

Arming Oncolytic Viruses with
Bispecific T Cell Engagers (BiTEs) for Expression
and Secretion into the Tumour Microenvironment



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Abstract

Oncolytic viruses exploit the cancer cell phenotype to complete their lytic life cycle, releasing progeny virus to infect nearby cells and repeat the process. We modified the oncolytic group B adenovirus EnAdenotucirev (EnAd) to express bispecific single chain antibodies, secreted from infected tumour cells into the microenvironment. The bispecific T cell engager (BiTE) binds to a selected antigen on target cells and crosslinks them to CD3 on T cells, leading to clustering and activation of both CD4 and CD8 T cells. BiTE transcription can be controlled by the virus major late promoter, limiting expression to cancer cells that are permissive for virus replication.

We first exemplified this approach using a BiTE that recognises EpCAM, which is highly expressed on many carcinomas and is recently identified as a marker of cancer-initiating cells. A second BiTE was engineered to target FAP on cancer-associated fibroblasts, demonstrating the power of such an approach in targeting cancer-promoting stromal cells of the tumour microenvironment. This strategy can potentiate the cytotoxicity of EnAd and we show using primary pleural effusions, peritoneal malignant ascites and solid prostate tumour specimens that infection of cancer cells with the BiTE-expressing EnAd leads to activation of endogenous T cells to kill endogenous tumour cells or fibroblasts despite the immunosuppressive environment. In this way, we have armed EnAd to combine both direct oncolysis and T cell-mediated killing, yielding a potent therapeutic that should be readily transferred into the clinic.

Declaration of Authentication

The material presented in this thesis was performed under the supervision of Professor Leonard Seymour, Department of Oncology, Division of Medical Sciences, University of Oxford. This work is entirely my own, and any contribution to the thesis made by others has been acknowledged. The work submitted here does not form part of another thesis in this or any other university.

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List of Abbreviations

ACT	Adoptive cell therapy
Abs	Absorbance
Ad	Adenovirus
ADCC	Antibody-dependent cell-mediated cytotoxicity
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
APC	Antigen-presenting cell
Asc	Peritoneal ascites
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
BiTE	Bispecific T cell engager
bp	Base pairs
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
CML	Chronic myeloid leukaemia
CMV	Cytomegalovirus
CPE	Cytopathic effect
CTLA-4	Cytotoxic T lymphocytes associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
Da	Daltons
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
dH ₂ O	Deionised water
DMEM	Dulbecco's Modified Eagles Media
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
E:T	Effector:Target ratio
EC ₅₀	Half maximal effective concentration
EDTA	Ethylenediamine tetra-acetic acid
EGFR	Epithelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EnAd	Enadenotucirev
EpCAM	Epithelial cell adhesion molecule
EphA2	Ephrin type-A receptor 2
ERK1/2	Extracellular signal-regulated kinase 1/2
Ex.Asc	Expanded ascites cells
FAP	Fibroblast activation protein
FBS	Fetal bovine serum

FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FHA	Filamentous hemagglutinin
Fv	Fragment variable region
g	Gravity
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HER2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1 protein
HRP	Horseradish peroxidase
HSP70	Heat Shock Protein 70
HSV1	Human herpes simplex virus type 1
IC ₅₀	Virus dose required to kill 50% of cells
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN γ	Interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin isotype G
IHC	Immunohistochemistry
IL	Interleukin
ImmTACS	Immune mobilising monoclonal T-cell receptors Against Cancer
I.T.	Intratumoural
I.V.	Intravenous
LAG3	Lymphocyte-activation gene 3,
LDH	Lactate dehydrogenase
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
MLP	Major late promoter
MPE	Malignant pleural effusion
MT110	An EpCAM-targeted BiTE
NHL	Non-Hodgkin lymphoma
NK cell	Natural killer cell
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
OD	Optical density
OV	Oncolytic virus
P2A	Porcine teschovirus-1 2A self-cleaving peptide
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1
PDL1	Programmed death-ligand 1
PEI	Polyethylenimine
PFU	Plaque forming units
pSF	Snap fusion plasmid

qPCR	Quantitative PCR
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute media
scFv	Single chain variable fragment
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T cell	T lymphocyte
TAA	Tumour-associated antigen
TAL	Tumour-associated lymphocyte
TAM	Tumour-associated macrophage
TCID ₅₀	50% tissue culture infectious dose
TCR	T cell receptor
TGF β	Transforming growth factor beta
Th	T helper cell
TIL	Tumour infiltrating lymphocyte
TIM3	T-cell immunoglobulin and mucin-domain containing-3
TME	Tumour microenvironment
TNF α	Tumour necrosis factor alpha
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VH	Heavy chain variable domain
VL	Light chain variable domain
ρ	Density

1. Introduction

1.1 Cancer Treatment

1.1.1 Traditional Approaches to Cancer Therapy

Cancer is a group of diseases characterised by abnormal cell proliferation and an ability to invade other tissues (Hanahan & Weinberg, 2011). The lifetime risk of cancer for people born in the United Kingdom after 1960 has now surpassed 50% (Ahmad et al, 2015), with cancer currently responsible for nearly one in three deaths (Office for National Statistics, 2014). Whilst the incidence of cancer is steadily rising, advances in treatment, detection and screening methodologies are helping to combat against many malignancies, reducing mortality rates in lung, colorectal, breast, and prostate cancers (Siegel et al, 2013). Indeed, the number of people surviving 5+ years has risen by over 21% in the past five years (Maddams et al, 2012). However, for the majority of patients, metastatic cancer remains an incurable disease and conventional therapies possess significant limitations in its treatment. Surgery is often effective as a means to debulk primary tumours but is unable to fight against micro-metastasis. Whilst radiation therapy can be a potent treatment regimen for removing larger masses, distal lesions are similarly difficult to target, and damage to surrounding tissue can be severe. Chemotherapy can be administered systemically and is able to target distant metastases, however the lack of specificity results in significant unwanted side effects. Even with combinatory treatments, in the majority of cancers the effect of the traditional therapies can be modest, and patients often show disease recurrence.

With a deeper understanding of the molecular mechanisms of cancer pathogenesis, the introduction of mechanism-based targeted therapies was heralded as a new era in the treatment of cancer (Sawyers, 2004). Many of the targeted therapies developed have been directed against specific molecular targets that are (putatively) essential to cancer biology, and this approach has been incredibly effective in treating a subset of tumour types (e.g. Imatinib for chronic myeloid leukaemia (CML) and acute myeloid leukaemia (AML)) (Baselga, 2001; Cohen et al, 2002). Whilst this specificity of action and fewer associated toxicities are highly desirable; in most cases when treating advanced highly heterogenic solid tumour, clinical responses have been transient and are followed by near-inevitable relapse (Engelman & Settleman, 2008; Pao & Chmielecki, 2010).

1.1.2 Immunotherapy of Cancer

Defined as the treatment of cancer by generating or augmenting an immune response against it, cancer immunotherapy is now recognised as a powerful anti-cancer approach. The idea that the immune system can play a fundamental role in controlling tumour growth dates back over half a century. First proposed by Ehrlich, Burnet and Thomas, the concept of immunosurveillance suggests that, whilst the inception of malignant cells is a common event, these cells are rapidly eliminated by the host immune system prior to any clinical manifestation (Burnet, 1971; Ehrlich, 1956). In 1957, Burnet stated:

“It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence.”

This theory proved contentious for decades due to a lack of direct experimental evidence, and the use of less sophisticated treatments such as chemotherapy and radiotherapy predominated. Supporting evidence has, however, steadily accumulated. Robust infiltration of cytotoxic T cells into tumours has been correlated with decreased recurrence and better survival in patients with colorectal cancer (Galon et al, 2006), ovarian carcinoma (Hwang et al, 2012), melanoma (Clemente et al, 1996) and non-Hodgkin lymphoma (Ansell et al, 2001). Numerous mouse models with specific immunodeficiencies (e.g. RAG2, TCR β , TCR γ , STAT1) have demonstrated increased tumour formation (Dunn et al, 2002). Both human immunodeficiency virus (HIV)- and drug-induced immunodeficiencies predispose patients to the development of cancers, including those with no known viral origin (Hollenbeak et al, 2005; Shiels et al, 2009). The most explicit evidence, however, was provided with the identification of T cells specific for peptides derived from tumour-associated antigens, isolated both from the bone marrow and infiltrating the tumour itself (Beckhove et al, 2004; van der Bruggen et al, 1991). By 2003, this concept was widely accepted and, over the past decade, cancer immunotherapy has been shown to significantly improve overall survival of patients with various advanced cancers in large scale phase III clinical trials, and is now reaching clinical regulatory approvals (Hodi et al, 2010; Maude et al, 2015; Robert et al, 2015; Topp et al, 2015).

The current interest in cancer immunotherapy is unprecedented, and whilst a wide spectrum of strategies are in development (summarised in Table 1.1), the clinical outcomes to date remain somewhat disappointing for the majority of patients. The reasons for this are diverse but are often linked to (i) the induction of a tolerance to cancer antigens, (ii) the contribution of the tumour microenvironment (TME) to tumour

progression and immune suppression, and (iii) the extraordinary intra-tumour heterogeneity. Therefore, in developing new interventions, it is essential to appreciate these complexities.

Table 1.1. An overview of immunotherapy strategies available. An overview of the spectrum of available immunotherapy strategies, and the major advantages and disadvantages of each approach. This list is not exhaustive. Rather, it gives an idea of the range of cancer immunotherapeutic interventions that are currently approved for clinical use or are undergoing clinical trials. Table modified from (Farkona et al, 2016).

Strategy	Basic mechanism and major advantages	Major disadvantages
Cytokines		
IL-2	- Stimulates the host's immune system.	- Low response rates. - Significant risk of serious systemic inflammation.
IFN γ	- Stimulates the host's immune system. - Durable responses (from a small subset of melanoma patients).	- Low response rates. - High-dose toxicity.
Cell-based therapies		
Vaccines	- Stimulates the host's immune system. - Minimal toxicity (e.g. sipuleucel-T). - Administered in the outpatient clinic.	- Lack of universal antigens. - Lack of ideal immunisation protocols. - Difficulty in breaking tolerance.
Adoptive cell therapy	- Omits the task of breaking tolerance to tumour antigens. - Produces a high avidity in effector T cells. - Genetic T cell engineering broadens TIL to malignancies other than melanoma.	- TIL therapy restricted to melanoma. - Poor persistence of infused T cells limits efficacy and lack of long lasting responses. - Difficulty in selecting tumour-specific antigens. - Safety issues and serious adverse effects. - Expensive/complicated procedure.
Monoclonal antibodies		
Inducing ADCC/CDC	- Stimulates the host's immune system.	- Good safety profiles. - Lack of long-lasting response.
Immune checkpoint inhibition	- Exhibits potent anti-tumour properties. - Therapeutic responses in patients within a broad range of human cancers. - Good clinical responses which are often durable. - Potential to trigger new immune responses.	- Safety issues and serious adverse effects. - Only a relatively small fraction of patients obtain clinical benefit.
T cell engagers		
ImmTACS	- Stimulates the host's immune system. - Omits the task of breaking tolerance to tumour antigens.	- Difficulty in selecting tumour-specific antigens. - Safety issues and potential for serious adverse effects. - Dependent on tumour HLA expression and antigen presentation. - Complicated production procedure.
Bispecific T cell engagers	- Stimulates the host's immune system. - Omits the task of breaking tolerance to tumour antigens. - Independent of HLA expression.	- Difficulty in selecting tumour-specific antigens. - Safety issues and potential for serious adverse effects.
Oncolytic viruses		
	- Stimulates the host's immune system. - Potential to trigger new immune responses. - Good safety profile. - Opportunity to encode transgenes for localised expression.	- Host anti-virus immunity may limit response. - Mostly intra-tumoural administration.

1.2 Basic Principles of Cancer Immunology

1.2.1 Immune Surveillance: The Cancer-Immunity Cycle

The immune response to cancer encompasses a series of finely regulated events or steps - collectively termed “the cancer-immunity cycle” - that must occur for anti-tumour immunity to develop (Figure 1.1) (Chen & Mellman, 2013). In brief, and summarised in Chen & Mellman 2013, the cycle is initiated when cancer-specific antigens are released from tumour cells (step one) and captured by dendritic cells (DCs) for processing and presentation (step two). These antigens can be derived from genetic mutations (neoantigens), foreign agents such as oncogenic viruses or the aberrant expression of specific developmental-stage proteins (Pardoll, 2003). For antigen presentation on major histocompatibility complex (MHC) I and MHC II to proceed, the DCs must receive specific immunogenic signals such as proinflammatory cytokines or damage-associated molecular patterns (DAMPs) released by dying tumour cells or the gut microbiota. In the absence of these signals, peripheral tolerance to the tumour antigen may be induced. Successful presentation, alongside upregulation of co-stimulation receptors, results in the priming and activation of effector T cells against the cancer-specific antigens (step three). Activated T cells traffic to and infiltrate the tumour via chemokine gradients and adhesion molecules (steps four and five). Within the tumour bed, effector T cells specifically recognise and kill their target cancer cell through interaction of TCR and cognate antigen in complex with MHC I (steps six and seven). Cancer cell death leads to release of new tumour antigens that enter the cycle and initiate another revolution, increasing both the potency and breadth of the response. Crucially, in normal circumstances, this cycle leads

to the eradication of a developing tumour and the establishment of durable and adaptable tumour-specific memory.

In cancer patients, however, this cycle is not completed effectively. Although mutated cells may induce at least a transient anti-tumour response, cancers can evade destruction by deregulation of any one of these events. Indeed, a growing tumour develops numerous strategies to minimise tumour antigenicity and to subvert anti-tumour immunity.

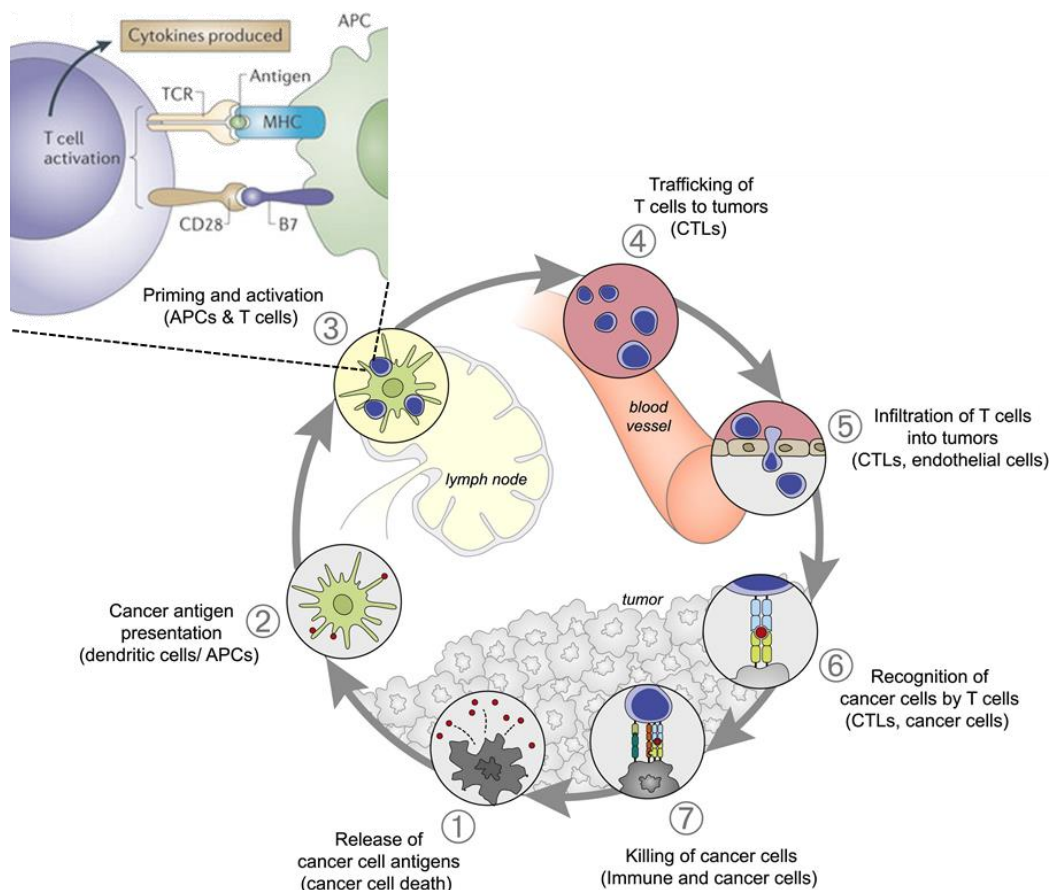


Figure 1.1. The cancer-immunity cycle. The generation of an anti-cancer response is cyclical process that can be divided into seven major events. It starts with the release of cancer cell antigens and ends with the killing of cancer cells by T cells. Each of these steps are described above, with the primary cell types involved listed. The cycle is also modulated by many stimulatory and inhibitory factors that act at each step to promote immunity or reduce immune activity. APCs, antigen presenting cells; CTLs, cytotoxic T lymphocytes. Image adapted from (Chen & Mellman, 2013; Sharma et al, 2011).

1.2.2 Immune Evasion: Tumour-Induced Tolerance

It should first be considered that – independent of active immunosuppressive mechanisms – the initiation and magnitude of an anti-cancer immune response may still be inadequate. Immune recognition is severely limited by the fact that tumour cells arise from an organism's own tissue and hence it follows that many tumour antigens – being 'self' or 'modified-self' – are protected by central tolerance mechanisms (Hogquist et al, 2005; Houghton et al, 2001). This would manifest as the thymic deletion of T cells capable of recognising and responding to tumour antigen-derived epitopes, with only low-avidity T cells remaining (Mapara & Sykes, 2004). A further challenge is the suboptimal presentation of tumour antigens. Tumour cells lack the co-stimulatory molecules required to make them effective antigen-presentation cells (Gong et al, 1997). Further, in the absence of the necessary activation signals (such as cytokines or Toll-like receptor agonists), immature DCs fail to mature and provide sufficient co-stimulation, leading to anergy of tumour antigen-specific T cells (Hawiger et al, 2001; Steinman et al, 2003).

1.2.3 Immune Selection: Escape of Non-Immunogenic Tumour Cells

If an anti-tumour immune response is initiated, one of the best documented evasion mechanisms is to avoid immune recognition (*'immunoselection'*) via the selection of non-immunogenic tumour cell variants. The down-regulation or loss of expression of HLA class I molecules has been reported in up to 90% of human tumour types (Garrido et al, 1997). Moreover, cancer cells have been reported to down-regulate proteins involved in antigen processing and presentation (TAP1, LMP2 (Singal et al, 1996)) and develop

mechanisms to avoid being killed by cytotoxic T cells, such as overexpression of SERPINB9 or mutation of caspase-8 (Medema et al, 2001).

1.3 The Tumour Microenvironment in Immune Evasion

1.3.1 Cancer: A Wound That Never Heals

Solid tumours are akin to complex tissues, containing many distinct non-malignant cell types such as fibroblasts, vascular cells, bone marrow-derived inflammatory cells and immune cells, along with a dense extracellular matrix (Hanahan & Coussens, 2012) (Figure 1.2). In 1986, the remarkable similarities between the stroma of a tumour and that of healing skin lesion led Harold Dvorak to postulate that "tumors are wounds that do not heal" (Dvorak, 1986). Whilst the importance of the stromal microenvironment in the regeneration of normal tissue has been recognised for over a century (Jones & Rous, 1914), the dynamic and often cancer-promoting role of the stromal cells of the TME is only recently being appreciated. Considerable evidence now supports a role for the stroma in cancer initiation, progression, metastasis and resistance (reviewed in (Quail & Joyce, 2013)), and it is suggested that epithelial tumours can initiate this process by exploiting the wound-healing response. In brief, this involves complex heterotypic interactions between tumour cells and the stroma, leading to the recruitment and activation of fibroblasts, matrix remodelling, development of a vascular system and the migration of immune cells.

One of the most significant effects of the stroma of a healing wound is its influence on immune function – partially modulated by activated fibroblasts, infiltrating regulatory T cells (Tregs) and macrophages (Schafer & Werner, 2008). To prevent auto-rejection caused by the chronic inflammatory and proliferative state of the wound, these cells adopt an immunosuppressive phenotype, with many molecular parallels to those within a tumour (Schafer & Werner, 2008). Accumulating clinical evidence now suggests that stromal-induced immunosuppression strongly contributes to the limited activity of previous immunotherapeutic approaches and that modulation of the TME will be an important component of any successful immunotherapy.

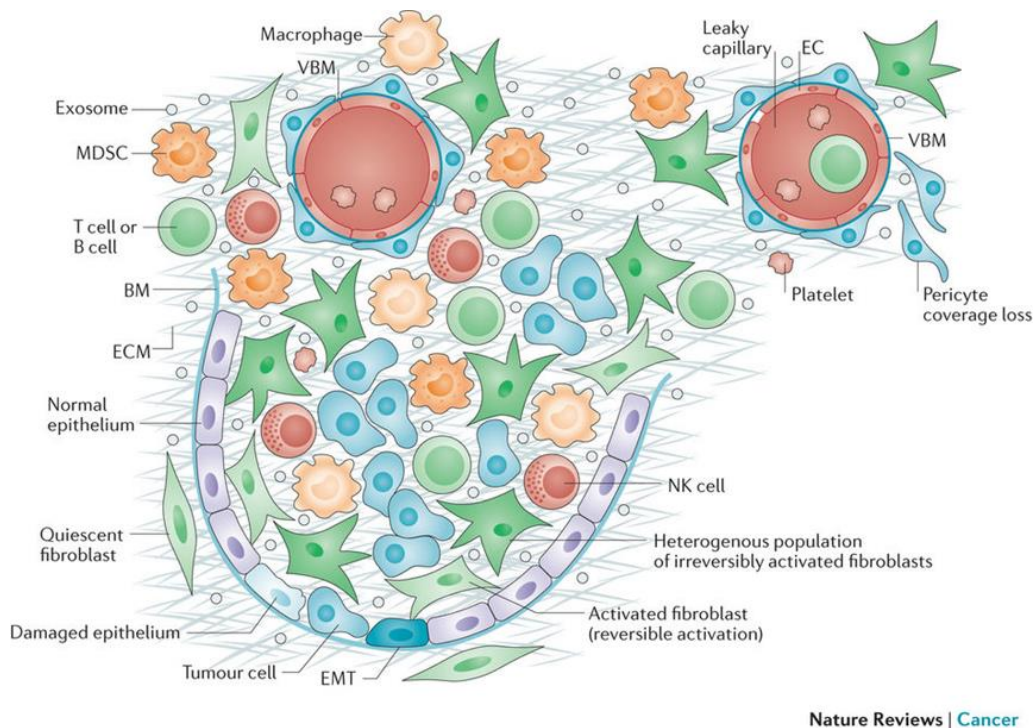


Figure 1.2. Schematic illustration of the tumour microenvironment. The formation of a tumour involves the co-evolution of cancer cells alongside an extracellular matrix, and vascular endothelial, stromal and immune (innate and adaptive) cells. These cells form a complex regulatory network that supports tumour growth, invasion, metastasis and therapeutic resistance. Another significant effect is the influence on immune function. Cells of TME enable cancers to evade immune surveillance and destruction by numerous immunosuppressive mechanisms. Image acquired from (Kalluri, 2016).

1.3.2 Immunosubversion: Stroma-mediated Immune Suppression

Many mechanisms by which cells of the tumour stroma mediate immunosuppression have now been elucidated. Whilst it has long been observed that low infiltrates of T cells into a tumour is correlated with reduced survival, recent evidence has revealed the active exclusion of T cells from the tumour bed (*'immune ignorance'*). Multiple mechanisms have now been revealed, including the production of a dense collagenous matrix, the down-regulation of CD8 T cell-attractant chemokines (CXCL9, and CXCL10) and the synthesis of CXCL12, shown to repel T cells at high concentrations (Harlin et al, 2009; Kraman et al, 2010; Salmon et al, 2012)

Alternatively, tumour cells can actively suppress an immune response (*'immunosubversion'*). The TME is often an area of powerful and localised immune suppression, thus providing an explanation for the observation that tumours often progress in the presence of substantial infiltration of tumour-specific T cells. Accordingly, considerable evidence suggests that these tumour-infiltrating lymphocytes (TILs) are functionally defective, incompletely activated and include regulatory subtypes (Bai et al, 2008; Chiou et al, 2005). The molecular mechanisms by which this is mediated are diverse and wide-ranging, and include the expression of inhibitory cytokines interleukin- (IL-) 10 and transforming growth factor beta (TGF β), inhibitory checkpoint proteins such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and inhibitory enzymes such as arginase I and indoleamine 2,3-dioxygenase (IDO) (de Vries, 1995; Gorelik & Flavell, 2001; Munn et al, 1999; Postow et al, 2015; Rodriguez et al, 2004). Three of the cell types that have received the greatest interest in cancer biology are cancer-associated fibroblasts (CAFs),

macrophages and Tregs, and the contribution of each to the suppression of anti-cancer immune responses will be briefly discussed in the subsequent sections.

1.3.2.1 **Cancer-Associated Fibroblasts**

Cancer associated fibroblasts (CAFs) play a pivotal role in the development of solid tumours by facilitating invasion (Itoh et al, 2017), coordinating angiogenesis (Tang et al, 2016), and maintaining an immune suppressive environment (Harper & Sainson, 2014). The impact of CAFs on immune activity within tumours occurs through a myriad of direct and indirect pathways. For example, CAFs are capable of expressing the tryptophan-depleting and T cell-suppressive enzyme IDO directly and inducing other stromal cells or leukocytes such as macrophages to do so via IL-6 signaling (Chen et al, 2014; Soliman et al, 2010). CAF-mediated TGF β expression, implicated in suppression of T cell function (Li et al, 2007) and macrophage reprogramming (Gong et al, 2012), can be so extensive that the precursor protein accumulates on the extracellular matrix as a long-term reservoir (Taipale et al, 1998), influencing incoming naive immune cells as they infiltrate into the lesion (Flavell et al, 2010). Increasing evidence regarding the immune regulatory function of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) now join the more established roles of IL-10 and TGF β , all produced by CAFs (Henriksson et al, 2011; Kojima et al, 2010) in helping to shape the tumour immune environment. The structural matrix protein, tenascin-C, itself laid down by CAFs (O'Connell et al, 2011), has an independent role in immune suppression. Furthermore, high concentrations of CAF-produced CXCL12 have been reported to block entry of CD8⁺ cells into the tumour microenvironment altogether whilst simultaneously attracting

Tregs (Fearon, 2014). Fibroblast activation protein (FAP) is selectively expressed on cancer-associated fibroblasts, in addition to reactive stromal fibroblasts in other inflammatory tissues. Critically, depletion of FAP⁺ stromal cells in tumour-bearing mice induced necrosis of the tumour mediated by IFN γ - and TNF α -expressing effector T cells (Kraman et al, 2010).

1.3.2.2 Tumour-Associated Macrophages

Macrophages are innate immune cells that are abundant in most human tumours, with often a strong correlation between macrophage density and poor patient prognosis (Leek et al, 1996; Lissbrant et al, 2000). In response to environmental stimuli, macrophages adopt distinct functional phenotypes that, in normal tissues, have historically been categorised under the M1/M2 paradigm (Mosmann et al, 1986). M1 macrophages are ‘classically activated’ with a phenotype that is pro-inflammatory, bactericidal and anti-tumour. At the opposite end of the spectrum, M2 macrophages induce immunosuppression, tissue remodelling and angiogenesis (Ferrante & Leibovich, 2012; Gordon & Taylor, 2005). Tumour-associated macrophages (TAMs) are more M2-like and adopt a pro-tumoural phenotype. They promote many features of tumour progression including cancer cell proliferation, angiogenesis, tumour cell invasion and metastasis and importantly, the suppression of T cell responses (Hagemann et al, 2005; Kuang et al, 2009; Takanami et al, 1999; Wyckoff et al, 2007). In response to tumour-derived signals, TAMs express an array of cytokines and enzymes that attenuate the anti-tumour immune response, including IL-10, TGF β and arginase (Colegio et al, 2014; Pollard, 2004). T cell effector functions can also be inhibited by TAM expression of the ligands for checkpoint

receptors, such as PD-1 and CTLA-4, in addition to non-classical HLA-G, which is reported to inhibit both NK and T cells (Biswas & Mantovani, 2010; Kren et al, 2010). Furthermore, TAMs can trigger apoptosis of both T cells and NK cells by direct contact via the expression of death receptors FAS and TNF-related apoptosis-inducing ligand (TRAIL). Blocking the immunosuppressive, pro-tumour effects of TAMs, whilst enhancing their anti-tumour activities, represents a significant clinical challenge. Therapeutic strategies aimed at achieving this include using antibodies or small-molecule inhibitors to deplete TAM populations, or alternatively inducing their polarisation to a more (M1) tumouricidal phenotype (Coussens et al, 2013; De Palma & Lewis, 2013).

1.3.2.3 **Regulatory T Cells**

Increased numbers of infiltrating regulatory T cells (Tregs) are often associated with poor clinical outcomes in cancers including breast, gastric, brain and ovarian (Bates et al, 2006; Curiel et al, 2004; Jacobs et al, 2010; Sasada et al, 2003). Tregs are potent immunosuppressive cells that enable cancer progression via their ability to induce peripheral tolerance and limit anti-tumour immunity. Characterised by expression of forkhead box P3 (FoxP3) and CD25, they are broadly classified into thymically-derived natural Tregs (nTregs) or inducible Tregs (iTregs), generated from CD4⁺ T cell precursors under tolerogenic conditions. Key mechanisms in their induced suppression of effector T cells are the secretion of IL-10, TGFβ and surface expression of CTLA4 (Asseman et al, 1999; Nakamura et al, 2004; Tai et al, 2012). These mediators prevent the expansion, cytokine production (*e.g.* IFNγ, tumor necrosis factor alpha (TNFα)), and cytotoxic activities of effector cells critically important for the control of tumour growth, in addition

to polarising DCs towards tolerogenic phenotypes (Tadokoro et al, 2006). Additionally, Tregs can induce apoptosis of NK and T cells directly via granzyme B- and TRAIL-dependent cytolytic functions, a phenomenon which was demonstrated to suppress CD8⁺ T cell-mediated clearance of cancer in multiple models (Cao et al, 2007). Due to their contribution to immune suppression, the elimination or modulation of Tregs in tumours may be considered an important component of any effective immunotherapy. Importantly, there are now several examples of such interventions translating to improved anti-cancer responses. For example, in various clinical trials, anti-tumour efficacy has been achieved via direct intra-tumoural Treg depletion (Dannull et al, 2005; Rech et al, 2012), blocking Treg trafficking into tumours (Ishida et al, 2012) or modulating their activity for example with low dose cyclophosphamide (Ghiringhelli et al, 2004).

1.4 General Strategies in Cancer Immunotherapy

1.4.1 Monoclonal Antibodies

The introduction of monoclonal antibody (mAb)-based immunotherapies represented a huge advance in cancer treatment, effective at reducing tumour load burden and prolonging overall survival in many cases. The unprecedented clinical efficacy of rituximab – the first success story of its kind – and its granting of a Food and Drug Administration (FDA) license in 1997 paved the way for the approval of many other therapeutic mAbs. The killing of tumour cells using mAbs arises through multiple mechanisms. Rituximab (Rituxan) exploits cells of the innate immune system, stimulating NK cells to eliminate CD20⁺ B cells (van Meerten et al, 2006). Bevacizumab (Avastin)

sequesters VEGF and directly inhibits pro-angiogenic signalling (Ferrara et al, 2005). Other monoclonal therapies target tumour-associated antigens (TAAs) expressed on tumour cells. Trastuzumab (Herceptin) binds to HER2 and Cetuximab (Erbix) to EGFR, each suppressing downstream growth signalling (Hudis 2007; Specenier & Vermorken, 2011).

1.4.2 Cancer Vaccines

Despite their success in a subset of patients, the efficacy of mAb treatment is hindered by the inherent genomic instability common to cancers, which facilitates escape from such targeted therapies (see 1.2.2). As a result, the outgrowth of resistant clones has been widely reported for each of the aforementioned mAb therapies (Reslan et al, 2009). Consequently, the mobilisation of the adaptive immune system to generate a powerful anti-cancer response – that is adaptable, durable and self-propagating - is a much more attractive prospect. CD8 T cell responses have long been the goal of the vast majority of immunotherapeutic strategies and harnessing their cytotoxic potential has received considerable attention over recent decades. Historically, the major approach to activating endogenous T cells against tumour antigens was by cancer vaccine therapy – propelled by the knowledge that patients can harbor CD8⁺ and CD4⁺ T cells specific for cancer antigens (Beckhove et al, 2004; van der Bruggen et al, 1991).

Vaccine therapy for infectious diseases is arguably the greatest medical intervention of the last century, and vaccination is responsible for the eradication of smallpox and the control of polio, measles and tetanus. Whilst there has been clinical success in the

prevention of cancer causing infectious diseases, including hepatitis B virus and human papillomavirus, vaccines against tumour antigens have demonstrated limited efficacy in human patients (Melero et al, 2014). In contrast to infectious diseases, vaccination for cancer faces considerable obstacles. Firstly, most cancer antigens – being ‘self’ or ‘modified-self’ - are protected by both central and peripheral immune tolerance (Hogquist et al, 2005; Houghton et al, 2001). Breaking tolerance is a significantly more challenging objective than inducing an immune response against a foreign antigen. Secondly, many key T cell effector functions are attenuated by the numerous mechanisms of immune suppression exploited by solid tumours (Zou, 2005). Consequently, any successful induction of a tumour-specific T cell population will be severely compromised in its efficacy, translating to poor clinical responses. Despite these hurdles, vaccine therapy for cancer has experienced some modest success in the form of the FDA-approved sipuleucel-T, in which patient-derived DCs are loaded with a fusion protein consisting of the antigenic prostatic acid phosphatase (PAP) and granulocyte-macrophage colony stimulating factor (GM-CSF), which helps APCs to mature. The activated DCs are then re-infused back into the patient for the treatment of metastatic prostate cancer (Kantoff et al, 2010).

1.4.3 Adoptive Transfer and Genetically Modified T Cells

Adoptive cell transfer (ACT) is a promising form of immunotherapy in which large numbers of tumour antigen-specific T cells are administered to the patient to eradicate primary and metastatic lesions. This strategy, in theory, circumvents the considerable challenge of breaking tolerance to tumour antigens. Indeed, over recent decades,

autologous T cell therapies have demonstrated potential in inducing remarkable clinical responses (Hinrichs & Rosenberg, 2014). Historically, the initial approach involved the transfer of *ex vivo* expanded tumour-infiltrating lymphocytes. Whilst multiple large-scale trials have reported consistently high and durable clinical response rates, the application of TIL ACT therapy has been restricted to melanoma, with immune tolerance significantly limiting the acquisition and exploitation of tumour-specific T cells targeting other malignancies.

Advances in gene-transfer based strategies, and the generation of engineered T cells presenting artificial TCR α/β heterodimers or chimeric antigen receptors (CARs), has considerably expanded the repertoire of available tumour-reactive T cells. CAR T cells - consisting of a single-chain variable fragment (scFv) fused to the CD3 ζ domain of the T cell receptor – dispense of the requirement for cancer cells to achieve functional antigen processing and MHC presentation, and instead target T cells to surface antigens. Moreover, second-generation CARs that contain additional co-stimulatory domains (CD28 and 4-1BB) have demonstrated further augmentation of T cell function and extension of T cell lifespan (Yu et al, 2017). CAR T cells have achieved unprecedented success in the treatment of haematological cancer, with proof-of-concept for the potential of this approach provided by the recent FDA approval of Kymriah (tisagenlecleucel) – which targets B cell antigen CD19 – for ALL (Makita et al, 2017). In fact, CAR T cells have now demonstrated clinical efficacy against a number of B cell malignancies, melanoma, synovial sarcoma, and trials are ongoing for many other forms of cancer (Wang et al, 2017).

Clinical responses in solid tumours, however, have been less encouraging with multiple reported cases of a lack of therapeutic response and/or severe dose-limiting toxicities (Brudno & Kochenderfer, 2016). Indeed, treatment of solid tumours presents four unique hurdles. Firstly, it is now well documented that tumours can limit the anti-tumour response by deregulating T cell recruitment and infiltration into the tumour, and this is likely to impede access of CAR T cells into the tumour (Harlin et al, 2009). Indeed, there is a large diversity in the magnitude of T cell infiltration with tumours ranging from immunologically ‘cold’ (poor infiltration) to immunologically ‘hot’ (high infiltration). Second, the microenvironment is significantly more immunosuppressive and mediates the functional impairment of infiltrating CAR T cells (Enblad et al, 2015). Thirdly, the ‘the on-target, off-tumour’ toxicities associated with targeting solid tumour antigens are often dose-limiting and can be lethal to the patient. For example, treatment of a metastatic colorectal cancer patient with HER2-specific CAR T cells triggered severe respiratory distress, and death five days later (Morgan et al, 2010). In another example, administration of carboxy-anhydrase-IX (CAIX)-targeted CAR T cells to patients with renal cancer induced dose-limiting liver toxicities at the lowest dose of infusion (2×10^8 T cells) (Lamers et al, 2006). In each case, subsequent examination attributed these toxicities to ‘on-target, off-tumour’ activity in healthy lung or liver tissues, which revealed low levels of target antigen expression.

A fourth challenge for CAR T cell therapy – common to the treatment of haematological and solid tumours – is the reported suboptimal retention of T cells following infusion. The persistence of CAR T cells is likely a crucial factor in determining the efficacy of the anti-tumour response. Consequently, most clinical trials to date have not detected durable responses in the majority of treated patients, demonstrated by disease progression and

transient B cell aplasias (Ahmed et al, 2015; Davila et al, 2014; Lee et al, 2015). In some cases, CAR T cells were undetectable within 28 days of treatment (Turtle et al, 2016).

1.4.4 Activating Endogenous T Cells by Immune Modulation

The concept of cancer immune modulation is founded on the striking observation that tumour regression, in some instances, can be induced by using monoclonal antibodies to block receptors involved in negatively regulating the T cell response (Pardoll, 2012). This, in theory, should stimulate T cell function and unleash pre-existing anti-cancer T cell responses. In fact, antibody blockade of a single molecule, CTLA4, results in effective T cell-mediated tumour reduction in multiple malignancies (Hodi et al, 2010; Merchant et al, 2016). The CTLA4-targeting Ipilimumab (Yervoy) obtained FDA-approval in 2011 for the treatment of metastatic melanoma, sparking huge interest in developing mAbs against other immune checkpoint proteins, most notably PD1, PDL1, T cell immunoglobulin and mucin-domain containing-3 (TIM3) and lymphocyte-activation gene 3 (LAG3) (Brignone et al, 2009; Robert et al, 2015; Sakuishi et al, 2010). Of these, the most clinically advanced are mAbs targeting the PD1-PDL1 axis, with PD1 pathway blockade generating both greater patient response rates and improved toxicity profiles (lower grade >3 adverse events) compared to targeting the CTLA4 checkpoint (Larkin et al, 2015). Moreover, to date, over 500 clinical studies have now been conducted using therapeutics that block PD1 signalling, with two mAbs (Nivolumab and Pembrolizumab) currently approved by the FDA for treatment of advanced melanoma, NSCLC, renal cell carcinoma, among other malignancies (Iwai et al, 2017). Alternatively, strategies to induce activation mechanisms are also being pursued, and targeting of co-

stimulation receptors OX40, 4-1BB and GITR have shown promise in pre-clinical and clinical studies (Curti et al, 2013; Schaer et al, 2012; Segal et al, 2014).

These studies clearly demonstrate that activating the endogenous T cell compartment, on its own, can achieve long-term remissions. A therapeutic strategy that directly activates tumour-resident T cells bypasses the requirement for the *ex vivo* pre-stimulation and infusion of T cells and obviates the complication of poor persistence. Moreover, the majority of immune modulation therapies are antibody-based, and as a result, considerably more practical than ACT from a clinical perspective. Yet, a relatively small subset of patients obtain clinical benefit from treatment with immune modulation therapies (Hodi et al, 2010), and the down-regulation of HLAs by tumour cells (by immune selection) is reported to be a common mechanism of resistance (Kakavand et al, 2017). An alternative approach, which circumvents the requirement for tumour antigen presentation, is to use T cell engaging antibodies. This group of multi-specific antibodies includes bispecific T cell engagers (BiTEs) and ImmTACs and their use as immunotherapies for cancer is explored in the next section.

1.5 T cell Engagers

1.5.1 T Cell Activation with Bispecific Antibodies: BiTEs and ImmTACs

T cell activation requires T cell receptor (TCR) crosslinking and formation of an immune synapse, which can be achieved by presenting anti-CD3 antibodies on the surface or nanoparticle [41]. Bispecific antibodies and ImmTACs are novel immunotherapeutic

agents that exploit this mechanism to polyclonally activate T cells to kill target cells, albeit via different mechanisms. ImmTACs are bi-specific biologics comprising a high-affinity TCR fused to an anti-CD3 scFv. Using modified TCRs enables intracellular antigens (presented by HLA) to be targeted with picomolar affinity, and ImmTACs have been reported to lyse tumour cells presenting fewer than 50 epitopes (Liddy et al, 2012). After promising early signs of efficacy in a phase I trial, the most advanced ImmTAC IMCgp100 has now progressed to phase IIa for melanoma (Pentier et al, 2016). Although ImmTAC-based therapies show great promise, they are limited in scope both by their HLA restriction and the frequency of HLA down-regulation in solid tumours.

Bispecific T cell engagers (BiTEs) represent an alternative and more versatile system (Figure 1.3). Composed of the binding domains (Fv) of two human IgG antibodies of different specificities, one arm targets CD3, the potent signalling co-receptor of the T cell receptor (TCR). This allows the engagement of any antigen-experienced T cell as a potential effector cell regardless of its TCR specificity. By simultaneously engaging CD3 and the target cell antigen, BiTEs mediate the clustering of CD3 and the formation of an immunological synapse between the two cell types. This leads to rapid release of perforin and granzyme B and subsequent apoptosis of the target cell. (Brischwein et al, 2006; Wolf et al, 2005). Importantly, binding of the second arm to a TAA ensures recruitment and subsequent elimination is restricted to only those cells expressing the specific TAA. In fact, the versatility of the platform ensures that a BiTE can also be raised against a HLA-peptide antigen complex, similarly to ImmTACs (Dao et al, 2015). Further, the requirement for multivalent CD3 engagement ensures that T cell signalling is only stimulated in the presence of BiTE-coated target cells, and not BiTE alone.

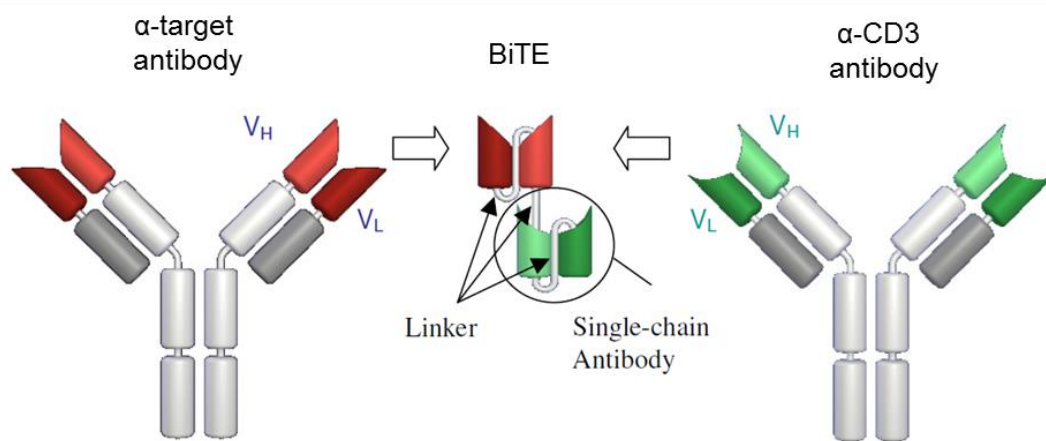
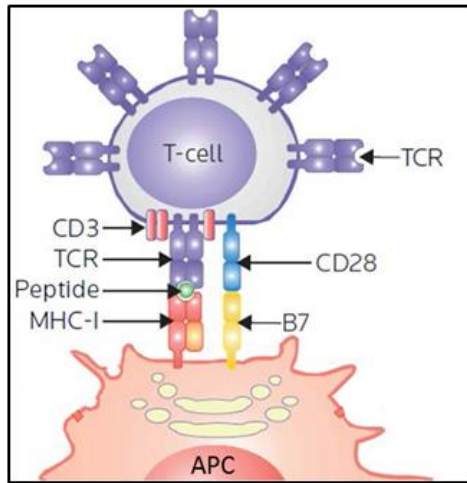


Figure 1.3. Schematic of the structural relationship of a BiTE molecule to its two parental antibodies. A BiTE construct was generated via recombinant DNA technology. The variable V_H and V_L domains of each monoclonal antibody are joined by a short non-immunogenic peptide linker to form a single chain antibody with dual antigen specificity. Image acquired from (Baeuerle & Reinhardt, 2009).

Unlike most other T cell-based therapies, the physical tethering of T cell to target cell renders BiTE-mediated tumour cell lysis independent of normal T cell activation - a complex process requiring the processing and presentation of antigens by APCs, CD28-CD80 co-stimulation and an antigen-specific TCR (Dreier et al, 2002) (Figure 1.4). This allows the potent cytotoxicity of T cells to be harnessed with numerous immune-escape mechanisms unlikely to impact (Baeuerle & Reinhardt, 2009). Not only CD8 cells, but also CD4 are capable of mediating cell lysis, and there is potential to convert the immunosuppressive activity of Tregs to cytotoxicity (Choi et al, 2013).

Conventional activation:



BiTE-mediated activation:

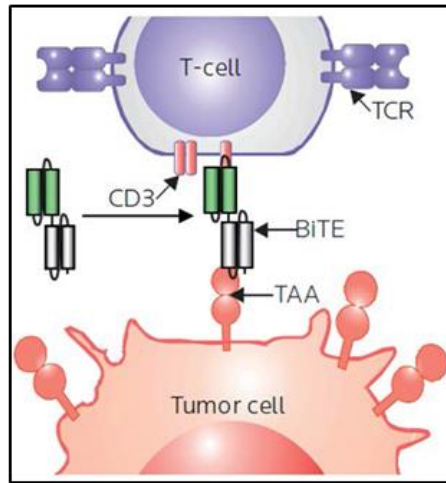


Figure 1.4. BiTE-mediated killing bypasses elements of normal cytotoxic T cell activation. Conventional T cell activation (left) requires the binding of the T cell receptor (TCR) to its cognate peptide antigen loaded on to the major histocompatibility complex (MHC) I. Simultaneous co-stimulation is also necessary for activation; provided by engagement of CD28 with B7, which is restricted to professional antigen-presenting cells (APCs). BiTEs (right) circumvent the requirement for cognate MHC-TCR engagement, providing a link between any CD3⁺ T cell and tumour-associated antigen (TAA)-bearing tumour cell, independent of antigen processing, presentation and co-stimulation, leading to the formation of an immunological synapse and subsequent target cell lysis. Image acquired from (Baeuerle et al, 2009).

1.5.2 Clinical Development of BiTEs Targeting Haematological Malignancies

Several BiTEs are under clinical development (Table 1.2). Blinatumomab, a BiTE targeting CD3 and CD19, is currently the clinically most advanced. Major responses have been demonstrated in various B cell malignancies including acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL) (Bargou et al, 2008; Topp et al, 2011), establishing the principle that polyclonal T cell engagement against tumour targets is both feasible and effective [27]. Having presented encouraging results from a phase II/III trials for patients with relapsed/refractory ALL, Blinatumomab was granted full

FDA approval for this indication (Kantarjian et al, 2017; Przepiorka et al, 2015), and is now undergoing clinical trials for other CD19 malignancies.

Pharmacologically, BiTEs possess a number of advantages and disadvantages. BiTEs can induce T cell activation and target lysis with remarkable potency, and half-maximal effective concentrations (EC₅₀) *in vitro* are typically in the picomolar range (Friedrich et al, 2014). In a phase I clinical trial for patients with NHL, Blinatumomab induced partial tumour regression at doses as low as 15 µg/m²/day (Bargou et al, 2008) and this dose proved sufficient in phase II trials to eradicate minimal residual disease in 80% of patients with ALL (Topp et al, 2011). Major toxicities in trials include cytokine release syndrome and central nervous system (CNS) events, which are attributed to the T cell-induced inflammation in the brain (Nägele et al, 2017; Teachey et al, 2013; Topp et al, 2015). Due to their small size (≈55 kDa), BiTEs demonstrate short serum half-lives in the range of 1.25 hours (for Blinatumomab (Klinger et al, 2012)) and therefore require continuous intravenous infusion. Importantly, this allows BiTE concentrations to be finely controlled and the frequency of any systemic toxicities to be constrained.

Table 1.2. BiTE biologics in clinical development as anticancer agents. An overview of BiTE molecules with completed or ongoing clinical trials. Table modified from (Yuraszeck et al, 2017).

BiTE	Targets	Phase	Indication
Blinatumomab	CD19 × CD3	Approved	Acute lymphoblastic leukemia (ALL)
AMG 211/MT 211	CEA × CD3	Ph 1	Gastrointestinal cancer
AMG 110/MT 110	EpCAM × CD3	Ph 1	Solid tumours
MEDI-565/MT 111	CEA × CD3	Ph 1	Gastrointestinal adenocarcinoma
AMG 330	CD33 × CD3	Ph 1	Acute myeloid leukemia (AML)
AMG 420	BCMA × CD3	Ph 1	Multiple myeloma
AMG 212/ MT 112	PSMA × CD3	Ph 1	Prostate cancer

1.5.3 Solid Tumour Immunotherapy with BiTEs

The success of BiTE therapy for treating haematological malignancies has so far not been replicated for solid tumours. BiTEs have been designed targeting different solid tumour-associated antigens including epithelial cell adhesion molecule (EpCAM), Her2/neu, EGFR, CEA, EphA2, PSCA and MCSP (Baeuerle & Reinhardt, 2009; Yuraszeck et al, 2017). To date, three have undergone clinical evaluation. The most advanced, Solitomab (MT110), targeting EpCAM, which is highly expressed on many carcinomas and is recently identified as a marker of cancer-initiating cells, has previously demonstrated preclinical efficacy *in vitro* and in demanding xenograft models (Brischwein et al, 2006; Terris et al, 2010). These encouraging results did not translate to the clinic, with poor clinical responses according to RECIST criteria (Fiedler et al, 2012). Dose-limiting toxicities were reported, with on-target off-tumour targeting of EpCAM⁺ healthy tissues and off-target CNS-related events (Fiedler et al, 2012). Meanwhile a BiTE targeting carcinoembryonic antigen (AMG 211) has entered a phase I clinical trial based on encouraging preclinical results (Osada et al, 2010). The trial, including patients with advanced gastrointestinal adenocarcinoma with AMG 211 administered intermittently, was recently completed (NCT02291614) with no objective responses observed. Finally, a prostate-specific membrane antigen (PMSA)-targeting BiTE (AMG 212) demonstrated efficacy in xenograft models (Friedrich et al, 2012), leading to the launch of a phase I clinical trial in patients with castration-resistant prostate adenocarcinoma (NCT01723475).

1.5.4 Challenges of Targeting Solid Tumours with BiTEs

Targeting solid tumours with BiTE therapy presents three unique challenges. In all clinical trials thus far, BiTEs are administered to the patient by intravenous delivery. The unique challenge for BiTE therapy is that the rapid clearance of the biologic by metabolism and in the urine, preventing sufficient circulatory time to allow for effective diffusion at the tumour site, may prove problematic. Indeed, as reported by Rakesh Jain, ‘for diffusion of IgG in tumours, the time constant is of the order of 1 hour for 100 μ m distance, ~2 days for 1 mm distance and ~7-8 months for 1 cm distance’ (Jain, 1990). Moreover, extravasation of macromolecules can be further restricted by heterogeneous blood flow and high interstitial pressure – both characteristic of solid tumours. Whilst the small size of the BiTE should permit more efficient penetration into solid tumour tissue, poor extravasation is still likely to considerably limit overall efficacy. In fact, the manufacture of clinical grade BiTE is an intensive and expensive process, and the requirement for continuous intravenous infusion to achieve therapeutic levels causes further complications.

A second key challenge – shared with CAR T cell therapy - is the selection of suitable target antigens. The availability and selection of an antigen that is only expressed in solid tumours and not in normal tissues is rare. In fact, most solid tumour antigens are also present at low levels on normal cells, and these levels may be sufficient to trigger BiTE-mediated immune responses against the tissues (Stone et al, 2012). This proves increasingly problematic following intravenous delivery, and toxicity caused by targeting of healthy EpCAM tissue has been reported in a phase I clinical trial for Solitomab (Fiedler et al, 2012). Further insights into the potential consequences of on-target off-

tumour activity of BiTE therapy is limited by the availability of clinical data. However, as discussed previously, there have been considerable systemic toxicities reported in clinical trials of CAR T cell targeting solid tumours, including high frequencies of life-threatening (grade four) adverse events and death (Lamers et al, 2006; Morgan et al, 2010).

Advanced solid tumours develop several strategies to impair T cell homing and infiltration (Sackstein et al, 2017). Importantly, TIL frequency, density, spatial localisation and subset ratio within melanoma and other solid tumours correlates with a good prognosis and immunotherapeutic outcomes (Haanen et al, 2006; Lee et al, 2016). Thus, although BiTE therapy can overcome numerous immune evasion mechanisms, their efficacy in solid tumours hinges upon the successful homing and infiltration of sufficient numbers of effector T cells into the tumour. For instance, tumour-directed aberrancies of chemokine profiles, endothelial vessels and adhesion molecule expression may disfavor the penetrance of T cell (Griffioen et al, 1996; Harlin et al, 2009). Tumours can also exploit stromal cells to further shape the infiltration of T cells. CAFs frequently form a thick layer around tumour vasculature forming a direct barrier between the bloodstream and the tumour bed, and remodeling of the tumour ECM can further anchor T cells to these stroma-rich regions (Salmon et al, 2012; Turley et al, 2015). CAFs can also shape infiltration by the generation of exclusion signals. For example, CAF-produced CXCL12 is demonstrated to limit infiltration of effector T cells and enhance the recruitment of Tregs (Vianello et al, 2006; Yan et al, 2011), with expression levels correlated with poor prognosis and immunotherapeutic outcome (Yan et al, 2011). Numerous other mechanisms have now been elucidated, and tumours possess a considerable diversity in

the number and location of their T cell infiltrates. The success of BiTE immunotherapy is likely to strongly depend on this T cell infiltration.

1.6 Oncolytic Viruses

1.6.1 Introduction to Oncolytic Viruses

Oncolytic viruses (OVs) infect and selectively replicate in tumour cells, leading to lytic cell death without causing harm to normal cells (Figure 1.5). The potential of using viruses to treat cancer was first considered in the late 1800s, with anecdotal observations that cancer patients who had contracted an infectious disease experienced transient periods of remission. For example, one widely cited report described a myelogenous leukemia patient who went into remission after contracting an influenza infection (Dock et al, 1904). Early clinical trials were conducted in the mid-20th century in attempt to treat cancer using a range of wild-type or attenuated viruses, including adenoviruses, hepatitis, yellow fever and dengue fever (Kelly & Russell, 2007). However, disappointing efficacy and concerns about safety due to the inability to control virulence led to near-abandonment of the field.

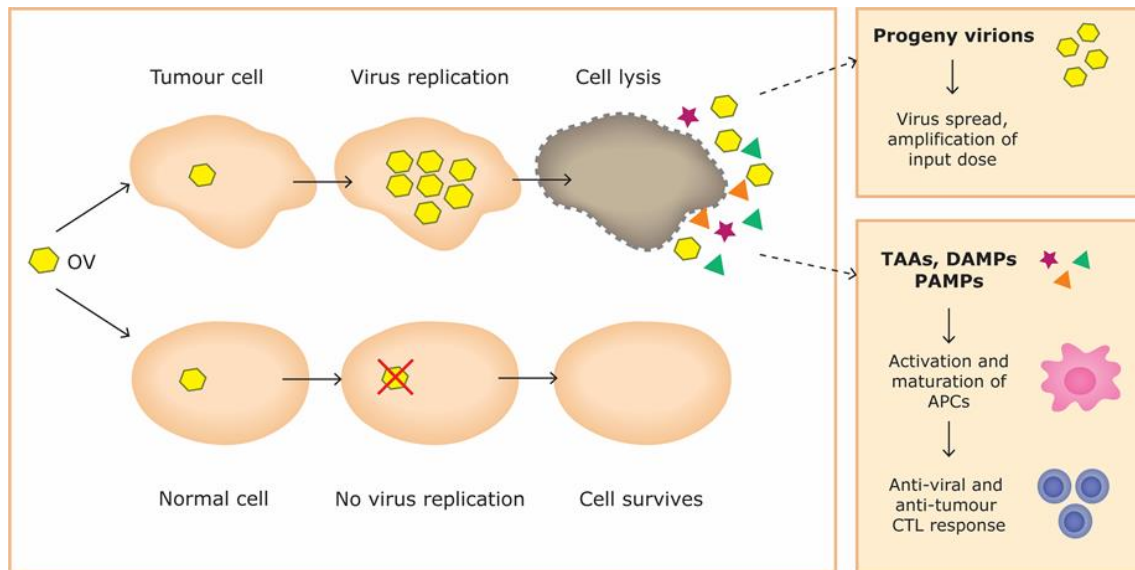


Figure 1.5. The rationale behind oncolytic virus therapy. Oncolytic viruses enter both normal and malignant cells, but selectively replicate in cancer cells. Replication leads to lysis of the cancer cell and release of daughter virions, resulting in amplification of the input dose and spread through the tumour. This selective targeting of cancer cells prevents harm of non-transformed tissue, in which viral replication is inhibited. Additionally, OV-mediated cell death is immunogenic, and is associated with the release of danger-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), and tumour-associated antigens (TAAs). Together, these promote the activation and maturation of antigen-presenting cells (APCs), leading to an adaptive cytotoxic T cell (CTL) response. Image acquired from (Scott et al, 2017).

The arrival of genetic engineering and a deeper understanding of tumour and virus biology – permitting the design of viruses with higher cancer specificity - brought about a renaissance in oncolytic virotherapy. Since then, more than 100 clinical trials using oncolytic viruses have been completed. Proof-of-concept for the potential of this approach is provided by Imlygic, an oncolytic herpes simplex virus (HSV)-1 encoding GM-CSF (Andtbacka et al, 2015). Having met its primary endpoints for a phase III clinical trial for melanoma - demonstrating an objective response rate of 26% - Imlygic represents the first success story of its kind (and the first agent to receive an FDA product license) (Andtbacka et al, 2015). Imlygic is paving the way for the clinical advancement of many other OVs, with the most clinically advanced including Reolysin, a wild-type reovirus; JX-594, a thymidine kinase (TK)-deleted vaccinia virus (VV) expressing GM-

CSF; CG0070, a E3-deleted adenovirus expressing GM-CSF; and Enadenotucirev, a chimeric group B adenovirus.

1.6.2 Targeting OV's to Cancer Cells

OVs possess a specificity for tumour cells that may be natural or engineered. Indeed, many of the OVs undergoing clinical development have a natural tropism for cell surface receptors that are expressed by cancer cells. HSV1, for example, enters a cell via herpesvirus entry mediator (HVEM), which is overexpressed on cancer cells in melanoma and various carcinomas (Yu et al, 2005). Other wild-type viruses with an intrinsic preference for replication in cancer cells include reovirus, Newcastle disease virus, myxoma virus, coxsackievirus and poliovirus (Kaufman et al, 2015).

Oncotropism may also be achieved by genetic manipulation. Viruses can be modified to directly target cancer-specific cell surface receptors, for example adenovirus Ad5/3- Δ 24 has been modified to bind to integrins that are overexpressed on cancer cells (You et al, 2001). The deletion of key viral genes required for replication in normal, but not in cancer cells, has also successfully introduced cancer-selectivity to numerous virus types. This includes vaccinia virus (e.g TK deletion), HSV (e.g. ICP34.5 deletion) and adenovirus (E1A deletion), all of which are currently being investigated in clinical trials (Singh et al, 2012).

An alternative approach is to use bioselection, wherein OVs with the greatest replicative and/or lytic capabilities are selected by serial passage (Kuhn et al, 2008). The promise of

this approach is the potential to select for viruses that target the cancer phenotype, rather than a specific oncogenic pathway. Several such OV's are currently under development, but one of the most advanced is Enadenotucirev (EnAd), a chimera of group B adenoviruses type 3 and type 11p, which is currently undergoing clinical evaluation for a range of metastatic carcinoma types (Calvo E & Salazar R, 2014; Illingworth et al, 2017; Kuhn et al, 2008). To generate EnAd, colon cancer cells were infected with a pool of seven adenovirus serotypes at high multiplicity of infection (MOI) to encourage recombination of the viral genomes, followed by selection for efficient replication and oncolysis. As displayed in Figure 3.12, the resulting oncolytic virus is an Ad11p-based virus with a chimeric Ad3/11p E2B region and deletions in E3 and E4orf4 and is both more potent and selective than either parental virus. Whilst the exact molecular mechanism is unresolved, the observed phenotype is likely a consequence of the mosaic E2B region encoding for a more efficient viral DNA polymerase, and the deletions in early genes involved in immune modulation (E3) and host cell metabolism and stress response (E4orf4). EnAd will be employed throughout my thesis and this virus will be described in further detail in subsequent sections.

1.6.3 OV-targeting of Disseminated Cancer

One of the major goals of OV therapy is the targeting of disseminated tumour deposits via systemic delivery. Whilst the majority of clinical trials currently employ intratumoural injection, there are notable examples of OV's, including EnAd, that have demonstrated successful intratumoural replication after system delivery. For example, in a study where EnAd is given intravenously a few days prior to resection of primary colon cancer,

subsequent immunohistological assessment of tumour sections showed that the virus had reached regions throughout the tumours and gave strong intranuclear hexon signals, indicating successful infection and virus replication selectively in tumour cells (Figure 1.6). This confirms preclinical data (Di et al, 2014; Illingworth et al, 2017) indicating that this virus is stable in whole human blood and should be capable of tumour-targeted infection of disseminated and metastatic malignancies in human patients.

1.6.4 Mechanisms of Oncolytic Virus Immunotherapy

Apoptosis, necrosis and autophagy – all conventional routes of cell death – tend to be inadequate in describing the complex cytotoxicity processes elicited by oncolytic viruses. This is because oncolytic viruses typically hijack the molecular cell death machinery of the infected cell, and death is only permissible after the cellular resources have been maximally exploited for virus replication (Kaminsky & Zhivotovsky, 2010). In fact, a key advantage of using oncolytic viruses in cancer treatment is their ability to kill infected cancer cells through multiple different mechanisms, which are distinct from those induced by chemotherapy and radiation (Green et al, 2009). The first is by direct oncolysis of the infected cells. Following entry into the cell, the parental virus replicates and induces the

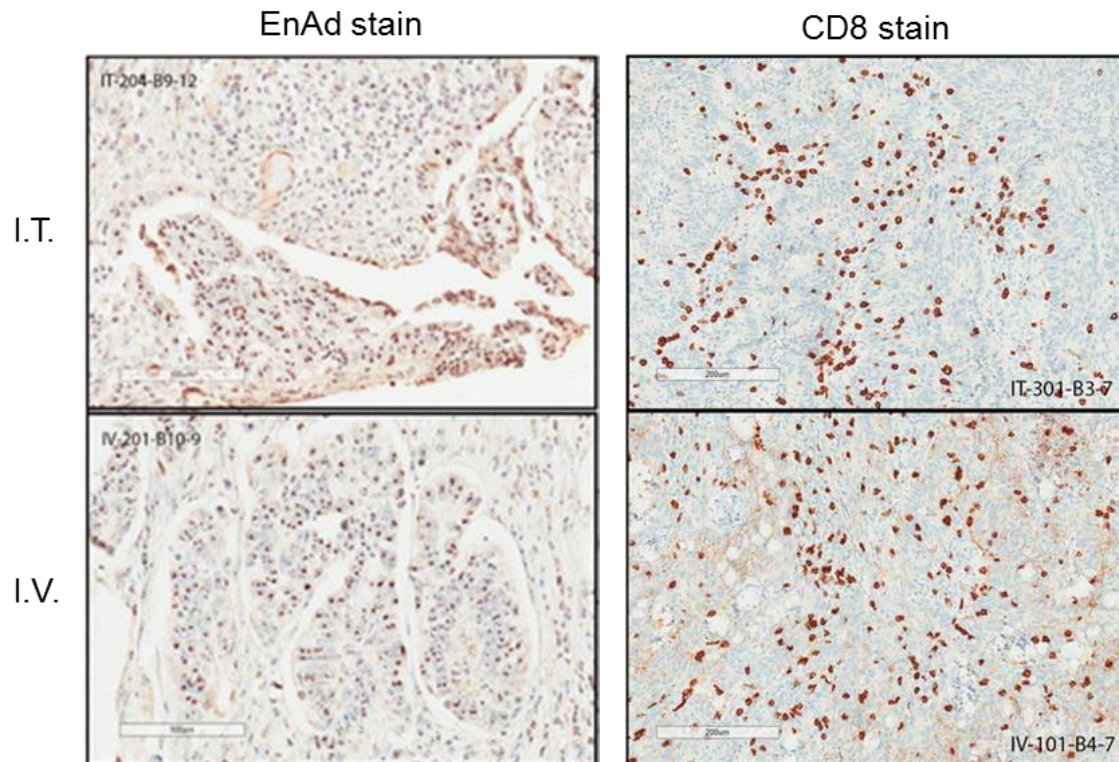


Figure 1.6. Examples of resected human colorectal tumours showing EnAd infection and associated CD8⁺ infiltration. Data from a phase 1 study of intravenous (I.V) administration of EnAd to patients with colorectal cancer. For comparison, a cohort of patients received EnAd via intratumoural (I.T) injection. For I.V, patients received three doses of virus (1×10^{12} virus particles). For I.T, one dose was administered ($\leq 3 \times 10^{11}$ viral particles). Tumours were resected on day 8-15 and immunohistochemistry (IHC) was performed to assess EnAd infection (by hexon staining) and CD8⁺ infiltration. Image adapted from (Garcia-Carbonero et al, 2017).

amplification of virus particles, followed by the active cell lysis. This leads to the release of high concentrations of daughter virions that spread through the tumour and continue this process (Fukuhara et al, 2016).

The induction of virus-mediated cell death can also promote the initiation of systemic immune responses. Indeed, there is emerging evidence that specific oncolytic viruses can kill cells by a pro-inflammatory process known as oncosis or immunogenic cell death (ICD) (Green et al, 2009), and this is likely pivotal to the ability of the virus to promote an effective anticancer immune response. This non-apoptotic death mechanism causes

release of several pro-inflammatory cellular components, such as ATP, HMGB1 and exposure of calreticulin (known as damage-associated molecular patterns, DAMPs) (Weerasinghe & Buja, 2012). This translocation of calreticulin to the plasma membrane promotes phagocytosis by DCs, which are further stimulated by extracellular ATP and HMGB1 (Gardai et al, 2005; Idzko et al, 2002; Rovere-Querini et al, 2004), and ultimately triggers a more powerful immune response.

Adenovirus, HSV, measles virus and vaccinia virus have all been shown to induce ICD (Guo et al, 2014). Indeed, the induction of an antiviral T cell response is a well-documented consequence of virus infection, and therefore, it is plausible that OV-triggered inflammation could counteract tumour immunosuppression. Animal models have lent support to this and demonstrate that infection of tumour cells with OVs can modify the cytokine milieu and the type of immune cells within the TME. Although more limited, clinical trial data complements these findings (Benencia et al, 2005; John et al, 2012; Zamarin et al, 2014). Analysis of resected tumour samples from metastatic breast cancer patients treated in a phase I trial with HSV-1 variant, HF10, revealed intratumoural infiltration of CD8⁺ cells (Sahin et al, 2012). Similarly, in a phase I trial of refractory melanoma patients with GM-CSF-encoding vaccinia virus, there was evidence of extensive infiltration of injected lesions with CD4 and CD8 T cells (Mastrangelo et al, 1999). In a phase II trial, increased infiltration of tumour-specific T cells was observed in resected tissue specimens from melanoma patients treated with Imlygic (Kaufman et al, 2010). Finally, data from a phase I trial of EnAd, suggests that the virus promotes a strong influx of CD8⁺ T cells into colorectal tumour tissue in 80% of test tumour samples, and that these T cells appeared to be activated (Figure 1.6). The aspiration is that these

changes promote the recognition and elimination of tumour cells, leading to tumour-associated antigen and epitope spreading.

Despite the potential of OV's to induce an anti-cancer immune response, clinical responses for the majority of patients are disappointing. One promising approach to improve efficacy is to encode and express other anticancer biologics. Secretion of the transgene by infected cancer cells ensures the biologic is at its highest concentration within the TME. Significantly, the biologic can be placed under transcriptional control of a virus late promoter, and expression will only be activated in cells that are permissive to virus replication. Indeed, this also provides the opportunity to facilitate the delivery of therapeutic agents in which systemic delivery is a challenge, for example those that suffer from poor pharmacokinetics or dose-limiting toxicities. Proof-of-concept has so far been provided by Imlygic, which encodes GM-CSF, however the potential for arming viruses is virtually limitless and provides many exciting opportunities to design multimodal therapeutic strategies with additive or synergistic anticancer effects (de Gruijl et al, 2015; Hermiston & Kuhn, 2002).

1.6.5 BiTE-Armed Oncolytic Viruses

The opportunity for (i) systemic targeting of metastatic disease, (ii) tumour-restricted transgene expression, in combination with (iii) virus-induced intratumoural infiltration of T cells, renders oncolytic viruses as a very appealing strategy for the delivery of BiTEs. The arming of OV's with BiTEs has potential to exploit the best features of each modality, which could overcome many challenges encountered by either strategy alone. Delivery

by OV increases the availability of the BiTE within the tumour, enabling the BiTE to direct T cell cytotoxicity to the target cell, independent of antigen presentation, whilst also minimising potential systemic toxicities. Indeed, the tumour-restricted delivery of a BiTE provides the exciting opportunity to deliver BiTEs that target more promiscuous target antigens – not necessarily limited to the tumour – with the potential to kill non-transformed cells of the stroma. It is noteworthy that both cytotoxic strategies that are immediately engaged by BiTE-armed oncolytic viruses operate independently of MHC I, and therefore can be employed to kill cancer cells even when tumour cells have lost MHC expression. From an OV perspective, BiTEs may improve the efficacy of the virus by acting as an ‘immune decoy’. By engaging anti-viral tumour infiltrating T cells, BiTEs can redirect their cytotoxicity towards target cells, attenuating viral clearance and extending the life-time of the tumour infection (Schmittnaegel et al, 2015). What is more, this strategy may protect against the emergence of cancer cells that are resistant to one of these platforms.

This rationale has led to several pre-clinical studies involving the development of OV-armed BiTEs. In the first, Yu *et al* have armed a double TK-deleted Western reverse strain vaccinia virus with an EphA2-targeted BiTE (Yu et al, 2014). The expression of the BiTE was placed under the control of a late viral promoter, F17R, and therefore production was restricted to cells in which the virus is replicating. In co-culture experiments, EphA2-BiTE secretion from infected cells lead to the activation of human peripheral blood mononuclear cells (PBMCs)-derived T cells and the lysis of uninfected A549 cells. *In vivo*, intravenous delivery of EphA2-BiTE-VV, but not the control virus, alongside PBMC significantly increased survival in a xenograft mouse model using A549 cells. T

cell proliferation was increased *in vitro* and *in vivo*, though only when supplemented with exogenous IL-2.

In a separate study, an oncolytic adenovirus, ICOVIR-15K, was encoded with a BiTE targeting EGFR under the control of the major late promoter (Fajardo et al, 2017). Fajardo *et al* demonstrated successful genome replication of EGFR-BiTE-ICOVIR-15K, leading to tumour cell oncolysis and secretion of EGFR-BiTE. *In vitro*, the BiTE-armed virus induced T cell activation and bystander killing of virus-resistant cells. Using two xenograft mouse models, intratumoural or systemic administration of EGFR-BiTE-ICOVIR-15K improved efficacy, relative to the unarmed virus, though this was dependent on the transfer of human PBMC. Encouragingly, and in contrast to the previous study, the authors described strong proliferation of T cells in the absence of supplemented IL-2.

Whilst having presented encouraging results in xenograft mouse models, one limitation of these previous studies is that their models poorly reproduce the complexities of the tumour microenvironment. Indeed, with very little stromal development, it is likely that these fast-growing xenografts lack many of the numerous mechanisms of immune suppression prevalent in solid human tumours. What is more, BiTE-armed OV's present the exciting opportunity to use each platform to target different cell types – for example, redirecting T cell cytotoxicity towards non-transformed cells of the stroma. These studies did not explore the potential of this approach.

1.7 Thesis Overview

1.7.1 Our Proposed Strategy

Oncolytic viruses offer an intriguing new strategy to combine several therapeutic modalities within a single targeted, self-amplifying agent (Keller & Bell, 2016; Seymour & Fisher, 2016). One central aim of oncolytic virus therapy is to create an anticancer T cell response that recognises patient specific neoantigens as well as “public” tumour associated antigens. Lytic viruses may do this by stimulating improved antigen presentation by lysing tumour cells in the context of DAMPs alongside virus-related PAMPs. Recently, it was reported that EnAd kills by a mechanism independent of caspase and p53, and instead, infected cells demonstrated strong increases in surface calreticulin and release of pro-inflammatory cellular components HSP70, ATP and HMGB1 in a death mechanism similar to ICD (Dyer et al, 2017). *In vitro*, EnAd infection led to the stimulation of DCs and CD4⁺ cells in mixed-lymphocyte reactions (Dyer et al, 2017). These findings are complemented by early clinical data, in which immunohistochemical staining of resected colon tumours, following I.V. delivery of EnAd, suggests the virus promotes a strong influx of CD8⁺ T cells into tumour tissue (Garcia-Carbonero et al, 2017), potentially converting an immunologically “cold” tumour into one that is immunologically “hot”.

Moreover, as a clinical candidate, EnAd offers high cancer specificity and excellent safety profiles, with the potential to target disseminated tumours. Exploiting the known blood-stability and systemic bioavailability of the virus, EnAd has now been evaluated in several early phase clinical trials showing successful delivery of the virus to the tumour. Pre-

clinically, multiple reports establish EnAd as a promising strategy for tumour-restricted transgene expression, with previous examples including DNAase, hyaluronidase (Tedcastle et al, 2016), Bevacizumab and Ipilimumab (data not published).

Encoding BiTEs within EnAd provides a particularly appealing strategy to achieve targeted expression in disseminated tumours, so that therapeutic efficacy may be maximised while minimising systemic toxicities. Combined with a virus-mediated influx of T lymphocytes into the TME, and the potential of the BiTEs to prolong the functional lifetime of the virus infection, we expect each platform to complement the other, with synergistic anticancer effects.

Therefore, the overarching aim of this thesis is to assess the possibility of encoding BiTEs within the clinically advanced candidate oncolytic adenovirus, EnAd, as a strategy to improve its anti-cancer efficacy. The utility of this platform to target cell types that are resistant to EnAd oncolysis – for example non-cancer stromal cells - will also be investigated for the first time. To test this, BiTEs targeting tumour or cancer-associated fibroblast antigens will be developed, and candidate EnAd-BiTE viruses will be engineered, rescued and validated. *In vitro* studies will functionally evaluate whether viral-mediated BiTE expression can activate primary T cells and induce the lysis of the chosen target cell. For maximum translational relevance and reflecting also the complete absence of immune-competent animal models for group B adenoviruses, we have focused our studies on human cell lines and primary biopsies maintained *ex vivo* – including human liquid tumour samples such as malignant peritoneal and pleural exudates, and solid human tumour specimens isolated from resected prostate tumours.

1.7.2 Thesis Hypotheses

1. Encoding an EpCAM BiTE within EnAd can potentiate the killing of human EpCAM⁺ cancer cell lines or tumour cells in clinical cancer biopsies by exogenous PBMC or endogenous T cells.
2. Arming EnAd with a FAP-targeted BiTE can mediate the killing of FAP⁺ cancer-associated fibroblasts *in vitro* or within patient biopsy samples.
3. Encoding BiTEs under the transcriptional control of the adenovirus major late promoter can restrict BiTE expression to EnAd-permissive tumour cells.
4. BiTE-mediated cytotoxicity can overcome many tumour-associated immune suppressive mechanisms in primary malignant exudate samples.

1.7.3 Thesis Overview

1. Engineering, production and characterisation of EpCAM BiTE-armed oncolytic adenoviruses (chapter three).
2. Generation and characterisation of fibroblast-targeting oncolytic adenoviruses expressing a FAP-specific BiTE (chapter four).
3. Targeting T cell cytotoxicity in human malignant exudate samples using BiTE-expressing oncolytic adenoviruses (chapter five).
4. Activation of tumour-infiltrating T cells and fibroblast targeting in human solid tumour samples using FAP BiTE-expressing oncolytic adenoviruses (chapter six).

2. Materials and Methods

2.1 Molecular Biology

2.1.1 Production of heat shock competent *E. coli* cells

XL10 Gold Ultra-competent *Escherichia coli* (*E.coli*) bacteria were used for plasmid amplification. Large batches of competent cells were prepared from fresh aliquots of XL10 cells (Stratagene, USA). A scraping from the top of a frozen aliquot was inoculated into 3 mL of 2YT media and cultured at 37°C using an orbital incubator (SI-300R, Medline Scientific, UK). This culture (and all further cultures at 37°C) were incubated with agitations at 200 rpm. Once reaching an optical density (OD) of 0.3 at 550 nm, the 3-mL culture was transferred to a fresh culture of prewarmed 200 mL 2YT media and cultured further until the OD₅₅₀ reached 0.480. The cultures were incubated on ice for 5 minutes, followed by pelleting by centrifugation at 4°C for 20 minutes. Cells were resuspended in 80 mL sterile transformation buffer I (TFBI; see Materials for recipe) and kept on ice for a further 5 minutes. The bacterial cells were spun as above and were resuspended in 8 mL transformation buffer II (TFBII; see Materials for recipe) and incubated on ice for 15 minutes. Competent cells were aliquoted in 100 µL volumes into pre-chilled Eppendorf tubes, frozen on dry ice and transferred to -80°C for long term storage.

2.1.2 Bacterial transformation

Recombinant plasmid DNA was amplified by transformation of *E.coli*. Transformation was performed by thawing an aliquot of competent *E.coli* cells on ice, followed by the

addition of 10 – 100 ng plasmid DNA. Cells were incubated on ice for a further 20 minutes and then underwent heat-shock by transferring tubes to a water bath at 37°C for 30 seconds. Up to 1 mL of Luria Bertani (LB) broth (20 g/L) was added to each tube and incubated at 37°C for 20 minutes (for ampicillin-resistant vectors) or 60 minutes (for kanamycin-resistant vectors). Samples were decanted and spread on to LB agar plates (35 g/L) containing the relevant antibiotic for selection (50 µg/mL ampicillin; 50 µg/mL kanamycin) and incubated at 37°C overnight to allow the isolation of individual colonies. Single colonies were picked and grown overnight at 37°C in LB broth (3 mL for mini-prep or 200 mL for maxi-prep) prepared with the relevant selection antibiotic. Samples were incubated at 37°C with agitations (200 rpm) using an orbital incubator (SI-300R, Medline Scientific, UK).

2.1.3 DNA purification and precipitation

Plasmid DNA were purified from mini-prep cultures of bacterial cells using QIAprep Spin Miniprep Kit (Qiagen, UK). For larger scale applications, plasmid DNA was purified from maxi-prep cultures using the Qiagen High Speed Maxi-prep kit (Qiagen, UK). Selected plasmid clones were screened by diagnostic restriction digest and agarose gel electrophoresis, and further validated by Sanger sequencing (Source Bioscience, UK). Where PCR amplification or digestions were performed for use in molecular engineering, DNA fragments of the desired size were visualised by agarose gel electrophoresis and purified from excised gel bands using the MinElute Gel Extraction Kit (Qiagen UK). Alternatively, where it was unnecessary to select for DNA fragments of a specific size, DNA was purified directly from the digestion reaction by QIAprep Spin Miniprep Kit.

In order to purify large fragments (> 20 kb) of DNA, in which commercial column-based purification kits were unsuitable, it was necessary to precipitate the DNA using isopropanol. A volume of isopropanol equivalent or exceeding 1.5 volumes of the DNA solution was added to the DNA, mixed thoroughly, and stored at -80 °C for 60 minutes. The DNA was pelleted by centrifugation at 15,000 g for 30 minutes at 4°C, and the supernatant decanted by pipette. The DNA pellet was air-dried thoroughly and resuspended in nuclease-free water (Thermo Fisher, UK).

2.1.4 DNA restriction digests

Restriction endonucleases were used to generate diagnostic digestion patterns or to produce DNA (for molecular engineering) with ‘sticky’ overhanging ends for subsequent ligation or Gibson assembly reactions. All digestion reactions were performed according to manufacturer’s instructions (New England Biolabs, UK). Briefly, 5 µg DNA, 2 µL enzyme in 100 µL reaction volume were incubated at 37°C for 3 hours.

2.1.5 Polymerase chain reaction (PCR) amplification

PCR amplifications were performed using 10 µM custom synthesised primers (Sigma-Aldrich, UK), the relevant DNA template (10 ng) and Phusion DNA polymerase Master Mix (Life Technologies, UK) according to manufacturer’s instructions. In most cases, this involved an initial denaturation step (98°C for 30 seconds), 30 repeated cycles of denaturation (98°C for 10 seconds), annealing (temperature dependent on annealing temperature of primers for 10 seconds), and extension (72°C for 15 seconds per kb), and terminated with a final extension step (72°C for 10 seconds). PCR reactions were performed using a PTC-225 Peltier Thermal Cycler DNA Engine Tetrad (MJ Research, USA).

2.1.6 Agarose gel electrophoresis

Agarose gel electrophoresis enabled visualisation of DNA from purified plasmids, fragments from restriction digests or PCR reactions. By relative comparison to commercially available molecular weight marker Hyperladder I (Bioline Ltd, UK), the size of DNA molecules could be estimated. DNA was fractionated by size on 1% (w/v) agarose gels containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich, UK) in 0.5 × TAE buffer (see Materials for recipe). DNA was loaded into wells and subjected to gel electrophoresis (10 volts per cm gel length, 30 – 120 minutes) in DNA electrophoresis gel tanks (Bio-Rad Laboratories Ltd, UK). If DNA was to be excised for purification and subsequent use, bands were visualised under blue light using Dark Reader trans-illuminator (GRI, UK). For all other purposes, DNA was visualised under ultraviolet light using a fluorescent imaging system (AlphaImager, Alpha Innotech Corporation, USA).

2.1.7 Quantification of purified DNA

The quantity of DNA was determined using a Nanodrop spectrophotometer (Thermo Scientific, UK). Prior to analysis, the Nanodrop was blanked using distilled water, and a background reading was collected using the buffer in which the DNA was dissolved.

2.1.8 DNA ligation

Insert and vector DNA were digested with single or double restriction enzymes to generate compatible ‘sticky’ overhangs for complementary base-pairing and subsequent ligation of insert to vector. The digested insert was selectively purified by extraction from excised agarose gel bands or if resolution of different sized fragments was unnecessary, purified directly from the digestion reaction using QIAprep Spin Miniprep Kit. To reduce the number of vector re-ligations, and therefore the number of false positive colonies, the

vector was dephosphorylated by treatment with 2 μ L of calf intestinal alkaline phosphatase (CIAP, Invitrogen, USA) for 2 hours at 37°C, and DNA purified as above. All purified DNA was eluted in nuclease-free water. Ligation reactions were performed using T4 DNA Ligase (New England Biolabs, UK), with a ratio of 1 vector: 3 insert, according to manufacturer's instructions.

2.1.9 Gibson assembly

Gibson assembly allows the combination of multiple DNA molecules in a single isothermal reaction. To be suitable for Gibson assembly, DNA fragments must possess a 20-40 pair overlap with adjacent fragments. The overlapping fragments were generated by PCR amplification or digest of plasmid DNA with restriction endonucleases, and DNA was purified as described above. Gibson assembly reactions were performed using Gibson assembly master mix (New England Biolabs, UK). Briefly, 100 ng of plasmid DNA was mixed with three-fold molar excess of fragments in 10 μ L volume. Ten μ L of Gibson assembly Master Mix is added, and samples are incubated in a thermocycler at 50°C for 15 minutes.

2.2 Cell Culture

2.2.1 Maintenance of cell lines

Cell lines were cultured in the appropriate media (Table 2.1) and maintained at 37°C in a humidified incubator with 5% CO₂. Where necessary, growth media was supplemented with fetal bovine serum (FBS, Gibco, UK). For sub-culturing adherent cells, monolayers were washed with PBS, treated with 0.05% trypsin (Sigma-Aldrich, UK) and incubated

at 37°C for several minutes until cells were raised from the surface of the culture flask with gentle agitation. Five volumes of growth medium containing FBS (10% v/v) was added to the trypsin-treated cells to neutralise the trypsin. Cells were then resuspended and clumps disaggregated by sequential pipetting for subsequent passage or analysis. When experiments required cell surface proteins to remain intact (e.g. for flow cytometry), cells were detached from the culture surface using cell-dissociation buffer (Gibco, USA). If resuspended cells were to be seeded into microplates, the concentration of cells was determined using a haemocytometer and cell number adjusted with growth medium prior to application to the microplate. All cell lines were authenticated by short tandem repeat (STR) profiling (CRUK Cambridge Institute, Cambridge) and were routinely tested for mycoplasma using MycoAlert™ Mycoplasma Detection Kit (Lonza, Switzerland).

2.2.2 Cryopreservation of cells and cell recovery

Cells for cryopreservation were resuspended in freezing medium (FBS + 10% DMSO), the medium acting as a cryoprotectant to prevent the formation of crystals. One mL of cell suspension was added to the cryovial, which was then transferred into freezing containers and stored at -80°C. To recover cryopreserved cells, frozen vials were thawed in a water bath at 37°C and cells were immediately transferred to a 15-mL falcon tube. Warm culture medium was added, and the cells were pelleted by centrifugation at 350 g and resuspended in fresh growth medium prior to transfer to culture flasks for culturing.

2.2.3 DNA transfection

DNA transfection of cells was performed on a small-scale using Lipofectamine 2000 (Life Technologies, UK) following the manufacturer's instructions. In brief, DNA and lipofectamine were mixed in OptiMEM (Life Technologies, UK) at a ratio of 1 µg DNA

Table 2.1. Characteristics and growth conditions of cell lines used in this study.

Cell line	Description	Source	Culture medium
A431	Human epidermoid carcinoma cells derived from skin/epidermis. Adherent.	ATCC, USA.	DMEM + 10% FBS.
A549	Human carcinoma cells derived from lung. Adherent.	ATCC, USA.	DMEM + 10% FBS.
CHO	Initiated from a biopsy of an ovary of an adult Chinese hamster. Adherent.	ATCC, USA.	RPMI + 10% FBS.
DLD1	Human adenocarcinoma cells derived from colon. Adherent.	ATCC, USA.	DMEM + 10% FBS.
HEK293A	An adherent subclone of the 293 cell line. HEK293 were derived from human embryonic kidney cells transformed with adenoviral E1A gene. Adherent.	ATCC, USA.	DMEM + 10% FBS.
HEK293T	A subclone of the 293 cell line also containing the SV40 large T antigen. Adherent.	ATCC, USA.	DMEM + 10% FBS.
HT29	Human adenocarcinoma cells derived from colon. Adherent.	ATCC, USA.	DMEM + 10% FBS.
NBE	Human normal bronchial epithelial cells isolated from epithelial lining of the lung. Adherent.	Lonza, USA.	RPMI + 10% FBS.
NHDF	Human normal dermal fibroblasts isolated from the dermis of normal human adult skin. Adherent.	Lonza, USA.	DMEM + 10% FBS.
SKOV3	Human adenocarcinoma cells derived from human ovaries. Adherent.	ATCC, USA.	DMEM + 10% FBS.
TIG1	Human normal fetal fibroblasts derived from the lung of a five-month foetus. Adherent.	Department of Oncology	DMEM + 10% FBS.

to 2.5 μ L lipid transfection reagent. Following incubation at room temperature for 20 minutes, the solution was added to wells or flasks containing target cells to be transfected. For large-scale transfection, *polyethylenimine* (PEI, linear, MW 25000; Polysciences, USA) was used. Optimisation conditions for PEI transfection were determined using pSF-

CMV-GFP reporter plasmid (Oxford Genetics, UK), as demonstrated in Figure 2.1. PEI was mixed with DNA at a ratio of 3 μ g DNA to 6 μ g PEI in OptiMEM and solutions were incubated for 20 minutes before being added to the cells to be transfected.

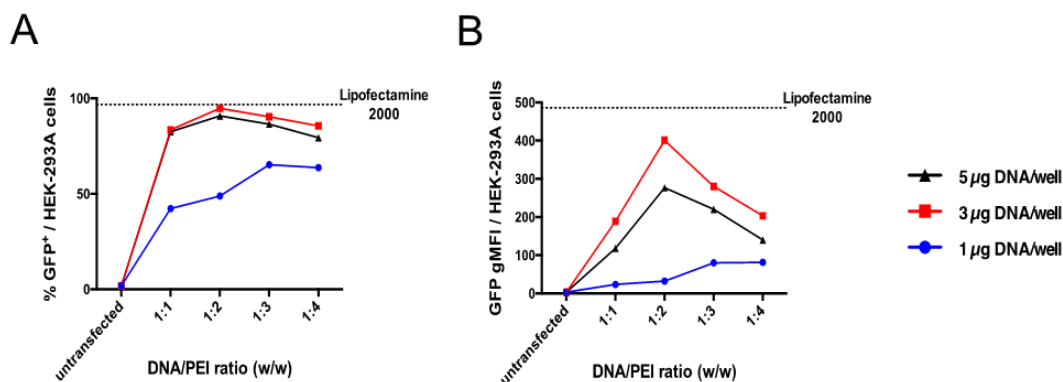


Figure 2.1. Optimisation of PEI transfection of HEK293A cells. (A, B) HEK293A cells were transiently transfected with pSF-CMV-GFP, using linear 25,000 MW PEI. Various DNA/PEI (w/w) ratios and DNA concentrations were tested as indicated. Percentage of GFP-positive cells (A) and geometric mean fluorescence intensity (gMFI; B) were measured by flow cytometry to determine the optimal transfection conditions. Transfection with Lipofectamine 2000 according to manufacturer's instructions with a DNA/Lipofectamine ratio of 1 μ g: 2.5 μ L and a DNA concentration of 2.5 μ g per well served as positive control. This study was completed in collaboration with Joachim Hagel (MSc student).

2.2.4 Production of lentiviral constructs

Recombinant lentiviruses were used to facilitate the generation of stably transduced cell lines expressing the gene of interest. EpCAM and FAP DNA sequences (Appendix 1) were generated from obtained proteins sequences (EpCAM ID 4072, FAP ID 1149, NCBI database), followed by gene synthesis (Genewiz, UK). The gene sequences were inserted into lentivirus expression vectors (pSF-Lenti; Figure 2.2A), containing a CMV promoter to drive transgene expression and PGK promoter driving puromycin resistance. Recombinant plasmids were screened for correct insertion of the transgene into the lentivirus plasmids by diagnostic enzymatic digestion with PshA + NheI (Figure 2.2B). Lentiviruses were generated by Lipofectamine 2000 transfection of 293T cells with

lentivirus plasmids in parallel with three packaging plasmids (pSF-CMV-HIV-Rev, pSF-CMV-VSVG, pSF-CMV-HIV-Gag-Pol, Oxford Genetics, UK) containing the necessary accessory genes for lentivirus production. After 48 hours in a humidified chamber at 37°C, cell supernatants were harvested and filtered through a 0.45 µm filter to remove cellular debris. The media containing lentivirus was then aliquoted and stored at -80°C until future use.

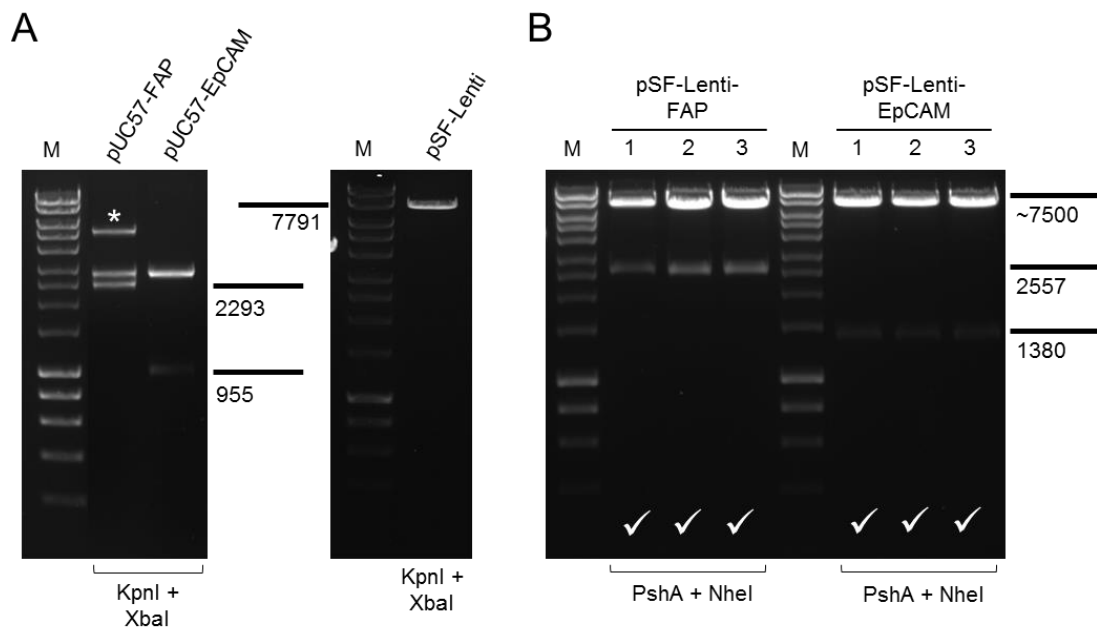


Figure 2.2. Engineering of EpCAM and FAP lentivirus vectors for stable transgene expression. (A) Double restriction enzyme digest of the pUC57-FAP and pUC57-EpCAM plasmids gave bands of the expected molecular weight (left). Plasmid DNA that had undergone a single cleavage is indicated with an asterisk (*). The desired fragments were gel-extracted and ligated into the cleaved pSF-Lenti vector (right). M, Hyperladder 1 kb DNA ladder. **(B)** Purified plasmids isolated from colonies generated by ligation of the products from (A) were screened for transgene insertion by digestion with PshA and NheI. Colonies with the correct digestion pattern are indicated with a tick. M, Hyperladder 1 kb DNA ladder. This study was completed in collaboration with Joachim Hagel (MSc student).

2.2.5 Generation of cell lines by lentivirus infection

Lentivirus infection was performed by adding serial dilution of lentivirus to CHO or HEK293A cells (2×10^5 cells/well of a 6 well plate). To enhance the efficiency of infection, the lentivirus-containing media was mixed with polybrene (Sigma-Aldrich,

UK), a polycation that reduces the charge repulsion between the lentivirus and cell membrane, at a final concentration of 4 µg/mL. Plates were incubated at 37°C in humidified chambers for 24 hours, with media subsequently replaced with DMEM supplemented with 10% FBS and puromycin as the selection antibiotic. The appropriate concentration of puromycin (8 µg/mL for CHO and 2 µg/mL for HEK293A) required for selection was pre-determined by setting up a puromycin kill curve. Colonies were allowed to form over 1-2 weeks, at which point the surviving cells were harvested and re-seeded at serial dilutions in order to isolate individual clones (in medium containing puromycin). Clones were then expanded and tested for transgene expression by flow cytometry (Figure 2.3; positive clones labelled with a tick).

2.3 Assessment of BiTE Expression and Functionality

2.3.1 Recombinant protein sample collection

Protein samples were obtained by DNA transfection or viral infection of HEK293A cells. After 48 hours incubation, supernatants were collected and centrifuged for 5 minutes, 300 g and concentrated 50-fold using Amicon Ultra 10k MWCO centrifugal filter units (EMD Millipore, USA). Cells were then washed with PBS, followed by treatment with radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher, UK), freeze-thawing and centrifugation at 18000 g at 4°C to remove cell debris. The quantity of protein in the cell lysate were determined using BCA protein assay kit (Thermo Fisher, UK), following the manufacturer's instructions.

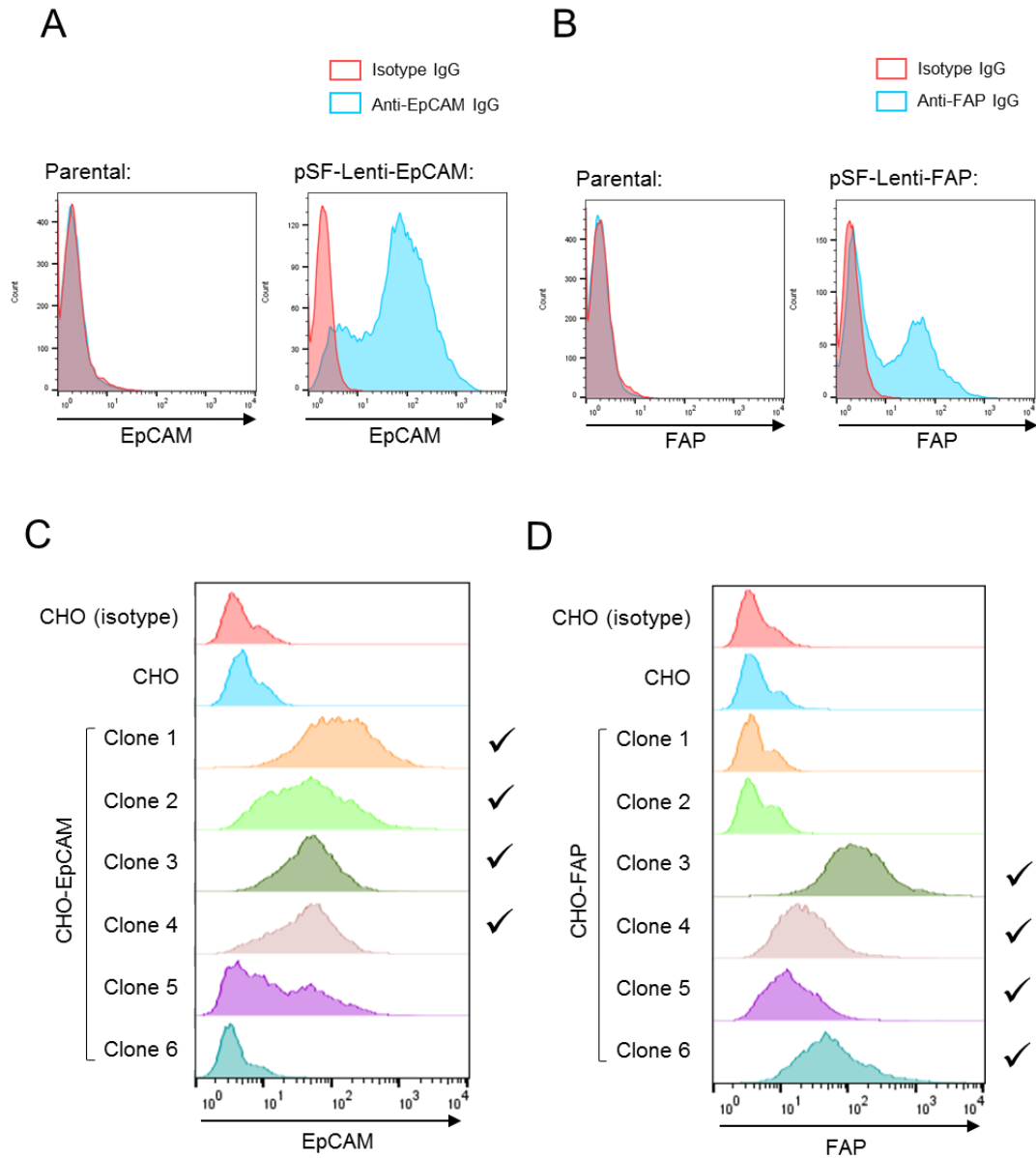


Figure 2.3. Isolation of single cell antigen-positive clones from a polyclonal population of lentivirus-infected CHO cells. (A, B) Polyclonal populations of stable EpCAM- (A) and FAP-expressing (B) CHO cell lines following selection with puromycin. Surface EpCAM or FAP expression was assessed by antibody staining and flow cytometry. An isotype control antibody was included (red) to measure background fluorescence and unspecific binding. **(C, D)** Levels of EpCAM (C) or FAP expression (D) of expanded single clones isolated by serial dilution of the polyclonal population in (A, B).

2.3.2 Immunoblotting

Western blotting was used to confirm expression and secretion of BiTE protein. Supernatants and cell lysates were fractionated by SDS-PAGE (10% polyacrylamide gel, 160 V, 1 hour) and transferred to a nitrocellulose membrane according to manufacturer's protocols (Bio-Rad). The membrane was air-dried, blocked with 5% milk and probed overnight at 4°C with α -6xHis (C-terminus) antibody (1:5000, clone 3D5, Invitrogen, UK, #46-0693) for detection of C-terminally His-tagged proteins. The membrane was further washed and incubated with anti-mouse secondary antibody (1:10000, Dako, Denmark, #P0161) before visualisation and film development using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher, UK).

Dot blot was used to measure the concentration of recombinant (decahistidine-tagged) BiTE produced from plasmid transfections. Two-fold serial dilutions of each BiTE and of a protein standard (10 x His-tagged (C-terminus) human Cathepsin D, Biolegend, US, #556704) were prepared. The molar concentration of the protein standard was adjusted to represent a BiTE concentration of 100 μ g/mL. Two μ L of each sample and protein standard were directly applied onto a nitrocellulose membrane. The membrane was air-dried, and further treated identically to that of western blotting protocol above.

2.3.3 Enzyme-linked immunosorbent assay (ELISA) to assess binding of BiTE to recombinant target protein

High protein binding MaxiSorp 96 well plates (Nunc, Denmark) were prepared by coating overnight at 4°C with human EpCAM protein (50 ng/well, Sino Biological, China, #10694-H02H-50) or FAP protein (50 ng/well Sino Biological, China, #10464-H07H). Unbound antigen was removed by washing three times with PBS/0.05% Tween (PBS-T,

Fisher Scientific, UK). Plates were blocked for 1 hour at ambient temperature with 5% BSA (Sigma-Aldrich, UK) diluted in PBS, followed by incubation with diluted supernatants (2 hours, room temperature). Where necessary, a primary antibody specific for the target protein was used as a positive control. Plates were washed five times with PBS-T and subsequently after every future binding step. Plates were incubated with anti-His (C-term) antibody (1:5000, clone 3D5, #46-0693, Invitrogen, UK) for 1 hour, room temperature, followed by HRP conjugated anti-mouse-Fc (1:1000 in PBS/5% milk, Dako, Denmark) for 1 hour at room temperature. HRP detection was performed using 3,3',5,5'-teramethylethylenediamine (TMB, Thermo-Fisher, UK) and stop solution was used for terminating the reaction. Absorbance at 450 nm was measured using a Wallac 1420 Victor² plate reader (Perkin Elmer, USA).

2.4 Flow Cytometry

2.4.1 Overview of flow cytometry methods

For adherent cells, media was removed from culturing flasks or plates and cells were washed with PBS before the addition of enzyme free cell dissociation buffer (Gibco, USA) to prevent the digestion of cell surface antigens. When cells were detached from the culture surface, they were suspended in PBS and transferred to V-bottom 96-well plates for antibody staining. For non-adherent cells (e.g. T cells), cells were collected from culture plates by resuspension with a pipette and directly transferred to V-bottom 96-well plates.

For surface staining, cells were washed with 200 μ L PBS, centrifuged (350g, 5 minutes) and resuspended in 50 μ L of antibody mix diluted in PBS. Staining was performed for 1 hour at 4°C, followed by washing twice with PBS to remove non-bound antibody. Cells were re-suspended in 200 μ L of MACS buffer (see Materials for recipe) for analysis or subsequent intracellular staining (cell surface staining must always be performed prior to intracellular stains).

For intracellular staining, cells were resuspended in 100 μ L Cytofix/Cytoperm Solution (BD Biosciences, USA) for 20 minutes at room temperature, followed by permeabilisation with Perm/Wash buffer (BD Biosciences, USA) for IFN γ (Table 2.2) or Perm buffer III (BD Biosciences, USA) for Phosflow P-ERK1/2 (Table 2.2). Cells were resuspended with the appropriate antibody in Perm/Wash buffer or PBS for 1 hour at 4°C, washed twice with PBS and finally resuspended in MACS buffer for analysis. In each case, the appropriate isotype control antibody was used to assess non-specific antibody binding. Flow cytometry analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, USA) and data processed with FlowJo v10.0.7r2 software (TreeStar Inc., USA). A representative example of analysis of flow cytometry data is shown in Figure 2.4.

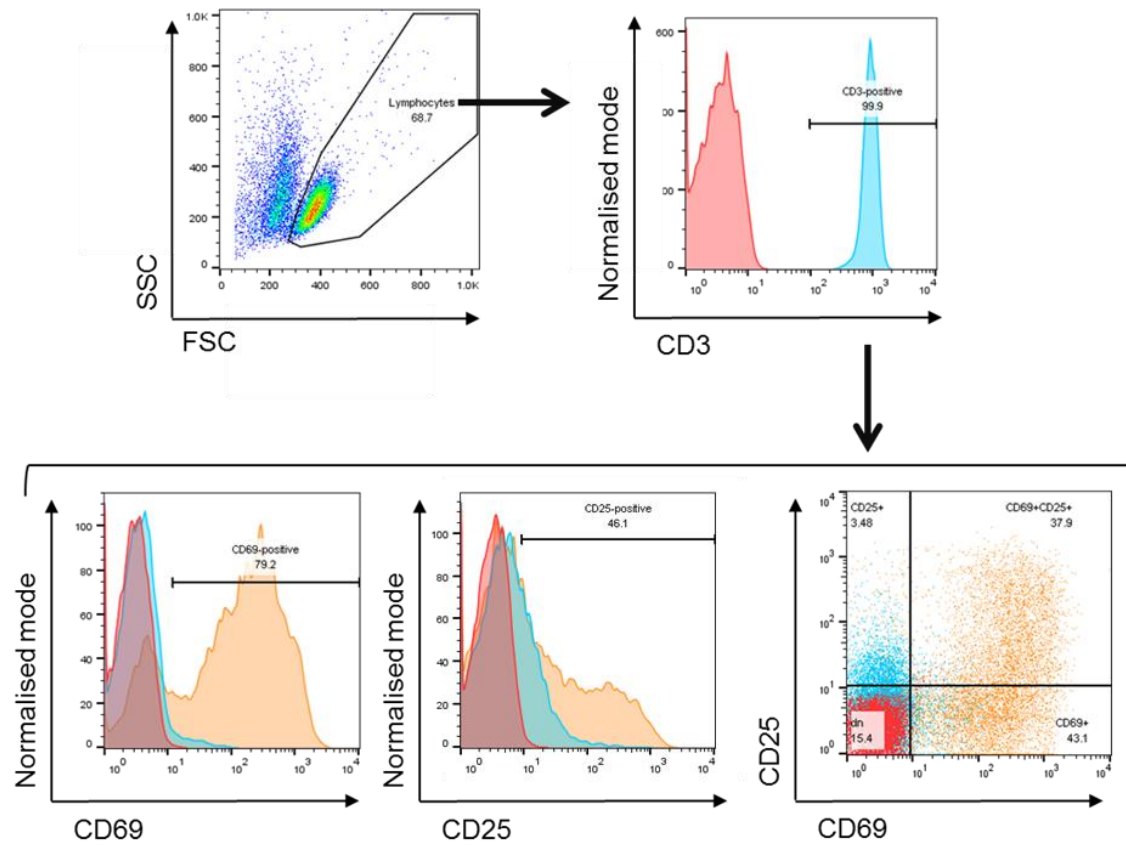


Figure 2.4. Example analysis of flow cytometry data. PBMC-purified T cells were stimulated with anti-CD3/CD28 beads or left untreated. After 24 hours, cells were harvested and stained for CD3, CD69 and CD25. Cell debris was excluded by analysing FSC and SSC properties (top left panel). Within this gate, CD3-positive cells were selected (top right panel) for further analysis of their expression of activation markers CD69 and CD25 (bottom panels). Isotype controls for CD69 and CD25 were used to measure background fluorescence and establish suitable gates (red). Increases in CD69 and CD25 expression were observed upon stimulation (orange) compared to untreated (blue).

2.4.2 Antibodies used in flow cytometry

Table 2.2. An overview of the antibodies used in flow cytometry.

Antibody	Species/Isotype	Clone	Dilution	Company
CD3	Mouse IgG2a	HIT3a	1:200	Biolegend, UK
CD4	Mouse IgG2b	OKT4	1:200	Biolegend, UK
CD8a	Mouse IgG1	HIT8a	1:200	Biolegend, UK
CD11b	Mouse IgG1	ICRF44	1:200	Biolegend, UK
CD25	Mouse IgG1	BC96	1:200	Biolegend, UK
CD56	Mouse IgG1	HCD56	1:200	Biolegend, UK
CD64	Mouse IgG1	10.1	1:100	Biolegend, UK
CD69	Mouse IgG1	FN50	1:200	Biolegend, UK
CD86	Mouse IgG2	IT2.2	1:100	Biolegend, UK
CD107a	Mouse IgG1	H4A3	1:100	Biolegend, UK
CD163	Mouse IgG1	GHI/61	1:100	Biolegend, UK
CD206	Mouse IgG1	15-2	1:100	Biolegend, UK
EGFR	Mouse IgG1	AY13	1:200	Biolegend, UK
EpCAM	Mouse IgG2b	9C4	1:200	Biolegend, UK
ERK1/2 (pT202/pY204)	Mouse IgG1	20A	1:10	BD Biosciences, USA
FAP	Mouse IgG1	427819	1:50	R&D Systems, USA
IFN γ	Mouse IgG1	4S.B3	1:100	Biolegend, UK
LAG-3	Mouse IgG1	11C3C65	1:200	Biolegend, UK
PD-1	Mouse IgG1	EH12.2H7	1:200	Biolegend, UK
PD-L1	Mouse IgG2b	29E.2A3	1:200	Biolegend, UK
TIM-3	Mouse IgG1	F38-2E2	1:200	Biolegend, UK

2.5 Microscopy

2.5.1 Brightfield and fluorescence imaging

Brightfield and fluorescence images were obtained on a Zeiss Axiovert 25 microscope and captured with a Nikon DS5M camera using the Zen software package. For time-lapse sequences, images were obtained on a Nikon TE 2000-E Eclipse inverted microscope and captured with a Hamamatsu Orca-ER C4742-95 using MetaMorph imaging software.

Images were collected at intervals of 15 minutes, with videos (12 frames/second) generated using ImageJ software (NIH, USA). Where appropriate, cells were stained with CellTracker Orange CMTMR Dye (Life Technologies, USA, #C2927) and CellTrace Violet Cell Proliferation Kit (Life Technologies, USA, #C34557), and apoptosis was visualised using CellEvent Caspase 3/7 detection reagent.

2.6 Production of Recombinant Adenoviruses

2.6.1 Virus cloning strategy

Recombinant EnAd viruses were generated using the pEnAd2.4 cloning vector (Figure 2.5) containing the EnAd genome, kindly provided by PsiOxus Therapeutics Ltd. BiTE transgene was encoded immediately downstream of the fiber gene, by direct insertion of the transgene cassette into the EnAd backbone by Gibson assembly. The transgene cassette contained a 5' short splice acceptor sequence (CAGG) or an exogenous CMV promoter, followed downstream by the EpCAM/FAP or control BiTE cDNA sequence (appendix 1) and a 3' polyadenylation sequence. A schematic of the EnAd genome containing the inserted transgene cassette is shown in Figure 3.12.

2.6.2 Virus rescue and amplification

Modified pEnAd2.4 plasmids containing the EnAd genome and encoded transgenes were linearised by digestion with the restriction endonuclease AscI. Digested DNA was purified by isopropanol precipitation and resuspended in nuclease-free water. HEK293A cells were seeded in T25 culture flasks to reach confluence of 80–90% prior to transfection. For each T25 flask, 5 µg of DNA was complexed with 15 µL Lipofectamine

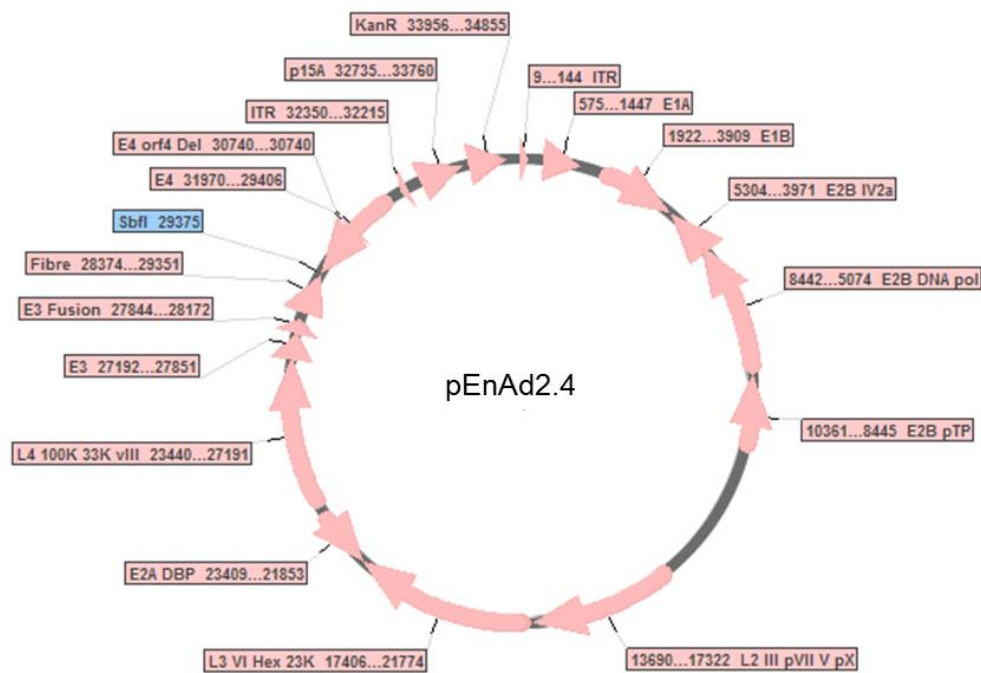


Figure 2.5. Plasmid map of pEnAd2.4 vector. Schematic of the pEnAd2.4 vector containing the EnAd genome, and used for engineering recombinant viruses. Transgene cassettes can be inserted downstream of the fibre gene by using the SbfI cleavage site (blue). The plasmid contains all the necessary genes for plasmid propagation in bacteria and virus production in mammalian HEK293A cells. ITR, inverted terminal repeat; E, early; L, late; Hex, hexon; p15A, origin of replication; KanR, Kanamycin resistance cassette.

2000 reagent, and the HEK293A monolayer transfected as described previously. After 4 hours, the transfection mix was replaced with DMEM supplemented with 2% FBS and flasks incubated at 37°C in humidified chambers for up to 14 days. The production of virus was monitored by observation of cytopathic effect (CPE) in the cell monolayer. Once extensive CPE was observed the virus was harvested from HEK293A cells by three freeze-thaw cycles. Single virus clones were selected by serially diluting harvested lysate and re-infecting HEK293A cells in 96-well culture plates, and harvesting wells containing single plaques.

Serial infections of HEK293A cells (in flasks of increasing size) were performed once an infection had reached full CPE to amplify the virus stocks. For the final stage of amplification, a 10-layer hyperflask (Corning, USA) was seeded with HEK293A cells harvested from a confluent T175 flask. After 2-3 days, the cells were infected with infectious supernatant. HEK293A cells were detached by gentle agitation when CPE was observable but infected cells were not lysed. Cells were collected from the flask, pelleted and resuspended in 10 mL of infected supernatant (final volume of approximately 12 mL) and stored at -80°C for purification of virus by banding on caesium chloride gradients.

2.6.3 Virus purification by caesium chloride gradients

Banding of virus by ultracentrifugation over caesium chloride (CsCl) gradients allows the purification of viral particles based on particle size and density. Cell preparations containing adenovirus to be purified were lysed by three freeze-thaw cycles to release virus particles. The mixture was centrifuged (500 g, 15 minutes, 4°C) to pellet cellular debris, and the supernatant containing free virus particles was harvested and loaded onto centrifuge tubes containing pre-poured discontinuous caesium chloride gradients (Figure 2.6). In brief, the gradient consists of 3 mL of CsCl $\rho = 1.32$, underlaid with 2 mL of CsCl $\rho = 1.45$, followed by an overlay of 2 mL 40% glycerol (see Materials for recipes). Finally, the virus lysate supernatant is added on top of the glycerol. The centrifuge tubes were then centrifuged at 25,000 rpm at 10°C for 120 minutes using a Beckman L8-70M Ultracentrifuge with SW40TI rotor. After centrifugation, two discrete bands were obtained – the higher band containing ‘empty’ viral particles and a lower band containing intact (infectious) viral particles. The intact virus was harvested by puncturing the tube using an 18G needle and extracting the lower band into a syringe.

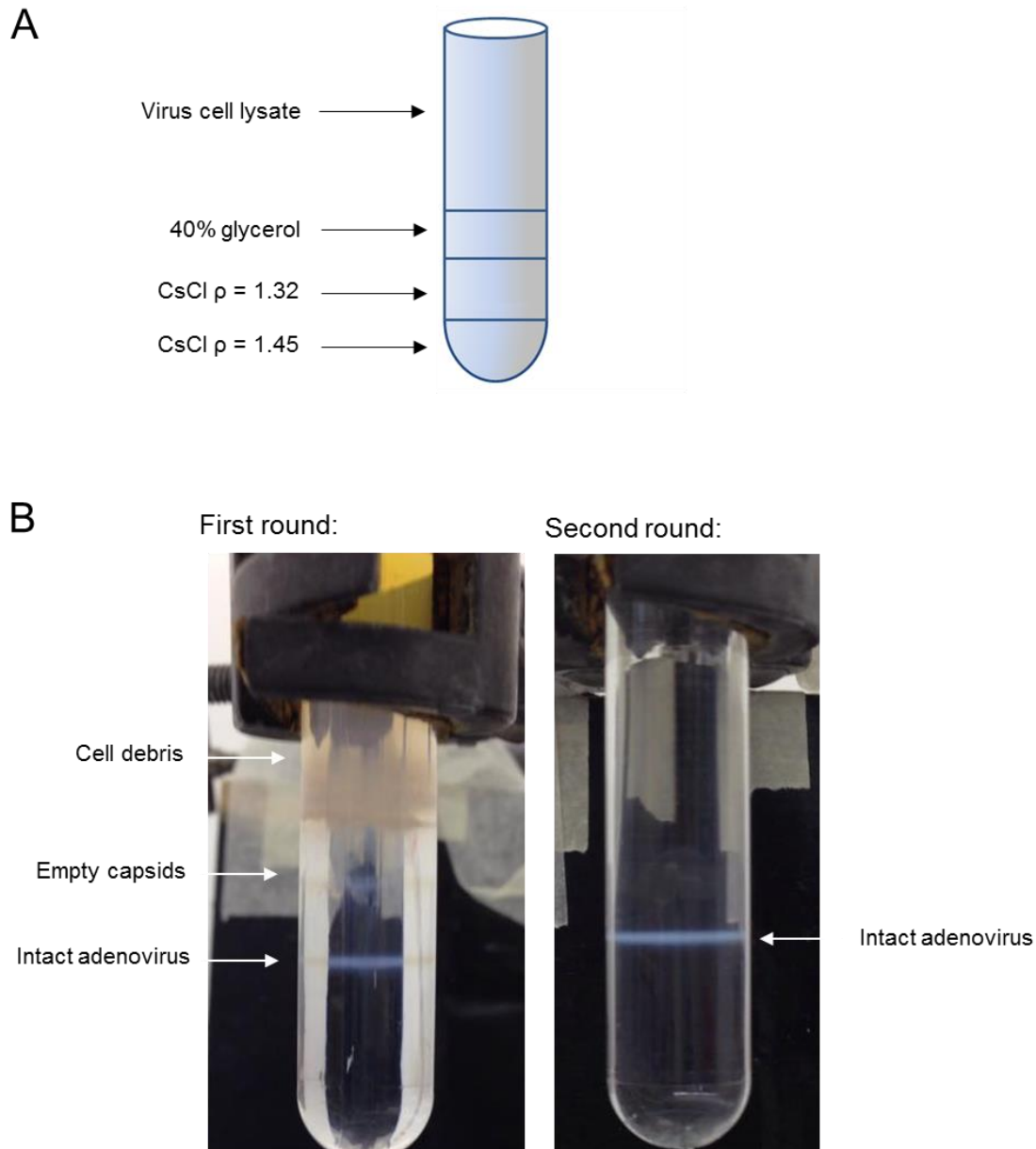


Figure 2.6. Caesium chloride (CsCl) gradient column for banding and purification of adenovirus preparations. (A) Schematic illustrating the CsCl gradient column for virus purification. The gradient consists of 3 mL of CsCl $\rho = 1.32$, underlayed with 2 mL of CsCl $\rho = 1.45$, followed by an overlay of 2 mL 40% glycerol. Finally, the virus lysate supernatant is added on top of the glycerol. (B) Representative photographic images of CsCl banding of virus preparations. Each adenovirus underwent two rounds of purification. The left image displays the column following the initial banding of the crude preparation. Cell protein and membranes are visible above the two bands of virus. The higher, more diffuse band corresponds to empty viral capsids (CsCl_{(ρ 1.32);glycerol interface) and the lower, larger band is intact virus (CsCl_{(ρ 1.45); CsCl_{(ρ 1.32) interface). The right shows the same sample after dialysis, benzonase treatment and the second round of banding. The only remaining band corresponds to intact virus particles.}}}

Caesium chloride was subsequently removed by two rounds of dialysis in PBS, followed by one round in PBS with HEPES using a 3-15 mL dialysis cassette (Slide-A-Lyzer Dialysis cassette, Thermo Fisher, UK). Virus was recovered, and unpackaged DNA (cellular or viral) was lysed by addition of $MgCl_2$ and treatment with Benzonase DNA nuclease (EMD Millipore, USA) for 30 minutes at room temperature.

A second round of banding by CsCl gradient was performed as described above, and extracted virus was similarly dialysed in PBS using 0.5-3 mL cassettes (Slide-A-Lyzer Dialysis cassette, Thermo Fisher, UK). Virus obtained after the final dialysis was supplemented with glycerol (as a cryoprotectant) to a final concentration of 40% and aliquots stored at $-80^{\circ}C$ for future use.

2.6.4 dsDNA measurement for adenovirus titre quantitation

The DNA concentrations of the purified adenovirus preparations were determined using the PicoGreen dsDNA assay (Invitrogen, UK), which exploits a nucleic acid probe that fluoresces upon binding to dsDNA. Virus stock samples were diluted in $1\times TE$ buffer with 0.5% SDS and incubated at $56^{\circ}C$ for 30 minutes to disrupt viral particles and release the genome. The quantity of dsDNA was then determined as per manufacturer's instructions using a standard curve constructed from bacteriophage lambda DNA. The concentration of viral particles in each sample was calculated based on $1\ \mu g$ DNA being equal to 2.7×10^{10} adenovirus particles.

2.6.5 Assessment of infectious virus dose by TCID₅₀

TCID₅₀ (50% tissue culture infectious dose) is a technique for measuring the number of infectious viral particles. Prior to infection, A549 cells were seeded in 96-well culture

plates at a density of 1×10^4 cells/well. Virus stocks were subject to 10-fold serial dilutions ranging from 10^{-1} to 10^{-12} in DMEM media supplemented with 2% FBS, with a minimum of ten replicate wells for each virus dilution. Culture media was replaced with 200 μ L of the virus dilutions, with virus-free media as a negative control, and plates were incubated at 37°C in humidified chambers. After five days, wells were scored for the presence of plaques using a Zeiss Axiovert 25 microscope; either designated to have evidence of CPE (or plaques) or be CPE-free. For each virus dilution, a ratio of the wells with CPE to those without was calculated. From this data, the TCID₅₀ was calculated using the KARBBER statistical method:

$$\text{Titre (TCID}_{50}/\text{mL)} = 10^{1 + D(S - 0.5)}$$

D = log(d); d = dilution factor
S = sum of the ratios of wells with CPE to CPE-free wells

2.7 Characterising Viral Genome Replication

2.7.1 qPCR to measure virus genome replication

As a surrogate readout for viral replication, adenovirus genomes were measured by quantitative PCR (qPCR) using primers and probe against the hexon gene. Prior to infection, DLD cells were seeded in 24-well culture plates (1.5×10^5 cells/well) for 18 hours at 37°C, 5% CO₂. Culture media was replaced with virus-containing media (100 vp/cell), and the total cell and supernatant harvested at the specified time-point. DNA was extracted using PureLink genomic DNA mini kit (Invitrogen, UK). Total viral genomes were quantified by qPCR against EnAd hexon using the specific primer-probe set (primers: TACATGCACATCGCCGGA/CGGGCGAACTGCACCA, probe:

CCGGACTCAGGTACTCCGAAGCATCCT) and the qPCRBIO kit. In brief, primers and probe were mixed with samples and added to QPCRBIO probe mix Hi-Rox (PCR Biosystems) master mix. The qPCR was run on an ABI PRISM 7000 (Applied Biosystems), with details of setup previously reported (Jiang et al, 2011).

2.8 Preparation of Human PBMC and T Cells

2.8.1 Isolation of PBMC from human blood and purification of T Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Boyum, 1968) from whole blood leukocyte cones obtained from the NHS Blood and Transplant UK (Oxford, UK). Blood was diluted 1:2 with PBS and layered onto Ficoll (Ficoll-Paque Plus, GE Healthcare, UK) before centrifugation at 400 g for 30 min at 22°C with low deceleration to preserve phase separation. After centrifugation, the PBMCs form a band above the Ficoll layer, as illustrated in Figure 2.7. Using a Pasteur pipette, the PBMCs were collected and washed twice with PBS (300 g for 10 minutes at room temperature) and resuspended in RPMI-1640 medium supplemented with 10% FBS.

For extraction of CD3-positive T cells from PBMCs, non-CD3 cells were depleted using Pan T Cell Isolation Kit (Miltenyi Biotec, Germany), according to the manufacturer's protocol. For further isolation of CD4- and CD8-positive T cells, CD3 T cells underwent another round of purification using CD4⁺ Microbeads (Miltenyi Biotec, Germany) and CD8⁺ Microbeads (Miltenyi Biotec, Germany).

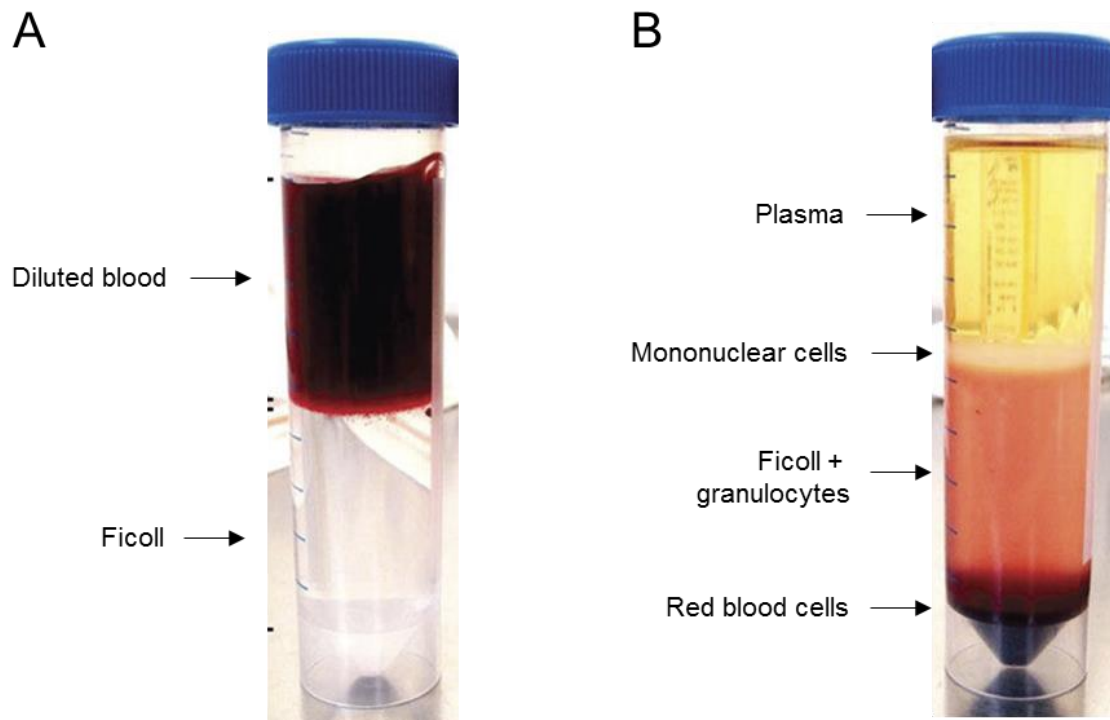


Figure 2.7. Ficoll density gradient for the isolation of PBMCs from whole blood. Schematic (A) and photographic image (B) showing the layering of diluted blood on Ficoll and the resultant separation of blood after centrifugation. PBMC are enriched in the interphase between Ficoll layer and plasma. The red blood cells and granulocytes are pelleted. Photos are adapted from (Gomes et al, 2015).

2.9 *Ex vivo* Therapy of Malignant Peritoneal and Pleural Clinical Biopsy Sample

2.9.1 General statement

Primary human malignant ascites and pleural effusion samples were received from the Churchill Hospital, Oxford University Hospitals (Oxford, UK) following informed consent from patients with multiple indications of advanced carcinoma, including but not limited to ovarian, pancreatic, breast and lung. This work was approved by the research

ethics committee of the Oxford Centre for Histopathology Research (Reference 09/H0606/5+5).

2.9.2 Processing patient-derived peritoneal ascites and pleural effusions

Upon receipt, cellular and fluid fractions were separated by two rounds of centrifugation at 350 g for 15 minutes at room temperature. Fluid was used immediately, or aliquots stored at -20°C for future analysis. The cellular fraction was treated with red blood cell lysis buffer (Roche, UK), and the remaining cells were counted and viability was determined by trypan blue stain. Cells were used immediately, or aliquots frozen in cryopreservative and stored as described previously.

In some cases, adherent cells from peritoneal isolates were expanded and purified by serial passaging of ascites cells upon receipt. Initially, unpurified ascites cells were seeded in T25 culture flasks, in equal parts of fluid from the same exudate sample and RPMI media supplemented with 10% FBS. At confluency, culture flasks were washed three times with PBS to discard dead or non-adherent cells. Adherent cells were re-suspended by treatment with 0.05% trypsin and transferred to two T25 culture flasks (one in two split) and cultured in RPMI media (10% FBS). This process was repeated once more, with the adherent primary cells transferred to a T75 culture flask. Cells were allowed to grow to confluency, before use for further experimentation.

2.9.3 Screening process to determine the cellular composition of biopsy samples

Upon receipt, and prior to experimentation or cryopreservation, each sample was screened for the cellular composition. The cell types present in each sample were

determined by cell-surface antibody staining for EpCAM, EGFR, FAP, CD45, CD11b, CD56, CD3, CD4, CD8, PD1, TIM3, LAG3 and CTLA4 (Table 2.2). Samples were analysed on a FACSCalibur flow cytometer (BD Biosciences, USA), as described previously.

2.9.4 Characterisation of protein and cytokine levels in exudate fluids

The concentration of total protein in patient-derived peritoneal or pleural fluid samples was measured to assess fluids as either transudate (< 30 g/L) or exudate (> 30 g/L). Frozen samples were thawed in a water bath at 37°C and applied to a 70 µm cell strainer to remove coagulated masses. Samples were diluted 500-fold in PBS, and the protein concentration was determined using BCA protein assay kit (Thermo Fisher, UK), as described previously.

The levels of IL-10, TGFβ, IFNγ and IL-2 in the patient fluids was measured by ELISA. Frozen samples were thawed at 37°C in a water bath and passed through a 70 µm cell strainer to remove clotted material. For IL-2, IFNγ and IL-2, samples were diluted two-fold in assay diluent and ELISAs were performed, according to manufacturer's instructions (IL-10, Human IL-10 ELISA MAX kit; IFNγ, Human IFNγ ELISA MAX kit; IL-2, Human IL-2 ELISA MAX kit; Biolegend, UK). For measuring TGFβ, fluid was diluted five-fold in PBS. Samples were treated with 0.2 volumes of 1 N HCL TGFβ for 10 minutes at room temperature, which activates the latent (precursor) form of TGFβ, followed by neutralisation with 0.2 volumes of 1 N NaOH. An ELISA to measure active TGFβ was performed following manufacturer's instructions (TGF beta-1 Human/Mouse ELISA Kit, Thermo Fisher, UK).

2.9.5 Characterisation of cytokines produced from expanded malignant ascites cells

The levels of various cytokines and growth factors produced by primary adherent cells isolated from peritoneal ascites samples were measured. Primary adherent cells were cultured until confluency in T75 flasks. The culture media was replaced with 15 mL RPMI supplemented with 2% FBS, and flasks incubated at 37°C in humidified chambers for two days. The media was harvested, centrifuged at 500 g for 10 minutes to remove cellular material, aliquoted and frozen for future analysis.

The levels of IL-10, IL-6, TGF β and CXCL12 were measured by ELISA, according to manufacturer's instructions (IL-10, Human IL-10 ELISA MAX kit; IL-6, Human IL-6 ELISA MAX kit, Biolegend UK; TGF β , TGF beta-1 Human/Mouse ELISA Kit, Thermo Fisher, UK; CXCL12, Human CXCL12/SDF-1 α ELISA Kit, R&D Systems, USA).

2.10 *In Vitro* or *Ex Vivo* Co-Culture Experiments

2.10.1 Experimental conditions

This section describes all *in vitro* co-culture experiments with PBMC and cell lines, or those established using cells isolated *ex vivo* from liquid tumour biopsies. All co-culture experiments were performed as follows, unless stated otherwise. PBMC- or patient-derived T cells were seeded with the appropriate target cell (effector:target ratio of 5) in flat bottom 96-well plates in a total of 100 μ L culture medium. Target cells were prepared with cell-dissociation buffer to preserve cell surface antigen structure, and the use of trypsin was avoided. Alternatively, unpurified total cells from ascites or pleural effusions

were seeded in equal parts of fluid from the same exudate sample and RPMI supplemented with 10% FBS (final concentration exudate of 50%).

To assess T cell activation by supernatants from BiTE-transfected (300 ng/mL) or virus-infected cells, co-cultures were treated with 100 μ L of the supernatants. For virus-mediated T cell assays, cells were infected with 100 vp/cell of virus in 100 μ L medium. Where appropriate, CD3/CD28 Dynabeads (Thermo Fisher, UK) or soluble phorbol myristate acetate (PMA)/ionomycin cell activation cocktail (Biolegend, UK) were included as positive controls for T cell activation, according to manufacturer's instructions. Plates were incubated at 37°C in humidified chambers at 5% CO₂, and non-adherent cells were harvested at the specified time point by pooling the culture media, followed by a successive PBS wash. If adherent cells were also required, cell-dissociation buffer was added to the well, and the plates were incubated at 37°C until the cells were visibly detached from the well surface. The wells were further washed with PBS, which was collected and pooled with the non-adherent cells.

2.11 Characterisation of Human T Cell Activation *In Vitro* or *Ex Vivo*

This section includes all *in vitro* co-culture experiments with PBMC and cell lines, or those established using cells isolated *ex vivo* from liquid tumour biopsies.

2.11.1 Activation markers

The expression of surface markers on T cells can be measured by flow cytometry, with T cells showing increased surface expression of CD69 and CD25 when activated. For T cell activation marker assays, co-cultures were established as described above and incubated for 24 hours, unless stated otherwise. Cells were stained for surface expression of CD69, CD25, CD4 and CD8 (Table 2.2) and analysed by flow cytometry, as described previously.

2.11.2 T cell proliferation

The proliferation of T cells can be assessed by measuring the dilution (by 2-fold) of a fluorescent CFSE dye, which upon each successive division of the cells manifests as peaks in the intensity of fluorescence. PBMC-derived T cells were labelled with 5 μ M CFSE dye (CellTrace CFSE kit, Invitrogen, USA), according to manufacturer's instructions. CFSE-stained T cells were co-cultured with target cells, as described above, and incubated for five days. Non-adherent cells were harvested, stained for surface expression of CD4 and CD8 (Table 2.2) and analysed by flow cytometry. The pattern of CFSE fluorescence can be used to model and quantify the proliferation of the T cell population using the proliferation function of FlowJo v7.6.5 software. Data is presented as the percentage of original cells that entered a proliferation cycle (% divided) or the average number of cell divisions that a cell in the original population has undergone (Division Index). If appropriate, absolute numbers of T cells per well can be measured by adding 5000 Precision counting beads (Biolegend, UK) to each sample prior to analysis.

2.11.3 Degranulation of T cells

The frequency of degranulating T cells can be quantitated by the expression of surface CD107a. PBMC-derived T cells and target cells were cultured and stimulated in the presence of an antibody specific for CD107a (added directly to the well; Table 2.2). In parallel, a well for each experimental condition was incubated with an isotype control antibody to measure non-specific binding or uptake. Cells were incubated for 1 hour at 37°C in humidified chambers, after which GolgiStop (containing monensin; BD Biosciences, USA) was added at a final concentration of 6 µg/mL. Monensin prevents the acidification of endocytic vesicles and inhibits the degradation of reinternalised CD107a from the surface. After an additional five hours of incubation, non-adherent cells were harvested, stained for surface expression of CD4 and CD8 (Table 2.2) and fluorescence analysed by flow cytometry.

2.11.4 IFN γ expression by flow cytometry

To assess the production of IFN γ by T cells, co-cultures were established as described above, and incubated at 37°C for six hours. After the first hour of incubation, GolgiPlug (containing Brefeldin A; BD Biosciences, USA) was added to the wells at a final concentration of 10 µg/mL. Brefeldin A prevents the exocytosis of cytokine-containing vesicles, ensuring IFN γ -positive cells are detected. Non-adherent cells were harvested and stained for surface expression of CD4 or CD8 and intracellular expression of IFN γ (Table 2.2). The frequency of IFN γ -positive cells was then quantitated by flow cytometry analysis.

2.11.5 Cytokine secretion by ELISA

The levels of IL-2 and IFN γ cytokines in harvested supernatants was measured by ELISA. CD3-purified PBMC and target cells were co-cultured as described above. At the stated time point, the culture supernatant was harvested and centrifuged at 350 g for 5 minutes to remove cells or cellular debris. The quantity of IL-2 and IFN γ within these supernatants was measured using the Human IL-2 ELISA MAX kit (Biolegend, UK) or Human IFN γ ELISA MAX kit (Biolegend, UK).

2.11.6 Cytokine multiplex array

Multiplex cytokine bead array allows the quantitation of multiple cytokines in one sample. Culture supernatants from 48-hour co-cultures were harvested, and cellular material removed by centrifugation at 350 g for 5 minutes. A flow cytometric multiplex bead immunoassay was performed on these supernatants using LEGENDplex Th Kit (Biolegend, UK), according to the manufacturer's instructions. This kit allows the quantification of IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, IFN γ and TNF α . This assay uses multiple populations of beads, with each population conjugated to capture antibodies specific for one of the cytokines. Each bead within a population has a defined level of fluorescence, and therefore each bead has a distinct fluorescence profile than can be resolved on a flow cytometer. After incubating supernatants with the beads, biotinylated detection antibodies were added, followed by streptavidin conjugated to PE. The level of fluorescence from the PE fluorophore is measured on a flow cytometer and is compared to standard curves made with known quantities of cytokines, allowing the quantity of the cytokines in the supernatant to be determined.

2.12 Evaluation of Target Cell Cytotoxicity Assay *In Vitro* or *Ex Vivo*

This section includes all *in vitro* co-culture experiments with PBMC and cell lines, or those established using cells isolated *ex vivo* from liquid tumour biopsies.

2.12.1 LDH release cytotoxicity assay

The release of lactate dehydrogenase (LDH) from the cytosol of a cell into the supernatant can be used as a quantitative measure of cell lysis. A LDH release cytotoxicity assay was performed using CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega, USA). The reagent contains a tetrazolium salt, which is converted to a red formazan product in a coupled enzymatic reaction when LDH catalyses the conversion of lactate to pyruvate. In brief, and unless stated otherwise, 1.5×10^4 target cells were seeded with 7.5×10^4 T cells (E:T ratio of 5) and incubated with BiTE-containing supernatants or virus. Culture medium, effector spontaneous, target spontaneous and target maximum controls were included (described in the manufacturer's protocol). After the specified time, 50 μ L of supernatant was transferred to a new flat-bottom 96-well plate, and 50 μ L of assay reagent added to the well. Plates were incubated at room temperature in the dark for 30 minutes, followed by addition of 50 μ L stop solution and absorbance at 490 nm was measured using a Wallac 1420 Victor² plate reader (Perkin Elmer, USA). The cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} * 100$$

2.12.2 MTS

Cell viability was determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), according to manufacturer's instructions. The reagent contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling agent (phenazine ethosulphate; PES). The MTS tetrazolium compound is bio-reduced in viable cells into a coloured formazan product, presumably by NADPH or NADH produced by dehydrogenase enzymes within the metabolically active cells. In brief, and unless stated otherwise, 1.5×10^4 target cells were seeded with 7.5×10^4 T cells (E:T ratio of 5) and incubated with BiTE-containing supernatants or virus. After the specific time, the total supernatant was removed, and 80 μ L PBS and 20 μ L reagent were added per well. Plates were incubated at 37°C in a humidified chamber for 1–4 hours, and absorbance at 490 nm was measured using a Wallac 1420 Victor² plate reader (Perkin Elmer, USA).

2.12.3 xCELLigence

xCELLigence RTCA technology (ACEA Biosciences) allows the viability of an adhered cells to be monitored in real-time. The system uses specialised E-plates (ACEA Biosciences) with electrodes embedded at the base of the well. The magnitude of the impedance (or cell index) is proportional to the number of cells adhering to the well surface, or the quality of this adherence, allowing the cytotoxicity of the cell monolayer to be observed. The cell viability assay was performed according to manufacturer's instructions. In brief, 10^4 target cells were seeded and incubated at 37°C in a humidified chamber for 18 hours. Cells were infected with virus (100 vp/cell) or treated with BiTE-containing supernatants (300 ng/mL), and incubated for a further two hours, before CD3-

purified PBMC (E:T = 5) were added. Cell index values were monitored every 15 minutes for 120 hours.

2.12.4 Flow cytometry

The number of residual target cells, as measured by flow cytometry, was used as a quantitative readout for cytotoxicity of specific target cells within mixed cell populations, including unpurified ascites or pleural effusion cells. Cultures were established as described previously. After five days incubation, total cells were harvested, stained for surface expression of CD4, CD8, CD25 and EpCAM or FAP (Table 2.2) and analysed by flow cytometry, as described previously.

2.13 *Ex vivo* Therapy of Resected Solid Tumour Samples

This section includes all *ex vivo studies* established using tissue isolated from solid tumour biopsies.

2.13.1 General statement

Human malignant and benign prostate tissue was received from the John Radcliffe Hospital, Oxford University Hospitals (Oxford, UK) with informed consent from patients undergoing radical prostatectomy surgery. This work was approved by the research ethics committee of the Oxford Centre for Histopathology Research (Reference 09/H0606/5+5).

2.13.2 Prostate tissue slicing and culture

The specimens for experiments were transported to the lab from surgery in RPMI medium supplemented with 10% FBS, where they were stored on ice until slicing (within two hours of surgery). Tumour tissue was embedded in UltraPure low melting point agarose (1% w/v, Thermo Fisher, UK), and mounted to the specimen plate with superglue. Tissue slices of 300 μm were prepared under semi-sterile conditions using a vibratome (Leica VT 1200S, Leica Microsystems, Germany). Each slice was transferred to a 0.6 cm^2 PTFE cell culture insert (EMD Millipore, USA) in 24-well plates containing 1 mL of custom designed cultivation media for prostate tissue (see Materials for recipe). A schematic demonstrating this process is shown in Figure 2.8. Slices were incubated overnight at 37°C in humidified chambers. The next day, the prostate media was replaced, and slices treated with BiTE-containing supernatants or recombinant virus. On day one, 30% of the culture supernatant was collected, frozen and replaced with fresh prostate media, followed by a further two days of culturing. On day four, 30% of the supernatant was harvested, frozen and replaced. On day seven, the total supernatant was harvested and frozen for future analysis. Additionally, slices were fixed in paraformaldehyde (4%) and embedded in paraffin for immunohistochemical analysis.

2.13.3 Characterisation of T cell activation

The levels of IL-2 and IFN γ cytokines in the supernatant harvested from slice culture was measured by ELISA. Tissue slices were cultured and treated as described above. The quantity of IL-2 and IFN γ within slice culture supernatants was determined using the Human IL-2 ELISA MAX kit (Biolegend, UK) or Human IFN γ ELISA MAX kit (Biolegend, UK), according to manufacturer's instructions.

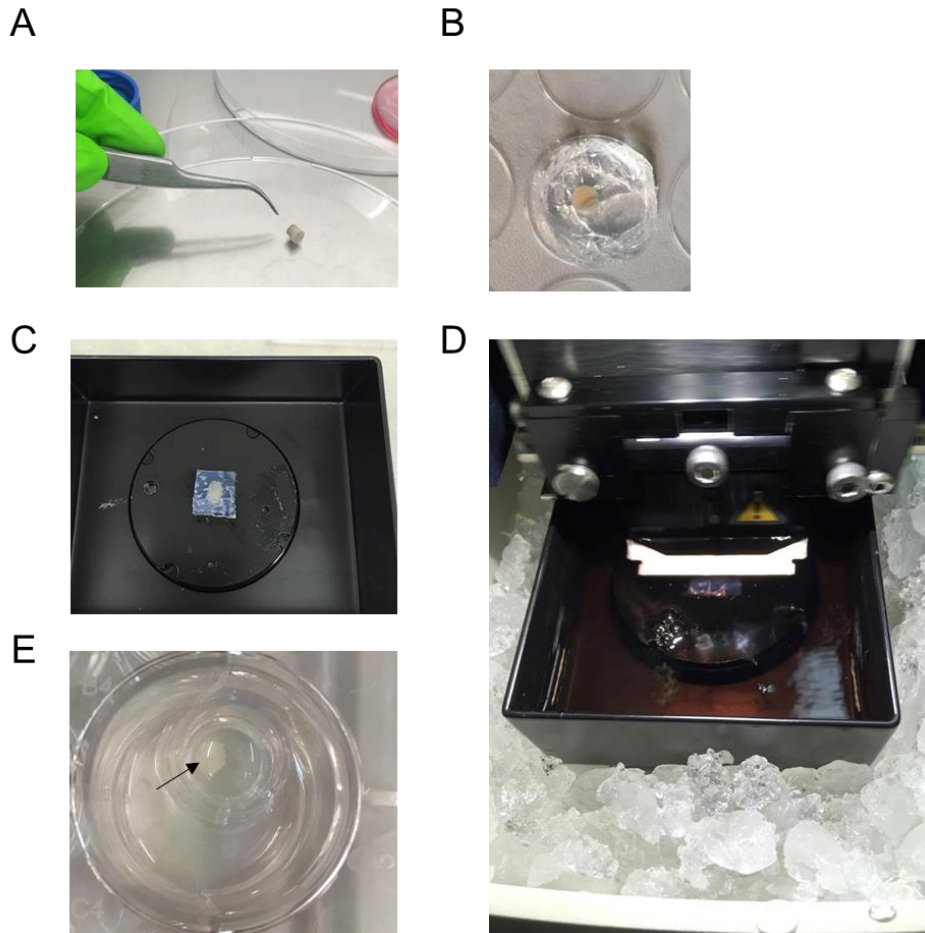


Figure 2.8. Photographic images of the tissue slice acquisition and culture. Cores were obtained from radical prostatectomy specimens (A) and embedded in low melting point agarose (B). A scalpel was used to cut away excess agarose and the block was mounted to the base of the specimen plate using superglue (C). Tissue slices of 300 μm were prepared using a vibratome (D) and each slice (indicated with an arrow) was transferred to a 0.6 cm^2 PTFE cell culture insert in 24 well plates containing 1 mL prostate medium for subsequent culturing and treatment (E).

Automated staining was carried out with the Leica BOND-MAX autostainer (Leica, Microsystems) in collaboration with Leticia Campo. Antigen retrieval was performed at 100°C using either Epitope Retrieval Solution 2 (AR9640, Leica Biosystems, Germany) and primary antibody staining performed as stated in Table 2.3. Detection was performed using the BOND™ Polymer Refine Detection System (DS9800, Leica Biosystems) as per manufacturer's instructions. Alternatively, for the FAP primary antibody, detection was performed using Anti-Sheep HRP-DAB Cell & Tissue Staining Kit (R&D Systems,

CTS019) as per manufacturer's instructions. Sections were incubated with haematoxylin for visualisation of nuclei. Slides were imaged on an Aperio CS2 slice scanner (Leica Microsystems).

Table 2.3. An overview of the antibodies used for immunohistochemistry.

Ab	Company/	Ag Retrieval	Ab dilution	Primary Ab incubation
CD25	Atlas Antibodies, HPA054622	20mins ER2	1/500	30mins
EpCAM	Biolegend, 118201	20mins ER2	1/100	30mins
FAP	R&D Systems, AF3715	20mins ER2	1/200	30mins
CD8	Dako, M7103	20mins ER2	1/100	30mins

2.13.4 Evaluation of BiTE-mediated cytotoxicity

The cytotoxicity of the slice could be visualised and assessed in live culture using CellEvent Caspase 3/7 detection reagent (Thermo Fisher, UK), and captured using a Zeiss Axiovert 25 microscope. The release of LDH into the supernatant was used to assess cytotoxicity of the tissue slice. The quantity of LDH in harvested supernatants was measured using CytoTox96 Non-Radioactive Cytotoxicity Assay Kit (Promega, USA), as described above.

2.14 Statistics

Where experiments produced two datasets, significance was evaluated using a student's two-tailed t-test. In all cases of more than two experimental conditions being compared, statistical analysis was performed using a One-way ANOVA test with Tukey's *post hoc* analysis or Two-way ANOVA test using Bonferroni *post hoc* analysis. All data is presented as mean $\pm$ SD. The significant levels used were $p = 0.01 - 0.05$ (*), $0.001 - 0.01$ (**), $0.0001 - 0.001$ (***). All *in vitro* experiments were performed in triplicate, unless stated otherwise.

3. Engineering, production and characterisation of EpCAM BiTE-armed oncolytic adenoviruses

3.1 Introduction

Oncolytic viruses (OVs) can be ‘armed’ to express therapeutic transgenes, and secrete them from infected tumour cells (Seymour & Fisher, 2016). Arming oncolytic viruses to express BiTEs combines two quite distinct therapeutic mechanisms, with the former providing lytic death of tumour cells that are permissive for virus infection, and the latter targeting T cell cytotoxicity via a specific chosen antigen, independent of MHC/TCR engagement. This provides considerable flexibility in the design of a therapeutic approach, perhaps using the BiTEs to deliver cytotoxicity to tumour-associated cells that are uninfected or those that are relatively resistant to kill by the virus directly. In this chapter, we assessed whether EnAd could be engineered to express and secrete a BiTE from infected cancer cells that would activate T cells to bind and kill epithelial cell adhesion molecule (EpCAM)-positive tumour cells.

EpCAM is a pan-carcinoma marker, highly expressed on cancer cells and recently identified as a marker of cancer-initiating stem-like cells (Terris et al, 2010). As an activator of the wnt pathway, EpCAM has been implicated in direct tumour progression. Overexpression in breast and gall bladder cancer correlates with decreased survival (Gastl et al, 2000), and evidence indicates EpCAM signalling up-regulates myc, cyclins A/E,

and NFκB (Munz et al, 2004). Its differential expression on healthy and malignant tissue potentially provides a therapeutic window for treatment of a wide variety of EpCAM⁺ epithelial cancers (Went et al, 2004) .

The Amgen-developed EpCAM-targeted BiTE, Solitomab, has demonstrated potent anti-tumour activity *in vitro* and in diverse and demanding xenograft models (Brischwein et al, 2006). BiTE-mediated CD3 engagement induced polyclonal activation and proliferation of primary human T cells, which were directed to serially lyse EpCAM⁺ cells with extreme potency; half-maximal target cell lysis was observed at picomolar quantities (Brischwein et al, 2006). The NOD/SCID mouse model used metastatic solid ovarian tissue presenting the full histological heterogeneity found in late-stage human cancer, likely including many T cell escape mechanisms. Notably, all treated mice presented a substantial reduction in tumour burden with no relapse observed, with T cell response relying solely on the tumour-resident human T cells present in the tissue. Whilst showing remarkable potency and specificity pre-clinically, Solitomab demonstrated poor efficacy in phase 1 clinical trials, due to poor pharmacokinetics and reported on-target off-tumour systemic toxicities (Fiedler et al, 2012).

Delivery via EnAd could overcome these challenges, and Solitomab expression is anticipated to augment the anti-cancer activity of the virus. To test this core hypothesis, it was first necessary to engineer expression plasmids. This expression system was designed to produce free BiTE protein, which could be used to determine if the EpCAM-targeted BiTE was functionally active. Of equal importance, the BiTE protein enabled the establishment of suitable *in vitro* experimental systems to measure the activation of primary human T cells - via activation markers, proliferation, cytokine production and

degranulation – and the cytotoxicity of EpCAM⁺ target cells, using release of lactate dehydrogenase and an electrical impedance-based system. The antigen-specificity of BiTE activity and the responding T cell subsets were also assessed.

BiTE-armed EnAds were prepared by molecular cloning to produce the modified EnAd genomes, followed by virus rescue, and large-scale production and purification by caesium chloride banding. We explored the use of different promoters to regulate the expression of the BiTE – including a cytomegalovirus (CMV) immediate early promoter and the adenovirus major late promoter (MLP). The coupling of BiTE expression to MLP ensures that gene expression is activated only in cells that are permissive to virus replication (Carette et al, 2005). Finally, ability of the armed viruses to activate primary T cells and mediate EpCAM⁺ cell cytotoxicity was assessed to determine if arming with the BiTE improved OV efficacy and enabled the targeting of EnAd-resistant cell lines.

3.2 Chapter aims

1. Engineer plasmid DNA vectors for expression of an EpCAM-targeted BiTE.
2. Measure the activity of the BiTEs in activating primary human T cells and inducing lysis of EpCAM⁺ target cells.
3. Produce modified EnAd viruses encoded with the EpCAM BiTE.
4. Assess the ability of EpCAM BiTE-armed EnAd to induce T cell activation and direct T cell cytotoxicity to EnAd-resistant EpCAM⁺ cell lines.

3.3 Results

3.3.1 Generation and production of a BiTE targeting EpCAM

A BiTE targeting EpCAM was engineered by joining two single-chain antibody fragments (scFvs) specific for CD3 ϵ and EpCAM with a flexible glycine-serine (GS) linker. BiTE-mediated T cell activation relies on this dual specificity to ensure T cells are only activated upon CD3 cross-linking by clustered BiTE (by EpCAM on the target cell surface or artificial surfaces), not soluble BiTE alone. A control BiTE, recognising CD3 ϵ and an irrelevant antigen, filamentous haemagglutinin adhesin (FHA) of *Bordetella pertussis*, was also produced. FHA is unlikely to be present in the experimental models used and acts as a surrogate for unclustered BiTE. Both BiTEs were engineered to contain an N-terminal signal sequence for mammalian secretion and a C-terminal decahistidine affinity tag for detection and purification (Figure 3.1A). To characterise the functionality of the recombinant BiTEs, the EpCAM BiTE and control BiTE DNA sequences were synthesised from obtained proteins sequences and cloned into expression vectors under transcriptional control of the cytomegalovirus (CMV) immediate early promoter, yielding pSF-CMV-EpCAMBiTE and pSF-CMV-controlBiTE, respectively (Figure 3.1B).

Adherent HEK293 cells (HEK293A) were transfected with the pSF-CMV-EpCAMBiTE and pSF-CMV-controlBiTE expression vectors using Lipofectamine 2000. In parallel, cells were transfected with pSF-CMV-GFP to confirm adequate transfection efficiency (Figure 3.2A) whilst also providing a mock-transfected control for subsequent analysis.

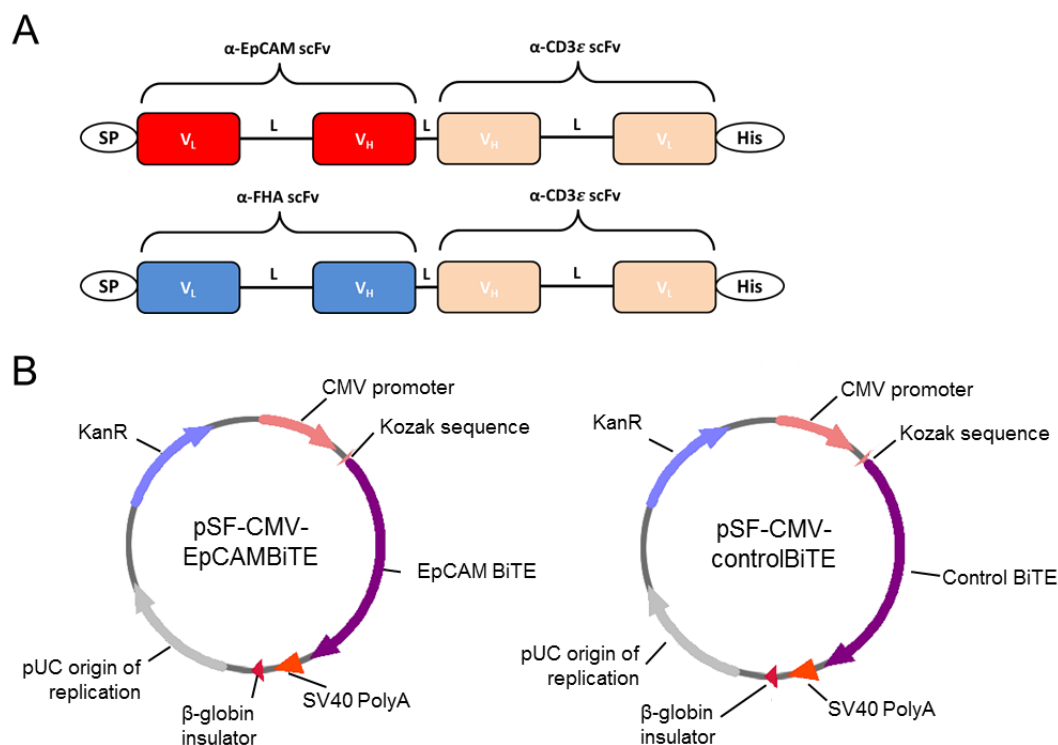


Figure 3.1. Generation of EpCAM and control BiTE vectors for mammalian expression. (A) Schematic representation of the structure of the two BiTEs used in this chapter. Each BiTE is composed of two single chain antibody fragments (scFvs) joined by a flexible glycine-serine linker. The EpCAM-targeted and irrelevant (filamentous haemagglutinin adhesion, FHA-targeted) control BiTE share a second specificity for the CD3ε chain of the T cell receptor complex. Each BiTE possesses an N-terminal immunoglobulin signal peptide and C-terminal decahistidine affinity tag for secretion and detection, respectively. SP, signal peptide; VL, variable light chain; VH, variable heavy chain; L, flexible linker; His, decahistidine affinity tag. **(B)** Schematic of expression plasmids for mammalian expression of the EpCAM-targeted BiTE and control BiTE under the control of a CMV promoter. KanR, Kanamycin resistance cassette; CMV, Cytomegalovirus; SV40, Simian vacuolating virus 40.

At 48 hours, supernatants and cell lysates were harvested for further analysis by SDS-PAGE and western blotting using an anti-His antibody to detect the BiTEs. For each transgene, equivalent amounts of cell lysate and supernatant were loaded to assess the distribution of the recombinant BiTEs in the lysate and supernatant. The EpCAM-targeted and control BiTEs were detected mostly in the supernatants of plasmid-transfected cells, demonstrating successful expression and secretion (Figure 3.2B). The expression and secretion of the EpCAM-targeted BiTE appeared to be higher than that of the control BiTE, likely reflecting differences in the amino acid sequence itself or the codon

optimisation of each protein. To confirm that these bands, which had an apparent mobility of 65 kDa, were the EpCAM and control BiTE transgenes (a theoretical size of 55 kDa), the protein bands were excised from a polyacrylamide gel, digested with trypsin and analysed by mass spectrometry (Figure 3.3). Each band was confirmed to contain peptide sequences identical to those of the corresponding BiTE sequence.

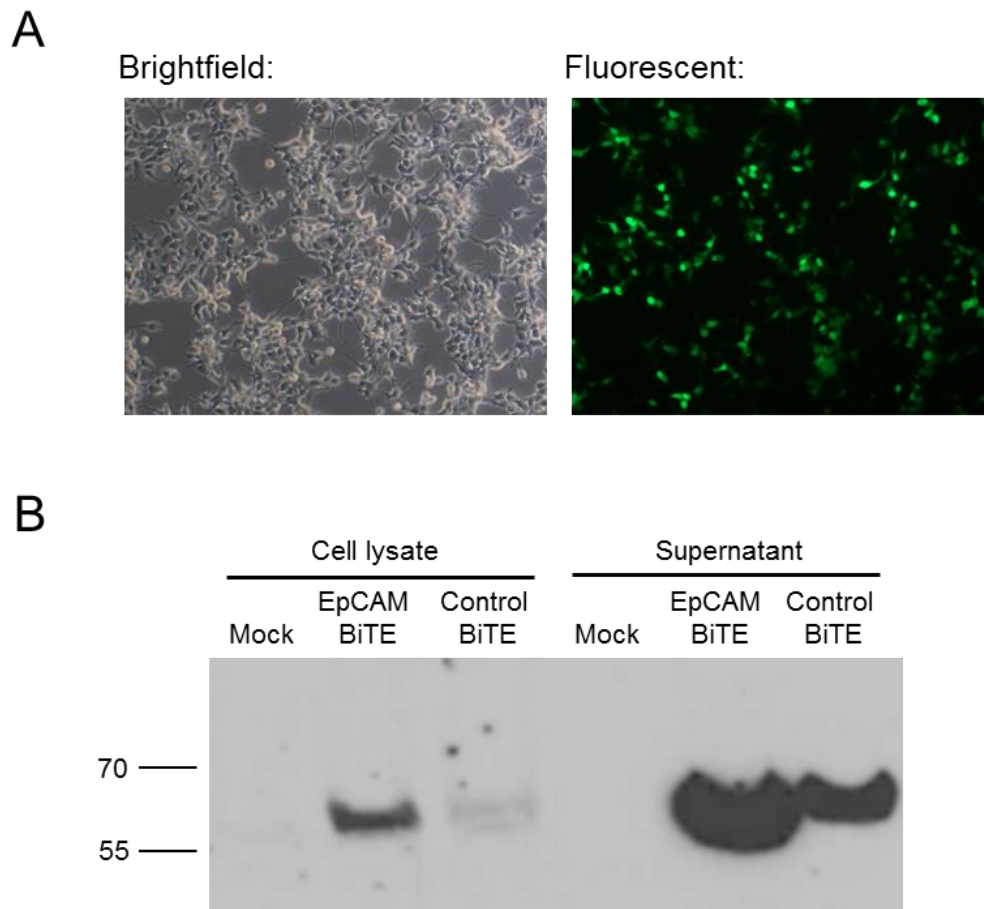
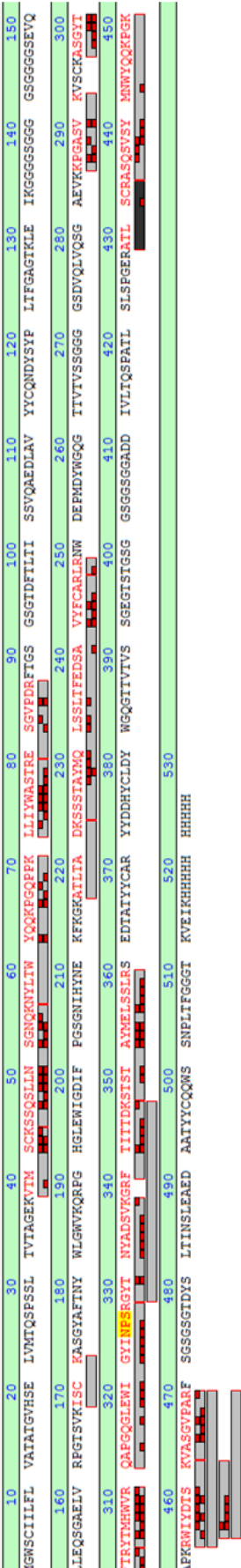


Figure 3.2. Expression and secretion of EpCAM and control BiTE from HEK293A cells. (A) GFP expression of pSF-CMV-GFP plasmid-transfected HEK293A cells were used to ensure adequate transfection efficiency. (B) HEK293A cells were transfected with pSF-CMV-EpCAMBiTE and pSF-CMV-controlBiTE plasmid DNA. After 24 hours, cell lysates and supernatants were harvested and assessed for BiTE expression by immunoblot analysis using an anti-his (C-term) antibody. pSF-CMV-GFP-transfected cells were used as a negative control for BiTE expression ('mock').

EpCAM BiTE:



Control BiTE:

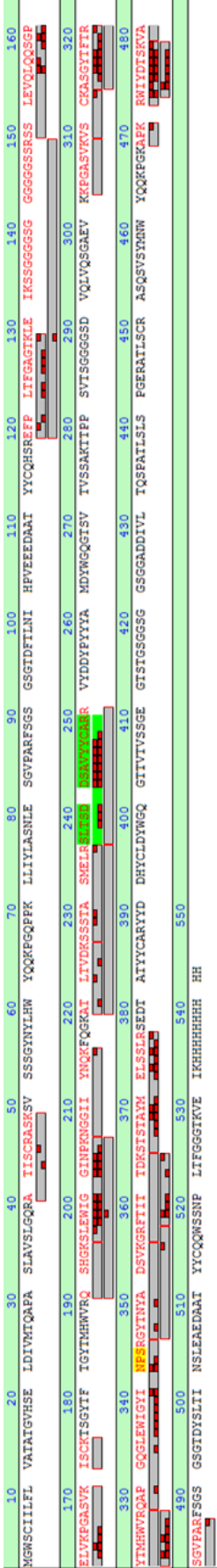


Figure 3.3. Mass spectrometry (MS) analysis of proteins of apparent mobility of 65 kDa. Supernatants from (i) pSF-CMV-EpCAMBiTE or (ii) pSF-CMV-controlBiTE-transfected HEK293A cells were separated by SDS-PAGE. Bands at 65 kDa were excised. In-band proteins were digested by trypsin and analysed by mass spectrometry. Grey boxes, sequence coverage MS; Red boxes, sequence coverage MS/MS (b and y ions). Yellow highlight; N-glycosylation consensus sequence.

With successful transgene expression and secretion confirmed, BiTE-containing supernatants were produced in large-scale preparations for further analysis and characterisation. HEK293A cells in T175 flasks were transfected with BiTE expression plasmids using *polyethylenimine* (PEI, linear, MW 25000, Polysciences, USA; see Materials & Methods). At 48 hours post-transfection, supernatants were harvested and concentrated 50-fold before storage in aliquots at -80°C. To estimate the amount of BiTE produced, samples were serially diluted and evaluated using a dot blot with an anti-His antibody (Figure 3.4A). Decahistidine-tagged cathepsin D was used as a standard. From 1.8×10^7 HEK293A cells, we estimated that approximately 20 µg/mL of recombinant BiTEs were secreted into the supernatant 48 hours post-transfection. Specific binding of the EpCAM BiTE and not the control BiTE to recombinant EpCAM protein was demonstrated by ELISA, similarly exploiting the His-tag for detection (Figure 3.4B).

3.3.2 Characterisation of human primary T cell activation by recombinant EpCAM BiTE

The ability of recombinant EpCAM BiTE to activate PBMC-derived T cells was evaluated by adding unstimulated human CD3-purified PBMC cells to a culture of human DLD colorectal carcinoma cells (effector:target, E:T ratio of 5:1), which are known to express EpCAM on their surface (confirmed by flow cytometry; Figure 3.5A). Addition

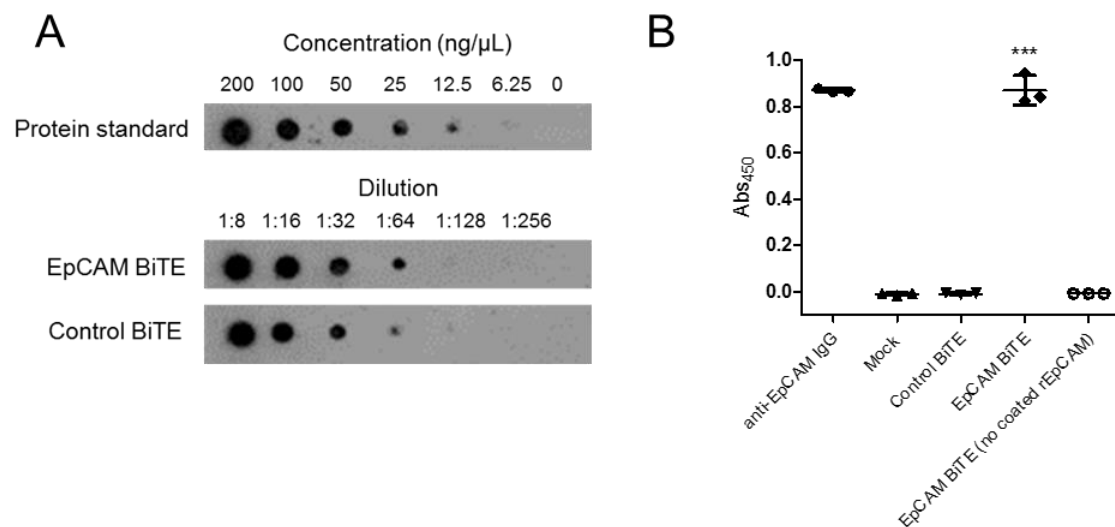


Figure 3.4. Quantification and functional validation of recombinant EpCAM and control BiTE. **(A)** Dot blot to estimate the quantity of EpCAM BiTE produced by transfected HEK293A cells using C-terminal decahistidine-tagged cathepsin D as a protein standard. **(B)** ELISA measuring the level of binding to recombinant EpCAM protein by EpCAM-targeted or control BiTE. Binding by the BiTE was determined by probing with a mouse anti-histidine (C-term) detection antibody, followed by an HRP-conjugated anti-mouse secondary antibody. Each condition was measured in technical triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'mock', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of 2.5 $\mu\text{g/ml}$ EpCAM BiTE, as supernatant from transduced HEK293A cells, led to a significant increase in T cell activation markers CD69 and CD25 at 24 hours (Figure 3.5B and C). T cells cultured with DLD cells alone or with control BiTE did not have elevated levels of CD69 nor CD25. Exposure of T cells to the EpCAM BiTE in the absence of tumour cells resulted in a very modest increase in CD69 and CD25, indicating that antibody-mediated clustering of CD3 is essential for full activation by this anti-CD3 binding. As demonstrated in Figure 3.5C, CD69 and CD25 follow the same trend upon T cell activation. For subsequent experiments, both markers will be used to assess activation where possible, however in some instances, one will be used. Consistent with the induction of activation markers, only primary T cells in co-culture with both DLD and EpCAM BiTE underwent phosphorylation of ERK1/2, involved in the Ras signalling

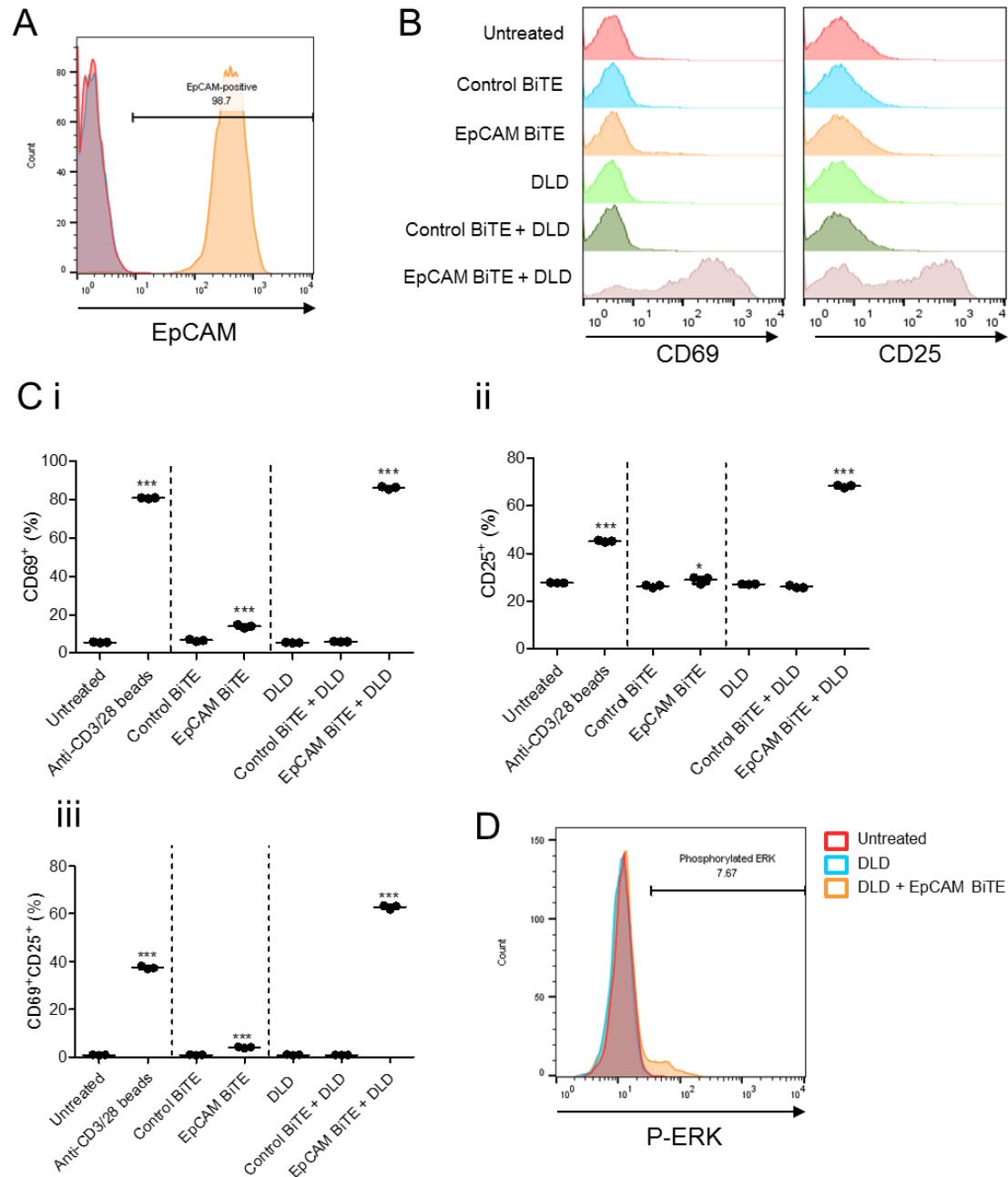


Figure 3.5. EpCAM BiTE induces activation of PBMC-derived T cell. (A) Level of EpCAM expressed on the surface of DLD carcinoma cells was measured by flow cytometry (orange). Unstained (red) and cells stained with an isotype control antibody (blue) were used to determine background fluorescence and unspecific binding, respectively. (B) BiTE-mediated activation of PBMC-derived T cells in co-culture with DLD (5:1) and BiTE-containing supernatants. Activation was assessed by expression of activation markers CD69 and CD25 and measured by flow cytometry at 24 hours. (C) A graphical representation of the data from (B). T cells were assessed for activation based on positivity for (i) CD69 alone, (ii) CD25 alone or (iii) dual positivity. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Phosphorylation of ERK1/2 in primary human T cells in co-culture with DLD alone or with BiTE-containing supernatants. Phosphorylation status was determined by intracellular staining with PhosFlow Anti-ERK1/2 (pT202/pY204) antibody and measured by flow cytometry

cascade activated by TCR engagement. Incubation with DLD alone had no effect (Figure 3.5D).

Stimulation with the EpCAM BiTE in the presence of tumour cells also induced a significant increase in T cell proliferation (Figure 3.6A). Proliferation was determined by measuring the dilution (by two) of the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) upon each successive division, manifesting in flow cytometry as peaks with differing fluorescence intensity. Proliferation of the T cell population was modelled and quantified using the pattern of fluorescence. This was then used to determine the division index and percentage of the parental population entering proliferation (Figure 3.6B). CFSE fluorescence in T cells in culture with the control BiTE did not decrease, indicating a lack of proliferation. This data is supported by an increase in absolute T cell numbers, as measured using precision counting beads, in co-cultures with DLD and EpCAM BiTE-containing supernatant (Figure 3.6C).

The release of IFN γ and IL-2 are associated with activation of CD4 Th1 and CD8 cytotoxic T cells. We characterised the expression of each cytokine by ELISA using supernatants harvested following EpCAM BiTE-mediated activation of T cells in the presence of DLD cells (Figure 3.7A). As before, the control BiTE had little effect, whilst the EpCAM BiTE induced the release of high quantities of IFN γ and IL-2 within 24 hours. Interestingly, whilst BiTE-mediated activation induced 9-fold higher levels of IFN γ than T cells stimulated with CD3/CD28 beads, the levels of IL-2 were reduced 10-fold. The reason for this is unclear but may be a consequence of stronger CD3 stimulation by the EpCAM BiTE or lack of additional CD28 co-stimulation. *De novo* expression of IFN γ by T cells was assessed by intracellular staining and flow cytometry after 6 hours co-culture

with DLD and BiTE-containing supernatants, confirming the T cells to be the source of IFN γ (Figure 3.7B). For a more comprehensive analysis, supernatants at 48 hours post-infection were assessed using a multiplex cytokine bead array. The bead array allowed

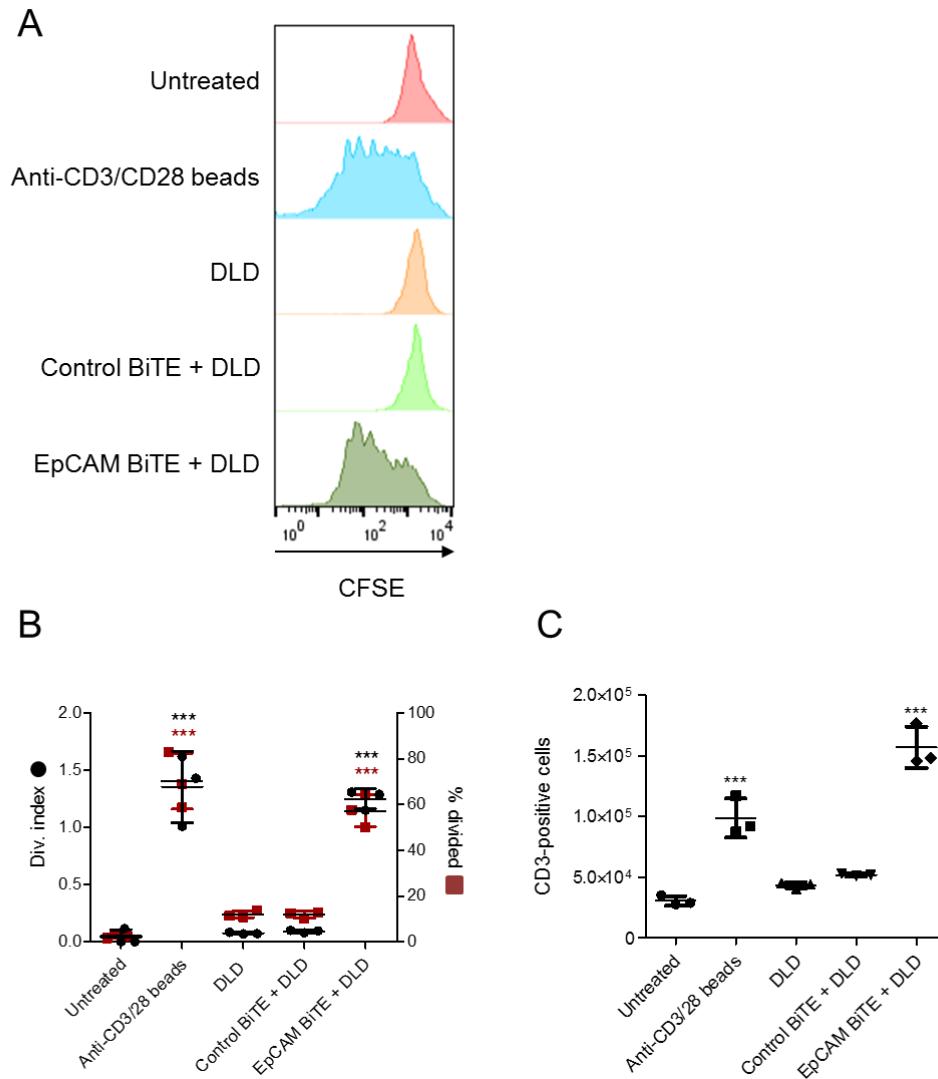


Figure 3.6. EpCAM BiTE induces proliferation of PBMC-derived T cells. (A) Flow cytometry plots showing proliferation of CFSE-stained T cells in co-culture with DLD cells (5:1) and BiTE-containing supernatants. Fluorescence was measured by flow cytometry five days after co-culture. (B) The proliferation in (A) can be modelled using pattern of fluorescence (and the FlowJo proliferation tool) and is represented as division index and percentage of parental population that entered proliferation ('% divided'). (C) The total cell number of CD3-positive T cells per well. T cell count was measured by flow cytometry, and normalised to determine total cell number using precision counting beads. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', *p < 0.05, **p < 0.01, ***p < 0.001.

quantification of 13 key cytokines, collectively secreted by Th1, Th2, Th9, Th17, Th22 and T follicular cells. While incubation with the control BiTE triggered little activity, EpCAM BiTE induced the release of several cytokines, including high levels of IL-2, IL-6, IL-10, IL-17, IFN γ and TNF α (Figure 3.7C). Production of IL-2, IFN γ and TNF α are generally associated with a Th1 response, whereas IL-6 and IL-10 are more often linked to a Th2 response (Mosmann & Sad, 1996). These results suggest that both Th1 and Th2 populations are activated in the presence of EpCAM BiTE.

3.3.3 EpCAM-targeted BiTE induces T cell-mediated killing of tumour cells

The aim of T cell activation by BiTEs is to cause degranulation-mediated cytotoxicity of target cells. After just six hours, expression of the degranulation surface marker CD107a (Aktas et al, 2009) was strongly upregulated by the EpCAM BiTE but not the control BiTE when added to co-cultures of PBMC T cells with DLD cells (Figure 3.8A). This EpCAM BiTE-mediated degranulation translated to DLD cytotoxicity, demonstrated visually by microscopic observation (Figure 3.8B, dead cells indicated by white triangles) and quantified at 24 hours by release of lactate dehydrogenase (LDH) into the supernatant, indicating target cell lysis (Figure 3.8C). This cytotoxicity was dependent on both the presence and number CD3-purified PBMC (Figure 3.8C) and the quantity of EpCAM BiTE (Figure 3.8D), producing a sigmoidal dose-response curve with an EC50 of 3 ng/mL, comparable to that reported in the literature (Brischwein et al, 2006). A similar curve demonstrating a dose-response relationship between T cell activation (CD69⁺/CD25⁺) and the concentration of EpCAM BiTE was also obtained (Figure 3.8E).

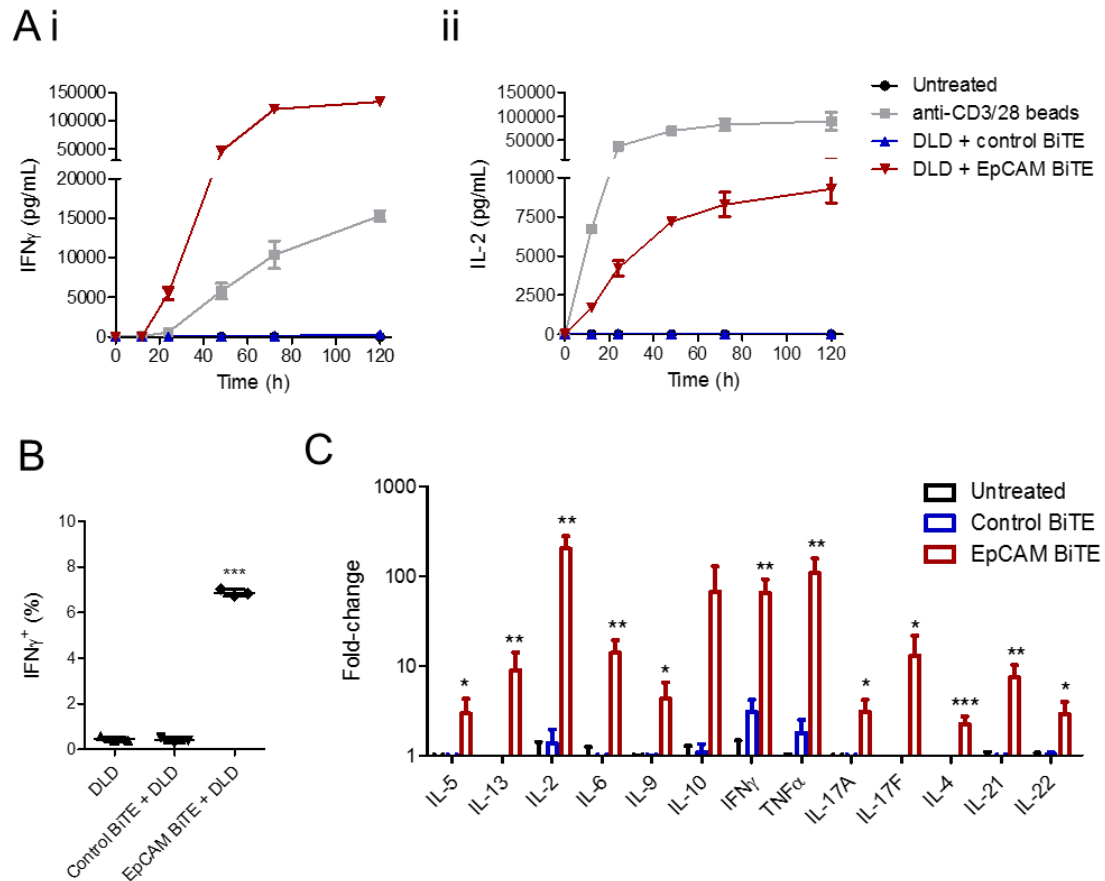


Figure 3.7. EpCAM BiTE-mediated T cell activation induces secretion of Th1 cytokines. (A) Levels of (i) IFN γ and (ii) IL-2 were measured by ELISA using supernatants from co-culture of CD3-purified PBMC with DLD (5:1) and BiTE-containing supernatants for various durations. Anti-CD3 and CD28 Dynabeads were used as a positive control for T cell activation. (B) Intracellular staining of T cells for IFN γ expression after 6 hours in co-culture with DLD cells (5:1) and BiTE-containing supernatants. (C) Cytokine levels were measured by a human multiplex Th cytokine panel using supernatants from co-cultures of T cells with DLD cells (5:1) in the presence of BiTE-containing supernatants for 48 hours. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated' or 'DLD', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

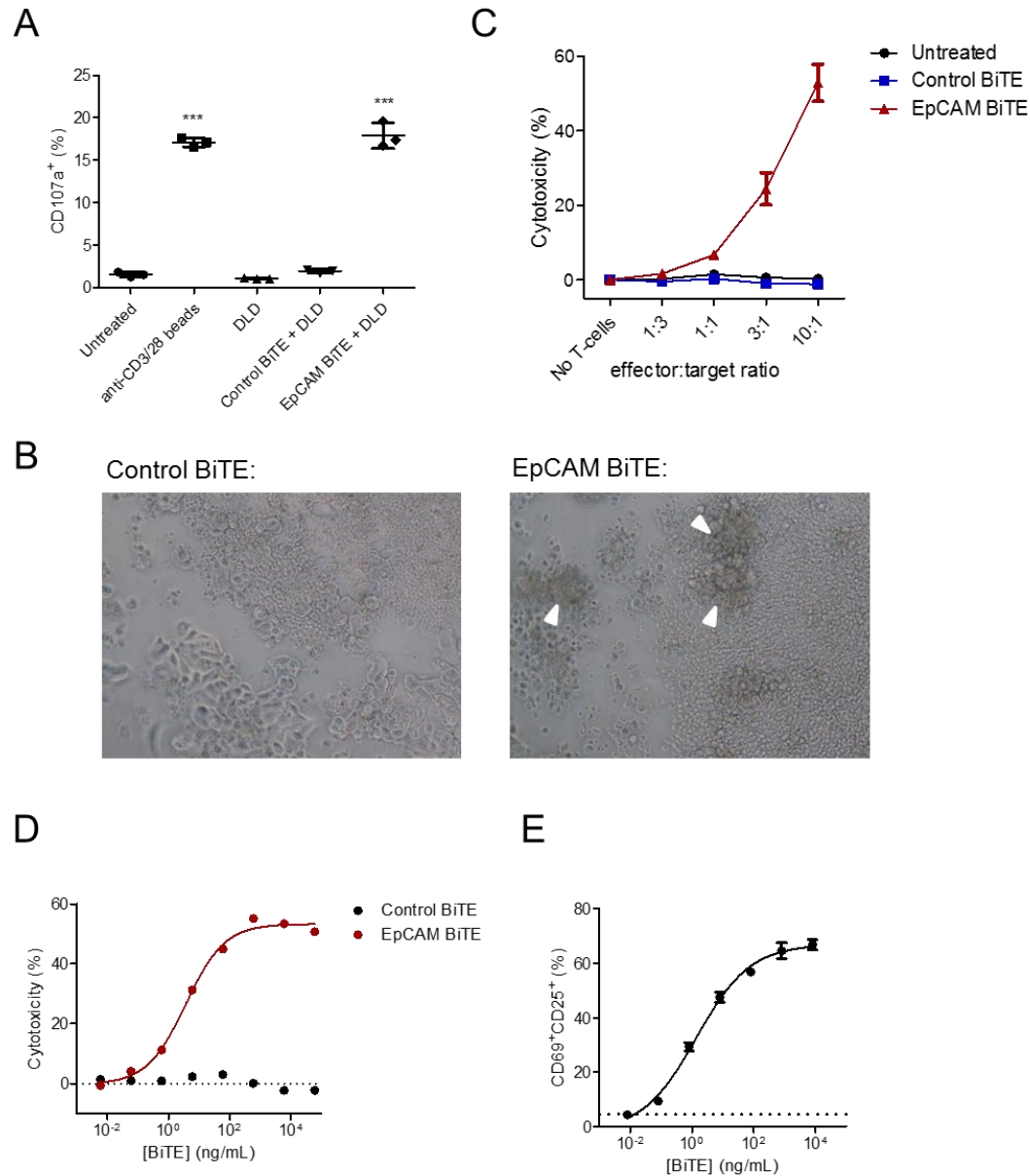


Figure 3.8. EpCAM-targeted BiTE induces T cell degranulation and specific cytotoxicity of EpCAM⁺ DLD cells. (A) Degranulation, measured by CD107a externalisation, of CD3-purified PBMC in co-culture with DLD cells (5:1) and BiTE-containing supernatants. Externalisation was assessed by co-culture with a CD107a-specific antibody for 6 hours followed by flow cytometry analysis. (B) Representative microscopy images of co-cultures of PBMC-derived T cells and DLD (5:1) incubated with control or EpCAM BiTE protein. White triangles show DLD cells that appear to have been killed. (C) Cytotoxicity of DLD cells in co-culture with PBMC-derived T cells and BiTE-containing supernatants at various E:T ratios. Cytotoxicity was assessed by measuring LDH release into the supernatant after 24 hours co-culture. (D) Lysis of DLD cells in co-culture with T cells (5:1) and increasing concentrations of BiTE. Target cell lysis was determined by LDH release into the supernatant. Each condition was measured in a single well. Spontaneous lysis is displayed by the dotted line. (E) Activation (by CD69/CD25 dual positivity) of CD3 purified PBMC in co-culture with DLD and recombinant EpCAM BiTE at increasing concentrations. Percentage of CD69⁺/CD25⁺ of unstimulated cells is displayed by the dotted line. (A, C, E) Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.4 Specificity of recombinant EpCAM BiTE

As most human epithelial cells express EpCAM, the antigen specificity of the EpCAM BiTE was assessed using Chinese Hamster Ovary (CHO) cells engineered using a lentiviral vector to express human EpCAM on their surface (Figure 3.9A; see Materials & Methods for their production). In the presence of EpCAM BiTE and CHO-EpCAM cells, exogenously added PBMC-derived T cells were strongly activated, as demonstrated by CD25 expression (Figure 3.9B) and cytotoxicity (Figure 3.9C). Activation and cytotoxicity were not seen with parental CHO control cells nor control BiTEs, confirming the antigen specificity of EpCAM BiTE-mediated cytotoxicity.

We assessed whether EpCAM BiTE could kill a range of tumour cells and whether EpCAM BiTE-mediated cytotoxicity levels were dependent on EpCAM expression (Figure 3.10A and B). T cell-mediated cytotoxicity in the presence of the EpCAM BiTE was measured in six different carcinoma cell lines (Figure 3.10C). The greatest cytotoxicity was observed in DLD and A431, and the lowest in A549 and PC3. Cytotoxicity was significantly associated with EpCAM surface levels, as determined by flow cytometry (Pearson correlation coefficient, $r = 0.7993$; $p = 0.0312$), where A549 and PC3 cells had the lowest levels of surface EpCAM, while DLD, the highest (Figure 3.10B). These results suggest that the presence and level of EpCAM expression do influence the degree of cytotoxicity, though other factors, such as the intrinsic resistance of cells to granzyme-mediated apoptosis, could also play a role in determining the overall level of cell killing.

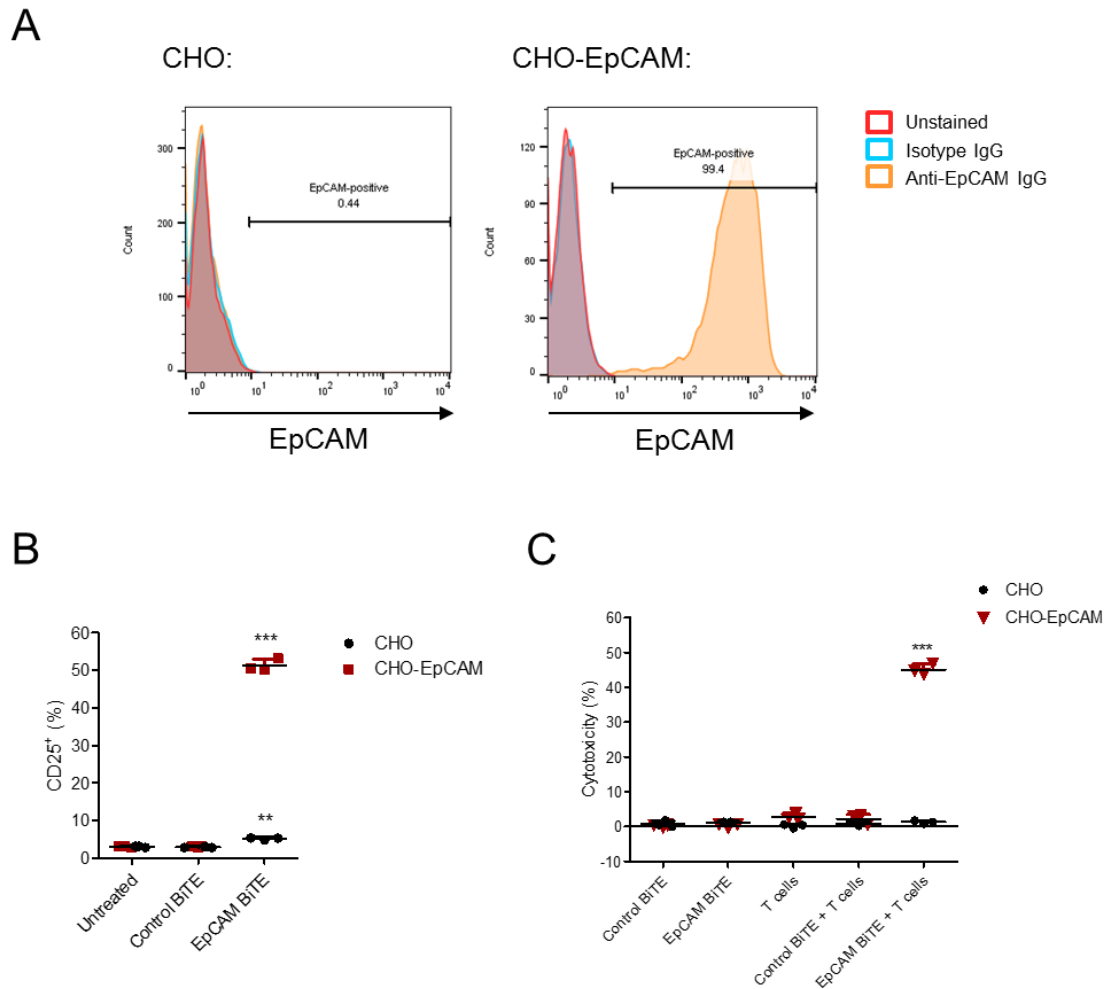


Figure 3.9. Assessment of antigen-specificity of EpCAM BiTE-mediated T cell cytotoxicity. (A) Flow cytometry spectrum of surface EpCAM expression on parental CHO and CHO-EpCAM cell lines. Isotype control antibodies were used to determine background fluorescence and unspecific binding. **(B)** Induction of activation marker CD25 on CD3⁺ T cells in co-culture with CHO or CHO-EpCAM cells (5:1) and BiTE-containing supernatants, measured by flow cytometry after 24 hours of co-culture. **(C)** Cytotoxicity of CHO or CHO-EpCAM cells cultured with BiTE-containing supernatants alone or in co-culture with T cells. Cytotoxicity was assessed by release of LDH into the culture supernatants after 24 hours of incubation. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated' or 'T cells', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

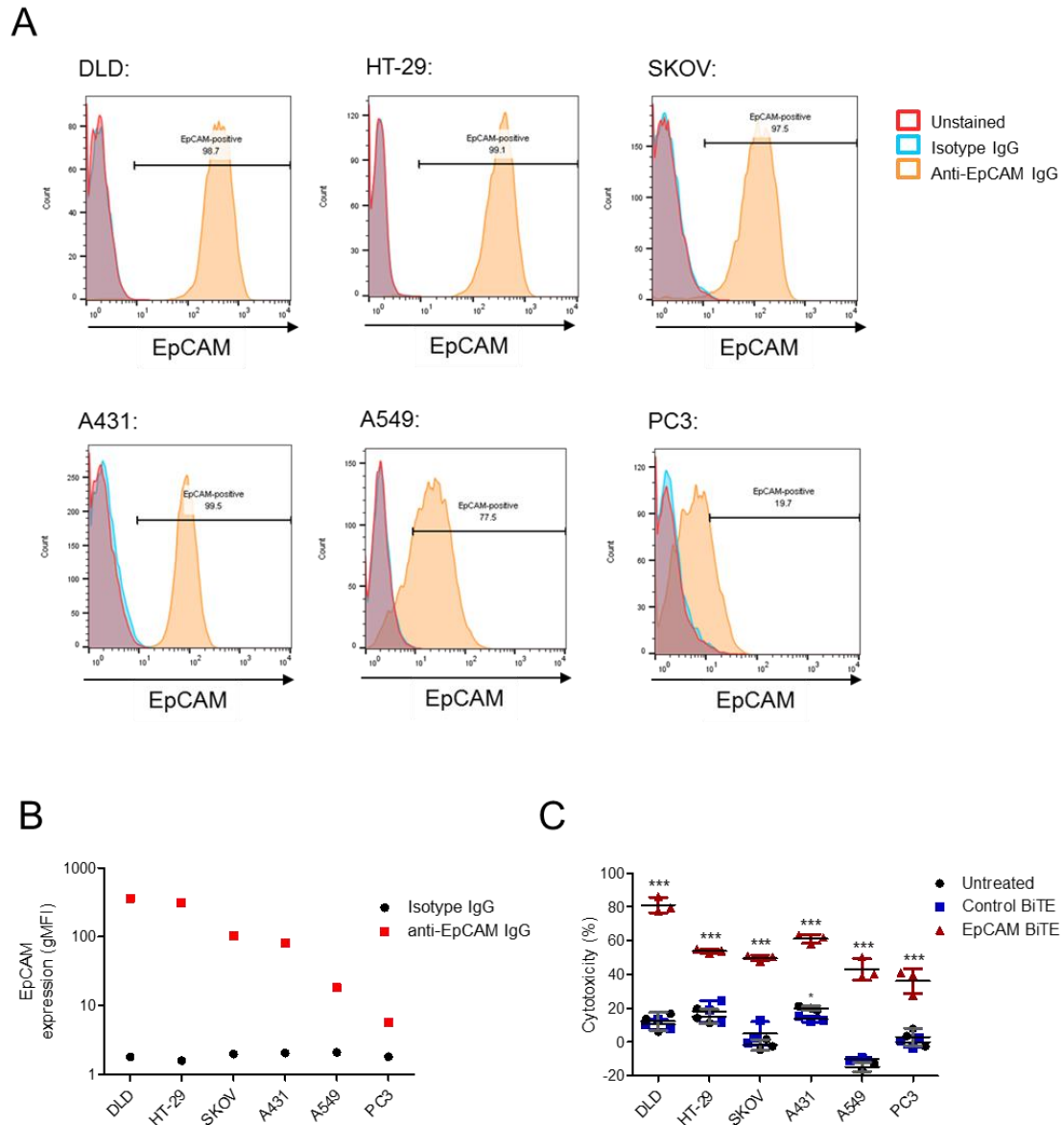


Figure 3.10. Association of BiTE-mediated cytotoxicity and the level of EpCAM expression on the target cell. (A) Flow cytometry plots showing levels of EpCAM expressed on the surface of a panel of carcinoma cell lines. An isotype control was used to determine background fluorescence and unspecific binding of each cell line. Colorectal, DLD, HT-29; prostate, PC3; ovarian, SKOV3; squamous, A431; lung, A549. (B) A graphical representation of the EpCAM expression levels (geometric mean fluorescence intensity, gMFI) of each cell line measured in (A). (C) Cytotoxicity of multiple EpCAM-positive carcinoma cells after 24 hours in co-culture with T cells (5:1) and BiTE-containing supernatants. Viability was measured by MTS assay after 24 hours of co-culture. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.5 BiTE-mediated activation of CD4⁺ and CD8⁺ T cell subsets

To determine which T cell types are activated by EpCAM BiTE, PBMC-derived T cells were incubated with DLD cells and activated using EpCAM-BiTE prior to flow analysis. At 24 hours, both CD4⁺ and CD8⁺ cells had high levels of expression of CD69 and CD25 (Figure 3.11A), although the percentage of activated CD4 cells was generally slightly greater. EpCAM BiTE-mediated T cell proliferation was assessed using CFSE (Figure 3.11B) and degranulation by surface CD107a expression (Figure 3.11C). Again, similar levels of activation were seen for both CD4⁺ and CD8⁺ cells. Finally, purified CD4⁺ and CD8⁺ T cell-mediated tumour cell cytotoxicity levels were assessed. All T cell preparations caused similar cytotoxicity (Figure 3.11D), indicating that both CD4⁺ and CD8⁺ cells contribute to BiTE-mediated cytotoxicity.

3.3.6 Expression of the EpCAM BiTE from the oncolytic adenovirus Enadenotucirev

Enadenotucirev (EnAd) is an oncolytic chimera of adenovirus group B types 11 and 3. EnAd has a mosaic E2B region, a nearly complete E3 deletion and a smaller E4 deletion mapped to E4orf4 (Figure 3.12A). Currently undergoing several early phase clinical trials for treatment of cancer, EnAd combines good systemic pharmacokinetics and promising clinical activity with the possibility to encode and express transgenes (Calvo E & Salazar R, 2014; Seymour & Fisher, 2016). The DNA sequence of EpCAM BiTE was amplified by PCR and inserted into a SbfI-linearised virus backbone by Gibson assembly immediately downstream of the fibre gene (Figure 3.12B and C). The EpCAM BiTE

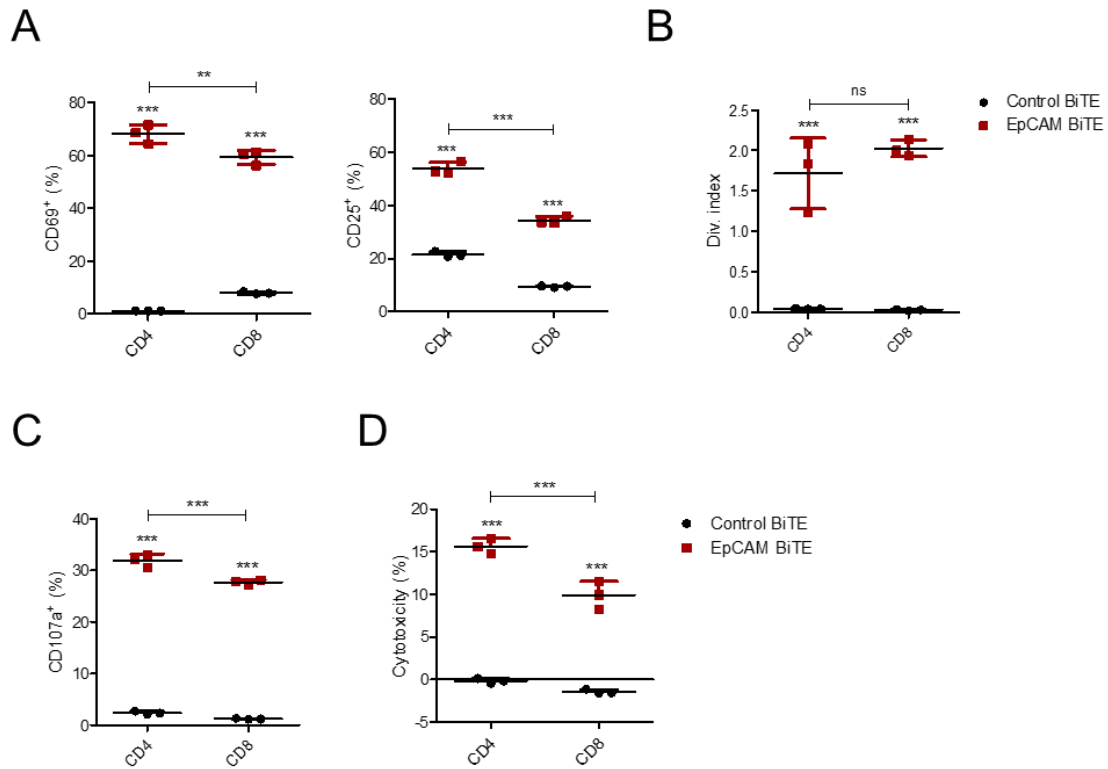


Figure 3.11. Identification of T cell subsets responsible for BiTE-mediated cytotoxicity. (A) BiTE-mediated T cell activation of CD4 and CD8 cells 24 hours after co-culture of CD3 T cells with DLD cells (5:1) and BiTE-containing supernatant. Activation was assessed by surface expression of CD69 and CD25 and measured by flow cytometry. (B) Proliferation of CFSE-stained CD4 and CD8 T cells in co-culture with DLD and incubated with BiTE-containing supernatants. Fluorescence was measured after 5 days incubation by flow cytometry. (C) Degranulation of CD4 and CD8 cells following 6 hours co-culture with DLD cells and BiTE-containing supernatants. A CD107a-specific antibody is added to the culture media for the duration of the co-culture and degranulation is assessed by flow cytometry. (D) Cytotoxicity by either the CD4 or CD8 T cell subset is assessed by LDH release into supernatant, following 24-hour incubation of DLD cells with CD4- or CD8-purified T cells (5:1) and BiTE-containing supernatant. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using an unpaired, two-tailed t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

transgene was placed either under transcriptional control of a CMV immediate early promoter (EnAd-CMV-EpCAMBiTE) or positioned downstream of a splice acceptor site for the adenovirus major late promoter (MLP; EnAd-SA-EpCAMBiTE). In the former configuration, the BiTE should be expressed and secreted whenever the virus successfully infects a cell, whereas expression from the MLP splice acceptor site will occur later in the infection cycle only when the MLP is activated in cells that are permissive to virus

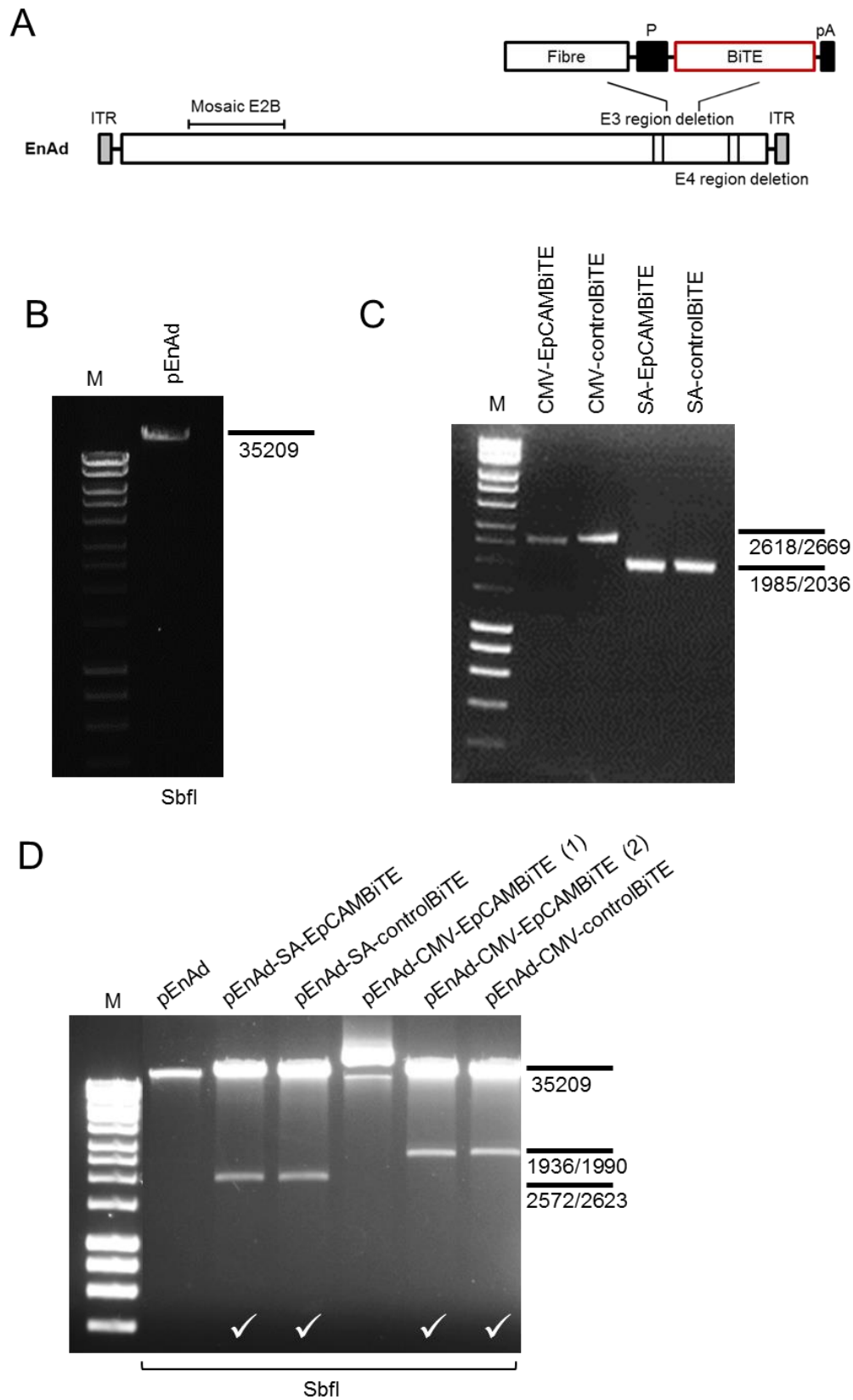


Figure 3.12. Engineering of BiTE-armed oncolytic adenoviruses. (A) Schematic representation of Enadenotucirev (EnAd) engineered to express the BiTE transgenes downstream of a CMV promoter or splice acceptor site (P) followed by a polyadenylation sequence (pA). (B) EnAd2.4 vector was linearised by digestion with SbfI. M, Hyperladder 1 kb DNA ladder. (C) PCR amplification of the EpCAM and control BiTE was performed to generate fragments with the CMV promoter or a splice acceptor site incorporated upstream of the transgene. The BiTE fragments were subsequently inserted into the linearised vector from (B) by Gibson assembly. M, Hyperladder 1 kb DNA ladder. (D) Recombinant plasmids generated from the ligation of the products in (B) and (C) were screened for correct transgene insertion by digestion with SbfI. The correct colonies are indicated with a tick. M, Hyperladder 1 kb DNA ladder.

replication. A control BiTE, recognising CD3 and FHA, was also introduced to create two corresponding control viruses. Recombinant plasmids containing the modified virus genomes were screened for correct transgene insertion by SbfI digestion (Figure 3.12D) and DNA sequencing.

The recombinant viral genome plasmids were linearised using AscI to remove all extra-viral genes required for the cloning process and transfected into HEK293A cells. Successful virus replication, packaging and production ('rescue') induced a cytopathic effect (CPE) in infected cells. The CPE manifested as the rounding and swelling of cells, lifting of the cell monolayer from the culture surface, and plaque formation (Figure 3.13A). All recombinant viruses were rescued in HEK293A cells and cell lysates were harvested after CPE had progressed to an advanced stage. Single clones of each virus were selected following infection of HEK293A with serially-diluted lysates in a 96-well plate and the subsequent harvest of wells containing a single plaque. For each virus, five clones were selected for western blotting using an anti-His antibody to assess BiTE secretion into supernatants after CPE formation (Figure 3.13B).

One clone of each recombinant virus (indicated with a tick) was selected for large-scale preparation in hyperflasks of HEK293A cells and purified twice by caesium chloride

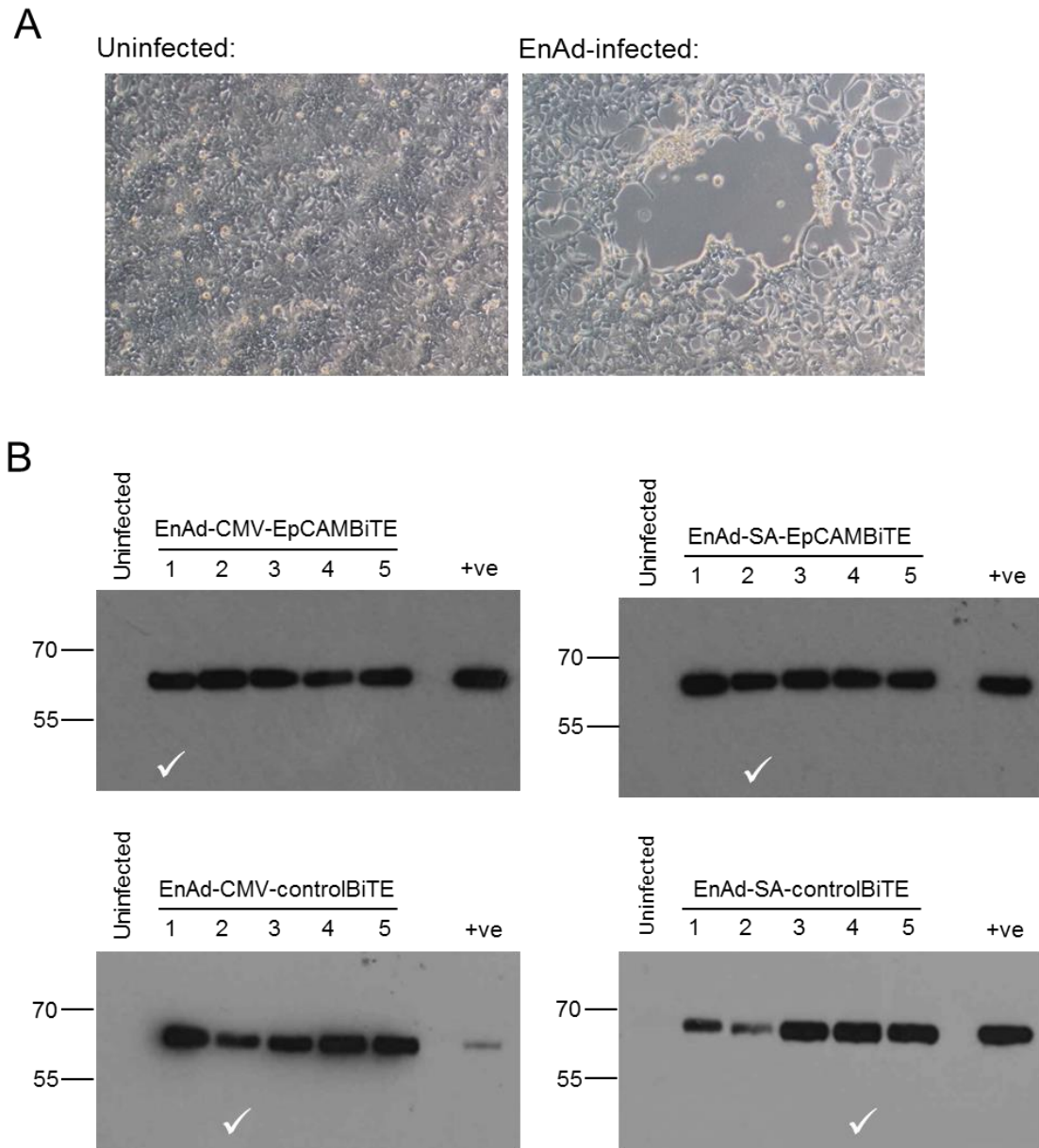


Figure 3.13. Rescue and selection of EpCAM and control BiTE-expressing EnAd clones. (A) Representative microscopy image showing the cytopathic effect of EnAd-infected HEK293A and the formation of plaques. In this case, cells were either untreated or transfected with the linearised genome of EnAd-CMV-EpCAMBiTE, and images were collected on day 7. **(B)** Supernatants from HEK293A cells infected with several different clones of EnAd expressing BiTEs (following CPE) were evaluated for secretion of BiTE by western blot analysis using an anti-His antibody. Transgene expression of five clones of each virus were assessed, with uninfected cells as negative control. Recombinant EpCAM or control BiTE protein was used as a positive control ('+ve'). The clones chosen for large-scale production are indicated with a tick.

banding (see Materials & Methods). Retention of the inserted transgenes was confirmed by DNA extraction from purified virus stocks and digestion with BglII, with all viruses displaying the expected cleavage products (Figure 3.14A). The virus titre and tissue culture infectious dose of each was determined by dsDNA Picogreen assay and serial titration on A549 cells, respectively (Figure 3.14B).

3.3.7 BiTE viruses are not impaired in genome replication or tumour cell oncolysis

Next, we investigated the replication and oncolytic properties of the viruses in the absence of human T cells. Infection of DLD with parental EnAd and the recombinant BiTE viruses (100 virus particles (vp)/cell; 2×10^7 vp/mL) yielded similar amounts of viral genomes, as measured by qPCR, indicating the BiTE transgene did not interfere with the viral replication kinetics (Figure 3.15A). The cytotoxicity of DLD cells infected with EnAd or recombinant BiTE viruses at increasing vp/cell was measured by MTS assay five-days post-infection. All recombinant viruses, irrespective of BiTE specificity or promoter, showed cytotoxic activity indistinguishable from the parental virus EnAd, indicating that genetic modification did not alter the intrinsic oncolytic activity of the virus (Figure 3.15B).

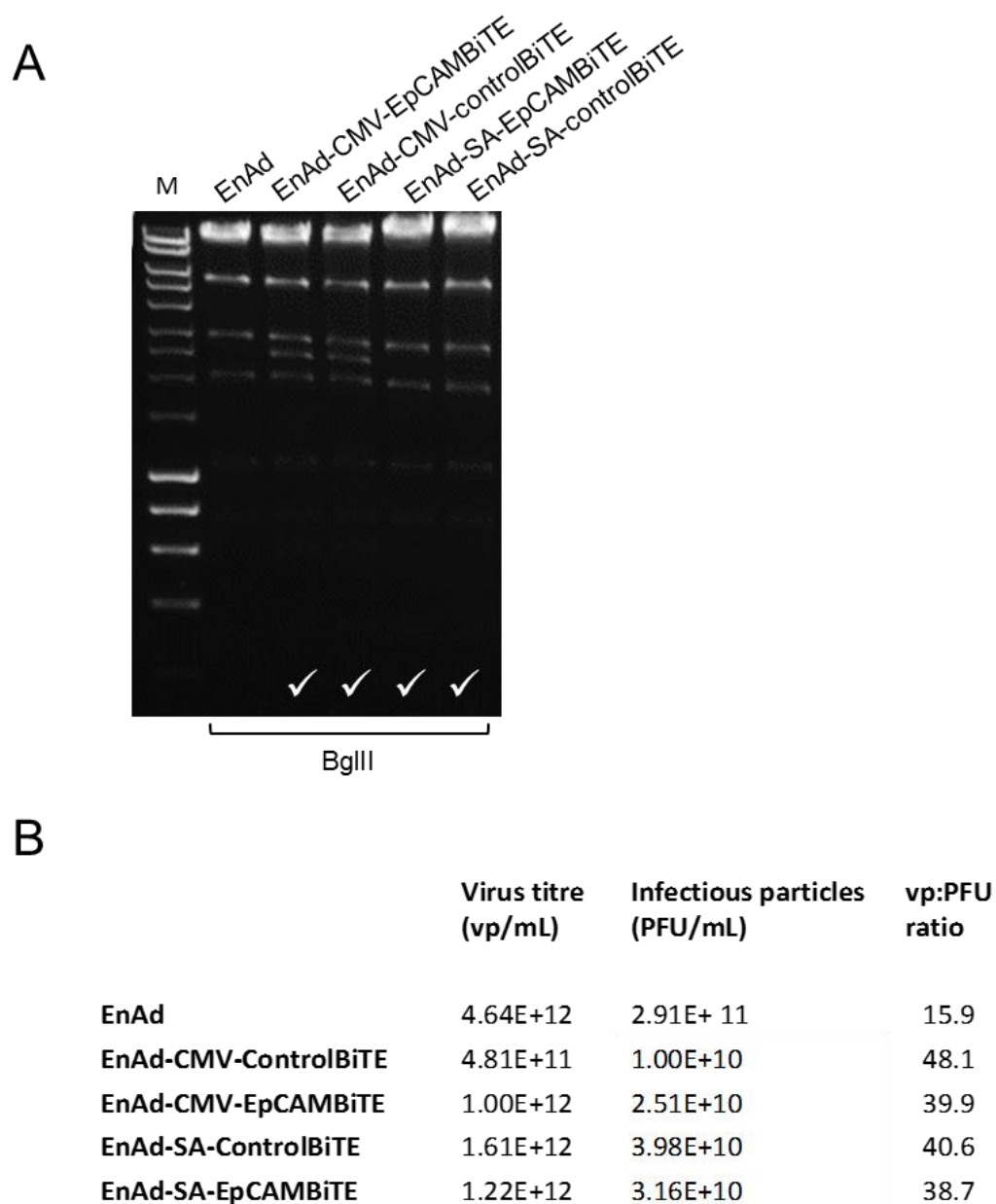
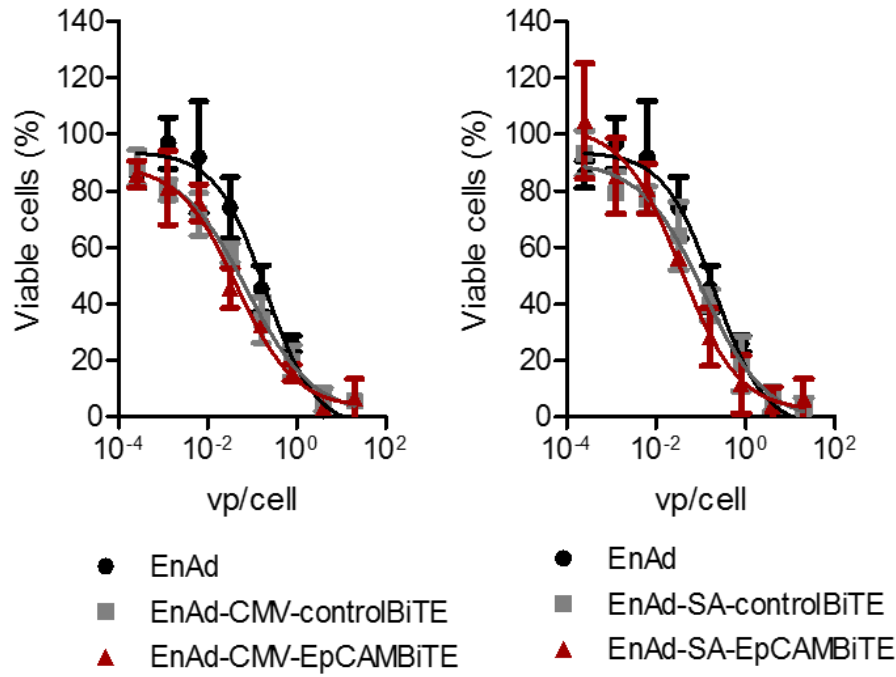


Figure 3.14. Large-scale production and purification of EpCAM and control BiTE-expressing oncolytic adenoviruses. (A) DNA extracted from recombinant virus were screened for correct transgene insertion by digestion with BglII. Colonies producing the expected pattern of cleavage products are marked with a tick. **(B)** Table outlining the virus titre and tissue culture infectious dose of each recombinant virus, determined by dsDNA Picogreen assay and serial titration on A549, respectively.

A



B

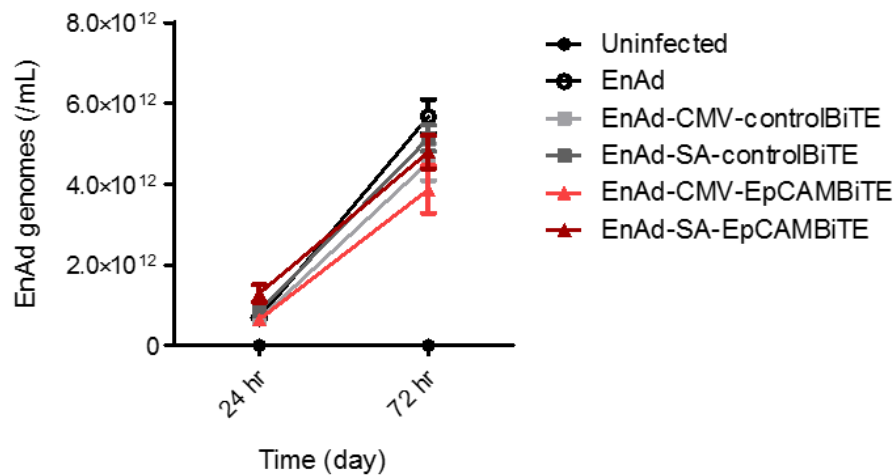
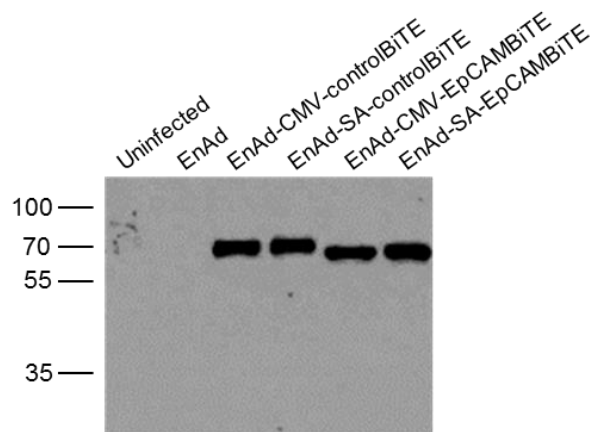


Figure 3.15. Assessment of the oncolytic properties of BiTE expressing EnAd viruses *in vitro*. **(A)** Cytotoxicity of DLD cells infected with EnAd or recombinant virus at increasing concentrations of virus. Cytotoxicity was measured by MTS assay after incubating for five days. **(B)** DLD cells were infected with parental EnAd or recombinant virus (100 vp/cell). Wells were harvested at 24 or 72 hours. Genome replication was assessed by using qPCR primers and probes against Ad11 hexon. Each condition was measured in biological triplicate and represented as mean $\pm$ SD.

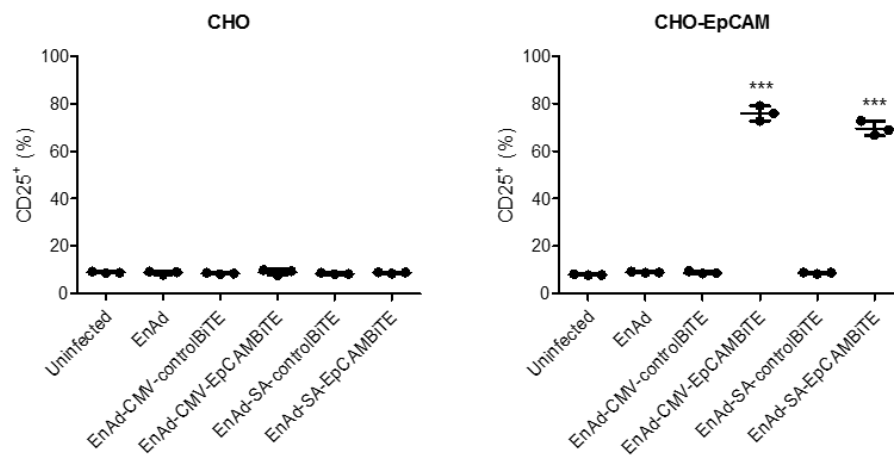
3.3.8 Selective killing of EpCAM positive cells by virally produced EpCAM BiTE

To assess BiTE expression and secretion, HEK293A cells were infected with the BiTE-expressing EnAd viruses. At 72 hours post-infection, supernatants were examined by western blotting using an anti-His antibody. All four viruses, including the two control BiTE viruses and the two EpCAM BiTE viruses, secreted similar levels of BiTE into the supernatant (Figure 3.16A). Supernatants from EnAd-EpCAM BiTE-infected HEK293A cells were added to cultures of CHO and CHO-EpCAM cells, with or without PBMC-derived T cells. T cell activation and cytotoxicity of the CHO or CHO-EpCAM cells was measured after 24 hours. In the presence of CHO, there was no increase in T cell expression of CD25 (Figure 3.16B) nor cytotoxicity observed with any treatment (Figure 3.16C). However, T cells incubated with CHO-EpCAM had substantially increased CD25 expression using supernatants from HEK293A cells infected with either EnAd-CMV-EpCAM BiTE or EnAd-SA-EpCAM BiTE (Figure 3.16B). As expected, this translated into cytotoxicity of CHO-EpCAM cells only when T cells were added in the presence of supernatant from HEK293A cells that had been infected with either EnAd-CMV-EpCAM BiTE or EnAd-SA-EpCAM BiTE viruses (Figure 3.16C). Crucially, no cytotoxicity was observed in the absence of T cells or with supernatants from EnAd-CMV-controlBiTE- or EnAd-SA-controlBiTE-infected HEK293A.

A



B



C

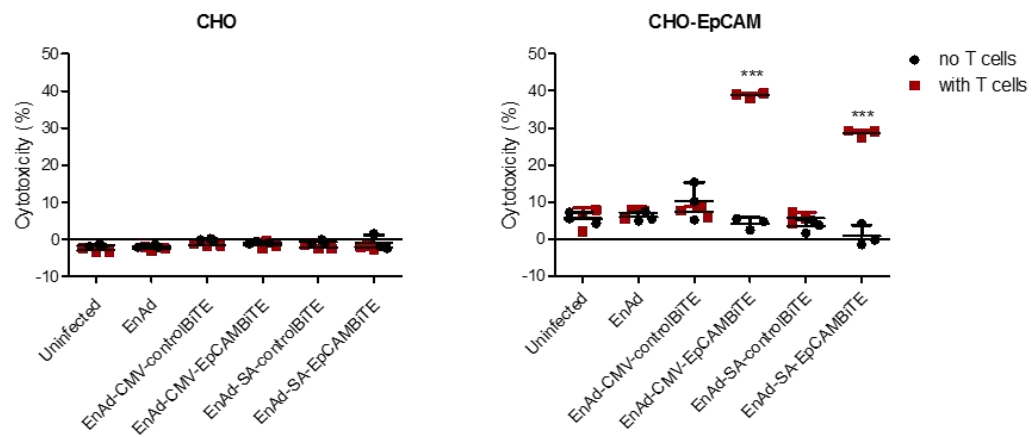


Figure 3.16. Characterisation of oncolytic virus EnAd expressing EpCAM BiTE using cell lines and PBMC-derived T cells. (A) Supernatants from uninfected or virus-infected HEK293A cells three days post-infection were assessed for transgene expression by immunoblot analysis and probed with an anti-His antibody. (B) Induction of activation marker CD25 of CD3-positive T cells cultured with CHO or CHO-EpCAM (E:T 5:1) and diluted HEK293A supernatants from (A). Activation was measured by surface expression of CD25 by flow cytometry. (C) Cytotoxicity of CHO or CHO-EpCAM cells incubated with HEK293A supernatants from (A) alone or in co-culture with CD3-purified PBMC (E:T 5:1). HEK293A supernatants were diluted 300-fold. Cytotoxicity was assessed by LDH released into the supernatant after 24-hour incubation. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * p <0.05, ** p <0.01, *** p <0.001.

3.3.9 Superior cytotoxicity of EnAd expressing EpCAM BiTE

EnAd kills most carcinoma cells quickly by direct oncolysis (Kuhn et al, 2008), though some cells – notably SKOV3 ovarian carcinoma cells – are partially resistant and killed more slowly. We therefore reasoned that the consequences of arming EnAd to secrete EpCAM BiTE, leading to cytotoxic activation of T cells might be particularly evident in SKOV3 cells. Cells were infected with 100 vp/cell of EnAd, EnAd-CMV-EpCAMBiTE, EnAd-SA-EpCAMBiTE or the corresponding control viruses 24 hours after seeding and cell death monitored by the xCELLigence system, which measures electrical impedance as a measure of cell viability. PBMC-derived T cells were added to SKOV3 cell cultures two hours post-infection. In the absence of T cells, SKOV3 grew for approximately 72 hours, as evidenced by an increasing cell index (Figure 3.17A). After 72 hours, cell growth plateaued and remained stable, independent of virus infection, up until at least 160 hours post-infection. All tested viruses, including parental EnAd, induced no observable target cell cytotoxicity during the time measured. However, in a co-culture with PBMC-derived T cells, both the EpCAM BiTE-armed viruses induced rapid SKOV3 lysis, with EnAd-CMV-EpCAM BiTE-driven lysis within 16 hours, and EnAd-SA-EpCAM BiTE within 44 hours following addition of T cells (Figure 3.17B). Importantly,

EnAd or the control BiTE viruses demonstrated no target cell lysis in this time frame despite the addition of T cells. This result was confirmed by LDH release into the supernatant at 24, 48 and 96 hours post-infection (without T cells, Figure 3.18A; with T cells, Figure 3.18B). To confirm that target cell cytotoxicity was mediated via T cell activation, CD3 cells were harvested at each time-point and activation status was

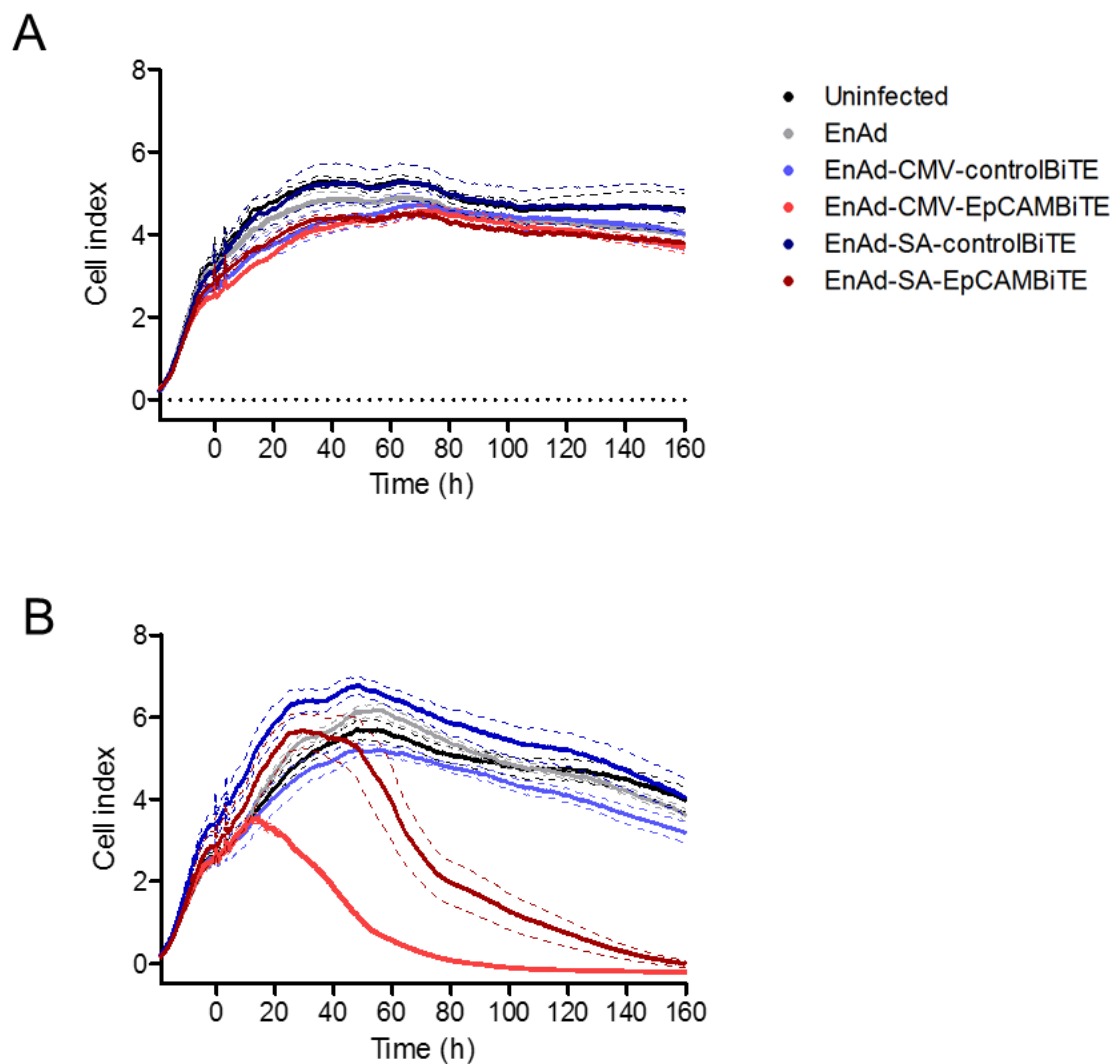


Figure 3.17. Superior potency of EnAd expressing EpCAM BiTE in partially EnAd-resistant SKOV3 cells. (A, B) Viability of SKOV3 cells was monitored in real-time over 160 hours by the xCELLigence-based cytotoxicity assay. SKOV3 cells were seeded and infected with EnAd or BiTE-armed EnAd viruses at zero hour. Uninfected cells were used as a negative control. In (B) CD3-purified PBMC (5:1) were added two hours post-infection and impedance was measured at 15-minute intervals. Each condition was measured in biological triplicate and represented as mean $\pm$ SD.

determined by CD69 and CD25 expression using flow cytometry. The kinetics of both activation markers were similar to those seen for cytotoxicity (Figures 3.18C and D).

These results were further supported by similar findings in DLD cells, in which EpCAM BiTE-expressing viruses induced T cell activation (CD69 and CD25 expression; Figure 3.19A and B) and cytotoxicity of DLD cells (measured by LDH release; Figure 3.19C and D) at a significantly quicker rate than the control BiTE viruses.

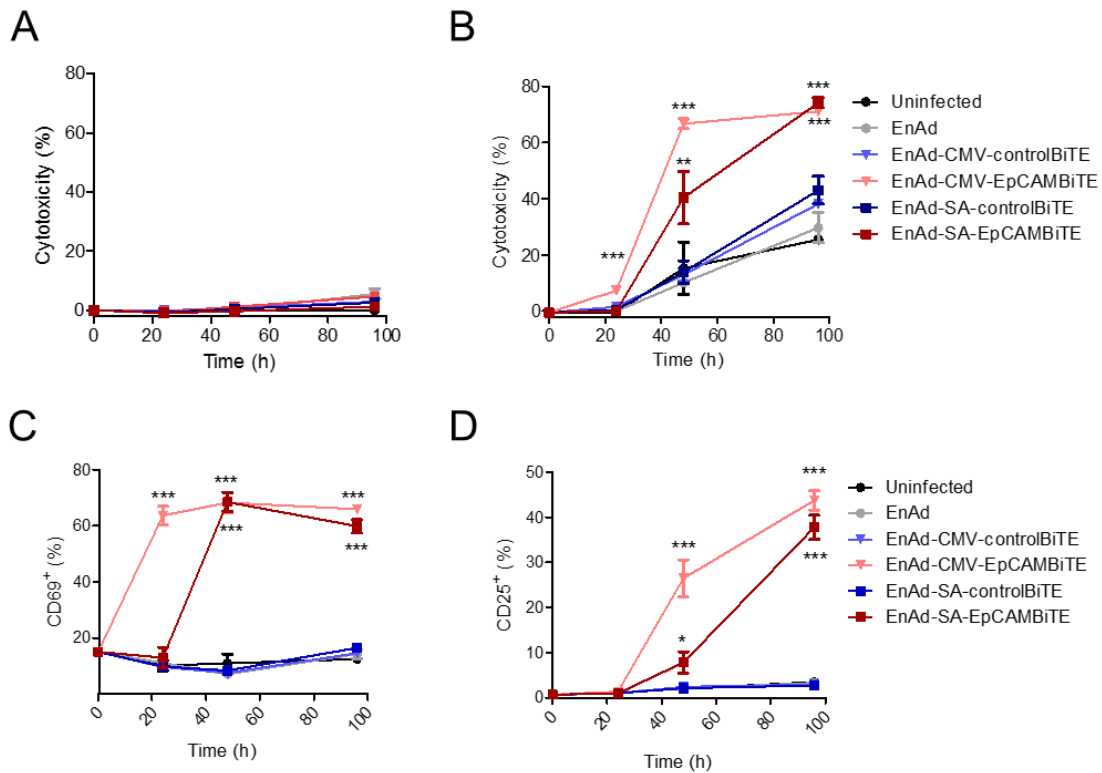


Figure 3.18. EnAd-EpCAMBiTE-mediated oncolysis induces activation of primary T cells. (A, B) SKOV3 cells were incubated with EnAd or recombinant viruses in the absence (A) or presence (B) of T cells. Cytotoxicity was measured by LDH release at the specified time-points. **(C, D)** CD3-purified PBMC were cultured with SKOV3 cells (5:1) that were infected with parental EnAd or recombinant BiTE-armed viruses. At each time-point, T cells were harvested and analysed for surface expression of CD69 (C) or CD25 (D) by flow cytometry. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'uninfected', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

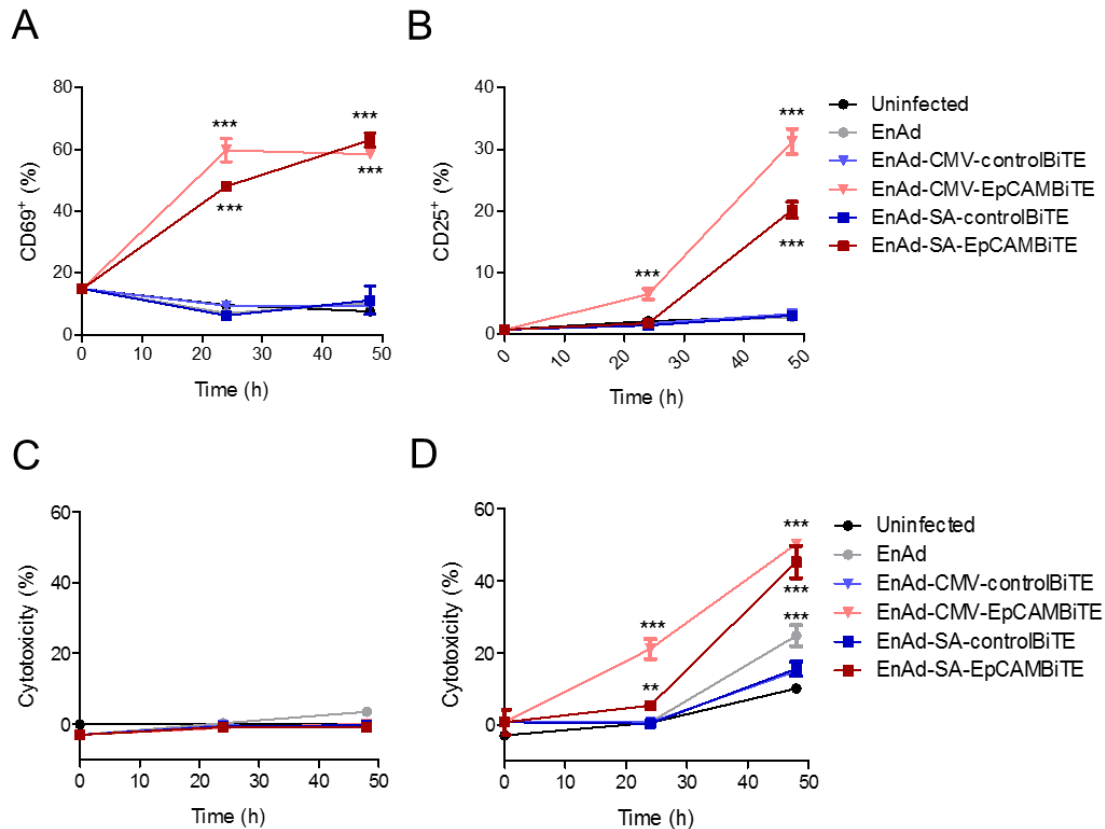


Figure 3.19. Cytotoxicity and T cell activation by EpCAM BiTE-expressing EnAd infection of DLD cells. (A, B) Induction of activation markers (A) CD69 and (B) CD25 on T cells in co-culture with DLD cells and infected with EnAd or EnAd expressing BiTE viruses (100 vp/cell). (C, D) Cytotoxicity for DLD cells infected with parental or armed viruses in (C) absence or (D) presence of PBMC-derived T cells (E:T 5:1). LDH release into the supernatant was used as a readout for cytotoxicity, measured from independent wells at each time point. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The approximate quantity of active EpCAM BiTE produced from infected DLD cells was determined by comparing cytotoxicity (assessed by Abs₄₉₀) induced by supernatants from EnAd-infected DLD to cytotoxicity (Abs₄₉₀) induced by known quantities of recombinant BiTE (i.e. by creating a standard curve). DLD in co-culture with CD3-selected PBMC were incubated with recombinant BiTE (Figure 3.20A) or diluted supernatants harvested from DLD cells infected for 72 hours with the specified virus (Figure 3.20B). The release of LDH into the supernatant was measured at 24 hours to assess cytotoxicity. We found that EpCAM BiTE was produced at 165 μ g and 50 μ g per million DLD cells for EnAd-

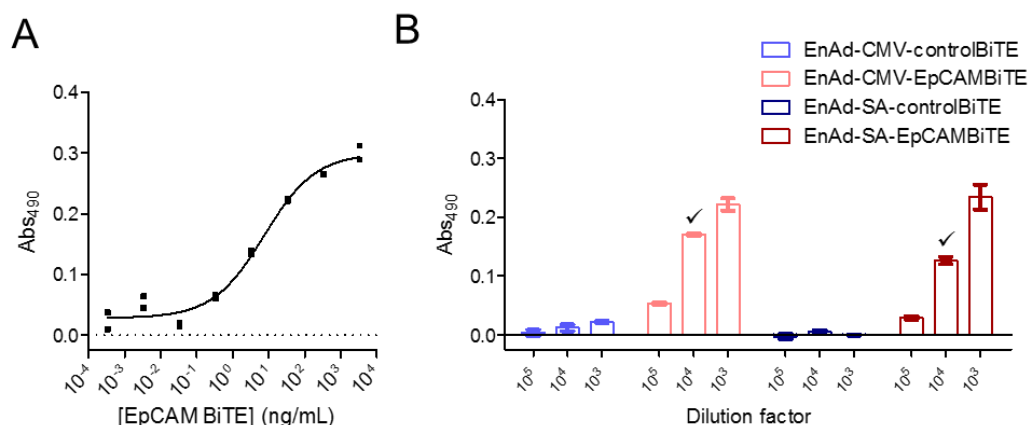


Figure 3.20. Quantification of EpCAM BiTE produced from DLD cells infected with recombinant viruses. (A) Standard curve of LDH released (Abs₄₉₀) into supernatants after 24 hours co-culture of DLD and CD3-purified PBMC (E:T 5:1) with varying quantities of recombinant EpCAM BiTE. (B) In parallel, DLD and T cell co-cultures were incubated with diluted DLD supernatants three days post-infection with the indicated virus. The dilution used to determine the quantity of EpCAM BiTE is indicated with a tick. Each condition was measured in biological duplicate and represented as mean $\pm$ SD.

CMV-EpCAMBiTE and EnAd-SA-EpCAMBiTE, respectively. As the EC₅₀ for the EpCAM BiTE is 3 ng/ml (Figure 3.8D), EpCAM-BiTE is produced by the recombinant virus at levels that are likely to reach therapeutic doses.

Cytotoxicity of EpCAM BiTE-expressing EnAd was visualised by time lapse video microscopy. SKOV3 tumour cells (unlabelled) were co-incubated with normal human dermal fibroblasts (NHDF; EpCAM-negative, labelled red, serving as non-target control cells) and PBMC-derived T cells (labelled blue) in the presence of a caspase stain added into the culture medium. The caspase stain, CellEvent Caspase 3/7 reagent, fluoresces green upon caspase activation. Co-cultures of SKOV3, exogenous T cells and EpCAM BiTE-expressing EnAd, but not parental EnAd, exhibited dramatic cytotoxicity and strong induction of apoptosis in SKOV3 cells. Importantly, the EpCAM-negative NHDF in co-culture remained viable throughout. Representative fluorescent images at 0, 30 and 48 hours post-infection are shown in Figure 3.21, and full time-lapse sequences are also available.

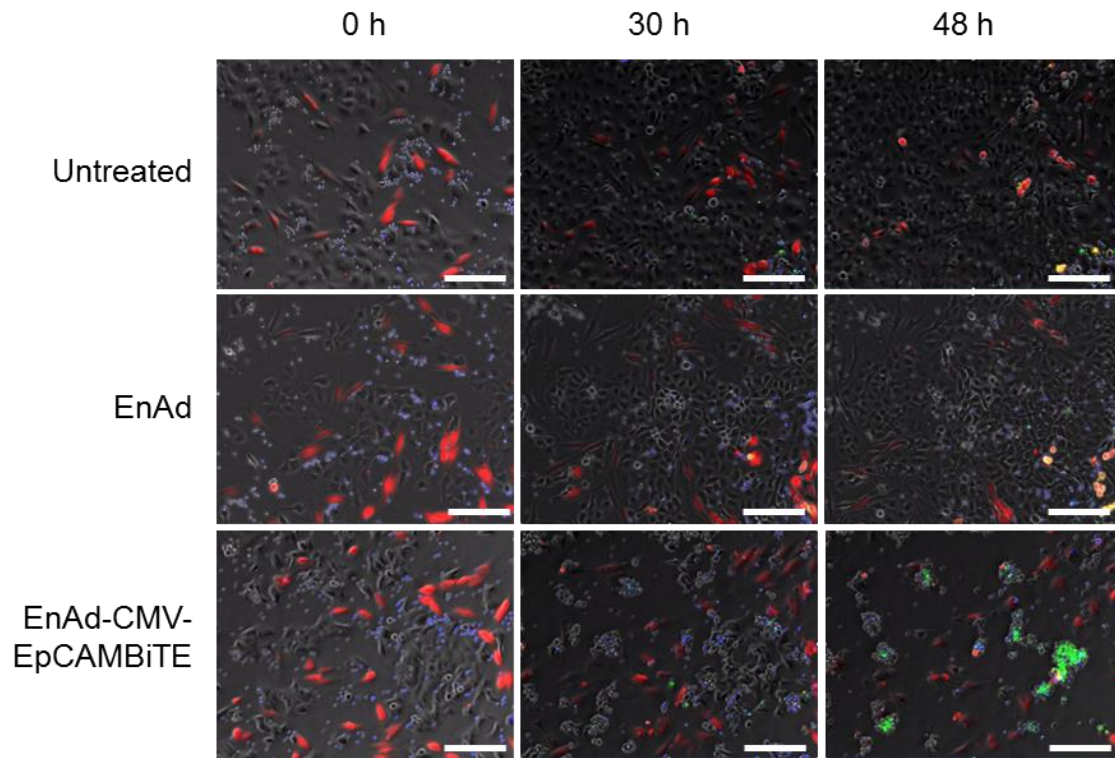


Figure 3.21. EnAd expressing EpCAM BiTE induces T cell-mediated apoptosis of EnAd-resistant SKOV3 cells. Time-lapse sequences showing co-cultures of SKOV3 carcinoma cells (unstained), NHDF fibroblasts (red) and CD3-purified PBMC (blue). Cells were infected with EnAd, EnAd-CMV-EpCAMBiTE or uninfected. Apoptosis was visualised using CellEvent Caspase 3/7 detection reagent (green). Images were taken on a Nikon TE 2000-E Eclipse inverted microscope at intervals of 15 minutes covering a period of 96 hours. Representative images were recorded at the times displayed; original magnification $\times 10$; scale bar 100 μm . Full time-lapse sequences are available.

3.4 Chapter conclusions

1. EnAd can be ‘armed’ without losing oncolytic potency.
2. EpCAM BiTE-expressing EnAd can activate PBMC-derived T cells and mediate targeted killing of EpCAM⁺ tumour cells.
3. EnAd encoded with EpCAM BiTE can direct rapid cytotoxicity to EnAd-resistant cells.

3.5 Chapter discussion

Encoding BiTEs within oncolytic viruses provides a powerful means to activate T cells to become cytotoxic and lyse antigen-positive target cells, providing a completely separate therapeutic modality from the effects of direct viral lysis. In this study we have shown that EpCAM BiTE-induced activation of T cells is robust, inducing proliferation, degranulation and up-regulation in activation markers. Indeed, BiTE-mediated activation induced a broad expression profile of cytokines with a likely Th1 bias, with strong secretion of IFN γ , IL-2 and TNF α (Figure 3.7). Further, BiTE-targeted cytotoxicity is fully antigen-specific (Figure 3.9) and can be mediated by both CD4 and CD8 T cells (Figure 3.11). Importantly, the EpCAM BiTE could be incorporated into an oncolytic adenovirus (Figure 3.13), leading to the rapid T cell-mediated lysis of EnAd-resistant cell lines (Figure 3.16 and 3.17).

The possibility to encode BiTEs within oncolytic viruses has been previously explored using an oncolytic vaccinia virus with an Ephrin A2-targeting BiTE (Yu et al, 2014). This agent showed that the Ephrin BiTE could mediate activation of PBMCs and antigen-targeted killing of tumour cells both *in vitro* and *in vivo*. Intriguingly, although the BiTE could activate T cells it did not lead to T cell proliferation without the addition of exogenous IL-2, whereas the BiTE used in the current study led to extensive proliferation of primary human T cells. This is consistent with Fajardo *et al*, who armed an oncolytic adenovirus, ICOVIR-15K, with EGFR-targeted BiTE, and also report strong proliferation of T cells in the absence of supplemented IL2 (Fajardo et al, 2017). The reason for this difference in behavior is currently unknown, although it may reflect the different BiTE design, the different oncolytic virus used or perhaps depend on the antigen density giving sufficient crosslinking of CD3 on the T cells.

BiTEs could be encoded by EnAd without any loss of oncolytic virulence, with no observable effect on either oncolytic activity or the replication of viral genomes (Figure 3.15). This is reflective of the considerable transgene packaging capacity of the virus. The presence of the transgene will not affect the physicochemical properties of the virus particles; hence the modified viruses should exhibit exactly the same clinical pharmacokinetics as the parental agent, and – when using the adenovirus major late promoter - should be capable of expressing the encoded BiTE selectively within tumours throughout the body.

In the majority of the experiments, anti-CD3/CD28 Dynabeads were used as a positive control for T cell activation. Interestingly, disparate responses were generated depending on the mode of stimulation. One of the most distinct, and perhaps surprising, was the large

discrepancy in cytokine production. Whilst BiTE-mediated activation induced 9-fold higher levels of IFN γ than T cells stimulated with CD3/CD28 beads, the levels of IL-2 were reduced 10-fold. The reason for this is unclear, however it is likely to be a consequence of the varying levels of CD3 stimulation or the presence of CD28 co-stimulation. Consistent with this, it is reported that CD28 mediates a myriad of functions, such as proliferation and survival. In fact, the CD28-PI3K-AKT and CD28-GRB2-Ras pathways may be the predominant signalling mechanisms for IL-2 expression (Janardhan et al, 2011), with CD28 ligation reported to augment production of IL-2 (Boise et al, 1995). Hence, whilst BiTEs are reported to lack a dependence on co-stimulation for T cell activation, this data may suggest that EpCAM BiTE-mediated activation is insufficient to fully induce all T cell effector functions, for example, production of IL-2. This phenomenon highlights the complexities of T cell signalling and activation. In particular, it raises the possibility of rationally designing BiTEs or alternative engagers to allow stronger or a more targeted modulation of specific T cell effector functions – for example, by providing co-stimulation or blocking checkpoint receptors.

4. Engineering, production and characterisation of an oncolytic adenovirus expressing a fibroblast-targeted BiTE

4.1 Introduction

Whilst we have exemplified the technology in chapter three using a BiTE that recognises a carcinoma-associated antigen (EpCAM), it is also possible to use the BiTE approach to target cytotoxicity to stromal cells of the tumour microenvironment (TME). One particularly attractive target is the cancer-associated fibroblast (CAF). There is a strong rationale for this. There is increasing awareness of the roles of CAFs in promoting tumour growth and mediating immune suppression, with therapeutic targeting of CAFs leading to significant benefit in preclinical models (Kraman et al, 2010; Santos et al, 2009; Wen et al, 2010). Stromal cells are also less genetically unstable than tumour cells, and selection of antigen-negative cells – a common cause of resistance to therapy - is less likely to occur. As the mechanisms by which CAFs supports tumourigenesis are common to most cancers, targeting such cells could provide therapeutic benefit in a broad spectrum of cancers. Thus, the use of OV_s armed with anti-CAF BiTEs offers a powerful means to target both tumour cells and stromal fibroblasts, with the added advantage of potentially synergistic activity (Figure 4.1).

While stromal interventions have a strong therapeutic rationale, implementation can be difficult due to the lack of unique CAF surface markers. Although they do have

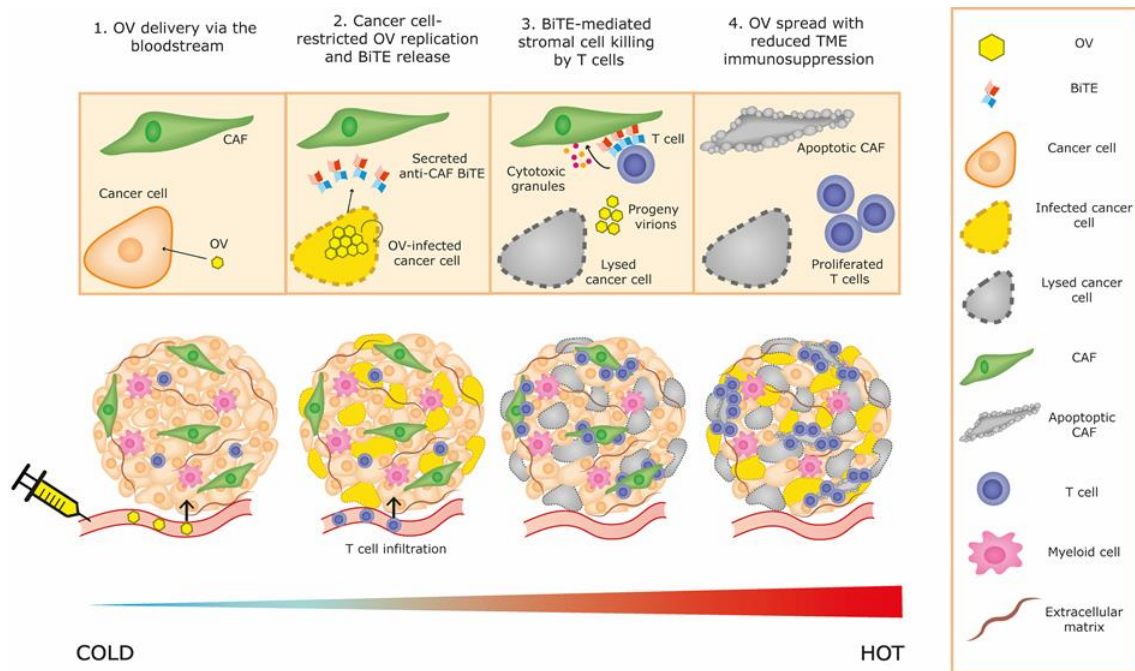


Figure 4.1. Targeting tumour cells and CAFs by arming EnAd with a CAF-targeted BiTE. Infection of cancer cells with a BiTE-armed OV leads to tumour-restricted expression of the BiTE. The BiTE will direct the cytotoxicity of tumour-infiltrating lymphocytes to CAFs, leading to reduced CAF numbers and the potential lifting of CAF-induced immune suppression. Additionally, virus replication and spread through the tumour will target cancer cells, and possibly induce the infiltration of more T cells. In this way, infection with OVs encoding CAF-targeted BiTEs may transform an immunologically ‘cold’ tumour into one that is ‘hot’. Image acquired from (Scott et al, 2017).

characteristic features or upregulated receptors, these are often shared with normal fibroblasts undergoing physiological functions elsewhere in the body. Fibroblast activation protein (FAP), for example, is highly and consistently expressed on CAFs across a broad range of cancer indications (Garin-Chesa et al, 1990) but it is also found on fibroblasts in some connective tissue in the muscle, gall bladder, bladder and bone marrow (Roberts et al, 2013).

Preclinical studies eliminating FAP positive cells from murine tumour models with antibodies or FAP-targeted CAR-T cells generally demonstrate positive results, particularly when combined with interventions such as cancer vaccines (Wang et al,

2014). For example, Kraman *et al* showed that depletion of FAP⁺ stromal cells in tumour-bearing mice induced necrosis of the tumour mediated by IFN γ - and TNF α -expressing effector T cells. While most reports conclude anti-FAP therapies are well tolerated, at least one study showed extensive bone marrow toxicity and cachexia (Tran *et al*, 2013). The authors of this study suggest toxicity is related to the potency of the immune response against the FAP-positive cells within these compartments.

Therefore, a potent therapeutic approach targeted towards a ubiquitously expressed stromal markers such as FAP should not be given systemically but generated locally within each tumour nodule. Oncolytic viruses are a powerful means to achieve this objective. Coupling BiTE production to virus replication allows expression of the FAP-targeted BiTE to be spatially restricted to the tumour, minimising systemic toxicities and providing a two-pronged approach that could yield a more complete therapeutic response.

In this chapter, we describe the engineering and expression of a novel FAP-targeted BiTE and its encoding in oncolytic adenovirus, EnAd. We show that by coupling expression to the adenovirus major late promoter, the FAP BiTE is selectively expressed and secreted from cancer cells, and that BiTE expression leads to the T cell-mediated destruction of FAP⁺ fibroblasts in mixed cell populations.

4.2 Chapter aims

1. Engineer and produce a novel fibroblast-targeted BiTE.
2. Assess the ability of the FAP-targeted BiTE to activate primary human T cells and kill FAP⁺ fibroblast cell lines.
3. Encode the FAP BiTE within EnAd and evaluate the ability of the armed virus to activate T cells and induce lysis of fibroblast cell lines.
4. Explore the prospect of coupling FAP BiTE transcription to the adenovirus major late promoter for tumour-restricted expression.

4.3 Results

4.3.1 Engineering and production of a fibroblast-targeted BiTE

A FAP-targeted BiTE was produced by the joining of two single chain antibody fragments (scFvs) recognising FAP or CD3 ϵ with a flexible glycine-serine linker. In this case, the CD3 ϵ binding domain was derived from I2C monoclonal antibody, different to the L2K-derived scFv within the EpCAM BiTE. Additionally, the fusion BiTE protein was engineered to contain an N-terminal immunoglobulin signal sequence for mammalian secretion and a C-terminal decahistidine affinity tag for detection. A schematic of the structures of the FAP-targeted and control BiTE is shown in Figure 4.2A. To achieve this, a FAP-specific T cell engager sequence was generated from an obtained protein sequence and the gene synthesised. The BiTE transgene-containing pUC57 plasmid was digested with NcoI and XbaI to yield a 1607 bp fragment (Figure 4.2B, left panel) and ligated into cleaved and dephosphorylated pSF-CMV vector (Figure 4.2B, right panel) yielding a plasmid in which the BiTE transgene is placed under transcriptional control of the immediate-early CMV promoter (pSF-CMV-FAPBiTE; displayed in Figure 4.2C). The correct insertion of BiTE transgene was confirmed by restriction digest screen (Figure 4.2D) and DNA sequencing.

To evaluate the expression and secretion of the FAP BiTE protein, 2×10^7 HEK293A cells were transfected with the pSF-CMV-FAPBiTE expression vector using Lipofectamine 2000. At 48 hours, supernatants were harvested and concentrated 50-fold. In parallel, cells were transfected with CMV promoter-driven expression plasmids for GFP and the control BiTE, serving as a negative (mock) and positive control,

respectively. Supernatants were separated by SDS-PAGE and analysed by western blot using the histidine affinity tag for BiTE detection. For each transgene, equivalent proportions of cell lysate and supernatant were loaded to allow visual assessment of the proportion of BiTE in each compartment. FAP BiTE protein was exclusively present in the supernatant, demonstrating its successful expression and secretion (Figure 4.3A). However, the level of expression appeared to be considerably lower than that of the

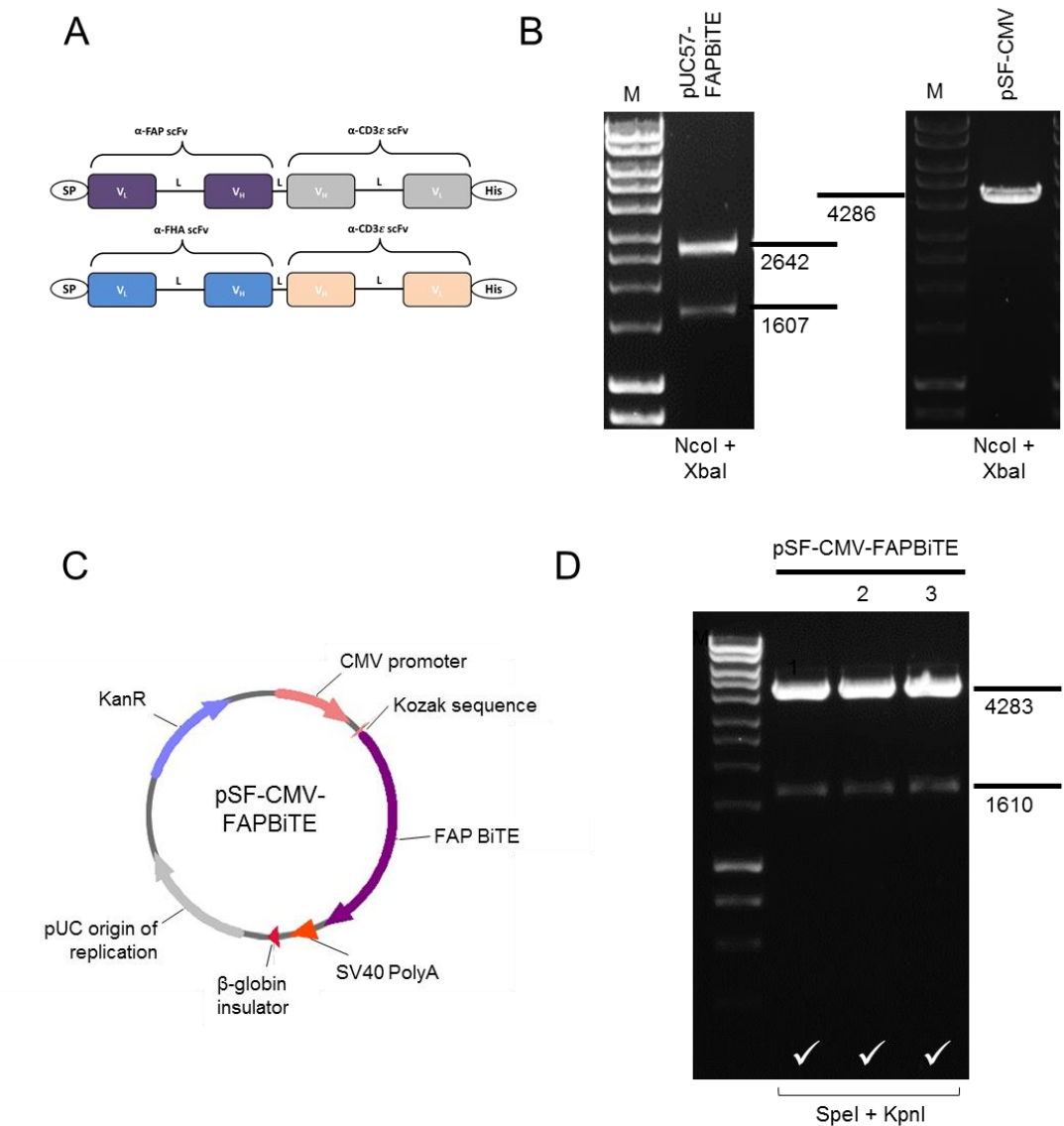


Figure 4.2. Engineering of a mammalian expression vector for FAP-targeted BiTE expression. (A) Schematic representation of the structure of the BiTEs used in this chapter. Each BiTE is composed of two single chain antibody fragments (scFvs) of different specificities, joined by a flexible glycine serine (L) linker. The FAP and control (filamentous haemagglutinin adhesion, FHA-targeted) BiTE share a second specificity for CD3 ϵ of the T cell receptor complex. Each BiTE possesses an N-terminal immunoglobulin light chain signal peptide (SP) for secretion and a C-terminal decahistidine affinity tag for detection (His). VL, variable light chain; VH, variable heavy chain. **(B)** Schematic of the expression plasmid engineered for mammalian expression of the FAP BiTE under transcriptional control of a CMV promoter. KanR, Kanamycin resistance cassette; CMV, Cytomegalovirus; SV40, Simian vacuolating virus 40. **(C)** Double digest of the pUC57-Kan-FAPBiTE gave bands of the expected molecular weight (left), with the 1.6 kb fragment gel-extracted and ligated into the cleaved pSF-CMV expression vector (right). M, Hyperladder 1 kb DNA ladder. **(D)** Purified plasmids isolated from colonies generated in the ligation of the products from (C) were screened for transgene insertion by digestion with SpeI and KpnI, with the correct insertion giving bands at 4283 and 1610 bp. Colonies with the correct digestion pattern are indicated with a tick. M, Hyperladder 1 kb DNA ladder.

control BiTE. In addition to this, the FAP BiTE demonstrated a higher mobility despite both proteins having a similar molecular weight. To investigate if such a discrepancy in mobility is due to differences in post-translational modification (PTM), the BiTE transgenes were subcloned into pET-16b plasmids for bacterial expression (data not shown). Proteins expressed from Rosetta™ 2 (BL21) cells are predicted to lack N- and O-linked glycosylation, and therefore the observed differences in mobility may be eliminated if due to PTM. Indeed, the mobility of bacterially-expressed BiTE was identical to that in mammalian cells with a similar discrepancy in mobility (data not shown). This suggests that glycosylation is not the cause, and the disparity in mobility is likely reflective of intrinsic structural- or sequence-specific differences between the proteins. As shown in Figure 4.3B, specific binding to recombinant FAP protein by the FAP BiTE and not control BiTE was confirmed by ELISA.

The filamentous hemagglutinin (FHA)-binding control BiTE generated in chapter three and used thus far possesses a scFv (specific for CD3 ϵ) derived from the L2K monoclonal antibody, which differs from that of the I2C-derived scFv of the FAP BiTE (highlighted

in Figure 4.2A). To act as a true surrogate for unclustered BiTE, the control BiTE and FAP BiTE must share an identical CD3 ϵ -binding scFv domains. Therefore, the FAP BiTE protein was modified to possess this L2K scFv (Figure 4.4A). FAP- and CD3 ϵ -binding DNA sequences were amplified from the relevant template by PCR, yielding bands of the expected molecular weight (Figure 4.4B). The 898 and 821 bp fragments were gel-extracted and inserted into linearised expression vectors (Figure 4.4B) by Gibson assembly. Correct insertion of the modified FAP BiTE was screened by digestion (Figure 4.4C) and DNA sequencing.

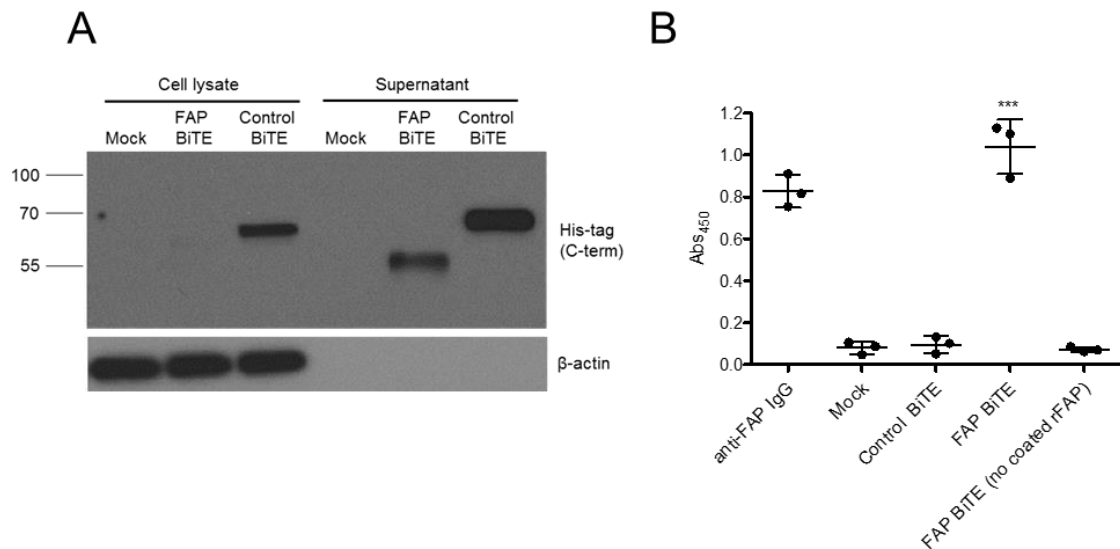


Figure 4.3. Expression, secretion and target binding of a FAP-targeted BiTE. (A) Western blot analysis showing secretion of the FAP BiTE. HEK293A cells were transfected with pSF-CMV-FAPBiTE or pSF-CMV-controlBiTE expression plasmids. After 24 hours, supernatants and cell lysates were harvested, concentrated, and assessed for the presence of BiTE protein by SDS-PAGE and western blot. Blots were probed with a mouse anti-histidine (C-terminal) antibody, followed by an HRP-conjugated anti-mouse secondary antibody. (B) ELISA measuring the binding of the FAP and control BiTE to recombinant FAP protein. BiTE binding was detected using an anti-histidine (C-terminal) antibody, followed by an anti-mouse secondary antibody. Each condition was measured in technical triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'mock', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

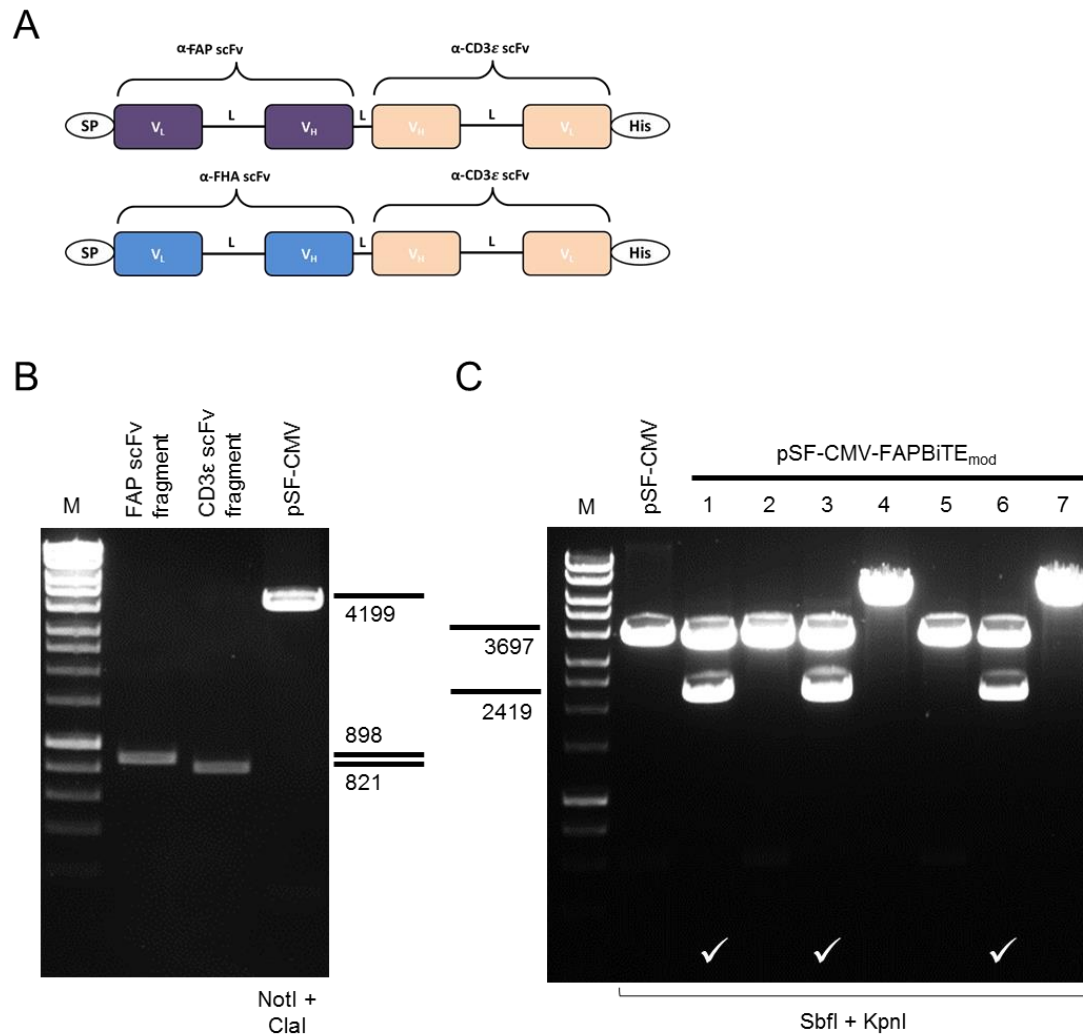


Figure 4.4. Engineering of FAP-targeted BiTE possessing the L2K scFv specific for CD3 ϵ . (A) Schematic illustration of the domain structure of the modified FAP BiTE and irrelevant control BiTE. (B) The FAP- and CD3 ϵ -binding scFv domains were amplified by PCR using pSF-CMV-FAPBiTE and pSF-CMV-controlBiTE, respectively. Bands of the expected size were gel-extracted, and inserted into a linearised a CMV expression vector by Gibson assembly. M, Hyperladder 1 kb DNA ladder. (C) Recombinant plasmids generated from the Gibson assembly reaction of the products in (B) were screened for successful transgene insertion and orientation. The correct plasmids show bands at 3697 and 2419 bp and colonies with the correct digestion pattern are indicated with a tick. M, Hyperladder 1 kb DNA ladder.

With successful assembly confirmed, the expression and secretion of the modified FAP BiTE was evaluated by western blot using BiTE-containing supernatants prepared from transfected HEK293A cells. As with the original FAP BiTE, the modified FAP BiTE was detected nearly exclusively in the supernatants of transfected cells, demonstrating

successful expression and secretion (Figure 4.5A). Large scale preparations of FAP and control BiTE-containing supernatants were produced for further characterisation. BiTE expression plasmids were transfected into HEK293A cells (3×10^7 cells in a T175 flask) using polyethylenimine (PEI, linear, MW 25000). After 48 hours, supernatants were harvested and cellular components removed by centrifugation. Supernatants were concentrated 50-fold and aliquots stored at -80°C until further analysis. To estimate the amount of BiTE produced, supernatants were serially diluted and evaluated by dot blot using decahistidine-tagged cathepsin D as a protein standard (Figure 4.5B). The modified FAP BiTE, showed high levels of expression (estimated at $15 \mu\text{g/mL}$), comparable to the EpCAM and control BiTE, and at least 10-fold higher than the original FAP BiTE (Figure 4.5B). Comparable binding (manifest as increase in Abs_{450}) to recombinant FAP protein by the original and modified FAP BiTE was confirmed by ELISA (Figure 4.5C) suggesting no detrimental effect of modification on FAP binding. Hereafter, all instances of “FAP BiTE” refer to the modified FAP BiTE.

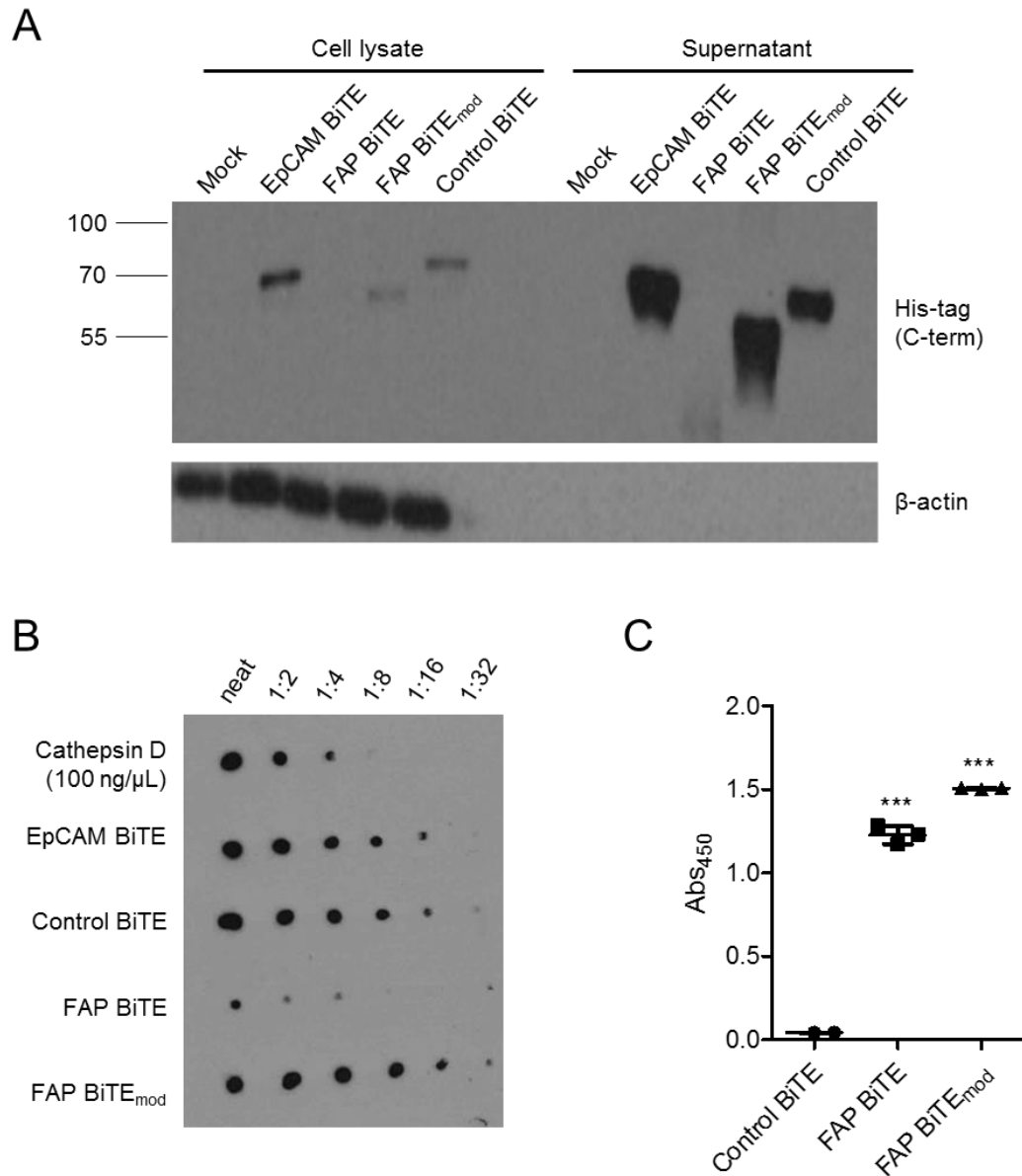


Figure 4.5. The modified FAP BiTE is expressed in larger quantities than the unmodified protein from HEK293A cells. (A) Expression and secretion of all BiTEs used in this study were evaluated by western blot. Supernatants and cell lysates were harvested from transfected HEK293A cells. BiTE expression was evaluated by western blot using an anti-histidine antibody for detection. (B) A dot blot was performed to estimate the quantity of FAP BiTE protein secreted from transfected HEK293A cells. Decahistidine-tagged Cathepsin D at known quantities was used as a protein standard to determine the quantity of FAP BiTE protein. (C) An ELISA demonstrating binding of the modified FAP BiTE to recombinant FAP protein. Binding was measured using a mouse anti-histidine antibody, followed by probing with an HRP-conjugated anti-mouse secondary antibody. Abs₄₅₀ was used to assess successful binding. Each condition was measured in technical triplicate and represented as mean ± SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'control BiTE, *p<0.05, **p<0.01, ***p<0.001.

4.3.2 Free FAP BiTE induces polyclonal activation of primary human T cells

The ability of recombinant FAP BiTE to induce T cell activation was evaluated in co-cultures of CD3-purified PBMC with normal human dermal fibroblasts (NHDF), which acquire an ‘activated’ phenotype and express FAP on their surface when cultured in growth media supplemented with high (10%) serum (confirmed by flow cytometry in Figure 4.6A). Incubation of NHDF and PBMC with 2.5 $\mu\text{g/ml}$ of FAP BiTE from supernatants of transfected HEK293 cells induced a significant increase in T cells expressing activation markers CD69 and CD25 at 24 hours, with the control BiTE having no effect (Figures 4.6B and C). Importantly, no activation of T cells was seen when they were incubated with the FAP BiTE in the absence of NHDF, suggesting that CD3 clustering is essential for activation (similarly demonstrated for the EpCAM BiTE in

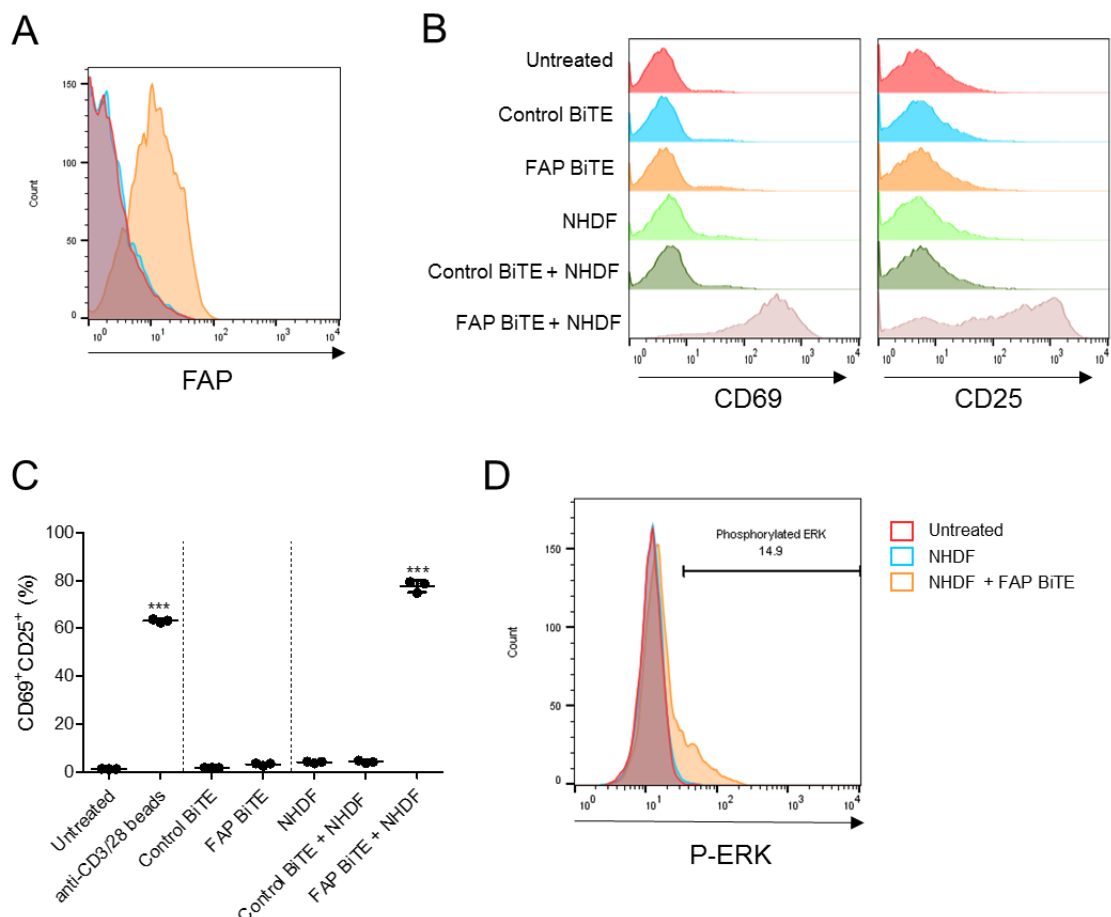


Figure 4.6. FAP BiTE-containing supernatants activate primary human T cells. (A) Flow cytometry spectra assessing the expression levels of surface FAP (orange) on NHDF cells. Unstained (red) and cells stained with an isotype control (blue) were included to determine background fluorescence and unspecific binding, respectively. **(B)** BiTE-mediated activation of primary human T cells in co-culture with NHDF (5:1) and BiTE-containing supernatants. Activation was assessed by expression of activation markers CD69 and CD25 and measured by flow cytometry at 24 hours. Anti-CD3 and CD28 Dynabeads were used as a positive control for T cell activation. **(C)** Percentage of CD69⁺ CD25⁺ T cells derived from the flow cytometry data collected in (B). Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', *p<0.05, **p<0.01, ***p<0.001. **(D)** Phosphorylation of ERK1/2 in PBMC-derived T cells was measured by intracellular staining with a PhosFlow Anti-ERK1/2 (pT202/pY204) antibody. T cells were co-cultured with NHDF cells (5:1) and BiTE-containing supernatants and phosphorylation status analysed by flow cytometry 30 minutes after stimulation.

chapter three). These results are further supported by the phosphorylation of ERK1/2 (downstream of TCR signalling) in primary T cells cultured with NHDF and FAP BiTE, but not NHDF alone (Figure 4.6D).

Moreover, culturing CFSE-labelled PBMC T cells with NHDF and the FAP BiTE induced multiple rounds of proliferation (Figure 4.7A), assessed by the dilution of the dye upon each round of division, and evident as peaks in flow cytometry histogram, allowing a quantitative analysis of T cell proliferation. Treatment with the FAP BiTE, but not control BiTE, induced 34.5% of parental population to enter a proliferative cycle after five days, with each cell of the total population completing 0.8 cycles on average (displayed as division index) (Figure 4.7B). The addition of counting beads to each sample prior to flow cytometric analysis allowed absolute T cell numbers to be determined, demonstrating a 3.8-fold increase in the number of T cells when cultured with NHDF and FAP BiTE-containing supernatants (Figure 4.7C).

To further characterise the activation of primary T cells by the FAP-targeted BiTE, the production of IFN γ and IL-2 (associated with a Th1 CD4 and CD8 response) was

evaluated. Cytokine levels were determined by ELISA using supernatants from BiTE-stimulated primary T cells in co-culture with NHDF (Figure 4.8A). As before, the control BiTE had no observable effect, whereas FAP BiTE-mediated activation induced substantial increases in IFN γ and IL-2 with levels peaking at 100 ng/mL (at 72 hour), and 500 pg/mL (at 24 hours), respectively. Like EpCAM BiTE-mediated stimulation, the FAP BiTE induced 10-fold higher IFN γ production and substantially less IL-2 than that induced by CD3/CD28 Dynabeads. Interestingly, the levels of FAP BiTE-induced IL-2 were reduced six-fold compared to incubation of T cells with the EpCAM BiTE and DLD cells. This is possibly due to potential differences in the density of antigen on the target cell or variation in the binding affinities of BiTE for FAP or EpCAM. Each potentially leads to varying magnitudes of CD3 ϵ signalling and will be the focus of future research. T cells were shown to be the source of this IFN γ , as assessed by intracellular staining and flow cytometry after six hours of co-culture with BiTE-containing supernatants and NHDF cells (Figure 4.8B).

The milieu of cytokines produced upon activation can provide hints as to the phenotype of the T-cells that are activated. A multiplex cytokine bead array was used to characterise the expression of 11 key cytokines (Figure 4.8C). The control BiTE did not induce significant changes in any cytokine. However, the FAP-targeted BiTE led to strong increases in IFN γ , IL-2, TNF α , IL-17 and IL-10, consistent with the stimulation of different T cell subsets. Taken together, the induction of activation markers, proliferation and cytokines was not observed in any setting upon incubation with control BiTE or in the absence of NHDF target cells, consistent with the necessity of CD3 ϵ clustering to induce full activation of T cells.

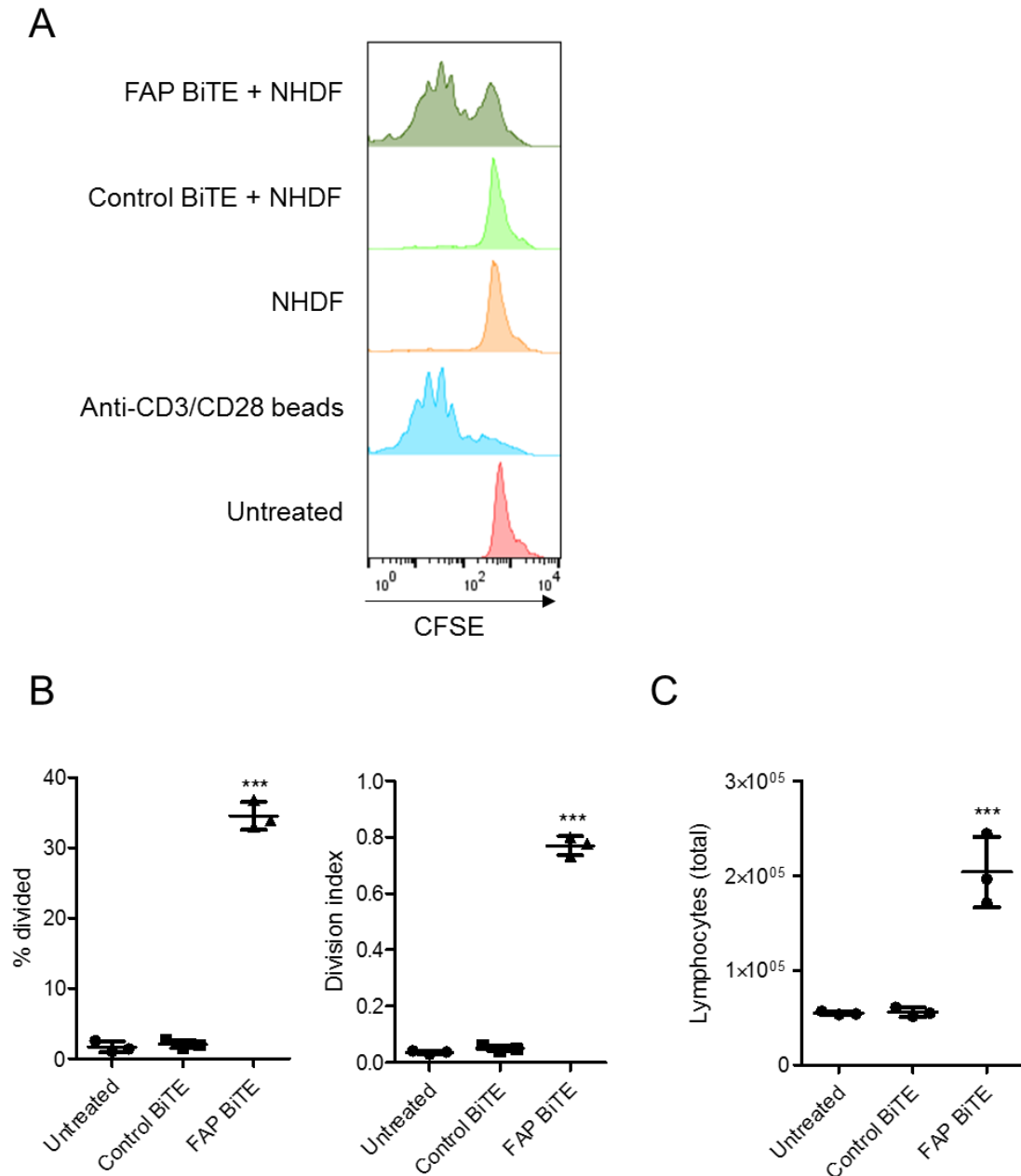


Figure 4.7. Stimulation with FAP BiTE induces proliferation of primary human T cells. (A) Flow cytometry histogram demonstrating proliferation of CFSE-stained T cells in co-culture with NHDF cells (5:1) and BiTE-containing supernatants. Each division produces two daughter cells with half the quantity of CFSE and therefore fluorescence. Fluorescence was measured by flow cytometry five days after co-culture. (B) The proliferation observed in (A) was modelled and is displayed here as division index and percentage of parental population that entered proliferation ('% divided'). (C) The total number of lymphocytes per well. Total numbers were determined by addition of counting beads to samples immediately prior to analysis by flow cytometry. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

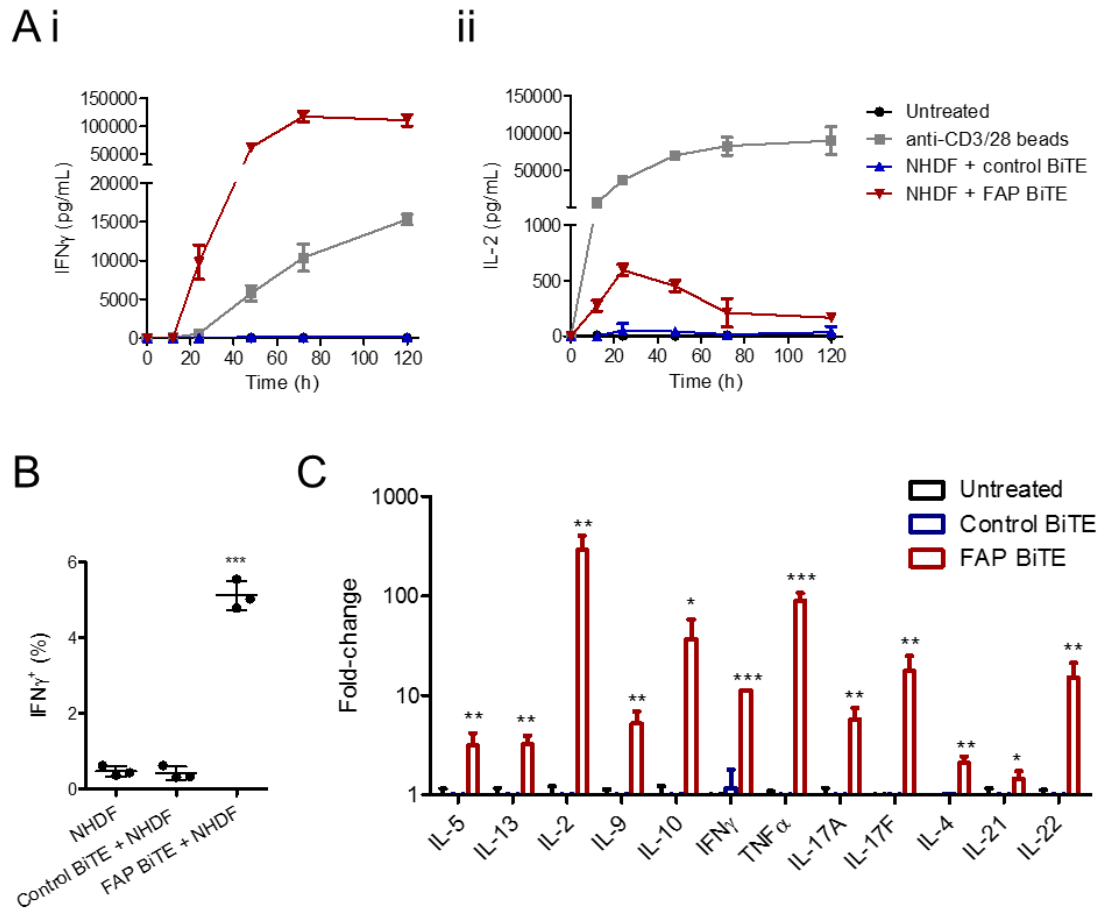


Figure 4.8. FAP BiTE induces production of inflammatory cytokines by primary human T cells. (A) ELISAs were performed to measure levels of IFN γ (i) and IL-2 (ii) in supernatants harvested from co-culturing PBMC-derived T cells with NHDF (5:1) and BiTE-containing supernatants. Anti-CD3 and CD28 Dynabeads were used as a positive control for T cell activation. (B) Percent of IFN γ -positive T cells after 6 hours in co-culture with NHDF cells (5:1) and BiTE-containing supernatants. (C) Production of 11 cytokines were evaluated by a multiple human Th cytokine panel. Supernatants were harvested from 48-hour co-cultures of T cells with NHDF cells (5:1) and BiTE-containing supernatants. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated' or 'NHDF', * p <0.05, ** p <0.01, *** p <0.001.

4.3.3 Characterisation of T cell induced fibroblast cytotoxicity by recombinant FAP BiTE

In addition to T cell activation, the artificial BiTE-mediated linkage of T cell to target cell should lead to T cell degranulation and target cell cytotoxicity. Stimulation with the FAP BiTE, but not control BiTE, in the presence of NHDF cells induced rapid degranulation (within 6 hours) of T cells, as determined by the externalisation of CD107a on the surface (Figure 4.9A). This induction of degranulation by the FAP BiTE translated to potent T cell-dependent fibroblast lysis, as observed by microscopy demonstrating the lifting and clumping of previously-adherent fibroblasts (Figure 4.9B). To quantify this cytotoxicity, the level of LDH released into the supernatant (correlating with the lysis of target cells) at 24 hours was assessed in co-cultures of primary T cells with NHDF and BiTE-containing supernatants. As expected, stimulation with the FAP BiTE induced significant T cell activation (by CD25 expression) and simultaneous target cell lysis (by LDH release), observed using T cells from all six donors (Figure 4.9C). This cytotoxicity was dependent on the effector: target ratio (Figure 4.9D) and the quantity of the FAP-targeted BiTE, producing a sigmoidal dose-response curve with an EC_{50} of 2.5 ng/mL (Figure 4.9E; left panel). The induction of activation marker CD25 on T cells was also dependent on FAP BiTE dose, producing a similar sigmoidal curve with an EC_{50} of 16 ng/mL (Figure 4.9E; right panel). No cytotoxicity was induced by the control BiTE, consistent with T cells remaining in an inactivated state.

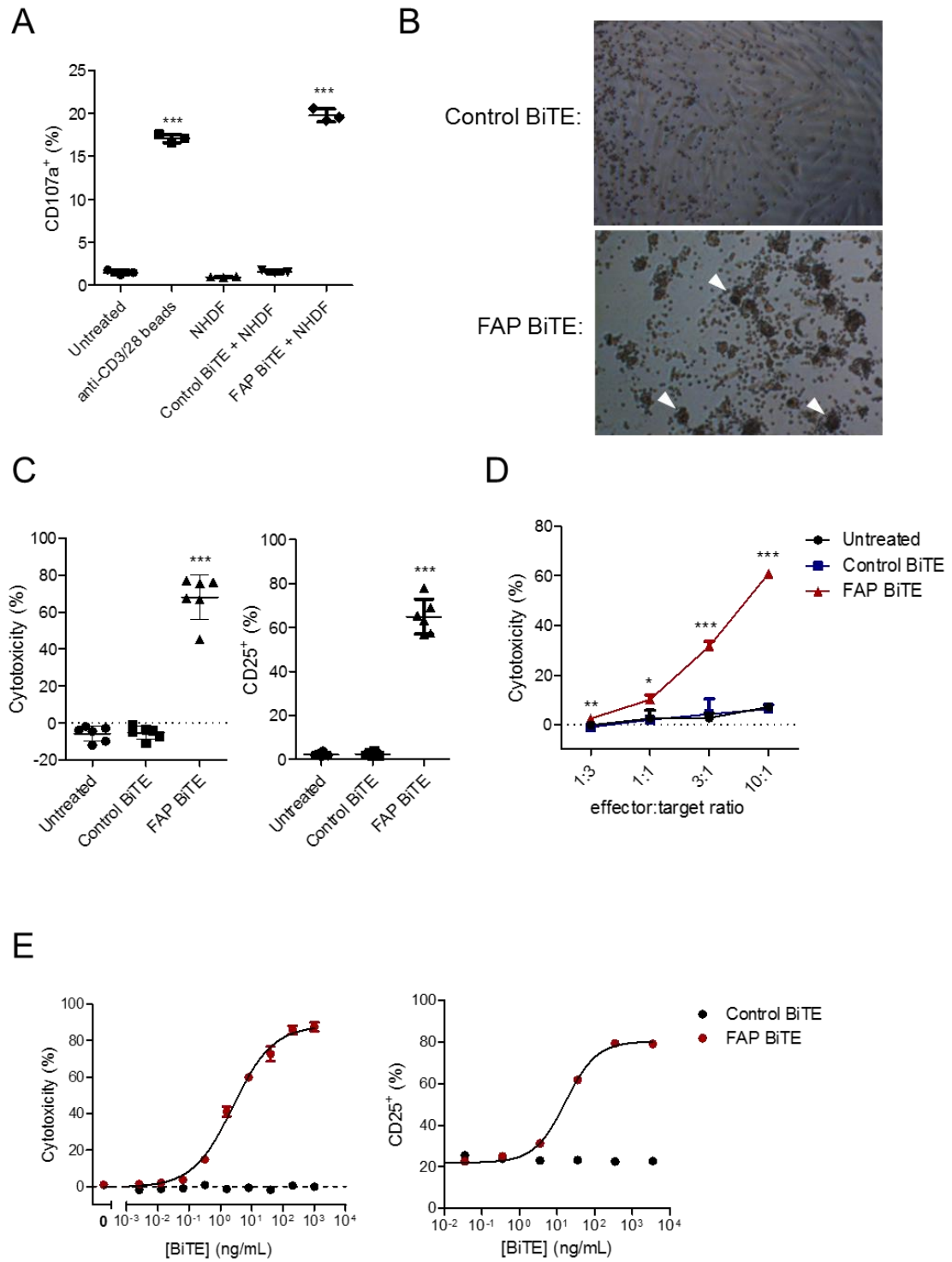


Figure 4.9. FAP-targeted BiTE induces T cell degranulation and targets cytotoxicity to FAP⁺ cells. (A) Degranulation of T cells in culture with NHDF cells (5:1) and BiTE-containing supernatants. Degranulation was assessed by externalisation of CD107a after six hours culture with a CD107a-specific antibody and measured by flow cytometry. CD3/CD28 Dynabeads were used as a positive control for T cell degranulation. (B) Representative microscopy images of co-cultures of primary T cells and NHDF (5:1) after 24 hours of stimulation with BiTEs. Dead fibroblasts are indicated with white triangles. (C) Lysis of NHDF as measured by LDH release (left panel) and CD25 induction on T cells (right panel) was assessed after 24 hours co-culture with PBMC-derived T cells (E:T 5:1) from six healthy donors and BiTE-containing supernatants. (D) T cell-mediated cytotoxicity of NHDF cells was evaluated. NHDFs were cultured with BiTE-containing supernatants and PBMC-derived T cells at various E:T ratios. Cytotoxicity was assessed by LDH release into culture supernatants after 24 hours co-culture. (E) Cytotoxicity of NHDF cells (left) and activation of CD3-purified PBMC (right) cultured for 24 hours at an E:T of 5 with 10-fold serial dilutions of BiTE-containing supernatants. Cytotoxicity was assessed by LDH release into culture supernatants and T cell activation. T cell activation was assessed by surface CD25 expression. (A, C, D) Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.4 Antigen-specificity of T cell activation and target lysis induced by FAP

BiTE

To assess the dependence of surface antigen expression for BiTE-mediated T cell activation, we first established antigen-negative and antigen-positive cell lines. Chinese Hamster Ovary (CHO; FAP-negative) cells were transduced with a lentivirus transduction system for stable surface FAP expression (Figure 4.10A; see Materials and Methods for their production). Culturing of primary T cells with FAP BiTE and CHO-FAP cells induced strong T cell activation (Figure 4.10B), which was associated with target cell lysis (Figure 4.10C). Neither T cell activation nor lysis were observed with parental CHO cells or control BiTE, demonstrating the necessity of surface antigen expression for T cell activation.

Previous characterisation of the EpCAM-targeted BiTE demonstrated the amount of EpCAM expression influences the degree of BiTE-mediated cytotoxicity (chapter three). To investigate whether there is a similar relationship between level of surface FAP

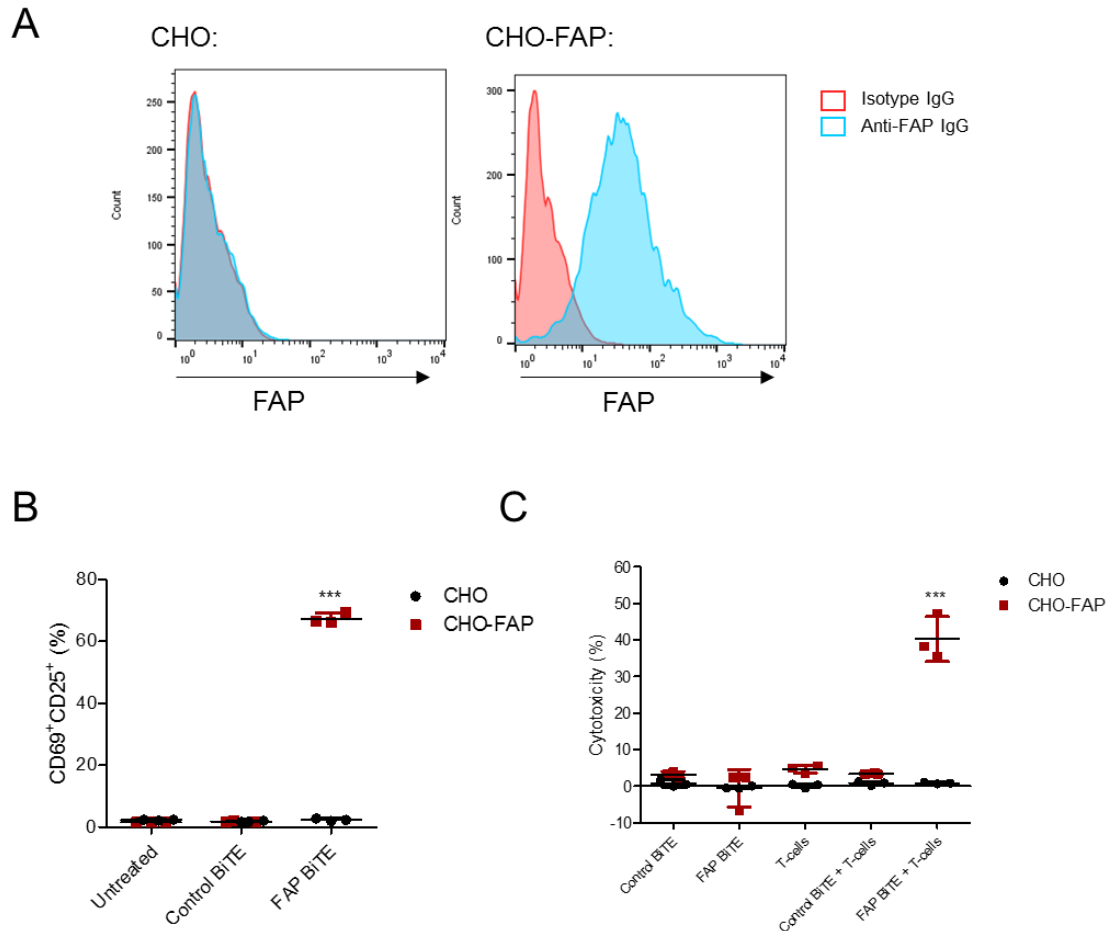


Figure 4.10. FAP BiTE-mediated cytotoxicity is antigen-specific. (A) Flow cytometry histogram of surface FAP expression (blue) on parental CHO and CHO-FAP cell lines. Isotype control antibodies were used to determine background binding in the absence of FAP (red). (B) Proportion of dual CD69/CD25-positive primary CD3 cells cultured with BiTE-containing supernatants and CHO or CHO-FAP cells (E:T 5:1). After 24-hour culture, cells were harvested and activation markers were measured by flow cytometry. (C) Cytotoxicity of CHO or CHO-FAP in (B) was assessed by LDH released into culture supernatants after 24-hour incubation. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated' or 'T cells', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

expression and FAP BiTE-induced lysis, cytotoxicity assays were performed using two CHO-FAP clones, selected for high or low FAP expression (as determined by flow cytometry; Figure 4.11A). Incubating CHO-FAP cells with primary T cells and serially diluted FAP BiTE-containing supernatants induced similar levels of cytotoxicity at all tested BiTE concentrations (Figure 4.11B). Whilst dependent on the presence of surface FAP (parental CHO cells were not lysed), the degree of cytotoxicity was unaffected by

the level of this expression with comparable EC₅₀ for each clone. It is possible that both clones expressed sufficient surface FAP for maximal cytotoxicity, and clones with lower levels of FAP expression should be selected and evaluated in future.

4.3.5 Characterisation of T cell subsets activated by recombinant FAP BiTE

As reported in chapter three, a BiTE targeting EpCAM could activate both CD4 and CD8 T cells. Perhaps more surprisingly, EpCAM BiTE-activated CD4 cells mediated tumour

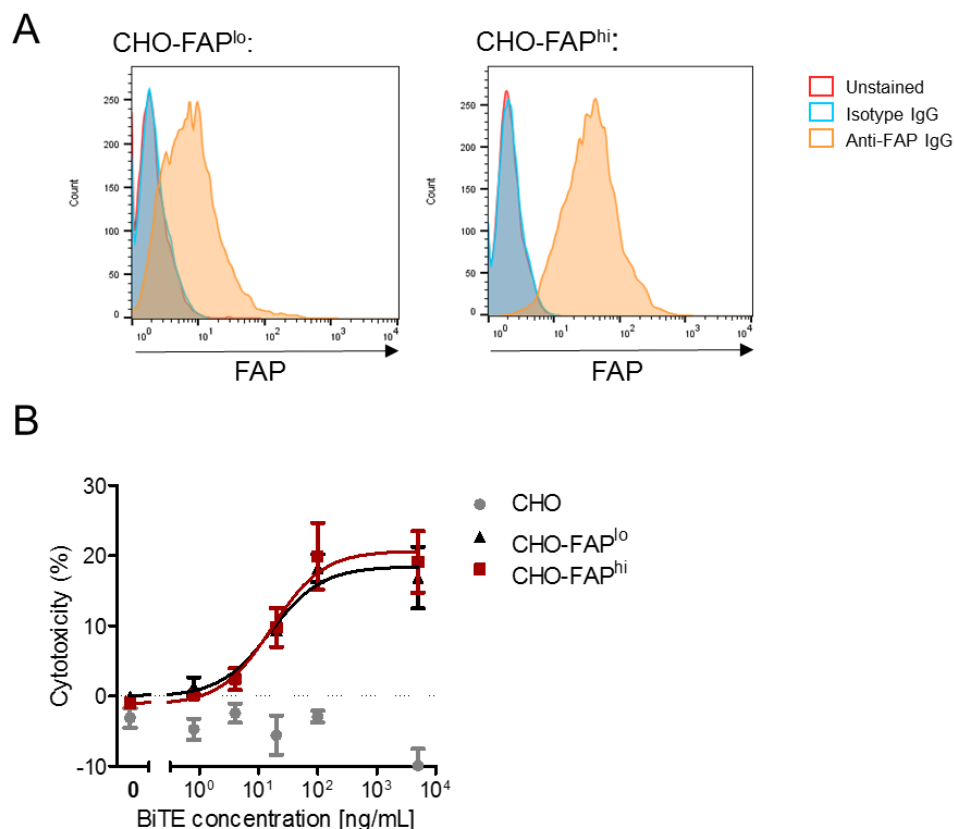


Figure 4.11. Influence of levels of FAP expression on BiTE-mediated cytotoxicity. (A) Flow cytometry spectrum of surface FAP expression on two selected CHO-FAP clones. Isotype control antibodies were used to assess unspecific binding of the antibody. **(B)** Cytotoxicity of CHO, CHO-FAP^{lo} or CHO-FAP^{hi} cells in co-culture with CD3-purified PBMC and 5-fold serial dilutions of FAP BiTE-containing supernatants. Cytotoxicity was assessed by LDH released into culture supernatants after 24-hour incubation. Each condition was measured in biological triplicate and represented as mean $\pm$ SD.

cell lysis comparable to that effected by CD8 cells. Similarly, both CD8 and CD4 cells cultured with the FAP BiTE and NHDF showed high levels of CD69 and CD25 expression, with higher induction in the CD4 subset (Figure 4.12A and 4.12B). FAP BiTE-mediated degranulation of each subset was compared via externalisation of CD107a, with similar levels of degranulation observed for both (Figure 4.12C). This translated to comparable levels of FAP BiTE-mediated cytotoxicity of NHDF cells (Figure 4.12D) indicating that both populations can contribute to FAP BiTE-mediated cytotoxicity.

4.3.6 Generation of FAP BiTE-armed oncolytic adenoviruses

Enadenotucirev (EnAd) is a conditionally-replicating chimeric group B adenovirus with promising clinical activity and the potential to encode and express transgenes. We armed EnAd with the FAP-targeted BiTE inserted downstream of the fibre gene with expression under the transcriptional control of either an exogenous CMV promoter, resulting in high and constitutive levels of transgene expression, or downstream of a splice acceptor (SA) site for the viral major late promoter (MLP), coupling transgene expression to the late phase of adenovirus infection. A schematic of modified viral genome is illustrated in Figure 3.12A. In the former arrangement, the BiTE will be expressed upon successful infection of a cell, whereas expression under the MLP will occur only in cells permissive to virus replication. To construct the modified viral genomes, the FAP BiTE fragments were generated by PCR from each respective vector containing the CMV promoter or an SA sequence was introduced directly upstream (Figure 4.13A, left panel). Bands of the expected size were ligated into an SbfI-linearised virus backbone by Gibson assembly

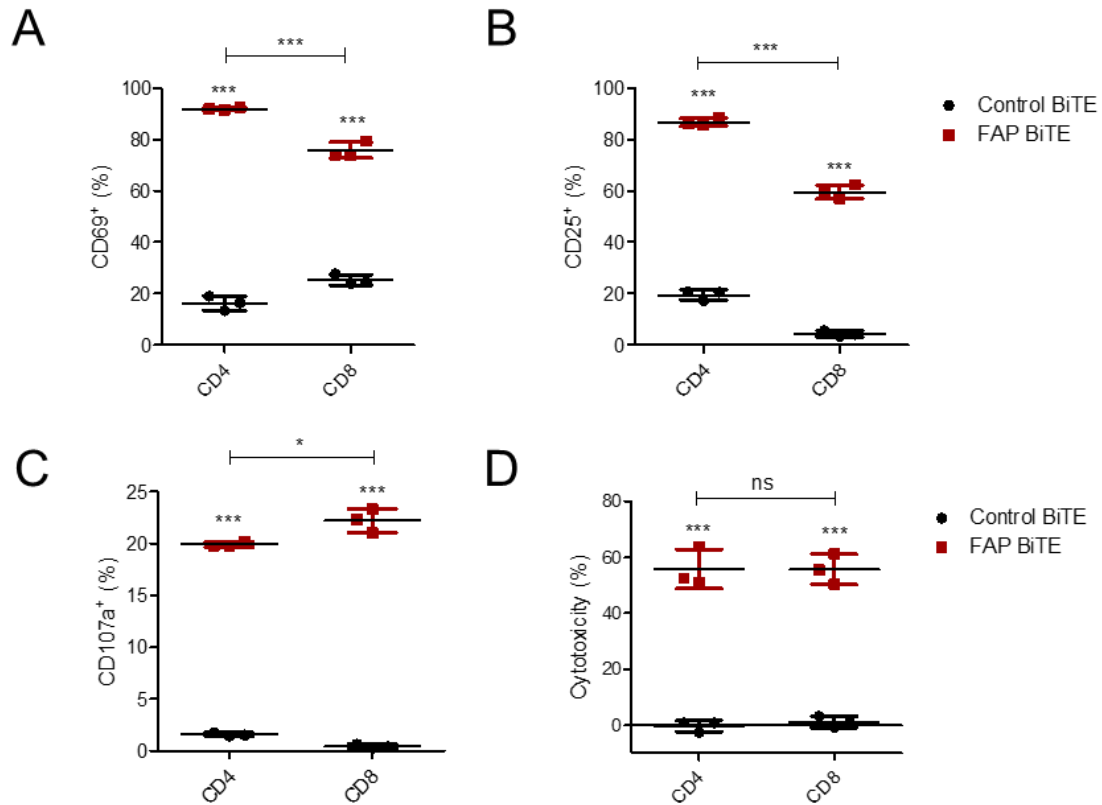


Figure 4.12. FAP BiTE activates and redirects cytotoxicity of both CD8 and CD4 T cells. (A, B) Induction of CD69 (A) and CD25 (B) on CD4 and CD8 T cells in co-culture with NHDF (5:1) and BiTE-containing supernatants. T cells were harvested after 24-hour co-culture and activation was assessed by flow cytometry. (C) Degranulation of CD4- and CD8-purified PBMC cells after six hours co-culture with NHDF (5:1) and BiTE-containing supernatants. Degranulation was assessed by adding a CD107a-specific antibody directly to the culture well at time zero, and measured by flow cytometry. (D) Cytotoxicity of NHDF cells by either CD4- or CD8-purified T cells cultured with BiTE-containing supernatants. Cytotoxicity was assessed by release of LDH into the culture supernatant at 24 hours. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using an unpaired, two-tailed t-test, * p <0.05, ** p <0.01, *** p <0.001.

(Figure 4.13A, right panel), and recombinant plasmids were screened for successful transgene insertion by digestion with BglII (Figure 4.13B; correct colonies marked with a tick). The resulting plasmids were denoted EnAd2.4-CMV-FAPBiTE and EnAd2.4-SA-FAPBiTE.

The modified viral genome plasmids were linearised using AscI to remove all non-viral genes required for molecular cloning and transfected into HEK293A cells to recover

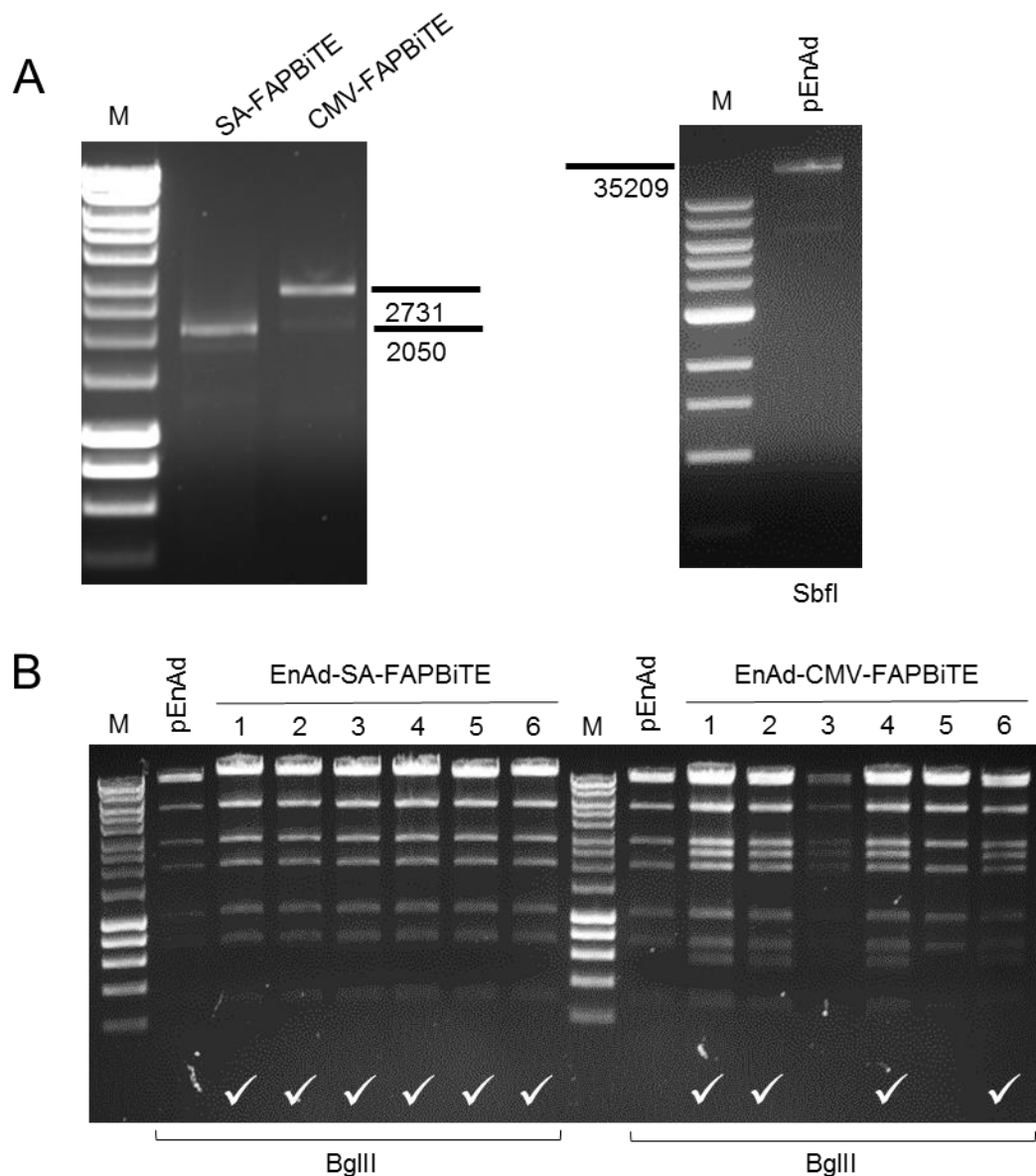


Figure 4.13. Generation of FAP BiTE-armed oncolytic adenoviruses. (A) PCR amplification of the FAP BiTE transgene was performed using pSF-CMV-FAPBiTE (left) for insertion by Gibson assembly into linearised and purified EnAd2.4 vector (right). Fragments generated by PCR included the CMV promoter or a splice acceptor sequence introduced directly upstream. A polyadenylation sequence was included downstream of the transgene in both cases. M, Hyperladder 1 kb DNA ladder. (B) Recombinant plasmids isolated from the Gibson assembly reaction in (A) were screened by digestion with BglII for correct insertion and orientation of the transgene. Colonies with the correct digestion pattern are indicated with a tick. M, Hyperladder 1 kb DNA ladder.

infectious virus. Recombinant viruses were successfully rescued, and five clones of each virus were selected. To assess BiTE expression and secretion from the modified virus clones, supernatants from infected HEK293A cells were evaluated 72 hours post-

infection by western blotting using an anti-His antibody (Figure 4.14A). BiTE expression was observed in all clones. One clone of each recombinant virus (indicated with a tick) was selected for batch production in hyperflasks and double purification by caesium chloride banding. As shown in Figure 4.14B, the titres of purified virus stocks were determined by the Picogreen dsDNA assay. The tissue culture infectious dose of each virus was determined by serial titration on A549 cells.

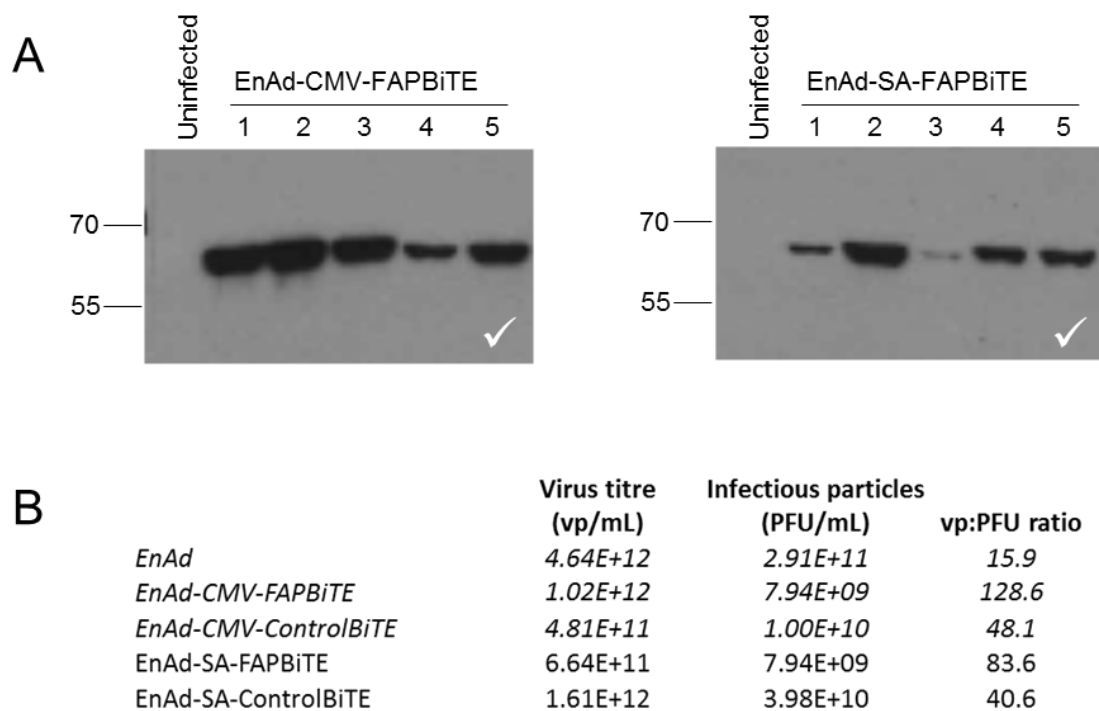


Figure 4.14. Clonal selection and production of FAP BiTE-armed oncolytic adenoviruses. (A) BiTE secretion of virus-infected HEK293A cells was assessed by western blot using an anti-His antibody. For each virus, transgene expression from five clones was evaluated after sufficient cytopathic effect had occurred. Supernatants from uninfected cells were used as a negative control. The clones chosen for large-scale production are indicated with a tick. **(B)** Table showing the virus titres and tissue culture infectious dose of the FAP BiTE expressing viruses, determined by dsDNA Picogreen assay and titration on A549 cells, respectively. The corresponding control viruses have been included for comparison (italic).

4.3.7 EnAd-BiTE viruses retain their oncolytic properties

To study the replication kinetics of the recombinant virus genomes, DLD colorectal adenocarcinoma cells were infected with 100 vp/cell of EnAd, or the FAP BiTE or control BiTE armed viruses (2×10^7 genome/mL), and viral genome copies quantified using qPCR primers against Ad11 viral hexon (Figure 4.15A). Within 24 hours of infection, the number of viral genome increased by 5,000-fold, demonstrating successful replication of viral DNA. Importantly, the yields of the recombinant viruses were indistinguishable from the parental virus showing no effect of BiTE ‘arming’ on viral replication kinetics. Next, we assessed the oncolytic properties of the viruses *in vitro*. DLD cells were infected with virus with increasing amounts of virus particles per cell and viability was measured on day five by MTS assay. All recombinant viruses had comparable levels of oncolytic activity compared to EnAd (Figure 4.15B). Collectively, this showed that the modification of the viral genome did not interfere with viral replication or intrinsic oncolytic activity.

4.3.8 Selective T cell activation and killing of FAP-positive cells by virally produced FAP BiTE

To confirm the expression and secretion of the FAP BiTE by infected cells, HEK293A cells were infected with EnAd, EnAd-CMV-FAPBiTE, or EnAd-SA-FAPBiTE, with the corresponding control viruses included as positive controls. Analysis of 72-hour supernatants by immunoblotting using an anti-His antibody confirmed that both FAP

BiTE-expressing viruses secreted similar levels of FAP BiTE into the supernatant (Figure 4.16A).

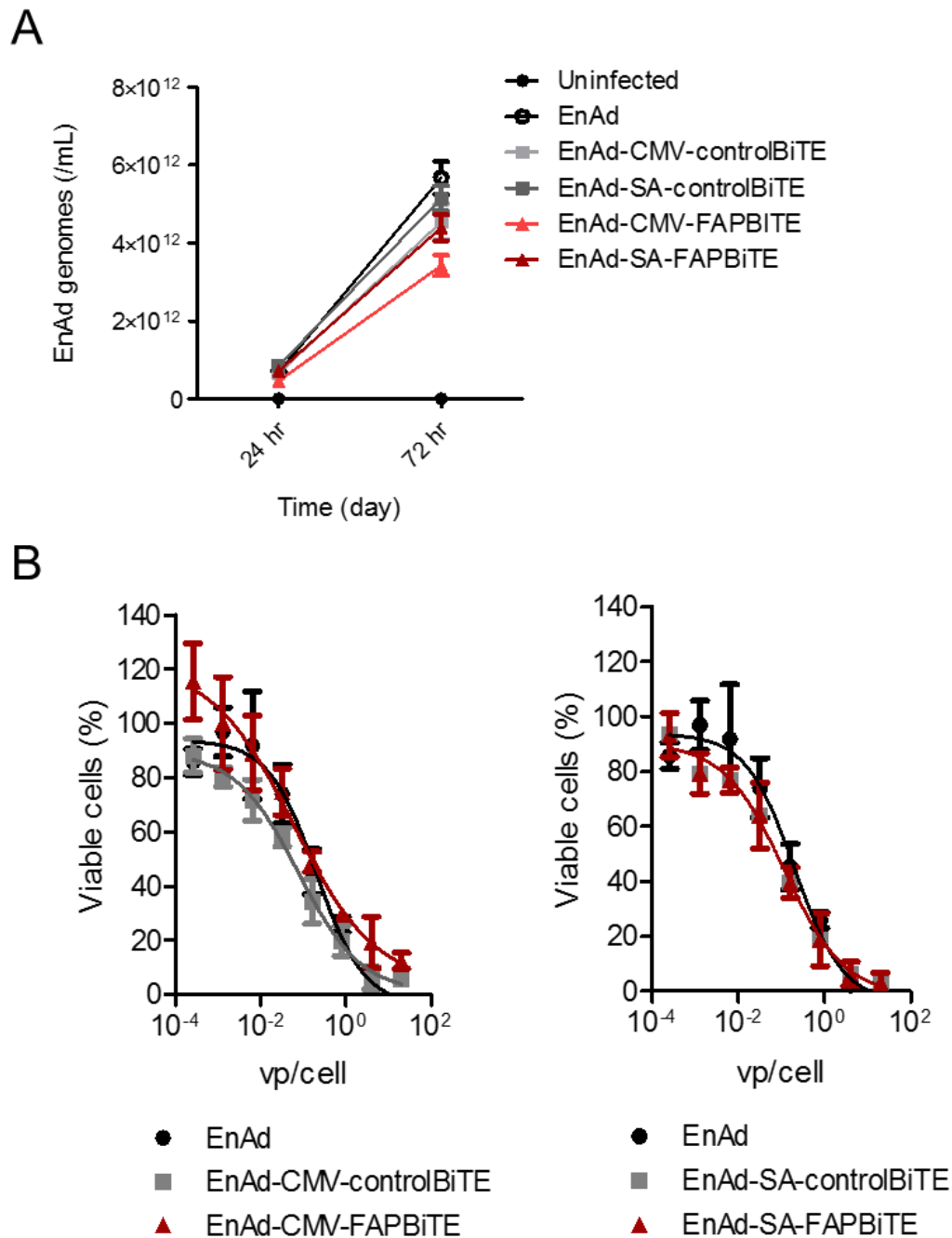


Figure 4.15. Characterisation of oncolytic properties of FAP BiTE expressing viruses. (A) Genome replication of parental EnAd or recombinant BiTE viruses. DLD cells were infected with virus (100 vp/mL) and wells harvested at 24 and 72 hours post-infection, with replication assessed by measuring viral genomes copies using qPCR primers against Ad11 viral hexon. **(B)** Oncolytic activity of recombinant BiTE-encoding viruses were evaluated by cytotoxicity of DLD cells infected with EnAd or recombinant virus at increasing quantities of virus. Viability was measured by MTS assay after five days of infection. Each condition was measured in biological triplicate and represented as mean $\pm$ SD.

To investigate the functionality of secreted BiTEs, supernatants from uninfected or virally infected HEK293A cells 72 hours post-infection were added to co-cultures of CD3-purified PBMC with CHO or CHO-FAP cells. T cell activation was evaluated by measuring CD25 expression at 24 hours post-treatment (Figure 4.16B). None of the treatments induced CD25 expression on T cells when incubated with CHO cells. However, T cells in culture with CHO-FAP cells showed strong induction of CD25 when incubated with supernatants from cells infected with EnAd-CMV-FAPBiTE and EnAd-SA-FAPBiTE. This FAP-dependent activation of T cells led to lysis of CHO-FAP cells, but not CHO cells, when supernatants from EnAd expressing FAP BiTEs were used (Figure 4.16C). Importantly, no activation or cytotoxicity was observed when supernatants from cells infected with parental or control BiTE viruses were used, nor in the absence of T cells (Figure 4.16B and C).

4.3.9 EnAd-FAPBiTE-mediated oncolysis induces a T cell-mediated bystander killing of fibroblasts

EnAd kills most carcinoma cell lines by direct oncolysis. However, it appears unable to replicate in and kill fibroblasts and other non-epithelial stromal cells (Illingworth et al, 2017). We expect the encoding and secretion of a FAP-targeted BiTE will allow the targeting of stromal fibroblasts by T cells upon infection of tumour cells by recombinant EnAd. Therefore, we evaluated whether the BiTE viruses could mediate the bystander killing of fibroblasts in a setting that better mirrors the oncolytic process, by culturing fibroblasts together with tumour cells (acting as BiTE producers) and T cells. SKOV3 ovarian carcinoma cells and NHDF cells were plated at a ratio of 1:4 and infected with

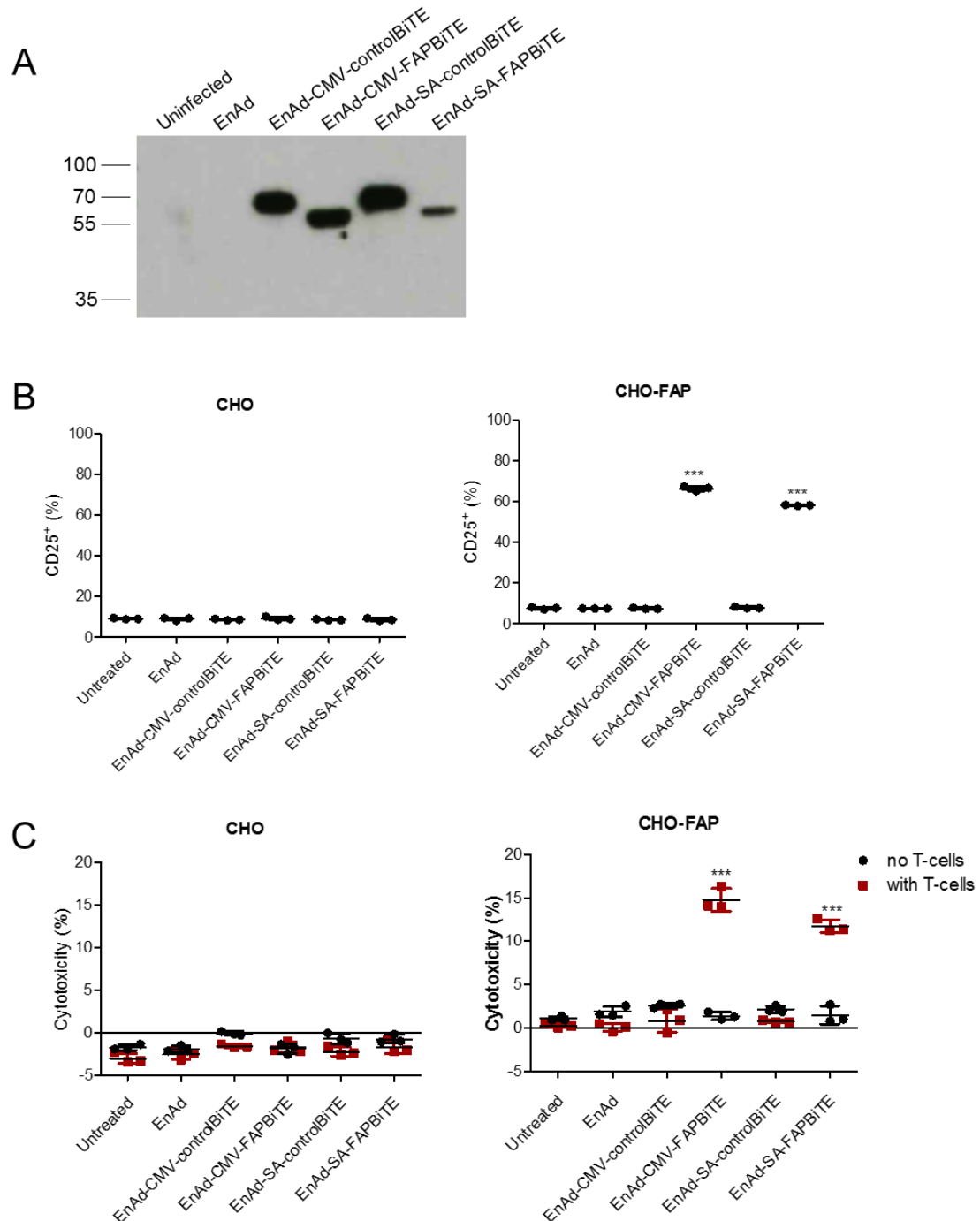


Figure 4.16. Functional characterisation of EnAd expressing FAP BiTE using cell lines and PBMC-derived T cells. (A) Supernatants from uninfected or virus-infected HEK293A cells three days post-infection were assessed for BiTE expression by western blot using an anti-His tag antibody. (B) T cell activation was measured in co-cultures of CHO or CHO-FAP cells (E:T 5:1) and virally-infected HEK293A supernatants after 24 hours. Activation was assessed by CD25 induction and measured by flow cytometry. (C) Cytotoxicity of CHO or CHO-FAP cells incubated with infected HEK293A supernatants alone or in co-culture with T cells (E:T 5:1). Cytotoxicity was measured by release of LDH into culture supernatants following 24 hours co-culture. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

100 vp/cell of EnAd, EnAd-CMV-FAPBiTE, or EnAd-SA-FAPBiTE or the corresponding control BiTE viruses. The number of viable cells, as measured by cell index, was monitored in real-time by an xCELLigence-based cytotoxicity assay, either in the absence (Figure 4.17A) or presence of PBMC-derived T cells (Figure 4.17B). In the absence of T cells and virus, cells continued to grow before reaching a plateau at around 120 hours, with similar levels of growth for virally-infected cells. Remarkably, the combination of T cells and FAP BiTE-expressing virus led to complete cytotoxicity of NHDF (and SKOV3), with lysis observed within 22 hours of infection by EnAd-CMV-FAPBiTE and 42 hours for EnAd-SA-FAPBiTE. Crucially, no cytotoxicity was observed when cultures were infected with EnAd or EnAd expressing the control BiTE, in both the absence and presence of added T cells.

Lysis of target cells was confirmed when similar co-cultures were established and release of LDH into the supernatant was observed only when co-cultures were infected with FAP BiTE viruses (Figure 4.18A). To determine if T cells mediate this target cell lysis, cells were harvested at 24, 48 and 96 hours post-infection and activation status (CD69 and CD25) of T cells determined by flow cytometry (Figure 4.18B and C). The kinetics of T cell activation paralleled that of NHDF cytotoxicity, with no observable increases in CD69 or CD25 in the presence of EnAd or EnAds expressing the control BiTE. Importantly, the FAP BiTE-encoded viruses failed to induce CD25 expression in the absence of NHDF, further demonstrating the requirement of FAP⁺ cells for BiTE-mediated T cell activation (Figure 4.18D).

To approximate the yield of active FAP BiTE produced from virally-infected DLD cells, the cytotoxicity (measured by LDH release; Abs₄₅₀) of NHDF by T cells cultured with

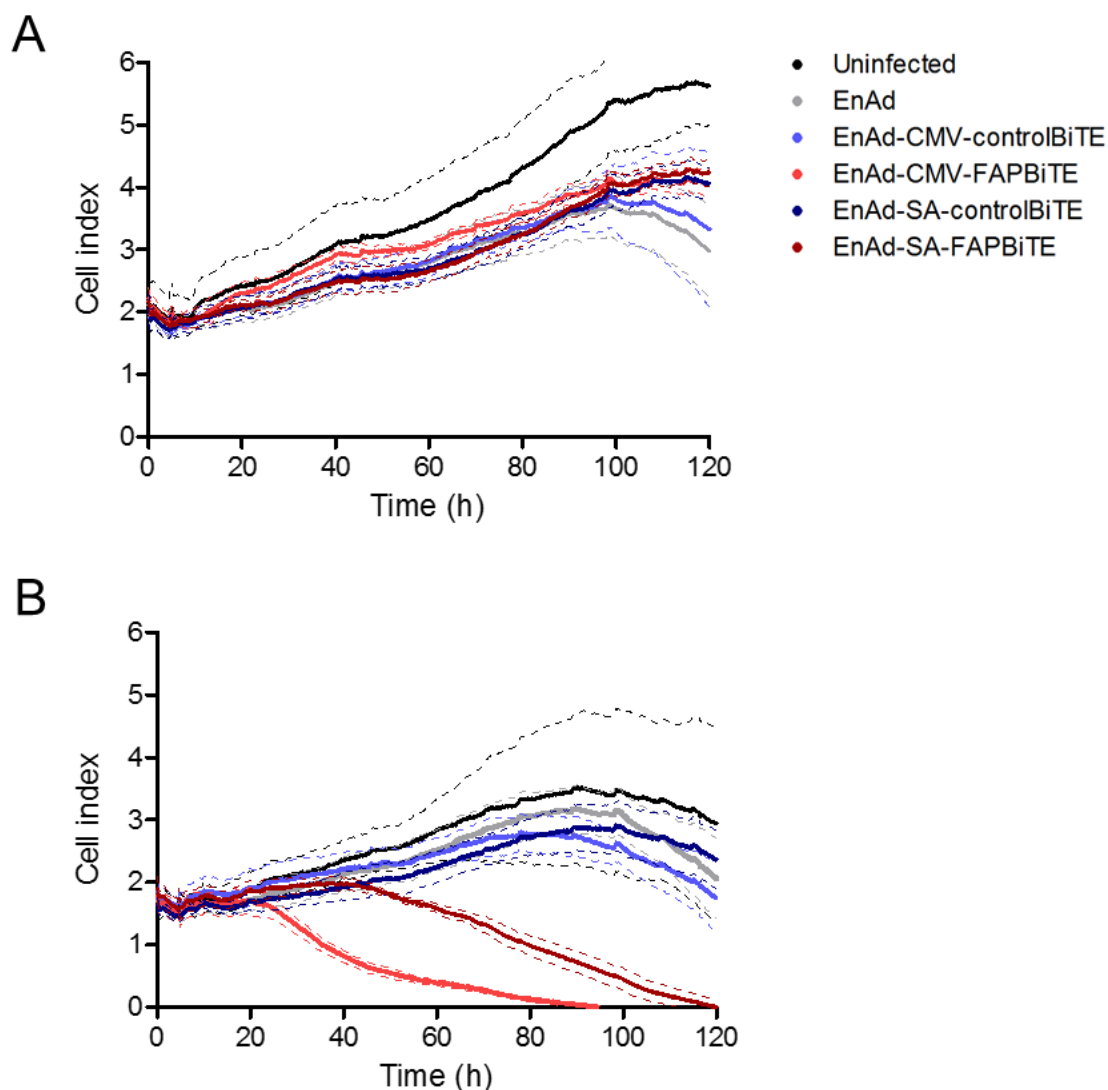


Figure 4.17. EnAd expressing FAP-targeted BiTE induces T cell-dependent cytotoxicity of stromal fibroblasts. (A, B) Viability of NHDF and SKOV3 carcinoma cells were monitored in real time over 120 hours by a xCELLigence-based cytotoxicity assay in the absence (A) or presence (B) of CD3-purified PBMC. NHDF and SKOV3 cells were seeded at a ratio of 4:1 and infected at time-point zero. In B, T cells were added 2 hours post-infection (CD3:NHDF, 5:1). Electrical impedance was measured at 10-minute intervals. Each condition was measured in biological triplicate and represented as mean $\pm$ SD.

known quantities of recombinant FAP BiTE (Figure 4.19A) was compared to that induced by (diluted) supernatants from infected DLD cells (Figure 4.19B). This allowed us to determine that EnAd-CMV-FAPBiTE and EnAd-SA-FAPBiTE viruses expressed approximately 10 and 50 μ g active FAP BiTE per million DLD cells, respectively. As the EC_{50} of the FAP BiTE is 2.5 ng/mL, we expect the viruses will produce sufficient

quantities to achieve therapeutic doses, as BiTE activity was detected even when infected supernatants were diluted 10,000-fold (Figure 4.19B).

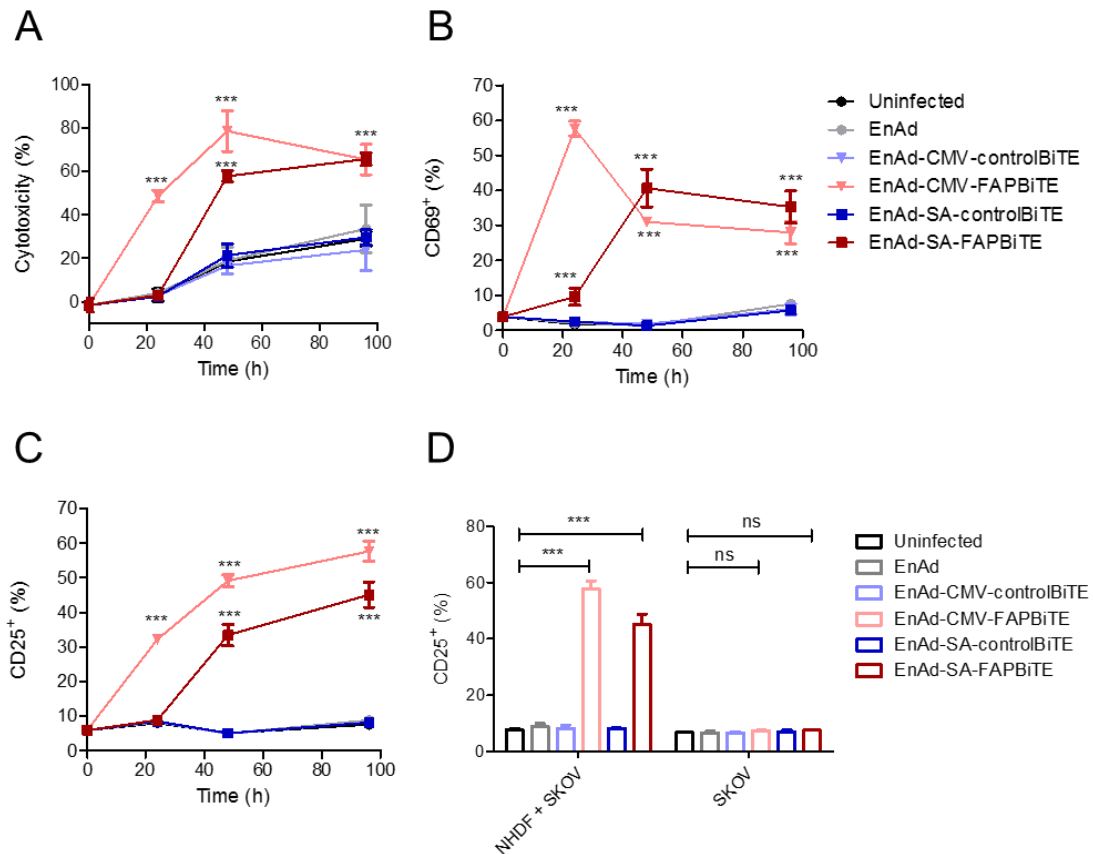


Figure 4.18. EnAd-FAPBiTE-mediated oncolysis induces FAP-dependent activation of primary human T cells. (A) Lysis of NHDF and SKOV3 cells infected with EnAd or recombinant virus and co-cultured with primary T cells. At each time point, supernatants were harvested and cytotoxicity measured by LDH. (B, C) Activation of T cells was assessed in the co-cultures established in (A). Activation status was assessed by surface levels of markers CD69 (B) and CD25 (C) and measured by flow cytometry at the specified time-point. (D) T cell activation (assessed by CD25 up-regulation) of PBMC-derived T cells in co-culture with SKOV alone or in combination with NHDF cells. After seeding, cells were infected with parental EnAd or armed virus and CD25 on T cells measured at 72 hours post-infection. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'uninfected', * p <0.05, ** p <0.01, *** p <0.001.

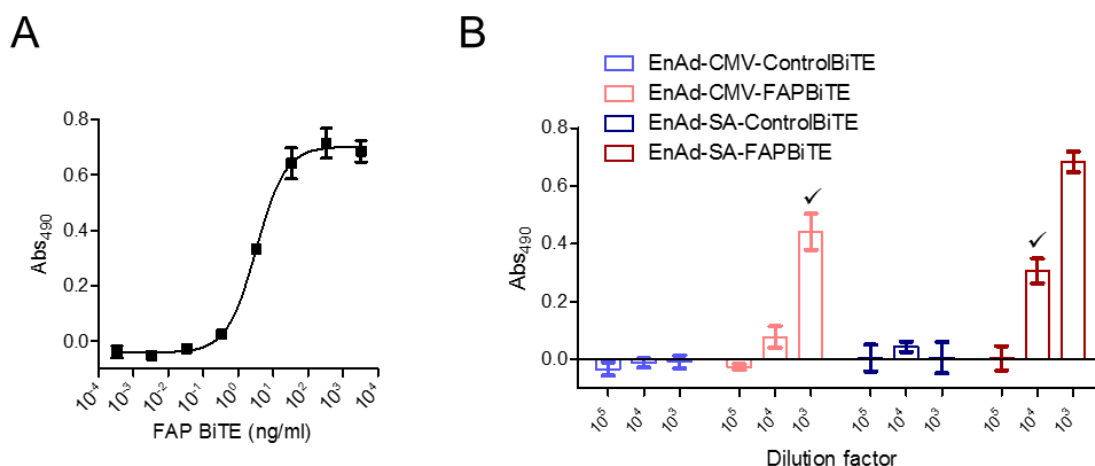


Figure 4.19. Quantitation of FAP BiTE secreted from virus-infected DLD cells. (A) A standard curve of cytotoxicity (LDH release; Abs₄₅₀) of NHDF cells in co-culture with PBMC-derived T cells (E:T 5:1) for 24 hours. To generate the standard curve, cells were incubated with known quantities of FAP BiTE-containing supernatants. **(B)** Corresponding co-cultures were established, with NHDF and T cells incubated with diluted supernatants harvested from DLD cells infected with the indicated virus for 72 hours. The dilution used to determine the quantity of FAP BiTE is indicated with a tick. Each condition was measured in biological duplicate and represented as mean $\pm$ SD.

This BiTE-induced cytotoxicity of stromal fibroblasts by T cells was captured by time-lapse microscopy. NHDF fibroblasts (labelled red; FAP⁺, resistant to viral oncolysis) were co-cultured with DLD tumour cells (unlabelled; susceptible to viral oncolysis) and PBMC T cells (labelled blue). An apoptosis stain (CellEvent Caspase 3/7 detection reagent), which produces green fluorescence in cells in which caspases 3/7 are activated, was also added. In uninfected controls, DLD and NHDF cells grew continuously for 48 hours without hindrance. However, infection with parental EnAd induced dramatic cytotoxicity of DLD cells within 24 hours. Importantly, NHDFs remained viable throughout. Only upon infection with EnAd-CMV-FAPBiTE or EnAd-SA-FAPBiTE did NHDF cells undergo strong T cell-mediated induction of apoptosis, turning yellow. This was accompanied by DLD cytotoxicity, as observed with parental EnAd. Representative images from 0 and 48 hours post-infection are shown in Figure 4.20, and full time-lapse sequences are also available.

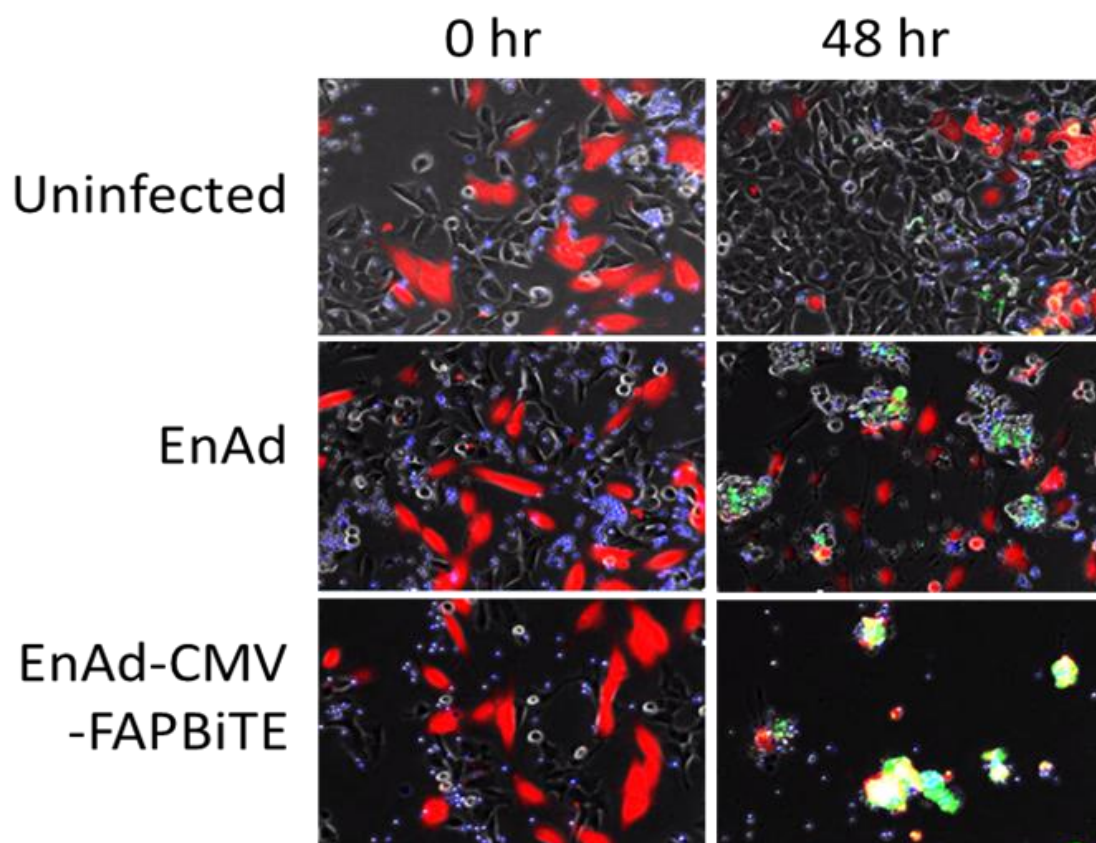


Figure 4.20. Infection with EnAd expressing FAP BiTE mediates apoptosis of stromal fibroblasts. Stills from time-lapse sequences showing co-culture of NHDF (red), DLD carcinoma cells (unstained, black) and T cells (blue). Cells were infected with EnAd or BiTE armed virus, or uninfected. Apoptosis was visualised using CellEvent Caspase 3/7 detection reagent (green). Images were taken on a Nikon TE 2000-E Eclipse inverted microscope at intervals of 15 minutes covering a period of 96 hours. Representative images were recorded at the times displayed; original magnification $\times 10$. Full time-lapse sequences are available.

4.3.10 EnAd-SA-FAPBiTE-mediated T cell activation and target cell lysis is tumour-selective

Immunotherapeutic approaches targeting FAP⁺ fibroblasts have been reported to be associated with bone marrow toxicities and cachexia (Tran et al, 2013). These side-effects may result from induced immune responses against FAP⁺ cells in the connective tissue of bone marrow and muscle. To address this, BiTE expressing viruses were engineered with

transgene expression under transcriptional control of the major late promoter (SA viruses; Figure 3.13). We hypothesised that coupling expression of the BiTE to virus replication would restrict immunotherapeutic activity to within the tumour.

To assess the activity of the recombinant viruses in normal human fibroblasts, NHDF cells were infected with EnAd, EnAd-CMV-FAPBiTE, EnAd-SA-FAPBiTE, or the corresponding control BiTE viruses in the presence of primary T cells but no producer cells. Cytotoxicity was assessed after 72 hours. As expected, infection with EnAd-CMV-FAPBiTE induced strong cytotoxicity (measured by LDH release) of NHDF cells (Figure 4.21A). Crucially, no lysis was observed when cells were infected with EnAd-SA-FAPBiTE, consistent with the reported inactivity of EnAd in non-epithelial cells (Illingworth et al, 2017).

To better emulate the multiple cell types present in a tissue, NHDF fibroblasts were cultured with exogenous T cells and either normal epithelial cells (normal bronchial epithelial, NBE) or ovarian carcinoma cells (SKOV3) and subsequently infected with EnAd-SA-FAPBiTE or the corresponding control virus. In the presence of normal epithelial cells, neither the FAP BiTE or control virus induced upregulation of CD69 or CD25 (Figure 4.21B and C). However, T cells in culture with NHDF and SKOV3 tumour cells, showed substantial activation of T cells when infected with EnAd-SA-FAPBiTE, but not the corresponding control BiTE virus. This SKOV3-dependent activation of T cells led to the selective lysis of target cells (confirmed to be NHDF by microscopy, not shown), with negligible levels of cytotoxicity detected in cultures with normal epithelial cells (Figure 4.21D).

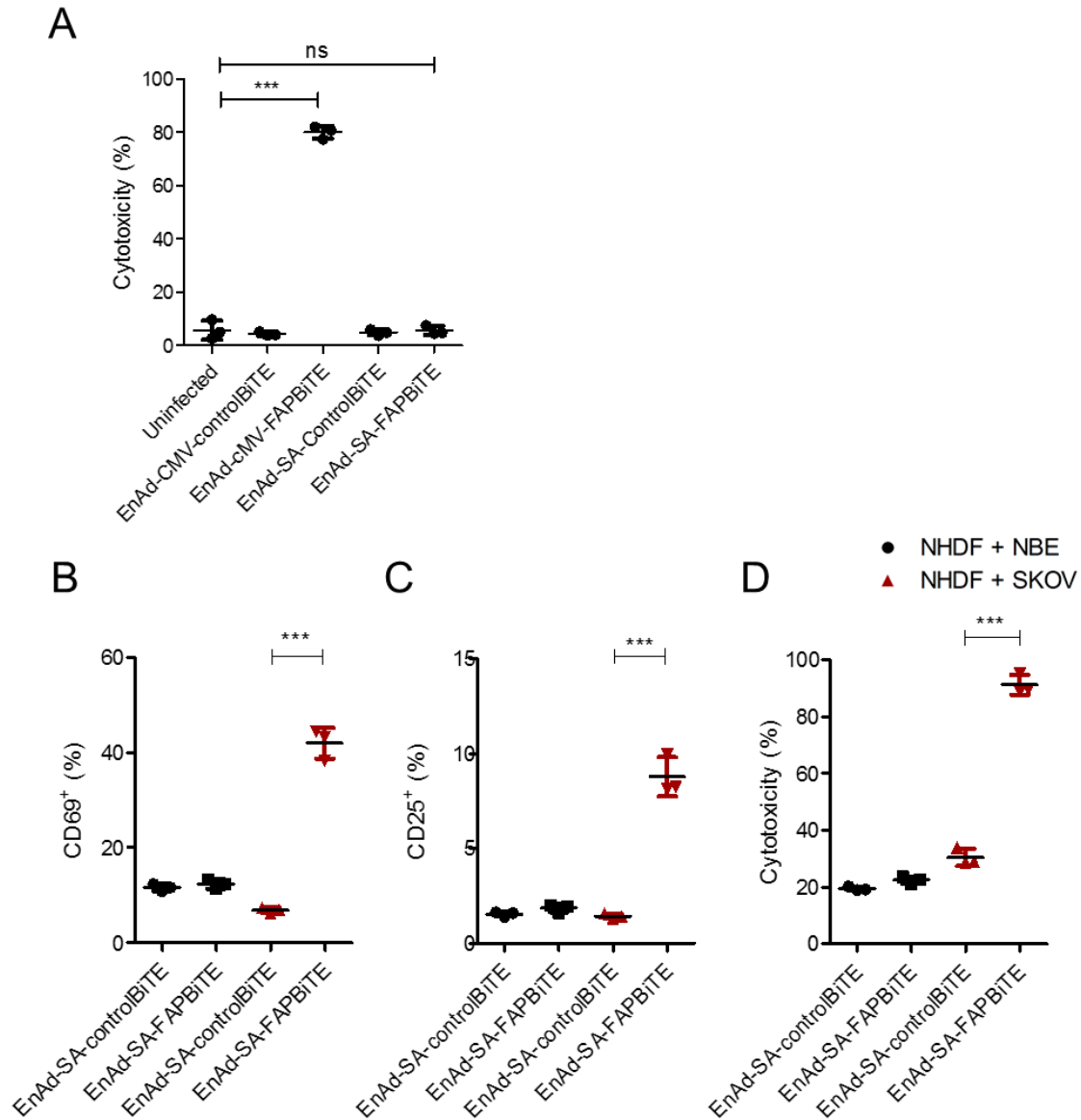


Figure 4.21. FAP BiTE expression from adenovirus major late promoter-driven recombinant virus is tumour-selective *in vitro*. (A) Cytotoxicity in T cell and NHDF co-cultures (E:T 5:1) was assessed by LDH release into supernatant 72 hours post-infection with recombinant BiTE-expressing viruses. (B, C) T cell activation in co-culture with NHDF and NBE (black) or SKOV3 (red) cells. NHDF and NBE or SKOV3 were seeded at 5:1, infected with BiTE-armed viruses. T cells were added two-hour post-infection. Activation of T cells was assessed by induction of CD69 (B) and CD25 (C) at 72 hours post-infection. (D) Cytotoxicity of NHDF cells was assessed in co-cultures established in (B, C). Cytotoxicity was measured by LDH release into culture supernatants at 72 hours post-infection. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'uninfected' or 'EnAd-SA-controlBiTE', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As mentioned previously, FAP-targeted therapeutics are reported to induce toxicity within the bone marrow compartment (Tran et al, 2013). To address this concern, the activity of the EnAd-SA-FAPBiTE was evaluated in a fresh human bone marrow sample. The cell types present in the bone marrow biopsy were screened by flow cytometry (Figure 4.22A), demonstrating that the sample contained CD3⁺ T cells (15%) and CD11b⁺ cells (monocytes and granulocytes), although no detectable FAP⁺ cells. Therefore, total bone marrow cells were co-cultured with NHDF fibroblasts for the duration of this study. Encouragingly, neither EnAd-SA-FAPBiTE or EnAd-SA-controlBiTE induced the activation of endogenous T cells (by induction of CD25; Figure 4.22B) or cytotoxicity of NHDF (release of LDH; Figure 4.22C). Addition of SKOV3 tumour cells lead to the induction of both CD25 expression on T cells and the lysis of NHDF following EnAd-SA-FAPBiTE infection.

Taken together, these data suggest that when under transcriptional control of the major late promoter, the activity of the FAP BiTE is selectively expressed by tumour cells, as assessed both *in vitro* and *ex vivo* using cells isolated from fresh bone marrow.

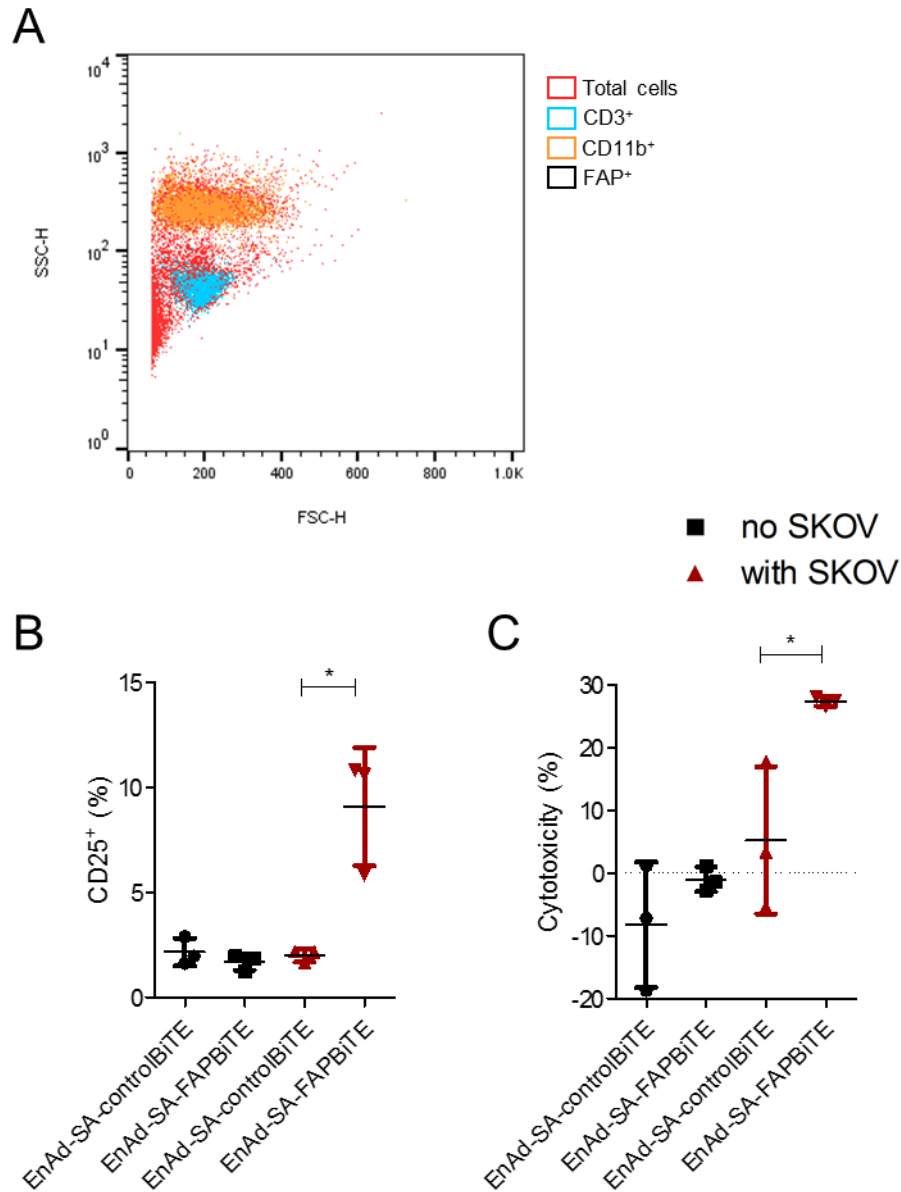


Figure 4.22. EnAd-SA-FAPBiTE virus induced no activation of T cells or lysis of FAP⁺ cells in a fresh human bone marrow sample. (A) Flow cytometry dot plot of forward versus side scatter demonstrating screening of bone marrow cells for cellular composition. Red, total cells; Blue, CD3⁺ cells; Orange, CD11b⁺ cells; Black, FAP⁺ cells. (B) T cell activation in fresh bone marrow sample infected with FAP BiTE-expressing EnAd was assessed. Prior to infection, bone marrow cells were plated with NHDF cells in the presence (red) or absence (black) of SKOV3 tumour cells at a ratio of 50:5:1 (bone marrow:NHDF:SKOV3). Expression of CD25 was measured by flow cytometry at 72 hours post-infection. (C) Target cell lysis was assessed by LDH release into supernatant in cultures established in (B). Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using an unpaired, two-tailed t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4 Chapter conclusions

1. Engineered and produced a novel and fully functional FAP-targeted BiTE.
2. EnAd armed with the FAP BiTE demonstrated no loss in oncolytic potency and is able to activate primary human T cells and mediate lysis of FAP⁺ fibroblasts.
3. Coupling transgene expression to the adenovirus major late promoter lead to tumour-selective BiTE expression.

4.5 Chapter discussion

There is now mounting evidence supporting a crucial role for cancer-associated fibroblasts (CAFs) in tumour progression and immune suppression. Indeed, the extent to which CAFs impact on immune response is so broad and exhaustive, it is difficult to envisage cancer immunotherapy reaching its full potential without directly addressing these cells as part of a combined therapeutic approach. In a study by Feig *et al*, it was demonstrated using a mouse model of pancreatic cancer, that genetic depletion of CAFs was essential in achieving a reduction in tumour growth and improving survival using anti-PDL1 and CTLA-4 antibodies. Without this depletion, tumours were refractory to checkpoint inhibition (Feig et al, 2013). This is one example of many recent studies that demonstrate benefit in targeting the CAF compartment of a tumour (Lee et al, 2005; Liao et al, 2009; Loeffler et al, 2006).

However, due to the lack of unique CAF surface markers, targeting CAFs has proved challenging. The ability to delivery BiTEs via an oncolytic virus (OV) presents the exciting opportunity to target such cell types. In this chapter, we investigated the potential of arming EnAd with a BiTE targeting fibroblast activation protein (FAP), which is strongly up-regulated by CAFs, but also suggested to reside in most normal adult tissue (Roberts et al, 2013; Tran et al, 2013).

A new FAP-targeted BiTE was first designed and engineered, and successful expression and secretion was demonstrated using a plasmid-based expression system (Figure 4.5). This BiTE protein was fully functional and could activate primary human T cells - manifest by increase in activation markers, proliferation, cytokine production and degranulation (Figure 4.6-4.9). Importantly, these activated T cells were able direct their cytotoxicity to FAP⁺ fibroblasts in mixed cell cultures (Figure 4.9). Similar to the EpCAM BiTE, the FAP BiTE could induce the degranulation of CD4 T cells, which were able to induce similar levels of target cell lysis as CD8 cells (Figure 4.12). The FAP BiTE demonstrated remarkable specificity. T cells were only activated in the presence of antigen-positive cells, indicating the requirement for BiTE to cluster on a target cell surface (Figure 4.10). Susceptibility to BiTE-mediated lysis appeared to be independent of the level of FAP expression, though, this could be due to sufficiently high FAP expression on both cell lines tested (Figure 4.11).

Although the FAP BiTE was able to induce the proliferation of T cells, we did not observe levels comparable to that induced by the EpCAM BiTE (Figure 4.7). Similarly, T cells stimulated with the FAP BiTE produced over 10-fold lower quantities of IL-2, which peaked at 24 hours, whereas the EpCAM BiTE-activated T cells sustained production for

at least five days (Figure 4.8 and 3.7). These two observations are likely linked (Boyman & Sprent, 2012), and suggests that the amount of IL-2 produced by FAP BiTE-mediated activation of T cells is not sufficient to sustain T cell proliferation. Given that both BiTEs induce activation by binding to CD3 alone, this surprising disparity indicates that the EpCAM BiTE is able to partially compensate for the absence of co-stimulation – possibly due to higher levels of CD3 stimulation. Interestingly, and despite this, both BiTEs induced comparable levels of IFN γ , which may suggest that less stimulation is required for IFN γ . These observations are consistent with work by Davis, who has established a hierarchy of T cell functions, with different functions requiring different levels of stimulation (Irvine et al, 2002; Wei et al, 2013).

We next incorporated the FAP BiTE into the EnAd genome (Figure 4.13). BiTE-armed viruses were successfully rescued, without any loss in oncolytic potency (Figure 4.15). These represent the first examples of oncolytic adenoviruses armed with stroma-targeted BiTEs. Importantly, EnAd-FAPBiTE-mediated oncolysis of tumour cells lead to the polyclonal activation of primary human T cells, leading to the rapid T cell-mediated lysis of NHDF fibroblasts (Figure 4.17 and 4.18). BiTE-activated T cells also appeared to induce the bystander killing of partially EnAd-resistant SKOV3 when co-cultured alongside FAP⁺ NHDF cells (Figure 4.17), with similar observations reported elsewhere (Ross et al, 2017).

FAP BiTE viruses were engineered to express and secrete BiTEs only in cells supporting virus replication (using a SA promoter). Indeed, the coupling of BiTE expression to the adenovirus major late protein should ensure that expression should be restricted to the tumour, minimising potential systemic toxicities. We evaluated the selectivity of this

approach in *in vitro* and *ex vivo* models. EnAd-SA-FAPBiTE only induced activation of primary human T cells when co-cultured with fibroblasts (NHDF) and ovarian carcinoma cells (SKOV3), but not normal epithelial cells (NBE) (Figure 4.21). Additionally, infection of a human bone marrow sample with EnAd-SA-FAPBiTE induced no significant activation of endogenous T cells or lysis of FAP⁺ fibroblasts, unless SKOV3 cells were added (Figure 4.22). Whilst these models may not reproduce the complexities of a human tissue, they do begin to provide preclinical evidence for the improved safety profile and therapeutic potential of delivering BiTEs using OVs. As such, the use of primary bone marrow cells was adopted to directly address the reports of bone marrow toxicity induced by systemic FAP-targeted treatments (Roberts et al, 2013; Tran et al, 2013). If necessary, it is feasible to introduce microRNA binding sites into the virus to further prevent expression in normal tissues, further improving its cancer tropism (Bofill-De Ros et al, 2015). One study, for example, reports exploiting the liver-specific expression of miRNA 122 to attenuate adenoviral E1A expression in liver cells, overcoming the liver toxicity often associated with systemic oncolytic adenovirus delivery (Ylösmäki et al, 2008).

5. Targeting T cell cytotoxicity in human malignant exudate samples using BiTE-expressing oncolytic adenoviruses

5.1 Introduction

Ascites is the accumulation of fluid in the peritoneal cavity. Pleural effusion is excess fluid in the pleural cavity. A substantial minority of reported cases are associated with malignancy; ovarian and gastrointestinal carcinoma are the most common for malignant ascites (Ayantunde & Parsons, 2007), and cancer of the lung and breast for malignant pleural effusions (MPE) (Leuallen & Carr 1955). The development of both ascites and pleural effusion is a sign of advanced disease and correlates with poor prognosis (Ayantunde & Parsons, 2007; Zamboni et al, 2015). Survival in both populations is poor, however quality of life is improved by palliative treatments, with puncture of the cavity and drainage of the fluid the most common.

Ascites and pleural effusion samples represent an extremely valuable resource for translational research. Primary isolates drained from patients are an easily accessible and rich source of late stage tumour tissue, with samples typically including cancer cells, lymphocytes, fibroblasts and macrophage, and thus, represent a highly clinically relevant model of the tumour microenvironment. Indeed, mounting evidence suggests that ascites and pleural effusion are sites of substantial immune suppression. Compared to matched plasma, ascites fluids showed elevated IL-10, IL-6 and VEGF, and reduced levels of IL-

2, IL-17 and RANTES (Giuntoli et al, 2009); higher IL-10 has been shown to correlate with shorter progression-free survival (Matte et al, 2012). In numerous studies, the fluid was highly immunosuppressive to T cells, manifest as reduced proliferation, IFN γ production, cytotoxic potential and decreases in NF- κ B and NFAT activation (Simpson-Abelson et al, 2013). Partial or complete loss of HLA expression on tumour cells is widely reported, as well as down-regulation of numerous proteins involved in antigen processing (E.g. LMP2, LMP7, TAP1 and TAP2) (Kaklamanis et al, 1995; Seliger et al, 2000). It is likely that these mechanisms of immune escape represent a considerable limitation to the majority of T cell therapies (vaccines, checkpoint inhibitors, TIL).

We postulate that BiTE-armed OV therapy is able to overcome many of these immune evasion mechanisms. For example, BiTEs are able to mediate T cell activation entirely independent of antigen processing and MHC presentation (Figure 1.4). Less widely reported, however, is the ability of the BiTE to facilitate the formation of a tumour cell-T cell immunological synapse that is larger than that induced by normal cognate TCR-MHC interactions (Patrick Baeuerle, presentation at EMBL cancer immunotherapy conference, 2017). It is plausible that this larger surface of interaction induces stronger activatory signalling that may be able to counteract negative signals mediated by the ascites- or MPE-derived immune suppression. Moreover, we predict that BiTE-mediated T cell activation and the secretion of proinflammatory cytokines (Figure 3.7 and 4.8) may fully or partially reverse the immune inhibitory milieu within the exudate fluids.

Therefore, for maximum translational relevance, and reflecting also the complete absence of immune-competent animal models for group B adenovirus, we chose to focus our studies on primary patient biopsies maintained *ex-vivo*. We approached this initially by

evaluating the effect of BiTE treatment on exudate-derived target cells and lymphocytes individually. To do this, we used many of the experimental systems developed in chapters three and four to measure both T cell activation and target cell cytotoxicity. The effect of ascites and MPE fluids on BiTE-mediated T cell activation was also explored. Finally, and perhaps most importantly, the activity of the BiTE-armed OV6s in multiple whole malignant exudate samples was assessed.

5.2 Chapter aims

1. Assess the influence of exudate fluids on BiTE-mediated T cell activation and killing.
2. Evaluate the ability of BiTE-armed EnAds to activate tumour-associated T cells and mediate the lysis of tumour cells and fibroblasts within cancer biopsy samples.
3. Explore whether BiTE activity can reverse or induce changes to the immune inhibitory milieu within the exudate fluids.

5.3 Results

5.3.1 Processing and initial characterisation of malignant exudate samples

Primary human malignant ascites and pleural effusion samples were received from the Churchill Hospital, Oxford University Hospitals (Oxford, UK) following informed consent from cancer patients and ethical review (ORB 09/H0606/5+5.). Table 5.1 outlines the 38 patient exudates used in this chapter (17 ascites and 21 pleural effusion), with samples labelled in order of the date of receipt. Samples were isolated from patients with multiple indications of advanced carcinoma (Table 5.1) including but not limited to ovarian (32%), breast (16%) and lung (11%). Upon receipt, cellular and fluid fractions were separated by centrifugation, and if necessary, red blood cells were eliminated. Each sample was screened by flow cytometry for cellular composition, using fluorescently-labelled antibodies for cell-specific markers. These included tumour (EpCAM and EGFR), fibroblast (FAP), myeloid (CD11b), T cell (CD3, CD4 and CD8), NK cell (CD56) and T cell exhaustion (PD1) markers, although not all stains were included in the initial screen for each sample. A representative example of the gating strategy employed in the analysis of flow cytometry data is shown in Figure 5.1, highlighting some of the characteristic cell types present in a typical sample (including tumour cells, macrophages and T cells).

As outlined in Table 5.2, 75% (12/16) of ascites and 33.3% (7/21) of pleural effusions samples contained a population of EpCAM⁺ cells (0.2 – 47.3, mean of 9.4%). Interestingly, a significantly smaller proportion (4.8%, 1/21) of pleural effusions specimens possessed FAP⁺ fibroblasts compared to 44% (7/16) of ascites samples (0.2 –

Table 5.1. A list of the human malignant exudate samples used in this chapter. All the malignant exudate samples collected for this study are presented in the table below. The following nomenclature has been used; Peritoneal ascites, Asc.01-17; pleural effusate, MPE.01-21. Samples are labelled in order of date of receipt.

Sample	Date received	Cancer type
Asc.01	26/11/2015	Ovarian
Asc.02	13/05/2016	Ovarian
Asc.03	16/06/2016	Ovarian
Asc.04	16/06/2016	Ovarian
Asc.05	24/06/2016	Breast
Asc.06	24/06/2016	Breast
Asc.07	07/07/2016	Breast
Asc.08	26/08/2016	Ovarian
Asc.09	17/01/2017	Pancreatic
Asc.10	24/01/2017	Ovarian
Asc.11	24/01/2017	Ovarian
Asc.12	21/02/2017	Ovarian
Asc.13	22/02/2017	Ovarian
Asc.14	28/03/2017	Melanoma
Asc.15	30/03/2017	Ovarian
Asc.16	18/05/2017	Ovarian
Asc.17	25/05/2017	Ovarian
MPE.01	08/09/2016	Squamous cell tongue
MPE.02	12/09/2016	Breast
MPE.03	19/09/2016	Lung
MPE.04	19/09/2016	Breast
MPE.05	18/10/2016	Mesothelioma
MPE.06	01/11/2016	Colorectal
MPE.07	01/11/2016	Follicular lymphoma
MPE.08	15/11/2016	Lung
MPE.09	15/11/2016	Mesothelioma
MPE.10	29/11/2016	Mesothelioma
MPE.11	29/11/2016	Lymphoma
MPE.12	20/12/2016	Mesothelioma
MPE.13	20/12/2016	Colorectal
MPE.14	10/01/2017	Oesophageal
MPE.15	06/02/2017	Lung
MPE.16	07/03/2017	Ovarian
MPE.17	07/03/2017	Mesothelioma
MPE.18	21/03/2017	Lung
MPE.19	21/03/2017	Breast
MPE.20	28/03/2017	Mesothelioma
MPE.21	28/03/2017	Mesothelioma

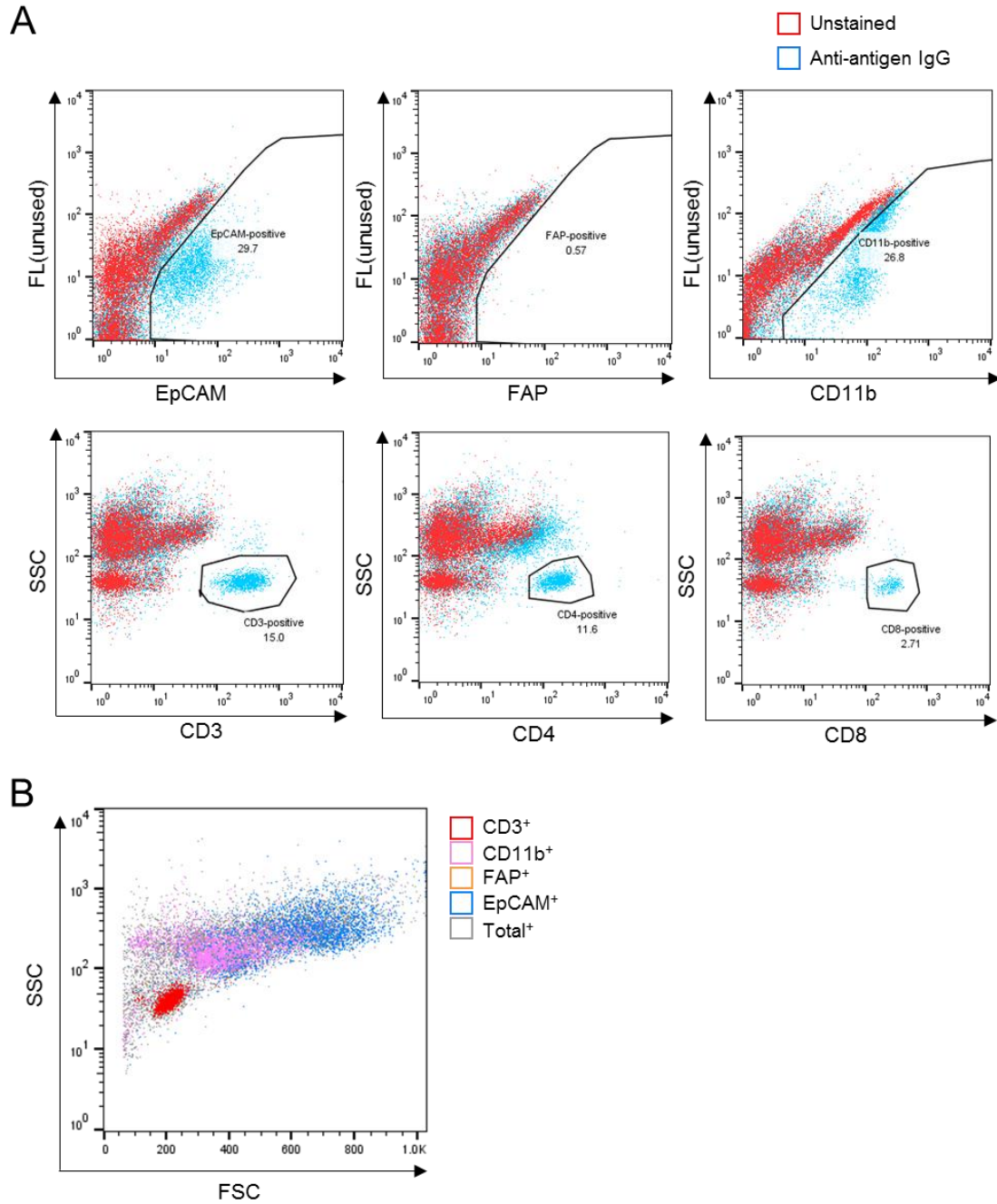


Figure 5.1. Representative example of the screening of exudate samples to determine cellular composition by flow cytometry. (A) Flow cytometry dot plots (Asc.15, see Table 5.2) demonstrating the gating strategy used to assess cellular composition. Upon receipt, the cellular and fluid fractions are separated, followed by elimination of red blood cells from the isolated exudate cells. Cell types present in each sample were determined by antibody staining for EpCAM (tumour), EGFR (tumour), FAP (fibroblast), CD11b (myeloid), CD3 (T cell), CD4 (T cell), CD8 (T cell), CD56 (NK cell) and PD1 (T cell exhaustion) and analysed by flow cytometry. This example is non-exhaustive, and not all stains and gating strategies are displayed. **(B)** Representative FSC-SSC flow cytometry dot plot displaying the characteristic cell types present in a typical sample, generated using the analysis in (A).

18.9, mean of 7.2%). All samples contained a population of CD3⁺ lymphocytes, with CD4/CD8 ratio ranging from 0.58 to 7.22 and above 2.2 in 54% (7/13) of ascites samples and in 100% (10/10) of pleural effusions. Plasma CD4/CD8 ratios between 1.6 and 2.2 are considered normal (Stedman, 2012), and a ratio above 1.6 has been demonstrated to be associated with significantly reduced overall survival (Giuntoli et al, 2009). The authors suggest that a possible explanation for this finding is the enrichment of immunosuppressive CD4⁺CD25⁺FOXP3⁺ Tregs, however a more comprehensive analysis would be needed to conclude this. Taken together, this data agrees with previous studies suggesting ascites and pleural effusions may represent environments of immune suppression.

5.3.2 Expansion of patient-derived cancer cells and fibroblasts

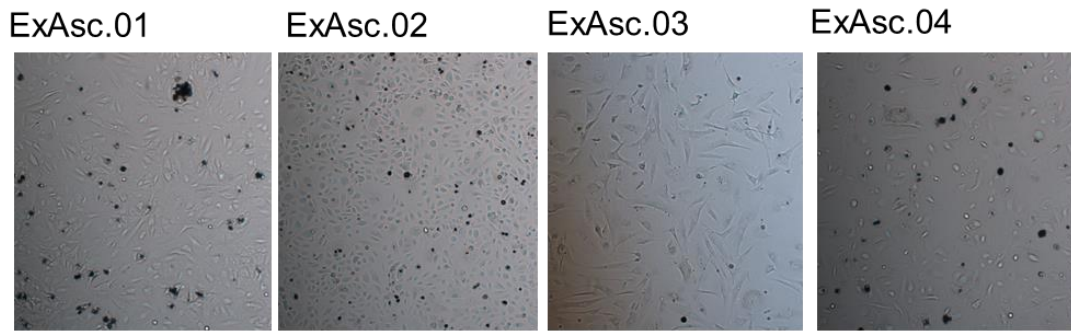
Four clinical samples of malignant peritoneal ascites (from chemotherapy refractory patients) containing EpCAM⁺ tumour cells and/or FAP⁺ primary fibroblasts were expanded *ex vivo*. These samples did not undergo a screen for cellular composition upon receipt and are therefore not included in Table 5.2. Sequential passages in tissue culture allowed enrichment of a mixed population of adherent cells as assessed by microscopy (Figure 5.2A). Flow cytometry was used to measure the proportion of EpCAM⁺ and FAP⁺ cells, showing a considerable expansion in the proportion of fibroblasts in all samples (Figure 5.2B and C). Analysis of conditioned media harvested from ascites-derived cells and untransformed fibroblasts (NHDF and TIG1) was performed by ELISA, and confirmed expression of immune suppressive cytokines TGFβ, IL-6 and IL-10 from the ascites cells (Figure 5.3). CXCL12, which is reported to be largely expressed by cancer-

associated fibroblasts, was not detected in conditioned media collected from any of the ascites populations, though levels may be lower than the sensitivity of the ELISA kit (150 pg/mL).

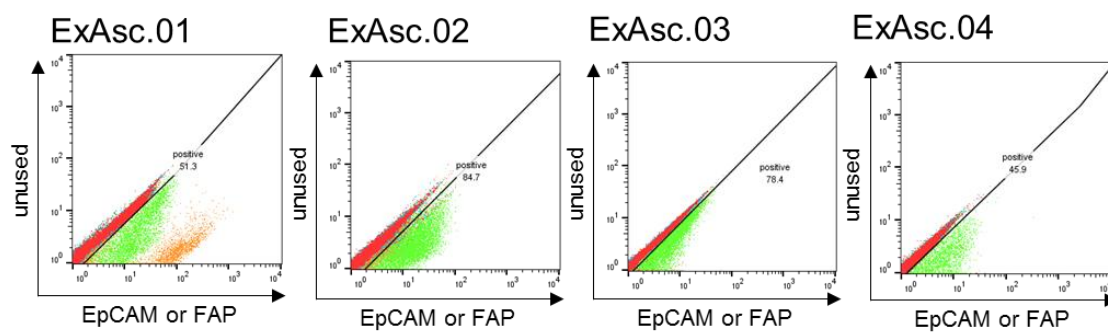
Table 5.2. Cellular composition of the human malignant exudate samples used in this study. The cellular composition of the samples outlined in Table 5.1 are displayed below. The cell types present in each sample were assessed by flow cytometry and are displayed as a percentage of the total cells in the sample. The following nomenclature has been used; Peritoneal ascites, Asc.01-17; pleural effusion, MPE.01-21. Samples are labelled in order of date of receipt. A hyphen (-) is shown if the stain was omitted from the initial screen and the data is unavailable.

Sample	Cancer type	Cell type (%)							
		EpCAM ⁺	FAP ⁺	EGFR ⁺	CD11b ⁺	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD56 ⁺
Asc.01	Ovarian	25.3	0	-	-	12.8	9.2	4.25	-
Asc.02	Ovarian	2	0	-	-	73.2	49.6	24.9	-
Asc.03	Ovarian	0	0	0.4	25.7	34	25.2	14	23.9
Asc.04	Ovarian	0.4	0.2	0	15.6	70.1	-	-	1.55
Asc.05	Breast	0.2	4.64	6.4	18.6	55	32.2	24.4	5.92
Asc.06	Breast	0.6	11.7	11.8	56.9	7	3.27	2.62	3.23
Asc.07	Breast	0	1	1	5.4	79.4	63.1	16.5	3.92
Asc.08	Ovarian	-	-	-	-	74	-	-	-
Asc.09	Pancreatic	0.38	9.7	-	28.2	22.1	-	-	-
Asc.10	Ovarian	0	0	-	33	27.3	14.9	11	-
Asc.11	Ovarian	2.9	0	-	33.2	53.2	18.4	37.4	-
Asc.12	Ovarian	26.8	0	-	33.3	6.5	5.42	1.24	-
Asc.13	Ovarian	4.86	0	-	55.8	17	13.7	3.2	-
Asc.14	Melanoma	0	0	-	13.2	77.4	61.8	17.7	-
Asc.15	Ovarian	29.7	0	-	26.8	15	11.6	2.71	-
Asc.16	Ovarian	0.2	18.9	-	20.1	46.2	13.2	22.8	-
Asc.17	Ovarian	47.3	4.6	-	19.5	32.7	15.6	6	-
MPE.01	Squamous cell tongue	0	0	-	32.9	48.5	-	-	-
MPE.02	Breast	1.4	0	-	19.6	45.3	-	-	-
MPE.03	Lung	0	0	-	-	78.7	-	-	-
MPE.04	Breast	0	0	-	-	0.7	-	-	-
MPE.05	Mesothelioma	1.11	0	-	10.6	37.2	-	-	-
MPE.06	Colorectal	7.5	0	-	2.77	2.89	-	-	-
MPE.07	Follicular lymphoma	0.8	0	-	2.26	50.4	-	-	-
MPE.08	Lung	3.44	0	-	16.4	15.3	-	-	-
MPE.09	Mesothelioma	0	0	-	4.7	84.9	-	-	-
MPE.10	Mesothelioma	0	0	-	1.2	95.1	-	-	-
MPE.11	Lymphoma	0	0	-	0.4	88.1	-	-	-
MPE.12	Mesothelioma	0	0	-	0	60.6	52.5	9.25	-
MPE.13	Colorectal	0	0	-	0	94.6	79.4	15.1	-
MPE.14	Oesophageal	0	0	-	10.3	81.8	55.2	23.6	-
MPE.15	Lung	0	0	-	5.2	74.2	63.1	8.73	-
MPE.16	Ovarian	22.2	1.2	-	34.1	37.9	27.4	10.7	-
MPE.17	Mesothelioma	0	0	-	5.76	90.3	62.6	27.2	-
MPE.18	Lung	0	0	-	10.5	55.2	38.9	16.3	-
MPE.19	Breast	1.25	0	-	2.1	79.8	56.9	23	-
MPE.20	Mesothelioma	0	0	-	19.1	59.1	46.2	12.6	-
MPE.21	Mesothelioma	0	0	-	0.8	87.2	69.5	17.8	-

A



B



C

	Cancer type	EpCAM ⁺ (%)	FAP ⁺ (%)
ExAsc.01	Breast	23.9	51.3
ExAsc.02	Ovarian	4.5	84.7
ExAsc.03	Breast	0	78.4
ExAsc.04	Breast	1.74	45.9

Figure 5.2. *Ex vivo* expansion of exudate cells isolated from chemotherapy pre-treated patient ascites samples. Four clinical samples of malignant peritoneal ascites containing EpCAM⁺ tumour cells and/or primary FAP⁺ fibroblasts were expanded by successive passage of the adherent cells. The mixed primary cell populations were examined by microscopy (A), and cellular composition assessed by flow cytometry (B and C).

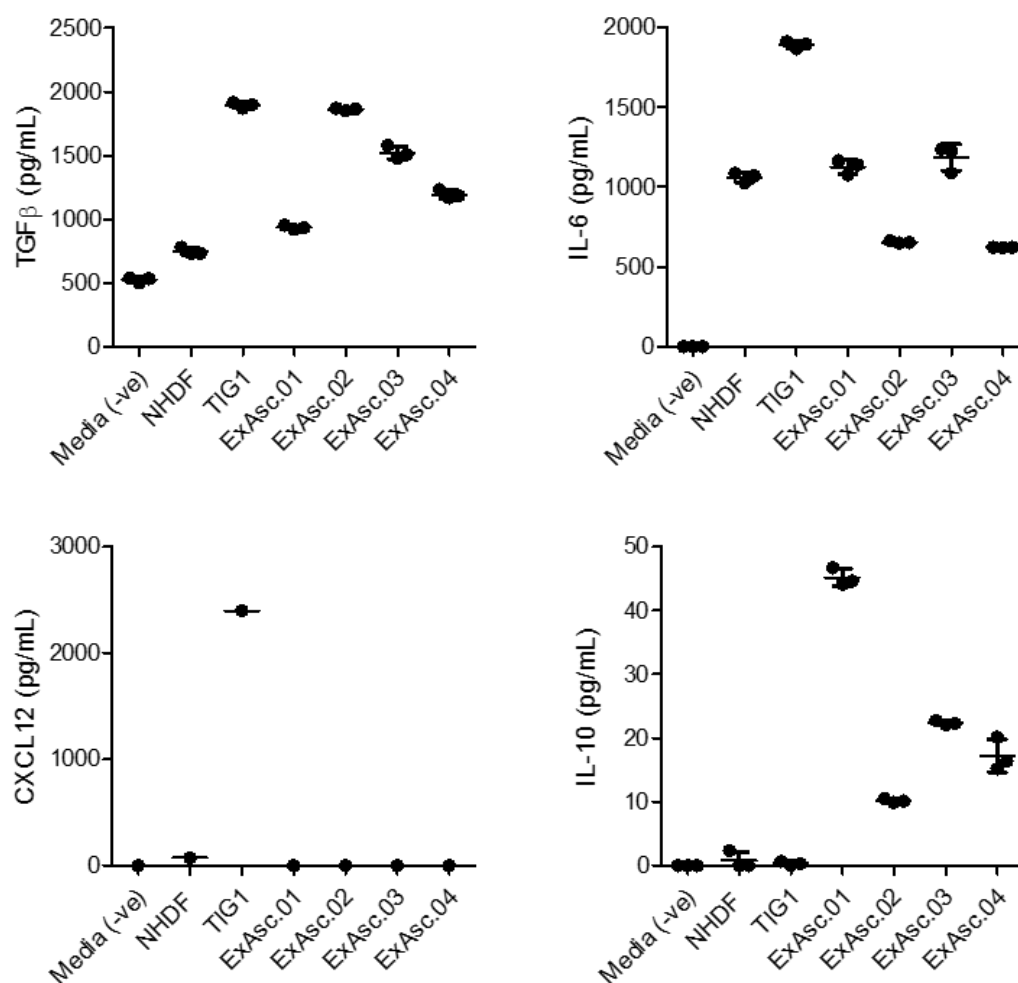


Figure 5.3. Expanded peritoneal ascites cells secrete TGFβ, IL-6 and IL-10. Production of TGFβ, IL-6, CXCL12 and IL-10 by the ascites cells was evaluated by ELISA. Cells at 90% confluency were washed with PBS and incubated in fresh low-serum media for 48 hours. Conditioned media was harvested and ELISA performed. For quantitation of TGFβ, IL-6 and IL-10, each condition was measured in technical triplicate and data represented as mean ± SD. CXCL12 levels were measured once.

5.3.3 BiTE expressing EnAd can direct PBMC to kill patient-derived tumour cells and fibroblasts from chemotherapy refractory patients

We first assessed the effects of BiTE treatment using this expanded multicellular and patient-derived model. By using fluorescently labelled antibodies and staining for EpCAM or FAP, flow cytometry allows the number of residual EpCAM⁺ or FAP⁺ cells

in a mixed cell population to be measured after treatment and is therefore indicative of cytotoxicity. However, it was first important to establish if the binding of EpCAM or FAP BiTE to their target antigen occludes binding of the detection antibody. Whilst pre-incubation of DLD (EpCAM⁺) or NHDF (FAP⁺) with high concentrations of BiTE (20,000 and 10,000 ng/ml) does reduce the fluorescent signal (Figure 5.4A and B), the effect on the number of detectable EpCAM⁺ and FAP⁺ cells is modest (Figure 5.4C and D). Crucially, at quantities of BiTE less than 4000 or 2000 ng/mL (for EpCAM and FAP, respectively) there is no significant attenuation in target cell detection. Therefore, free BiTE is to be used at 1000 ng/mL for assays requiring this means of detection. In a separate experiment, ascites cells infected with EnAd-CMV-FAPBiTE for five days produced BiTE at less than 500 ng/mL (data not shown), and therefore, levels of BiTE are not expected to reach sufficient quantities to impede detection.

To test whether the recombinant BiTE expressing viruses could kill the corresponding target cells, expanded ascites cells derived from three patient samples (ExAsc.01, ExAsc.02 and ExAsc.04) were incubated with PBMC-derived T cells and treated with free BiTE or 100 vp/cell parental or BiTE encoding recombinant viruses in culture medium. Sample ExAsc.03 had no detectable EpCAM⁺ cells and was omitted from the study. The viruses used in this chapter were from the same batch as those produced and characterised in chapter three and four. After 72 hours, the level of EpCAM⁺ cells (Figure 5.5A) or FAP⁺ fibroblasts (Figure 5.5B), and the activation of T cells (Figure 5.5C) were measured by flow cytometry. Treatment of the samples with the EpCAM BiTE-expressing viruses led to strong T cell activation (measured by CD25 expression), and a depletion of EpCAM⁺ tumour cells to background levels. Importantly, FAP⁺ (EpCAM-negative) fibroblasts showed no change in numbers. This demonstrates that the EpCAM

BiTE (encoded within an oncolytic virus) can mediate activation of PBMC-derived T cells and result in selective cytotoxicity to human tumour cells in malignant peritoneal ascites.

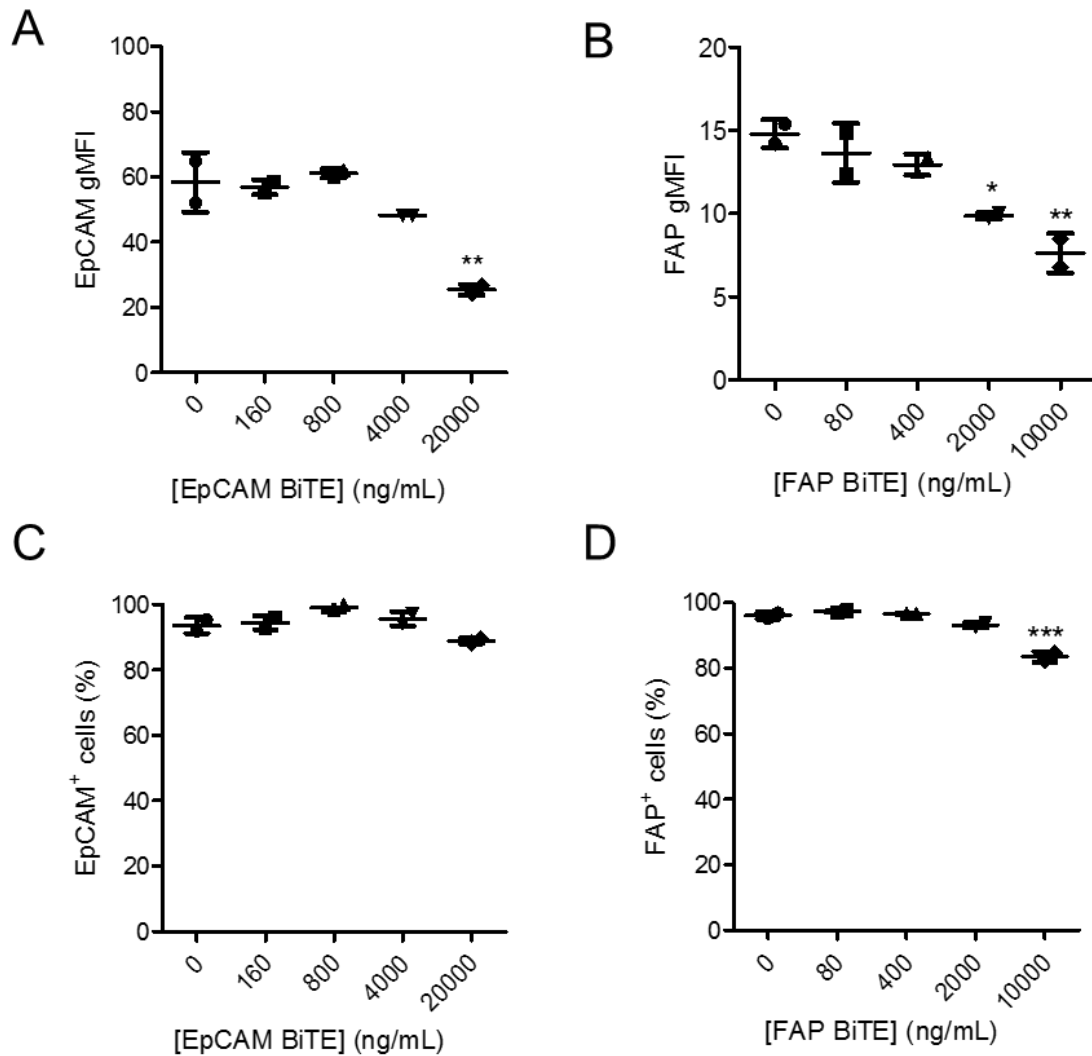


Figure 5.4. The detection of target cells using EpCAM- or FAP-specific antibodies and flow cytometry is occluded by BiTE binding at high BiTE concentrations. EpCAM⁺ SKOV3 or FAP⁺ NHDF cells were pre-incubated with the specified BiTE concentration and then stained with the corresponding antibody. Flow cytometry was performed and the geometric mean fluorescence intensity (gMFI; A + B) and percentage of antigen-positive cells (C + D) determined. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to '0 ng/mL', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Similarly, the FAP BiTE expressing viruses induced T cell activation and FAP⁺ cell depletion, with no significant change in EpCAM⁺ tumour cells. Critically, in a repeat experiment, this lead to a strong reduction in TGFβ detected in supernatants (Figure 5.5D), Although EpCAM-armed viruses were omitted from this follow-up study, this result suggests FAP⁺ cells to be the source of this TGFβ. This was observed in all the patients' samples, and none of the other treatments (using parental virus or virus encoding control BiTEs) showed any T cell activation or cytotoxicity.

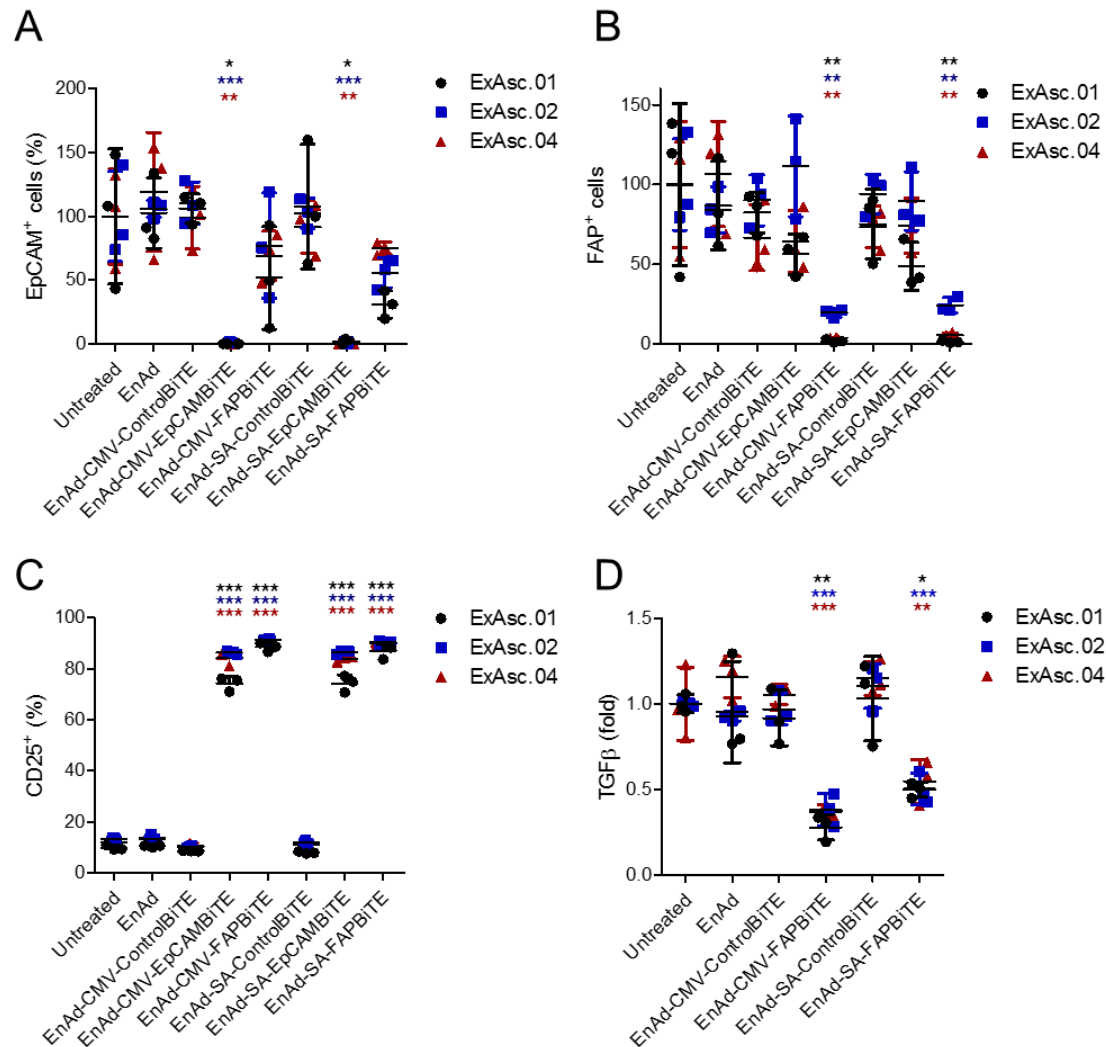


Figure 5.5. EnAd expressing BiTE can selectively kill the corresponding target cell from expanded patient samples. (A, B) Cytotoxicity of EpCAM⁺ (A) or FAP⁺ cells (B), first isolated from three patients' ascites and expanded *ex vivo*. Cells were cultured with PBMC-derived T cells (E:T 5:1) and infected with EnAd or recombinant virus, or uninfected. Cytotoxicity was measured by flow cytometry after five days. (C) Induction of activation marker CD25 on T cells from (A + B) was determined by flow cytometry. (D) An ELISA was performed to measure the level of TGFβ in supernatants harvested from a repeat experiment to that displayed in (A – C). Each condition was measured in biological triplicate and represented as mean ± SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', *p<0.05, **p<0.01, ***p<0.001.

5.3.4 Characterisation of tumour-associated lymphocytes in exudate samples

Tumour-associated lymphocytes (TALs) isolated from ovarian cancer patient ascites are reported to have enriched expression of PD1 and impaired effector functions – including cytotoxicity and IFNγ production (Matsuzaki et al, 2010). We assessed the expression of PD1 by endogenous T cells in the exudate samples (following their initial isolation), and whilst only 10-20% of PBMC-derived T cells express PD1, the malignant exudate T cells were on average 65% positive for PD1 and on occasion reached as high as 92.5% (Table 5.3 and Figure 5.6A). To further characterise T cells in the exudates, ten patient samples were recovered from cryopreservation and the percentage CD3⁺ cells and their levels of LAG3 and TIM3 expression assessed by flow cytometry (Figure 5.6B-D). Fifty percent of TALs samples had significantly stronger expression of TIM3 than PBMCs, indicative of a more exhausted phenotype (Jones et al, 2008). For LAG3, however, there was no discernible pattern, with 2/10 samples showing higher expression, 1/10 with lower, and the rest within the normal range.

Table 5.3. Expression of PD1 on malignant exudate CD3⁺ T cells. The expression of PD1 by exudate T cells following their initial isolation from ascites and pleural effusions was assessed by flow cytometry and is displayed as a percentage of the total CD3⁺ cells in the sample.

Sample number	Cancer type	Cell type	
		CD3 ⁺	CD3 ⁺ PD1 ⁺
Asc.05	Breast	55	60.9
Asc.07	Breast	79.4	50.5
Asc.08	Ovarian	74	62.8
Asc.09	Pancreatic	22.1	73.6
Asc.10	Ovarian	27.3	77
Asc.11	Ovarian	53.2	70.9
Asc.12	Ovarian	6.5	78.5
Asc.13	Ovarian	17	89.3
Asc.14	Melanoma	77.4	28.9
Asc.15	Ovarian	15	84.7
MPE.01	Squamous cell tongue	48.5	69.8
MPE.02	Breast	45.3	45
MPE.03	Lung	78.7	55
MPE.05	Mesothelioma	37.2	47.2
MPE.06	Colorectal	2.89	73
MPE.08	Lung	15.3	92.5
MPE.09	Mesothelioma	84.9	74.6
MPE.10	Mesothelioma	95.1	30.2
MPE.11	Lymphoma	88.1	43.4
MPE.12	Mesothelioma	60.6	64.6
MPE.13	Colorectal	94.6	64.6
MPE.15	Lung	74.2	36.3
MPE.16	Ovarian	37.9	80.6
MPE.17	Mesothelioma	90.3	71.1
MPE.18	Lung	55.2	68.2
MPE.19	Breast	79.8	67.9
MPE.20	Mesothelioma	59.1	51.5
MPE.21	Mesothelioma	87.2	55.8

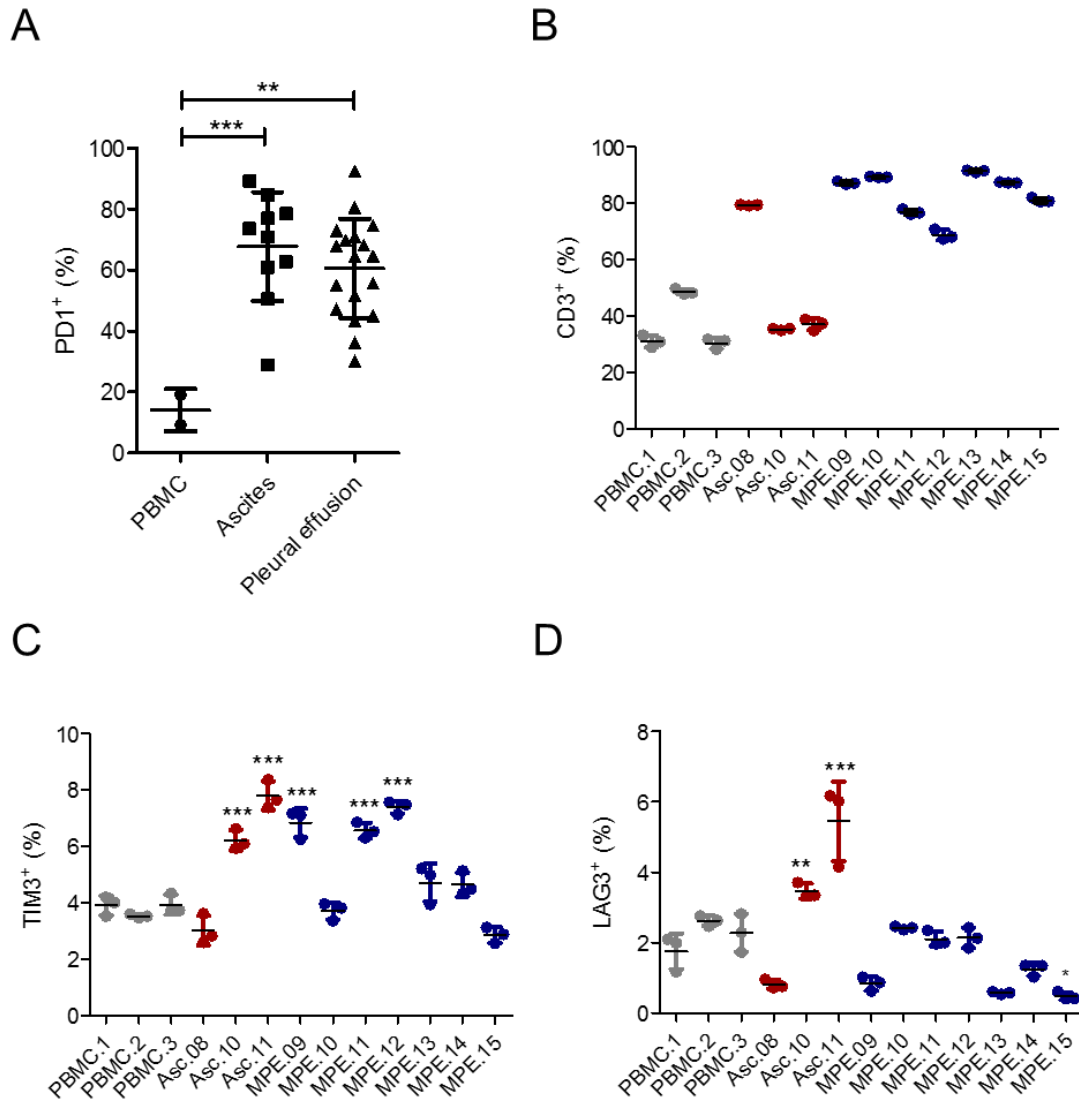


Figure 5.6. Characterisation of the endogenous T cells in malignant exudate samples. (A) Graphical representation of the data displayed in Table 5.3. The expression of PD1 by endogenous T cells was assessed by flow cytometry in the initial screen and is displayed as a percentage of the total CD3⁺ cells in the sample. (B - D) Unpurified total cells from PBMC, ascites (Asc) or pleural effusion (MPE) samples were recovered from cryopreservation, rested overnight, and the proportion of CD3⁺ cells (B) was assessed by flow cytometry. The percentage of CD3⁺ T cells that were TIM3⁺ (C) and LAG3⁺ (D) was also evaluated. Each condition was measured in biological triplicate and represented as mean ± SD. Significance was assessed by comparison to 'PBMC' using a one-way ANOVA test with Tukey's Post Hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

5.3.5 BiTEs can activate tumour-associated lymphocytes and direct cytotoxicity to tumour and fibroblast cell lines

To assess whether T cells within the cancer biopsy samples could be directed against target cells, BiTE-induced cell death was monitored in real-time by a xCELLigence-based cytotoxicity assay. PBMCs from three donors were used, and BiTE-mediated cytotoxicity was compared to that effected by T cells in six exudate samples (three ascites and three pleural effusion), including five that had significantly elevated levels of TIM3. To assess the activity of the EpCAM BiTE, DLD tumour cells were plated and unpurified PBMCs or exudate cells were added with BiTE-containing supernatants. In the absence of added cells, DLDs continued to grow before peaking at around 80 hours (Figure 5.7A). Remarkably, all exudate samples (red and blue) induced complete cytotoxicity of DLD cells when the EpCAM BiTE was added, with similar rates of lysis – with the exception of Asc.10 - observed when effected by PBMC and exudate T cells. Importantly, the irrelevant BiTE control demonstrated no target cell lysis in this time frame with the addition of any of the PBMC or exudate cells.

A similar pattern was demonstrated using the FAP BiTE, in which all exudate samples induced complete lysis of NHDF cells by 90 hours post-treatment (Figure 5.7B). In the presence of the control BiTE, there was no observable cytotoxicity mediated by 5/6 of the exudate samples. The exception, Asc.11, showed complete lysis of NHDF that was BiTE-independent, with BiTE-mediated cytotoxicity of DLD and NHDF (by Asc.11 cells) also the most rapid of all exudate and PBMC samples. One plausible explanation for this is a high proportion of NK cells within the patient sample, though additional sample analysis (for example, CD56⁺ population) would be required to confirm this.

Alternatively, whilst this phenomenon can't be explained by the number of CD3⁺ cells (the sample has the lowest %CD3 of the six exudate samples; see Figure 5.6B), Asc.11 has a CD4/CD8 ratio of 0.49. This ratio is the lowest of all 24 measured samples, considerably lower than the average of 3.0, and this high proportion of CD8 cytotoxic T cells – which kill more rapidly than CD4 cells - may explain the potency of response.

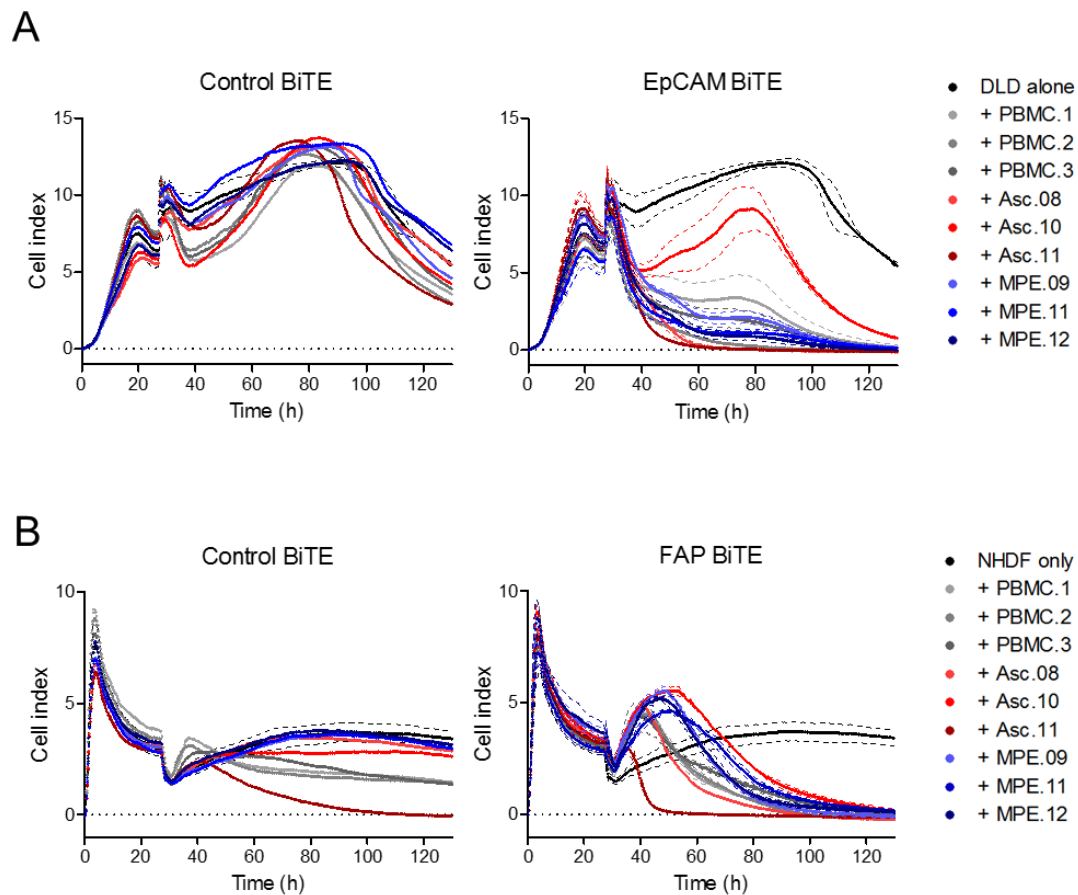


Figure 5.7. BiTEs direct T cells within patient biopsy samples to lyse EpCAM⁺ DLDs or FAP⁺ NHDF cells. (A) Viability of DLD cells in co-culture with PBMC or total exudate cells (E:T 5:1). Cell viability, displayed as cell index, was monitored in real time over 130 hours by a xCELLigence cytotoxicity assay. Control BiTE (left panel) or EpCAM BiTE (right panel) was added with PBMC or biopsy cells at 26 hours. **(B)** In parallel to (A), the viability of NHDF cells was also assessed. Cultures identical to above were established, and control BiTE- (left panel) or FAP BiTE-containing (right panel) supernatants were added. Treatment with EpCAM or FAP BiTE was measured in biological duplicate and represented as mean $\pm$ SD. Treatment with control BiTE was measured once. PBMC samples are displayed in grey, ascites in red and pleural effusion in blue.

To further investigate BiTE-mediated activation of these tumour-associated T cells, supernatants were harvested at 130 hours and IFN γ and IL-2 levels were measured by ELISA (Figure 5.8). EpCAM BiTE-mediated activation induced cytokine production in all exudate samples, though both IFN γ and IL-2 production appeared to be attenuated quite drastically in 2/6 cancer biopsy (Asc.10 and Asc.11) compared to PBMCs (Figure 5.8A). Interestingly, whilst the FAP BiTE similarly induced IFN γ production in all exudate samples, it was significantly reduced in the three pleural effusion samples (Figure 5.8B). This pattern is in stark contrast to that observed with EpCAM BiTE stimulation, in which Asc.10 and Asc.11 showed reduced expression. Stimulation with the FAP induced 300-fold lower levels of IL-2 than the EpCAM BiTE; a similar trend to that demonstrated *in vitro* in chapter four.

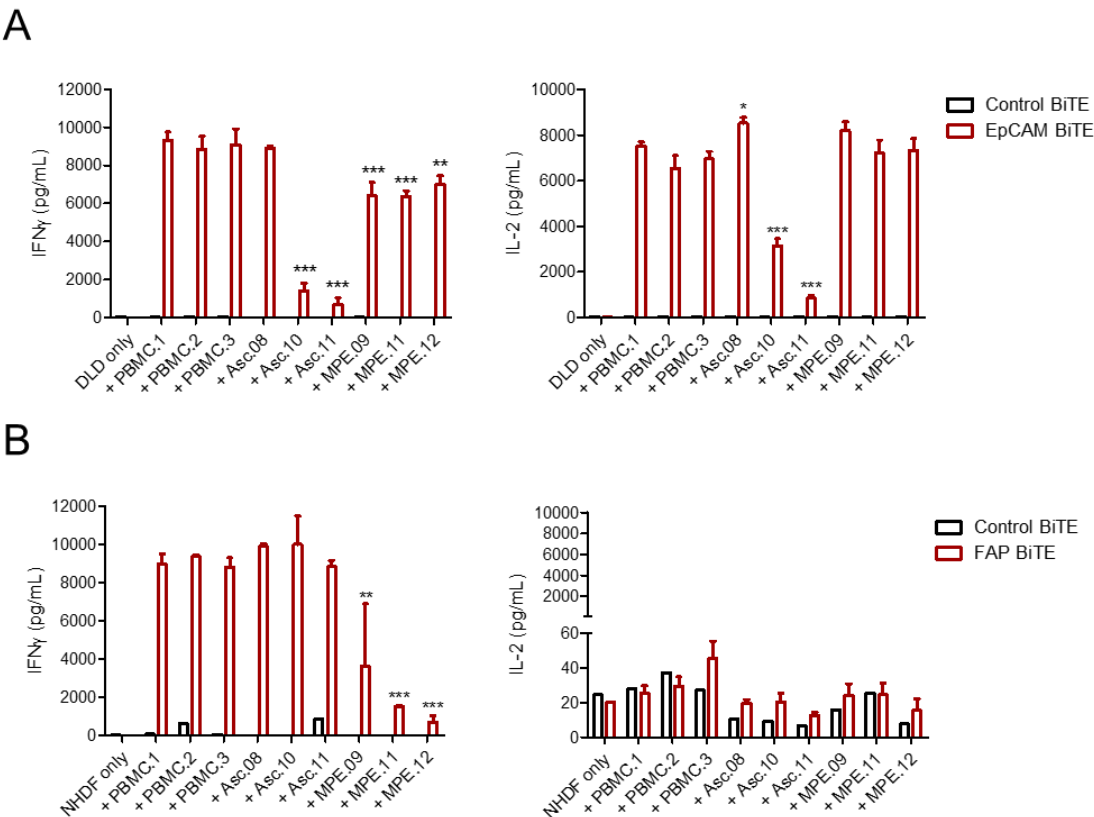


Figure 5.8. BiTEs activate endogenous T cells within patient biopsy samples. (A) Levels of IFN γ and IL-2 in culture supernatants harvested from the experiment described in Figure 5.7A. Supernatants were harvested at 130 hours and cytokine levels were quantitated by ELISA. (B) In parallel to (A), culture supernatants were harvested from the experiment displayed in Figure 5.7B, and IFN γ and IL-2 levels were similarly measured by ELISA. Treatment with EpCAM or FAP BiTE was measured in biological duplicate and represented as mean $\pm$ SD. Treatment with control BiTE was measured once. Significance was assessed by comparison to ‘PBMC’ using a one-way ANOVA test with Tukey’s Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.6 Characterisation of the fluid component of exudate samples

Malignant exudates represent an environment of potential immune tolerance with suppressed immune responses commonly observed in patients with late-stage metastatic cancer. The patient fluids to be used in this study were confirmed to be exudates by measuring protein concentration with all fluids having quantities in excess of 50 mg/mL (Figure 5.9A; transudate, < 25 mg/mL; exudate, > 30 mg/mL (Gupta et al, 1995)). The levels of IL-10 and TGF β , considered to be anti-inflammatory cytokines, were measured in serum from three healthy donors and malignant exudate fluids from 12 patients with peritoneal (Asc) and pleural (MPE) malignancies (Figure 5.9B and 5.9C). Consistent with the hypothesis that the exudates represent an environment of T cell immune suppression, IL-10 levels in the exudates (88.1 – 633.4 pg/mL, mean 375 pg/mL) were far in excess of those measured in normal serum (7.2 – 10.0 pg/mL, mean 8.9 pg/mL). However, in contrast, the levels of TGF β in the exudate fluids were significantly lower than that of normal serum. Whilst initially unexpected, this result was comparable to that reported in similar studies (Coupes et al, 2001; Santin et al, 2001). Indeed, platelets are well-documented to constitute one of the largest pools of TGF β – containing 40 to 100 times more than other cells– which they release upon activation (Ahamed et al, 2008; Assoian et al, 1983). It is likely that the blood collection and preparation process used to generate

the normal serum leads to rapid release of TGF β from latent pools, and the measured quantity doesn't mirror the true amount present in the circulation (Coupes et al, 2001). Therefore, the levels of total TGF β is unlikely to offer a useful measure of immune suppression within the fluid samples.

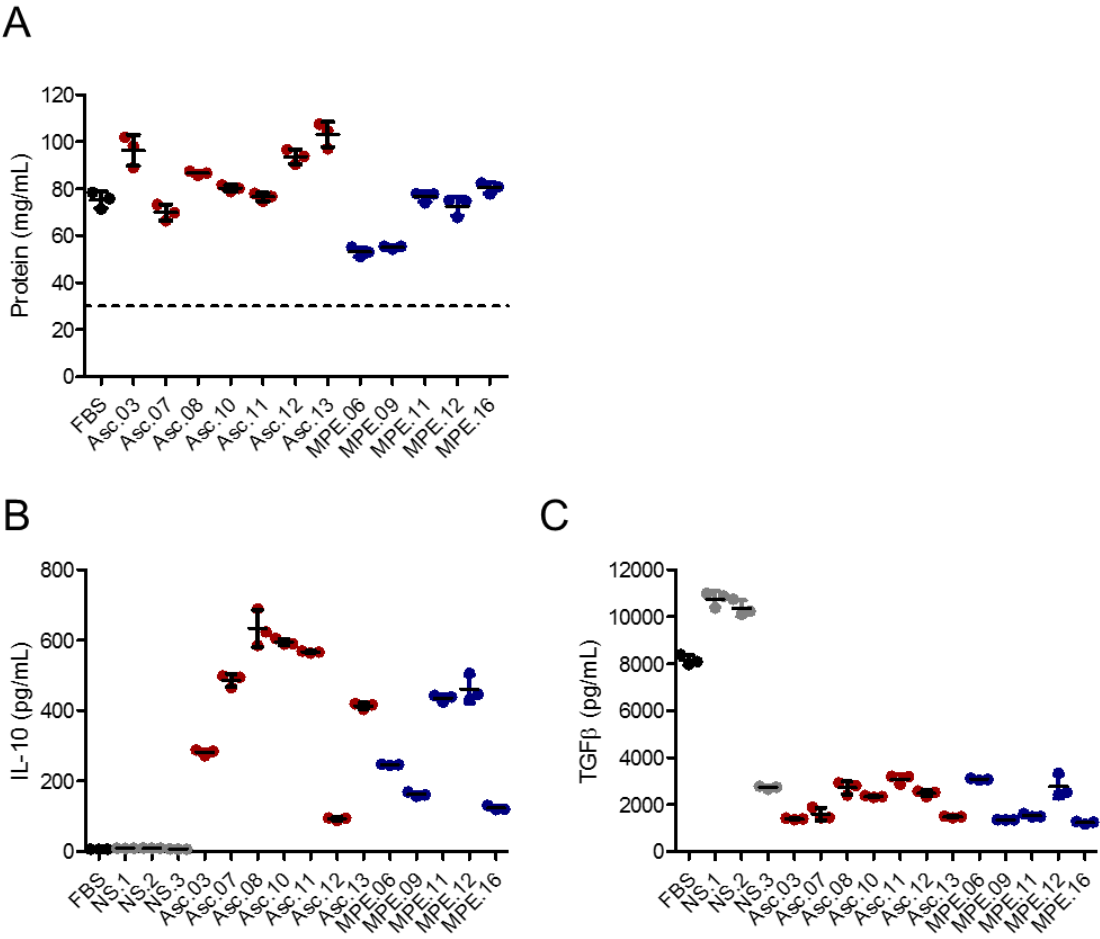


Figure 5.9. Characterisation of patient-derived malignant exudate fluids. (A) The concentration of protein in fluid samples was quantitated by bicinchoninic acid (BCA) assay. Fluids were classified as exudates if the protein concentration was greater than 30 mg/mL (Gupta et al, 1995; Hooper et al, 2010). NS, normal serum; Asc, ascites fluid; MPE, pleural effusion. (B, C) Quantity of human IL-10 (B) and total (active and latent) TGF β (C) in primary malignant exudates. NS, normal serum; Asc, ascites fluid; MPE, pleural effusion.

5.3.7 BiTE-mediated T cell activation can overcome immune suppression within malignant exudate fluids

The effects of these fluids on T cell activation was investigated by polyclonally stimulating PBMC-derived T cells with anti-CD3/CD28 beads, that mimic *in vivo* T cell activation, in the presence of normal serum, ascites or pleural fluid (all 50%). Whereas in normal serum the anti-CD3/CD28 beads reproducibly gave approximately 60% of T cells dual positivity for both CD25 and CD69, the presence of ascites fluid attenuated T cell activation in 6/12 fluids (Figure 5.10A). This was strongly correlative with a suppression in T cell degranulation (Pearson co-efficient, $r = 0.8951$; $p < 0.0001$), with CD107a-positive T cells reduced in 10/12 fluids and was particularly pronounced for patient fluids Asc.10, Asc.11, Asc.13, MPE.11 and MPE.12 (Figure 5.10B). Importantly, T cell degranulation negatively correlated with levels of IL-10 in the patient fluids (Pearson co-efficient, $r = -0.7645$; $p = 0.0038$; Figure 5.10C). This supports our hypothesis that components of malignant ascites and pleural fluid may exert an immune suppressive or tolerising effect.

T cell activation via BiTEs may be able to bypass tumour microenvironment-associated mechanisms of T cell immunosuppression (Nakamura & Smyth, 2016). We therefore investigated the ability of PBMC-derived T cells and EpCAM BiTE to mediate T cell activation (CD69, CD25) and degranulation (CD107a) in the presence of normal serum or seven patient malignant exudate samples, of which six were known to be immunosuppressive to T cell activation by the CD3/CD28 beads (Figure 5.10A and 5.10B). Remarkably, 0/7 fluids attenuated the BiTE-mediated increase in dual positive CD69⁺/CD25⁺ cells, with four supporting modest increases in BiTE-mediate T cell

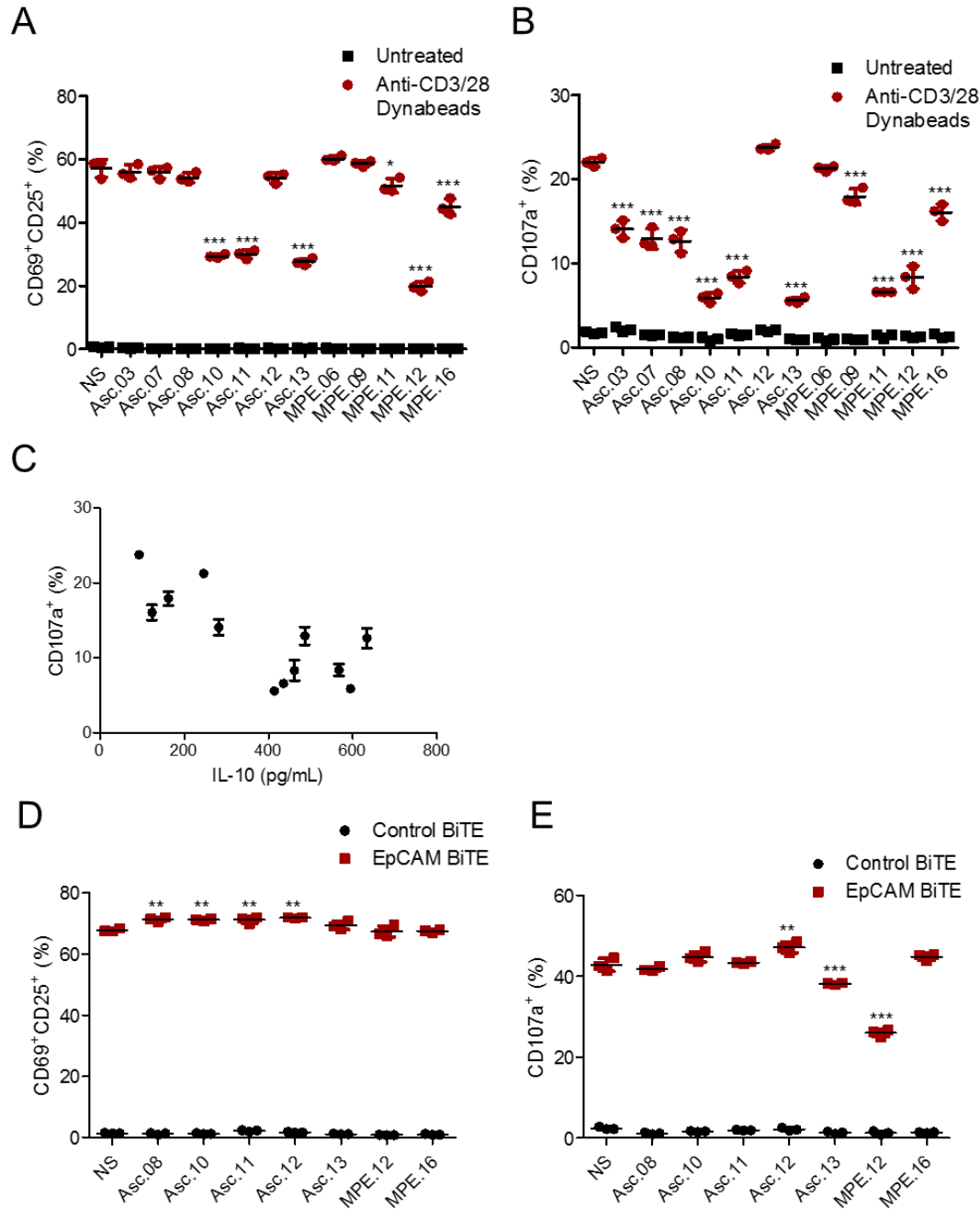


Figure 5.10. Free EpCAM BiTE can overcome immune suppressive effects of pleural effusion and ascites fluid. (A-B) PBMC-derived T cells were incubated with anti-CD3/CD28 Dynabeads in normal serum or exudate fluid (50%) from 12 cancer patients (Asc, ascites; MPE, pleural effusion). Induction of T cell activation markers CD69 and CD25 at 24 hours (A) and degranulation at six hours (B) were analysed using flow cytometry. (C) Correlation analysis of the degranulation of T cells and the quantity of IL-10 in the tested exudate fluid (see Figure 5.9B). (D-E) CD3-purified PBMC were co-cultured with SKOV3 (5:1) and EpCAM or control BiTE in the presence of normal serum or either pleural effusion or ascites fluid (50%) from seven patients. CD69 and CD25 dual positivity (D) and CD107a externalisation (E) on CD3⁺ T cells were measured by flow cytometry at 24 and six hours respectively. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed by comparison to 'untreated' or 'control BiTE' using a one-way ANOVA test with Tukey's Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

activation compared to normal serum (Figure 5.10D). Two fluids (Asc.13 and MPE.12) reduced T cell degranulation (measured by CD107a), however the decrease induced by Asc.13 was modest (Figure 5.10E). Notably, BiTE-mediated T cell activation was more potent than CD3/CD28 beads, giving higher levels of dual positive CD69⁺/CD25⁺ cells (67.6% vs 58.9%, Figure 5.10A and D) and degranulation measured by CD107a (42.9% vs 22% Figure 5.10B and E).

We assessed the ability of BiTEs to activate T cell cytotoxicity to SKOV3 target cells (a cell line derived from ovarian ascites) in the presence of immunosuppressive ascites or pleural fluid following infection with EnAd-SA-controlBiTE or EnAd-SA-EpCAMBiTE. PBMC-derived T cells were incubated with normal serum (50%) or four immunosuppressive patient fluids (Asc.10, Asc.11, Asc.13, MPE.12, 50%; see Figure 5.11A for reduced CD107a activity) and cytotoxicity evaluated by an xCELLigence-based cytotoxicity assay. All samples showed similar profiles of cytotoxicity to SKOV3 cells, indicating that the virus-encoded BiTEs can overcome the immune-suppressive components of the malignant effusions (including samples Asc.13 and MPE.12) and mediate functional cytotoxicity of T cells (Figure 5.11B).

We next investigated whether the FAP BiTE has a similar ability to induce activation of PBMC-derived T cells in the presence of immunosuppressive exudate samples (all at 50%). Whereas in normal serum the anti-CD3/CD28 beads (which mimic *in vivo* T cell activation) gave 74% of T cells dual-positivity for both CD25 and CD69 at 24 hours, the presence of ascites fluids significantly attenuated T cell activation in 3/5 tested samples (Figure 5.12A). When stimulated with the FAP BiTE, however, there was no observable exudate-mediated suppression of T cell activation (Figure 5.12B), demonstrating that the

FAP-targeted BiTE may similarly overcome immunosuppressive mechanisms to activate T cells.

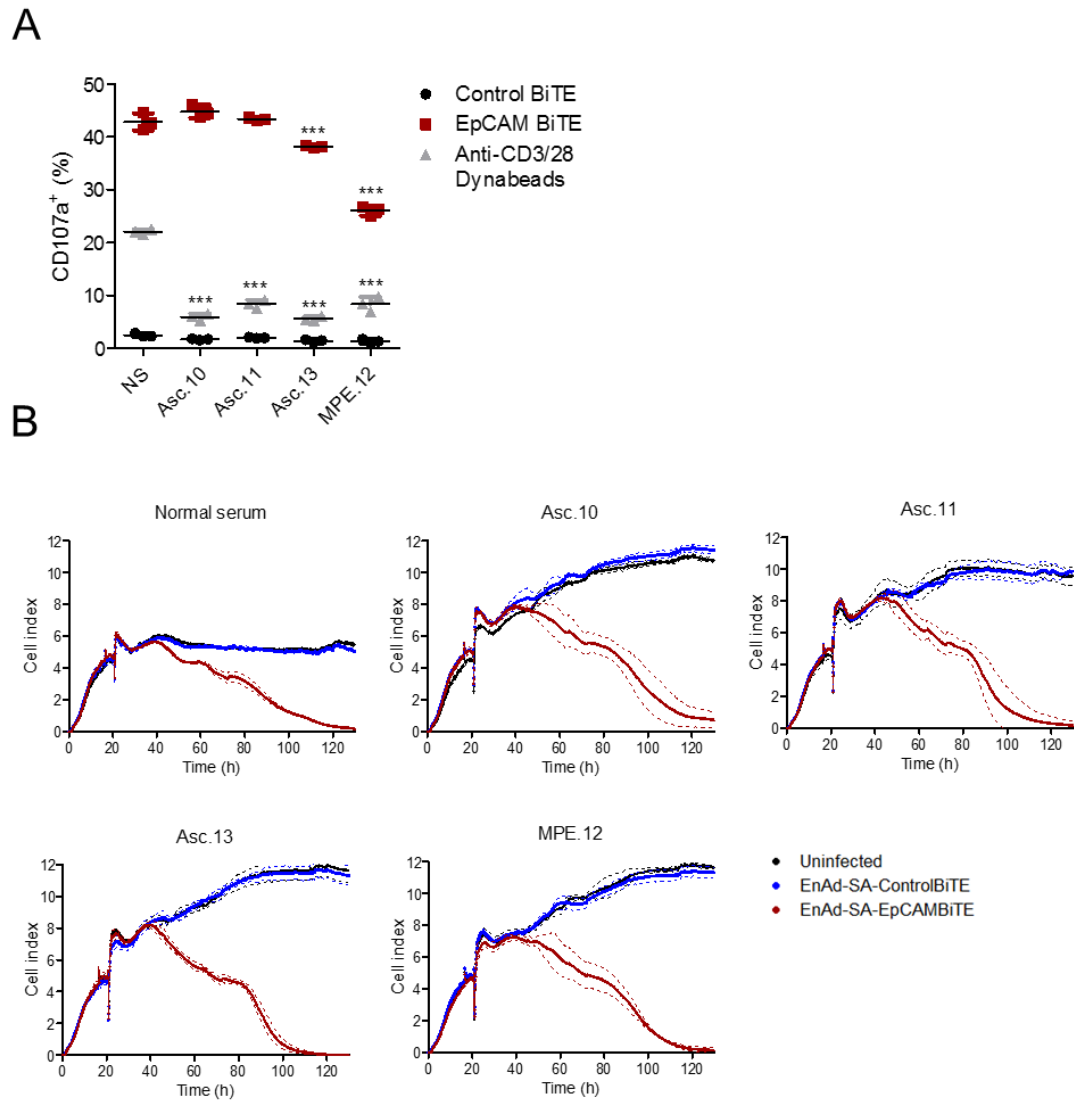


Figure 5.11. EnAd-SA-EpCAM BiTE induces unhindered T cell-mediated lysis of SKOV3 cells despite immune suppressive effects of exudate fluids. (A) Degranulation of stimulated PBMC-derived T cells incubated in normal serum (NS) or the malignant exudate fluids to be used in (B). Data is originally shown in Figure 5.10, but the relevant data is included here for clarity. **(B)** Viability of SKOV3 cells were monitored in real-time over 130 hours by a xCELLigence-based cytotoxicity assay. SKOV3 cells were seeded and incubated with EnAd-SA-controlBiTE or EnAd-SA-EpCAMBiTE at 16 hours. Uninfected cells served as a negative control. CD3-purified PBMC (5:1) were added 2 hours post-infection in the presence of culture medium or either pleural effusion or ascites fluid (all 50%). Impedance was measured at 10-minute intervals. Each condition was measured in biological triplicate and represented as mean $\pm$ SD.

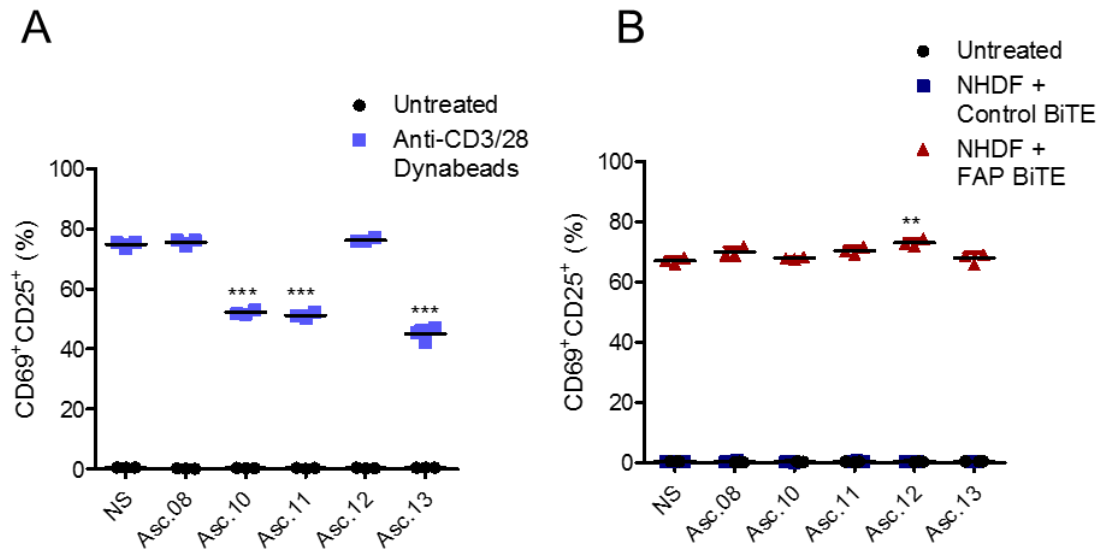


Figure 5.12. Free FAP BiTE can overcome immunosuppression in ascites fluids to activate primary T cells. (A) CD3-purified PBMC were incubated with anti-CD3/CD28 Dynabeads in normal serum (NS) or ascites fluid (all 50%) from five patients. CD69 and CD25 dual positivity was measured by flow cytometry at 24 hours post-treatment. (B) Induction of CD69/CD25 on PBMC-derived T cell cultured with NHDF (E:T 5:1) and BiTEs in the presence of normal serum or ascites fluid (all 50%). Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed by comparison to 'NS' using a one-way ANOVA test with Tukey's Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.8 EnAd-EpCAMBiTE can activate endogenous T cells and kill endogenous tumour cells within malignant cancer biopsies

In addition to the immune suppressive fluid and tumour cells present, ascites and pleural effusion samples contain tumour-associated lymphocytes and supporting cells of the tumour stroma, providing a unique tumour-like model system to test BiTE-mediated activation of endogenous patient-derived T cells. Following incubation of total endogenous cells (from patient sample Asc.15; E:T ratio 0.49) and ascites fluid with free recombinant BiTE or virus encoding BiTE, activation of endogenous patient T cells was assessed. After 24 hours incubation, the EpCAM BiTE (but not the control BiTE) induced CD69 and CD25 expression, similarly to that observed in culture RPMI medium (Figure 5.13A). This effect was persistent, and after five days only ascites cells receiving free

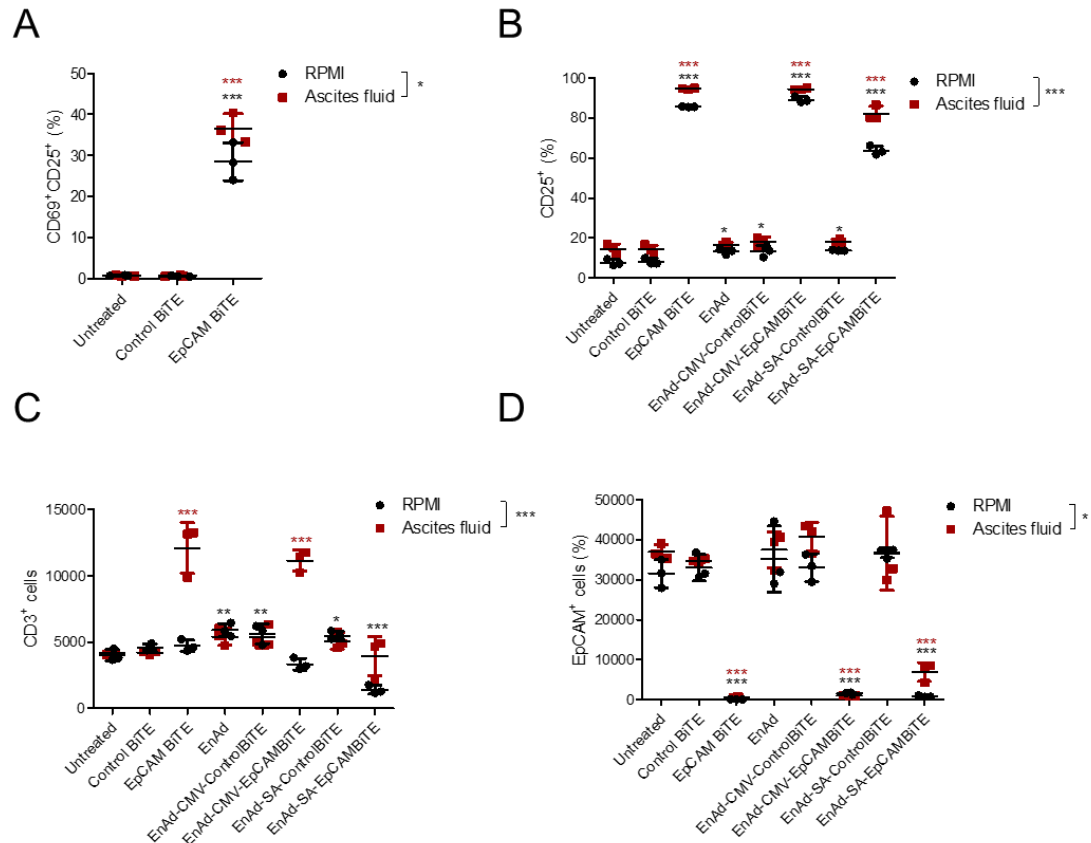


Figure 5.13. EnAds armed with EpCAM BiTE can activate endogenous T cells to kill endogenous tumour cells in both culture medium and malignant exudate fluid. (A) Endogenous unpurified ascites cells (from Asc.15) were incubated in RPMI culture medium or 50% ascites fluid in the presence of free EpCAM or control BiTE. After 24 hours, the total cell population was harvested, and the number of CD69⁺CD25⁺ T cells measured by flow cytometry. (B-D) Total ascites cells (from Asc.15) were incubated in RPMI culture medium or 50% ascites fluid and treated with free BiTE, EnAd or EnAd expressing BiTE. After five days, CD25 expression of endogenous T cells (B) and the quantity of CD3⁺ (C) and EpCAM⁺ (D) cells were measured by flow cytometry. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance within each fluid treatment was assessed by comparison to ‘untreated’ using a one-way ANOVA test with Tukey’s Post Hoc analysis. Significance between fluid treatments was assessed by two-way ANOVA test with Bonferroni Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

EpCAM BiTE or virus encoding EpCAM BiTE showed CD25 expression on T cells, with slightly higher levels seen when cells were cultured in the ascites fluid than in RPMI media (Figure 5.13B). Interestingly, free EpCAM BiTE and EnAd-CMV-EpCAMBiTE mediated proliferation of T cells in whole ascites fluid, and not in basic RPMI media, suggesting that basic culture media may lack factors required to support viability or

proliferation of the ascites T cells (Figure 5.13C). The absence of an increase in CD3⁺ cells following EnAd-SA-EpCAMBiTE treatment at this time point may reflect the later onset of expression kinetics relative to the CMV promoter. Cytotoxicity was assessed by measuring residual level of EpCAM cells by flow cytometry. Free EpCAM BiTE and the virus encoding EpCAM BiTE (CMV and SA) caused a similar depletion of EpCAM cells when the experiment was performed in basic medium and also in 50% ascites fluid (Figure 5.13D). These data show that the EpCAM BiTE can overcome the immune suppressive effects of peritoneal ascites or pleural fluid to activate endogenous T cells to kill endogenous tumour cells.

5.3.9 Identification of the cell types within exudate samples that express EnAd encoded transgenes

To determine which cells were infected by the EnAd virus, and which cells would produce and secrete BiTEs, we evaluated infection patterns using reporter viruses (EnAd-CMV-GFP and EnAd-SA-GFP). The viruses were added to whole cells isolated from MPE.16 by centrifugation and resuspended in 50% exudate fluid. After 72 hours, fluorescence microscopy showed that EnAd-CMV-GFP (which is expected to express GFP transgene in all cells that are infected) showed expression in cells with a range of morphologies (Figure 5.14A), whereas EnAd-SA-GFP (which is designed to express GFP transgene only in cells supporting virus replication) was restricted to plump round cells. Flow cytometry showed that the GFP expression from EnAd-CMV-GFP was found in many macrophages (CD11b⁺) as well as EpCAM⁺ cells, whereas EnAd-SA-GFP expression was restricted to EpCAM⁺ cells (Figure 5.14B). This supports the hypothesis that EnAd-

CMV-EpCAM BiTE will express BiTEs in a range of cells within the malignant effusions, whereas EnAd-SA-EpCAM BiTE will be restricted mainly to tumour cells.

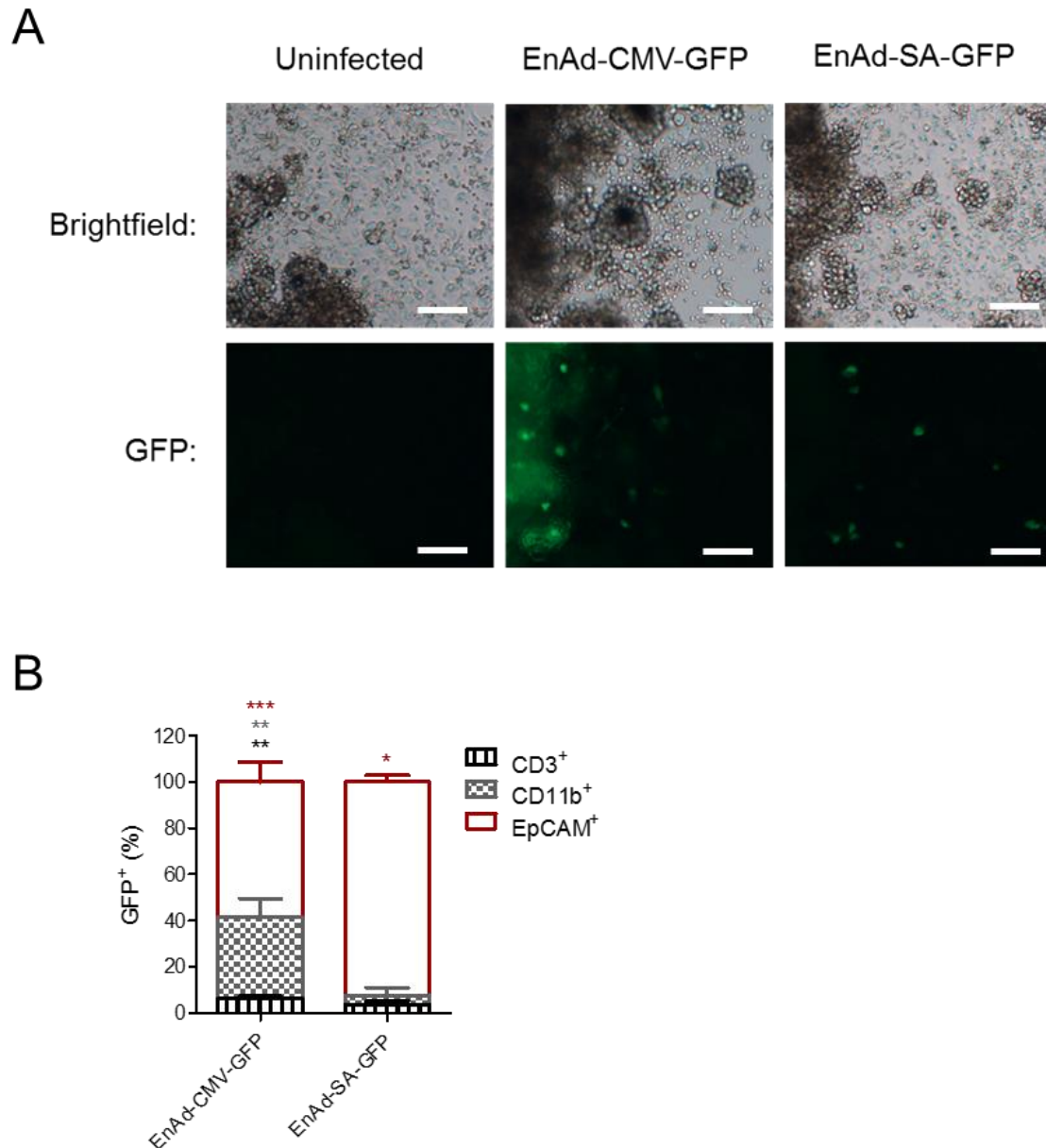


Figure 5.14. GFP expression from major late promoter (SA)-driven recombinant EnAd is selectively expressed in EpCAM⁺ tumour cells. Total cells from a peritoneal ascites sample (MPE.16) were incubated with EnAd-CMV-GFP or EnAd-SA-GFP, with uninfected cells serving as a negative control. After three days, wells were imaged by brightfield or fluorescent microscopy. Original magnification $\times 10$; scale bar 100 μm (A). Total cells were then harvested, and the proportion of GFP⁺ cells that were CD3⁺, CD11b⁺ or EpCAM⁺ was measured using flow cytometry (B). Each condition was measured in biological quadruplicate and represented as mean $\pm$ SD. Significance was assessed by comparison to ‘uninfected’ control samples using a one-way ANOVA test with Tukey’s Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.10 EnAd-EpCAMBiTE shows reproducible activity within a range of malignant peritoneal and pleural exudates

To assess inter-patient variability of the EpCAM BiTE-expressing viruses in this highly clinically-relevant setting, we assessed activity in several samples of pathological exudates from patients with a range of malignancies. At initial screening samples considered suitable for further analysis were those containing CD3⁺ and EpCAM⁺ cells, with a wide range of E:T (CD3⁺:EpCAM⁺) ratios (0.25 to 33.5, see graph legend of Figure 5.15C).

Total exudate cells (prepared as above) were incubated at fixed concentrations in 50% exudate fluid in the presence of 500 ng/mL free EpCAM BiTE or 100 vp/cell virus encoding BiTE. After five days, the total cell population was harvested, and the total number of CD3⁺ cells (Figure 5.15A) was measured. Compared to untreated controls, only samples receiving the free EpCAM BiTE or EnAd encoding EpCAM BiTE showed T cell proliferation. This confirms that the EpCAM BiTE was binding to the EpCAM target and crosslinking CD3 to stimulate endogenous T cells. Treatment with the EpCAM BiTE, EnAd-CMV-EpCAM BiTE and EnAd-SA-EpCAM BiTE typically expanded the T cell numbers by factors of up to 30-fold. The free EpCAM BiTE also induced significant T cell activation of tumour-associated lymphocytes (assessed by CD25 expression) in all patients' samples, even within the likely immune-tolerising environment of the malignant exudate fluid (Figure 5.15B). There was noticeable variation between patients, with activation ranging from 50% to 95% dependent on the donor. Similarly, samples treated with EnAd expressing the EpCAM BiTE showed high activation in some patients (ranging from 10-20% up to 95%, for EnAd-SA-EpCAM

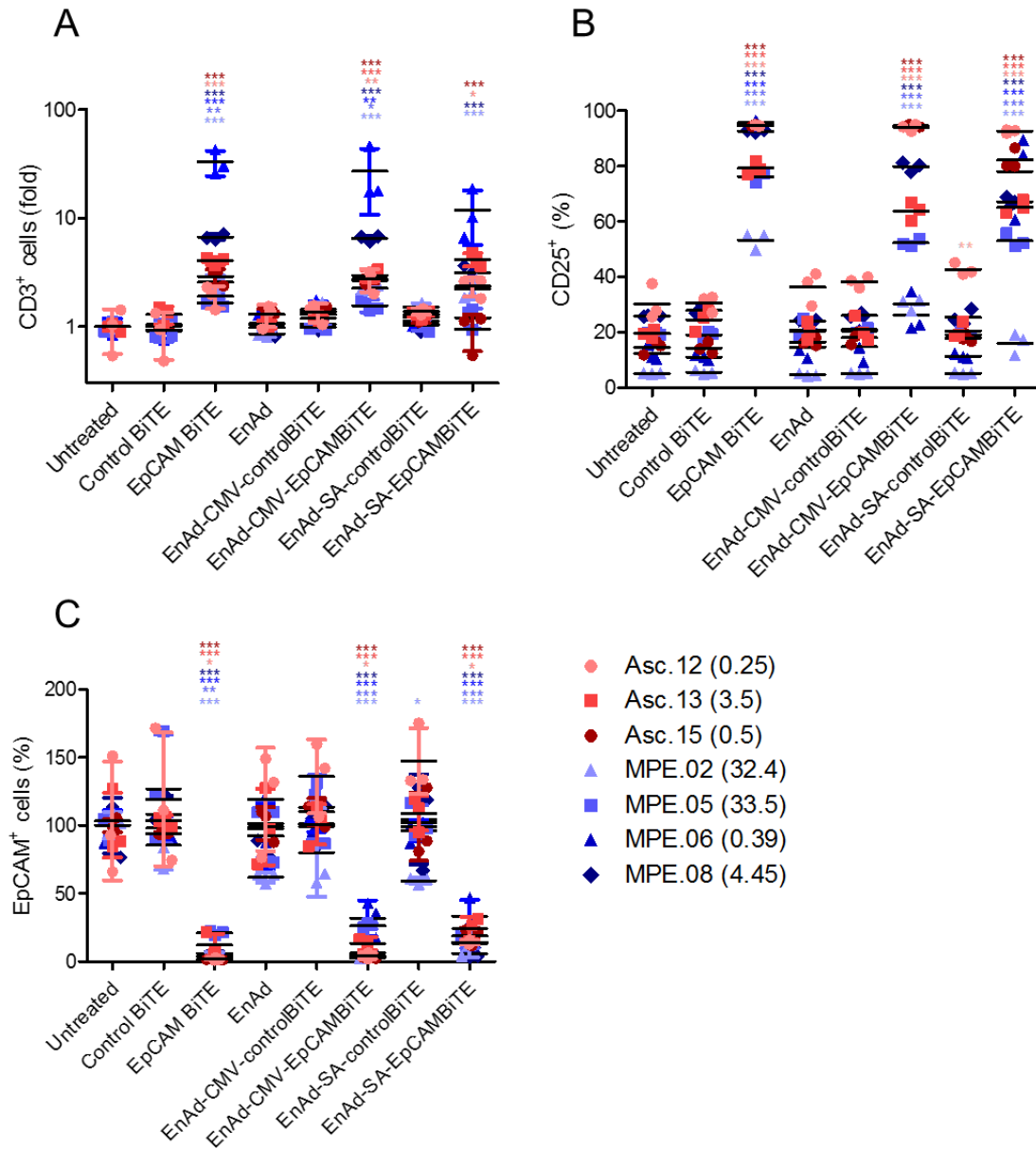


Figure 5.15. EnAd-EpCAMBiTE shows reproducible activity within an expanded cohort of patient malignant peritoneal and pleural exudates. (A-B) Unpurified total cells from ascites or pleural effusions (from seven different patients; pleural effusion, blue; peritoneal ascites, red) were incubated in 50% fluid from the same exudate sample in the presence of free BiTE, EnAd or recombinant virus. After five days, the total cell population was harvested, and the number of (A) CD3⁺ T cells and those which were (B) CD25⁺ were quantified. **(C)** The number of residual EpCAM⁺ cells from (A) was measured using flow cytometry. E:T (CD3⁺:EpCAM⁺) ratio upon receipt is indicated in brackets for each sample. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed by comparison to ‘untreated’ using a one-way ANOVA test with Tukey’s Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

BiTE). This may reflect the broad range of E:T ratios between patients (ranging from 0.25 to 33.5). Indeed, this ratio significantly negatively correlated with the level of induced CD25 expression (Pearson co-efficient, $r = -0.8479$; $p = 0.0159$), with T cells likely receiving higher stimulation at lower E:T ratios. Parental EnAd, or EnAd expressing control BiTEs, or free control BiTEs caused no stimulation above background in any sample.

We assessed the ability of the BiTE-expressing viruses to mediate EpCAM-targeted cytotoxicity by measuring residual levels of EpCAM positive cells by flow cytometry at the end of the five-day incubation (Figure 5.15C). The free EpCAM BiTE, and the two viruses encoding EpCAM BiTE, caused a marked depletion of autologous EpCAM-expressing cells in every case, whereas the other treatments had little or no effect on the level of EpCAM⁺ cells. In most patients, EnAd expressing the EpCAM BiTE showed similar levels of T cell activation and targeted cytotoxicity independent of whether BiTE expression was regulated by the CMV promoter or the SA system. However, one patient (MPE.06) showed higher T cell expression of CD25 using the EnAd-SA-EpCAMBiTE compared to EnAd-CMV-EpCAMBiTE, although the levels of T cell proliferation and associated cytotoxicity to EpCAM⁺ cells were similar for both. The reason for this finding is currently unclear, although it might indicate that even partial T cell activation is sufficient to eradicate tumour cells in this patient sample.

The different effects of parental EnAd and EnAd-SA-EpCAM BiTE are shown by microscopy in Figure 15.16 where expression of the BiTE decreases the presence of tumour cells (seen as large, plump cells) and expands the T cell population (small round

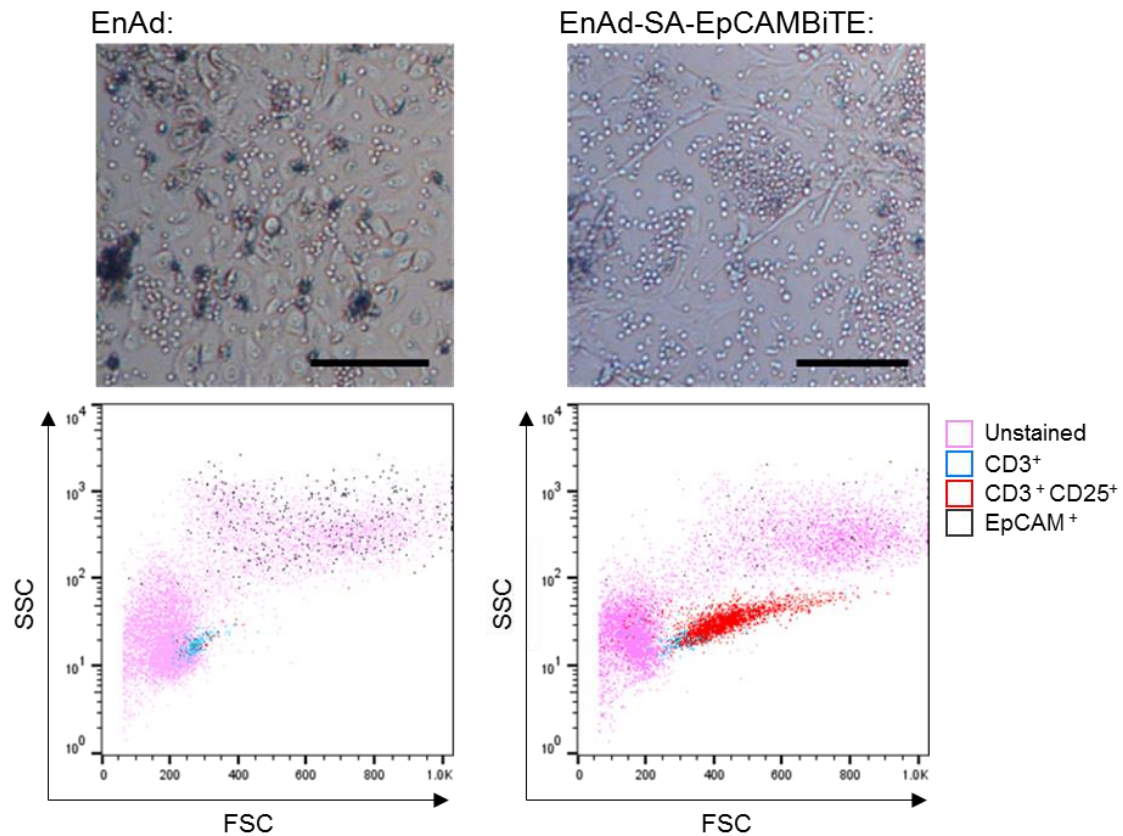


Figure 5.16. Representative microscopy images displaying the proliferation of T cells and elimination of tumour cells induced by EnAd-SA-EpCAMBiTE. Representative microscopy images (magnification $\times 10$; scale bar 100 μm) and flow cytometry analysis of sample MPE.06 (cancer cells and lymphocytes) following treatment with EnAd or EnAd-SA-EpCAM BiTE. E:T ($\text{CD3}^+:\text{EpCAM}^+$) ratio of this sample upon receipt was 0.39.

cells). The associated flow cytometry plots confirm the substantial expansion and activation of T cells following treatment with the EpCAM BiTE-expressing virus.

Finally, we characterised the effects of the various treatments by measuring the levels of key Th1 cytokines produced using a multiplex cytokine bead array (Figure 5.17). By far the greatest fold increases were in $\text{IFN}\gamma$, which rose nearly 1000-fold following treatment with the free EpCAM BiTE or EnAd encoding EpCAM BiTE. These two treatments also caused approximately 10-fold increases in expression of IL-5, IL-13, $\text{TNF}\alpha$, IL17A and

IL17F, characteristic of activated T cells. EnAd alone (or expressing the control BiTE) also caused a 10-fold rise in IFN γ , but otherwise no treatments caused any appreciable changes in cytokine expression.

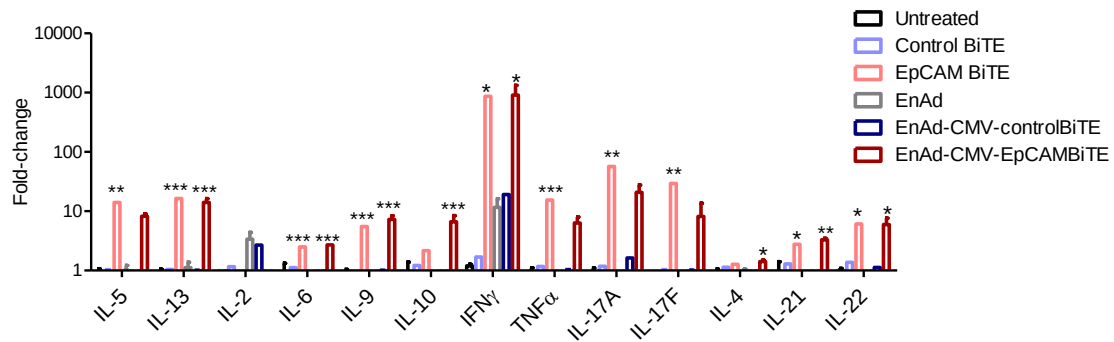


Figure 5.17. EnAd-CMV-EpCAMBiTE induces secretion of Th1 and Th17 cytokines from endogenous T cells in pleural effusion sample. Cytokine levels in supernatants harvested from the experiment displayed in Figure 5.15 (MPE.05) were measured by multiplex human Th cytokine panel. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance was assessed by comparison to ‘untreated’ using a one-way ANOVA test with Tukey’s Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.11 EnAd-FAP BiTE reproducibly activates tumour-associated lymphocytes to kill endogenous fibroblasts within malignant ascites isolates

Similar studies were established assessing the ability of FAP BiTE expressing viruses to induce T cell-mediated cytotoxicity of primary fibroblasts in exudate cultures. Total (and unpurified) cells from three FAP⁺ patient biopsy samples were plated in 50% ascites fluid and treated with free BiTE or 100 vp/cell parental of BiTE encoding virus. After five days incubation, T cell activation (by CD25 expression; Figure 5.18A) and absolute number of CD3⁺ and FAP⁺ cells were measured by flow cytometry (Figure 5.18B and C). In all

patient samples, free BiTE and EnAd-CMV-FAP BiTE induced strong T cell activation, with up to 80% of patient-derived T cells activated, and a marked depletion of FAP⁺ fibroblasts. Interestingly, EnAd-SA-FAPBiTE induced no activation of T cells or associated fibroblast cytotoxicity in Asc.07. This is likely due to the absence of tumour cells (EpCAM⁺ cells) in this sample (Table 5.2) and is consistent with the requirement for tumour cells for splice acceptor-driven transgene expression. Taken together, this data demonstrated that EnAd expressing FAP BiTE can reproducibly activate tumour-associated T cells to kill endogenous fibroblasts in malignant biopsy samples.

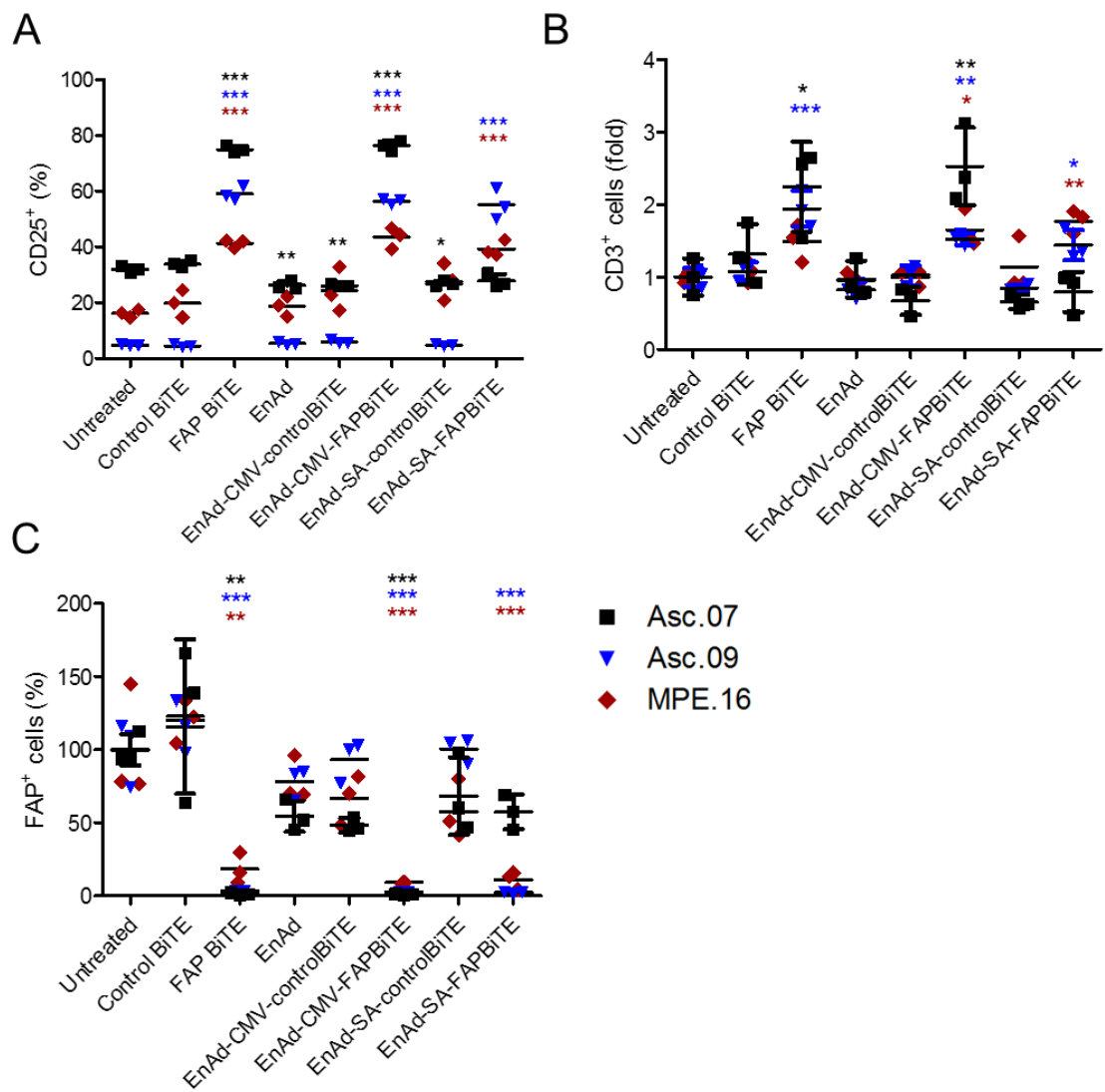


Figure 5.18. EnAd-expressing FAP BiTE activates endogenous T cells and induces FAP⁺ cell lysis in malignant exudate samples. Induction of CD25 (A) on endogenous T cells following treatment with free BiTEs or EnAd expressing BiTE. Total unpurified cells were plated and treated in 50% exudate fluid from the same biopsy sample and harvested and analysed by flow cytometry five days post-treatment. Absolute quantity of CD3⁺ (B) and residual FAP⁺ cells (C) was also measured. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance within each fluid treatment was assessed by comparison to 'untreated' using a one-way ANOVA test with Tukey's Post Hoc analysis, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3.12 Blockade of PDL1 has little impact on BiTE mediated cytotoxicity

Tumour cells and cancer-associated fibroblasts are reported to express PDL1 on their surface, one mechanism in which they may induce T cell exhaustion (Juneja et al, 2017; Nazareth et al, 2007). We investigated whether BiTE activity could be improved by blocking the PD1 checkpoint in patient biopsy samples. Ascites cells isolated from a patient with PDL1⁺ EpCAM⁺ cells (in which T cells were 73.6% PD1⁺; Figure 5.19A and B) were incubated with free EpCAM BiTE or EpCAM BiTE expressing virus, in the presence or absence of a blocking antibody for PDL1. After two days, total cells were harvested and residual EpCAM⁺ cells and T cell activation was measured. Whilst there was a modest increase in CD25 induction (Figure 5.19C) following addition of PDL1 antibody, cytotoxicity of EpCAM⁺ cells (Figure 5.19D) was unaffected – with near complete target lysis by day two in either setting. This suggests that EpCAM BiTE-mediated T cell activation and resulting cytotoxicity are little effected by the PD1/PDL1 checkpoint.

The effect of PD1 blockade was analogous for the FAP-targeted BiTE. A similar culture to above was established using a FAP⁺ ascites sample (Figure 5.20A and B) treated with free BiTE or FAP BiTE expressing virus. The inclusion of a PDL1 blocking antibody

with EnAd-CMV-FAPBiTE induced a slight increase in T cell activation (CD25; Figure 5.20C), though comparably to EpCAM BiTE-mediated lysis, there was no effect on target cell cytotoxicity (Figure 5.20D). Interestingly, production of IFN γ increased two-fold with PD1 blockade (Figure 5.20E), and this is possibly reflective of PD1 signalling having differential effects on individual T cell effector functions (Wei et al, 2013).

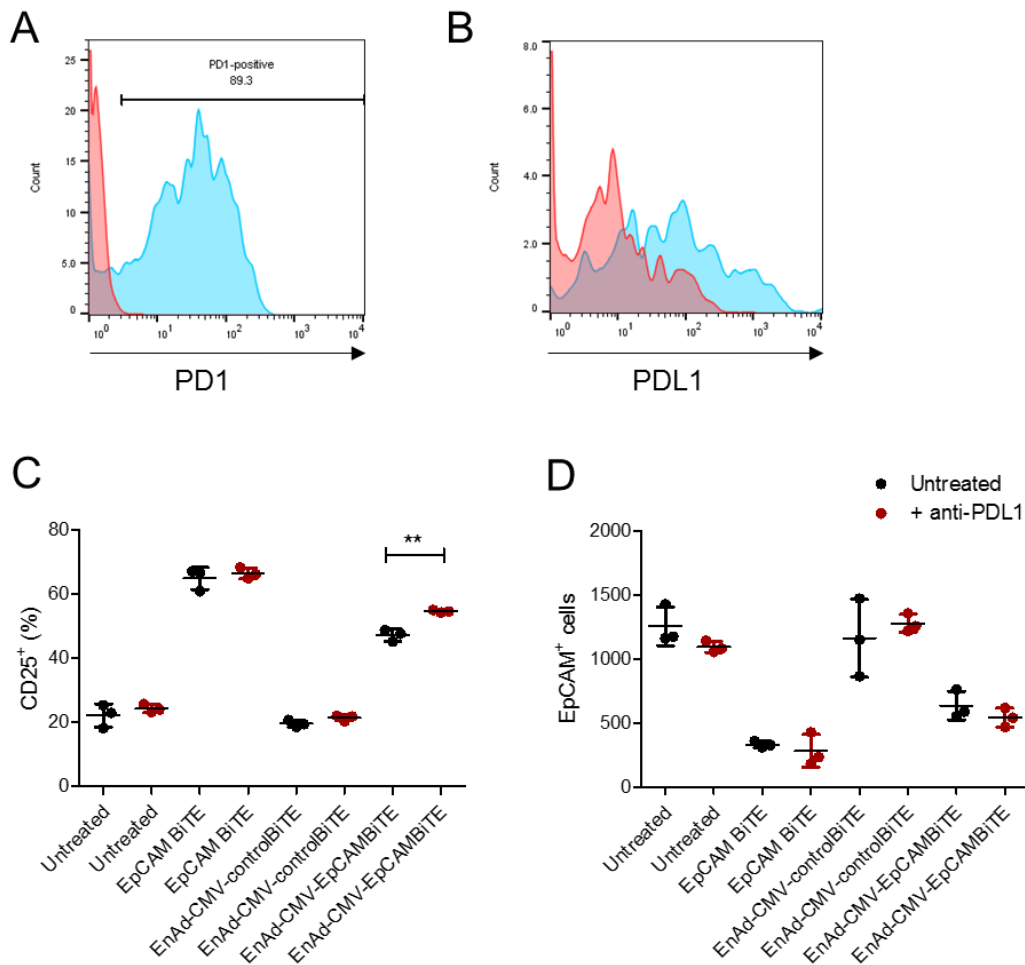


Figure 5.19. PDL1 blockade has little effect on EpCAM BiTE-mediated T cell activation in ascites sample. (A, B) The expression of PD1 (blue) by endogenous CD3⁺ cells (A) and PDL1 (blue) on EpCAM⁺ cells (B) following their initial isolation from a peritoneal ascites sample (Asc.13) was assessed by flow cytometry. Isotype control antibodies were used to determine background fluorescence and unspecific binding (red). (C) Total cells from peritoneal ascites were incubated in 50% fluid from the same exudate in the presence of free BiTE, EnAd or recombinant virus, with or without anti-PDL1 blocking antibody. After two days, the total cell population was harvested, and the number of CD25⁺ T cells was quantified by flow cytometry. (D) The absolute number of EpCAM⁺ cells in (C) was measured using flow cytometry. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance within each fluid treatment was assessed by comparison to ‘untreated’ using a one-way ANOVA test with Tukey’s Post Hoc analysis, * p <0.05, ** p <0.01, *** p <0.001.

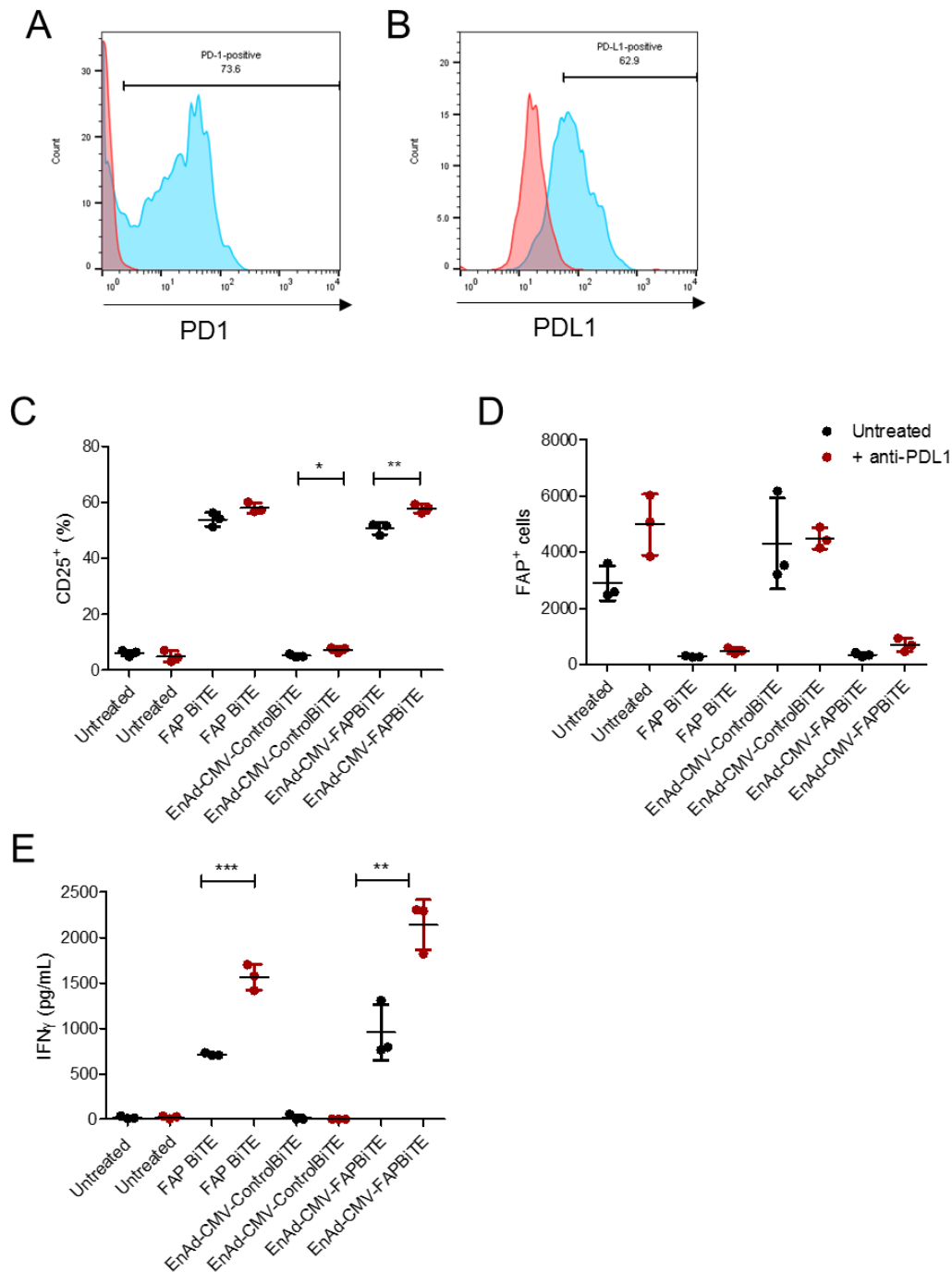


Figure 5.20. The effect of PDL1 blocking antibodies on FAP BiTE-mediated T cell activation in ascites sample. (A, B) The expression of PD1 (blue) by endogenous CD3⁺ (A) and PDL1 (blue) on FAP⁺ cells (B) following their initial isolation from a peritoneal ascites sample (Asc.09) was assessed by flow cytometry. Isotype control antibodies were used to determine spectra of background fluorescence (red). **(C)** Unpurified total cells from peritoneal ascites were incubated in 50% fluid from the same exudate in the presence of free BiTE, EnAd or recombinant virus, with or without anti-PDL1 blocking antibody. After two days, the total cell population was harvested, and the number of CD25⁺ T cells was quantified by flow cytometry. **(D)** The absolute number of FAP⁺ cells in (C) was measured using flow cytometry. **(E)** The quantity of IFN γ in culture supernatants from (C) was measured by ELISA. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance within each fluid treatment was assessed by comparison to 'untreated' using a one-way ANOVA test with Tukey's Post Hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

5.3.13 EnAd-FAPBiTE can induce the polarisation of tumour associated macrophages in patient ascites to a more M1-like phenotype

BiTE-activated T cells produce large quantities of Th1 cytokines, including IFN γ , TNF α and IL-2. It is plausible that this change in cytokine milieu may sufficiently induce a shift in the tumour microenvironment from immunosuppressive and pro-oncogenic state towards anti-tumour activity. We investigated this by evaluating the polarisation (and potential reprogramming) of CD11b⁺CD64⁺ macrophages in a patient-derived FAP⁺ cancer biopsy isolate (Tamoutounour et al, 2012). Total unpurified cells (Asc.16) were plated in 50% ascites fluid and treated with free BiTE or infected with EnAd-SA-control BiTE or EnAd-SA-FAPBiTE virus (at 100 vp/cell). Cells were treated in parallel with IFN γ to induce an activated macrophage phenotype. After three days incubation, activation status (CD25) of T cells was measured by flow cytometry (Figure 5.21A, black circles). Treatment with FAP BiTE and EnAd-SA-FAPBiTE induced CD25 expression of up to approximately 60% of CD3⁺ T cells and this activation translated to large quantities of IFN γ in culture supernatants (Figure 5.21A, red squares). No increase above background by the control BiTE or control virus was observed for CD25 or IFN γ expression.

To determine if T cell activation and the subsequent production of IFN γ affect TAM polarisation, the expression levels of CD64 and CD86 (M1 or ‘activated’ markers) and CD206 and CD163 (M2 or TAM markers) on macrophages were measured (Figure 5.21B). Treatment with free FAP BiTE or EnAd expressing FAP BiTE appeared to induce a more activated phenotype – manifested by significant increases in CD64 expression, and strong decreases in CD206 and CD163 – similar to that observed when recombinant

IFN γ was added in. Whilst treatment with free FAP BiTE or control virus induced no change in CD86 above background, the EnAd expressing FAP BiTE induced a large increase in CD86 expression – indicating possible synergism between virus infection and FAP BiTE activity (Figure 5.21C). Interestingly, IFN γ treatment seemed to induce a modest decrease in CD86, and this suggests that the strong increase observed by EnAd-SA-FAPBiTE may be via an IFN γ -independent mechanism or that IFN γ alone is insufficient.

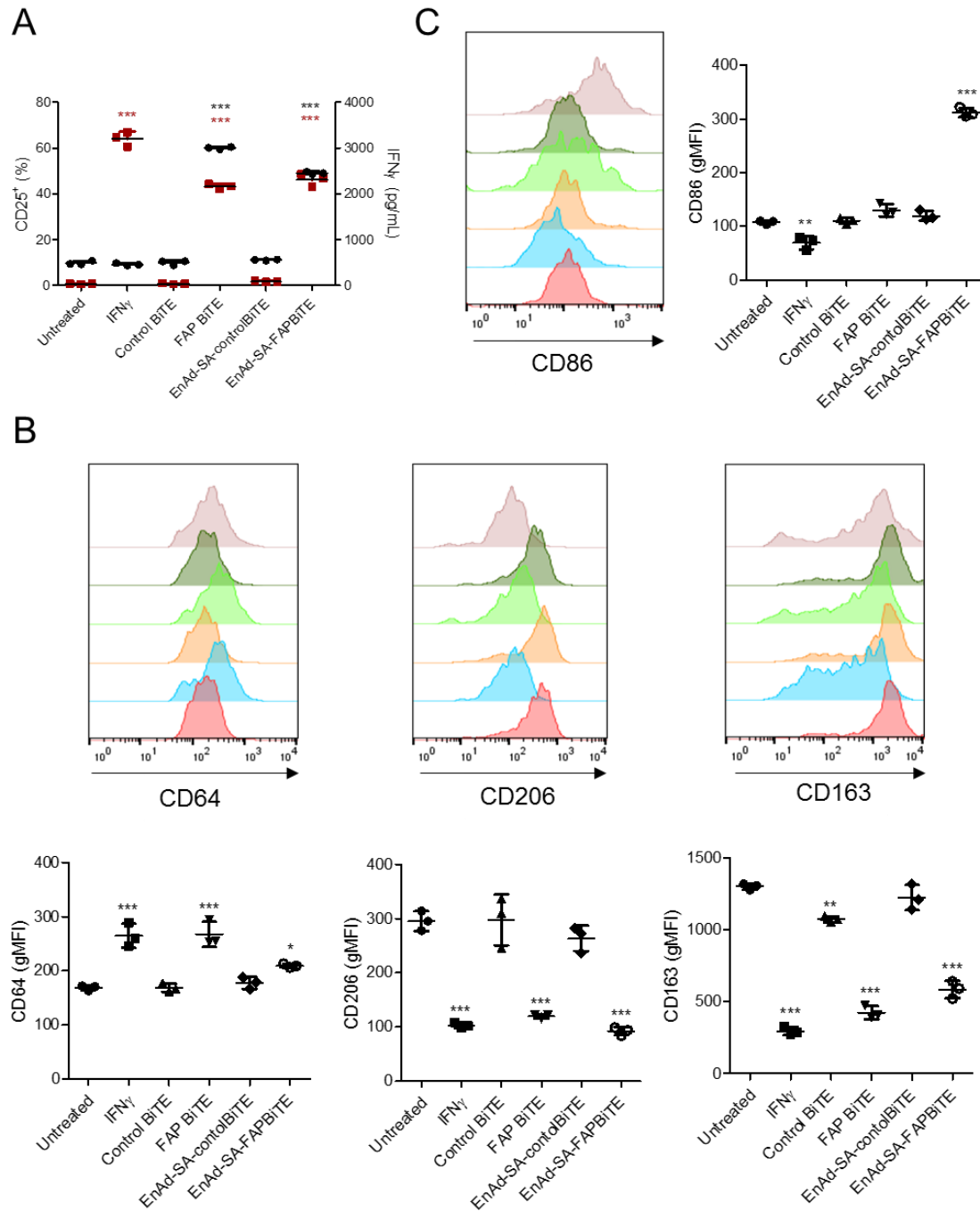


Figure 5.21. EnAd-SA-FAPBiTE mediates the polarisation of CD11b⁺CD64⁺ cells in patient ascites to a M1-like phenotype. (A) Total cells from ascites sample (Asc.16) were incubated in 50% ascites fluid in the presence of free BiTE or BiTE expressing virus. IFN γ treatment (25 ng/mL) was used as a positive control for M1 polarisation. After three days, the total cell population was harvested and the induction of activation marker CD25 on CD3⁺ cells was measured by flow cytometry (black circles). ELISA was performed on the harvested culture supernatants to measure the levels of IFN γ (red squares). (B, C) At three days, the expression levels of CD68, CD206, CD163 (B) and CD86 (C) on CD11b⁺CD64⁺ cells from (A) were measured by flow cytometry. Representative flow cytometry spectra from triplicate wells is shown alongside the complete data set. Each condition was measured in biological triplicate and represented as mean $\pm$ SD. Significance within each fluid treatment was assessed by comparison to ‘untreated’ using a one-way ANOVA test with Tukey’s Post Hoc analysis, * p <0.05, ** p <0.01, *** p <0.001.

5.4 Chapter conclusions

1. BiTE-expressing virus can mediate targeted killing of tumour cells and fibroblasts in clinical cancer biopsies by activating exogenous PBMC or endogenous T cells.
2. BiTE-mediated cytotoxicity is independent of exudate-associated immune suppression.
3. The adenoviral major late promoter drives transgene expression selectively in EpCAM⁺ tumour cells.
4. BiTE armed OV_s can induce the polarisation of TAMs in patient ascites to a more M1-like phenotype.

5.5 Chapter discussion

The current study shows, for the first time, that endogenous T cells within liquid cancer biopsies can be activated by BiTEs and virus-encoded BiTEs and can kill endogenous EpCAM⁺ tumour or FAP⁺ stromal cells without any additional stimulation or reversal of immune suppression (Figure 5.15 and 5.18). Importantly, this can happen even in the primary fluids that comprise the microenvironment of peritoneal ascites or pleural effusions, acting as surrogates for the immune suppressive microenvironment of solid tumours (Figure 5.10 and 5.12).

The use of BiTE-armed OV_s in patient exudate samples demonstrated the remarkable ability of BiTEs to induce the activation of T cells in a highly clinically relevant model

system. Despite evidence to suggest T cell dysfunction, we observed strong activation (measured by CD25 upregulation) of endogenous T cells in all patient samples. Additionally, both BiTEs were highly effective at mediating complete and consistent target cell lysis at E:T ratios as low as 0.25 (Figure 5.15 and 5.18). Unarmed parental virus appeared ineffective at killing EpCAM⁺ cells – possibly due to the use of sub-clinically relevant virus doses. Strikingly, the EpCAM BiTE was able to expand T cell numbers by factors of up to 30-fold and without the requirement for supplemented IL-2. The FAP BiTE, however, induced smaller increases. This was consistent with similar observations *in vitro* (Figure 3.6 and Figure 3.7) and may be explained by the ability of the FAP BiTE to only induce very modest increases in IL-2 production by either exudate- or PBMC-derived T cells (Figure 5.8). IFN γ production, however, was stimulated by both the EpCAM- and FAP-targeted BiTE, but this appeared to be significantly attenuated in a subset of samples (Figure 5.8). Despite this observation, in one ascites samples, treatment with the EpCAM BiTE-armed OV induced 1000-fold increases in IFN γ along with 10-fold increases in expression of IL-5, IL-13, TNF α , IL17A and IL17F (Figure 5.17). Taken together, this data suggests that whilst T cells from exudate samples are able to kill with equal potency to that effected by PBMC T cells, in some cases they may be impaired in their ability to produce pro-inflammatory cytokines.

Importantly, the data presented here supports our hypothesis that BiTE-armed OV therapy may be able to overcome many of the immune evasion mechanisms prevalent in solid tumours – including HLA down-regulation, as well as strategies that actively disable T cell responses. Whilst we didn't measure the expression of HLA on target tumour cells, others have reported a down-regulation or complete loss of HLA (and associated proteins) in up to 100% of analysed samples (Seliger et al, 2000). It is plausible, therefore, to

envisage that tumour cells within at least a subset of the exudate samples used in this chapter had experienced a similar loss in antigen presentation capability. It is noteworthy that given this loss, the majority of T cell therapies (vaccines, OV, checkpoint inhibition) would be completely limited in their ability to induce an effective anti-tumour response. BiTEs, however, induced potent T cell activation and near-complete lysis of tumour cells in all tested samples (Figure 5.5 and 5.15).

Indeed, whilst the majority of cell-free exudate fluids suppressed the degranulation of T cells when stimulated with anti-CD3/CD28 Dynabeads, BiTE-mediated activation was able to overcome this in nearly all cases (Figure 5.10 and 5.12). This phenomenon may be explained by the atypical manner in which a BiTE activates a T cell. One proposed mechanism of ascites-mediated T cell inhibition is via the modulation of phosphorylation patterns of distal TCR signalling molecules, leading to down-regulated signalling and the attenuation of NF- κ B and NFAT activation (Simpson-Abelson et al, 2013). It is possible that such a mechanism may be ineffective in scenarios of extraordinarily powerful T cell stimulation, like that artificially induced by a BiTE. Notably, and in support of this proposition, we also observed that BiTE-mediated T cell activation was significantly more potent than CD3/CD28 beads, giving both higher levels of dual-positive CD69⁺/CD25⁺ cells and degranulation measured by CD107a. Assuming this is true, it follows that PD1 ligation – also demonstrated to attenuate downstream T cell signalling – may similarly be unable to sufficiently dampen BiTE-mediated signalling to levels required to inhibit T cell functions (Parry et al, 2005). This is consistent with our data suggesting that PD1 blockade has little effect on BiTE-mediated T cell activation or cytotoxicity (Figure 5.19 and 5.20), with similar observations reported elsewhere (Deisting et al, 2015).

One of the ultimate goals of arming EnAd with BiTEs is to induce the polyclonal activation of tumour-infiltrating lymphocytes. This may facilitate a global shift from an environment that is immune-suppressive to one that is pro-inflammatory. Indeed, in this chapter we have demonstrated the BiTE-induced secretion of large quantities of Th1 cytokines (Figure 5.8 and 5.17) and the polarisation of tumour-associated macrophages (TAMs) to a more 'classically activated' M1 phenotype - manifested as increased CD64 and strong decreases in CD206 and CD163 (Figure 5.21) (Mosser, 2003). Whilst we haven't yet extended this study to evaluate macrophage function, M1 macrophages are reported to be potent killers of tumour cells, in addition to producing large quantities of pro-inflammatory cytokines (Mills et al, 2000). A more functional-based assessment (e.g. nitric oxide phagocytosis, cytokines secretion, antigen presentation) will be the focus of future research.

In the case of CD86, the combination of virus and FAP BiTE (EnAd-SA-FAPBiTE) was required to induce an up-regulation on macrophages (Figure 5.21). Neither FAP BiTE, control EnAd or supplemented IFN γ alone had any effect. Indeed, adenovirus has been demonstrated to activate antigen-presenting cells (including macrophages) via Toll-like receptor (TLR) binding to CpG DNA or TLR-independent cytosolic detection of adenoviral DNA (Zhu et al, 2007). Therefore, it is possible that whilst BiTE-induced IFN γ provides the first signal, the presence of virus PAMPs provides an important second signal that is essential for full macrophage activation. This highlights the potential for synergism between virus infection and FAP BiTE activity and further strengthens the rationale for combining these two immunotherapeutic strategies.

6. Activation of tumour-infiltrating T cells in human solid tumour samples using FAP BiTE-armed EnAd

6.1 Introduction

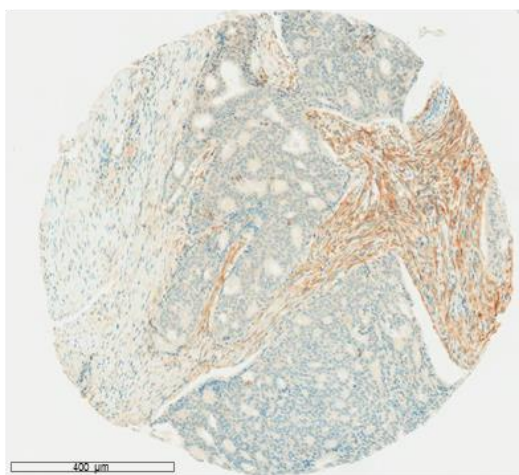
It has been reported that only 7.5% of anticancer treatments tested in Phase I clinical trials eventually obtain approval (Toniatti et al, 2014). One of the main reasons for this is the use of poor preclinical models that do not reflect the complexity of a tumour. Indeed, cancers can be described as analogous to a ‘pathological organ’, in which there is an intricate interplay between highly heterogeneous tumour cells, immune infiltrates and non-malignant cells of the stroma (Figure 1.2). Mounting evidence now implicates these stromal cells in tumour progression, immune suppression and response to therapy (Turley et al, 2015). Primary cell cultures - using malignant ascites and pleural effusion samples - share many of the different cell types present in a solid tumour and have thus far enabled the high throughput analysis of BiTE/OV efficacy in clinically relevant patient samples. Despite these merits, such cultures are not able to reproduce the complex tumour morphology, microenvironment and signalling dynamics within a solid tumour. For example, many mechanisms of immune suppression require cell-to-cell contact and the accumulation of high concentrations of anti-inflammatory factors in the interstitial space. These elements can’t be recapitulated using liquid tumours.

Organotypic tissue slices, on the other hand, retain the complex architecture of a solid tumour. This approach involves the fine slicing of resected human solid tumour tissue and their subsequent culture. In this setting, tumour cells and the surrounding microenvironment are largely undisturbed, providing realistic preclinical models of diverse and heterogenic tumour tissue (Davies et al, 2015). Success of this approach has now been demonstrated in many different tumour tissues, including prostate, breast, testicular and liver (Bläuer et al, 2008; Carranza-Torres et al, 2015; Laughlin et al, 2010; Zimmermann et al, 2009). Indeed, in the absence of adequate mouse models, this represents an excellent system for evaluating BiTE-armed OV. Crucially, such a model would also possess nearly all the immunosuppressive mechanisms present in a solid tumour, and uniquely would enable the evaluation of (i) BiTE delivery using OV, (ii) the activation of often-anegetic TILs and (iii) physical changes in microenvironment architecture following CAF-targeting.

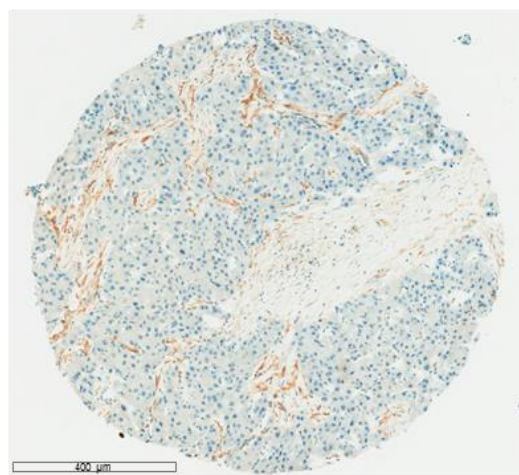
This chapter seeks to establish a tissue slice culture methodology to assess the activity of the FAP BiTE-armed EnAd viruses in solid tumour tissue. Whilst absent in the majority of liquid biopsy samples (Table 5.2), FAP⁺ stromal cells are reported to be present in almost all adenocarcinomas (Figure 6.1) (Garin-Chesa et al, 1990). Moreover, fibroblasts/myofibroblasts are reported to be the predominant stromal cell type in prostate tumours (Tuxhorn et al, 2002). For this reason - in addition to its ability to support EnAd replication and the likelihood of lymphocyte infiltrates - prostate tumour tissue was utilised for the experiments described in this chapter (Strasner & Karin, 2015). To aid in this analysis, new FAP BiTE-armed reporter viruses were engineered to express red fluorescent protein (RFP). This modification should enable the visualisation of virus infection and transgene expression in solid tumour tissue cultured *ex vivo*. The effect of

these BiTE-armed EnAd viruses on solid tumour tissue was then assessed using prostate tumour slices, with analysis focusing on the activation of TILs and potential BiTE-induced cytotoxicity.

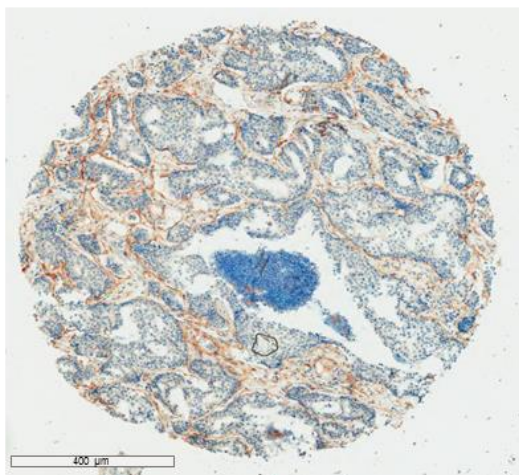
Prostate



Liver



Colorectal



Breast

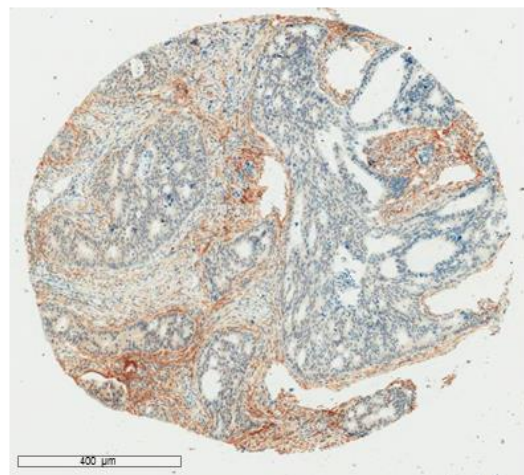


Figure 6.1. Expression of FAP in multiple carcinoma tumour types. A tissue microarray was used to assess the expression of FAP (brown) on stromal fibroblasts in multiple indications of epithelial adenocarcinoma.

6.2 Chapter aims

1. Establishment of a prostate tumour tissue slicing and culture methodology.
2. Engineering and production of FAP BiTE-armed EnAd viruses, with BiTE expression coupled to that of RFP.
3. Evaluate the ability of BiTE/RFP-armed EnAds to activate tumour-infiltrating T cells and direct cytotoxicity to fibroblasts within prostate tumour slices.
4. Assess the specificity of FAP BiTE-mediated activation by comparing virus activity in malignant and benign prostate tissue.

6.3 Results

6.3.1 Engineering and functional validation of FAP BiTE armed reporter viruses

To enable accurate assessment of virus infection and transgene expression in solid tumour tissue *ex vivo*, fluorescent reporter viruses were genetically engineered and produced. The expression of a transgene (for example, a BiTE) can be linked to that of a second protein (such as red fluorescent protein (RFP)) by removing the stop codon of the former and fusing the transgenes with a P2A self-cleaving peptide site, discovered in some members of the picornavirus family (Ryan et al, 1991). The two proteins will be expressed as a single polypeptide chain that undergoes ‘self-cleavage’ (or ribosome skipping, to be more precise) immediately downstream of the last glycine of the 21-residue P2A sequence to generate two individual mature proteins. Assembling the BiTE upstream of RFP ensures that RFP signal is only detected when BiTE is expressed. Since the cleavage site occurs between the two final amino acids, the BiTE transgene will have 20 additional residues at its C-terminus. To eliminate any potential detrimental effects by this extra sequence, a furin cleavage site (RRKR) was included immediately downstream of the first transgene. A schematic of the assembled expression cassette is illustrated in Figure 6.2A.

To construct the modified sequence, the RFP fragment was generated by PCR amplification using a RFP template vector (pSF-CMV-DNAase-RFP; kindly provided by Dr Margaret Duffy) including the furin and P2A site introduced directly upstream. Similarly, the FAP BiTE fragment was amplified from pSF-CMV-FAPBiTE vector, and both fragments were inserted into linearised pSF-CMV backbone by Gibson assembly

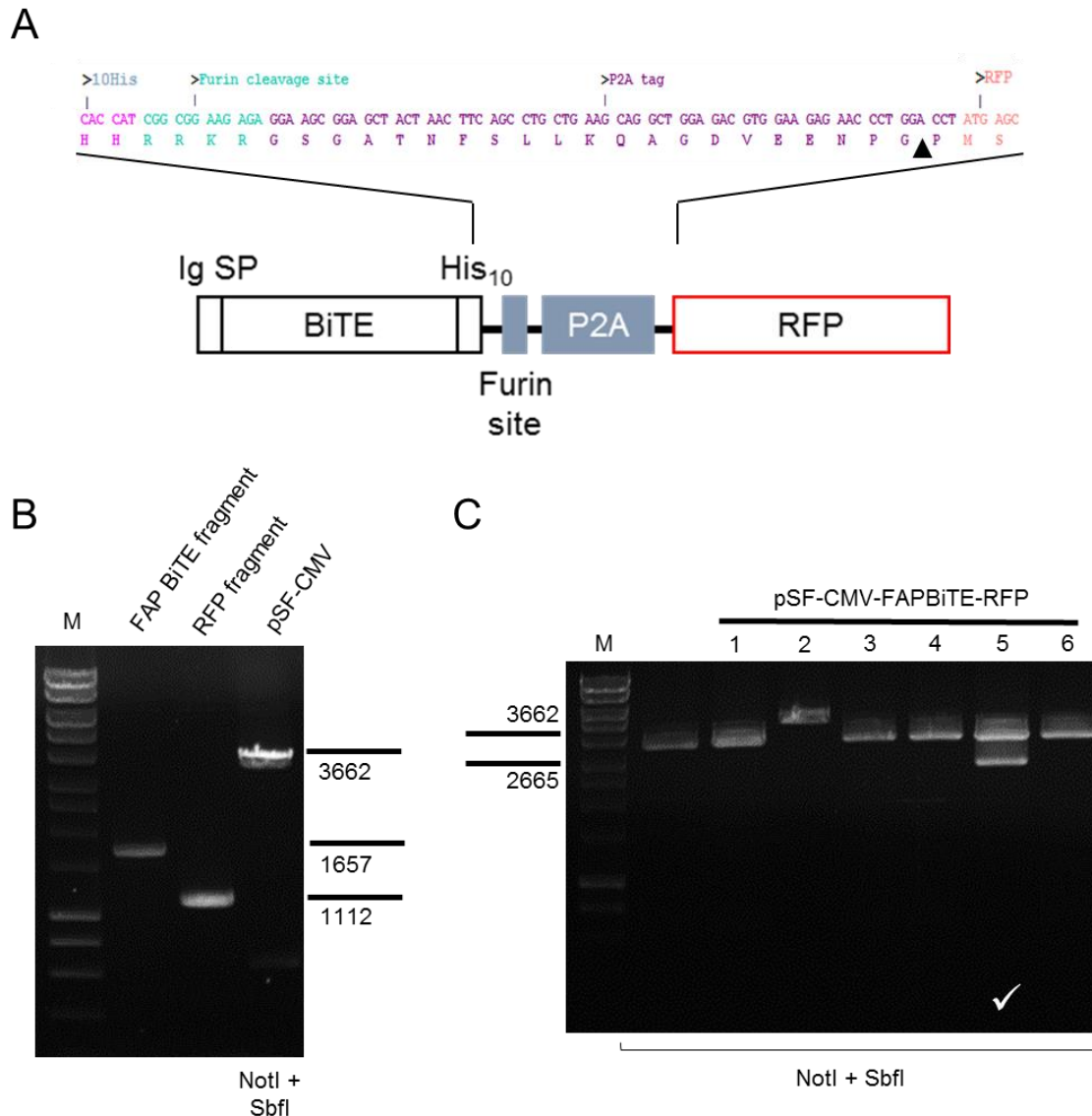


Figure 6.2. Engineering of a FAP BiTE and RFP fusion protein and encoding in a mammalian expression plasmid. (A) Schematic representation of the FAP BiTE-RFP expression cassette. The FAP BiTE and RFP are joined by a P2A sequence with an upstream furin cleavage site. A GSG spacer was included at the N-terminus of the P2A site to improve cleavage efficiency. The BiTE protein possesses an N-terminal immunoglobulin light chain signal peptide (Ig SP) for secretion and a C-terminal decahistidine affinity tag for detection (His₁₀). Black triangle; P2A self-cleavage site. **(B)** FAP BiTE and RFP fragments were generated by PCR amplification producing bands at expected sizes of 1657 and 1112 bp, respectively (left). Fragments were inserted into linearised CMV expression vectors (right) by Gibson assembly. M, Hyperladder 1 kb DNA ladder. **(C)** Recombinant plasmids from the reaction in (A) were screened by digestion with NotI and SbfI for correct assembly of FAP BiTE-P2A-RFP fusion protein, giving bands at 3662 and 2665 bp. Colonies with the correct digestion pattern are indicated with a tick. M, Hyperladder 1 kb DNA ladder.

(Figure 6.2B). While the recombination reaction appeared to be somewhat inefficient, a single colony appeared to show correct assembly, and was confirmed by digest screen (Figure 6.2C; bands at 3662 and 2665 bp) and DNA sequencing. To assess transgene expression, HEK293A cells were transfected with pSF-CMV-FAPBiTE-RFP or pSF-CMV-FAPBiTE vectors using Lipofectamine 2000, or were left uninfected. At 48 hours, cells were observed by bright-field and fluorescence microscopy, demonstrating successful expression of RFP from the BiTE-RFP expression plasmid (Figure 6.3A). As shown in Figure 6.3B, western blot analysis using 48-hour supernatants confirmed that similar levels of FAP BiTE were secreted from each FAP BiTE-containing plasmid.

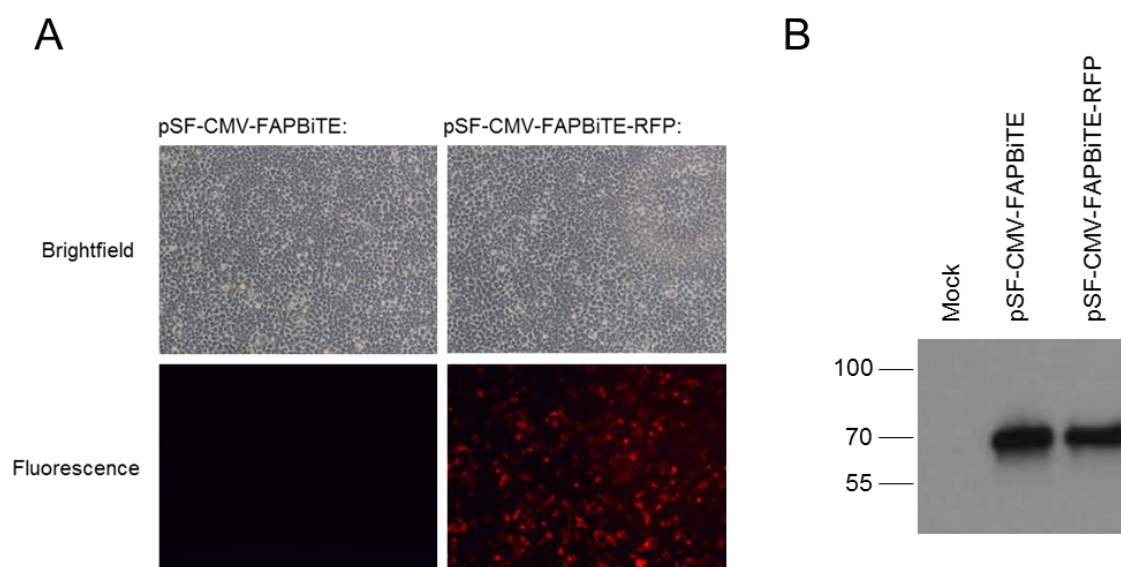


Figure 6.3. Expression of FAP BiTE and RFP from a CMV expression plasmid. (A) Fluorescence microscopy images of HEK293A cells transfected with pSF-CMV-FAPBiTE or pSF-CMV-FAPBiTE-RFP plasmids. Images were collected 48 hours post-transfection using a Zeiss Axiovert 25 microscope. (B) BiTE secretion was assessed by western blot analysis of 48-hour supernatants from the plasmid-transfected HEK293A cells in (B). Blots were probed with a mouse anti-histidine antibody, followed by an HRP-conjugated anti-mouse secondary antibody.

Next, the EnAd genome was modified to encode the FAPBiTE-P2A-RFP cassette downstream of the fibre gene, with transgene expression under transcriptional control of a CMV promoter or the major late promoter (using a splice acceptor (SA) sequence). As described previously, MLP-driven expression will be limited to cells permissive to virus replication, whereas the CMV promoter will induce expression in all infected cells. The respective transgene cassette fragments were amplified by PCR (Figure 6.4A) and inserted into linearised EnAd2.4 vector (as shown in Figure 3.12B) by Gibson assembly. Successful insertion was confirmed by digest screen using ScaI (Figure 6.4B; correct colonies highlighted with a tick), and DNA sequencing.

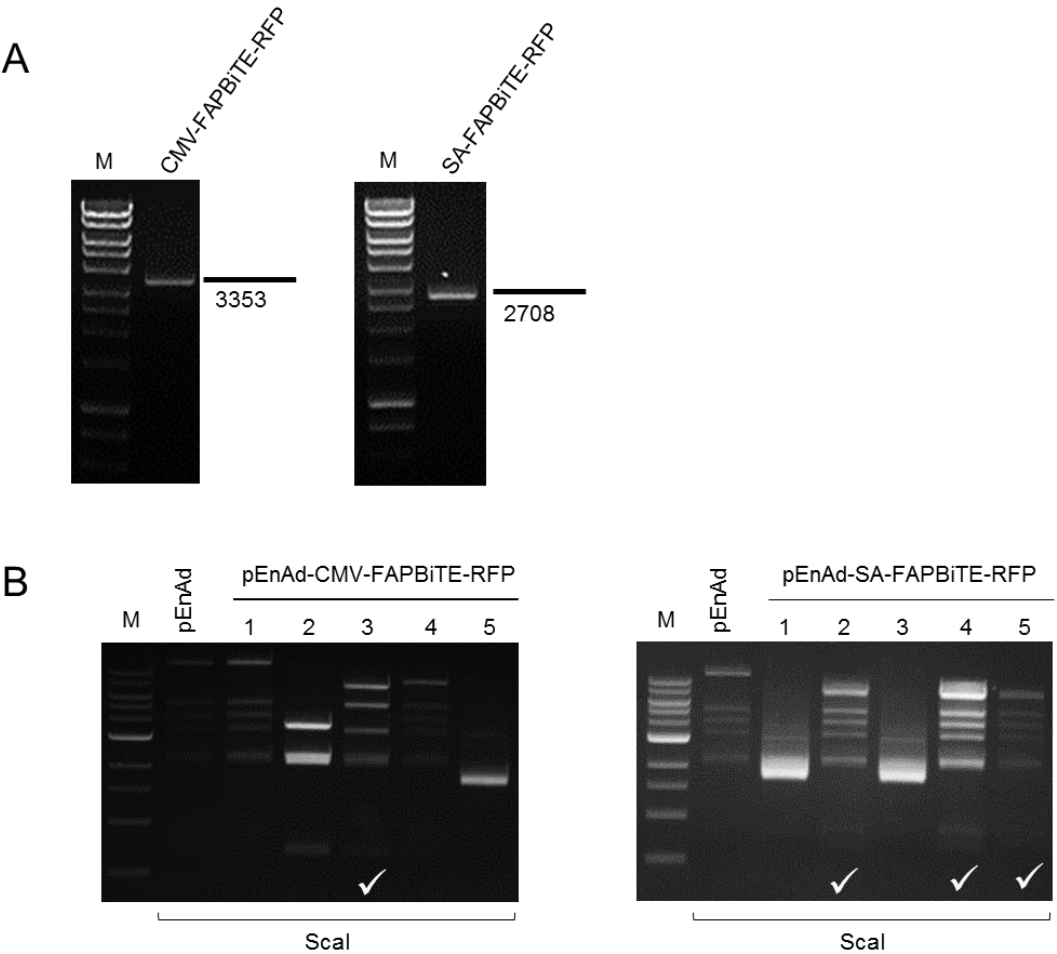


Figure 6.4. Engineering of oncolytic adenoviruses expressing FAP BiTE and RFP. (A) PCR amplification of FAP BiTE-P2A-RFP expression cassette was performed to generate fragments for insertion by Gibson assembly into the linearised EnAd2.4 vector in Figure 3.12A. Fragments were generated with the CMV promoter included (left), or with an added splice-acceptor sequence directly upstream of the transgene (right). **(B)** Recombinant plasmids generated from Gibson assembly of EnAd2.4 vector and PCR products in (A) were evaluated for successful assembly by digest screen (ScaI). Colonies producing the expected pattern of cleavage products are marked with a tick. M, Hyperladder 1 kb DNA ladder.

The recombinant viral plasmids were linearised using AscI and transfected into HEK293A. Rescue of viable virus was confirmed by induction of CPE (as assessed by brightfield microscopy in Figure 6.5A), with both CMV and SA viruses demonstrating expression of RFP transgene (fluorescence microscopy, Figure 6.5A). Five clones of each were isolated following infection of HEK293A cells with serially-diluted lysates and harvesting of wells containing a single plaque. Next, expression of FAP BiTE was assessed by western blotting using the harvested supernatants (Figure 6.5B) and a single clone was selected (indicated with a tick) and amplified for large-scale purification by CsCl gradient (see Materials & Methods).

To assess newly purified recombinant virus RFP expression and T cell-mediated lysis of FAP⁺ fibroblasts, time-lapse videos were obtained showing co-cultures of NHDF (green), SKOV3 tumour cells (unlabelled) and T cells (blue) (Figure 6.6). Cells were infected with EnAd-CMV-FAPBiTE-RFP and EnAd-SA-FAPBiTE-RFP, with the expression of RFP used as an indicator of viral infection and replication, respectively. Cells were also infected with EnAd-SA-RFP (without FAP BiTE, and kindly provided by Dr Margaret Duffy), acting as a negative control for fibroblast cytotoxicity. Treatment with all recombinant viruses lead to RFP expression, by 12 and 28 hours for the CMV-driven and SA-driven viruses, respectively. Only when the FAP BiTE was also encoded, was the onset of RFP expression swiftly followed by cell death of the fibroblasts (manifest by rounding up; indicated with white arrows), with NHDF lysis not observed prior to

expression of RFP. Taken together, these data show the successful engineering and rescue of viruses with linked expression of RFP and a FAP-targeted BiTE.

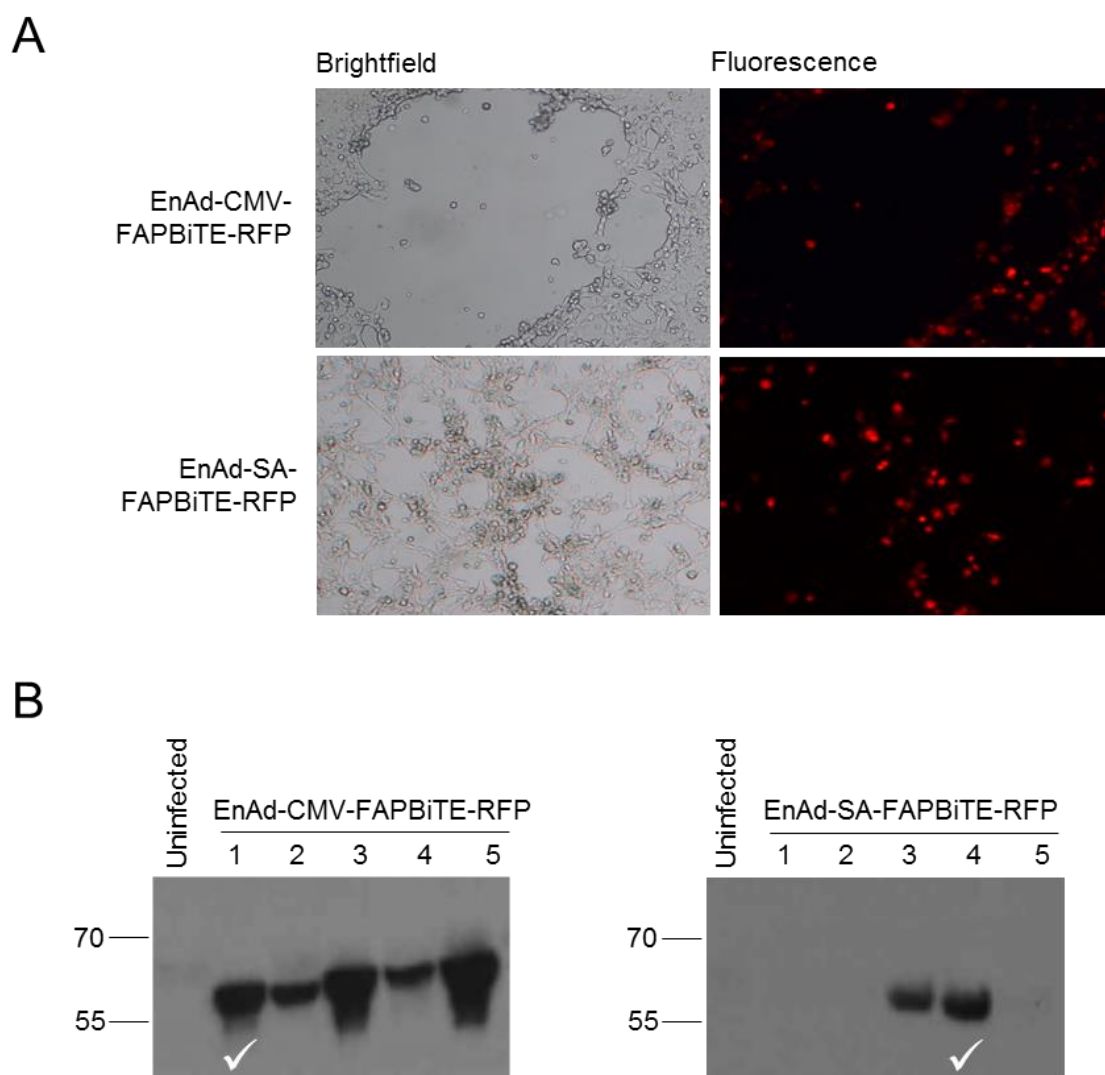


Figure 6.5. Rescue and selection of EnAd clones expressing FAP BiTE and RFP. (A) Bright-field and fluorescence microscopy was used to confirm rescue of the recombinant viruses following transfection of HEK293A cells with *Asc*I-linearised virus genome. (B) Western blotting was used to confirm BiTE expression from HEK293A cells infected with the recombinant viruses rescued in (A). Blots were probed with a mouse anti-histidine (C-terminal) antibody for detection. Transgene expression from five virus clones was assessed for each virus, with uninfected cells as a negative control. The clones chosen for large-scale virus production are indicated with a tick.

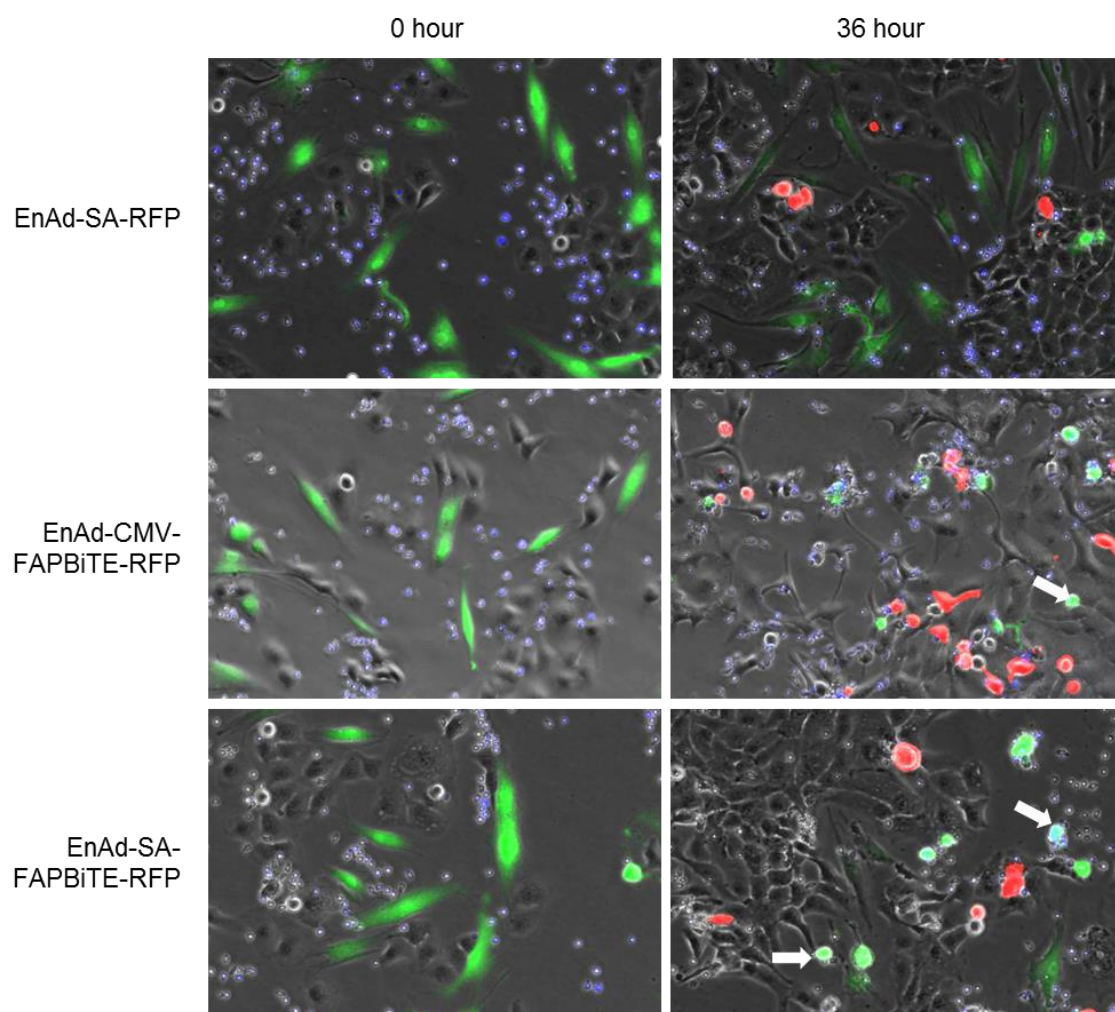


Figure 6.6. Infection with FAP BiTE expressing RFP reporter viruses induces cytotoxicity of stromal fibroblasts *in vitro*. Still images from time-lapse sequences showing co-culture of NHDF (green), SKOV carcinoma cells (unstained) and T cells (blue) infected with EnAd or recombinant BiTE virus (expressing RFP, red). Full time-lapse sequences are available. Killed NHDF cells are marked with a white arrow.

6.3.2 Determination of conditions for slicing, culture and processing of solid prostate tumour samples

Primary human solid prostate samples were received from the John Radcliffe Hospital (Oxford University Hospitals, Oxford, UK) following informed consent from patients undergoing radical prostatectomy surgery to remove advanced prostate carcinomas. Table

6.1 outlines details of the patient samples described in this chapter - including the Gleason score as assessed by Dr Clare Verill (consultant pathologist, OUH). Specimen tissue was obtained immediately post-surgery, and 8 mm cores taken from putatively benign and malignant regions; as assessed by macroscopic analysis (and later verified using immunohistochemistry by Dr Clare Verill, see Table 6.1). The cores were precision-cut in semi-sterile conditions to 300 μ m thickness using a VT1200S vibrating blade microtome (vibratome) and slices were transferred onto polytetrafluoroethylene inserts with a pore size of 0.4 μ m inserts in 24-well plate and cultured in 1 mL of freshly prepared cultivation medium. This media was supplemented with rEGF, BPE, insulin-transferrin-selenium, hydrocortisol, R1881 androgen, primocin and FBS (see Materials and Methods for recipe), as a similar formula was previously reported to maintain prostate tissue slices for up to seven days in culture (Davies et al, 2015; Maund et al, 2014).

Table 6.1. A list of the human prostate tumour samples used in this chapter. All the solid prostate samples collected for this study are presented in the table below. Samples are labelled in order of date of receipt. The Gleason grade was scored by a qualified pathologist at the John Radcliffe Hospital, Oxford University Hospitals.

Sample number	Surgery date	Date of birth	Tumour Gleason	Classification by IHC
1	30/03/2017	25/08/1948	4+3(7)	Malignant & benign
2	08/05/2017	17/10/1946	4+5(9)	Malignant & benign
3	18/05/2017	14/07/1955	3+4(7)	Malignant & benign
4	21/06/2017	17/01/1953	4+4(8)	Malignant & benign
5	17/07/2017	20/12/1943	4+3(7)	Benign & benign
6	31/07/2017	01/10/1952	4+3(7)	Malignant & benign

To assess whether the slicing of the core induces cytotoxicity, the quantity of LDH released into the supernatant (which measures cytotoxicity) after the first 24 hours of culture was assessed. As shown in Figure 6.7A, the slicing procedure induces release of LDH (Abs₄₉₀) from all slices, and to varying levels. This is likely to make subsequent

post-treatment evaluation of cytotoxicity (using LDH release) difficult, and therefore the media was replaced with fresh media after the first day of culture. It is important that the tissue remains viable for the duration of the studies, and to assess this, two malignant slices (1 x untreated and 1 x in the presence of EnAd-CMV-control BiTE) were cultured for up to six more days after the first media change. On days one, three and six, one third of the media was harvested and stored (and replaced). Replenishment of the medium should enhance tissue viability by maintaining sufficient levels of supplements and removing waste products. In the absence of treatment, the slice remained viable for the duration of culture with little increase in LDH by six days (Figure 6.7B). Encouragingly, when infected with EnAd-CMV-controlBiTE, the slice remained viable until day three, and with potential EnAd-associated cytotoxicity evident by day six.

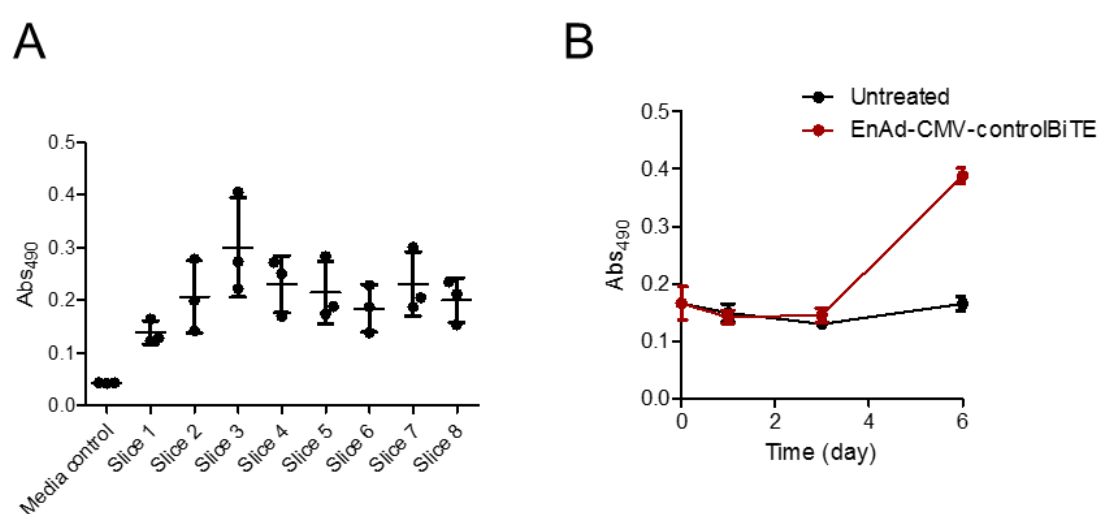


Figure 6.7. Prostate cancer slices remain viable in culture for up to six days. (A) Release of LDH into the supernatant from freshly cut slices after the initial 24 hours of culture. **(B)** Cytotoxicity of prostate tumour slice in culture for a further six days. Culture medium was replaced with fresh medium after 24 hours initial incubation and slices were infected with EnAd-CMV-controlBiTE or were left untreated. Cytotoxicity was assessed by LDH released into the supernatant at the specified timepoints. Data shown here was obtained using patient specimen number two. Each condition was measured in technical triplicate and represented as mean ± SD.

6.3.3 Histological characterisation of malignant prostate tissue

The long-term preservation of slice morphology was evaluated by haematoxylin and eosin stain and immunohistochemistry using malignant slice tissue. Following culture for six days, the slices displayed well-preserved prostate morphology, showing the characteristic glands and intervening stroma. This is most clearly illustrated in Figure 6.8 by staining for EpCAM (epithelial cell or tumour marker) and FAP (activated fibroblast marker). All tested specimens showed cells positive for CD8 confirming the presence of tumour-infiltrating CD8 T cells.

It is noteworthy that the fibroblasts showing the strongest FAP expression are (reproducibly) those adjacent to islands of malignant epithelial cells (round blue nuclei; Figure 6.8 and 6.9A), supporting similar findings that FAP is highly expressed on those fibroblasts associated with tumour cells (Henriksson et al, 2011). Consistent with this, FAP expression was limited to malignant tissue (Figure 6.9A), with very little staining in benign prostate from the same patient (Figure 6.9B).

6.3.4 EnAd expressing FAP BiTE activates tumour-infiltrating lymphocytes within malignant solid tumour tissue

After establishing a culturing procedure that maintained the viability and cellular composition of the native tissue, we next assessed the ability of the RFP reporter viruses to infect and replicate in the solid tissue slices. Following the first day of culture (after slicing), the supernatant was replaced with fresh media containing FAP BiTE-expressing

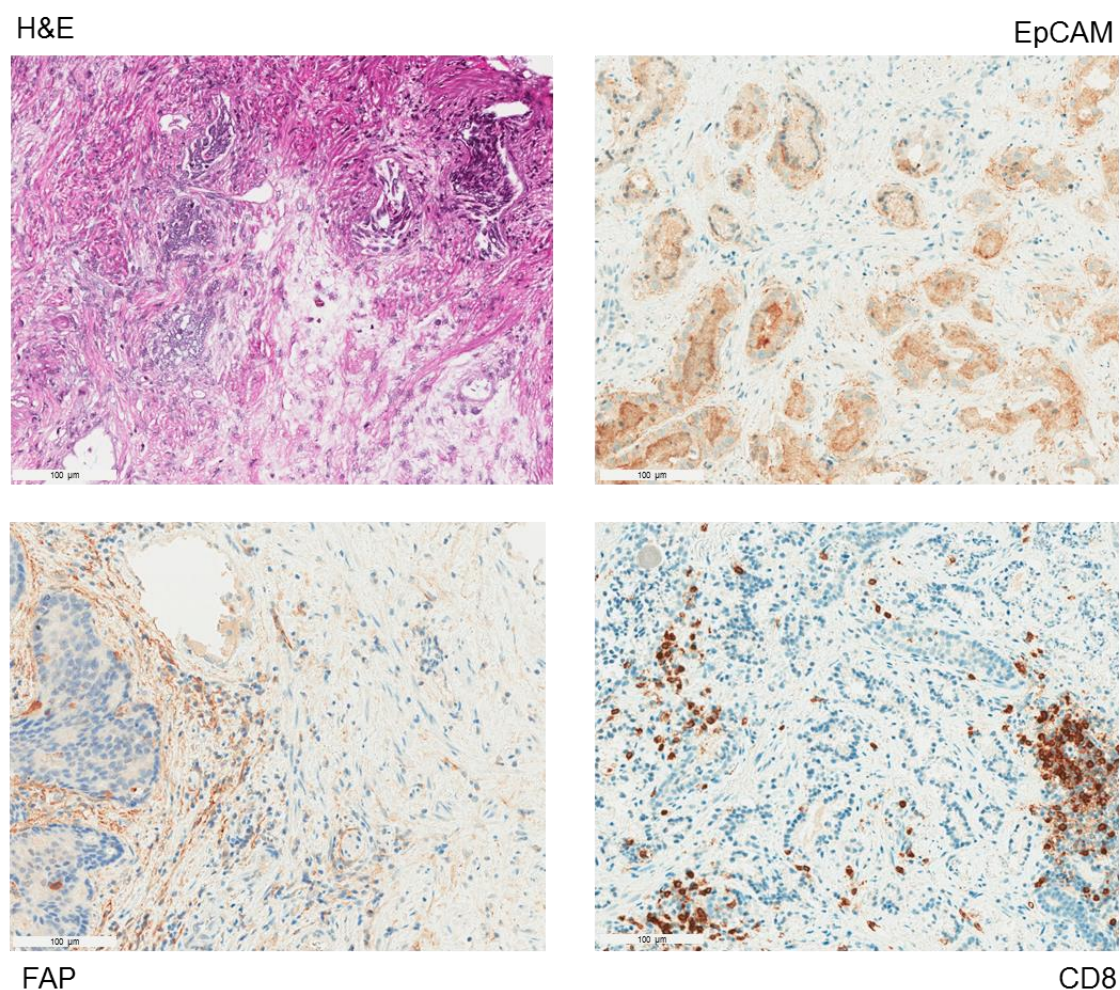


Figure 6.8. Histology of prostate cancer tissue slice cultures. Haematoxylin and eosin (H&E) stained section of patient prostate tumour slice showing characteristic glands and intervening stroma. Immunohistochemistry was performed to determine cellular compositions of prostate cancer tissue slice cultures. Cellular markers of epithelial cells (EpCAM), activated fibroblasts (FAP) and cytotoxic T cells (CD8) were appropriately expressed in prostate cancer tissue slices after five days in culture. Data shown here was obtained using patient specimen number one.

EnAd. After 96 hours, brightfield and fluorescence microscopy images were collected. In parallel cultures – and to control for virus infection or FAP BiTE expression - slices were left untreated or infected with EnAd-CMV-ControlBiTE, respectively. As shown in Figure 6.10, both RFP expressing viruses showed successful infection of cells within the solid tissue, with expression of RFP from the MLP-driven promoter (within the SA virus) confirming viral replication. The viruses, however, demonstrated different patterns of

RFP expression with CMV-driven expression displaying a much higher number of RFP-positive cells. Strikingly, RFP expression induced by the SA virus was restricted to cells in circular structures, similar in morphology to the EpCAM⁺ (and likely malignant) rings of epithelial cells shown in the immunohistochemical analysis in Figure 6.8. Importantly, because FAP BiTE expression has been linked to that of RFP, the observation of RFP-positive cells also indicates the expression of BiTE transgene.

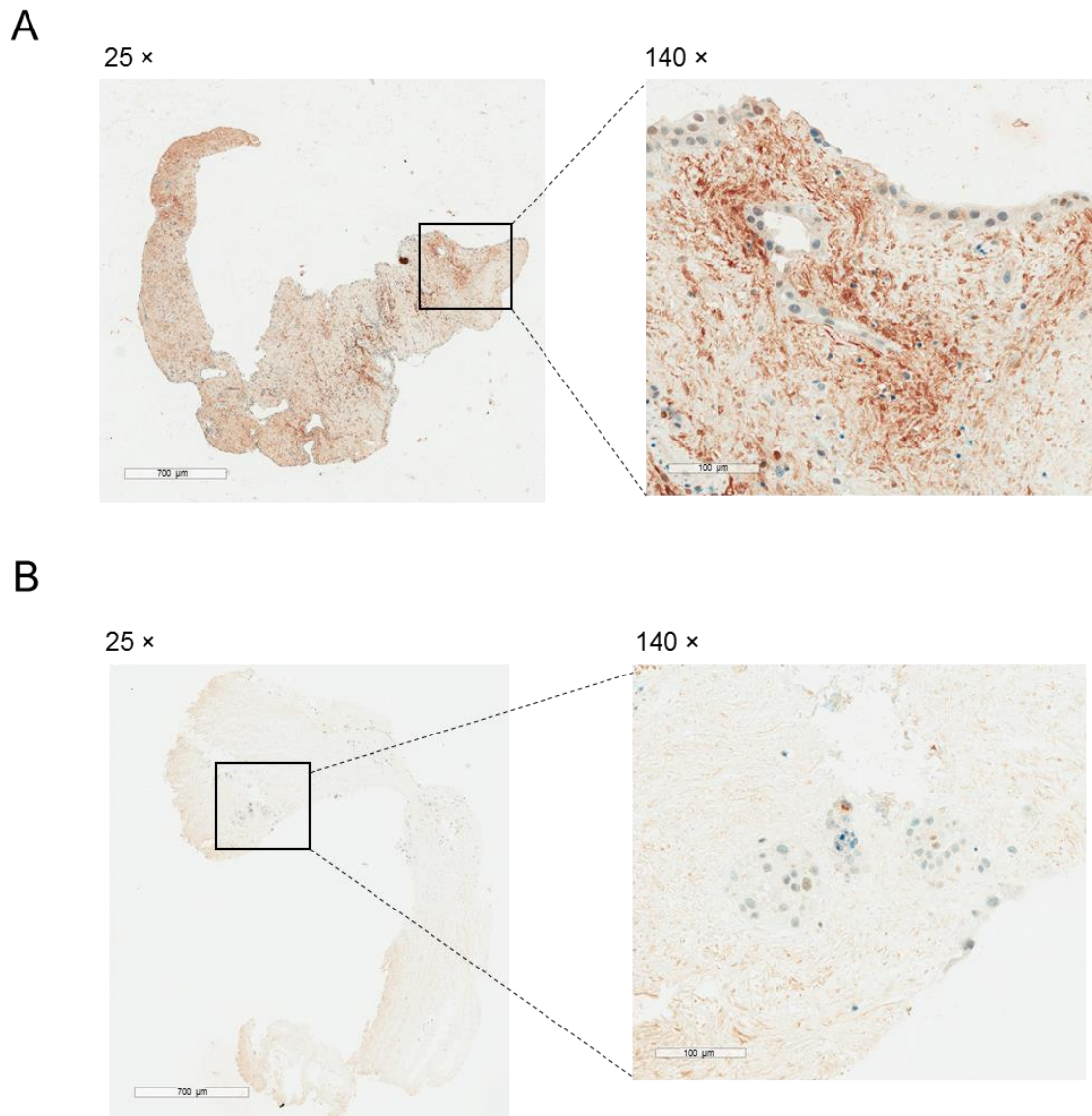


Figure 6.9. FAP expression on stromal fibroblasts in benign and malignant prostate tissue slices. Immunohistochemistry was used to determine expression patterns of FAP in malignant (A) and benign (B) prostate slices cultured for seven days. A $\times 140$ magnification insert shows strong expression of FAP staining on stromal cells in malignant slices. Data shown here was obtained using patient specimen number four.

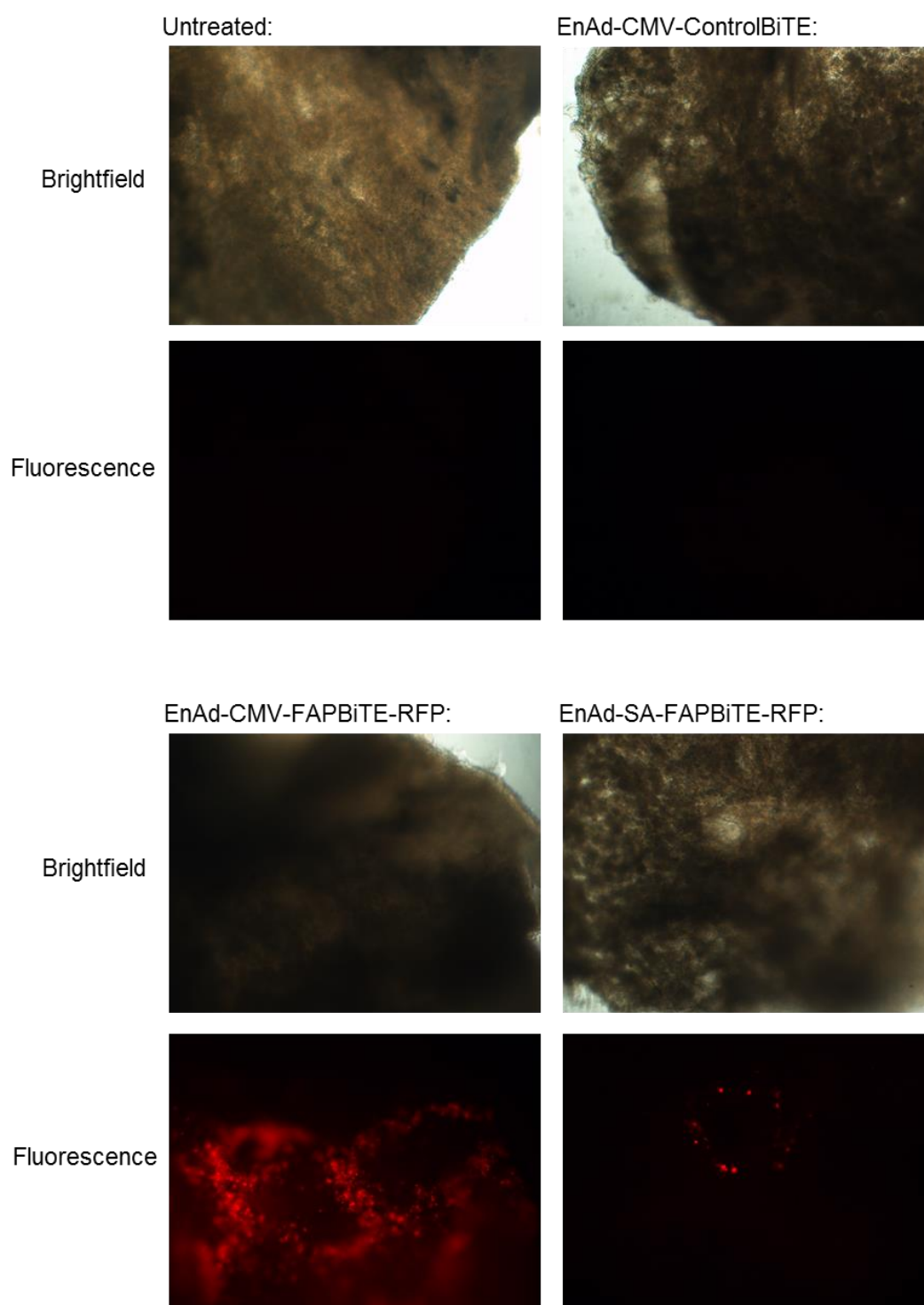


Figure 6.10. Infection and replication of armed EnAd viruses in prostate tissue slices. Representative bright-field and fluorescence images showing uninfected prostate tissue slice or slices infected with recombinant EnAd viruses. RFP transgene expression was evaluated, providing a surrogate marker for visualisation of virus infection, replication and BiTE expression. Images were collected on day four using a Zeiss Axiovert 25 microscope. Data shown here was obtained using patient specimen number four and is representative of three individual patient samples.

To explore the effects of successful virus infection and potential FAP BiTE expression, the activation of tumour-infiltrating lymphocytes was assessed. To do this, malignant tissue slices were infected and cultured identically to those described above. After six days of culture, the slices were harvested and processed for immunohistochemical analysis of the expression of CD25, a T cell activation marker (Figure 6.11). Neither untreated slices, or those infected with EnAd-CMV-controlBiTE showed detectable CD25-positive cells. However, tissue infected with the FAP BiTE expressing EnAds showed increases in the frequency of cells expressing CD25; suggesting activation of tumour-infiltrating lymphocytes. This FAP BiTE-mediated activation of T cells appeared to be the trend in most cases, however in one sample, EnAd-CMV-controlBiTE induced similar levels of CD25 expression, suggesting that the virus may have an innate (patient-dependent) ability to activate T cells (data not shown).

Importantly, this activation of T cells translated to the production of the effector cytokines, IFN γ and IL-2, both associated with activated CD4 Th1 and CD8 cytotoxic T cells. Cultures similar to above were established, however to assess the specificity of virus/BiTE-mediated activation, benign and malignant tissue slices from a patient sample with clear FAP⁻ and FAP⁺ tissue (see Figure 6.9) were utilised and infected. Supernatants were harvested on day three and six and the expression of IFN γ and IL-2 was assessed by ELISA. Crucially, in the case of the benign tissue, there was no increase in either IFN γ or IL-2 with any of the treatments (Figure 6.12A). However, malignant tissue infected with EnAd-CMV-FAPBiTE-RFP or EnAd-SA-FAPBiTE-RFP demonstrated significant increases in IFN γ , with the latter also inducing IL-2 production (Figure 6.12B). This same pattern, of the SA virus inducing higher levels of cytokine expression, was observed in

3/4 of the patient samples, and is also apparent in the level of CD25 expression in Figure 6.11.

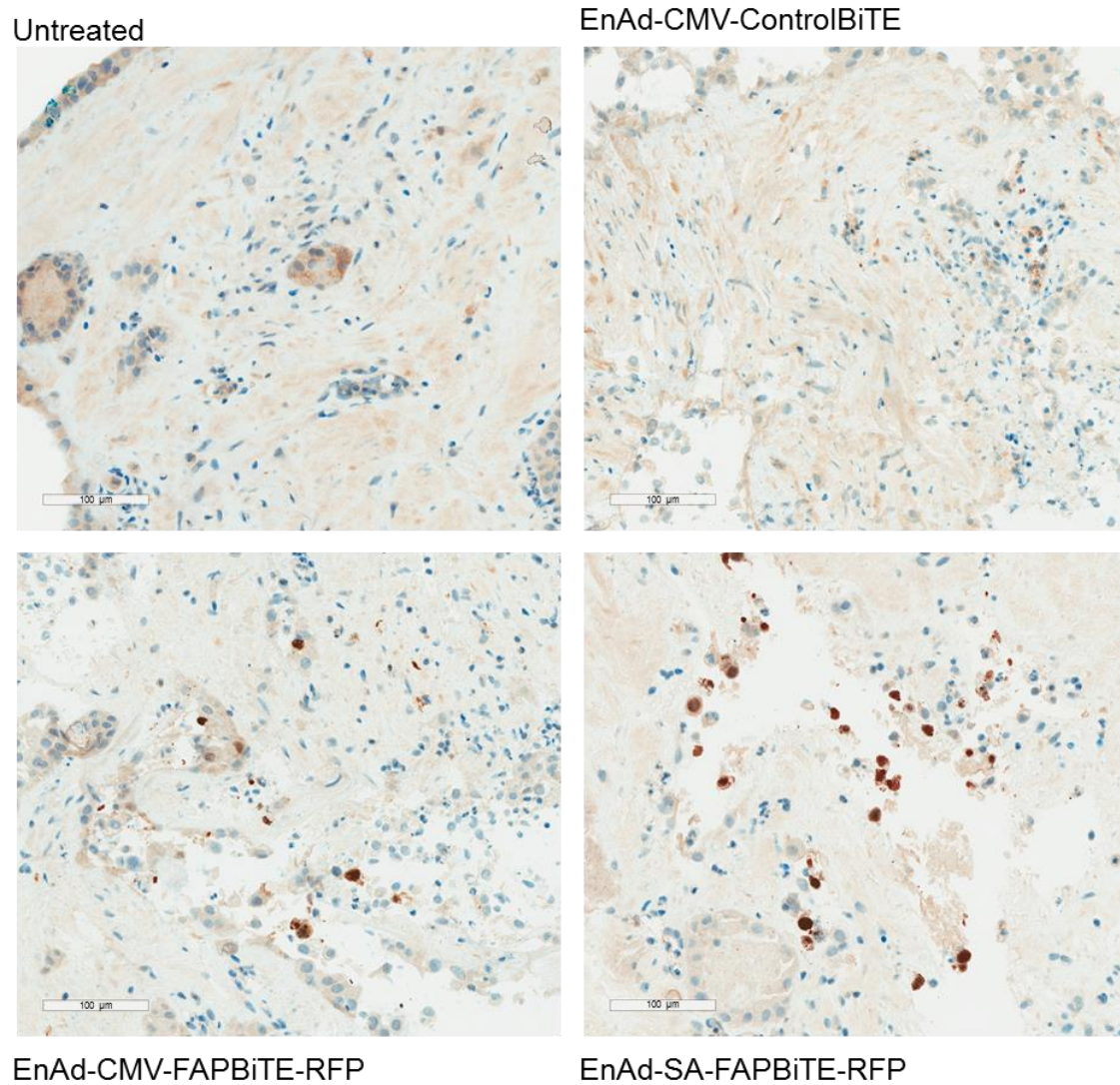


Figure 6.11. EnAd expressing FAP BiTE viruses activates tumour-infiltrating T cells in malignant prostate slices. Activation status of tumour-infiltrating T cells in prostate slices was assessed by immunohistochemistry. Slices were harvested six days after EnAd infection and evaluated for expression of CD25. Data shown here was obtained using patient specimen number four and is representative of three individual patient samples.

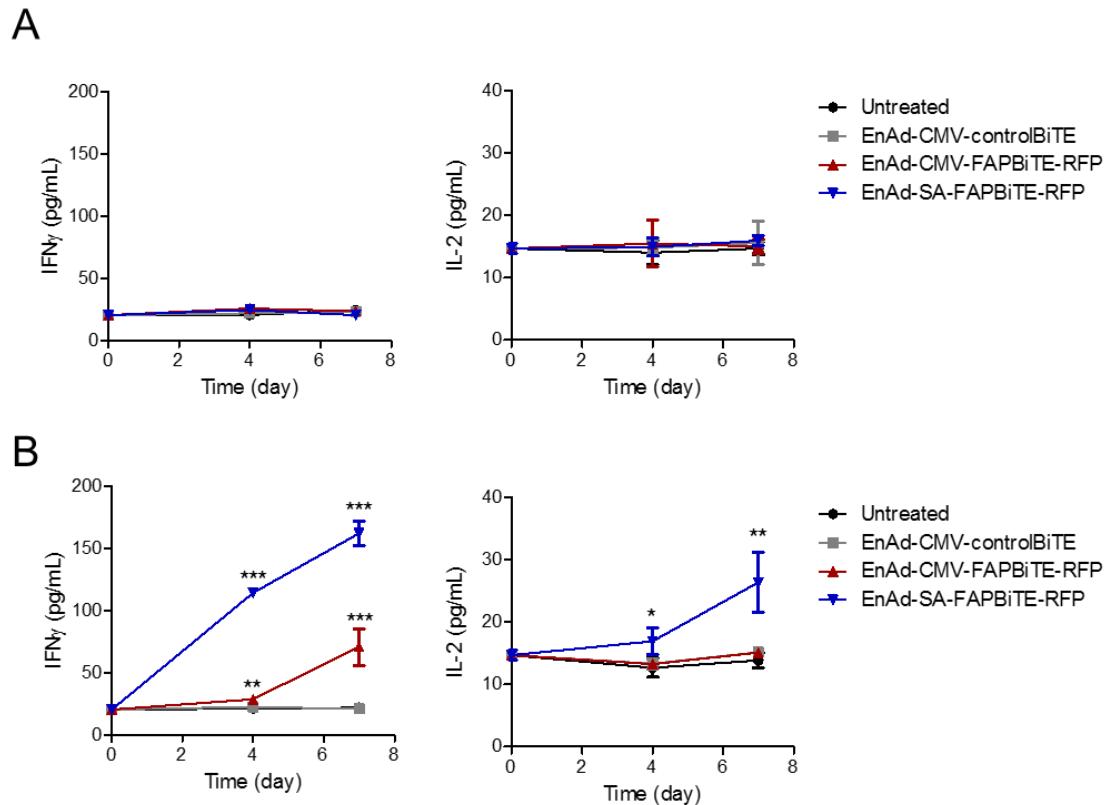


Figure 6.12. EnAd expressing FAP BiTE induces secretion of Th1 cytokines from endogenous T cells in malignant prostate slices. Levels of IFN γ and IL-2 produced from benign (A) and malignant (B) prostate slices infected with recombinant EnAd or uninfected. Cytokines levels were assessed by ELISA using supernatants harvested at the specified timepoints. Data shown here was obtained using patient specimen number four and is representative of three individual patient samples. Each condition was measured in technical triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In previous chapters, stimulation of T cells *in vitro* (PBMC) or *ex vivo* (liquid biopsies) with recombinant BiTE induced stronger activation (CD25, proliferation, IFN γ) than treatment with BiTE expressing virus. Interestingly, in a comparative study using malignant solid prostate slices, FAP BiTE expressed from a virus induced stronger levels of activation than free FAP BiTE. As demonstrated in Figure 6.13, the FAP BiTE induced slight increases in IFN γ by day four that appeared to plateau, whereas the EnAd expressing FAP BiTE induced continual increases in cytokine production. Given that EnAd-CMV-controlBiTE induced no measurable increase, this result cannot be explained

by virally-induced IFN γ production alone but may suggest a synergistic effect between viral infection or delivery and the activity of the BiTE. As before (Figure 6.12), SA-driven BiTE expression induced the highest level of activation (as assessed by IFN γ).

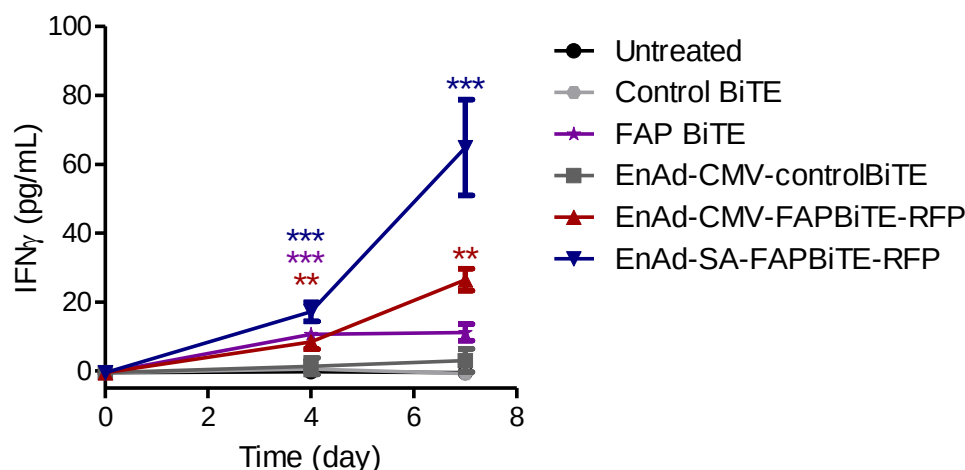


Figure 6.13. EnAd expressing FAP BiTE induces higher levels of IFN γ than free FAP BiTE. Levels of IFN γ produced from untreated malignant prostate slices or cultured with free FAP BiTE or FAP BiTE expressing EnAds. ELISAs were performed on supernatants harvested at the specified time point. Data shown here was obtained using patient specimen number six and is representative of two individual patient samples. Each condition was measured in technical triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * p <0.05, ** p <0.01, *** p <0.001.

6.3.5 FAP BiTE expressing EnAds induce greater cytotoxicity than parental virus in malignant prostate tissue slices

One of the ultimate goals of arming EnAd with a FAP-targeted BiTE was to induce cytotoxicity of FAP⁺ cells and improve overall therapeutic efficacy. To explore this, we first assessed cytotoxicity of the slices by the release of LDH into the supernatant. Benign and malignant tissue from the same patient specimen was sliced and cultured for one day. As discussed previously, replacing the supernatant after the first day of culture removes

the LDH released in the slicing process, ensuring LDH levels are comparable between slices at the start of treatment. Slices were left untreated or infected with EnAd-CMV-control BiTE virus or those expressing FAP BiTE (CMV and SA), and the level of LDH assessed at the specified timepoints (Figure 6.14). Untreated benign and malignant slices appeared to remain viable for the duration of culture, with no observable increase in LDH released. Infection of malignant tissue with EnAd-CMV-controlBiTE, however, induced a small but significant rise in LDH release, possibly reflective of the virus's innate oncolytic activity (Figure 6.14A). Significantly higher levels of cytotoxicity were induced by the FAP BiTE expressing viruses – with the SA-driven FAP BiTE mediating more potent cytotoxicity than the CMV. This phenomenon, in addition to no treatment inducing an increase in cytotoxicity within the first four days of treatment, was similar to the trend observed for IFN γ and IL-2 production. Crucially, in the case of benign tissue, none of the viruses appeared to induce cytotoxicity within the seven-day duration of the study (Figure 6.14B).

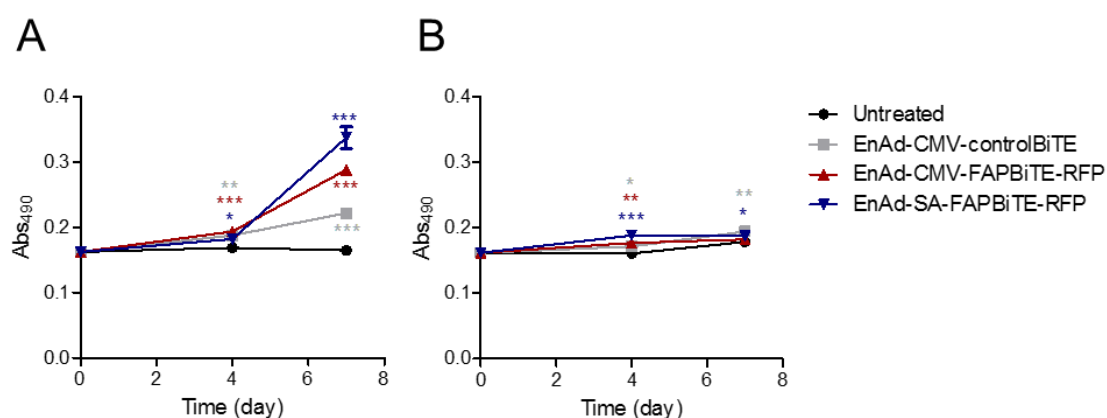


Figure 6.14. EnAds expressing FAP BiTE induce superior cytotoxicity to parental EnAd in malignant prostate slices. Cytotoxicity of malignant (A) or benign (B) prostate slice untreated or infected with recombinant EnAd. Culture medium was harvested at the specified time-points and cytotoxicity was assessed by release of LDH into the supernatants. Data shown here was obtained using patient specimen number six and is representative of two individual patient samples. Each condition was measured in technical triplicate and represented as mean $\pm$ SD. Significance was assessed using a one-way ANOVA test with Tukey's Post Hoc analysis with each condition compared to 'untreated', * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

To visualise cells undergoing apoptosis in real-time within the live tissue, similar slice cultures to above were established, and caspase 3/7 detection reagent was added directly to the media. This reagent is an intrinsically non-fluorescent substrate for activated caspase-3/7, and once cleaved, is able to bind to DNA producing a (green) fluorogenic signal. As shown in Figure 6.15, infection with EnAd-SA-FAPBiTE-RFP induced a similar pattern of RFP-positive rings, as observed previously (Figure 6.10). Encouragingly, there was an apparent association of these infected (red) cells with apoptotic nuclei (green), suggesting viral expressed BiTE may be inducing the proximal cytotoxicity of the surrounding cells. There were also several clear instances of red cells possessing yellow nuclei indicating potential virus-mediated cytotoxicity of the infected cell (highlighted with white arrows; Figure 6.15).

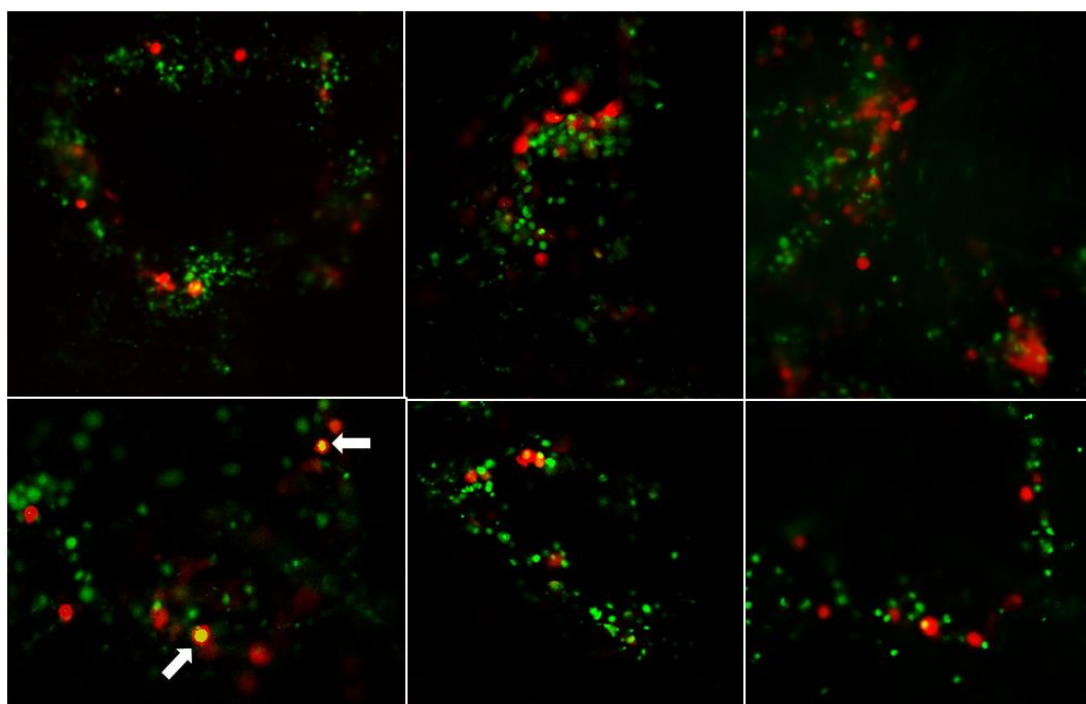


Figure 6.15. Fluorescence microscopy of active caspase 3 in virus-infected malignant tissue slices. Fluorescence images showing malignant prostate tissue infected with EnAd-SA-FAPBiTE-RFP. Expression of RFP (red) allowed for visualisation of virus replication and acted as a surrogate for FAP BiTE expression. Apoptosis was visualised using CellEvent Caspase 3/7 detection reagent (green) added directly the culture medium. Images were obtained using an Axiovert 25 microscope after four days of infection. Data shown here was obtained using patient specimen numbers four and five.

This induced cytotoxicity proved difficult to visualise by immunohistochemistry. However, in one sample, there appeared to be clear loss of tissue integrity surrounding activated T cells in tissue infected with the FAP BiTE expressing EnAd. Malignant slices infected with EnAd-CMV-FAPBiTE-RFP or cultured alone, were fixed, processed and stained for CD25 expression, allowing the architecture of the tissue to also be assessed. As demonstrated for other samples, untreated tissue showed no CD25⁺ cells (Figure 6.16A), with FAP BiTE expressing EnAd strongly inducing the expression of CD25 (Figure 6.16B). Amongst regions of high T cell activation, there was a clear absence (or degradation) of surrounding tissue or stroma, with the tissue architecture remaining wholly intact in the untreated sample. Whilst encouraging, further work is required to determine whether this is reproducible, or simply an artefact.

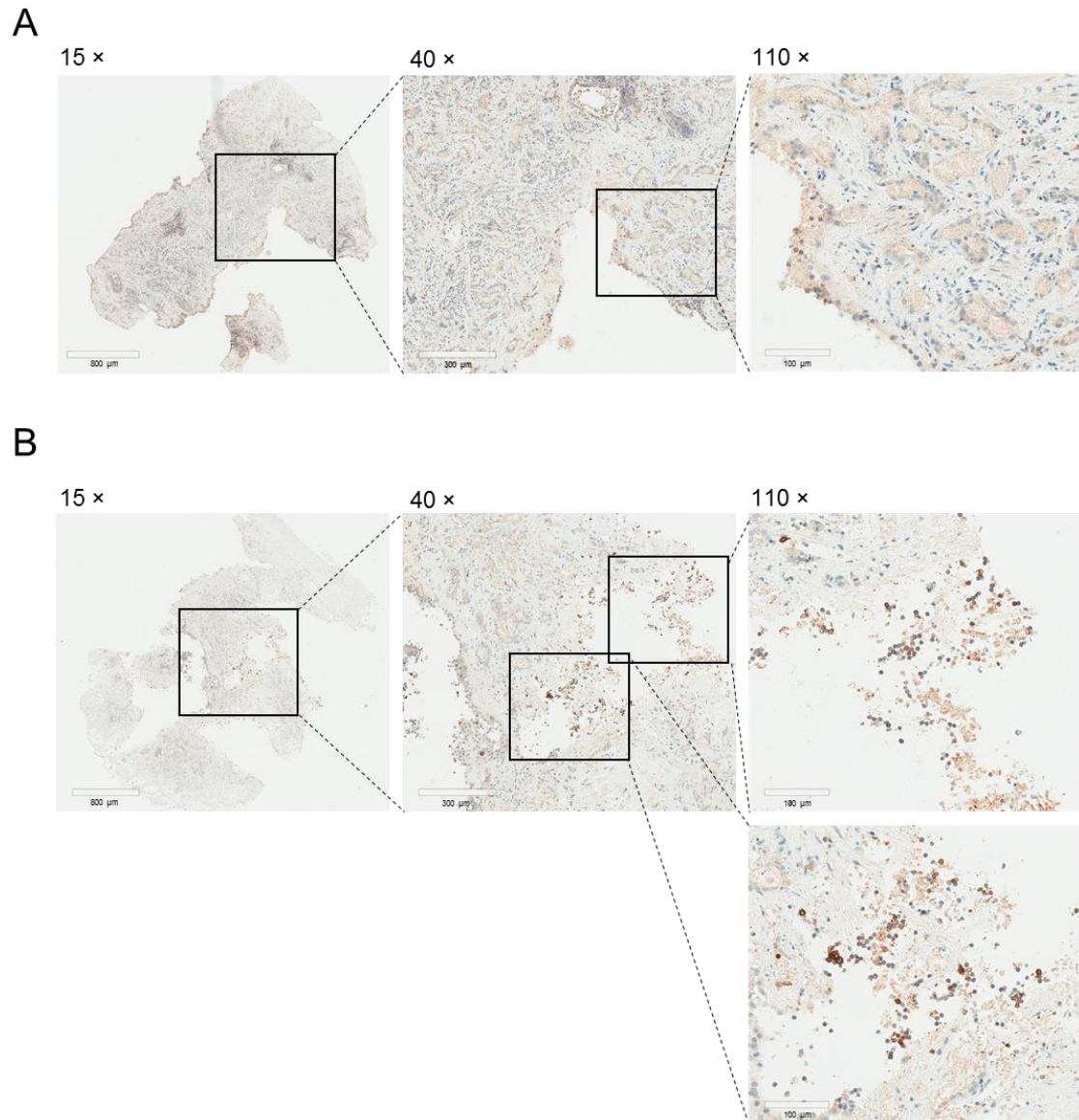


Figure 6.16. Histology and activation of endogenous T cells in virus-infected malignant prostate slices. Activation status of tumour-infiltrating T cells in prostate slices was assessed by immunohistochemistry. Slices were either (A) uninfected or (B) infected with EnAd-CMV-FAPBiTE and tissue harvested on day four and stained for CD25 expression. Data shown here was obtained using patient specimen number one.

6.4 Chapter conclusions

1. FAP BiTE-armed EnAd demonstrated infection and replication within malignant prostate tumour tissue.
2. FAP BiTE-expressing EnAd can activate tumour-infiltrating lymphocytes, and induces superior cytotoxicity compared to control virus.
3. TIL cell activation and associated cytotoxicity was dependent on the presence of FAP expression within the prostate tissue.
4. BiTE expression mediated by the adenoviral major late promoter (i.e. SA virus) induces higher levels of activation and cytotoxicity than CMV-driven expression.

6.5 Chapter discussion

The use of organotypic tissue slices represents an invaluable system to assess the activity of BiTE-armed OV_s in a realistic preclinical model. As expected, the prostate tissue slices were shown to retain the complex and heterogenic architecture expected of a solid human tumour (Figure 6.8). Crucially, given that these results were obtained using freshly collected patient biopsies with minimal manipulation, they may offer a preview of the efficacy that may be achieved in advanced-stage cancer patients

In this chapter, we demonstrate for the first time that tumour-infiltrating lymphocytes in solid tumour tissue can be activated by BiTE-armed OV_s. This T cell activation was FAP-dependent (Figure 6.9) and manifested as an upregulation in CD25 expression and

secretion of pro-inflammatory cytokines IFN γ and IL-2 (Figure 6.11 and 6.12). It is worth noting that the quantity of cytokines measured in the culture supernatant is likely to be a considerable underestimation. Firstly, the volume of the tissue slice is approximately 300-fold smaller than of the surrounding media. Moreover, assuming that the extracellular volume is assumed to range from 10-30% of total tumour volume (Bandula et al, 2013), this may suggest local interstitial concentrations of over 100,000 pg/mL IFN γ and 15,000 IL-2 pg/mL. In light of multiple reports that show T cells within prostate tumours to be strongly immune suppressed, this data is demonstrative of the remarkable activatory potential of the BiTE approach (Silvestri et al, 2015; Thakur et al, 2013).

Consequently, this BiTE-mediated activation appeared to translate to increased cytotoxicity, as demonstrated by increases in LDH release (Figure 6.14). Specifically measuring the cytotoxicity of FAP⁺ cells, however, proved more difficult to evaluate. Whilst the FAP BiTE-dependent release of LDH indicates a FAP BiTE-mediated increase in cell lysis, it does not show which cell types have been killed. Additionally, whilst the association of caspase-positive (green) cells alongside RFP-expressing (and therefore, infected) cells is encouraging, this similarly does not reveal which cell types have been killed. An approach that utilises immunohistochemistry may represent a more informative approach, with the potential to perform dual stains of both FAP and a cytotoxicity marker.

One of the central justifications of using OV_s to express BiTEs is the potential to maximise efficacy by continuous and localised delivery of the BiTE directly to the tumour microenvironment. The use of solid tumour slices may provide a unique opportunity to evaluate this. Indeed, treatment with the free FAP BiTE protein appeared to be significantly less stimulatory than each of the FAP BiTE-armed viruses (Figure 6.13).

Initially, this result did seem to be explained by improved BiTE delivery into the tissue. However, given that the thickness of the slice is 300 μm - and therefore diffusion of the BiTE through the tissue should be of the order of only one hour (Jain, 1990) - this explanation falls short. The striking trend in the majority of patient samples demonstrating that EnAd-SA-FAPBiTE induces a superior response to that of the corresponding CMV virus – despite producing considerably less RFP/BiTE - suggests a potentially more compelling and complicated explanation is required (Figure 6.12 and Figure 6.14).

Indeed, a second rationale for arming OV_s with BiTE_s is the potential for synergism between the BiTE and OV – and such a phenomenon may provide an explanation for this trend. This would be consistent with the observation in chapter five, in which both EnAd and FAP BiTE were required to induce the up-regulation of CD86 on ascites macrophages (Figure 5.21). To understand the trend in Figure 6.12, however, it is possibly of significance that CMV- and MLP-driven BiTE expression differs in (i) the magnitude, (ii) location and (iii) temporal onset of transgene expression. It is plausible that the delayed onset of transgene expression using the MLP allows virus-mediated modulation to occur within the microenvironment prior to the production of BiTE. Whilst it is not immediately clear what modulation or ‘priming’ may be occurring, virus infection could potentially ‘prime’ the microenvironment in numerous ways. For example, in a report analysing TILs isolated from human prostate tumour tissue, it was suggested that the presence of CpG-A (virus-associated dsDNA) can reverse Treg induced immune suppression of effector T cells (Kiniwa et al, 2007). Other possibilities include virus-induced alterations in the chemokines and cytokine milieu, as well as the activation of tumour-resident DCs (Benencia et al, 2005).

Whilst using the tissue slice system has many advantages over the use of mouse models – such as the ability to support EnAd replication and act as a true model of a solid human tumour – one major drawback is the lack of a systemic immune system. The situation in a patient would provide a continual source of T cells, especially if, as expected, there is an immune response initiated against the virus. Indeed, given the low number of T cells compared to fibroblasts in the slices (Figure 6.8), it is plausible that the BiTE-armed OVAs could mediate an even more powerful response *in vivo* with the virus-induced T cell infiltration. Such a possibility may be investigated by the development of a perfusion system allowing the continuous supply of immune cells.

7. Final Discussion

7.1 Overview of this Thesis

Oncolytic viruses offer an intriguing new strategy to combine several therapeutic modalities within a single targeted, self-amplifying agent (Keller & Bell, 2016; Seymour & Fisher, 2016). As they replicate selectively within cancer cells and spread from cell to cell, some oncolytic viruses are thought to mediate cell death by non-apoptotic death pathways (Ingemarsdotter et al, 2010; Li et al, 2013), as part of the process allowing virus particles to escape from dying cells. EnAd, in particular, kills cells by a pro-inflammatory process known as oncosis or ischemic cell death (Dyer et al, 2017). This non-apoptotic death mechanism causes release of several pro-inflammatory cellular components (or DAMPs), such as ATP, HMGB1 and exposure of calreticulin (Weerasinghe & Buja, 2012), and is likely pivotal to the ability of the virus to promote an effective anticancer immune response. In addition to the consequences of direct lysis, however, viruses offer the potential to encode and express other anticancer biologics, obviating delivery challenges and ensuring the biologic achieves its highest concentration within the tumour microenvironment. Imlygic encodes GM-CSF, however the potential for arming viruses is virtually limitless and provides many exciting opportunities to design multimodal therapeutic strategies with additive or synergistic anticancer effects (de Gruijl et al, 2015; Hermiston & Kuhn, 2002).

Encoding BiTEs within oncolytic viruses provides a powerful means to activate tumour infiltrating lymphocytes to become cytotoxic and lyse antigen-positive target cells,

providing a completely separate therapeutic modality from the effects of direct viral lysis. What is more, BiTEs may improve the efficacy of the virus by acting as an ‘immune decoy’. By engaging anti-viral tumour infiltrating T cells, BiTEs can redirect their cytotoxicity towards target cells, attenuating viral clearance and extending the life-time of the tumour infection. Potential in this approach has already been demonstrated in the successful antibody-mediated redirecting of CMV-specific T cells against tumour cells (Schmittnaegel et al, 2015).

In this study we have shown that BiTE-targeted cytotoxicity is fully antigen-specific and can be mediated by both CD4 and CD8 T cells. BiTEs could be encoded by EnAd without any loss of oncolytic virulence, reflecting the considerable transgene packaging capacity of the virus. The presence of the transgene will not affect the physicochemical properties of the virus particles; hence the modified viruses should exhibit exactly the same clinical pharmacokinetics as the parental agent and should be capable of expressing the encoded BiTE selectively within tumours throughout the body. In addition, the current study shows, for the first time, that endogenous T cells within human liquid and solid prostate cancer biopsies can be activated by BiTEs and virus-encoded BiTEs and can kill both endogenous tumour cells and cancer-associated fibroblasts without any additional stimulation or reversal of immune suppression. Importantly, this can happen despite the immunosuppressive microenvironment of the solid tumour and primary exudate fluids.

Targeting the tumour microenvironment with BiTE-armed OV

Arming oncolytic viruses to express BiTEs combines two quite distinct therapeutic mechanisms, with the former providing lytic death of tumour cells that are permissive for virus infection, and the latter targeting T cell cytotoxicity via a specific, chosen, antigen. BiTE technology has now even been demonstrated to target tumour-specific intracellular antigens in the context of HLA molecules (Dao et al, 2015). This provides considerable flexibility in the design of a therapeutic approach, perhaps using the BiTEs to deliver cytotoxicity to tumour-associated cells that are relatively resistant to kill by the virus directly. For example, whilst we originally exemplified the technology here using a BiTE that recognises a carcinoma-associated antigen (EpCAM), we have also demonstrated that it is possible to use the BiTE approach to target cytotoxicity to FAP⁺ cancer-associated fibroblasts. Indeed, even when the targets for BiTE-recognition are not restricted to expression in the tumour microenvironment, by linking BiTE production to virus replication allows expression of the BiTE to be spatially restricted to the tumour, minimising systemic toxicities. This is important, as BiTEs administered intravenously show relatively short circulation kinetics (Klinger et al, 2012) and are often associated with considerable on-target off-tumour toxicities (Teachey et al, 2013). With this in mind, BiTEs targeting other non-malignant cells of the tumour microenvironment are now under investigation in our laboratory.

Overcoming immunosuppressive mechanisms using BiTEs-armed OV

The induction of an adaptive anti-tumour immune response is one of the central goals of cancer immunotherapy. However, even if successful, adaptive T cell responses are ultimately dependent on the expression of MHC class I antigens by tumour cells, to allow targeted killing. Loss of MHC expression is a well-documented immune evasion strategy for tumours (Garrido et al, 2016). It is significant that both cytotoxic strategies that are engaged by BiTE-armed oncolytic viruses operate independently of MHC class I by the tumour cells, and therefore can be employed to kill cancer cells even when tumour cells have lost MHC expression. Accordingly, BiTE-armed OV were able to induce near-complete lysis of tumour cells in all tested exudate samples, despite others reporting of a down-regulation or loss of HLA in up to 100% of analysed cancer biopsy samples (Seliger et al, 2000). Importantly, given this potential loss in HLA, the majority of alternative T cell therapies (vaccines, OV, checkpoint inhibition) would be highly limited in their ability to induce an effective anti-tumour response in these patients.

It is also feasible that BiTEs may be able to overcome alternative tumour-associated immune-suppressive effects that limit physiological immune responses, simply by providing excessive stimulation to T cells through simultaneous clustering of multiple CD3 molecules. Indeed, this is consistent with our observations that BiTEs are (i) able to induce stronger activation than CD3/CD28 beads, and (ii) also unaffected by both exudate-fluid mediated immune suppression and PD1 ligation. Moreover, others have also demonstrated that the EpCAM-targeted BiTE, Solitomab, is little effected by IDO, PDL1, TGF β and IL-10 (Deisting et al, 2015).

Since BiTEs employ an antibody structure to recognise cell surface antigens, without the need for HLA presentation, the number of antigens recognised by an individual BiTE may be very large. For example, EpCAM is expressed at levels up to 1×10^6 copies/cell on malignant exudates (Yao et al, 2013). In contrast, most class I HLA molecules are present at less than 100,000 copies per cell, and each can contain just one from thousands of different HLA-associated peptides (Schellens et al, 2015), meaning that the number of targets for a specific T cell will likely be less than a hundred per cell (Purbhoo et al, 2006). In normal circumstances, this is sufficient for activation, with cytokine production requiring ten MHC complexes to be triggered, and killing as little as three (Wei et al, 2013). It follows that T cell regulation, including checkpoint-mediated suppression, has likely evolved to be active in this range of stimulus – for example, Wei *et al* demonstrated that ligation of PD1 induced a change in which 30 engaged MHC were required for full activation, rather than 10. Hence it may be possible for BiTE-mediated recognition of targets to overcome pathophysiological inhibition mechanism simply by excessive stimulatory T cell signalling. This would not be possible with the use of vaccines, checkpoint inhibitors or adoptive transfer of TILs, which all depend on classical TCR-MHC interaction.

Consequently, the ability of the BiTE to cross-link CD3 receptors and stimulate T cell signalling is likely dependent on the density of antigen-bound BiTE presented to the T cell, and therefore both on the level of antigen on the target cell surface and also the quantity of the BiTE expressed and delivered to the tumour microenvironment. These factors need to be considered when designing new BiTE-armed OVs.

Modulation of the Tumour Microenvironment by BiTE-armed OV

It is noteworthy that both strategies employed by arming an OV with a BiTE are reported to induce widespread changes within the tumour microenvironment. Clinically, there are now numerous reports of OVs inducing the infiltration of T cells into the tumour bed (Kaufman et al, 2010; Mastrangelo et al, 1999; Sahin et al, 2012). In the absence of clinical data, BiTEs have also been demonstrated to induce the persistence and infiltration of T cells into mouse xenograft tumours (Dao et al, 2015; Fajardo et al, 2017).

In this study, we reported similarly important changes. We have demonstrated the BiTE-induced secretion of large quantities of Th1 cytokines *in vitro*, in liquid biopsy samples, and in solid prostate tumour specimens. It is plausible that this may facilitate a global shift from an environment that is immune-suppressive to one that is pro-inflammatory. Accordingly, within a patient ascites samples, we observed the BiTE-mediated polarisation of tumour-associated macrophages (TAMs) to a more ‘classically activated’ phenotype. Directing BiTEs against cells of the TME adds an additional opportunity to reverse immune suppression, for example, by eliminating CAFs, which lead to a knock-down in TGF β expression in three patient samples. Indeed, a rationale for arming OVs with BiTEs is the potential for synergism between the BiTE and OV. BiTE-armed EnAd was able to induce strong upregulation of co-stimulatory receptor CD86 on TAMs in an ascites samples, with BiTE or EnAd alone having little effect. Moreover, BiTE expressed from a virus achieved much stronger T cell activation in solid prostate tumour sample than either of the monotherapies. Strikingly, in the latter example, MLP-driven BiTE expression appeared to produce superior T cell activation than CMV-mediated

expression. This could suggest that there may be benefit in allowing virus replication or other virus-mediated effects to occur prior to BiTE expression.

Induction of secondary immune responses

One central aim of oncolytic virus therapy is to create an anticancer T cell response that recognises patient specific neoantigens as well as “public” tumour associated antigens. Lytic viruses may do this by stimulating improved antigen presentation by lysing tumour cells in the context of DAMPs alongside virus-related pathogen-associated molecular patterns (PAMPs). Such an effect has now been documented in multiple clinical reports, including a phase I trial using a GM-CSF-encoded oncolytic adenovirus (Kanerva et al, 2013).

Whilst the immediate immune-stimulating activity of a BiTE is entirely independent on the generation, expansion and epitope spreading of specific T cells clones (Figure 1.4), BiTEs have also demonstrated the potential to induce secondary, specific T cell responses against other tumour antigens. For example, Dao *et al* reported that using an *in vitro* autologous system, they could induce T cell responses against antigens that were different to the specificity of the BiTE (Dao et al, 2015). They suggest that this did not require cross-presentation by APCs and was a consequence of the reactivation and expansion of pre-existing – and potentially anergic - T cells within the patient. Whilst *in vivo* or clinical data is lacking, such a phenomenon is well-documented with the use of vaccines and TIL infusions – and responding patients have demonstrated strong expansions in pre-existing and new T cells against various antigens (Corbiere et al, 2011; Hunder et al, 2008). In the

case of vaccines, it is hypothesised that this response is mediated by the killing (via apoptosis) of tumour cells by vaccine-specific T cells, which are then phagocytosed by APCs, leading to the activation of T cells against new tumour antigens. Given that EnAd also induces tumour cell lysis, though by immunogenic cell death, there is arguably even more potential with this BiTE-armed oncolytic virus approach. Crucially, such an effect would not be possible using CAR T cells, in which target specificity has been genetically redirected to only the chosen antigen.

Enhancing the BiTE and OV platforms

Given the versatility of the BiTE technology and the considerable transgene packaging capacity of the virus, there is much scope for further augmenting efficacy and minimising potential toxicities. From an efficacy perspective, the potency of a BiTE-induced T cell response appears to be dependent on the interaction between BiTE and antigen. This is supported by the variable induction of T cell proliferation and IL-2 production depending on whether the EpCAM or FAP-BiTE – which share a CD3 ϵ -binding domain - were used. Additionally, whilst the EGFR-BiTE featured in Fajardo *et al* induced secretion of high quantities of IL-2, Yu *et al* reported no appreciable increase in IL-2 with an EphA2-BiTE. There are numerous potential explanations for this – including the different densities of antigen on the target cell and the affinity of the BiTE for the target.

Nevertheless, IL-2 and proliferation can be strongly induced by co-stimulation (Boise *et al*, 1995), and this raises the possibility of engineering a BiTE to feature CD28-activating domains, potentially leading to a molecule that can induce robust T cell proliferation. The

generation of second- and third-generation CAR T cells – featuring added co-stimulatory domains – achieve such an effect, with demonstrable improvements in proliferative capacity and persistence of modified T cells (Ritchie et al, 2013). The recent FDA approval of a third generation anti-CD19 CAR T cell for ALL demonstrates the huge potential of this approach. Other promising strategies to improve efficacy of BiTEs include encoding cytokines (for example IL-12), blocking checkpoint receptors or recruiting alternative effector cells, such as NK cells and macrophages (Stein et al, 2012). On the other hand, additional transgenes can be encoded within an oncolytic adenovirus to stimulate a stronger T cell response. GM-CSF has proved effective in the FDA-approved Imlygic, with the encoding of T cell chemoattractants (e.g. CXCL9 and CXCL10) also an attractive strategy.

Safety is also an important consideration. Trispecific antibodies have been developed that allow targeting of an effector cell on one end, and the dual targeting of tumour antigens on the other. This approach increases the specificity for cancer cells, and reduces the likelihood of targeting normal tissues (Kugler et al, 2010). Systemic toxicities can also be lessened by altering the pharmacokinetics of the BiTE molecules; BiTE can be modified to be less stable or cleared more rapidly from the circulation. Indeed, the virus can also be engineered for improved safety. For example, it is feasible to introduce microRNA binding sites into the virus to further prevent expression in normal tissues (Bofill-De Ros et al, 2015)

Concluding remarks

This thesis aimed to demonstrate the potential of arming the oncolytic virus and clinical candidate, Enadenotucirev, with a BiTE. The promise of each individual strategy has previously been exemplified by the FDA approval of Imlygic for melanoma and blinatumomab for ALL. However, the possibility of combining these approaches offers the opportunity to combine the advantages of each, with potential for synergy. OV-delivered BiTEs are able to induce potent and MHC-independent activation of TILs – potentially re-activating anergic T cells – whilst avoiding undesirable systemic toxicities. Moreover, a virus-induced immune response would be redirected towards a chosen target cell, extending the lifetime of the virus, with potential for further BiTE-mediated epitope spreading. Crucially, arming OVs with BiTEs provides a powerful means to target cells of the tumour stroma, providing a completely separate therapeutic modality from the effects of direct viral lysis. With increasing awareness of the numerous roles of non-transformed cells in contributing to tumour growth and immunosuppression, it difficult to envisage cancer immunotherapy reaching its full potential without addressing these cells as part of a combined therapeutic approach such as this. Given the stability of EnAd in human blood, and its ability to target disseminated malignant disease in cancer patients, it is anticipated that BiTE-armed EnAds may well display potent therapeutic efficacy in future clinical trials.

Appendix A: Materials

TFB1 – In water: 30 mM $\text{KC}_2\text{H}_3\text{O}_2$, 100 mM RbCl, 10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 50 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 15% v/v glycerol. Adjust to pH 5.8 using 0.2 M acetic acid. Filter sterilise.

TFB2 – In water: 10 mM MOPS, 10 mM RbCl, 75 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15% v/v glycerol. Adjust to pH 6.6 using 5 M KOH. Filter sterilise.

TAE – In water: 40 mM Tris-Acetate, 1 mM EDTA.

MACS buffer - In PBS: 0.5% BSA and 2 mM EDTA.

Prostate media – In keratinocyte serum-free media (Thermo Fisher), 2.5 ng/mL rEGF (Thermo Fisher), 30 µg/mL BPE (Thermo Fisher), 10 µg/mL insulin (Thermo Fisher), 500 ng/mL hydrocortisol (Sigma-Aldrich), 1.42 µg/mL (Sigma-Aldrich), 100 µg/ml primocin (Invivogen, USA) and 2% FBS. Filter sterilise.

CsCl $\rho = 1.32$ - 32.0g CsCl, 6.8 mL of 0.5 M Tris-HCl at pH 7.9, 61.2 mL dH₂O.

CsCl $\rho = 1.45$ - 20.5g CsCl, 2.9 mL 0.5 M Tris-HCl at pH 7.2, 28.5 mL dH₂O,

40% glycerol - 40 g glycerol, 2 mL of 0.5 M Tris-HCl at pH 7.9, 0.5 mL of 0.2 M EDTA at pH 8.0, made up to 100 mL using dH₂O,

Appendix B: DNA and amino acid sequences

EpCAM amino acid:

MAPPQVLAFGLLLAAATATFAAAQEEVCENYKLAVNCFVNNNRQCQCTSVGAQNTVICSKLAA
KCLVMKAEMNGSKLGRRAKPEGALQNNNDGLYDPDCDESGLFKAKQCNGTSMCWCVNNTAGVRRTD
KDTEITCSERVRTYWIIEELKHKAREKPYDSKSLRTALQKEITTRYQLDPKFITSILYENNVIT
IDLQVNSSQKTQNDVDIADVAYYFEKDVKGESLFHSHKMDLTVNGEQLDLDPGQTLIYYVDEKA
PEFSMQGLKAGVIAIVVVVIAVVAGIVVLVISRKKRMAKYEKAEIKEMGEMHRELNA

EpCAM DNA:

ATGGCTCCGCCGCAAGTGCTCGCCTTTGGACTGCTCCTGGCCGCTGCTACTGCCACCTTTGCTG
CCGCTCAGGAAGAATGCGTGTGCGAAACTACAACTGGCAGTGAAGTCTTCGTGAACAATAA
CCGCCAATGCCAGTGCACCAGCGTGGGCGCGCAGAACACTGTCATCTGCAGCAAGCTGGCCGCA
AAATGCCTGGTCATGAAAGCGGAGATGAACGGATCGAAGCTGGGTCGGCGCGCTAAGCCGGAAG
GAGCGCTACAAAATAACGACGGGCTGTACGATCCGGATTGCGACGAAAGCGGTCTGTTTAAGGC
AAAGCAGTGTAATGGGACCAGCATGTGTTGGTGCGTGAACACTGCCGGAGTGCGCCGCACTGAC
AAGGATACCGAAATTACGTGCTCGGAGCGCGTGAGAACGTATTGGATCATCATCGAGTTGAAGC
ATAAAGCTAGAGAGAAGCCGTACGATAGCAAGTCCCTGCGCACCGCTCTCCAAAAAGAAATTAC
CACTAGATACCAACTCGACCCGAAATTCATCACTTCAATCCTGTACGAAAACAACGTGATTACG
ATCGACCTGGTGCAGAACTCCAGCCAAAAGACGCAGAACGACGTGGACATTGCAGACGTGGCTT
ACTACTTTGAAAAGGACGTGAAGGGGAATCACTATTTTATTCCAAGAAAATGGACCTGACCGT
CAACGGAGAGCAGCTCGACCTGGACCCAGGTCAGACTCTGATCTACTACGTGGACGAGAAGGCA
CCGGAGTTTTTCGATGCAGGGCCTGAAAGCCGGAGTCATCGCCGTGATCGTGGTGGTGGTATTG
CCGTCGTCGCCGGGATCGTGGTGCTCGTGATTAGCCGCAAAAAGAGAATGGCTAAGTATGAAA
GGCAGAGATCAAGGAAATGGGAGAAATGCACCGCGAACTCAACGCATAG

FAP amino acid:

MKTWVKIVFGVATSAVLALLVMCIVLRPSRVHNSEENTMRALTLKDILNGTFSYKTFPPNWISG
QEYLHQSadnnivlynietgqsytilsnrtmksvnasnyglspdrqfvyleSDYsklwrySYTA
TYIYDLsngefVRGNELPRPIQYLCWSPVGSKLAYVYQNNIYLKQRPDPFPQITfNGRENKI
fNGIPDWVYEEEmLATKYALWwSPNGKFLAYAEfNDTDIPVIAYSYYGDEQYPRtINIpyPKAG
AKNPVVRIFIIDTTYPAYVGPQEVVPAMIASSDYfFSWLTWVtDERVCLQWLKRVQNVSVLSI
CDFREDWQTwDCPKTQEHIEESRTGWAGGfFVSTPVfSYDAISYYKIFSDKDGYKHIHYIKDTV
ENAIQITSGKWEAINIFRVTQDSLfySSNEfEEYPGRrNIYRISIGSYPPSKKCVtChLRKERC
QYYTASfSDYAKYYALVCYGPgIPISTLHDGRtDQEIKILEENKELENALKNIQLPKEEIKKLE
VDEITLWYKMILPPQfDRSKKYPLLIQVYGGPCSQSVRSVFAVNWISYLASKEGMVIALVDGRG
TAFQGDkLLYAVYRKLGVYEVEDQITAVRKFIEMGfIDEKRIAIWGSYGGYVSSlALASGTGL
FKCGIAVAPVSSWEYYASVYTERfMGLPTKDDNLEHYKNSTVMARAEYFRNVdYLLIHGTADDN
VHFQNSAQIAKALVNAQVDFQAMWYSDQNHGLSGLSTNHLYTHMTHfLKQCfSLSD

FAP DNA:

ATGAAGACCTGGGTGAAAATCGTGTTTGGAGTCGCAACCAGCGCCGTGCTGGCCCTGTTGGTGA
TGTGCATCGTGCTGCGCCCGTCACGCGTGCATAACAGCGAAGAAAACACCATGAGAGCTCTGAC
GCTAAAGGACATCCTGAATGGCACTTTTTCCTATAAGACTTTCTTCCCGAATTGGATTAGCGGG
CAGGAGTACCTGCACCAATCGGCGGATAACAATATCGTGCTCTACAACATCGAACTGGCCAGA
GCTACACCATCCTGTGCAATAGAACTATGAAATCGGTGAATGCGTCGAACTACGGACTTAGCCC
GGACCGCCAGTTTCGTGTACCTAGAATCGGACTACAGCAAGCTGTGGCGGTACAGCTACACTGCT
ACGTACTACATCTACGACCTGTGCAATGGCGAGTTTCGTGAGAGGAAACGAACTCCCCCGCCGA
TCCAATACCTGTGCTGGAGCCCTGTGGGAAGCAAGCTGGCGTACGTGTATCAGAACAAATCTA
CCTCAAGCAGAGGCCGGGCGACCCACCTTTCCAGATCACTTTTAACGGTCGGGAGAAACAAGATT
TTCAATGGAATCCCGGATTGGGTGTACGAAGAAGAGATGCTGGCGACCAAATATGCGCTCTGGT
GGTCGCCAAACGGAATTTCTAGCATACGCCGAGTTCAATGACACCGACATCCCCGTCATCGC
CTACAGCTACTACGGAGATGAGCAATACCCACGCACTATTAATATCCCGTACCCGAAAGCCGGC
GCTAAAAATCCGGTCGTGCGGATCTTCATCATCGACACTACCTATCCAGCTTACGTGGGACCGC
AAGAGGTCCCTGTGCCAGCCATGATCGCCAGCAGCGATTACTACTTCTCCTGGCTGACTTGGGT
CACTGACGAGAGGGTGTGCCTCCAATGGCTCAAGCGCGTGCAGAACGTGAGCGTGCTGTCCATC
TGCGACTTCAGAGAAGATTGGCAAACGTGGGACTGTCCGAAAACCTCAAGAGCATATCGAGGAGA
GCAGAACCGGATGGGCAGGGGGTCTTCGTGTCCACCCCGGTGTTTCAGCTACGATGCGATCTC
GTACTACAAGATTTTCTCCGATAAGGACGGGTACAAACATATCCACTACATCAAGGACACCGTG
GAGAATGCGATCCAAATCACTAGCGGAAAATGGGAAGCGATTAACATTTTCCGGGTGACGCAAG
ACTCATTGTTCTATTTCATCCAATGAATTTGAAGAGTACCTGGCCGGAGGAACATCTACCGCAT
CTCCATCGGATCATAACCCACCATCAAAGAAGTGCGTGACCTGCCATCTCCGCAAGGAGCGCTGC
CAATACTACACCGCGTCTCTTCTCCGATTACGCGAAGTACTACGCCCTGGTGTGCTACGGCCCGG
GAATCCCAATCTCGACCCTACATGATGGGCGCACCGACCAGGAGATCAAAATCCTGGAGGAAAA
CAAGGAGCTCGAAAATGCTCTGAAAAATATTCAACTACCGAAGGAGGAAATCAAGAAGCTGGAG
GTGGATGAAATTACTCTGTGGTACAAAATGATCCTGCCGCCACAGTTCGACCGCTCCAAGAAGT
ACCCGCTGCTGATCCAGGTGTACGGAGGACCCTGCTCACAGTCGGTGCGCTCAGTGTTTGCTGT
GAATTGGATCAGCTACCTCGCATCAAAGGAGGGGATGGTCATCGCGCTTGTGGACGGAAGAGGT
ACTGCATTCCAGGGGGACAACTGCTCTACGCGGTGTACCGCAAGCTGGGCGTCTACGAGGTGG
AGGACCAAATCACGGCGGTGAGAAAGTTCATCGAGATGGGTTTCATCGACGAGAAGAGGATCGC
CATCTGGGGCTGGTCGTACGGAGGTTACGTGTCGTCACTCGCTCTGGCGAGCGGGACTGGATTG
TTCAAATGCGGTATCGCAGTCGCCCCCGTGTGTCGTATGGGAATACTACGCCTCGGTGTATACCG
AACGCTTTATGGGACTCCCTACTAAAGACGATAACCTAGAGCACTACAAAACTCGACGGTGAT
GGCACGCGCAGAGTACTTTTCGCAATGTGGATTACCTCCTGATCCACGGAACCGCCGATGATAAT
GTCCACTTCCAGAACTCAGCCCAAATCGCGAAAGCCCTGGTGAACGCTCAAGTGGACTTTCAAG
CAATGTGGTATTTCAGACCAGAACCACGGACTATCCGGCCTATCGACTAATCACCTGTACACCCA
TATGACCCATTTCTTAAACAGTGTTTCAGCCTGTCCGATTAG

EpCAM BiTE amino acid:

MGWSCIIILFLVATATGVHSELVMTQSPSSLTVTAGEKVTMSCKSSQSLNSGNQKNYLTWYQQK
PGQPPKLLIYWASTRESGVPDRFTGSGSGTDFLTIISSVQAEDLAVYYCQNDYSYPLTFGAGTK
LEIKGGGSGGGGSGGGGSEVQLLEQSGAELVRPGTSVKISCKASGYAFTNYWLGWVKQRPBGH
LEWIGDIFPGSGNIHYNEKFKGKATLTADKSSSTAYMQLSSLTFEDSAVYFCARLRNWDEPMDY
WGQGTTVTVSSGGGSDVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHWVRQAPGQGLEWI
GYINPSRGYTNYADSVKGRFTITTDKSTSTAYMELSSLRSEDATYYCARYYDDHYCLDYWGQG
TTVTVSSGEGTSTGSGSGSGGADDIVLTQSPATLSLSPGERATLSCRASQSVSYMNWYQQKP
GKAPKRWIYDTSKVASGVPARFSGSGSGTDYSLTINSLEAEDAATYYCQQWSSNPLTFGGGTKV
EIKHHHHHHHHHH

EpCAM BiTE DNA:

ATGGGATGGAGCTGCATCATCCTATTCCCTCGTGGCGACGGCCACTGGAGTGCATAGCGAACTGG
TGATGACTCAGTCCCCGTATCCCTGACGGTGACCGCCGGCGAGAAGGTCACCATGTCGTGCAA
GTCCTCGCAAAGCCTGCTTAACAGCGGCAACCAGAAAACTACCTCACGTGGTATCAGCAAAAG
CCAGGTCAACCCCCAAAACCTGCTCATCTACTGGGCGAGCACCCGCGAGTCGGGGGTGCCAGACC
GGTTCACCGGCTCCGGGTGAGGAAGTATTCACCCTAACCATCAGCTCGGTGCAAGCGGAGGA
CCTGGCCGTGTACTACTGCCAAAATGATTACTCGTACCCTCTGACCTTTGGAGCGGGCACCAG
CTCGAAATCAAGGGCGGTGGAGGAAGCGGGCGGGGAGGCTCAGGTGGGGGAGGATCAGAAGTCC
AACTGCTGGAGCAGTCAGGAGCCGAACCTGGTCCGCCCGGGAACCTCCGTCAAGATTTCCCTGTAA
GGCTTCCGGCTACGCTTTTACCAATTACTGGCTGGGCTGGGTCAAGCAAAGACCGGGGCATGGC
CTGGAGTGGATCGGCGACATCTTCCCAGGGAGCGGCAACATCCACTACAACGAGAAGTTCAAGG
GGAAAGCGACTCTGACTGCCGACAAATCATCCAGCACCGCCTACATGCAGCTGTCGTGCTCAC
TTTCGAAGACAGCGCGGTGTACTTTTGTGCTCGGCTCCGGAACCTGGGATGAACCAATGGACTAC
TGGGGACAAGGAACCTACCGTGACCGTCTCCTCCGGCGGCGGTGGAAGCGATGTGCAACTCGTGC
AGTCCGGTGCGGAAGTGAAAAAGCCGGGCGCGAGCGTCAAAGTGTGATGCAAGGCGTCAGGATA
TACGTTTACTAGATACTATGCACTGGGTGCGCCAGGCACCTGGTCAGGGCCTTGAATGGATC
GGCTACATCAATCCGTGAGAGGCTACACTAATTACGCGGACTCAGTCAAAGGGCGCTTCACGA
TTACGACCGACAAGTCCACCTCGACTGCATACATGGAACCTGTCCTCGCTGAGAAGCGAGGACAC
CGCTACTTACTACTGCGCTAGATACTACGATGATCACTACTGCCTCGATTACTGGGGCCAGGGA
ACCACTGTACGGTGTATCGGGAGAGGGCACCTCAACCGGATCAGGGGGATCGGGAGGCTCGG
GCGGCGCAGACGACATCGTCCTGACCCAGTCGCCCCGCCACCTTGTGCTGTCCCCAGGAGAAAG
AGCGACCCTGTCATGCCGGGCGTCGCAAAGCGTGAGCTATATGAATTGGTATCAGCAGAAGCCA
GGAAAGGCGCCGAAGAGATGGATCTACGACACCTCCAAGGTCGCTTCAGGTGTCCCGGCTAGAT
TCTCAGGATCGGGATCAGGAACGGAACCTCCCTGACCATCAATTCAGTGAAGCAGAAGATGC
GGCCACCTACTACTGTGAGCAGTGGTCCTCCAACCCGCTGACTTTCGGAGGCGGAACCTAAGGTC
GAGATCAAGCATCACCATCACCATCACCACCATCACCATTAGCATCGATTTCTGGATCCGCAGG
CCTCTGCTAGCTTGACTGACTGA

Control BiTE amino acid:

MGWSCIIILFLVATATGVHSELDIVMTQAPASLAVSLGQRATISCRASKSVSSSGYNYLHWYQQK
PGQPPKLLIYLASNLESGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSREFPLTFGAGTK
LEIKSSGGGGSGGGGGSSRSSLEVQLQQSGPELVKPGASVKISCKTSGYTFTGYTMHWVRQSH
GKSLEWIGGINPKNGGIIYNQKFQGKATLTVDKSSSTASMELRSLTSDDSAVYYCARRVYDDYP
YYYAMDYWGQTSVTVSSAKTTPPSVTSGGGSDVQLVQSGAEVKKPGASVKVSCKASGYTFTR
YTMHWVRQAPGQGLEWIGYINPSRGYTNADSVKGRFTITTDKSTSTAYMELSSLRSEDTATYY
CARYYDDHYCLDYWGQGTITVTVSSGEGTSTGSGSGSGGADDIVLTQSPATLSLSPGERATLS
CRASQSVSYMNWYQQKPGKAPKRWIYDTSKVASGVPARFSGSGSGTDYSLTINSLEAEDAATYY
CQQWSSNPLTFGGGTKVEIKHHHHHHHHHH

Control BiTE DNA:

ATGGGTTGGTCATGCATCATCCTGTTTCTCGTCGCAACTGCGACCGGCGTGCATTTCGGAAGTGG
ACATCGTGATGACCCAGGCACCTGCATCACTGGCAGTGAGCCTGGGACAGAGAGCCACCATTTC
ATGCCGGGCCAGCAAGAGCGTGTCGTCATCCGGATACAATTATCTGCACTGGTATCAGCAGAAA
CCAGGACAACCTCCAAAGCTACTCATCTACCTGGCGTCGAACCTCGAATCGGGCGTCCCAGCTA
GATTCTCAGGGAGCGGTTTCGGGAACCGATTTCACCCTGAACATCCACCCTGTGGAGGAGGAAGA
CGCGGCAACGTACTATTGCCAGCATTCCCGCGAGTTCCCTCTCACTTTCGGTGCGGGAACCTAAG
CTGGAGATCAAGTCCAGCGGCGGCGGAGGTAGCGGTGGAGGTGGCGGAGGAAGCTCCCGGTCTGT
CGCTGGAGGTGCAGCTGCAACAATCCGGCCCCGGAAGTGGTCAAGCCAGGCGCATCCGTCAAGAT
TTCATGCAAGACCTCGGGGTACACCTTCACCGGGTACACGATGCATTGGGTGAGGCAGAGCCAC
GGCAAGTCCCTGGAATGGATCGGAGGAATCAACCCCCAAAACGCGGCGCATCATCTACAACCAAA
AGTTCCAGGGAAAAGCCACTCTGACCGTGGACAAGTCGTCGAGCACGGCCAGCATGGAGCTGCG
GTCCCTCACTTCCGACGACTCAGCCGTGTATTACTGCGCGAGACGGGTCTACGATGACTACCCA
TACTACTACGCTATGGACTACTGGGGACAAGGAACCAGCGTGACCGTCTCATCGGCGAAAACCTA
CTCCGCCGTGCGTGACGTCGGGAGGTGGTGAAGCGACGTGCAGCTCGTCCAGTCGGGTGCCGA
GGTGAAGAAGCCAGGAGCCTCCGTGAAGGTCTCGTGCAAAGCCAGCGGCTACACTTTTACTAGG
TACACTATGCACTGGGTGCGGCAAGCGCCGGGACAAGGTCTGGAGTGGATCGGATACATCAATC
CGTCGCGGGGATACACTAATTACGCGGACTCCGTCAAGGGACGGTTTACTATCACTACGGATAA
GTCCACTAGCACCGCCTACATGGAAGTGTCTCGCTGCGGTGCGAAGACACTGCGACCTACTAC
TGCGCTAGATATTACGATGACCACTACTGCCTCGACTATTGGGGGCAGGGCACTACGGTCACCG
TCTCGTCGGGAGAAGGAACCTCAACTGGATCGGGCGGATCGGGAGGCTCCGGAGGAGCCGACGA
CATCGTGCTTACCCAGTCGCCTGCGACCTGTCCCTGTCCCCAGGAGAGAGAGCGACTCTTTCA
TGCAGGGCTTCCCAATCAGTCTCCTACATGAATTGGTATCAACAAAAACCCGGCAAGGCCCCGA
AACGCTGGATCTACGATACTTCAAAGGTGGCCAGCGGTGTGCCTGCCCGCTTCTCCGGGTGCGG
GTCCGGCACCGATTACTCGTTGACTATCAATAGCCTGGAGGCCGAGGACGCTGCAACTTACTAC
TGCCAGCAGTGGTCTTCAACCCTCTCACCTTCGGAGGCGGGACCAAGGTGGAAATCAAACATC
ACCATCACCATCACCACCATCACCATTAGCATCGATTTCTGGATCCGCAGGCCTCTGCTAGCTT
GA

FAP BiTE (original) amino acid:

MGWSCIIILFLVATATGVHSDIVMTQSPDSLAVSLGERATINCKSSQSLLYSRNQKNYLAWYQQK
PGQPPKLLIFWASTRESGVPDRFSGSGFGTDFTLTISSLQAEDVAVYYCQQYFSYPLTFGQGTK
VEIKGGGSGGGGSGGGGSQVQLVQSGAEVKKPGASVKVSCKTSRYTFTEYTIHWVRQAPGQRL
EWIGGINPNNGIPNYNQKFKGRVTITVDTSASTAYMELSSLRSEDVAVYYCARRRIAYGYDEGH
AMDYWGQGLTVTVSSGGGGSEVQLVESGGGLVQPGGSLKLSCAASGFTFNKYAMNWVRQAPGKG
LEWVARIRSKYNNYATYYADSVKDRFTISRDDSKNTAYLQMNNLKTEDTAVYYCVRHGNFGNSY
ISYWAYWGQGLTVTVSSGGGSGGGGSGGGGSQTWTQEPSTVSPGGTVTLTCGSSTGAVTSGN
YPNWWQQKPGQAPRGLIGGTFKFLAPGTPARFSGSLLGGKAALTLSGVQPEDEAEYYCVLWYSNR
WVFGGGTKLTVLHHHHHHHHHH

FAP BiTE (original) DNA:

ATGGGCTGGAGCTGCATCATCTTGTTCTTGGTTCGCAACTGCTACCGGAGTCCATTTCGGACATCG
TCATGACCCAAAGCCCTGACTCGCTCGCTGTGTCACTGGGAGAGCGGGCGACTATCAACTGCAA
ATCATCCCAGAGCCTGCTGTATTCACGCAATCAGAAAACTACCTGGCCTGGTATCAGCAGAAG
CCGGGCCAGCCTCCCAAGCTGCTGATCTTCTGGGCCTCCACCCGCGAAAGCGGCGTGCCGGACC
GCTTCAGCGGAAGCGGATTTCGGAAGTGAAGTCTTACTCTGACCATTAGCTCCTTGCAGGCGGAGGA
CGTGGCCGTCTACTACTGCCAGCAGTATTTCTCCTATCCGCTCACCTTTGGGCAAGGCACCAAG
GTGGAGATTAAGGGAGGGGGCGGCAGCGGGGGAGGCGGCAGCGGCGGGGGGATCGCAGGTCC
AGCTCGTCCAAATCCGGAGCCGAAGTCAAGAAGCCGGGAGCGTCCGGTCAAGGTGAGCTGCAAAAC
TTCGCGCTACACCTTCACTGAGTACACGATCCACTGGGTCCGCCAGGCGCCCGGCCAGCGGCTG
GAGTGGATCGGCGGGATCAACCCAAACAACGGAATCCCAAATTACAATCAGAAATTTAAAGGGC
GGGTGACTATCACCGTGGATACCTCGGCCTCCACGGCGTACATGGAGCTCTCATCACTCAGATC
GGAGGACACCGCGGTCTATTACTGCGCCCCGCCCGCGGATCGCTTATGGATACGATGAAGGACAT
GCGATGGATTACTGGGGCCAGGGCACCTCGTCACGGTGTGTCAGGAGGCGGCGGTTTCAAGAG
TGCAACTGGTTCGAGTCGGGAGGGGGACTGGTGAACCGGGGGGATCACTAAAGCTCAGCTGTGC
TGCGAGCGGATTCACTTTCAATAAATACGCAATGAATTGGGTGCGGCAAGCGCCGGGAAAAGGA
CTGGAGTGGGTGCGCCAGGATTCGCTCGAAATACAATAATTACGCGACTTACTACGCTGATAGCG
TGAAAGACCGGTTTACCATCTCGCGCGACGATAGCAAGAATACTGCCTACCTGCAGATGAATAA
CCTGAAGACCGAAGACACCGCAGTGTATTACTGCGTCAGACACGGCAATTTTGGAACTCCTAC
ATTTCTCTATTGGGCTTACTGGGGACAGGGCACTCTGGTGACCGTCTCGTCAGGCGGCGGAGGGT
CGGGAGGCGGAGGATCGGGCGGAGGCGGTTCACGACTTGGACTCAGGAACCGAGCCTCACTGT
GTCCCCGGGCGGGACCGTCACTCTGACTTGCGGCAGCAGCACTGGAGCCGTGACTTCCGGGAAC
TACCCGAAGTGGGTCCAAACAAAAGCCTGGACAGGCACCGCGCGGACTGATCGGAGGCACCAAT
TCCTTGCCCCCGGAAGTCCGGCTAGATTTTCCGGATCACTCCTGGGGGGCAAGGCTGCGCTGAC
GCTCAGCGGTGTCCAGCCGGAAGACGAGGCGGAATACTATTGTGTGTTGTGGTACAGCAACAGG
TGGGTGTTTGGTGGCGGAAGTAAAGCTGACTGTCCTGCACCATCACCAACCACCACCACCATC
ACTAG

FAP BiTE (modified) amino acid:

MGWSCIIILFLVATATGVHSDIVMTQSPDSLAVSLGERATINCKSSQSLLYSRNQKNYLAWYQQK
PGQPPKLLIFWASTRESGVPDRFSGSGFGTDFTLTITSSSLQAEDVAVYYCQQYFSYPLTFGQGTK
VEIKGGGSGGGGSGGGGSQVQLVQSGAEVKKPGASVKVSCKTSRYTFTEYTIHWVRQAPGQRL
EWIGGINPNNGIPNYNQKFKGRVTITVDTASASTAYMELSSLRSEDVAVYYCARRRIAYGYDEGH
AMDYWGQGLTVTVSSGGGSDVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHWVRQAPGQG
LEWIGYINPSRGYTNVADSVKGRFTITTDKSTSTAYMELSSLRSEDVAVYYCARYYDDHYCLDY
WGQGTITVTVSSGEGTSTGSGSGSGGADDIVLTQSPATLSLSPGERATLSCRASQSVSYMNWY
QQKPGKAPKRWIYDTSKVASGVPARFSGSGSGTDYSLTINSLEAEDAATYYCQWSSNPLTFGG
GTKVEIKHHHHHHHHHH

FAP BiTE (modified) DNA:

ATGGGCTGGAGCTGCATCATCTTGTTTCCTGGTCGCAACTGCTACCGGAGTCCATTTCGGACATCG
TCATGACCCAAAGCCCTGACTCGCTCGCTGTGTCACTGGGAGAGCGGGCGACTATCAACTGCAA
ATCATCCCAGAGCCTGCTGTATTACGCAATCAGAAAACTACCTGGCCTGGTATCAGCAGAAG
CCGGGCCAGCCTCCCAAGCTGCTGATCTTCTGGGCCTCCACCCGCGAAAGCGGCGTGCCGGACC
GCTTCAGCGGAAGCGGATTTCGGAAGTGAAGTCTTACTCTGACCATTAGCTCCTTGCAGGCGGAGGA
CGTGGCCGTCTACTACTGCCAGCAGTATTTCTCCTATCCGCTCACCTTTGGGCAAGGCACCAAG
GTGGAGATTAAGGGAGGGGGCGGCAGCGGGGGAGGCGGCAGCGGCGGGGGGATCGCAGGTCC
AGCTCGTCCAAATCCGGAGCCGAAGTCAAGAAGCCGGGAGCGTCGGTCAAGGTGAGCTGCAAAAC
TTCGCGCTACACCTTCACTGAGTACACGATCCACTGGGTCCGCCAGGCGCCCGGCCAGCGGCTG
GAGTGGATCGGCGGGATCAACCCAAACAACGGAATCCCAAATTACAATCAGAAATTTAAAGGGC
GGGTGACTATCACCGTGGATACCTCGGCCTCCACGGCGTACATGGAGCTCTCATCACTCAGATC
GGAGGACACCGCGGTCTATTACTGCGCCCCGCCCGCGGATCGCTTATGGATACGATGAAGGACAT
GCGATGGATTACTGGGGCCAGGGCACCTCGTCACGGTGTGTCAGGAGGCGGCGGTTTTCAGATG
TGCAACTCGTGCAGTCCGGTGCAGGAAGTGAAGAAAGCCGGGCGCGAGCGTCAAAGTGTGATGCAA
GGCGTCAGGATATACGTTTACTAGATACACTATGCACTGGGTGCGCCAGGCACCTGGTTCAGGGC
CTTGAATGGATCGGCTACATCAATCCGTCGAGAGGCTACACTAATTACGCGGACTCAGTCAAAG
GGCGCTTCACGATTACGACCGACAAGTCCACCTCGACTGCATACATGGAAGTGTCTCGCTGAG
AAGCGAGGACACCGCTACTTACTACTGCGCTAGATACTACGATGATCACTACTGCCTCGATTAC
TGGGGCCAGGGAACCACTGTCACGGTGTGATCGGGAGAGGGCACCTCAACCGGATCAGGGGGAT
CGGGAGGCTCGGGCGGCGCAGACGACATCGTCTGACCCAGTCGCCCCGCCACCTTGTGCTGTC
CCCAGGAGAAAGAGCGACCTGTGATGCCGGGCGTCGCAAAGCGTGAGCTATATGAATTGGTAT
CAGCAGAAGCCAGGAAAGGCGCCGAAGAGATGGATCTACGACACCTCCAAGGTGCTTTCAGGTG
TCCCGGCTAGATTCTCAGGATCGGGATCAGGAACGGACTACTCCCTGACCATCAATTCAGTGA
AGCAGAAGATGCGGCCACCTACTACTGTCAGCAGTGGTCTTCCAACCCGCTGACTTTTCGGAGGC
GGAAGTAAAGGTGAGATCAAGCATCACCATCACCATCACCACCATCACCATTAG

FAP BiTE-P2A-RFP cassette amino acid:

MGWSCIIILFLVATATGVHSDIVMTQSPDSLAVSLGERATINCKSSQSLLYSRNQKNYLAWYQQK
PGQPPKLLIFWASTRESGVPDRFSGSGFGTDFTLTISSLQAEDVAVYYCQQYFSYPLTFGQGTK
VEIKGGGSGGGGSGGGGSQVQLVQSGAEVKKPGASVKVSCKTSRYTFTEYTIHWVRQAPGQRL
EWIGGINPNNGIPNYNQKFKGRVTITVDTSASTAYMELSSLRSEDTAVYYCARRRIAYGYDEGH
AMDYWGQGTLLTVSSGGGSDVQLVQSGAEVKKPGASVKVSCKASGYTFTRYTMHWVRQAPGQG
LEWIGYINPSRGYTNVADSVKGRFTITTDKSTSTAYMELSSLRSEDTATYYCARYYDDHYCLDY
WGQGTITVTVSSGEGTSTGSGGSGGGGADDIVLTQSPATLSLSPGERATLSCRASQSVSYMNWY
QQKPGKAPKRWIYDTSKVASGVPARFSGSGSGTDYSLTINSLEAEDAATYYCQQWSSNPLTFGG
GTKVEIKHHHHHHHHHRRKRKSGATNFSLLKQAGDVEENPGPMSELIKENMHMKLYMEGTVNN
HHFKCTSEGEKPYEGTQTMKIKVVEGGPLPFAFDILATSFMYGSKAFINHTQGIPTDFKQSF
EGFTWERITTYEDGGVLTATQDTSFQNGCIIYNVKINGVNFPSNGPVMQKKTRGWEANTEM
LYPADGGLRGHSQMAKLKLVGGGYLHCSFKTTRYRSKKPAKNLKMFGFHFVDHRLERIKEADKET
YVEQHEMAVAKYCDLPSKLGHR

FAP BiTE-P2A-RFP cassette DNA:

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CACGAGATGGCTGTGGCCAAGTACTGCGACCTCCCTAGCAAACCTGGGGCACAGATGA

Appendix C: Publications

1. Freedman JD, Hagel J, Scott EM, Psallidas I, Gupta A, Spiers L, Miller P, Kanellakis N, Ashfield R, Fisher KD, Duffy MR, Seymour LW (2017) Oncolytic adenovirus expressing bispecific antibody targets T-cell cytotoxicity in cancer biopsies. *EMBO Molecular Medicine* **9**: 1067-1087
2. Scott EM, Duffy MR, Freedman JD, Fisher KD, Seymour LW (2017) Solid Tumor Immunotherapy with T Cell Engager-Armed Oncolytic Viruses. *Macromolecular Bioscience*: 1700187-n/a
3. A second primary paper titled 'Targeting T cells to cancer associated fibroblasts using an oncolytic virus expressing a FAP specific T cell engager' is to be submitted for publication.

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