metabolism/viability in response to CVA21 at 1 pfu/cell, although not significant, and a significant reduction in metabolism/viability following 5FU treatment, which was conserved in the group treated with both CVA21 and 5FU. As with SW480, the pre-treatment of SW620 cells with 10 Gy radiation enhanced both of these treatment arms (**Figure 4-18**). In summary, these data suggest that combination of CVA21 and

10 Gy radiation is as, if not more effective, at reducing the cell metabolism/viability of CRC than a combination of 5FU and 10 Gy radiation, although this warrants further investigation.

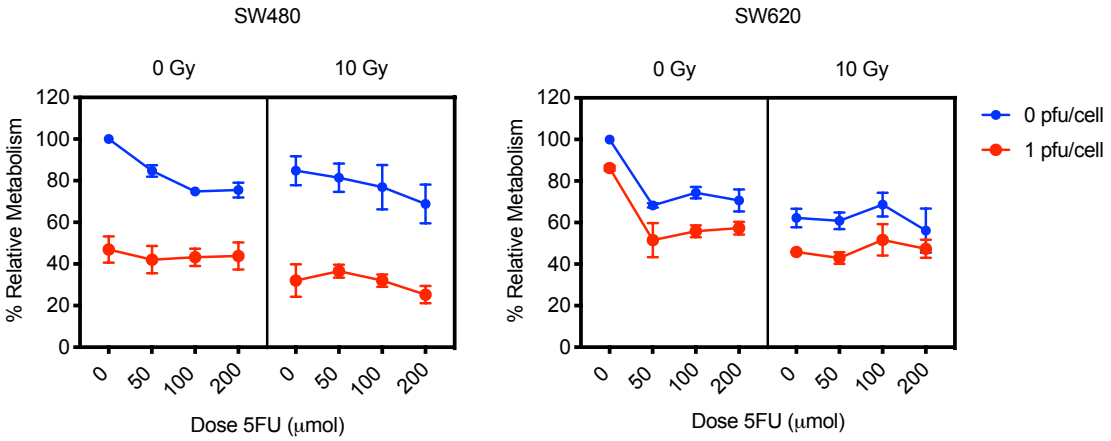


Figure 4-18: MTT Comparison of CVA21 and Radiation Against 5FU and Radiation in CRC Cell Lines. CRC cells were treated with (mock) 0 Gy, or 10 Gy radiation and then seeded in triplicate in 96 well plates at a volume of 200μL and a concentration $4x10^4$ cells/mL. Cells were incubated for 24 hours prior to treatment with +/-CVA21 and +/- 5FU. Graphs show % relative metabolism compared control experiment. Data show mean results ±SEM, n = 3.

4.8 NanoString Data

To confirm whether *in-vitro* expression of ICAM-1 on CRC cells is synonymous with population variation and the more complex molecular picture observed *in-vivo*, we investigated ICAM-1 expression in CRC tissue obtained from patient samples who had received either SCRT or LCRT prior to surgical resection. Access was given to archival CRC tissue from 53 patients, both pre-treatment biopsy and post treatment resection specimen, from the Division of Pathology at St James University Hospital, Leeds (Research Ethics Committee No 08/H0903/62). In a collaborative project with the Institute of Cancer Research (ICR), London, the gene expression, including ICAM-1, of these patient samples was analysed extensively in both the pre-treatment biopsy and post-treatment specimens. Gene expression analysis was undertaken using NanoString nCounter Technology (NanoString Technologies, Seattle, WA). This method involves directly recoding the presence of target ribonucleic acid (RNA) by labelling specific codes with a target probe complex and recording relative counts on an automated fluorescence microscope. Unlike other methods of gene analysis, there is no creation of cDNA or amplification steps required, a step which can increase variability within data. In order to ensure quality control, samples were normalized using positive and negative controls on 'housekeeping genes' which were included in the NanoString panel [189]. Only genes with non-zero expression in 75% or more of the samples were retained to ensure that any absence of ICAM-1 was not due to poor quality RNA.

From the original 53 patient specimens, 49 pre-treatment biopsy samples contained sufficient tissue for NanoString analysis. These samples revealed a wide range of ICAM-1 expression (read counts ranging from 23 to 247, mean 74), with the majority of samples expressing read counts between 40 – 100 (**Figure 4-19**). Due to the historical nature of the samples, matched normal colonic tissue was not available to be analysed for comparison. However, what is also important to consider is that, although this dataset is composed of a large patient number, all the samples have originated from patients who were deemed to have resectable disease and were being treated with curative intent. The data set is therefore intrinsically biased towards this demographic. In keeping with previous literature, the samples analysed showed

good expression of ICAM-1 in patients with CRC [197, 209, 250] implying that CVA21 treatment has a potential clinical role in the treatment of this disease.

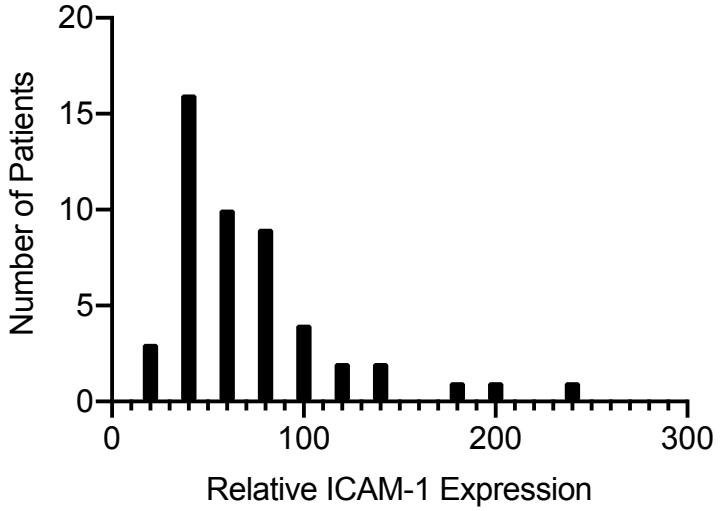


Figure 4-19: Frequency Distribution of ICAM-1 Expression on Patient Biopsy of Untreated Primary CRC Tissue. ICAM-1 gene expression of 49 diagnostic, historical, formalin fixed, CRC patient biopsy samples with successful RNA extraction suitable for NanoString analysis are displayed. Relative tissue ICAM-1 expression is denoted by individual counts of ICAM-1 mRNA normalized to reference genes.

As described in section 1.2.4, prognosis in colorectal cancer is directly related to tumour stage, presence of metastatic disease and tumour differentiation, with 5-year survival being statistically lower in patients with more advanced stage of disease and in those with metastatic disease [38, 49, 63, 251]. It has also been long established that patients with poorly differentiated CRC have a poorer prognosis in terms of increased resistance to standard treatments, increased risk of recurrence after treatment and a poorer survival [252-255]. Although reported evidence describes that CRC cells express higher levels of ICAM-1 in comparison to normal colonic or rectal epithelium [197, 204, 256], membrane and stromal bound ICAM-1 is found to be at relatively lower levels in tumours with a poorer differentiation and more advanced stage [205, 250, 257]. We therefore evaluated the levels of ICAM-1 within the patient specimens according to disease stage (**Figure 4-20**), metastasis (**Figure 4-21**) and histological differentiation (**Figure 4-22**). This was in order to investigate whether there were statistically significant variations in ICAM-1 expression according to these disease characteristics and to evaluate whether CVA21 would be an appropriate treatment in patients with advanced or metastatic disease and poor prognostic factors.

Of the 49 biopsy samples suitable for analysis, records of full clinical pathological staging were available for 46. Upon assessment of ICAM-1 by CRC stage, there was an apparent increase in expression seen with increasing T-stage of disease, although this was not statistically significant (one-way ANOVA) (**Figure 4-20**). Again, the data was skewed as the majority of samples investigated were T3 or T2 disease (n = 31 and n = 11, respectively), with only 2 samples each at stage T1 or T4. As mentioned previously, all the specimens originate from patients with resectable disease and the inclusion of biopsy results from patients with advanced, metastatic or inoperable disease would have increased the statistical power of these comparisons. As there were no patients within the specimen cohort with synchronous metastatic disease, the specimens were divided into those with local and nodal disease. Again, similar to evaluation of ICAM-1 levels for stage of disease, analysis of ICAM-1 values did not reveal any statistical difference (unpaired t-test, $p = 0.7686$) between local or nodal disease (**Figure 4-21**).

Upon assessment of the effect of tumour differentiation, there appeared to be some difference in ICAM-1 expression (**Figure 4-22**). Mean Log2 ICAM-1 expression for well,

moderately and poorly differentiated tumours was 5.185, 6.028 and 6.187, respectively. The difference in means however, was again not statistically significant (one-way ANOVA). It should also be noted that the majority of the samples investigated were from moderately differentiated tumours as opposed to poor or well differentiated (n = 38, n = 7, n = 1, respectively) making statistical comparison difficult. The suggestion from this data therefore is that the potential effectiveness of CVA21 on ICAM-1 in CRC targets would not be inhibited in patients with advanced or poor prognostic disease.

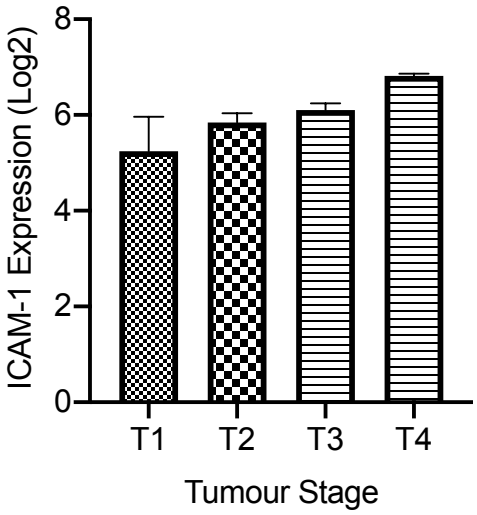


Figure 4-20: ICAM-1 Expression in CRC Biopsy According to Tumour Stage. ICAM-1 gene expression of 46 historical CRC patient biopsies was assessed using NanoString. Histological reports of the specimens revealed a range of samples with histological stage of T1 (n = 2), T2 (n = 11), T3 (n = 31) and T4 (n = 2). Values displayed are Log2 ICAM-1 expression, n = 46.

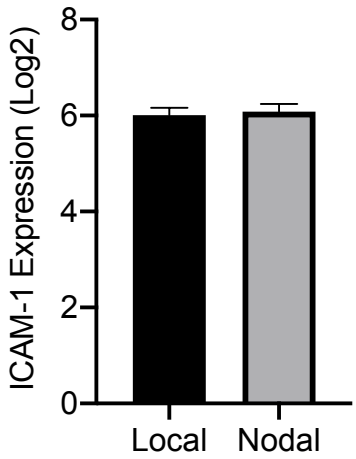


Figure 4-21: ICAM-1 Expression in CRC Biopsy Samples from Patients with Local or Nodal Disease. Of 49 historical, formalin fixed biopsy samples of patients with CRC, 46 had access to full pathological staging. The number of samples with local disease was 30 and the number of samples with nodal disease was 16. Gene expression analysis was undertaken using NanoString and results show Log2 values of relative expression normalized to reference genes.

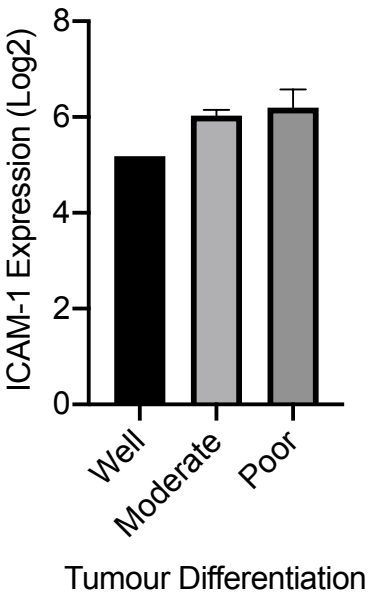


Figure 4-22: ICAM-1 Expression of CRC Biopsies in Moderately and Poorly Differentiated Tumours. ICAM-1 gene expression of 46 historical CRC patient biopsies was assessed using NanoString. Histological reports of the specimens revealed 1 well differentiated, 38 moderately differentiated and 7 poorly differentiated tumours. Gene expression analysis was undertaken using NanoString and results show Log2 values of relative expression normalized to reference genes. Values displayed are Log2 ICAM-1 expression, n = 46.

As already mentioned earlier in this chapter (section 4.1, 4.3), radiation is known to increase expression of ICAM-1. As we have already hypothesised that radiation would increase the effectiveness of CVA21 treatment in the context of CRC, the specimens available were subdivided by clinical treatment into those receiving SCRT (n = 27) and those receiving LCRT (n = 14) for further analysis of the effects of radiotherapy on ICAM-1 expression. Only specimens with both matched pre-treatment biopsy and post-resection specimen were used for this part of the analysis given the wide variation in ICAM-1 expression between individual patients which has already been demonstrated (**Figure 4-19**). In patients that received SCRT, ICAM-1 expression appeared to increase slightly following treatment (mean 6.08 for biopsy increasing to 6.26 at resection, n = 27), although no statistical significance was observed (*p = 0.3571*, paired t-test) (**Figure 4-23**). Moreover, no difference in ICAM-1 expression was observed in patients that had been treated with LCRT prior to surgical resection (**Figure 4-24**). Interestingly when the samples were subdivided according to radiological response (poor; n = 8, moderate; n = 8 or good; n = 11) using change in tumour-cell density (ΔTCD) (undertaken by ICR [189]) there is an interesting and notable relationship between ICAM-1 levels before and after SCRT in the biopsy (n = 48) and resection specimens (**Figure 4-25b**). For example, patients that demonstrated a good response to SCRT exhibited the lowest levels of ICAM-1 in pre-treatment biopsies when compared to moderate and poor responders. Furthermore, the level of ICAM-1 was significantly increased subsequent to treatment within this group (*p = 0.0298*, 2way ANOVA) (**Figure 4-25b**). An almost identical pattern is seen amongst the subgroup following LCRT, although in this instance the increase in ICAM-1 levels demonstrated in the good responders was not statistically significant (**Figure 4-25c**). Data that shows ICAM-1 expression in all biopsy samples, irrespective of radiation modality seems to confirm the finding that tumours with the lowest ICAM-1 levels have the best response to radiotherapy (**Figure 4-25a**). Interestingly, within the group of patients that demonstrated good response to radiotherapy, subsequent to surgical resection, a total of 4 of these patients developed distant metastases and disease recurrence. Despite having a good response to systemic therapy, the fact that these patients developed disease recurrence means that exploring novel drug targets or treatments to reduce the chance of metachronous disease continues to be an important research avenue.

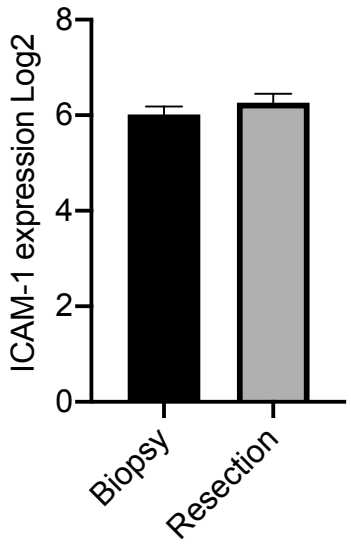


Figure 4-23: Changing ICAM-1 Expression in CRC Patients Following SCRT. CRC tissue from patients that had undergone short course radiotherapy of 25Gy in 5 fractions over 5 days, and subsequently proceeded to surgical resection were analysed for gene expression of ICAM-1 in pre-operative biopsy and post-operative resection specimens. Value displayed are Log2 ICAM-1 and n = 27.

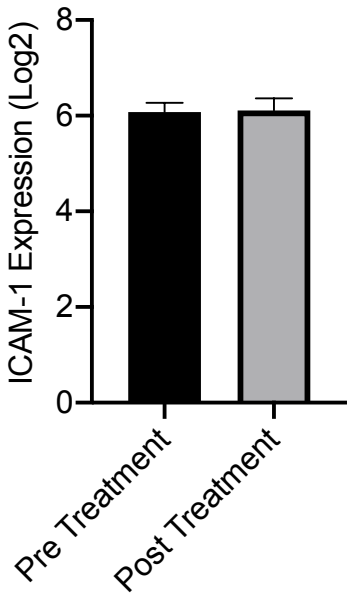


Figure 4-24: ICAM-1 Expression in CRC Patients Following LCRT. CRC tissue from patients that had undergone long course chemoradiotherapy, and subsequently proceeded to surgical resection were analyzed for gene expression of ICAM-1 in pre-operative biopsy and post-operative resection specimens. Value displayed are Log2 ICAM-1 and n = 15.

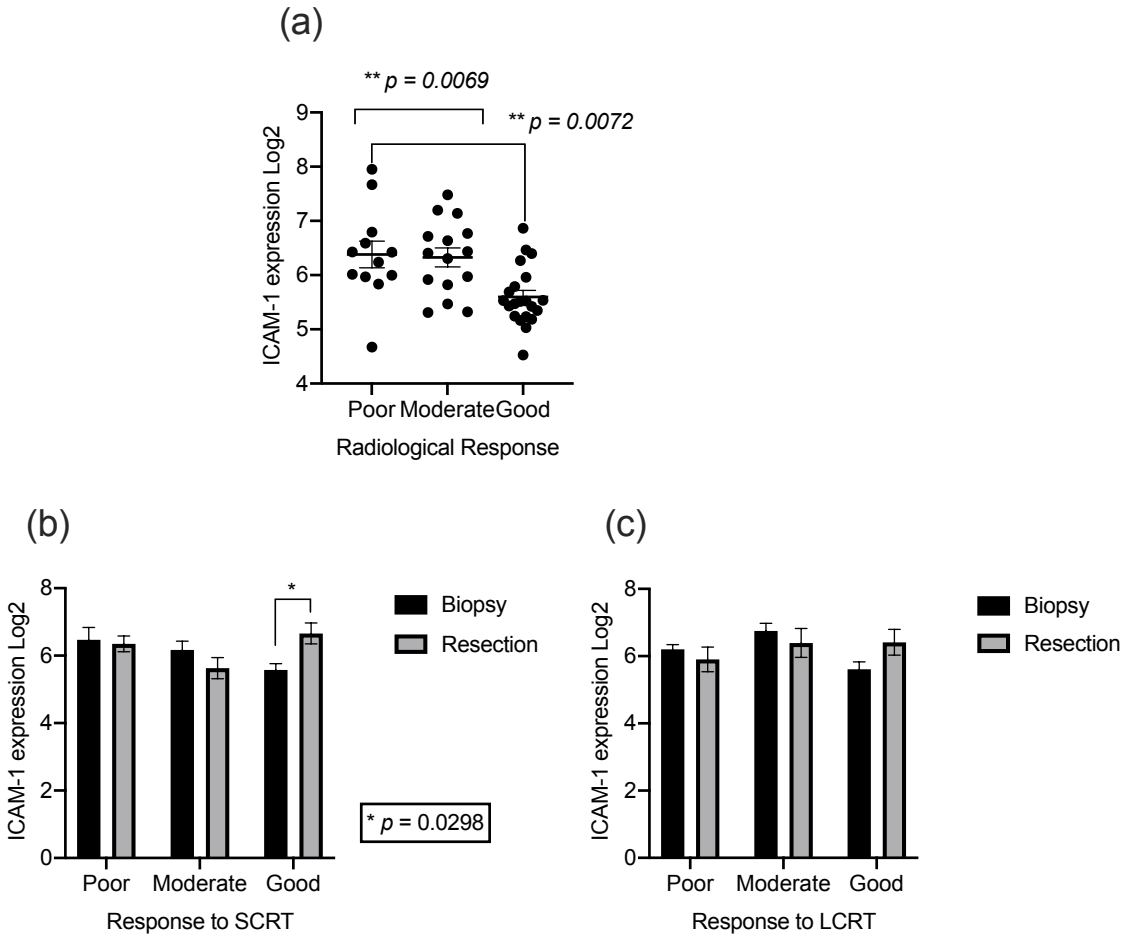


Figure 4-25: ICAM-1 Expression in Patients with Poor, Moderate and Good Response to Radiotherapy. (a) ICAM-1 expression in biopsy of CRC tissue in patients according to their subsequent response to radiotherapy as determined by ΔTCD, n = 48. (b) Change in ICAM-1 expression between pre-treatment biopsy and post treatment resection specimens following SCRT. (c) Change in ICAM-1 expression between biopsy and resection specimens subsequent to LCRT.

4.9 Discussion

Within this chapter we have investigated the potential therapeutic consequences of combining CVA21 with radiotherapy in CRC, with a working hypothesis that radiation could increase ICAM-1 expression in CRC cancer cells and may therefore enhance the direct cytotoxicity (*Error! Reference source not found.*) of CVA21 treatment. This modality of combination therapy was chosen for investigation due to the benefit seen in regard to other OVs combined with radiation, as well as the fact that radiotherapy is a standardised treatment in CRC, discussed in 1.2.5.3. Importantly, upregulation of ICAM-1 following both radiotherapy and mitomycin-c treatment has previously been reported in bladder tumours and improved tumour cell susceptibility to CVA21 toxicity [171] as a consequence. To date, there is no published work with regard to the combination of CVA21 and radiotherapy for the treatment of CRC, and very little in regard to the combination of CVA21 and radiotherapy other than the aforementioned study.

As a proof of principle, we first assessed if we could upregulate ICAM-1 in CRC cells following treatment with TNFα, as suggested by previously published literature [243, 244, 258]. Our findings confirmed that TNFα treatment can result in ICAM-1 upregulation in CRC cell lines, with an increase demonstrated within all 4 cell lines used, an effect which was statistically significant in two. These changes resulted in a consequential increase in CVA21-mediated cytotoxicity of CRC cells as assessed using two separate modes of investigation, LIVE/DEAD® and MTT. Based on these promising findings we continued to investigate the potential sequalae of combining radiation with CVA21 treatment of CRC.

Our initial experiments used flow cytometry to investigate whether radiation could be used to upregulate the expression of ICAM-1 on CRC. Upregulation of ICAM-1 occurs in response to inflammatory stimuli and is known to occur subsequent to radiation[243, 259, 260]. ICAM-1 transcription is stimulated by the presence of inflammatory cytokines such as IL-1β, TNFα, interferon γ and reactive oxygen intermediates (ROI). These mediators activate the transcription factor NFkB, which participates in the induction of ICAM-1 [261, 262].

To investigate whether the upregulation of ICAM-1 seen in our *in vitro* studies would translate into clinically significant changes, we proceeded to examine biopsy and resection specimens from patients that had received treatment for CRC. All the samples investigated originated from patients that had undergone SCRT or LCRT prior to surgical resection. For the purpose of our analysis matched samples were available for pre-treatment biopsies and post-treatment resections. NanoString examination of these samples was undertaken in collaboration with a team at ICR, London. The analysis demonstrated that CRC biopsy and resection samples for ICAM-1 expression showed high levels of expression, but with a wide range of data, which is in keeping with the published literature [197, 202, 204, 256]. Levels of ICAM-1 which originated from patients with local or nodal disease did not differ, however there seemed to be an increase in ICAM-1 expression in patients with poorly differentiated tumours and increasing stage of disease, again in keeping with previous findings [209, 250, 257]. In contrast to our *in vitro* data, there was no apparent difference in ICAM-1 expression between pre-radiation or post-radiation samples in either the entire cohort, or when data was separated into those patients who underwent SCRT and those who received LCRT. However, an interesting caveat to this data is that, when the group of patients was divided into poor, moderate and good responders to radiotherapy, a technique undertaken by Wilkins et al [189], the data revealed significant differences with respect to ICAM-1 expression. For example, patients that had a good response to radiotherapy had the lowest levels of ICAM-1 expression in pre-treatment biopsies compared to those with moderate or poor responses; these differences were statistically significant. In addition to this, patients with a good response to radiotherapy increased ICAM-1 expression following both SCRT and LCRT. These are interesting findings given that within the literature, low levels of ICAM-1 in CRC tumour samples have been associated with a higher chance of metastasis and worse prognosis and higher levels were associated with more stable disease [202]. Moreover, there has been documentation of higher levels of tumour infiltrating lymphocytes (TILs) associated with high levels of ICAM-1 expression and this has been associated with a better prognosis in CRC [205, 209, 263]. What is important to consider is that the 'radiological response' of our data is a histological description, not a measure of clinical course of the patient's disease.

As previously mentioned, the combination of OV and radiation has been investigated for a variety of cancers including prostate, pancreatic [229], ovarian [228], lung [226] and CRC

[233], all of which demonstrate enhanced tumour cell death as a result of the combination. Within this chapter we have demonstrated that the radiation-induced upregulation of ICAM-1 that we demonstrated on CRC cell lines correlated with increased cell death and impairment of relative viability/metabolism subsequent to CVA21 treatment. This increased efficacy is most likely as a result of increased CVA21 cell entry, facilitated by increased ICAM-1 expression. Further evidence to this theory is that viral replication, as demonstrated by plaque assay, was also enhanced in 3 out of 4 cell lines. With respect to SW620 we were even able to demonstrate de novo replication of CVA21 following pre-treatment with radiation. Interestingly, this cell line was resistant to CVA21 mediated toxicity throughout our investigations, possibly as a consequence of its relatively low DAF expression and failure to accumulate CVA21 at the cell surface. This theory would be supported by the fact that both HCT116 and HT29 cells expressed higher levels of DAF and demonstrated more marked improvement in treatment response in the dual treated group. Despite this, the fact that CVA21 replication in SW620 cells can be facilitated following radiation shows that the combination treatment has potential even in the most CVA21 resistant cell lines.

Some studies have successfully reported the increased effectiveness observed with OVs and additional treatments as a synergistic rather than additive effect. For example, the combination of HSV and radiation on pancreatic cell lines has been reported to synergistically enhance cell death. In this study, increased viral replication was not demonstrated, and it was hypothesised that radiation sensitized the cells to virus-mediated cell death [229]. In addition, oncolytic HSV has also been shown to synergise with radiation against lung cancer cell lines, this was hypothesised to be the result of radiation-induced growth arrest and DNA damage inducible (GADD) genes, which increased HSV cytotoxicity and viral proliferation [226]. Moreover, Twigger et. al. have shown in pre-clinical models that Reo can synergise with radiotherapy, and reported enhanced cellular cytotoxicity in CRC cell lines, SW480 and HCT116, in response to dual treatment [236]. Unlike our work, this study treated cells with Reo prior to 5 Gy radiation as they had shown that Reo was not inactivated by clinically relevant doses of radiation. Finally, treatment of CRC cells with a TRAIL-armed Adenovirus (rAD-TRAIL) subsequent to a radiation dose of up to 8 Gy reported increased viral replication and cell death in a synergistic manner in HCT116, HT29, SW480 and SW620 cells. When this combination treatment was extrapolated to an *in vivo* xenograft using SW480 cells, tumour

volumes were smaller, and survival was improved in animals that received the combination treatment compared to control or monotherapy groups [233]. Given that similar synergy has been observed with many other OVs requiring different receptors for cell entry, it is possible that increasing ICAM-1 may not be the only factor related to increased cytotoxicity and viral replication observed in this study. Unfortunately, it is difficult to establish from the work presented within this chapter if the benefit of combining CVA21 with radiotherapy is due to an additive or synergistic response. Although, upon review of the normalised data which controlled for the effects of radiation alone, it is possible that there could be a degree of synergism under some conditions. However, to confirm this we would need to establish more precise dose response curves for radiation and CVA21 treatment for the CRC cell lines; we would then be able to further decipher the nature of the relationship by using computational tools such as isobologram analysis. This form of statistical analysis allowed Dilley et. al to show synergy between radiation and adenovirus against prostate cell cancer lines [264].

Despite further work being required to explore the precise mechanism responsible for the enhanced efficacy of combination treatment using CVA21 and radiation against CRC, this chapter has suggested that the mechanism of ICAM-1 upregulation is crucially important. The radiation-induced increase in ICAM-1 expression in CRC cells results in an increased ability of CVA21 to infect and replicate in CRC cells, thereby increasing CVA21-mediated cellular toxicity. Moreover, we have shown using MTT, that this combination approach of using radiation with CVA21 has the potential to be as efficacious in the treatment of CRC cell lines as the combination of radiation and 5FU, a common combination treatment in clinical practice. It would be interesting therefore, to expand this study to investigate the potential therapeutic benefit of combining CVA21 with other chemotherapeutics and biologic treatments for CRC.

This improvement in cell death, as a result of radiotherapy and CVA21, is in keeping with other findings that have investigated OVs in combination with radiotherapy [226, 228, 229, 264]. Whilst further investigation is required, combination of CVA21 with radiation lends itself to a clinical study either using intra-tumoral or systemic treatment of CVA21 alongside radiotherapy. Although, during the proof of principle experiments we demonstrated that TNFα treatment upregulated ICAM-1 expression of CRC cells and also resulted in enhanced

cytotoxicity when combined with CVA21, this combination would not offer a realistic clinical option. This is because many previous studies have demonstrated significant toxicity and lack of efficacy with TNFα as an anti-cancer agent[265, 266]. Upcoming trials with regard to combining radiotherapy and OV treatment for CRC currently look promising. At the present time a phase I trial combining Edadenotucirev with chemoradiotherapy in locally advanced rectal cancer (CEDAR) has recently been registered [267]. Ultimately, the exciting finding that radiation can sensitise CRC to OVs, including CVA21, offers not only a novel treatment, but also the potential to reduce the clinical dose of radiation that is required to see clinical benefit. This would have the exciting prospect of potentially limiting the dose related side effects of radiotherapy, such as wound dehiscence, surgical site infection (SSI) and anastomotic leak, low anterior resection syndrome (LARS), and genitourinary dysfunction [268-270].

5 Indirect Immune-Mediated Effects of CVA21 in Colorectal Cancer

5.1 Introduction

The direct cytotoxic effects of CVA21 on CRC, and the enhancement of these following radiation treatment have been discussed in the previous two chapters. However, as yet we have not discussed the potential anti-cancer effects elicited by the indirect mechanism of CVA21, for example, through activation of innate and adaptive immune responses. Systemic, immune-mediated killing of tumour cells is one of the principle mechanisms of OVs [110, 129], and the induction of host immune responses is dependent on the type of OV, mode of cell death and the pattern of danger signals released from virally-infected cells [110]. Typically, infection of cells with an OV results in cellular stress and an anti-viral response with upregulation of reactive oxygen species (ROS) and cytokine release, typically type I interferons (IFNs) [271]. This results in the activation and recruitment of macrophages, CD8+ T cells and NK cells [272]. OV-mediated tumour cell death causes the release of TAAs which promote an adaptive immune response, as well as result in the release of DAMPS, PAMPs and further cytokines which causes maturation of APCs [129]. The cross presentation of TAAs by mature APCs stimulates the generation of tumour specific CTLs which migrate to the tumour site and induce an anti-tumour response [273].

The specific mechanisms used by CVA21 to induce anti-tumour immune responses are currently under investigation [124, 170, 176, 179], although more detail is required to generate a full understanding. CVA21 infection is known to modulate the tumour environment and mediate anti-tumour immune response [274]. For example, following intra-tumoral injection of CVA21 in patients with melanoma in the CALM study, there was evidence of regression, and even complete resolution, of non-injected lesions [274]. Moreover, CVA21 infection of bladder cancer cell lines results in the induction of two key DAMPs, calreticulin and HMGB1 [171]. Calreticulin is critical for the recognition of dying tumour cells by DCs [275] and secreted HMGB1 recruits inflammatory cells and can activate macrophages, NK cells and DCs [276]. Elevated IL23, a cytokine with significant anti-tumour effects associated with activation of CTLs and NK cells, was also elevated following CVA21 treatment of bladder tumours [176]. Elevated levels of IL-8, known to be secreted by macrophages, and IFN-γ, produced by cytotoxic T-cells and NK cells, was also identified in patient serum, even in the

absence of circulating CVA21 [274]. In another recent study it was also reported that CVA21 can potentiate both innate and adaptive anti-tumour immunity [124]. The authors demonstrated cytokine-mediated bystander killing against CVA21 resistant AML cell lines, as well as enhanced NK cell-mediated killing cells and priming of tumour specific CTLs. CVA21 is also now being investigated alongside immune checkpoint inhibitors, due to the observation that CVA21 infection results in increased TILs and PD-L1 expression [136, 179].

Within this chapter we aim to investigate the potential exciting mechanisms of indirect cytotoxicity of CVA21 within the context of CRC. Following the conclusion that radiation enhances the direct cytotoxic effects of CVA21, we will also aim to investigate how radiation could affect any immune mediated killing of CRC cells.

5.2 Activation of NK Cells

NK cells, a component of the innate immune system, are effector lymphocytes, derived from haematopoietic progenitor cells, which undergo terminal maturation in secondary lymphoid tissues [277]. They are primarily responsible for immune surveillance and are a major component of innate immunity, contributing a substantial role in the killing of both virally infected and tumour cells [278], killing target cells by directed exocytosis of cytotoxic granules or by the induction of death-receptor-mediated apoptosis [279]. They mount a cytotoxic response, in the form of cytolysis, and also result in cytokine and chemokine production, to perceived abnormal cells ('non-self') in the absence of previous antigen exposure. In doing so they are able to interact with components of the adaptive immune system and support the generation of CTLs [280]. The potential importance of the role of NK cells in the complex mechanisms of OV-induced immunogenic cell death has been highlighted in many papers [112, 124, 281-283].

Here we sought to investigate the role of NK cell function against CRC cell lines following treatment with CVA21 through assessing phenotypic activation, degranulation and killing of target cells. Mature NK cells are identified by the absence of T-Cell receptor complex (CD3-) and presence of neural cell adhesion molecule (CD56+) [284]. Therefore, NK cell activation following CVA21 treatment was assessed via expression of C-type lectin receptor 69 (CD69) on NK cells (CD3-, CD56+) obtained from healthy donor PBMCs following *in vitro* treatment with the virus. CD69 is a membrane protein found on human lymphocytes and is an early indicator of NK cell activation [285]. CVA21 activated NK cells as demonstrated by an increase in CD69 expression following CVA21 treatment for 24 hours (**Figure 5-1**); in each PBMC donor NK cell activation was observed in response to CVA21, even at the lower dose of 0.1 pfu/cell. Understandably there was variation in the levels of expression of CD69 detected due to individual donor variation (**Figure 5-1a**), however the data combined showed a statistically significant activation of NK cells in response to 0.1 pfu/cell and 1 pfu/cell CVA21 ($p = 0.0332$ and $p = 0.0497$, respectively) (**Figure 5-1b**).

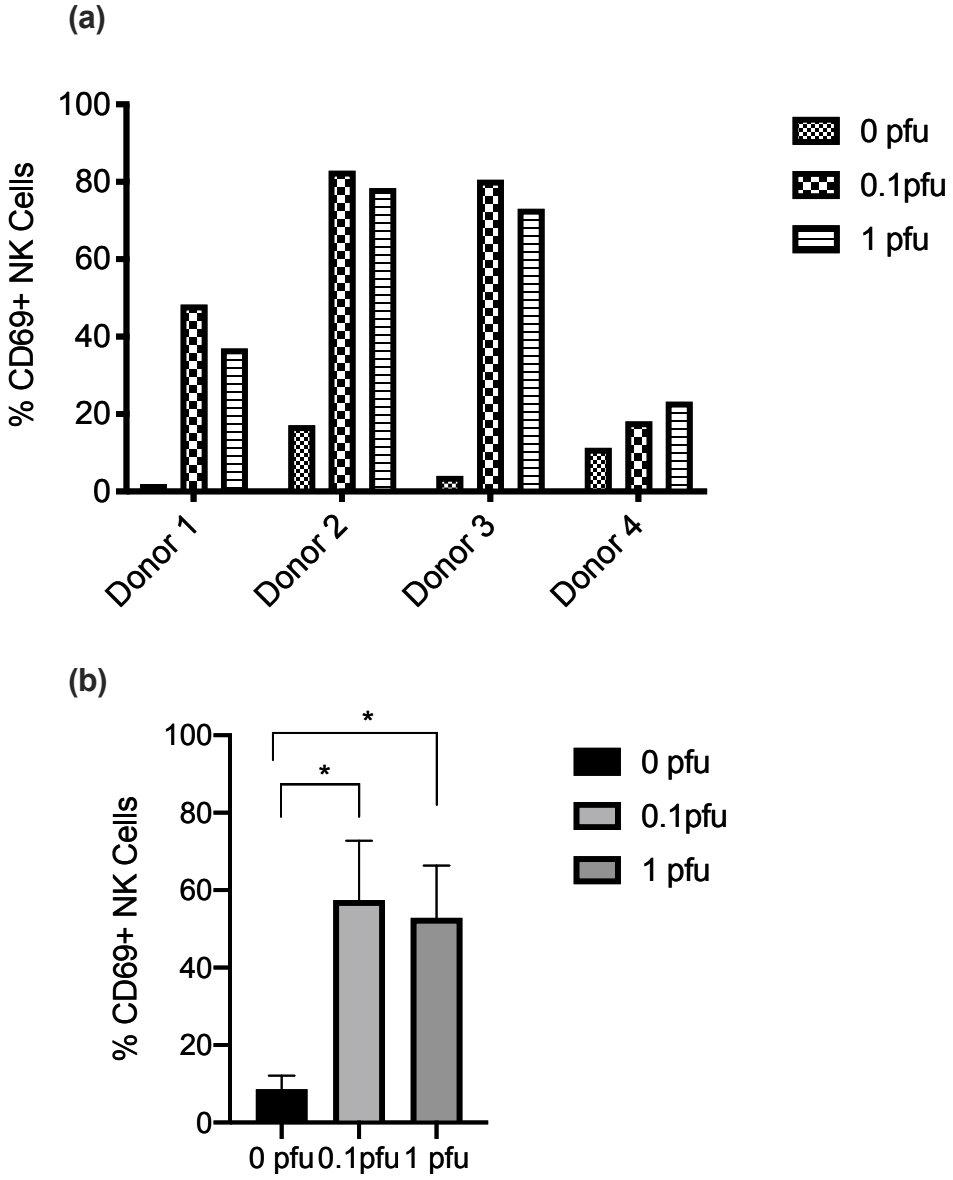


Figure 5-1: NK Cell Activation in Response to CVA21. Healthy donor PBMCs from 4 different donors were treated with CVA21 at doses of 0, 0.1 and 1 pfu/cell for 24 hours. Activation of NK Cells (CD3-, CD56+) was measured as the percentage of NK cells expressing CD69 using flow cytometry (cytoflex). (a) % activation of NK cells within individual donors (b) Summary data of NK cell activation across all donors (n = 4). Statistical significance is demonstrated, where * represents $p \leq 0.05$ (calculated using One-way ANOVA).

Next, to explore the changes in NK cell function following CVA21 treatment, healthy donor PBMC were treated with CVA21 for 24 hours, co-cultured with CRC target cells, and the percentage of NK cells expressing CD107 was determined in order to quantify secretory lysosome release. CD107a is present in the membrane of perforin containing cytotoxic granules and is transiently expressed on the cell surface membrane when granules fuse with the cell membrane [286]. Importantly, CVA21 significantly enhanced NK cell degranulation against all 4 CRC cell lines tested (**Figure 5-2**), suggesting that CVA21 has the capacity to enhance immune-mediated killing of CRC cells, even in the absence of direct oncolysis, as is evident for SW620 cells (**Figure 5-2b**). Paradoxically, there also appeared to be a greater level of degranulation associated with the majority of cell lines at the lower dose of CVA21 (0.1 pfu/cell) used.

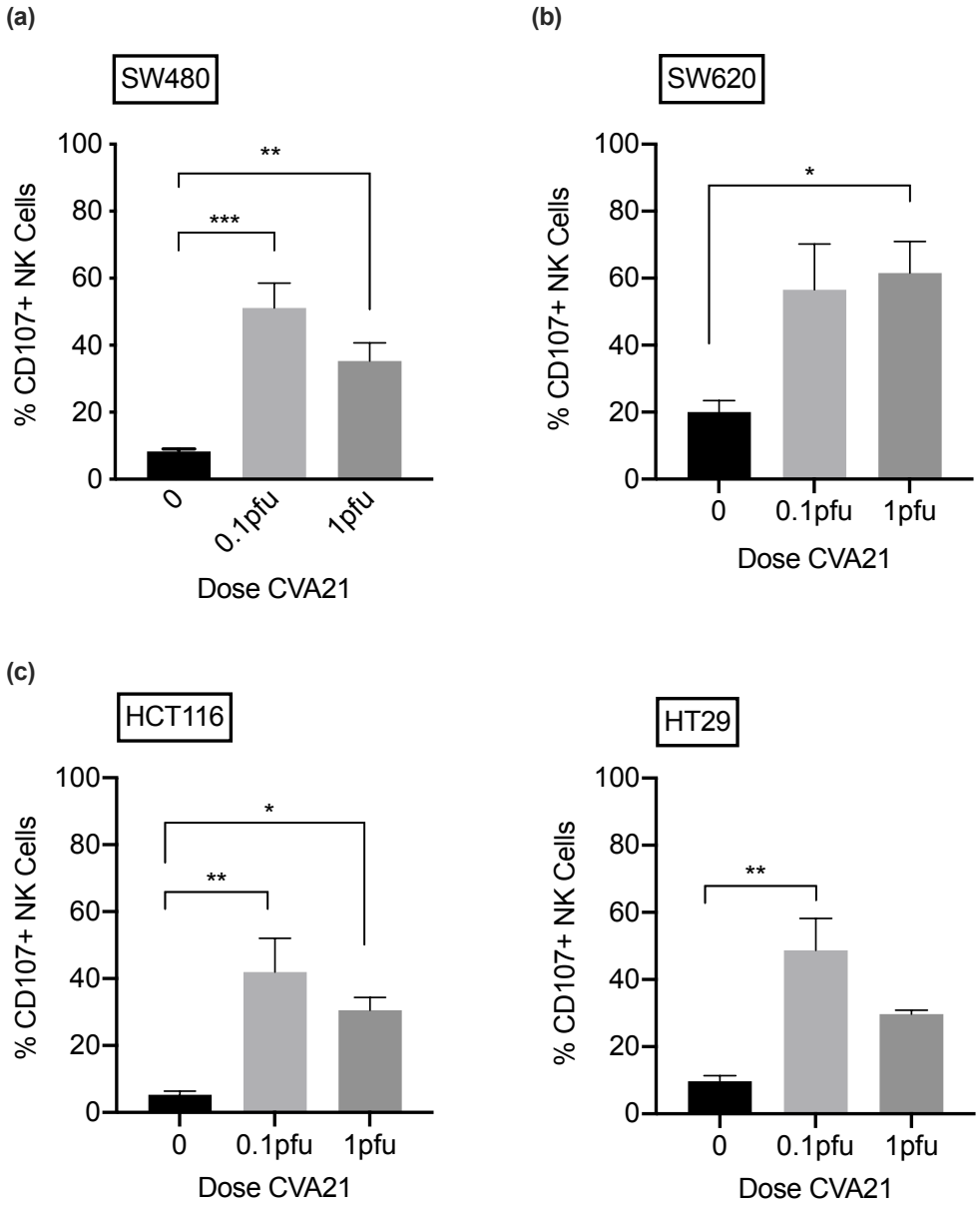


Figure 5-2: NK Cell Degranulation in CRC. Healthy donor PBMCs from 4 different donors were treated with CVA21 at doses of 0, 0.1 and 1 pfu/cell for 24 hours. Degranulation of NK Cells (CD3-, CD56+) was measured via CD107 using flow cytometry (cytoflex). (a) Degranulation against SW480 target cells, which are known to be sensitive to CVA21 direct cytotoxicity. (b) Degranulation of NK cells against SW620 CRC cells which were insensitive to cytotoxic effects of CVA21 and (c) Degranulation of NK cells against HCT116 and HT29 CRC cancer cells, both of which display moderate sensitivity to CVA21 infection. Data shown where n = 4 ±SEM and statistical significance is calculated using one-way ANOVA, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represents $p \leq 0.001$.

After confirming that CVA21 increased the CD107 degranulation of NK cells against CRC cell lines, we next sought to confirm that increased cytotoxic granule release correlated with an increase in killing of each of the CRC cell lines. NK cell mediated death was seen across three out of the four cell lines investigated, with the greatest cell death observed in SW480 (38%), followed by SW620 (33%) and then finally, HCT116 (21%). The only cell line apparently resistant to NK cell-mediated cell death was HT29 (**Figure 5-3**). Importantly, all of the three CRC cell lines that demonstrated susceptibility to NK cell-mediated cell death exhibited an even greater degree of cell death when co-cultured with PBMCs that had been activated with CVA21. In contrast to the higher level of degranulation that was seen associated with a low dose of 0.1 pfu/cell (**Figure 5-2**), the percentage of death of CRC cells occurred in a dose dependent manner (**Figure 5-3**). These findings are particularly important for the HCT116 and SW620 CRC cell lines which display only intermediate sensitivity, and almost complete resistance to direct CVA21 oncolysis, respectively (**Figure 3-2**, **Figure 3-4**). The evidence of NK cell-mediated death within these cell lines, which is significantly enhanced by CVA21, demonstrates the promise of immune-mediated cytotoxicity against CRC following CVA21 treatment.

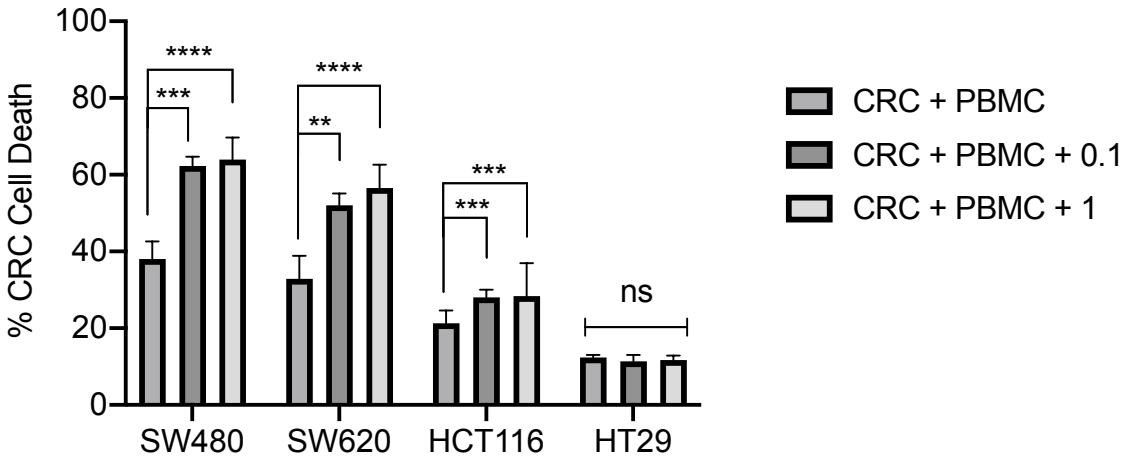


Figure 5-3: NK Cell Mediated Death of CRC Cell Lines: CRC cell lines +/- pre-treatment were co-cultured with healthy donor PBMCs which had been treated with 0, 0.1 or 1 pfu/cell CVA21 for 24 hours. Co-cultures were undertaken at a ratio of 20 PBMC:1 target cells. Cell tracker green was used to identify CRC cells, with subsequent LIVE/DEAD® analysis using flow cytometry (cytoflex). Statistical significance is denoted, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$, $p \leq 0.001$ and *** represents $p < 0.0001$ (two-way ANOVA, Turkeys multiple comparison). Error bars represent SEM, n = 3.

5.3 Irradiation Enhances NK Cell Mediated Killing of Colorectal Cancer

Since we had earlier concluded that CRC cells treated with radiotherapy showed an enhanced susceptibility to the direct cytotoxic effects of CVA21 (**Figure 4-6**, **Figure 4-10**), we next sought to establish what effect radiation would have on the NK cell-mediated cytotoxicity that was displayed within the majority of our panel of CRC cell lines (Figure 5-3), to determine whether radiation would affect the ability of CVA21-activated NK cells to kill CRC cells. We therefore investigated NK cell-mediated killing after CRC cells had been irradiated with 0 or 10 Gy radiation for a period of 48 hours prior to co-culture with PBMCs (**Figure 5-4**). After treatment of CRC cells with 10 Gy radiation, co-culture with CVA21 activated PBMCs resulted in significant death of all four cell lines (Figure 5-4), ranging from around ~25% mean cell death in HT29 cells to as high as ~70% in SW480 cells.

Of the four cell lines investigated, SW480, HCT116 and HT29 showed an increase in NK cell mediated death following treatment with 10 Gy radiation of the cell lines prior to co-culture with PBMC (**Figure 5-5**). Of particular note is that one of the cell lines, HT29, was previously resistant to immune mediated killing, but subsequent to the pre-treatment with radiation, these cells demonstrated significant NK cell-mediated death, albeit on a comparatively small degree of cell death relative to the other cell lines investigated. Importantly, SW620, the only cell line that did not demonstrate this increased sensitivity to NK cell-mediated cytotoxicity, did not show any inhibition or resistance as a result of the radiation.

The progressive increase in NK-cell mediated killing of CRC cells that is observed with pre-treatment of PBMCs with increasing doses of CVA21 in SW480, SW620 and HCT116 (**Figure 5-3**) is a pattern that is maintained with statistical significance in only the first two of these cell lines subsequent to dosing with 10 Gy radiation. Although falling short of significance in HCT116, there remained an apparent, progressive, dose dependent increase in cell death following CVA21 treatment of PBMCs. Excitingly, despite resistance to cell death attributable to 10 Gy radiation within these cell lines at the time point used (48 hours) (**Figure 4-6**), NK-cell mediated death following co-culture with CVA21-activated PBMCs was significantly

higher when the CRC cells were irradiated, which would imply that, not only does radiation improve the cytotoxic ability of NK cells, but it also enhances the activation ability of CVA21-activated NK cells to kill. This is particularly evident in the HT29 cells, which are resistant to NK cell mediated death in the absence of radiation; the combination of CVA21 activated PBMCS following 10 Gy radiation achieved significant death.

In summary, by pre-treating CRC cells with radiation, immune-mediated killing was demonstrated in every cell line investigated, and cell death continued to be enhanced by pre-conditioning the PBMCs with CVA21 prior to co-culture with CRC cells.

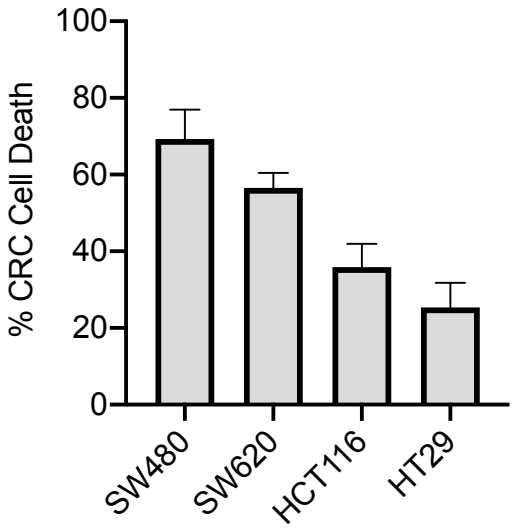


Figure 5-4: Death of Irradiated CRC Cells Following Treatment with CVA21 Activated PBMC. CRC cells were pre-treated with a single dose of 10Gy radiation 48 hours prior to co-culture with healthy donor PBMCs which had been activated with 1 pfu/cell CVA21 for 24 hours. Co-cultures were undertaken at a ratio of 20 PBMC:1 target cells. Cell tracker green was used to identify CRC cells, with subsequent LIVE/DEAD® analysis using flow cytometry as a marker of viability. Data show mean cell death error bars represent SEM, n = 3.

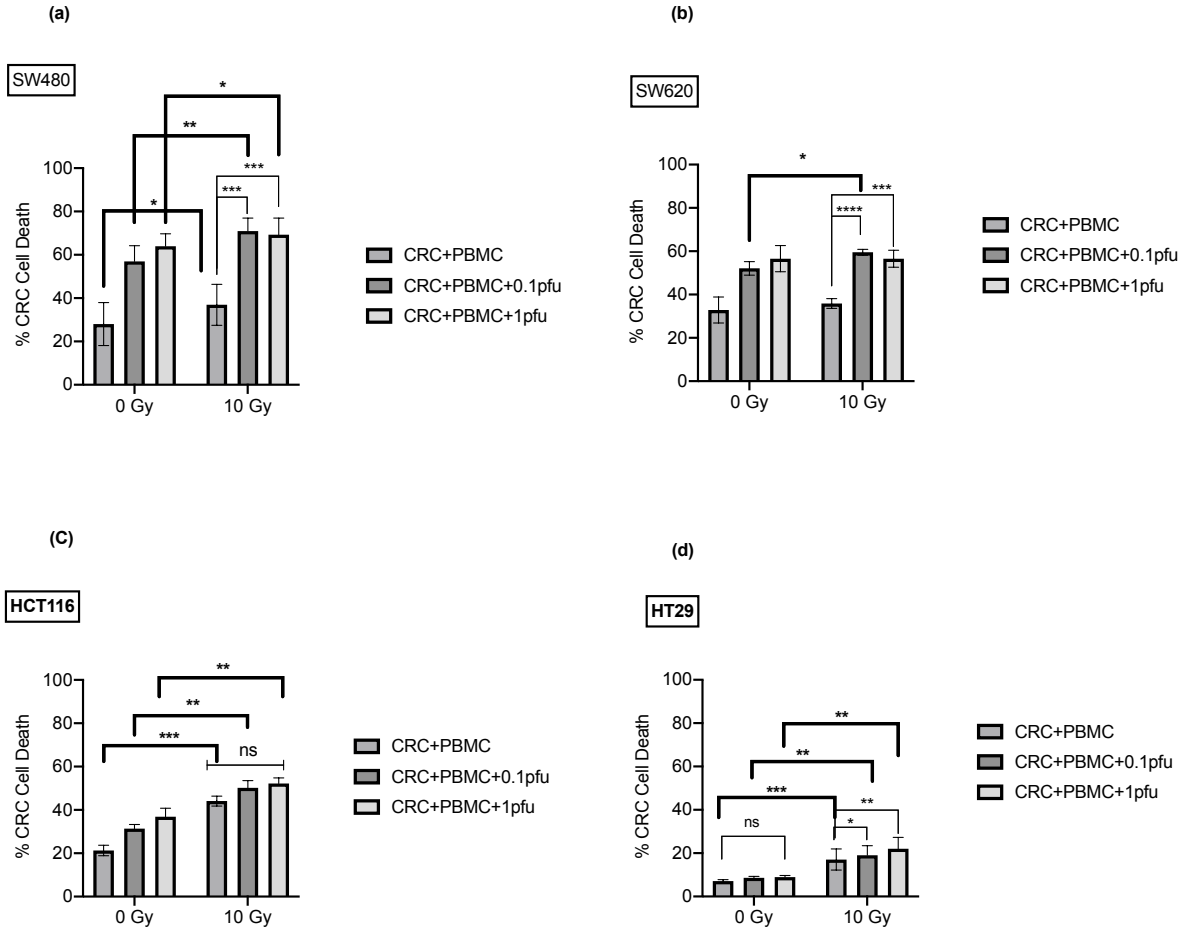


Figure 5-5: NK Cell-Mediated Death of Irradiated CRC Cell Lines. CRC cells were pre-treated with a single dose of 10Gy radiation 48 hours prior to co-culture with healthy donor PBMCs which had been activated with 0, 0.1 or 1 pfu/cell CVA21 for 24 hours. Co-cultures were undertaken at a ratio of 20 PBMC:1 target cells. Cell tracker green was used to identify CRC cells, with subsequent LIVE/DEAD® analysis using flow cytometry as a marker of viability. Data shown is % death normalized for radiotherapy effect to investigate any enhancement of NK cell mediated death. Statistical significance is denoted, where * represents $p \leq 0.05$, ** represents $p \leq 0.01$ and *** represents $p \leq 0.001$ (two-way ANOVA, Turkeys multiple comparison). Error bars represent SEM, n = 3.

5.4 The Effect of sICAM-1 on CVA21 Immune-Mediated Killing of CRC

Given that we know that patients with CRC not only have higher expression of ICAM-1 within the tumour, but also demonstrate higher serum levels of sICAM-1 compared to a healthy population, and that these higher levels may be associated with an increased likelihood of metastatic disease, we sought to establish if sICAM-1 could impair the immune activation that we had demonstrated against CRC cell lines. Since sICAM-1 could inhibit interaction of CVA21 with immune cell component and thus prevent NK cell activation; moreover, it could also act as a circulating ligand to bind with LFA-1 of Leukocytes, thereby making them less available for binding with ICAM-1 on target cells. Having identified that CVA21 can activate NK cells (**Figure 5-1**, **Figure 5-2**), we next sought to determine whether immune activation by CVA21 would be abrogated by sICAM-1, at concentrations that would be evident within a patient demographic.

Interestingly, increasing concentration of sICAM-1 supplementation seemed to inhibit NK cell activation (CD69 expression) at the lower dose of 0.1 pfu/cell, reaching significance at the maximal dose of sICAM-1 used (400 ng/mL), a dose designed to simulate serum of a patient with metastatic disease. However, this effect was not apparent at the higher dose of 1 pfu/cell CVA21 (**Figure 5-6**). Therefore, these data suggest that sICAM-1 can inhibit the phenotypic activation of NK cells at lower doses and that higher doses of CVA21 may be required to overcome any potential inhibitory effects of sICAM-1 in CRC patients, particularly those with metastatic disease. By contrast, upon investigating NK cell degranulation against CRC targets, there was no difference in the level of NK cell degranulation with increasing sICAM-1 concentrations (two-way ANOVA), suggesting that the functional activity of NK cells (in the presence of CVA21) is not affected (**Figure 5-7**).

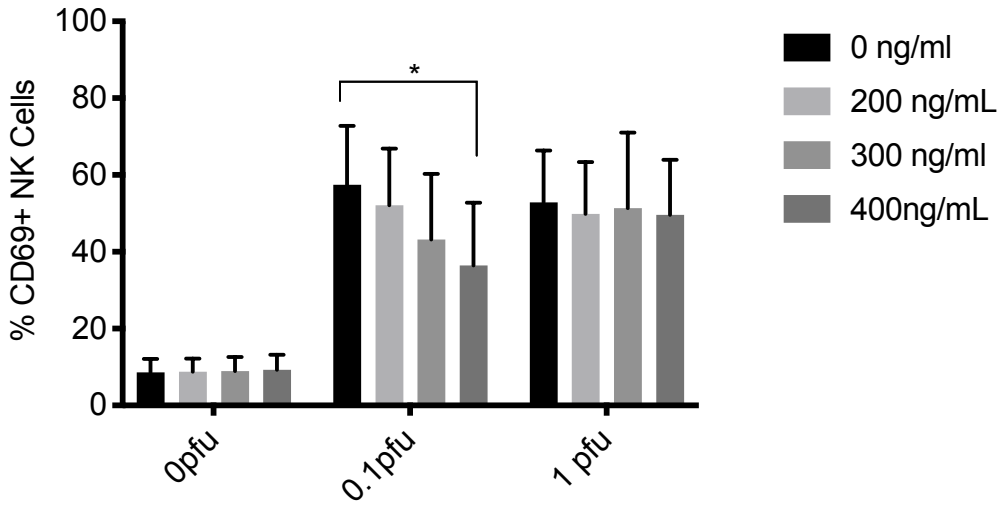


Figure 5-6: NK Activation in the Presence of sICAM-1. Healthy donor PBMCs from 4 different donors were treated with CVA21 at doses of 0, 0.1 and 1 pfu/cell for 24 hours in the presence of increasing doses of sICAM-1 in order to replicate levels found within plasma of patients with and without CRC. Activation of NK Cells (CD3-, CD56+) was measured as the percentage of NK cells expressing CD69 using flow cytometry (cytoflex). Statistical significance is denoted, where * represents $p \leq 0.05$ (two-way ANOVA with Turkeys multiple comparisons test), error bars represent +SEM, n = 4.

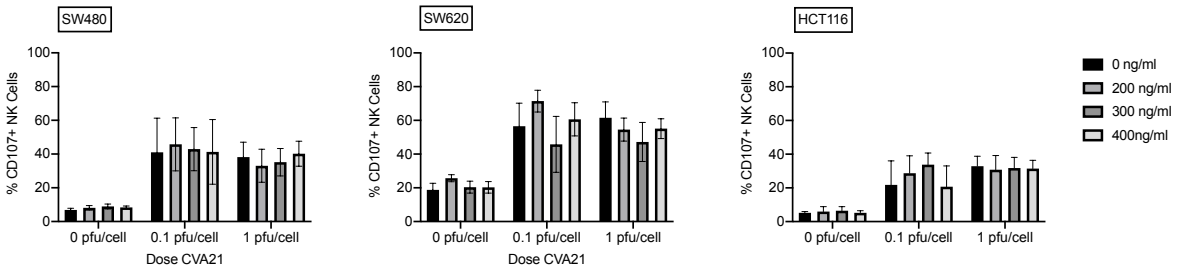


Figure 5-7: Increasing sICAM-1 Concentrations Does Not Alter NK Cell Degranulation against CRC Targets. Healthy donor PBMCs from 3 separate donors were treated with CVA21 at doses of 0, 0.1 and 1 pfu/cell for 24 hours in increasing doses of sICAM-1 conditioned media, in order to replicate levels found within plasma of patients with and without CRC. Degranulation of NK Cells (CD3-, CD56+) against CRC targets was measured via CD107 using flow cytometry (cytoflex). Data shown represents mean for n=3 separate experiments, with error bars to represent +SEM.

5.5 Discussion

As already mentioned earlier within this chapter, the potential of immune-mediated tumour cell death as a result of CVA21 treatment has previously been highlighted by evidence of inflammatory infiltrates within CVA21-injected lesions [136], as well as in non-injected distal lesions [287]. The precise mechanisms by which CVA21 can achieve these responses are in the early stages of research. Within this chapter we have primarily investigated the role of NK cells with respect to CVA21 treatment, with a primary focus on treatment of CRC. NK cells are circulating, lymphatic, cytotoxic cells that are a component of the innate immune system, playing an important role in the defence of viral infections, tumour surveillance and tumour cell death [280]. They are derived from haematopoeietic cells within bone marrow and undergo maturation in secondary lymphoid tissues [288]. Once these cells are activated by pro-inflammatory cytokines such as IL-12 and type I interferons, they mediate cytotoxicity and produce cytokines including TNFa and IFNg, GM-CSF and chemokines, which can modulate the function of both innate and adaptive cells [289]. They thereby possess an integral role as a key intermediary within the immune system and their activation by CVA21 may herald important information as to how this OV may result in its anti-cancer effects.

There is evidence to support that, despite their role in elimination of virally infected cells [290], NK cells are important in immune-mediated OV function. For example, mice bearing NSCLC xenografts infected with coxsackievirus B3 (CVB3) elicited significant tumour regression, and tumours were shown to have higher levels of NK cells and increased levels of CD107 compared to untreated tumours [291]. Similarly, infection of pancreatic cancer cell lines with a parvovirus, H-1PV, resulted in significantly greater cell death as a result of IL-2 stimulated NK cells, than un-infected tumour cells [281]. This same virus, which has limited direct killing of CRC cell lines, has also been shown to elicit enhanced NK cell-mediated cytotoxicity against these virus resistant cells [292] demonstrating that, even in the absence of direct cytotoxicity, OVs can elicit an NK cell mediated anti-tumour immune response. There is further evidence to support the importance of the role of NK cells in immune-mediated cytotoxicity from trials with Reovirus. Serum from patients with CRC involved in a clinical trial whom received intravenous Reovirus demonstrated rapid expression of CD69 on NK cells

within 48 hours of treatment [293], and a separate study also found that Reo treated PBMCs expressed high levels of degranulation marker CD107 following co-culture with CRC cell lines [220]. Depletion this subset of NK cells greatly reduced innate tumour cell killing, thereby highlighting the pivotal role of NK cell in immunogenicity of Reo [220].

The first stage of our investigation of the potential role of CVA21 as an OV was to investigate possible activation of NK cells *in vitro*. Following treatment of PBMCs from healthy donors with increasing doses of CVA21, we demonstrated that NK cells (CD3-, CD56+) were phenotypically activated, even at a low dose of CVA21. This finding was ubiquitous among all the healthy donors investigated and consistent with data demonstrated by Muller et.al. [124] which not only showed the same increase in *in vitro* samples, but also reported a peak in CD69 expression on NK cells obtained from patients with advanced malignancy that had been treated with CVA21 as part of the STORM study [124]. NK cells are known to express CD69 after cytokine stimulation such as IL2 and IFNα [294], and its expression induces cytolytic activity of these lymphocytes [295].

Following the demonstration that CVA21 infection induces CD69 expression on NK cells, we found that co-cultures of PBMCs with CRC targets resulted in increased expression of CD107, a marker of degranulation, against all four of our CRC cell-lines when PBMCs were activated with CVA21. This degranulation of CVA21 activated NK cells translated into a significant increase in cell death in CRC cell lines, with the exception of HT29 which remained resistant. Interestingly, despite the process of immune escape leading to solid tumours often being able to evade or be resistant to NK cells [296], there is previous published data showing NK-cell mediated death of CRC cells [297, 298]. Contrary to our data, one study also reported that HT29 cells show high susceptibility to NK cell-mediated death. However, it should be noted that the authors of this paper had pre-activated NK cells with IL-2 [292]. Of note, we did find that following radiation treatment of HT29 cells, there was significant increase in NK-cell mediated death, which could possibly be attributable to the observation that increased IL2 levels can be found in patient serum following irradiation [83, 299], although we did not analyse supernatants for its presence within our experiments to confirm this hypothesis. It is possibly more likely that ionizing radiation may be inducing NK activatory ligands on CRC cells resulting in the increased NK cell-mediated we observed. This increase in activatory ligands

on cancer cell lines following radiation treatment has previously been reported [87]. Mechanisms of NK cell resistance are well described and are common amongst cancerous cells; there can be a failure of target cell recognition through interaction of activation and inhibitory signals, or failure of NK cell to destroy a recognized target [300]. Since all the CRC cell lines investigated resulted in significant degranulation of NK cells, it seems the failure of resultant cell death in HT29 cells from NK cells would likely be attributable to the latter of these two mechanisms. This could be the result of impaired perforin binding to the target cell, a mechanism which has been demonstrated in leukaemia cell lines [301], or through increased expression of a protease inhibitor which would prevent granzyme function and has been reported in lymphoma cells [302, 303].

The increase in NK cell mediated death seen in SW480, SW620 and HCT116 cells when PBMCs were pre-treated with CVA21 demonstrates a virally induced enhancement of NK cell-mediated death. Moreover, what is important to consider here is that the SW620 cell line, which is almost completely resistant to CVA21 direct cytotoxicity, was susceptible to NK cell-mediated cell death. This implies that even in absence of direct oncolysis, CVA21 may have an important role as treatment in CRC. The possible production of type I IFN in response to CVA21 treatment would be promising as, in the context of CRC, IFNα has been shown to have antiproliferative and pro-apoptotic effects resulting in inhibition of growth of CRC cells [304]; furthermore, IFNα also inhibited tumour growth and liver metastases in human xenograft models of CRC [305]. Interestingly, CVB infection has previously been shown to result in NK cell production of IFNγ [306] which has, similarly to IFNα, can inhibit CRC cell proliferation and colony forming potential [307] as well as initiating apoptosis [308]. In summary, we have confirmed that CVA21 not only results in activation of NK cells but induces NK cell degranulation and enhances NK cell mediated cytotoxicity of CRC cells. Although the precise mechanisms behind these results have not been investigated within this chapter, given previous literature relating to OV activation of NK cells, a role for type I IFN is likely [124, 309].

There is documented evidence to show that NK cell counts are significantly reduced in the CRC tumour micro-environment and lower NK cell levels are associated with a more advanced stage of disease [310]. Interestingly, low numbers of NK cells are found in CRC when compared with normal adjacent tissue, and pre-malignant adenomas also demonstrate less

NK cell infiltration than surrounding normal tissue [166]. In addition, it has also been reported that NK cells are either low or completely absent in CRLM [166]. Importantly, it is known that decreased levels of NK cells present in CRC has implications on disease prognosis which has been highlighted by analysis of histopathologic samples obtained following surgical resection of CRC in treatment naïve patients whom underwent surgical resection. For example, there was significant improvement in overall survival ($p = 0.0032$), and improved disease-free survival ($p = 0.0083$) in the group of patients with extensive NK cell infiltration when compared to the group with little or moderate infiltration [311]. Moreover, a separate study which analysed percentage NK cells within peripheral blood of over 400 patients with CRC also found that patients with a higher percentage of circulating NK cells had a longer survival time when compared with those with a low percentage NK cells [312]. Further studies have also not only highlighted decreased NK cell activity in the serum of patients with CRC [313] but also a decrease in NK cell activating ligands, compared to healthy controls, with a further decrease in these levels associated with lymph node metastasis, worse histological grade and increasing depth of invasion of tumour [314]. This important relationship between NK infiltration and activation in CRC could imply that if CVA21 treatment created an inflammatory environment that improves recruitment and activation of NK cells, as demonstrated within this work, then it could be an important treatment to improve patient outcomes, even in the scenario where the primary tumour is resistant to the direct mediated cytotoxicity of CVA21. Indeed Reo has previously been observed to result in recruitment of both innate and adaptive immune effectors, with significant increase in NK cell recruitment following intra-tumoral injection of Reo into a murine model of prostate cancer [315].

NK cell activity is regulated by a balance of activating and inhibitory signals conferred by the binding of NK cell receptors to ligands expressed on target cells. The three main NK cell receptors include natural cytotoxicity receptors (NCRs), C-type lectin (CD94/NKG2) and killer cell immunoglobulin-like receptors (KIRs). NCRs are activating receptors and C-type lectin and KIRs can be activating or inhibitory. Of the most important NK cell receptors are KIRs and NKG2A because they recognise MHC on target cells as evidence of “self”, leading to inhibition of NK cell activity [316]. NK cells are known to recognize and kill cells with low or absent MHC class I expression, a marker of ‘self’ [280] [317], and could therefore potentially clear tumour cells which fall into this category. In theory, the greatest susceptibility to NK cell mediated

death would occur where MHC class I is downregulated and there is an upregulation of activatory ligands.

It has previously been reported that MHC class I is downregulated in CRC. In an analysis of 88 colorectal tumours, 72% were shown to have down regulation of MHC-1 expression, however tumour-infiltrating lymphocytes were predominately CD8 and CD4+ lymphocytes rather than NK cells [318]. In contradiction to this data, a different study reported that, in rectal tumours specifically, HLA class I expression was high in more than 80% of resected tumours and there was no difference in expression between irradiated and non-irradiated tumours [319]. The tissue samples for this study were obtained as part of the wider Dutch TME trial, a large randomised controlled, multi-centre study which investigated the value of short-term radiotherapy in combination with total mesorectal excision (TME). The samples were therefore obtained from patients with surgically-resectable disease and did not have any significant metastatic disease burden [320]. From these samples it was established that patients with low expression of MHC I had worse overall survival and disease-free survival, irrespective of treatment. However, this predictive value was lost in multivariate analysis, possibly explained by the group of tumours with low HLA class I expression having significantly more advanced stage disease and a higher proportion with positive resection margins. Other studies have reported that ionizing radiation has been shown to upregulate MHC I expression in cancer cells [321, 322] and more specifically CRC cells [244], however this is not necessarily detrimental to NK cell cytotoxicity or immunogenicity. Reits *et. al.* suggested that directed radiotherapy of cancer can improve the efficacy of tumour immunotherapy [323]. They demonstrated a dose dependent increase in MHC class I expression following treatment with radiation of a melanoma cell line using transgenic mice demonstrated increased sensitivity to antigen-specific cytotoxic T cell killing; enhanced CD8+ killing of CRC cell lines which had upregulated MHC class I following sub-lethal doses of radiation have also been reported [244].

Another important aspect to consider is that ionizing radiation has been shown to increase the expression of NKG2D ligands, which can render cells susceptible to NK cell attack [324]. Furthermore, in addition to the altered balance between activating and inhibitory NK cell ligands, it could be considered that the cytokine milieu induced by radiation treatment could

also enhance or support NK cell function, such as via IFNγ secretion [325]. Although it should also be noted that we did not investigate the effects of radiation on NKG2D ligand on CRC cell lines, DNA damage induced by radiation can result in its increased expression; increased expression of this NKG2D is associated with NK cell mediated cytotoxicity. Levels of NKG2D have been shown to increase in cancer cells, including a CRC cell line, as a result of ionizing radiation [326] and this increase resulted in enhanced sensitivity to NK cell-mediated cytotoxicity. Further evaluation of the effect of radiation on CRC cells and how the overall balance of activatory or inhibitory signals, and their effect on NK function in the presence of CVA21 infection would therefore be interesting to determine.

Further important considerations for immune mediated enhancement seen with radiation of CRC and CVA21 treatment is the pivotal role of ICAM-1 expression (also crucial for direct cytotoxicity of CVA21, reported in chapter 3). It is known that tumour infiltrating lymphocytes are found at higher levels in CRC tumours exhibiting greater levels of ICAM-1 [202]. In addition, ICAM-1 is one of the natural ligands to the lymphocyte function-associated antigen-1 (LFA-1) which is expressed on all leukocytes; however, LFA-1expression is two to three times greater on activated T-cells compared to resting T-cells. Binding of LFA-1 to ICAM-1 induces an activation signal which enhances the cytotoxicity and cell growth of NK cells. The irradiation of CRC cell lines, with the resultant increase in ICAM-1 levels (reported in chapter 4) may allow for increased NK cell-mediated death, since there is more availability of ICAM-1 for LFA-1 binding. Jeong et al., demonstrated increased NK cell-mediated killing following upregulation of ICAM-1 subsequent to radiotherapy treatment in other human cancer cell lines [327]. Overall, our earlier findings have shown a statistically significant increase in ICAM-1 in SW480 cells in response to radiation, and the suggestion of an increase in SW620 and HCT116 (**Figure 4-4**, **Figure 4-5**) and this could explain the increase in NK-mediated cell death observed following irradiation of human CRC cell lines. Crucially, we have shown that NK activation and NK-mediated cell death is not impaired despite increasing concentrations of sICAM-1 as may be encountered in patients with more advanced or metastatic CRC.

In summary, our findings have shown that CVA21 treatment induces NK cell activation. Co-culture of CVA21 treated PBMCs with CRC cell lines results in degranulation of NK cells and consequently facilitates NK cell-mediated death of CRC cells, even in cell lines which are

resistant to direct cytotoxicity of CVA21 treatment. When CRC cells are treated with sub-lethal doses of radiation prior to co-culture with CVA21 treated PBMCs, a further enhancement of NK cell-mediated death is observed. These findings show that, not only does CVA21 treatment of CRC result in immune-mediated tumour cell death, but its concurrent use with pre-existing treatments such radiotherapy can further enhance the anti-tumour immunity elicited by CVA21.

6 Conclusions and Future Work

Although there have been significant advances in the treatment of CRC over the last few decades, its incidence continues to rise and the prognosis of patients with advanced or recurrent disease remains poor. Whilst numerous OVs are in the early stage of research for CRC, CVA21 has not previously been investigated in the context of this disease. This thesis therefore examined the ability of CVA21 to mount a direct cytotoxic and indirect, immune-mediated, cytotoxicity against CRC and further examine how these effects may be altered in the presence of existing treatments of CRC, namely radiotherapy. To date, this is the first study to examine the role of CVA21 in the treatment of CRC.

Within this study, we have demonstrated that CRC cell lines show varying degrees of sensitivity to direct cytotoxicity as a result of treatment with CVA21. As studies have previously shown with respect to other types of cancers, the susceptibility of CRC cells to the direct cytotoxic effects of CVA21 is proportional to the degree of ICAM-1 expression that they display. Although only one CRC cell line (SW480) showed significant cell death on LIVEDEAD® studies, the more subtle effects of impaired metabolism/cell viability were present on additional cell lines (HCT116 and HT29), both of which express high levels of the secondary receptor for CVA21, DAF, and suggests a more important role for this often-overlooked receptor. This important finding was further highlighted by evidence of CVA21 replication and impaired clonogenic ability within these same cells.

In addition to investigating direct cytotoxicity of CVA21 against CRC, this study has established that CVA21 can result in immune-mediated killing of CRC cells. We have demonstrated that CVA21 treated NK cells, from healthy donor blood, resulted in CD69 activation, NK cell degranulation and increased cytotoxicity against CRC targets. Crucially, following treatment with CVA21, NK cells demonstrated degranulation and death of CRC targets even in a cell line (SW620) which was entirely resistant to the direct cytotoxic effects of CVA21.

It is important to consider how OVs may be affected by their concurrent use with standard treatments for cancer, partly as they are unlikely to be utilised independently within the

setting of clinical practice, and also since combination therapy has been shown to confer synergistic benefit. Not only has this study been the first to investigate CVA21 in CRC, but it is the first time that CVA21 has been investigated in combination with radiation for CRC. The use of these treatments together not only enhanced the direct cytotoxic effects of CVA21 but also resulted in amplifying the degree of NK cell-mediated death observed. Excitingly, the use of combination therapy resulted in CVA21 being able to replicate in a previously resistant cell line (SW620) and NK cell-mediated death being observed in HT29 cells which were resistant to this mode of cell death in the absence of radiation treatment. These findings highlighted that the combination use of CVA21 may be an important tool in optimising CVA21 as a treatment.

Although this study has demonstrated an exciting potential role for CVA21 treatment in CRC, the results have primarily been derived from in vitro work with a small panel of CRC cell lines and healthy donor PBMCs. Given the genetic variability and heterogeneity seen within primary and metastatic tumours, it would be interesting as a first step to expand the panel of cell lines used within this study to establish a more comprehensive picture of the potential clinical effectiveness of CVA21 in terms of both its direct and indirect cytotoxicity against CRC. As a further expansion of this it would be useful to extrapolate to a more complex model of CRC such as 3-dimensional spheroids or, preferably, murine in vivo studies. This would not only further clarify the effectiveness of CVA21 as a treatment for CRC but also further examine the beneficial results seen when combining CVA21 with radiation.

This study has demonstrated that CVA21 has the capacity to elicit immune mediated cellular death of CRC by way of activation of NK cells, part of the innate immune system. In order to expand on this, further investigations to study potential recruitment of the adaptive immune system is required. This could be undertaken by investigation of potential activation of DCs and priming of colorectal-specific CTLs in the presence of CVA21.

For further investigation of CVA21 as a combination treatment with radiation against CRC, it would be interesting to firstly establish more accurate dose response relationships to assess whether we are observing an additive or synergistic benefit. It would also be of significance to investigate other possible mechanisms behind the enhancement of both direct and indirect

cytotoxicity of CVA21. For example, cytokine milieu, NK activating ligands and other cellular signalling pathways that may impact CVA21 infection and replication. It would also be important to investigate the optimal timing of using these two treatments together, for example, would CVA21 replication be inhibited if radiation treatment followed CVA21 dosing?

Although the Nanostring analysis of colorectal cancer biopsies demonstrated a variety of ICAM-1 expression, which is in keeping with previous published literature, the data was somewhat limited by the bias of selecting patients which had gone on to have treatment with curative intent. These human tissue samples also gave us an insight into the changes of ICAM-1 expression following radiotherapy treatment, although again, the same bias applied. Therefore, it would be important to expand this aspect of the study to include tissue specimens of a wider range of patients, with different disease stages, including patients undergoing palliative treatment. It would also be of relevance to correlate tumour ICAM-1 expression with circulating sICAM-1. This would give us a greater idea of how the two correlate and if ICAM-1 shedding affects prognosis in CRC and how CVA21 cytotoxicity may be affected in terms of direct and indirect cytotoxicity; essentially does unstable ICAM-1 impact immune evasion of CRC and could CVA21 treatment counteract this?

In summary, we have demonstrated for the first time that CVA21 not only has significant potential as a treatment for CRC, but when used in combination with radiation, demonstrates further enhanced direct and immune-mediated killing of CRC cells. This early *in-vitro* data provides important information with which to design further studies into this combination treatment for CRC. For example, early phase clinical trial could potentially be approached in a number of different ways. The ideal format however would be to use either intra-tumoural or systemic delivery during a period of SCRT with subsequent surgical resection as the treatment pathway and timings are relatively uniform which would deliver a degree of uniformity within the trial conditions.

Ultimately, the success of this combination of CVA21 and radiotherapy may further enhance tumour response to treatment, even in the context of CVA21 resistant tumours, and holds the potential of promoting an enduring immunological response to minimise disease

recurrence. The encouraging evidence which we have presented within this study warrants further research into the use of CVA21 as a treatment for CRC.

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