Macrophage delivered oncolytic virotherapy in triple negative breast cancer

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Oncology and Metabolism

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I, Amy Kwan, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not previously been presented for an award at this, or any other, university.

Work towards this thesis was conducted during the COVID-19 pandemic.

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Table of Contents

List of figures and tables

The list below relates to figures and tables which are not contained within publications.

Figures

Tables

Abbreviations list

AML	Acute myeloid leukaemia
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BC	Breast Cancer
BRCA1	Breast Cancer gene 1
BMDM	Bone marrow derived macrophage
CAF	Cancer associated fibroblast
CAR	Chimeric antigen receptor
CCL2	C-C motif chemokine ligand 2
CD	cluster of differentiation
CDK4/6	cyclin-dependent kinases 4 and 6
CI	Confidence interval
CL	Clodronate liposomes
CNS	Central nervous system
CSFR1	Colony-Stimulating Factor-1 Receptor
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
CXCL13	C-X-C Motif Chemokine 13
DAPI	4’6’-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ER	Oestrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HER2	human epidermal growth factor receptor 2
HMGB1	High mobility group box 1
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cells
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IT	Intratumoural
IV	Intravenous
IVIS	In Vivo Imaging System
LAG3	Lymphocyte-activation gene 3
MDM	Monocyte derived macrophages
MDSC	Myeloid derived stem cells
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MRI	Magnetic resonance imaging
NICE	National Institute for Clinical Excellence
NK	Natural Killer
NSCLC	Non-small cell lung cancer

OCT	Optimal cutting temperature
ORR	Overall response rate
OS	Overall survival
OV	Oncolytic virus
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed cell death ligand 1
PDO	Patient derived organoid
PDX	Patient derived xenograft
PFA	Paraformaldehyde
PFS	Progression free survival
PFU	Plaque forming unit
PPP2R2B	Protein phosphatase 2 regulatory subunit Beta
RMPI	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
TAM	Tumour associated macrophages
TILS	Tumor-infiltrating lymphocytes
TME	Tumour microenvironment
TNBC	Triple negative breast cancer
VEGFR	Vascular endothelial growth factor receptor

Summary

Background:

Triple negative breast cancer (TNBC) is a high cause of morbidity and mortality. Novel treatments for TNBC are needed to extend the overall survival of patients with metastatic TNBC where the prognosis remains poor. A high density of tumour associated lymphocytes and macrophages in TNBC is the foundation for exploration of immunotherapy-based treatments such as oncolytic virotherapy. Oncolytic virotherapy initiates immune cell activation against cancer cells through direct cell lysis and the induction of immunogenic cell death. The overarching hypothesis of this thesis is to assess the suitability of an HSV-1 derived virus, HSV1716, in TNBC and whether the delivery of HSV1716 can be enhanced through packaging viral particles within macrophages prior to administration.

Methods

Firstly, suitable metastatic TNBC models were identified. Next, primary and metastatic immunocompetent models of TNBC were used to assess the efficacy of HSV1716 with a focus on immune cell activation. Finally, macrophage delivery oncolytic virotherapy was assessed in several metastatic immunocompetent in models.

Results

A TNBC model using the 4T1 cell line in BALB/c mice which allowed for immune cell analyses and captured the aggressiveness of TNBC was identified. HSV1716 was found to be efficacious in controlling tumour burden in primary and metastatic models and immune cell activation was confirmed. Macrophages were found to be permissive to HSV1716 and proliferating cell nuclear antigen was found to support viral replication. Macrophage delivery was found to enhance the efficacy compared to naked virus alone.

Conclusion

Oncolytic Virotherapy is a novel branch on immuno-oncology that has the potential to provide new treatment options for cancer patients. This PhD describes experiences with oncolytic virotherapy using intravenous HSV1716 in several *in vivo* TNBC models and forms the foundation for discussion about the feasibility of clinical trials within this area.

Format of thesis

This thesis has been written in a publication format and includes 2 original papers, with supplementary material, and 3 reviews within the main text. Papers are highlighted in the contents page, text and contain a page border to differentiate these from the main text.

This PhD was undertaken as a part time candidate alongside clinical work and commenced in 2016 where the mainstay of treatment for triple negative breast cancer (TNBC) was limited to chemotherapy. However, oncology is a continually advancing field and the management of TNBC has significantly changed since the commencement of this PhD. A notable change is the inclusion of immune checkpoint inhibitors in combination with chemotherapy in patients with TNBC. Despite this, TNBC still accounts for the highest mortality of all breast cancer subtypes. The clinical picture of TNBC, with a high incidence of systemic metastases, brain metastases in particular, remains unchanged and research into this field is as pertinent today as it was 8 years go. Chapter 1 consists of rational and scientific foundations leading to the conception and development of this project to place the PhD in its historical context. The method and materials presented in Chapter 2 are supplements to the material and methods described in the published original work. Result chapters 3, 4 and 5 contain published work with all figures and supplementary figures included within this document. The PhD concludes with a discussion about the impact of this work and its limitations in the field of TNBC, oncolytic virotherapy and the future role proliferating cell nuclear antigen (PCNA), an antigen found to be over expressed with macrophages infected with HSV1716.

Publications

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Chapter 1

1 Background

1.1 Breast cancer

Breast cancer is the most common cancer in the UK. It accounts for over 15% of all new cancer diagnoses and is the fourth most common cause of cancer related deaths between 2017-2019 (1). Although it is more common in older women, it is increasing in incidence in premenopausal women ((2). In this population, it is more common to see aggressive features of breast cancer such as lack of expression of hormone receptors, increased expression of human epidermal growth factor receptor 2 (HER2) receptors and higher stage at presentation (3). Over the last 30 years, the incidence of breast cancer has risen by a quarter (24%) in females although more recent predictions have suggested a rise of around 1% between 2023-2025 (1).

Clinically, breast cancer is diagnosed through mammogram screening or through the rapid access breast clinic after the palpation of an unexpected breast mass. Patients undergo a triple assessment which includes clinical examination, imaging and histological confirmation. Clinically, breast cancers are classified based on disease spread, which is helpful to guide the suitability for surgical resection, however, does not inform whether a patient will respond to systemic therapy.

Histologically, breast cancer is found to express several biomarkers including the oestrogen receptor (ER) and HER2 receptor which broadly allows stratification of treatment and can be used to guide prognosis. Knowledge about breast cancer subtypes has significantly changed over the last 40 years and stratification of the subtypes by biomarkers have helped to identify subgroups of patients suitable for targetable therapies against the ER and the HER2 receptors. These treatments have significantly altered the course of ER+ and HER2+ cancers in both the early and metastatic setting. In particular, ER positive breast cancer has been

transformed with the use of hormone treatments, such as aromatase inhibitors like anastrozole, and CDK4/6 inhibitors. HER2+ breast cancers a treatment challenge 20 years ago now have a number of treatment options which specifically target the HER2 receptor.

However, around 15-20% of breast cancer patients do not have up-regulation of the ER or HER2 receptor and these are classified as triple negative breast cancer (TNBC) (4).This particular group of patients, poses a clinical challenge to treat as tumours are highly aggressive, leading to high mortality, display different innate biology therefore and they do not possess an obvious targetable receptor.

1.2 TNBC

TNBC represents a heterogeneous subtype of breast cancer which poses a unique clinical challenge (5). They are often seen in women under 40 (6), are more common in women with Afro Caribbean ancestry (7) and hereditary breast cancer (8). Clinically TNBCs can present localised to the breast and local lymph nodes (early disease), or with distant metastases (advanced disease). In the early setting, despite optimal surgical and radiotherapy treatment, they have high rates of tumour recurrence (30-40% at 5 years following resection (9)) and decreased overall survival when compared to other breast cancer subtypes (10). In advanced disease metastases are common to the lungs, liver, brain and bone (11). At the commencement of this PhD, treatment of TNBCs was limited to cytotoxic chemotherapies where they were often treated with combination chemotherapy with an anthracycline and/or taxane backbone, in the early setting (11–13)and single agent chemotherapy on recurrence (**Table 1**). Hormone therapy and HER2 targeted therapy do not work for this breast cancer subtype and is one of the reasons for the high incidence of recurrence. Advances in the understanding of the molecular features of TNBC has led to several novel agents being developed with varying success rates including PARP and immune checkpoint inhibitors (**Table 2**). Although these are important

advances that are changing the progression of TNBC, despite these more recent advances, the overall survival of patients with metastatic TNBC remains low, with an average overall survival of less than 2 years (14,15) and therefore novel treatments for this breast cancer subtypes remains an area of unmet need.

TNBC has a distinct histological and clinical picture in comparison to other breast cancer subtypes. It is a notoriously aggressive and infiltrating immune cell rich disease where the development of visceral and brain metastases is more common. There is a niche for a therapy that has a fast onset on action with tolerable toxicity and for this be targeted to conventionally difficult to treat metastases such as brain metastases. One of the landmark discoveries for the treatment of some solid cancers are the development of immunotherapies, such as checkpoint inhibitors. However, checkpoint inhibitors can take months to have an effect, with studies describing pseudo-progression (apparent enlargement of tumours on imaging that is due to immune cell engorgement rather than cancer growth) if scans are done too early(16). In addition, breast cancer it has been more challenging to effectively harness the immune system with response rates less than 20% with single agent checkpoint inhibitors (17). The following sections describe a review of the literature surrounding subtypes of breast cancer and immunotherapy directed at TNBC. This chapter concludes with a review article about oncolytic virotherapy and the barriers to this novel treatment which lays the foundations for the hypothesis of this PhD which explores whether a novel immunotherapy, oncolytic virotherapy, can cause tumour cell lysis and immunological medicated effects against TNBC.

Table 1: *Treatment options for early and advanced TNBC at the time of commencement of the PhD.*

Setting	**Line of treatment**	**Treatment options**	**Example of regimen**
Early breast cancer	Neoadjuvant	1) Taxane and anthracycline based combination chemotherapy (12,13) 2) Platinum based combination chemotherapy (18,19)	T-FEC
	Adjuvant	1) Taxane and anthracycline based combination chemotherapy (12,13) 2) Platinum based combination chemotherapy	FEC-T
Advanced	1st line	Single agent chemotherapy which patient has not received so far (20)	Capecitabine, Oral Vinorelbine, Eribulin, weekly Taxol
	2nd line	Single agent chemotherapy which patient has not received so far (20)	Capecitabine, Oral Vinorelbine, Eribulin, weekly Taxol
	3rd line	Single agent chemotherapy which patient has not received so far (20)	Capecitabine, Oral Vinorelbine, Eribulin, weekly Taxol
Site specific metastasis	Brain metastases	Dependant on size, number and location of lesion(s) ((NGC), 2011)	Radiotherapy (stereotactic or WRBT) Surgical resection
	Bone metastases	Radiotherapy + supportive treatment with anti-reabsorptive agents (21)	Bisphosphonates (e.g. zoledronic acid) Denosumab

Table 2: *Novel NICE approved treatments for TNBC since the start of this PhD*

Molecular target	Subtype	Treatment	Clinical outcomes and use
PARP inhibition	BRCA1 OR 2 mutation /BRCA-ness	Olaparib	NICE approved May 2023 for adjuvant treatment of HER2-negative high-risk early breast cancer that has been treated with neoadjuvant or adjuvant chemotherapy (22). Reported 3yr disease free survival of 85.9% in the olaparib group and 77.1% in the placebo group (23).
		Talazoparib	NICE approved Feb 2024 for BRCA 1/2 mutated HER2-negative locally advanced or metastatic breast cancer after prior chemotherapy (24). Improved PFS (8.6 months vs. 5.6 months (25)) although no change in OS (26).
Check point inhibition	All subtypes	Pembrolizumab	NICE approved December 2022 for use in the neoadjuvant or adjuvant setting in early or locally advanced TNBC (27). At 36 months, event-free survival was 84.5% in the pembrolizumab–chemotherapy group versus 76.8% in the placebo–chemotherapy group (28). NICE approved June 2022 for use with chemotherapy in previously untreated unresectable or metastatic TNBC (29)Reported median OS of 23.0 months in the pembrolizumab–chemotherapy group versus 16.1 months in the placebo–chemotherapy group (14).
		Atezolizumab	NICE approved for use with nab-paclitaxel for treating unresectable, locally advanced or metastatic TNBC whose tumours express PD-L1 at a level of greater than 1% (30). Reported median OS of 21·0 months with addition atezolizumab and 18·7 months with nab-paclitaxel alone (31).
antibody–drug conjugate targeting the human trophoblast cell-surface antigen 2 (Trop-2)	All subtypes	Sacituzumab govitecan	NICE approved for use in unresectable TNBC after 2 or more systemic therapies, at least 1 of which was for advanced disease (32). Reported median OS of 12.1 months sacituzumab govitecan versus 6.7 months with standard chemotherapy (33).

1.3 The Immune environment of TNBC

Although TNBC has chemotherapeutic options which prove efficacious in the primary setting, early recurrence of disease is common in patients who have not achieved a histological complete response after these treatments (34). The tumour microenvironment (TME) of TNBC has some unique features which open the potential for targeting with immunotherapeutic agents, notably the finding of a high proportion of lymphocyte infiltration within the tumour microenvironment, increased PD-L1 expression in comparison to other breast tumour types and an increase in neoantigen expression due to more genomic instability.

Tumour infiltrating lymphocytes (TILs) are immune cells which are key players in directing an anti-tumour response. It has been reported that patients with early TNBC whose TME contains high numbers of TILs have an improved responses to treatments and better overall survival outcomes. A large, pooled analysis by the German Breast Cancer Group (35) analysed the histology of 906 women with primary TNBC treated with neoadjuvant chemotherapy and classified TILs proportion into low (<10%), intermediate (11-59%) and high (60%). Within these groups, those with high TILs achieved a 50% complete pathological response to neoadjuvant chemotherapy compared to only 30% of patients with low or intermediate TILs. This has led to TIL numbers being put forward as useful surrogate markers to response to treatments in patients with TNBC. This has been more recently confirmed in a few studies with a large retrospective analysis of 1966 participants with early-stage TNBC treated with locoregional therapy where survival rates were close to 90% for patients with a high TIL level of 50% or greater, compared with 72% for patients with a low TIL level of less than 30% at 5-year follow-up (36). Others have confirmed the significance of TILs as a prognostic biomarker (37,38) however this has not reached routine clinical practice.

There are also differences in the mutation make up of TNBC. Bioinformatics studies have shown that the expression of PPP2R2B, a phosphatase and tumour suppressor, is

downregulated in TNBC. Downregulation of this gene is correlated with a shorter survival time. Interestingly the PPP2R2B expression is positively associated with CD8+T cells, Th1 CD4+ cells and M1 macrophages which imply that TNBC may present with a more immunosuppressive tumour microenvironment (39) which contrasts the thought that the mere presence high TILs imply TNBC is a suitable niche for immunotherapy use.

In addition, certain genetic mutations found particularly in inherited TNBC (e.g. BRCA 1 or BRCA 2) predispose to genomic instability which make the cells more suspectable to undergoing cell death. Should this process occur in an immunogenic way, the neo- antigen release could activate the immune system to respond in a cytotoxic manner. This is known as immunogenic cell death. Interestingly however, with conventional chemotherapy treatments no differences in survival were found between those with or without BRCA mutation in the young breast cancer group (40). Perhaps the inherent immunosuppressive microenvironment of breast cancer, a disease which is said to immunogenically cold, leads to increase immune evasion through T cell exhaustion and M2 like macrophages and therefore one can speculate that neoantigen release alone does not stimulate the immune response. T cell exhaustion, a term given to “burn out” and poorly functioning cytotoxic T cells, is characterised classically by high PD-L1 and LAG3 levels, is found to be present in T cells with high expression of CXCL13 on T cells and MHC1 on tumour cells(41). Targeting the PD-L1 or LAG3 pathways may reverse T cell exhaustion and immune evasion. The most well studied pathway of immune evasion encompasses immune checkpoint expression through the PD-1/PD-L1 pathway. This has led to the investigation of PD-1/PD-L1 checkpoint inhibitors within this setting and PD-L1 expression is often used as a biomarker to predict response. However, it is noted that when single-cell transcriptomics combined with imaging mass cytometry was used to systematically study immune environments in breast cancers they found that PD-L1 expression did not correlation with cytotoxic “inflammatory” immune environment (41). In this case high PD1 expression was the most predictive factor.

Aside from the intrinsic immune evasive ability of the TNBC cancer cell, the TME plays a crucial role in the activation of the immune system. As discussed above, the TME in TNBC tends to have a high number of infiltrating lymphocytes which would lend towards improved ability to be recognised by the immune system, however the balance of co-existing cells such as tumour associated macrophages (TAMs), cancer associated fibroblasts and cancer associated adipose can lead to immunosuppression the cytotoxic T cells. TAMs are derived from embryonic and bone marrow monocytes which are recruited by inflammatory factors released from tumour cells and differentiate into different TAMs phenotypes; M1 and M2. M1 like TAM are stimulated by IFN gamma and lipopolysaccharide and classical express high levels of proinflammatory cytokines and nitric oxide. They are associated with a pro-inflammatory and cytotoxic tumour microenvironment. Conversely, M2 like TAMs are stimulated by exposure to cytokine such as IL-4, IL-10, or IL-13 and promote a Th2 cytokine response resulting in immunoregulation, tumour promotion and angiogenesis. Studies of patient derived tumours have shown an increase in tumour progression and metastases and a decrease in overall survival is associated with increasing M2 like (CD68+CD163+) TAMs (42,43). This is particularly marked if it is also associated with low CD4+CD8+CD20+ TILs suggesting a key interaction between macrophages and lymphocytes in modulating the immune response (44,45). TAMs are very abundant in breast tumour and can constitute up to 50% of tumour bulk. They are often CD163+ M2 polarised macrophages which released IL-10, a cytokine which has been described as restricting the activity of CD8+ T lymphocytes. The resultant effect is increase in cancer proliferation and spread compounded by the maintenance of an immunosuppressive TME. Furthermore, they have been shown to influence the effects of a range of anti-cancer treatments including immunotherapies, chemotherapies and radiotherapy. The interaction of radiotherapy and macrophages is interesting. Radiotherapy has been shown to increase the expression of CCL2 by cancer cells. CCL2 promotes macrophages to a more immunosuppressive M2 phenotype. In models of pancreatic ductal adenocarcinoma, a

particular radiotherapy insensitive tumour, radiotherapy treatments have been shown to increase CCL2 and cause and increase of immunosuppressive macrophages within the TME (46). However, in the more radiotherapy sensitive rectal adenocarcinoma tumours, an alternative radiotherapy regime (short course, neoadjuvant radiotherapy) was found to lead to a shift in TAM polarity towards an M1-like pro-inflammatory phenotype in *ex vivo* tumour samples (47). With chemotherapy, a redistribution of macrophages is seen with an increase presence of M2 like macrophages in the perivascular areas which has been correlated with an increased risk of tumour relapse (48).

The existence of macrophages within the human tumour microenvironment is much more complex than the existence of 2 macrophage subtypes with some groups suggesting up to 9 different archetypes of macrophages, co-existing in synergy depending on the status of cancer development (49,50). The plasticity of macrophages is recognised with shifts between subtypes observed depending on environmental conditions. An understanding of macrophages and their role in cancer growth development and treatment, may help direct these unique cells to enhance cancer outcomes. Indeed, a couple of main strategies have been investigated. These broadly either reduce the number of macrophages within the TME or the reprogramme macrophages to change their functionality into a more cytotoxic phenotype. The number of TAMs within the TME can be reduced either through depletion of pre-existing TAMs or prevention of macrophage recruitment. The most well documented way of eliminating TAMs within the TME is to decrease their survival through the blockage of CSFR1. This not only causes decreases in macrophage survival, but it also prevents the differentiations of monocytes into macrophages (51) and increases reprogrammes the phenotype of macrophages from an M1 like phenotype to an M2 like phenotype (52). It may also improve the efficacy of other agents such as PD-L1 inhibitors.

1.4 Immuno-modulatory therapy

The immune system is important for clearing “non-host” material, including microbes and cancer cellas. There is a complex interplay between malignant cells and immune cells which can result in both tumour growth or tumour suppression. Immune system evasion is facilitated by tumour associated macrophages and CD4+T regulatory cells. Additionally, the cancer cell itself can upregulate receptors such as PD-L1 which mask the cancer from the immune response. This immune evasion is recognised as one of the hallmarks of cancer cells (53). Although immunotherapy has been a popular concept for several decades, it has only been within the last 10 years that this has entered routine clinical use. Currently there are an increasing number of ways that the immune system can be modulated to tip the balance of the immune system in favour of cytotoxicity. These are summarised in **Figure 1.** Checkpoint blockade and CAR-T cells are currently in clinical use in several solid and haematological malignancies, including breast cancer, although immune activation is not guaranteed and a deeper understanding in to how to prime the immune system is needed to optimise response.

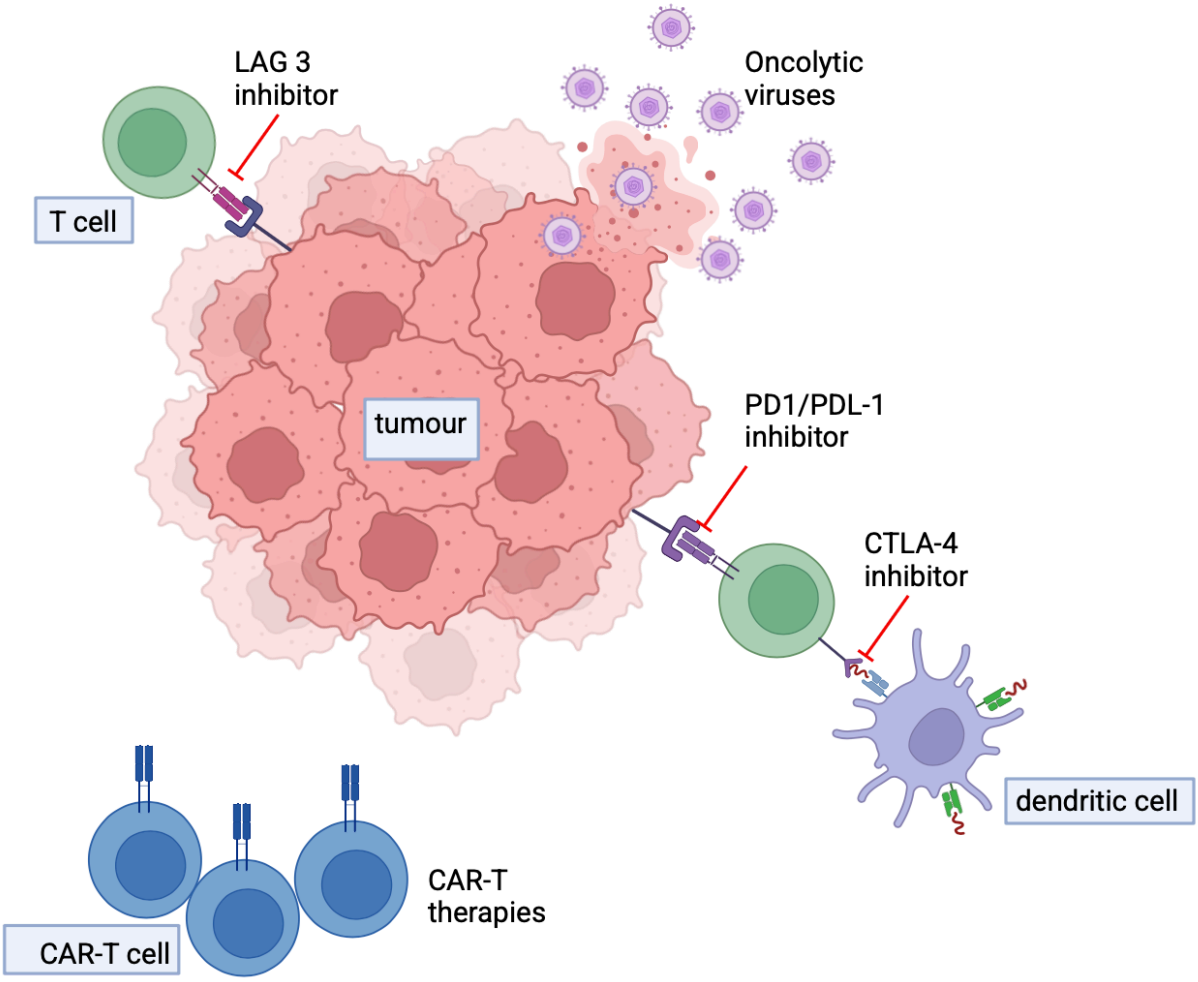

Figure 1: Immunological targets against cancer. *A schematic representation of the immune system modulators which are currently available to target cancer cells. These are checkpoint inhibitors, which act through blockade of either CTLA-4, PD-1/PD-L1, LAG3, infusion of CAR-T cells or oncolytic virotherapy..*

1.5 Immunotherapy and TNBC

Immunomodulatory therapy has been traditionally applied to cancers, which have immunogenic potential (e.g. melanomas). Breast cancer is not classically immunogenic; however, studies have found 44% of primary breast cancers have a high percentage (>10%) of stromal tumour infiltrating lymphocytes with this rising to 78% of TNBC (54,55). This makes TNBC an attractive target for checkpoint inhibitor which act on T cell exhaustion receptors such as PD-L1 and LAG-3. However, within the study by Althobiti, higher levels of stromal TILs were found to be associated with poor prognostic factors like advanced stage at diagnosis and lymph node positivity and were associated with high mortality during recurrent disease in all breast cancer subtypes (54). Although the significance of high level of TILs is not fully understood, higher levels of TILs have been associated with pathological complete response and increased overall survival. This is supported by a study by Wang et al where TNBC patients with high TILs in their primary breast cancer had both an improved in overall survival and that the increased number of TILs (particularly CB8+ T cells) predicted response to chemotherapy and improved survival(55). Perhaps this is due to immunogenic cell death that can be stimulated by some chemotherapies, such as anthracyclines, which are commonly used in early breast cancer treatment regimes. Together, these findings support the use of immunomodulatory therapies in TNBC.

1.5.1 Checkpoint blockade

Immune cells are constantly involved in immune tumour surveillance where on presentation and detection of tumour-associated antigens, they are primed towards a cytotoxic response. This involves a complex signalling pathway between many different immune cells. To prevent over activation of the immune system, a number of regulatory checkpoints exist. Understanding the mechanism of action of two such checkpoints has led to the development of

a handful of agents which block the inhibitory signal of these regulatory checkpoints and consequently release the breaks of the cytotoxic drive. These agents are divided broadly into three subgroups: the CTLA-4 inhibitors, the PD-1 inhibitors and the PD-L1 inhibitors. CTLA-4 is a receptor ligand that is found to be expressed on T lymphocytes (56). It binds selectively to CD80 and CD86 (also known as B7-1 and B7-2). On binding to these, it exerts an inhibitory response for T cell recognition and activation in response to a cancer associated antigen. Therefore, inhibition of the CTLA-4 – B7 interaction through use of a monoclonal antibody (mAb) to CTLA-4 causes increased cytotoxic T cell response (57). The most clinically evaluated CTLA-4 mAb is ipilimumab. This is currently available for the treatment of advanced melanomas with response rates of around 20% (58). Increased clinical experience has unveiled high toxicity associated with ipilimumab, particularly with immune related side effects such as colitis, which may be life threatening (59,60).

The PD-1 receptor is expressed on activated T cells and its ligand (PD-L1) is expressed on antigen presenting cells. Interaction between PD-1 and PD-L1 results in an inhibitory signal that suppresses T cell activation and cytotoxicity. Cancer cells overexpress PD-L1 which results in decreased immune recognition of these cells by activated T cells. Interruption of the binding of PD-1 to PD-L1 unveils the cancer to the immune system and monoclonal antibodies to both PD-1 or PD-L1 are currently standard of care in most tumour types. Within, breast cancer the evidence is strongest for three agents: pembrolizumab, atezolizumab and avelumab. Pembrolizumab is a humanized monoclonal IgG4 kappa antibody to the PD-1 receptor and blocks the interaction with PD-L1 and PD-L2 on tumour cells. It now sits as one of the standard treatment options for both early and metastatic breast cancer based on the results of 2 significant KEYNOTE trials. In the KEYNOTE 355 trial (61), 847 patients with advanced untreated TNBC were randomized to receive either chemotherapy plus placebo or chemotherapy plus pembrolizumab. In final interim analysis after 44.1 months of follow up, patients stratified by PD-L1 with a high combined PD-L1 score of over 10 had a significant

increase in overall survival from 16.1 to 23 months (P=0.0185). Additionally, the use of neoadjuvant pembrolizumab in early breast cancer has become a new standard of care in the UK following the KEYNOTE 522 trial, which revealed a decreased in cancer related event free survival in patients treated with combination neoadjuvant chemotherapy and pembrolizumab compared to chemotherapy verses placebo (62).

Atezolizumab is a humanized monoclonal antibody immune checkpoint inhibitor that selectively binds to PD-L1. It was initially assessed in combination with paclitaxel for all breast cancers which included 45% of patients had TNBC. The results from this shows promise in the phase II trial with a median overall survival of 21.3 months with atezolizumab plus nab-paclitaxel and 17.6 months with placebo plus nab-paclitaxel (63). It was this that initially led to early FDA approval for the use, however use of this was withdrawn a couple years later after the phase 3 trial IMpassion131 revealed that adding atezolizumab to paclitaxel did not improve PFS in the PD-L1–positive population with a PFS of 6.0 months for patients who received atezolizumab and paclitaxel compared with 5.7 months for patients who received placebo and paclitaxel (64). More recently Atezolizumab has been trialled in a phase 2 trial in combination with carboplatin for patients with metastatic TNBC (TBCRC 043)(65). Here, PFS was increased by from 2.2 to 4.1 months which is a similar benefit to the results from KEYNOTE 355. Interestingly, patients with high TILs, high mutation burden and prior chemotherapy received greater benefit to the addition of Atezolizumab to carboplatin and those who were luminal androgen receptor positive TNBC fared worse. The phase 3 trial is currently recruiting, and it will be of interest to see how this changes the landscape of breast cancer management in the future.

Avelumab, another monoclonal IgG1 antibody directed against PD-L1, is currently in the early phase of clinical investigation for use in breast cancer patients. At present there are reported phase I and phase II trials of Avelumab alone and in combination with other agents which shown promise (66–68). Of interest the combination of Avelumab and a PARP inhibitor,

talazoparib, has been reported in a phase 1b and 2 non-randomized controlled trial, in patients with advanced solid tumours stratified by tumour type. The patients with breast cancer the ORR was 18.2% (95% CI, 5.2%-40.3%) in patients with TNBC; 34.8% (95% CI, 16.4%-57.3%) in patients with ER-positive, HER2 negative breast cancer; and 63.6% (95% CI, 30.8%-89.1%) in patients with platinum-sensitive, BRCA1/2 mutated breast cancer (66). These results may suggest a niche for this combination of treatment and larger phase III trials would be needed to help define this. Avelumab has also just completed trials in combination with radium 223 to specifically target patients with predominant bony metastatic disease (Trial ID: EUCTR2018-003620-37-GB).

1.5.2 Adoptive T cell therapy using T-CAR cells

T cell response is normally triggered against a cell if a peptide antigen is presented on this target cell in association with major histocompatibility complex (MHC) proteins. The down regulation of MHC by tumour cells is a known mechanisms used to evade tumour immune surveillance (69) and therefore T cell effector function may not be appropriately triggered against tumour cells. T-CAR cells are T cells which have been reprogrammed to express an artificially engineered receptor known as the chimeric antigen receptor CAR). These receptors allow the recognition of protein antigens, independent of MHC proteins, on the target cell. From a practical level the basic clinical protocol for adoptive cell transfer of T-CAR cells involves isolation of T lymphocytes from the affected patients, transducing CAR genes using retroviruses and then clonal expansion of the genetically engineered T-CAR cells before re-infusion, often with IL-2 support, into the patient (70). Patients are often pre-conditioned with chemotherapy before re-infusion. The structure of the CAR used has been refined over the past 15 years and currently third generation CARs are in development. These contain a costimulatory CD28 domain, and another costimulatory domain fused with an activation

domain. This refinement results in a similar cytotoxic effect to previous generations of CARs with increased levels of cytokine production and T cell proliferation (70). Clinical trials of T-CAR cells have been focused on haematological malignancies; however, a number of papers have reported a potential effect in *in vivo* models of solid malignancies. Once such paper describes the use of T-CAR cells which target folate receptor-alpha expression in TNBC. In this study immunodeficient mice bearing MDA-MB-231 tumour xenografts were treated with human derived T cells engineered to express FR-alpha CAR (71). This showed a modest effect and the potential problem with this, and many other *in vivo* T-CAR trials, is the effects to not translate well to immune competent mouse models using murine derived T-CAR cells. Another challenge in T-CAR therapy is the potential for off target effects. Tumour specific antigens are rare, and many antigens are also found in normal tissue albeit at significantly lower levels physiologically (70). T-CAR cells are highly specific and low levels of antigen can also trigger a powerful immune response. In the clinical setting, this could be devastating resulting in a cytokine storm and patient mortality (72,73).

However, this novel treatment has started to translate to meaning clinical use with a recent study in T-CAR cells (74) describing the outcomes of 42 patients with treatment refractory metastatic breast cancer who underwent surgical resection of a metastatic lesion(s), isolation of TIL cultures, identification of exomic nonsynonymous tumour mutations, and immunologic screening for neoantigen reactivity. Following this, 13 of the 42 patients were found to be suitable for T cell transfer and 6 patients were recruited to a phase II pilot trial of adoptive cell transfer of selected neoantigen-reactive TILs, with a short course of pembrolizumab. Of these, objective tumour regression was noted in three patients, including one complete response (over 5.5 years) and partial response in 2 (6 and 10 months). The time involved and cost of screening for such patients is high and further refinement of CAR T therapy is needed before it reaches mainstream adoption.

1.5.3 Vaccines

Cancer vaccines, consisting of tumour antigens of various forms, have been postulated to both treat cancers in situ or to prevent recurrence. Breast cancer vaccines can be categorized into the following groups: peptide vaccines, nucleic acid (DNA/RNA) vaccines and cell-based vaccines. With dendritic cell-based vaccines tumour antigen is phagocytosed *in vitro* and then then injected into the host. They are an area of growing preclinical interest. One of these is the vaccine Nelipepimut-S (NeuVax) which is comprised of a human epidermal growth factor receptor 2 (HER2) peptide and granulocyte-macrophage colony-stimulating factor (GM-CSF) (75). Despite expressing a HER2 antigen, this vaccine demonstrates activity in all breast cancer subtypes in the phase 2 trial which led to much excitement, however the subsequent phase 3 trial of NeuVax (PRESENT) was terminated early due to futility due to interim analyses showing a high proportion of image detected recurrence in the NeuVax arm (76). Interestingly NeuVax has also been trialled with trastuzumab in the phase 2 setting inpatients who are HER2 low or negative (77). In this study patients were randomised to receive adjuvant trastuzumab for 12 months in combination with NeuVax (vaccine group) or GM-CSF (placebo group) every 3 weeks for 6 doses, starting with the third dose of trastuzumab. After 26.1 months of follow up those with TNBC were found to have a significantly improved clinical response in comparison to the ER+ group. Post hoc analysis reveal that this was further delineated where biomarkers of response included prior neoadjuvant chemotherapy, HLA-A24 positivity, age over 51 years and stage 1-2 disease(78–80). These features support the consideration of a phase 3 trials in TNBC of NeuVax and trastuzumab in the adjuvant treatment of TNBC, although how given the plethora of adjuvant chemotherapy and targeted therapies how these fits into the current treatment pathway is unclear.

Another vaccine which has completed a phase 2 trial in patients with all subtypes of metastatic breast cancer is called PANVAC. This pox-virus derived vaccine revealed some

early sign of potential synergy when combined with docetaxel with a median PFS of 7.9 months in the combination arm vs 3.9 months with docetaxel alone (81). Reported toxicities were all similar in both groups suggesting this was mainly docetaxel related however there was a significant difference in oedema (grade 1) and infusion site reactions (grade 1-2) in the combination arm. Limitations around this study relate mainly to size as this was a small phase 1-2 trial involving only 45 patients and larger phase 3 trial would be needed to provide conclusive evidence. Within the design of this, it would be helpful know if patients were immunosuppressed or had received prior immunotherapy treatments as this may influence response to a cancer vaccine.

1.5.4 Oncolytic viruses

An oncolytic virus is one that has the ability, either intrinsically but more commonly genetically altered, to preferentially divide and replicate in cancer cells rather than non-cancer cells. Although the mechanism of how individual viruses deliver their cytotoxic effect differs, the broad effect of the virus is to directly lyse tumour cells and generate immunogenic cell death, thus presenting tumour antigens, which will be recognised and targetable by the host's own immune system. Immunogenic cell death is the term given to the release of a stream of pro-inflammatory markers including calreticulin, heat shock proteins, ATP and HMGB1. This environment is particularly appealing to dendritic cells that phagocytose tumour associated antigens and present these to T cells.

The number of oncolytic viruses described in the literature is vast. A recent systematic review revealed 1450 descriptions of oncolytic viruses within which 47 different oncolytic viruses were described (82). The most studied of these are adenoviruses, reoviruses and herpes simplex viruses. These have been trialled in several tumour types with preclinical and clinical efficacy. The leading tumour subtype to be treated with virotherapy is advanced melanoma

with the first FDA approved oncolytic virus (T-VEC) in 2015. T-VEC is a modified HSV that carries the transgene for GM-CSF. The landmark phase 3 virotherapy trial (OPTIM) described increased response and overall survival in patients treated with this in comparison with GM-CSF alone (83).This virus, and many others, are given intratumorally due to the challenges of systemic therapy although other options are possible e.g. intrapleural (84). Other areas to refine within this field could include identifying a virus which can be delivered systemically without the need for intratumoural injection or a vector (stability in blood), genetically changing the virus so it hones more specifically to target cancer cells and thus generate an appropriate downstream immune response and trying to enhance response on virotherapy with combination treatments. In breast cancer, the oncolytic virotherapy experience is a growing steadily with several preclinical studies and early phase studies showing promise. Some of these are summarised in **Table 3**.

Table 3: *A selection preclinical studies and clinical trials of oncolytic virotherapy in breast cancer*

Name of virus	**Type of model**	**Other treatments**	**Outcome**	**Ref**
Pre-clinical studies				
Rhabdovirus Maraba-MG1	*in vitro* and *in vivo* using metastatic immunocompetent murine models (EMT6, 4T1 and E0771) n =5 per group	Paclitaxel chemotherapy	Increased viral replication with chemotherapy and increased overall survival with combination compared to either no treatment or single treatment	(85)
HSV G47Δ	*in vitro* using cancer stem cells from primary human breast cancers and SK-BR-3 cells (HER 2 positive).	n/a	G47Δ inhibited the growth of established tumours *in vitro* and *in vivo*	(86)

	n=10 per group			
Name of virus	**Type of model**	**Other treatments**	**Outcome**	**Ref**
Pre-clinical studies				
GLV-1h164 carrying an anti-angiogenic scAb (GLAF-2) given intratumourally	*in vitro* and *in vivo* using immunosuppressed mammary fat pad models (MDA-MB-231) n= 11 per group	n/a	Durable tumour regression in all treated animals compared to PBS treated animals	
OVV-CXCR4-A-mFc	*in vivo* where orthotopic growing 4T1 primaries were treated with systemic virus and CXCR4 antagonist n= 6 per group	CXCR4 antagonist	Inhibition of tumour growth and destruction of tumour vasculature Decrease in tumour free interval and development of metastases	(87)
Oncolytic paramyxoviruses	*in vitro* co culture model (MDA-MB-231-LUC) n= 3 per experiment	Human derived macrophages	Macrophages enhance oncolytic paramyxovirus-mediated tumour-cell killing	(88)
HSV derived NV1066	*in vitro* repeated in triplicate (MDA-MB-231, HCC1806, HCC38, HCC1937, HCC1143) *In vivo* in an MDA-MB-231 model (n= 10 per group)	MEK inhibitor PD98059	Tumour suppression *in vitro* and in vivo with synergistic effect between NV1066 and PD98059	(89)
mJX-594 (JX), a GM-CSF-armed oncolytic vaccinia virus	*MMTV-PyMT* transgenic mouse model (n = 7-11 per group)	PD-1 or CTLA-4 inhibitor	Intratumoural JX is effective alone, but in combination with systemic PD-1 or CTLA-4 inhibitors a further enhanced response is seen	(90)

Clinical trials				
Adenovirus ONYX-015	Phase 1 trial (n=8). Mixed tumours. 2 doses of intravenous virus alongside etanercept	Etanercept	2 metastatic breast cancer patients. Both has PD after treatment. No significant toxicity	(91)
Adenovirus RGD-4C (ICOVIR-7)	Phase 1 (n = 21) mixed tumour type single dose of virus given intratumorally via ultrasound guidance	n/a	3 metastatic breast cancer patients; 1 had a minor response 2 had PD. Grade 2 muscle pain/abdominal pain and fatigue, grade 3 anaemia in 1 of 21 patients	(92)
Adenovirus Ad5–D24– RGD	Phase 1 (n= 13) mixed tumour trial given via intratumoural route	n/a	2 metastatic breast cancer patients; 1 had minor response and 1 had PD. Grade 1-2 toxicities: fatigue, fever and injection site pain.	(93)
Adenovirus Ad5/3-D24-GMCSF (3 treatments over 10 weeks)	Phase 1 trial (n=14) with IT alone or combination of IT and IV arms	n/a	3/14 metastatic patients had SD or PR to the virus. No significant toxicities documented.	(94)
Herpes virus OncoVEXGM-CSF (intra- tumoural)	Phase 1 with a mixture of tumour types including metastatic breast cancer (n=30)	n/a	6/14 metastatic breast cancer patients had stable disease or flatter lesions. Well tolerated toxicity, more marked toxicity if seronegative for HSV	(95)
Herpes virus Talimogene laherparepvec (T-VEC)	Phase 2 trial (n=40). Stage 2-3 TNBC 5 intratumoural injections of T-VEC	Neoadjuvant chemotherapy; paclitaxel, doxorubicin and cyclophosphamide	Surgical clearance improved with RCB0 rate = 45.9% and RCB0–1 = 65% which authors conclude T-VEC may RCB0-1 rates. Similar toxicities between experimental and control arm.	(96)

Name of virus	**Type of model**	**Other treatments**	**Outcome**	**Ref**
Clinical trials				
Herpesvirus HF10	Pilot study (n=6). HSV antibody + Intratumoural injection of nodules	n/a	Patients needed 2 nodules to act as their own control End point: histology response reported as 30-100%. No adverse effects	(97)
Vaccinia virus VVDD	Phase 1 (n=16) Intratumoural injection	n/a	4 metastatic heavily pretreated breast cancer with clinical response described in 2 and virus found in non-injected sites in these. No dose limiting toxicity.	(98)
Reovirus Pelareorep	Phase II (n=74) Intravenous administration at multiple time points	paclitaxel chemotherapy	PFS increase by 0.4 months, OS increased by 7 months. Well tolerated with both arms showing similar toxicities.	(99)
Newcastle disease virus PV701	Phase 1 (n=16) 2 step tolerability study	n/a	2 metastatic breast cancer with 1 showing PD and 1 showing prolonged SD.	(100)

1.6 Oncolytic HSV

HSV-1 is a member of the herpesvirus family and belongs to the alpha-herpesvirus subfamily which are characterised as enveloped, double-stranded DNA viruses that can undergo both lytic and latent lifecycles. It affects over 50% of the adult population in the UK, although its incidence is decreasing (101). It is composed of three major structural elements: a nucleocapsid containing its genome, a lipid bilayer envelope embedded with glycoproteins, and a proteinaceous layer in between (102).

To undergo viral replication, HSV-1 binds to the glycoprotein D receptors (which include CD111 (Nectin 1), HVEM and 3-OS-HS) on the cell surface which permits fusion of the viral envelope to the cell membrane and the capsid is allowed to enter the cell (103). The capsid then migrates to the nucleus and the viral genome enters the nucleus where it undergoes 3 rounds of transcription: immediate early (α-genes), early (β-genes), and late (γ-genes). This is then translated in the cytoplasm where a new capsid is formed and then released out of the cell membrane through process known as budding. The HSV-1 group of viruses are particularly attractive for oncolytic virotherapy; they can infect a range cell types and are relatively safe to give with the most common reported side effects being grade 1-2 fever, myalgia, fatigue and transient liver enzyme derangement (104). Initial concerns of HSV induced encephalitis have not been reported in clinical trials to date and HSV oncolytic virotherapy has the further advantage having a well-known and effective rescue treatment (the antiviral acyclovir) which will reverse severe toxicity.

HSV replication (figure 2) takes place primarily within the nucleus of epithelial cells where viral and cellular factors interact with the 152 kbp HSV-1 genome to regulate viral DNA replication, transcription of viral genes, and viral genome packaging into capsids. HSV contains most of its own DNA replication machinery, which includes an origin binding protein (UL9), a single-stranded DNA binding protein (ICP8), DNA polymerase (UL30), a processivity factor (UL42), and a helicase/primase complex (UL5/UL8/UL52). UL9 initiates viral DNA replication when it binds to the viral origins of replication (oriS and oriL). At this point, ICP8 forms a complex with UL9 and this helps unwind the DNA into single strands by destabilising duplex DNA during the movement of the replication fork(105). This interaction recruits a heterotrimeric complex consisting of UL5 (helicase), UL8 (accessory protein), and UL52 (primase) which is known as the helicase–primase complex which helps further unwind the DNA (106). Recruitment of the viral DNA polymerase UL30 improves replication fidelity through allowing the removal or mismatch nucleotides and slow elongation rate (107). This is

coupled with the processivity factor UL42 which acts in a similar way as PCNA in normal cells to allow for HSV-1 replication. It works independently to PCNA, and, unlike PCNA, it acts as a monomer thus does not form a multimeric ring around the DNA. Interestingly, studies have shown that PCNA is needed for optimal viral replication and PCNA knockdown by siRNA results in reduced viral DNA replication (108). There is some speculation that PCNA may play a role in viral DNA repair during replication and thereby improve the fidelity of viral replication (109).

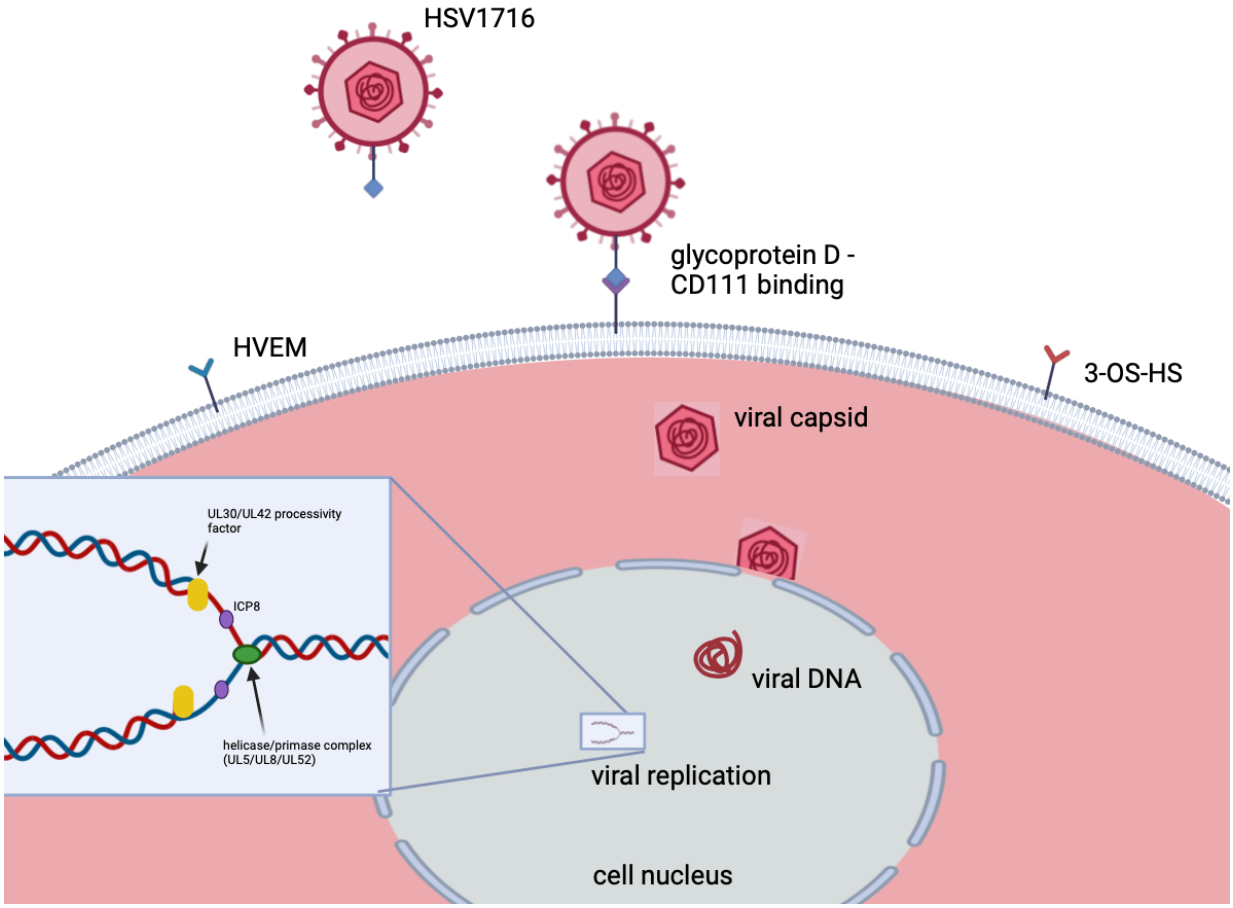

Figure 2: HSV1716 infection and replication. Infection and replication with HSV involves attachment to the cell surface, interaction with a specific entry receptor (glycoprotein D and its 3 potential cell surface receptors has been illustrated here), internalization of the viral capsid, and replication of the viral DNA within the cell nucleus. Viral replication takes place primarily within the nucleus of epithelial cells where HSV own DNA replication machinery leads to the transcription of viral genes and viral genome packaging into capsids which are then released when host cells are lysed or exit via the cell membrane through a process known as budding.

1.7 HSV1716

HSV1716 is an oncolytic virus derived from HSV-1 strain 17 developed by Virttu Biologics. This virus was designed to prevent neurotoxicity and fatal viral encephalitis which was achieved by the deletion of both copies of the neurovirulence gene γ34.5. HSV1716 maintains the capability of replication in tumour cells where ICP34.5 is not required for viral replication, such as cancer cells, whereas it fails to replicate in normal neurons where ICP34.5 is essential for this process(110,111). Interestingly this lack of ICP34.5 may lead to lowered viral replication as studies have shown that ICP34.5 helps facilitate the link between PP1 and eIF2α which thereby facilitates the protein synthesis and viral replication in normal cells (112). Within cancer cells, the higher prevalence of PCNA has been suggested to allow preferential replication (113). It maintains expression of thymidine kinase, targetable by administration of acyclovir and thereby providing a "therapeutic safety net" in case of an unlikely but potential adverse toxicity secondary to excess viral proliferation.

HSV1716 exerts its effect in 3 main mechanisms, firstly it causes direct oncolysis, secondly it may disrupt tumour vasculature and finally it heats up tumour microenvironments which recruits the immune system to the tumour sites.

Direct oncolysis of HSV affects a few cell death pathways including apoptosis and immunogenic cell death. Firstly, apoptosis is activated on viral entry to the cell as it is a host cell defence mechanism that limits viral infection by shutting down the cellular machinery through inhibition of protein synthesis which prevents viral reproduction. HSV1716 has modification to the anti-apoptotic pathway which results in preferential replication in cancer cells possibly through a process known as HSV-1-dependent apoptosis (HDAP) (114). Further to this HSV has been found to induce apoptosis both directly to and affecting up to 10% neighbouring cancer cells in a model of gastric cancer infected with NV1066 (an oncolytic HSV-1 mutant that contains the marker gene for enhanced green fluorescent protein)(115).

Apoptosis is non-immunogenic by nature. That is to say that is allows clearance of cellular material through means that do not alert the immune system of their presence. Immunogenic cell death of cancer cells can be stimulated by several chemotherapeutic agents, e.g. anthracyclines, and this mechanism enhances treatment cytotoxicity. Immunogenic cell death is initiated through a collection of molecules known as damage-associated molecular patterns (DAMPs). The most studied of these are Adenosine triphosphate (ATP), high mobility group box 1 (HMGB1), HSP (heat-shock protein) 70, HSP90 and calreticulin (CRT). Studies with oncolytic HSV in squamous cell cancers have shown an increase in HMGB1 and ATP within the first 24 hours of infection (116,117). Furthermore, in a murine model of HER-2/neu positive breast cancer using the TUBO cell line, it has been shown that the release of DAMPs and the subsequent recruitment of CD8+ T cells in the initial stages of immunogenic virus replication leads to more significant anti-tumour cytotoxicity than the cell lysis caused the viral replication alone (118).

Tumours can be classified as immunogenically “hot” or “cold” dependant on the balance of infiltrating immune cell populations. A tumour microenvironment which is “hot” is one where immunotherapies have been found to be most effective. These consistent of high CD8:CD4 T cell ratios and M1 skewered macrophages(119). In contrast, a “cold” tumour often contains Tregs, Bregs, and MDSCs, which prevent cytotoxic immune cells from penetrating into the TME. A few oncolytic HSV strains have been shown to modulate the tumour microenvironment with HSV1716 having data from several tumour models including melanoma (120), breast (121) and rhabdomyosarcoma (122) which demonstrate an increase in inflammatory immune cells, CD4+ T cells, CD8+ T cells, NK cells and macrophages, to areas of tumour. These changes can be integrated to enhance other treatments, such as immune checkpoint inhibition. Indeed, in a model of pancreatic ductal adenocarcinoma, a challenging cancer to treat clinically, administration of HSV was found to downregulate TAMs and increase the percentage of TILs, including activated CD8+ T cells and T helper (Th)1 cells, and

as a result extended the lifespan of the tumour-bearing mice significantly when in combination with a checkpoint inhibitor (123).

In addition to immune effects, HSV-glycoproteins, viral DNA and can cause damage to the vasculature structure and have been found on CD31-positive ovarian cancer vascular endothelium *in vivo* and HSV1716 was found to inhibit capillary tubular formation *in vitro* and *in vivo* (124). These glycoproteins were not present in the vasculature of other organs suggesting that these antiangiogenic effects may be involved in the mechanism of HSV1716 therapeutic effect. Of interest, HSV1716 has synergistic effects with other drugs that also cause vasculature damage including doxorubicin (a chemotherapy) and two VEGFR tyrosine kinases inhibitors, Sorafenib and Sunitinib, with data suggesting that the use of these drugs in combination sensitise cells to apoptosis by these drugs(125).

HSV1716 has been looked at in several preclinical and early clinical trials which support its safety and tolerability, particularly within gliomas. These are summarised in table 4. Other modified HSV have since developed from the HSV1716 backbone although the discussion of these is outside the remit of this PhD.

Table 4: HSV1716 in preclinical and clinical studies

Tumour type	Type of study	Study design	Results	Ref
Breast	Immunocompetent 4T1 model of breast cancer	2 or 3 doses of HSV1716 5.4×10^5 pfu or placebo	HSV1716 reduced lung metastases and increased survival time and partially inhibited 4T1 tumours developing on rechallenge	(121)
Head and neck squamous cell cancers (HNSCC)	*In vitro:* Three HNSCC cell lines were studied; UM-SCC 14C, UM-SCC 22A and UM-SCC 22B	HSV1716 alone and combined with cisplatin	HSV1716 alone and combined with cisplatin was efficacious in destroying head and neck squamous cell carcinoma cells.	(126)
Hepatocellular carcinoma	*in vitro* cell lines immunosuppressed xenograft models HuH7 and a luciferase-expressing variant of HepG2 cells	3 injections at 2-weekly intervals verses two injections on days 1 and 4.	Both treatments affected growth, but 3 x 2 weekly injections were better at affecting cures (50%) versus 2 injections on days 1 and 4 (25% cured).	(127)
Lung cancer	*In vitro* mainly with some *In vivo* mixing studies - immunocompetent and immunocompromised murine host	Lewis lung carcinoma cells were infected with HSV-1716 and implanted in the flanks of mice at varying ratios of infected to uninfected cells.	LLC were killed by HSV1716	(128)

Tumour type	Type of study	Study design	Results	Ref
Melanoma	*In vitro* and *in vivo* using SK-MEL-3 cell line Engineered HSV1716 with noradrenaline transporter (NAT) gene which allows targeted radiotherapy using radiolabelled 131I-MIBG.	1×10^7 PFU of HSV1716/NAT alone or in combination with 10 MBq of 131I-MIBG either simultaneously or 24 h later given to in vivo SK-MEL-3 models.	HSV1716/NAT and I-131-MIBG resulted in decreased tumour growth and some complete cures with enhanced survival relative to injection of either agent alone	(129)
Myeloma	*In vitro* and *in vivo* xenograft (JJN-3 cells in NSG mice) and syngeneic (murine 5TGM1 cells in C57BL mice) systemic models of myeloma	Intravenous HSV1716 (1×10^7 pfu 1 or 2 times per week with bortezomib	Combination of HSV1716 and bortezomib treatments prevented myeloma cell regrowth for up to 25 days compared to only transient cell growth suppression with bortezomib treatment.	(130)
Non-small cell lung cancer (NSCLC) cell lines	*In vivo*	NCI-H460 flank tumours were directly injected with HSV-1716 ($4 \text{ X } 10^6$ PFU) followed by intravenous MMC administration (0.17 mg/kg) 24 hr later.	After 3 weeks, the mean tumour weight in the combined treatment group was significantly less than either individual treatment individually. Synergistic effect of Mitomycin C (MMC)	(131)
Tumour type	**Type of study**	**Study design**	**Results**	**Ref**

Ovarian Cancer	mouse xenograft model	Intraperitoneal administration of 5 × 10^6 PFU	A significant reduction of tumour volume and spread and an increase in survival	(132)
Rhabdomyo-sarcoma	Immuno competent murine model	3 doses of intratumoural HSV1716 followed by 3 dose of intraperitoneal PD1 inhibitor	M3-9-M (MHC I high) but not 76-9 (MHC I low) tumours responded to HSV1716 + PD-1 blockade	((122)
High-grade glioma (resected)	Phase 1 clinical trial	12 patients post resection. 8-10 injections of HSV1716 into the tumour cavity. Adjuvant radio-therapy or chemotherapy	3 patients clinically stable at 15-, 18- and 22-months post-surgery and HSV1716 injection. Remarkably, the first patient in the trial, who had extensive recurrent disease pre-procedure, is alive at 22 months since injection of HSV1716 and 29 months since first diagnosis. No significant toxicity	(133)
High grade glioma (recurrent)	Phase 1 study	9 patients, intratumorally inoculation of doses up to $l0^5$ pfu	No significant toxicity. 4/9 patients currently alive and well 14–24 months.	(134)
High grade Glioma	Early phase “Proof of Principle" study	Intratumoural injection of 10^5 pfu of HSV1716 followed by tumour debulking 4-9 days later	HSV1716 replicates in HGG without causing toxicity in both HSV-seropositive and -seronegative patients	(135)
Tumour type	**Type of study**	**Study design**	**Results**	**Ref**

Mesothelioma	Phase I/II trial of intrapleural HSV1716 .	1×10^7 pfu HSV1716 injected via an indwelling intrapleural catheter (IPC) on one, two or four occasions a week apart	Intrapleural HSV1716 was well-tolerated and demonstrated an anti-tumour immune response in MPM patients.	(84)
Sarcomas, clival chordoma, malignant peripheral nerve sheath tumour (MPNST), and renal cell carcinoma	Phase 1 clinical study in paediatric patients	Intra- tumoral injection of HSV1716 heavily pretreated patients (2+ treatments prior)	Well, tolerated Grade 3 back pain in 2 patients No deaths due to toxicity 8/9 HSV seronegative Stable disease in 3 patients at 28 days but	(136)

1.8 Limitations of OV

One of the concerns of oncolytic HSV1716 was whether the virus could be delivered systemically to humans who have has past exposed to HSV-1 or HSV-2. Concerns about premature viral elimination or clearance in a pre-exposed host meant the focus of early trials was to deliver the viral directly into the tumour, though intratumoural injection. These concerns, and ideas to overcome these challenges, are discussed in the following review in section 1.9. Another observation is the differences in outcomes based on HSV seropositivity. Several trials using HSV derived viruses have screened for seropositivity at baseline and there have been some interesting reports to suggest that prior exposure to HSV may influence clinical outcome. Of clinical interest, when the oncolytic virus TVEC was used, Hu et al found that the maximum tolerated dose of the virus in patients who were HSV-1 seronegative was 1 ×

10^7 pfu/mL, whereas there was no maximum tolerated dose in patients who had prior HSV exposure suggesting differences in toxicity may also be (95). No such reports have looked at efficacy, but this would be a consideration if taking HSV1716 to clinical trial.

Given the concerns regarding the delivery of oncolytic virotherapy and to aid understanding of the barriers to therapy which may affect the efficacy of virotherapy clinically, an extensive review of the literature was performed. This is documented in the following review published on June 20221 in the journal “Viruses” (137).

1.9 Review Article: Oncolytic Virotherapy Treatment of Breast Cancer: Barriers and Recent Advances

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Abstract

Oncolytic virotherapy (OV) is an emerging class of immunotherapeutic drugs. Their mechanism of action is two-fold: direct cell lysis and unmasking of the cancer through immunogenic cell death, which allows the immune system to recognize and eradicate tumours. Breast cancer is the most common cancer in women and is challenging to treat with immunotherapy modalities because it is classically an immunogenically "cold" tumour type. This provides an attractive niche for OV, given viruses have been shown to turn "cold" tumours "hot," thereby opening a plethora of treatment opportunities. There has been a number of pre-clinical attempts to explore the use of OV in breast cancer; however, these have not led to any meaningful clinical trials. This review considers both the potential and the barriers to OV in breast cancer, namely, the limitations of monotherapy and the scope for combination therapy, improving viral delivery and challenges specific to the breast cancer population (e.g., tumour subtype, menopausal status, age).

1. Introduction

Neoplastic disease accounts for one in six deaths globally, with cancer being the second leading cause of death worldwide. The most frequently occurring subtype of malignant cancer

found in woman globally is breast cancer, with statistics showing roughly 1000 women dying per month in the UK [**1**]. Even though breast cancer is localized, it can be described as a heterogeneous disease, exhibiting multiple phenotypic variations [**2**]. These genetic variations, combined with other factors including tumour size, grade, and morphology alongside hormone receptor expression, are also used for diagnostic purposes to stratify patients' prognosis and treatment regime [**3**]. The most abundant subtype of breast metastases stems from adenocarcinomas, accounting for 95% of all invasive breast cancer cases recorded. From this population, 55% will present with invasive ductal carcinoma (IDC), which is characterised by the uncontrolled neoplastic proliferation of epithelial cells, which are localized to the ducts or lobules of the mammary gland [**3**]. Of these IDC patients, 15% will develop a triple negative breast cancer (TNBC) status (Cancer Research UK, 2017). TNBC is a highly metastatic disease that has been known to spread to distant organs such as the brain, bone, and the lungs. The inability to remove residual cells from the primary site after the initial treatment is ceased increases the risk of multi-drug resistance (MDR) due to genetic heterogeneity, enabling tumour progression [**4**]. Moreover, the genetic variations of this cancer subtype leave the individuals negative for the hormone receptors human epidermal growth factor (HER-2), oestrogen (ER), and progesterone, resulting in a lack of response to traditional hormone therapies and elevating the problems associated with breast cancer therapies [**3**]. The current therapy options available for the treatment of aggressive breast cancers consists fundamentally of hormone therapies, human epidermal growth factor receptor 2 (HER2)+ receptor targets, and chemotherapies, used sometimes in combination with immunotherapies. Unfortunately, evidence still suggests that some patients die as a result of harsh treatments, with harmful side effects overshadowing patient benefit [**4**]. Such evidence highlights the desperate need for research to uncover new, alternative therapies in the fight against breast cancer.

In the new era of scientific innovation, genetic engineering has led the way in the field of cancer research, with oncolytic viruses sparking new interest. Multiple viruses have been utilized

within the field of oncology, including adenovirus, reovirus, measles, herpes simplex, Newcastle disease, vaccinia, and Myxoma viruses. Oncolytic viruses are either inherently tumour specific (e.g., Myxoma virus) and display a natural tropism towards tumours cells or modified to enhance tumour specificity. The relationship between virus and tumour cells was first discovered in the early 1800s, when patients with leukaemia and lymphoma exhibited tumour regression after contracting the measles virus [**5**]. Since then, genetic engineering has enabled researchers to enhance viral tumour specificity through the deletion or insertion of essential genes within their genomic structure. For example, the most recent virotherapy to be approved by the Food and Drug Administration (FDA) and the European Medicines Agency was a herpes simplex virus (HSV-1) known as Talimogene laherparepvec (T-Vec). The T-Vec virus was attenuated to express high levels of granulocyte macrophage colony-stimulating factor, which is an essential cytokine for the production and stimulation of new infection-fighting white blood cells [**6**]. It was first used as a single therapy for the treatment of aggressive melanoma. However, research has since looked at its effects in combination with immunotherapies. Sun et al. (2018) [**7**] recently investigated the combination effects of a checkpoint inhibitor and T-Vec in unresectable stage III-IV melanoma. The study was conducted on a small number of patients (n = 10) with over half (n = 6) experiencing complete response after intratumoural injection of T-Vec, with an additional two patients seeing a response in not just the primary lesion but off-target lesions as well. Follow-up blood work showed that in patients exhibiting a response, CD4+ and CD8+ T cells were elevated seven -months post treatment, suggesting good synergy between the two therapies and a possible treatment for enhancing immune activation in cancer [**7**]. Nevertheless, the sobering fact is that at present such immunotherapies work in a limited patient population with a given cancer type, and in some types of cancer they have little or no effect. Breast cancers have historically been amongst the hardest to treat and so far, immunotherapies including checkpoint inhibitors have resulted in little success in clinical trials [**8**,**9**]. This highlights the

need for new combinational therapies that help target cancers that are specifically immunosuppressed, using checkpoint inhibitors.

The method by which oncolytic viruses specifically kill tumour cells is still under investigation; however, it is thought that the main mode of cancer killing is through direct oncolysis and increased tumour immunogenicity culminating in neoplastic cell elimination. The ability for oncolytic viruses to generate multiple daughter virions upon infecting one tumour cell is one of the more attractive qualities of using virotherapies. This "self-implication" property enhances rapid tumour lysis and increases the possibility of single or lower dosing regimens for patients. The fact that cancerous cells deviate from their original homeostatic signals enables great oncolytic viral sensitivity [**10**]. Besides oncolysis, immune activation is another key aspect to tumour eradication and is arguably the most important. Therefore, virotherapy can switch a previously immunogenically "cold" tumour into a "hot" one through the exposure of tumour-associated antigens (TAA) from the primary cancer to the circulatory system, thus eliciting an anti-tumour response from the immune system [**11**]. TAA stimulation of the immune system via OV offers the potential for long-lasting tumour immunisation from re-occurring cancers by generating tumour-specific T cells [**10**]. This is a major advantage and has potential to make tumours more responsive to immunotherapies that would ordinarily not work.

Although vaccines of viral components, in the form of gene therapy, can often be thought of as an OV, this review focuses on the use of live, replicating viruses and their potential role in breast cancer treatment.

2. Preclinical Evidence of OV in Breast Cancer

Breast cancer is a heterogeneous disease where the majority of tumours are immunologically "cold," and therefore the use of immunotherapeutics within breast cancer appeals to be less compelling than those of melanoma or lung cancer. However, OV has been shown to modulate the tumour microenvironment (TME), causing an increase in pro-

inflammatory cells (e.g., cytotoxic T cells, macrophages) that could potentially turn a "cold" tumour into a "hot" one (**Figure 1**). In breast cancer, there is a number of preclinical studies using oncolytic virotherapy as well as a few early-phase clinical studies with promising early markers of response [**12**,**13**,**14**,**15**,**16**,**17**,**18**]. This was reviewed recently by O'Bryan and Mathis [**19**], Martini [**9**], and Chaurasiya and Fong [**20**].

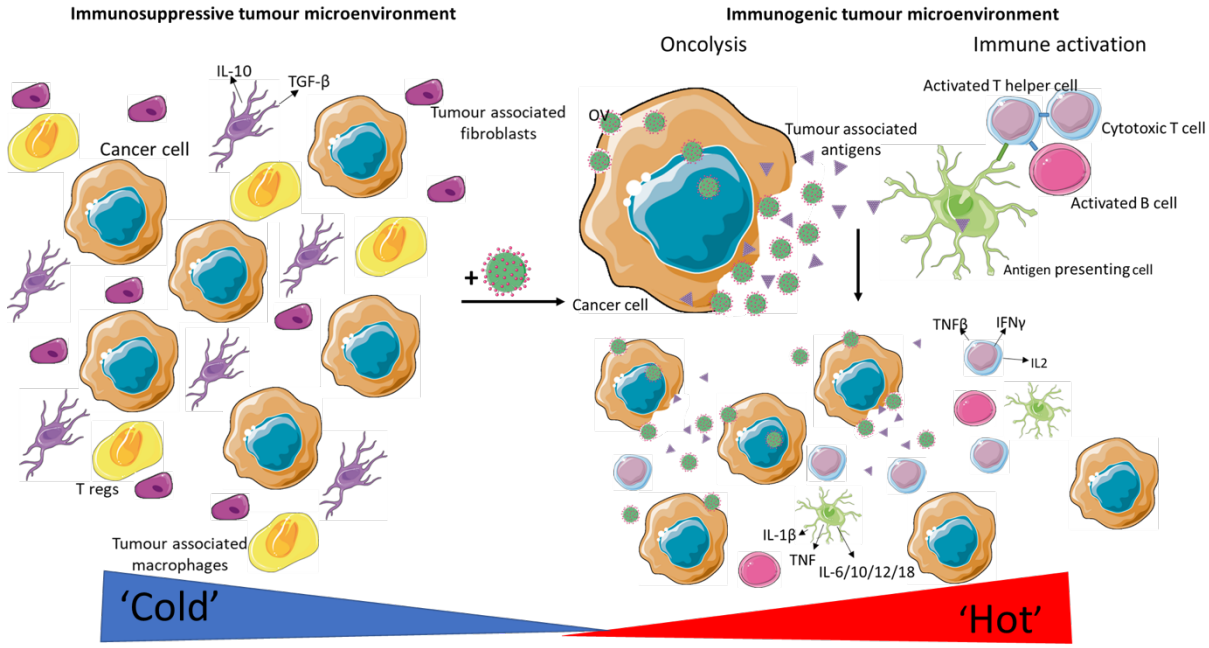

Figure 1. Immunogenic stimulation secondary to oncolytic viruses: Upon viral entry, replication and tumour cell lysis occurs and the innate and adaptive immune systems are activated. The killing of tumour cells via oncolysis releases tumour-associated antigens (TAA) into the circulation. Tumour debris stimulates the activation of resident and circulating antigen-presenting cells, resulting in their maturation. Mature antigen-presenting cells prime both B and T lymphocytes against specific TAAs, leading to long-term immunisation.

Some cancers such as melanoma and lung cancer demonstrate high response rates to immune checkpoint inhibitors and are commonly referred to as "hot tumours." These are in sharp contrast to tumours with low immune infiltrates, called "cold tumours" or non-T-cell-inflamed cancers, such as breast cancer. OV has the potential to make "cold" tumours "hot" by

reprogramming the TME in patients whose tumours are cold. The virus can inflame the tumour, attract immune antigen-presenting cells, and recruit T cells that can kill tumour cells.

Oncolytic viruses can be broadly categorized into DNA and RNA viruses. The DNA viruses most commonly investigated in breast cancer are adenovirus, HSV, and varicella. Oncolytic adenoviruses may be easily modified by the insertion of tumour-specific promoters to ensure replication occurs only with tumour cells. This can be used to improve breast cancer targeting. The receptor by which most adenoviruses enter the cell is the human Coxsackie receptor (hCAR); however, this is found in low numbers on breast cancer cells. Therefore, a study recently attempted to improve breast cancer targeting by the placement of an Ad3 component into the Ad5 backbone, which allows the adenovirus to enter cells through non-hCAR routes. Furthermore, an oncolytic adenovirus coding for GM-CSF was engineered and shown to lead to clinical stable disease in 8/12 patients who were advanced refractory to conventional treatments, with resolution of pleural fluid and ascites in some patients [**21**]. No breast cancer patients were included in this small study, and therefore we are unable to speculate whether this response would have been seen for this group.

Additionally, promoters may be inserted to enhance viral efficacy. Insertion of a promoter to the transcriptional factor E2F-1 and IL15 showed decreased tumour growth in both *in vitro* and *in vivo* models of TNBC using MD1-MB-231 cells inoculated in nude mice. Furthermore, a dual cancer-specific oncolytic adenovirus Ad-Apoptin-hTERTp-E1A (Ad-VT) with the apoptin and hTERT promoter was constructed and showed improved cytotoxicity compared to non-modified virus in MCF-7 breast cancer spheroids grown *in vitro* [**22**].

Herpes simplex type 1 (HSV-1) is a double-stranded DNA virus that fuses to the plasma membrane of host cells. To limit viral replication to cancer cells, the ICP34.5 gene is deleted, which results in an inability to replicate within neurons. Additional modifications can be made to the HSV-1 envelope to allow increased specificity to breast cancer cells. For example, the HSV G47(delta) has additional mutations in ICP6 and (alpha)47. This variation has

demonstrated *in vitro* and *in vivo* effectiveness. Of particular interest is the potential for the use of this virus in tamoxifen-resistant breast cancer [**23**].

A naturally occurring variant of HSV, HF10, has been explored in breast cancer in the preclinical and clinical settings, with patients showing decreased tumour size and increased CD8+ T-cell infiltration in the TME. In one such study, patients were given a dose of HF10 into one metastatic nodule and a dose of placebo into another [**24**]. The authors commented that the nodule treated with HF10 had signs of immune activation with increases in infiltration of CD8+ T cells and increased apoptotic markers. In our work with HSV1716, we have found systemic immune responses to OV treatment in a number of immunocompetent murine models of TNBC. This did, however, appear to be more marked when treatment was delivered systemically as opposed to locoregionally [**25**].

A number of RNA viruses have also been studied for use in breast cancer. Reovirus is a double-stranded RNA virus that is naturally non-pathogenic to humans and therefore used in an unmodified form. In particular, the type 3 Dearing reovirus strain is naturally oncolytic, and several studies have been performed that used it against breast cancer [**26**,**27**]. Due to early promise, intralesional administration of reovirus has undergone a phase 1 trial in advanced cancers, of which three patients had advanced breast cancer. As this revealed tolerable side effects, further studies including breast cancer patients have involved combinations of reovirus with other therapies, including phase 1 combinations with chemotherapies (docetaxel [**28**] and gemcitabine [**29**]), and *in vivo* studies with immunotherapies (PD-1 inhibitor) [**30**]. The combination of OV and immunotherapies has shown most promise and is discussed later.

3. Barriers to OV

Although OV appears promising, there are several limitations. These can be broadly categorized into efficacy as monotherapy and challenges with delivery (**Figure 2**).

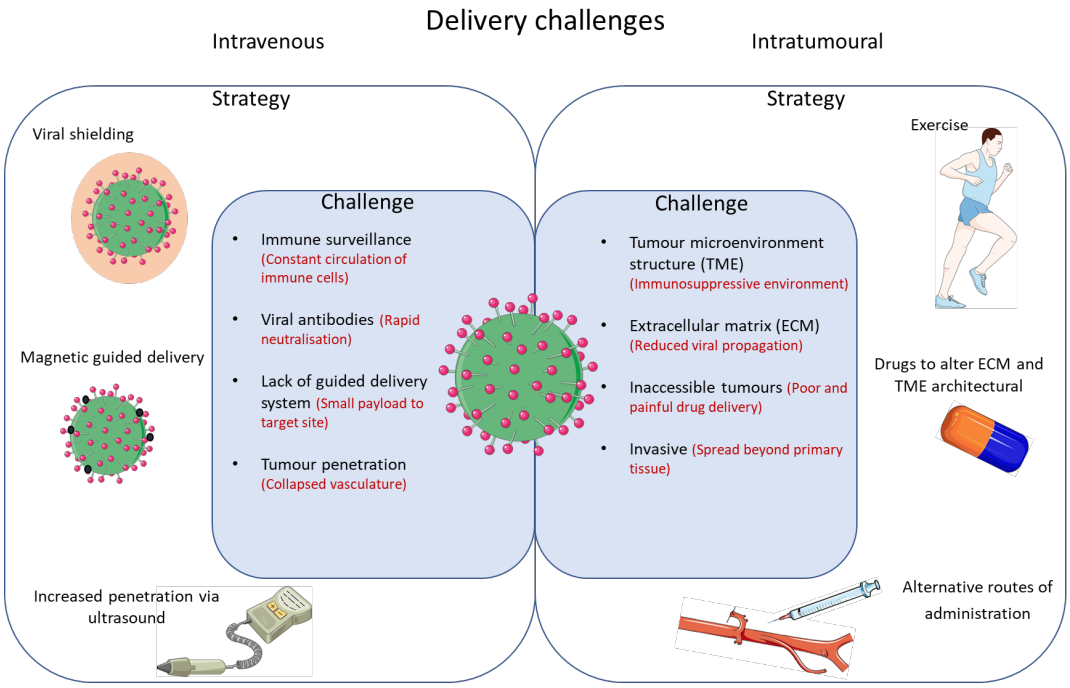

Figure 2. Outlined are some of the challenges and strategies in overcoming restricted delivery of OVs.

The approach to many novel therapies has been to ascertain safety, efficacy, and biological mechanisms of action through rigorous testing of oncolytic viruses as monotherapies. However, the actual efficacy of OV on its own is limited, as is the case with more established immunotherapies such as checkpoint blockade. **Figure 2** outlines the challenges that need to be overcome and potential strategies for improved delivery of oncolytic viruses.

This includes neutralizing oncolytic viruses in the bloodstream, sequestration of oncolytic viruses in the liver/spleen leading to decreased viral titre arriving at the site of the tumour, imbalance within the TME leading to poor tumour penetration, and suboptimal TAA production (which may be enhanced through a combination of OV and conventional treatments).

Additionally, cancer therapies, including virotherapy, carry their own risk and the way in which these treatments are administered can greatly reduce these risks. Most oncolytic viruses, especially those undergoing clinical trials, are predominantly administered through intratumoural (IT) injection. This helps overcome one of the main obstacles associated with intravenous administration, such as viral neutralisation and sequestration. Many studies,

including those by Andtbacka et al. [**31**] and Cripe et al. [**32**], utilised IT delivery to establish large viral titres within the TME and generate an anti-tumour response.

The following sections will address the developments that have been made to overcome these challenges and ascertain whether breast cancer itself may pose a barrier to OV therapy.

4. Combination with Chemotherapy

Patient stratification is not only used for determining patient prognosis but also for identifying the best treatment regime. Before determining the best course of action for the patient, decisions need to be made on whether the breast cancer can be eradicated with surgery or mastectomy, and whether chemotherapy should be given prior to the surgery (neoadjuvant therapy) or after (adjuvant treatment). Personalising chemotherapy treatments with breast cancer subtypes is essential for patient survival, with many chemotherapies targeting different phenotypic variations within the cancer. Such chemotherapies are used for the management of excessive cell proliferation by targeting DNA repair mechanisms with platinum, causing DNA damage with anthracycline agents and inhibiting P53 synthesis with taxanes [**33**]. However, many of the current therapies used within the clinic today lack the ability to target tumour cells specifically and therefore are administered in large doses in order to eliminate residual breast cancer cells. Unfortunately, this approach usually leaves the patient with poor quality of life due to harmful off-target side effects resulting from a lack of treatment specificity. Therefore, combinational therapies comprising OV and lower doses of chemotherapy could be a potential alternative treatment option for breast cancer patients.

At present, there are very few chemo-virotherapeutics making headway in breast cancer clinical studies. However, promise has been seen in other cancer types. In advanced phase II clinical studies, Karapanagiotou et al. [**34**] investigated the synergy of a reovirus type 3 Dearing (RT3D) virus, which is attenuated to specifically replicate in RAS-transformed cells and carboplatin/paclitaxel in patients with relapsed or metastatic solid tumours. Previous studies

conducted by the same group determined optimal dosing regimens prior to the phase II trial mentioned above. Patients received a combinational therapy of intravenously administered chemovirotherapy (virus ((RT3D virus) given over five days, whilst chemotherapy (carboplatin/paclitaxel) was given three times a week. Tumour response was evaluated alongside any evidence of an anti-tumour response (n = 19). The results demonstrated a complete response in one patient, whereas eight went on to experience a partial response to treatment. In the remaining patients, nine had a stable disease state and eight had disease progression. The safety profile of the study showed that patients tolerated treatment extremely well, with minimal to no known adverse effects, and was considered a good treatment option for patients with head and neck cancer [**34**]. Furthermore, a randomized study of pelareorep and paclitaxel in advanced breast cancer did not show a difference in progression-free survival, but rather a significantly longer OS with the combination [**35**]. So far, most clinical investigations have focused on the treatment of solid tumours in easily accessible areas, where the oncolytic virus can be injected directly into the tumour. This leaves breast cancer on the outskirts of clinical research. However, promising pre-clinical investigations are currently being conducted to help overcome this. Berry et al. [**36**] developed a doxorubicin conjugation reovirus (re-dox) for controlled drug release whilst simultaneously enabling viral lysis of tumour cells for the treatment of TNBC. The group used a hetero-bifunctional crosslinker (succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate), which enables covalent bonds to form between the doxorubicin and virus and enables the simultaneous release of viral particles and doxorubicin within the tumour after intratumoural administration. The results demonstrated *in vitro* show that the combination therapy increased mRNA expression of innate immune activation markers, including interferon—IFNL1, IFNB1, and IFNG in MDA-MB-231 cells—whereas treatment with re-dox still retained its infection and DNA-damaging abilities. Re-dox also significantly reduced tumour burden in mice with TNBC (4T1 model) implanted in the hind flank, resulting in a reduction in metastatic disease, predominantly within the lungs [**36**]. In addition, Bourgeois-Daigneault et al.

looked at the combination of oncolytic rhabdovirus Maraba-MG1 and paclitaxel for the treatment of murine TNBC in two established cell lines, 4T1 and E0771, *in vitro* and *in vivo* [**12**]. *In vitro* results demonstrated synergistic behaviour between the two therapies, with elevated viral propagation in 4T1 tumour cells. Further investigation also evidenced that paclitaxel does not affect the infection or replicative ability of the oncolytic virus. Mice were implanted with 4T1 and EMT6 cells in the second left mammary fat pad for *in vivo* experiments with treatments administered via the intraperitoneal or intratumoural route. The results showed significant tumour killing compared to controls when combination treatments were used over individual treatment [**12**]. Data such as these demonstrate the potential for OV in combination with chemotherapies as a future treatment option for breast cancer. However, other combinations need to be investigated to identify which options are most effective for breast cancer, including radiotherapy and immunotherapy.

5. Combination with Radiotherapy

Radiotherapy is a modality of primary treatment in 50% of cancers. It is an effective treatment on its own and causes cell death through DNA damage that may not be repairable in areas of malignancy. In a review by O'Cathail et al., they described how the combination of an oncolytic adenovirus and radiotherapy can be used to sensitise tumours to radiotherapy treatment. They postulate that this is due to the adenovirus preventing DNA repair following DNA damaging radiotherapy treatment leading to cancer cell death [**37**].

However, to date, there is little clinical data about the effectiveness of such treatment and no such data in relation to breast cancer. T-Vec, as mentioned earlier, is an HSV-derived virus that has been approved for use in melanoma. Within this population of patients, the combination of radiotherapy and T-Vec has been explored in preclinical animal models, with promising results of synergistic effects [**38**]. In addition, a phase II trial of intratumorally administered T-

Vec in combination with hypofractionated radiotherapy in melanoma and other tumours commenced in 2016, with results expected later this year.

Other promising trials in active recruitment include the Chemoradiation with Enadenotucirev as a radiosensitiser in locally Advanced Rectal cancer (CEDAR) trial, which is a dual-endpoint, dose-escalation phase I trial of an intravenously administered adenovirus, enadenotucirev, in combination with radiotherapy in colorectal cancer. This route of administration may be advantageous in the cancer population, as it allowed multiple areas to be sensitized to radiotherapy following a single dose of OV treatment [**39**].

The mechanism of this synergy is not fully clear. Many studies speculate on the combination of DNA damage secondary to radiotherapy and the viral properties of preventing DNA repair. However, some groups have found that the addition of radiotherapy to OV treatment results in enhanced viral replication, viral yield, and viral release. In this study of adenovirus dl520, the addition of radiation inhibited the growth of subcutaneous U373 glioblastoma tumours in a xenograft mouse model through an increase in YB-1, a protein required for viral replication [**40**]. Given the widespread use of radiotherapy in curative and metastatic breast cancer, the potential for a synergistic therapy, and the paucity of available studies, it would seem pertinent to assess this combination of OV and radiotherapy in breast cancer.

6. Combination with Immunotherapies

In a recent publication, we showed that the breast TME becomes primed for immunogenic killing through the use of oncolytic viruses. In particular, the phenotype of tumour-associated macrophages (TAMs) becomes re-educated from a tumour-promoting M2 subtype to a more inflammatory and cytotoxic M1 phenotype. These TAMs also enhance the recruitment of cytotoxic CD8+ T cells that directly eradicate cancer cells. However, we also showed that although OV may increase the number and activation of cytotoxic immune cells within the TME (CD8+, M1-like macrophages), they also upregulate the expression of PD-L1 within the tumour,

which causes an arrest to this immunological killing [**25**]. This led to speculations that OV therapy in combination with PD-L1 or PD1 inhibitors may release this break, allowing the immune system to respond more actively to OV.

Bourgeois-Daigneault et al. described the use of a Maraba virus prior to the removal of breast tumours in immunocompetent murine models [**12**]. Here, they administered a course of OV treatment 7 days prior to mammary tumour resection, followed by an adjuvant course of PD1 inhibitor treatment. They demonstrated that the use of virotherapy prior to surgery allowed for the sensitization to immune checkpoint therapy given adjuvantly and that on rechallenge, the immunological effects were long lasting [**41**]. This novel, neoadjuvant approach to treatment is appealing to clinical trial design, where the degree of response to neoadjuvant treatment can guide whether further adjuvant treatment is required.

Similarly, Mostafa et al. demonstrated that the oncolytic effect of reovirus can be enhanced with the addition of a PD-1 inhibitor in an EMT6 immunocompetent murine model of breast cancer, causing a reduction in tumour growth in comparison to monotherapy [**30**]. Furthermore, this is likely due to the positive cytotoxic changes in the immune TME, including an increase in CD8+ cells and a decrease in CD4+ T cells. A synergistic effect of OV and PD-L1 inhibitor was seen in the study by Chaurasiya et al., where a pox virus, CF33-hNIS-ΔF14, was used in combination with an immune checkpoint inhibitor (anti-PD-L1) using the EO771 murine model of breast cancer. Here they observed no significant change in tumour size with treatments as monotherapy, but the combination of both treatments lead to a 50% survival rate for animals at 100 days post treatment. They also noted the change in the TME, with an increase in pro-inflammatory cytokines and activated CD8+ T cells [**42**]. Given the promising preclinical data (**Table 1**), there are a few ongoing clinical trials combining oncolytic viruses with a checkpoint inhibitor in breast cancer. Viral groups of investigation at present include HSV, vaccinia, and reovirus, with the likelihood that other viral groups will be included as data mature.

OV	Checkpoint inhibitor	Cancer	Reference
Oncolytic vaccinia virus coexpressing a mouse PD-L1 inhibitor and GM-CSF.	PD-L1 inhibitor	Py230 breast cancer	[43]
Oncolytic reovirus - non modified	PD-1 inhibitor	immunocompetent, syngeneic EMT6	[30]
Polio:rhinovirus recombinant (PVSRIPO)	PD1/PD-L1 axis	E0771	[44]
Marabavirus - non modified	Anti-PD-1 (clone RMPI-14, BioXCell) and anti-CTLA4 (clone 9D9, BioXCell)]	EMT6, E0771, 4T1 immunocompetent, syngeneic neoadjuvant models	[41]
Modified measles virus (MV-NAP)	PD-1/PD-L1 blockade	Phase 1 trial on going (Mayo Clinic Breast Cancer SPORE)	https://grantome.com/grant/NIH/P50-CA116201-12

Table 1. Combination of oncolytic viruses and checkpoint inhibition in breast cancer.

7. Overcoming Barriers of Intravenous Delivery OV

The haemodynamics of the TME is a key aspect associated with drug delivery, with most therapies relying on the "leaky" vasculature of the tumour for drug uptake. Major efforts have been exerted on the enhanced permeability and retention (EPR) effect, also referred to as the

"royal gate" of drug delivery, since its importance was first highlighted in the late 1980s [**45**]. The combination of vascular fenestration and collapse, the heterogeneity of the basement membrane, the dense coverage of pericytes, and the lack of lymphatic blood vessel formation all contribute to elevated interstitial fluid pressure (IFP) within tumours. Increased IFP leads to interstitial hypertension within the TME, which restricts the network of connective transport systems available for OV perfusion and extravasation, diminishes the EPR effect [**46**], and provides a challenge for intravenous (IV) delivery. The importance of the EPR effect for efficient drug delivery was demonstrated following the systemic delivery of oncolytic viruses in heterogeneous intratumoural (IT) perfusion states within the TME [**47**]. Within the study, animals bearing myeloma tumour cells were administered with oncolytic VSV whilst undergoing physical exertion. Exercise is known to decrease splanchnic circulation, preventing viral sequestration and enhancing the rate of blood flow, which increases IT perfusion pressure. The results indicated significant amplification in the amount of "infection centres" and greater homogenous infection distribution throughout the tumours. This correlated with greater overall survival [**47**]. However, exercise is not a feasible option for all patients, making IT a better delivery option for oncolytic viruses. The successful delivery of therapies in breast cancer remains a major obstacle in clinic, with the TME arguably being the main candidate that drives drug resistance. Shee et al. [**48**] recently published a study stating that factors secreted by the TME, mainly the angiogenic cytokine fibroblast growth factor 2 (FGF2), which promotes tumour progression via irregular vascular formation, was highly overexpressed within ER+ breast cancer cells after *in vivo* experiments of immunocompromised mice bearing MCF-7 xenografts. The results demonstrated that FGF2 modulated resistance to fulvestrant and other PI3K–mTOR pathway inhibitors in anti-oestrogen-resistant ER+ breast cancer [**48**]. This provides a possible therapeutic targeting strategy via FGF2 mediated pathways, which could remove the TME as an uptake barrier for oncolytic viruses and highlights a need for personalised treatment in relation to individual patient TME status.

However, IT delivery poses challenges of its own, including being a limited delivery option for inaccessible tumours or small metastatic lesions that cannot be reached with a needle. In cases where IT delivery is not possible, IV delivery is the next best option. Therefore, "camouflaging" oncolytic viruses is of utmost importance to increase therapeutic efficacy in patients, particularly as many of us have already been exposed to these viruses and will therefore carry pre-existing neutralising antibodies (NAb) that will prevent the virus from entering the tumour. The encapsulation of oncolytic viruses through both synthetic and biological agents such as immune cells, copolymers, nanoparticles, and biodegradable materials is the main contender for viral "cloaking" [**49**,**50**,**51**,**52**]. Research carried out by Muthana et al., (2011) demonstrated the potential of cell carriers, namely, "macrophages," as biological protectors of a prostate-specific adenovirus [**53**]. It is well known that TAMs are abundant within the TME and are recruited to enhance the immunosuppressive environment [**54**]. Given that high numbers of macrophages are home to tumours, the researchers opted to exploit this and used macrophages to deliver oncolytic viruses to prostate tumours grown in spherical cell complexes that mimic the TME *in vitro* and in xenograft models of prostate cancer. The macrophage–virus complex successfully delivered the virus to the tumours, resulting in efficient viral replication under hypoxic conditions, tumour oncolysis, and inhibition of tumour growth in mice. More importantly, when co-cultured with high-titre NAb in human serum, the macrophages protected the virus, and was significantly more effective than adenovirus on its own, which was completely neutralised [**53**]. Additional cellular carriers have also been explored. For example, Melzer et al. showed that CD8+ T central memory cells (CD8+ T cm) can be efficiently loaded with VSV and transport virus to tumour cells without compromising their own viability or antitumor reactivity [**55**]. Furthermore, mesenchymal stromal cells have been shown to systemically deliver a binary vector containing an OAd together with a helper-dependent Ad (HDAd; combinatorial Ad vector (Cad)) that expresses interleukin-12 (IL-12) and checkpoint PD-L1 (programmed death-ligand 1) blocker [**56**]. Alternatively, chemical agents can also be used as protective "camouflage" for

OV, and this was demonstrated by Nosaki et al. [**50**]. Ionic polymer coating made via polyethyleneimine hydrochloride was used to encapsulate a measles virus. The coated oncolytic virus was administered to mice bearing LL/2 lung cancer cells. The study demonstrated the enhanced oncolytic activity in the presence of NAb (mice were pre-immunised 3 weeks prior to treatment), with significantly decreased tumour burden. *In vitro* analysis showed reduced neutralisation of coated MV in multiple cell lines, including MDA-MB-231s (breast), WiDr (colon), and A549 (lung) cells, highlighting the potential for synthetic polymers as effective "shields" against immune elimination [**50**].

8. Overcoming Barriers Using a Targeted Delivery Approach—Magnetic Guided Delivery

Within biomedical applications, synthetic magnetic nanoparticles (MNPs) are easily generated at high yield for a low cost. This, coupled with their strong magnetic properties, makes them ideal candidates as effective drug delivery systems that can be guided via an external magnetic force. The use of magnetic nanocarriers for the delivery of OV was recently reviewed by Howard and Muthana 2020 [**51**]. So far, this research has mainly used MNPs to investigate the movement of magnetically labelled chemotherapies both *in vitro* and *in vivo* or to bind and block viral entry into the cell as an anti-infection treatment [**57**]. Magnetic guidance could also be used for targeting oncolytic viruses to tumours. Almstatter et al. were the first to publish the *in vivo* application of MNPs linked to an oncolytic vesicular stomatitis virus (VSV) [**58**]. The anti-cancer properties of the magnetically labelled virus, along with its MRI contrast properties, were investigated in rats bearing orthotopic hepatocellular carcinoma. Good bioavailability was demonstrated by large aggregates of armed MNPs at the tumour site after visualisation using MRI. Within the experimental process, the tissue-mimicking phantom properties of the MNP–VSV complex indicated the potential for a sustainable contrast agent, which could be visualised for up to 24 h post treatment. In addition, good complex stability was observed *in vivo* when rats

were subjected to an external magnetic gradient (1.5 T and 3 T) for 30 min [**58**]. Unfortunately, in this study they did not investigate systemic delivery of the magnetised oncolytic virus, as the complex was injected directly into the tumour and the magnetic field was used to keep the complex in the tumour for longer periods. Therefore, it is impossible to predict whether application of magnetic gradients would have improved the targeting of the virus following systemic delivery, or whether the MNPs protected the virus from NAbs.

Tresilwised et al., in an attempt to combat multi-drug resistance (MDR) in cancer, also magnetically labelled an oncolytic adenovirus [**59**]. As Almstatter et al. [**58**] did, the particles were intracellularly internalised by 181RDB cells (a MDR pancreatic carcinoma cell line). Ultrastructural analysis showed excellent structural stability and good anti-tumoural killing. Mouse tumour pancreatic xenografts, which were treated IT with the magnetically labelled adenovirus, showed a statistically significant reduction (49%) in tumour burden compared to untreated mice, and in mice treated with adenovirus alone (without a magnetic gradient), 25 days post administration. The magnetically targeted virus was localised via an external static magnetic gradient over the right flank of the mouse to direct the magnetic gradient as much as possible over the pancreas [**59**]. Similarly to Almstatter et al. [**58**], no IV administered data of the complex were investigated in this study.

Muthana et al. [**60**] also investigated whether magnetic guidance could be used to improve targeting of systemically delivered macrophages armed with oncolytic HSV1716 in order to increase tumour specificity. Here the researchers exploited the gradients of MRI scanners to generate a controlled magnetic gradient to non-invasively "steer" the magnetically labelled macrophages from circulation into tumours. This exciting study not only used MRI to guide the therapy to the tumour but to also track delivery using MRI in its conventional imaging modality. They demonstrated a significant increase in drug delivery and reduction in tumour burden in mice with both primary and secondary prostate tumours, after IV administration with the magnetically labelled macrophage–HSV complex compared to virus alone [**60**].

These studies show that magnetic targeting increases viral titres within the tumours and could be used to enhance the delivery and retention of oncolytic viruses to tumours. The applicability of this approach to breast cancer remains to be investigated.

9. Overcoming Breast Cancer-Specific Challenges

Aside from the generic barriers with delivery and enhancing efficacy, breast cancer has some unique challenges. These can be subdivided into host specific (immune system differences in females and the effect of menopause on the immune system) and tumour specific (ER/HER2 status and use of anti-oestrogen therapy). However, research into this interesting area is limited and this section is based on the evidence with immunotherapies in the general cancer population.

Differences in immune response to checkpoint inhibition have been observed between males and females in the treatment of lung cancer [**61**] and melanoma [**62**]. This is felt to be because oestrogen acts as a steroid hormone on the immune system, perhaps modulating the tumour environment by promoting a tumourigenic landscape. Oestrogen is thought to play an important role in the adaptive immune system and upregulation of the ER has been observed in T cells and B cells. Furthermore, it may be that disease progression is a consequence of the mobilization of myeloid-deprived suppressor cells and enhancement of their immunosuppressive effects *in vivo* [**63**]. Within lung cancer, the use of anti-oestrogens to modulate the immune environment is currently under exploration [**64**].

Breast cancer is almost exclusively diagnosed in women the vast majority of whom are oestrogen dependent with upregulation of the oestrogen receptor, yet it is in this group that we see the least response to immunotherapies. However, there appears to be a potential role for the use of combination anti-oestrogen therapy, a well-established treatment modality for these cancers and checkpoint inhibition in breast cancer [**65**].

As a consequence of this, several clinical trials are currently underway with the goal of evaluating the added benefit of oestrogen-modulating drugs to immune checkpoint inhibitors in

the context of breast cancer (NCT02997995, NCT02778685, NCT03280563, NCT02990845, NCT02971748, NCT02648477, NCT02971761, NCT02997995 [**66**]). Within these trials, all combine inhibitors targeting checkpoints CTLA-4, PD-1, or PD-L1 with agents that target the oestrogen pathway, such as fulvestrant or exemestane. Interestingly, oestrogen may also be combined with oncolytic viruses. In a study by Stiles et al., the addition of oestrogen to the oncolytic HSV-1 NV1066 enhanced the lytic effect of the virus, with an MCF-7 cell death of 95% and 97% *in vitro* at MOIs of 0.1 and 0.5, respectively, compared to 53% and 87%, respectively, in the absence of estrogen [**67**]. This finding may be used to target the more immunotherapy-resistant ER+ breast cancers and is worth potential investigation.

The other difference between males and females is the predisposition of females to present with autoimmune disease. T helper (Th) cells are postulated to be responsible for this, and females have been found to have a Th1 bias [**68**]. This may have implications in terms of response to treatment and potential toxicity. Indeed, our own unpublished data have suggested that predisposition to a particular Th cell response generated opposite results. Inoculation with oncolytic HSV had a more marked systemic response in tumour-laden Th2-biased BALB/c mice, but a protective one in Th1-biased C57Bl/6 mice (unpublished data).

Breast cancer is also a disease in older women, and there may be an impact of age and response to immunotherapies. Firstly, the changes in an ageing immune system have been extensively covered in the literature [**69**]. This predisposes the older population to a number of age-related diseases, including a predisposition to Th1-biased diseases such as atherosclerosis and leading to the elderly being poor stimulators of the adaptive immune response and antibody production. In particular, ageing leads to an increased susceptibility to acquiring viral infections and an inadequate immune response [**70**]. Of interest, ageing leads to an increase in T reg cells, which play a role in masking the cancer from the host's immune system. This change may be potentially reversed with OV.

Furthermore, hormonal changes associated with menopause have been described as provoking immune-related changes. For example, post-menopausal women show elevated levels of pro-inflammatory cytokines MCP1, TNFalpha, and IL-6 [**71**]. In many breast cancer patients, menopause is artificially induced with chemotherapy or chemical castration using LHRH antagonists. What is interesting is that in the premature menopause setting, circulating immune cells of the adaptive immune system are modified. Kumru et al. [**72**] and Giglio et al. [**73**] analysed the peripheral blood of breast cancer patients who had undergone menopause surgically or naturally, respectively. In both studies a decrease in CD4 T cells and B cells was observed as well as a corresponding increase in CD8 T cells peripherally. These consistent differences raise the question of whether pre- and post-menopausal women respond differently to immunotherapies including OVs, a question that has yet to be addressed in clinical trials.

The next most common subtype of breast cancer is the HER2+ subgroup and accounts for 15–19% of breast cancers [**74**]. This patient group may respond to immunotherapy and there is some data to suggest that HER2 receptor blockers may cause long-term disease-free survival through this route. To enhance this, there has been interest in developing HER2 vaccinations that consist of HER2 antigens to stimulate an immune response. Morse et al. described a pilot study of one such vaccine administered with dendritic cells to boost immunogenic stimulation [**75**]. They co-administered dendritic cells (derived from patients' peripheral blood mononuclear cells) with HER2 antigens and recorded acute and long-term toxicity and response. They describe an impressive 4.5-year survival in all patients, with only one recurring with a single pulmonary lesion at 4 years post treatment. Results from later phase clinical trials have not been reported. Additionally, as HER2 is a targetable receptor, it can be exploited in OV. For example, to specifically target HER2+ breast cancer, the monoclonal antibody trastuzumab is often used clinically to block the ERB receptor. To achieve this, in a preclinical study one group engineered an oncolytic virus where the human trastuzumab antibody heavy- and light-chain genes were uncoded within a serotype 5 adenovirus, Ad5/3-Δ24-tras. This allowed viral oncolysis and

assembly and release of the trastuzumab antibody within the TME, with impressive results *in vivo* [**76**].

10. Conclusions

Breast cancer is a heterogeneous disease and although conventional treatments have evolved significantly over the past few years, treatment resistance invariably occurs. Training the immune system to recognize and target cancers has proved curative for select patients in a number of solid tumour types. The hallmark of response appears to be a favourable "inflammatory" TME. OV, through the production of TAA and immunogenic cell death, may be the platform to sensitise breast cancer to other immunotherapies. However, further work needs to be done to overcome the barriers to OV and personalise treatment for this particular group of patients. This review highlights the areas of current development, which include optimising delivery of oncolytic viruses, combining OV with conventional breast cancer therapeutics, and targeting breast cancer-specific challenges.

Author Contributions

Conceptualization, A.K. and M.M.; methodology, A.K., N.W. and M.M.; writing—original draft preparation, A.K. and N.W.; writing—review and editing, A.K., N.W. and M.M.; supervision, M.M.; funding acquisition, A.K. and M.M. All authors have read and agreed to the published version of the manuscript.

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Abbreviations: interleukin (IL), interferon (IFN), tumour growth factor (TGF), and T regulatory cells (Tregs).

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Amendment to published work to the above paper following viva examination:

1) The sentence: “This leaves breast cancer of the outskirts of clinical research” has been reworded to “Due to the location of metastatic disease, the applicability of such treatments in breast cancer have been limited.”

2) The sentence “Breast cancer is almost exclusively diagnosed in women the vast majority of whom are oestrogen dependent with upregulation of the oestrogen receptor, yet it is in this group that we see the least response to immunotherapies.” has been reworded to “Breast cancer is predominantly diagnosed in women, where the largest subtype consists of these are oestrogen dependent, with upregulation of the oestrogen receptor, yet it is in this group that we see the least response to immunotherapies.”

1.10 Summary of literature review

Through this review the clinical need for a novel therapy within TNBC has been highlighted. The characteristics of TNBC have identified and a role of the immune system in the TNBC TME shows immunotherapy, particularly oncolytic virotherapy, may have potential as a novel treatment for TNBC. Much of oncolytic virotherapy research in breast cancer has been trying to improve targeting of the virus to the cancer, ways of improving immunogenicity of the virus or provide enhanced cytotoxicity through combination treatments. What has become apparent is that a single agent approach to treating TNBC may not provide the required effect.

This PhD will explore HSV virotherapy for TNBC alone and in a trojan horse style cell delivery system, with the view that this will help overcome some of the issues with virotherapy alone, to form the foundations needed for an early phase clinical trial and the translation of this novel therapy to the clinic.

1.11 Hypothesis and aims

The overarching hypothesis of this PhD is that oncolytic virotherapy treatment can be enhanced by the use of macrophage delivery for primary and metastatic TNBC. To assess this hypothesis, the aim of the PhD is to demonstrate firstly that the oncolytic virus HSV1716 can trigger a cytotoxic and immune response to aid treatment of TNBC and secondly, that macrophages can be used as cellular carriers to improve HSV1716 delivery.

To achieve the above aims the following objective were identified:

1. Establish an appropriate immunocompetent model for the assessment HSV1716 to treat TNBC in both primary and metastatic setting (described in chapter 3)
2. Demonstrate that intravenous HSV1716 is effective in a variety of TNBC models (described in chapter 4)
3. Assess and explore the immune cell infiltration within the TME in response to HSV1716 treatment to identify differences between control and treatment groups (described in chapter 4)
4. To assess the response of macrophage delivered HSV1716 in primary and metastatic disease (described in chapter 5).

Chapter 2

2 Materials and Methods

To avoid duplication, the materials and methods included in this chapter pertain to those which are not described in detail within the publications include in the chapter 4 and 5.

2.1 List of chemicals and regents

Reagent	**Company**
1% Eosin	Sigma-Aldrich
1% Penicillin and Streptomycin	Lonza BioWhittaker Ltd
10% Fetal bovine serum (FBS)	Gibco
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	Invitrogen
Absolute Ethanol	Thermo Fisher Scientific
Acetone	Thermo Fisher Scientific
Agarose	Sigma-Aldrich
Baytril	Bayer
Bovine serum albumin	Vector laboratories
Clodronate liposomes	Liposoma
Control liposomes	Liposoma
Cryo-M Bed Optimal Cutting Temperature Compound (OCT)	VWR International
Crystal Violet	Sigma-Aldrich
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich
Dispase II	Gibco
DPX Mounting medium	Sigma Aldrich
Dulbecco's Modified Eagle's Medium (DMEM) Ultraglutamine, 4.5g/L glucose Lonza DMEM medium	Lonzo BioWhittaker Ltd
Ficoll-Hypaque	GE Healthcare
Goat serum	Vector laboratories

Haematoxylin Solution Gill No. 2	Sigma Aldrich
Hibiscrub	Mölnlycke
Human AB serum (2%)	Lonza BioWhittaker Ltd
Iscove's Modified Dulbecco's Medium (IMDM)	Lonza
Isoflurane	Med-Vet
L-Glutamine (4mM)	Lonza BioWhittaker Ltd
Luciferin	Perkin Elmer
Matrigel	Corning
Metacam	Boehringer Ingelheim
Nuclease free water	Qiagen
Paraformaldehyde	Sigma Aldrich
Phosphate Buffered Saline	Lonza BioWhittaker Ltd
PrecisionPlus qPCR mastermix with SYBR green and ROX	Primer Design
ProLong Gold Antifade mountant	Invitrogen
Roswell Park Memorial Institute (RPMI) mediumRPMI medium	Lonzo BioWhittaker Ltd
RPMI	Lonza BioWhittaker Ltd
Super PAP pen	Thermo Fisher Scientific
TO-PRO-3	Thermo Fisher
Trypsin/EDTA	Sigma Aldrich
TWEEN 20	Thermo Fisher Scientific
Zombie UV Fixable Viability kit	Biolegend

2.2 List of antibodies

Antibody	**Company**	**Conjugate**	**Clone**	**Dilution**
F4/80	Biolegend	488	C1:A3.1	1:50
PCNA	Biolegend	-	PC10	1:75

CD8	Biolegend	53-6.7	PE	1:100
CD4	Biolegend	GK1.5	AF488	1:50
FOX P3	Biolegend	MF14	AF647	1:100
MRC1	Abcam	-	ab64693	1:200
CD3	BD Pharmingen	APC	145-2C11	1:200
PD1	Biolegend	APC	EH12.2H7	1:100
PD-L1	Biolegend	PE/CY7	29E.2A3	1:100
DAPI	Life technologies	-	-	1:100

2.3 List of Equipment and Apparatus

Equipment and apparatus	**Company**
Automated Cell Counter	BIO-RAD
Bench centrifuge	SANYO
Compound light microscope	Olympus
Cryostat	Leica CM1900
Incubator	SANYO
Laminar airflow hood	Heraeus
Micropipette	Eppendorf
PIPETBOY	INTEGRA
Refrigerator	BioCold
Water bath	Grant
IVIS –Lumina II	Caliper Life Sciences
Applied Biosystems 7900 Real-time PCR machine	Applied Biosystems
FACSCalibur	Becton Dickinson
Light microscopy	Leica

LSR II Flow cytometer	BD Bioscience
Nikon A1 Confocal	Nikon
Plate reader	Thermo Scientific
Sensitive balance	Sartorius
Shaking platform	Luckham
Sonicater	Bioruptor
Spectrophotometer	Varian Associates
Coverslips	Scientific Laboratory Supplies
Fisherbrand 384-well skirted PCR plate	Thermo Fisher Scientific
Superfrost Plus Microscope Slides	Thermo Fisher Scientific
Tissue culture flasks (Nunc EasYFlak)	Thermo Fisher Scientific
Micro-fine insulin needles	BD

2.4 Commercial Experimental Assays

Kit	**Company**
Nitric Oxide Assay Kit (ab65328).	Abcam
RNeasy Mini Kit	QIAGEN
Precision 2X q-PCR Mastermix	PrimerDesign

2.5 List of software

Software	**Company**
Image J	Fiji
Graphpad prism	Graphpad Inc
Flow Jo	TreeStar Inc
BioRender	BioRender
Image scope	Aperio

2.6 Methods

2.6.1 Preparation of cell culture medium

- RPMI complete medium: Roswell Park Memorial Institute (RPMI) medium was supplemented with 10% Foetal Bovine Serum (FBS) and 4 mM L-Glutamine (200mm in 0.85% sodium chloride solution).
- DMEM complete media: Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 10% FBS.
- DMEM F-12 was supplemented with 10% FBS and 1% Penicillin Streptomycin.
- IMDM was supplemented with human AB serum and glutamine.

All culture medium was stored at 4^{o}C.

2.6.2 Breast cancer cell lines

All human and murine metastatic BC cell lines used are listed in **Table 5.** MDA-MB-231, PyMT-TS1 and 4T1 cell lines were grown in DMEM complete medium, whereas the SK-Br-3 cell line was grown in RPMI complete medium. Murine luciferase labelled 4T1 brain seeking cells (LUC-4T1-BR) were obtained from Prof Sanjay Srivastava, University of Texas Tech University Health Sciences Center. PyMT TS1 cells were obtained from Prof Johanna Joyce, Memorial Sloan Kettering Cancer. Both cell lines were maintained in DMEM supplemented with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and were used within 25 passages and negative for mycoplasma. The cells were routinely passaged using trypsin (0.05%) upon reaching maximum cell confluence (~80-90%).

Table 5: List of breast cancer cell lines used in this PhD

Cell line	Host source	Obtained from
MDA-MB 231	Human	ATCC
MCF7	Human	ATCC
SKBR3	Human	ATCC
4T1	Murine	ATCC
Luc-4T1-BR	Murine	University of Texas Tech University Health Sciences Center
EO771	Murine	ATCC
PyMT-TS1	Murine	Memorial Sloan Kettering Cancer

2.6.3 Vero cells

Vero Cells are a kidney epithetical cell line derived from an African Green Monkeys that are susceptible to viral infection as they contain a deletion in type I interferon genes resulting in the inability to produce interferon(138). They retain intact cell contact inhibition, therefore requiring regular passaging to maintain cell viability. Vero cells within this PhD were used to assess viral replication through plaque assays described below. They were maintained with DMEM F-12 complete medium.

2.6.4 L929 cells

L929 cells are derived from mouse fibroblasts. They secret M-CSF and their medium has often been isolated and used to stimulate macrophage growth following bone marrow extraction (139). L929 cells, previously stored in liquid nitrogen, were thawed quickly over a warm water bath and brought up in DMEM complete media in a T25 flask. On confluency, trypsin was applied to detach cells from the flask and cells were moved to a T75 flask. The

cells were allowed to develop to approximately 70-80% confluency and then media was harvested. This media was centrifuged and passed through a 0.45 μm filter before freezing at -20. The remaining cells were grown continuously until adequate frozen stocks had been obtained.

2.6.5 Murine bone marrow derived macrophages

Bone marrow was obtained from both femurs and tibias of BALB/c mice under aseptic conditions as described in Toda et al (140) . Here, the lower abdominal cavity and both hind limbs of humanly sacrificed mice were sprayed with 70% ethanol before tibias and femurs were isolated. Joints were taken from above the pelvis and the muscles removed by blunt dissection and then cleaned with sterile gauze. Femurs were transported in sterile PBS to the tissue culture lab where they were washed in sterile 70% ethanol. The epiphyses of bones were cut, and the shaft of the bones were flushed with 2ml of sterile DMEM using a sterile 25G needle until the bone marrow appears white. The bone marrow was then centrifuged at 1000rpm for 5 minutes. The pellet was re-suspended and incubated in differentiation DMEM which contained 10% heat-inactivated FCS, and 20% L929 cell-conditioned medium. Non-adherent cells were washed on day 5 and adherent cells were used for infection on day 7.

2.6.6 Monocyte derived macrophages

Mononuclear cells were isolated from waste buffy coats using Ficoll separation. Blood was diluted with PBS in a ratio of 2:3 (Blood:PBS) and then very slowly introduced into sterile 50ml falcon tubes containing 20ml of Ficoll. Falcon tubes were then placed into the centrifuge and spun at 1400 RPM (no brake) for 40 minutes. Ficoll allowed the separation of erythrocytes and peripheral blood mononuclear cells (PBMCs) (see figure 3). After removal of most of the plasma layer, the PBMC layer was collected and seeded in T75 flasks in IMDM medium

supplemented with 1% glutamine and 2% human AB serum. Cells were incubated at 37°C in 5% CO_2. Non-adherent cells were washed, and macrophages were allowed to fully differentiate until use from day 5-7.

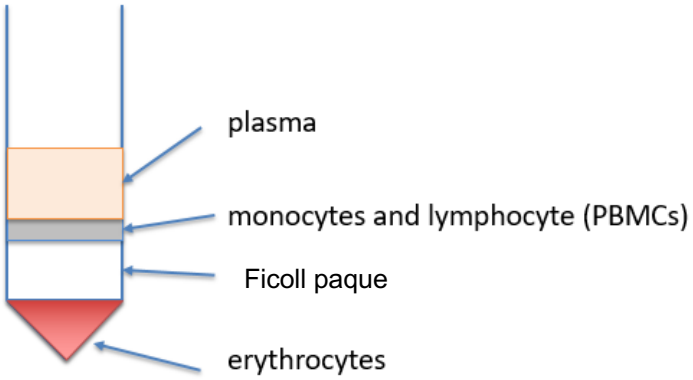

Figure 3: Ficoll separation*. A schematic representation of the layers of blood products which are seen through Ficoll separation after centrifuging. The grey layer, containing monocytes, is carefully removed and seeding into flasks to allow maturation of monocycles to macrophages.*

2.6.7 Cell counting

Cell numbers were calculated by an automated method using trypan blue exclusion. In brief, cell suspensions were centrifuged at 1000rpm for 5 minutes using a benchtop centrifuge (SANYO, London, UK). The pelleted cells were resuspended and either sample were used directly or diluted further at 1:10 or 1:100 and placed into a counting slide with trypan blue (0.4%). The number of live cells/ml was determined via the automated cell counter.

2.6.8 Cell cryopreservation

Cells were counted as described in 2.6.7, re-pelleted and re-suspended in freezing medium (90% FBS +10% Dimethyl sulfoxide) at a concentration of $2x10^6$ cells/ml. Cells were distributed into cryotubes at 1ml per tube and placed in a NALGENE cryo 1 freezing container

(ThermoFisher Scientific Inc, Loughborough, UK), which was filled with propan-2-ol and stored at -80°C until the following day. Cryotubes were retrieved and stored long term in liquid nitrogen.

2.7 Virus production and handling

HSV1716 (unlabelled) and HSV1716-GFP (GFP labelled) was obtained from our collaborators (Sorrento Pharmaceutics) in stock concentrations of $1x10^8$ PFU in PBS. All vials were stored at -80ºC and freshly thawed before each experiment. A HSV sheep antibody, used in immunofluorescent staining, was also obtained from Virttu Biologics. This was generated by four consecutive monthly injections of 1×10^6 PFU HSV1716 as described in the paper by Connor et al (141)

2.7.1 Infection of macrophages

Macrophages were washed in 1 ml of PBS and medium was changed to RPMI serum free medium (500ul for a 6 well plate and 3ml for a T75 flask). Viral stocks were freshly thawed, on ice, from the -80ºC freezer and added to the medium at an MOI of 5 or 10 (depending on the experiment). Virus was allowed to permeate macrophages for 2 hours in serum free RPMI medium and then additional RPMI medium (supplemented with 10% FBS and glutamine) was added (500ul for a 6 well plate at 3ml for a T75 flask). Macrophages were then incubated for a further 2 hours.

2.7.2 Alamar blue cytotoxic evaluation

Alamar blue is a unique non-toxic cell viability assay which allows the assessment of cell viability without disruption of the electron transport chain and subsequent cell death. The

reagent works by using a weakly fluorescent cell permeable compound known as resazurin. Upon entering the cell, this is actively reduced into resorufin, a highly fluorescent component. Viable cells will continuously convert resazurin into resorufin within the incubation period, resulting in a greater fluorescent intensity (142).

Breast cancer cells (in 100ul of media) were seeded into 96 well plates and were treated with either HSV1716 or check point inhibitors in reducing doses. To assess cell viability, 10µl (10%) of alamar blue reagent was added to each well and incubated for 4 hours (1-4 hours is the optimal incubation time to allow for sufficient time to convert the resazurin into resorufin). If greater detection is required, for instance if there are only a small number of cells present per well, a greater incubation period is recommended (24 hours). The cells were read on a fluorescent plate reader, excitation peak 570nm and emission peak 585nm. After which, the alamar blue reagent was removed and the wells washed in PBS before being replaced with 100µl of complete medium.

2.7.3 Cytotoxic analysis of HSV1716 on aggressive BC cell lines

Alamar blue was used to determine the cytotoxic effects of HSV1716 on multiple aggressive BCs at 24, 48, 72, 120 and 168 hours post infection. The time frame was chosen to assess the acute effects of HSV1716, and its ability to self-replicate after 1 week without repeat administration. The virus was allowed to gradually thaw on ice before infection. The plate was infected with HSV1716 using a serial dilution of multiplicity of infection (MOI) (MOI 10, 5, 1, 0.5, 0.1 and 0.05). Multiplicity of infection equates to the number of viral particles (pfu) per cell. Therefore, an MOI of 1 would equate to a 1:1 ratio of plaque forming units to cells.
An assessment of cell number was performed on the day of infection by taking an average cell count of 4 random wells in each 96 well plate. This allows the correct calculation of MOI.

Prior to the addition of the viral particles, the medium was removed, and cell monolayers washed in PBS. The PBS was removed and replaced by 100µl of serum free DMEM or RPMI to ensure cellular senescence and prevent non-viral induced gene or cytokine production. Cells were allowed to incubate for 2 hours to allow sufficient time for the virus to infect the cells. After 2 hours, complete DMEM or RPMI medium was used to replace the serum free medium, and cells were incubated for a further 2 hours to complete infection. Assessment of cell viability following infection was assessed using the alamar blue assay described above.

2.7.4 Plaque assay

The plaque assay protocol used with this PhD are described in the paper by Baer and Kehn-Hall (143). Vero cells ($4x10^5$ per well) were seeded into 6 well plated in 3ml of medium and incubated overnight until confluent. HSV1716 was defrosted on ice and serial dilutions of virus in PBS were made. Diluted virus (0.1ml) was added dropwise to get an even coverage across the monolayer and cells where then incubated for 2 hours. Following incubation, the viral inoculum was removed, and the agarose overlay was prepared. A sterile solution of 4% agarose in distilled water was prepared by autoclaving at 120^{o}C for 20 minutes. This was then equilibrated in a 65^{o}C water bath and media was added whilst swirling to make up a 1:10 dilution of agarose:medium. A pipette was used to add 3mls of this agarose/medium mixture to each well. The agar was allowed to solidify for 15mins at room temperature and then the plate was moved to a humidified incubator (37^{o}C) for 72 hours. To fix cells, 4% PFA was applied for an hour. Agarose plugs were then removed and washed with PBS. Cells were finally stained with 1ml crystal violet stain for 5 minutes, rinsed with cold tap water and the dried with a paper towel. The number of plaques were counted per well and viral titre was determined as described by Baer and Kehn-Hall (143).

2.7.5 Nitric oxide assay

Macrophages were isolated following the protocol above. On day 7 the macrophages were seeded into 6 well plates and allowed to adhere overnight. Medium was then replaced by RPMI supplemented with 10% FBS and glutamine and left in the incubator overnight. The required wells were infected with HSV1716 at an MOI of 5 or PBS for 4 hours as described in section 3.5. Nitric oxide quantification was performed using an Abcam Nitric Oxide Assay Kit (ab65328). Cells were prepared as per Abcam protocol (without the deproteinization step). Cells were combined with 200ul of ice-cold assay buffer and scraped off using a cell scraper. They were homogenized via vigorous pipetting and spun on a cold centrifuge for 5 minutes. Supernatant was then removed. A 96 well plate was set up with standards and sample wells. To these, 5 ul of nitrate reductase and 5ul of cofactor were added and this was incubated at room temperature for 1 hour. Subsequently 5ul of enhancer was added and left at room temperature for 10 mins. Finally, 50ul of Griess regents 1 and 2 were added to each well and this was left for 15 minutes at room temperature. All plates were read on a spectrophotometer at 562 OD.

2.8 Murine triple negative breast cancer models

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with the approval of the University of Sheffield Ethical Committee (PPL70/8670). Home office licence was obtained for Dr Amy Kwan to carry out the work (PIL number: I3DFA8E11). The number of animals per experiment were consider and the law of diminishing returned was considered to estimate appropriate sample size.

Humane end points for all experiments were the following:

- A body weight decreased of over 20% baseline.

- Significant disease burden (e.g. extensive lung disease on IVIS)/tumour volume over 1500mm^3.
- The posture of the animal demonstrated marked physical discomfort/distress.
- Any symptom to suggest the animal was suffering.

All measurements were made by the same digital callipers and two perpendicular diameters were recorded, and tumour volume was estimated using the following formula;

$$\text{volume} = (\text{length} \times \text{width}^2)/2.$$

2.8.1 Primary Model

Dr Emer Murphy carried out the murine primary PyMT-TS1 model, and subsequent post-mortem analysis and all the remaining animal studies following this were conducted by me during this PhD project. Primary mammary tumours were developed by subcutaneous implantation of 1x10^6 PyMT-TS1 cells in 50µl (50:50 matrigel:cells) into the 4th mammary fat pad of 6-7 week old female FVB mice (N=5/group). When tumour volumes reached 500mm^3, mice were divided into 5 groups and received a single injection of the following treatments: group 1 received 100 µl PBS, group 2 received in intratumoural (IT) injection of 1x10^7 PFU of HSV1716, group 3 received an intravenous injection of 1x10^7 PFU of HSV1716, group 4 received macrophages alone and group 5 received HSV1716 loaded BMDMs. Mice were culled after 9 days of treatment or when diameters reached the maximum permitted size (15mm diameter). Primary tumours, organs and blood were collected for postmortem analysis. Half the tumours and organs were fixed in 4% paraformaldehyde for 2-4 hours prior to freezing and the other half was snap frozen in OCT freezing media.

2.8.2 Metastatic models

As TNBC is most difficult to treat following the development of metastatic disease, a selection of different metastatic models were explored to evaluate which would be the most applicable for use in this PhD. These are described as the "resection model", the "intracardiac inoculation mode" and the "intracranial inoculation model". The scope of this PhD did not allow for full refinement of all models. Animals were obtained from Charles River Laboratory, unless specified, and acclimatised in the biological services laboratory for 7 days prior to the procedure.

2.8.2.1 Resection model

In the resection model, (illustrated in figure 4), 10 BALB/c mice were orthotopically implanted with 1x10^6 4T1-LUC-BR cells in a 50:50 PBS:matrigel mixture into the right 4th mammary fat pad. Mice were observed and weighed daily to ensure no complication of implantation, and the 4th mammary fat pad was inspected. On signs of tumour development mammary lesions were measured using callipers.

Tumours were resected under the guidance of Prof. Penelope Ottewell 14 days after implantation. At this point they were roughly 250-350mm^3. However, many of the tumours were impossible to resect due to invasion of the peritoneal cavity and only 3 of the 10 animals were resected. In the animals where resection was possible, IVIS imaging for metastatic spread was performed every 2-3 days until metastatic disease was seen. Animals were culled when a humane end point was reached or 28 days post resection.

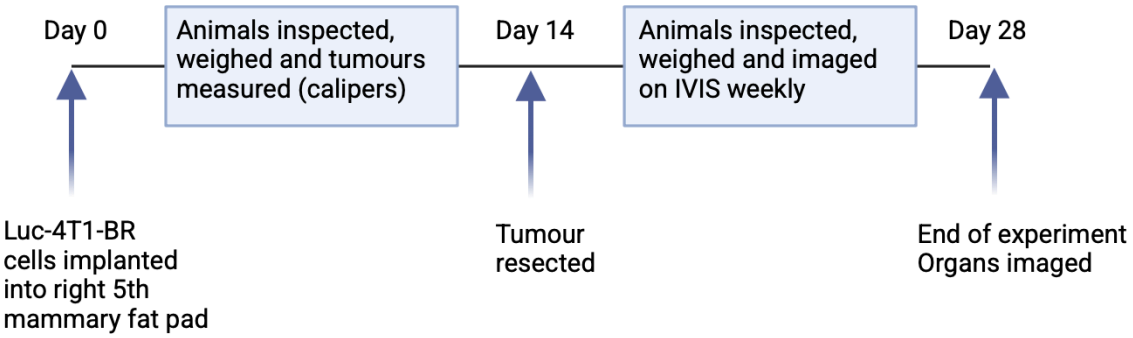

Figure 4: Resection Model. *A schematic representation of the timeline for the resection model experiment.*

2.8.2.2 Intracardiac inoculation model

Female BALB/c mice, 6–8-week-old, were anesthetised using 3-4% isoflurane in 70:30% N_20:0_2. Whilst under anaesthesia, 1 x10^5 LUC 4T1-BR cells were filtered and injected via the intra-cardiac route into the left cardiac ventricle. All animals were allowed to recover in a heated chamber. This work was carried out under the supervision and guidance of Prof. Penelope Ottewell. Initial experiments followed the rate of development of metastatic disease on the IVIS only. This was repeated for a total of 17 animals.

As this model recapitulated the aggressiveness of TNBC, this model was used to explore treatment with HSV1716. In this experiment, 24 animals were inoculated with 1 x10^5 LUC 4T1-BR cells as described above. On day 5 days following tumour cell injection, animals were assigned to one of 5 groups as described below (n=6 per group):

Group 1: 100ul of intravenous PBS at day 5 only

Group 2: 1x10^7 PFU of HSV1716 (intravenously in100ul volume) at day 5 only

Group 3: 1x10^7 PFU of HSV1716 (intravenously in 100ul volume) at days 5,7 and 9

Group 4: BMDM loaded with HSV1716 at an MOI of 10 (intravenously in100ul volume) at days 5,7 and 9

All intravenous administration was performed by tail vein injection. Additionally, six mice were sacrificed to allow harvesting of bone marrow derived macrophages. These were grown as described in the methods section and used on day 7. Macrophages were infected 2 hours before use. Fresh macrophage harvest was performed prior to inoculation. Due to a low number viable of macrophage immediately prior to the planned study the dose of infected macrophages administered per inoculation was as followed. was as follows; day 5: $7.95x10^5$, day 7: $4.59x10^5$, day 7: $1.04x10^5$. All macrophages were infected with HSV1716 at an MO1 of 10. The ethical issues around the animals involved in the experiment with the above changes were considered and discussed with Dr Muthana. It was felt that on consideration of the options, proceeding as above would, on balance, provide to be most ethical.

Animals were imaged using the Lumina II *in vitro* imaging system (IVIS) on day 5 and every 2-3 days later to monitor the growth of metastases. Luciferin was made up from stock solution (15mg/ml) prior to each imaging session and administered at a concentration of 10ul/g by intraperitoneal injection 10 minutes prior to imaging. Animals were observed for a maximum of 50 days. A summary timeline for this experiment is shown in **figure *5***.

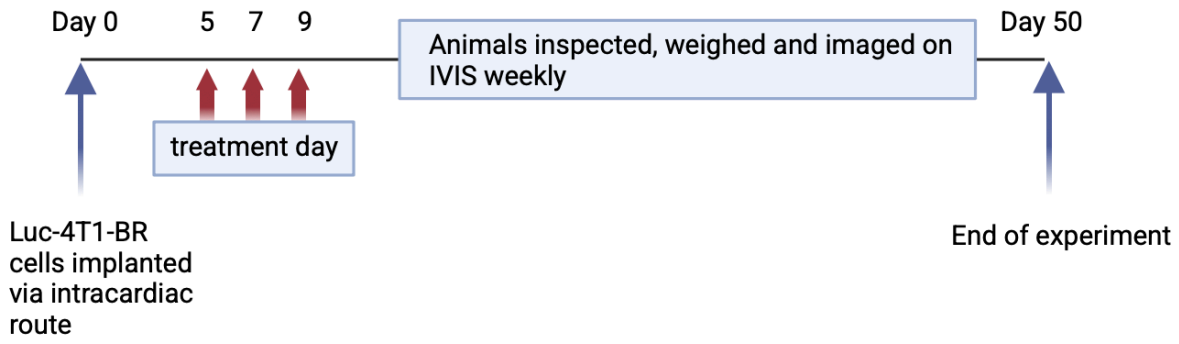

Figure 5: The intracardiac inoculation model. *A schematic representation of treatment schedule within the intracardiac inoculation model*

2.8.2.3 Intracranial inoculation model

Female BALB/c mice, 6–8-week-old, were obtained from Charles River Laboratory and acclimatised in the biological services laboratory for 7 days prior to the procedure. All mice were anesthetised using 3-4% isofluorane in 70:30% oxygen: nitrous oxide. Whilst under anaesthesia and held within a stereotactic frame, 1 x10^4 LUC 4T1-BR cells were injected intracranially. All animals were allowed to recover in a heated chamber. Animals were given 2 doses of analgesia via subcutaneous injection, Metacam (5% solution with 20ul injected subcutaneously), just prior to intracranial inoculation and at 24 hours post recovery. Animals were also given a single dose of antibiotic (Baytril 2.5% solution for injection, diluted 1:10 in saline and injected 20ul intramuscularly) just following the procedure (Figure 6).

Animals were observed daily for 48 hours after the procedure and tumour growth was tracked on the IVIS every 2-3 days. When luminescence of an intracranial lesion was seen, animals were assigned to one of 3 groups (see below). All intravenous administration was performed by tail vein injection.

Group 1: PBS (100ul)

Group 2: HSV1716 (3 doses, 1 x10^5PFU)

Group 3 Macrophage infected with HSV1716 (3 doses, MOI 10)

Animals were observed every 2-3 days with tumour growth progression visualised on the IVIS. All animals were culled 26 days post first intracranial tumour implantation, or if they met a humane end point prior to this.

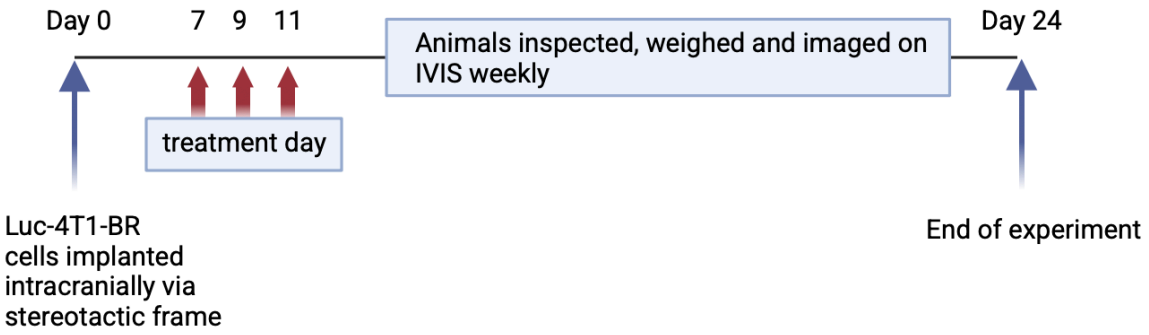

Figure 6: *The "intracranial inoculation model" experiment outline.*

Brain, lung, liver and spleen of all animals were taken and embedded in OCT freezing media. Samples of virus injected and macrophage laden with virus were frozen at -80^{o}C until a plaque assay was done to estimate actual viral concentration given.

2.8.2.4 Clodronate Liposome experiment

To investigate if macrophages are needed for HSV1716 activity, suppression of macrophages through use of clodronate liposomes was assessed. Clodronate, a bisphosphonate, has been used in its liposomal form in a number of studies to selectively deplete macrophages. It has been shown to be internalised by osteoclasts *in vivo* leading to potential bone toxicity. In high concentrations however, clodronate has also been shown to kill RAW 264 macrophages when ingested through initiation of programmed cell death/apoptosis and alveolar macrophages in lungs. Although clodronate can be administered intravenously for systemic depletion and locally, e.g. lungs to target select population; to achieve high concentrations to be toxic to macrophages it can be encapsulated within a liposome. In this form, the liposomes are phagocytosed into macrophages and form a phagolysosome where the liposome is broken down and free clodronate is released. After release clodronate crosses the phagolysosome membrane into the macrophage's cytosol where is its converted to a non-hydrolysable ATP

analogue, adenosine 5'-(β,γ -dichloromethylene) triphosphate (AppCCl 2 p) and irreversibly binds to the ATP/ADP translocase in the membrane of mitochondria (144,145). The mitochondrial membrane depolarises and proapoptotic mediators are released which triggers macrophage cell death and depletion.

This clodronate liposome experiment was designed based on a report protocol which confirmed macrophage depletion 48 hours after administration of one dose of clodronate liposomes (146).

In our study, primary mammary tumours were developed orthotopically through the intranipple inoculation of 50,000 PyMT-TS1 cells in a 1:1 mix of PBS:matrigel into the 4^{th} and 5^{th} mammary fat pads of 6–8-week-old female FVB mice (Charles River laboratory). Animals were anaesthetised for the procedure with 2% isoflurane in 70:30% N20:02. Animals were weighed and primary tumour growth was measured every 2-3 days. When tumours reached $100mm^3$ in volume, they were divided into 4 groups (2 control groups and 2 experimental groups) as follows:

Group 1: Intravenous injection of control liposomes (1 dose – 100ul)
Group 2: Intravenous injection of clodronate liposomes (1 dose – 100ul)
Group 3: Intravenous injection of HSV1716 (1 dose, 1 $x10^6$ PFU)
Group 4: Intravenous injection of clodronate liposomes followed by intravenous HSV1716 (1 dose, 1 $x10^6$ PFU).

This is also shown schematically in **figure 7.** Assessment of the animal's health occurred every 2-3 days and tumour size was assessed using digital callipers. When tumour volume exceeded $800mm^3$ animals were culled. Primary tumours and lungs were embedded in OCT freezing medium and stored at -80°C.

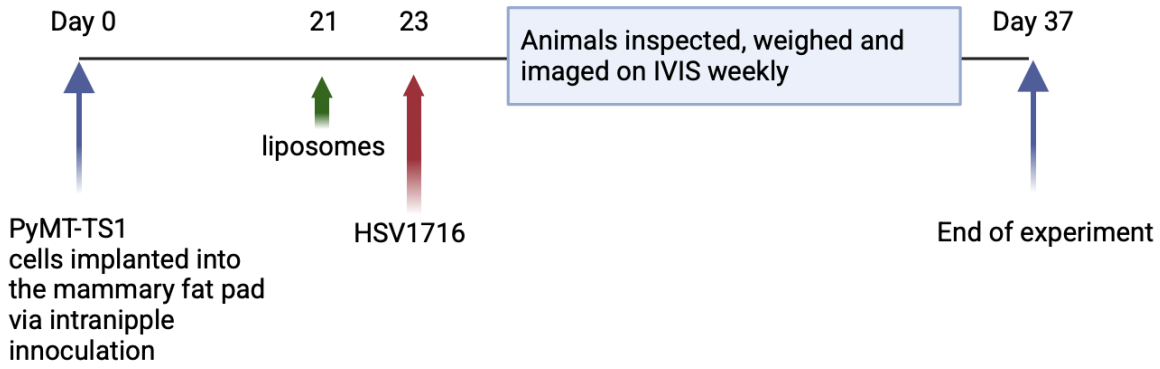

Figure 7: The clodronate liposome experiment timeline. *Primary mammary tumours were developed orthotopically through the intranipple inoculation. Macrophages were then suppressed through administration of clodronate liposomes 48 hours prior to HSV1716 treatment. Tumour growth was assessed by calliper measurement and animals were culled when tumour reached a humane end point.*

2.9 Histology

2.9.1 Cryostat sectioning

Tissue blocks were transferred from -80ºC to -20ºC overnight. They were kept on dry ice whilst waiting to be cut. Blocks were then allowed to gently thaw within the cryostat chamber. The cryostat was used to cut frozen sections of 10um thickness. Tumour blocks were shaved until the sectioned tissue was large enough to be adequately sectioned. Where sites of metastases were uncertain 5 sections were taken every 100um. Sections were transferred onto glass slides and slides were kept in -80 until use.

2.9.2 Haematoxylin and Eosin (H and E) staining

Haematoxylin and Eosin (H and E) staining was performed on both frozen and paraffin embedded sections.

Paraffin embedded formalin fixed slides were initially dewaxed in 2 washes with xylene and then gradually rehydrated using ethanol solutions of decreasing concentrations. Haematoxylin was applied to slides for 2 minutes and then washed in tap water. Slides were then dehydrated through ethanol solutions in increasing concentrations. Eosin in 95% ethanol was applied to the slides for 1 minute. Slides were then rinsed in 100% ethanol and xylene.

Frozen sections were initially air dried for 30 minutes on a slide show. Slides were then transferred to a slide rack and fixed with a 50:50 mix of methanol and acetone for 15 minutes. Slides then underwent 2 washes with PBS. Haematoxylin was applied for 1 minute and washed off with tap water. Slides were dehydrated through ethanol in increasing concentrations (70%, 90%) and then eosin in 95% ethanol was applied for 30 seconds. Finally, slides were rinsed in 100% ethanol and xylene.

All slides were mounted with DPX mounting medium and a glass cover slip. Slides were left to dry for at least 48 hours and then converted to digital format at the Sheffield Institute for Translational Neuroscience (Sitran) Building by Daniel Fillingham. All analysis was performed using the Aperio Image scope software system.

2.9.3 Immunofluorescent staining

Immunofluorescence was performed on samples from the PyMT-TS1 primary breast cancer model. Pre-cut tumour sections were taken from -80^{o}C and allowed to defrost and air dry at room temperature. Fresh frozen samples in OCT freezing medium required air drying and fixing in acetone for 10 minutes prior to blocking. Samples that had been fixed in paraformaldehyde (PFA) prior to freezing did not require acetone fixation. PBST (0.05%) was used to dilute the antibody, for all washings and applied to slides to keep moist.

Prior to staining all sections were blocked with 1% bovine serum albumin (BSA) and 5% goat serum in PBST for 30 minutes at room temperature. Primary antibody (Appendix 4) was then applied for 60 mins at room temperature or overnight at 4ºC. Slides were then washed three times in PBST. Secondary antibody was applied for 40 mins if needed. Slides were then washed a further three times and DAPI was applied for 3 mins. Slides were washed in PBST one final time and mounted using anti-fade Prolong Gold. All slides were dried at room temperature overnight and then transferred to the 4 ºC fridge until they were visualised.

Slides were visualised on a Nikon A1 confocal microscope. An 40x objective lens was used for imaging and at least 5 randomly selected fields of view were captured per tumour. Fiji (image J) was used to analyse the images. Staining was noted to be intracellular, extracellular or cytoplasmic.

2.10 Statistical considerations

The primary study objective was to develop an exploratory model (described in chapter 3) to assess the relationship between the tumour microenviroment and HSV1716 alone (described in chapter 4) or with macrophage delivery (described in chapter 5) with the outcomes of development of an inflammatory tumour microenviroment and treatment efficacy defined by tumour volume, overall survival and the development of metastatic disease. To address this, several studies as described in the methods were conducted to test the hypotheses as described in the aims. *In vitro* experiments were repeated in triplicate to minimise random/type 1 errors and the mean of these were used in calculation of significance. For *in vivo* experiments, the number of animals per experiment were consider and the law of diminishing returned was considered to estimate appropriate sample size as described in the paper by Charan and Kantharia (147). Here, the value E is calculated using the following formula.

E = (total number of animals) – (total number of groups). An E of between 10-20 is considered adequate. For the experiments a part of this PhD, this estimated a minimum of 5 animals would be appropriate, however, to correct for attrition, a minimum of 6 animals was used. Formal power calculations were not used as the effect size was unknown. In general, Student T tests were performed when 2 variables were under comparison. Statistical analysis of data was performed using GraphPad Prism 8 (GraphPad Inc,San Diego, USA) and expressed as mean and SEM. Further details of individual statistical tests are described in the figure legends. A p-value of <0.05 was deemed statistically significant. For multi variate analysis, a 1-way ANOVA was performed using GraphPad Prism 8 (GraphPad Inc,San Diego, USA).

Chapter 3

3 Development of a preclinical model to assess the efficacy of HSV1716 in TNBC.

3.1 Introduction

Traditional breast cancer therapeutics have been developed incrementally with the increasing in understand of the cancer biology. Early chemotherapy treatments targeted the high mitotic rate seen in cancer and therefore focused on terminating cell division. Other treatments encouraged apoptosis. The models used for these treatments are good models to assess cell division or apoptosis but did not initially allow for stratification based on tumour biology. Delineating whether a mammary tumour is hormone or HER2 positive has led to a correlation with clinical features and the development of targeted treatment which has led to landmark changes in the efficacy of treatments within these groups. Those that do not express either hormone (ER or PR) receptors or the HER2 receptor are known as triple negative. Within this group there is marked tumour heterogenicity and has been historically difficult to find treatments for patients with these tumours. Novel treatments are of increasing interest and oncolytic virotherapy, which encompasses the dual modality of action of direct tumour lysis and stimulation of downstream immunogenicity, may help bridge this gap.

The choice of mammary model used to assess a new therapy can significantly influence the success, or failure, of a therapeutic (148). Since significant investment goes into the development of a potential therapeutic, it is pertinent to consider model type prior to testing efficacy. Model choice is dependent upon the hypothesis of the experiment and should consider whether these either mimic what is seen biologically on a microscopic level, or clinically at a macroscopic level. For virotherapy, it's dual mode of action needs to be considered. For

example, immuno-deficient “nude” mice will not stimulate the host’s immune system in the same fashion as a clinic patient.

Since our group’s experience with OV was predominantly in prostate cancer (149), this chapter initially sought to explore and a variety of TNBC models. The overarching aim of this work would allow for the optimisation of a suitable breast cancer models for this work and thereby achieve the first objective of the PhD; to identify a suitable breast cancer model. To begin, an extensive review of the literature was carried out and this summarised in the published review in section 3.2.

3.2 Review Article: An Overview of the Bench to Bedside Models of Breast Cancer in the Era of Cancer Immunotherapy

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Abstract

One of the barriers to novel treatment developments within breast cancer is the ability to prove efficacy in the preclinical setting before moving on to clinical trials. Preclinical models range from single cell monolayers to more sophisticated humanised PDXs systems each with their set of advantages and limitations. Modelling the immune environment in cold tumours, such as breast cancer can also be challenging as are currently no clearly defined markers that can stratify patients based on treatment response. Immune checkpoints receptors such as PD-L1 may not show predictive outcomes in this tumour type. Furthermore, the heterogeneity of breast cancer may be difficult to recapitulate at the bench side. In this review, we provide an overview of the available *in vitro*, *in vivo* and *ex vivo* models of breast cancer with consideration of how these may extrapolate to the investigation of the role of the immune system and immunotherapy developments in breast cancer.

Introduction

Breast cancer is the most common cancer in women. It is incredibly heterogeneous and has been classified into several subtypes based on cell receptor status which allow for stratification of treatments and prediction of prognosis. The invasiveness and metastatic potential of tumours is largely dependent on their subtype and treatment designated accordingly. The outcomes on early breast cancer have improved significantly over the last 25 years due to optimization of chemotherapy regimens and the addition of targeted therapies such as hormonal treatments and antibodies against the HER2 receptor. However, in the metastatic setting, although there is a trend to improved outcomes (150–152) particularly in the HER2+ patients, long-term prognosis remains poor and there is a clear need for more effaceable treatment options for these patients.

Development of novel therapies is dependent on the ability to prove efficacy in the preclinical setting. However, a large proportion of promising therapies have never made it to clinical trial. Part of this failure may be due to the limitations of preclinical models to mimic the complexity of the heterogeneous tumour microenvironment. The number of breast cancer models are vast, and the choice of model is often based on the question posed. Immortalized cell lines, for example, can be used to correctly identify new treatments, such as tamoxifen in ER+ breast cancer (153). In humans, 80% of triple negative breast cancers (TNBC) are basal by molecular profiling (154,155) and the majority of the cell lines described have a basal molecular class which means treatments can be directed to the most common groups. However, some treatments may not produce effects within a 2D model, necessitating the use of more complex 3D modelling systems (156). In addition, data published by Hollern et al (157) described that despite the vast heterogeneity within mouse models of breast cancer and these may or may not represent what is seen clinically. Furthermore, exploring the immune system within conventional models may pose more of a challenge as the immortalized cell lines used are

often derived from a human source and are therefore only grown in immunosuppressed animal hosts.

The intention of this review is to provide an overview of the current available models of breast cancer (Table I) and the advantages, limitations, and challenges that each face when applied to novel and immunotherapeutic drug discoveries and how this may be extrapolated into future clinical trials. In this, readers will develop an understanding of the current *in vitro, in vivo* and *ex vivo* techniques.

Table 1: Current models of cancer

Origin of cells	Model type	Advantages	Disadvantages	Examples of use with immunotherapies
Immortalized cell lines	2D monolayer	-Easy and cheap - Can look for direct cell to cell changes	-Unable to replicate TME -Cell lines may not be truly representative of patient's cancer	Detection of CTLA-4 receptors on a number of immortalized cell lines allowed testing of a CTLA-4 inhibitor on these cell lines with a promising response (158)
	3D Spheroid	-Easy and cheap -Necrotic center -Direct cell to cell interactions	-Unable to replicate TME - Not truly representative -Lacks heterogenicity	Spheroids of HGC27 were incubated with T cells in a model to evaluate the cytotoxicity of the PD-1 blockade. These provided useful information about T cell cytotoxicity within this system (159).
	3D scaffold	- Some replications of TME -Can assess diffusion	-No vasculature structures -Cell lines may not be truly representative of patient's cancer	Using a hydrogel scaffold, mutant Ad5-3Δ-A20T infected pancreatic stellate cells indicating improved viral spread within the microenvironment in this 3D hydrogel model that would have not been discernable in 2D culture (160).
	3D microfluidics	Able to assess perfusion or flow of substances using micro vessels	-Cell lines may not be truly representative of patient's cancer -Lacks heterogenicity	A multicellular tumor-on-a-chip platform involving breast cancer cell lines, monocytes and endothelial cells within a gelatin hydrogel was infused with T cells to assess T cell movement and cytokine release (161).
	Immunodeficient mouse model	- Allows more complex modelling of substances - Can use human derived cell lines	- Lacks immune system - Cancers may not be truly representative of patient's cancer	Female NOD/SCID mice were used to general a model of pancreatic cancer which demonstrated immune cells enhanced the activity of gemcitabine, erlotinib and NK cells (162).
	Immunocompetent mouse model	-Allows more complex modelling of substances - Immune system present	-May not be truly representative of patient's cancer -Differences in mouse and human immune response	C57BL/6 female mice were inoculated with a B16 melanoma cell line and treated with a CTLA-4 blockers and GMCSF vaccines which demonstrated efficacy and toxicity with autoimmune depigmentation (163)
	Humanized mouse model	- complex modelling of Immune system - human derived cell lines	-May not be representative -Dampened immune response -Autoimmunity against host	HCC827, NCI-H1975, HSC4, RKO PD-L1 positive cell lines were engrafted on to humanized NOG mice deficient for mouse FcγR genes to evaluate the anti-cancer effects of nivolumab (164).

GEMMs		-complex modelling - intact immune system present	-Cancers may not develop or respond in the expected way due to genetic alterations	Transgenically bred PD-1-deficient mice were used as part of this study to confirm that the administration of an anti-PD-L1 antibody suppressed tumour growth in a myeloma cell line (165).
Patient derived	Dispersed cells (*ex vivo*)	-quick screening -associated cells e.g. fibroblasts	-Short life span of cells -Immune cells unlikely to be present	-*Ex vivo* co-culture models assessed immunotherapy in patients with colorectal cancer (166)
	Organoid	-Able to represent the patient's TME	-Short life span -lacks vasculature and immune cells -Suitable tissue hard to source	Paired melanoma and lymph node specimens from patients with advanced melanoma formed viable organoids (90%). Treatment with pembrolizumab, nivolumab, ipilimumab and dabrafenib/trametinib matched clinical response (85%)(167).
	PDX – humanized models	-Able to represent patient's TME -Immune system present	-Cost and time consuming -Suitable issue hard to source -High rate of failure to take grafts	Partially human leukocyte antigen matched TNBC PDX cells formed tumours in humanized IL2Rγnull (hNSG) mice. Human CD45+ cells were detectable in PDXs models and anti-PD-1 antibody therapy caused reduction in tumor growth and increased survival consistent with clinical findings (168).

Immunotherapy in breast cancer

Historically the mainstay of breast cancer treatment has been a strong chemotherapy backbone with clinical responses elicited from many chemotherapy agents including anthracyclines, taxanes, carboplatin and fluorouracil. Understanding of the cancer heterogenicity and selective targeting of aberrant pathways have increased the treatment repertoire. Currently treatment can be classified into chemotherapies, targeted therapies (including hormonal targets) and immunotherapies and are often administered in combination regimes to increase efficacy and decrease the occurrence of cancer resistance. Immunotherapies are a relatively new addition to the breast oncologist's tool kit and, if it's use mirrors other tumour types, it is likely to feature more prominently in coming years.

Modulation of the immune system using immune checkpoint inhibitors has transformed the treatment of solid malignancies such as melanoma, renal and lung cancer (169). It's use in breast cancer was initially contentious as some argue that the lack of an inflamed immunogenically hot tumour microenvironment, particularly within the ER+HER2- group, will preclude a response to such treatments. As such, clinical studies of immunotherapies have been primarily limited to TNBC with proven clinical trial success.

Immunotherapy agents which have reached clinical trial stage can be classified into 3 areas: immune check point inhibitors, adoptive cell transfer and oncolytic viruses. Checkpoint inhibition has been the most well researched with 3 main agents described in breast cancer: pembrolizumab, atezolizumab and avelumab. Pembrolizumab is a humanized monoclonal IgG4 kappa antibody to the PD-1 receptor and blocks the interaction with PD-L1 and PD-L2 on tumour cells. It now sits as one of the standard treatment options for both early and metastatic breast cancer based on the results of 2 significant KEYNOTE trials. In the KEYNOTE 355 trial (61), 847 patients with advanced untreated TNBC were randomized to

receive either chemotherapy plus placebo or chemotherapy plus pembrolizumab. In final interim analysis after 44.1 months of follow up, patients stratified by PD-L1 a high combined PD-L1 score of over 10 had a significant increase in overall survival from 23 to 16.1 months (P=0.0185). Additionally, the use of neoadjuvant pembrolizumab in early breast cancer has become a new standard of care in the UK following the KEYNOTE 522 trial, which revealed a decreased in cancer related event free survival in patients treated with combination neoadjuvant chemotherapy and pembrolizumab compared to chemotherapy verses placebo(62)

Atezolizumab is a humanized monoclonal antibody immune checkpoint inhibitor that selectively binds to PD-L1. It was initially assessed in combination with paclitaxel for all breast cancers which included 45% of patients had TNBC. The results from this shows promise in the phase II trial with a median overall survival of 21.3 months with atezolizumab plus nab-paclitaxel and 17.6 months with placebo plus nab-paclitaxel (63). It was this that initially led to early FDA approval for the use, however use of this was withdrawn a couple years later after the phase 3 trial IMpassion131 revealed that adding atezolizumab to paclitaxel did not improve PFS in the PD-L1–positive population with a PFS of 6.0 months for patients who received atezolizumab and paclitaxel compared with 5.7 months for patients who received placebo and paclitaxel (64). More recently Atezolizumab has been trialled in a phase 2 trial in combination with carboplatin for patients with metastatic TNBC (TBCRC 043)(65). Here, PFS was increased by from 2.2 to 4.1 months which is a similar benefit to the results from KEYNOTE 355. Interestingly, patients with high TILs, high mutation burden and prior chemotherapy received greater benefit to the addition of Atezolizumab to carboplatin and those with luminal androgen receptor positive TNBC fared worse. The phase 3 trial is currently recruiting, and it will be of interest to see how this changes the landscape of breast cancer management in the future.

Avelumab, another monoclonal IgG1 antibody directed against PD-L1, is currently in the early phase of clinical investigation for use in breast cancer patients. At present there are reported phase I and phase II trials of Avelumab alone and in combination with other agents which shown promise(66–68). Of interest the combination of Avelumab and a PARP inhibitor, talazoparib, has been reported in a phase 1b and 2 nonrandomized controlled trial, in patients with advanced solid tumors stratified by tumour type. The patients with breast cancer the ORR was 18.2% (95% CI, 5.2%-40.3%) in patients with TNBC; 34.8% (95% CI, 16.4%-57.3%) in patients with ER-positive, HER2 negative breast cancer; and 63.6% (95% CI, 30.8%-89.1%) in patients with platinum-sensitive, BRCA1/2 mutated breast cancer(66). These results may suggest a niche for this combination of treatment and larger phase III trials would be needed to help define this. Avelumab has also been trialled in combination with radium 223 to specifically target patients with predominant bony metastatic disease.

Adoptive cell transfer, most commonly CAR T cells, have been of increased clinical interest due to the specificity and personalization of treatment. Here they can be used alone, or in combination with other immunotherapy agents. One such study (74) includes a description of 42 patients with treatment refractory metastatic breast cancer who underwent surgical resection of a metastatic lesion(s), isolation of TIL cultures, identification of exomic nonsynonymous tumor mutations, and immunologic screening for neoantigen reactivity. Following this, 13 patients were found to be suitable for T cell transfer and 6 patients were recruited to a II pilot trial of adoptive cell transfer of selected neoantigen-reactive TILs, with a short course of pembrolizumab. Of these, objective tumor regression was noted in three patients, including one complete response (over 5.5 years) and partial response in 2 (6 and 10 months). The time involved and cost of screening for such patients is high and further refinement of CAR T therapy is needed before it reaches mainstream adoption.

Oncolytic viruses are treatments which cause both direct tumour lysis and stimulation of an immunogenic response. They exist as many forms and can have a de novo or engineered preference for replication within cancer cells. There are many phase 1 trials of dose escalations and tolerability for viruses within the breast cancer setting, however, phase II trials are limited to a reovirus(170), herpes virus(171) and oncolytic vaccinia virus(172). The draw of oncolytic virotherapy is the changes that are observed within the tumour microenvironment (TME) as this has been shown to induce the inflammation of the tumour microenvironment by the initiation of immunogenic cell death(173). Together this tactic can be potentially used to sensitize otherwise refractory TNBC to immune check point inhibitors and increase response to oncolytic virus treatment (174). Clinical studies of combinations of immunotherapies in breast cancer are therefore emerging.

Despite the advances described above, there are still many unknowns, why does immunotherapy work for some patients and not others? Are there any ways that we can improve response rates to treatment? These questions may be answered through further understanding about the tumour biology and the tumour microenvironment explored through the selection of the appreciate breast cancer model.

In vitro techniques

Utilising cell cultures make it possible to understand cell biology, tissue morphology, mechanisms of diseases, drug action, protein production and the development of tissue engineering (175). Traditionally, cancer drug discovery started by assessment of response using a monolayer of immortalized, well characterized cell lines. They can be derived from a number of hosts however human and murine derived are most common. These cell lines were established from aggressive primary tumours or their metastatic sites and some date back to the 1950s. Over time they have kept their malignant potential and are cryopreserved in liquid

nitrogen until needed. They are at risk of contamination and changes in baseline characteristics with repeated passages such that cell lines are often replaced when the passage number exceeds 30. Some are engineered to express proteins such as GFP and luciferase which allow dynamic quantification of the cells of interest. These cell lines can be used to generate both 2D and 3D models of breast cancer within matrices of varying complexities. However, given the heterogeneity of breast cancer, there is a significant loss of generalizability of the data from these cell lines to the clinic.

Two-dimensional cell culture systems

In two dimensional (2D) models, monolayers of single cell lines are grown in tissue culture plates and passaged when confluent. The advantages of this model type are the simplicity, reproducibility, and low cost. This is of particular importance in high throughput screening. The access to these cell lines are widely available and there have been a number of reviews summarising the key characteristics and behaviour of these established cell lines (176–178). To assess immune function 2D cancer cell cultures can be enriched or co-cultured with immune cells and immune cell mediators which can simulate the TME. Immunogenic cell death is a feature that lends well to be studied through 2D cell culture systems. Here, co-cultures of cancer cells and immune cells of interest can be co-cultured(179–181). Cancer cells can then be stimulated to undergo immunogenic cell death and the phagocytosis, effect and maturation, activation of the immune cell of interest can be assessed through flow cytometric measure of cell surface markers or ELISA of cytokines such as HMGB1, IL-17 and type 1 IFN (182,183). To simulate the heterogenous make-up of the TME, co-cultures can be made more sophisticated through the addition of numerous cell lines. In our laboratory, lymphocytes are isolated from waste buffy coats before co-culturing with monocyte derived macrophages and 2D cultured human derived TNBC cells. We have shown that within this co-culture mixed lymphocytes show activation when exposed to an oncolytic virus treatment(173).

The main advantage of 2D models is the effect on cells can be directly observed and variables can be controlled to confirm causality rather than association. However, as the external conditions in which cells are grown does not mimic the natural host's system, behaviour of the cells may be different to those seen *in vivo* and changes in cell morphology, due to adherence to the bottom of plastic plates, cause cells to be longer and flatter which changes their exposure to the culture medium. Additionally, an assessment of hypoxia is not possible as monolayers receive a uniform homogenous amount of nutrients that is replenished with each passage (175). Furthermore, monolayers of cell lines are often grown in isolation and therefore do not recapitulate the innate tumour cell heterogeneity and tumour microenvironment as they lack the cell-to-cell interactions, tissue structure and surrounding cellular components of the tumours such as fibroblasts, macrophages, and other immune cells. Traditionally, researchers have moved straight from 2D models to *in vivo* assessment of novel therapeutics. However given these limitations, this may not be ethical and incurs financial and time expenses, therefore there is a growing interest in advanced cell culture techniques which involve the inclusion of a structure for cells to adhere to, co-culture with non-epithelial cells or a diffusion gradient created through microfluidics.

Three-dimensional cell culture systems

Three-dimensional (3D) cell cultures systems have developed in response to a growing awareness of the importance of the interactions between tumour cells and the extracellular matrix (ECM) they are suspended in. Therefore, although 2D cell culture is useful in high-throughput screening of drug in plates to assess sensitivity to differing agents, 3D cell culture may be more useful in drug discovery and can potentially lessen the importance of *in vivo* work. The extracellular matrix consists of a milieu of different protein structures and growth factors that facilitate interconnections between cells. Alterations in ECM composition may influence drug response through altered drug availability, expression of drug targets, or

changes in cellular defence (184). The advantages and disadvantages of 3D over 2D cell culture systems is summarized in table 1 in the paper by Kapałczyńska et al (175) Importantly, 3D culture allows the possibility of co-culturing cancer cells with other cell types within an infrastructure that can reproduce the challenges of delivering treatments to the TME. For example, the co-culture of cells of either different type or origin can allow for the assessment of cross talk between these cells. Arrigoni et al., describe a systematic review of breast cancer metastasis towards bone and how the interaction between the bone microenvironment and tumour cells can be stimulated by co-culturing bone and cancer cells in numerous ways. This included incubating cells in tumour conditioned medium, directly mixing bone and cancer cells, or allowing cancer cells to permeate an artificial bone membrane and track into the monolayer of bone cells (185).This concluded that advancement in understanding of bone metastasis was only possible because of the precision and control of co-culture *in vitro* systems which would not have been possible in an *in vivo* system alone.

Three-dimensional cell cultures have also started to expand into the world of addressing immune system modelling. For example, an immunogenic 3D breast cancer model was recently described using MDA-MB-231 cells and patient derived immune cells cultured at ratio of 1:1 (186). The addition of patient-derived cells more accurately represents the TME the crosstalk between both cell types to be studied for up to 10 days, as well as the assessment of antitumour immune responses to immunotherapies. These experiments have shown differences in response between the tumour cells alone, tumour and immune cells and immune cell alone groups (unpublished observation) which support the need to use advanced cell culture techniques when exploring immunotherapeutic efficacy. Tevis et al. generated a TNBC 'heterospheroid', containing breast tumour cells and macrophages embedded in a collagen gel (187). This model displayed increased secretion of anti-inflammatory IL-10, suggesting that the macrophages adopt a more M2-like phenotype upon co-culture with MDA-MB-231 cells.

Furthermore, this model exhibited resistance to paclitaxel treatment in comparison with MDA-MB-231 monoculture spheroids.

Although there are several 3D cell culture systems in the literature, they can be broadly divided into scaffold dependent or scaffold independent. Scaffold independent systems rely on the self-aggregation/organization of cells when placed in specialized culture plates or media. For drugs where hypoxia is important, spheroids can be created with their own ECM and grown to a size where they demonstrate a hypoxic gradient within its core. The main disadvantage of spheroids and other non-scaffold systems is challenges in the reproducibility of these cell models in terms of size and culture conditions. An exploration of the substrate in which breast cancer cells are grown have led to the development of hydrogels. One particular model describes the use of an alginate/Matrigel hydrogel to study invasion of TNBC MDA-MB-231 cells. Malignant cellular morphology such as invadopodium, actin-based protrusions of the plasma membrane through which cells anchor to the extracellular matrix was demonstrated (188). This feature is thought to be key in the development of metastatic potential of malignant cells and may provide insight into how this mechanism may be addressed with cancer therapies. Interestingly, these 3D models can also be used to model the inflammatory microenvironment where different cell types can be incorporated e.g. adipose-derived stromal cell (189) thus also enabling obesity in breast cancer to be investigated (190).

In essence 3D models have a potential to provide small, controlled environments to repeated assess with immune cells for a short duration of time. Within these environments the mix of cell lines, cell types and even tumour microbiome can be altered to further mimic the heterogeneous nature of the TME. These models can be made more sophisticated using microfluidic assays.

Microfluidic Assays

Microfluidics assays (MFAs) are a branch of three-dimensional models that are intended to recreate *in vivo* microenvironments *in vitro*. These MFA devices or chips can vary in design but are commonly made from transparent moulded or engraved materials suitable for cell culture to allow the imaging and real-time tracking of cells introduced into the MFA devices using confocal microscopy. They may focus upon the internal dynamics and structure of the vessel lumen or on the external perivascular microenvironments but are based on the application of fluid flow to channels through the device. MFAs have been adapted to form 'organs on chips' by growing micro vessels in gels that mimic the extracellular matrix (ECM) of various tissues or organs (191) .They are now increasingly being used to look at events within and close to micro vessels in defined environments like gels *in vitro*. They have already been used to extensively monitor both the intravasation and extravasation of cancer cells through micro vessels (192) and are now increasingly being used to explore the role of immune cell subsets with one another and micro vessels (193,194).

There are typically two kinds of MFA models, those that use a precast pattern or network, in which endothelial cells are seeded in to coat the exposed surfaces and models that rely on vasculogenesis, in which the micro vessel network is formed by mixing endothelial cells in ECM gels. Bischel and colleagues used a precast device to generate micro vessels within a collagen/Matrigel hydrogel. Briefly, the channels of an MFA device were filled with polymerised ECM gel. Media was then pumped through this until it had re-created channels through the gel, and then lined these with HUVECs to produce vessel-like structures (195). The Kamm lab(196) also used HUVECs and a gel to form a microvascular network in an MFA device. With their model, the microvascular network could also be formed in the presence of human lung fibroblasts (HLFs) (196,197). Here, the MFA device consisted of 3 parallel channels, the centre channel, designed to hold the hydrogel, was separated from the

outer media channels by trapezium posts. These held in the pre-polymerised hydrogel and stopped it leaking into the media channels. To produce a microvascular network, HUVECs and HLFs were seeded in a co-culture in a fibrin gel. Then over a 4/5-day period they formed vessel-like structures with lumens that span the central gel region and connected the flanking media channels(196).

Microfluidic systems are often used to access flow. In the immunotherapy environment the bystander effect of oncolytic virus therapy is of interest. Lee et al designed a link system of two microfluidic-based models which mimic the delivery of oncolytic viruses through the blood stream to cancer deposits. The flow condition used were similar to flow level that can generate the interstitial flow and shear stress for simulating the *in vivo* microenvironment and the dispersal of oncolytic virus within the system can be observed. However, this system was limited by the lack of immune cells with the assay (198).

Other groups have adapted these MFAs to investigate the 3D interaction of human monocytes/macrophages with human tumour cells. This has shown how the model can be used to monitor the interaction between human tumour cells and immune cells - and identify the mechanisms of their interaction. In one such example, a single media channel was lined with HUVECs and introduced macrophages and A549 lung carcinoma spheroids into the collagen gel in the middle channels. Prior to embedding in the gel macrophages were preconditioned into a M0, M1, M2a, M2b and M2c phenotype, this was done by treating macrophages derived from buffy coat isolated monocytes for 24 h. Macrophages were left untreated (M0), grown with LPS and IFN-γ (M1), IL-4 (M2a), human IgG and LPS (M2b) or IL-10 (M2c).

Macrophage infiltration towards tumour spheroids and the effect of differences in macrophage phenotype on dispersion for the A549 aggregates in the gel were observed. Of note, culturing

with macrophages of an M1 or M2b phenotype showed the greatest dispersion of tumour spheroids, these effects were seen with and without HUVECs lining the media channel. Dispersion of tumour cells was seen to be promoted by contact dependent mechanism involving CD11a and CD11b. When there are blocked macrophages or its receptor, ICAM-1 on A549 cells, a significant decrease in the dispersion of tumour spheroids is observed (199). A more recent study by the same group showed that human monocytes infused into MFAs extravasate through the micro vessels into the perivascular region. Here the role of the CCR2 signal is correlated to relate to tumour growth and invasion through promotion of angiogenesis, recruitment of M2 like macrophages and suppression of CD8+ T cells. It is reported that a higher number of inflammatory, $CCR2^{+}$ monocytes were able to extravasate through the vasculature than those which were $CCR2^{-}$. Moreover, following extravasation $CCR2^{+}$ monocytes begin to upregulate MRC1. However, this was not linked to extravasation, as monocytes that were seeded directly into the fibrin gel, with HUVECs and HLFs also displayed the same levels of MRC1 upregulation (200). Studies like the above have since led to the development of advanced MFAs to study human breast cancer cell extravasation into an actively secreting bone microenvironment generated by embedding human bone marrow-derived mesenchymal stem cells (MSCs), endothelial cells (ECs) and osteoblast-differentiated cells (OBs) using a gel system. The ECs form vasculature, whereas MSCs and OBs create a bone microenvironment. Cancer cells introduced in the vessel extravasate into the organ-mimicking gel which can be used as a drug screening platform(201). Microfluidics can also be used to test drug sensitivity to treatment. In a novel model involving a co culture of MDA-MB-231 cells with HMEpiC cells, cancer cell migration was assessed through assays of IL-6 and CK14. In this model, treatment with anti-cancer agents paclitaxel and tamoxifen were shown to decrease migration.

Although there are several advantages to *in vitro* models, namely the convenience, reproducibility, lack of animal work and potentially cost savings, the use of a host allows the exploration of a medications effect on the whole-body system. This is particularly important for immunotherapies where key benefits of treatment are to induce systemic anticancer effects through stimulation of the immune cascade.

In vivo **models**

The complexity of the human vasculature and drug clearance is best assessed within a living model and *in vivo* studies are felt to be a required "gold standard" before clinical trials.

In vivo breast cancer models can be formed through genetic modification, spontaneous/chemically induced tumorigenesis or implantation of human, animal derived (predominantly murine) or human cell lines. There are significant differences between human, humanised and murine breast cancer cell lines notably in relation to the tumour environments compared to patient derived xenografts(202). In particular, the tumour immune interactions (both at the site of the tumour and systemically) can be observed, and additionally the stromal components of the tumour (e.g. fibroblasts) are of tumour origin. These differences mean that the true nature of immunotherapies may not be best exposed in their models.

Immunocompetent animal models of breast cancer are limited. Murine breast cancer can either arise sporadically and spontaneously in fully immunocompetent non transgenic mice mimicking the de novo presentation of human breast cancer, be induced through inoculation of a known murine breast cancer cell line or arise spontaneously in mice that been engineered with transgenic genetically engineered mouse models (e.g. GEMMs.). Below is a discussion of the use of patient derived and genetically engineered mouse models.

Genetically Engineered Mouse Models

Genetically engineered mouse models are created by random integration of a transgene into the genome, which results in gene overexpression in transgenic mice, gene deletion in conventional and conditional knock-out mice or targeted insertion of the transgene in a specific position known as knock-in mice (203).

Conventional knock-out mouse models are advantageous due to enabling the study of specific oncoproteins and allowing the analysis of the interactions between protein domains and mutations and how they contribute to the progression of disease. The immune system remains intact, and different stages of tumour progression can be studied, including metastatic disease. Regarding immunotherapy an increase in the mutational burden can lead to the formation of neoantigens that can be recognized by immune cells (204) and this can lead to the evolution of the anti-cancer immune response, and studies of how this may affect the effectiveness of immunotherapy. Limitations arise with regards to the process being time consuming and expensive, as well as the consistency of the models being executed, this is due to the knocked-out genes being switched off, in all cells, at all times. Knock-out mice also do not truly represent human tumour development, due to the mouse microenvironment.

Conditional knock-out mice are models where chemically generated transcription factors or deletion of tumour suppressor genes can be controlled, with regards to when the target gene is turned on or off(205). This gives advantage over conventional knock-out models due to the decreased risk of the mice displaying developmental abnormalities, which increases the consistency of models being executed with aids in reproducibility. A summary of genes which have been targeted in breast cancer models are shown in figure 1 and table 2.

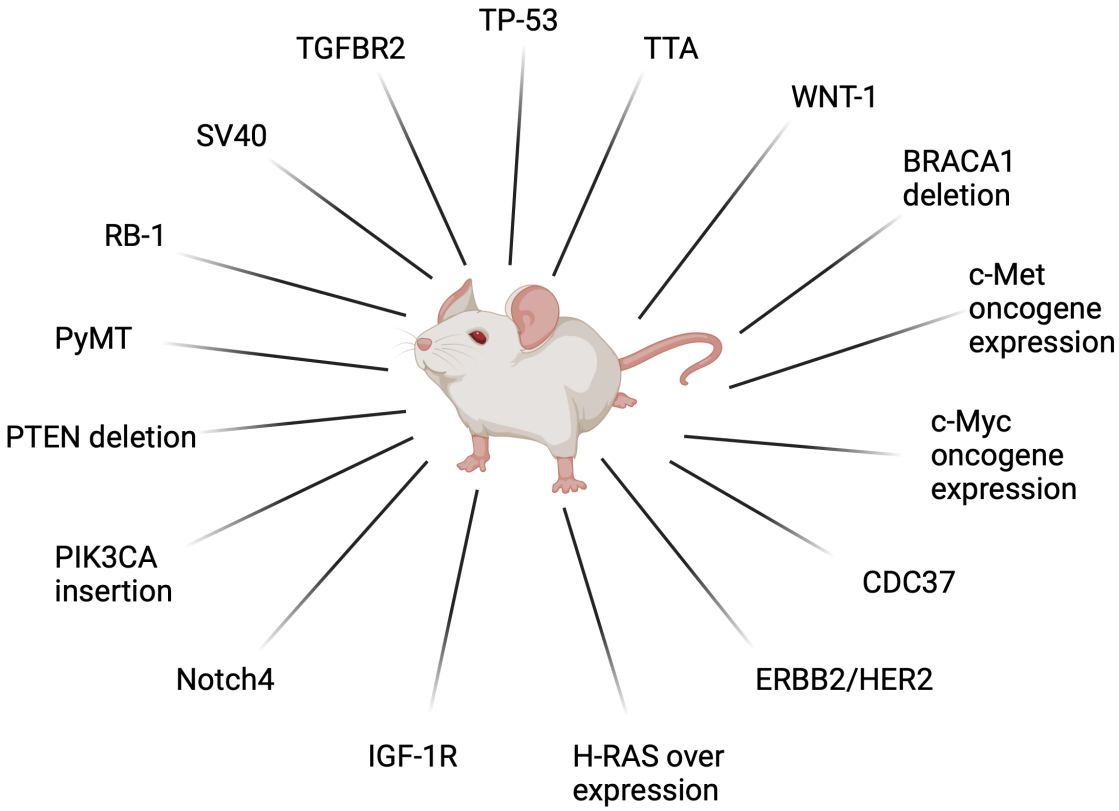

Figure 1: *The spectrum of genes that can be engineered to be over or under expressed within mice to form GEMMs. Genes can be deleted singularly or in combination.*

Table 2: *A selection of published transgenic mice strains available in breast cancer and their features.*

Gene	Strain	Model name	Features	Ref
BRCA1 deficient	Mixed	BLG-Cre;Brca1F22 – 24/F22 – 24;p53+/−	Basal ER negative, HER2 +	(206)
expression of c-Met oncogene	FVB	Met^{mut} (M1248T/L1193V)	Met receptor expression	(207)
Expression c-Myc oncogene	FVB	MTV/MYC fusion gene		(208)
Over expression CDC37	Mixed		Poorly metastatic	(209)
ERBB2/HER2	FVB	FVB/N-Tg(MMTV-ErbB2*)NDL2-5Mul mouse	Expresses PDL-L1 and responds to PD-l1 inhibitors	(210)
H RAS over expression	FVB	MMTV-v-Ha-ras	Salivary and lymphoid and mammary tumours	(211)
IGF-1R	Mixed		Produces salivary gland tumours Weakly ER/PR +	(212)
PIK3CA insertion	FVB	Pik3ca(H1047R);MMTV-Cre mice	Multiple tumour subtypes	(213)
PTEN deletion	C57/BL6	Mammary specific PTEN deletion		(214)
PyMT	FVB	E.g. MMTV-PyMT FVB/NJ strain uses MMTV-LTR to drive expression of PyMT	Loss of ER, variable overexpression of HER2. Immune cell infiltration is high. Lung metastases common.	(215)
RB1	Mixed	MMTV-Cre:Rbfl/fl	Latency of 18.4 month. ER negative, luminal B or basal like tumours. Lung metastases in 50%.	(216)
Sv40	FVB	PSBP C3(1) 5′ flanking sequence to drive expression of Tag	Invasive ductal cancer from 16 weeks age. 15% lung metastases. Loss of ER. Responds to 1L-12 immunotherapy	(217)
TGFBR2	C57/BL6	Truncated transforming growth factor beta receptor 2	Invasive cancers with lung metastases	(218)
WNT 1	FVB/mixed	MMTV-Wnt1	2 subtypes reported: early (more cellular) and late (more vascular) tumours.	(219)

TTA	FVB/C57/BL6	Tet-op-Esr1MMTV-tTA/tet-op-SV40-TAg	ER+ adenocarcinomas latency of 11 months. Lymphocytes present in TME.	(220)
deletion of p53 and Brca1	FVB K14-cre mice	K14-Cre; p53f/f Brca1f/f	human basal-like breast cancer Propensity to have immune cell infiltrates. Sensitive to carboplatin and paclitaxel.	(221)
Amplification of MYC and deletion of PTEN		Myc;Ptenfl RosaLSL-Myc/LSL-Myc;Blg-Cre strain with the Ptenfl/fl-conditional knockout	TNBC histology Complex tumour immune environment described	(222)

Genetically engineered mouse models have contributed to the understanding of the immune systems role in the early development of breast cancer where the use of the mouse model of mammary tumour progression that expresses the Polyomavirus middle T (PyMT) and Cre recombinase (Cre) in a doxycycline-inducible fashion (MMTV-MTB/TetO-MIC) revealed the importance of STAT3 in creating the immunosuppressive environment which enables the immune system evasion in the early stages of tumour growth and metastatic breast cancer. In this model the conditional STAT3 allele is deleted in the mammary epithelium through induction with doxycycline. Stat3 deficient mice were found to have a profound delay in mammary tumour onset and the tumours that emerge did not reach their full metastatic potential. Furthermore, an increase in activated T cells and macrophages was observed postmortem(223).

Genetically engineered mouse models can also be used to ascertain response to check point blockade therapy. In a study by Hollern et al (224), two different GEMMs: Tp53−/− tumour syngeneic transplant derived cell line (T11) and a cell line from a K14-Cre;Tp53f/f; Brca1f/f tumour (KPB25L) were used to identify genomic signatures which suggest treatment response or resistance. Through this they developed new mutagenized models for studying

immunotherapy in TNBC as conventional GEMM mammary tumour models were resistant to immune check point blockade and possible due to low tumour mutational burden. They correlated these with findings within human breast and melanoma models and found higher representation of B cell populations seemed to predict response. Further to this, they described a B/T cell sub population which has the potential to be used as a biomarker to suggest response to treatment. B cells are an increasing area of interest in immuno oncology, and it will be interesting to see if these studies will provide translatable clinical effects in the years ahead.

Patient Derived Xenograft Models

Patient Derived Xenograft (PDX) models are generated using human tissue samples, which are taken directly from a tumour biopsy, in a sterile environment. The sample taken is divided into fragments and inserted directly into the cells of a highly immunocompromised mouse - this mouse is termed the first passage. Once established, the sample is then transplanted into multiple mice, as a subsequent passage and so forth.

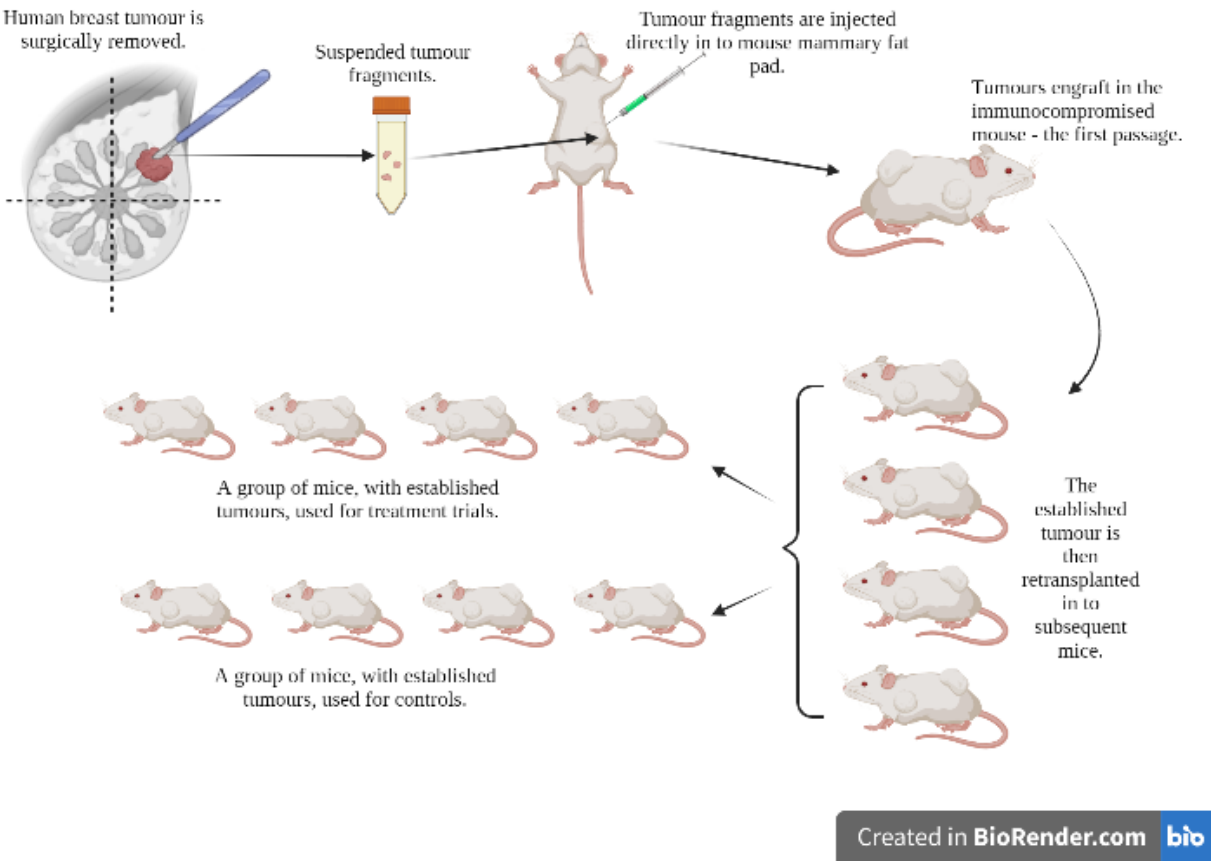

Figure 2: *The creation of a PDX model. Cancer containing tissue is removed en bloc and fragmented but not digested to maintain tissue structure. These are then implanted into an immunocompromised host and following tumour engraftment, are repassaged until tumours stably reproduced. A number of PDX lines can be used in series to provide a heterogenous variety of test subjects. Mice of the same strain are used to provide the control.*

A large amount of PDX models can be generated at one time, which enables numerous medications to be screened together. Research can be carried out to find the most effective treatment, with the best response rate, at the optimum dose, this also allows study into any treatment resistance (225). Patient derived xenografts allow for multiple biopsies at different points of treatment, this means that treatment can be specifically adapted, and genetic changes can be studied throughout. Most breast cancer PDX models are orthotopic which means that the primary tumour site from the human, is imitated within the mouse, for example, a TNBC tumour sample would be inserted into the mouse mammary tissue, by injecting tumour cell suspension directly into the mammary fat pad. Recently, this model has been improved by

injecting PDXs directly into the mammary duct, enabling interaction of tumour cells with the mammary gland and formation of heterogenous tumours that histologically mimic the original patient tumour(226). Utilising this method also increases the chances of mammary tumours metastasising to the same site as observed in the patient. Importantly, implanting human bone into immune compromised mice before intra-ductal injection of PDXs provides a human specific site for which PDXs can metastasise to and also results in human specific haematopoiesis generating human specific immune cells, however, the functionality of these cells remains to be determined (226).

What is particularly of interest is the validation of treatment responses of PDX models in comparison to clinical response observed. Pettersen et al described established a PDX model from fragments of patients with breast cancer and were able to observe human tumour cells within the implanted tissues (227). Furthermore, the response to paclitaxel treatment in in the animals correlated with observed clinical responses suggesting if the implant is successful this could be used to assess and predict treatment in a proactive manner ahead of when a patient may need it.

Advantageous in comparison to other models, as the sample comes directly from a human, it maintains the genetic heterogeneity of the original tumour, as well as histological structure of the patient. However, PDX models are expensive and time consuming – sometimes requiring months to establish adequate tumour engraftment, the validity of a biopsy could be doubted on its relevance to human structure, due to the timescale required. Due to these limitations, PDX models are only able to give a part representation of the development of the tumour and its microenvironment, at present. This means that further study into the long-term effect of treatments is difficult.

Other limitations arise, due to the use of immunodeficient mice, though this is currently the best model to represent a human tumour, without rejection, immunotherapy cannot be studied due to the lack of immune system(225). It is already understood that the immune system can be targeted to help support tumour eradication. Further study is needed to replicate the human immune system, within PDX models, without the risk of rejection of the sample. A closer representation of a tumour's microenvironment could then be simulated, with regards to the immune system involvement. Immunotherapy could then be studied and tested on PDX models to possibly support combination therapies which are currently already routinely used in other types of cancers.

Humanised Patient Derived Xenograft models

The introduction of human hematopoietic stems cells in NSG or BRG mice has led to the creation of a hybrid "humanised" model. In this process mice are first irradiated with whole body gamma irradiation between 5-10 weeks of age, and subsequently human CD34+ stem cells are intravenously injected and allowed to engraft. This is monitored via flow cytometry at around 10-12 weeks where successful engraftment is considered when mice have more than 25% human CD45+ cells in circulation (228). Once established human tissue can be introduced as per figure 2. As these mice are now as semi-immunocompetent host, their immune can be assessed(229) and immunotherapies can be introduced. What is particularly promising about these models is the suggestion that the TME can be preserved. Morton et al., describes that human immune cells were able to infiltrate engrafted head and neck tumours within a humanised mouse model. Furthermore, these cells were able to induce lymphangiogenesis and sustain the original gene expression profile of the PDX (230).

Humanised models show promise for investigation of immunotherapy treatment with checkpoint inhibitor therapy has been assessed in humanized NSG mice for bladder

cancer(231), hepatocellular carcinoma (HCC) (232), melanoma(233,234), non-small-cell lung cancer (NSCLC) (235,236), autologous renal cell carcinoma (RCC) (237), and TNBC (234,238). In a humanised nasopharyngeal cancer model with NSG mice, Liu et al have interestingly observed matching clinical and preclinical responses to the combination immunotherapy of nivolumab and ipilimumab with significantly increased IFN-γ and IL-6 production and decreased the CD4/CD8 ratio in a humanised PDX model compared to their non humanised PDX model(239).

A TNBC PDX-engrafted HSC-humanised NSG mouse model was designed to show TNBC patients positive for programmed death-ligand 1 (PD-L1) can benefit from the anti-PD1 immune checkpoint inhibitors atezolizumab or pembrolizumab in combination with chemotherapy (240,241). In these studies, some mice had reduced tumour growth upon treatment with the anti-PD1 pembrolizumab or nivolumab, while no effect was observed upon anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) ipilimumab treatment. Furthermore, these humanised models can confirm the checkpoint receptor expression which may result in further treatments to be targeted towards a certain population. An example would be the results of immune checkpoint profiling in a group of humanized breast cancer mice which has shown co-expression LAG-3/PD-1/TIM-3(242). Perhaps this will form the foundation to trials to investigate the use of a LAG3 inhibitor and PD-L1 inhibitor in breast cancer as has recently been approved for melanoma. Additionally, as a reliable ER positive model has been difficult to conventionally develop and there has been great interest in humanised breast cancer mouse models in the ER+ group and a number have been described. One such is the immune-humanized ER+ model where the HCI-013 PDX line (a metastatic, endocrine resistant ER+ model of lobular breast cancer)(243).

Limitations of PDX models revolve around costs and difficulties with engraftment - more aggressive breast cancers have high engraftment rate (244). In one centre the overall 'take rate', defined as PDX growth for at least two generations, was only 29%. Primary tumours were found to be more challenging to engraft (25% of 102 attempts) than metastases sites (36% of 50). ER positive PDXs were the most difficult to develop, with a take rate of 9% for primary ER positive tumours ($n = 32$ attempts) in contrast to TNBC with a take rate of 58% for primary tumours ($n = 12$ attempts) (245). Despite this, these models have started to help bridge translation research the gap between bench and bedside (246).

There are concerns about the number of animals needed for the generation of PDX models with protocols often requiring multiple animals per patient and low engraftment rates. Organisations such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) are therefore supporting *ex vivo* technologies as alternatives to our dependence of animal models as gold standards.

Patient derived explant models (ex-vivo models)

The use of *ex vivo* based models that use fresh surgically resected tumour or biopsy material has seen a resurgence since their first use in the 1950s. In order to better replicate the effects of drugs within the patient, that aren't seen within cell line models, the development of drug screening assays that utilize patient material has begun to take off, with multiple different approaches now been taken to develop models that will predict drug resistance, biomarker discovery and drug development. By using patient derived material, this is the next step in tailoring personalized medicine, allowing data to be cross-referenced with the diagnostic and patient outcome. Currently there are a range of approaches to patient derived material in an *ex vivo* setting which are summarised in figure 3 and described below.

Preserved microenvironments

Ex vivo assessment using breast cancer specimen using a perfusion bioreactor has been shown to maintain both tumour and immune cell viability for 7 days. Using $2mm^3$ cut fragments, cultured between a collagen scaffold, these are perfused at a constant flow rate with supplemented culture medium. Tumours were treated with Fulvestrant, Pertuzumab, anti PD-L1 and anti-CTLA4 by adding to the culture media. Samples were fixed and embedded before staining was performed on sections for assessment. Higher cell viability was seen is perfused culture vs static cultures as measured through negative caspase 3 staining. Fulvestrant treatment on ER+ tumours, significantly reduced epithelial cell viability compared to untreated controls. With an effect also seen when HER2+ tumours were treated with Pertuzumab. Treatment with anti PD-L1 and anti-CTLA4 in 3 TNBC tumours showed a significant decrease in cancer cell viability after 7 days of treatment hen the controls of lymphocytes and normal breast tissue remained unaffected (247).

In a separate study, looked at the paclitaxel treatment on using 200um thick breast tumour slices (248). These were then cultured for 24 hours before the slices were treated with vehicle or paclitaxel for a further 48 to 72hrs. Samples were then fixed embedded and stained for cell death markers and proliferation. Paclitaxel treatment on explant cultures did not induce high levels of cell death during the experiment timeframe, but an increased uptake was seen in tumour cells, it was suggested that cell death from paclitaxel would have occurred, but this was not observed due to the time limits on the explant culture. Treatment of samples with another microtubule inhibitor, Vincristine, did elicit a response. This indicates that in some instances, some commonly used standard care drugs, may not be suited to the *ex vivo* environment. Response to drug screening using patient material has also been seen in NSCLC. This method, using fresh surgical tumour tissue, adds a pre recovery phase where the tumour is cut into 2-3mm^2 pieces and cultured for 16-20 hours prior to treatment. These tumour pieces are then

transferred for culture with the therapeutic compounds for 24 hours before being fixed, embedded and sectioned for analysis through IHC/IF. Using cisplatin, this was shown from 26 patients, the response of NSCLC to Cisplatin using this assay saw a link to patient outcome. Using cPARP staining as a measure of cell death to the highest levels of Cisplatin, they determined a cut-off for sensitive and resistant tumours. The sensitive samples identified were shown to correlate with patient survival, although there was no minimum follow-up period for the patient data (249).

Patient derived organoids

Patient derived organoids are 3D reconstruction of patient derived tissues within an *ex vivo* environment that tries to stimulate the environment *in vivo*. The advantages of this over 3D scaffold systems is that the organoids can self-renew and differentiate into different cell lineages. However, they lack a vascular system and therefore can only be sustained for a limited number of passages (250). The diversity of cell milieu within the organoid in both the heterogeneity of the cancer cells suggest that this may provide a useful model to screen for drug sensitivity prior to treatment. Moreover the receptor status of over 100 cases of breast cancer organoids continued to be well matched in histopathology, hormone receptor status, and HER2 expression to their original tumours (251). In support, the fidelity of cell lines, PDX, PDOs and genetically engineered mouse models were assessed using and AI assisted programme which suggested that general, genetically engineered mice and PDOs reveal higher transcriptional fidelity than PDX and cell lines(252).

Unfortunately, culture mediums and the lack of vasculature on formation of the organoid means that immune function can be difficult to ascertain. Some groups have published protocols to co-culture organoids with lymphocytes and CAFs (253,254), but these have yet to

be investigated in trials. There have however, been successful reports of PDO being used to assess the specificity and enhance efficacy of CAR-T cells (255).

A similar 3D concept can be seen in within the development of patient derived spheroids. In patients with several histological subtypes check point blockade can be shown to have similar response in a PDOT *ex vivo* microfluidic based model. Here authors also despite cytokine profiling within this model which has led to a suggestion that those with immunosuppressive cytokine expression (CCL19/CXCL13) had a decrease in clinical PFS survival. This is of particular clinical interest as outcome prediction tools to allow more personalised treatment can help clinical weigh up the risks and benefits of certain treatments (256).

Dispersed methods

Keeping the tumour environment intact does have its advantages in that the changes can be seen in situ. However, if the same drug response is also given once the tumour microenvironment is dispersed and still correlates to the patient response, then this is a potentially more powerful tool. There is greater scalability of drug screening assays by removing the limitations on the number of compounds tested, while response to compounds can be seen using a fraction of the cells used in organoids and preserved microenvironments. Although well suited to haematological cancers where promising results have already been seen (257) this is increasingly being used within solid tumours(258).

Unlike *ex vivo* methods that look to preserve the tumour microenvironment, assessment of the dissociated tumour microenvironment allows for greater scalability of drug screening assays. Although these models may lack the environmental aspects, they retain the heterogeneity of cells. Dispersed models are well suited for drug discovery screening assays, through analysing the effects of the drugs on the healthy and the cancerous population. Where

multiple mutations exist within the tumour cells, this can also be picked up from large-scale screening platforms.

One study that used imaged based analysis of a single cell population in AML showed that 15 out of 17 patients had an overall response when using imaged based analysis to guide their treatment compared to 4 of 17 when compared to their previous treatment given(257). It is, however, tricky to stimulate the effect of the immune system on single cell populations in culture, but could perhaps screen if patients samples are positive for PD-L1, LAG3 and other check point markers and therefore help guide which patients would benefit from immunotherapy treatments.

With the breast cancer setting *ex vivo* work is of increasing interest in both the diagnostic and therapeutic field. A recent proof of concept study to assess a novel *ex vivo* anthracycline sensitivity assay revealed that 75% patients had matching assay and clinical MRI responses to anthracyclines. A similar study assessing screening for cisplatin and docetaxel sensitivities have also been described (259). Although the sensitivity and specificities have the potential for further refinement this is a promising use of *ex vivo* screening which would allow therapy to be targeted (260).

Challenges with patient derived cell lines

Despite the translational benefits of working with patient derived material, there are multiple challenges associated with it. The time it can take from the sample being resected, processed by histopathology and then being made available for processing in the lab, can affect the overall viability and quality of the sample with environmental conditions not being kept optimal. Even if conditions are kept optimal, burning/scarring from surgical procedures used to

resect tissue can greatly hinder the viability of the cells, leading to inter patient variability between samples.

Biobanks hold an increasing number of viable cryopreserved samples, many accompanied by clinical data. As such, these may be a great source of retrospectively validating assays but work still needs to be undertaken to understand the effects of cryopreservation on the cells and if this influences any changes in the drug response. Each assay has its own limitations that have still to be defined, such as the quantity of tissue needed, the time each assay is run and the reproducibility within the assay itself.

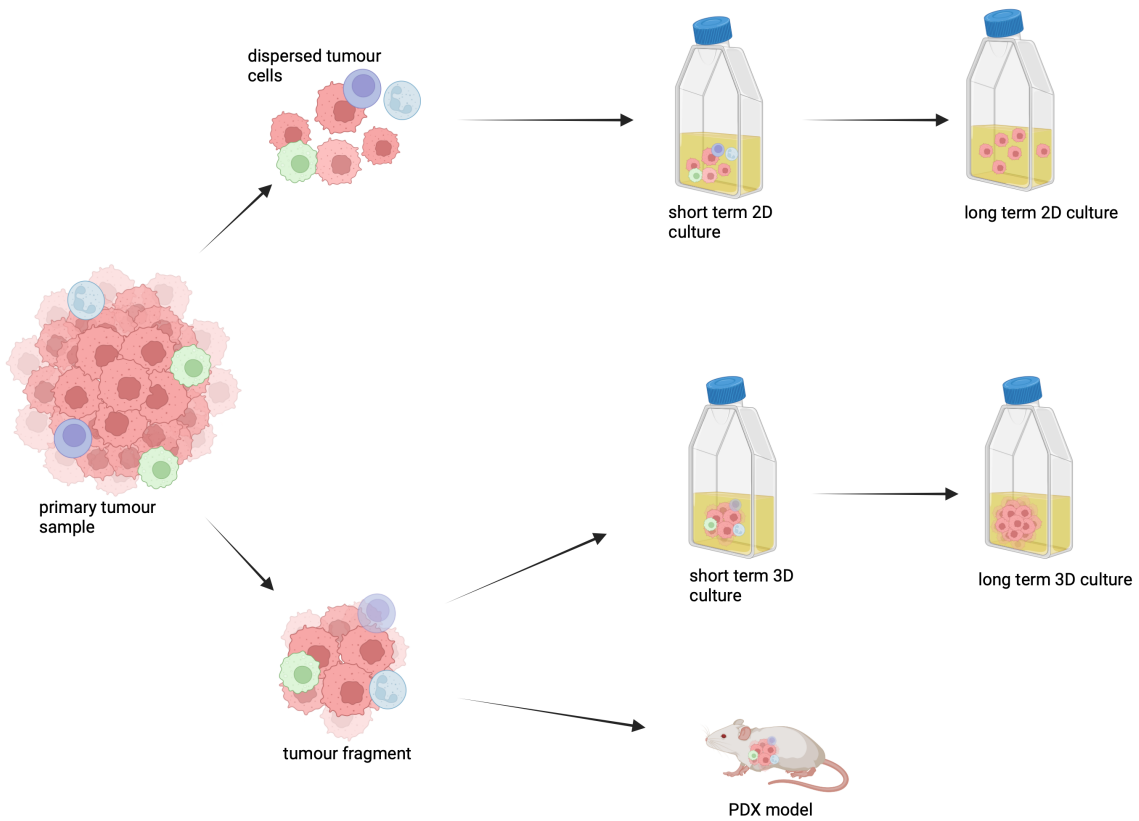

Figure 3: *Use of patient derived cells in breast cancer models. Patient derived tumour cells contain a milieu of cell types. A short-term model of the immune make up of tumours can be derived for dispersed immune cells. However, culture mediums are not able to retain immune cells. Alternatively primary tumours can be divided into fragments which can then be cultured as organoids or implanted into humanized mouse models. Organoid models allow for more complex TME modelling and assessment of penetration, but again become deplete of immune cells over a short duration of time. PDX models are an attractive alternative however studies may be limited by cost and poor engraftment rates. Created with BioRender.com*

Conclusions

The overarching aim of a preclinical model is to try and simulate the complexity and heterogenicity of a patient's TME, but yet still be consistently reliable and reproducible. This is a tall ask, and yet, models may "make or break" a treatments success in the clinical trial setting. The development of Tamoxifen as an anti-oestrogenic breast cancer treatment is a good example of this. Originally developed as an effective "morning after pill" in laboratory rodents, it was found to be a poor contraceptive in humans. Additionally, it was also noted to have an anti-oestrogenic effect in rats and primates, however a pro-oestrogenic effect in mice (261). The uncertainty of whether this treatment would be effective for breast cancer patient was anticipated, and yet, now this hormonal treatment is one of the backbone treatments for ER positive breast cancers.

Likewise, the widely used immunotherapeutic drug Pembrolizumab, was discovered when looking for a drug to treat autoimmune disease (262). These coincidental discoveries compel us to ask whether the challenge we face as scientists is picking the right model or have a general knowledge of the body to the extent that you can see potential between disease sites. The landscape of breast cancer treatment has changed significantly over the last 50 years. Although we see improvements in survival because of patients being diagnosed and treated early and the availability of treatment from a larger pool of drugs, we also see a difference in history of presentation such as late relapses and an increase in unusual sites of metastases, e.g. brain metastases.

The flexibility of models to adapt to these changes have allowed continued development of novel treatments. However, the dream model would allow personalization and dynamic testing of patient cells and the use of *ex vivo* breast cancer screening of drugs is of significant interest

at present. There are signs that *ex vivo* testing can be used to screen responses to chemotherapy however we are yet to see if this can be extrapolated to immunotherapy. This review highlights the currently available models and their potential to stimulate an environment suitable to evaluate the immune system in breast cancer and paves the way to the development of future breast cancers studies.

Conflict of interest: "The authors have no conflicts of interest to declare"

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3.3 Modelling primary breast cancer

3.3.1 Immunocompetent verses immunocompromised animal models

The evolution of immunocompromised animal hosts has led to the use of human cell lines to model disease and thus there are number of stages of immunodeficiency These include nude mice which lack T lymphocyte(263), NOD mice which lack an innate immune system (deficient in macrophages, NK cells and reduced complement) (264), SCID mice which are deficient in B and t lymphocytes but retain NK cells due to an aberrant *Prkdc* gene and NOD/SCID mice(265), which lack T and B cells and have reduced phagocytic activity of macrophages cell, the -killing activity of NK cells, and complement activity. As a high incidence of lymphoma of the thymus was seen in the latter group, a further group, NOD/SCID rg^{null} , was developed by knocking out the IL-2 receptor gamma chain (IL-2 rg) which results in a lack of B, T and NK cells as well as reduced activity of macrophages, dendritic cell and complement function (266,267).

Immunocompetent mouse models have intact murine immune systems although each mouse strain has its own unique characteristics and profile. BALB/c mice are known to be susceptible to tumour induction and have strong Th2 response secondary to immunization. C57BL/6 mice are known to have a predominant Th1 response, and these differences manifest in the clinical response to immune cell stimulation with macrophages from C57BL/6 mice displaying higher levels of TNF-alpha and IL-12 compared to BALB/c mice after stimulation with macrophage-activating lipopeptide-2 or lipopolysaccharide(268).

The merits the immunological status of the host was considered in the choice of the model for this PhD, and it was felt, on balance, that an immunocompetent host would be preferable.

3.3.2 TNBC growth curves

Three triple negative mammary cancer cell lines, PyMT-TS1, 4T1 and EO771, were used to form primary mammary tumours in FVB, BALB/c and C57BL/6 immunocompetent mice respectively. Optimisation of the PyMT-TS1 model and the EO771 models were performed by Dr Emer Murphy and Dr Faith Howard post-doctoral researcher in this team. Permissions have been granted to describe these within this PhD. The subsequent post-mortem analysis and all the remaining animal studies following this were conducted during this PhD project.
Primary PyMT-TS1 mammary tumours were developed by subcutaneous implantation of $1x10^6$ PyMT-TS1 cells in 50µl (50:50 Matrigel: cells) into the 4th mammary fat pad of 6-7 week old female FVB mice (N=10). Animals were observed for changes in their health on a daily basis and digital calliper measurement of tumour size was performed every 2-3 days. A growth curve for this model is shown in figure 8a. Mice were culled on reaching a humane endpoint or if tumour diameters reached the maximum permitted size (15mm diameter). Primary tumours, organs and blood were collected for postmortem analysis. Tumours and organs were fixed in 4% paraformaldehyde for 2-4 hours prior to freezing in OCT freezing media. Finally, tumours were sectioned with use of the cryostat at a thickness of 10um.

To ascertain size and necrosis they were stained using H and E. These tumours revealed high levels of necrosis with an average percentage of necrosis per tumour of 44.6% (range 24.4 -74.4%, n=5). The lungs and liver of animals were stained with H and E and metastases were quantified and only found within lungs. A representative image of H+E staining is illustrated in figure 8b and 8c.

Tumour growth modelling was also performed using a 4T1 cell line in BALB/c mice and the EO771 cell line in C57 mice. Growth curves are illustrated in figure 9. As the BALB/c tumours appeared most aggressive and, as the literature around this cell line (269,270)

suggested it was highly metastatic to multiple organs, all subsequent models were based around this cell line only.

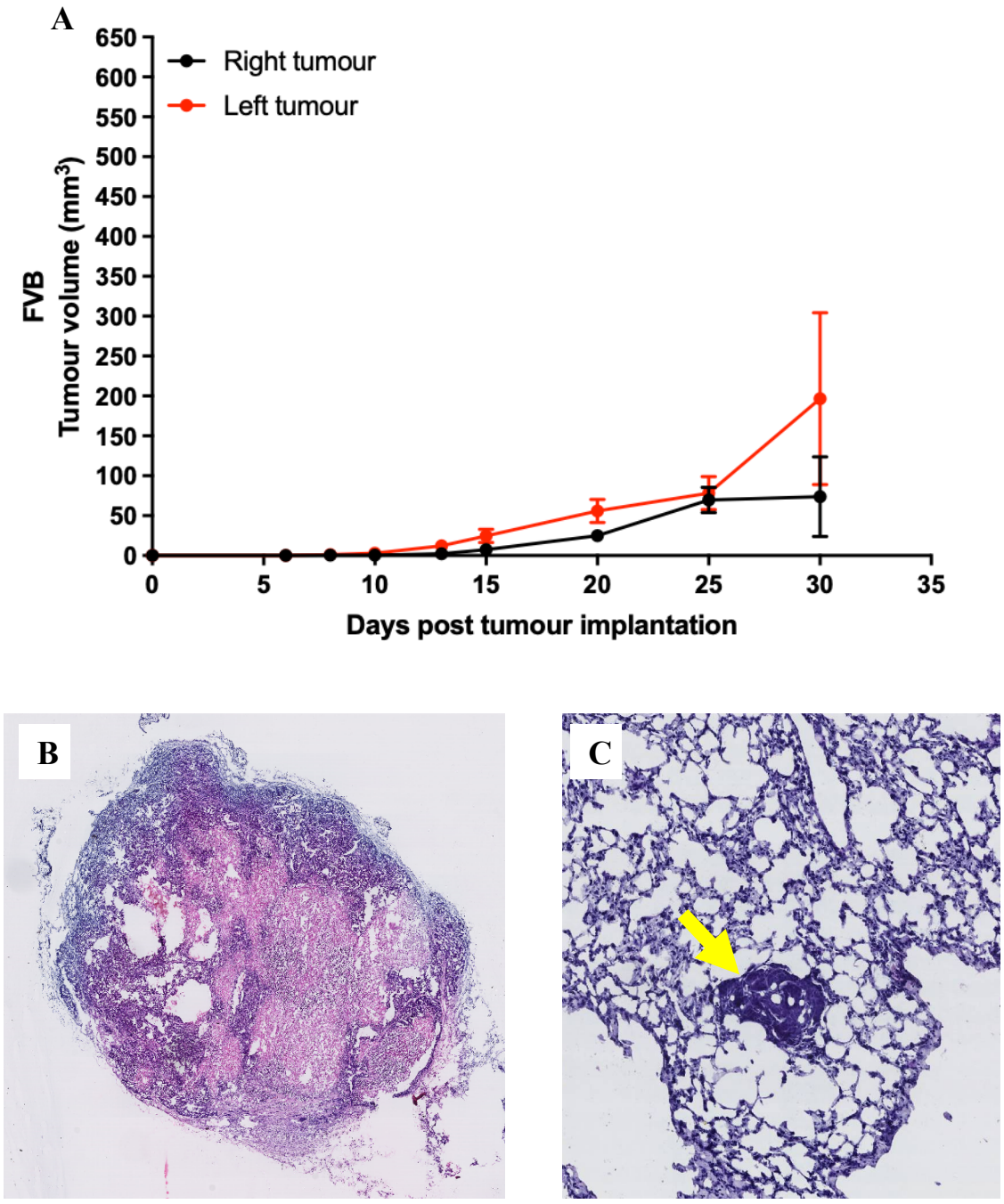


Figure 8: The PyMT-TS model of primary TNBC. *(a) A growth curve showing the average growth of tumours following implantation of PyMT-TS1 cells in FVB mice (n=6) obtained from Dr Faith Howard. Mice were culled when tumour volume exceeded 500mm^3 and half the postmortem tumours were embedded in paraffin with the other half fresh frozen. Metastases was assessed at postmortem. A representative H+E stained section of mammary tumours derived from PyMT-TS1 cells. (b) a cross section through a paraffin embedded primary mammary tumour with visible central necrosis (pale pink). (c) a section from a frozen lung specimen with lung metastases marked (arrow).*

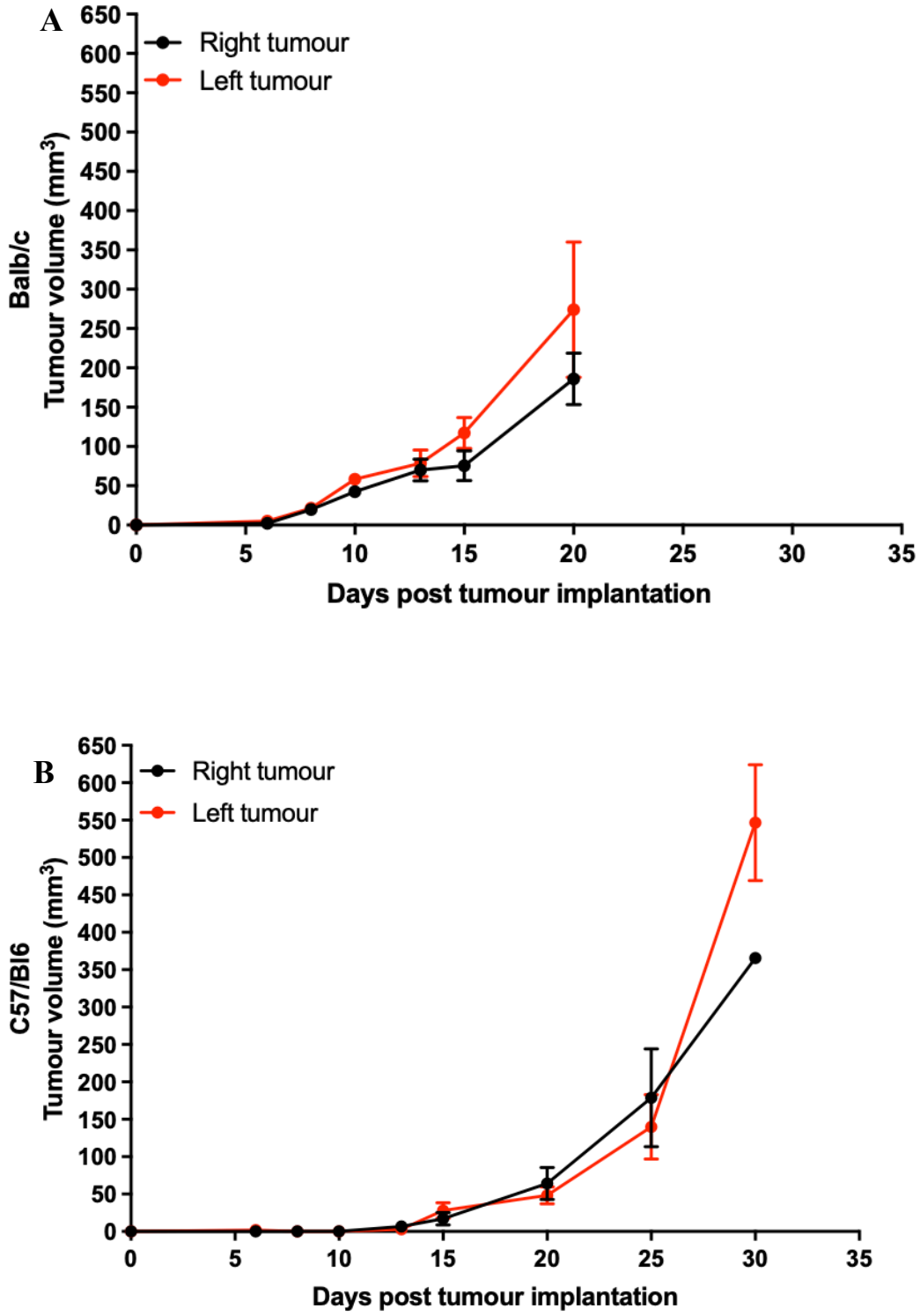


Figure 9: 4T1/BALB/c and EO771/C57 models of primary mammary cancer*. (a) A growth curve showing the average growth of tumours following implantation of 4T1 cells in BALB/c mice. (b) A growth curve showing the average growth of tumours following implantation of EO771 cells in C57 mice. Both growth curves were plotted using an average of calliper measurements from 6 mice.*

3.4 Modelling Metastatic Breast Cancer

Although only 5% of TNBC present as de novo metastatic disease, TNBC is the most common breast cancer subtype to recur following definitive surgery for early-stage disease (271). In addition to this, the aggressive nature of the disease means a higher proportion of patient experience brain metastases during their journey with breast cancer. Due to limited treatments, after the development of brain metastases, the overall survival is an average of only 7 months(272). It is therefore noted that developing treatments, and effective models to assess these, addresses an area of clinical need. The models in the next few sections describe experience within this PhD to identify an appropriate model with this clinical niche in mind.

3.4.1 Characteristics of metastatic seeding model

To try and mimic the widespread aggressive disease seen in metastatic triple negative breast cancer, this model investigated inoculation of LUC-4T1-BR cells via the left ventricle. Inoculation of cells through the left ventricle allows passage and seeding of the cells into organs such as bone, liver and brain. This cell line was selected as it was described in a paper by Ranjan *et al* to be a brain seeking model (273), which addresses the metastatic site of interest. Additionally, a luciferase labelled cell line was used so we could dynamically track a response to treatment.

The model was created by injecting 6-8 week old BALB/c mice with $1x10^5$ LUC-4T1-BR cells into the left ventricle of each mouse via an intracardiac route (n=17, 5 acquired from Envigo laboratories and 12 acquired from Charles River laboratories). Cells were filtered just before inoculation to prevent clumping. All mice developed widespread metastatic disease in a pattern consistent with human TNBC with presumed involvement of the lungs, liver, brain and bones (**Figure 10**).

Mice were observed for a maximum of 14 days or sacrificed earlier if disease burden was significant. Luciferase labelled cells allowed for dynamic monitoring of tumour development in these mice. Mice were imaged using the IVIS every 2-3 days. Animals were presumed to have lung metastases if these were located in the chest and liver metastases if there was flux signal in the dorsal view of the upper right sided of the mouse's abdomen. The location of metastases were confirmed postmortem. Tumour sites and organs were harvested for postmortem analysis; with 50% of the tissue snap frozen and 50% of the tissue fixed in 10% formalin before embedding with paraffin. Mice developed histologically confirmed metastases in the lungs and liver (figure 10 -13). Splenic enlargement was also noticed in the majority of the mice postmortem. The significance of this is unclear, although spleens were frozen in OCT for further analysis if needed. Femurs of mice were taken and decalcified, however these were not stained and visualised for disease, and should this work be repeated, this would be a high recommended.

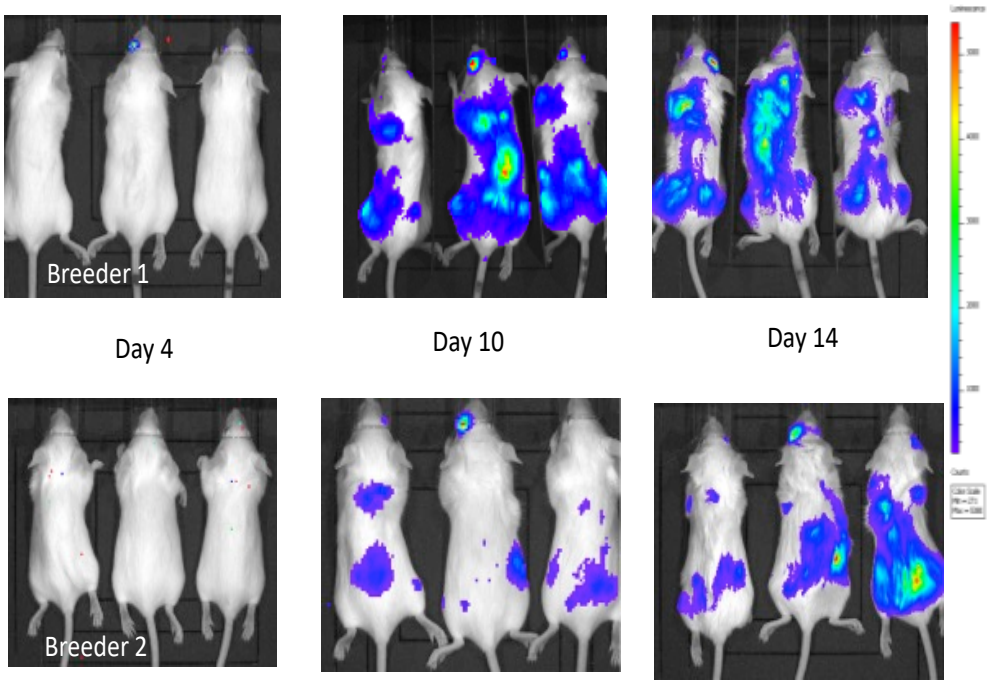


Figure 10: Development of metastatic disease between animals from different breeders. *(a) 1x10^5 LUC-BR-4T1 cells were implanted via the intracardiac route into BALB/c mice from 2 different breeders. The top panel shows representative mice from the breeder Envigo and, the lower panel shows mice from Charles River laboratories. A difference in the rate of development of metastatic disease was seen between breeders. Animals were administered with 10ul/g of luciferin before imaging on the IVIS on auto exposure.*

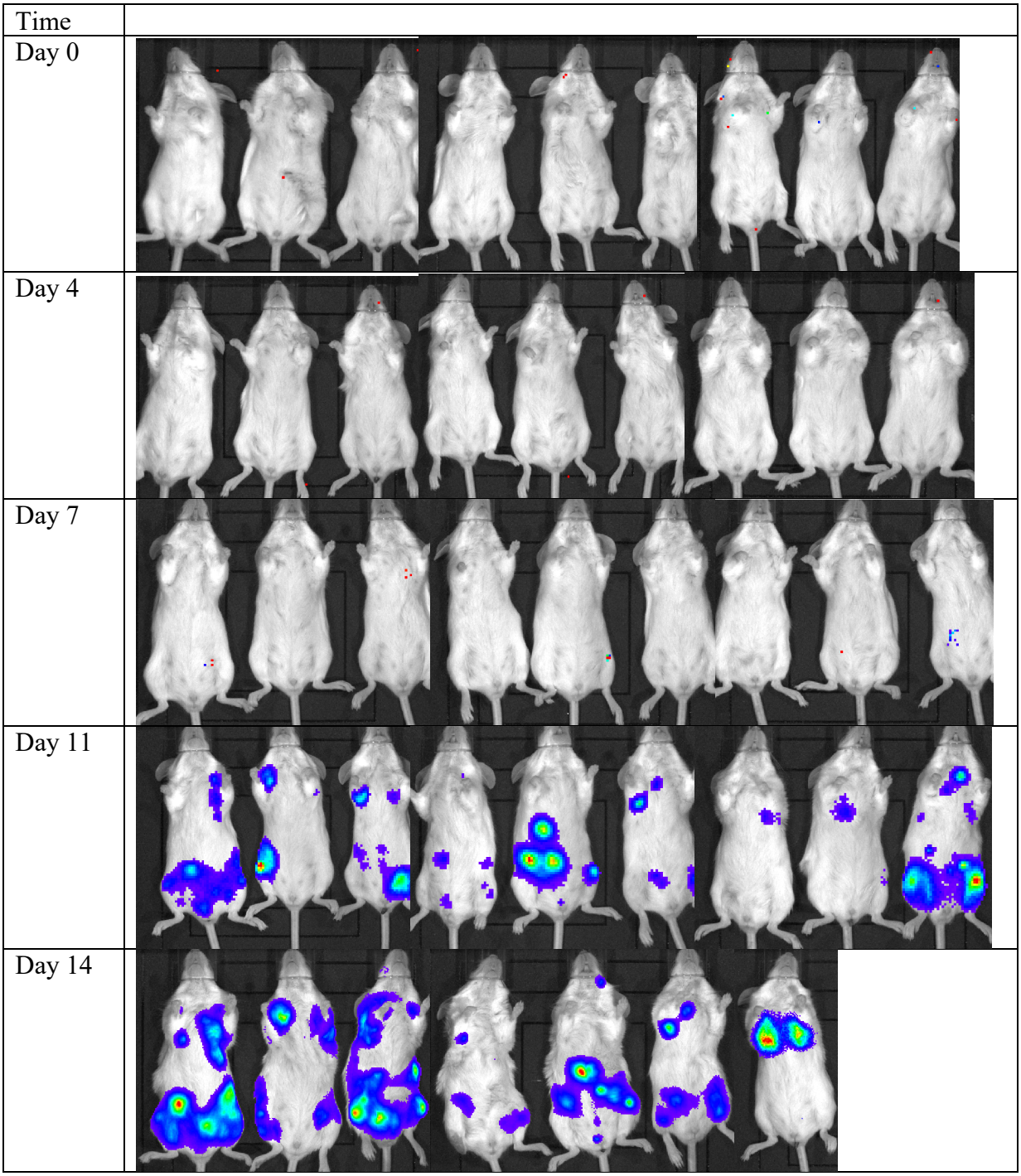


Figure 11: Ventral view: development of metastatic disease following intracardiac inoculation of LUC-Br-4T1 cells over time. *$1x10^5$ LUC-BR-4T1 cells were implanted via the intracardiac route into 6–8-week-old female BALB/c mice (n=9). Animals were imaged regularly using the IVIS. Ventral views of each mice are displayed here in time order with the corresponding animal vertically above. Animals were found to have a widespread pattern on metastatic disease. Unfortunately, organs were not dissected and images using the Lumina II IVIS on this occasion.*

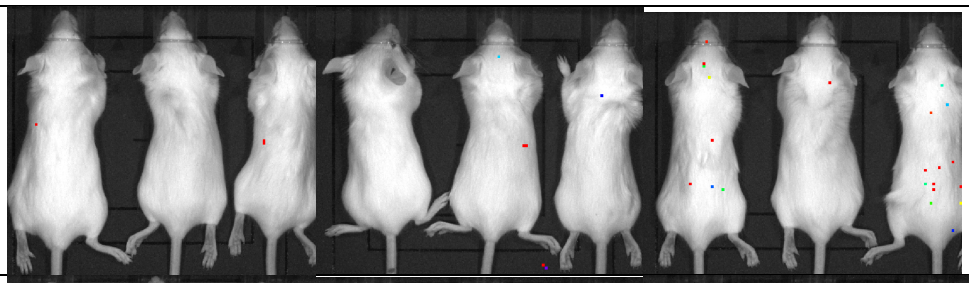
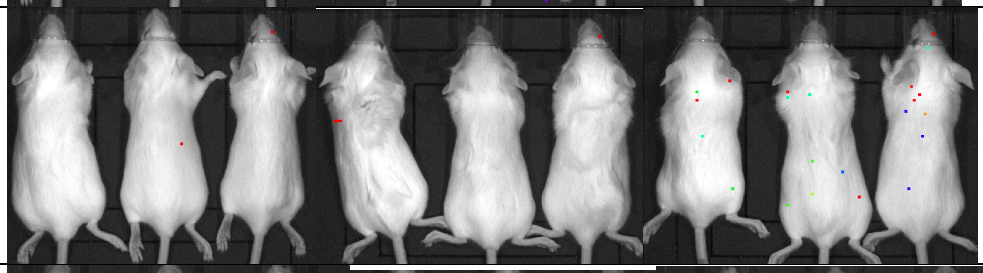
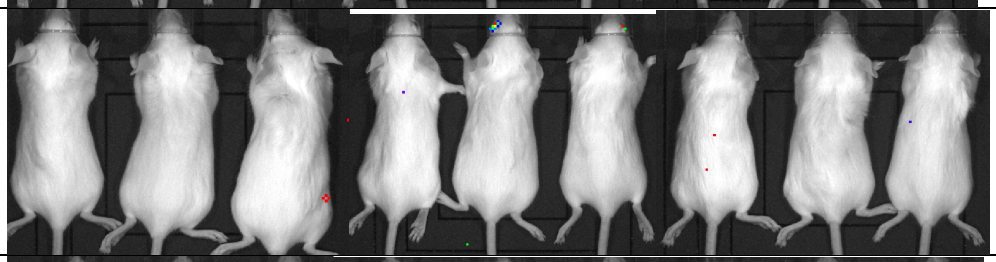
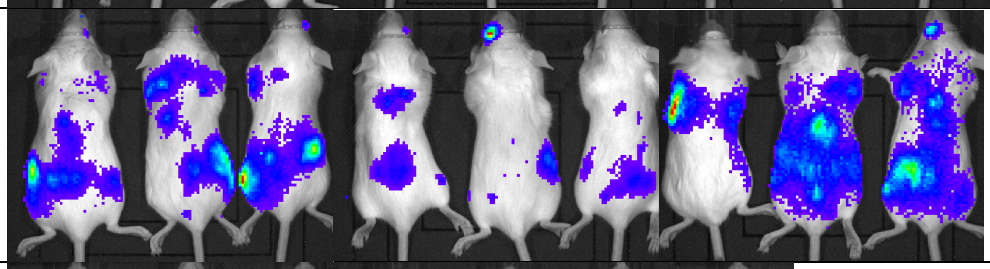
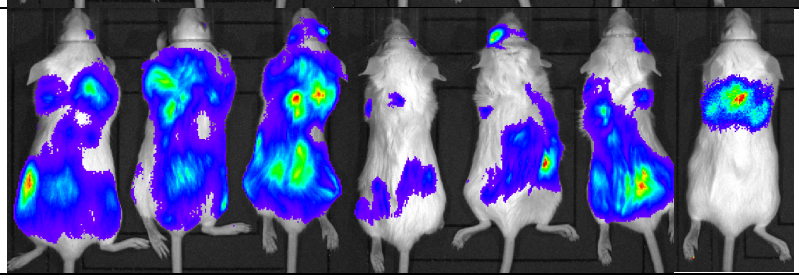
Time point	
Day 0	
Day 4	
Day 7	
Day 11	
Day 14	

Figure 12: Dorsal view: development of metastatic disease following intracardiac inoculation of LUC-Br-4T1 cells over time. *$1x10^5$ LUC-BR-4T1 cells were implanted via the intracardiac route into 6–8-week-old female BALB/c mice (n=9). Animals were imaged regularly using the IVIS. Dorsal views of each mice are displayed here in time order with the corresponding animal vertically above. Animals were found to have a widespread pattern on metastatic disease. Unfortunately, organs were not dissected and images using the Lumina II IVIS on this occasion.*

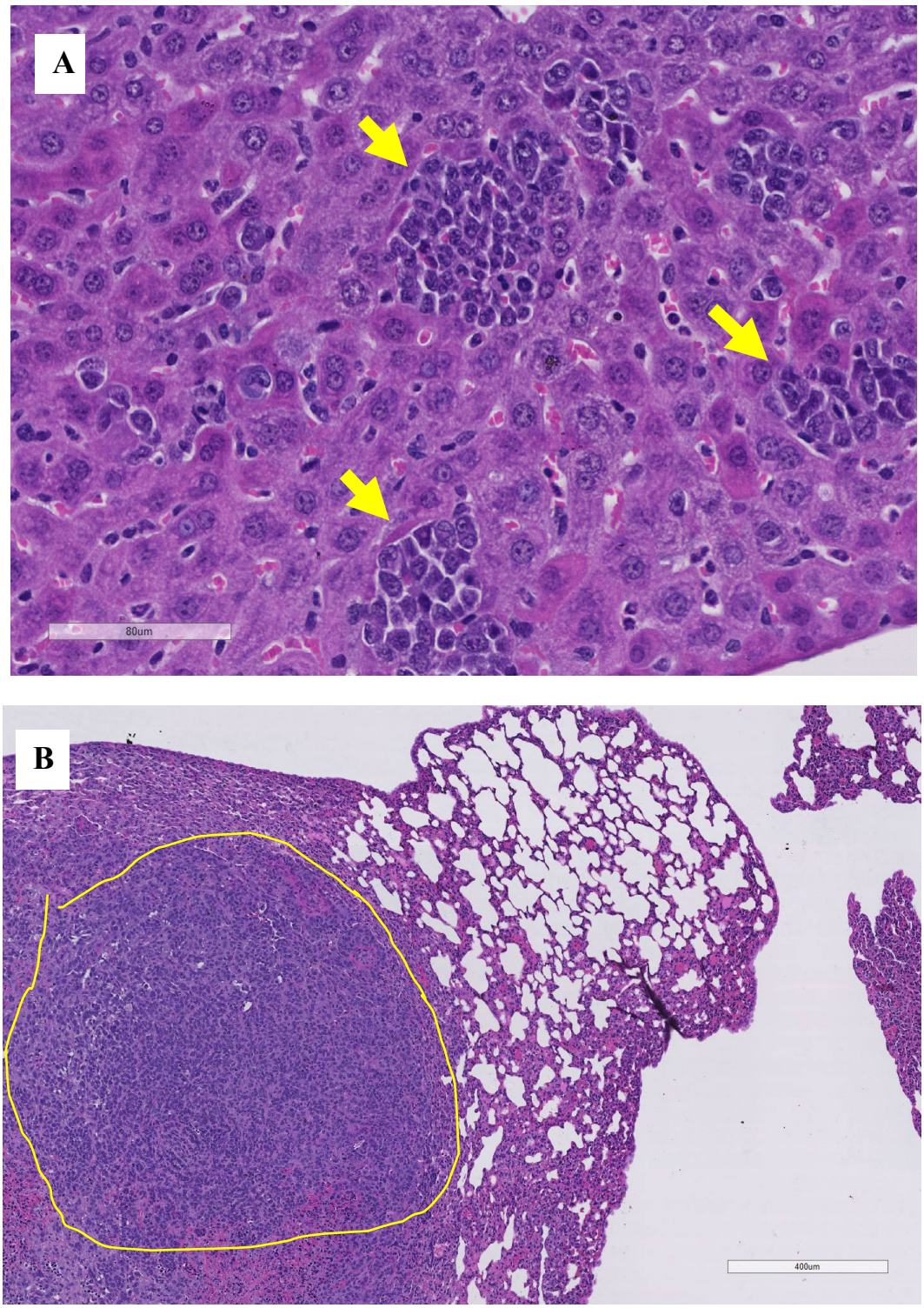


Figure 13: 4T1 liver and lung metastases *(a) a representative postmortem H and E paraffin embedded section of murine liver showing numerous liver metastases (arrows identify three of these). (b) a representative postmortem H+E stained paraffin embedded section of murine lung showing a large lung metastases (circled).*

This model recapitulates many of the traits that makes treatment of TNBC challenging, multiple metastatic sites, aggressive disease and short time course. However, differences in the time to develop metastatic disease and aggressiveness of the tumours observed were noted between breeders. This was an unexpected finding and has not been reported in the literature. At the age, sex and breed of the mice were identical from the breeders and the dose of cell line and housing conditions were identical between these groups we were unable to explain this finding. Given the differences in the time to develop metastatic disease were seen between the two animal breeders and the Charles River laboratory was selected as breeder of choice for any further animal work. This selection allowed a more manageable time frame from inoculation to metastatic disease thus making it easier schedule and assess the effect of repeated treatments.

3.4.2 The resection model proved unpredictable in nature for our experiments

There is some data to suggest that spontaneous metastases (those that form from primary lesions) may represent a more representative metastatic lesion than forced metastases and this can be modelled within the 4T1 cell line (274). However, when attempting this resection model, it was found that when cells were implanted the primary tumours became large, very quickly making a number of animals unsuitable for resection as the tumours were adherent to the peritoneum. Perhaps this was due to the number of cell inoculated and had it been felt to be necessary and resources allowed, further work could be done to optimise the number of cells needed to implant so tumours develop more gradually, however it was felt that this model will not be used again in this PhD due to a number of pitfalls.

The first is the unpredictable nature of the model both in providing consistent sites of metastases, and when the animals developed metastatic disease that was visible by bioluminescence it was often very poorly. This would mean the timing and assessment of treatments would have been challenging. Secondly, it was thought that given the duration to

development metastases and time needs to optimise both the number of cells to inoculate and the timing of surgery, this model could potentially be more costly and other models are likely to produce similar metastatic spread with less financial burden. Finally, interestingly given this cell line is thought to be brain seeking, metastatic disease was often preferentially visualised in bony regions on IVIS images. Ex-vivo, organs were visualised (see **14**). Of note, the spleens on the 4T1 mice were found to be significantly enlarged and contain metastatic disease. This disease was not visible during *in vivo* analysis using the Lumina II bioluminescence system (IVIS).

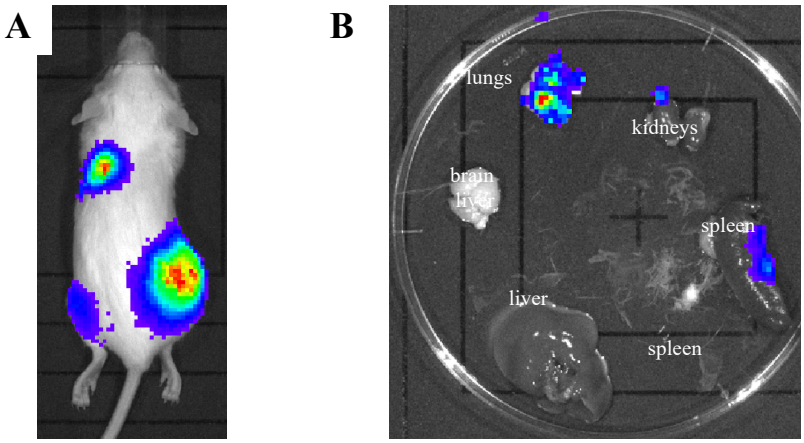


Figure 14: The resection model. *1x10^5 LUC-BR-4T1 cells were implanted into the 4th fat pad of BALB/c mice. These were resected after 7 days and animals were imaged every 2-3 days to assess metastatic spread (a) an in vivo image of one the animals at day 7 post resection and (b) ex vivo images of organs in resection model*

3.4.3 Characteristics of the stereotactically implanted brain metastases model

A topical area of unmet need is the treatment of breast cancer brain metastases. In the intracardiac inoculation model brain metastases were formed in approximately 70% of animals. However, these presented late and were consistently a sign of advanced disease. This resulted in a very narrow window for treatments to be initiated and take effect. Immunotherapy treatments take some time to show a first response, for examples patients receiving either

single or combination check point blockade for melanoma only showed first response after a median of 2.7 months (range 2-12 months) of treatment (275,276). Dr Priya Patel, a post-doctoral member of our group, had success with intracranial inoculation of a murine glioblastoma cell line and this was therefore explored in our breast cancer cell lines. This was performed by Mr Gary Shaw, a technician at the University of Leeds. Here 50,000 Luc-4T1-BR cells were inserted intracranially with the use of a stereotactic frame (Digital Just for Mice Stereotaxic Instrument - 51725D Stoelting) (277). Animals were imaged on the IVIS every 2-3 days until intracranial lesions were visible and then visualised daily. Brain disease was visible on the IVIS from 6 days after inoculation. The development of intracranial lesions over time and is shown in **figure 15**. Interestingly, the intracranial lesions were best picked up using auto exposure as fixing the exposure to 60 seconds did not pick up small lesions. Brains were fresh frozen embedded in OCT medium, and histology was analysed using H+E staining to confirm size and location of tumours. Of note, some brains were fragile to handle as tumours grown were diffuse and an example of this is shown in **figure 16**.

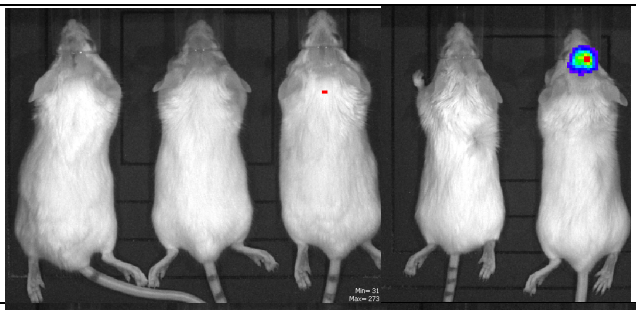
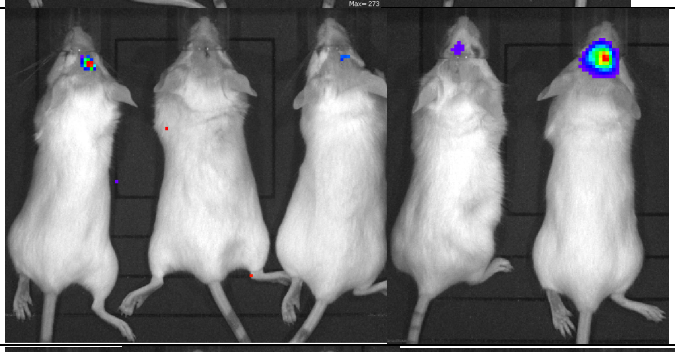
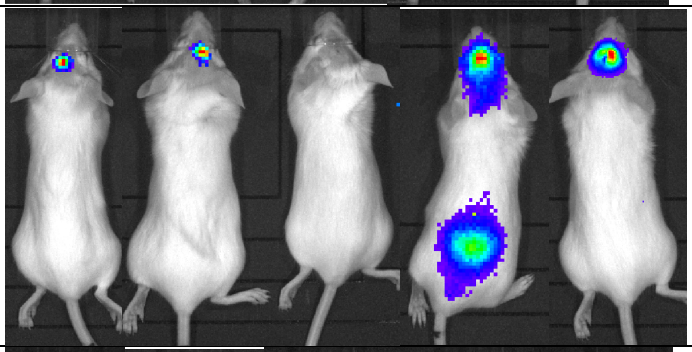
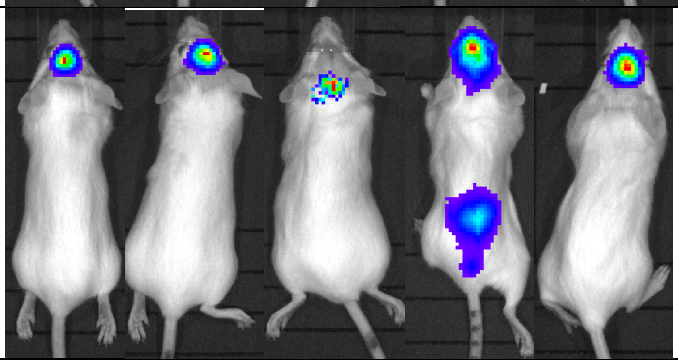
Timepoint	
Day 3	
Day 7	
Day 9	
Day 12	

Figure 15: intracranial lesion following stereotactic inoculation of LUC-4T1-BR cells*. 1x10^4 LUC-4T1-BR cells in PBS were implanted using a stereotactic frame. Images taken using the Lumina II IVIS system 10 mins following administration of 10ul/g of luciferin, on auto exposure, 12 days after inoculation of cells. Mice were imaged in dorsal view only. Brains were dissected and analyses postmortem, but were not imaged on the Lumina II IVIS.*

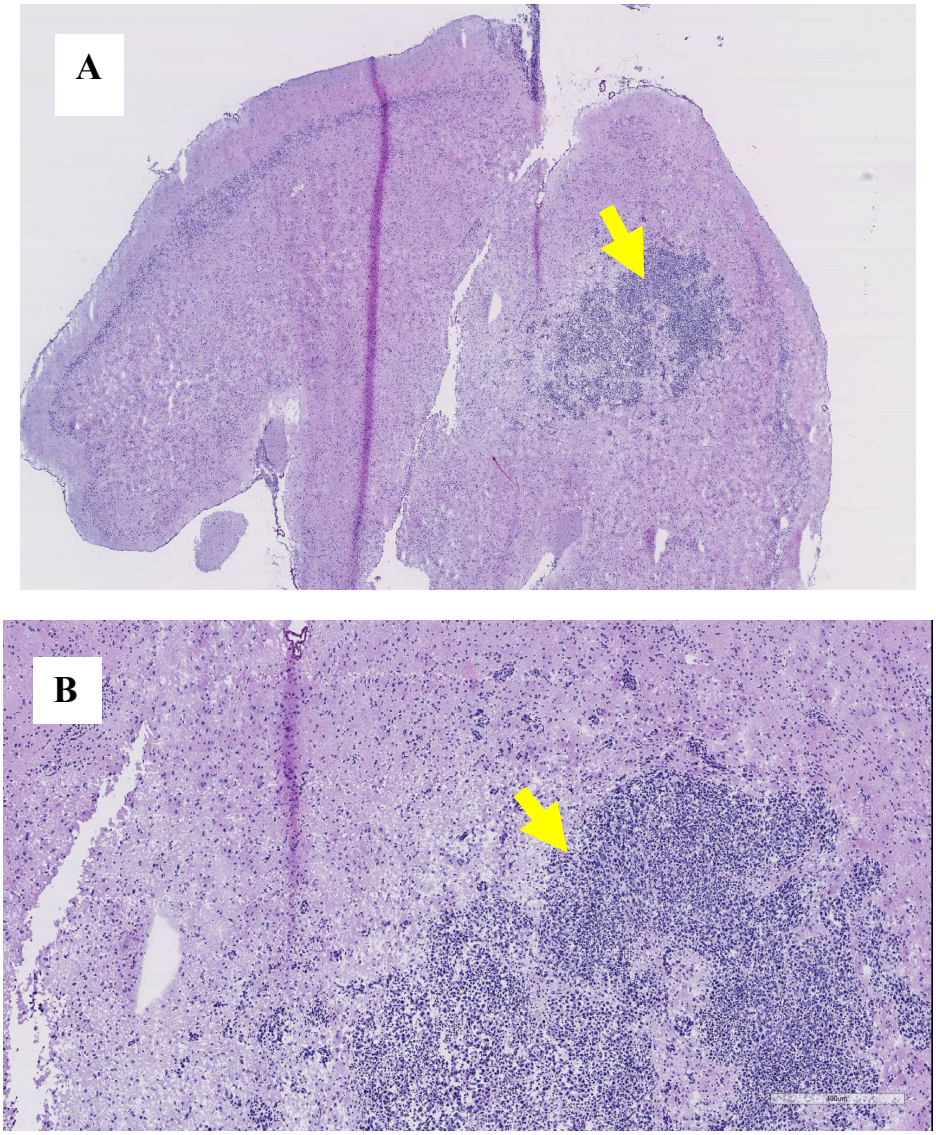


Figure 16: intracranial lesion following stereotactic inoculation of LUC-4T1-BR cells*. 1x10^4 LUC-4T1-BR cells in PBS were implanted using a stereotactic frame. Images taken using the Lumina II IVIS system 10 mins following administration of 10ul/g of luciferin, on auto exposure, 12 days after inoculation of cells.*

3.5 Summary of models and their role in this PhD

Clinically TNBC is a fast growing widely metastatic disease, and the murine models explored above, particularly the 4T1 model, seem to recapitulate this. These models will be taken further in the next few chapters and used to form the foundations for *in vivo* experiments of HSV oncolytic virotherapy. Although outside the scope of this PhD, experiences with these

in vivo models could be further developed through exploration of the number and type of cells per inoculation and whether this changes the natures of the metastatic disease. Another area to explore would be the use of patient derived cells. At the start of this PhD, the University of Sheffield did not have a biobank of patient derived breast cancer cells, although this has changed recently with an increasing amount of *ex vivo* work being performed within the oncology department. With regards to metastatic areas of interest, patient derived brain metastases have been used to form subcutaneous flank lesions in NSG mice with libraries being formed (278) and future work could involve introduction of these samples with stereotactic intracranial injection to see if this improves modelling. Patient derived models often require immunosuppressed hosts and as oncolytic virotherapy, which exerts it effects through the immune system, it was felt important for this PhD to focus on immunocompetent models with an intact vascular and immune system. Therefore, the models describe form a solid foundation to take this work forward.

In particular, the unmet need of targeting TNBC brain metastases was a conscious decision given personal clinical experience in this area and recognises the significance in patient morbidity and mortality. Dissemination of TNBC to the brain is a complex multifactorial process and is unfortunately outside the scope of this PhD, however a suitable and reproducible model to assess HSV oncolytic virotherapy has been established.

A significant consideration has been made to the N3Rs in the experimental design for this PhD. Thought has been given to use of 3D *in vitro* models and their potential to address the PhD's hypothesis. In particular, scaffold structures to form organoids with a mixture of immune, connective tissue cells (e.g. fibroblasts) and tumour cells may illustrate the interactions between virotherapy and cancer cells. However, as the remits of this PhD was to focus on the efficacy of HSV1716 delivery, an *in vivo* system, with intact vasculature, immune system and blood brain barrier was felt to be a necessity. Non animal based models of cancer are of interest, and this could be an area of future work.

The next chapters present two peer reviewed published original articles describing the work performed as part of this PhD. Chapter 4 pertains to experiments to confirm the oncolytic virus HSV1716 has activity in TNBC. Here, the administration route of HSV1716 was found to have similar outcomes when administered intravenously or intratumorally, and HSV1716 efficacy was confirmed in a number *in vitro* and *in vivo* of breast cancer models. In addition, the mechanism of action of HSV1716 was confirmed to be immunogenic with an increase in cytotoxic leukocyte infiltration and a decrease in T reg cells. Interestingly and unexpectedly, macrophages were found to play an important role in HSV1716 replication, and this established support for macrophage delivered virotherapy as described in Chapter 5.

Chapter 4

4 Macrophages Mediate the Antitumour Effects of the Oncolytic Virus HSV1716 in Mammary Tumours

4.1 Introduction

The following paper (section 4.2) and supplementary figures (section 4.3) formed the bulk of the experimental work during this PhD. This chapter demonstrates the effectiveness intravenously delivered HSV1716 alone against three triple negative breast cancer cell lines in both *in vitro* and immunocompetent *in vivo* models. The immunological changes including leucocyte infiltration and the reprogramming on TAMs are confirmed. Furthermore, the finding that macrophages may support HSV1716 replication (and therefore enhance HSV1716 efficacy) is described with evidence to show that an increase in cytosolic PCNA may mediate this.

The data presented in this paper has been collected and analysed by a number of members of the tumour targeting group. During this PhD, my role was to perform, oversee and analyse the experiments from the 4T1 and TS1 cell lines *in vitro*, the experiments involved in the production, maintenance and infection of monocyte derived macrophages and bone marrow derived macrophages, the conceptualisation, design, data collection and analyses of *in vivo* work, the preparation of slides, histological analysis through H+E staining and immunofluorescence and flow cytometry and the finally the collation and writing the original research paper.

4.2 Original Research: Macrophages Mediate the Antitumor Effects of the Oncolytic Virus HSV1716 in Mammary Tumors

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Abstract

Oncolytic viruses (OV) have been shown to activate the antitumor functions of specific immune cells like T cells. Here, we show OV can also reprogram tumor-associated macrophage (TAM) to a less immunosuppressive phenotype. Syngeneic, immunocompetent mouse models of primary breast cancer were established using PyMT-TS1, 4T1, and E0771 cell lines, and a metastatic model of breast cancer was established using the 4T1 cell line. Tumor growth and overall survival was assessed following intravenous administration of the OV, HSV1716 (a modified herpes simplex virus). Infiltration and function of various immune

effector cells was assessed by NanoString, flow cytometry of dispersed tumors, and immunofluorescence analysis of tumor sections. HSV1716 administration led to marked tumor shrinkage in primary mammary tumors and a decrease in metastases. This was associated with a significant increase in the recruitment/activation of cytotoxic T cells, a reduction in the presence of regulatory T cells and the reprograming of TAMs towards a pro-inflammatory, less immunosuppressive phenotype. These findings were supported by *in vitro* data demonstrating that human monocyte-derived macrophages host HSV1716 replication, and that this led to immunogenic macrophage lysis. These events were dependent on macrophage expression of proliferating cell nuclear antigen (PCNA). Finally, the antitumor effect of OV was markedly diminished when TAMs were depleted using clodronate liposomes. Together, our results show that TAMs play an essential role in support of the tumoricidal effect of the OV, HSV1716-they both host viral replication via a novel, PCNA-dependent mechanism and are reprogramed to express a less immunosuppressive phenotype.

Introduction

Although modulating the immune system to target cancer has been a successful treatment for some solid malignancies, various forms of breast cancer are immunogenically cold (1) in that they exhibit a decreased mutational load and neoantigen expression. This leads to a lower infiltration by activated cytotoxic T cells and is often accompanied by a highly immunosuppressive tumor microenvironment (TME), resulting in an intrinsic resistance to immunotherapies. The TME consists of cancer cells, tumor vasculature, fibroblasts, mesenchymal stem cells, adipocytes, extracellular matrix, and immune system elements such as lymphocytes and tumor-associated macrophages (TAM). TAMs are a key component of the TME that contribute to immune evasion, suppress lymphocyte activity, and support tumor growth (2, 3). In particular, the accumulation of perivascular M2-skewed TAMs on the abluminal surface of tumor blood vessels has been shown to drive tumor relapse following

radiotherapy (4) and chemotherapy (5), perhaps contributing to the eventual resistance of these standards of care (5, 6). A shift in the composition of the TME, together, with a burst in the release of tumor antigens, may turn a “cold” tumor into a “hot” one and therefore allow the host’s immune system to recognize and halt tumor growth and metastasis (7).

Oncolytic viruses (OV) are a promising class of anticancer therapeutics, which replicate in malignant cells and stimulate antitumor responses by initiating immunogenic cancer cell death (ICD), activating T cells and inducing protective antitumor immunity. Preclinical and early-phase clinical studies, in a number of solid tumor types including breast cancer have shown OV to have therapeutic efficacy with minimal toxicity (8–10).

HSV1716 is an OV derived from the Herpes Simplex Virus HSV-1 strain 17. It possesses a deletion in the RL1 genes encoding ICP34.5. Mutants lacking ICP34.5 are selectively replication competent in cancer cells. The subsequent lysis of these cells induces antitumor immune responses both directly, through cell lysis, and indirectly, via the induction of immunogenic cell death (ICD) and stimulation of adaptive immunity (11). As HSV1716 maintains expression of thymidine kinase, its toxicity is reversible by administering the antiviral acyclovir, thereby providing a “therapeutic safety net” to clinical toxicity. Phase I/II trials in over 100 pediatric and adult patients with solid malignancies have demonstrated minimal systemic toxicity when HSV1716 is administered intratumorally, intravenously, or loco- regionally (12, 13).

Here we show that HSV1716 effectively reduces primary and metastatic mouse breast tumors *in vivo*, in part, by replicating within and reprograming macrophages in the TME.

Materials and Methods

Cell lines and culture

Murine PyMT-TS1 [a kind gift from Prof Johanna Joyce, Memo- rial Sloan Kettering Cancer Center (MSKCC); ref. 14], E0771 (obtained from Dr Jessalyin Ubellacker (Harvard University, USA), and LUC-4T1-BR (obtained from Prof Sanjay Srivastava, University of Texas, Texas; ref. 15) mammary cancer cells were used *in vivo*. Human MCF-7, MDA-MB-231, MCF10DCIS, and SKBR3 cells, murine 4T1, EO771, and PyMT-TS1 cells and African Green Monkey Vero cell lines were used *in vitro*. Unless specified, all cell lines were purchased from the ATCC between 2015 and 2018 and used within 30 passages. Murine E0771 and human MDA-MB-231 cells were maintained in RPMI supplemented with 10% v/v FBS and 1% L-glutamine. Murine Luc-4T1-BR cells (4T1 cells transfected to express luciferase) were maintained in DMEM supplemented with 10% v/v FBS. All cells were used within 20 passages and were cultured at 37C in 5% v/v CO_2.The identities of all cell lines were regularly confirmed using microsatellite analysis and were tested to be mycoplasma free. All culture reagents were purchased from Lonzo BioWhittaker Ltd.

Preparation of monocyte-derived macrophages (MDM)

Human monocytes were isolated from mononuclear cells derived from human buffy coats obtained from the NHS Blood and Transplant Unit, Sheffield, as described previously (16). Briefly, the peripheral blood mononuclear cell (PBMC) layer was collected following centrifugation over Ficoll (Sigma-Aldrich) and seeded overnight in Iscove's Modified Dulbecco's Medium (IMDM; Sigma-Aldrich) and 2% v/v human AB serum (Sigma Aldrich). Nonadherent cells were removed, and macrophages allowed to fully differentiate.

Virus production and handling

HSV1716 (unlabelled) and HSV1716-GFP (in which GFP is driven by a CMV promoter) were obtained from Virttu Biologics in stocks of 1 10^8 particle forming units (PFU) in compound

sodium lactate (Hartmann's solution) with 10% v/v glycerol. All vials were stored at 80C and freshly thawed on ice immediately before each experiment.

HSV1716 infection of MDMs *in vitro*

MDMs were washed in PBS, suspended in 500 mL serum-free RPMI medium and then incubated with HSV1716 virus (MOI 5) for 2 hours at 37 C in 5% v/v CO_2. Noninfected virus was washed off and cells were analyzed 24 to 72 hours after infection. For plaque assays supernatants were removed at each time point and added to Vero cells as described previously.

Mixed lymphocyte reaction

A mixed lymphocyte reaction (MLR) was used to identify leukocyte activation and proliferation in an autologous reaction. MDMs were obtained from human buffy coats and cultured as above. Lymphocytes, from the same donor, were frozen down in 90% v/v FBSþ10% v/v DMSO until needed. Once mature, MDMs alone or MDMs infected with HSV1716 for 4 hours were co-incubated with lymphocytes at a ratio of 1:6, as described previously (17). MDA-MB-231 cells were also added, if needed, at a 1:1 ratio with MDMs. Lymphocytes were co- cultured with macrophages for 24 hours before analysis by flow cytometry.

Flow cytometric analysis

MDMs (24 hours after infection) and dissociated mammary tumors were stained with fluorescent antibodies (Supplementary Table S1; ref. 5). All antibody incubations were performed for 1 hour at 4C and the samples were analyzed using an BD LSR II flow cytometer (BD Biosciences) and data analyzed processed using FlowJo Flow Cytometric Data Analysis Software (BD Biosciences). The mouse immune cell populations analyzed included: neutrophils ($CD45^{þ}CD11b^{þ}Ly6G^{þ}$), monocytes ($CD45^{þ}CD11b^{þ}Ly6G^{neg}Ly6C^{þ}$ $F4/80^{Lo}$),

macrophages ($CD45^{þ}CD11b^{þ}Ly6G^{neg}Ly6C^{Lo}F4/80^{Hi}$), THelper ($CD45^{þ}CD3^{þ}CD4^{þ}$), and cytotoxic T-cells ($CD45^{þ}CD3^{þ}$ þCD8). The membrane impermeant, fixable, amine reactive dye Zombie UV Fixable (BioLegend Inc.) was used to discriminate between live and dead cells. All data are presented as the proportion of viable leukocytes.

Real-time PCR (qPCR)

Total mRNA was extracted from cultured MDMs or murine tissues using the RNeasy Mini Kit (Qiagen). A list of primer sequences is given in Supplementary Table S2. The Ct values generated from these samples were normalized to a housekeeping gene. Relative gene expression to untreated macrophages was estimated via normalization of the gene of interest to the housekeeping gene followed by the comparative Ct ($2^{\Delta\Delta Ct}$) method.

Western blot analysis

Protein detection by SDS-PAGE was carried out on MDMs (18). Protein samples were denatured at 70C and loaded onto a gel with Laemmli sample buffer. The gel was transferred onto a nitrocellulose membrane (Invitrogen) using aniBlot gel transfer device. The mem- brane was blocked in 5% v/v milk for an hour and incubated at room temperature, with primary antibodies for 90 minutes and secondary antibodies for an hour. Membranes were probed with enhanced chemiluminescence (ECL; Bio-Rad) and visualized using a Chemidoc 2011 (Bio-Rad).

HSV1716-induced cell lysis

To assess cell lysis induced by HSV1716, various cell lines were seeded at 1 10^5 cells/well in a 24-well plate and infected with HSV1716 at MOI 5 (unless otherwise stated). At 24 and 48 hours, cells were stained with 2 mg/mL propidium iodide (PI). Cell numbers and PI positivity

were analyzed on the BD LRSII flow cytometer (Thermo Fischer Scientific). FlowJo software was used to analyze cell death based on a change in fluorescence against FL3-H for PI and FL1-H for GFP expression.

Viral replication: qPCR

RNA was isolated from infected MDMs using the RNeasy Mini Kit (Qiagen) followed by cDNA synthesis using Super Script III Reverse Transcriptase (Life Technologies). cDNA was analyzed using viral replication genes ICP0, ICP8, and gB with GAPDH as the housekeeping gene using SYBR Green (Primer Design; see Supplementary Table S2).

In vivo viral detection

IHC was carried out on fixed tissue sections stained using a polyclonal sheep HSV antibody at a dilution of 1:500 (kind gift from Virttu Biologics) for 1 hour. Staining was then visualized using a sheep VECTASTAIN ABC HRP (Horseradish Peroxidase) Kit (Vector Laboratories). Slides were counter stained, mounted, and sections scanned using Hamamatsu NanoZoomer XR (Hamamatsu).

Plaque assays

These were performed as described previously by Baer and Kehn-Hall (19). Briefly, confluent monolayers of Vero cells were inoculated with serial dilutions of supernatants derived from infected macrophages as described previously. After 2 hours, supernatants were removed and monolayers were overlaid with 4% w/v agarose:culture medium (1:10), which was allowed to solidify for 15 minutes at room temperature before incubation in a humidified incubator for 72 hours at 37C. Paraformaldehyde (4% w/v) was applied to agarose plugs for 1 hour to fix the cell monolayers before their removal. Cells were washed with PBS, stained with 1 mL crystal

violet for 5 minutes, and rinsed with tap water. Once dried, plaques were counted per well and viral titer determined.

Analysis of MDM death

To understand how HSV1716 mediated the oncolysis of MDMs *in vitro,* the expression of a panel of cell death markers was determined using qPCR. cDNA was analyzed using a predesigned apoptosis and survival array (tier 1) (Bio-Rad) and then validated by qPCR using a panel of apoptotic genes (FASL and BCL2), autophagy genes (ATG5 and LC3B), and ICD genes (HMGB1 and CalR), with GAPDH as the housekeeping gene (see Supplementary Table S2). Analysis using a HMGB1 ELISA Kit II (Shino-Test) and an ENLITEN ATP assay (Promega) confirmed the presence of ICD.

Knockdown of PCNA expression in MDMs

MDMs were transfected with PCNA or nonspecific siRNAs (Accell Human PCNA siRNA SMARTpool, 10 nmol; Thermo Scientific/ Dharmacon). For this, MDMs were aliquoted into a six-well plate ($0.5\ \ 10^5$ macrophages/well) and incubated with 1.5 mL of Accell delivery medium containing 1 mmol/L siRNA, after which they were incubated at 37C for 72 hours. Protein or mRNA knockdowns were confirmed by Western blot analysis and qPCR and viability deter- mined using the MTS CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

In vivo studies

Animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 with approval from the UK Home Office approval (PPL70/8670), the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines, and the University of Sheffield Animal Welfare Ethical Review Body (AWERB). All female mice were obtained

from Charles River Laboratory at 6 to 8 weeks and acclimatized in the Biological Services Laboratory for 7 days prior to experimentation. Animals were anesthetized using 3% to 4% isofluorane in 70:30% N_2O:O_2.

Orthotopic mammary tumor model

Mammary cancer cells (1 10^6 PyMTTS1 cells in 50 mL of 1:1 Matrigel) were implanted into the fourth mammary fat pads of 6- to 7-week-old syngeneic FVB mice (n 1⁄4 10/group). E0771 and 4T1 cells were implanted via intraductal injection to the mammary fat pads of syngeneic C57BL/6 and BALB/c mice, respectively. Mammary tumor growth was assessed by digital caliper measurement every 2 to 3 days and when tumors reached 150 mm^3 mice were randomly divided into groups and received a single 100 mL injection of PBS or HSV1716 ($1x10^6$ PFU) intravenously via tail–vein injection. Of note, a similar treatment schedule was performed to compare intravenous and intra- tumoral injections in the PyMT TS1 model. Of note, mice in the PBS groups became unwell at day 9 and therefore some mice, in both groups, were culled early for postmortem comparison of tissues. Excised tissues (tumors, brain, liver, lungs, kidney, and spleen) were embedded in OCT freezing media or paraffin wax for immunocytochemical and histologic labeling studies. Tumors were dispersed by enzymatic digestion after first dicing into approximately 1 mm^3 pieces. These pieces were incubated for 40 minutes at 37C in serum-free IMDM (VWR International) supplemented with 2 mg/mL dispase, 0.2 mg/mL collagenase IV (Sigma-Aldrich), and 100 U/mL DNase (Merck Millipore). Dispersed tumors were passed through 70 mm nylon filters (Becton Dickinson) and maintained on ice in PBS or cryo- preserved in 90% v/v FBS and 10% v/v DMSO for flow cytometric analysis.

Experimental metastasis model

To model the metastatic seeding seen in patients with breast cancer, we used a metastatic 4T1 cell line which metastasizes to brain, lung, liver, and bones when administered via intracardiac route (15). For this model, 1 10^5 LUC-4T1-BR cells were filtered and injected into the left ventricle of 6- to 7-week-old female BALB/c mice. Tumors were allowed to grow for 5 days following which animals were randomly allocated (n 1⁄4 6 per group) and received either PBS, one dose of HSV1716 (1 10^7) or three doses of HSV1716 (1 10^7 given on day 1, 3, and 5). Animals were imaged two to three times a week using a luminescence *in vivo* imaging system (IVIS Lumina II imaging; Caliper Life Sciences) following intraperitoneal injection of luciferin (150 mg/kg). Mice were sacrificed if they reached a humane endpoint (weight loss over 20%), signs of distress (e.g., breathlessness or pain), or the experimental endpoint of 50 days following tumor inoculation. Weight loss was the most common cause of premature sacrifice.

Macrophage depletion model

Macrophages were depleted using a single dose of clodronate liposomes (CL; Liposoma B.V.) 48 hours prior to viral administration in the primary mammary model. When mammary tumors reached 150 mm^3, animals were divided into four groups, n 1⁄4 5/group. Control groups received either one dose of PBS (100 mL) or one dose of CLs (100 mL). Treatment groups received an intravenous injection of HSV1716 (one dose, 1 10^6 PFU) or intravenous injection of CL followed by intravenous HSV1716 (one dose, 1 10^6 PFU). Animals were monitored and primary tumors measured every 2 to 3 days using calipers. All animals were culled as soon as one animal had a tumor of 800 mm^3.

Tissue analysis

Frozen tumor sections were blocked with 1% w/v BSA and 5% v/v goat serum for 30 minutes and incubated at room temperature, with the relevant primary antibodies (Supplementary Table

S1), for an hour. Alexa fluor–conjugated anti-rabbit or anti-rat secondary anti- bodies (as appropriate) were used to detect primary antibody binding. Nuclei in all tumor sections were counterstained with DAPI. Slides were visualized using a Nikon Dual Cam system microscope, a Nikon A1 Confocal Laser Microscope and an EVOS Cell Imaging System (Thermo Fisher Scientific). Formalin-fixed, paraffin-embedded tissues were rehydrated, peroxidase blocked, antigen retrieved, serum blocked, and then incubated with primary antibodies for 1 to 2 hours. Primary antibodies were detected with ABC or Polymer Detection Kits followed by chromogen staining with 3^0-diaminobenzidine (DAB).

Following hematoxylin and eosin staining, slides were visualized using the Hamamatsu NanoZoomer XR scanner (Hamamatsu) and staining in five randomly selected fields of view per tumor quantified using ImageScope (Leica Biosystems).

NanoString nCounter gene expression analysis

Amplification-free gene expression profiling of tumor tissue using a NanoString nCounter FLEX platform and the murine PanCancer Immune Profiling Panel, which consist of 750 immune-related genes and 20 housekeeping genes (NanoString Technologies Inc.) was undertaken in the John van GeestCancer Research Centre (Notting- ham Trent University). Total mRNA was extracted from cultured MDMs or murine tissues using the RNeasy Mini Kit (Qiagen), and quality controlled using a NanoDrop 8000 Spectrophotometer. For gene expression profiling, 150 ng of total RNA from each sample was used for NanoString probe hybridization, which was undertaken overnight (20 hours) at 65C in a PCR machine with heated lid [each reaction mixture contains 5 mL of RNA solution (150 ng), 8 mL of reporter probe and 2 mL of capture probe]. After overnight hybridization, excess probes were removed using the NanoString nCounter Prep Station and magnetic beads, hybridized mRNA/probe were immobilized on a streptavidin-coated cartridge. The processed cartridge was subsequently

scanned, and raw data generated at high resolution (555 fields of view, fov) using a NanoString nCounter digital analyzer platform and processed using nSolver Data Analysis Software (V.4.0). Imaging quality control (QC), mRNA positive control QC and normalization QC were checked, and all the samples were with the quality parameters of NanoString gene expression assays. Differential expression, pathway, and cell type scoring was performed using the nSolverAdvanced Analysis Module v.2.0.115. Data normalization was performed using the geNorm algorithm for the selection of the best housekeeping genes. Genes which showed ≥2, fold change in their expression with a BY (Benjamini–Yekutieli procedure) P value ≤0.05 were considered significantly different between the groups.

Apoptosis and pro-survival array

The apoptosis and pro-survival tier 1 array (Bio-Rad) was used to assess cell death. For this, cDNA was synthesized from control or infected MDMs using the Precision nanoScript2 Reverse Transcription Kit (PrimerDesign). cDNA was plated into the 386-well qPCR plate and processed using an Applied Biosystems 7900 Real-Time PCR System.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 and the tests are described in the figure legends. Data are mean SEM (as indicated) and P values of <0.05 were considered statistically significant.

Results

HSV1716 has antitumor activity in breast cancer models

The susceptibility of breast cancer cells to HSV1716 infection (Supplementary Fig. S1A) and virus-mediated death (Supplementary Fig. S1B) was demonstrated *in vitro* using a panel of

human and murine breast cancer cells lines (MCF7, MDA-MB-231, SKBR3, MCF10DCIS.com, 4T1, EO771, and PyMT-TS1).

The cytotoxic potential of HSV1716 was then assessed in three *in vivo* models of primary breast cancer. First, we investigated the best route of HSV1716 delivery in the PyMT-TS1 model. For this, animals were randomly assigned into one of three treatment groups: control (PBS), intratumoral (IT) HSV1716, and intravenous (IV) HSV1716, with a reduction in primary mammary tumor growth being observed in both the IT and IV groups (Supplementary Fig. S2A). IT and IV administration of HSV1716 also reduced pulmonary metastasis and increased tumor necrosis (Supplementary Figs. S2B and S2C). Given the positive response to IV HSV1716, and that the IV route is currently the preferred modality to deliver breast cancer chemotherapies, the remainder of the study focused on IV delivery of HSV1716.

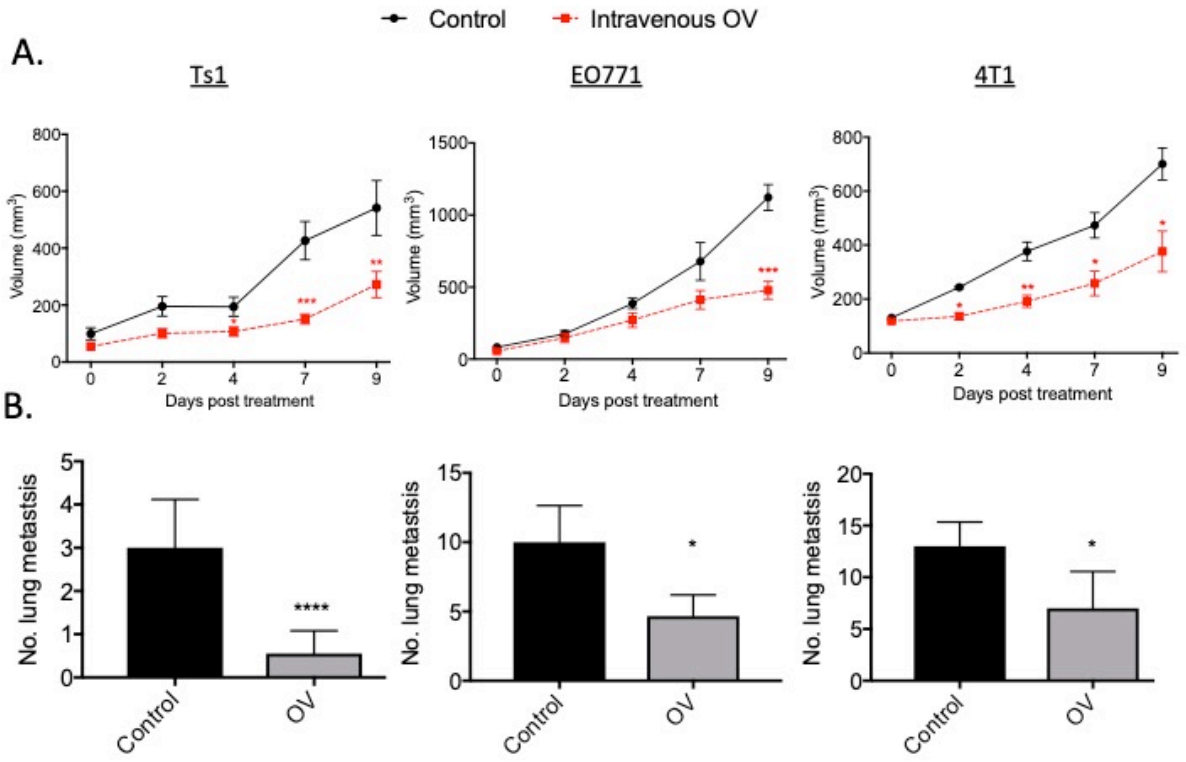

*Figure 1: HSV1716 treatment inhibits primary mammary tumor growth and metastatic spread. PyMT-TS1, EO771, and 4T1 cells were implanted into the fourth mammary fat pad of immuno-competent syngeneic female mice. When tumors reached ~150 mm3, mice received intravenous HSV1716 (dose 1 × 106 PFU) or PBS. Tumor volume was assessed by caliper measurements in vivo and pulmonary metastases were measured postmortem by hematoxylin and eosin staining. HSV1716 (grey line) significantly reduced (A) mammary tumor growth and (B) subsequent development of lung metastases in all three models. Data shown are mean ± SEM, n = 10 mice/treatment group and statistical significance analyzed using multiple t tests, where *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with control (untreated cells).*

In three models of primary breast cancer (PyMT-TS1, E0771, and 4T1), we demonstrated IV HSV1716 to significantly slow the growth of orthotopically implanted tumors (Fig. 1A). The number of subsequent spontaneous lung metastases in these animals was also significantly reduced in the IV treated groups (Fig. 1B). Furthermore, we observed that early introduction of HSV1716 prevented the formation of breast cancer metastases and thereby increased overall survival in the met- astatic 4T1 model. In this model, luciferase-labelled 4T1 cells were injected into the left ventricle of the heart. Five days later, mice were treated with PBS, HSV1716 (one dose), or HSV1716 (three doses). Doses were repeated every 48 hours (Fig. 2A). Tumor growth and spread were monitored using bioluminescent *in vivo* imaging two to

three times weekly (Fig. 2B). A survival advantage was seen in the HSV1716-treated groups over the PBS control, and a significant number of animals showed no sign of disease by day 50 (Fig. 2C). As shown, this was more marked with repeated doses of the virus, in which instance, all mice survived to the experimental endpoint (50 days) compared with an average survival of 31 days for one dose and 24.5 days for PBS mice (P 1⁄4 0.0002; CI, 15.49–35.18). This may be because the immunomodulatory effect of the virus takes time to develop and repeated dosing allows for this to occur, or that repeat dosing does not allow circulating tumor cells or micro-metastases to develop. The presence of brain, liver, and lung metastasis was assessed and a significant reduction in the number of lung metastases in the groups treated with HSV1716 observed, with a trend to less metastases in the brain and liver (Fig. 2D). Together, these exciting results support the possible use of HSV1716 to treat breast cancer.

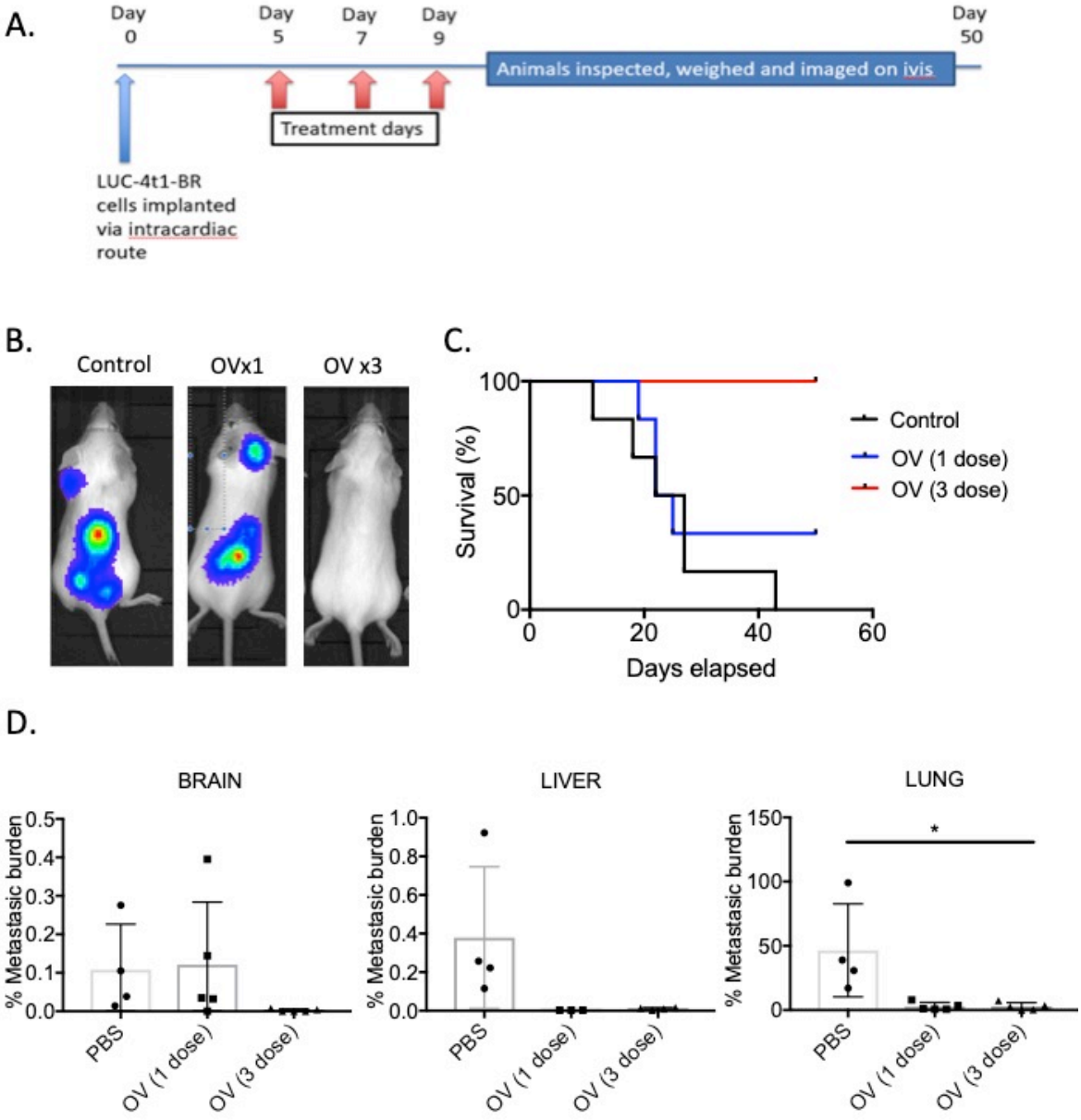

Figure 2: HSV1716 treatment prevents tumor spread in a model of metastatic seeding. *Luciferase labeled 4T1 cells were injected into the left ventricles of female BALB/c mice. At day 5, mice were treated with PBS, HSV1716 (1 × 107 PFU, single dose) or HSV1716 (1 × 107 on days 5, 7, 9). A, Schematic representation of the treatment schedule was shown. B, Representative images of metastatic burden in different treatment groups at day 20. Tumor burden was inhibited in the OV-treated group with no disease observed in the three-dose group at the end of experiment. C, Overall survival was also increased in all mice that received virus. There is a statistically significant reduction in the survival between the control (PBS, treated) and the group that received three doses of HSV1716 (P = 0.0002; CI, 15.49–35.18) and between the OVx1 and OVx3 group (P = 0.01; CI 5.401–31.93). D, Burden of metastases was calculated as the percentage of the organ with metastatic involvement; this was calculated as an average between four slides in two to four different sections of organ, over 100 µm apart. Data shown are mean ± SEM, n = 6 animals. Statistical significance was assessed by one-way ANOVA, where *P < 0.05.*

HSV1716 stimulates leukocyte infiltration into tumors

The influence of HSV1716 treatment on the immune content of the TME, including immune infiltrates was examined using the Nano- String nCounter Mouse PanCancer Immune Profiling Panel. This large targeted gene panel comprised of specific gene sets to understand different immune cell types and their functions in the TME. As shown in Fig. 3A, treatment with HSV1716 induced the differential regulation of 282 genes (where P 1⁄4 0.05). The top 20 genes and their biological functions are highlighted in Supplementary Table S3, and these include genes involved in innate and adaptive immune responses (e.g., CD55, IL21, TXk, Thbs1), immune cell function (CD22, CD37, Blnk, Sell, CD247, IL7R, Dpp4, Btla, CD247, Thbs1), and the TNF pathway (TNFrsf13b, Ltb). Supplementary Fig. 3A shows genes related to upregulated pathways including innate and adaptive immunity, inflammation, cytokines and receptors, apoptosis, and cell type scores (dendritic cell, natural killer, macrophages, and T cells) that were significantly upregulated following intravenous HSV1716 (Supplementary Fig. 3B).

To confirm the presence of these immune cells in the TME, flow cytometric analysis of dispersed PyMT primary tumors demonstrated a significant increase in $CD11b^{+}Ly6C^{+}$ monocytes, $CD11b^{+}$, $LY6G^{+}$ neutrophils, $CD3^{+}$ T cells, and $CD8^{+}$ cytotoxic T cells following HSV1716 treatment (Fig. 3B). This is consistent with other published studies using OV (20). Triple immunofluorescence analysis of tumor sections revealed an increase in activated $CD8^{b+}$ cytotoxic T cells (i.e., $IFNg^{+}$ or $PD\text{-}1^{+}$ $CD3^{+}CD8^{+}$ cells) in this mouse model after HSV1716 treatment (Fig. 3C). Furthermore, HSV1716 treatment led to a reduction in the number of $CD4^{+}FOXP3^{+}$ T regulatory (Treg) cells (Fig. 3D).

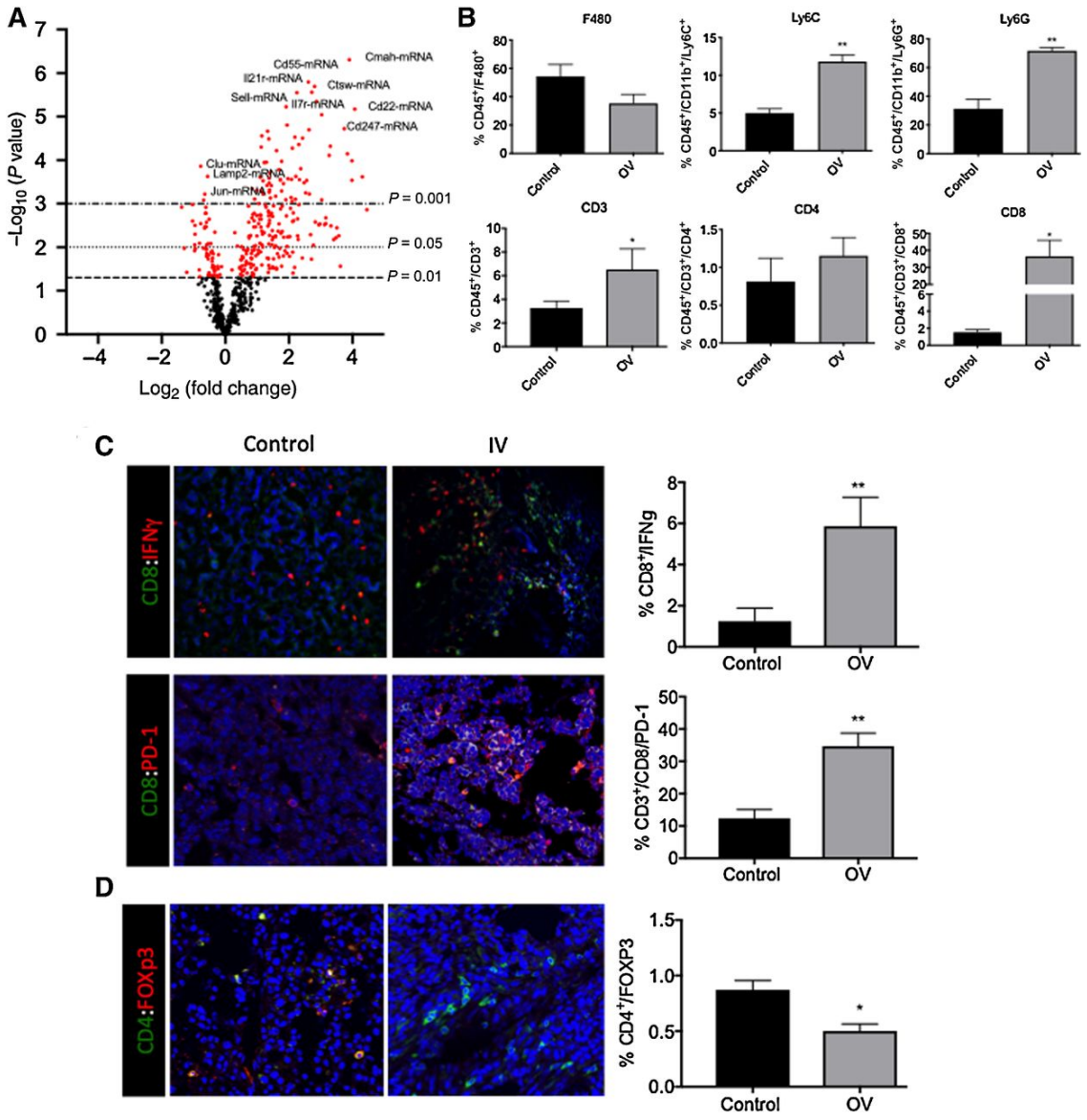

Figure 3: HSV1716 treatment induces inflammation within the TME. *PyMT tumors were grown in syngeneic female FVB mice and randomized into two treatment arms, control (PBS) and intravenous HSV1716 treatment (*n *= 5–10 per arm).* ***A,*** *RNA was isolated from tumors and analyzed using the NanoStringnCounter murine Pan-Cancer Immune Profiling Panel. Volcano plots show genes that were upregulated or downregulated following IV HSV1716 treatment compared with tumors in PBS-treated mice (*n *= 3). The data were processed and analyzed using nSolver Analysis Software, using the Advanced Analysis module.* ***B,*** *Flow cytometric data from these enzymatically digested tumor specimens are shown from* n *= 10 mice per group. Only viable cells (UV-) were used in this analysis. Each immune cell population was gated upon based on CD45 expression and the respective immune cell marker. This shows changes in the percentage of infiltrating immune cells [myeloid cells ($F480^+$), monocytes ($Ly6C^+$), neutrophils ($Ly6G^+$), T cells ($CD3^+$, $CD4^+$, $CD8^+$)] within the TME.* ***C,*** *Immunofluorescent staining of respective tumors (five fields per view per slide) confirmed that these T cells become activated in response to HSV1716 treatment, as illustrated by increased expression of IFNg and PD1 and (**D**) a decrease in the prevalence of $CD4^+FOXP3^+$ immunoregulatory T (Treg) cells. Data shown are mean ± SEM, analyzed by Student* t*tests, where* $P < 0.05$.

HSV1716 reprograms TAMs to a more pro-inflammatory and perivascular phenotype

NanoString nCounter gene profiling revealed an upregulation in the macrophage function scores (Supplementary Fig. 3B). Despite this, we observed the average number of TAMs within tumor samples (% of $CD45^{þ}F4/80^{þ}$ per 100,000 events of viable cells) was not significantly altered following HSV1716 treatment (Fig. 3B), but that there was a marked change in their phenotype. First, HSV1716 treatment significantly decreased the prevalence of "tumor promoting" perivascular macrophages (i.e., $F4/80^{þ}$ TAMs directly in contact with $CD31^{þ}$endothelial cells) in PyMT mammary tumors (Fig. 4A). Second, HSV1716 treatment significantly increased the number of $F4/80^{þ}$ TAMs expressing pro-inflammatory, M1-like markers, IL12 and iNOS, relative to matched controls (no virus). Furthermore, HSV1716 treatment significantly reduced ($P < 0.05$) the number of $F4/80^{þ}$ TAMs expressing the M2-like marker "MRC1" (Fig. 4B). This reprogramming of TAMs has the potential to change the balance in the TME, in that M2-like TAMs become M1-like, thereby promoting the recruitment of adaptive immune cells and cytotoxic potential.

Our *in vitro* experiments also demonstrated that human MDMs infected with HSV1716 undergo a transformation to a more inflammatory phenotype, specifically a greater expression of M1-like markers ($CD80^{hi}$, $CD86^{hi}$, $PD\text{-}L1^{hi}$) and lower expression of M2-like markers ($CD64^{lo}$, $CD163^{lo}$, and $CD206/MRC1^{lo}$; Supplementary Fig. S4A) and increases in the expression of pro-inflammatory versus anti-inflammatory markers at the mRNA level (Supplementary Fig. S4B). Infected macrophages also secreted pro-inflammatory cytokines including IL6, IL12, and TNFa (Supplementary Fig. S4C) and increased levels of nitric oxide (Supplementary Fig. S4D).

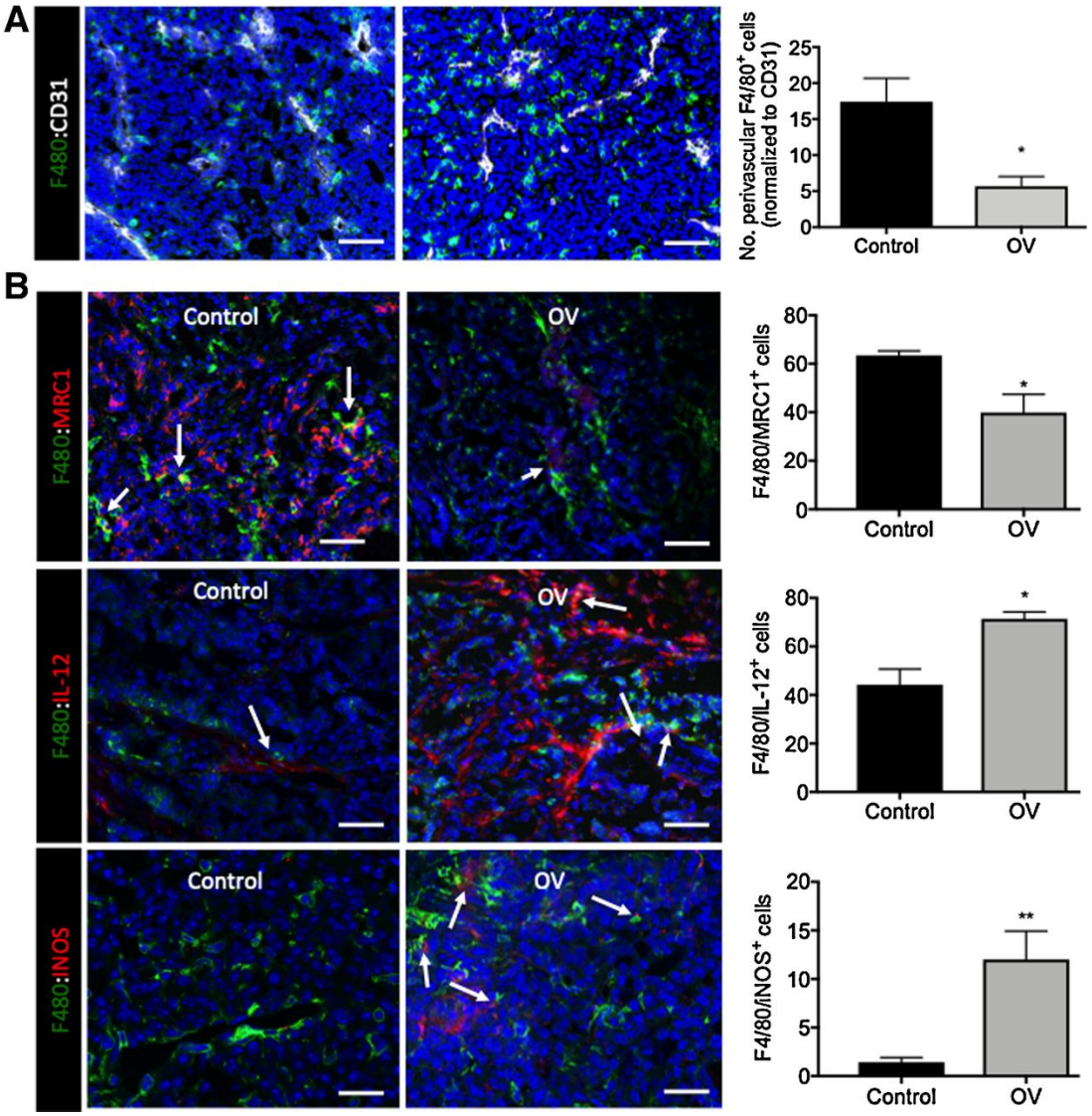

Figure 4: HSV1716 treatment reprograms TAMs to become less perivascular and more pro-inflammatory. *A, Sections of primary tumors, derived from the PyMT-TS1 model, treated with intravenous HSV1716 (OV) or PBS show a significant decrease in the number of perivascular (CD31+) macrophages after HSV1716 treatment (n = 10 animals). These were quantified by only counting the F4/80+ cells in direct contact with CD31+ cells. B, HSV1716 treatment also causes a shift in macrophage phenotype with a downregulation in F4/80+MRC1+ cells and a significant increase in presence of F4/80+IL12+ and F4/80+iNOS+ cells. Images were taken using the Nikon A1 confocal microscope and scored using FIJI image J software. Colocalization between F4/80 and MRC1, IL12, or iNOS was determined and quantified using the Cell Counter tool from ImageJ (Fiji; NIH) and five randomly selected fields of view were imaged per tumor. Data shown are mean ± SEM, analyzed by Student t tests, where $P < 0.05$.*

Macrophages support HSV1716 replication and undergo ICD

The observation that HSV1716 treatment led to a colocalization of F4/80^{+} TAMs and HSV1716 in the TME (Fig. 5A) prompted the question as to whether macrophages are permissive to HSV1716 infection and, if so, what is the nature of this relationship? To investigate this, MDMs derived from human buffy coats were infected with the HSV1716 for 2 hours, following which excess virus was washed off, cells incubated for a further 24 hours and infection confirmed on the basis of GFP expression using the reporter virus (HSV1716:GFP; Fig. 5B). Plaque assays determined viral titers within the supernatants of these virally infected MDMs. An increase in viral titer sat 24, 48, and 72 hours was seen following infection of the HSV permissive Vero cell line (Fig. 5C). Furthermore, expression of genes required for early (ICP0), mid (ICP8), and late (gB) viral replication was quantified using qPCR (Fig. 5D). These studies confirmed that HSV1716 went through all stages of viral replication within MDMs *in vitro* and suggest that MDMs infected with HSV1716 supported active viral replication.

As we saw a significant drop in macrophage numbers 24 hours after treatment *in vivo* (Supplementary Fig. S5A), we next assessed the impact of HSV1716 on macrophage viability and sought to identify the cause of this in human MDMs *in vitro*. First, we noted that human MDMs infected with HSV1716 undergo enhanced levels of cell death compared with noninfected cells *in vitro* (Supplementary Fig. 5B). To ascertain the mechanism of this, a cell death array was performed (Supplementary Table S4). The mechanism of macrophage cell death and signalling pathways were largely apoptotic and immunogenic, with an increase in Fas ligand and HMGB1 expression, and a downregulation of HSP genes (validated by qPCR; Supplementary Fig. S5C). These data were supported by Western blots showing an increased production of apoptotic proteins, FADD, FASL, and caspase 3 (Supplementary Fig. S5D) and

ELISA assays showing an increased secretion of immunogenic proteins, HMGB1 (Supplementary Fig. S5E) and extra- cellular ATP (Supplementary Fig. S5F).

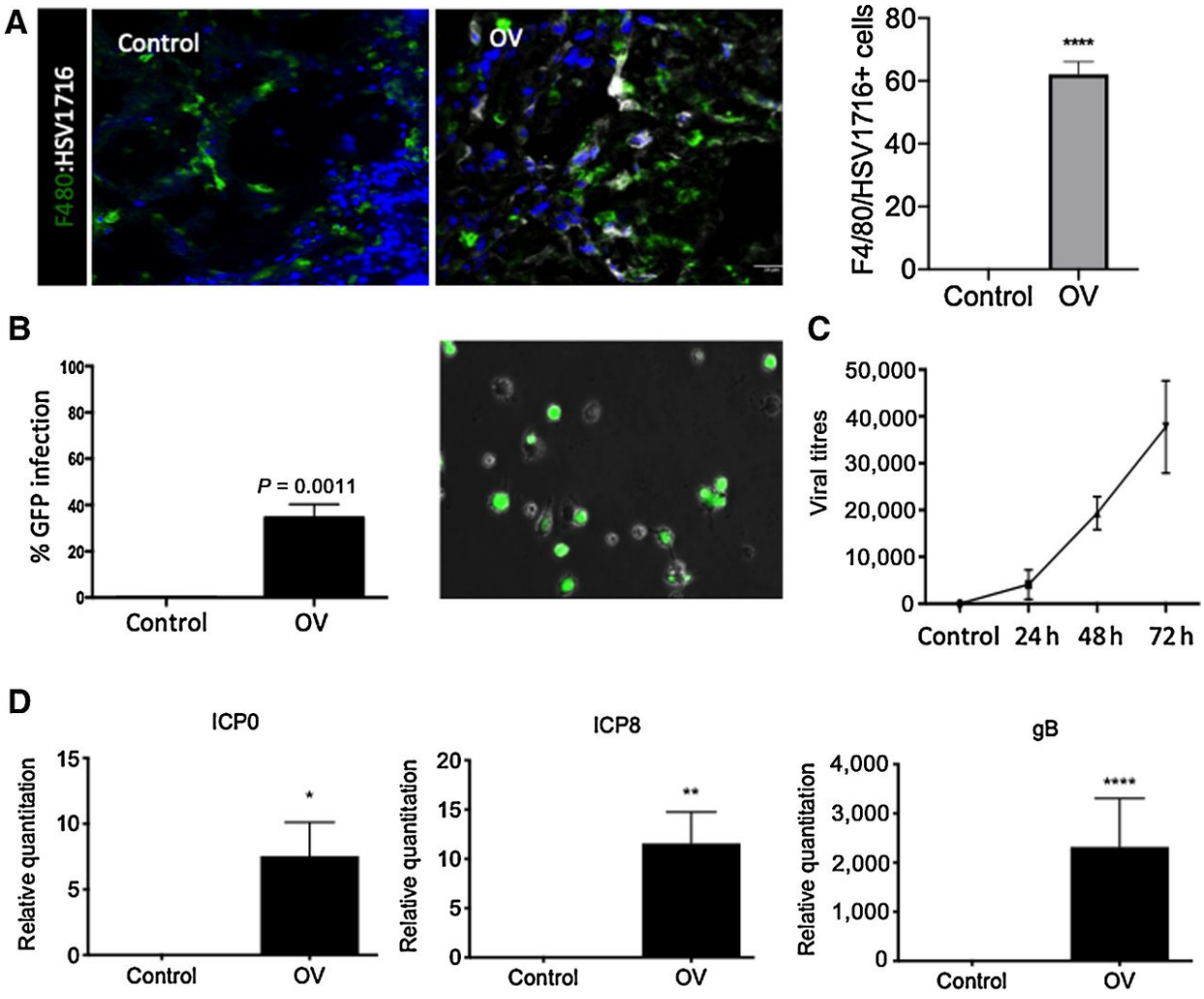

Figure 5: MDMs are permissive to HSV1716 infection and replication. *A, Representative sections from primary PyMT TS1 tumors in control (PBS) and HSV1716-treated mice showed colocalization of F4/80+ macrophages (green) and HSV1716 (white). This was quantified in n = 10 animals, five fields per view. B, MDMs were infected with HSV1716 expressing GFP at MOI 5. In vitro infection was assessed by flow cytometry (left image) and fluorescence (right image). C, Supernatants taken from these cultures were assessed by the plaque-forming assay on Vero cells and an increase in viral titers over time suggested active viral replication within these macrophages. D, qPCR analysis of MDMs at 24 hours after infection resulted in the mRNA expression of the viral early-ICP0 (left), mid-ICP8 (center), and late-gB (right) replication genes. Data shown are mean ± SEM, analyzed by Student t test, where $P < 0.05$, n = 5 independent*

<u>Proliferating cell nuclear antigen (PNCA) mediates HSV1716 replication in TAMs</u>

Oncolytic virus replication is known to occur within dividing tumor cells and therefore we were keen to determine why HSV1716 was replicating within macrophages (i.e., terminally differentiated cells). Wild type HSV has been shown to initiate proliferation in non-proliferative cells such as neurones by interacting with the cellular protein, PCNA (21), although this has not previously been demonstrated in macrophages. Interestingly, we found that HSV1716-infected PyMT- TS1 tumors contained significantly more PCNA^{þ} TAMs (Fig. 6A).

PCNA expression in HSV1716 infected and noninfected MDMs was measured *in vitro* at both the gene and protein level. MDMs infected with HSV1716 exhibited significant relative increases in PCNA mRNA (11-fold increase, P 1⁄4 0.0137) and protein expression (P 1⁄4 0.0173) compared with untreated MDMs (Supplementary Fig. S6A). This increased expression was more marked when MDMs were infected with HSV1716 in the presence of tumor-conditioned medium (TCM; Fig. 6B). Given the increase in PCNA following infection, we wanted to ascertain whether HSV1716 replication was possible in the absence of PCNA. PCNA knockdown (PCNA^{KD}) was carried out using Accell siRNA (Supplementary Fig. S6B). This had no effect on viral infectivity and the viability of macrophages compared with the nontargeting control (Supplementary Figs. S6C and S6D). Indeed, in the absence of PCNA, HSV1716 was unable to undergo viral replication within infected macrophages. This is evidenced by the lack of cell death in MDA-MB-231 cells following incubation with supernatants taken from infected macrophage cultures following PCNA^{KD} (Fig. 6C) and a reduction in viral replication genes (Fig. 6D). We therefore speculate that the presence of PCNA is likely the cause of viral replication and macrophage cell death after infection.

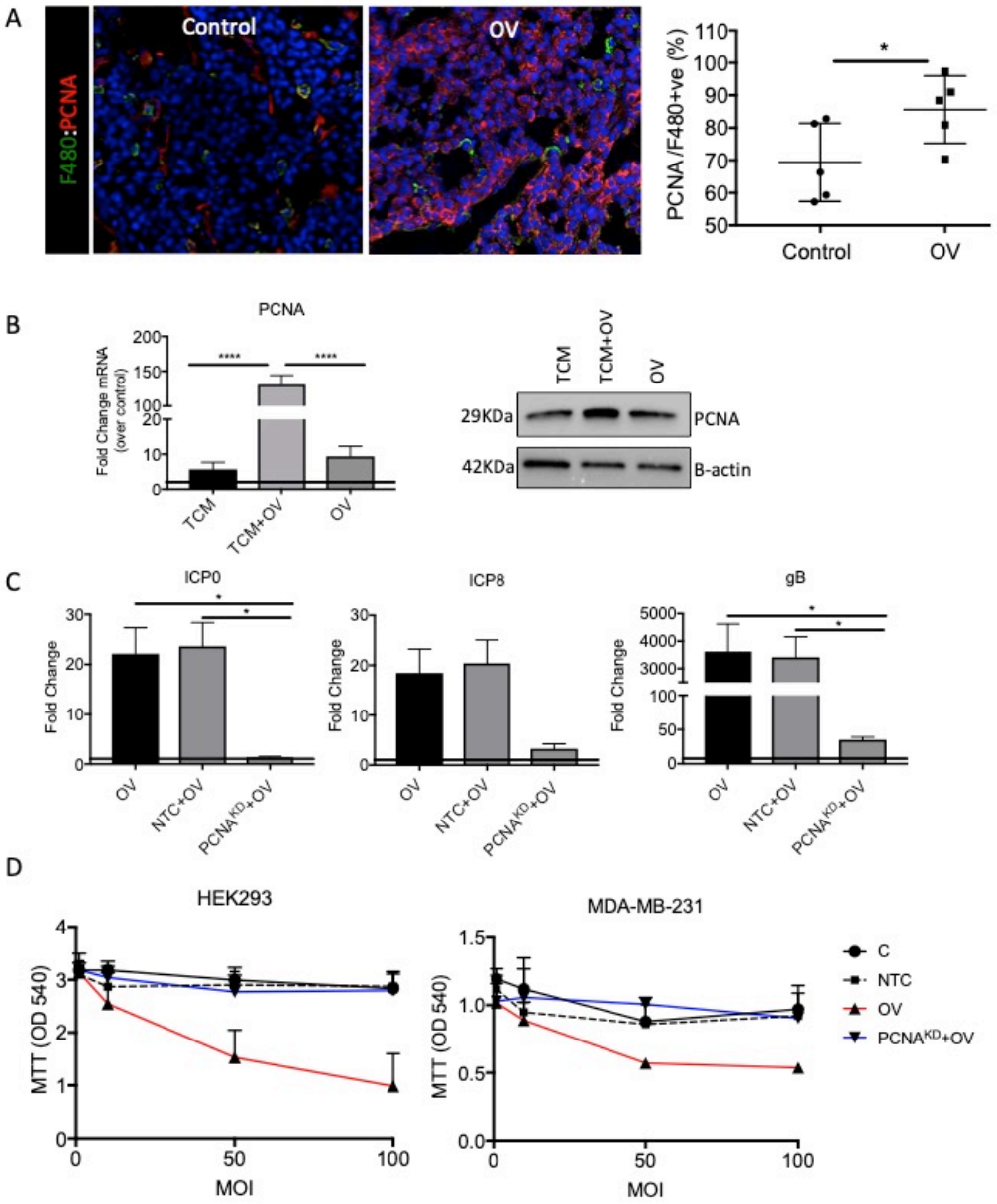

Figure 6: PCNA mediates HSV1716 replication in MDMs. *Female FVB tumor-bearing mice (PyMT-TS1) received a single dose of intravenous HSV1716 at 10^6 PFU (OV) or PBS (control).* ***A,*** *PCNA expression by TAMs (arrowed) is significantly upregulated in response to HSV1716 at day 9 posttreatment (*n = *5 mice per group).* ***B,*** In vitro *studies confirmed that PCNA expression at both the RNA and protein level by MDMs significantly increases after infection.* ***C,*** *AccellsiRNA was used to knockdown PCNA ($PCNA^{KD}$) within MDMs. The human breast cancer cell line MDA-MB-231 was treated with supernatants from MDMs infected with HSV1716 (with or without $PCNA^{KD}$) at different MOIs. At 72 hours, we see inhibition of viral induced cell death in cultures where PCNA is knocked down.* ***D,*** *PCNA knockdown also suppressed replication of virus infected cells (MOI 5), as shown by qPCR of MDMs at 72 hours after infection with suppression of early-ICP0 (left), mid-ICP8 (center), and late-gB (right) viral replication genes. Key: OV = oncolytic virus and $PCNA^{KD}$ + OV = PCNA knockdown and OV. Data shown are mean ± SEM and analyzed using a one-way ANOVA, where* $P < 0.05$, n = *5 experiment.*

Given the interactions between HSV1716 and macrophages, we then investigated the role of macrophages in the antitumor responses seen with this oncolytic virus in our *in vivo* model. To explore this, circulating monocytes and macrophages were eliminated *in vivo* using CLs (Fig. 7A; ref. 22). CLs induce the apoptosis of macrophages and others have shown that this to trigger antitumor activity by inducing changes in the TME (23). We administered a single dose of clodronate at 48 hours prior to treatment with HSV1716 and noticed a modest reduction in macrophage number control (PBS) 69.4 11.4, clodronate alone 33.9 11.6, P 1⁄4 0.0001. As expected, IV HSV1716 decreased tumor growth in comparison with PBS controls. However, this effect was lost when monocytes/macrophages were depleted, in that there was an increase in primary tumor growth (Fig. 7B) and a development of lung metastases when data for HSV1716 treatment are compared in the presence (OVþCL) or absence (OV) of CLs for simplicity (Fig. 7C). Of note, a greater number of lung metastases were seen in this experiment compared with the data presented in Fig. 1B. This is likely due to the endpoint in this experiment being later (day 14) as opposed to day 9 in Fig. 1B. T-cell subsets in these two groups were examined using immunofluorescence post-mortem. An increase in the number of CD4þ T cells and a decrease in CD8þ T cells were seen in animals in which macrophages had been eliminated (Fig. 7D). These data suggest that TAMs are key to the cytotoxicity of HSV1716 infection *in vivo* and may mediate this through their regulation of T-cell subsets in the TME. The interaction between macrophages and T cells was also confirmed in a human mixed lymphocyte population whereby the introduction of HSV1716, in the presence of MDMs, resulted in a shift to activated CD4þ and CD8þT cells with an increase in the expression of the costimulatory receptor 4-1BB, OX40 (TNF superfamily members), and PD-1(Supplementary Figs. S7A and S7B). No T-cell activation was noted in cultures with uninfected macrophages.

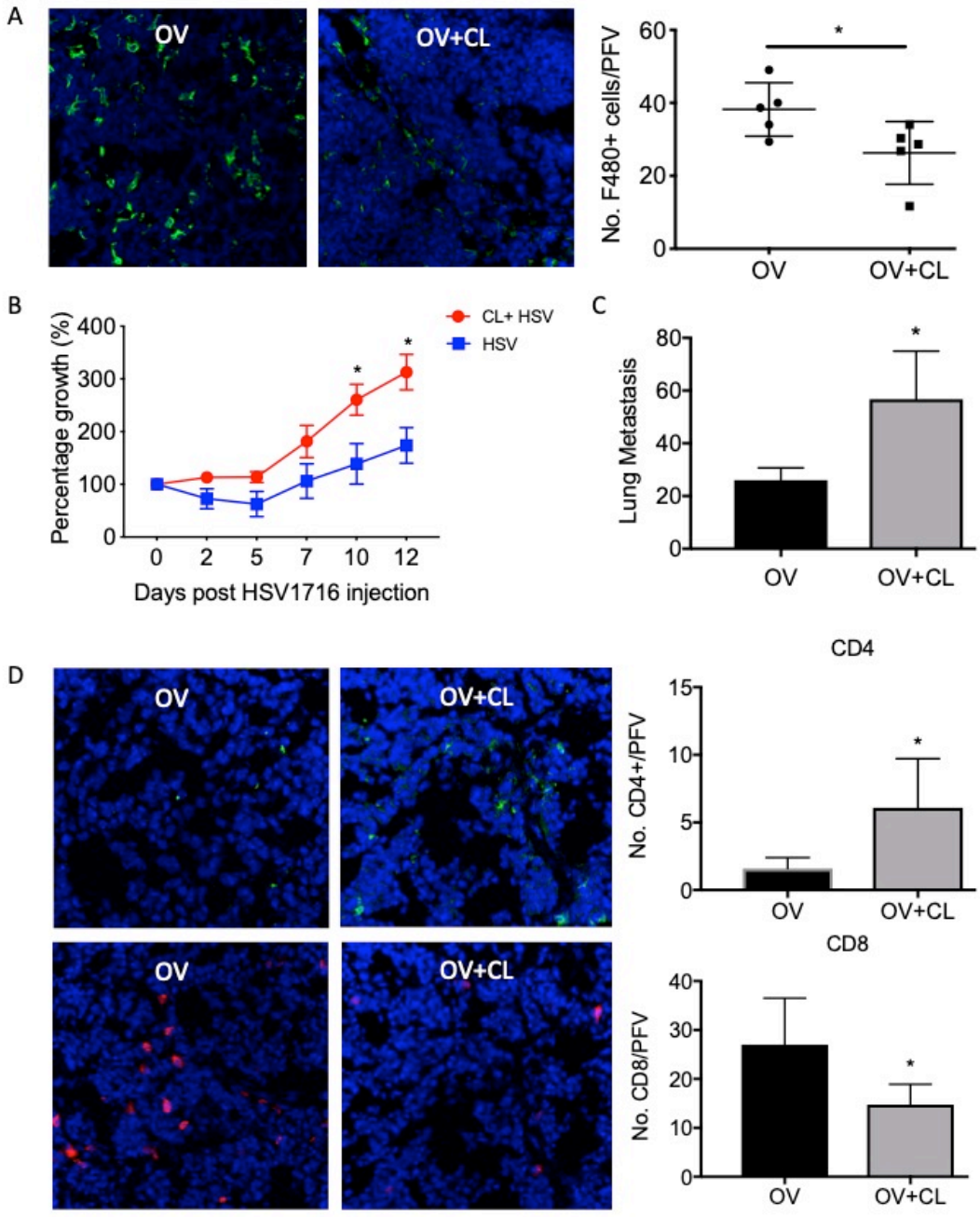

Figure 7: HSV1716 cytotoxicity is dependent on macrophages and transforms immunogenically "cold" tumors to "hot" tumors. *Orthotopically implanted PyMT-TS1 tumors were grown immunocompetent syngeneic female FVB mice. Macrophages were eliminated by intravenous administration of CLs 48 hours before administration of HSV1716 (*n *= 5 animals, one section, five fields per view per section).* ***A,*** *CLs decrease TAMs within the TME (brighter cells = F480$^+$ cells). This depletion of TAMs attenuated the influence of HSV1716 treatment on the growth of primary tumors (**B**) and development of pulmonary metastases (**C**) in vivo. (**D**) An increase in helper CD4$^+$ T cells and a reduction in cytotoxic CD8$^+$ cells was observed after HSV1716 treatment (brighter cells). Data shown are mean ± SEM, one-way ANOVA, where* $P < 0.05$, n *= 5 experiments.*

Discussion

Previous studies have investigated the effect of OV on the survival of malignant cells and the number and activation status of lymphocyte subsets in tumors (24–26). Although the effect of OVs on TAMs has not been fully ascertained, primary brain tumors of patients that received intravenous oncolytic reovirus had an unexpected increase in TAMs, suggesting that the role of TAMs in OV therapy should be investigated further (9). Here we show that TAMs play an important role in mediating the antitumor effects of HSV1716. The virus had pronounced effects on the phenotype of these cells and their depletion within the TME reversed the tumoricidal effect of the OV.

TAMs are abundant in most breast tumors (27) and high numbers correlate with reduced patient survival (28–31). This accords with various studies in mice showing that these cells stimulate angiogenesis and metastasis in mammary tumors (32, 33). Macrophages show high plasticity and can move along a continuum between two polarized activation states; “classically activated,” antitumor, “M1-like” TAMs, and “alternatively activated,” tumor-promoting immunosuppressive, “M2-like TAMs” (34). The latter have been shown to limit tumor responses to various treatments. In our work, we show that although macrophage numbers are stable, there is a marked reduction in the MRC1þ and perivascular macrophages within our tumors after treatment with HSV1716.

In contrast, M1-like TAMs can stimulate cytotoxic T cells by presenting cancer cell antigens to them (3). This creates a pro- inflammatory tumor microenvironment through the release of cytokines (IL1, IL2, IL6, and IL12), superoxide anions and nitrogen free radicals. Generally, studies have shown TAMs to be associated with poor patient prognosis and may help facilitate cancer growth (2, 35, 36). In our study, it is unclear whether the TAM variation is causally linked to the size of the tumors. However, like others, we see the re-education of TAMs from

an M2-like phenotype to more M1-like is associated with improved survival (37, 38). In addition, current evidence suggests that depletion or modification of TAMs alone may result in increased survival *in vivo* (39, 40).

The ability of an OV to reprogram macrophages from an immunosuppressive to an immunostimulatory phenotype opens the potential for OV to be used in conjunction with immunotherapies. Immune checkpoint inhibition involving the blockade of the PD1-PD-L1 path- way is currently being promoted in many tumor types. This acts to release an inhibitory break thus allowing T cells to perform their cytotoxic function. In breast cancer, checkpoint inhibitor monotherapy has a mixed response. This may be due to a failure of activation and migration of $CD8^{+}$ cytotoxic T cells within the TME. Given their ability to present antigen and regulate the antitumor functions of T cells, macrophage modification may be key to enhancing this. Indeed, it has been shown that combining PD-1 inhibition (to take the "brake" off T cells) and CSF-1 receptor blockade (to deplete TAMs within the TME) increases T-cell activation and recruitment within MMTV- PyMT–derived MET-1 tumors, thereby increasing the efficacy of the immunotherapy (41).

However, our data show that macrophages may also play an important role in enhancing OV cytotoxicity by supporting viral amplification. We have previously shown that macrophages can be loaded with OV *ex vivo* and, upon injection into the circulation of tumor-bearing mice, deliver it to tumors (16, 42). In this, we observed an HVS1716 appeared to be amplified by macrophages. Our current work confirms that macrophages not only have the ability to take up HSV1716, enable viral replication and release more HSV1716 particles *in vitro*, but also re-educate macrophages in the process. Therefore, we presume, the lysis of cancer cells by HSV1716 is mediated by direct effects on cancer cells, resulting in ICD, and indirect effects on T cells, via TAMs, in the TME.

The ability of HSV1716 to reduce the growth and spread of cancer in our mouse mammary models demonstrates that this may be useful for the treatment of breast cancer and warrants clinical evaluation. HSV1716 is reported to have a good safety profile in non-breast cancer early phase clinical trials (12, 13) and lends well to seamlessly moving towards translating this research from the bench to the bedside of patients with breast cancer. Our findings indicate that the efficacy of treatments such as checkpoint inhibitors, which require activated T cells to be present in tumors, may be enhanced when used in conjunction with HSV1716.

Previous studies have demonstrated the ability of macrophages to support the replication of highly infective viruses such as the RNA viruses, influenza (43) and simian immunodeficiency virus (44). We believe we are the first to describe that cancer-killing OV replication occurs within macrophages and that this may enhance virotherapy. Herein, we show that PCNA expression by macrophages supports the replication of HSV1716 in a PCNA-dependent manner. PCNA is an essential component of the "replication and repair" machinery of cells but also shown to be involved in the HSV replication cycle (45, 46). It has been proposed that neurovirulence factor ICP34.5 is needed to allow PCNA mediated viral replication in nondividing cells, with studies showing ICP34.5 deleted HSV strains are avirulent in nondividing central nervous system neuronal lines (21, 47). In tumor cells, PCNA is already "switched on" for cellular DNA replication and ICP34.5 is not required to initiate viral replication (45). Studies comparing the replication of wild-type and ICP34.5 deleted HSV1 in Vero (African Green Monkey kidney epithelial) cells demonstrated that viral replication was inhibited when PCNA expression had been knocked down (46). The implications of this are that PCNA plays a role in HSV DNA replication and that this might be independent of ICP34.5. In untreated breast cancer, high numbers of PCNA- positive TAMs correlate with an immunosuppressive TME and with poor patient prognosis (48–50). In our *in vitro* work, exposure of macrophages to tumor-conditioned medium increased their intracellular expression

of PCNA suggesting that cancer cells may stimulate TAMs to express a phenotype that supports viral replication. It remains to be seen whether patients with high numbers of PCNA-expressing TAMs respond better to HSV1716 than those with low numbers. If so, this could be a new way to stratify patients for such form of OV therapy.

Authors' Disclosures

E. Atkinson reports grants from Virttu Biologics during the conduct of the study. P.D. Ottewell reports a patent for use of IL1b binding antibody or its functional fragment for treating and/or preventing cancer in patients having partial inflammatory basis, or preventing lung cancer in patient issued and a patent for use of IL1b binding antibody in treatment and/or prevention of cancer, e.g., lung cancer, preferably non–small cell lung cancer, colorectal cancer, melanoma or gastric cancer having partial inflammatory basis in patient issued. A.G. Pockley reports other from multimmune GmbH outside the submitted work. J.E. Brown reports grants from Sheffield Hospitals Charity during the conduct of the study; grants from Amgen and Bayer; personal fees from Amgen, Novartis, Bayer, Ipsen, and Merck, Sharp, Dome outside the submitted work. J. Conner reports other from Virttu Biologics Ltd. outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

A. Kwan: Resources, data curation, formal analysis, validation, investigation, visualization, methodology, writing–original draft, project administration, writing– review and editing. N. Winder: Data curation, formal analysis, investigation, visualization, methodology. E. Atkinson: Resources, data curation, validation, investigation, methodology. H. Al-Janabi: Resources, data curation, validation, investigation, methodology. R.J. Allen: Resources, data curation, software, supervision, investigation, methodology. R. Hughes: Resources, data curation, supervision, methodology, writing–review and editing. M. Moamin: Data curation, validation, investigation, methodology. R. Louie: Resources, data curation, validation, investigation. D. Evans: Resources, data curation, validation, investigation. M. Hutchinson: Resources, data curation, validation, investigation. D. Capper: Resources, data curation, validation, investigation. K. Cox: Data curation, formal analysis, validation, investigation. J. Handley: Resources, data curation, validation, investigation. A. Wilshaw: Data curation, validation, investigation. T. Kim: Resources, formal analysis, investigation, visualization. S.J. Tazzyman: Resources, data curation, investigation, visualization, methodology, writing–review and editing. S. Srivastava: Resources. P. Ottewell: Resources, supervision, investigation, methodology, writing–review and editing. J. Vadakekolathu: Writing–review and editing. G. Pockley: Writing–review and editing. C.E. Lewis: Supervision, funding acquisition, writing–review and editing. J.E. Brown: Supervision, funding acquisition, writing–review and editing. S.J. Danson: Supervision, writing–review and editing. J. Conner: Conceptualization, resources, supervision, funding acquisition, writing–review and editing. M. Muthana: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

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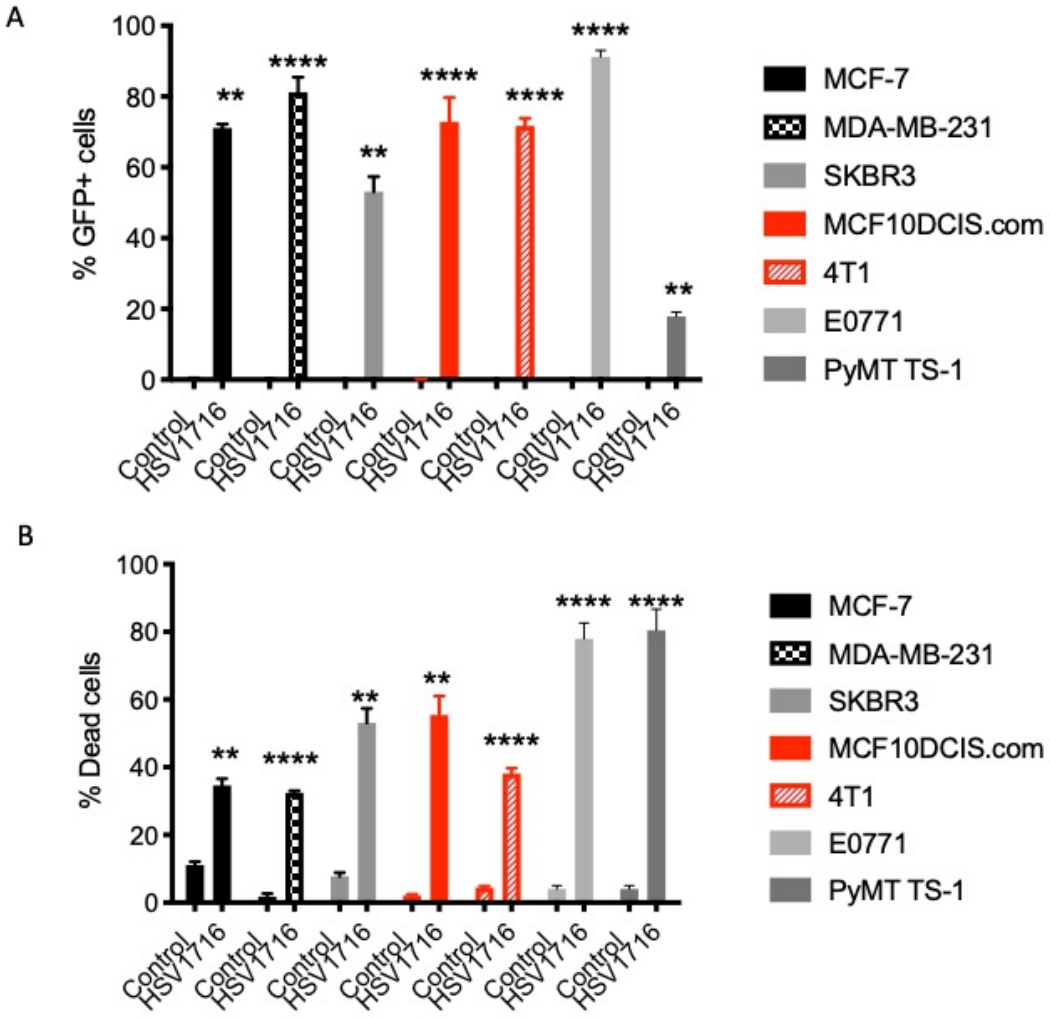
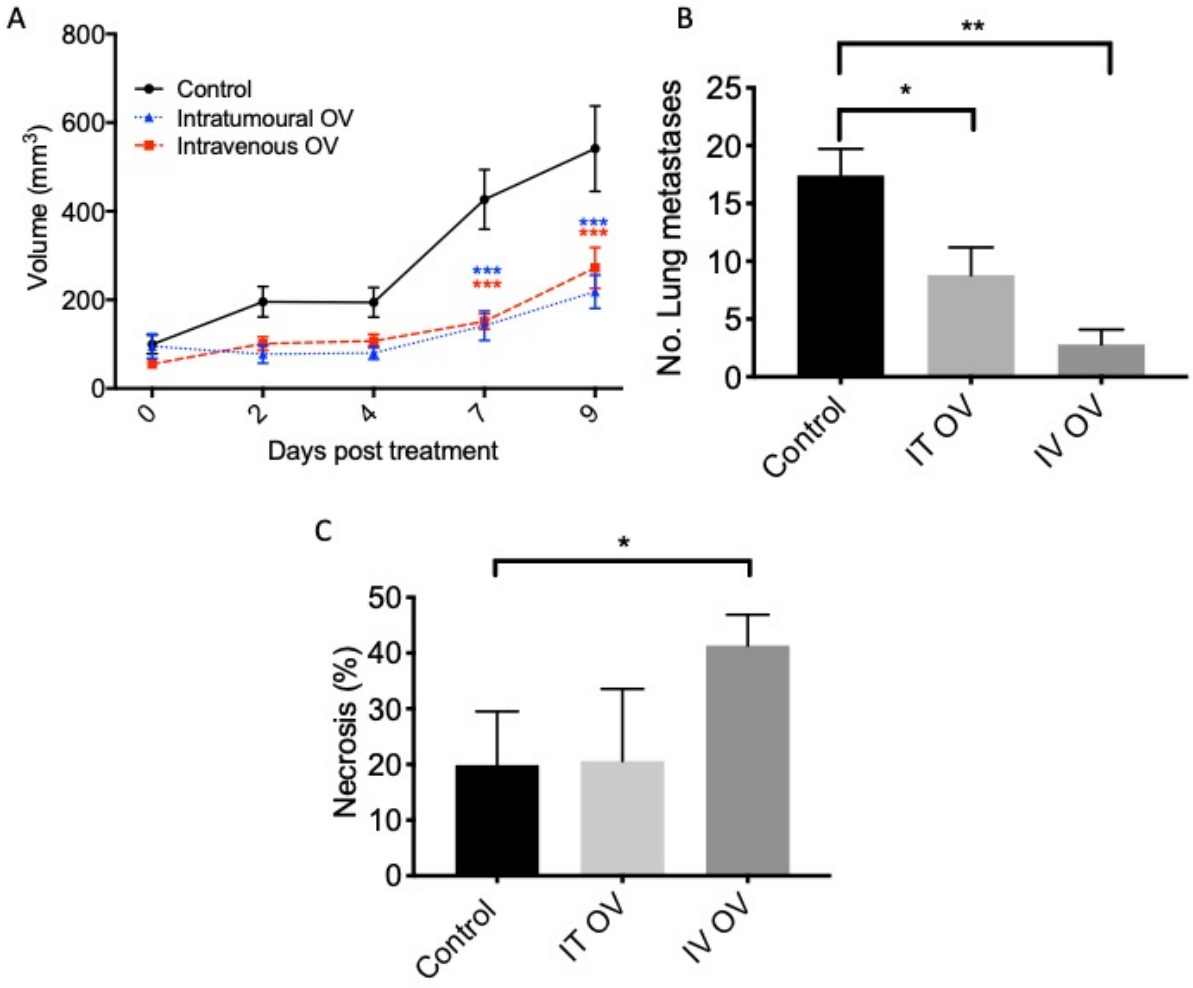
Supplementary Fig. 1. HSV1716 infects and kills human and murine breast cancer cell lines



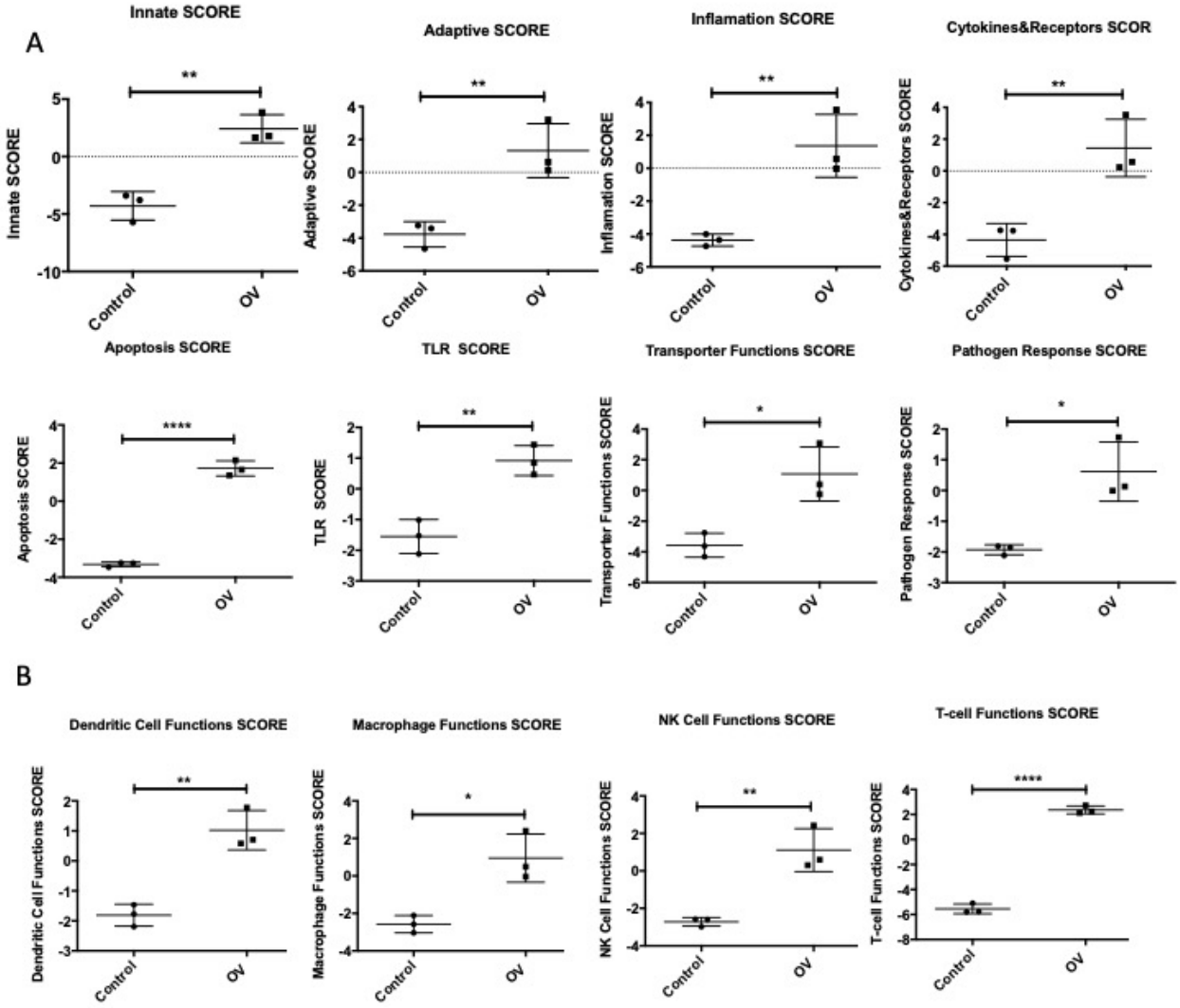
Supplementary Fig. S1: HSV1716 infects and kills human and murine breast cancer cell lines. *Cells ($5x10^5$) were seeded into 6 well plates and infected with HSV1716 at MOI 5. (A) Flow cytometry was used confirm OV infection (GFP expression) at 48 hours. (B) The viability dye, TOPRO3 was added just prior to analysis to assess cell death at the same time point. Data shown are mean ± SEM, analyzed using two way ANOVA with multiple comparisons where * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001 compared to control (untreated cells).*

Supplementary Fig. 2. Intravenous HSV1716 is as efficient as intratumoural HSV1716 in the inhibition of MMTV-PyMT-derived TS1 primary and metastatic tumour growth



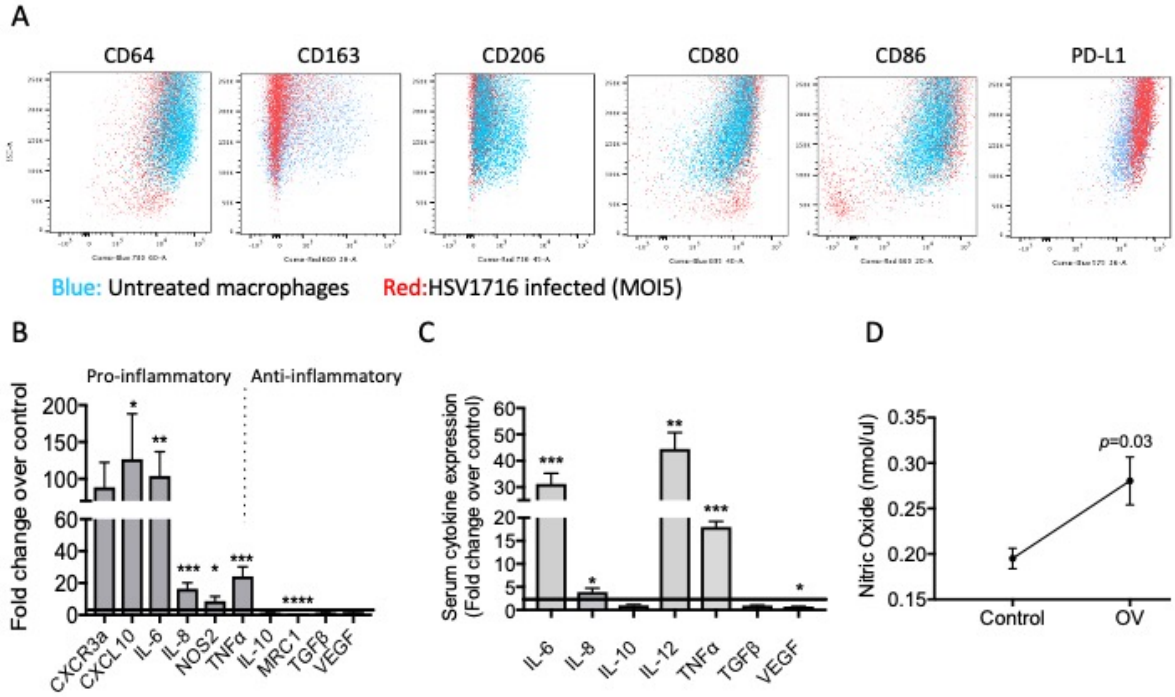
Supplementary Fig. S2: Intravenous delivery is as efficient as intratumoral delivery of HSV1716 in the inhibition of primary and metastatic mammary tumor growth. *PyMT-TS1 cells were implanted into the 4th mammary fat pad of immuno-competent female mice. When tumors reached 500mm³, mice received intravenous or intratumoral HSV1716 (dose 1x10⁶ PFU) or PBS. Tumor volume was assessed by caliper measurements in vivo and pulmonary metastases were measured postmortem by H&E staining. HSV1716 significantly reduced (A) mammary tumor growth and (B) subsequent development of lung metastases (C) Percentage tumor necrosis. Data shown are mean ± SEM, n= 10 animals, analyzed using one-way ANOVA where * = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001 compared to control (untreated cells).*

Supplementary Fig. 3. Pathway scores for genes involved in immune function in mammary tumors following intravenous HSV1716 versus control.



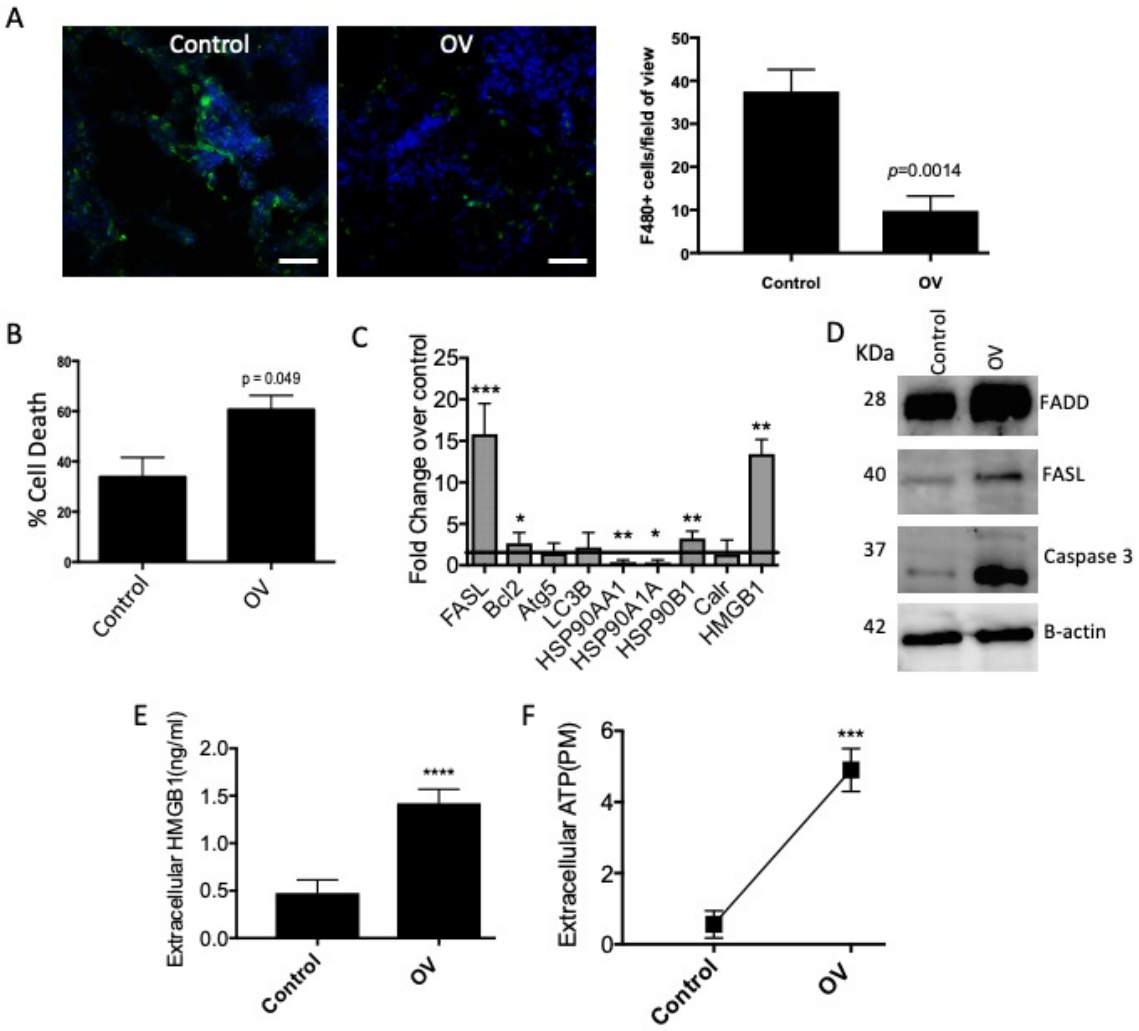
Supplementary Fig. S3: Pathway scores for genes involved in immune function in mammary tumors following intravenous HSV1716 versus control. *This involved hybridization of tumor RNA to the mouse pan cancer immune codeset (NanoString) followed by defining probe counts and bioinformatics analysis. The data was processed and analysed using nSolver Analysis Software, using the Advanced Analysis module. Three tumor samples per treatment were analysed and statistically significant differences between the HSV1716 treated over control are shown. Immune pathway scores, expressed in log_2 are graphically represented.* ***A.*** *Expression profiling of immune-activating-associated pathways in each treatment group.* ***B.*** *Profiling of tumor-associated immune cell type markers. Of note, data are the mean ± SEM (n=3) and statistical analysis was assessed using the student t test *p<0.05; **p<0.01; ***p<0.001 and ****p<0.001.*

Supplementary Fig. 4. HSV1716 reprograms human MDMs to become pro-inflammatory



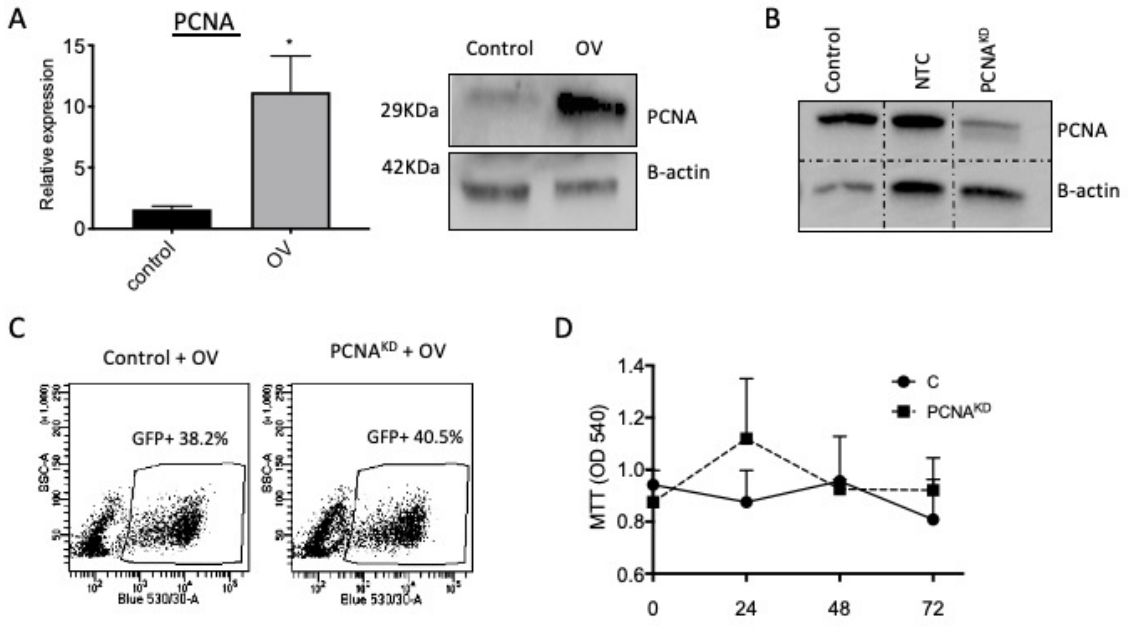
Supplementary Fig. S4: HSV1716 re-programs MDMs to become more pro-inflammatory. *A shift in phenotype was seen when MDMs were infected with HSV1716 in vitro (red) compared to untreated/control (blue). (A) Flow cytometric analysis shows macrophage phenotypic changes following infection with a decrease in typical "M2" markers (CD64, CD163 and CD206) with increased expression of pro-inflammatory "M1" like markers: CD80, CD86 and PD-L1. (B) HSV1716 infection also results in increased mRNA expression of pro-inflammatory genes and (C) the release of pro-inflammatory cytokines as assessed by cytokine bead array. (D) Nitric oxide quantification was performed using an Abcam Nitric Oxide Assay Kit (ab65328) following a 4 hour infection of macrophages with HSV1716. Data shown are mean ± SEM and analyzed using two way ANOVA with multiple comparisons (B,C) or student t test (D) where $p<0.05$, $n=5$ independent macrophage donors.*

Supplementary Fig. 5. HSV1716 triggers immunogenic cell death in infected macrophages



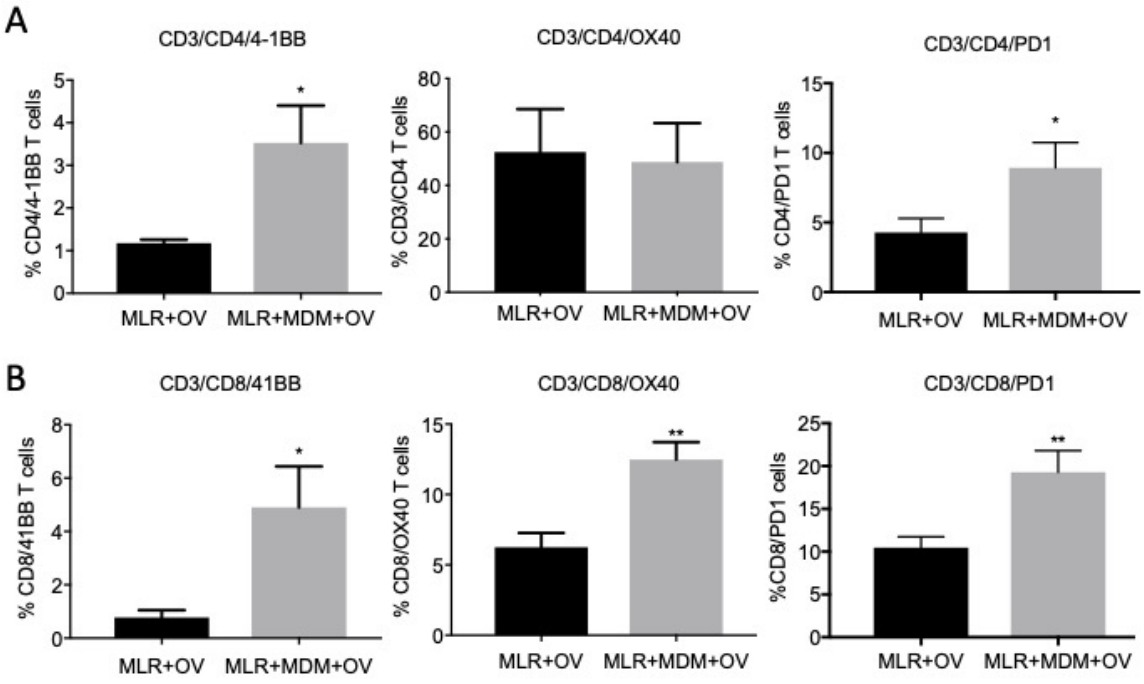
Supplementary Fig. S5: HSV1716 triggers immunogenic cell death in infected macrophages. *Female FVB tumor-bearing mice (Pymt-TS1) received a single dose of intravenous HSV1716 at 10^6PFU (OV) or were injected with saline (control). (A) Sections taken 24 hours after treatment show a significant reduction in the number of F4/80+ cells (green). (B) Human MDMs were infected for 24 hours with HSV1716 at MOI5, these undergo cell death following infection as confirmed by measuring the percentage of PI positive cells using flow cytometry. (C) A panel of cell death markers (qPCR) show an increase in apoptotic and immunogenic cell death markers at the mRNA level and (D) by western blotting. Immunogenic cell death was also confirmed by extracellular HMGB1 ELISA (E) and extracellular ATP (F). Data shown are mean ± SEM, analyzed using either student t test or two way ANOVA with multiple comparisons (C), where $p<0.05$, $n=5$ independent macrophage donors.*

Supplementary Fig. 6. PCNA^{KD} does not prevent HSV1716 infection of macrophages



Supplementary Fig. S6: PCNA^{KD} does not prevent HSV1716 infection of macrophages. (A) MDM infected with HSV1716 have increased PCNA mRNA expression. (B) Accell siRNA was used to knockdown PCNA within MDMs and 72 h later protein was isolated from cells. PCNA knockdown was confirmed by western blot, where PCNA expression following siRNA knockdown was reduced compared to the non-targeting control (NTC) and untreated cells. (C) Despite PCNA knockdown, HSV1716 infection was unaltered compared to untreated cells. This was assessed by flow cytometry of GFP expression. (D) Additionally, PCNA knockdown has no effect on macrophage viability over time as determined using the MTS Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Supplementary Fig.7. Increased T Cell activation expression in mixed lymphocyte reactions after HSV1716 infection



Supplementary Fig. S7: HSV1716 increases T cell activation in MLRs in the presence of macrophages. *Flow cytometrical analysis of T cell activation markers in the mixed lymphocyte reaction in the presence of HSV1716 or HSV1716 infected macrophages. (A) A significant increase in CD4+ T cell expression of 4-1BB and PD1 is observed in the HSV1716-infected macrophage co-cultures as well as (B) an increase in 4-1BB, OX40 and PD1 expression in CD8+ T cells. Data shown are mean ± SEM, Data shown are mean ± SEM, analyzed using student t test, where $p<0.05$, $n=5$ independent macrophage donors.*

Supplementary Table S1. List of antibodies and reagents for flow cytometry

Antibody	Company	Suppliers	Conjugate	Clone	Dilution	Host	Reactive species
CD3/CD4/CD8	Biolegend	London, UK	PE- CY5/PE/FITC	UCHT1/RPA-T4/RPA-T8	1:5	Mouse	Human
CD3	Biolegend	London, UK	APC	17A2	1:20	Rat	Mouse
CD4	Biolegend	London, UK	FITC	RM4-5	1:20	Rat	Mouse
CD8	Biolegend	London, UK	PE	53-6.7	1:20	Rat	Mouse
CD12/23p40	Biolegend	London, UK	Unconjugated	C15.6	1:100	Rat	Human
CD8a	eBioscience™	Hatfield, UK	PE	53-6.7	1:20	Mouse	Human
CD11b	Biolegend	London, UK	PE Dazzle	M1/70	1:20	Rat	Mouse
CD25	Biolegend	London, UK	AF 700	PC61	1:20	Mouse	Human
CD31	Biolegend	London, UK	AF647	MEC13.3	1:100	Rat	Mouse
CD34	Biolegend	London, UK	FITC	561	1:20	Mouse	Human
CD56	Biolegend	London, UK	Brilliant Violet 421	HCD56	1:20	Mouse	Human
CD134	Biolegend	London, UK	PE-Dazzle	Ber-ACT35 (ACT35)	1:20	Mouse	Human
CD137 /41-BB	Biolegend	London, UK	APC-CY7	4B4-1	1:20	Mouse	Human
CD274	Biolegend	London, UK	PE -CY7	29E.2A3	1:20	Mouse	Human
CD273	Biolegend	London, UK	APC	24F.10C12	1:20	Mouse	Human
CD279	Biolegend	London, UK	APC	EH12.2H7	1:20	Mouse	Human
PCNA	Biolegend	London, UK	Unconjugated	PC10	1:50	Mouse	Ms/Hu/Rat
F4/80	Biolegend	London, UK	Brilliant violet 421	BM8	1:20	Rat	Mouse
F4/80	Bio-Rad	Oxford, UK	AF488	C1:A3-1	1:50	Rat	Mouse
FoxP3	Biolegend	London, UK	Alexa Fluor 647	FM14	1:75	Rat	Mouse
IFNg	Biolegend	London, UK	APC	XMG1.2	1:100	Rat	Mouse
Ly6-C	Biolegend	London, UK	APC CY7	HK1.4	1:20	Rat	Mouse
LY6-G	Biolegend	London, UK	PerCp Cy5.5	1A8	1:20	Rat	Mouse
MRC1	Abcam	Cambridge, UK	Unconjugated	NA	1:200	Rabbit	Mouse
Ant-rabbit 647	Abcam	Cambridge, UK	AF647	NA	1:1000	Goat	Rabbit
Anti-Rat 555	Biolegend	London, UK	AF 555	Poly4054	1:200	Goat	Rat
NOS2	Abcam	Cambridge, UK	NA	Polyclonal	1:50	Rabbit	Mouse, Rat
Normal Rabbit IgG Isotype Control	Invitrogen	BLEISWIJK, Netherlands	NA	NA	1:1000	Rabbit	NA
Goat IgG Anti-Rabbit IgG (H+L)-DyLight 550	ImmunoReagents	North Carolina, USA	DyLight 550	Polyclonal	1:100	Goat	NA
IgG2a k	eBioscience™ (TF S)	Hatfield, UK	PE-CY5	eBM2a	1:20	Mouse	NA
Zombie UV™	Biolegend	London, UK	NA	NA	1:100	NA	NA
AbC™ Anti-Mouse Bead Kit	ThermoFisher Scientific	Loughborough, UK	NA	NA	Not applicable	NA	Mouse

Supplementary Table S1. List of antibodies and reagents for flow cytometry

Antibody	Company	Suppliers	Conjugate	Clone	Dilution	Host	Reactive species
CD3/CD4/CD8	Biolegend	London, UK	PE- CY5/PE/FITC	UCHT1/RPA-T4/RPA-T8	1:5	Mouse	Human
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CD4	Biolegend	London, UK	FITC	RM4-5	1:20	Rat	Mouse
CD8	Biolegend	London, UK	PE	53-6.7	1:20	Rat	Mouse
CD12/23p40	Biolegend	London, UK	Unconjugated	C15.6	1:100	Rat	Human
CD8a	eBioscience™	Hatfield, UK	PE	53-6.7	1:20	Mouse	Human
CD11b	Biolegend	London, UK	PE Dazzle	M1/70	1:20	Rat	Mouse
CD25	Biolegend	London, UK	AF 700	PC61	1:20	Mouse	Human
CD31	Biolegend	London, UK	AF647	MEC13.3	1:100	Rat	Mouse
CD34	Biolegend	London, UK	FITC	561	1:20	Mouse	Human
CD56	Biolegend	London, UK	Brilliant Violet 421	HCD56	1:20	Mouse	Human
CD134	Biolegend	London, UK	PE-Dazzle	Ber-ACT35 (ACT35)	1:20	Mouse	Human
CD137 /41-BB	Biolegend	London, UK	APC-CY7	4B4-1	1:20	Mouse	Human
CD274	Biolegend	London, UK	PE -CY7	29E.2A3	1:20	Mouse	Human
CD273	Biolegend	London, UK	APC	24F.10C12	1:20	Mouse	Human
CD279	Biolegend	London, UK	APC	EH12.2H7	1:20	Mouse	Human
PCNA	Biolegend	London, UK	Unconjugated	PC10	1:50	Mouse	Ms/Hu/Rat
F4/80	Biolegend	London, UK	Brilliant violet 421	BM8	1:20	Rat	Mouse
F4/80	Bio-Rad	Oxford, UK	AF488	C1:A3-1	1:50	Rat	Mouse
FoxP3	Biolegend	London, UK	Alexa Fluor 647	FM14	1:75	Rat	Mouse
IFNg	Biolegend	London, UK	APC	XMG1.2	1:100	Rat	Mouse
Ly6-C	Biolegend	London, UK	APC CY7	HK1.4	1:20	Rat	Mouse
LY6-G	Biolegend	London, UK	PerCp Cy5.5	1A8	1:20	Rat	Mouse
MRC1	Abcam	Cambridge, UK	Unconjugated	NA	1:200	Rabbit	Mouse
Ant-rabbit 647	Abcam	Cambridge, UK	AF647	NA	1:1000	Goat	Rabbit
Anti-Rat 555	Biolegend	London, UK	AF 555	Poly4054	1:200	Goat	Rat
NOS2	Abcam	Cambridge, UK	NA	Polyclonal	1:50	Rabbit	Mouse, Rat
Normal Rabbit IgG Isotype Control	Invitrogen	BLEISWIJK, Netherlands	NA	NA	1:1000	Rabbit	NA
Goat IgG Anti-Rabbit IgG (H+L)-DyLight 550	ImmunoReagents	North Carolina, USA	DyLight 550	Polyclonal	1:100	Goat	NA
IgG2a k	eBioscience™ (TF S)	Hatfield, UK	PE-CY5	eBM2a	1:20	Mouse	NA
Zombie UV™	Biolegend	London, UK	NA	NA	1:100	NA	NA
AbC™ Anti-Mouse Bead Kit	ThermoFisher Scientific	Loughborough, UK	NA	NA	Not applicable	NA	Mouse

Supplementary Table S2. List of primer sequences used for qPCR analysis

Primer	Forward primer region	Reverse primer region
ATG5	TCTGGATGGGATTGCAAAATG	TTTCTTCTGCAGGATATTCCATG
BCL-2	GGAAGTGAACATTTCGGTG	GCCTCTCCTCACGTTCCC
B2M	CTGCTACGTAACACAGTTCCACCC	CATGATGCTTGATCACATGTCTCG
CALR	AGGTTCATGTCATCTTCAAC	CAGGTGTGTAAACTCATCATC
CXCR3a	CAACCACAAGCACCAAAGC	AACCTCGGCGTCATTTAGC
CXCL10	TCAAGTGGCATAGATGTGGAAGAA	TGGCTCTGCAGGATTTTCATG
GAPDH	ACAGTTGCCATGTAGACC	TTTTTGGTTGAGCACAGG
FASL	ATCCCTCTGGAATGGGAAGA	CCATATCTGTCCAGTAGTGC
GAPDH	ACAGTTGCCATGTAGACC	TTTTTGGTTGAGCACAGG
gB	TGTGTACATGTCCCCGTTTTACG	GCGTAGAAGCCGTCAACCT
HMGB1	TACGAAAAGGATATTGCTGC	CTCCTCTTCCTTCTTTTTCTTG
HSP90AA1	ATATCACAGGTGAGACCAAG	GTGACTGACACTAAAGTCTTC
HSP90A1A	AATTTCCTGTGTTTGCAATG	AAAATGGCCTGAGTTAAGTG
HSP90B1	TTCAAAGGAAAGTGATGACC	GCATCATATCATGGAAGTCG
ICP0	AAGCTTGGATCCGAGCCCCGCCC	AAGCGGTGCACGGGAAGGT
ICP8	GACATTACGTTCACGGCCTTCGAAGCCAG	GGCCGAGTTGGTGCTAAATACCATGGC
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-8	TGTGAAGGTGCAGTTTTGCCA	GGGGTGGAAAGGTTTGGAGTA
IL-10	AAGACCCAGACATCAAGGCG	AATCGATGACAGCGCCTAG
LC3B	GTGGAAGATGTCCGGCTCAT	TGGTCAGGCACCAGGAACTT
MRC1	GCAAATGGAGCCGTCTGTGC	CTCGTGGATCTCCGTGACAC
NOS2	TCCTGGACATTACGACCCCT	CTCTGAGGGCTGACACAAGG
PCNA	GCCGAGATCTCAGCCATATT	ATGTACTTAGAGGTACAAAT
TGFβ	AACCCACAACGAAATCTATG	CTTTTAACTTGAGCCTCAGC
TNFα	CCTTCACAGAGCAATGACTC	GTCTACTCCCAGGTTCTCTTC
VEGF	CAGGCTGCTGTAACGATGAA	CTTGGCGATTTAGCAGCAGA

Supplementary Table S3. Top 20 differentially regulated genes in mammary tumors following intravenous HSV1716 versus control

Gene	p-Value	Fold change	Biological process
Cmah	4.94E-07	3.9	Transporter Functions
Cd55	1.60E-06	2.61	CD molecules, Complement Pathway, Innate
Ctsw	2.02E-06	2.81	CD molecules
Il21	2.75E-06	2.72	CD molecules, Cytokines & Receptors
Sell	2.82E-06	2.25	Adhesion, Apoptosis, CD molecules, T-Cell Functions
Cd247	4.46E-06	2.87	CD molecules, NK Cell Functions, T-Cell Functions
Il7r	5.93E-06	1.91	B-Cell Functions, CD molecules, Cytokines & Receptors, T-Cell Functions
Cd22	6.73E-06	4.07	Adhesion, B-Cell Functions, CD molecules
Ltb	9.03E-06	3.03	Cytokines & Receptors, Interleukins, TNF Superfamily
Tnfrsf13b	1.57E-05	1.94	B-Cell Functions, CD molecules, TNF Superfamily
H2-DMb2	1.90E-05	3.74	Antigen Processing, MHC
Dpp4	2.00E-05	2.63	B-Cell Functions, CD molecules, T-Cell Functions
Thbs1	2.17E-05	1.33	Adaptive, Cell Cycle, Inflammation, Macrophage Functions
Cd37	2.77E-05	1.15	B-Cell Functions, CD molecules, Humoral
Il16	2.96E-05	2.19	Chemokines & Receptors, Cytokines & Receptors, Interleukins, Leukocyte Functions
Txk	3.11E-05	2.43	Adaptive, Cytokines & Receptors, Interleukins, T-Cell Functions
Cd84	3.25E-05	1.37	Adhesion, CD molecules
Blnk	4.37E-05	2.31	B-Cell Functions, Humoral
Btla	4.80E-05	3.3	B-Cell Functions, CD molecules, T-Cell Functions
Pecam1	5.01E-05	1.13	Adhesion, CD molecules, Leukocyte Functions, Transporter Functions

Supplementary Table S3: Top 20 differentially regulated genes in mammary tumors following intravenous HSV1716 versus control. This involved hybridization of tumor RNA to the NanoString mouse pan cancer immune codeset (NanoString) followed by defining probe counts and bioinformatics analysis, as described in Materials and Methods. The data was processed and analysed using nSolver Analysis Software, using the Advanced Analysis module.

Supplementary Table S4. Apoptotic and survival array data

Gene	Signalling Pathway	MDM+OV over control
RB1	nAChR inhibition	45.9
TP53	P53 signalling	163.1
NFKB1	TNFR signalling	238.9
LCN2	Caspase Cascade	32.8
IGF1	Caspase Cascade	25.5
FAS	FAS signalling	14.4
FASL	FAS signalling	11.7
CAS3	Caspase Cascade	8.2
CAS8	Caspase Cascade	11.8
CAS9	Caspase Cascade	16.3
HMGB1	RAGE signalling pathway	5.5
HSP90AA	IAP-protein inactivation	12.6
CTNNB1	Wnt Signalling	22.1
HSPA1	IAP-protein activation	54.2
HSPD1	Caspase Cascade	4.8
HSPA5	IAP-protein activation	11.9
MYC	ESR1 signalling	32.1
FGFR2	PI3K/Akt Signalling	9.9
ERBB3	PI3K/Akt signalling	17.3

Supplementary Table S4: Apoptotic and survival array data. *A 90 gene apoptotic and pro-survival array Tier 1 (Bio-Rad) was performed. Human MDMs were in infected with HSV1716 for 24hrs. The mRNA expression of Apoptosis and Pro-survival genes relative to untreated cells was assessed using the 2-ΔΔCt method following normalization of the data to the house keeping gene GAPDH. Genes presented in this table were differentially regulated in response to HSV1716 infection. Expression relative to untreated cells was calculated using the 2-ΔΔCt method following normalization of the data to the house keeping gene GAPDH. Data is presented as the Mean fold change for n=2 independent experiments.*

Amendment to published work to the above paper following viva examination:

1) Amendment to legend for figure 1:

Figure 1: HSV1716 treatment inhibits primary mammary tumor growth and metastatic spread. *PyMT-TS1, EO771, and 4T1 cells were implanted into the fourth mammary fat pad of immuno-competent syngeneic female mice. When tumors reached ~150 mm3, mice received intravenous HSV1716 (dose 1 × 106 PFU) or PBS. Tumor volume was assessed by caliper measurements in vivo and pulmonary metastases were measured postmortem by hematoxylin and eosin staining. HSV1716 (grey line) significantly reduced (A) mammary tumor growth where data shown are mean ± SEM, n = 10 mice/treatment group and statistical significance analyzed using multiple t tests, where the Bonferroni correction was applied to reduce type 1 errors. Significance is coded as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with control. (B) Subsequent development of lung metastases in the respective models at postmortem. Data was analysed by a student T test where significance is coded as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with control.*

2) Amendment to legend of figure 7:

Figure 7: HSV1716 cytotoxicity is dependent on macrophages and transforms immunogenically "cold" tumors to "hot" tumors. *Orthotopically implanted PyMT-TS1 tumors were grown immunocompetent syngeneic female FVB mice. Macrophages were eliminated by intravenous administration of CLs 48 hours before administration of HSV1716 (*n = *5 animals, one section, five fields per view per section).* ***A,*** *CLs decrease TAMs within the TME (brighter cells = F480$^+$ cells). This depletion of TAMs attenuated the influence of HSV1716 treatment on the growth of primary tumors (**B**) and development of pulmonary metastases (**C**)* in vivo. *(**D**) An increase in CD4$^+$ T cells and a reduction in CD8$^+$ cells was observed after HSV1716 treatment (brighter cells). Data shown here are mean ± SEM, Student T tests performed for 2 groups and one-way ANOVA when more than 2 where p < 0.05,* n = *5 experiments.*

3) Amendment to legend of Figure S5

Supplementary Fig. S5: HSV1716 triggers immunogenic cell death in infected macrophages. Female FVB tumor-bearing mice (Pymt-TS1) received a single dose of intravenous HSV1716 at 10^6PFU (OV) or were injected with saline (control). (A) Sections taken 24 hours after treatment show a significant reduction in the number of F4/80+ cells (green). (B) Human MDMs were infected for 24 hours with HSV1716 at MOI5, these undergo cell death following infection as confirmed by measuring the percentage of PI positive cells using flow cytometry. (C) A panel of cell death markers (qPCR) show an increase in apoptotic and immunogenic cell death markers at the mRNA level and (D) by western blotting. Caspase 3 antibody (H-277, Santa Cruz, sc-7148) was used which represents a full length proprocaspase-3 of human origin. Immunogenic cell death was also confirmed by extracellular HMGB1 ELISA (E) and extracellular ATP (F). Data shown are mean ± SEM, analyzed using either

student t test or two way ANOVA with multiple comparisons (C), where $p<0.05$, n=5 independent macrophage donors.

4.3 Summary of findings

This chapter presents work completed as part of my PhD investigating the role of HSV1716 as a therapeutic agent against TNBC. This was assessed with a number of *in vitro* and *in vivo* models. Firstly, HSV1716 infection and cell lysis was confirmed in murine and human derived breast cancer cell lines (MCF7, MDA-MB-231, SKBR3, MCF10DCIS.com, 4T1, EO771, and PyMT-TS1). Immunocompetent mouse models confirmed cytotoxicity of HSV1716 against 3 murine TNBC cell lines, 4T1, EO771 and PyMT-TS1, with a reduction in metastatic disease with repeated doses. Intravenous and intratumoral delivery of HSV1716 was explored and resulted in similar primary tumour reduction and reduction in lung metastases. The mechanism behind which intratumoral HSV1716 is thought to be to due systemic immune system activation with studies reporting that following local infiltration of oncolytic virotherapy, distant tumor infiltration of activated CD8+ and CD4+ effector but not regulatory T cells was seen and was dependent on CD8+ cells, NK cells and type I interferon (83,279). These findings support the dual effect of virotherapy cytotoxicity: direct tumour lysis and immune cell activation. In view of the acceptability of intravenous delivery in TNBC treatments and the inaccessibility some TNBC metastases, namely brain and bone metastases, IV delivery was selected to be explored for further work. Dosing differences between single dose and multiple dose regimes was explored and metastatic disease suppression was found to be greater when HSV was given in multiple doses every 48 hours leading to a significantly extended the overall survival of mice. Within the models described we confirmed immune cell activation with increased intratumoral CD8+ and decreased CD4+ populations.

Interestingly, it was also found that HSV1716 was able to replicate within TAMs. It is unclear from the work done as to what proportion of replication occurs in TAMs or tumour cells and exact quantification would have been difficult with e current study design. This may be

explored with animals culled at set time points and quantification of circulating virus and concentrations of virus at the site of tumour. Although immunofluorescent or flow cytometry analysis may suggest an association of virus and either TAMs or tumour cells, and a rough quantification of viral replication could be confirmed through the presence of viral replication genes, teasing out which cell allows for a greater degree of viral replication would be tricky *in vivo*.

It was also noted that viral replication was dependent on PCNA. This was evidenced by an increase in PCNA within TAMs infected with HSV1716 and a lack of viral replication within TAMs that have undergone PCNA knockdown. The mechanism of how PCNA knockdown inhibits viral replication has not been specifically explored within this paper, however PCNA is one of the most enriched proteins on the HSV-1 replication fork (280) and it has been described that PCNA inhibition using the small molecular inhibitor PCNA I1 reversibly inhibits HSV-1 DNA replication (281), thus supporting our findings. The mechanism of action was explored by the used of 2 different PCNA inhibitors; PCNA-I1 and T2AA. PCNA-I1 stabilizes the PCNA homotrimer and reduces the repair of double strand breaks by homologous recombination and suppresses nucleotide excision repair (282). T2AA inhibits interactions between proteins that contain a PCNA interacting protein (PIP)-Box motif and the PCNA IDCL or mono-ubiquitinated K164 (283). They found that inhibition of PCNA causes a decreased in key protein recruitment to viral genomes and that in addition to inhibiting DNA replication, it was also noted that PCNA inhibition resulted in decreased late HSV-1 gene expression and decreased infective virus production. This paper considered the potential to use PCNA targeted therapies to treat persistent HSV-1 infections.

As one uncovered the complexity and interplay between macrophages and the immune system as a whole, it was interesting to discover studies exploring the role of PCNA and

immune cells. A review into this been performed by myself with a consideration about how this may shape oncolytic virotherapy. This summarised in **figure 17**

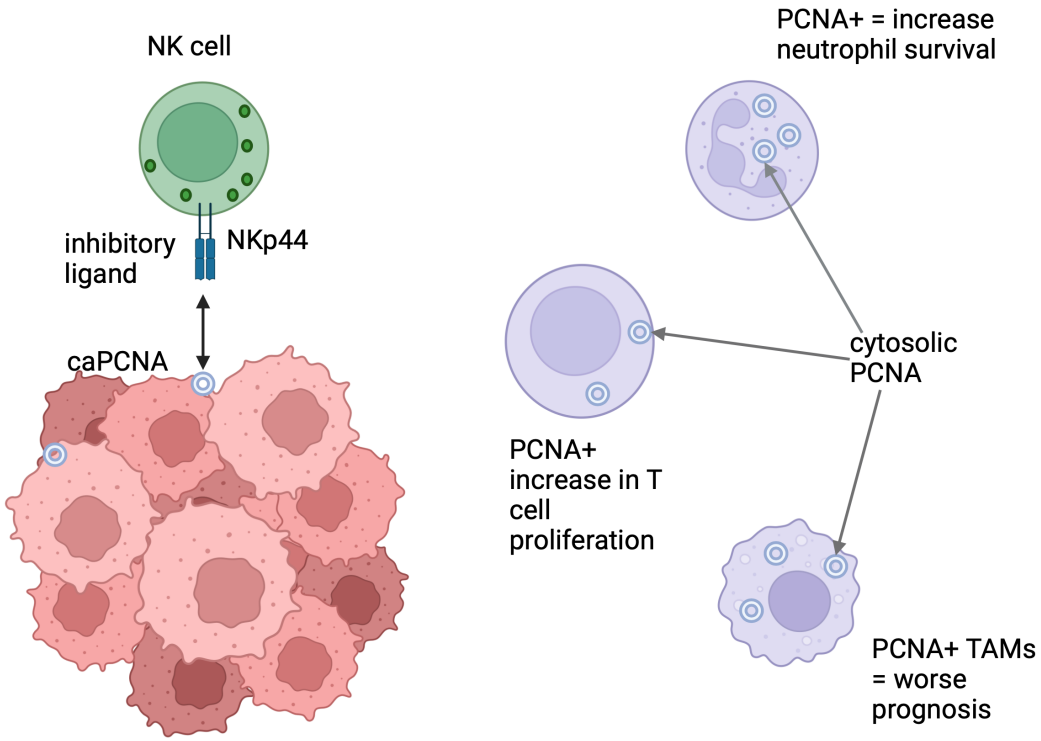

Figure 17: PCNA and its role in the tumour microenvironment*. PCNA is present on the cell surface of cancer cells and its role is to enhance immune evasion by prevention of NK cell activation and degranulation through the inhibitory receptor NKP44. PCNA is found in the cytosol of neutrophils, T cells and tumour associated macrophages. In neutrophils the function of PCNA I to prolong survival through modulation of glycolysis. In TAMs the function of PCNA is unknown however these TAMs express more M1 like markers which is normally associated with cancer cytotoxicity. Interestingly a higher prevalence of PCNA + TAMs suggest poorer prognosis and further studies are needed to understand these findings. Little information is available about lymphocytes although the role of PCNA in the proliferation of T cells is documented.*

Data from my paper, has raised the question of the role of macrophages in facilitating HSV1716 replication. An exploration of the role of TAMs and PCNA is limited in published literature. It has been described that when PCNA+ TAM genes were compared to those of M1 and M2 macrophage phenotypes, it was found the PCNA+ TAMs shared significantly more genes with the M1 phenotype than the M2 (284). This was a little conflicting as PCNA+ TAMs have also been found to be associated with poor prognosis in breast tumours (285)and high M1 macrophage number often represent a pro-inflammatory and therefore cytotoxic microenvironment. Although, there are many questions relating to PCNA+ TAMs that have not been clear, a growing consensus supported, by a recent study, suggest that there are more than two phenotypes of TAMs and compartmentalization of TAMs into only two categories is an oversimplification of the complexities involved in phenotypic partitioning (286).

Given the abundance of TAMs within breast cancer tumour mass, the potential ability of HSV1716 to replicate within macrophages and work published from within our group by Dr Muthana illustrating the feasibility of macrophage delivery to enhance viral cytotoxicity, the focus of future work was to explore whether macrophage delivery virotherapy would be both feasible and effective within TNBC. The following chapter describes this work.

Chapter 5

5 Macrophage Delivered HSV1716 Is Active against Triple Negative Breast Cancer

5.1 Introduction

Optimising the delivery all cancer therapeutics is a topical area of research as the mode of delivery can affect parameters of effectiveness, tolerability and ultimately patient compliance, Administration of clinically approach oncolytic HSV strain is primarily via the intratumoral route due to concerns around neutralisation of virus by the immune system. However, as illustrated by the results from chapter 4, delivery via the intravenous route is feasible and can show efficacy in the murine model setting. However, the animals used in these experiments did not have prior exposure to HSV and this may have influence results. In the human population, HSV is a common cause of low-grade infections with an estimate of over 3500 million adults having HSV type 1infection and 491 million with HSV-2 infection (287). Although some studies have suggested an increase tolerability of virotherapy with prior HSV exposure (83), the possibility of acquired resistance to the immunogenic effect of virotherapy through premature neutralisation by preexisting antibodies may lead to a decrease in efficacy and survival(288). The barriers to oncolytic virotherapy and the potential strategies to overcome these were reviewed in Chapter 1 and this identified that cellular packaging of therapeutics including oncolytic viruses is feasible and may lead to better overall comes (137,289).

Another drive to explore macrophage delivered virotherapy is to address difficult to reach metastases. One of particularly personal clinical interest is regarding breast cancer brain

metastases (BCBM). Triple negative breast, once metastatic, sees 50% of patients develop BCBM, compared to other breast cancer subtypes, BCBM in TNBC has a poorer outcome with an average survival of around 6 months. One of the factors contributing to poorer outcomes is the challenges of delivering therapies across the blood brain barrier. The blood brain barrier is a milieu of cells which acts as a physical structure to prevents unwanted toxins, microbial organisms and some medicals crossing into brain parenchyma. It was historical felt to be an immunological privileged site; however, it is not impenetrable to peripheral cells and microorganisms. There are 3 ways of organism may cross; transcellular (290)paracellular (291), and via infected phagocytic cells (292). HSV-1 has been shown to enter the brain primary via retrograde transport along peripheral nerves . It then either exerts undergoes viral replication exerting an acute inflammatory reaction with the release of cytokines and chemokines leading to encephalitis, necrosis and haemorrhage or lies latent with parenchymal cells. HSV1716 has been modified to remove the neurovirulence gene, which prevents viral replication with the CNS, however, does not prevent viral entry. Increasing the permissiveness of the brain to HSV1716, through use of macrophage delivered virotherapy by creating dual modalities of entry, may be a potential therapeutic advantage.

The following paper describes our experience of delivering HSV1716 using a macrophage cell carrier (293). The data presented in this paper has been collected and analysed by a number of members of the tumour targeting group. During this PhD, my role pertained to the assessment of TNBC cell viability following infection with HSV1716, the conceptualisation, design, data collection and analyses of *in vivo* work described, the preparation of slides and histological analysis through H+E staining and immunofluorescence and the finally the collation and writing the original research paper.

5.2 Original Research: Macrophage Delivered HSV1716 Is Active against Triple Negative Breast Cancer

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Abstract

Oncolytic viruses (OV) promote anti-tumour responses through the initiation of immunogenic cancer cell death which activates the host's systemic anti-tumour immunity. We have previously shown that intravenously administered HSV1716 is an effective treatment for mammary cancer. However, intravenous administration of a virus has the potential to result in neutralization and sequestration of the virus which may reduce efficacy. Here, we show that the oncolytic virus HSV1716 can be administered within a cellular carrier (macrophages). PyMT and 4T1 murine mammary cancer cell lines were implanted into immuno-competent murine models (orthotopic primary, early metastatic and brain metastasis models). HSV1716 or macrophages armed with HSV1716 (M-HSV1716) were administered intravenously, and tumour size was quantified using caliper measurement or bioluminescence imaging. Administration of M-HSV1716 led to tumour shrinkage and increased the survival of animals. Furthermore, these results were achieved with

a 100-fold lower viral load, which has the potential for decreased toxicity. Our results demonstrate that M-HSV1716 is associated with activity against murine mammary cancers and provides an alternative platform for the systemic delivery of OV.

Introduction

Breast cancer is the most commonly occurring cancer and the leading cause of cancer-related death worldwide, with an estimated 2.3 million new cases and 684,996 deaths in 2020. It accounts for 15.5% of cancer death in females [**1**], and of these, the triple-negative subtype of breast cancer (TNBC) is often one of the most challenging to treat.

Tumour-associated macrophages (TAMs) are common in breast cancers, and it has been described that the breast cancer microenvironment may educate macrophages to become more "M2" like [**2**]. Furthermore, high numbers of TAMs in TNBC have been associated with an increase in tumour aggressiveness and metastases [**3**]. The increased tumour progression and decreased survival may be manipulated if TAMs are reprogrammed, aiding the immune system's recognition of cancer and enhancing the response to cancer therapeutics. The use of macrophages as a cell-based therapy is increasing in popularity within the healthcare setting. One such example is the use of macrophages to reverse and repair the damage caused by liver cirrhosis [**4**]. Within the cancer setting, macrophage-based therapies have been investigated with varying success. Therapies include the modification of macrophage numbers through depletion within the tumour site or interfering with TAM recruitment, and macrophage reprogramming using inhibitors of receptors/protein involved in the innate immune response including CD47, CD40 and toll-like receptors [**5**]. We have recently shown that macrophages can also be reprogrammed using an oncolytic virus (HSV1716) resulting in decreased tumour burden and increased survival in murine breast cancer models [**6**]. In this study, we describe the changes in macrophage

phenotype from “M2-like” to “M1-like”, and if macrophages were depleted using clodronate liposome, anti-tumour efficacy was suppressed.

Oncolytic viruses (OV) are a novel class of cancer therapeutics which have the ability, either intrinsically or more commonly in genetically altered form, to preferentially divide and replicate in cancer cells rather than non-cancer cells. Although the mechanism behind individual viruses delivering their cytotoxic effect differs, the broad effect of the virus is to directly lyse tumour cells and generate immunogenic cell death, thus presenting tumour antigens which will be recognised and targetable by the host’s own immune system. Immunogenic cell death is the term given to the release of host of pro-inflammatory markers including calreticulin, heat shock proteins, ATP and HMGB1. This environment is particularly appealing to dendritic cells which then phagocytose tumour associated antigens and presents these to T cells.

The most commonly studied of these are adenoviruses, reoviruses and herpes simplex viruses (HSV). These have been trialled in a number of tumour types with preclinical and clinical efficacy, whilst advanced melanoma represents the leading tumour subtype to be treated with the first FDA-approved herpes simplex virus (T-VEC). T-VEC is a modified HSV that carries the transgene for granulocyte colony-stimulating factor (GMCSF). The landmark phase 3 virotherapy trial (OPTIM) described increased response and overall survival in patients compared with GM-CSF alone [**7**]. In breast cancer, oncolytic virotherapy is an expanding area of interest, with a number of preclinical studies and a few early phase studies showing early markers of response [**8**,**9**,**10**,**11**,**12**,**13**,**14**]. However, to date, most clinical studies using OV have relied on intratumoural injection due to the challenges of systemic therapy.

Injection of OV into the circulation may lead to suboptimal viral concentrations reaching the tumour and exerting its anti-tumour effects. This is mainly a result of neutralisation via the

patient's immune system and non-specific uptake in tissues, predominantly the liver and spleen [**15**,**16**]. Intratumoural injection of OV has been used in melanoma metastases, however, intratumoural injection would prove technically challenging in treating TNBC metastases, which are primarily located in the liver, lung, brain and bone. One of the approaches to systemic delivery of virotherapy is to exploit the cellular components of the tumour microenvironment. A number of cell carriers have been reported to provide viral protection and carry the virus to the tumour in a "Trojan horse" style. These cells include T cells, dendritic cells, mesenchymal stem cells, neural progenitor cells, endothelial progenitor cells and macrophages [**17**]. Of these, macrophages are of particular interest, as they are present in high numbers in tumours [**18**] and may have a prognostic implication in breast cancer [**19**,**20**]. Using the macrophage as a vector for virotherapy, also known as macrophage virotherapy, has been shown to protect the virus in circulation and target inaccessible prostate tumours in murine models [**21**,**22**]. Others have recently published data of an *in vitro* co-culture model of monocyte-derived macrophages, a paramyxovirus and TNBC [**23**]. Here we describe the potential for macrophage virotherapy using an oncolytic HSV1716 virus within murine primary and metastatic breast cancer.

Results

Characterisation of M-HSV1716

Bone marrow was isolated from immune-competent mice as described in **Section 4.3**. At day 0 the majority of cells were found to be undifferentiated monocytes (**Figure 1**A). After 7 days of incubation in macrophage differentiation medium, these cells demonstrated a shift towards F4/80-expressing macrophages, with over 95% of adherent cells expressing CD45+ CD11b+ F4/80+ and a loss of Ly6G expression (**Figure 1**B). These bone marrow-derived macrophages (BMDM) were infected with HSV1716 at an MOI of 10 in serum-free medium for 2 h. Of note, an MOI of 10 was used by the group as this allowed for high viral infectively and moderate viability. It was used because at higher MOI's (greater than MOI 10) BMDMs were

susceptible to cell death (over 90% of macrophages died at MOI 25 with 50% cell death at MOI 5) at 96 h following infection (data not shown). Unbound virus was washed off, and cells underwent an additional 2 h incubation; flow cytometry revealed a viral infection efficiency of 53% s ($p = 0.0004$, **Figure 1**C). Patency of the virus within BMDMs (M-HSV1716) was demonstrated by a significant difference in the expression of genes responsible for viral replication (**Figure 1**D) compared to control BMDMs 24 h post-infection. We also confirmed viral replication in the standard plaque assay using Vero cells (**Figure 1**E). Viral concentration of M-HSV1716 demonstrated 100-fold fewer viral particles compared to HSV1716. This was most likely associated with the infection efficiency during the preparation of the treatment. Due to the retrospective nature of quantifying infectious inoculations, together with the loss of viability associated with the storage and preparation of such treatments, a matching virus-alone control is problematic. We have, however, demonstrated that the cytotoxic activity of HSV1716 is dose dependent, with 99% of TS1 cells succumbing 7 days post infection ($p < 0.0001$, **Figure 1**F). Additionally, we have previously shown that HSV1716 can infect and lyse a number of breast cancer cell lines *in vitro* [**6**]. To examine whether this can be replicated with M-HSV1716, PyMT-TS1 spheroids were co-cultured with HSV1716 or M-HSV1716 for 72 h hours, and tumour cell lysis was assessed via flow cytometry with TOPRO-3. Here, equivalent cell death was seen with both HSV1716 alone and when delivered via macrophages; however, macrophages alone did not induce any significant cell death over the untreated control cells (**Figure 1**G).

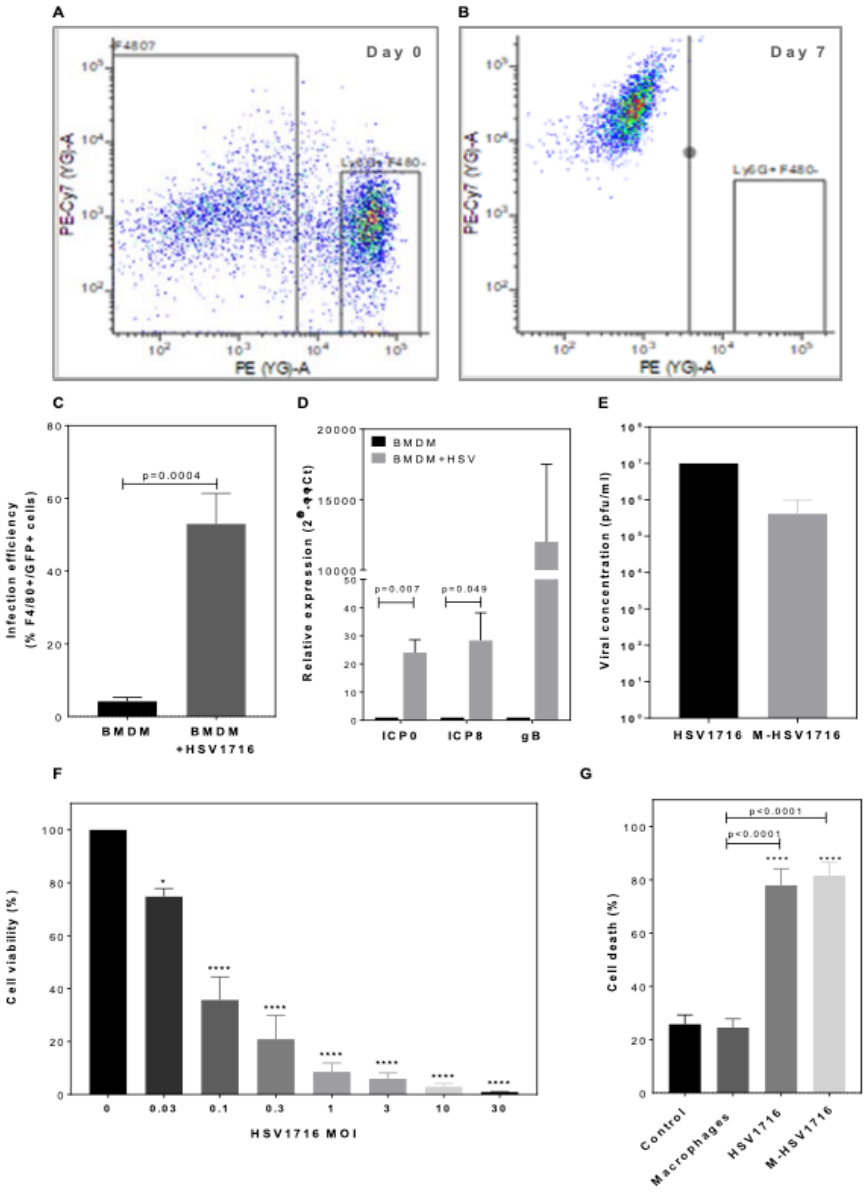

Figure 1. *Characterisation of M-HSV1716. Isolation of BMDM at day 0 was characterised as predominantly undifferentiated monocytes (**A**), x-axis—Ly6G (neutrophil marker), y-axis—F480 (macrophage marker). Following 7 days of incubation, these differentiated into F4/80-expressing macrophages (**B**). The infection efficiency of BMDM with HSV1716 (MOI 5) was quantified by flow cytometry of dual-stained F4/80 macrophage marker with GFP-expressing HSV1716 (**C**). Low-level non-specific binding of antibody was noted in untreated cells. (**D**) HSV1716 patency within BMDMs was assessed by measuring viral replication genes 24 h post infection. M-HSV1716 was prepared by incubating BMDM with HSV1716 at MOI 25 for 2 h. Cells were harvested and washed in PBS prior to administration, and plaque assays were performed to ascertain viral titre of treatment groups (**E**). (**F**) HSV1716 was used to infect PyMT-TS1 cells at a concentration range of MOI 0.03–30, and the effects on cell viability were determined by Alamar blue assay 7 days post infection. (**G**) FACS data showing equivalence in cell death (TOPRO-3) when spheroids were infected with HSV1716 and M-HSV1716. Data represents the mean ± SEM of assays performed in triplicate.*

*Statistical significance was analysed by unpaired t-test (**C**,**D**). * = p < 0.05, **** = p < 0.0001 one-way ANOVA (**F**,**G**).*

Macrophage-Mediated Delivery of HSV1716 Slows Primary Breast Tumour Growth

Previously, we have shown that HSV1716 alone slows the rate of breast cancer growth in three murine models of primary breast cancer [**6**]. This oncolytic effect resulted from both direct cell lysis and changes in the tumour microenvironment, notably a change in macrophage behaviour and T-cell activation, which has been shown to result in immunogenic cell death. Additionally, viral replication within macrophages was observed, resulting in the use of this strategy to enhance HSV1716 delivery to tumours in this study.

In this study we investigated the effect of M-HSV1716 treatment in a primary model of breast cancer. PyMT-TS1 cells were implanted into the mammary fat pads of BALB/c mice. Treatment was initiated when tumours had grown to an average of 500 mm^3 (**Figure 2**A). The effect on the breast tumour growth post-treatment was quantified by caliper measurements, whereby M-HSV1716 significantly slowed tumour growth compared with HSV1716 ($p < 0.001$) and BMDM ($p < 0.05$) alone up to day 11 post-treatment (**Figure 2**B). Notably, our recent studies demonstrate that HSV1716 needs to be administered repeatedly in order to suppress tumour growth; despite this, once the treatment stops, tumours did regrow [**6**]. We did not expect BMDM to have an inhibitory effect, as these cells were not stimulated to be anti-tumour-like, and their purpose was only for delivery of HSV1716.

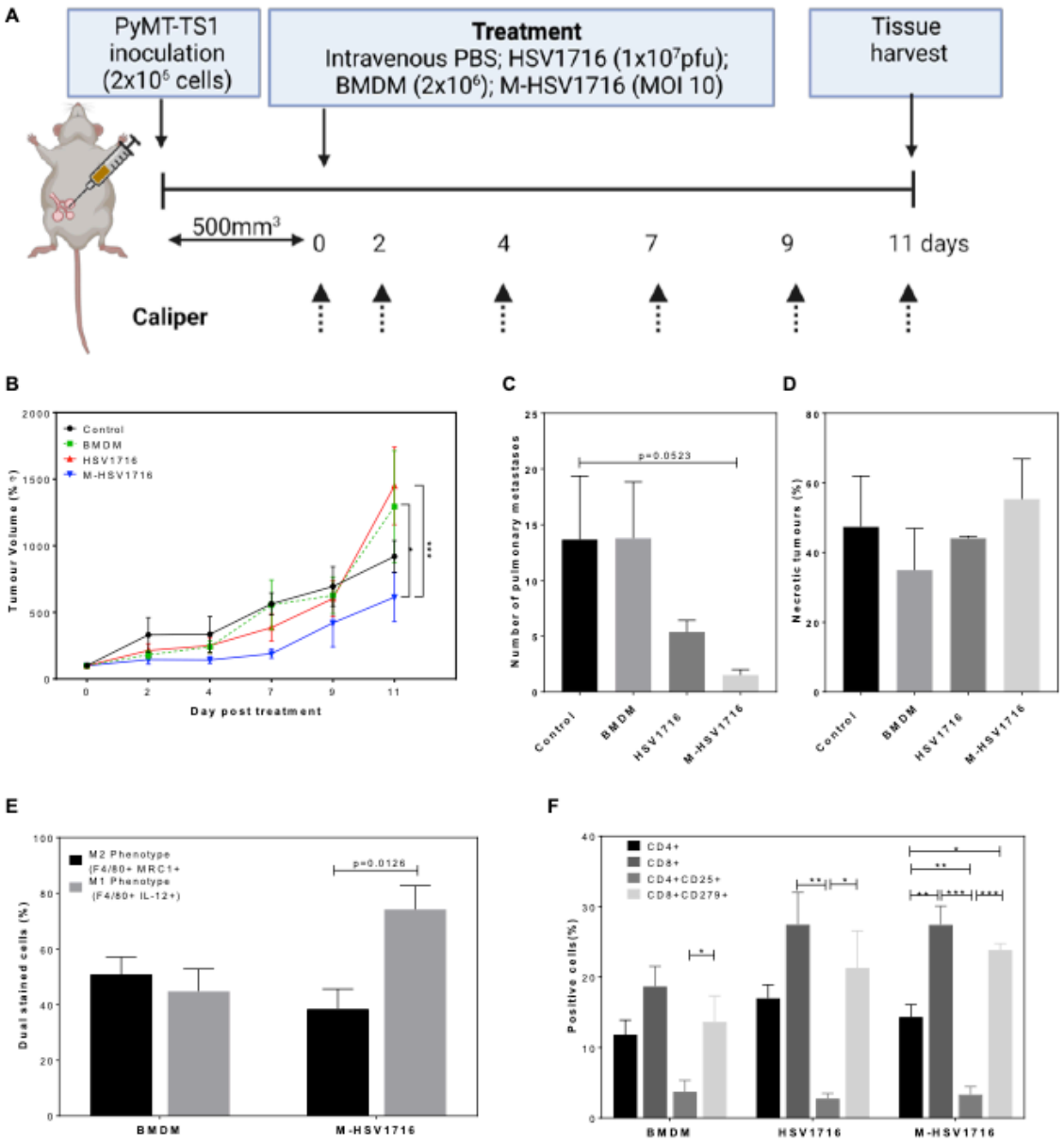

Figure 2. M-HSV1716 reduces tumour burden in a primary mouse model. *PyMT-TS1 cells were implanted into the 4th mammary fat pads to assess the effectiveness of HSV1716 or M-HSV1716 in primary tumours (**A**). Tumour growth was measured by digital calipers for 11 days following treatment (**B**). Pulmonary metastases (**C**) and necrosis (**D**) were quantified histologically postmortem from an average of 2–4 different sections of lung tissue over 100 µm apart from 2–4 slides. Immune cell changes within the TME were seen when M-HS1716 was given compared to macrophage alone. Flow cytometric analysis of dispersed tumours showed a skewing to M1 macrophage phenotype (**E**) and an increase in CD8+ T cells (**F**). Data are mean ± SEM, n = 6–8 mice per group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ analysed using one-way ANOVA. Panel **A** was created with BioRender.com, accessed on 6 October 2022.*

A difference in the number of lung metastases was also seen between the control (untreated) and M-HSV1716 group ($p = 0.0523$), as well as a trend for improvement between the HSV1716 and M-HSV1716 group (**Figure 2**C). There was no difference in tumour necrosis between groups (**Figure 2**D). As viral titre was significantly lower in the M-HSV1716 group compared to HSV1716 treatment alone (**Figure 1**E), we speculate that this may be a result of improved tumour targeting and shielding of the virus from immunosurveillance whilst in the circulation in comparison to naked virus (as described in our previous work [**21**,**22**]) or an increase in viral replication within the macrophage carriers [**6**].

Interestingly, a greater proportion of M1-like macrophages were seen when phenotyping M-HSV1716 compared to BMDM ($p = 0.0126$, **Figure 2**E). Macrophage expression of MRC1 decreased from 51% to 32%, whilst the percentage of F4/80+ cells expressing IL12 within tumours increased from 48% to 74% following treatment with M-HSV1716 (**Figure 2**E). Additionally, M-HSV1716 mirrors the changes in CD8+ T cell numbers and phenotype which we have previously described (**Figure 2**F). These changes are not seen when macrophages alone are given. In view of this, we did not include macrophage controls for subsequent studies.

Equivalence of HSV1716 and M-HSV1716 in an Early Metastatic Model despite Decrease in Viral Load

To investigate whether M-HSV1716 could prevent the development of metastatic disease, an early metastatic model was performed [**6**]. In this study, a highly metastatic 4T1 cell line was used to model the aggressive nature of TNBC. Luciferase-labelled 4T1 cells were implanted into the left ventricle of female BALB/c mice. Five days later treatment was initiated, and animals were monitored regularly for tumour burden using an *in vivo* luminescent imaging system (IVIS) (**Figure 3**A). We have shown previously that multiple dosing of HSV1716 improved metastatic disease compared to single dosing [**6**]. Therefore, comparison with multi-dosing HSV1716 and

M-HSV1716 was performed in this experiment. This consisted of 3 treatments given 48 h apart. A significant improvement in disease survival was seen in the HSV1716 and M-HSV1716 treatment groups as compared to controls ($p < 0.0001$, **Figure 3**B). At day 50 we saw no development of luciferase-measurable metastatic disease in either group (**Figure 3**C,D), suggesting that both regimes effectively prevented metastatic disease in this early setting.

Metastatic burden was quantified by H&E histological staining in the lung, liver and brain (**Figure 3**E–G). This confirmed that there was no metastatic disease in both the HSV1716 and the M-HSV1716 treatment arms.

Macrophage Mediated Delivery of HSV Improves Survival of Mice with Stereotactically Inserted Brain Metastases

Brain metastases pose a therapeutic challenge in TNBC. Once developed, these are often aggressive and difficult to treat systemically. As viruses are small, there is a potential they may cross the blood–brain barrier. We performed a survival model of an aggressive brain metastatic breast cancer cell line, inoculating luciferase-labelled brain-seeking 4T1 cells into the brain parenchyma of female BALB/c mice (**Figure 4**A). In this small study, $n = 5$ mice per group were implanted with tumours; once these were visible using IVIS (**Figure 4**B), mice were treated with intravenous treatments of either PBS control, IV HSV1716 or M-HSV1716 combination. Mice demonstrated a median survival time of 9, 14 and 15.5 days when treated with PBS, HSV1716 or M-HSV1716, respectively. Treatment with M-HSV1716 demonstrated a survival advantage when compared to control mice ($p = 0.0167$, Mantel–Cox test) (**Figure 4**C).

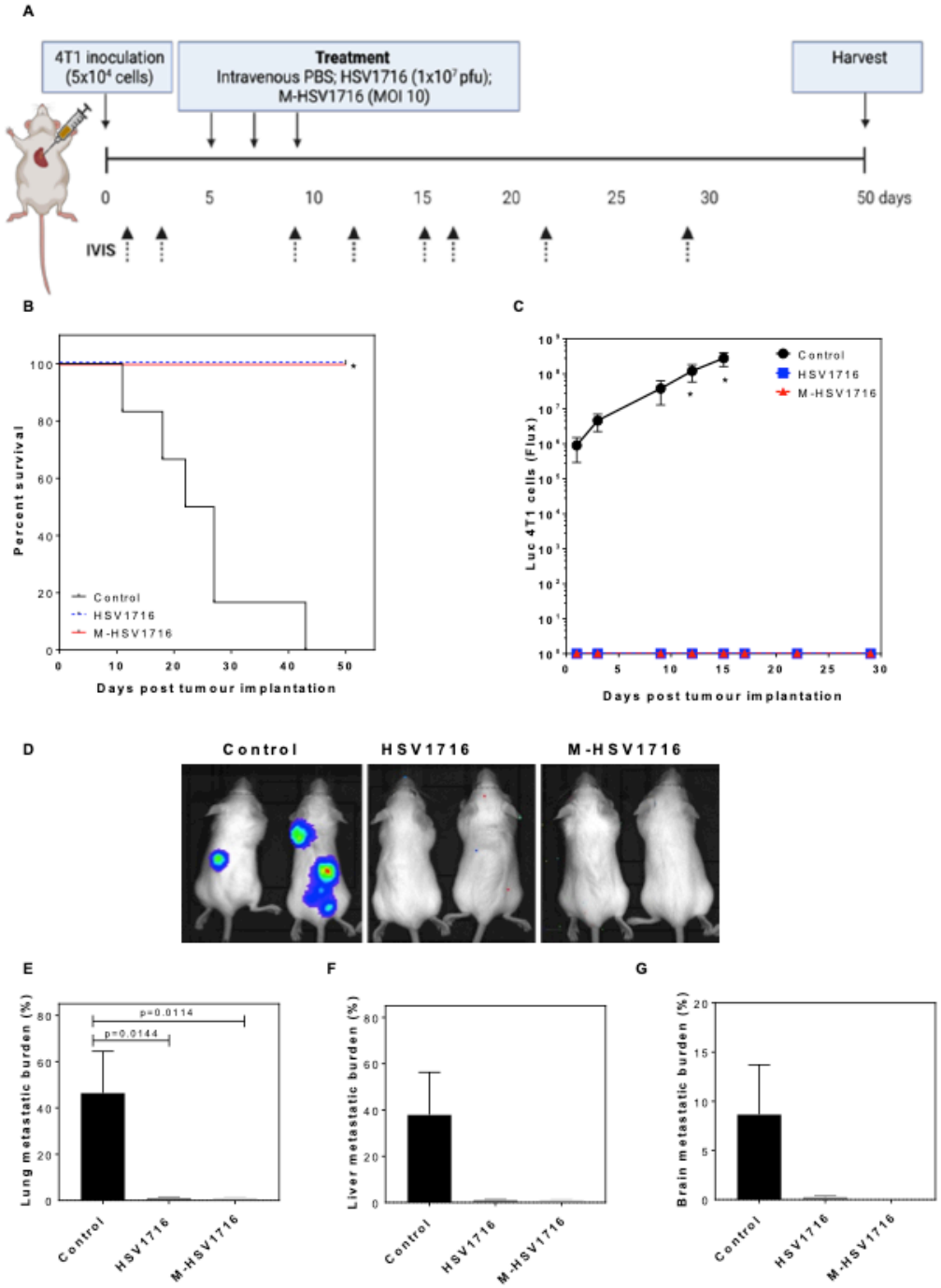

Figure 3. *M-HSV1716 is equivalent to intravenous HSV in an early metastatic model. Luciferase-labeled 4T1 cells were injected into the left ventricle of the heart via intracardiac injection of female BALB/c mice. At days 5, 7 and 9, mice were treated with PBS, HSV1716 (1 × 10^7 pfu/mL) or M-HSV1716 ((1 × 10^6 macrophages infected at MOI 10), and the development of metastases was monitored by IVIS up to day 50 (**A**). M-HSV1716 provided a survival advantage (**B**, Log-Rank test) corresponding with an absence of visible metastatic disease quantified by flux analysis of luciferase-expressing 4T1 cells (**C**,**D**). The burden of metastases was calculated as the percentage of the organ with metastatic involvement calculated as an average of 2–4 different sections of lung (**E**), liver (**F**) and brain (**G**), over 100 µm apart from 2–4 slides. Data shown are mean ± SEM, n = 4–6 animals. Panel **A** was created with BioRender.com, accessed on 6 October 2022.*

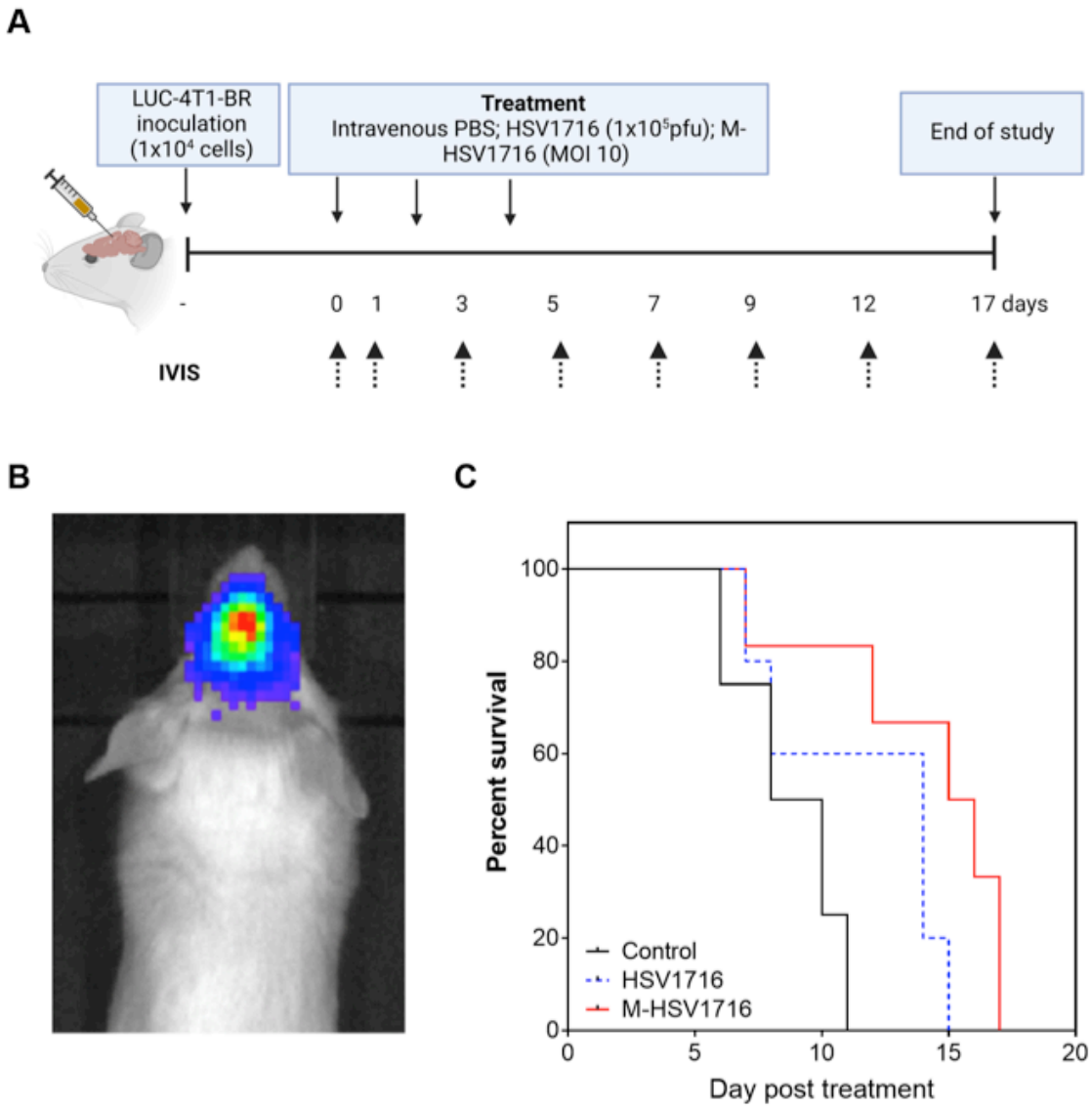

Figure 4. *M-HSV1716 improves survival of mice with stereotactically implanted brain metastases. A brain metastases model was formed by the inoculation of 1 × 10^4 LUC-4T1-BR cells intracranially, to a depth of 3 mm, using a Hamilton syringe. Animals were imaged until the intracranial lesion was visible and then treated with either PBS, HSV1716 or macrophage-HSV1716 (**A**). (**B**) A representative image of a brain metastases as seen on IVIS. (**C**) A Kaplan–Meier curve demonstrated a survival advantage with M-HSV1716 compared to other treatment groups (Log-Rank test). Mice were culled when tumours reached the maximum permitted size as determined by the IVIS, at which point tissues were harvested. Only mice treated with M-HSV1716 survived beyond day 15. Data shown are mean ± SEM, n = 5 animals. Panel **A** was created with BioRender.com, accessed on 6 October 2022.*

Discussion

Understanding the immune system and enhancing immunotherapies is a topical area of research. Here we show that improvements in treatment efficacy are seen when macrophages are used to deliver an oncolytic viral immunotherapy to murine mammary tumours. Of particular interest is that the viral dose carried within macrophages was found to be 100-fold less than that of the intravenous dose and still showed similar efficacy. This has the potential of reducing a patient's viral-associated off-target effects by both allowing for more-specific tumour targeting and enabling a lower dose of viral particles to be administered for the desired effect. Therefore, although there are costs involved in the production of M-HSV1716, these costs may be offset by a reduction in the dose of treatment required and the downstream costs of patient-related hospital admissions secondary to toxicities. One possible explanation of this finding could be related to the ability of some viruses to replicate within macrophages. Studies have shown that this occurs in pathological infections with viruses such as influenza [**24**] and HIV [**25**]. Indeed, we recently described how the oncolytic herpes virus HSV1716 has the ability replicate within MDM *in vitro* [**6**]. Perhaps this could account for the increased effectiveness of macrophage-delivered virus over virus alone, especially in view of the discrepancy in viral doses.

Nevertheless, cell delivery of anticancer drugs is a growing area of interest [**17**]. Research carried out by Muthana et al. [**21**,**22**], demonstrated the potential of cell carriers, namely macrophages, as vectors to deliver a prostate-specific adenovirus. Given that high numbers of macrophages are present in the hypoxic areas of prostate tumours, the researchers opted to exploit this and used macrophages to deliver OV to prostate tumours grown in spherical cell complexes that mimic the TME *in vitro* and in xenograft models of prostate cancer. The Macrophage–OV complex successfully delivered the virus to the tumours resulting in efficient viral replication under hypoxic conditions, tumour oncolysis and inhibition of tumour growth in mice. More importantly, when co-cultured with high-titre neutralising antibodies in human

serum, the macrophages protected the virus, and this was significantly more effective compared to adenovirus on its own, which was completely neutralised [**21**].

As we know, high concentrations of virus are required for an efficacious anti-tumour response when delivered intravenously due to its rapid neutralisation by circulating antibodies and removal by the reticular endothelial system. However, our group has observed dose-dependent tolerability issues in murine models resulting in recoverable subacute piloerection, pallor and reduced mobility to death in the most severe cases. This phenomenon is seen in tumour-bearing mice only and is also dependent on mouse strain. The maximum tolerated dose of HSV1716 has been elucidated (unpublished observation) in these models, and we are confident that the shielding of viral epitopes by macrophage delivery negates immunosurveillance thus facilitating the use of lower doses resulting in the absence of observable toxicities. Both of these observations support the use of macrophages as vectors to improve viral delivery (**Figure 5**).

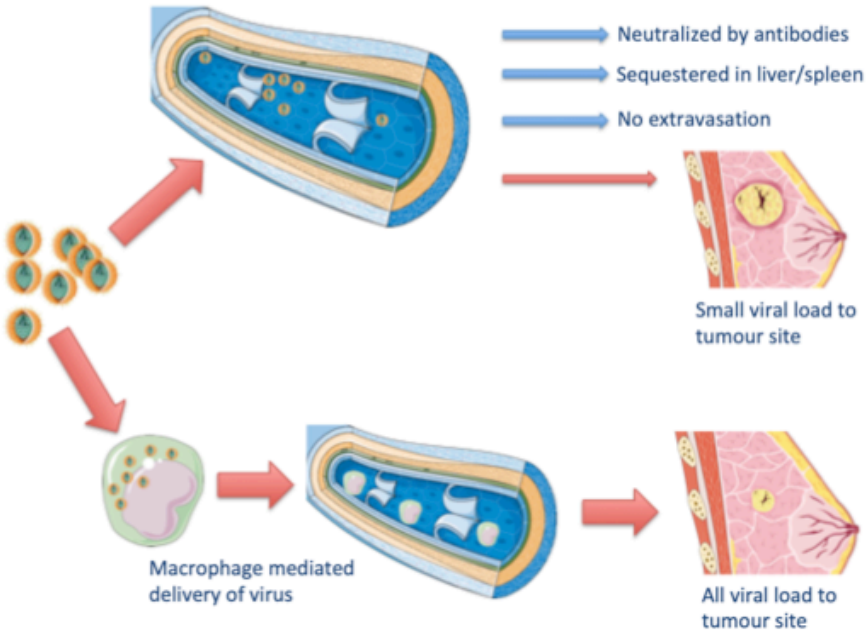

Figure 5. *Potential for M-HSV1716 in TNBC. A schematic representation of how macrophages with virus may be used to target breast cancer.*

In other studies, improved tumour growth *in vivo*

was shown when macrophages were used to transport chemotherapy particles, SN38, a derivative of irinotecan [**26**]. Another showed that loading M1-polarised macrophages with a sorafenib nanoparticle improved drug targeting and was effective at treating hepatocellular cancer *in vitro* and *in vivo* [**27**]. Furthermore, non-malignant disease areas are exploring the use of macrophage carriers to treat diseases such as atherosclerosis [**28**] and liver cirrhosis [**29**].

However, the reduction of breast tumours was not curative with virus alone. This suggests that perhaps virus alone is unable to stimulate a sufficient immune response despite improved delivery with macrophage carriers. Combination therapy of OV and established cancer therapeutics is of significant interest to a number of research groups. Of particular promise are the trials involving checkpoint inhibition and OV. For example, Bourgeois–Daigneault et al. [**8**] described the use of a rhabdovirus Maraba virus prior to the removal of a breast tumour in immunocompetent murine models. Here, they administered a course of intravenous OV treatment 7 days prior to mammary tumour resection, followed by an adjuvant course of checkpoint inhibitor treatment. They demonstrated that use of virotherapy prior to surgery allowed for sensitisation to immune checkpoint therapy given adjuvantly, and that on re-challenge, the immunological effects were long lasting [**8**]. Similarly, Mostafa et al. [**30**] demonstrated that the oncolytic effect of reovirus can be enhanced with the addition of a PD-1 inhibitor in an EMT6 immunocompetent murine model of breast cancer, causing a reduction in tumour growth in comparison to monotherapy, and this change resulted from cytotoxic changes in the TME including an increase in CD8+ cells and a decrease in CD4+ T cells. Given these promising preclinical data, there are a few ongoing clinical trials combining oncolytic viruses with a checkpoint inhibitor in breast cancer. Viruses under investigation at present include HSV, vaccinia and reovirus, with the likelihood that other viral groups will be included as data matures.

Another area this research highlights is the potential for targeting brain metastases. We show that macrophage-delivered treatment enhances the survival of animals treated for brain metastases. The potential to cross the blood–brain barrier, a clinically challenging niche, needs to be explored further. Macrophages have been used as carriers to effectively transport nanoparticles loaded within them to target gliomas in the brain [**31**]. Furthermore, some groups are exploring the use of macrophages as vector-carrying nanoparticles which enhance photo thermal ablative therapies to the brain [**32**,**33**].

Materials and Methods

Cell Lines

PyMT-TS1 [**34**] (a kind gift from Prof. Johanna Joyce, MSKCC, Manhattan, NY, USA), and LUC-4T1-BR [**35**] (obtained from Prof. Sanjay Srivastava, University of Texas, Austin, TX, USA) were used *in vivo*. Vero cells were purchased from the ATCC. Cells were maintained in DMEM supplemented with 10% FBS. All cells were used within 20 passages and were cultured at 37 °C in 5% CO_2. All cell lines were routinely tested for mycoplasma and microsatellite analysis. All culture reagents were purchased from Lonzo BioWhittaker Ltd.

Virus Production and Handling

HSV1716 was obtained from Virttu Biologics (Glasgow, UK) in stocks of 1×10^8 particle-forming units (PFU) in compound sodium lactate (Hartmann's solution) with 10% glycerol. All vials were stored at −80 °C and freshly thawed on ice before each experiment.

Production of Murine Bone Marrow Derived Macrophages

Bone marrow was obtained from both femurs and tibias of BALB/c mice under aseptic conditions. The lower abdominal cavity and both hind limbs of animals were sprayed with 70% ethanol before tibias and femurs were isolated. Joints were taken from above the pelvis and the

muscles removed by blunt dissection and then cleaned with sterile gauze. Femurs were transported in sterile PBS to the tissue culture lab, where they were washed in sterile 70% ethanol. The epiphyses of bones were cut, and the shafts of the bones were flushed with 1 mL of DMEM using a sterile 25G needle. The bone marrow was then centrifuged at 1000 rpm for 5 min. The pellet was resuspended and incubated in differentiation DMEM which contained 10% heat-inactivated FCS and 20% L929 cell-conditioned medium. Non-adherent cells were removed on day 5, and adherent cells were matured by incubating at 37 °C, 5% CO_2 ready for infection on day 7.

HSV1716 Infection of Macrophages *In Vitro*

Virus was allowed to gradually thaw on ice. Cells were washed in PBS, and 500 µL serum free RMPI medium was added to each well followed by virus at the desired multiplicity of infection (MOI). Macrophages were incubated, at 37 °C, 5% CO_2, with virus for 2 h and then free virus was removed by washing with PBS. M-HSV1716 complex was incubated for 2 more hours before use in experiments. Cells were analyses 24–48 h post infection.

Spheroids

For tumour spheroid experiments, 96-well plates were coated with DMEM containing aga rose prior to seeding with TS1 cells at 2×10^4 cells/well. Following incubation for 5 days and an approximate cell density of 10^5 cells, spheroids were inoculated with HSV1716 or M-HSV1716 at an MOI of 5. For these experiments HSV1716 GFP was used, and for controls, spheroids were either untreated or infiltrated with BMDM at a concentration of 1×10^5. Spheroids were monitored by light microscopy (Leica DM1000) at ×10 magnification. Flow cytometry as described above was used to assess cellular death and GFP infection on day 3 post-infection.

Flow Cytometric Studies

Dissociated mammary tumours were stained with fluorescent antibodies [**36**]. Cell viability was determined using the Zombie UV™ Fixable Viability Kit (Biolegend) and ABC™ Anti-Mouse Bead Kit (ThermoFisher Scientific, Loughborough, UK). The following antibodies were used: CD4 (cat no. 100510), CD8 (cat no. 100707), CD25 (cat no. 101909), CD279 (cat no. 109110), LY6-G (127616), F4/80 (AbD Serotec, cat no. MCA497A488, clone C1:A3-1), IL-12 (cat no 505207), GFP (Abcam, Cambridge, UK, cat no. ab290), TOPRO3, MRC1(Abcam, ab64693). All antibodies were purchased from Biolegend unless otherwise stated. All FACS data were analyzed on an LSR II flow cytometer (BD Biosciences, Wokingham, UK) and processed using Flow software (Tree Star, Texas, USA).

PCR

RNA was isolated from infected MDMs using the RNeasy Mini Kit (Qiagen) followed by cDNA synthesis using SuperScript III reverse transcriptase (Life Technologies, Paisley, UK). cDNA was analyzed using the following viral replication genes: ICP0 (Forward primer region AAGCTTGGATCCGAGCCCCGCCC, reverse primer region AAGCGGTGCACGGGAAGGT), ICP8 (Forward primer region GACATTACGTTCACGGCCTTCGAAGCCAG, reverse primer region GGCCGAGTTGGTGCTAAATACCATGGC) and gB (Forward primer region TGTGTACATGTCCCCGTTTTACG, reverse primer region GCGTAGAAGCCGTCAACCT) with GAPDH (forward primer region ACAGTTGCCATGTAGACC, reverse primer region TTTTTGGTTGAGCACAGG) as the housekeeping gene using SYBR Green (Primer Design, Chandler's Ford, UK). q-PCR reaction was performed in a 384-well plate (three wells per gene and sample), and a 7900HT AbiPrism sequence detection system (Applied Biosystems) was used. The fold change in gene expression between treatment groups was analysed by inserting Ct values into Data Assist V3.01 software (Applied Biosystems), and changes in gene expression were only analysed for genes with a Ct value of ≤ 25.

Viral Quantification

As previously described, plaque assays were performed as described in Baer and Kehn-Hall [**37**]. Briefly, confluent monolayers of Vero cells were inoculated with serial dilutions of HSV1716 for 2 h. Following removal of the viral inoculum, monolayers were overlaid with 1:10 4% agarose:culture medium and allowed to solidify for 15 min at room temperature before the plate was moved to a humidified incubator (37 °C) for 72 h. A total of 4% PFA was applied to agarose plugs for 1–2 h to fix cell monolayers before their removal. Cells were washed with PBS, stained with 1 mL crystal violet for 5 min and rinsed with tap water. Once dried, plaques were counted per well and viral titre was determined.

In Vivo Studies

All animal procedures were carried out in accordance with the UK Animals Scientific Procedures Act 1986 and with the approval of the University of Sheffield Ethical Committee (PPL70/8670). All mice were obtained from Charles River Laboratory and acclimatised in the biological services laboratory for 7 days prior to the procedure. Animals were anesthetised using 3–4% isofluorane in 70:30% N20:02.

Primary mammary tumours: 1×10^6 PyMT TS1 in 50 µL of 1:1 matrigel:cells, were implanted into the 4th mammary fat pad of 6–7-week-old FVB mice (n = 10/group) as described in [**6**]. When tumours reached ~500 mm^3, mice were randomly divided into groups and received a single injection of 100 µL PBS, 2×10^6 BMDMs, HSV1716 (1×10^7 PFU) or M-HSV1716 (1×10^6 macrophages infected at MOI 10) intravenously. Mice were culled on day 9 for postmortem comparison of tissues or when tumour volumes exceeded the maximum permitted size (1500 mm^3) for survival studies. Excised tissues including tumours, brain, liver, lungs, kidney and spleen were embedded in paraffin wax for histological studies. Tumours were also

digested, and single-cell suspensions were cryo-stored in 90% FBS and 10% DMSO for flow cytometry.

Metastatic model: 1×10^5 LUC 4T1 cells were filtered and injected via the intracardiac route into the left ventricle of female BALB/c mice as described in [**6**]. All animals were allowed to recover in a heated chamber. Animals were randomly allocated (n = 6/group) and received either PBS, 1 dose of HSV1716 (1×10^7) or 3 doses of HSV1716 (1×10^7) or M-HSV1716 ((1×10^6 macrophages infected at MOI 10)) given on day 1, 3 and 5). Animals were imaged weekly using the *In Vivo* Imaging Systems (IVIS Lumina II imaging, Caliper Life Sciences, Preston Brook, UK) following intra-peritoneal injection of luciferin (150 mg/kg). Mice were culled on reaching a humane end point (over 20% weight loss, respiratory distress, physical signs of discomfort) or after 50 days from treatment. Brain, liver, lungs, and spleen were embedded in paraffin wax for histological studies.

Brain metastatic model: Within a stereotactic frame, 1×10^4 LUC 4T1-BR cells were injected intracranially into female BALB/c mice. All animals were allowed to recover in a heated chamber. Animals were given 2 doses of analgesia via subcutaneous injection, Metacam (5% solution with 20 µL injected subcutaneously), just prior to intracranial inoculation and at 24 h post recovery. Animals were also given a single dose of antibiotic (Baytril 2.5% solution for injection, diluted 1:10 in saline and injected 20 µL intramuscularly) just following the procedure.

When luminescence of an intracranial lesion was observed, animals received intravenous treatment of either control (PBS), 3 doses of HSV1716 (1×10^7) or M-HSV1716 ((1×10^5 macrophages infected at MOI 10). Animals were observed every 2–3 days with tumour growth progression visualised on the IVIS. All animals were culled if a humane end point was reached or after 26 days post first intracranial tumour implantation and brain and lung were cryopreserved in OCT freezing medium.

Tissue Analysis

Haematoxylin and Eosin staining was carried out to determine necrosis and metastasis in FFPE tissue (2–4 sections per sample, 10 microns thickness and 1000 microns apart). Slides visualised using the using Hamamatsu NanoZoomer XR (Hamamatsu, Hertfordshire, UK), and quantified using Image Scope (Leica Biosystems, Newcastle Upon Tyne, UK).

Statistics

All statistical analyses were performed using GraphPad Prism 8, and the tests used are described in the Figure legends. Data are means ± SEMs, and p values of < 0.05 were considered to be significant.

Conclusions

Breast cancer is a heterogeneous disease, and although conventional treatments have evolved significantly over the past few years, treatment resistance invariably occurs. Training the immune system to recognize and target cancers has proved curative for select patients in a number of solid tumour types. The hallmark of response appears to be a favourable "inflammatory" TME. Oncolytic viruses are an expanding group of immunotherapeutics with the potential to enhance the TME. Here we show that macrophages may be able to further enhance their effect through improved delivery of virus. Future work involving the combination of M-HSV1716 with other chemotherapeutics or immunotherapies would enhance this study.

Author Contributions

Conceptualization, M.M. and A.K.; methodology, M.M., G.C.S., A.K. and P.D.O.; validation, A.K., F.H. and N.W.; formal analysis, A.K. and F.H.; investigation, A.K.; data curation F.H., N.W., A.K., A.J., E.A., R.A. and P.B.P.; writing—original draft preparation A.K. and F.H.; writing—review and editing, F.H., M.M., J.E.B., C.L., P.D.O., G.C.S., C.W., J.C., S.K.S. and S.J.D.; supervision, M.M., C.W. and S.J.D.; project

administration, A.K.; funding acquisition, M.M., J.E.B., C.L. and A.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with the approval of the University of Sheffield Ethical Committee (PPL70/8670).

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5.3 Summary of findings

The paper above describes the feasibility of macrophage delivered HSV1716 therapy, where in comparison to placebo, macrophage virotherapy significantly improves outcome. There is a suggestion that macrophage delivered therapy may improve outcomes compared to the HSV1716 deliver treatment at the doses given, however this is not statistically different. Of particular interest is that the viral dose carried within macrophages was found to be 100-fold less than that of the intravenous dose and still showed similar efficacy. This has the potential of reducing a patient's viral-associated off-target effects by both allowing for more-specific tumour targeting and enabling a lower dose of viral particles to be administered for the desired effect. However, cellular therapy is not without complications and consideration for the potential for cumulative toxicities to the patient have to be weighed into logistics. HSV-1 based viruses have shown relatively modest toxicities for patients in studies to date, and early phase studies for HSV1716 in particular describe the treatment to be very well tolerated, when given locally or systemically. With regard to cellular therapy, concerns have been realised about the possibility of cytokine release syndrome, Cytokine release syndrome is a clinical and biochemical diagnosis characterised by fever, hepatosplenomegaly, progressive liver failure with coagulopathy, cytopenia, and hyperferritinemia which is secondary to the systemic release of pro-inflammatory cytokines, such as IL-1, IL-6, interferon-γ (IFN-γ), tumor necrosis factor (TNF), and IL-18. Macrophages have been shown to be key mediators to the development of cytokine release syndrome and awareness of how to diagnose and manage this will be pertinent to the developments of cellular therapies (294,295).

Another factor to consider is that although we see effectiveness of macrophage delivery therapy, the potential to progress to new treatments clinically, is also weighed on the feasibility of manufacturing and the cost this entails. At present, cellular therapies, such as CAR T cells have to be manufactured at appropriate sites and in the UK and these are few and far between.

An explorative cost analysis, based in a German academic center, approximately that costs per patient per treatment could be around $60,000 (USD) however use of reagents may be able to bring costs down to $33,000. In addition to the manufacturing costs, the outlay cost of constructing an appropriate facility may be in the region of $580,000 (296).

In addition to the cost of cell isolation and purification, there are the added costs of producing virus alone, which can be extrapolated from viruses currently in use clinically. T-VEC, an HSV-1 virus that has shown efficacy in metastatic melanoma was initially marketed by Amgen to cost around $64,000 although cost price varies between countries due to local negotiations (297). HSV1716, is an older and less engineered version of HSV-1 compared to T-VEC and perhaps this will prove favourable with costing.

These pragmatic and practical reflections will help to shape the direction of both oncolytic virotherapy and cell-based delivery systems.

Chapter 6

6 Discussion

At the time of conceptualising this PhD, the landscape of breast cancer management was vastly different to what it is at present. Treatments were limited to chemotherapy, hormone therapy and targeted treatments such as trastuzumab. Since completion of the data collection and publication from this PhD, we now see the introduction of checkpoint inhibition as a standard of care in TNBC in the early and metastatic setting (298). In addition, newer targeted agents have been introduced in all breast cancer subtypes. For example, in ER+ setting cyclin-dependent kinase 4 and 6 inhibitors (CDK4/6i) such a palbociclib have been shown to double progression free survival when used in combination with an aromatase inhibitor(299). Trastuzumab deruxtecan in the HER2+ setting (300)has added a third line of treatment to patients who are HER2+ and PARP and immune checkpoint inhibition are continuing the change the landscape in the TNBC setting. This is an exciting time to be involved in the breast oncology field. However, where does that leave this PhD and where do we go from now?

In this PhD a novel form of immunotherapy was investigated to assess the potential for using this to treat TNBC. This exploited the now known responsiveness of TNBC to immune targeted therapies, such as checkpoint inhibition. Oncolytic viruses have several desirable characteristics namely the ability to both cause direct tumour lysis and immune system activation with minimal toxicities in patients. In Chapter 1, the background to the PhD was outlined. Here, the subtypes of breast cancer, the need for novel targets for TNBC and the ways the immune system was being utilised to target cancers is discussed. Clinically breast cancers are divided based on receptor status where a subgroup of those that are negative for ER/PR and HER2 receptors showed a high potential for response to oncolytic virotherapy. This TNBC subtype was felt to be the more appropriate target for immunotherapy treatments given the more inflamed tumour microenvironment, enriched with TILs and high mutational burden

which enables a higher proportion of cells to undergo immunogenic cell death. This view was shared amongst other investigators with bench side and clinical trials limiting immunotherapy to this subgroup of patients, thus checkpoint inhibition has become a standard of care in the management of TNBC with significant improvement in clinical outcomes(298). Immunotherapy treatments in many tumour types have been focused primarily on immune checkpoint inhibition, however the modulation of the immune system's response to cancer has been explored through cancer vaccines (79), CAR-T cells and more recently oncolytic virotherapy with mixed results. Perhaps these novel treatments are limited by understanding the process of immune system sensitisation, recognition, activation and ultimately it may need multiple immune system targets to generate the desired effect. Certainly, clinical outcomes are more marked when dual immune checkpoints are blocked simultaneously (301), however with the dual inhibition, an increase in immune related toxicity is observed.

Chapter 2 summarises the list of materials and methods used in addition to those described in the papers. As our group has not previously worked with breast cancer, knowledge about the different breast cancer model was extensively researched before commencing work. Model choice is important as it can aid the success and add to the robustness of the hypothesis in question. It is felt that inappropriate model choice can lead to failure to progress to clinical trials and early termination of potentially useful treatments. Given the emphasis minimising inappropriate animal studies and the relevance of the work of the NC3Rs, a strong consideration was given towards alternative models. Chapter 3 highlights the thought processes behind choice of model to help design a trial of an immunotherapy in breast cancer. Namely, it highlights the breast cancer subtypes and how models can be developed to allowing a representation of the immune system. From this research, given the timeframe, costs and restrictions due to Covid-19, a number of immunocompetent murine models of primary TNBC and metastatic models were identified. The choice to focus on immunocompetent *in vivo* models was namely because these allow the exploration of tumour burden reduction and

immune pathway assessment, primary models with a PyMT derived TS-1 cell line and 4T1 cells, an aggressive TNBC cell line were established. As the 4t1 cell line has more potential for metastatic spread and the niche area of interest in brain metastases was identified, a number of metastatic model were trialled. Firstly, a metastatic seeding model was explored whereby luciferase labelled brain seeking 4T1 cells were inoculated into the arterial circulation of BALB/c mice through intracardiac injection. The number of cells inoculated was based on previous experience by Dr Penny Ottewell. This model was highly productive in terms of the development of metastases after 4 days as observed on the IVIS, however the metastatic burden in lungs and bones was far greater than intracranial disease. It is postulated that animals were culled before intracranial disease was able to be established. In view of this, a resection model was proposed. The plan was to firstly establish mammary primaries through intranipple inoculation of 4T1 cells followed by resection of the primary to allow for secondary intracranial disease to develop. Unfortunately, due to the aggressive nature of the cell line the tumours grew too rapidly to resect with clear margins and the timetable and budget did not allow for a full refinement of this model. As neoadjuvant clinical trials, particularly within breast cancer, are becoming increasingly popular this resection model may be of interest to refine this further as part of future work. Finally, a primary intracranial inoculation model was explored. Within this model, luciferase labelled brain homing 4T1 cells were stereotactically implanted into the cerebral cortex of BALB/c mice. The advantage of this model is the intracranial lesions were guaranteed however there is some debate as to the appropriate number of cells needed for implantation as our brain metastases developed quickly limiting the window for the administration of therapy. There are also suggestions that large tumour burden can negatively impact the outcome of immunotherapy-based treatments (302). Further work with this model would include optimization of the number of cells needed per cell line and use this to guide treatment administration regimes. An alternative is to consider other methods for the generation of brain metastases such as through inoculation of cells into the carotid artery(303),

however reports suggest brain lesions were mainly found in the hippocampus which is an unusual site for metastases in breast cancer patients.

Oncolytic virotherapy has the potential to stimulate the immune systems response to cancer cells through cell lysis and immunogenic cell death. Chapter 4 describes the use of a modified virus, HSV1716, as a therapeutic agent against TNBC. Here, the susceptibility of breast cancer to HSV1716 infection and cell lysis was confirmed against a range of murine and human derived breast cancer cell lines (MCF7, MDA-MB-231, SKBR3, MCF10DCIS.com, 4T1, EO771, and PyMT-TS1). Immunocompetent mouse models confirmed cytotoxicity of HSV1716 against 3 TNBC cell lines, 4T1, EO771 and PyMT-TS1. Intravenous and intratumoral delivery of HSV1716 was explored and resulted in similar primary tumour reduction and reduction in lung metastases. In view of this, intravenous delivery was selected to be explored for further work as this would allow an easier translation to clinical delivery. The prevention of metastatic disease development was found to be greater when HSV was given in multiple doses every 48 hours which significantly extended the overall survival of mice. Activation of the immune system through infiltration of leukocytes and changes in TAMs which support a more pro-inflammatory and cytotoxicity immune microenvironment were seen in the mice treated with HSV1716. Interestingly, it was found that HSV1716 was able to replicate within TAMs and that this replication was dependent on PCNA. This was evidenced by an increase in PCNA within TAMs infected with HSV1716 and a lack of viral replication within TAMs that have undergone PCNA knockdown (chapter 4, figure 6). PCNA been shown to be necessary for HSV-1 replication and has been shown to be associated with HSV-1 replication forks (280) and therefore is needed for HSV viral replication. Additionally, inhibition of PCNA function using 2 different PCNA inhibitors, PCNA-I1 and T2AA, is shown to limit HSV-1 infection (281). This explains why after PCNA knockdown a reduction in viral replication is observed (108). What is less clear is the role of macrophages in the viral replication process. The observation that PCNA staining is increased within the TAMs

after infection with oncolytic HSV1716 and that PCNA knockdown within MDMs resulted in decreased viral replication when coinfected with HSV1716 compliments the above knowledge that PCNA is key to HSV replication. As higher levels of PCNA is often seen in more aggressive cancers (304) perhaps this will prove to be a natural therapeutic advantage for oncolytic virotherapy. However, given the multifaceted role of PCNA in the development of cancer cells and metastasis and its role in immune cells, future exploratory work would be of interest to ascertain if combination of inhibitor of PCNA or cancer related PCNA and immunotherapies would prove complementary or antagonistic. An interesting angle may be to explore primary and metastatic mammary tumour growth when HSV1716 is given alongside two different PCNA inhibitor, one which is specific for cancer related PCNA. This would elucidate if cancer related PCNA targeting causes a decrease in HSV1716 replication within tumour and thereby inhibiting the cytotoxic effect of HSV1716.

As PCNA and its role in healthy cells, cancerous and immune cells have not been explored in the context of oncolytic virotherapy, a review was completed and published alongside this PhD (305).

(283,306)(307,308)(309)(310)(311)(312)(313)(314)(315)(316,317)(318)(319)(320)

6.1 Oncolytic virotherapy and its place in cancer therapeutics

The relationship between cancer, viruses and the immune system has been long established. In some cases, the aetiology of cancer is a result of chronic viral infection (e.g. HPV in neck and neck cancers (306)) perhaps due to a chronic T cell exhaustion. As our knowledge about the mechanism by which cancer starts growing and evading the immune system increases, perhaps we can use this understanding to develop vaccinations to circumvent cancer development. T cell exhaustion itself is an interesting subject with understanding growing at a rapid rate (307,308). Given the interplay between T cell exhaustion in chronic infection and its role in cancer development, as well as how this can be manipulated using immune checkpoint inhibition, it may be postulated that combinations of oncolytic virus and checkpoint inhibitors would be extremely synergistic.

Clinical trials of such combinations have been reported for a handful of tumour sites with promising phase 2 results(309). The most robust data, however, comes from trials involving combinations of the virus, TVEC and RPI. A phase 3 trial of T-VEC (a modified herpes virus) and the checkpoint inhibitor Pembrolizumab(310). Here 692 patients with previously untreated unresectable stage II/IV melanoma were given T-VEC at a dose of 4×10^6 plaque-forming unit (PFU) and pembrolizumab (200mg) every three weeks. However, there was no significant improvement in the overall survival between the combination and single agent pembrolizumab arms. This was clearly a disappointment for drug developers; however, authors note that the combination is still under investigation for those who become refractory to checkpoint inhibition. More recently, early data has been presented in abstract form is the combination of the virus RP1 (replimune) and the checkpoint inhibitor, Nivolumab. Here, phase 1/2 trial data showed a 33.6% overall response rate for RP1 plus nivolumab in melanoma patients who have processed after first line PD1 targeted treatment, raising the thought that perhaps oncolytic

virotherapy can reignite a tumour's sensitivity to checkpoint resistance (311). The modifications to the tumour microenvironment where particularly interesting. Patients were found to have an increase in CD8 T cell infiltration, PD-L1 expression within tumours by immunohistochemical staining, and increased gene expression associated with immune activation. Although a phase 3 trial (NCT06264180) is undergoing recruitment, in view of these exciting findings, the FDA has recently announced a prioritized review of RP1 and nivolumab for advanced melanoma, with the possibly of approval by July 22, 2025, a potential landmark for combination oncolytic virotherapy. As more trials of oncolytic virus and immunotherapy emerge and show promise and the results of how these will shape the landscape of cancer treatment these are eagerly awaited (312). Within breast cancer, there have been no reported clinical studies exploring the use of oncolytic virotherapy and checkpoint inhibitors. In the preclinical setting there are a couple of notable studies that describe meaningful results and therefore may pave the way to early in the future. In an immunocompetent breast cancer model (EMT6), the efficacy of an oncolytic reovirus was demonstrated to be increased with PD-1 blockade. Interestingly T regulatory increased with reovirus alone but was reversed with the addition of the check point inhibitor, a benefit synergistic effect (313). Bourgeois-Daigneault et al, describe a novel neoadjuvant murine model where the timing of Maraba virus was placed prior to surgical resection of 4T1, EO771 and EMT6 flank tumours (314). A second tumour was implanted on the opposite flank after surgery following which intraperitoneal check point inhibitors were dosed. Authors describe significant suppression of secondary tumour growth when the combination of virus and check point inhibitors was administered. This is an unusual scheduling as it doesn't follow any clinical evidence. Given that check point inhibition has only recently been approved for the treatment of TNBC it is likely that combination trials of OV and checkpoint inhibitors may be delayed. There are however a number of clinical trials which have reported combination of

virotherapy more established treatments like chemotherapy or targeted therapy and these are described below.

In a phase 2 trial, patients with early stage TNBC were given five intratumoral T-VEC injections alongside standard of care chemotherapy, paclitaxel, doxorubicin and cyclophosphamide neoadjuvant setting followed by surgery (315). The complete pathological response rate was 45.9% which is similar to reported trials of chemotherapy alone and therefore difficult to fully extrapolate the benefit of the addition of the virus as there is no control arm within this study. However immune activation was seen in the tumours in patients and the long-term recurrence rate for these patients is not known. One can only speculate that early activation of the immune system, whilst cancer burden is low, will promising in terms of disease recurrence prevention.

Interestingly there have also been cases of unexpected treatments combinations that have led to a clinical response. Yuan et al describe a case report of a patient with heavily pretreated metastatic TNBC who was recruited for a phase 1clinical trial of an oncolytic virus CF33-hNIS-anti-PD-L1 (CHECKvacc)(316). After no signs of clinical response, they were discontinued off treatment and unusually given trastuzumab deruxtecan, a drug which in the UK is only administered to patients with confirmed HER2 receptor positivity. The patient had a significant clinical response and a significant improvement in overall survival of 10 months which was unexpected. The authors postulate that combination of virus and trastuzumab deruxtecan may have influenced response. In the UK, this case would not be possible to repeat, however a clinical trial looking at trastuzumab deruxtecan in combination with oncolytic virotherapy in HER2+ patients would be a potential avenue to explore.

Novel treatment delivery strategies are also of interest. Oncolytic viruses can be packed and delivered using cell carriers, such a macrophages as described in chapter 5 and reported by Muthana et al(149,317), linked to magnetic nanoparticles(318) or encapsulating in a liposome (319). Alternatively given their cancer homing tendencies they can also be used as vectors to

carry other treatments. CD40 stimulation is felt to complement anti-cancer immune responses through production of TNF, ROS and reactive nitrogen species and thereby activation of macrophages and other proinflammatory immune cells, however on-target toxicities of these agents are high when given directly via the intravenous routes. The oncolytic adenovirus NG-350A is a tumour-selective and blood stable adenoviral T-SIGn vector expressing a potent fully human IgG agonistic anti-CD40 antibody which has completed phase 1 trials with and without pembrolizumab treatment (320,321). In these studies, which are currently only reported as abstract form, it was seen that the intravenous delivery a viral vector expressing a CD40 antibody allowed the same downstream effects as administering the antibody intratumourally without any systemic circulation of CD40 antibody and thereby reduction toxicity. The addition of pembrolizumab to this novel virotherapy did not significantly increase side effects and NG-350A was able to induce a meaningful clinical outcome (stable disease) to 3/6 heavily pre-treated metastatic epithelial cancer patients at the 4th dose level although this dose had not been combined with pembrolizumab.

6.2 Optimising delivery of HSV1716

Oncolytic virotherapy delivery is dependent on the nature of the virus and the possibility of prior exposure to a similar virus which will trigger immunogenic clearance of a known pathogen. HSV-1 based virus, such as HSV1716, are considered to have promising clinical applications owing to its potent lytic ability, the broad spectrum of infected cells, ease of engineering and induction of long-term cellular immune responses (322). However, it is also widely accepted that the majority of patients will have been exposed to HSV at some point in their life. In view of this, the delivery of HSV-1 viruses in clinical trials for cancers have often been administered via the intratumoral route. This may limit the use of these drugs in metastatic breast cancers, where the majority of disease is not readily accessible as it is found

in the bones, liver and lungs. In chapter 5, a novel way of packaging oncolytic HSV1716 into macrophages is described. Here, HSV1716 is inoculated with MDMs prior to delivery. Given the phagocytic nature and natural tumour taxis of macrophages, this is taken up and transported via the bloodstream to the TME where virus is unpacked and released for cytotoxic effect. The publication in this PhD reports the first description of this method being used for the delivery of an oncolytic HSV in triple negative breast cancer and a visual summary of the changes are illustrated in figure 18. Myeloid derived cells have also been explored in other tumour sites. One such study explores the effect on the systemic delivery of a measle virus (323). Here authors describe how rapidly measles is neutralised when administered systemically and how they cover come using monocyte cell carriers. What is particularly interesting, and something not considered in this PhD, was their data suggesting that viruses could be found on the surface of carrier cells, and these were fully susceptible to antibody and complement neutralisation. Prior to *in vivo* treatments, the group washed cell carriers in a strong neutralising cocktail of antibodies and to avoid any triggering immune system effects due to cell surface associated viruses. Another account of macrophage delivered therapy is that of Muthana et al (317), here authors used an E1A-dependent adenovirus, whose proliferation is restricted to prostate tumour cells using prostate-specific promoter elements from the TARP, PSA, and PMSA genes, and co-transduced these with macrophages with a hypoxia-regulated E1A/B construct. The resultant effect is a very targeted tumour selective transport system which releases virus in areas of tumour hypoxia. This more cancer specific approach is clinically interesting as immunotherapeutic agents, including oncolytic virotherapy, have the potential to induce many undesirable side effects which have long lasting effects of patient morbidity and mortality.

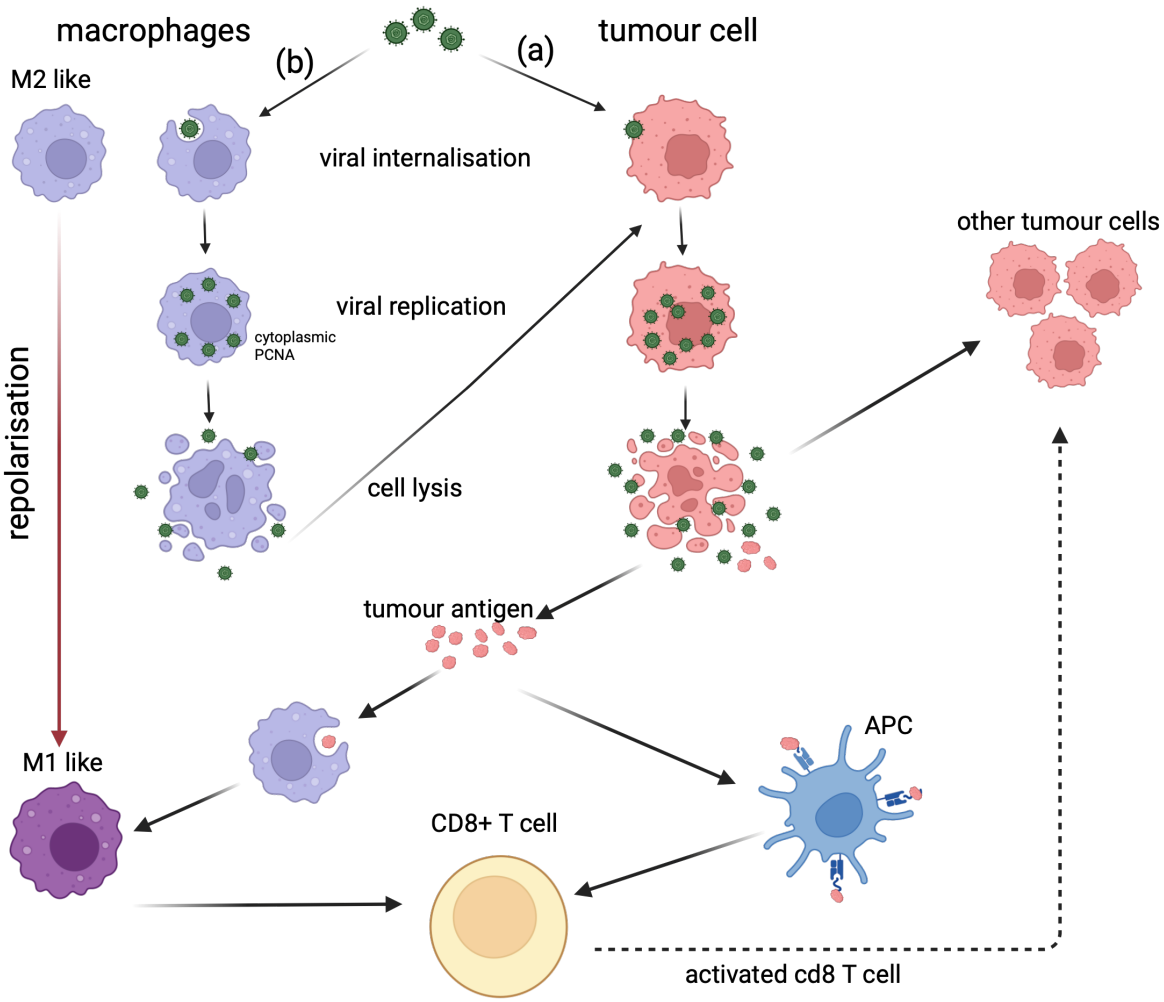

Figure 18: Visual summary of immune system changes following administration of HSV1716. *HSV1716 is administered (a) intravenously or (b) loaded into macrophages. HSV1716 is internalised and replicates within both tumour and macrophage cells. When these cells undergo immunogenic cell death, viral progeny is released into the surrounding tissue which causes a further cycle of infection and replication. Additionally, tumour antigens are released into the bloodstream where they can be detected by antigen presenting cells (dendritic cells or macrophages) and presented to CD8+ T cells. This primes the T cells to become cytotoxic and activated against the cancer cells leading to further tumour cell death.*

An alternative cellular vector for viral loading are lymphocytes. These can be administered de novo or primed to be sensitive to tumour antigens (CAR-T cells). An example includes the systemic administration of an oncolytic herpes simplex virus-1 mutant R3616 onto

lymphocytes harvested from mice with acquired antitumor immunity(324). It was demonstrated that the mice with disseminated peritoneal tumours lived longer when treated with this virus in comparison to virus loaded on non-sensitised lymphocytes, lymphocytes and virus alone suggesting improved outcomes when tumour specific lymphocytes were obtained.

Although, the work of this PhD and the work of others have shown cellular vector have the potential to overcome the challenges in systemic delivery, mainly neutralisation, however work needs to be done to investigate the logistics around the clinical isolation of cellular vectors and the ratio of viral particles to vectors to allow adequate dosing at the site of tumours. Interestingly, we found that although a dose of $1x10^6$ PFU of HSV1716 was administered in initial studies, this appeared to have the same effect as $1x10^4$ PFU loaded into macrophages suggesting that the use of macrophages may extend beyond being a simple trojan horse to deliver virotherapy and may indeed enable viral replication and therefore enable a higher dose of virus to the target site within tumours. At the time of completing the *in vivo* work for this PhD, there were no local facilities to support the set-up of a phase 1 clinical studies of macrophage delivered oncolytic virotherapy. There have been an increase in CAR-T technologies over the past 5 years and CAR-macrophage therapy has been recently reported (325,326). The chimeric antigen receptor (CAR) has become a promising personalised approach to enhance the capacity of immune cells to recognise and kill cancer cells. Both T cells and macrophages can be developed with CAR constructs. Given the effectiveness of TAMs to penetrate the TME, CAR-M cells were thought to possess a significant potential novel therapy. Indeed, within breast cancer, CAR-M therapy have resulted in early phase clinical trials in patients with HER2+ disease following positive results from vivo work by Klichinsky et al after the refinement of a second generation anti-HER2 CAR-M (327). There are several concerns with CAR-M therapy including how to manage macrophage plasticity, how to prevent off target toxicity and, as the liver has the highest concentration of

macrophages with the body, how to focus treatment to prevent potential hepatotoxicity. This concerns, have thus far, limited the mainstream use of CAR-M therapies.

Despite this, given the advances in the isolation, culturing and expanding of macrophages required for the development of CAR-M, the technology needed for optimisation macrophage delivered therapies is in existence. Now may be the time to consider the feasibility of macrophage virotherapy. The practicalities behind this would involve patient venesection, harvesting of macrophages from donated blood, incubation of these macrophages with oncolytic virus and then reinfusion of loaded macrophages intravenously (see figure 19). There are, however, several potential limitations to macrophage delivered therapy, it is very personalised which may limit the number of patients one is able to treat due to lack of processing sites and laboratories. Patient's blood samples may also have limited macrophage numbers, and therefore allogenic blood may have to be considered which may put strain on limited resources. Therefore, at the present time, it seems that a phase 1 trial is a little premature.

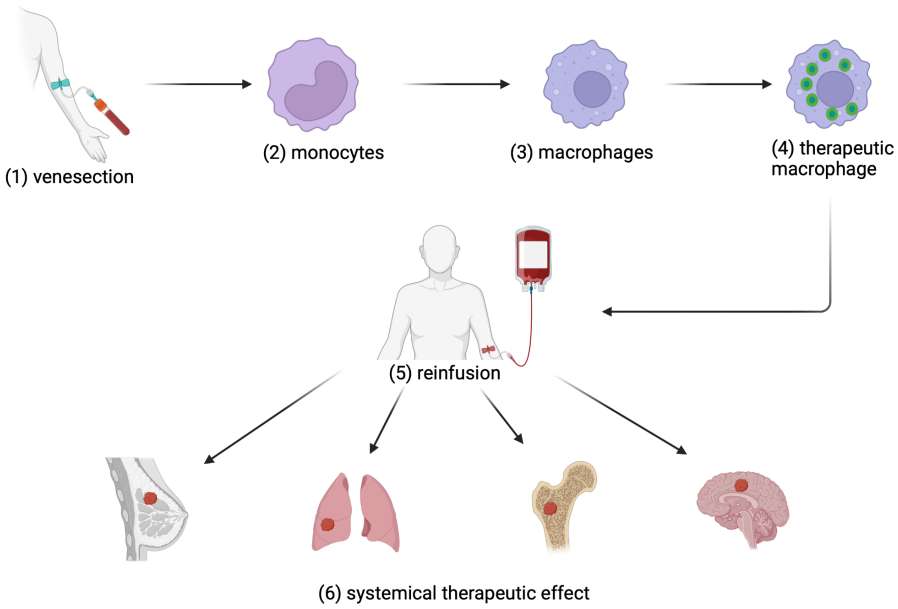

Figure 19: Steps for clinical use of oncolytic virotherapy. *(1) Venous blood is obtained peripherally through venesection. (2) monocytes are isolated from patient blood through density gradient centrifugation. (3) monocytes are allowed to mature into macrophages. (4) Macrophages are co-cultured with HSV1716 for 4 hours to allow HSV1716 engulfment to form a therapeutic macrophage. (5) Therapeutic macrophages are re-infused into the donor patient using a blood transfusion kit. (6) Therapeutic macrophages are transported via the bloodstream to the areas of malignant disease to exert their therapeutic effect.*

6.3 Role of TME and macrophages

The tumour microenvironment is composed of many different immune cell lineages and within this monocytes and macrophages makes up the largest component (328). The role of TAMS within this has been of interest to many. The origin of TAMS, their multiple personalities and the potential to influence these through the use of check point inhibitors is central to this interest. The origins of TAMs have shaped the understanding about the development of the cancer process. For example, in lung adenocarcinomas using lineage tracing, tissue resident macrophages were found to be closely associated with tumour cells in the early phases of carcinogenesis providing a nurturing niche for epithelial-mesenchymal

transition and signalling a regulatory T cell response, whereas monocyte-derived macrophages were recruited to the tumour site at a later stage further accelerating tumorigenesis (329).

In chapter 4, the heterogenicity of macrophages with murine mammary models in response to oncolytic virotherapy were described. Here it is seen that macrophages exist in two broad states, one classically described as “M1”, a proinflammatory state which can alert the innate and adaptive immune system to respond to a threat and a “M2” state which is signals a need for immunosuppression rather than response. It is observed that through treatment with immune stimulating agents such as the HSV1716 virus, that we can flip the switch between the two states leading to an increase in awareness and activation of the pathways that result in immune surveillance and cytotoxicity.

We have also known that macrophage inhibition using clodronate liposomes results in increase cancer cell growth despite treatment with oncolytic virotherapy. This suggests there is a role for macrophages in mediating this immunes response. Clinically we see that different tumour types respond variably to immune check point inhibition with melanoma and renal cells having a higher response rate than breast cancer. Perhaps this could be related to the macrophage density or signature within the tumour type. Cheng et al describe the generation of a single cell transcription analysis of myeloid cells within 15 different cancer types (330). Here they note the differences in expression of receptors from monocytes in bloods, at the periphery of cancer tissues and within the tumour microenvironment themselves. They also note that within the TME macrophage co-exist in both M1 and M2 phenotypes and the balance of these subsets vary between cancer type and that these form specific heterogeneous transcriptomic patterns, thus need to be analysed separately to better describe their properties. It can be postulated that this transcription pattern may result in a predictive model to assess immune system sensitivity and thus the group analysed scRNA-seq data derived from melanoma patients treated with immune checkpoint inhibitors. These were categorised into two groups, responders and non-responders and the proportion of VCAN+ angiogenesis-associated TAMs

were found to be significantly lower in the responder group, suggesting that this could be a potential biomarker to predict response to immunotherapies.

The heterogenicity of the tumour myeloid compartment has previous been shown to be influential in predicting response of resistance to immunotherapies in TNBC models. Here, in the paper by Kim et al, 2 distinct immune subtypes" of TNBC were described: neutrophil-enriched (NES) and macrophage-enriched subtypes (MES) (331). Authors found that the MES associated tumours has a high response rate to check point blockade whereas the NES subtype showed an inherent tumour resistance. They described conversion between the MES subtype to the NES subtype led to an acquired resistance to check point blockade. To take this further, it would be interesting to explore the role of oncolytic virotherapy in conjunction with immune check point blockade to see if any of this immune resistance can be reversed.

6.4 Use of macrophages in cancer therapeutics

Emergence of the programmable nature of macrophages and their role in cancer development and prolongation, many have started to investigate the use of macrophages themselves in the cancer therapeutic pathway. These can be broadly divided into their use as cellular carriers (as described in this PhD) and as macrophages equipped with CAR constructs (CAR-M) (332). The concept of CAR-M was based on the popularity and success of CAR-T lymphocyte adoptive cell transfer where T cells are primed in the laboratory again patient specific cancer antigens. It is of interest to groups early on and have led to a number of small clinical trials in a number of solid tumour types (333–335)However, the data from these are not entirely convincing and this be due to the macrophages not been appropriately primed due to the limitations in the technology 30 years ago. Currently the design of viral vectors and genome editing technologies allow for a precise modification of cells and this has led to a resurgence of interest in CAR-M focused therapies. Within breast cancer CAR-M have been

invested in immunocompetent murine models of TNBC (using the 4T1 cell line) and these *in vivo* studies have confirmed the cytotoxicity of this (336,337). Phase I trials have started to be reported for CAR-M therapies (338) and there are also a number of novel start up technology companies that are keen to capitalise on this promising novel therapy. It is expected to be an area of potential growth in the future.

6.5 Limitations and future work

One of the challenges with the studies that we have done as part of this work was that the tolerated dosing of HSV1716 varied between mouse strain. For our initial *in vivo* studies, the FVB mouse strain was used. This revealed that these mice were moderately sensitive to immediate viral inoculation and therefore around 50% of mice were lost to immediate, unexplained side effected. The company who had supplied the virus hadn't reported any adverse effects in their animal experiments. Our group next experience with HSV1716 dosing was in the C57bl/6 mice who tolerated the same dose of virus without experiencing any immediate toxicity. However, the standard dose of virus to the BALB/c mice used in the majority of experiments in this PhD proved lethal to all our the BALB/c mice and as a result experiments had to be modified quickly to prevent further mortality. This unexpected event causes an interruption to my *in vivo* work and the direction of the PhD. These observations have been followed up by a post-doctoral fellow within our group who has since published her findings that this variability may be linked to a T helper profiling (339).

Other challenges faced during the completion of this PhD was directly linked to the Covid-19 pandemic. The effect of lock down as a result of the pandemic caused early termination of an experiment which resulted in a financial loss and change of PhD direction. The terminated experiment was due to look at combination check point inhibition and oncolytic virus. At the time, very few groups had explored this strategy. However, more

recently a number of groups have report promising preclinical and early phase results and it will be interesting to see how these develop in the future.

Adapting to and overcoming challenging is an important process to learn as a PhD student, although it recognised that it was hoped by the end of the PhD one would be in a position to discuss the possibility of a phase 1 trial with HSV1716. Aside from Covid-19, progression to a clinical trial was limited by the resources available at the time, namely the isolation and purification of patient macrophages had not yet been optimised and the company providing HVS1716 changed ownership weren't in a position to supply clinical grade products.

The work in this PhD was also limited by financial resources. To complete further work as discussed above, additional funding would need to be acquired. The murine models were proposed where the most resource effective (cost, time and expertise) however in an ideal work, model with humanised (immunocompetent) PDX models may give a different picture. Additionally, there are also a few experiments that in hindsight would have been helpful to perform. The question of how efficient macrophages are at both transporting HSV1716 and unpacking HSV1716 to the site of tumours is unknown. This is of particular interest in brain tumours which is notoriously difficult to treat due to the highly selective blood brain barrier. Future work could include labelling macrophages to confirm they cross the blood brain barrier and quantifying viral titre within tumours (both in and outside the brain) and bloods samples at set time points following infection to ascertain the pharmacokinetics of HSV1716 . Furthermore, macrophages have shown to transport other viruses without degradation to cause pathogenesis (Rigden et al., 2002)and exploration about which viruses are more amenable macrophage carriers and which virus, as in the case of HSV1716, can replicate within macrophages would help appropriately select oncolytic viruses that are suited for macrophage assisted delivery.

Further to this, to add to the data found in the PhD, it would have been interesting to explore biomarkers alongside clinical response and with hindsight, it would have been helpful to provide additional data about known biomarkers e.g. TILs and tumour mutation burdens in our experiments.

6.6 Conclusion:

Whilst oncolytic viruses have yet to reach their full potential in clinic, the work done as part of this PhD has contributed to the understanding of HSV based oncolytic virotherapy in TNBC, the use of cellular carriers as a vector to deliver oncolytic viruses and added to the limited information about PCNA and its role within macrophages.

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